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Research Article

Co-application of QX-314 and Lidocaine in Rabbit Brachial Plexus Block Using a Nerve Stimulator

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Abstract: We aimed to assess the efficacy and safety of QX-314, and lidocaine co-application (QX/Lid) in rabbit brachial plexus block (BPB) using the neurostimulation method to improve the selectivity and quality of induced regional anesthesia. Fifteen female rabbits (1.5-2 kg) randomly assigned into three groups: Lidocaine: 5 mg/kg; QX-314: 35 mM and QX/Lid: 35 mM + 5 mg/kg. The relevant anesthetic solution injected into the BP using a nerve stimulator (NS) and the BPB was evaluated by assessing sensory and motor functions. Based on the reaction to painful stimuli and the loss of bearing weight on the treated forelimb, respectively, the onset and duration of sensory and motor block were measured. The quality of sensory block assessed by using the scoring method. Blood samples collected before and at 5 min after drug injection up to180 min to measure the plasma concentration of QX-314 by HPLC. The QX/Lid resulted in a significantly faster onset of sensory and motor block, significantly decreased the duration of motor block and improved the quality of sensory block. QX-314 peak plasma concentration observed at 5 and 45 min in QX-314 and QX/Lid groups, respectively, and there was no behavioral evidence of systemic toxicity. In conclusion: QX/Lid co-application accelerate the onset of BP block with a greater quality of sensory and motor blockade separation and can be safely performed in rabbits using the NS method.

Keywords: *Brachial plexus block, Lidocaine, QX-314, Rabbits*

Bir Sinir Stimülatörü Kullanılarak Gerçekleştirilen Tavşan Brakiyal Pleksus Blokajında QX-314 ve Lidokainin Birlikte Uygulanması

Öz: Regional anestezinin seçiciliğini ve kalitesini artırmak için nörostimülasyon yöntemi kullanılarak tavşan brakiyal pleksus blokajında (BPB) QX-314 ve lidokainin birlikte uygulamasının (QX/Lid) etkinliğini ve güvenliğini değerlendirmeyi amaçladık. On beş adet dişi tavşan (1.5-2 kg); 5 mg/kg Lidokain, 35 mM QX-314 ve 35 mM + 5 mg/kg QX/Lid olmak üzere rastgele üç gruba ayrıldı. İlgili anestezik, bir sinir stimülatörü (NS) kullanılarak brakiyal pleksusa (BP) enjekte edildi ve BPB duyusal ve motor fonksiyonlar yönünden değerlendirildi. Anestezik uygulanan ön ayakta ağrı oluşturan uyaranlara verilen tepki ve üzerinde ağırlık taşıma kaybı temel alınarak, sırasıyla duyusal ve motor blokajın başlangıcı ve süresi ölçüldü. Duyusal blokajın kalitesi puanlama yöntemi ile değerlendirildi. QX-314'ün plazma konsantrasyonunun HPLC ile ölçülmesi amacıyla enjeksiyondan önce ve enjeksiyondan 5 dak. sonrasında 180. dak.'ya kadar kan örnekleri alındı. QX/Lid, duyusal ve motor blokajı anlamlı derecede daha hızlı başlattı, motor blokaj süresini anlamlı derecede kısalttı ve duyusal blokajın kalitesini artırdı. QX-314 ve QX/Lid gruplarında, QX-314'ün plazma pik konsantrasyonları sırasıyla 5 ve 45. dak.'larda gözlendi ve sistemik toksisiteye dair davranışsal bir kanıt bulunmadı. Sonuç olarak, QX-314'ün Lidokain ile birlikte uygulanması, daha yüksek kalitede duyusal ve motor blokaj ayrımı sağlayarak BP blokajının oluşmasını hızlandırmakta ve NS yöntemi ile tavşanlarda güvenle kullanılabilmektedir.

Anahtar Sözcükler: *Brakiyal pleksus bloğu, Lidokain, QX-314, Tavşan*

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Introduction

By reversibly blocking the formation and propagation of action potentials in both motor and sensory fibers, local anesthetics (LAs), as opposed to systemic analgesics, provide total analgesia and paralysis with fewer adverse effects. In the clinical use, LAs with fast onset, long action, selective effect and low local and systemic toxicities are desired. The combination of LAs is one of the methods that was used to improve the quality of induced analgesia, and decreases the toxicity effect by LAs dose reduction [1,2].

QX-314 is a membrane-impermeable quaternary derivative of lidocaine, which produces rapid onset and long-lasting nociceptive selective block. Due to its weak capacity to permeate the lipid tissues as a result of its persistent positive charge, QX-314's action is constrained. The delivery of QX-314 into nociceptors has been found to occur when transient receptor potential (TRP) channels are activated. Therefore, capsaicin, acidic solution and various LAs, such as lidocaine and bupivacaine are combined with QX-314 as TRP activators in regional nerve blocks [3-5].

Brachial plexus (BP) block is an effective method to provide anesthesia to the upper limb from the shoulder to fingertips depending on the block indication, surgery procedure, and anatomy variation [6,7]. BP block can be done with an injection of LAs around the peripheral BP nerves using different methods, including blind needle placement, ultrasound-guided, and nerve stimulator-guided. Neurostimulation is an alternative direct method to identify the peripheral location of the motor component of nerves using continuous electrical currents that produce muscular contraction and decrease required LAs for nerves block [6-9].

In rabbit, which is accepted as the BP experimental model in human and in pets, information regarding BP block by using NS method is lacking. In the present study, we aimed to evaluate the efficacy and safety of QX-314 and Lidocaine co-application (QX/Lid) in rabbit BP block using neurostimulation method to improve the selectivity and quality of induced regional anesthesia.

Material and Methods

Ethical Approval

The study was carried out based on the guidelines for the care and use of laboratory animals. All experimental procedures were approved by the Committee of Scientific Research and Institutional Animal Experimental Ethics, Shahid Chamran University of Ahvaz, Iran, (Approval no: EE/98.24.3.26578).

Animals and Study Design

Fifteen female adult New Zealand white rabbits (weighing 1.5-2 kg, 3- to 5-month-old) were purchased from Razi Vaccination, and Serum Center of Iran. Rabbits were single-housed with free access to food and tap water. The rabbits were randomly assigned into one of three groups: 1) Lidocaine [(Lid), 5 mg/kg]; 2) QX-314 [(QX), 35 mM]; and 3) QX-314 combined with lidocaine [(QX/Lid), 35 mM + 5 mg/kg]. Lidocaine and QX-314 alone injection served as controls. Drug solutions were freshly prepared before use, and the solution pH was measured by a laboratory pH meter (Crison pH meter; Basic 20$^+$, Barcelona, Spain). The rabbits fasted for 12 h before experiments, but water was available at all times. The right limb was selected for block, and the left was used as control.

Brachial Plexus Block

The rabbit's axillary region was prepared in the supine position. The location of BP nerves was determined using a nerve stimulator as described by Boogaert et al.[10]. In brief, the positive electrode was attached to the skin at a distance of 5-7 cm from the shoulder joint. A 23-gauge insulated needle (Pole Needle, Equip Medical, Holland) was connected to the other pole. Alongside the artery, the insulated needle was introduced into the skin and subcutaneous tissue. Electrical stimulations were provided until the limb's muscles began to contract, at which point the current was reduced and the needle was moved to produce the maximum twitch with the least amount of current (0.4-0.5 mA, 0.1 ms, 2 Hz). After the aspiration, a freshly prepared anesthetic solution (in total volume of 1ml) was injected slowly until the twitch disappeared. To block all branches of the BP, the needle was moved to different points and the time required to complete the procedure from the insertion of needle into the skin was recorded *(Fig. 1)*.

Assessment of Nerve Block

After the administration of drugs, the rabbits were kept in a standing position and the onset and duration of motor block was determined by the loss of bearing weight on treated forelimb. Based on the absence of response to painful stimuli, such as superficial and deep pin pricks with a 25-gauge needle and pinching of skin with a hemostat clamp closed to the first ratchet for 1-2 sec, the onset and duration of forelimb analgesia (sensory block) inside and below the elbow joint were recorded. The procedure was repeated every 10 min until a response was observed. Any obvious clinical signs related to local anesthetic toxicity, including extensor rigidity, muscle twitching, and convulsions were not monitored and recorded. The same investigator assessed analgesia in all cases and was blinded to given anesthetics.

Analgesia Maintenance Quality Score (0-3)

0- No analgesia, with obvious signs of discomfort made

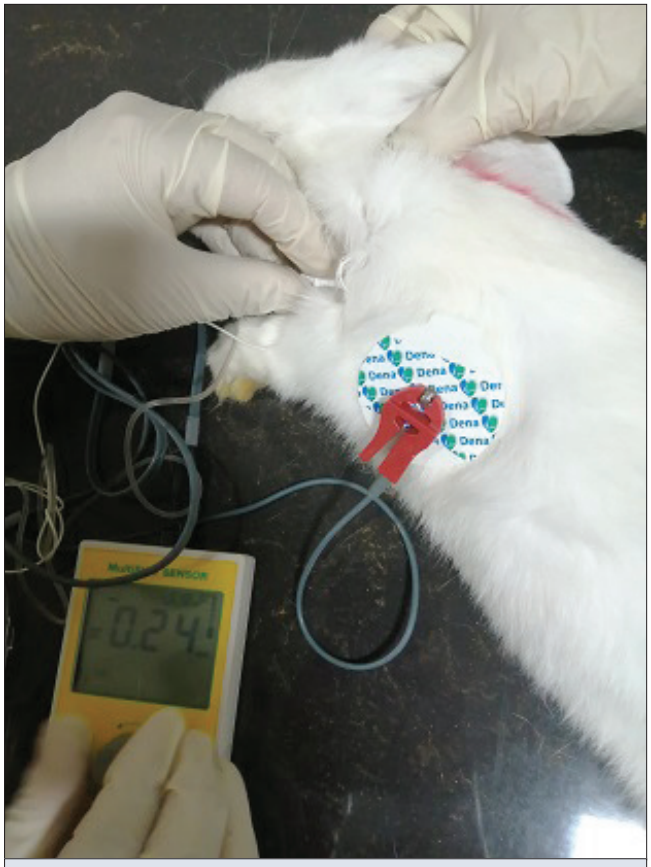

Fig 1. Brachial plexus block by using the neurostimulation method in rabbit

worst by firm pressure, the tendency to struggle and escape from their restraint.

1- Moderate analgesia, with some overt signs of the discomfort which were made worse by painful stimuli, exhibit signs of anxiety, such as whining.

2- Good analgesia, with no overt signs of discomfort but reaction to painful stimuli, teeth grinding.

3- Complete analgesia, with no overt signs of discomfort and no reaction to painful stimuli.

Measurement of QX-314 Plasma Concentration (HPLC)

Femoral artery blood samples were collected before and at 5, 10, 25, 45, 60, 90, 120, and 180 min after drug injection. The plasma concentration of QX-314 was measured by HPLC using the method that was previously described with some modifications [11]. In summary, plasma of blood samples was separated by centrifuge at 1500 rpm for 10 min at 4°C and were stored immediately at -20°C until further analysis. For sample preparation, 10 µL of perchloric acid was added to 50 µL of plasma samples and mixed using a vortex mixer for 1 min, centrifuged at 12000 rpm for 5 min at 20°C, and then filtrated. The final solution was diluted by mobile phase containing 60% of 50 mMol phosphate buffer with pH = 4, 30% of methanol, and 10% of acetonitrile with 0.16% trimethylamine. Then, 100 µL of the solution was injected into the HPLC system (Knauer HPLC system, Germany). The HPLC system consisted of an L-2100 pump, L-2300 column oven, and UV detector. The analytical column was a C18 column of 50-mm length and 4.6-mm diameter (Shiseido, Japan). The temperature was maintained at 40°C for the column and the flow rate was 0.7 mL/min. The wavelength of the detector was 210 nm and the retention time for QX-314 was 11.3 min. The detectable concentration of QX-314 was 300 ng/mL.

Statistical Analysis

Data are expressed as mean ± SEM. All statistical analyses were performed using GraphPad Prism software (Version 9.0.0) and data were analyzed for normality. The statistical significance of differences was calculated by one-way of variance (ANOVA) with Bonferroni's test, and paired t-tests for differences from the control values. Differences were considered statistically significant at ($P<0.05$).

Results

Age, body weight, American Society of Anesthesiologists (ASA) score, and the mean time of drug injection showed no significant differences among the groups. Moreover, there were no observed side effects in rabbits receiving QX-314. We assessed the effectiveness of BP block by measuring sensitivity and motor functions following QX-314 and QX/Lid injections. The results of the recorded data are presented here.

The Onset Time of Forelimb Analgesia and Paralysis

Table 1 shows the mean onset time of BP sensory and motor block. Lidocaine induced forelimb analgesia and

Table 1. *The mean onset time and duration of brachial plexus sensory and motor block*

Groups	Sensory Block		Motor Block	
	Onset Time (min)	Duration (min)	Onset Time (min)	Duration (min)
QX	-	-	-	-
Lid	7.3±2.82	26.25±3.14	2.75±1.94	25.5±3.63
QX/Lid	2.1±0.87	30.33±1.30	Less than 1 min (0.5±0.58)	14.6±0.28

QX (QX-314, 35mM), Lid (Lidocaine, 5 mg/kg), and QX/Lid (QX-314 in combination with Lidocaine (35mM+5 mg/kg)) (N = 5). Data are presented as mean ± SEM

paralysis at 7.30±2.8 and 2.7±1.94 min, respectively. QX-314 alone showed no significant effect on sensory or motor functions even at high doses (5-100 mM) over the measured time. In contrast, QX/Lid injection induced significantly faster sensory (2.1±0.87 min) (P<0.001) and motor (less than 1 min, 0.5±0.5 min) (P<0.05) block when compared to lidocaine. The onset of motor block was significantly faster than the onset of sensory block in Lid and QX/Lid groups (P<0.05) *(Fig. 2)*.

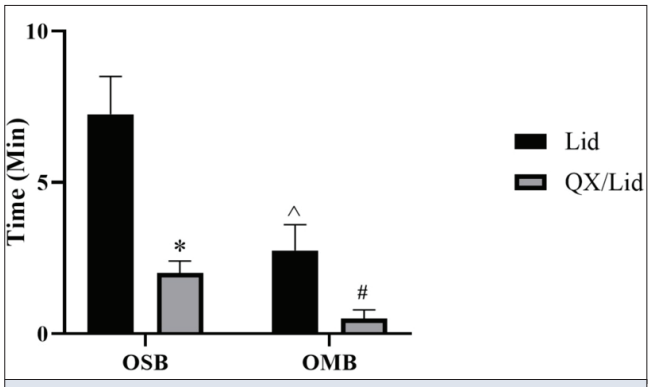

Fig 2. The mean onset time of brachial plexus sensory and motor block. Lid (Lidocaine, 5 mg/kg), and QX/Lid (QX-314 in combination with lidocaine (35 mM + 5 mg/kg)) (N=5). **OSB:** onset of sensory block; **OMB:** Onset of motor block. Data are presented as mean ± SEM. *P<0.001 vs Lid OSB, ^P< 0.05 vs Lid OSB and #P< 0.05 vs Lid OMB

Duration of Forelimb Analgesia and Paralysis

The duration of BP block for sensory and motor function is presented in *Table 1* and *Fig. 3*. QX/Lid combination significantly decreased the duration of forelimb paralysis as compared to Lid (14.6±0.28 vs 25.5±3.63 min) (P<0.05). Although there were no significant differences in the duration of forelimb analgesia among the groups, QX-314 improved the quality of induced analgesia as compared to Lid (Grade 3 vs 2/3; data not shown).

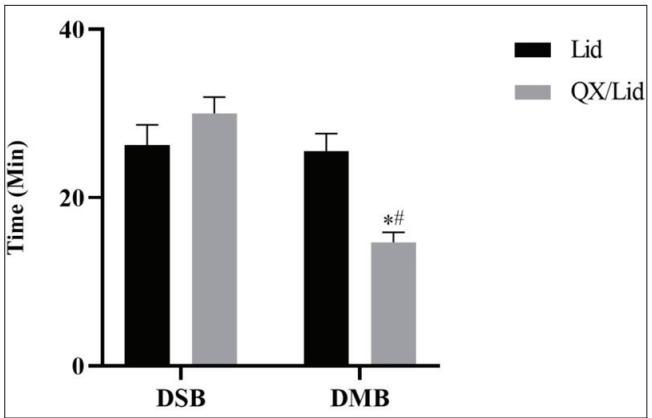

Fig 3. Duration of brachial plexus sensory and motor block. Lid (Lidocaine, 5 mg/kg), and QX/Lid (QX-314 in combination with lidocaine (35 mM + 5 mg/kg)) (N=5). **DSB:** Duration of sensory block; **DMB:** Duration of motor block. Data are presented as mean ± SEM. *P<0.05 vs Lid DMB, *P<0.001 vs QX/Lid DSB

QX-314 Plasma Concentration

The plasma concentration-time profile of QX-314 was determined by HPLC method and data are presented in *Fig. 4-A,B*. QX-314 peak plasma concentration observed at 5 and 45 min after injection in QX-314 (0.74±0.09 µg/mL) and QX/Lid (0.65±0.13 µg/mL) groups, respectively. Cmax of QX-314 showed no significant differences between two groups. However, the absorption of QX-314 was more rapid when injected alone as compared to injection in combination with lidocaine.

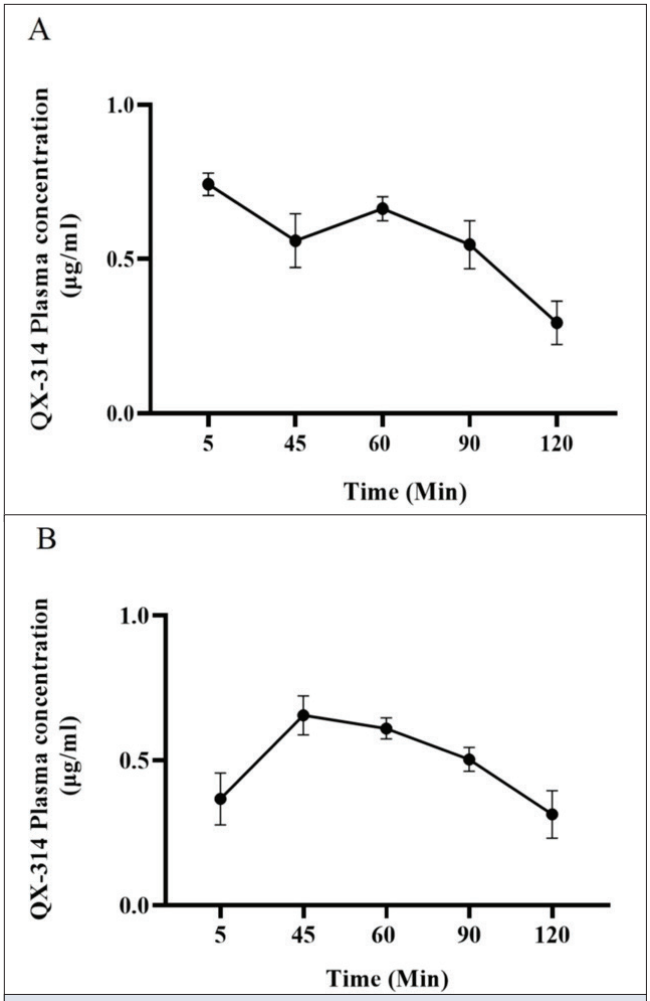

Fig 4. Mean concentration-time profile of QX-314 in plasma after brachial plexus injection. The plasma concentration of QX-314 was measured in **(A)** QX and **(B)** QX/Lid groups within 2 h after injection. N=5 per time point. Data are presented as mean ± SEM

Discussion

We evaluated the effect of QX-314 in combination with lidocaine on BP block (In both sensory and motor function) using nerve stimulator method in rabbits. The combined administration of QX-314 and Lidocaine dramatically sped up the start of BP nerve block, enhanced the quality of forelimb analgesia, and successfully separated BP

sensory from BP motor function, according to the results. Indeed, QX/Lid significantly decreased the duration of forelimb paralysis with no significant effect on sensory blockade duration, in contrast to nonselective effect of lidocaine when administrated alone.

Currently, differential nerve block is required for the surgical and non-surgical situation such as labor process, control of chronic pain and assessment of motor function during surgery. In such cases, it is necessary to provide pain relief without reducing motor function. These demands can be partially achieved with the available anesthesia methods which depend on the technique used and the types and concentration of local anesthetic.

The blockade of Brachial plexus is an effective technique to provide the surgical anesthesia and postoperative analgesia in the upper limb from the shoulder to fingertips depending on the indication and approach used [12]. BP block in rabbits can be induced by the desensitization of cervical (C5, C6, C7, and C8) and the first thoracic spinal (T1) nerves. Due to complex branching and crossing innervations, there are conflicting reports regarding rabbit BP innervation and anatomical structure; which is needed for surgical planning of experiments [12,13]. A combination of methods is needed for describing BP innervation and distinguishing between sensory and motor nerves. Despite the conflicting anatomical and innervation data, it was suggested that rabbit BP models human nerve injuries [6,7]. The BP block has been administered using a variety of techniques, including blind needle insertion, ultrasound guidance, and nerve stimulator guidance. Locating the nerve roots directly, lowering the dosage of LA, and improving the efficacy of BP block are all possible using peripheral nerve stimulator (NS). Reports indicate that the success rate of BP block using NS method is high and stable over time when compared to other methods [12-15]. In small animals LAs toxicity is common and the reducing the volume of the local anesthetic can makes it safer method. In rabbits, information regarding the clinical use of NS method for BP block is lacking, therefore, we aimed to use this method in present study.

Local anesthetics (LAs) are widely used techniques for the desensitization of a localized area of the body, which allows surgical procedures to be performed in the conscious animal. The efficacy of anesthetics can be improved by providing a faster onset time, creating selective effects, prolonging the duration of action, and decreasing side effects [16,17]. LAs vary in their ability to block sensory versus motor fibers and this differential pattern is affected by various factors, including the type of fiber, frequency of stimulation, length of nerve exposed to local anesthesia, and choice and concentration of LAs. When the nerve trunk and large nerves like the brachial plexus are targeted, the somatosensory arrangement of nerve fibers also affects the progression of the block. Therefore, combination of different LAs, the use of additive drugs, and different drug release methods are used to achieve the above-mentioned goals in LAs [17].

QX-314 is a membrane-impermeable quaternary derivative of lidocaine, which was shown to produce long-lasting regional anesthesia in animal models. It was confirmed that QX-314 is a selective sensory blocker that can induce motor block with a duration that is shorter than the sensory block [4,5,18,19]. However, due to its inadequate capacity to penetrate the plasma membrane due to its constant positive charge, its effectiveness in nerve block is restricted. Therefore, different methods and additives were used to enhance the diffusion of QX-314 across the lipid barriers. Activation of TRP channels was reported to specifically deliver QX-314 into nociceptors, so as to produce a rapid and long-lasting nociceptive-selective blockade without affecting motor function [3,20]. TRPV1 plays a critical role in peripheral nociceptor activation and management of acute and chronic pain. QX-314 has a biphasic regulatory effect on TRPV1 channels. At low concentrations (micromolar) it inhibits and at high concentrations (millimolar) activates TRPV1 channels [21]. Acidic solution, capsaicin, surfactants and various LAs, such as the lidocaine and bupivacaine, are combined with QX-314 as TRP channels activators [3,20-22]. Lidocaine, the most wildly used local anesthetic in veterinary medicine, at clinically relevant concentration is a potential non-irritative activator of TRPV channel. It can selectively deliver the QX314 to nociceptors and produce selective regional blocked [3,14,15,23,24].

Lidocaine has a variety of analgesic and anti-inflammatory properties but its application is limited due to its non-selectivity and short duration of action, especially in the postoperative pain management and induces longer duration of a motor block as compared to sensory block [14,15,23,24].

Beside the type of LAs, two items were mentioned for successful peripheral nerve block, appropriate LA concentration and injection solution volume to expose the critical length of the nerve. Injection of smaller volume and higher concentration has been suggested as critical parameters for a successful nerve block rather than the LA dose. A larger volume is suggested when nerves are poorly accessible and are not well located [16,17,25]. The total volume of LAs solution for performing the successful BP block was reported in dogs (0.25-1.0 mL/kg), cats (0.2-0.6 mL/kg), goats (0.3-0.4 mL/kg), and sheep (0.25 mL/kg). The minimum volume of LAs that is required to perform BP block in rabbits is 0.8 mL [26]. We injected 0.34±0.25 mL of lidocaine 2% in 0.65 mL QX-314 solution around the BP nerve using a nerve stimulator. Thus, the total amount

of the injectable medication solution was consistent with earlier research. The observed short duration of anesthesia is explained by the total dose of administered lidocaine, which was (7 mg/mL). Low concentration of lidocaine was just chosen as a TRP activator to accelerate the entrance of the QX-314 into nociceptive nerves to improve the BP block with fewer side effects.

The local tissue toxicity of QX-314 is concentration-dependent, and it is safe at concentrations below 35 mM. Therefore, a safe and effective concentration of QX-314 was used in combination with lidocaine, which resulted in a significantly faster sensory and motor block as compared to lidocaine. QX/Lid combination significantly decreased the duration of motor block and improved the quality of sensory block (grade 3 vs 2/3) as compared to lidocaine, which may be explained by the activation of TRP channels by lidocaine. However, the precise underlying process is yet unknown and requires more research. Increased BP block duration in TRPV1 KO mice after QX/Lidocaine administration suggests that lidocaine may aid QX-314 entrance into neurons through other major polar channels or potentially by enhancing fluidity of the neuronal membrane [20,22,27]. Surprisingly, the duration of the sensory block in our study did not differ among the groups and was lower than it was expected. This may be in terms of the low concentration of the injected drugs and/or drug-drug interactions in an aqueous solution, which reduce the effective concentration of each component. In any real solution, interactions occur among the components and reduce the effective concentration of the solution. In a solution, a rapid balance forms for lidocaine between its uncharged and charged status but QX-314 is permanently charged [23,24,28].

Injection methods may interfere with drug activity in the solution. The drug-drug interactions at the site of action or even during the injection process through microcatheters and possibly small gauge needles result in poor mixing of drugs in BP nerve roots, which affects administrated dose value and needs further studies. Although other factors such as the sensitivity of sensory and motor evaluation methods, injection technical errors, and drug absorption rate should be considered.

Furthermore, the plasma concentration of QX-314 was measured using HPLC method. Results indicated that when QX-314 was administrated alone, the peak plasma concentration occurred very fast and was observed within 5 min of injection. Because QX-314 cannot easily cross the cell membrane it may enter the blood circulation. The combination of QX-314 and lidocaine caused a slower absorption of QX-314 into blood circulation that peaked later at 45 min. In other words, lidocaine decelerated the absorption of QX-314 from the injection site into the blood. Vascularity and the binding of LAs to tissues are the factors that affect the initial rate of absorption into blood circulation. Previous studies reported that in the presence of lidocaine, QX-314 diffuses and binds to neural and also non-neural cells, which may explain the obtained results [3,30]. However, the pace of local anesthetic systemic absorption relies on a number of variables, including the drug's physicochemical qualities and formulation, the injection location, the speed of the injection, the supplied dosage, the presence of additives, and the agent's vasoactivity [28,29]. One of the main influencing factors can be the effect of LAs on local blood flow. At present, there is no referable data about the QX-314 effect on peripheral blood flow, which should be further studied. Lidocaine induces vasocontraction activity in low concentrations, but not at commonly used clinical concentrations. Therefore, future studies should investigate the vasoactivity of LAs when they use in combination forms, which affects drug concentration at the injection site.

The present study has some limitations, including the drug interaction analysis, dose optimization and the sensitivity of evaluation of sensory and motor blocks. The use of static and dynamic methods for sensory and motor block assessments is recommended.

The Combination of QX-314 and Lidocaine at the minimum effective concentration of both anesthetics, significantly accelerate the onset of BP nerve block, improved the quality of forelimb analgesia and satisfactory separate the BP sensory and motor function of. Future studies should identify the optimal ratio concentration of QX/Lid and injection volume to improve clinical success. QX-314 alone had no significant effect on the rabbit's brachial plexus block, measured by the sensory and motor performance and show a rapid absorption rate from injection site in compare to combination form. No side effects were observed in rabbits that received QX-314 alone even at high doses.

Availability of Data and Materials

The datasets analyzed during the current study are available from the corresponding author (M. Ezzati Givi) on reasonable request.

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Author Contributions

ME and HI conceived and supervised the study. SE, ME and HI collected, and analyzed data. SE, ME and HI performed the anesthesia, brachial plexus function examinations and HPLC analysis. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

Conflicts of Interest

The authors declare no conflict of interest. The authors alone are responsible for the content and writing of paper.

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RESEARCH ARTICLE

Breeding Soundness Evaluation in Alpaca *(Vicugna pacos)* Males: A Long Retrospective Study of the Effects of Cystic Masses and Environmental Temperatures on Scrotal Measures [1]

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Abstract: Male alpacas have reproductive peculiarities that can influence the breeding soundness evaluation (BSE). This study was aimed to analyse the frequency of epididymal and testicular cystic lesions in alpacas *(Vicugna pacos)* retrospectively during different environmental temperatures. A total of 45 subject from 120 males were evaluated with linear probe (10 MHz; MyLab VetONE) between at 5 different alpaca farms in Italy. Semen was collected via artificial vagina and semen parameters were evaluated. Twenty % (9/45) of the males demonstrated the presence of abnormal testicular and epididymal cyst. Those cystic lesions could represent a simple result of drainage defect of the fluid produced (ectasia of the rete testis), congenitally derived from a probable hereditary pattern (2/9) or affecting epididymal structures (3/9). Cystic lesions can increase the scrotal volumetric asymmetry due to the most common mono-lateral lesions ($P<0.05$). Some diagnostic biochemical parameters such as the seminal plasma alkaline phosphatase was higher in affected males ($P<0.05$). Besides, environmental temperature can influence scrotal swelling independently by the presence of cystic lesions thus alter the scrotal volume. The ultrasonography can provide more specific information about the presence of epididymal and testicular anomalies influencing the BSE results.

Keywords: *Alpaca, Breeding soundness evaluation, Ectasia, Cystic degeneration, Epididymal cyst, Ultrasonography*

Alpaka *(Vicugna pacos)* Erkeklerinde Üreme Sağlamlığı Değerlendirmesi: Kistik Kitlelerin ve Çevresel Sıcaklıkların Skrotal Ölçümler Üzerindeki Etkilerinin Uzun Bir Retrospektif Çalışması

Öz: Bu çalışmanın amacı epididimal ve testiküler kistik lezyon frekanslarının, farklı ortam sıcaklıklarında retrospektif olarak incelenerek ortaya koyulmasıdır. İtalya'da bulunan 5 farklı işletmeden toplam 120 erkekten seçilen 45 birey USG lineer prob (10 MHz; MyLab VetONE) muayene edildi. Sperma örnekleri suni vajinayla alındı ve sperma (Visközite, renk, konsantrasyon ve motilite) parametreleri değerlendirildi. Erkeklerin %20'si (9/45) anormal testis ve epididimal kisti varlığı gösterdi. Bu kistik lezyonlar, üretilen sıvının drenaj defekti (rete testis ektazisi), konjenital bir kalıtsal patern (2/9) veya epididimal yapıları etkileyen (3/9) bir problem olarak değerlendirildi. Kistik yapılar, en sık görülen monolateral lezyonlar nedeniyle skrotal hacimi ile asimetriyi artırdığı belirlendi ($P<0.05$). Seminal plazmada bulunan bazı biyokimyasal tanı parametrelerden, alkalin fosfataz patolojik erkeklerde daha yüksek bulundu ($P<0.05$). Ayrıca, çevresel sıcaklık, kistik lezyonların varlığından bağımsız olarak skrotal ödemi etkileyebilmekte ve böylece skrotal hacmi değiştirebilmektedir. Sonuç olarak, ultrason, ÜSM sonuçlarını etkileyen epididimal ve testis anomalilerinin varlığı hakkında daha spesifik bilgi sağlayabilmektedir.

Anahtar sözcükler: *Alpaka, Üreme sağlamlığı değerlendirmesi, Ektazi, Kistik dejenerasyon, Epididimal kist, Ultrason*

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Introduction

Breeding soundness evaluation (BSE) is a standard protocol used to classify domestic animals breeders into five categories: exceptional, satisfactory, questionable, unsatisfactory and deferred. Despite a fair amount of research on alpaca male reproductive characteristics [1,2], there is a limited amount of information available on reference ranges of semen quality and productivity [3-5]. Today, ultrasonography (US) is routinely used as a collateral diagnostic approach in addition to the traditional visual assessment and trans- scrotal palpation to evaluate the male reproductive content. Among the abnormalities that can be detected by US, scrotal swelling (SS) and cystic masses (CM) are commonly observed. Scrotal swelling can have various causes, including transudative or exudative origins, and can represent a simple reduction of blood flow when CM are present at the testicular or epididymal level. This pathology has been reported in multiple species, including canines, bovines, equines, and humans [6-10].

Scrotal CM can be differentiated into three types: tubular ectasia of the rete testis (TERT), cystic degeneration of the rete testis (CD) and epididymal cysts (EC). Rete testis is an intra-testicular area that originates from the testicular hilum and contains numerous anechoic areas. The typical US image represents the enlargement of the numerous canaliculi of the rete testis. The exact cause of TERT is not yet fully understood, however, possible causes include mechanical obstruction, primary congenital deformity, ischaemic degeneration of the efferent ducts (from the rete testis to the epididymis) and hormonal mechanisms, particularly androgen deficiency. Pathogenesis wise, during embryogenesis, the mesonephric duct forms the efferent ducts, epididymis and vas deferens, whereas the germinal epithelium separately forms the rete testis and the testicular cord. The connection between the mesonephric duct and germinal epithelium occurs at the rete testis level and CD could be related to a disorder of the connection between the mesonephric duct and germinal epithelium. Cystic degeneration has ultrasonographic similarities with TERT, but it is a congenital deformity [11]. Interestingly, different studies reported scrotal cystic lesions and specific anatomical features indicating the unknown origins in South American Camelids [2,5,12,14]. Bott et al.[5] reported an incidence of cystic lesions of 18.5 % considering a total of 173 male alpacas scheduled for castration. Sumar [14], considered the importance of culling these males with CM lesions from the breeding plan, since it may have a hereditable predisposition. Hence, the integration of US as a collateral tool for breeding soundness evaluation is becoming essential, as it can give useful information about the quality in terms of breeder utility [14,15].

Therefore, our aim was to retrospectively analyse collected data from BSE in male alpacas, in addition to characterizing scrotal cystic lesions during scrotal ultrasonography under different environmental temperatures and assessing impact on scrotal measurements.

Material and Methods

Ethical Statement

This study was conducted during routine clinical visits to Alpaca di Marano in Piacenza, Antico Feudo in Prato, Poggio Piero in Grosseto, and Piani degli Alpaca in Viterbo, which are private farms. As such, the study did not require ethical permission, as it involved no additional interventions or procedures beyond what was necessary for the normal veterinary care provided to the animals. Moreover, as a European country, Italy has certain exemptions for animal experiments under the EU Directive 2010/63/EU, which includes procedures that are part of veterinary practice or routine monitoring of animal health. As a result, the study did not meet the criteria for a research study that requires ethical permission. The study was conducted in an ethical and responsible manner, with the protection of the welfare and rights of the animals as the top priority.

Animals

In the initial phase of our study, we conducted a retrospective analysis of ultrasonography (US) images collected from South American Camelids raised in Italy between 2010-2018. We carefully selected and evaluated individual data and US images of males to examine and classify the intra-scrotal anomalies observed. A total of 45 out of 120 males were selected for analysis in our study. These individuals were chosen due to their classification as breeders and the fact that they underwent a complete physical examination, including the use of scrotal ultrasonography as a collateral diagnostic tool.

In the second phase of the study, we analyzed data collected from a total of 9 males (6 normal, without lesions, and 3 abnormal, with lesions). These males were chosen due to their origin from a single farm and their completion of a full breeding soundness evaluation (BSE), including semen collection and evaluation, as well as collateral diagnostic exams (scrotal and accessory gland ultrasonography, and evaluation of seminal plasma biochemical composition). In our study, semen collection and ultrasonography (US) evaluation were conducted twice, each time under different environmental-seasonal temperatures. This was done as the first BSE was classified as deferred. The study was conducted on 5 farms, with 3 located in northern Italy and 2 in central Italy. All the farms presented a balanced diet consisting in *ad libitum* hay (Dry substance - DS - 63-91.71%, Fat 1.12-2.95% DS, Protein 6.61-14.91% DS,

Ash 8.21-11.43% DS, NDF 57.26-59.71% DS, ADF 32.98-38.32% DS), small amount (around 250 g) of concentrate during the breeding times (Protein 16.3%, Crude fiber 10%, Fat 2.7%, Ash 10.3, Vit. A 100000 UI, Vit. D_3 1000 UI, Vit. E 174 mg, Niacine 900 mg, Vit. B_1 43.6 mg, Vit. B_2 9.5 mg, Vit. B_6 32.4 mg, Vit. B_{12} 0.72 mg, Pantothenic acid 72 mg, Vit K 6 mg, Choline chloride 2.25 mg, Fe 73.6 mg, I 6 mg, Co 3.2 mg, Mn 120 mg, Zn 148 mg, Se 1.04 mg) and mixed pasture.

Scrotal Measurements

The length, width, and thickness of both testes were measured using calipers, testicular volume was calculated using the following formula [16]: Testicular volume: length x width x height x 0.5236.

Scrotal and Accessory Glands Ultrasonography

Ultrasonography images were collected using MyLab VetONE (Esaote®, Italy) with linear array probe (10 MHz; Gain max, Deepness 6 cm). The scrotal content was examined with sagittal and transversal sections of the testicular parenchyma and the appearance of the epididymal structures of 45 males. To collect the images of accessory glands, feces were removed manually, and the US probe was inserted and localized above the upper portion of the prostate and bulbourethral glands of 9 males. In our study, we used 10 MHz probe, changing only the deepness from 6 cm to 4 cm for testicle and accessory glands respectively. Each US image was classified depending on the normality or abnormality of testicular, epididymal and accessory glands structures. Abnormal findings were classified as SS, TERT and CD and EC.

Semen Collection

Nine adult males were studied to compare normal semen (n=6) to that of males with cystic lesions in the testicles (n=3). The males were abstinent from mating for at least two months prior to semen collection. Semen was collected twice from each male, under both high and low environmental temperatures, using a modified ovine artificial vagina in the presence of a teaser female [17]. The artificial vagina was equipped with a foam structure resembling a cervix and a glass tube for semen collection. The artificial vagina was heated to 38°C during semen collection and immediately afterward, the semen was placed in a water bath at 37°C. Standard semen parameters such as volume, consistency, concentration, and percentage of motile sperm were evaluated. The percentage of motile sperm was determined by using a microscope (Olympus CX-31) with a magnification of 400x. The samples were observed under a 18mm x 18mm coverslip on a warm glass slide maintained at 37°C. There is very little to no progressive motility in normal alpaca semen due to the high viscosity. Therefore, sperm motility, detected as an oscillatory motion of the flagellum, is given as the percentage of motile spermatozoa. Sperm concentration was measured using a Cell VU Sperm counting chamber, and motility was determined by the percentage of sperm displaying oscillatory motion. After analysis, the semen samples were centrifuged, and the seminal plasma was separated and stored at -20°C for further biochemical analysis. Environmental temperatures were recorded using a thermos-hygrometer. (Oregon Scientific THGR122NX).

Seminal Plasma Biochemistry

Biochemical analysis of the seminal plasma was conducted using an automatic analyzer and specific kits (912 Automatic Analyzer; Hitachi Boehringer Mannheim, Mannheim, Germany). The concentrations of alanine aminotransferase (ALT), g-glutamyl transferase (GGT), and alkaline phosphatase (ALP) were measured, as well as levels of glucose, total protein, triglycerides, cholesterol, phosphorus, and calcium.

Data Analysis

Reproductive ultrasound images were used to classify the males in the study. The first phase involved determining the frequency of pathological conditions affecting the male reproductive tract. In the second phase, data collected was analyzed using the GLM function in SIGMASTAT 2.03 software. A two-way ANOVA was performed, with testicular volume and semen quality parameters as dependent variables and the presence/absence of testicular dysplasia and seasonal changes in environmental temperature as independent variables. A P-value of less than 0.05 was considered statistically significant.

Results

A total of 9 out of the 45 males (20%) had cystic lesions in their scrotums. *Fig. 1*, *Fig. 2*, *Fig. 3* and *Fig. 4* show the ultrasound appearance of these cystic lesions. *Table 1* lists the age of the subjects and the type of lesions they had. Epididymal cysts, accounting for 6.67% of scrotal lesions, were found in males of different ages (2.5, 3.5, and 4 years) and could occur as either a single (1/3) or bilateral (2/3) lesion.

Cystic degeneration and TERT together accounted for 13.33% of the frequency of cystic masses at the scrotal level. The classification of cystic degeneration instead of TERT was considered in two juvenile cases (case 1 and 2) because their conditions were like that of their father (case 8). Testicular cystic masses were classified as cystic degeneration/TERT when the congenital origins were not known. *Table 2* shows the variability of the parameters during the two collection times during semen collection.

All adult males used as breeders (3 out of 9) had a

Table 1. *List of scrotal pathological conditions in Alpaca males*

Male ID	Alpaca	Age	Type	Figure
1	Huacaya	1.5	Cystic degeneration RT	1-A
2	Huacaya	1.5	Cystic degeneration RT	1-B
3	Huacaya	2.5	Tubular Ectasia/Cystic degeneration RT	2-A
4	Huacaya	3	Tubular Ectasia/Cystic degeneration RT	2-B
5	Huacaya	2.5	Epididymal cyst	3-A
6	Huacaya	3	Epididymal cyst	3 B
7	Huacaya	4	Epididymal cyst	3 C
8	Huacaya	6	Tubular Ectasia/Cystic degeneration RT	4 A
9	Huacaya	10	Tubular Ectasia/Cystic degeneration RT	4 B

Table 2. *Value of the parameters evaluated (normal vs pathological) during low and high environmental temperature*

Parameter	Normal (12)		Abnormal (6)	
	Low Temp.	High Temp.	Low Temp.	High Temp.
Age (year)	7.5 (5.56-10.58)	7.91 (4.84-13.6)	9.68 (6.40-12.08)	8.92 (5.61-11.37)
BCS	3.2±0.13	3.13±0.17	3±0.25	2.83±0.08
Right Testicular Volume	7.23±1.08[a]	16.69±2.28[b]	10.36±2.08[a]	25.48±4.53[a]
Left Testicular Volume	9.45±1.77[a]	18.38±2.39[b]	11.68±1.73[a]	25.45±0.47[b]
Volume (ml)	1.78±0.52	1.10±0.28	1.77±0.14	1±0.5
Viscosity	2	1.67±0.21	2.67±0.33	2.33±0.66
Colour	1.75±0.2	2.33±0.33	2.67±0.66	2.00
Concentration ($1x10^6$/ml)	46.17±2.08[a]	7.67±2.39[b]	120.83±15.02[a]	43.33±21.86[b]
Motility (%)	20	16.67±9.19	50.00±5.77	46.33±21.85

Different number of asterisks indicates a significant difference between low and high temperature within normal and pathological conditions [a,b]*= P<0.05*

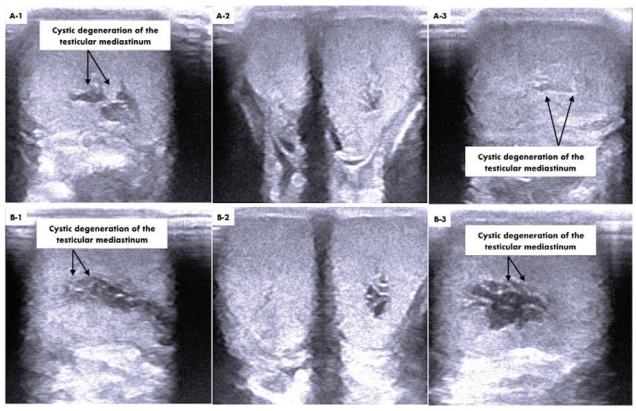

Fig 1. Juvenile mono-lateral cystic degeneration of the rete testis. Case 1 and 2 are classified as juvenile origin due to brotherhood, also being male offspring of case 8. Case no 1. A-1 Longitudinal left section; A-2 Transversal section; A-3 Longitudinal right section. Case no 2. B-1 Longitudinal left section; B-2 Transversal section; B-3 Longitudinal right section

unilateral, intra-testicular lesion. The presence of cystic masses affected the testicular volume, reducing it by around 40% *(Table 3)*. Most of the lesions causing this decrease in volume were located on the left testicle. There was a significant difference in environmental temperature between seasons, ranging from 22°C (15-30°C) in the summer to 12°C (7-16°C) in the spring. The testicular volume was more affected by the hot environment, with a 130% and 145% increase for normal and pathological cases, respectively *(Table 3)*.

Sperm concentration was found to be affected by temperature, regardless of the presence of cystic masses. High temperatures caused a decrease of around 80% in sperm concentration *(Table 2)*. Some seminal plasma parameters also varied between seasons, with an increase observed during high temperatures.

Table 3. *Variability of the parameters considered during BSE of alpaca males with normal and pathological intra-scrotal contents*

Parameter	Normal (12)	Abnormal (6)	Average Value
Age (year)	7.73 (4.84-13.60)	9.30 (5.61-12.08)	8.28 (4.84-13.60)
BCS	3.16±0.36	2.92±0.3	3.07±0.35
Right Testicular Volume	12.39±6.55	17.92±9.92	14.34±8.06
Left Testicular Volume	14.32±6.82	18.56±7.79	15.82±7.24
Testosteronemia (ng/mL)	205.50±116.67	178.50±7.78	192.0±69.29
Volume (mL)	1.37±0.97	1.38±0.71	1.38±0.86
Viscosity	1.80±0.42	2.50±0.84	2.06±0.68
Colour	2.1±0.74	2.33±0.82	2.19±0.75
Concentration (1x10^6/mL)	26.92±40.36	64.50±63.87	45.71±54.59
Motility (%)	17.14±20.59	46.67±25.03	30.77±26.6

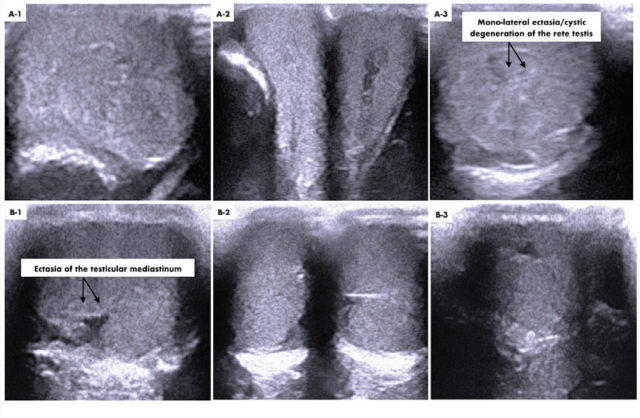

Fig 2. Mono-lateral ectasia-cystic degeneration of the rete testis. Case no 3. A-1 Longitudinal left section; A-2 Transversal section; A-3 Longitudinal right section. Case no 4. B-1 Longitudinal left section; B-2 Transversal section; B-3 Longitudinal right section

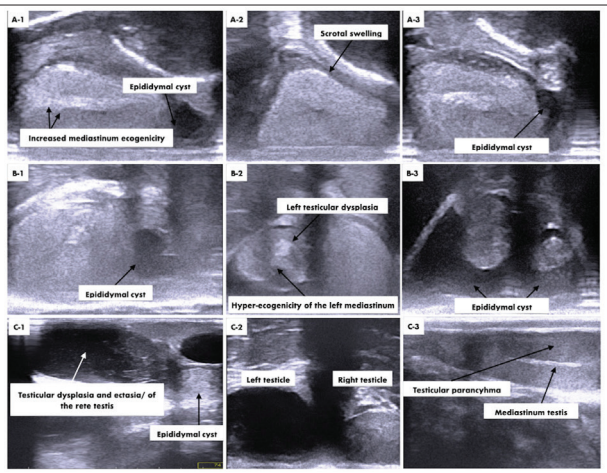

Fig 3. Bilateral epididymal cyst, scrotal swelling and increased echogenicity of left testicular mediastinum. Case no 5. A-1 Longitudinal left testicular and epididymal section; A-2 Transversal section; A-3 Longitudinal right testicular and epididymal section. Case no 6. B-1 Longitudinal left testicular and epididymal section; B-2; Transversal section; B-3 Transversal section of emi-scrotal areas. Case no 7. Mono-lateral epididymal cyst and extreme testicular dysplasia and ectasia of rete testis. C-1 Longitudinal left testicular and epididymal section; C-2 Transversal scrotal section; C-3 Longitudinal right testicular and epididymal section

Fig. 5 reports the US appearance of the accessory glands (prostate and bulbourethral glands) which is not influenced by the seasons. *Fig. 6* is clearly showing the presence of scrotal swelling (hydrocele) during the hot season.

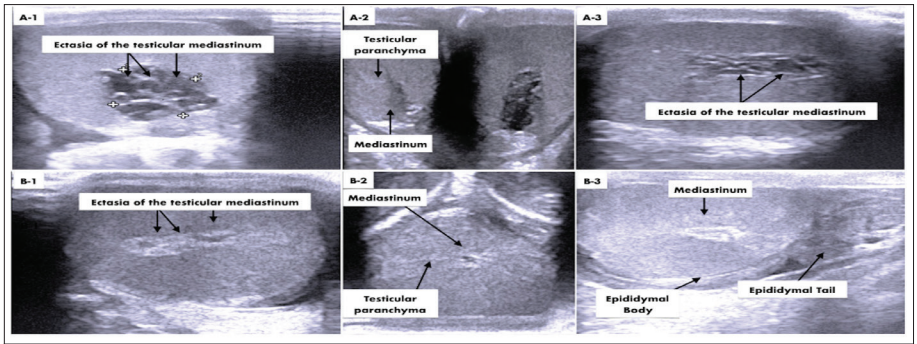

Fig 4. Mono-lateral epididymal cyst and extreme testicular dysplasia and ectasia of the rete testis. Case no 8. Adult male father of the cases 1 and 2. A-1 Transversal scrotal section; A-2 Transversal left testis advanced; A-3 Longitudinal left testicular section. Case no 9 Bilateral ectasia/cystic degeneration of the rete testis; B-1 Longitudinal right testicular section; B-2 Transversal right testicular section; B-3 Longitudinal testicular and epididymal left section

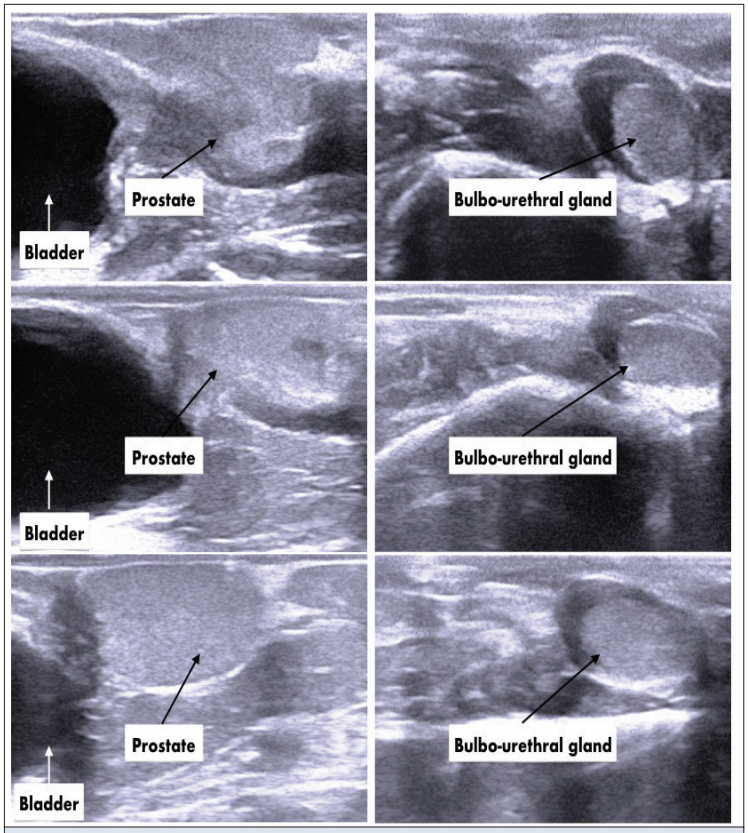

Fig 5. Accessory glands of male alpacas

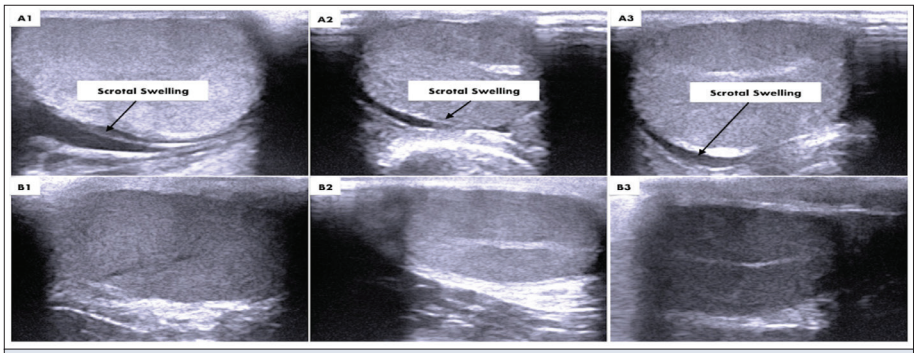

Fig 6. Scrotal swelling in alpacas

Seminal alkaline phosphatase (ALP) was found to be the most indicative parameter, showing a 140% increase in level between normal and pathological cases and a 244% (normal) to 520% (pathological) increase between seasons *(Table 4)*. The levels of other seminal plasma components were also reported in *Table 4*.

Discussion

Breeding soundness evaluation is an essential protocol to classify breeder males. It is based on physical measurements such as scrotal circumference or testicular diameters. However, in alpaca males, obtaining these measurements can be difficult due to their species-specific position of the scrotum and a high percentage of cystic masses. In the past, there was a general agreement on the relationship between testicular size and productivity. However, in males affected by cystic masses, this relationship may not hold as the testicular volume may be higher due to the cystic masses, even if the productive tissue is partially absent. Therefore, ultrasonography measurements are considered the most appropriate approach to determining testicular volume in these cases.

The most significant finding of our study is the higher sperm concentration and motility in individuals diagnosed with reproductive anomalies such as tubular ectasia of the rete testis (TERT), cystic degeneration of the rete testis (CD), and epididymal cysts, compared to

Table 4. *Seminal plasma biochemical parameters evaluated (normal vs pathological) during low and high environmental temperature*

Parameter	Normal (12)		Abnormal (6)		Average
	Low Temp.	High Temp.	Low Temp.	High Temp.	
Glucose (mg/dL)	0.85± 0.37	0.85±0.17	1.74± 0.71[a]	0.26±0.14[b]	1.02±0.28
Cholesterol (mg/dL)	2.24± 0.96	1.11±0.43	2.75± 1.68	2.97±1.41	1.96±0.63
Triglycerides (mg/dL)	14.8± 3.99	29.72±13.39	14.78± 4.95	41.38±16.68	23.23±7.53
Total Protein (mg/dL)	1.94± 0.6	4.3±1.98	3.91± 1.09	6.91±1.02	3.83±1.13
GGT (UI/L)	32.6±20.68	122.2±53.7	141.6±52.25	247.1±90.6	115.11±36.38
ALP (UI/L)	67.9±43.6[a]	234.2±128.7[a]	184.1±101.8[a]	1142.2±336.1[b]	255.83±118.60
Ca (mg/dL)	11.55± 0.91	10.26±2.77	16.3±2.36	13.39±1.54	12.38±1.74
P (mg/dL)	0.28± 0.05	0.71±0.31	0.33±0.04	0.63±0.06	0.50±0.17
Mg (mg/dL)	4.45± 0.52	2.99±0.44	4.15±0.56	2.54±0.58	3.61±0.4
Creatinine	0.72± 0.30	0.59±0.17	0.49±0.03	0.36±0.04	0.58±0.14
Urea	39.8±12.2	40.59±4.70	47.67±6.92	45.97±5.41	42.62±5.27

Different number of asterisks indicates a significant difference between low and high temperature within normal and pathological conditions [a,b] *= P<0.05*

those classified as normal. This increase is believed to be caused by an enlarged scrotal volume and obstructed sperm ducts. However, these anomalies also result in higher sperm viscosity. Although these conditions are not detectable by macroscopic or manual examination, they can be observed through ultrasound. We therefore suggest that alpaca males undergo scrotal and testicular examination with ultrasound as part of their reproductive health assessment. It should be noted that the standard deviation among sperm findings in this study was substantial, due to the unique biology of alpaca males during sperm retrieval, handling, viscosity, semen analysis, and evaluation method. The consirable variations in the results are consistent with those from other studies [18,19], with an average ejaculate volume of 1.8±0.8 mL and sperm concentration of 17.6±26.1x10^6 sperm/mL reported by Flores et al.[20] without evaluating sperm motility. It has been reported that repeated sperm retrieval reduces these standard deviations [21].

In veterinary practice, B-mode ultrasonography is the most used method, and it is easily performed with minimal restraint or discomfort for the animal. It provides highly sensitive information using a 5-7.5 MHz probe, with up to 100% accuracy in diagnosing hydroceles, haematoceles, and para-testicular masses, but less informative for testicular abscesses or epididymo-orchitis. Higher MHz probes (7-10 MHz) with or without color flow Doppler are the preferred probes for evaluating the testicles, epididymal structures, and spermatic cords. The study found an increased testicular volume during the summer collection, which was independent of the presence of cystic masses at different levels. This increase in volume during the summer months could be due to local circulatory impairment because of a general thermoregulatory response during the hot season. The cystic masses were reported as unilateral in most cases, which contributed to the testicular asymmetry. The seminal plasma biochemical composition indicated a testicular/epididymal response to elevated environmental temperatures.

In addition to the absence of a relationship between testicular volume and cystic masses, the study found that the effect of environmental temperature on the thermoregulatory response was evident with a decrease in semen quality and biochemical composition of the seminal plasma. Spontaneous recovery of simple testicular cysts has been reported only in humans [22]. In the study population, almost all the cases were unilateral and had a probable degree of heritability based on congenital cystic degeneration in two half-brothers. Epididymal cysts in alpacas have been reported by other authors [3,13], and little is known about their persistence or natural regression, as reported for humans [22-24]. These lesions are usually visible unilaterally. The cystic transformation of the rete testis is often associated with ipsilateral renal agenesis or other renal dysplasia. Congenital epididymal cysts (EC) are the most frequently observed anomaly of the male internal genitalia of Wolffian origin. However, traumatic, and inflammatory causes are also possible. The efferent ducts are part of the head of the epididymis because of their embryological origin from mesonephric/Wolffian structures. In the study population of alpaca males, one case had an EC lesion that increased the influence on the rete testis and a derived TERT for 18 months without showing any distress due to the enlarged testicle (case 8). To the best of our knowledge, this study reported the highest incidence of cystic masses compared to previously published percentages [5], and even though the study did not include histological investigations as the

animals were not scheduled for castration, it aimed to identify the effects of cystic masses on testicular/scrotal functionality and breeding soundness evaluation results. However, there is an interesting report by Barrios et al.[25], and reproductive anomalies such as small testicles, uni- and bilateral epididymal cysts were found in 102 out of 177 alpaca males in a slaughterhouse evaluation in 2011, which has higher rates than our study.

During the second phase of the study, data regarding sperm motility was found to range between 5% and 80%, with high levels of variation among the males, and mean motility was found to be quite low (35%). The mean sperm motility in this study was within the range reported for alpaca semen collected using an artificial vagina (15.3% - 63.7%) in previous studies [21, 27]. Additionally, only oscillatory motility was observed in the collected samples, which agrees with previous studies that also described the oscillatory motion [26-30]. The study found that semen concentration was negatively influenced by high environmental temperature, with a decrease in values between seasonal changes. The increased testicular volume and decreased semen concentration seem to be linked by a pathophysiological cause, such as an impairment of the thermoregulatory vascularization of the scrotal content. Semen volume and concentration have shown clear tendencies, identifying, independently of pathological conditions, the negative effect of seasonal high temperature.

The most interesting biochemical parameter in the study was seminal plasma alkaline phosphatase (ALP). The value of this enzyme in seminal plasma is an important indicator of membrane damage and changes in membrane function resulting from increased sperm production [17, 26]. ALP is also an important parameter for testing the health and functionality of accessory glands. In azoospermic canine and equine ejaculates, high ALP levels can indicate a testicular origin of azoospermia, while low ALP levels may correspond to bilateral outflow obstruction [31,32]. In a study conducted on bulls, it was observed that ALP enzyme levels decreased in chronic efferent duct obstruction that causes ectasia in the epididymal, ampullary canal, and ductus deferens. In the study, the ALP value measured in the seminal plasma of animals with cystic lesions was 140% higher than in healthy animals [15]. This value was also affected by high temperature, with an increase of 244% in healthy animals and 520% in animals with cystic lesions.

Cystic masses in Alpaca are very common compared to other species. The presence of these lesions may influence testicular measurement and breeding soundness evaluation. Differential diagnosis between cystic dysplasia of the rete testis and simple ectasia should be further investigated to exclude carriers from breeding programs. More research is needed to identify the hereditary origin and any potential ipsilateral renal problems, making this species a potential animal model for human pathology. Breeding soundness evaluation in alpaca should be carried out during periods when environmental temperature is not high, as high temperatures can influence scrotal swelling and give false information on testicular dimensions. Furthermore, the use of ultrasound is essential to rule out the presence of cystic masses and to calculate the correct parenchymal testicular volume.

Availability of Data and Materials

The data presented in this study are available on request from the corresponding author (K. Tekin). The data are not publicly available due to the agreement with funding bodies.

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Conflict of Interest

The authors declare no conflict of interest.

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Research Article

Evaluating the Effect of Drinking Saline Water on Fermentation Kinetics, Methane Production and Nutritional Value of Alfalfa Hay and Barley Grain Using *In Vitro* Gas Production Technique in Sheep

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Abstract: The aim of this study was to determine the effect of drinking saline water on fermentation kinetics, methane emission and nutritional value of alfalfa hay (AH) and barley grain (BG) using *in vitro* gas production technique in sheep**.** Rumen liquor collected from eight rumen cannulated Shal rams, which had received different levels of saline water as four treatment containing 480, 4000, 8000 and 12000 ppm total dissolved solids (TDS). The results showed that there were significant differences between the experimental treatments in terms of the amount of methane produced as well as total gas production and relevant parameters ($P<0.05$). The lowest amount of methane production in AH and BG was observed at the treatment containing 4000 ppm TDS. The treatment containing 12000 ppm TDS, had the highest amount of gas production in AH at the most of incubation times. Short chain fatty acids (SCFA), digestible organic matter (DOM), metabolisable energy (ME), net energy for lactation (NE_L) of AH and BG significantly differ between treatments ($P<0.05$), with the highest amount at the highest salinity level. In a general conclusion, drinking water salinity seems to affect fermentation kinetics and nutritive value of AH and BG depending on the level of salinity and the type of feedstuffs.

Keywords: *Fermentation, Gas production, Methane emission, Sheep, Water salinity*

Koyunlarda *In Vitro* Gaz Üretim Tekniği Kullanılarak Tuzlu Su İçiminin Yonca Kuru Otunun ve Arpa Tanesinin Fermantasyon Kinetiği, Metan Üretimi ve Besin Değeri Üzerine Etkisinin Değerlendirilmesi

Öz: Bu çalışmanın amacı, koyunlarda *in vitro* gaz üretim tekniğini kullanarak tuzlu su içmenin, yonca kuru otu (AH) ve arpa tanesinin (BG) fermantasyon kinetiği, metan emisyonu ve besin değeri üzerine etkisini belirlemektir. 480, 4000, 8000 ve 12000 ppm toplam çözünmüş katı madde (TDS) konsantreli tuzlu su verilerek oluşturulan 4 sağaltım grubuna ait 8 adet rumen kanüllü Shal koçundan rumen sıvı örnekleri toplandı. Bulgular, üretilen metan miktarının yanı sıra toplam gaz üretimi ve ilgili parametreler açısından çalışma grupları arasında önemli farklılıklar olduğunu gösterdi ($P<0.05$). AH ve BG'de en düşük metan üretim miktarı 4000 ppm TDS içeren grupta gözlendi. 12000 ppm TDS içeren grupta, inkübasyon sürelerinin çoğunda AH'de en yüksek gaz üretimi gerçekleşti. AH ve BG'nin kısa zincirli yağ asitleri (SCFA), sindirilebilir organik madde (DOM), metabolize edilebilir enerji (ME), laktasyon için net enerji (NEL) değerleri, en yüksek tuz seviyesine sahip grupta en yüksek olmak üzere, gruplar arasında önemli ölçüde farklılık gösterdi ($P<0.05$). Sonuç olarak, içme suyunun tuzluluğu, tuzluluk seviyesine ve yem maddelerinin türüne bağlı olarak AH ve BG'nin fermantasyon kinetiğini ve besleyici değerini etkiliyor görünmektedir.

Anahtar Sözcükler: *Fermantasyon, Gaz üretimi, Metan emisyonu, Koyun, Su tuzluluğu*

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Introduction

Due to climate change worldwide, the incidence of water scarcity and drought will increase in many regions, especially in arid and semi-arid regions [1,2]. Climate changes are reflected in heating and rainfall reduction, which successively may increase the salinity of both soil and water [3]. Saline water available in these areas may contain high concentrations of total dissolved solid (TDS), sometimes reaching levels above 30000 ppm TDS. High water salinity can have certain consequences on the animals. Sheep were reported to tolerate saline drinking water containing up to 1.3% sodium chloride without ill effects [4]. Tolerance of animals to salinity varies based on their water requirements, species, age, physiological condition, besides of time of the year, and salt content in the total diet [5,6]. Excessive level of salts may counteract one another at higher concentrations lead to limiting their availability for rumen microorganisms. As a result, the microbial activities as well as nutrients utilization may shift [4]. McGregor [7] reported that the period needed for animals to adapt to high salinity is still ambiguous. The rumen consists of complex anaerobic microbial populations such as methanogens which constitute 10^8–10^9/mL. Ruminants lose about 2-15% of their ingested energy solely as methane. Methane comprises between 20 and 30% of total gases produced within the rumen [8]. The production of methane gas in the rumen depends on factors such as pH, SCFA, diet, animal species and environmental conditions. The increased rumen passage rate due to increase osmotic pressure may reduce methane emission [8].

The *in vitro gas* production method is a useful technique for feed evaluation, which is cost effective, fast and easy to determine and suitable for use in developing countries. This method also can predict fermentation kinetics, microbial nitrogen supply, and amount of short chain fatty acids, carbon dioxide, methane production and metabolisable energy as well as organic matter digestibility of feeds for ruminants [9].

In general, water quantity and quality have a significant effect on rumen performance, and research on the effects of saline water on rumen fermentation has been neglected. Thus, the aim of this study was to determine the effect of drinking saline water on fermentation kinetics, methane emission and nutritional value of alfalfa hay (AH) and barley grain (BG) using *in vitro* gas production technique in sheep.

Material and Methods

Animals and Management

This experiment was carried out at the Animal Science Research Institute, Agricultural Education, and Extension Research Organization, Karaj, Iran; according to the "*Guide to the Care and Use of Experimental Animals" prepared by Iranian Council of Animal Care, Isfahan University of Technology, Isfahan.* Eight adult fistulated Shal rams with an initial body weight (BW) of 76±2.5 kg were used in this study. Ten days preliminary period was allowed for adaptation, feeds were offered twice daily at 8:00 and 16:00 h at a rate of 10% higher than the maintenance limit according to the standard tables of the NRC [10], and the salt-free diet was included 70% forage (alfalfa hay and wheat straw), and 30% concentrate (barley grain, soybean meal and cottonseed, mineral and vitamin supplements).

Treatments

Control group was consumed fresh water and other treatment groups contain, 3.5, 7.5, 11.5 g of salt per liter which was equal to 480 ppm total dissolved solids (TDS) for control treatment and 4000, 8000, 12000 ppm TDS for 2nd, 3rd, and 4th treatments, respectively. All rams had free access to drinking water according to their treatment. The electrical conductivity (EC) of these treatments was measured by the EC meter in the Chemical Laboratory, Institute of Animal Sciences. The value of TDS using EC data was calculated by the equation TDS = 640 * EC where TDS with ppm unit and EC with ds/m unit [5]. Chemical contents of the water showed in *Table 1*.

Table 1. *Chemical components of the fresh water (control)*

Component	Value
Na (mg/L)	119
Ca (mg/L)	39
Mg (mg/L)	7.9
Cl (mg/L)	35
SO_4 (mg/L)	116
HCO_3 (mg/L)	241
TDS (ppm)	480

TDS: total dissolved solids

Chemical Analysis

Chemical composition including dry matter (DM), ether extract (EE), crude protein (CP) and crude ash (CA) content of AH and BG were determined according to AOAC [11]. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were measured by procedures proposed by Van Soest et al.[12]. The non-fibrous carbohydrates (%NFC = 100 – (%NDF + %CP + %EE + %CA) were calculated as proposed by NRC [13].

In vitro Gas Production

Rumen fluid obtained from fistulated Shal rams before morning feeding when animals well adapted to drinking saline water (after 10 days adaptation period). Approximately 200 mg samples of dry feedstuff were weighed in triplicate and placed in a 100 mLcalibrated glass syringe. Feeds samples were incubated *in vitro* with rumen fluid-buffer mixture (30 mL) was transferred into the glass syringes of 100 mL according to the method of Menke and Steingass [14]. The samples were incubated in 100 mL syringe in a shacking incubator at 39°C. Volume of gas production was recorded at 2, 4, 6, 8, 12, 24, 48, 72 and 96 h of incubation times and corrected for blank. In order to measuring methane (CH_4) production, after reading the syringes at the time of 24 h incubation, 4 ml of NaOH (10 M) was added to syringes and after 10 minutes, the said syringes were read again and removed. The NaOH (10 M) was introduced from the latter into incubated contents, thereby avoiding gas escape. Mixing of content with NaOH solution allowed for the absorption of CO_2, with the gas volume remaining within the syringe considered to be CH_4 [15].

Equations, Calculations and Statistical Analyses

Net gas production data were fitted to the model outlined by Ørskov and McDonald [16] and gas production parameters were estimated by the Fitcurve software version 6:

$P = A\ (1\text{-}e^{-ct})$; Where, A = potential gas production, c = the gas production rate constant for the insoluble fraction (b), t = the incubation time (h), P = the gas production at the time t

The digestible organic matter (DOM), net energy for lactation (NE_L) and metabolisable energy (ME) for tested feedstuffs were estimated using equations of Menke and Steingass [14], and short chain fatty acids (SCFA) was estimated using equation of Makkar [17].

Equations Used for Alfalfa Hay

DOM (%) = 0.889 GP + 0.45 CP + 0.651 CA + 14.88

ME (MJ/kg DM) = 0.136 GP + 0.057 CP + 0.00286 EE^2 + 2.2

NEL (MJ/kg DM) = 0.096 GP + 0.038 CP + 0.00173 EE^2 + 0.54

SCFA (mmol) = 0.0222 GP – 0.00425.

Equations Used for Barley Grain

DOM (%) = 0.9991 GP + 0.595 CP + 0.181CA +9

ME (MJ/Kg DM) = 0.157 GP + 0.084 CP + 0.22 EE – 0.081 CA + 1.06

NE_L (MJ/Kg DM) = 0.115 GP + 0.054 CP + 0.14 EE – 0.054 CA – 0.36

SCFA (mmol) = 0.0222 GP –0.00425

Where, GP was gas production volume at 24 h of incubation time (mL/200 mg DM). General linear model (GLM) procedure of SAS [18] software was used in order to statistical analysis of data from gas production. The experiment and statistical analysis designed and performed based on complete randomized design (CRD) with four treatment and three replicates for each treatment. Treatment means was compared by Duncan multiple range tests.

Results

The chemical composition of AH and BG is given in *Table 2*. The gas production in experimental treatments at the different times of incubation of AH and BG showed in *Table 3*. There are significant differences between salinity levels and control treatment on AH and BG regarding gas production ($P<0.05$). Also, according to the results, the highest amount of AH gas production in most of the incubation times was observed in the 12000 ppm TDS treatment and the lowest in the treatment containing 4000 ppm TDS compared to the control treatment. But also, BG gas production was decreased at incubation times of 2, 4, 6, 8 and 96 in the treatments containing different levels of salinity water compared to the control treatment. The estimated kinetic parameters by exponential model are presented in *Table 4*. The significant difference was observed in A of AH ($P<0.05$). So that the highest amount of A was observe in the treatment containing 12000 ppm TDS, and the lowest amount was, in 4000 ppm TDS, but BG does not have significant differences between treatments. Significant difference was observed in c parameter of AH and BG ($P<0.05$). So that in AH, treatment containing 4000 ppm TDS has more than other groups in this fraction, and c of BG was significantly highest in the treatment containing 12000 ppm TDS and lowest in 8000 ppm salinity levels compared to the control treatment. The amounts of methane production from AH and BG under salinity levels and fresh water showed in *Table 4*. Different levels of salinity significantly affected methane emission ($P<0.05$). So that lowest methane emission of AH and BG was observed in the treatment containing

Table 2. *Chemical composition of alfalfa hay and barley grain (%)*

Constituents	Alfalfa Hay (AH)	Barley Grain (BG)
DM	93.1	92.5
CP	16.7	13.3
EE	1.1	1.8
Ash	10.1	2.7
NDF	37.0	16.5
ADF	26.5	6.7
NFC	35.1	65.5

DM: dry matter, CP: crude protein, EE: ether extract, NDF: neutral detergent fiber, ADF: acid detergent fiber, NFC: non-fibrous carbohydrates

Table 3. *Effects of drinking water salinity (TDS as ppm) on gas production volume (mL/200 mg DM) at different incubation times*

Feedstuffs	Salinity Level	Incubation Time (h)								
		2	4	6	8	12	24	48	72	96
Alfalfa hay (AH)	Con. (480)	11.2±0.01	19.4±0.28^{a}	26.8±0.58^{a}	32.9±0.76^{a}	38.5±1.00^{a}	45.5±1.15^{b}	57.0±1.15^{b}	61.3±1.15^{b}	62.3±1.15^{b}
	4000	8.5±0.50^{c}	15.5±0.75^{b}	22.7±1.00^{c}	28.5±0.75^{c}	35.5±0.75^{b}	43.7±1.25^{b}	52.2±1.75^{d}	52.7±2.25^{d}	53.5±2.50^{d}
	8000	9.3±1.00^{c}	16.4±0.50^{b}	22.0±1.00^{c}	27.5±1.00^{c}	34.4±1.00^{b}	44.8±1.00^{b}	53.5±0.75^{c}	56.2±1.25^{c}	57.2±1.25^{c}
	12000	13.0±0.22^{a}	18.7±0.25^{a}	24.6±0.37^{b}	30.0±0.23^{b}	37.90±0.35^{a}	50.6±0.60^{a}	61.10±1.10^{a}	64.9^{a}±1.35	65.9^{a}±1.35
	SEM	0.032	0.282	0.454	0.428	0.473	0.596	0.717	0.903	0.957
	P- value	<0.0001	<0.0001	0.0003	<0.0001	0.0008	0.0002	<0.0001	<0.0001	<0.0001
Barley grain (BG)	Con. (480)	9.7±0.50^{a}	21.2±0.01^{a}	35.5±0.01^{a}	45.2±0.01^{a}	54.5±0.01^{a}	68.7±0.50^{ab}	85.2±1.5	92.0±2.00	95.0±1.00^{a}
	4000	6.7±0.2^{b}	15.2±0.03^{b}	27.2±0.50^{bc}	39.2±0.50^{b}	53.2±0.50^{a}	70.5±1.00^{a}	85.5±1.00	91.2±0.75	94.2±0.75^{a}
	8000	6.8±0.50^{b}	15.1±0.25^{b}	26.0±0.0^{c}	37.0±1.00^{c}	48.9±0.50^{b}	67.6±0.63^{b}	83.1±0.63	89.7±0.75	92.0±0.50^{b}
	12000	8.0±1.75^{ab}	15.5±1.5^{b}	28.5±1.50^{b}	41.0±1.75^{b}	53.5±1.75^{a}	70.5±1.25^{a}	84.2±0.5	89.2±0.50	90.3±0.45^{c}
	SEM	0.550	0.439	0.457	0.600	0.545	0.517	0.570	0.670	0.410
	P- value	0.01	<0.0001	<0.0001	<0.0001	0.0004	0.01	0.07	0.06	0.0002

a-d: *Means within a column with different subscripts differ (P<0.05),* ***Con:*** *control group, 4000: 4000 ppm salinity water, 8000:8000 ppm salinity water, 12000: 12000 ppm salinity water,* ***SEM:*** *Standard error mean*

Table 4. *Effects of different levels of drinking water salinity (TDS as ppm) on gas production parameters and methane production*

Feedstuffs	Salinity Level	A	c	CH_4 (%)	CH_4 (mL/200 mg)	CH_4 (mL/g DM)	CH_4 (mL/g OM)
Alfalfa hay (AH)	Con. (480)	60.6±0.49^{b}	0.0682±0.003^{b}	16.3±1.27^{a}	9.0±0.50^{a}	45.0±2.5^{a}	50.0±2.78^{a}
	4000	52.0±1.05^{d}	0.0912±0.005^{a}	9.8±1.65^{b}	4.3±1.04^{b}	21.6±5.20^{b}	24.0±5.78^{b}
	8000	56.2±0.90^{c}	0.0709±0.001^{b}	15.8±2.23^{a}	8.6±1.52^{a}	43.3±7.63^{a}	48.1±8.48^{a}
	12000	65.3±0.35^{a}	0.0604±0.002^{c}	14.7±2.91^{a}	7.1±1.75^{a}	35.8±8.78^{a}	39.8±9.75^{a}
	SEM	0.891	0.0017	1.219	0.750	3.754	4.171
	P- value	<0.0001	<0.0001	0.019	0.008	0.008	0.008
Barley grain (BG)	Con. (480)	91.3±0.88	0.0714±0.002^{b}	14.7±1.36^{a}	9.5±1.32^{b}	47.5±6.61^{b}	51.0±7.11^{b}
	4000	91.3±0.25	0.0721±0.003^{b}	9.6±2.90^{b}	5.3±1.53^{c}	26.6±7.64^{c}	28.6±8.21^{c}
	8000	89.9±0.85	0.0652±0.001^{c}	16.3±0.85^{a}	14.3±1.04^{a}	71.6±5.20^{a}	77.0±5.60^{a}
	12000	90.7±1.35	0.0781±0.001^{a}	15.6±0.66^{a}	11.6±0.58^{b}	58.3±2.89^{b}	62.7±3.10^{b}
	SEM	1.13	0.0013	0.975	0.677	3.38	3.64
	P -value	0.81	0.0009	0.005	<0.0001	<0.0001	<0.0001

a,b,c: *Means within a column with different subscripts differ (P<0.05);* ***Con:*** *control group (480 ppm), 4000: 4000 ppm salinity water, 8000:8000 ppm salinity water, 12000: 12000 ppm salinity water;* ***A:*** *potential of gas production,* ***c:*** *the gas production rate constant for the insoluble fraction,* ***CH_4:*** *Methane emission;* ***SEM:*** *Standard error mean*

4000 ppm TDS. The predicted SCFA, DOM, ME and NE_L are presented in *Table 5*. Significant differences were observed in AH between salinity levels and the control treatment (P<0.05). So that the highest amount of these factors in AH observed in 12000 ppm TDS and also in BG do not have significantly difference between salinity levels and control treatment, but there was significantly difference among of salinity levels.

Discussion

The chemical composition of the tested AH and BG in most cases are within the range of several studies [19-22]. Our results demonstrate that the gas production of AH and BG at different incubation times in a control treatment were in the range of previous reports [19,23-25]. The gas production parameters (*A* and *c*) for AH and BG in the control treatment was in range of the previous researches [19,20,23,26,27]. The SCFA in the control treatment of AH were higher than that of findings of Safaei et al.[20] and in agreement with Aghajanzadeh-Golshani et al.[19]. Values of SCFA production for BG (control treatment) in the present study were in the range of previous finding [25,27]. The amount of ME, NE_L and DOM of AH and BG in the control treatment were in the range of some other reports [20,24,26,27]. Regarding experimental treatment with different salinity levels, total gas production, SCFA,

Table 5. *Effects of different levels of drinking water salinity (TDS as ppm) on the amount of digestible organic matter (DOM), net energy for lactation (NEL) and metabolisable energy (ME) and short chain fatty acids (SCFA)*

Feedstuffs	Salinity Level	SCFA	DOM	ME	NE_L
Alfalfa hay (AH)	Con. (480)	1.00±0.03^{b}	69.5±1.03^{b}	9.3±0.16^{b}	5.5±0.11^{b}
	4000	0.96±0.03^{b}	67.8±1.11^{b}	9.1±0.17^{b}	5.3±0.12^{b}
	8000	0.99±0.02^{b}	68.8±0.89^{b}	9.2±0.14^{b}	5.4±0.10^{b}
	12000	1.12±0.0 1^{a}	74.0±0.53^{a}	10.0±0.08^{a}	6.0±0.06^{a}
	SEM	0.013	0.530	0.081	0.057
	P-value	0.0002	0.0002	0.0002	0.0002
Barley grain (BG)	Con. (480)	1.52±0.01^{ab}	86.1±0.50^{ab}	13.0±0.08^{ab}	8.3±0.06^{ab}
	4000	1.57±0.02^{a}	87.9±1.00^{a}	13.4±0.16^{a}	8.6±0.12^{a}
	8000	1.49±0.01^{b}	85.0±0.62^{b}	12.8±0.10^{b}	8.1±0.07^{b}
	12000	1.56±0.03^{a}	87.8±1.25^{a}	13.3±0.20^{a}	8.5±0.14^{a}
	SEM	0.011	0.516	0.081	0.059
	P-value	0.01	0.01	0.01	0.01

a,b: Means within a column with different subscripts differ (P<0.05); ***Con:*** *control group, 4000: 4000 ppm salinity water, 8000:8000 ppm salinity water, 12000: 12000 ppm salinity water;* ***SCFA:*** *short chain fatty acid (mmol);* ***DOM:*** *organic matter digestibility (%);* ***ME;*** *metabolisable energy (MJ/Kg DM);* ***NE_L;*** *net energy for lactation (MJ/Kg DM);* ***SEM:*** *Standard error mean*

DOM, ME and NE_L of the present study for AH and BG were similar, *A* parameter higher and *c* fraction lower than that of Gozali [28]. Methane emissions of AH at control treatment of the current study was in line with Safaei et al.[20] and Bhatta et al.[29]. Methane production of BG in the control treatment were in the range of Halimi-Shabestari et al.[30] and Fant et al.[31]. The difference in the results obtained in the control treatment of the present study with other studies may be due to the different chemical composition of feedstuffs, inter-laboratory variations, microbial origin and donor animals. Since the values of DOM, ME and NEL are calculated from the GP as well as CP, EE and CA content, varying amount in any of these factors can change estimated nutritive value of tested feedstuffs [19,32]. Based on the review of literatures, there is a limited number of studies regarding to the effect of water salinity on the rumen fermentability of feedstuffs. The experiments regarding the effect of salinity on the rumen fermentation process and consequently the fermentability and energy of the feeds showed that high consumption of salt, leads to an increase in salt concentration in the rumen and decrease in the ruminal production of SCFA [33]. High consumption of salt has led to a decrease in the number of bacteria, pH and rumen ammonia in cattle, but this condition does not exist in sheep, so that in sheep it can even increase the number of bacteria. However, the diversity of bacteria in cattle remains unchanged, but in sheep, rumen microbial diversity decreases [33,34]. It is expected that changes in rumen osmotic pressure in sheep that consume saline water are caused by changes in the concentration of electrolytes in the rumen, especially in the concentration of sodium and potassium. Also, high consumption of salt leads to an increase in rumen chloride concentration and osmotic pressure. Regarding to changes in rumen function, the observed effects appear to be related to increased ruminal fluid passage rates resulting from increased fluid intake [35]. All bacteria and some protozoa need sodium and potassium to grow and their tolerance to amounts of salt is different. Most rumen microorganisms have maximum growth and production in normal salt concentrations in the rumen and in some cases the number of bacteria is increased by adding a small amount of salt to the diet [33,34]. Various acid producing bacteria such as *Streptococcus bovis* can survive in salt-containing environments and it is likely that they are the dominant microbial population in sheep fed with high salinity [34]. With a sharp increase in salt concentration, *Selenomonas* will be the dominant bacteria in the rumen and the number of *Bacteroides* will decrease. *Bacteroides* are one of the major producers of succinate in the rumen and decrease in the number of this population results in a decrease in propionate production [33,34]. The results of Costa et al.[36] showed that cellulose and glucose fermenting bacteria are more sensitive to salinity than starch fermenting bacteria. So that starch fermenting bacteria were much more resistant than other microorganisms at high levels of salt in water (16000 mg/L). The population of cellulolytic bacteria decreased linearly with the increase of water salinity. The highest microbial protein production was obtained at the sodium chloride concentration of 8800 mg/L. Thomas et al.[37] also observed that with the increase of salt concentration in the rumen, the bacteria population decreases and as a result, the rumen performance is affected. Alves et al.[38]

also showed that with increasing salt concentration in the diet, the digestibility of NDF in cows, and therefore the acetate concentration in the rumen decreases linearly with increasing sodium chloride in water. The results of Costa et al.[39] also confirmed this and showed that when the available substrate for microorganisms is starch or glucose at different levels of salinity, the production of microbial protein will increase. Oliveira et al.[40] stated that fibrolytic microorganisms have an acetate pathway, so the decrease in the concentration of this volatile fatty acid in the environment can be justified by reducing NADH, reduction-oxidation, microbial growth and increasing the concentration of sodium chloride. Butyric acid producing bacteria are also sensitive to salt concentration. The imbalance of sodium, potassium, and chlorine in the rumen environment can disrupt the pH balance of the rumen and disrupt the supply of nutrients for microorganisms, thereby causing the death of some microbial populations [41].

Nowadays, methane production has received global attention due to its role as a greenhouse gas and global warming. Ruminants produce significant amounts of CH_4 as a byproduct of rumen fermentation under the anaerobic conditions and lose up to 12% of gross energy intake [42]. Ruminal methanogens are able to tolerate 1.5 percent of sodium chloride. These methanogens live with protozoa and the change in the population of protozoa can affect the number of methanogens [34]. Newbold and Ramos-Morales [43] reported that decreasing ruminal protozoan populations resulted in reduced methane production, which is often accompanied by a decrease in rumen pH. Alhraishawi et al.[44] showed that methanogenic bacteria are affected at a salt concentration of 6 grams per liter. The proportion of methane decreases significantly by increasing the dose of salt to between 10-15 g of salt. An increase in salt can increase SCFA and decrease the pH of the anaerobic digesters such as rumen. Usually, the chemical content in the feedstuffs affects the production of methane. High amounts of soluble carbohydrates in high-energy concentrates increase the production of propionate in the rumen, which prevents the growth of *methanogens* and thus reduces the production of methane per unit of fermented organic matter. Propionate acts as a hydrogen scavenger and reduces the supply of hydrogen for methane gas production [45]. In addition, the high content of ether extract helps to reduce methane because some fatty acids, especially medium chain fatty acids are toxic to *methanogens* [46]. The type of feed can also affect methane production. Alfalfa contains crude protein with high digestibility, which leads to primary fermentation and gas production. The observed difference in methane output between feedstuffs is attributed to their nutrient composition because the slow digestion of feed is associated with higher methane production [29]. With these interpretations, several factors such as breed, age, salt content in water and diet, type of feeds and diet, population of protozoa, rumen pH and rumen passage rate can affect enteric methane production [7,29,34,44].

In an overall conclusion, drinking water salinity at the level of 12000 ppm TDS led to increase gas production and SCFA, DOM, ME and NE_L of AH. Methane production was affected by saline water consumption, so that the lowest amount of methane emission in AH and BG was observed in the treatment containing 4000 ppm TDS. Different levels of salinity did not affect the gas production and amount of SCFA, DOM, ME and NE_L of BG compare with control treatment. It seems that, drinking water salinity affect fermentation kinetics and nutritive value of AH and BG depending on the level of salinity and the type of feedstuffs. The results of current study showed that saline water up to12000 ppm TDS may be used for adult sheep without negative effects on nutritional value of consumed feedstuffs. It should be noted that the high level of salt in the diet can affect the current results.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflict of Interest

There is not conflict of interest with any person or institute/ organization regarding this manuscript.

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Authors Contribution Statement

R.S.D.N. and N.M.S. were as the supervisors (designed the experiments and interpretation of the results as well as leading the manuscript writing and revising), M.A.P.M. operating the the experiments, collecting data and writing the manuscript, A.R.S. and A.A.G. were the thesis advisors and helped in all process of experiment and manuscript preparation as well as statistical analyses.

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Research Article

Radiogrametric Analysis of the Metapodial Bones in English Setters

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Abstract: Radiography is one of the most preferred imaging methods when it comes to obtaining bone-related data by veterinarians on subjects such as clinical cases and education. This study was carried out to discover the sex differences on the metacarpal and metatarsal bones of English Setter hunting dogs. It aims to reveal the relationship between each bone forming the metapodiums and differences between male and female individuals. Twenty-eight English Setter dogs (16 females, 12 males) were included in the study. Actively hunting dogs were selected for the study, aged between 1 and 8. The average weight of the dogs were 18.79±4.12 kilograms. X-ray images were obtained from dogs positioning fore limb in dorso-palmar and hind limb in dorso-plantar position. For metacarpus measurements, males had higher values than females. In contrast to the metacarpal bones, females had higher measurements than males at some values (MT3: BP; MT4: SD; MT5: SD, BD) for metatarsal bones. The most distinguishing bone between males and females for the metacarpus was MC3 with the $P<0.05$ value at all measurements taken from the bone, and MT3 for the metatarsus ($P<0.05$ for three out of five measured values). In conclusion, metapodiums had statistically significant differences in terms of gender.

Keywords: *Dog, Metacarpus, Metatarsus, Radiogrametric analysis, X-ray*

İngiliz Setterlerde Metapodial Kemiklerin Radyogrametrik Analizi

Öz: Klinik vakalar ve eğitim gibi konular göz önünde bulundurulduğunda, veteriner hekimler tarafından kemikle ilgili verilerin elde edilmesi söz konusu olduğunda radyografi en çok tercih edilen görüntüleme yöntemlerinden birisidir. Bu çalışma, İngiliz Setter ırkı av köpeklerinin metacarpal ve metatarsal kemiklerindeki cinsiyet farklılıklarını ortaya koymak amacıyla yapılmıştır. Bunun yanında metapodyumları oluşturan her bir kemik arasındaki ilişki değerlendirilmiş ve farklılıklar araştırılmıştır. Çalışmaya 28 adet İngiliz Setter ırkı köpek (16 dişi, 12 erkek) dahil edilmiştir. Çalışma için yaşları 1 ile 8 arasında değişen ve aktif olarak avlanan av köpekleri seçilmiştir. Köpeklerin ortalama ağırlığı 18.79±4.12 kilogramdır. Köpeklerin ön bacakları dorso-palmar ve arka bacakları dorso-plantar pozisyonda konumlandırılarak röntgen görüntüleri elde edildi. Metacarpus ölçümlerinde erkeklerin dişilerden daha yüksek değerlere sahip olduğu görüldü. Metacarpal kemiklerin aksine, metatarsale kemiklerde dişilerin bazı değerlerinin (MT3: BP; MT4: SD; MT5: SD, BD) erkeklerden daha yüksek ölçümlere sahip olduğu görüldü. Erkekler ve dişiler arasında metacarpus için en ayırt edici kemiğin MC3 (ölçülen tüm değerlerde $P<0.05$) ve metatarsus için MT3 (ölçülen beş değerden üçü için $P<0.05$) olduğu tespit edilmiştir. Sonuç olarak, metapodyumlar cinsiyet açısından istatistiksel olarak anlamlı farklılıklar göstermiştir.

Anahtar sözcükler: *Köpek, Metacarpus, Metatarsus, Radyogrametrik analiz, X-ray*

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Introduction

Radiogrametric analysis and measurements are the most practical and budget-friendly method for obtaining bone-related data from live animals. Veterinarians frequently use x-ray imaging techniques for clinical cases [1-4]. In addition to two-dimensional radiological imaging techniques, three-dimensional imaging techniques are also among the imaging techniques that are increasing in use today [5,6]. Although radiologic imaging techniques are not only used for treatment and/or diagnosis in veterinary medicine, but also used as educational materials as well [7,8].

Metapodial bones consists metacarpal and metatarsal bones. Dogs have 5 metacarpal and 5 metatarsal bones. Metacarpal bones are named as os metacarpale 1, os metacarpale 2, os metacarpale 3, os metacarpale 4 and os metacarpale 5 from medial to lateral. Os metacarpale 1 is the shortest and thinnest besides os metacarpale 5 is the thickest of the metacarpal bones [9]. In metatarsal bones, however, the os metatarsale 1 is very small and does not have a phalanx at its tip [10]. Metapodial bones have attracted the attention of scientists in many different scientific fields and many studies are done on them. These studies include obtaining morphometric data specific to animal breeds [11], evaluation of bones obtained in archaeological excavations [12-14] and metapodial fractures [15].

For dogs, metapodial bones are often important for orthopedic diseases and fractures. Orthopedically, fractures in the metacarpal and metatarsal bones are usually encountered due to road accidents, falling objects, falling from a height, fighting, crush injury and track injury, and fractures may include one or more metapodial bones [16]. English Setter breed dogs, which are preferred as hunting dogs, also have the possibility of encountering some orthopedic cases mentioned above. In order to increase the condition of life of these dogs, which are in our lives as companion animals or working animals, and to provide them with better recovery opportunities, the studies on metapodiums are of great importance as our study focuses on the relationship between each bone forming the metapodiums, differences between male and female individuals.

Material and Methods

Ethical Statement

This study was approved by the Istanbul University-Cerrahpaşa, Faculty of Veterinary Medicine Animal Experiments Local Ethics Committee (Approval no: 2022/22).

Animals

Twenty-eight clinically sound English Setter dogs (16 females, 12 males) actively hunting were included in the study. The dogs' ages ranged from 1 to 8. They had an average weight of 18.79±4.12 kg. Weights were taken just before the X-ray image by using a digital scale. Standard physical, orthopedic and neurological examinations were performed before conducting the experiment.

Radiographic Images

X-rays were taken via "Gierth TR 90/30" portable x-ray

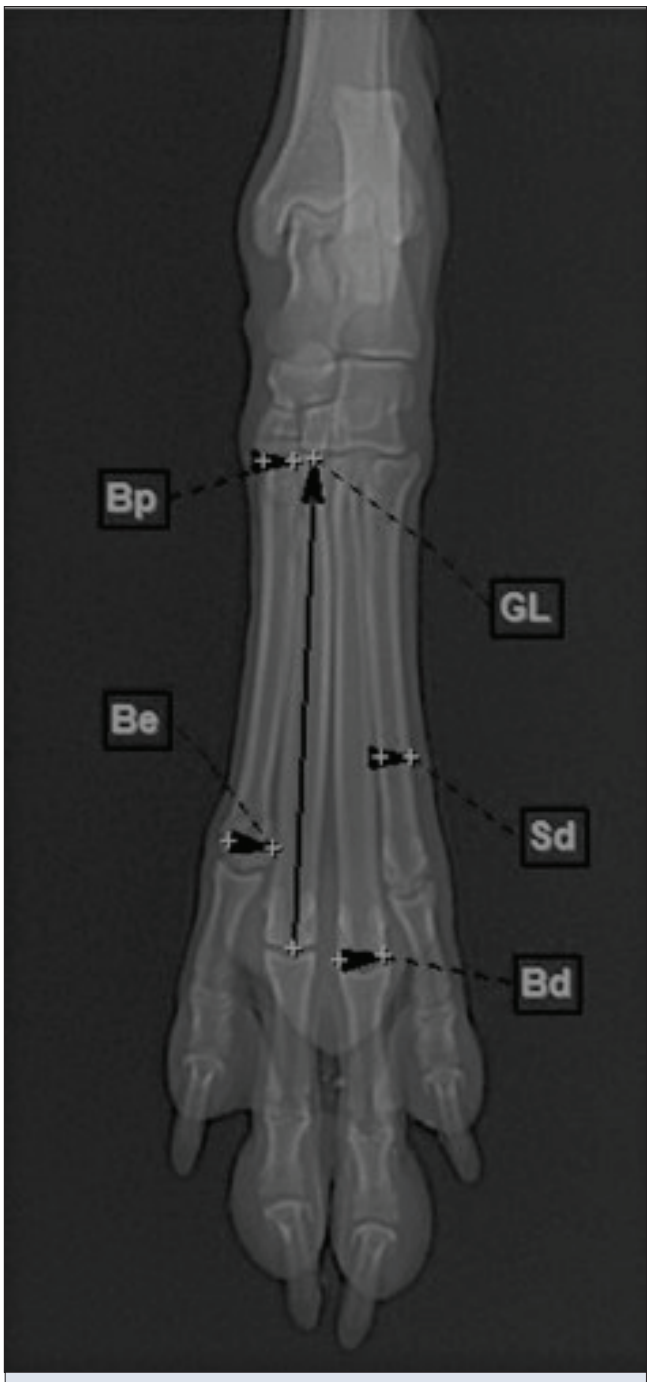

Fig 1. Radiogrametric measurements of metapodial bones (metatarsus). Bp: Width of proximal end; Sd: Smallest width of diaphysis; Bd: Width of distal end; GL: Greatest length; Be: Greatest width of metaphysis

device with 85 kW, 1.5 mAs settings from 1 meter distance to cassette. Radiographs are taken in dorso-palmar position for the fore limbs and dorso-plantar for the hind limbs. Attention was paid to make sure that all metacarpus and metatarsus were clearly visible while the images were taken. X-ray images of the dogs were also examined in terms of deformities in the metapoidiums and joint areas, fractures or cracks, and orthopedically healthy dogs were included in the study. Sedative or anesthetic agents were not used on dogs in the study.

Radiogrametric Measurements

Some measurements that are frequently used in publications [17-19] related to metapodium radiogrametric measurements *(Fig. 1)* are listed below with their explanations:

Width of proximal end (BP): Length of the widest point at the proximal end of the bone.

Smallest width of diaphysis (SD): Length of the narrowest part on diaphysis.

Width of distal end (BD): Length of the widest point at the distal end of the bone.

Greatest length (GL): The maximum length between proximal and distal end of the bone.

Greatest width of metaphysis (BE): Length of the widest point on metaphysis.

Radiogrametric measurements were obtained via "RadiAnt DICOM Viewer" using the digital ruler in the software program.

Statistical Analysis

Statistical analysis were performed using "IBM SPSS Statistics V21.0". Mean, STD (standard deviation) and P values are obtained and "One-way ANOVA" (Analysis of Variance) was performed to statistically determine the difference between gender groups via statistical program mentioned above. Independent t-test was used to analyze relationship between radiogrametric measurements of metacarpus and metatarsus regardless of sex discrimination. The effects of age and weight on radiogrametric measurements were also analyzed with bivariate correlation test via "IBM SPSS Statistics V21.0"

Results

Radiogrametric measurements of metapodial bones for male and female are given in *Table 1* and *Table 2*. BP value and gender discrimination in all metacarpal bones were statistically different. In general, considering the genders, it was observed that there were statistically significant differences in GL values of metapodiums. Except for MC5 (P: 0.08), the P values were 0.01 for the MC2, MC3 and MC4. For the MC5, the biggest difference was in BP. The most distinguishing bone between female and male was MC3. The difference between male and female for MC3 was statistically different in all measurements taken. In addition, the longest bone for both female and male individuals was MC3 (GL; male: 69.37±4.01 mm, female: 66.14±2.18 mm)

The results of metatarsus measurements for males and females are given in *Table 2*. It was observed that the metatarsal bones were not as deterministic as the metacarpus in terms of gender. Most of the metatarsal bone measurements were high in male. In contrast to the metacarpal bones, females had higher measurements than males at some values (MT3: BP; MT4: SD; MT5: SD, BD). In terms of gender, the greatest difference for metatarsus

Table 1. *Results of radiogrametric measurements of metacarpal bones in Setter dogs*

Measurement	Sex	MC2			MC3			MC4			MC5		
		Mean	STD	P	Mean	STD	P	Mean	STD	P	Mean	STD	P
BP (mm)	Male	5.83	0.84	0.02	5.44	0.73	0.03	5.92	0.93	0.01	9.89	0.93	0.01
	Female	5.34	0.72		4.98	0.81		5.34	0.73		8.62	1.06	
SD (mm)	Male	5.30	0.75	0.03	6.10	0.59	0.01	5.49	0.63	0.04	5.44	0.74	0.11
	Female	4.88	0.63		5.39	0.71		5.14	0.58		5.11	0.78	
BD (mm)	Male	7.25	0.76	0.02	7.39	0.88	0.02	7.24	0.74	0.06	7.30	0.72	0.01
	Female	6.76	0.80		6.84	0.88		6.86	0.74		6.75	0.84	
GL (mm)	Male	58.86	3.26	0.01	69.37	4.01	0.01	68.53	4.08	0.01	55.58	2.81	0.08
	Female	55.85	1.94		66.14	2.18		65.20	2.40		53.97	3.63	
BE (mm)	Male	9.08	1.17	0.06	8.93	0.88	0.01	8.77	1.19	0.12	9.26	1.07	0.01
	Female	8.52	1.06		8.55	0.79		8.38	0.67		8.53	0.84	

BP: Width of proximal end; SD: Smallest width of diaphysis; BD: Width of distal end; GL: Greatest length; BE: Greatest width of metaphysis; MC2: Metacarpus 2; MC3: Metacarpus 3; MC4: Metacarpus 4; MC5: Metacarpus 5; STD: Standard deviation

Table 2. *Results of radiogrametric measurements of metatarsal bones in Setter dogs*

Measurement	Sex	MT2			MT3			MT4			MT5		
		Mean	STD	P	Mean	STD	P	Mean	STD	P	Mean	STD	P
BP (mm)	Male	5.28	0.71	0.05	4.76	0.60	0.95	5.56	1.02	0.34	9.10	1.17	0.01
	Female	4.87	0.78		4.78	1.14		5.34	0.69		8.34	0.89	
SD (mm)	Male	4.60	0.76	0.02	5.91	0.87	0.44	4.80	0.71	0.72	4.24	0.69	0.66
	Female	4.18	0.60		5.76	0.66		4.87	0.76		4.36	1.19	
BD (mm)	Male	6.63	0.97	0.11	7.49	0.94	0.01	7.06	1.03	0.47	5.92	0.97	0.91
	Female	6.24	0.85		6.85	0.79		6.89	0.73		5.94	0.50	
GL (mm)	Male	63.36	5.44	0.16	74.90	6.48	0.05	76.92	7.33	0.08	66.22	6.95	0.16
	Female	61.54	4.19		72.04	4.15		73.83	5.70		63.91	5.05	
BE (mm)	Male	8.22	0.94	0.14	9.06	1.00	0.05	8.57	1.00	0.25	7.74	1.00	0.81
	Female	7.87	0.80		8.61	0.68		8.29	0.77		7.68	0.70	

BP: Width of proximal end; SD: Smallest width of diaphysis; BD: Width of distal end; GL: Greatest length; BE: Greatest width of metaphysis; MT2: Metatarsus 2; MT3: Metatarsus 3; MT4: Metatarsus 4; MT5: Metatarsus 5; STD: Standard deviation

Table 3. *Radiogrametric measurements of metacarpal bones, gender disregarded (independent t test)*

Measurement	MC2	MC3	MC4	MC5
BP (mm)	5.55[a]	5.18[b]	5.59[a]	9.17[c]
SD (mm)	5.06[a]	5.69[b]	5.29[a]	5.25[a]
BD (mm)	6.97[a]	7.08[a]	7.02[a]	6.99[a]
GL (mm)	57.14[a]	67.53[b]	66.63[b]	54.66[d]
BE (mm)	8.76[a]	8.72[a]	8.55[a]	8.84[a]

BP: Width of proximal end; SD: Smallest width of diaphysis; BD: Width of distal end; GL: Greatest length; BE: Greatest width of metaphysis; MC2: Metacarpus 2; MC3: Metacarpus 3; MC4: Metacarpus 4; MC5: Metacarpus 5
[a,b,c,d] *Values within a line with different superscripts are significantly different (P<0.05)*

was in the BD measurement of MT3 (P<0.01). For female and male, the longest bone in the metacarpus was MC3, but for the metatarsus, the longest bone was MT4 (GL; male: 76.92±7.33 mm, female: 73.83±5.70 mm). No radiogrametric measurements of MT4 were enough for sex-determination.

Statistical analysis of metacarpus and metatarsus bones without gender discrimination are given in *Table 3* and *Table 4*. The longest bone for the metacarpus was MC3 (67.53 mm). In terms of length (GL), MC3 and MC4 were not statistically different. However, GL results and MC2 and MC5 were statistically different from each other as well as from MC3 and MC4. The shortest bone was MC5 (GL: 54.66 mm). The greatest difference for metacarpus measurements was in BP value. BP values of MC5 were considerably higher than other metacarpus bones (9.17 mm). And this difference was statistically significant. BE values were close to each other in all metacarpus bones. There was no statistical difference for BE value between bones.

The longest bone for the metatarsus was MT4 (GL: 75.15 mm). However, as with metacarpus values, there was no statistical difference between MT4 and MC3 values. The highest difference was seen in the BP value among the metatarsus bones. BP values of MT5 were higher than other metatarsus bones, and this difference was statistically significant *(Table 4)*.

The correlation results of measurement values with age and weight are given in *Table 5*. It was observed that the weight showed a positive correlation on all radiogrametric values. The highest correlation was seen between weight and BE measurement (correlation value: 0.353). For age, only the correlation with BE value was statistically significant (correlation value: 0.195). When the measurement values were evaluated within themselves, the highest correlation was seen between BE and BD (correlation value: 0.634).

Discussion

The study is carried out on the metapodium of English Setter dogs, and the measured radiogrametric values were evaluated both between sexes and bones. Radiogrametric data obtained from X-ray images is of great value in terms of obtaining morphological data and determining the ways to be followed in the treatment process of orthopedic

Table 4. *Radiogrametric measurements of metatarsal bones, gender disregarded (independent t test)*

Measurement	MT2	MT3	MT4	MT5
BP (mm)	5.05[a]	4.77[a]	5.44[c]	8.67[d]
SD (mm)	4.36[a]	5.8[b]	4.84[c]	4.31[a]
BD (mm)	6.40[a]	7.13[b]	6.96[b]	5.93[d]
GL (mm)	62.32[a]	73.27[b]	75.15[b]	64.90[d]
BE (mm)	8.02[a]	8.81[b]	8.41[c]	7.71[a]

BP: Width of proximal end; SD: Smallest width of diaphysis; BD: Width of distal end; GL: Greatest length; BE: Greatest width of metaphysis; MT2: Metatarsus 2; MT3: Metatarsus 3; MT4: Metatarsus 4; MT5: Metatarsus 5
[a,b,c,d] *Values within a line with different superscripts are significantly different (P<0.05)*

Table 5. *Correlation results between radiogrametric measurements, age and weight*

Measurements	SD	BD	GL	BE	Age	Weight
BP	-.057	-.061	-.312**	.057	.023	.173**
SD		.404**	.184**	.397**	-.002	.208**
BD			.238**	.634**	.069	.240**
GL				.116*	-.020	.176**
BE					.195**	.353**
Age						.233**

**P<0.05, **P<0.01*

cases. Considering the differences between the sexes, as known, the bone structures of male individuals are expected to be larger than female individuals, and the metacarpus measurements in our study supported this thesis. Metatarsal data were found to have higher values in males than females in general, but this situation changed in some measurements (MT3: BP; MT:4 SD; MT5: SD, GL). Although the values of female individuals were measured to be higher than male individuals in the above-mentioned values, it was observed that the difference between the sexes was statistically insignificant (P>0.05). It is thought that the reason why the significant size differences seen in metacarpus are not evident in metatarsus is related to the weight on the forelimb being more than the weight on the hindlimb. Considering that males under the same conditions are heavier than females, it is thought that the weight difference on the forelimb may be greater than the weight difference on the hindlimb.

Dursun [9] and Bahadır and Yıldız [10] stated that MC3 and MC4 are the longest bones for carnivores that information is consistent with our study. However, both authors mentioned carnivores in general and no specific breed was stated as in our study. Hence, the breed-specific values and results in our study are important in terms of determining the differences in carnivores. In a study on marten, which is a carnivore, the longest metacarpus was reported to be MC3 [20] as in English Setter dogs. In addition, in cats [6] it is stated that MC3 is the longest bone followed by MC4, when the metacarpus' are evaluated. Although the aforementioned cat is a domestic cat, lynx [21], a wild cat, had also similar features to the dogs used in our study. For metatarsus of lynxes [21], it is seen that MT3 and MT4 were the longest, but the longer one between them was unclear. Similar results are also found in a metatarsus study on badger [22], an omnivore, states that MT3 and MT4 had nearly same length. It is seen that similar length measurements between the two bones are also suitable for marten [23] a carnivore. Results of our study shows that the longest bone is MT4 and MT3 comes right after it for the English Setter dogs. As in the studies given above, the longest metatarsus was found to be one of MT3 and MT4. When it comes to the longest bone, although there are differences in the fore and hind limb, the difference between MC3 - MC4 and MT3 - MT4 was statistically insignificant. Considering the metapodiums, it is seen that the bones that carry the weight are the bones numbered 3 and 4, as in other animals, and these bones are more developed than the others (MC1, MT1, MC2, MT2, and MC5, MT5). Cracks, fractures, etc., which occur as a result of trauma in these bones, that carry the majority of the weight, is more likely to occur than other metapodiums [15] also supports the results of our study. When the gender

factor was ignored, the difference in BP values between each metapodium was found to be statistically significant. This suggests that the effects of body weight on each metapodium are a biomechanically related consequence.

The correlation of measurements with weight is positive and statistically significant, indicating the effect of body weight on metapodial bones. In addition, the positive correlation between BE value and age show that the epiphyseal region thickens with age. The positive correlation between BE and BD also supports the thesis above, in conclusion our study shows that the distal part of the metapodial bones shows parallel increase with age.

The study, which was carried out on English Setter dogs on x-ray images, the differences between the sexes of metapodiums and the relations between the bones themselves were examined radiogrametrically. One of the difficulties encountered during the study was to be able to take x-rays in right position while the animals did not receive any anesthetic or sedative substances. This is an appropriate approach when considering ethics and animal welfare, but we would like to point out that animals should have calm characters to maintain the right image. In addition, many of the X-rays are taken more than once to obtain the appropriate image during the study. Classical osteometric studies are usually done with bones of dead animals [20-23]. However, thanks to the radiological imaging techniques, data of bone morphometry of the animals that are still alive can be obtained [1-4]. In general, both metacarpus and metatarsus mean values for males were higher than females, but when the difference between the sexes was statistically evaluated, it was significant for metacarpus in many measurements, but this was not the case for metatarsus. It was observed that the longest bone among the metacarpus was MC3, and MT4 among the metatarsus. Although the number is increasing, studies on animals belonging to a certain breed are insufficient. For future scientific studies, it is recommended to study race-specific morphological data, if possible. It is hoped that the basic data obtained with our study results will be useful in many fields such as education, archeology, anatomy and especially for veterinary clinical practice, such as veterinary orthopedists, that our results can be the basis for further surgical, therapeutic applications and research.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author (D. Aydın Kaya) on reasonable request.

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There is no funding support.

Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Ethical Statement

This study was approved by the Istanbul University-Cerrahpaşa, Faculty of Veterinary Medicine Animal Experiments Local Ethics Committee (Approval no: 2022/22).

Authors Contributions

Z.M. and M.K.: Conceived and supervised this study; Y.A., D.O.E. and D.A.K.: Collection of the data; E.Ö. and E.G.: Statistical analysis. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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Research Article

Tarantula Cubensis Extract and Low-Level Laser Therapy: A Histopathological, Radiological and Serological Analysis of Bone Repair on an Experimental Bone Defect Model

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Abstract: In this study, it was aimed to investigate the effects of *tarantula cubensis* extract (TCE) and low-level laser therapy (LLLT) on cancellous and cortical bone repair. A circular unicortical bone defect was created on both the cancellous and cortical regions of the tibia of each subject. The subjects, totaling 54 mature New Zealand rabbits, were randomly allocated into three groups (n:18 each): control, LLLT (Galium-aliminum-arsenide laser at a wavelength of 780 nm and 4 J/cm^2, 5 min/day) and TCE (1 µg/kg) groups. Relative optic density (ROD) level was higher in the TCE group than the control group on day 28 as radiological (P<0.05). In both cancellous and cortical bone, inflammatory cell densities were less on the 14 and 21st day in the TCE and LLLT groups, bone tissue formation and qualities were higher 7 and 14th days in TCE and LLLT and collagen maturation were higher 28th day in the TCE group as histopathological (P<0.05). In this study, TCE accelerates bone repair as much as LLLT and more than the control group. In conclusion, that TCE is an effective, easier to apply and more economical supportive treatment in bone defects like LLLT.

Keywords: *Bone repair, Homeopathy, Low-level laser therapy, Tarantula cubencis extract, Rabbit*

Tarantula Cubensis Ekstraktı ve Düşük Seviye Lazer Tedavisi: Deneysel Kemik Defekti Modelinde Kemik Onarımının Histopatolojik, Radyolojik ve Serolojik Analizi

Öz: Bu çalışmada *tarantula cubensis* ekstraktı (TCE) ve düşük seviyeli lazer tedavisinin (LLLT) süngerimsi ve kortikal kemik onarımı üzerindeki etkilerinin araştırılması amaçlandı. Her deneğin tibiasının hem süngerimsi hem de kortikal bölgelerinde sirküler unikortikal kemik defekti oluşturuldu. Toplam 54 olgun Yeni Zelanda tavşanından oluşan denekler rastgele üç gruba ayrıldı (her biri n:18) kontrol, LLLT (780 nm dalga boyunda ve 4 J/cm^2, 5 dk/gün'de Galium-alüminyum-arsenid lazer) ve TCE (1µg/kg) grupları. Rölatif optik yoğunluk (ROD) düzeyi radyolojik olarak 28. günde TCE ve LLLT gruplarında kontrol grubuna göre daha yüksekti (P<0.05). Histopatolojik olarak hem süngerimsi hem de kortikal kemikte, inflamatuvar hücre yoğunlukları 14. ve 21. günde TCE ve LLLT gruplarında daha azdı kemik dokusu oluşumu ve kalitesi 7. ve 14. günlerde TCE ve LLLT gruplarında daha yüksekti ve kollajen olgunlaşması 28. günde TCE grubunda daha yüksekti (P<0.05). Bu çalışmada TCE, kemik onarımını LLLT kadar ve kontrol grubuna göre daha fazla hızlandırmaktadır. Sonuç olarak, LLLT gibi kemik defektlerinde TÇE'nin etkili, uygulaması kolay ve ekonomik bir destekleyici tedavi olduğu sonucuna varıldı.

Anahtar sözcükler: *Kemik onarımı, Homeopati, Düşük seviye lazer tedavi, Tarantula cubencis ekstraktı, Tavşan*

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Introduction

Today, both bone metabolism the disorders and the impairment of bone integrity due to traffic and work accidents have made bone healing an important health problem in both humans and animals. The main targets in the treatment of these bone defects in orthopedic clinics are to make the damaged area painless by performing the necessary conservative or surgical intervention as soon as possible and to help the patient return to normal life by helping the bone repair as quickly as possible [1,3].

To accelerate bone repair, biomaterials, bone morphogenetic proteins (BMP), physical stimulation such as ultrasound, electromagnetic fields, and laser applications and chemical stimulants such as drugs and homeopathic remedies are used [4,5]. Tarantula cubensis extract (TCE), a venom of spider, as an injectable agent that is potentized (6x) according to the German Homeopathic Pharmacopeia after maceration in alcohol, has been used as homeopathic in many animal species in recent years. TCE, which has a peptide structure, removes necrotic tissues and thus accelerates epithelialization and heals the wound [6,7]. It has been used in the treatment of breast tumors, pododermatitis, painful abscesses involving gangrene, septicemia, and toxemia by creating a demarcation line around the lesion [8,10]. In addition, it causes a decrease in inflammation in rats, accelerates uterine involution, treats genital microbial diseases, oral ulcers, cutaneous and oral papillomatosis [6,8-10]. It has also been reported to be effective for tendon, nerve, and wound healing [10-12]. However, studies on bone tissue are very scarce [13].

Low-level laser therapy (LLLT) is a supportive treatment application that aids biological tissue regeneration processes such as bone repair, skin lesions and muscle tissue repair [14,15]. With LLLT, photobiomodulation (nonthermal phototherapy) occurs in the tissues by creating reactions such as increased cell proliferation, osteoblast activity, vascularization, bone formation and collagen deposition in bones and various tissues [15-18]. LLLT also accelerates the healing process with its analgesic and anti-inflammatory effects [19,20]. Most studies using LLLT have been planned for the repair of defects formed in the cortical bone [15,19,21]. Although the repair of cancellous bone is known to be faster than that of cortical bone [22], it is not evident whether there is a difference between the repair of LLLT and TCE on both cortical and cancellous bone defects at the same time.

Briefly, since bone healing is basically connective tissue healing [1], it is thought that TCE may positively affect healing by accelerating collagen synthesis from fibrin networks and fibroblasts that fill the defect area in the early stages of bone repair. In addition, due to the vascularization-enhancing effect of TCE in the repair area [12], it may cause an increase in VEGF (Vascular endothelial growth factor), which stimulates new vessel formation, and increases bone stabilization in the reparative phase of the bone healing process, leading to the formation of fibrocartilagenous callus. Because of the above-mentioned features, TCE is thought to be able to provide a faster repair of the bone by accelerating angiogenesis, collagen synthesis and anti-inflammatory effect, which have a primary role in bone repair like LLLT. Moreover, LLLT and TCE are thought to be important in terms of revealing the expression and localization changes of BMPs with osteoinductive properties in the repair of bone and their roles in regeneration. In light of these hypotheses, it was aimed to find answers to the following questions. First, does TCE increase the expression of VEGF receptors (flt1/fms, flk1/KDR and flt4, respectively) and BMPs in damaged bone tissue? Second, is there a difference in the efficacy of TCE and LLLT on cancellous and cortical bone regions? Third, since homeopathic agents are known to significantly reduce cost compared to conventional agents [23], does TCE as effective in bone repair as LLLT, which requires specialized equipment and cost?

Material And Methods

Ethical Statement

This study was conducted with the principles of animal welfare in mind. All experimental protocols were thoroughly revised and approved by the Animal Experiments Local Ethics Committee, Dicle University, Diyarbakır (Approval No: 2016/8, 13.04.2016)

Animals

Healthy 54 male mature New Zealand white rabbits, which have a tibial diameter of 0.5-0.7 cm, were included in this study. Rabbits were purchased from the experimental animal production unit for the University of Dicle. The rabbits were properly housed in individual cages in the rabbit room with the same humidity and temperature in the same unit before and after the surgical procedures. Standard feed and water were provided *ad libitum*. The acclimation period before surgery for all the rabbits was one week.

Anesthesia and Surgical Procedure

Before the surgery, rabbits were anesthetized with ketamine HCl (50 mg/kg, IM) (Ketasol®, Richter-Pharma AG, Wels, Austria) following premedication with xylazine HCl (10 mg/kg, IM) (Rompun® 2%, Bayer, Turkey) and butorphanol (0.3 mg/kg, IV) (Butomidor®, Richter-Pharma AG, Wels, Austria). After providing asepsis-antisepsis of the operation area of all rabbits under general anesthesia, the right tibias of the rabbits were opened surgically with an appropriate anatomical surgical approach, then

a unicortical bone defect was created on the cortical and cancellous bones of the right tibia with a 3.5 mm diameter drill. Drilling was performed slowly to avoid possible bone necrosis, accompanied by saline irrigation [19]. After the defected area was irrigated with saline to remove bone debris, the muscles and skin were covered with 3-0 suture material. Rabbits were administered 400.000 IU of procaine penicillin (Procaine Pen® 400.000, Tümekip İlaç, İstanbul) for 5 days for postoperative antibiotherapy and carprofen (1.5 mg/kg/day, SC) (Rimadyl®, Zoetis, İstanbul) for 2 days for postoperative analgesia. Since unicortical defects were performed, there was no need to apply any support bandage on the respective legs of the rabbits since no problems with walking were expected. All rabbits received daily wound care for 10 days. If spontaneous fracture in the defect areas and development of osteomyelitis were accepted as exclusion criteria in the experimental process.

Study Design and Treatment Protocols

After the experimental bone defect model was created, the rabbits have randomly allocated into 3 main groups as follows:

Group 1: LLLT (n = 18): Low-level laser therapy was applied with a [Galium-aliminum-arsenide-GaAlAs-Chattanooga Intellect-USA] laser at a wavelength of 780 nm and 4 J/cm^2, 5 min/day] at the same time for 4 weeks starting on the 4th postoperative day [21].

Group 2: TCE (n = 18): *Tarantula cubensis* alcoholic extract (Homeopathic group) (Theranekron® D6, 1 mg/mL, Richter-Pharma AG, Wels, Austria) was administered to rabbits subcutaneously (SC) on the 3rd, 7th, 10th, and 15th days at 1 µg/kg on the skin of the defect-created tibia [11].

Group 3: Control (n = 18): Rabbits were administered SC saline only once after the surgical procedure, and these rabbits were used as the control group.

The three main groups then were allocated into 3 subgroups according to the duration of treatment (7, 21 and 28 days), with six animals in each subgroup.

Laboratory (Serological) Evaluations

Serum samples were obtained from the rabbits by centrifugation of blood from the ear vein on preop (0) and postoperative 7th, 14th, 21th and 28th days. Bone Alkaline phosphatase (B-ALP), Osteocalcin (OC), Prokollagen I N-terminal peptide (PINP) bone formation factors, and Interleukin-1β (IL-1β), Interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) proinflammatory cytokines were analyzed in the serum samples using rabbit-specific commercial ELISA kits (Elabscience Biotechnology Co., Ltd, Wuhan, China).

Radiological Evaluation

Each animal underwent radiological evaluation just postoperatively and once a week for 4 weeks. The radiological technique was performed according to Matos et al.[24]. In contrast to conventional radiological evaluation to confirm the assessment and follow-up of fracture repair, the Fracture Healing Monitor Module of the Relative Optical Density Image Analysis (RODIA) a system developed by Polish researcher Glinkowski [25] was utilized for image evaluation. For this purpose, the web-based Fx-Expert 2014 program was used, which is called Orthopedic Intelligence System v.1.0. [26] *(Fig. 1-A).* This program works on the principle of determining the optical density (OD) of a fracture or defect area on the

Fig 1. The Fx-Expert program was used to determine the relative optical density (ROD) value and the sequence of images showing the ROD analysis with the program. A: Accessing the program via the internet, B: Opening the X-ray image in the program; C: Displaying the solid and defect regions on the x-ray image; D: Measuring the OD values in the solid and defect regions, and the program calculating the ROD value automatically *(green arrow)*

radiological image transferred to the computer. Briefly, digital DICOM X-ray images obtained with a CR system were transferred to a personal computer. The images were then opened with the Fx-Expert program running on the web *(Fig. 1-B)*. The optical density of the intact region adjacent to the defect area was first determined on the images *(Fig. 1-C)*. The optical density of the defect was then determined. After these two OD values were determined, the program automatically determined the relative optical density (ROD) value with the ratio of the OD value of the defective region to the intact region *(Fig. 1-D)*. These analyzes were performed separately for the defect site in both the cancellous and cortical bone. All analyzes were performed by a blinded researcher.

Histopathological Analysis

The rabbits were euthanized end of the experiment in days postoperative 7th, 14th, 21th, and 28th days after administration of a high-dose anesthetic (50 mg/kg) (Pentothal Sodium®, Tümekip İlaç, İstanbul) and pancuronium (1 mg/kg) (Pavulon®, Organon, İstanbul). Histopathologic sections were prepared according to Pretel et al.[27]. For the histopathological and immunohistochemical examinations, serial sections of 5 µm thickness were taken from the prepared blocks. Histopathological sections were stained with Crosmann's triple and Solochrome Cyanine. In the areas where new bone formation occurred in Crosmann triple stained preparations, collagen fibrils, distribution and morphology of trabecular structure, determination of the presence of osteoblasts and osteocytes, inflammatory cell density, blood vessels, etc. were evaluated. In the preparations stained with Solochrome Cyanine, osteids, mineralized and calcified bone areas of newly formed bone tissue were evaluated.

Immunohistochemistry Staining

The streptavidin peroxidase method was used to determine the localization and expression of VEGF and its receptors, osteopontin and BMPs in the bone tissue of the rabbits. Immunohistochemical staining procedures were performed according to the staining principles in our previous study [28]. All sections were treated according to the same protocol.

After staining, the preparations were photographed on a Nikon-Eclipse 400 DSRI Nikon digital camera (NIS Elements Imaging Software-version 3.10) attachment research microscope and evaluated.

Semi-Quantitative Evaluation

The scoring method belonging to Pretel et al.[27] was used to evaluate the histopathologic differences in the formation stages of new bone tissue after sections were stained with Crosmann's triple and Solochrome Cyanine staining. Histopathologic analysis was performed under a light microscope. Each prepared sample was evaluated blindly by two independent observers. In cases of disagreement, the samples were re-evaluated and a consensus was reached among independent observers.

Statistical Analysis

The data were analyzed with the SPSS 24.0 program and are presented as mean ± SD. P-value less than 0.05 was considered statistically significant. The Kruskal-Wallis test was used to determine the difference between treatment days and groups and Mann-Whitney-U test was used to determine the group that showed differences in histopathologic data. Bone-specific markers and radiological ROD evaluations were performed using One-Way ANOVA, and the Post-hoc Duncan test for multiple comparisons was used to detect differences. Two independent samples test (student t-test) was used to evaluate the difference between cortical and cancellous bone defects.

Results

There were no postoperative complications in any of the rabbits during the experimental period.

Clinical and Radiological Findings

Clinically, the rabbits had no problems walking during their daily controls, and there were no signs of discharge, swelling, etc. in the surgical wounds. In radiological evaluations, conventional radiographs were presented *Fig. 2* and statistical data of the ROD analysis obtained by

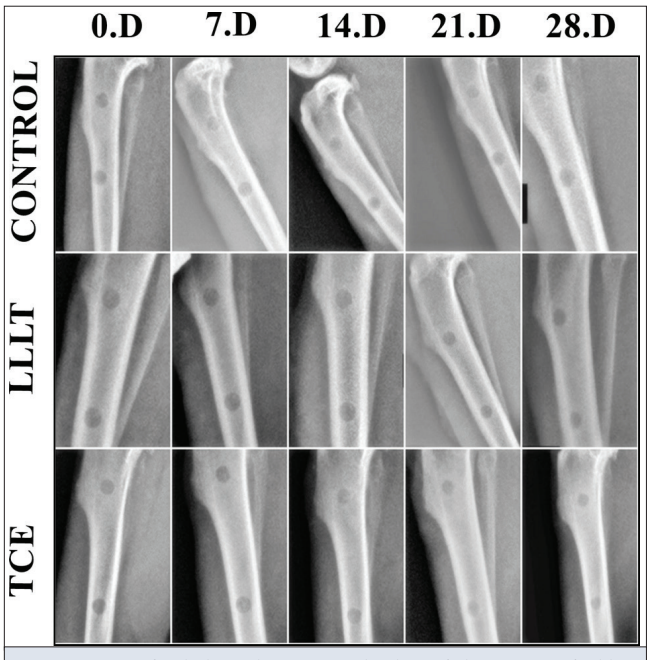

Fig 2. A series of radiological images on the days of observation of rabbits from each group

rabbits immediately after the surgery and on the 7th, 14th, 21st, and 28th days are presented in *Table 1*.

Histopathological Findings

The histopathological results obtained by scoring techniques are summarized in *Table 2*. Histopathological images of tissues are given on *Fig. 3* and *Fig. 4*.

Immunohistochemical Findings

Intensity scores of osteopontin, BMPs, VEGF and VEGF receptors (flt1/fms and flk1/KDR) expression in the cortical and cancellous bone defect during the applications are presented in *Table 3* and immunohistochemical staining of VEGF are shown in *Fig. 5*.

Laboratory (Serological) Findings

The results of the ELISA test carried out on the serum samples are presented in *Table 4*.

Discussion

In clinical and experimental studies, it has been revealed that LLLT increases vascularization in damaged tissue with its photobiological and photochemical effects, accelerates reepithelization, and accelerates tissue healing by increasing fibroblast activity and collagen synthesis [14,15,17,19]. Clinically, it is known that LLLT have beneficial effects on pain management and bone formation [14,29]. In this study, it was tried to determine whether there was a difference in the effects of LLLT on cancellous and cortical bone. By direct histopathological examination of the bone tissue, cells can be determined quantitatively and functionally, structural changes in the cortical and cancellous bone can be analyzed, and changes in the fracture healing cycle, bone diseases, and bone development cycle can be determined most accurately [30,31]. Since the research was an experimental study, changes in the bone healing process, such as the inflammatory phase, cellular proliferation

Table 1. *Statistical data (Mean±SD) of radiological relative optical density (ROD) analysis*

Bone Tissue	Groups	0. Day	7. Day	14. Day	21. Day	28. Day
Cancellous	C	0.80 ± 0.01^{D}	0.84 ± 0.02^{C}	0.87 ± 0.03^{B}	0.89 ± 0.03^{AB}	0.91 ± 0.03^{Abc}
	TCE	0.81 ± 0.01^{D}	0.85 ± 0.03^{C}	0.90 ± 0.03^{B}	0.92 ± 0.03^{AB}	0.95 ± 0.03^{Aa}
	LLLT	0.80 ± 0.02^{D}	0.85 ± 0.01^{C}	0.88 ± 0.02^{B}	0.90 ± 0.02^{B}	0.94 ± 0.02^{Aab}
Cortical	C	0.77 ± 0.01^{C}	0.79 ± 0.03^{C}	0.83 ± 0.02^{B}	0.86 ± 0.02^{A}	0.88 ± 0.02^{A}
	TCE	0.78 ± 0.01^{D}	0.82 ± 0.02^{C}	0.86 ± 0.03^{B}	0.88 ± 0.03^{B}	0.91 ± 0.02^{A}
	LLLT	0.77 ± 0.02^{C}	0.79 ± 0.02^{C}	0.83 ± 0.03^{B}	0.87 ± 0.02^{A}	0.89 ± 0.03^{A}

Superscripts in the same line (A, B, C, D) and in the same column (a, b, c) are statistically significant (P<0.05).
C: *Control,* ***TCE:*** *Tarantula cubensis extract,* ***LLLT:*** *Low-level laser therapy*

Table 2. *Scores of histopathological findings in the defect area in cortical and cancellous bone according to the follow-up periods (Means ± SD)*

Histopathological Scores	Follow-up Periods	Cortical Bone			Cancellous Bone		
		Control	LLLT	TCE	Control	LLLT	TCE
Inflammation	7	2.80 ± 0.447^{a}	2.40 ± 0.547^{a}	2.60 ± 0.547^{a}	2.90 ± 0.457^{a}	2.50 ± 0.547^{a}	2.60 ± 0.547^{a}
	14	1.60 ± 0.547^{bA}	0.60 ± 0547^{cB}	0.40 ± 0.547^{cB}	1.50 ± 0.547^{bA}	0.40 ± 0547^{bB}	0.20 ± 0.547^{bB}
	21	0.20 ± 0.447^{cA}	0.0 ± 0.0^{dB}	0.0 ± 0.0^{dB}	0.20 ± 0.447^{cA}	0.0 ± 0.0^{cB}	0.0 ± 0.0^{cB}
	28	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{d}	0.0 ± 0.0^{c}	0.0 ± 0.0^{c}
Formation and quality of bone tissue	7	0.50 ± 0.20^{aA}	1.60 ± 0.547^{aB}	1.80 ± 0.836^{aB}	0.70 ± 0.30^{aA}	1.70 ± 0.447^{aB}	1.90 ± 0.836^{aB}
	14	1.40 ± 0547^{bA}	2.80 ± 0.447^{bB}	2.60 ± 0.547^{aB}	1.60 ± 0547^{aA}	2.90 ± 0.447^{bB}	2.70 ± 0.447^{aB}
	21	3.20 ± 0.837^{c}	3.80 ± 0.447^{c}	3.60 ± 0.547^{b}	3.50 ± 0.547^{b}	3.90 ± 0.447^{c}	3.70 ± 0.447^{b}
	28	3.80 ± 0.447^{cd}	4.0 ± 0.0^{cd}	4.0 ± 0.0^{bc}	3.80 ± 0.447^{b}	4.0 ± 0.0^{c}	4.0 ± 0.0^{b}
Collagen maturation	7	1.40 ± 0.547^{a}	1.40 ± 0.547^{a}	1.80 ± 0836^{a}	1.40 ± 0.547^{a}	1.60 ± 0.547^{a}	1.80 ± 0836^{a}
	14	1.80 ± 0.447^{a}	2.40 ± 0.547^{b}	2.40 ± 0547^{a}	2.40 ± 0.547^{b}	2.60 ± 0.547^{b}	2.50 ± 0547^{a}
	21	2.80 ± 0.447^{bA}	3.60 ± 0.547^{cAB}	3.80 ± 0.447^{bBC}	2.80 ± 0.447^{bA}	3.70 ± 0.447^{cAB}	3.80 ± 0.447^{bBC}
	28	3.80 ± 0.447^{c}	4.0 ± 0.0^{cd}	4.0 ± 0.0^{bc}	3.60 ± 0.447^{b}	4.0 ± 0.0^{c}	4.0 ± 0.0^{b}

Different superscripts (a, b, c, d) in the same column indicate significant differences among treatment days (P<0.05). Different superscripts (A, B, C) in the same line indicate significant differences among treatment groups in cortical and cancellous bone (P<0.05)

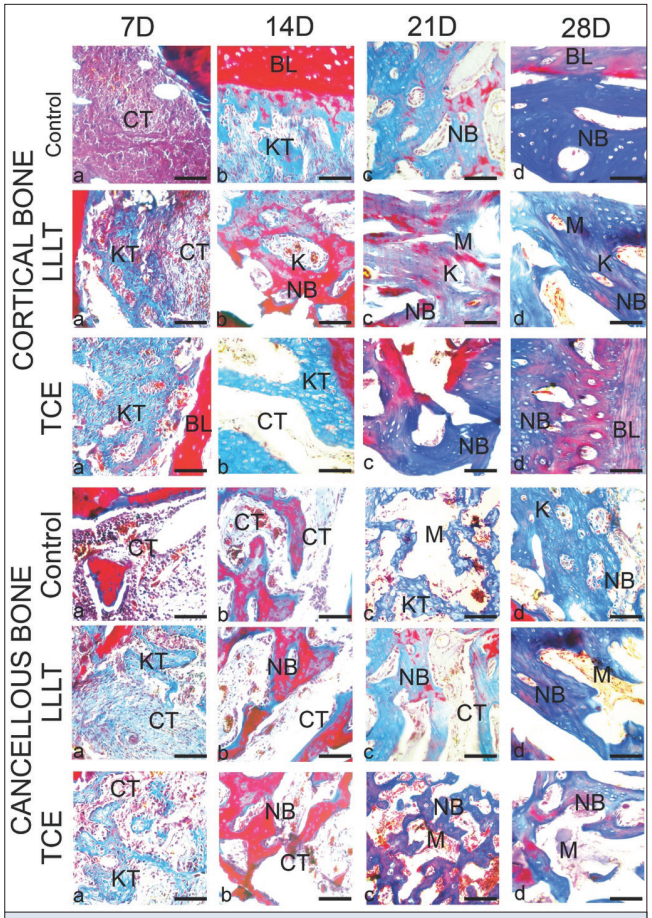

Fig 3. Histological sections of cortical and cancellous bone according to applications days after the bone defect is created**.** Cortical and Cancellous bone: repair process in bone tissue at 7 (a), 14 (b), 21 (c) and 28 (d) days in the control, LLLT and TCE groups. TCE groups; new bone tissue (NB) formed at the defect region, and observed cancellous bone matrix formation (M) and blood vessels (K) in the cortical bone. In the cancellous bone, on days 21 and 28, the defect is completely filled with new bone tissue (NB), the presence of bone marrow cells (M), and numerous capillaries in the TCE groups. CT; connective tissues, KT; cartilage tissue, BL; intact bone line, NB; new bone tissue, FT; fibrotic tissue, M; bone matrix formation and bone marrow cells, K; blood vessels. Crossman's triple stain, scale bars: 25 µm

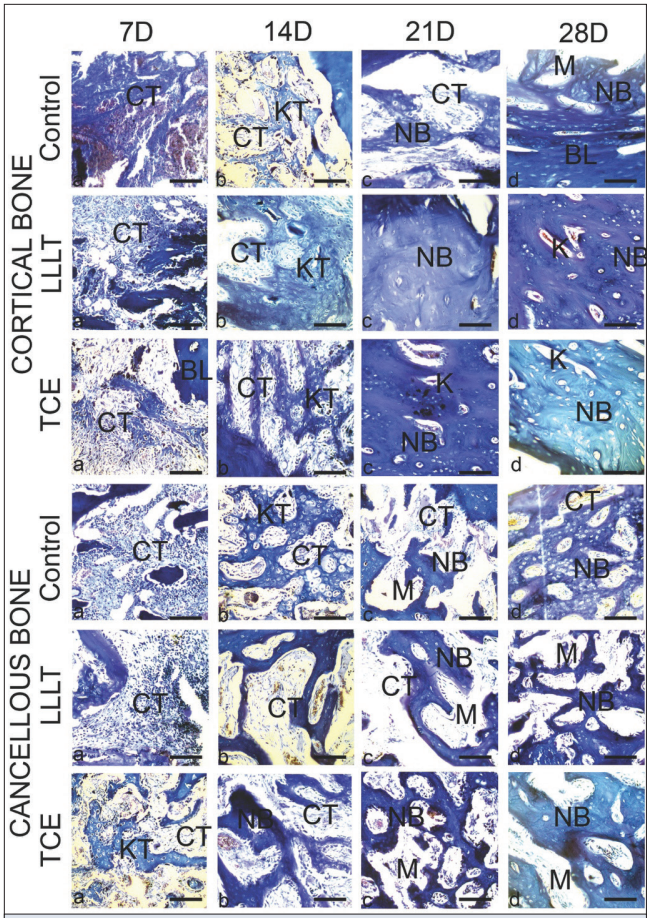

Fig 4. Histological sections of cortical and cancellous bone according to applications days after the bone defect is created. In both cortical and cancellous bones, the appearance of mineralized bone (M-light blue), calcification front (dark blue), and osteoids (K-light red) in the bone defect region during the repair on days 7, 14, 21, and 28 in the control, LLLT, and TCE groups. CT; connective tissue, KT; cartilage tissue, BL; bone line, K; osteoids, M: bone matrix, NB; new bone-mineralized bone. Solochrome cyanine R staining, Scale bars: 25 µm

or fibroplasia phase and bone remodeling phase, could be determined more easily by histopathological examination. It was noticed that compared to the control group, the LLLT group was found to be lower in terms of inflammatory cell density and to be higher bone tissue formation and collagen density as expected. In the study, it was also investigated whether TCE, a homeopathic agent whose effectiveness has been reported in many tissues other than bone, is effective and whether it can be an alternative to LLLT, which has equipment costs and difficulties in application. In the histopathological examination, bone tissue formation and quality 7th day and collagen maturation were significantly better on the 21st day in the TCE group compared to the control group. In the LLLT group, both cortical bone and cancellous bone showed histopathologically similar collagen density and bone tissue formation. These similarities observed in the study showed that TCE is at least as effective in bone tissue repair as LLLT.

Histopathologically, the increase in cell proliferation, osteoblastic activity, vascularization, new bone formation, and collagen deposition in bone tissue could accept as an indicator that TCE enables the amelioration of necrotic structures, a decrease of inflammatory cells and thus the healing of bone by accelerating osteogenesis [7-13]. This is also an indicator that LLLT has a photobiomodulatory effect on bone tissue [15,19,21]. In this study, the fact that bone tissue formation and quality were more prominent in the TCE and LLLT group on the 7th and 14th day than in the control group, and that collagen maturation was higher than in the control group on the 21st day. However, the great similarities between the TCE group and LLLT at all observation times indicate that the homeopathic regenerative effect of TCE is significant on bone tissue as

Table 3. *Intensity scores of osteopontin, bone morphogenetic protein-1, -2, -3, vascular endothelial growth factors, and its receptors (flt1/fms and flk1/KDR) expression in the cortical and cancellous bone defect regions during the applications*

Groups	Days	Cortical Bone							Cancellous Bone						
		OP	BMP-1	BMP-2	BMP-3	VEGF	Flt1/fms	Flk1/KDR	OP	BMP-1	BMP-2	BMP-3	VEGF	Flt1/fms	Flk1/KDR
Control	7	++	+	+	+	+++	+++	+++	+	+	+	+	+++	+++	+++
	14	++	+	++	+	++	++	++	++	+	++	+	++	++	++
	21	+++	++	+	++	+	+	+	+++	++	+	++	+	+	+
	28	+++	++	++	++	+	+	+	+++	++	++	++	+	+	+
LLLT	7	+	+	+	+	+++	+++	+++	+	+	+	+	+++	+++	+++
	14	++	+	+	+	++	++	++	++	+	+	+	++	++	++
	21	+++	++	+	++	+	+	+	+++	++	+	++	+	+	+
	28	+++	++	++	++	+	+	+	+++	++	++	++	+	+	+
TCE	7	+	+	+	+	+++	+++	+++	+	+	+	+	+++	+++	+++
	14	++	+	+	+	++	++	++	++	+	+	+	++	++	++
	21	+++	++	++	++	+	+	+	+++	++	++	++	+	+	+
	28	+++	++	++	++	+	+	+	+++	++	++	++	+	+	+

OP: *osteopontin,* ***BMP-1:*** *bone morphogenetic protein-1,* ***BMP-2:*** *bone morphogenetic protein-2,* ***BMP-3:*** *bone morphogenetic protein-3,* ***VEGF:*** *vascular endothelial growth factors,* ***flt1/fms:*** *fms related receptor tyrosine kinase 1,* ***flk1/KDR:*** *Tyrosine Kinase Growth Factor Receptor*

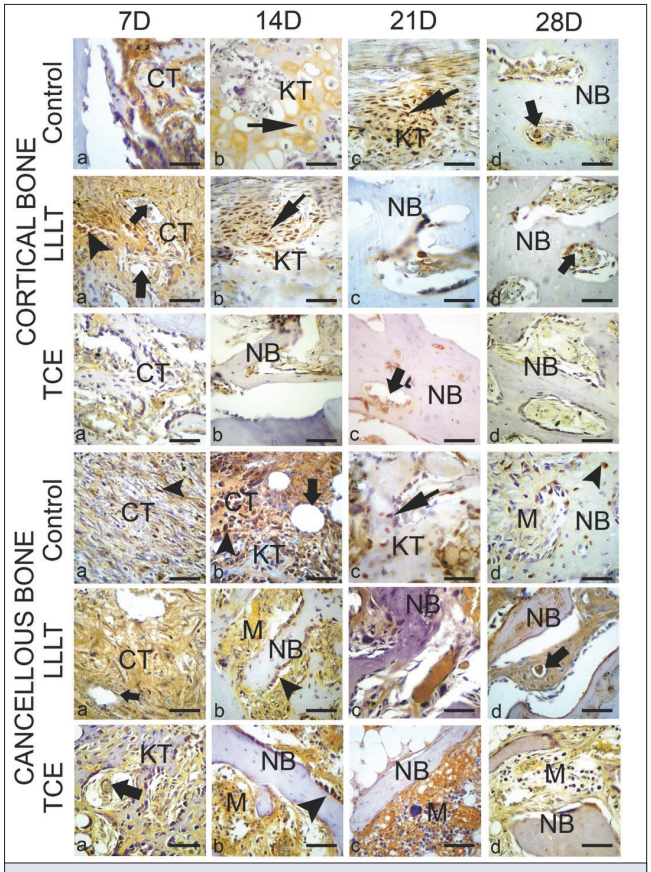

Fig 5. Immunohistochemical localization of vascular endothelial growth factor (VEGF) in the defect area and newly formed bone tissue according to the control, LLLT, and TCE groups in the cortical and cancellous bones. Expression of VEGF in connective tissue cells in the defect area and cartilage cells, osteoblasts, and osteocytes in newly formed bone tissue at 7, 14, 21, and 28 days of application. CT; connective tissue, KT; cartilage tissue, BL; bone line, M: bone marrow, NB; new bone-mineralized bone, K; blood vessel. Arrowhead; VEGF positive connective tissue cell, thin arrow; VEGF positive cartilage cell (chondrocytes or Chondroblast), thick arrow; VEGF positive osteocytes or osteoblast. Scale bars: 25 μm

well as in other tissues (tendons, skin, etc.). In addition, the fact that the inflammatory phase was less in the TCE and LLLT groups compared to the control group and that no inflammatory cells were observed on the 21st day may be evidence that the healing process starts more quickly with the anti-inflammatory effects of these applications.

Histopathological examination of bone tissue is important in terms of quantitative and functional determination of cells, analysis of structural changes in cortical and cancellous bone, and determining the borders of the bone healing cycle [11]. In clinical practice, histopathological examination by taking a biopsy sample from the fracture site or applying expensive high-resolution imaging techniques is not a very simple and convenient option in routine. Therefore, the clinician should be able to make this assessment with easier and more economical measures to determine the healing status. For this purpose, one of the most preferred methods to evaluate the fracture healing process is conventional radiography. Despite its advantages these radiographic features are not sufficient in the evaluation of clinical improvement (esp. mechanical strength), and quantitative evaluations are needed. On the other hand, it is known that there is a strong correlation between optical densitometric analysis and clinical evaluation in bone healing assessments performed on digital radiography [25,26]. For this reason, optical density analyzes have encouraged researchers to develop web-based methods, especially since they offer a quantitative evaluation opportunity and are non-invasive. Web-based assessment tools are a method that can generally be worked asynchronously, based on user-user interaction, offering various solutions for clinicians with different specialties, including orthopedic surgery. In the field of orthopedics,

Table 4. *Statistical data of proinflammatory cytokines and bone-specific markers measured by ELISA carried out on the serum samples*

Parameters	Groups	0. Day	7. Day	14. Day	21. Day	28. Day
TNF-α (pg/mL)	C	0.19 ± 0.06^{c}	0.43 ± 0.13^{Aa}	0.38 ± 0.17^{ab}	0.26 ± 0.12^{Bbc}	0.43 ± 0.12^{ABa}
	TCE	0.19 ± 0.06	0.23 ± 0.14^{B}	0.26 ± 0.13	0.22 ± 0.06^{B}	0.30 ± 0.08^{B}
	LLLT	0.19 ± 0.06^{b}	0.28 ± 0.14^{ABb}	0.34 ± 0.15^{ab}	0.52 ± 0.19^{Aa}	0.53 ± 0.15^{Aa}
IL-1β (pg/mL)	C	0.10 ± 0.04	0.23 ± 0.27	0.10 ± 0.02	0.16 ± 0.07	0.24 ± 0.10^{A}
	TCE	0.10 ± 0.04	0.07 ± 0.02	0.10 ± 0.05	0.11 ± 0.04	0.12 ± 0.04^{B}
	LLLT	0.10 ± 0.04^{b}	0.19 ± 0.07^{a}	0.12 ± 0.06^{b}	0.10 ± 0.03^{b}	0.09 ± 0.04^{Bb}
IL-6 (pg/mL)	C	3.08 ± 0.69^{b}	4.02 ± 1.0^{Aab}	5.23 ± 1.90^{a}	5.23 ± 2.10^{Aa}	5.82 ± 1.29^{a}
	TCE	3.08 ± 0.69^{b}	2.65 ± 0.5^{Bb}	3.75 ± 1.17^{b}	3.22 ± 0.67^{Bb}	5.87 ± 2.31^{a}
	LLLT	3.08 ± 0.69^{b}	4.10 ± 1.57^{Ab}	4.40 ± 1.20^{b}	4.12 ± 0.92^{ABb}	6.12 ± 1.46^{a}
OC (pg/mL)	C	0.38 ± 0.12	0.39 ± 0.16	0.52 ± 0.18	0.47 ± 0.13^{A}	0.38 ± 0.22
	TCE	0.38 ± 0.12	0.35 ± 0.11	0.30 ± 0.11	0.34 ± 0.06^{AB}	0.27 ± 0.05
	LLLT	0.38 ± 0.12	0.37 ± 0.15	0.33 ± 0.21	0.27 ± 0.08^{B}	0.28 ± 0.14
B-ALP (ng/mL)	C	3.21 ± 0.08^{a}	2.32 ± 0.72^{b}	2.31 ± 0.37^{Bb}	2.24 ± 0.35^{Cb}	2.37 ± 0.40^{b}
	TCE	3.21 ± 0.08^{a}	2.23 ± 0.36^{b}	2.49 ± 0.55^{ABb}	2.71 ± 0.31^{Bab}	2.60 ± 0.26^{b}
	LLLT	3.21 ± 0.08^{ab}	2.83 ± 0.48^{bc}	3.12 ± 0.36^{Aab}	3.40 ± 0.08^{Aa}	2.66 ± 0.34^{c}
PINP (ng/mL)	C	0.68 ± 0.80	0.68 ± 0.12	0.67 ± 0.07	0.69 ± 0.10	0.69 ± 0.14^{B}
	TCE	0.68 ± 0.80	0.76 ± 0.11	0.81 ± 0.28	0.91 ± 0.27	0.91 ± 0.23^{A}
	LLLT	0.68 ± 0.80^{ab}	0.68 ± 0.12^{ab}	0.77 ± 0.13^{a}	0.78 ± 0.20^{a}	0.55 ± 0.04^{Bb}

Tumor necrosis factor-alfa (TNF-α), Osteocalcin (OC), Interleukin 1-Beta (IL-1β), Interleukin-6 (IL-6), Procollagen I N-terminal peptide (PINP). The superscripts in the same line (a, b, c) and same column (A, B, C) are statistically significant (P<0.05)

telemedicine-style techniques are mostly performed on radiological images [25,26,31]. In contrast, Werkman et al.[5] performed optical density analysis with a free computer program called Image Tool (UTHSCSA Image Tool version 3.0) to evaluate the repair in the bone defect model they created in rats with osteoporosis, and they argued that the optical density analysis they performed did not take into account the differences between trabecular and lamellar structures in newly formed bone and did not fully explain the morphological course of the bone callus, since it added fibrous tissue to the analyzes, and therefore it was not significant in the evaluation of bone repair. Through a specially developed web-based program, it was determined the optical density in this study. In the study, the best group according to the results of ROD was the group in which TCE was applied, followed by the group in which LLLT was applied, and the group with the lowest ROD value was the control group. It was also observed that the ROD value in the cancellous bone was higher than in the cortical bone in all groups and on all follow-up periods. This elevation in optical density is generally thought to be related to the degree of mineralization of the callus after the reparative phase. Because bones hold 30-40 times more radiation than soft tissue. Since the mineralization level of a callus is determined by densitometry of x-ray images [32], it shows that an osseous callus is formed due to better mineralization in the TCE group. In addition, since these results show similarity with histopathological evaluations, contrary to Werkman et al.[5], optical density was thought to be useful in the evaluation of bone repair.

Clinically, fractures of the cancellous bone repair much more rapidly and have a different healing process compared to diaphyseal fractures in the cortical bone structure. While the periosteum is very important for the healing process in cortical bones, the trabeculae structures are important in cancellous bones. Compared to cortical bone fractures, cancellous ones have a larger internal contact surface area and greater blood supply capacity, allowing adequate fixation. In addition, the cancellous bone tissue is largely in contact with the bone marrow cells. Therefore, cancellous bone fractures have a tighter affinity with multiple sources of mesenchymal stem cells that can differentiate into the osteoclast-osteoblast cell line to promote cancellous bone healing [33,34]. Han et al.[33] reported in their study that less bone tissue necrosis, less hematoma formation, a more limited inflammation event, and no external callus were observed in the cancellous bone healing phase compared to cortical fractures. Moreover, unlike cortical bone healing, the healing cycle consists of five phases: hematoma or inflammatory phase, cell proliferation phase, secondary

bone formation phase, lamellar bone formation phase, and bone remodeling phase [33]. In the present study, it was not possible to make this histopathological distinction with precise limits since the evaluation was performed on only 4 different observation days (7, 14, 21, and 28 day). However, according to the histopathological evaluation data, the healing of the cancellous bone was better than the cortical bone, although it was not statistically significant. On the other hand, in terms of ROD values performed on radiological images, the mineralization level in the cancellous bone was found to be statistically better than in the cortical bone.

During the inflammatory phase of the fracture healing process, cellular debris and the fracture hematoma are bounded by a fibrous capsule consisting of irregular granulation tissue that is dense with type 3 collagen. At this early stage of bone healing, a small amount of type I collagen may be found in the callus in isolated areas close to the cortical bone. Collagen tissues are also found in high amounts in the primary callus and have very important functions, especially in the reparation phase of healing. Collagen types I, III, and V are more intense in the reparation phase, and collagen type I in the remodeling phase. Therefore, the presence of collagen tissue in tissue damage is very important for wound healing and healing quality [31,35,36]. In the present study, collagen tissue densities were evaluated both histopathologically and serologically in terms of PINP. Histopathologically, only their density in the repair phase was evaluated without making any distinction of collagen type. Accordingly, the collagen tissue density in both the cancellous and cortical bone defect areas in the TCE-treated group was found to be higher, although not significant, on the 7th day, but significantly better on the 21st day compared to the control group. Considering the PINP level, the TCE group showed an increase insignificant level of PINP on the 7, 14 and 21st days compared to the other two groups, but showed an increase significant level of PINP on the 28th day, which was consistent with the histopathological evaluation. Similar to its effects on tissues [11,12], it can be said that TCE accelerates repair in bone tissue. Moreover, the fact that collagen type I is more intense in the remodeling phase of the bone tissue [10,35] may be an indication of the completion of the healing phase in the TCE group.

Many inflammatory mediators, such as interleukins and TNF-α, are significantly elevated after fracture for several days. These pro-inflammatory mediators have a chemotactic effect on other inflammatory cells. After this process, angiogenesis occurs for fibrin deposition and a greater supply of nutrients. Vascular proliferation within the developing callus tissue is regulated by angiogenic factors such as FGF, VEGF, and angiopoietins. In the present study, the expression levels of VEGF and its receptors (Flt1/fms, Flk1/KDR), which are responsible for vascular proliferation, were examined immunohistochemically. Because the expression of VEGF is very important for endochondral bone healing [3,37,38]. As expected, the expression of VEGF and its receptors was observed at a higher level on the 7th day in both the cancellous and cortical bone defect areas. In the later phases, since these cells need to be expressed to provide the blood reserve required for repair activities [3,38], the expression continued, albeit at a low level. However, expression levels of VEGF and its receptors were similar between the groups at all follow-up periods (7, 14, 21, and 28 days), and no difference was found in terms of scoring. In the present study, VEGF and its receptors showed similar localizations and expressions in the defect areas shaped in new bone tissue, and the lack of relative differences in expression densities suggested that VEGF and its receptors together perform similar functions in the bone defect region in rabbits as mentioned above. However, further experimental studies are needed to reveal the precise physiological roles of VEGF and its receptors in bone tissue remodeling in rabbits.

Although activation of the fracture repair process appears to depend on an adequate pro-inflammatory response, the resolution of this inflammatory condition is critical for continued healing [39]. Pro-inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, which are molecular components that initiate the fracture healing process, first increase significantly for a few days after the fracture, and their levels decrease rapidly within 3 days. The fact that these cytokines are significantly high in the inflammatory phase is very important in terms of activating other inflammatory cells for the initiation of angiogenesis with fibrin deposition at the fracture line. In addition, they accelerate the healing and callus consolidation by being expressed again in the later stages of fracture healing, namely in the remodeling phase [3,39,40]. A situation that can be said to be a deficiency in this study is the lack of early postoperative period data. However, since the treatment started on the 3rd day at the earliest in our study, it is thought that the analysis of pro-inflammatory cytokines in this process would not make a difference between the groups. Therefore, it is not possible to comment on the approach of these cytokines to normal levels in the first 3 days. However, on the 7th day, TNF- α, one of these three pro-inflammatory cytokines, was found to be significantly higher in the control group compared to normal values (pre-op), whereas the other two cytokines were not. On the other hand, although the inflammatory cell density in the control group was not statistically significant on the 7th day, histopathologically, the fact that it was higher than in the other groups and the serologically higher TNF-α level could be evidence of the control group's continued

inflammation. During the remodeling phase of fracture healing, late cytokine expression accelerates healing and callus consolidation [39,40]. Therefore, it can be interpreted that repair of the bone is better in the LLLT group.

Since serum bone markers are less invasive and more economical than biopsies, they can be used to evaluate bone metabolism and diseases. These markers reveal bone formation, matrix degradation, and enzymatic activity of bone cells. Commercial serum markers are currently available for determining bone formation activity such as bone-specific alkaline phosphatase (B-ALP), procollagen type-I N-terminal propeptide (PINP), and osteocalcin (OC) [31,41]. Bone alkaline phosphatase (B-ALP), which are thought to play a role in bone formation and/or mineralization, is an osteoblastic ectoenzyme. Change in these enzyme levels, may be an indicator of collagen formation and bone formation in bones and osteoblast cells [15,41,42]. It was stated that B-ALP levels were higher in groups with better ossification with LLLT in bone injuries. In addition, it has been revealed that LLLT application modulates the inflammatory process and changes the level of osteoblasts and osteoclasts with alkaline phosphatase activity [15,39]. In contrast, Nissan et al.[29] suggested that LLLT on the bone defect created in the rat mandible was not different from the control group in terms of serum B-ALP levels at 1, 2, and 4 weeks. In the present study, serum B-ALP levels were observed to be significantly lower on days 7, 14, 21, and 28 compared to normal values (pre-op), except for days 14 and 21 in the LLLT group. It has been suggested that the observed early decrease in serum B-ALP activity may be related to the suppression of bone formation and/or mineralization during the bone remodeling phase in the defect area [41]. However, this situation does not show compatibility with the histopathological and radiological findings determined in the study. Therefore, it can be said that there was no significant change in terms of bone repair in this study, contrary to what is known, except for the group in which LLLT was applied serologically at the B-ALP level.

In conclusion, TCE may be a good alternative to LLLT in terms of accelerating bone repair. Thus, it could be an effective, easily applicable and relatively economical supportive treatment option in bone defects. In addition, since TCE accelerates the cancellous bone a little more than cortical bones, it could be said that the rehabilitation period may be shorter for cancellous bone defects. However, further studies are needed on the specific efficacy of TCE on VEGF, its receptors and BMPs.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author (S. Altan). The data are not publicly available due to privacy or ethical restrictions.

Conflict Interests Statement

The authors declare that they have no conflicting interests.

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Disclosure of Funding

None

Author Contribution

SA and HS conceived and supervised this study. SA, BEK, FA and RÇ completed the main experimental content. SA, HS, EA, BEK, and FA collected and analyzed data. SA wrote the first draft of the manuscript. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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Research Article

Comparison of the Effects of Ketamine-Diazepam, Tiletamine-Zolazepam and Propofol Infusion Anesthesia in Rabbits

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Abstract: In this study we evaluated the anesthetic effects of ketamine-diazepam (K-D), tiletamine zolazepam (T-Z), and propofol (P) infusion for one h in rabbits, and also the impact of these anesthetics on physiological and biochemical parameters, and the tissue structure of the liver, kidney, and lungs. 18 New Zealand white rabbits were allocated to three administration groups. All groups were premedicated with xylazine. For induction, ketamine and diazepam were administered intravenously in group K-D, tiletamine-zolazepam combination was administered in group T-Z, and propofol was administered in group P. After induction, infusion anesthesia was continued for one h by intravenously ketamine and diazepam in group K-D, tiletamine-zolazepam combination in group T-Z, and propofol in group P. The respiratory rate significantly decreased in the P group at 30, 45, and 60 min compared to the initial values ($P<0.05$). End-tidal CO_2 significantly increased in the P group at 30, 45, and 60 min compared to the initial values ($P<0.05$). All physiological and serum biochemical parameters were within the reference ranges. Infusions resulted in varying degrees of degenerative changes in the kidney, lung, and liver, but these changes did not affect the anesthesia status or biochemical parameters. We suggest that K-D, T-Z, and P can be applied safely as an infusion for one h anesthesia of rabbits as an alternative to traditional anesthesia regimens.

Keywords: *Infusion anesthesia, Ketamine-diazepam, Tiletamine-zolazepam, Propofol, Rabbit*

Tavşanlarda İnfüzyon Tekniği ile Ketamine-Diazepam, Tiletamine-Zolazepam ve Propofol Anestezi Uygulamalarının Etkilerinin Karşılaştırılması

Öz: Bu çalışmada tavşanlarda bir saat süreyle uygulanan ketamin-diazepam (K-D), tiletamin zolazepam (T-Z) ve propofol (P) infüzyonunun anestezik etkilerinin yanı sıra bu anesteziklerin karaciğer, böbrek ve akciğer dokularının üzerindeki etkilerini fizyolojik ve biyokimyasal parametreler yönünden değerlendirdik. 18 adet Yeni Zelenda tavşanı rastgele üç gruba ayrıldı. Tüm gruplarda, sedasyon için xylazine HCl, kas içi uygulandı. İndüksiyon için intravenöz olarak K-D grubuna ketamin-diazepam kombinasyonu, T-Z grubuna tiletamin-zolazepam kombinasyonu, P grubuna ise propofol uygulandı. İndüksiyondan sonra, intravenöz olarak K-D grubunda ketamin ve diazepam, T-Z grubunda tiletamin-zolazepam kombinasyonu ve P grubunda propofol uygulanarak infüzyon anestezisine bir saat boyunca devam edildi. Solunum hızı P grubunda başlangıç değerlerine göre 30., 45. ve 60. dakikada anlamlı olarak azaldığı görüldü ($P<0.05$). End-tidal CO_2, P grubunda başlangıç değerlerine göre 30., 45. ve 60. dakikada artış gösterdi ($P<0.05$). Her üç grupta da bir saatlik infüzyon süresinden sonra tüm fizyolojik ve serum biyokimyasal parametreleri referans aralıklarındaydı. İnfüzyonlar böbrek, akciğer ve karaciğerde değişen derecelerde dejeneratif değişikliklere neden oldu, ancak bu değişiklikler anestezi durumunu veya biyokimyasal parametreleri etkilemedi. Geleneksel anestezi protokollerine alternatif olarak tavşanların bir saatlik anestezisi için infüzyon olarak K-D, T-Z ve P' nin güvenle uygulanabileceğini öneriyoruz.

Anahtar sözcükler*: İnfüzyon anestezisi, Ketamin-diazepam, Tiletamin-zolazepam, Propofol, Tavşan*

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Introduction

Anesthesia is required for many animal experiments and is often provided by the injectable anesthetics such as ketamine, tiletamine, or propofol. These agents are also used for the anesthesia of rabbits [1]. Nevertheless, the effects of administering these drugs as infusions in rabbits remain unclear.

Potential intra- and postoperative mortality have been observed with various anesthetic drugs and therefore developing safe and effective anesthesia methods has often been difficult in rabbits. Rabbit anesthesia has always been considered a high-risk procedure, however, when done properly, the risks are low and do not outweigh the benefits. Rabbits are common companion animals widely used in experimental surgeries and biomedical research [2].

Ketamine and xylazine are two widely used anesthetics. Ketamine, a dissociative anesthetic, produces a state of cataleptic sedation and is usually administered in combination with xylazine or diazepam for inducing surgical anesthesia in rabbits [3-5]. Animals have been successfully immobilized with ketamine hydrochloride and sedative drugs. Inadequate muscle relaxation, convulsions, and prolonged recovery periods are the most common side effects of these drugs in animals. Diazepam is frequently used in anesthesia due to its muscle relaxant effect. When this positive effect is used together, it is beneficial to eliminate the cataleptic effects of ketamine [6-8].

Tiletamine and zolazepam have dissociative anesthetic and tranquilizing properties, respectively. Tiletamine-zolazepam has been used alone or in combination with other anesthetic agents in rabbits. Tiletamine does not relax the muscles or affect the cranial nerve and spinal reflexes, however the combination with zolazepam results in muscle relaxation [6]. This combination is commonly used as part of the anesthetic management of domestic animals. But, tiletamine is not an anesthetic substance with an antidote such as ketamine [9,10].

Propofol is a lipophilic anesthetic used widely for the induction and maintenance of anesthesia in humans and animals. It has acquired worldwide acceptance because of its rapid onset, short duration of action and clinical effect, and quality and rate of recovery. Its pharmacokinetics in most species makes it the preferred agent for induction and continuous infusion. It can maintain stable anesthesia for prolonged periods and the recovery is rapid [11,12].

In veterinary medicine, there is no alternative method to the intramuscular dissociative anesthesia for medium-term anesthesia (45 min-1 h) in rabbits under field conditions. The most significant disadvantage of dissociative anesthetics is that they do not have an antidote for use when complications occur. During monitorization of the infusion anesthesia, when deep anesthesia appears reducing the dose of anesthetic will prevent complications [13].

This study compares the anesthetic effects of intravenous infusion of ketamine-diazepam, tiletamine-zolazepam, and propofol for one h to create medium-term anesthesia in rabbits. We also investigated the effects of these anesthetics on the physiological, hepatic and renal biochemical parameters, and the histology of the liver, kidney, and lungs.

Materials and Methods

Ethical Statement

The study was started after the approval of the Ethics Committee on Animal Research of Bursa Uludag University (Approval No: 2021-03-01)

Animals

In this study we applied three anesthesia protocols on 18 clinically healthy male adolescent New Zealand white rabbits weighing 2.30-2.90 kg. The rabbits were single-housed in cages for at least 14 days for acclimatization before the experiments and were given commercial pellet food (Purina®) and water *ad libitum*. Before initiating the experiments, the animals were fasted for 12 h and water was removed 2 h in advance. The rabbits were randomly assigned to one of the three groups: Ketamine-diazepam (K-D), tiletamine-zolazepam (T-Z), and propofol (P) with six rabbits in each group.

Anesthesia and Monitorization Period

On the day of the experiment, rabbits were weighed and transferred from the housing room to the operating room using a covered pet carrier. The catheters (Vasofix®, 24-gauge, B. Braun, Istanbul, Turkey) were applied to the lateral ear veins for taking blood samples and for intravenous infusion administration. Rabbits were immobilized in sternal recumbent position, supplied with blow-by-nose oxygen, and sedated with xylazine (5 mg/kg IM, Rompun®, Bayer, Leverkusen, Germany) injected into the longissimus muscle. The following indicators determined sedation: absence of pedal withdrawal reflex in response to a toe pinch, stable reduced frequency of respiration (from more than 150 breaths per min to 30-60 evenly paced breaths per min), reduction of smooth muscle tone, and loss of righting reflex when placed in a lateral recumbent position. Once sedation was confirmed, rabbits were transferred to lateral position on the pad with a heating feature to maintain body temperature. One of the catheters was used for the anesthetic infusion when animals were completely sedated. The other catheter was used to provide warmed 0.9% sodium chloride at a dose of 10 mL/kg/h during anesthesia. First blood samples for the measurement of enzyme levels were obtained before infusion. Blood was placed into

heparinized tubes and centrifuged (Minifuge RF, Heraeus, Hannover, Germany) at 1200 g at 4°C for 20 min. Plasma was separated and stored frozen at -20°C until assayed. The second blood sample was taken with a similar procedure after the termination of infusion anesthesia.

For induction, 10 mg/kg ketamine (Alfamine®, Alfasan, Woerden, The Netherlands) and 0.25 mg/kg diazepam (Diazem®, Deva, Istanbul, Turkey) were administered intravenously in group K-D, 10 mg/kg tiletamine-zolazepam (Zoletil®, I.E. Ulagay, Istanbul, Turkey) combination was administered in group T-Z, and 10 mg/kg propofol (Propofol®, Fresenius Kabi, Austria) was administered in group P to achieve induction. All rabbits were then intubated via the orotracheal route with a size 2.5 cuffed endotracheal tube (Bicakcilar, Istanbul, Turkey) after the disappearance of the swallowing reflex by using the premature blade. After intubation, the animals were connected to the anesthesia device (Espire®, General Electric, Boston, MA, USA), and only oxygen ventilation was provided. After that, the infusion of the anesthetic substance was started by an infusion pump (Infusomat® Space - B. Braun Medical Inc, Melsunger, Germany). The infusion pump was operated for one h to administer the anesthetics determined for each group. For infusion administration, 25-30 mg/kg/h ketamine and 2.5-3.0 mg/kg/h diazepam were administered intravenously in group K-D, 25-30 mg/kg/h tiletamine-zolazepam combination was administered in group T-Z, and 30-50 mg/kg/h propofol was administered in group P for one h. Separate syringe infusion pumps were used for each drug for the suitable dose adjustment.

Monitoring devices were attached after induction, but before the infusion of anesthesia. The end-tidal CO_2 collector of the monitor (Cardiocap 5®, Datex-Ohmeda, Helsinki, Finland) was placed between the endotracheal tube and the anesthesia device's Y pipe. Then, the end-tidal CO_2 value was measured. At the same time, oxygen saturation (SpO_2) probe of the monitor was placed on the rabbit's tongue, and the saturation value was measured. Heart rate was determined from a lead II ECG recording by ECG electrodes (3M®, Istanbul, Turkey). Electrodes were placed on the skin on the medial aspect of the rabbits' upper forelegs and left hind leg. The respiratory rate was recorded along with the end-tidal CO_2 value. A rectal thermometer measured body temperature. Heart rate, respiration rate, SpO_2, and end-tidal CO_2 were recorded every 15 min using the monitor during anesthesia.

The depth of anesthesia was monitored utilizing pedal withdrawal, ear pinch, palpebral, and corneal reflexes. When assessing these reflexes, any movement was considered a positive response. A desired surgical plane of anesthesia was determined by the absence of a corneal or laryngeal response under maintenance of independent diaphragmatic breathing.

The anesthetic drug infusion was terminated after one h. After the infusion was completed, 1 mL of blood sample was taken for blood serum analysis. The levels of BUN, creatinine, AST, and ALT were determined from serum samples. Euthanasia was established by rapid intravenous administration of six times the induction dose of the drugs administered in each group.

Histopathological Examination

After euthanasia, tissue samples from the liver, kidneys, and lungs were taken into neutral, buffered formalin from all rabbits for histopathological examination. After routine tissue processing, 4 μm sections were cut and stained with hematoxylin&eosin. Four randomly selected areas were examined in each section under x400 magnification. Livers were examined for hepatocellular degeneration, and the presence of sinusoidal or portal inflammatory cell infiltrations. Kidneys were evaluated for tubular degeneration and inflammatory cell infiltrates. The lung tissues were examined for hyperemia, intraalveolar edema and hemorrhages, presence of inflammatory cells, and atelectasis. All parameters were scored as 1: mild, 2: moderate, and 3: severe. Other significant findings in the organs were also noted.

Statistical Analysis

Data for plasma BUN, creatinine, ALT, AST concentrations, and heart and respiratory rates, end-tidal CO_2, SpO_2 levels were analyzed using a one-way analysis of variance (ANOVA) and then Tukey's HSD post hoc test for equal variences with repeated measures in each group to assess changes with time, followed by Dunnett's *t*-test when a significant difference was indicated. Scores of the histopathological changes were analyzed using ANOVA. Differences were considered significant when $P<0.05$. A statistical software was used for the calculations (SPSS v22.0 Statistical Software, IBM Corp, CA, USA).

Results

All anesthetized animals survived the one-h infusion anesthesia. In each group, unconsciousness occurred in a brief time (42±8 sec [mean±SD]) with intravenous induction doses. No additional doses were needed in any group for induction.

Physiological Parameters During Infusion

The mean heart rate (beats per min) was lower in the T-Z group at the beginning of the infusion period than in the K-D group and in the P group. The heart rate increased slightly until the 30th min and returned to the initial value at the 60th min in the K-D group, whereas a slight decrease was observed over time in other groups ($P>0.05$). In any of the groups, the changes in heart rate over time could not be statistically substantiated ($P>0.05$) *(Table 1)*.

The respiratory rate significantly decreased in the P group at 30, 45, and 60 min compared to the initial values (P<0.05); the most significant decrease being observed at 60 min. There was no significant difference in the respiratory rates during the infusion period in the K-D and T-Z groups *(Table 1)*.

End-tidal CO_2 significantly increased in the P group at 30, 45, and 60 min compared to the initial values (P<0.05). There was no significant difference in oxygen saturation (SpO_2) during the infusion periods in any of the anesthesia groups (P>0.05) *(Table 2)*.

Reflexes Monitored

Anesthesia evaluation was based on reflex examinations without surgical intervention. Pedal withdrawal, ear pinch, and palpebral reflexes were abolished entirely in all groups. When a slight presence of the corneal reflex was observed, the infusion doses of the anesthetics was increased to their upper limit, maintaining the depth of anesthesia to the desired level.

Body Temperature

No significant changes were observed and the body temperature remained between 37.8-39.4°C in all rabbits.

Biochemical Parameters

The results of the plasma biochemical parameters are shown in *Table 3*. The hematological findings were within the reference values in all groups (P>0.05).

Table 1. *Heart rate and respiratory rate at various times in the ketamine-diazepam, tiletamine-zolazepam, and propofol groups during infusion periods **

Time (min)	Heart Rate (beats per min)			Respiratory Rate (breaths per min)		
	K-D (n=6)	T-Z (n=6)	P (n=6)	K-D (n=6)	T-Z (n=6)	P (n=6)
0	171.17±15.33	149.17±20.63	189.33±6.90	41.17±4.28	42.33±3.63	48.67±1.45[a]
15	173.17±13.73	145.00±19.01	183.33±7.98	40.33±4.84	43.33±5.58	48.67±2.33[a]
30	183.17±11.60	135.17±16.26	173.83±8.80	43.17±4.19	40.17±1.74	41.17±1.89[a,b]
45	179.17±6.83	126.67±10.43	172.17±5.91	40.33±2.74	39.17±2.22	42.17±2.22[a,b]
60	168.50±9.58	120.17±9.80	169.50±5.43	41.00±4.90	39.00±2.82	37.83±2.25[b]

** Data presented as mean ± SEM. Different superscripts within a column indicate a significant difference among the anesthesia groups (P<0.05).* ***K-D:*** *ketamine-diazepam,* ***T-Z:*** *tiletamine-zolazepam,* ***P:*** *propofol*

Table 2. *End-tidal CO_2 and SpO_2 (saturation) at various times in the ketamine-diazepam, tiletamine-zolazepam, and propofol groups during infusion periods**

Time (min)	End-Tidal CO_2 (mmHg)			SpO_2		
	K-D (n=6)	T-Z (n=6)	P (n=6)	K-D (n=6)	T-Z (n=6)	P (n=6)
0	25.67±4.00	26.67±2.30	27.50±1.48[a,b]	96.17±1.01	97.50±1.72	91.16±1.25
15	24.33±4.35	25.50±2.88	25.50±1.43[a]	96.00±1.34	93.33±3.32	91.33±1.56
30	25.83±3.56	24.17±4.12	31.83±1.56[b,c]	93.00±2.51	94.50±2.08	90.83±1.51
45	28.17±3.56	26.00±4.59	35.00±1.61[c]	95.17±1.40	95.67±2.39	93.67±1.05
60	30.50±4.25	25.50±5.30	36.33±1.80[c]	94.00±2.06	96.50±2.01	94.50±1.72

** Data presented as mean ± SEM. Different superscripts within a column indicate a significant difference among the anesthesia groups (P<0.05).* ***K-D:*** *ketamine-diazepam,* ***T-Z:*** *tiletamine-zolazepam,* ***P:*** *propofol*

Table 3. *Plasma ALT, AST, BUN, CRE concentrations, and BUN/CRE ratio in the ketamine-diazepam, tiletamine-zolazepam, and propofol groups before and after the infusion period**

Group/Time (n=6)		ALT (IU/L)	AST (IU/L)	BUN (mg/dL)	CRE (mg/dL)	BUN/CRE	Refereence Range ALT/AST/BUN/CRE
K-D	0 min	29.67±1.65	27.0±1.12	16.61± 1.29	0.84±0.04	19.80±1.32	(10-27)/(9-34)/(12-22)/(0.8-2.6) [14,15]
	60 min	25.03±1.59	28.17±1.72	16.40± 1.35	0.76±0.02	21.13±1.35	(10-27)/(9-34)/(12-22)/(0.8-2.6) [14,15]
T-Z	0 min	17.67±1.67	31.33±3.92	18.31± 1.86	0.71±0.12	33.35±10.39	(10-27)/(9-34)/(12-22)/(0.8-2.6) [14,15]
	60 min	17.50±4.10	32.17±3.30	18.78± 2.15	0.86±0.11	22.75±2.54	(10-27)/(9-34)/(12-22)/(0.8-2.6) [14,15]
P	0 min	36.33±3.29	20.13±2.32	20.13± 2.32	0.89±0.03	22.48±1.99	(10-27)/(9-34)/(12-22)/(0.8-2.6) [14,15]
	60 min	33.0±3.26	21.35±2.50	21.35± 2.50	0.82±0.04	25.38±2.28	(10-27)/(9-34)/(12-22)/(0.8-2.6) [14,15]

** Data presented as mean ± SEM. A column without superscripts indicates no significant difference among the anesthesia groups.* ***K-D:*** *ketamine-diazepam,* ***T-Z:*** *tiletamine-zolazepam,* ***P:*** *propofol,* ***ALT:*** *alanine aminotransferase,* ***AST:*** *aspartate aminotransferase,* ***BUN:*** *blood urea nitrogen,* ***CRE:*** *creatinine*

Histopathological Findings

Histopathology scores and the results of statistical analysis are shown in *Table 4*. Briefly;

Kidney: In all groups, varying degrees of vacuolar degeneration in the tubules and inflammatory cell infiltrates in the intertubular regions were observed.

Table 4. *Scores (mean ± SEM) and the comparison of the histopathological changes in the kidney, lung, and liver after the administration of ketamine-diazepam, tiletamine-zolazepam, and propofol*

Group (n=6)	Liver			Kidney		Lung			
	Vacuolar Degeneration	Sinusoidal Infiltration	Infiltration in Portal Areas	Tubular Degeneration	Infiltration	Hyperemia	Intraalveolar Edema and Hemorrhage	Infiltration	Atelectasis
K-D	1.50±0.22[a]	1.17±0.17[a]	2.00±0.00[a]	1.17±0.17[a]	1.17±0.17[ab]	1.83±0.40[a]	1.33±0.21[a]	1.83±0.17[a]	1.67±0.42[a]
T-Z	2.67±0.21[b]	2.33±0.21[b]	2.67±0.21[b]	2.00±0.26[b]	1.17±0.21[b]	1.00±0.00[b]	1.17±0.17[a]	1.50±0.22[a]	1.00±0.00[a]
P	1.67±0.33[a]	1.00±0.00[a]	1.83±0.17[a]	1.50±0.22[ab]	1.00±0.00[a]	1.67±0.21[ab]	1.83±0.30[a]	2.00±0.00[a]	1.33±0.21[a]
P Value	0.014	0.000	0.004	0.051	0.022	0.090	0.149	0.116	0.255

[a,b,c] *Values within a column with different superscripts differ significantly at P<0.05.* ***K-D:*** *ketamine-diazepam,* ***T-Z:*** *tiletamine-zolazepam,* ***P:*** *propofol*

Liver: Significantly more severe vacuolar degeneration was observed in the livers of the T-Z administered animals than in the K-D and P administered animals. It was determined that hepatocytes were swollen and sinusoids were narrowed in these animals. In all groups, vacuolar degenerations affected hepatocytes especially around the vena centralis, while hepatocytes close to the portal area had a healthier appearance *(Fig. 1)*. In terms of inflammatory cell infiltrates in the sinusoids, the animals in the T-Z group were more severely affected. The infiltrates were predominantly composed of lymphocytes. Inflammatory cell infiltrations in the portal areas were observed in almost all groups, but the infiltration severity was higher in the T-Z group than in the other groups. Multifocal necroses were observed in the liver parenchyma in two animals administered T-Z.

When evaluated in terms of both tubular degeneration and inflammatory cell infiltrations in interstitial areas, the effect was greater in animals treated with T-Z. It was also noted that in some areas the tubular epithelium was swollen, tubule epithelium nuclei were karyorhectic and karyolytic, and the tubule lumens were narrowed *(Fig. 2)*.

Lung: The vessels were hyperemic in all groups and the hyperemia was more prominent in the K-D and P groups than in T-Z group. No significant differences were observed regarding the edema and intraalveolar hemorrhage, interalveolar septal infiltration, and atelectasis scores among the groups. Besides these parameters intra-alveolar macrophages *(Fig. 3)*, hemosiderinophages and emphysematic areas were observed in all groups.

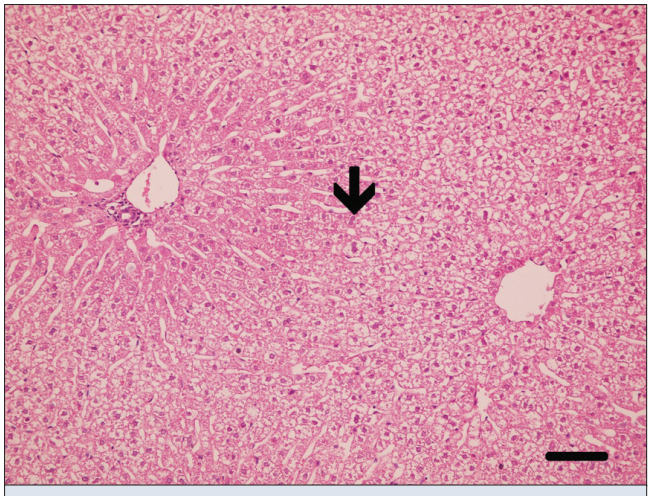

Fig 1. Vacuolar degeneration was the most significant change in the livers of all groups. Hepatocytes especially around vena centralis were more severely affected *(right and bottom of the arrow)*, while hepatocytes close to the portal area *(left of the arrow)* were less affected. Animal from the T-Z group, hematoxylin&eosin staining, bar=200 µm

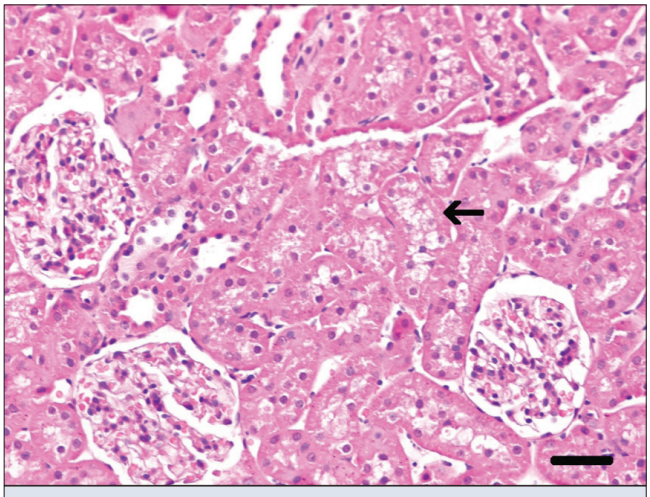

Fig 2. Tubular degeneration of varying degrees was observed in all groups. Cytoplasms of the tubular epithelium are vacuolar and some nuclei are karyolytic *(arrow)*. Lumens are narrowed due to the swollen tubular epithelium. Animal from the T-Z group, hematoxylin&eosin staining, bar=100 µm

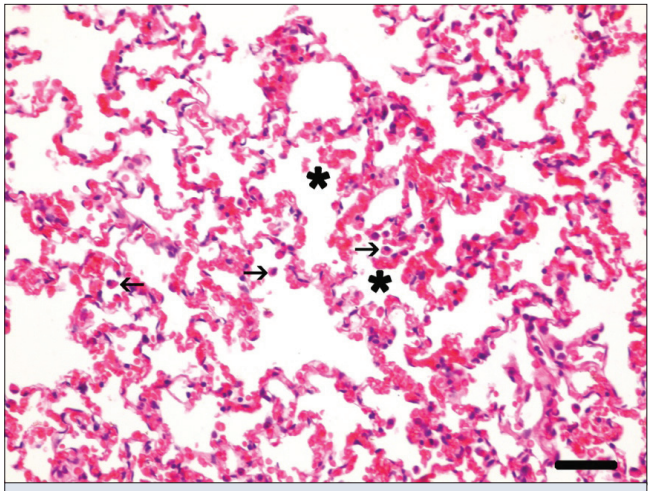

Fig 3. Hyperemia of the interalveolar capillaries was the most significant change in all groups. Intraalveolar hemorrhages *(*)* and intraalveolar macrophages *(arrows)* were also observed in some animals. Animal from the K-D group, hematoxylin&eosin staining, bar=100 µm

Discussion

Anesthetics may affect the physiological parameters, structure, and function of organ and biological systems, and these effects have been extensively studied. However, there is limited information about the effects of the infusion anesthetics in rabbits. We applied three different infusion anesthesia protocols and the results showed no negative effects on hematological and biochemical values and only minor changes on tissue structure of the liver, kidney, and lungs.

In experimental studies in rabbits, the effects of intramuscular ketamine and tiletamine anesthesia on different parameters were investigated [4,6,9,13]. Numerous studies present data from infusion anesthesia with propofol. Results of previous studies suggest that the necessary infusion rate for propofol in rabbits varies from 24-100 mg/kg/h [16,17]. We found that the propofol infusion rate of 30-50 mg/kg/h was sufficient to provide an optimal anesthesia level. Only in two rabbits corneal reflex was present, revealing superficial anesthesia, and the anesthetic dose was increased to 50 mg/kg/h.

Since the doses administered for induction were sufficient in all anesthetic groups, orotracheal intubation was achieved without the aid of topical lidocaine by using a disposable plastic endotracheal tube in our study. Then, the rabbits assumed lateral recumbency.

Heart rates were well-maintained in rabbits in the K-D and T-Z groups and there was no need for any anticholinergic drugs in any group. The heart rate decreased slightly over time in all groups, but the difference could not be substantiated statistically.

Ketamine or tiletamine administration increases heart rate in animals [10]. It has been reported that the slightly higher heart rate in the K-D and T-Z groups is due to the heart frequency-enhancing effects of the enhanced vagal activity of dissociative anesthetics-induced sympathomimetic effect [18]. In our study the heart rate increased slightly until the 30th min and returned to a slightly below initial value at the 60th min in the K-D group, while a slight gradual decrease was observed in the T-Z group. The difference between the aforementioned studies and our study can be due to the administration route of the anesthetics. While the anesthetics were used as a single dose intramuscularly in the studies mentioned, we applied the anesthetics in the form of an infusion for one h in our study.

An Another study revealed that propofol reduces sympathetic tone more than the parasympathetic tone, thus resulting in bradycardia from an unopposed parasympathetic response [19]. Heart rates were slightly lower in rabbits in the P group than in the K-D and T-Z groups throughout our study, but the statistical difference was insignificant.

Respiratory rates did not change in the K-D and T-Z groups from the beginning to the end of the infusion. However, a significant decrease in respiratory rate was observed from the 30th min of infusion in the P group, which affected the end-tidal CO_2 level in this group. The oxygen saturation did not change significantly over time in any of the groups.

Varying degrees of histopathological changes were observed in liver, kidney, and lung after all infusions. In the liver, T-Z infusion resulted in significantly more damage than in the other groups, as demonstrated by more severe vacuolar degeneration and inflammatory cell infiltrates both in the sinusoids and portal areas. In the kidney T-Z infusion resulted in slightly more severe changes than in the other groups. In the lungs all infusions resulted in similar edema and intraalveolar hemorrhage, interalveolar septal infiltration, and atelectasis scores. However, none of these organ changes unfavorably affected the anesthesia or biochemical parameters.

As a result of our study, we believe that ketamine-diazepam, tiletamine-zolazepam, and propofol can be safely administered as an intravenous infusion in healthy rabbits as an alternative to the single-dose intramuscular administration according to the hematological, serum biochemical and histopathological data.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author (O. Ates) on reasonable request.

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Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

AT, NYGS, OA and EMU planned the study, designed the experiments and helped manuscript writing; AT, NYGS, OA and EMU performed the this study; OA, EMU and OY collected blood and pathological samples and conducted laboratory process. ITC and OY worked on histopathological examination and writing phase. EMU analysed the statistics data. All authors read and approved the final manuscript.

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Research Article

Identification of the ERV Insertion of *APOB* Gene and Deletion of *TFB1M* Gene Associated with Lethal Haplotypes of Holstein Cattle Reared in Balıkesir Province, Türkiye

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Abstract: Cholesterol deficiency (CDH) and lethal haplotype 5 (HH5) are autosomal recessive genetic defects of Holstein cattle. HH5 and CDH are associated with embryonic mortality and calf loss, respectively, resulting in severe economic losses in the dairy cattle breeding. The aim of the present study was to investigate the presence of HH5 and CDH haplotypes and to determine the carrier and haplotype frequencies for these haplotypes in Holstein cattle reared in Balıkesir province of Türkiye. Polymerase chain reaction (PCR) was used to detect the deletion of the transcription factor B1 *(TFB1M)* gene and the retroviral insertion into the apolipoprotein B *(APOB)* gene, causing CDH and HH5, respectively. A total of 450 Holstein cows born between 2011 and 2018 were screened for causative mutations of both haplotypes. As a result, the carrier frequencies for CDH and HH5 were 4.67% and 7.56% and the haplotype frequencies were 0.023 and 0.038, respectively. 12.22% of the cows were identified to be carriers of at least one genetic defect. This study demonstrated the presence of CDH and HH5 haplotypes in Holstein cattle reared in Türkiye.

Keywords: *Holstein, Cholesterol deficiency, Lethal haplotype 5, Genetic defects*

Balıkesir'de Yetiştirilen Holştayn Irkı Sığırlarda Letal Haplotiplerle İlişkili *APOB* Geni ERV İnsersiyonu ve *TFB1M* Geni Delesyonunun Belirlenmesi

Öz: Kolesterol eksikliği (CDH) ve letal haplotip 5 (HH5) Holştayn ırkı sığırlarda görülen otozomal resesif genetik kusurlardır. HH5 ve CDH süt sığırı yetiştiriciliğinde önemli ekonomik kayıplara neden olan embriyonik ölüm ve buzağı kayıpları ile ilişkilidir. Bu çalışmanın amacı, Balıkesir'de yetiştirilen Holştayn ırkı sığırlarda HH5 ve CDH haplotiplerinin varlığını araştırmak, taşıyıcı ve haplotip frekanslarını belirlemektir. HH5 ve CDH'ye neden olan transkripsiyon faktörü B1 *(TFB1M)* geni delesyonu ile apolipoprotein B *(APOB)* geni retroviral insersiyonunu tespit etmek için polimeraz zincir reaksiyonu (PCR) kullanılmıştır. 2011-2018 yılları arasında doğan Holştayn ırkı toplam 450 inek haplotiplerin nedensel mutasyonları yönünden taranmıştır. Sonuçta, CDH ve HH5 için taşıyıcı frekansları sırasıyla %4.67 ve %7.56 ve haplotip frekansları 0.023 ve 0.038 olarak tespit edilmiştir. İneklerin %12.22'sinin en az bir genetik kusurun taşıyıcısı olduğu belirlenmiştir. Bu çalışma, Türkiye'de yetiştirilen Holştayn ırkı sığırlarda CDH ve HH5 haplotiplerinin varlığını ortaya çıkarmıştır.

Anahtar sözcükler: *Holştayn, Kolesterol eksikliği, Letal haplotip 5, Genetik kusurlar*

Introduction

In modern dairy cattle breeding, intensive selection and the widespread use of elite sires have led to significant genetic improvement in economically important traits, but also to increased inbreeding and reduced genetic diversity and survival traits worldwide. The accumulation of inbreeding has increased the frequency of lethal recessive alleles, resulting in a higher incidence of genetic defects in dairy cattle populations [1-3].

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The large-scale genomic data obtained as a result of the routine use of single nucleotide polymorphism (SNP) array genotyping in dairy cattle breeding programs over the last decade has enabled the identification of recessives. Lethal recessives can be discovered from haplotypes that, although common in the population, are never seen in the homozygous state in living animals [4-6]. After the discovery of the first three haplotypes (HH1, HH2, HH3) in the American Holstein in 2011 [4], 43 haplotypes (HH0-HH38, HHB, HHC, HHD, CDH) with reduced homozygosity have been identified in different Holstein populations so far [6]. Sixteen of these haplotypes, including CDH and HH5, have been listed in the Online Mendelian Inheritance in Animals (OMIA) database as likely causal variants for inherited traits and disorders in the Holstein breed [7].

Cholesterol deficiency a newly identified recessive inherited genetic defect in Holstein (OMIA: 001965-9913), that causes calf mortality in the dairy cattle breeding. The causative mutation of CDH results from a 1.3 kb insertion of an endogenous retrovirus (ERV) into the fifth exon of the apolipoprotein B gene on chromosome 11 (BTA11:77.959 kb). The premature stop codon in protein synthesis as a result of the insertion causes the amino acid number of the protein to remain below 140 [7-10]. The premature truncation of the protein results in an inability secretion of chylomicrons, and leads to malabsorption of dietary fat and fat-soluble vitamins in the intestine, and is assumed to impair cholesterol metabolism and transport in the circulation and liver [10,11]. The disease-associated haplotype descended from the Canadian Holstein sire Maughlin Storm (ID HOLCANM000005457798) born in 1991, suggesting autosomal recessive inheritance [12,13]. Clinical signs of the disease in homozygous calves include chronic diarrhea, insufficient development, and severe hypocholesterolemia. Affected calves do not respond to symptomatic treatment and usually die within the first 6 months of their life [10,14,15]. Heterozygous carriers (calves, bulls and nonlactating females) are clinically healthy but show reduced cholesterol and lipoprotein concentrations [11,16,17].

HH5 is an autosomal recessive inherited genetic defect in Holstein cattle (OMIA: 001941-9913), caused by a deletion affecting in the mitochondrial *TFB1M* gene, that affects mitochondrial protein translation and causes embryonic lethality before day 60 of gestation [2,7,10]. The deletion of 138 kb (93.233 kb to 93.371 kb on BTA 9) contains the entire *TFB1M* gene and was traced back to Thornlea Texal Supreme, born in 1957. It has been reported that the frequency of HH5 carriers in European and North American Holstein cattle is approximately 4-5% [10]. Recently, Häfliger et al.[6], found the strongest association to date between heifers non-return rate after 56 days and HH5.

HH5 and CDH are associated with embryonic loss and calf mortality, resulting in severe economic losses [18,19]. Balıkesir is one of the leading provinces in Türkiye for cattle numbers and is an important center for milk production. Therefore, the objectives of the present study were to investigate the presence of CDH and HH5 haplotypes and to determine the carrier and mutant allele frequencies for these haplotypes in Holstein cattle reared in Balıkesir province of Türkiye.

Material and Methods

Ethical Approval

This study was approved by the local ethics committee on animal experiments of Balıkesir University (protocol number: 2019/12-8).

Sample Collection and DNA Isolation

A total of 450 blood samples were collected randomly from Holstein cows, born between 2011 and 2018 and raised in five dairy farms in Balıkesir province of Türkiye. Blood samples were collected from the tail vein into a sterile tube containing K3 EDTA anticoagulant and stored at -20°C. Genomic DNA was isolated from whole blood using the PureLink genomic DNA mini kit (Invitrogen, CA, USA) according to the manufacturer's instructions.

Polymerase Chain Reaction (PCR)

To identify CDH and HH5 carriers in Holstein cows two PCRs were performed for each sample using a common forward primer and two reverse primers for mutant and wild-type alleles of the *APOB* gene [20] and the *TFB1M* gene [10], respectively. Information on the genetic defects and the nucleotide sequences of the primers used for PCR analysis are shown in the *Table 1*. All PCR amplification reactions were performed in a Biometra TAdvanced thermal cycler (Analytik Jena, Germany).

PCR amplification for the *APOB* gene was performed in a total volume of 25 µL reaction mixture containing 50 ng of genomic DNA, 6 pmol of each primer (Oligomer Biotechnology, Ankara, Türkiye), 12.5 µL of 2X Taq DNA polymerase master mix (Ampliqon Inc., Odense, Denmark), and 9.05 µL of nuclease-free water (Ambion Inc., Austin, TX, USA). Thermal cycler conditions consisted of an initial denaturation step at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec annealing at 62°C for 30 sec, extension at 72°C for 30 sec, and a final extension step at 72°C for 7 min.

PCR amplification for the *TFB1M* gene was performed in a final volume of 25 µL, containing 50 ng of genomic DNA, 5 pmol of each primer (Oligomer Biotechnology, Ankara, Türkiye), 12.5 µL of 2X Taq DNA polymerase master mix (Ampliqon Inc., Odense, Denmark), and 9.30

Table 1. Information on the genetic defects and the nucleotide sequences of the primers used for the PCR analysis

Locus[a]	Chr[b]	Gene	Mutation and Type of Variant[c]	Primer (Sequence 5′-3′[d]	Chromosomal Positions of Primers[c]	Amplicon Length	Tm[e] (°C)
HH5	9	*TFB1M*	g.93223651 to 93370998del; deletion	CF: AGATATGCTAAAGTTTACCTAGAAGAA	BTA9: 93371172 - 93371146	442 bp (Wild) 256 bp (Mutant)	57.5
				WT-R: CTGAAGCTCCATTCTGAGTCAT	BTA9: 93370731 - 93370752		
				Del-R: TGCTCTATGAATTTTGTGAATGGT	BTA9: 93232580 - 93232603		
CDH	11	*APOB*	g.77958995ins1.3kb; insertion	CF: TGCAAAGCCACCTAGCCTAT	BTA11:77958901 - 77958920	366 bp (Mutant) 171 bp (Wild)	62
				Ins-R: CACTCCTAATTGCCCAGGAA	within the insertion		
				WT-R: AGATGATGCCCCTCTTGATG	BTA11:77959071 - 77959052		

[a] *HH5, Holstein haplotype 5* [10]; *CDH, Cholesterol deficiency haplotype* [9,20]; [b]*Chromosome;* [c] *Genomic positions refer to the Bos taurus UMD 3.1 genome assemble;* [d] *CF, common forward primer; WT-R, wild-type reverse primer; mutant-type reverse primers: Del-R, deletion specific reverse primer, Ins-R, insertion specific reverse primer;* [e] *Tm: melting temperature*

µL of nuclease-free water (Ambion Inc., Austin, TX, USA) under the following conditions: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 57.5°C for 30 sec, extension at 72°C for 30 sec, and a final extension step at 72°C for 7 min.

A sample, provided by Professor Stanislaw Kamiński, known to be a carrier of CDH was used to control the amplification of the mutant allele in the PCR optimization. The first samples identified as CDH or HCD carriers were used for control of the mutant allele in PCR reactions (as a positive control). The PCR products of *APOB* and *TFB1M* genes were electrophoresed on 1.5% agarose gel stained with GelRed (Biotium, Hayward, CA, USA) in 0.5X TBE (Tris-borate-EDTA) buffer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and visualized under ultraviolet light.

Data Analysis

Carrier and haplotype frequencies of CDH and HH5 were estimated by direct counting. The distribution of carriers by date of birth was considered in periods of 2 years. Tables and graphs were generated using Microsoft Excel for Microsoft 365 (Microsoft Corporation, Redmond, WA).

Results

A total of 450 cows were analyzed by PCR for the detection of causative mutations for CDH and HH5. The PCR products of the *APOB* and *TFB1M* genes were analyzed by 1.5% agarose gel electrophoresis and visualized under ultraviolet light *(Fig. 1-A,B)*. As a result of electrophoresis, two DNA bands specific for both mutant and wild-type alleles were expected to be present in carrier animals, and only one DNA band specific for the wild-type allele in normal animals. Heterozygous carriers of CDH had two DNA fragments of 171 and 366 bp, whereas normal animals (wild-type homozygotes) had only one DNA fragment of 171 bp *(Fig. 1-A)*. HH5 carriers had two DNA fragments of 256 and 442 bp, whereas normal animals had only one DNA fragment of 442 bp *(Fig. 1-B)*.

CDH analysis revealed that of the 450 cows were genotyped, 429 were identified as normal and 21 were identified as carriers for CDH. As a result of the HH5 analysis of 450 cows, 416 were identified as normal and 34 were identified as carriers of HH5. As expected, no homozygous mutants were found for either haplotype. Carrier frequencies were found to be 4.67% and 7.56% for CDH and HH5, respectively, and the haplotype frequencies were calculated to be 0.023 and 0.038, respectively. While no cows carrying both haplotypes were found, 12.22% of the population was observed to carry at least one of the genetic defects.

It was observed that the CDH carrier frequency was highest (12.94%) in cattle born in 2011-2012 and followed a gradually decreasing trend in the following years, first decreasing to the range of 3.41-3.45% in cattle born between 2013 and 2016, and then decreasing to the lowest level (1.86%) in cattle born in 2017-2018 *(Fig. 2-A)*. The HH5 carrier frequency was observed as 2.35% in cattle born in 2011-2012, increased to 9.09% in cattle born in 2013-2014, reached the highest level (18.97%) in cattle born in 2015-2016 and then decreased to the lowest level (1.24%) in cattle born in 2017-2018 *(Fig. 2-B)*.

Discussion

The present study demonstrated the presence of defective alleles for HH5 and CDH in the Turkish Holstein population. HH5 carriers in the population were observed

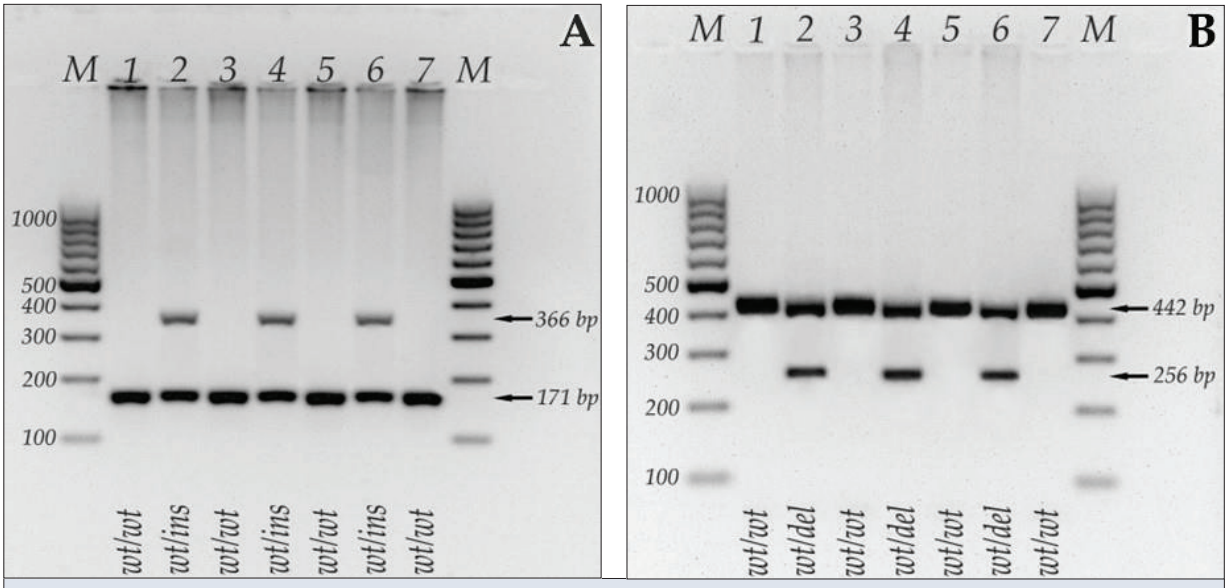

Fig 1. Agarose gel electrophoresis of the diagnostic PCR products for CDH and HH5, respectively. A- Lane M,100 bp DNA ladder; lane 1, 3, 5, 7 normal animals with one DNA fragment of 171 bp; lane 2, 4, 6 CDH carriers with two DNA fragments of 171 and 366 bp. B- Lane M,100 bp DNA ladder; lane 1, 3, 5, 7 normal animals with one DNA fragment of 442 bp; lane 2, 4, 6 HH5 carriers with two DNA fragments of 442 and 256 bp

to be more than CDH carriers. Studies investigating the prevalence of HH5 and CDH carriers in different countries have shown a wide variation. The frequency of CDH carriers in Türkiye (4.67%) was lower than previously reported for Holsteins in Germany (8.7% and 12.7%) [10,14], Kazakhstan (11%) [21] and Russia (5.66% and 7.76%) [22,23]. However, it was higher than that reported in Uruguay (2.61%) [2] and China (3.62%) [3]. The HH5 carrier frequency (7.56%) was found to be higher than previously reported for Holsteins in Germany (5.5%) [10], Russia (2.23%) [22], Uruguay (0.26%) [2] and China (4.30%) [3]. The results indicate that the prevalence of HH5 carriers in the Turkish Holstein population is higher than those of reported in other countries, while the prevalence of CDH carriers is lower than those of reported in most of the other countries. This may be related to the prevalence of the HH5 and CDH carriers among the sires that were allowed to import semen into Türkiye at that time. Consistent with the results of the present study, Inal and Cam [24], reported that among the 273 Holstein sires whose semen was allowed to be imported into Türkiye in 2015, there were 22 HH5 and 15 CDH carriers. The study, which was identified HH5 and CDH as the two most common haplotypes, respectively, by examining the records in the sire catalogs, also indicated that information on the genetic structure of about half of the sires could not be obtained [24].

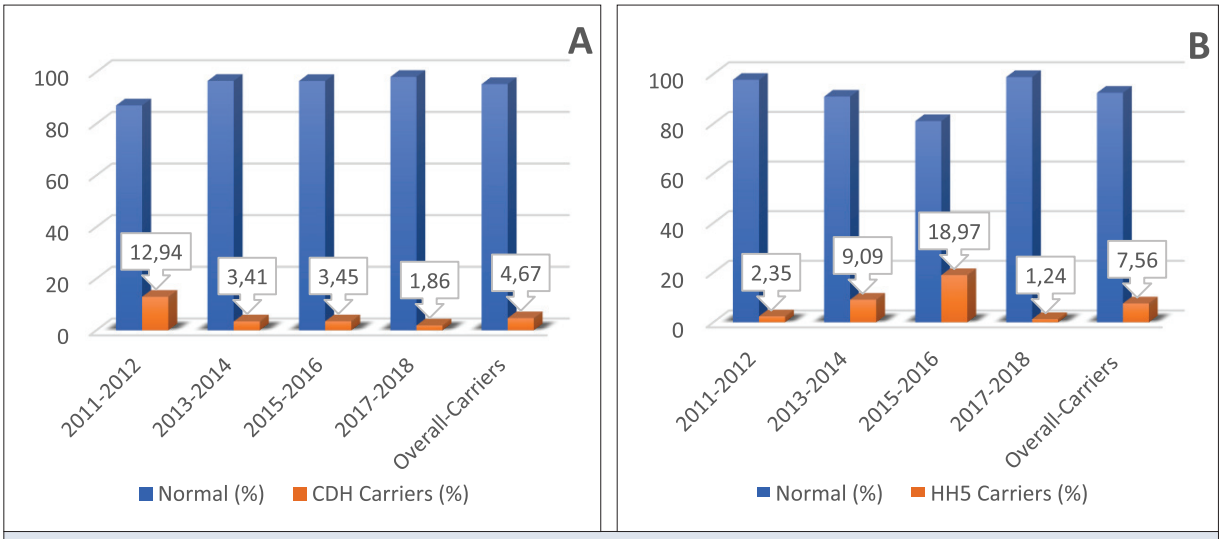

Fig 2. Distribution of carriers by year of birth. A- CDH carriers, B- HH5 carriers

Although the distribution of CDH and HH5 carriers in cattle born between 2011 and 2016 followed a different trend, the lowest carrier frequencies for both haplotypes were observed in cattle born in 2017-2018 *(Fig. 2-A,B)*. It can be said that the distribution of carriers according to birth dates between 2011 and 2018 is related to the prevalence of haplotypes both in the sires used for artificial insemination (AI) in Balıkesir from 2010 to the first months of 2018, and in the breeding cattle available in Balıkesir during the same period. During this period, there were no legal restrictions on the use of CDH or HH5 carrier sires for AI in Türkiye. Since the second half of 2018, the import of semen from HH5 carrier sires has been prohibited [25]. However, there are still no legal restrictions on the import of semen from CDH-carrier sires [26].

Recently, it has been suggested that CDH affects not only *APOB* mutant homozygotes, but also some heterozygous carriers by similar clinical signs due to incomplete penetrance. It has been reported that there is a significant difference between total cholesterol and triglyceride concentrations of clinically CDH-affected and non-affected *APOB* heterozygotes [27]. In this context, the potential impact of CDH on the dairy industry may be higher than estimated. It is important to raise awareness of this newly discovered haplotype among Turkish veterinarians to identify potential carriers and prevent the uncontrolled spread of the mutant allele of the *APOB* gene. DNA testing should be widely used for the identification of carriers and for the definitive diagnosis of affected calves.

In conclusion, this study demonstrated the presence of CDH and HH5 haplotypes in the Turkish Holstein population. It revealed the prevalence of the haplotypes in cows born between 2011 and 2018 in Balıkesir province. To prevent calf losses and reduce the future frequency of the haplotypes, carrier-to-carrier matings, which are expected to result in 25% calf losses and 50% carrier calves, should be avoided. The use of semen from carrier bulls in AI should be prevented to avoid the spread of the mutant allele throughout the country. Further studies should be conducted to identify haplotype carriers and investigate their prevalence in Holstein herds.

Availability of Data and Materials

The data used and analyzed in this study are available from the corresponding author (M. Gürses) on reasonable request.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contribution

MG designed and conducted the study. MG and ND collected the blood samples, carried out the extraction of genomic DNA, PCR analysis, and gel electrophoresis. MG drafted the manuscript. All authors have approved the final version of the manuscript.

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Research Article

Canine Adipose Tissue Stem Cells Induced With Toll-Like Receptor Agonists Exhibit Antibacterial Activity Against Multi Drug Resistant Pathogens

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Abstract: Infections caused by antibiotic-resistant pathogens pose a major threat worldwide. There is an urgent need to develop effective strategies to solve this problem. The antibacterial activity of adult mesenchymal stem cells (MSCs) has recently been determined against various bacterial isolates. New approaches, such as Toll-like receptor activation, were used to enhance their antibacterial potency. This study examines the antibacterial activity of TLR agonist (TLR2/TLR1 and/or TLR2/TLR6) activated adipose-derived canine MSCs (AD-MSCs) on multi-drug resistant isolates including *Staphylococcus aureus, Escherichia coli, Enterococcus faecalis,* and *Pseudomonas aeruginosa* regarding bacterial growth, and minimum inhibitory concentration (MIC) determination. Effects on bacterial morphology were assessed by electron microscopy. Our results showed that the AD-MSCs conditioned medium primed with different TLR agonists inhibited the growth of *E. coli* and *S. aureus*, but it had a decreased effect on *E. faecalis* and *P. aeruginosa*. Despite this, AD-MSCs conditioned medium prepared with the combination of TLR agonists exhibited antibacterial activity against all isolates. These findings were in parallel with MIC levels of conditioned media. We conclude that adipose-derived canine MSCs primed with TLR agonists (TLR2/TLR1 and TLR2/TLR6 combination) possess antimicrobial activity against multi-drug resistant isolates of *E. coli, S. aureus, E. faecalis* and *P. aeruginosa*. Further studies for testing in *in vivo* models are being planned to assess the potential application of AD-MSCs as an adjunct treatment modality for multi drug resistant infections.

Keywords: *Antibacterial agent, Gram negative bacteria, Gram positive bacteria, Mesenchymal stem cell, Multidrug resistance*

Toll-Benzeri Reseptör Agonistleri ile İndüklenen Köpek Adipoz Dokusu Kök Hücrelerinin Çoklu İlaca Dirençli Patojenlere Karşı Antibakteriyel Aktivitesi

Öz: Antibiyotik dirençli patojenlerin neden olduğu enfeksiyonlar dünya çapında büyük bir tehdit oluşturmaktadır. Bu sorunu çözmeye yönelik etkili stratejilerin geliştirilmesine acil gereksinim bulunmaktadır. Son dönemde, yetişkin mezenkimal kök hücrelerinin (MKH) çeşitli bakteri izolatlarına karşı antibakteriyel aktivite gösterdiği tespit edilmiştir ve antibakteriyel etkinliklerini artırmak için Toll benzeri reseptör aktivasyonu gibi yeni yaklaşımlar kullanılmıştır. Bu çalışmada, TLR agonisti (TLR2/TLR1 ve/veya TLR2/TLR6) ile aktive edilmiş adipoz türevli köpek MKH'lerin (AD-MKH) çok ilaca dirençli *Staphylococcus aureus, Escherichia coli, Enterococcus faecalis* ve *Pseudomonas aeruginosa* izolatları üzerindeki antibakteriyel etkisi, bakteri üremesi ve minimum inhibitör konsantrasyon (MİK) tayini ile belirlenmiştir. Bakteri morfolojisi üzerindeki etkiler elektron mikroskobu ile değerlendirilmiştir. Sonuçlarımız, farklı TLR agonistleri ile uyarılmış AD-MKH'lerden elde edilen şartlandırılmış besiyerinin *E. coli* ve *S. aureus* üremesini inhibe ettiğini, ancak *E. faecalis* ve *P. aeruginosa* üzerinde sınırlı bir etkiye sahip olduğunu gösterdi. Buna karşın, TLR agonistlerinin kombinasyonu ile hazırlanan AD-MKH şartlandırılmış besiyeri tüm izolatlar üzerinde antibakteriyel aktivite sergiledi. Bu bulgular, şartlandırılmış besiyerinin MİK seviyeleri ile paralellik göstermiştir. TLR agonistleri (TLR2/TLR1 ve TLR2/TLR6 kombinasyonu) ile uyarılmış adipoz türevli köpek MKH'lerinin, çoklu ilaca dirençli *E. coli, S. aureus, E. faecalis* ve *P. aeruginosa* izolatlarına karşı antibakteriyel aktiviteye sahip olduğu sonucuna vardık. AD-MKH'lerin çoklu ilaca dirençli enfeksiyonlar için ek bir tedavi yöntemi uygulaması olarak potansiyelini değerlendirmeye yönelik *in vivo* modellerde ileri çalışmalar planlanmaktadır.

Anahtar sözcükler: *Antibakteriyel ajan, Çoklu ilaç direnci, Gram negatif bakteri, Gram pozitif bakteri, Mezenkimal kök hücre*

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Introduction

Infections caused by antibiotic-resistant bacteria are a major global health problem and it is estimated that those infections result in nearly 5 million deaths each year [1]. Despite advances in healthcare-associated services, microorganisms evolve novel survival mechanisms and improve resistance through transferable genetic materials such as plasmids, integrons, and transposons [2]. The time required to develop a new antibacterial agent is between 10-15 years. On contrary, bacteria gain resistance rapidly. Authorities underline an urgent need to propose and develop alternative strategies against antibiotic-resistant bacterial pathogens, including multi-drug-resistant organisms. Bacteriophages, modified drugs, monoclonal antibodies, nanoparticles, anti-virulence agents, and antimicrobial peptides are among the novel therapies developing against antibiotic-resistant bacterial infections [3].

Mesenchymal stem cells (MSCs) are multipotent progenitor cells that have the potential to differentiate into osteogenic, chondrogenic, and adipogenic tissues. Moreover, MSCs exert anti-inflammatory, proliferative, and regenerative effects on tissue repair through angiogenesis, connective tissue formation, epithelialization, and production of inflammatory mediators [4]. All these features make them a promising therapeutic tool in regenerative medicine. The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposed the following criteria for MSCs definition; plastic surface adhesion, positive CD105, CD73 and CD90 expression, and negative surface markers, including CD45, CD34, CD14, or CD11b, differentiation to osteoblasts, adipocytes and chondroblasts *in vitro* [5]. MSCs can be isolated from different sources like bone marrow, adipose tissue, umbilical cord, placenta, dental follicles, etc.

Recent studies have revealed MSCs exert antimicrobial activity by directly secreting a range of antimicrobial peptides and indirectly triggering immune effector cells to call an innate immune response [6-9]. Antimicrobial peptide-based stem cell secretome is mainly induced by the activation of toll-like receptors (TLRs) found on the cellular surface. Adipose tissue and bone marrow-derived MSCs from humans and mice have been shown to express TLR 1 to 6 molecules [10].

Several studies have evaluated different TLR ligands to increase the antimicrobial and immunomodulatory properties of MSCs from different sources. In general, those studies used polyinosinic-polycytidylic acid (poly IC) and bacterial LPS to stimulate TLR-3 and TLR-4 receptors, respectively [11-13]. Activation of TLR receptors induces MSCs to secrete antimicrobial substances and also support innate immune cells for immunomodulation and clearance of pathogens [14]. In addition, studies have been performed for the antibacterial activity of TLR agonist (mainly with TLR3 ligand) stimulated canine adipose-derived MSCs against multi-drug resistant bacterial pathogens [9,15].

In this study, we aimed to examine the antibacterial effects of different TLR agonists (TLR2/TLR1 and/or TLR2/TLR6 agonists) primed canine adipose tissue mesenchymal stem cell (AD-MSC) conditioned media on antibiotic-resistant pathogenic isolates of *Staphylococcus aureus, Escherichia coli, Enterococcus faecalis,* and *Pseudomonas aeruginosa*.

Material and Methods

Stem Cell Culture and TLR Agonist Priming

Canine adipose-derived mesenchymal stem cells (AD-MSCs) were purchased commercially (Generon Ltd, Slough, UK). Cells ($2x10^6$ cells) were maintained in Dulbecco's Modified Eagle Medium (DMEM, PAN-Biotech, Aidenbach, Germany) supplemented with 20% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, 0.1 mg/mL streptomycin (PAN-Biotech) at 37°C in 5% CO_2. Subpassaging was performed at 80% confluency.

AD-MSCs at passage 3 and become 70-80% confluent were primed with TLR agonists. For priming, cells were seeded into 12-well tissue culture plates (10^5 cells/well) and incubated for 48 h in DMEM (PAN-Biotech) supplemented with 10% FBS and antibiotics at 37°C in 5% CO_2. Cells were washed twice with sterile phosphate-buffered saline (PBS) and fresh culture media without FBS and antibiotics were added. Synthetic TLR2/TLR1 agonist Pam3CSK4 (Invivogen, San Diego, CA, USA) and TLR2/TLR6 agonist Pam2CSK4 (Invivogen) were prepared in distilled water and used at 1.5 µg/mL final concentration for cell priming. Experiments were performed in triplicate.

Stem Cell Viability and Characterization After Priming

Canine AD-MSCs were verified that priming conditions did not reduce cell viability as detected by trypan blue staining. Briefly, cells from priming experiments were suspended in PBS and 0.1 mL cell suspension stained with 0.1 mL of 0.4% trypan blue solution (Merck KGaA, Darmstadt, Germany). Cells were counted on the Thoma cell counting chamber under a light microscope.

AD-MSCs were also characterized by flow cytometry analyses for the presence or absence of stem cell-specific surface markers. Cells were examined with the following monoclonal antibodies (BD Biosciences, San José, CA, USA) for flow cytometric immunophenotyping; CD73 APC (Allophycocyanin), CD90 FITC (fluorescein isothio-

cyanate), CD105 PerCP-Cy 5.5 (phycoerythrin-cyanine 5.5). A conjugated monoclonal antibody cocktail (BD Biosciences) containing CD34/ CD45/ HLA-DR/ CD11b PE (phycoerythrin) was used for negative markers. All experiments were performed with a FACSCalibur flow cytometer (BD Biosciences) equipped with the CellQuestTM software (BD Biosciences).

Preperation of Conditioned Media

Conditioned media was collected on 3rd day from AD-MSCs primed with each of TLR2/TLR1 or TLR2/TLR6 agonists or their combination in final concentrations at 1.5 µg/mL and 0.75 + 0.75 µg/mL, respectively. Samples were centrifuged for 5 min at 400g to remove cellular debris, then filtered through a 0.22 µm membrane filter and concentrated by freeze drying process (CoolSafe; LaboGene, Allerod, Denmark). Media from control wells without priming were also collected and processed. Samples were frozen at -80°C until further use in subsequent experiments.

Bacterial Strains and Antibiotic Susceptibility Determination

Bacterial strains including *S.aureus, E.coli, E.faecalis,* and *P.aeruginosa* were obtained from the culture collection of Marmara University Microbiology Laboratory. All isolates were grown on 5% sheep blood agar (bioMerieux, Marcy l' Etoile, France) at 37°C overnight in a bacteriological incubator. Microbiological identification of isolates was confirmed with MALDI-TOF Mass Spectrometry (Vitek MS; bioMerieux). Isolates were tested for antibiotic susceptibility with the disk diffusion method and results were evaluated by using breakpoint values from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical & Laboratory Standards Institute (CLSI) interpretation criteria [16,17].

Bacterial Growth Assay

Assessment of inhibition of bacterial growth by AD-MSCs conditioned medium was performed by spectrophotometric measurement. All bacterial isolates were grown at 37°C overnight in 5 ml tryptic soy broth (TSB) medium (Oxoid Ltd, Basingstoke, UK). Then the culture was diluted into fresh TSB medium to obtain 10^7 cfu/mL (approximately OD600=0.05) by using a densitometer (DEN-1; Biosan, Riga, Latvia). The resulting bacterial suspension was 1/10 diluted in AD-MSCs conditioned medium and transferred into a 96 well-plate for incubation at 37°C for 6 h. Bacteria with fresh cell culture medium was used as the positive control and medium alone was used as the negative control. Optical density measurements for each bacterial isolate were obtained by using a spectrophotometer (Synergy H1; BioTek Instruments, Winooski, VT, USA). Bacterial counts were quantitated by inoculating serial dilutions of samples taken from culture supernatants.

Assessment of Antibacterial Activity

The antibacterial activity of AD-MSCs conditioned media was tested by broth microdilution susceptibility test according to EUCAST [18]. Briefly, overnight cultures of bacterial isolates were diluted with 5 mL of Mueller Hinton broth (Oxoid Ltd, Basingstoke, UK) to obtain 10^6 cfu/ml. AD-MSCs conditioned media prepared with TLR agonists was added as 0.1 mL into a first well and diluted twofold in 8 wells of 96-well microplates. An equal volume (0.1 mL) of bacterial suspension was transferred to all wells containing conditioned media. Microplates were incubated at 37°C for 18 h. Results were evaluated visually and the highest dilution without visible bacterial growth was determined as minimum inhibitory concentration.

Electron Microscopy

Scanning electron microscopy (SEM) was used to examine the effect of AD-MSCs conditioned media prepared with TLR agonists on *E. coli* as a bacterial model. The bacterial cells (10^8 cells/mL) were incubated in a conditioned medium (0.5 mL) primed with Pam3CSK4/ Pam2CSK4 mixture (0.75 µg/mL for each) at 37°C for 4 h. After incubation, the suspension was centrifuged at 3000 rpm for 5 min and the pellet was washed twice with sterile PBS (0.05 M, pH: 7.4). Pellet was smeared on glass coverslips, and fixed with 2.5% glutaraldehyde for 2 h at ambient temperature. Samples were washed with PBS, then dehydrated with stepwise ethanol treatment. After drying at room temperature, coverslips were coated with gold and palladium in a sputter coater (Emitech SC7620; Quorum Technologies, Lewes, UK). Treated bacterial cells and untreated cells as control were subsequently analyzed with SEM (Evo MA 10; Carl Zeiss Microscopy GmbH, Jena, Germany).

Statistical Analysis

Statistical analyses were performed by using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). The data were analysed with an independent *t*-test to test the significance of the effect of conditioned media from AD-MSCs on bacterial growth. The data have been normalized against non-treated control and are expressed as means±standard error of the mean (SEM). The level of significance was considered as $P<0.05$.

Results

Cell viability and stem cell characterization were analyzed on the third day after adding TLR agonists to cell cultures. Canine AD-MSCs were observed as 70-80% confluency and determined as adherent cells on the culture flask surface with spindle shape presentation under

microscopical examination. Cell viability was tested with trypan blue dye exclusion assay and by 72 h, ranging from 84% to 96%. Cell viability rates were not significantly different between any of the TLR agonist-primed AD-MSCs groups *(Fig. 1)*. Canine AD-MSCs characterization was performed with flow cytometry analyses for surface markers. It resulted that all the AD-MSCs groups were positive for CD73 (99.6%), CD90 (99.8%), and CD105 (94.9%), but negative for CD35, CD45, CD11b, and HLA-DR (1.1%) *(Fig. 1-A,B,C,D)*. These data confirm that TLR-primed AD-MSCs can retain their characteristics similarly to MSCs.

Selected Gram-positive and Gram-negative bacterial strains were tested against to appropriate antibiotics suggested by EUCAST [16]. *Staphylococcus aureus* isolate was found to be cefoxitin and oxacillin resistant and identified as methicillin-resistant *S.aureus* (MRSA). *E. coli* isolate was detected as an extended-spectrum β-lactamases (ESBL) producer according to a double disk synergy test performed with ceftazidime, ceftazidime-clavulanic acid, and cefotaxime, cefotaxime-clavulanic acid disks. *Enterococcus faecalis* isolate was found to be vancomycin resistant. *Pseudomonas aeruginosa* isolate was detected as resistant against imipenem, meropenem, and doripenem, so-identified it as carbapenem-resistant. All antibiotics were tested with the disk diffusion method and respective inhibition zones were measured. Zone of inhibition (ZOI) measurements and antibiotic susceptibility profiles of study isolates evaluated according to EUCAST and CLSI are shown in *Table 1* [16,17].

Conditioned medium samples obtained from Pam3CSK4 (TLR2/TLR1 agonist), Pam2CSK4 (TLR2/TLR6 agonist), or the agonist combination (Pam3CSK4 and Pam2CSK4) activated canine AD-MSCs were tested for growth

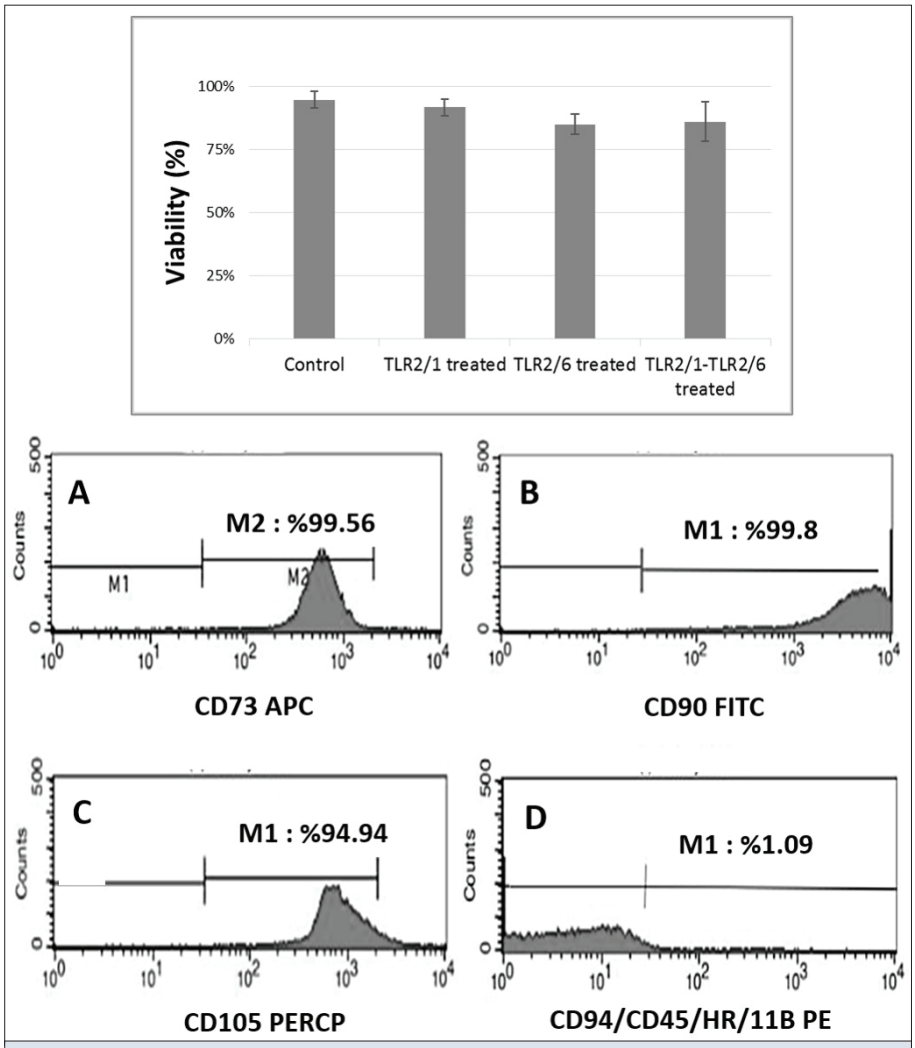

Fig 1. Cell viability rates and flow cytometric stem cell characterization of TLR agonist primed canine AD-MSCs. No significant difference was found in cell viability between any of the TLR-agonist primed AD-MSCs groups (P>0.05). Canine AD-MSCs analysed with flow cytometry for positive markers (A-C) and negative markers (D)

Table 1. *Antibiotic susceptibility profiles and resistance phenotypes of study isolates*

Bacterial Isolate	Antibiotic	Antibiotic Susceptibility[a]			Resistance Phenotype
		Zone of Inhibition (mm)	EUCAST Interpretation (breakpoint)	CLSI Interpretation (breakpoint)	
S. aureus	Cefoxitin	15	R (<22)	N/A	MRSA
	Penicillin	12	R (<26)	R (<28)	
	Erythromycin	11	R (<21)	R (<13)	
	Tetracycline	10	R (<22)	R (<14)	
	Fusidic acid	12	R (<24)	N/A	
	Mupirocin	8	R (<13)	N/A	
	Clindamycin	26	S (>22)	S (>21)	
	Linezolid	25	S (>21)	S (>21)	
	Tigecycline	24	S (>19)	N/A	
	Nitrofurantoin	19	S (>13)	S (>17)	
	TMP-SMX	18	S (>17)	S (>16)	
E. coli	Ampicillin	11	R (<14)	R (<13)	ESBL(+)
	Cefuroxime	11	R (<19)	R (<14)	
	Cefoxitin	12	R (<19)	R (<14)	
	Ceftazidime	14	R (<19)	R (<17)	
	Ceftriaxone	12	R (<22)	R (<19)	
	Cefazolin	16	R (<20)	R (<14)	
	Gentamicin	10	R (<17)	R (<12)	
	Ciprofloxacin	18	R (<22)	R (<21)	
	TMP-SMX[b]	9	R (<11)	R (<10)	
	Tigecycline	14	R (<18)	N/A	
	Amoxicillin/Clavulanate	22	S (>16)	S (>18)	
	Piperacillin/Tazobactam	26	S (>20)	S (>21)	
	Ertapenem	29	S (>25)	S (>22)	
	Meropenem	25	S (>22)	S (>23)	
	Amikacin	23	S (>18)	S (>17)	
	Nitrofurantoin	18	S (>11)	S (>17)	
E. faecalis	Ampicillin	6	R (<8)	R (<16)	VRE
	Gentamicin	7	N/A	N/A	
	Streptomycin	11	N/A	N/A	
	Vancomycin	10	R (<12)	R (<14)	
	Nitrofurantoin	11	R (<15)	R (<14)	
	Ciprofloxacin	9	R (<15)	R (<15)	
	Levofloxacin	10	R (<15)	R (<13)	
	Teicoplanin	21	S (>16)	S (>14)	
	Linezolide	25	S (>20)	S (>23)	
P. aeruginosa	Piperacillin/Tazobactam	13	R (<18)	R (<14)	Carbapenem resistant
	Ceftazidime	12	R (<17)	R (<14)	
	Imipenem	11	R (<20)	R (<15)	
	Meropenem	12	R (<14)	R (<15)	
	Doripenem	12	R (<22)	R (<15)	
	Cefepime	11	R (<21)	R (<14)	
	Ciprofloxacin	16	R (<26)	R (<18)	
	Levofloxacin	16	R (<18)	IM (15-21)	
	Ceftazidime- Avibactam	13	R (<17)	R (<20)	
	Amikacin	25	S (>15)	S (>17)	
	Tobramycin	23	S (>18)	S (>15)	

Antibiotic susceptibility was tested with disk diffusion method. ***EUCAST:*** *European Committee on Antimicrobial Susceptibility Testing;* ***CLSI:*** *Clinical & Laboratory Standards Institute;* ***TMP-SMX:*** *Trimethoprim-sulfamethoxazole;* ***MRSA:*** *Methicillin-resistant Staphylococcus aureus;* ***ESBL(+):*** *Extended spectrum beta-lactamase positive;* ***VRE:*** *Vancomycin-resistant Enterococci;* ***S:*** *Susceptible;* ***R:*** *Resistant;* ***IM:*** *Intermediate resistant;* ***N/A:*** *Not applicable*

inhibitory activity on bacterial isolates. Bacterial growth was interpreted by absorbance measurement of bacterial cultures at 600 nm and relatively compared to DMEM (negative control), which was accepted as 100%. Bacterial loads were confirmed with plating experiments. The most potent growth inhibition for all study isolates was obtained with agonist combination, followed by TLR2/TLR6 agonist Pam2CSK4. The growth of *E. coli* and *S. aureus* isolates was highly inhibited compared to *E. faecalis* and *P. aeruginosa*. *E.coli* was determined as the most susceptible isolate according to growth reduction rates *(Fig. 2)*.

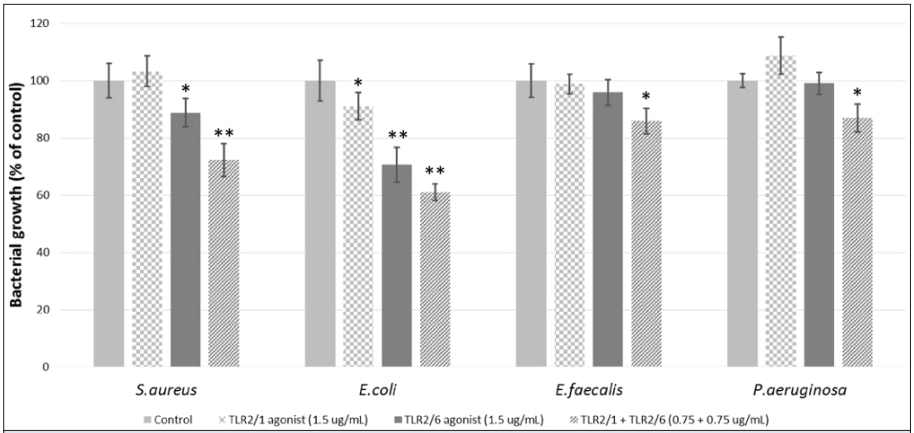

Fig 2. The activity of conditioned media from TLR agonist primed canine AD-MSCs on bacterial growth. Pam3CSK4 (TLR2/TLR1 agonist), Pam2CSK4 (TLR2/TLR6 agonist), and the agonist combination were tested for growth inhibitory activity on bacterial isolates. The data were expressed relative to bacterial growth in the control (non-conditioned medium). The data are shown as means±SEM. *P<0.5 and **P<0.05

The antibacterial activity of canine AD-MSCs conditioned media prepared with Pam3CSK4 (TLR2/TLR1 agonist), Pam2CSK4 (TLR2/TLR6 agonist), or the agonist combination (Pam3CSK4 and Pam2CSK4) was assessed in broth microdilution assay. Conditioned media was diluted in the range between 1/2 to 1/128 and inoculated with bacterial suspensions. TLR agonist combination (Pam3CSK4 and Pam2CSK4) primed conditioned media expressed solid antibacterial activity against *E. coli* and *S. aureus* isolates at 1/4 dilution and *E. faecalis* isolate at 1/2 dilution. However, it was active in *P. aeruginosa* only as undiluted. The undiluted AD-MSCs' conditioned medium primed with Pam2CSK4 (TLR2/TLR6 agonist) inhibited the growth of *E. coli* and *S. aureus* isolates, but it was not expressed antibacterial activity against *E. faecalis* and *P. aeruginosa*. On the other hand, Pam3CSK4 (TLR2/TLR1 agonist) conditioned medium was not shown antibacterial activity in any of the study isolates.

Scanning electron microscopy was used to observe the effects of TLR agonist combination (Pam3CSK4 and Pam2CSK4) primed canine AD-MSCs conditioned medium at 1/2 dilution on *E.coli* cell morphology. After 4 h of incubation, bacterial cells exposed to the conditioned medium showed unusual morphological changes, as observed by the perturbation of membrane and pore formation, in contrast, the bacterial cell in untreated control had a buxom shape and smooth cell surface (*Fig. 3*).

Discussion

In this study, we investigated the antibacterial effect of TLR2/1 and TLR2/6 primed canine adipose tissue-

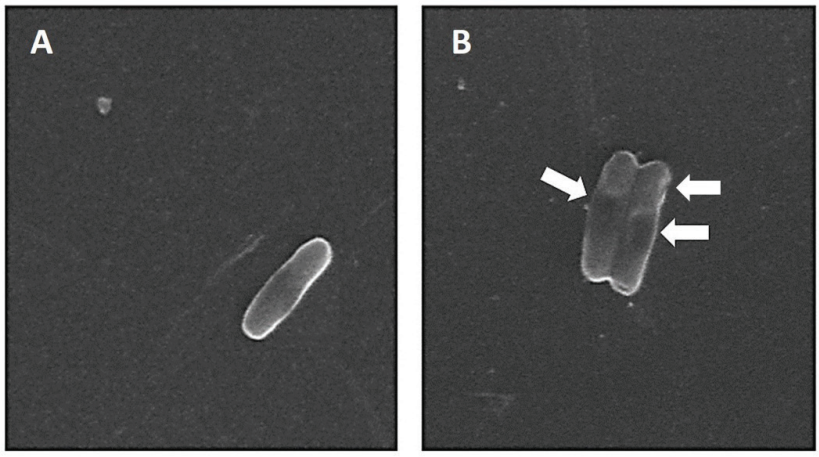

Fig 3. The effects of TLR agonist primed canine AD-MSCs conditioned medium on *E.coli* cell morphology were examined with SEM. A- Untreated control, B- Conditioned medium treated. Arrows indicate pore formation and membrane damage of bacterial cells

derived MSCs (AD-MSCs) on antibiotic-resistant bacterial pathogens. Priming with combined TLR agonists significantly increased the antibacterial activity of the AD-MSCs against multidrug resistant isolates of *S. aureus, E. coli*, *E. faecalis*, and *P. aeruginosa*.

Infections caused by multidrug-resistant (MDR) bacteria have progressively increased in recent years and exhibit a major health problem causing limited treatment options in humans and animals. One Health concept has been defined as the collaborative effort of multiple disciplines working locally, nationally, and globally to attain optimal health for people, animals, plants, and our environment. In this context, human, animal, and environmental health are interrelated and bound to each other; thus, MDR bacteria represent a significant threat to public health worldwide [19].

MDR pathogen-caused infections were accepted as epidemics in veterinary medicine, and indicate an important problem related to the transmission of these pathogens within veterinary environments [20]. Many published studies on this subject reported that the genera frequently associated with MDR infections in veterinary medicine contain Gram-positive and Gram-negative bacteria including methicillin-resistant staphylococci, vancomycin-resistant enterococci, and extended-spectrum beta lactamase (ESBL)-producing Enterobacteriaceae and *P.aeruginosa* [21-25]. Therefore, in this study, we selected four bacterial isolates; methicillin-resistant *S. aureus*, vancomycin-resistant *E. faecalis*, extended-spectrum beta lactamase (ESBL) producing *E. coli* and carbapenem resistant *P.aeruginosa* which were identified as multidrug-resistant in antibiotic susceptibility testing *(Table 1)*.

With the growing problem of antibiotic resistance in veterinary pathogens, the demand for alternative treatment approaches results in several non-antibiotic strategies such as novel nanoparticles, phage therapies, and antimicrobial substances released from stromal cells [3,25]. In this scope, mesenchymal stem cells (MSCs) have been considered a reliable choice for scientists in the treatment of resistant bacteria [26].

Toll-like receptors (TLRs) for defending against micro-organisms are widely expressed by the immune system cells as well as other body cells such as epithelial cells, endothelial cells, fibroblasts, and MSCs. The expression levels of TLR1 through TLR6 are stable between the different types of MSCs, while the expression of TLR7, TLR8, TLR9, and TLR10 show variance. TLR2 alone senses bacterial peptidoglycan, in heterodimeric form with TLR1 (TLR2/TLR1) or TLR6 (TLR2/TLR6) binds to triacylated and diacylated lipopeptides, respectively [27].

Data from the literature have previously presented that pre-activation of MSCs with TLR agonists can enhance antimicrobial activity against different species of bacteria or bacterial biofilms through *in vitro* and *in vivo* conditions. In these studies, TLR activation is mainly produced by native bacteria itself or TLR3 and TLR4 ligands such as polyinosinic-polycytidylic acid (pIC) and Gram-negative bacterial lipopolysaccharide (LPS) [7,11,15,28,29]. In line with those studies, our results confirm that TLR activation is involved in MSCs' antibacterial activity.

We used two approaches to examine the antibacterial activity of conditioned medium after TLR2/TLR1, and TLR2/TLR6 priming of canine adipose-derived MSCs, namely bacterial growth assay and minimal inhibitory concentration determination. Our data from *in vitro* bacterial growth study indicate that conditioned medium from TLR2/TLR1 primed AD-MSCs has only shown activity for *E. coli* with slightly decreased growth relative to control (8.9%). However, conditioned medium from TLR2/TLR6 primed cells reduced the growth of *S. aureus, E. coli,* and *E. faecalis* by 11.2%, 29.3%, and 4.1%, respectively. Moreover, conditioned medium primed with TLR2/TLR1 and TLR2/TLR6 combination expressed a significant growth inhibition on *S. aureus, E. coli*, *E. faecalis,* and *P. aeruginosa* isolates with a range of 27.7%, 38.9%, 14.1%, and 13.0%, respectively *(Fig. 2)*. We obtained concordant results with the MIC study; 1/4 dilution of conditioned medium prepared with TLR2/TLR1 and TLR2/TLR6 combination was detected as MIC against *E. coli* and *S. aureus* isolates. However, *E. faecalis* isolate had MIC at 1/2 dilution of the same conditioned medium; and undiluted conditioned medium expressed as MIC for *P. aeruginosa* isolate.

Pezzanite et al.[11] demonstrated equine MSCs stimulated with TLR-3, TLR-4, and NOD-like receptor (NLR) agonists produce bactericidal activity against multidrug-resistant *S.aureus* both in planktonic and biofilm forms. Compared to other ligands, activation with TLR-3 agonist (polyinosinic:polycytidylic acid, pIC) was most effective in triggering antibacterial activity and immunomodulatory cytokine production. In our study, two different TLR ligands were used solely and in combination for MSC stimulation. The antibacterial activity of stimulated MSCs' was most prominent with ligand combination followed by TLR2/TLR6 agonist, than TLR2/TLR1 compared to unstimulated control cells. Moreover, conditioned medium from MSCs primed with combined agonists exhibited lower minimal inhibitory concentration levels against pathogens.

Johnson et al.[9] reported the mouse and canine MSCs primed with TLR-3 ligand (pIC) reduced *S. aureus* bacterial count in mice with chronic infection and also in dogs with wound infections compared to antibiotics or MSCs alone. Interestingly, TLR3-activated canine MSCs were found to be ineffective for the enhancement of antibacterial activity and detected antibacterial effects

associated with constitutively secreted antimicrobial molecules from MSCs. This could be associated with the pathogens involved; our results notably demonstrated the antibacterial potential for TLR-activated MSCs' varied between bacterial pathogens, such as *E.coli* vs *P.aeruginosa* which showed the 3-fold difference in growth inhibition rates after treatment (38.9% vs 13.0%) *(Fig. 2)*.

In a recent study [15], the effectiveness of allogenic canine MSCs primed with TLR3 ligand was investigated for the treatment of naturally occurring drug-resistant infections in dogs. Results showed that TLR3 priming of canine MSCs caused enhancement of macrophage bactericidal activity and increased both MSCs' migration capacity and IL-8 secretion involved in innate immunity. The *in vivo* part of the study revealed an improved microbiological response against pathogens (methicillin-resistant *Staphylococcus pseudointermedius, Proteus mirabillis,* and *P. aeruginosa*) detected in half of the treated dogs. The authors concluded that TLR3 priming of canine MSCs caused an increase in indirect antimicrobial properties, especially which is much more important clinically. This statement suggests that TLR activation of MSCs can produce more effective treatment results in the clinical setting rather than in laboratory experiments.

We take into account that the differences between the results of studies are likely to be associated with methodological variations such as cell-culture techniques, TLR agonist concentration, exposure time, tested bacterial targets, the source and the number of exposed MSCs, etc.

Electron microscopy was employed to observe alterations of treated *E. coli* cells. The effects of conditioned medium prepared with TLR2/TLR1 and TLR2/TLR6 combination on *E. coli* morphology were similar to that obtained by cationic antimicrobial peptides [30]. Treated bacterial cells have shown perturbation of cell membranes and pore formation *(Fig. 3)*.

The results of this study demonstrate that TLR2/TLR1 and TLR2/TLR6 primed canine adipose-derived MSCs conditioned medium has antimicrobial activity against multidrug-resistant isolates of Gram-positive and Gram-negative bacteria. Moreover, compared with the single agonist stimulation, the conditioned medium prepared with the combination of TLR2/TLR1 and TLR2/TLR6 agonists was more potent with up to 4-fold decreased MICs against studied pathogens. These results should be evaluated by performing *in vivo* testing in clinical models of multidrug-resistant infections.

Availability of Data and Materials

The data that support the findings of this study are available on a reasonable request from the corresponding author (B. Aksu).

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Ethical Statement

This study does not need ethical approval because experiments did not involve any animals or humans. Animal cells were purchased from a commercial company.

Conflict of Interest

The authors declared that there is no conflict of interest

Author Contributions

BA and TA conceived and supervised this study. BA and OY completed the main experimental content. BA and OY collected and analyzed the data. BA and TA wrote the first draft of the manuscript. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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RESEARCH ARTICLE

The Effect of Vitamin E-Trace Mineral Treatments on Reproductive Performance in Morkaraman Sheep During the Breeding Season[1]

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Abstract: The aim of this study was to investigate the effect of vitamin E-trace mineral treatments before synchronization and $PGF_{2\alpha}$ injection one day before and on the day of sponge removal on reproductive parameters in Morkaraman ewes during the breeding season. Fifteen days before the synchronization, the sheep in Group I (n=11) and Group II (n=12) were given orally capsules containing a vitamin E-mineral combination. The sheep in Group III (n=10) and IV (n=14) weren't given. An intravaginal sponge was applied to all sheep to remain in the vagina for 6 days, and eCG was injected immediately in all groups after the sponge was removed. The sheep in Groups I and III were injected with $PGF_{2\alpha}$ only on the fifth day, and the sheep in Groups II and IV were injected with $PGF_{2\alpha}$ on both the fifth and sixth days. The highest rates of pregnancy (91.67%) were in Group II (P>0.05). The rate of multiple lambing was higher in Groups II (90.91%) and IV (87.5%) than in other groups (P>0.05). The lambing rate was lowest in Group III (60%, P>0.05). Embryonic mortality rates in the groups were found to be 14.29%, 9.09%, 20.00% and 12.50% in the Group I, Group II, Group III and Group IV, respectively. It was determined that litter size was 1.33, 2.3, 2.67, and 3.29 in the Group I, Group II, Group III and Group IV, respectively (P>0.05). It was determined that the average birth weight of lamb was highest in Group I (4.88±0.18 kg). It was determined that vitamin E-trace mineral treatments and double $PGF_{2\alpha}$ injection increased the pregnancy rate (P>0.05), especially double $PGF_{2\alpha}$ injection contributed to the formation of multiple pregnancies.

Keywords: *Double $PGF_{2\alpha}$ injections, Ewe, Trace minerals, Vitamin E, Reproductive performance*

Üreme Mevsimindeki Morkaraman Koyunlarında Vitamin E-İz Mineral Uygulamalarının Üreme Performansı Üzerine Etkisi

Öz: Sunulan çalışmada üreme mevsiminde bulunan Morkaraman koyunlarında senkronizasyondan önce Vitamin E ve mineral kombinasyonu içeren kapsüllerin ve sünger çıkarılmadan bir gün önce ve sünger çıkarıldığı gün $PGF_{2\alpha}$ enjeksiyonunun üreme parametreleri üzerine etkisinin araştırılması amaçlandı. Senkronizasyon protokolünden 15 gün önce Grup I ve Grup II'deki koyunlara vitamin E-mineral kombinasyonu içeren kapsüller ağızdan yutturuldu. Grup III ve Grup IV'teki koyunlara ise verilmedi. Tüm koyunlara 6 gün süre ile vaginada kalacak şekilde intravaginal süngerler yerleştirildi ve süngerin çıkarıldığı gün eCG enjeksiyonu yapıldı. Grup I ve III'teki koyunlara yalnızca beşinci gün, Grup II ve IV'teki koyunlara ise hem beşinci hem de altıncı gün $PGF_{2\alpha}$ enjeksiyonu yapıldı. Gebelik oranı (%91.67) en yüksek Grup II'deydi (P>0.05). Grup II (%90.91) ve Grup IV'te (%87.5) çoklu kuzulama oranı diğer gruplardan yüksekti (P>0.05). Kuzulama oranı en düşük Grup III'teydi (%60, P>0.05). Embriyonik ölüm oranları Grup I, II, III ve IV'te sırasıyla %14.29, %9.09, %20.00 ve %12.50 olduğu belirlendi. Doğum yapan koyun başına düşen kuzu sayısı gruplarda sırasıyla 1.33, 2.3, 2.67 ve 3.29 olduğu belirlendi. Ortalama doğan kuzu ağırlığının en yüksek Grup I'de olduğu saptandı (4.88±0.18 kg). Vitamin E ve mineral tedavisi ve çift $PGF_{2\alpha}$ enjeksiyonunun gebelik oranını artırdığı (P>0.05) tespit edildi, özellikle çift $PGF_{2\alpha}$ enjeksiyonunun çoklu gebelik oluşumuna katkı sağladığı belirlendi.

Anahtar sözcükler: *Çift $PGF_{2\alpha}$ enjeksiyonu, İz mineraller, Koyun, Üreme performansı, Vitamin E*

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Introduction

Reproduction is the most basic element for ensuring productivity in sheep [1]. Increasing the reproduction rate makes a great contribution to the economy by ensuring both the continuity of productivity and the increase in lamb production [2]. For many years, the breeding cycles of sheep have been interfered with artificially [3]. With these interventions, it is aimed to aggregate the estrus and birth time in a certain period, to provide uniformity in the offspring [4], and increase offspring productivity by stimulating multiple births [2]. They are the most basic methods used to stimulate reproduction and increase efficiency in various hormonal applications and changes in nutrition strategy. The use of $PGF_{2\alpha}$ to shorten the luteal phase during the breeding season or progestagen to artificially prolong the luteal phase is one of the most preferred methods [4-6].

The presence of various vitamins and minerals is essential for reproductive health in animals [7]. Vitamin E plays an important role in the management of oxidative stress. Oxidative stress compromises ovarian activity and follicular development. Vitamin E and selenium are synergistically effective [8]. Selenium is an important component of the enzyme glutathione peroxidase (GSH), which is involved in the detoxification of free radicals [1]. Graaf follicles and oocytes are reported to be highly sensitive to antioxidant status change, especially GSH [8] as the GSH level decreases, the rate of apoptosis in the antral follicle increases. Therefore, it is reported that embryonic and fetal deaths may be more likely in vitamin E and selenium deficiency [8]. It is known that trace elements such as copper, selenium, and cobalt are the most effective minerals for reproduction. These trace minerals affect the reproductive performance, as well as the survival of the embryo [9]. They are involved in the synthesis of many proteins and the activation of the enzyme system. Its deficiency affects the immune system during the recognition of pregnancy or implantation, changing embryonic growth. In farm animals, the incidence of premature embryonic death increases if they are deficient [9]. Sheep need cobalt more because they use a lot of propionic acid for gluconeogenesis, and they need sulfur-containing amino acids such as methionine for wool growth. It has been determined that subclinical cobalt deficiency causes a decrease in the ovulation rate in superovulated sheep [10].

The study is aimed to determine the effect of capsules containing Vitamin E-trace minerals 15 days before short-term (6 days) progestagen administration and double-dose $PGF_{2\alpha}$ injection on some reproductive parameters in Morkaraman sheep.

Material and Methods

Ethical Approval

The present study was approved by the Animal Research Ethics committee of the University of Kafkas (Ethics approval number: KAÜ-HADYEK, number 2021/157).

Animals

The study was performed during the breeding season of 2021 in Morkaraman sheep at the Iğdır University Application and Research Farm in Iğdır province, which is located at 39° north latitude and 44° east longitude. Forty-seven clinically healthy and non-lactating sheep in the breeding season were included in the study. The sheep were 2-4 years old, with a live weight of 55-65 kg. 4 rams, 2-3 years old, were used. 600 g of barley was given per sheep per day. Meadow grasses and water were offered ad libitum. In the last 1 month, close to birth, meadow grass, wheat straw, and clover straw were given *ad libitum,* and they were additionally fed with 500 g sheep milk feed.

Methods

The rams were separated from the herd 30 days before starting the synchronization protocol. 15 days before the synchronization in the breeding season, 2 capsules (Vit. E 500 IU, dicalcium phosphate 150 mg, sodium selenite 2.5 mg, copper sulfate 10 mg and cobalt sulfate 12.5 mg) containing vitamin and mineral combinations (Bakosel®, Ceva, Türkiye) were given orally for sheep in Groups I (n=11) and II (n=12). The sheep in Group III (n=10) and IV (n=14) were not given. An intravaginal sponge (20 mg flugeston acetate, Chronogest®, France) was applied to all sheep for 6 days. While sheep in Group I and III were injected with $PGF_{2\alpha}$ (1 mL, 5 mg, Dinoprost, Enzaprost®, France) on the fifth day (the day before removing the sponge), sheep in Group II and IV were injected with $PGF_{2\alpha}$ double, the day before removing the sponge and immediately after removing the sponge. After this, all sheep were given i.m. an injection of 600 IU of eCG (Chrono-gest/PMSG, Germany), and the four fertile rams joined the herd. After intravaginal sponges were removed, estrus was followed up 2 times a day (12 h intervals) for 30 min for 5 days. The dates and times of estrus were recorded. Estrus ewes were separated and mated naturally. Ewes were observed for mounting by the rams. They were tested for pregnancy detection on day 30 after mating using ultrasonography device (Hasvet model 838, HASVET, Türkiye) with 7.5 MHz linear array rectal transducer. The date of birth, multiple births, the sex of the offspring, and their body weight were recorded.

Blood was collected from the *v. jugularis* into serum tubes from all sheep on day 0 (the day the sponge was inserted), on day 6 (the day the sponge was removed), and on the

day of estrus. The collected blood was centrifuged at 3500 rpm for 10 min; the serum was removed, transferred to eppendorf tubes, and stored at -20°C until measurements were made.

Progesterone and estrogen analyses were performed quantitatively by direct chemiluminescence (CLIA) method using Siemens brand hormone determination device and the same brand ADVIA Centaur (Siemens®, Tarrytown, Newyork, USA) test analysis kits. The ADVIA Centaur test is a measurement technique with a sensitivity of 100% and a specificity of 95.5%. The sensitivity and test range for progesterone is 0.21-60 ng/mL, and for estrogen it is 11.8-3000 pg/mL.

The reproductive parameters were calculated from the findings obtained. These parameters are; The start time of estrus [removal of the sponge and observation of signs of estrus]

Estrus rate [Number of sheep showing estrus/Total number of sheep]x100

Pregnancy rate [Number of pregnant sheep/Total number of sheep]x100

Lambing rate [Number of lambing sheep/Number of pregnant sheep]x100

Fertility [Number of lambing sheep/Number of mating sheep]x100

Birth of a single offspring [Number of sheep lambing with a lamb/Number of sheep giving birth]x100

Multiple lambing [Number of sheep giving birth to two or more lambs/Number of sheep giving birth]x100

Number of lambs per mating sheep (fecundity) [Number of lambs born/Number of mating sheep]

Number of lambs per ewe giving birth (litter size) [Number of lambs born/number of sheep giving birth].

Statistical Analysis

Data analyzes were performed using the SPSS® (SPSS 18, IL, USA) program. Percentages between groups were compared using the Chi-square method. Normality tests of the data were analyzed with the Shapiro-Wilk test. One Way ANOVA and welch ANOVA were performed for the weights of the offspring. Tamhane's T2 Post hoc test was applied. The level of progesterone and estrogen was compared with the Mann-Whitney U test. The results were given as mean ± standard error (X±SE). P<0.05 was considered significant.

Results

It was found that the sponge fell only in one sheep in Group IV among the sheep that were applied intravaginal sponge. Slight vaginal inflammation was detected in all of the sheep included in the study when the sponge was removed. It was determined that the time between rams joined in the herd and onset of estrus symptoms varied between 18-83 h. Reproductive parameter results of the synchronization protocol performed during the season in Morkaraman sheep are presented in *Table 1*. The rate of estrus was found to be 90.91%, 100%, 80%, and 92.86% in Group I, Group II, Group III, and Group IV, respectively. It was determined that the highest rate of estrus and pregnancy was in Group II (P>0.05). The greatest number of embryonic mortality after pregnancy diagnosis was in Group III (20%, P>0.05). The highest rate of fecundity was in Group II. 6 of the offspring in Group IV (26.09%) were determined to have died after birth; all the others continued to live. It was determined that there was no statistically significant difference between the groups for these reproductive parameters (P>0.05). Pregnancy loss was determined to be 14.29% in Group I, 9.09% in Group II, 40% in group III, and 12.5% in Group IV.

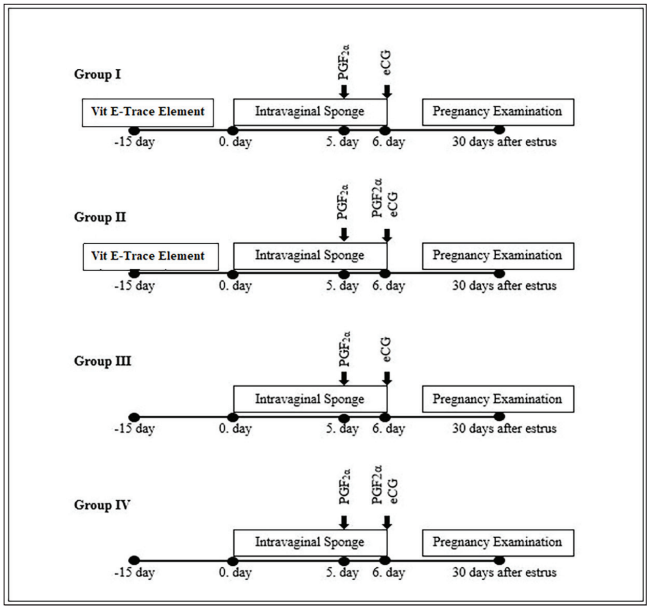

Fig 1. Application procedure in groups

It was determined that the highest rate of multiple births was in Group II and Group IV *(Table 2)*. The were 4 females and 4 males in Group I, 7 females and 16 males in Group II, 3 females and 2 males in Group III, 11 females and 12 males were born in Group IV.

The reproductive parameters obtained when the sheep were classified as Bakosel given and not given are presented in *Table 3*. It was determined that the rate of estrus, pregnancy, and lambing was higher in the group that received Bakosel than in the group that did not receive it (P>0.05). It was determined that the fecundity rate was higher in the Bakosel group (1.41) than in the non-Bakosel group (1.33). Litter size, on the other hand,

Table 1. *Reproductive parameters in groups*

Parameters	Group I	Group II	Group III	Group IV
Ewes joined (n)	11	12	10	14
Estrus rate (%)	10/11 (90.91)	12/12 (100)	8/10 (80)	13/14 (92.86)
Pregnancy rate (%)	7/11 (63.64)	11/12 (91.67)	5/10 (50)	8/14 (57.14)
Lambing rate (%)	6/7 (85.71)	10/11 (90.91)	3/5 (60)	7/8 (87.5)
Single lambing rate (%)	4/6 (66.67)	1/10 (10)	1/3 (33.33)	1/7 (14.29)
Multiple lambing rate (%)	2/6 (33.33)	9/10 (90)	2/3 (66.67)	6/7 (85.71)
Fecundity	8/10 (0.8)	23/12 (1.92)	5/8 (0.63)	23/13 (1.77)
Litter size	8/6 (1.33)	23/10 (2.3)	5/3 (1.67)	23/7 (3.29)
Survival rate (%)	100	100	100	73.91

Table 2. *Distribution of the number of offspring born in groups*

Parameters	Group I	Group II	Group III	Group IV
Number of lambs born	8	23	5	23
Single lambing rate	4/6 (%66.67)	1/10 (%10)	1/3 (%33.33)	1/7 (%14.29)
Twine lambing rate	2/6 (%33.33)	6/10 (%60)	2/3 (%66.67)	2/7 (%28.57)
Triple lambing rate	-	2/10 (%20)	-	1/7 (%14.29)
Quadruplet lambing rate	-	1/10 (%10)	-	1/7 (%14.29)
Quintuple lambing rate	-	-	-	1/7 (%14.29)
Six lambing rate	-	-	-	1/7 (%14.29)

was lower in the Bakosel group (P>0.05). Although there was a numerical difference in the reproductive parameters of these groups, this difference was not statistically significant.

The reproductive parameters obtained when the groups were classified as sheep with a single and double injection of $PGF_{2\alpha}$, was presented in *Table 4*. It was determined that the rate of estrus, pregnancy, and lambing were higher in the group that received a double injection of $PGF_{2\alpha}$ than in the group that received a single (P>0.05). It was found that the rate of multiple lambing was quite high in the group that underwent double $PGF_{2\alpha}$ (P>0.05). Fecundity and litter size was found to be higher in the double $PGF_{2\alpha}$ group (1.84 and 2.71, respectively) than in the other group (0.72 and 1.44, respectively), but this difference was not statistically significant.

Intravaginal sponge application is synchronized sheep (day 0), sponge removal (day 6), mean serum progesterone (P4) concentrations, and mean serum estradiol levels on the day of estrus are given in *Table 5*. It was determined that the progesterone level was >1 ng/mL in 76.60% (36/47) of sheep on the day of intravaginal sponge application. When the groups were compared in terms of progesterone levels, it was determined that there was a difference on day

Table 3. *Reproductive parameters in Bakosel given and not given sheep*

Parameters	Bakosel (+)	Bakosel (-)
Ewes joined (n)	23	24
Estrus rate (%)	22/23 (95.65)	21/24 (87.5)
Pregnancy rate (%)	18/23 (78.26)	13/24 (54.16)
Lambing rate (%)	16/18 (88.89)	10/13 (76.92)
Single lambing rate (%)	5/16 (31.35)	2/10 (20)
Multiple lambing rate (%)	11/16 (68.65)	8/10 (80)
Fecundity	31/22 (1.41)	28/21 (1.33)
Litter size	31/16 (1.98)	28/10 (2.8)
Mortality (lamb)	-	6

Table 4. The Reproductive parameters in sheep with a single injection of $PGF_{2\alpha}$ and double injection of $PGF_{2\alpha}$		
Parameters	**Single PGF**	**Double PGF**
Ewes joined (n)	21	26
Estrus rate (%)	18/21 (85.71)	25/26 (96.15)
Pregnancy rate (%)	12/21 (57.14)	19/26 (73.08)
Lambing rate (%)	9/13 (69.23)	17/22 (77.27)
Single lambing rate (%)	5/9 (55.56)	2/17 (11.76)
Multiple lambing rate (%)	4/9 (44.44)	15/17 (88.24)
Fecundity	13/18 (0.72)	46/25 (1.84)
Litter size	13/9 (1.44)	46/17 (2.71)

0 only between Group II and Group IV (P<0.05). There was no statistical difference in the level of progesterone and estrogen on the other measurement days. In 2 sheep in Group I, 1 sheep in Group II, 2 sheep in Group III, and 2 sheep in Group IV, the level of progesterone on the day of estrus were determined to be >1 ng/mL. It was determined that the level of progesterone on the day of estrus in the groups was between 0.61-0.92 ng/mL. The highest mean estrogen level was in Group I and Group III.

P=0.001 between Group III and Group IV. When the ewes were evaluated among themselves, a difference was found between Group I and Group IV and between Group III and Group IV. A statistical difference in rams was found between Group I and Group II, Group I and Group IV.

Table 5. Intravaginal sponge application (day 6), removal (day 0) and serum progesterone levels and serum estradiol levels on the day of estrus				
Groups	**Progesterone (ng/mL)**		**Estrus Day**	
	0. day	**6. day**	**Progesterone (ng/mL)**	**Estradiol (pg/mL)**
Group I (n=11)	3.83±0.71	1.38±0.45	0.92±0.22	56.29±7.97
Group II (n=12)	2.80±0.85*	1.67±0.51	0.69±0.16	48.19±3.54
Group III (n=10)	3.77±1.08	1.68±0.53	0.92±0.37	56.71±5.34
Group IV (n=14)	5.88±0.58*	1.72±0.46	0.61±0.18	54.09±8.03
· The difference between values with different letters in the same column is significant (P<0.05)				

Table 6. Average weight of offspring in groups			
Groups	**Average Lamb Weight (kg)**	**Average Female Lamb Weight (kg)**	**Average Male Lamb Weight (kg)**
Group I	4.74 ± 0.16^{a}	4.42 ± 0.28^{k}	4.88 ± 0.18^{x}
Group II	3.43 ± 0.16^{b}	3.36 ± 0.21^{kl}	3.47 ± 0.22^{y}
Group III	4.63 ± 0.23^{c}	4.72 ± 0.22^{mk}	4.41 ± 0.71^{xy}
Group IV	2.99 ± 0.30^{bd}	2.53 ± 0.40^{l}	3.34 ± 0.42^{yz}
a:d, k:m, x:z: The difference between values with different letters in the same column is significant, a:b:<0.001, a:bd: <0.001, b:c: 0.005, c:bd: 0.001, x:y: <0.001, x:z:0.03, k:l: 0.007, mk:l: 0.001			

The average weights of the lambs born are presented in *Table 6*. It was determined that the highest offspring weight was in Group I when the groups were compared in terms of weight. There was a statistical difference of P<0.001 between Group I-Group II and between Group I-Group IV, P=0.005 between Group II-Group III, and

Discussion

Since sheep bred in Turkey generally have low productivity, profitability decreases considerably [11]. Morkaraman sheep is a fat-tailed breed that is mainly bred in the east of Türkiye [11,12]. The factors that affect profitability the most are listed as fertility rate, litter size, and lamb's vitality [11]. The average weight of lambs in Morkaraman sheep is 3-4 kg; the lambing rate is 90%, the litter size is 1.13-1.28, the fertility rate is 0.92-1.07, and the twin lambing rate is 2% [13].

In recent years, short-term protocols have been preferred for the synchronization of breeding in sheep [2, 14-16]. These protocols use progestogen for 5 [17], 6 [2], 7 [14], and 9 days [11], which are generally performed by adding eCG and $PGF_{2\alpha}$ injection. It is reported that the fertility rate is higher in short-term protocols than in the long-term [18]. It is believed that low fertility in long-term protocols is due to the fact that the follicular wave in sheep is slower and stimulates the ovulation of the persistently dominant follicle. It is explained that pregnancy rates are higher since ovulation of newly growing follicles probably occurs in short-term progestogen treatment [18]. It was determined that the estrus rate was 100% in sheep treated with progestogen and $PGF_{2\alpha}$ analog for 5 days [17]. In the study conducted by Biehl et al [14], on the other hand, the rate of estrus was determined as 70.8% in sheep who were administered short-term (7 days, $PGF_{2\alpha}$, 300 IU PMSG) intravaginal progestogen. Najafi [19] determined the estrus rate as 80%. It has been reported that the pregnancy rate varies between 75-93.75% during the breeding season [14-16,19-21]. It was seen that the litter size ratio in sheep that received short-term progesterone supplementation and injected with $PGF_{2\alpha}$ and different doses of eCG varied between 1.24-2.20 [16-18,20,22]. Fertility rates have been reported to be between 75 and 87.5% [21]. In the present study, it was determined that the rate of estrus in the groups was between 80-100%. The pregnancy rates in the groups were 63.64%, 91.67%, 50%, and 57.14%, respectively. Although the multiple pregnancy rate was not statistically significant, it was determined that it was highest in Group II (90%). In all these studies, it is thought that the wide range in the values of the reproductive parameters is due to the breed of the animal, the synchronization protocol, the progesterone source used, the dose of $PGF_{2\alpha}$ and eCG, and the injection days.

It is known that due to the increasing number of animals and unconscious grazing in our country, the pastures are severely damaged, and the plant quality has decreased significantly [23]. There is insufficient protein intake in sheep grazing on low-quality pastures. Protein deficiency both reduces the absorption of trace elements and leads to a lack of amino acids necessary for the synthesis of antioxidant enzymes and, therefore to a deterioration in antioxidant capacity [24]. Vit E plays an important role in the management of oxidative stress [8]. Some trace elements, such as selenium [25,26] and copper, act as a structural element of many enzymes and hormones and act on fertility [25]. It is reported that a low copper level during estrus negatively affects the pregnancy rate [25]. In cobalt deficiency, it has been determined that ovulatory response decreases in superovulated sheep. In addition, it is reported that subclinical cobalt insufficiency affects both the pregnancy and conception rate and the survival and viability of the newborn [10]. In a study investigating the effect of Vit E and Selenium injection on reproductive performance in estrus synchronization, injections were made on the day the sponge was applied, the day the sponge was removed (600 IU PMSG), and 19 days after the sponge was removed. No difference was found between the injected group and the control group in terms of the lambing rate of twins or triplets. It was found that the pregnancy rate (86.8%) was higher in the vitamin-administered group than in the control group (63.9%) ($P<0.01$). It has been determined that vitamin-mineral administration reduces pregnancy loss. It has been determined that fecundity and prolificacy increase with vitamin administration, but this difference is not statistically significant [27]. According to the study conducted by Birdane et al.[28], it was found that the injection of vitamins A, D3, and E into sheep synchronized with progestagen and eCG increases the pregnancy rate. It was determined that the number of offspring per pregnant sheep (1.54) and the rate of multiple births (42.9%) increased in the vitamin-administered group compared to the control group (1.37 and 33.3%, respectively). However, no statistical difference was found in any of these values.

In the study conducted by Farahavar et al.[29], Vit E+Selenium injection was given to sheep 2 weeks before CIDR was administered (for 13 days), on the day CIDR was administered, and on the day CIDR was removed. There are 18 sheep in each group. In the first service period, the number of lambing sheep was 11 in the vitamin-administered group, 10 in the control group, and the number of lambs born was 13 and 11 in the groups, respectively. Single births were determined as 9 in both groups; twins or triplet births were determined as 2-to-1, male offspring as 7-to-8, and female offspring as 6-to-3. The fertility rate was determined as 61.11% to 55.56%, pregnancy loss as 38.88% to 44.44%, and productivity as 118 to 110%, respectively. According to Zonturlu et al.[7], it was found that bolus administration of trace amounts of vitamin E and minerals 14 days before synchronization increased the rate of multiple births. While the pregnancy rate and lambing rates were 75.4% and 75.4%, it was determined as 67.2% and 65.6% in the control group. The number of twin offspring was found to be significantly higher compared to the control group. In the study conducted by Musa et al.[30], Vit E injections were given to one group and Vit E and Selenium injections to the other group at 14-day intervals, while the third group received saline injections as the control group. CIDR is left in the body for 14 days. Estrus rate was 80%, 100%, and 100% in control, Vit E and Vit E + Selenium groups; the pregnancy rate was 75%, 80%, 80%, lambing rate was 66.6%, 100%, 100%, fecundity was 100%, 100%, 100% respectively. It is reported that Vit E and Selenium have a positive effect on fertility. Although there was no

statistically significant difference in the presented study, the rate of estrus (95.65%), pregnancy (78.26%), and lambing (88.89%) in the group given bolus containing vitamin E and minerals were compared to the group not given (87.5%, 54.16%, and 76.92% respectively) were determined to be higher. In addition, the fecundity rate was also higher in the vitamin-mineral group. The absence of a statistically significant difference between the groups may be due to the small number of animals. It is thought that this increase in fertility parameters may be due to the intracellular antioxidant properties of vitamin E and trace elements and their contribution to the protection of cell membranes from oxidative damage by cleansing reactive oxygen radicals. It is because reactive oxygen species play a role in folliculogenesis, oocyte maturation, ovulation, corpus luteum formation and regression, implantation, and fetal development [30]. In addition, it is thought that intravaginal sponge application may be one of the sources of oxidative stress in synchronized sheep and that Vit E + Selenium supplementation reduces oxidative stress [29] and thus contributes to reproductive performance.

Exogenous $PGF_{2\alpha}$ injection stimulates the production of utero ovarian $PGF_{2\alpha}$ [31]. In sheep, the corpus luteum responds to $PGF_{2\alpha}$ between days 4-14 of the cycle. It is explained that $PGF_{2\alpha}$ injection is needed at a dose of 6 mg [32] for a corpus luteum on days 2-4 of the cycle and 10 mg for a mature corpus luteum [33,34]. It is reported that the response rate increases as the dose of $PGF_{2\alpha}$ increases [35]. It has been reported that the maximum response in a single injection is at the dose of 20 mg [35], and it can be used in divided doses every 3 h [36], reducing the cost of medication [37]. It was determined that the progesterone level decreased within 24 h and the percentage of estrus within 1-5 days was 35% in sheep given $PGF_{2\alpha}$ injection at a dose of 5 mg. At doses of 15 mg and 20 mg, this ratio was found to be 70% and 95%, respectively [35]. Mekuriaw et al.[38] reported that the rate of estrus and the time of onset of estrus did not change depending on the dose, but the pregnancy rate was higher in the group that received a high dose of $PGF_{2\alpha}$ injection. In a study conducted by Dixon et al.[37], CIDR was applied intravaginally for 5 days. $PGF_{2\alpha}$ injections were given double (20 and 17 h before) a day before CIDR removal. During the first service period, the pregnancy rate was determined as 63.9% in the first group, the lambing rate was determined as 95%, the pregnancy rate was determined as 71%, and the lambing rate as 95% in sheep that received a double dose of $PGF_{2\alpha}$ injection 3-h intervals on the day the CIDR was removed. The pregnancy rate was found to be higher in cows who were administered the CIDR + cosynch protocol for 5 days; and in those who received $PGF_{2\alpha}$ injections every 8 h [39] and 12 h [40] compared to those treated with a single $PGF_{2\alpha}$ protocol [39]. It has been reported that luteolysis is not complete in a single injection of $PGF_{2\alpha}$ or that it is caused by less complete luteinization [39,40]. In the present study, by taking previous studies as an example [37,41,42], dinoprost was used at a dose of 5 mg for luteolysis. It was determined that the rate of estrus and pregnancy was higher in the group that received $PGF_{2\alpha}$ injections with 24 hours intervals compared to those who received a single dose of $PGF_{2\alpha}$. In addition, it was found that the rate of multiple lambing was higher in the group receiving a double dose than in the group receiving a single dose of $PGF_{2\alpha}$. It is believed that this increase may be because a double dose of $PGF_{2\alpha}$ fully provides luteolysis and acts on the follicular wave and ovulation.

It was reported that the average birth weight of Morkaraman lambs was 3.41±0.06 kg. It has been reported that there is a difference of 1.38 kg in favor of singles and 0.438 kg in favor of rams between rams and ewes [43]. In the presented study, it was determined that the average offspring weight ranged between 3.34 and 4.88 kg. It was determined that the average weight of the offspring was the least in Group II and Group IV. In these groups, lamb weights were determined to be lower due to the multiple pregnancies.

In the study, it was confirmed that the progesterone level was >1 ng/mL in 76.6% of sheep on the day of sponge application (mean 4.16±0.42 ng/mL); that is, the animals were in cyclicity. On the day the sponge was removed, the level of subluteal progesterone (1.61±0.24 ng/mL) was determined. A lower progesterone level on the day of removal is because chronogest, which contains synthetic progesterone, cannot be measured in the blood [22].

As a result, in this study, it was determined that Vit E-mineral therapy and double $PGF_{2\alpha}$ injection increased the reproductive performance numerically, but this difference was not statistically significant. In particular, it was determined that a double $PGF_{2\alpha}$ injection contributes to the formation of a complete luteolysis and increases fertility and productivity. In order to fully understand the effect of Vit E-mineral treatment and $PGF_{2\alpha}$ on reproductive performance, it is recommended that a more comprehensive study should be conducted by increasing the number of animals.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author (M.C. Demir) on reasonable request.

Ethical Approval

The present study was approved by the Animal Research Ethics committee of the University of Kafkas (Ethics approval number: KAÜ-HADYEK-2021/157).

Conflicts of Interest

The authors declare no conflict of interest. The authors alone are responsible for the content and writing of paper.

Author Contributions

SK and CK planned the study, designed the experiments, and

helped manuscript writing; SK, CK, GK and MCD performed this study; GK and MCD collected blood samples and conducted laboratory process. SK analyzed the statistics data. All authors read and approved the final manuscript.

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RESEARCH ARTICLE

Influence of Sheep Tail Fat and Autochthonous Starter Culture on the Formation of Volatile Nitrosamines in Sucuk

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Abstract: The study aimed to investigate the effect of sheep tail fat (STF) on the volatile nitrosamines in a dry fermented sausage (sucuk) with/without autochthonous starter culture *(Lactiplantibacillus plantarum* GM77 and *Staphylococcus xylosus* GM92). Beef fat (BF) was used as control. The production was carried out under controlled conditions with initial fermentation temperature of 22±1°C. After production, samples were subjected to pH, a_w, thiobarbituric acid reactive substances (TBARS), residual nitrite, instrumental color and nitrosamine analysis. According to results, the use of STF increased TBARS value, while it decreased L* value. The use of starter culture lowered the mean pH below 5.0. On the other hand, mean pH of 5.23 was found in the group without starter culture. Both starter culture and fat type had no significant effect on residual nitrite. N-nitrosopiperidine (NPIP) content was affected by STF. In contrast, STF showed no significant effect on N-nitrosodimethylamine (NDMA) and N-nitrosomethylethylamine (NMEA) content of sucuk. Starter culture caused an increase in NDMA levels, while decreasing N-nitrosopiperidine (NPIP). According to principal component analysis (PCA), pH, a_w, TBARS, NDMA and NPIP located on positive side of the principal component 1 (PC1), while NMEA, residual nitrite, L*, a* and b* values were in negative side of the PC1. In addition, NMEA showed more correlation with BF.

Keywords: *Lipid oxidation, Sheep tail fat, NDMA, NPIP, Sucuk, TBARS*

Kuyruk Yağı ve Yerel Starter Kültürün Sucukta Uçucu Nitrozamin Oluşumuna Etkisi

Öz: Bu çalışmada, yerel starter kültür *(Lactiplantibacillus plantarum* GM77 and *Staphylococcus xylosus* GM92) içeren veya içermeyen kuru bir fermente sosiste (sucuk), koyun kuyruk yağının (KKY) uçucu nitrozaminler üzerindeki etkisinin araştırılması amaçlanmıştır. Sığır et yağı kontrol olarak kullanılmıştır. Üretim, 22±1°C başlangıç fermantasyon sıcaklığı ile kontrollü koşullar altında gerçekleştirilmiştir. Üretimden sonra örnekler pH, a_w, tiyobarbitürik asit reaktif maddeler (TBARS), kalıntı nitrit, enstrümental renk ve nitrozamin analizlerine tabi tutulmuştur. Sonuçlara göre kuyruk yağı kullanımı TBARS değerini artırırken L* değerini azaltmıştır. Starter kültür kullanılması ortalama pH'yı 5.0'ın altına düşürmüştür. Buna karşın, starter kültür kullanılmayan grupta ortalama pH değeri 5.23 olarak saptanmıştır. Hem starter kültür hem de yağ çeşidinin kalıntı nitrit üzerinde önemli bir etkisi olmamıştır. N-nitrozopiperidin (NPIP) içeriği KKY'den etkilenmiştir. Buna karşın, KKY sucuğun N-nitrozodimetilamin (NDMA) ve N-nitrozometiletilamin (NMEA) içeriği üzerinde önemli bir etki göstermemiştir. Starter kültür, NPIP'yi düşürürken NDMA düzeylerinde artışa neden olmuştur. Temel bileşen analiz sonuçlarına göre pH, a_w, TBARS, NDMA ve NPIP temel bileşen 1 (PC1)'in pozitif; NMEA, kalıntı nitrit, L*, a* ve b* değerleri ise PC1'in negatif tarafında yer almıştır. Ayrıca, NMEA, sığır et yağı ile daha fazla korelasyon göstermiştir.

Anahtar sözcükler: *Lipit oksidasyonu, Koyun kuyruk yağı, NDMA, NPIP, Sucuk, TBARS*

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Introduction

Nitrite is a multifunctional curing agent, due to with its antimicrobial and antioxidant activites, as well as its contributions to the product in terms of color and flavor, has been debated for years due to health concerns. The hazard of nitrite lies in its capability to form N-nitrosamine rather than nitrite itself [1]. N-nitrosamines are compounds formed by reactions between nitrosating agents and secondary amines. Many factors affect the nitrosamine formation in cured meat products, including ingoing nitrite levels, residual nitrite, pH, inhibitor and catalyst compounds, spice (especially black pepper), cooking and storage conditions [2,3]. Among nitrosamines, N-nitrosodimethylamine (NDMA), N-nitrosopyrrolidine (NPYR) and N-nitrosopiperidine (NPIP) are the commonly found nitrosamines in fermented sausages including sucuk [2,4]. Examining the factors affecting the formation of nitrosamines in sucuk is of great importance concerning public health. In this regard, the effect of ascorbate and black pepper on nitrosamine formation have been investigated for this product, previously [3]. Sheep tail fat can be used solely or in formulation with beef fat as raw material in sucuk production [5]. Since sheep tail fat is more susceptible to oxidation, it was thought that it may pose a risk in terms of nitrosamine formation. Some researchers have claimed that nitrosamine precursors may be formed due to lipid oxidation during ripening [6]. Free fatty acids formed through lipolysis are precursors of lipid oxidation. Secondary metabolites such as aldehydes, ketones and alcohols are formed during lipid oxidation and among these metabolites, malondialdehyde (MDA) which is measured by thiobarbituric acid reactive substances (TBARS) test, is considered as an indicator of lipid oxidation [7]. Kurechi et al.[8] reported that in vitro conditions, MDA significantly affects nitrosamine formation and that this effect is related to pH. The same researchers also stated that nitrosamine formation decreased at pH:3 and increased between pH:4-7 in MDA present, depending on the concentration of aldehyde. However, research into the interaction between lipid oxidation and nitrosamine formation is still needed. In the study, it was aimed to investigate the effect of sheep tail fat on nitrosamine formation in sucuk in the presence or absence autochthonous starter culture. In addition, the relationship between physicochemical properties and nitrosamine levels of sucuk samples belonging to both groups was determined.

Material and Methods

Material

Lean beef, and beef fat (intermuscular fat) or sheep tail fat were used as raw material. After visible fat and connective tissue were removed from lean beef, it was cut into small pieces. Similarly, fat materials were also cut into small pieces. Then, all the raw material was vacuum packaged and separately stored at -18°C for a week.

As starter culture, authochotonous strains *(Lactiplantibacillus plantarum* GM77 and *Staphylococcus xylosus* GM92) were used [5]. *L. plantarum* GM77 was grown in De Man, Rogosa and Sharpe (MRS) broth (Merck, Darmstadt, Germany) and *S. xylosus* GM92 was grown in Tryptic Soy Broth (TSB) (Merck, Darmstadt, Germany) at 30°C for 24 h. *L. plantarum* GM77 was inoculated with 10^7 cfu/g, while *S. xylosus* GM92 was inoculated with 10^6 cfu/g.

Sucuk Production

Four independent sausage batches were prepared: A: Beef Fat + Lean Beef; B: Beef Fat + Lean Beef + Starter culture; C: Sheep Tail Fat + Lean Beef; D: Sheep Tail Fat + Lean Beef + Starter culture. Per kg lean beef and fat (beef fat or sheep tail fat), 0.15 g sodium nitrite, 20 g salt, 10 g garlic, 7 g red pepper, 2.5 g allspice, 9 g cumin, 5 g black pepper, 4 g sucrose and were used in the production. For each treatment, 4 kg of lean beef and 1 kg of fat were used. Sucuk batches were produced in a laboratory cutter (MADO type MTK 662, Dornhan/Black Forest). Three batches were independently prepared in different times for each treatment.

The sucuk batches were filled into collagen casings (Naturin Darm, 38 mm) using laboratory filling machine (MADO Typ MTK 591, Dornhan/Schwarzwald). The sausages were placed into a climate unit (Reich, Germany). The program was used for the ripening as following; for 1 day with a temperature of 22±1°C, relative humidity of 91±2%, and for the second day with a temperature of 20±1°C, relative humidity of 91±2%. On the following days (3^{th}, 4^{th} and 5^{th} days), the temperature was kept constant at 18±1°C, and the relative humidity was gradually reduced to 89±2%, 87±2% and 84±2%, respectively [5].

Physico-chemical Analysis

The a_w value was detected by using the a_w device (Novasina, TH-500 a_w Sprint). To determine the pH, a 10 g of sample was homogenized with 100 mL of distilled water using an Ultra-Turrax (IKA factory T 25, Germany). The pH was measured with a pH meter (ATI ORION 420, MA 02129, USA) after calibrated with 4.01 and 7.01 buffers at 22°C [5]. TBARS was detected according to the method by Kilic and Richards [9]. The results were expressed as µmol MDA/kg. Residual nitrite analysis was carried out as to the method given by NMKL [10]. The instrumental color values (L*, a*, b*) were determined by the colorimeter device (Minolta Co, Osaka, Japan) with a *C D65 illuminant, an aperture size of 8 mm and standard observed of 2° [11].

For nitrosamine analysis, ten g of sample and 1M NaOH solution was placed into a centrifuge tube, after sonication, methanol of 20 mL was added to homogenize.

Then the homogenate was centrifuged (4°C, 10.000 rpm). After filtration (glass microfiber filter, 70 mm diameter; Whatman, Maidstone, UK), 5 mL of 20% NaCl solution and 15 mL of filtrate were loaded onto a ChemElut column (20 mL unbuffered; Agilent, Santa Clara, CA). After equilibration, the column was eluted with dichloromethane. The eluent was concentrated to 1 mL with a Kuderna Danish equipment. The concentrate was evaporated using a stream of nitrogen (N-EVAP-111 evaporator, Organomation, Berlin, MA) at 40°C. Gas chromatography (GC) with mass spectrometry (MS) (Agilent 6890N/Agilent 5973; Agilent, Santa Clara, CA) was used for the detection of nitrosamines. Helium as carrier gas and DB-5MS (30 m x 0.25 mm x 0.25 µm, Agilent) as column were used in the system, and the mass spectrometer was operated in selective ion mode (SIM). N-Nitrosodipropylamine-d_{14} was used as an internal standard. The oven temperature of gas chromatography was set at 50°C for 2 min, and after that oven temperature was raised to 100°C at 3°C/min, held for 5 min, then increased to 250°C at 20°C/min. EPA 521 nitrosamine mix (Supelco, Bellefonte PA) was used for identification and quantification of volatile nitrosamines (N-nitrosodiethylamine (NDEA); N-nitrosodibutylamine (NDBA); N-nitrosomethylethylamine (NMEA); N-nitrosodipropylamine, (NDPA); NPYR; NDMA; NPIP) [12]. The results were given as µg/kg. Correlation coefficient (R^2) was determined 0.9999 for all nitrosamines. Linear range, limit of detection (LOD), limit of quantification (LOQ), linear equation, correlation coefficient (R^2), recovery and relative standard deviation (% RSD) values for nitrosamines are given in *Table 1*.

Statistical Analysis

Different fat type (sheep tail fat-STF or beef fat-BF) and autochthonous starter culture (with starter culture-"with SC" or without starter culture-"without SC") were taken as factors, and experiments were performed according to the randomised complete block design with three blocks. The results of analysis were subjected to two-way ANOVA analysis using the general linear model with SPSS version 24.0 statistical program (SPSS Inc., Chicago, IL, USA). Different fat type and starter culture were fixed factors and the replicates were random effect for all analysis. The means were compared using Duncan's multiple comparison test at the $P<0.05$ level. Means and standard errors were reported. Principal component analysis (PCA) was plotted to assess the relationships between physicochemical properties and nitrosamine levels of sucuk samples belonging to both groups. PCA was carried out with the Unscrambler software (CAMO software version 10.1).

Results

The overall effects of fat type and starter culture on the pH, a_w, TBARS, residual nitrite, color values and nitrosamine levels of sucuk are given in *Table 2*. Fat type had no significant effect on pH ($P>0.05$), on the contrary, the starter culture had a notable effect on pH ($P<0.01$). The mean a_w value of the groups with and without starter culture were 0.910 and 0.905, respectively, and the difference between groups were not significant ($P>0.05$). The mean TBARS value was higher in sucuk containing STF compared to the sucuk with BF ($P<0.05$). The starter culture had no significant effect on TBARS value ($P>0.05$). Residual nitrite was also not influenced by starter culture and fat type ($P>0.05$). Sucuk with STF had a lower mean L* value than the BF group. However, starter culture caused an increase in L* value ($P<0.05$). In addition, the starter culture increased the a* value and decreased the b* value ($P<0.05$).

As for volatile nitrosamines, the fat type had no significant effect on NDMA. However, the use of starter culture caused an increase in NDMA ($P<0.05$). Neither starter culture nor fat type affected NMEA level ($P>0.05$) *(Table*

Table 1. *Linear range, limit of detection (LOD), limit of quantification (LOQ), linear equation, recovery (%) and relative standard deviation (RSD %) values of volatile nitrosamines*

Volatile Nitrosamine	Linear Range (µg/L)	LOD (µg/L)	LOQ (µg/L)	Linear Equation	Recovery %	RSD %
NDMA	0.5-20	0.32	0.98	y=39157x-9242.9	101-104.37	4.44-6.15
NMEA	0.5-20	0.42	1.28	y=37559x-524.7	97-101.07	1.85-6.78
NDEA	0.5-20	0.44	1.34	y=37649x-637.8	94-101.36	3.06-7.42
NPYR	0.5-20	0.36	1.09	y=28751x-2272.5	95.43-103.17	2.98-7.12
NPIP	0.5-20	0.32	0.98	y=32293x-7776.8	99.73-100.83	0.95-4.56
NDBA	0.5-20	0.38	1.15	y=13616x-3859.9	96.97-99.79	1.26-5.81
NDPA	0.5-20	0.15	0.46	y=6376.7x-3529.3	97.17-99.79	0.77-5.77

NDMA: *N-nitrosodimethylamine;* ***NMEA:*** *N-nitrosomethylethylamine;* ***NDEA:*** *N-nitrosodiethylamine;* ***NPYR:*** *N-nitrosopyrrolidine;* ***NPIP:*** *N-nitrosopiperidine;* ***NDBA:*** *N-nitrosodibutylamine;* ***NDPA:*** *N-nitrosodipropylamine;* ***LOD:*** *Limit of detection;* ***LOQ:*** *Limit of quantification;* ***RSD:*** *Relative standard deviation*

Table 2. *The overall effects of fat type and starter culture on pH, a_w, TBARS, residual nitrite, color values and volatile nitrosamine levels of sucuk*

Group	n	pH	a_w	TBARS µmol MDA/kg	Residual Nitrite (mg/kg)	L*	a*	b*	NDMA (µg/kg)	NMEA (µg/kg)	NPIP (µg/kg)
Fat Type (FT)											
Beef Fat (BF)	12	4.94	0.905	5.55^b	12.10	35.74^a	12.55	9.17	0.67	0.69	1.42^b
Sheep Tail Fat (STF)	12	4.92	0.909	13.46^a	10.31	34.76^b	12.59	8.92	0.84	0.72	2.09^a
SEM		0.036	0.02	0.792	0.950	0.495	0.253	0.142	0.060	0.088	0.162
Significance		ns	ns	**	ns	**	ns	ns	ns	ns	*
Starter Culture (SC)											
Without SC	12	5.23^a	0.910	9.85	11.09	33.76^b	12.04^b	8.96	0.61^b	0.57	2.30^a
With SC	12	4.63^b	0.905	9.16	11.32	36.73^a	13.0^a	9.13	0.90^a	0.85	1.20^b
SEM		0.036	0.002	0.792	0.950	0.495	0.253	0.142	0.060	0.088	0.162
Significance		**	ns	ns	ns	**	*	ns	*	ns	**
FTxSC		ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

^{a,b} *Values within a column with different superscripts differ significantly at P<0.05;* ***ns:*** *not significant: P>0.05; * P<0.05, ** P<0.01;* ***SEM:*** *Standard error of mean;* ***FT:*** *Fat type;* ***BF:*** *Beef fat;* ***STF:*** *Sheep tail fat;* ***SC:*** *Starter culture;* ***TBARS:*** *Thiobarbituric acid reactive substances;* ***NDMA:*** *N-nitrosodimethylamine;* ***NMEA:*** *N-nitrosomethylethylamine;* ***NPIP:*** *N-nitrosopiperidine*

2). The group with starter culture gave a higher mean for NMEA, but the difference between the two groups was not statistically significant (P>0.05). NPIP content was found at higher level in group containing STF (P<0.05) *(Table 2)*.

PCA was applied to evaluate the relationships between factors (BF, BF_SC, STF and STF_SC) and nitrosamines, pH, a_w, TBARS and residual nitrite *(Fig. 1)*. The principal component1 (PC1) and principal component2 (PC2) were to explain 84% and 14% of the variation, respectively *(Fig. 1)*. The groups with STF and STF_SC situated on positive part of PC1, while the groups with BF and BF_SC placed on negative part of PC1 *(Fig. 1)*. In addition, pH, a_w, TBARS, NDMA and NPIP situated on positive part in PC1, while NMEA, residual nitrite, L*, a* and b* values were in negative part for PC1 *(Fig. 1)*.

Discussion

Lactic starter culture used in this study showed a good development during fermentation, lowering the pH. Similar results have been found in other studies [3,4,13]. The most important function of lactic acid bacteria during fermentation is to produce lactic acid from the sugars added to the batches. Acid formation during fermentation contributes to product safety and provide to the development of sensory and textural properties [11,14]. Therefore, the desired drying was also achieved in the sucuk without starter culture.

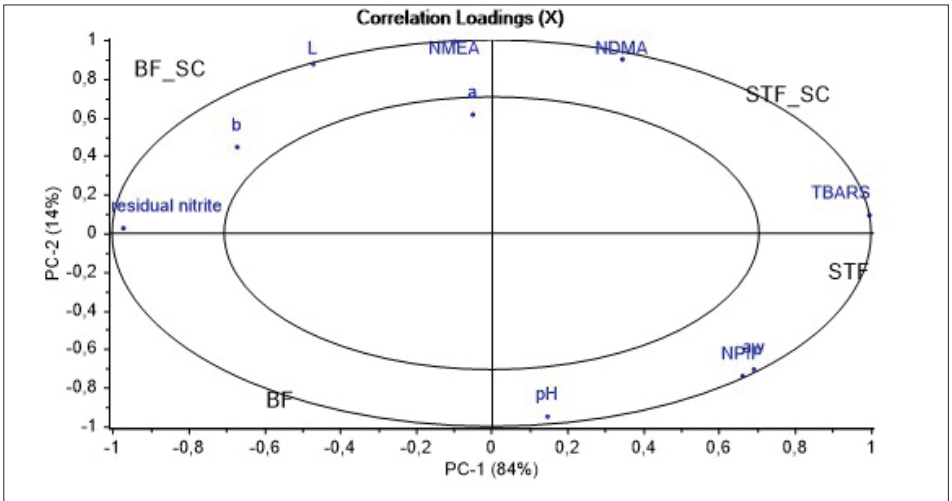

Fig 1. Principal component analysis biplot of the relationships between groups (starter culture and/or fat type) and nitrosamines (BF_SC: Beef fat with starter culture; BF: Beef fat without starter culture; STF_SC: Sheep tail fat with starter culture; STF: Sheep tail fat without starter culture)

In this study, the high TBARS value in the sucuk group containing STF is thought due to the fact that STF contains more mono- and polyunsaturated fatty acids than BF [15]. High levels of unsaturated fatty acids significantly accelerate lipid oxidation as the double bonds are easily oxidized, yielding a range of oxidation products including alcohols, ketones and aldehydes [7]. On the other hand, having no effect of starter culture on TBARS is probably due to the low pH difference between the STF and BF groups. pH also plays an important role in nitrite degradation in fermented sausages, and a decrease in pH increases nitrite degradation [11,14]. However, in the present study, although there was a significant difference between the groups with and without starter culture, no statistical difference between groups was found in terms of the residual nitrite. This result probably indicates that lowering the pH to around 5.3, as in the non-starter culture group, may be sufficient for nitrite degradation. On the other hand, starter culture addition increased a* and L* value in this study *(Table 2)*. Similar findings were also reported by Lorenzo et al.[16].

In the present study, increasing the NDMA level in sucuk group with starter culture was thought to be related with a sharp pH decrease by starter culture. The formation of volatile nitrosamine in fermented sausages can occur more easily as the pH of the product approaches optimum pH level (pH: 3.5) of the nitrosation reaction [17]. NDMA can be formed when dimethylamine reacts with the nitrosating agent [18]. In addition to pH effect, it is thought that proteolytic degradation products formed during ripening play a role in the increase of NDMA. Indeed, many researchers have stated that proteolysis degradation products are important precursors for NDMA formation [19]. Scanlan and Issenberg [20] also state that various micro-organisms produce substances that catalyze nitrosation. The mechanism of nitrosamine formation is quite complex. This mechanism is affected by many factors such as pH, oxidation-reduction potential, a_w, level of precursors, ingoing nitrite level, residual nitrite [19].

The factors examined in this study had no impact on NMEA and mean NMEA values were below than 1 µg/kg *(Table 2)*. Similar findings were reported by Herrmann et al.[2]. As seen in *Table 2*, the sucuk group containing STF gave a higher mean NPIP value. This result is linked to be due to the fact that sucuk groups produced with STF had higher TBARS values than those with BF *(Table 2)*. In studies conducted on model systems, it is also stated that MDA, an indicator of lipid oxidation, promotes the nitrosamine formation [8,21,22]. Lu et al.[23] also emphasized that lipid oxidation promotes the formation of nitrosamine. In the current study, the starter culture caused a decrease in NPIP level *(Table 2)*. This result suggested that the decrease in NPIP level by starter culture in sucuk might be associated with the reduction in its precursors.

PCA results indicate that as lipid oxidation increases, NDMA and NPIP levels increase. It also turns out that NDMA and NPIP are dependent on pH and a_w. Although the groups with starter culture (BF_SC and STF_SC) placed on the positive side of PC2, BF and STF groups placed on the negative side of PC2. These results explain that the starter culture showed a similar effect in both types of fat. Namely, PC2 separated the groups with (BF_SC and STF_SC) and without starter culture (BF and STF), regardless of fat type. On the other hand, PC1 separated the beef fat (BF and BF_SC) and sheep tail fat (STF and STF_SC) from each other. Based on these results, STF groups with and without starter culture positively affected NDMA and NPIP. In addition, NMEA more correlated with BF groups with and without starter culture *(Fig. 1)*.

In conclusion, the starter culture caused a significant decrease in pH value during fermentation of sucuk, in addition, it increased a* value. TBARS was not affected by the starter culture, but STF accelerated lipid oxidation. Addition of starter culture increased NDMA and decreased NPIP. STF significantly increased NPIP, however, NMEA was more correlated with BF. TBARS had a positive correlation with NDMA and NPIP as well as pH, a_w.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author.

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Ethical Statement

Ethics approval was not required for this research due to conducting *in vitro* in the laboratory.

Conflict of Interest

The author declared that there is no conflict of interest.

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RESEARCH ARTICLE

Evaluation of GV26 Electrical Acupuncture Stimulation on Anesthetic Recovery Time of Spur-thighed Tortoise *(Testudo graeca)*

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Abstract: Recovery time from anesthesia can be extended in reptiles, consequently patients undergoing general anesthesia, require prolonged monitoring period which increases the probability of postoperative complications. Therefore, prolonged recovery time following inhalant anesthesia is a common complication in chelonians. Ability to intracardiac shunting and bypassing blood from pulmonary circulation, may contribute to their unpredictable inhalant anesthetic recovery times. The acupuncture point Governing vessel (GV-26) has been demonstrated to reduce anesthetic recovery times from inhalant anesthesia in many species. In this study eight spur-thighed tortoises *(Testudo gracea)* were anesthetized by isoflurane administration for 60 min. The trial was performed in two parts with four weeks washout period. In the first time, once isoflurane administration was discontinued, the tortoises received no therapy in the recovery period and in the second part of the study, tortoises received GV-26 electroacupuncture stimulation. Physiologic variables, anesthetic parameters, time to first movement and time to extubation were recorded. Data were compared with the use of independent sample t tests. Tortoises receiving GV-26 electroacupuncture had a significantly reduced time to return of voluntary movement ($P<0.001$), and a significantly reduced time to extubation ($P<0.001$). Furthermore, time from first movement to extubation was significantly shorter ($P<0.05$). Hence, the use of GV-26 electroacupuncture results in significant reduction of anesthetic recovery time in spur-thighed tortoises which have received inhalant anesthetic.

Keywords: *Anesthesia, Electroacupuncture, GV-26, Recovery, Spur-thighed tortoise, Testudo graeca*

GV26 Elektriksel Akupunktur Stimülasyonunun Mahmuzlu Kaplumbağanın *(Testudo graeca)* Anesteziden Uyanma Süresi Üzerine Değerlendirilmesi

Öz: Sürüngenlerde, genel anestezi altındakilerde, operasyon sonrası komplikasyon olasılığını artıran uzun bir izleme süresine ihtiyaç duyulduğundan, anesteziden uyanma süresi uzayabilir. Bu nedenle, inhalasyon anestezisini takiben uzayan uyanma süresi kaplumbağalarda yaygın bir komplikasyondur. İntrakardiyak şantın azaltılması ve pulmoner dolaşımdan kanın bypass edilmesi, inhalasyon anestezisinden uyanma sürelerine katkıda bulunabilir. Akupunktur noktasını yöneten damarın (GV-26) birçok türde inhalasyon anestezisinde anesteziden uyanma sürelerini azalttığı gösterilmiştir. Bu çalışmada, 8 adet mahmuzlu kaplumbağaya *(Testudo gracea)* izofluran uygulanarak 60 dakika süresince anestezi uygulandı. Anestezi işlemi, dört haftalık arınma periyodunu takiben iki kez uygulandı. İlk uygulamada, izofluran verildikten sonra, kaplumbağalar uyanma döneminde herhangi bir tedavi almadı. Çalışmanın ikinci bölümünde ise kaplumbağalara GV-26 elektroakupunktur stimülasyonu verildi. Fizyolojik değişiklikler, anestezik parametreler, ilk hareketliliğe kadar geçen süre ve ekstübasyona kadar geçen süre kaydedildi. Veriler, bağımsız örneklem t testleri ile karşılaştırıldı. GV-26 elektroakupunktur uygulanan kaplumbağalarda istemli hareketin geri dönme süresi ($P<0.001$) ve ekstübasyon süresi ($P<0.001$) önemli ölçüde azaldı. Ayrıca, ilk hareketten ekstübasyona kadar geçen süre anlamlı derecede kısaydı ($P<0,05$). Dolayısıyla, GV-26 elektroakupunktur uygulaması, inhalasyon anestezisi yapılan mahmuzlu kaplumbağalarda anesteziden uyanma süresini önemli ölçüde azaltmaktadır.

Anahtar sözcükler: *Anestezi, Elektroakupunktur, GV-26, Uyanma, Mahmuzlu kaplumbağa, Testudo graeca*

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Introduction

Chelonians may be anesthetized for diagnostic, therapeutic and research purposes. Inhalant anesthesia is safe and easy to administrate in veterinary medicine, therefore using these agents for reptile anesthesia are common. Although inhalant anesthesia regularly used, prolonged recovery times from anesthesia is a common complication in many reptiles such as chelonians [1,2]. Non-crocodilian reptiles as chelonians have a three-chambered heart including one ventricle and two atria. Also, a septum-like structure named muscular ridge divide the ventricle into two chambers. Muscular ridge is not complete anatomic separation and lack of complete separation enables intracardiac shunting. Different pressure between the pulmonary and systemic circulations regulates the size and direction of the shunts. The pressure differences are due to vascular resistance which could be controlled by cholinergic and adrenergic factors [1,3]. Right-to-left intra-cardiac shunting which occurs during anesthesia and is documented in chelonians is one theory for prolonged anesthetic recovery times. These shunts affect systemic arterial oxygen content, and also the uptake and elimination of anesthetic agent. Inhalant anesthetics in these species undergoes little metabolism prior to being eliminated from the body and the primary route of inhalant anesthetic elimination is exhalation via the respiratory tract. The circulatory system of chelonians could effectively act as an anesthetic storage site when blood is shunted away from pulmonary circulation during anesthesia [1,4]. Delayed anesthesia recovery time leads to extended monitoring period. Prolonged anesthesia monitoring can result in increased personal time, labor cost, morbidity and mortality. Apnea is the most common post-operative complication of reptile anesthesia and difficulties in monitoring is a frequent problem encountered during post anesthetic period. Consequently, establishing a safe and effective method that reduces right-to-left intracardiac shunting would decrease anesthetic recovery time [5,6]. Therefore, techniques such as acupuncture which is documented in many species, would be advantageous for patients, staff and clinicians [7,8]. The acupuncture Governing vessel 26 (GV-26), also known as Renzhong, is one of the most used emergency acupoints in humans and animals. GV-26 is used commonly as an adjunctive therapy in the treatment of shock, cardiopulmonary arrest and apnea. This point is generally located on midline below the nares [7]. An anatomic location for GV-26 has been illustrated in eastern box turtle *(Terrapene carolina carolina)* [4]. The aim of this study was to evaluate the efficacy of electrostimulation of the GV-26 point on reducing inhalant anesthesia recovery time in the spur-thighed tortoise *(Testudo graeca)*. The hypothesis was that time to extubation would be significantly faster following GV-26 electrostimulation in spur-thighed tortoises.

Material and Methods

Ethical Approval

All procedures were in accordance with relevant guidelines of the animal experimental ethics committee of IR.IAU. REC.1399.248.

Animals

Eight adult female spur-thighed tortoises *(Testudo graeca)* with a body mass ranging of 2.25±0.3 kg (Mean±SD) were obtained from wildlife rehabilitation center (Chamran wild animals park, Karaj, Alborz, Iran).

Study Design

They were housed individually in 100x80x80 cm containers with basking area and UV lamps (UVB 160W, JBL, China). The room humidity was maintained at 40-50%, and photoperiod kept under 12:12 light: dark cycle. The temperature gradient and humidity level of the enclosure were monitored twice daily (at 7:00 am and 7:00 pm) [9]. The animals had free access to water at all times and were fed with green leaf base (75%), vegetable (15%) and fruits (10%) once a day, five days a week. Food was withheld 12 h prior to anesthesia [10]. Tortoises were in good general health and condition based on a physical examination performed prior to experiment [11]. This study was carried out using the same tortoises with a washout period of four weeks. Thus, each tortoise was anesthetized twice, first time in control group they received only inhalant isoflurane with no additional treatment during recovery, and one month later, the same tortoises received GV-26 electroacupuncture immediately following discontinuation of isoflurane as the treatment group. Tortoises were manually restrained and face mask was used with 5% concentration of isoflurane with a flow rate of 1 L/min (Piramal Critical Care, USA) to induce with anesthesia machine (Leon plus Bad Ems, Germany). The anesthesia induction was continued until they reached a sufficient level of unconsciousness for intubation. The animals were intubated with a 2 mm (internal diameter) un-cuffed endotracheal tube. Following intubation, tortoises were connected to rebreathing anesthetic circuit and administered isoflurane 5% in 100% oxygen (1 L/min) to achieve a deep plane of anesthesia, then isoflurane level was reduced and maintained at 3% up to 60 min. Administration of anesthetic flow were discontinued 60 min from the intubation time. Each tortoise received similar anesthetic protocol in each of the two processes of study. In this study some sedation parameters such as palpebral, corneal and withdrawal reflexes and response to limb extension, in addition to vital parameters including heart rate (HR), respiratory rate (RR), saturation of peripheral oxygen (SPO_2), end-tidal CO_2 ($ETCO_2$) and cloacal body temperature were evaluated every 5 min up to 90 min, thereafter evaluated every 10

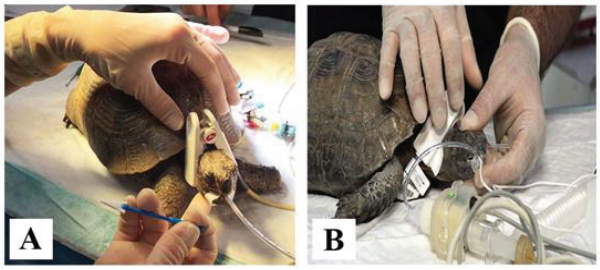

Fig 1. Anesthesia monitoring in spur-thighed tortoise anesthetized with an inhalant anesthetic. A. Evaluation of reflexes, B. GV-26 electroacupuncture needle insertion

min until extubation *(Fig. 1-A)*. In the first experiment, tortoises were received no treatment after they were disconnected from the anesthetic machine, and data were considered as control group. Animal were allowed to washout for 4 weeks before the next experiment. In the second experiment and as treatment group, animals were received electrical stimulation of the GV-26 point with electroacupuncture machine (Hwato SDZ-11, China) immediately after inhalant anesthetic was discontinued. GV-26 stimulation was performed by individual, trained by a certified acupuncturist. The acupoint (GV-26), which is located at midpoint of the ventral margin of the nares, was stimulated with 36-gauge stainless-steel disposable acupuncture needle (Suzhou, China) and a pair of wire ad joint to needle *(Fig. 1-B)*. Electrical stimulation was performed at a continuous 10-Hz frequency, until the time of recovery. Extubation was carried out after a tortoise showed spontaneous respiration and voluntary movement, and it was characterized as recovery.

Statistical Analysis

Data were analyzed using SPSS, version 26. Independent sample t-test was used to compare recovery time and anesthetic parameters for the control and treatment groups. Data were presented as mean ± SD and P<0.05 was considered statistically significant.

Results

No morbidity, mortality or complication were observed in any of the tortoise during the study period. All tortoises were returned to rehabilitation center and few months later they released into wild. Spur-thighed tortoise received GV-26 electroacupuncture had significantly faster times to return of voluntary movement than tortoises in the control group and also extubation was significantly faster following electroacupuncture stimulation of GV-26. The results and P values are displayed *(Table 1)* *(Fig. 2)*. Mean time from first movement to extubation was significantly faster following GV-26 electrical stimulation in the treatment group. Time from first movement to extubation for both groups is reported *(Table 2)*. Return of spontaneous respiration time recorded in the control and

Table 1. *Anesthetic recovery parameters for spur-thighed tortoises*

Recovery Parameter	Control Group M±SD	Treatment Group M±SD	P Value
Extubation (min)	105.50±8.35	82.87±8.36	P<0.001
First voluntary movement (min)	121.50±3.96	88.63±12.88	P<0.001

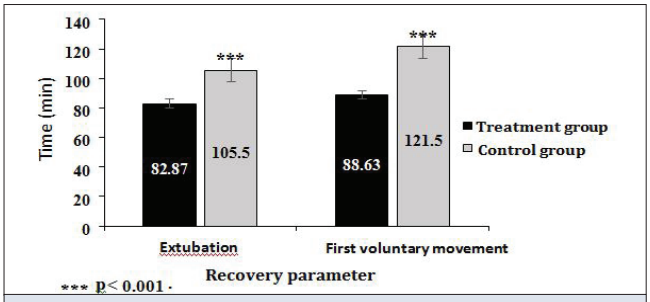

Fig 2. Comparison of mean extubation time and mean first voluntary movement time

treatment groups. Mean time to return of spontaneous respiration were compared and it was not significantly different between groups (P=0.751) *(Fig. 3)*. Return of corneal reflex, palpebral reflex, withdrawal reflex and response to limb extension were compared between the two groups. No significance was found when comparing the time to return of corneal and palpebral reflexes. Tortoises in the treatment group had significantly reduced time to return of withdrawal reflexes and response to limb extension. Result and P value are displayed *(Table 3)* *(Fig. 4)*. Heart rate at 5 min, 30 min, 60 min and 70 min were not significantly different between groups (P>0.05). Although

Table 2. *Anesthetic recovery parameters for spur-thighed tortoises*

Recovery Parameter	Control Group M±SD	Treatment Group M±SD	P Value
Time from first movement to extubation (min)	16.00±6.19	5.75±7.79	P<0.011

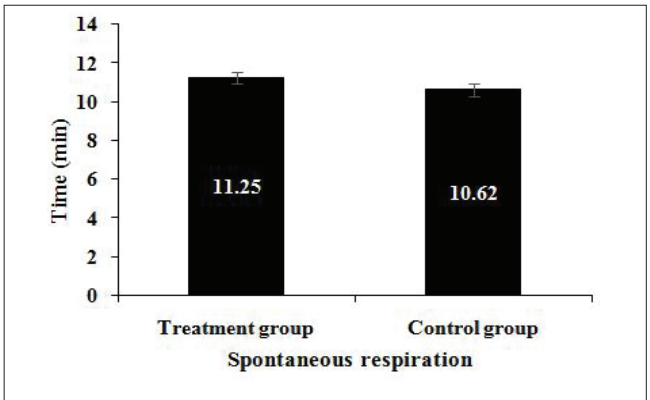

Fig 3. Comparison of return of spontaneous respiration

Table 3. *Comparison of return of reflexes*

Return of Reflexes	Control Group M (min)±SD	Treatment Group M (min)±SD	P Value
Corneal reflex	78.12±26.31	68.75±12.17	0.376
Palpebral reflex	86.87±21.87	73.75±9.54	0.152
Withdrawal reflex	98.75±12.46	80.00±9.64	0.005
Response to limb extension	107.50±4.63	83.12±11.00	<0.001

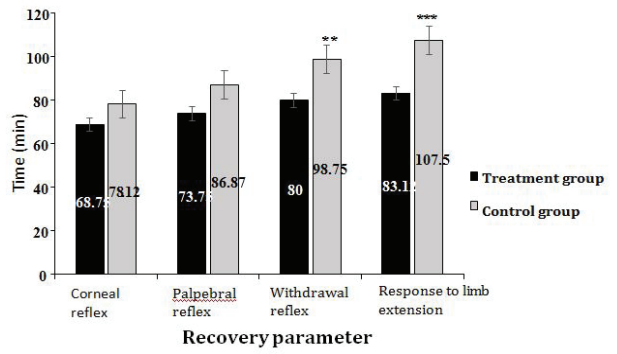

Fig 4. Comparison of mean time to return of reflexes (**P<0.01, ***P<0.001)

within 10 min following GV-26 electrostimulation, heart rate increased relative to control group, but this was not statistically significant (P=0.099) *(Fig. 5)*. Mean end-tidal CO_2 at 5 min, 30 min, 60 min and 70 min were not significantly different between control and treatment groups (P>0.05). Mean end-tidal CO_2 was 18.3± 8.99 and 16.6±18.75 just prior to extubation in treatment and control groups respectively. There was no significant difference between the two groups (P=0.792) *(Fig. 6)*. Mean respiratory rate compared between the control and treatment groups. Despite the fact that mean respiratory rate in treatment group increase faster following GV-26 electrical stimulation, the change of mean respiratory rate from 60th to 70th min was not significant (P=0.125). Furthermore, at time points of 5 min, 30 min, 60 min and 70 min, significant differences were not observed between groups (P>0.05) *(Fig. 7)*. SPO_2, and body temperature

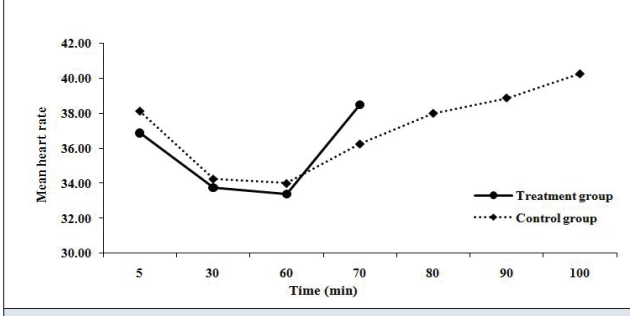

Fig 5. Distribution of heart rate over time

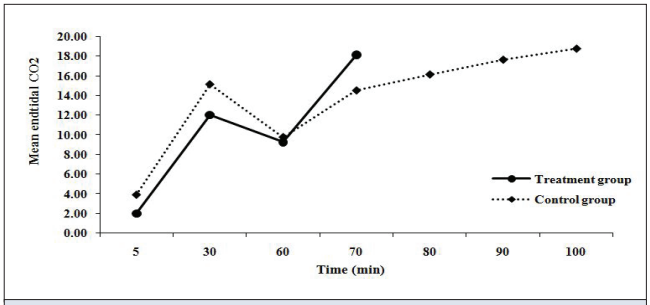

Fig 6. Distribution of Mean end-tidal CO_2 over time

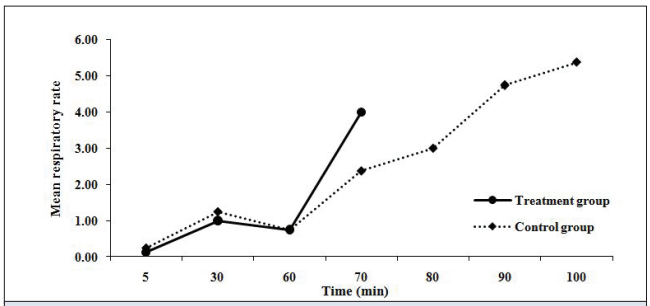

Fig 7. Distribution of Mean respiratory rate over time

were compared between the control and treatment groups at time points of 5 min, 30 min, 60 min and 70 min. These variables were not significantly different between groups at mentioned time points (P>0.05).

Discussion

Isoflurane is currently the inhalation anesthetic choice for reptiles; however, inhalant anesthesia in these species may result in a prolonged time to return of breathing. Intracardiac shunting occurs during anesthesia and these right-to-left shunts can affect the elimination of inhalant agents. Isoflurane undergoes extremely limited renal and hepatic metabolism. Isoflurane is excreted almost exclusively by the lungs and the respiratory excretion has negative influences on the recovery time in these species. Furthermore, because reptiles lack diaphragm, they rely on the thoracic muscles for ventilation. Both inspiration and expiration are active processes, so the respiratory depression associated with anesthesia may be more profound relative to mammalian species where expiration is a passive process. It is imperative that regular spontaneous respiration is in conjunction with movement of the limbs. It is established, re-sedation and apnea may occur, if the animal is not continually stimulated during recovery period [2,12]. Generally, chelonians have relatively lower metabolic rate between all reptile species. They are expected to metabolize and excrete drugs more slowly compared to other species. Furthermore, chelonians are faced with additional respiratory challenges since expansion of the thoracic cavity by movement of the ribs is not possible [1,3]. During apnea, parasympathetic

tone leads to increased pulmonary vascular resistance, bradycardia and reduction of pulmonary perfusion. These cardiovascular changes result in the development of right-to-left shunt. In contrast, ventilation is associated with reduction of pulmonary vascular resistance, tachycardia and increase in pulmonary perfusion. Therefore, sudden changes in the direction of the blood can lead to sudden changes in serum concentration of inhalant anesthetics which can markedly decrease recovery times [13]. Several studies have evaluated recovery in reptiles receiving various anesthetic protocols; however, few have evaluated methods specifically focused on shortening the recovery period [5,13,14]. The use of acupuncture in reptiles has been described but not objectively evaluated. Positive effects of GV-26 electrostimulation on resuscitation and anesthetic recovery in human and many animal species, make this acupoint of particular clinical interest. No complication was encountered, and no adverse effects have been noted by the authors [13-15]. In a study, electrical stimulation of GV-26 in common snapping turtles *(Chelydra serpentina)* reduced significant recovery times following inhalant isoflurane anesthesia. Also return of spontaneous ventilation and time to movement were significantly faster following the GV-26 electrostimulation in the turtles [14]. In another study, eastern box turtles *(Terrapene carolina carolina)* received either GV-26 acupuncture and GV-26 electroacupuncture following anesthesia with intramuscular administration of dexmedetomidine. Both of the treatments led to significantly reduced time to return of voluntary movement and a significant reduced time to anesthetic recovery [13]. Our results were parallel to aforementioned studies since stimulation of GV-26 with electroacupuncture was associated with significantly reduction of time to first voluntary movement and significantly reduced time to extubation, thus, significant reduction of anesthetic recovery time in isoflurane-anesthetized spur-thighed tortoises achieved. Furthermore, in the present study, time from first voluntary movement to extubation, time to return of withdrawal reflex and time to return of response to limb extension were significantly shorter. These results were both statistically and clinically significant. Following induction with isoflurane, tortoises became relaxed from cranial to caudal sides and motor function was returned in the opposite direction during the recovery time. Response to limb extension was observed in all animals prior to the return of voluntary movement. Similarly, another study on eastern box turtle indicated that animals received electroacupuncture had significantly reduced time to response to limb extension [4]. In the present study, despite the lack of statistical significance, heart rate and end-tidal CO_2 were increased faster following GV-26 electroacupuncture stimulation. End-tidal CO_2 also increased over time indicating increase of pulmonary blood flow. In another study, GV-26 electroacupuncture had similar effects on heart rate and end-tidal CO_2 in common snapping turtles *(Chelydra serpentina)* [7]. In this study, mean time for spontaneous respiration return was 10 min in the control group and 11 min in the treatment group, so there was no significant difference between the groups. The main reason for this event was probably the reduction of isoflurane concentration from 5% to 3% after few minutes from anesthesia induction. Since the mean breath per minute (BPM) was between zero to one until isoflurane was discontinued, it may consider that some tortoises were apnea in all time points and another reason is that the tortoises had just one breath in every few minutes due to their periodic respirations [2]. Although, the tortoises were maintained at a deep plane of anesthesia in order to encourage maximal pulmonary to systemic shunting. The absence of reflexes indicated the deep plane of anesthesia during the isoflurane administration. This study was considered more stringent than those normally used in a clinical setting but were chosen to maximize patient safety. Given the threatened conservation status of this tortoise, a larger study population was not possible. In eastern box turtle GV-26 acupuncture alone did not have a significantly reduced time to response of limb extension but receiving either GV-1 and GV-26 acupuncture or GV-1 and GV-26 electroacupuncture associated with significant changes. However, needle location is most likely responsible for the reported therapeutic benefits, but electroacupuncture is said to further stimulate the response of acupuncture. The mechanism of action responsible for faster anesthesia recovery with GV-26 stimulation has been attributed to a neurorespiratory or adrenergic mechanism [13,14]. The use of GV-26 electroacupuncture to shorten recovery periods in a clinical setting may result in reduced monitoring time, shorter hospitalization, decreased personal time, reduced client expense and fewer post anesthetic complications. The criteria used to define time to recovery in this study were considered more stringent than those normally used in a clinical setting but were chosen to maximize patient safety. GV-26 electroacupuncture can be used as an adjunctive method to reduce anesthetic recovery time in spur-thighed tortoises that have received inhalant anesthetic. This study represents an early step in investigation of clinical use of electroacupuncture in reptiles. Further neurophysiologic investigations are required to better explain the mechanism of GV-26 stimulation in reptiles and whether such benefits can be ascribed to a neurorespiratory or adrenergic mechanism [5,13,14].

In conclusion, GV-26 electroacupuncture can be used as an adjunctive method to reduce anesthetic recovery time in spur-thighed tortoises that have received inhalant anesthesia. Further investigations need to be

performed on other anesthetic protocols and additional research appears warranted in reptilian species which are predisposed to slow anesthetic recoveries.

Availability of Data and Materials

The data that support the finding of this study are available on asking from the corresponding authors. The data are not publicly available due to privacy or ethical restriction.

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Competing Interest

The Authors declare that there is no conflict of interest. The authors alone are responsible for the content and writing of this article.

Author Contributions

HSN carried out the preparation process and wrote the first draft of the manuscript. All of process of the project was under supervision of HF and BV. MM and AKR advised and co-supervised the project. The final revision of the manuscript was done by HSN, HF and BV.

HSN, HF, BV, MM and AKR approved the final version.

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RESEARCH ARTICLE

Promotives of Nano-Zinc Oxide as an Immune Stimulant in the Treatment of Lambs Suffering from Zinc Deficiency

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Abstract: Zinc nanoparticles have a positive effect in enhancing growth and improving immunity, so this work aimed to investigate the role of Nano-zinc oxide as an immune stimulant and treatment of zinc deficient lambs in comparison with zinc in ordinary size. Thirty lambs were used and divided into three groups, the 1st group (N=10) control healthy group, the 2nd group (N=10) lambs suffered from zinc deficiency and treated with Nano-zinc oxide (nZnO), the 3rd group (N=10) lambs suffered from zinc deficiency treated with zinc oxide (ZnO) ordinary size. Serum samples were collected at zero-day (0-day), 7th, and 15th days of experiment. The serum analysis results of the zinc-deficient groups at 0-days revealed hypozincaemia, hypovitaminosis A and E, hypoproteinemia, hypoalbuminemia, hypoglobulinemia, decreased antioxidant (SOD, TAC, CAT, GSH) and immunoglobulin (γ2, γ1, β2, β1), while increased MDA, α2, and α1 compared to control healthy group. Most of parameters revealed rapid recovery from the 7th day of experiment in the nZnO group rather than the ZnO group. Some parameters of SOD, total protein, globulin, γ2, γ1, α2, and α1 appeared on the 7th day of experiment of the nZnO group significantly increased compared to the control healthy group, which revealed an improvement in the immune response. In conclusion; nZnO induced rapid recovery, and improved both immunity and antioxidants than zinc oxide ordinary sized.

Keywords: *Nano-zinc oxide, Zinc oxide, Vitamins, Antioxidants, Protein, Electrophoresis*

Bir Sinir Stimülatörü Kullanılarak Gerçekleştirilen Tavşan Brakiyal Pleksus Blokajında QX-314 ve Lidokainin Birlikte Uygulanması

Öz: Çinko nanopartikülleri büyümeyi arttırmada ve bağışıklığı geliştirmede olumlu bir etkiye sahiptir, bu nedenle bu çalışma Nano-çinko oksidin bağışıklık uyarıcı olarak rolünü ve normal seviyedeki çinko ile karşılaştırıldığında çinko eksikliği olan kuzuların tedavisini araştırmayı amaçlamıştır. Çalışmada 30 kuzu kullanılmış ve kuzular; 1. grup (N=10) sağlıklı kontrol grubu, 2. grup (N=10) çinko eksikliği olan ve Nano-çinko oksit ile tedavi edilen grup (nZnO) ve 3. grup (N=10) çinko eksikliği olan ve normal dozajlı çinko oksit ile tedavi edilen grup (ZnO) olmak üzere 3 gruba ayrılmıştır. Serum örnekleri, deneyin 0. gün, 7. gün ve 15. günlerinde alınmıştır. Çinko eksikliği olan grupların 0. gün analiz sonuçlarına bakıldığında, kontrol grubu ile kıyaslandığında, serum örneklerinde hipozinkemi, hipovitaminoz A ve E, hipoproteinemi, hipoalbuminemi, hipoglobulinemi, antioksidan (SOD, TAC, CAT, GSH) ve immünoglobulin (γ2, γ1, β2, β1) değerlerinde azalma, MDA, α2 ve α1 değerlerinde ise artış tespit edilmiştir. Parametrelerin çoğu, ZnO grubundan ziyade nZnO grubunda deneyin 7. gününden itibaren hızlı bir iyileşme göstermiştir. Deneyin 7. gününde nZnO grubunun SOD, toplam protein, globulin, γ2, γ1, α2 ve α1 gibi bazı değerleri kontrol grubuna kıyasla önemli ölçüde artmış ve bu da bağışıklık yanıtında bir iyileşme olduğunu göstermiştir. Sonuç olarak; nZnO hızlı bir iyileşmeye neden olmuş ve normal dozajlı çinko okside kıyasla hem bağışıklığı hem de antioksidanları iyileştirmiştir.

Anahtar Sözcükler: *Nano-çinko oksit, Çinko oksit, Vitamin, Antioksidan, Protein, Elektroforez*

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Introduction

Zinc is one of the foremost essential trace elements needed for the metabolic activity of more than 300 co-enzymes and is crucial for the synthesis of protein, DNA, and immune system function [1]. Zinc has a vital role in impacting animal growth, reproduction and the immune system by affecting enzyme activity and protein gene expression [2]. Also, it is a crucial part of the body's antioxidant system, which can prevent cell membrane oxidation. Once zinc deficiency occurs, lipid oxidation increases, which leads to oxidative damage [3]. Zinc has an interaction with vitamin A, while absorption, metabolism, hepatic release, conversion, and tissue utilization of vitamin A may depend on zinc status. Zinc deficiency may lead to impair synthesis of the protein, hepatic synthesis of cellular retinol-binding protein (cRBP) and retinol-binding protein (RBP), and mobilization of vitamin A within cells and liver [4]. Zinc deficiency may lead to elevated serum malondialdehyde (MDA) and a decrease in total antioxidant capacity (TAC), catalase (CAT), reduced glutathione (GSH), and SOD activity in sheep [5,6].

Nano minerals technologies are widely utilized in wide-range of sectors as well as agriculture, livestock animals, and food systems [7]. Nano zinc oxide particles have better bioavailability, bigger specific surface area, higher surface activity, high catalytic potency, and stronger adsorbing ability [8], also because it is often easily carried up by the gastrointestinal tract (GIT) and utilized within the animal system and reach deeper tissues more efficiently than the larger sized particles [9]. Nano-ZnO is often prepared by various methods, like the traditional high-temperature solid-state methodology, chemical precipitation, sol-gel synthesis, and hydrothermal methodology [10]. Nano-ZnO have enhanced responses once fed to livestock conventional diet improving growth, immunity, reproduction and feed efficiency [11].

The research aimed to investigate the efficacy of Nano-zinc oxide as an immune stimulant and anti-oxidative activator during the treatment of zinc deficient lambs.

Material and Methods

Ethical Statement

This protocol was approved by the Research Committee of the Animal Health Research Institute and authorized by The Institutional Animal Care and Use Committee (ARC-IACUC)/Agricultural Research Center (ARC/AH/22/22).

Experimental Design

This work carried out on a private sheep farm. Thirty fattening lambs aged 12-15 months were used and divided into three groups. Control apparently healthy group (N=10) 1st group. Twenty diseased lambs showing a clinical symptoms of zinc deficiency (wool eating and para-keratosis and confirmed by decreased serum zinc level), were divided into two groups according to treatment used; diseased lambs (N=10) were treated by zinc oxide nanoparticles prepared solution 10 mL/animal (contains 2 mg of Nano-zinc oxide) one dose orally/day for 7 days, (nZnO) 2nd group. Diseased lambs (N=10) were treated by zinc oxide powder 20 mg/kg DM mixed ration daily for 7 days, (ZnO) 3rd group.

Sampling

Blood samples were collected from the jugular vein of all animals at the time of disease detected and the beginning of treatment (0-day), at the end of treatment (7th day of the experiment) and after a week from the end of treatment (15th day of the experiment). Serum samples were obtained by centrifuging the blood samples at 5000 rpm for 5 minutes. Clear sera were transferred into clean dry Eppendorf tubes and stored at -20°C till biochemical analysis.

Nano-Zinc Oxide (nZnO) Preparation

Nano-zinc oxide solution was prepared according to Wang et al.[10]. Zinc oxide (ZnO) nanoparticles were characterized by TEM, XPS, XRD, and UV-visible spectrophotometry. The morphological characteristics of nZnO were investigated by a scanning electron microscope (SEM) and a transmission electron microscope (TEM). X-ray photoelectron spectroscopy (XPS) was used to identify the chemical bonding states of the Zn and O. The UV spectrum of the ZnO nanoparticles was recorded with a UV-visible spectrophotometer, and the max excitation wavelength was 325 nm. The solution contains Nano-zinc oxide (2 mg/10 mL).

Serum Biochemical Analysis

Zinc levels were detected by atomic absorption Spectrophotometer according to the method described by Maret and Henkin [12]. Special kits were used for calcium (Ca) and inorganic phosphorus (P) detection by the method described by Roberts et al.[13]. Special kits (Bio diagnostic Company) CAT. No. SD2521 was used for Superoxide dismutase (SOD) activity estimation according to Nishikimi et al.[14] and CAT. No. TA2513 was used for total antioxidant capacity (TAC) estimation according to Koracevic et al.[15], reduced glutathione (GSH) level was detected by a method of Pleban et al.[16], catalase (CAT) activity was detected by a method of Aebi [17] and malondialdehyde (MDA) was detected calorimetrically according to Lahouel et al.[18].

Total Protein and Electrophoretic Protein Estimation

The serum total protein and electrophoretic pattern were

estimated according to Sonnenwirth et al.[19] and Davis [20], respectively and calculated according to SynGene S. No. 17292*14518 sme*mpcs.

Serum Vitamins Examination

Determination of Retinol (vitamin A) and α-tocopherol (vitamin E) concentrations in serum samples were performed by High-Performance Liquid Chromatography (HPLC).

Chemicals and Reagents

Retinol, α-tocopherol, and 2, 6-di-tert-butyl-4-methyl phenol (BHT) were provided by Sigma-Aldrich. The grade Methanol and hexane of HPLC were obtained from Fisher Scientific. HPLC-grade Ethanol was obtained from Carlo Erba and the Purified deionized water was prepared using a Milli-Q system.

Chromatographic Conditions

HPLC system (Agilent 1200 series, Software - Agilent Chemistation Version B.040.01) SP1 (Agilent Technologies, Germany), with a pump, degasser, autosampler, DAD detector and Chromatographic column - Agilent C18, 100A (4.6 x 250 mm, 5 µm) as the stationary phase was used. The chromatographic condition was set according to Bystrowska et al.[21].

Stock, and intermediate standard solutions of retinol and α-tocopherol were prepared according to Bystrowska et al.[21]. The serum calibration curve was created by spiking blank lamb serum with varying intermediate standard solution volumes at concentrations ranging from 0.1 to 100 µg/mL. Three different levels of quality control (QC) samples were prepared in blank lamb's serum and were used for achieving the method validation requirements.

Extraction Procedures

Samples prepared for extraction were performed by a liquid-liquid extraction technique according to the procedure described by Siluk et al.[22] which was a modified version of an earlier reported procedure by Aebischer et al.[23].

This method was validated according to USP [24] via the determination of method precision, recovery, linearity, the limit of detection, and quantification.

The HPLC method was accurate with high recovery (95-99%) of good linearity ($r^2 \geq 0.999$) with a low LOD and LOQ; as LOD were 0.29 µg and 2.0 µg and LOQ were 0.87 µg and 6.1 µg for retinol (Vit. A) and α-tocopherol (Vit. E), respectively. Specificity and selectivity were illustrated with chromatogram of retinol (Vit. A), at 22.28 min retention time *(Fig. 1)* and chromatogram of α-tocopherol (Vit. E), at 12.29 min retention time *(Fig. 2)*.

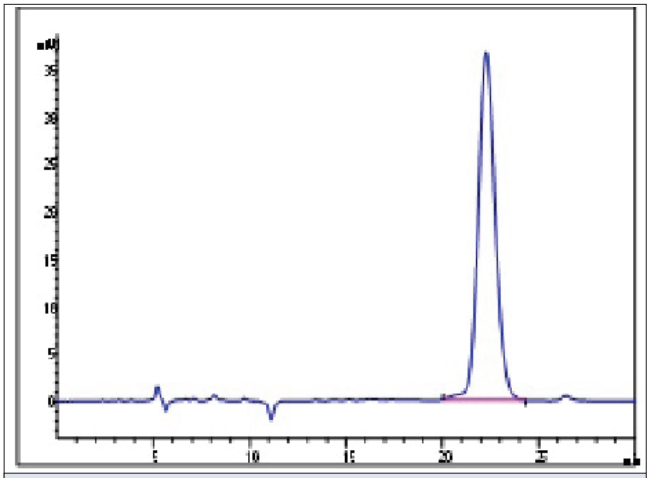

Fig 1. Chromatogram of Retinol (Vit. A), at 22.28 min retention time

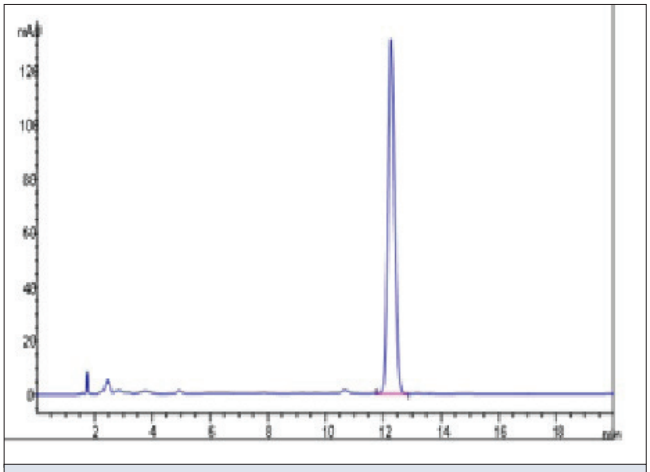

Fig 2. Chromatogram of α-tocopherol (vit. E), at 12.29 min retention time

Statistical Analysis

The data were statistically analyzed using two-way (ANOVA) by SPSS 22 software to test both the treatment and times. The results were demonstrated as means ± SE. The results were considered statistically significant at $P<0.05$.

Results

The results of serum minerals and vitamins analysis *(Table 1)* of the diseased groups on the day of detected zinc deficiency (0-day) revealed a significant ($P<0.05$) decrease in zinc levels and non-significant changes in calcium and phosphorus levels compared to the control healthy group. Treatment by Nano-zinc oxide (nZnO) revealed a significant ($P<0.05$) increase on the 7th day of experiment compared to the group treated by zinc oxide in ordinary size (ZnO) but non-significant with the control healthy group.

The serum vitamins analysis results *(Table 1)* revealed a significant decrease ($P<0.05$) in vitamins A and E at 0-day in diseased groups compared to the control healthy group. After treatment by (nZnO), there was a

Table 1. *The results of serum levels of minerals, vitamins, and anti-oxidants in the different experimental groups*

Parameters	Time	Groups		
		Control Healthy Group	Group Treated with nZnO	Group Treated with ZnO
Zinc (µg/dL)	0- day	105.98±9.33 ^{a,1}	75.83±9.62 ^{b,1}	76.49±9.33 ^{b,1}
	7th day	107.35±8.71 ^{a,b,1}	109.44±9.43 ^{a,2}	94.54±9.86 ^{b,2}
	15th day	106.57±10.16 ^{a,1}	108.47±10.24 ^{a,2}	103.75±8.46 ^{a,2}
Calcium (mg/dL)	0- day	8.774±0.466	9.294±0.612	9.292±0.403
	7th day	8.644±0.395	8.776±0.336	8.788±0.346
	15th day	8.74±0.34	8.704±0.278	8.732±0.33
Phosphorus (mg/dL)	0- day	4.868±0.325	4.69±0.3	4.67±0.34
	7th day	4.888±0.339	4.794±0.306	4.716±0.348
	15th day	4.854±0.303	4.824±0.261	4.794±0.276
Vitamin A (µg/mL)	0- day	57.6±4.04 ^{a,1}	3.61±1.08 ^{b,1}	3.82±1.04 ^{b,1}
	7th day	60±6.04 ^{a,1}	35.26±4.09 ^{b,2}	26.6±2.82 ^{c,2}
	15th day	59.6±5.55 ^{a,1}	60.8±4.55 ^{a,3}	51.52±3.04 ^{b,3}
Vitamin E (µg/mL)	0- day	52.6±4.67 ^{a,1}	2.016±0.849 ^{b,1}	2.064±0.748 ^{b,1}
	7th day	54±4.74 ^{a,1}	41.78±2.39 ^{b,2}	36.76±2.46 ^{c,2}
	15th day	53.4±4.72 ^{a,1}	54.2±3.27 ^{a,3}	47.8±3.35 ^{b,3}
SOD (U/mL)	0- day	35.314±4.359 ^{a,1}	21.918±4.319 ^{b,1}	22.894±3.785 ^{b,1}
	7th day	35.682±4.476 ^{b,1}	42.43±4.36 ^{a,2}	40.704±4.768 ^{a,b,2}
	15th day	36.704±4.556 ^{b,1}	45.204±4.222 ^{a,2}	41.908±4.312 ^{a,b,2}
TAC (mU/L)	0- day	1.778±0.08 ^{a,1}	1.058±0.171 ^{b,1}	1.104±0.123 ^{b,1}
	7th day	1.788±0.099 ^{a,1}	1.652±0.106 ^{a,2}	1.37±0.136 ^{b,2}
	15th day	1.784±0.116 ^{a,1}	1.802±0.084 ^{a,2}	1.618±0.121 ^{b,3}
CAT (U/mL)	0- day	16.97±0.98 ^{a,1}	12.664±1.193 ^{b,1}	12.762±1.048 ^{b,1}
	7th day	16.89±0.99 ^{a,1}	16.862±1.027 ^{a,2}	16.024±1.021 ^{a,2}
	15th day	17.044±0.944 ^{a,1}	17.036±1.121 ^{a,2}	16.884±1.069 ^{a,2}
GSH (U/mL)	0- day	5.302±0.473 ^{a,1}	3.746±0.569 ^{b,1}	3.764±0.565 ^{b,1}
	7th day	5.33±0.48 ^{a,1}	5.246±0.585 ^{a,2}	4.762±0.832 ^{a,2}
	15th day	5.352±0.465 ^{a,1}	5.362±0.589 ^{a,2}	5.23±0.7 ^{a,2}
MDA (nmol/mL)	0- day	1.764±0.159 ^{b,1}	2.228±0.185 ^{a,1}	2.208±0.193 ^{a,1}
	7th day	1.794±0.152 ^{a,1}	1.818±0.143 ^{a,2}	1.982±0.122 ^{a,2}
	15th day	1.784±0.162 ^{a,1}	1.766±0.158 ^{a,2}	1.796±0.132 ^{a,2}

Data are expressed as mean ± SE of 10 samples
$^{a, b, c}$ *Superscript letters: Mean significance difference between groups in the same time on P<0.05*
$^{1, 2, 3}$ *Superscript numbers: Mean significance difference among times of treatment in the same group on P<0.05*

gradually significant (P<0.05) increase in both vitamin A and E until become non-significant on the 15th day of experiment compared to the control healthy group and significantly (P<0.05) increased compared to (ZnO) group throughout treatment. The (ZnO) group revealed a gradually significant (P<0.05) increase in vitamin A and vitamin E levels but still significantly (P<0.05) decreased compared to the control healthy group.

The results of serum antioxidant *(Table 1)* of the diseased groups at 0-day revealed a significant (P<0.05) decrease in SOD, TAC, CAT, and GSH with a significant (P<0.05) increase in MDA in comparison with the control healthy group. After treatment with (nZnO), there was a significant (P<0.05) increase in SOD level on the 7th and 15th day compared to the control healthy group, at the same time non-significant in comparison with (ZnO) group, on the contrary, there was a non-significant change between (ZnO) group and control healthy at 7th and 15 days of experiment. Only the TAC level of (ZnO) group on the 15th day revealed a significant (P<0.05) decrease compared to either (nZnO) group or the control healthy

Table 2. *The results of serum levels of patterns of protein electrophoresis fractions and sub-fractions (g/dL) in the different experimental*

Parameters	Time	Groups		
		Control Healthy Group	Group Treated with nZnO	Group Treated with ZnO
Total Protein (g/dL)	0- day	5.18±0.31 ^{a,1}	3.458±0.255 ^{b,1}	3.44±0.22 ^{b,1}
	7th day	5.26±0.43 ^{b,1}	5.742±0.398 ^{a,2}	4.944±0.313 ^{b,2}
	15th day	5.39±0.31 ^{a,1}	5.428±0.324 ^{a,2}	5.136±0.216 ^{a,2}
Albumin (g/dL)	0- day	1.496±0.091 ^{a,1}	1.062±0.078 ^{b,1}	1.057±0.067 ^{b,1}
	7th day	1.52±0.12 ^{a,1}	1.576±0.109 ^{a,2}	1.224±0.078 ^{b,2}
	15th day	1.558±0.09 ^{a,1}	1.503±0.09 ^{a,2}	1.46±0.062 ^{a,3}
Globulin (g/dL)	0- day	3.68±0.22 ^{a,1}	2.396±0.177 ^{b,1}	2.383±0.151 ^{b,1}
	7th day	3.74±0.305 ^{b,1}	4.223±0.292 ^{a,2}	3.72±0.236 ^{b,2}
	15th day	3.83±0.22 ^{a,1}	3.925±0.234 ^{a,2}	3.676±0.155 ^{a,2}
Gamma 2 (γ2) (g/dL)	0- day	0.49±0.03 ^{a,1}	0.192±0.014 ^{b,1}	0.191±0.012 ^{b,1}
	7th day	0.495±0.04 ^{b,1}	0.54±0.04 ^{a,2}	0.46±0.03 ^{b,2}
	15th day	0.508±0.029 ^{a,1}	0.503±0.03 ^{a,2}	0.44±0.02 ^{b,2}
Gamma 1 (γ1) (g/dL)	0- day	1.362±0.082 ^{a,1}	0.541 ±0.04 ^{b,1}	0.538±0.034 ^{b,1}
	7th day	1.384±0113 ^{b,1}	1.639±0.113 ^{a,2}	1.326±0.084 ^{b,2}
	15th day	1.419±0.082 ^{b,1}	1.562±0.093 ^{a,2}	1.42±0.06 ^{b,2}
Beta 2 (β2) (g/dL)	0- day	0.682±0.041 ^{a,1}	0.358±0.026 ^{b,1}	0.356±0.023 ^{b,1}
	7th day	0.693±0.057 ^{a,1}	0.681±0.047 ^{a,2}	0.689±0.044 ^{a,2}
	15th day	0.71±0.04 ^{a,1}	0.674±0.04 ^{a,b,2}	0.648±0.027 ^{b,2}
Beta 1 (β1) (g/dL)	0- day	0.618±0.037 ^{a,1}	0.49±0.036 ^{b,1}	0.487±0.031 ^{b,1}
	7th day	0.628±0.051 ^{a,1}	0.616±0.043 ^{a,2}	0.498±0.032 ^{b,1}
	15th day	0.644±0.037 ^{a,1}	0.63±0.038 ^{a,2}	0.401±0.017 ^{b,2}
Alpha 2 (α2) (g/dL)	0- day	0.285±0.017 ^{b,1}	0.486±0.036 ^{a,1}	0.483±0.031 ^{a,1}
	7th day	0.29±0.024 ^{b,1}	0.394±0.027 ^{a,2}	0.409±0.026 ^{a,2}
	15th day	0.297±0.017 ^{b,1}	0.291±0.017 ^{b,3}	0.409±0.017 ^{a,2}
Alpha 1 (α1) (g/dL)	0- day	0.245±0.015 ^{b,1}	0.329±0.024 ^{a,1}	0.327±0.021 ^{a,1}
	7th day	0.249±0.02 ^{c,1}	0.296±0.021 ^{b,2}	0.334±0.021 ^{a,1}
	15th day	0.255±0.015 ^{b,1}	0.265±0.016 ^{b,3}	0.351±0.015 ^{a,1}

Data are expressed as mean ± SE of 10 samples
$^{a, b, c}$ *Superscript letters: Mean significance difference between groups at the same time on P<0.05*
$^{1, 2, 3}$ *Superscript numbers: Mean significance difference among times of treatment in the same group on P<0.05*

group. The other antioxidants (CAT, GSH, and MDA) had non-significant changes in both groups of treatment compared to the control healthy group from the 7th day of experiment.

Total protein, albumin, and globulin analysis *(Table 2)* of the diseased groups at 0-day revealed a significant (P<0.05) decrease in total protein, albumin, and globulin compared to the control healthy group. After treatment with nZnO on the 7th day of experiment, there was a significant (P<0.05) increase in total protein compared to either (ZnO) group or the control healthy group, while there were non-significant changes between groups in the 15th day of experiment. There was a significant (P<0.05) increase in albumin level in the (nZnO) group on the 7th day compared to the (ZnO) group, but non-significant in comparison with the control healthy group. The albumin level in the (ZnO) group gradually increased until become non-significant in comparison with either (nZnO) group or the control healthy group on the 15th day of experiment. The globulin level revealed a significant (P<0.05) increase in the (nZnO) group on the 7th day of experiment compared to either the (ZnO) group or the control healthy group, while non-significant changes between groups in globulin level on the 15th day of experiment.

Serum protein electrophoresis results *(Table 2)* of the diseased groups at 0-day revealed a significant (P<0.05)

decrease in (γ2, γ1, β2, β1) and significant (P<0.05) increase in (α2, α1) compared to control healthy group. After treatment with nZnO, there was a significant (P<0.05) increase in γ2 on the 7th day of experiment compared to either the (ZnO) group or the control healthy group. On the other hand, there was a significant (P<0.05) increase on the 15th day of experiment compared to the (ZnO) group but non-significant compared to the control healthy group. There was a significant (P<0.05) increase in γ1 of the (nZnO) group on the 7th and 15th day compared to either the (ZnO) group or the control healthy group, at the same time there were non-significant changes between the (ZnO) group and control healthy group. There were non-significant changes in the β2 levels between groups on the 7th day of experiment but only there was a significant (P<0.05) decrease in the (ZnO) group on the 15th day of experiment compared to the control healthy group and non-significant changes compared to (nZnO) group. There was a significant (P<0.05) decrease in the β1 level of the (ZnO) group on the 7th and 15th day of experiment compared to either the (nZnO) group or the control healthy group. There was a significant (P<0.05) gradually decreased in α2 and α1 levels of the (nZnO) group toward non-significant compared to the control healthy group on the 15th day and at the same time revealed significant (P<0.05) decrease compared to the (ZnO) group.

Discussion

Reduced zinc concentration in lamb serum are commonly used as a biomarker for zinc deficiency [6,25]. This result may be attributed to a decreased zinc level in the ration and/or an elevated calcium level in the ration which decreases zinc absorption and metabolism [5,6,25]. The rapid recovery of serum zinc level in the (nZnO) group on the 7th day of the experiment rather than the (ZnO) group that recovered on the 15th day of the experiment referred to the greater bioavailability, bigger specific surface area, higher surface activity, and rapid adsorption ability of Nano-zinc oxide particles [8].

The serum vitamins A and E levels showed decreased levels in 0-day of diseased groups with respect to the healthy. This observation is in accordance with Serdar and Funda [26] in calves suffering from dermatophytosis and decreased zinc levels and Nguta [27] in lactating cow. As absorption, metabolism, hepatic release, conversion, and tissue utilization of vitamin A may depend in zinc status, so zinc deficiency may lead to impairing synthesis of the protein, hepatic synthesis of cRBP and RBP, and mobilization of vitamin A within cells and from the liver [4]. Zinc deficiency may inhibit lipid absorption and/or vitamin E (lipid soluble vitamin) [28]. The recovery of vitamins A and E on the 15th day of treatment in the nZnO group before the ZnO group may be referred to nano-zinc can be rapidly absorbed by the GIT and reached deeper tissues and be utilized in the animal system, more efficiently than the larger sized particles [9].

The serum antioxidant results of the diseased groups at the 0-day revealed decreased SOD, TAC, CAT, and GSH while increased MDA were agree with Song and Shen [5] and Yousif et al.[6]. These results may be attributed to zinc deficiency which increasing lipid oxidation, and may provide oxidative damage [3]. After treatment with either (nZnO) or (ZnO) groups, there was an increase in SOD level compared to the healthy group, as zinc has an important role in the stability of cell membranes and protein as it helps in balancing reactive oxygen species (ROS) production because its presence in superoxide dismutase (SOD) [29]. The result of the recovery of antioxidants in the (nZnO) group is in accordance with Song et al.[30] who recorded similar results after zinc supplementation in buffalo calves. These results may be attributed to the role of zinc is a crucial component of the body's antioxidant system [3]. The rapid recovery of antioxidants (TAC, CAT, GSH, and MDA) in the (nZnO) group may be attributed to the rapid effectiveness role of nano-zinc in improving the antioxidant system in ewes and lambs [31].

In the present work, the results of decreased total protein, albumin, and globulin were in agreement with Fouda et al.[32]. Langenmayer et al.[33] recorded a significant decrease in serum albumin in zinc-deficient calves. These decreases may be attributed to suppression in feed consumption, decreased appetite, and disruptions in protein synthesis in the liver [34]. The decreased globulin level is agreed with El Maghraby and Mahmoud [35] who recorded similar results in zinc-deficient neonatal calves. This result may be related to the reduction of gamma globulins (γ2, γ1) and beta globulins (β2, β1) levels, which may be attributed to B-prolymphocyte mitogenesis dysfunction. Also attributed to a decrease in the serum immunoglobulin concentration and antibody response to T-dependent antigen [36]. Since the role of zinc in inducing the B cells to secrete globulin, and improving the immune function of animal B cell and enhancing the immunoglobulin synthesis ability, so zinc deficiency will lead to disorder of the immunoglobulin secretion [37]. The results of increased total protein and globulin in the (nZnO) group than either the (ZnO) group or the control group, similar to results were recorded in ewes and lambs [31] and goats [38]. These results may be referred to the higher elevation of gamma globulins (γ2, γ1) and beta globulins (β2, β1) levels in the nZnO group, which may be attributed to the role of Nano-zinc oxide in improving growth and immunity in livestock [11]. Also, Nano zinc oxide proved to be better than traditional zinc (ZnO) for improving growth performance [39], as well as zinc oxide nanoparticles can

be rapidly absorbed by GIT and reached to deeper tissues and utilized in the animal system with more efficacy than the larger sized particles [9], also attributed to the greater bioavailability, bigger specific surface area, and higher surface activity of zinc oxide nanoparticles [8].

In conclusion; Zinc has a crucial role in the body's physiology and important trace element required for most of the body's enzymes, metabolic activities, and immune system functions. Zinc deficiency leads to impairment of the antioxidant system and biochemical changes that affect immune response. Nano-zinc oxide supplementation has a rapid and effective recovery more than zinc oxide in ordinary size, not only but also, zinc oxide in nanoparticles has a higher and strong effect on immunity and antioxidants.

Availability of Data and Materials

The data sets analyzed during the current study are available from the corresponding author H. M. Yousif on reasonable request.

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Conflict of Interest

No potential conflict of interest was reported by the authors. Authors only are responsible for the content and writing of the paper.

Author Contributions

H. M. Yousif designed the research and collect the samples. M. K. Mansour, H. M. Yousif, R. A. A. Rezk and A.M. El Mahdy performed the experimental duties of this study and analyzed the data. M. K. Mansour and H. M. Yousif did the statistical analyses. All authors participate in writing and approved the final version of the manuscript.

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RESEARCH ARTICLE

Study on the Skeletal Muscle Transcriptomics of Alxa Gobi Camel and Desert Camel

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Abstract: In order to explore the reasons for the difference in meat production performance between Alxa Gobi camel and Desert camel, we used high-throughput transcriptome sequencing technology, HTSeq, DEGseq and Gene Ontology (GO) and Encyclopedia of Genes and Genomes (KEGG) databases to compare the skeletal muscle gene expression between the two types. A total of 484 classification items were significantly enriched with Gene Ontology (GO) function, among which 246 were related to biological processes, accounting for 50.8%. One hundred eighty-four were related to molecular function, accounting for 38.1%. Fifty-four were related to cell components, accounting for 11.1%. Gene Ontology (GO) function was significantly enriched to 339 up-regulated genes and 108 down-regulated genes. There were 6116 differentially expressed genes annotated in Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Pathway significance enrichment analysis revealed that these genes were involved in 250 biological metabolic pathways, among which 19 had extremely significant differences, 37 were involved in skeletal muscle development, and 13 were involved in fat metabolism. The results showed that Alxa Gobi camel evolved more genes and signaling pathways related to skeletal muscle development and fat deposition than that of Alxa Desert camel during the long evolutionary process, which changed the traits of Alxa Gobi camel and improved its meat performance.

Keywords: *Alxa Bactrian camel, Gene ontology, Kyoto Encyclopedia of genes and genomes, Skeletal muscle transcriptomics*

Alxa Gobi Devesi ve Çöl Devesinin İskelet Kası Transkriptomiği Üzerine Çalışma

Öz: Alxa Gobi devesi ile Çöl devesi arasında et üretim performansındaki farkın nedenlerini araştırmak amacıyla, iki tür arasındaki iskelet kası gen ekspresyonunu karşılaştırmak için yüksek verimli transkriptom dizileme teknolojisi, HTSeq, DEGseq ve Gen Ontolojisi (GO) ve Genler ve Genomlar Ansiklopedisi (KEGG) veri tabanlarını kullandık. Toplam 484 sınıflandırma öğesi, Gen Ontolojisi (GO) işlevi ile önemli ölçüde zenginleştirildi, ki bunların 246'sı biyolojik süreçlerle ilgiliydi ve %50,8'ini oluşturuyordu. Bunların 184'ü moleküler işlevle ilgiliydi ve %38,1'ini oluşturuyordu. Elli dördü ise hücre bileşenleriyle ilgiliydi ve %11,1'ini oluşturuyordu. Gen Ontolojisi (GO) işlevi, 339 upregüle gen ve 108 downregüle gen için önemli ölçüde zenginleştirildi. Kyoto Genler ve Genomlar Ansiklopedisi (KEGG) veri tabanında 6116 farklı eksprese edilmiş gen bulunmaktaydı. Yolak önem zenginleştirme analizi, bu genlerin 250 biyolojik metabolik yolakta yer aldığını, bunlardan 19'unun son derece önemli farklılıklara sahip olduğunu, 37'sinin iskelet kası gelişiminde ve 13'ünün yağ metabolizmasında yer aldığını ortaya koydu. Sonuçlar, Alxa Gobi devesinin uzun evrimsel süreç boyunca Alxa Çöl devesine kıyasla iskelet kası gelişimi ve yağ birikimi ile ilgili daha fazla gen ve sinyal yolu geliştirdiğini, bunun da Alxa Gobi devesinin özelliklerini değiştirdiğini ve et performansını artırdığını göstermiştir.

Anahtar sözcükler: *Alxa Bactrian devesi, Gen ontolojisi, Kyoto Genler ve Genomlar Ansiklopedisi, İskelet kası transkriptomiği*

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Introduction

The camel belongs to the genus *Camelus* in the *Camelidae* of the order *Artiodactyla* of the *Mammalia* class. The Dromedary camel and the Bactrian camel are divided by the number of humps. Bactrian camels are mainly distributed in Central and Northeast Asia, northern China and Mongolia [1]. China is one of the mainly breeding countries of Bactrian camels, and there are about 411.000 Bactrian camels [2], among which Alxa Bactrian camels are the most numerous and widely distributed, with 220.000 camels, accounting for about half of the whole country. China's Alxa region is the main rearing area for the Alxa Bactrian camels [3]. Alxa Bactrian camel is divided into two types: Gobi camel and Desert camel. Gobi camel is mainly distributed in the Gobi region, while Desert camel is mainly distributed in the desert region [4].

At present, there are many studies focus on their milk, hair and biological characteristics of the Alxa Bactrian camel [4], but there are few reports on its skeletal muscle traits [3-6]. Compared with Desert camels, Gobi camels have higher meat production performance (the net meat weight is 90 kg more than that of Desert camel), smaller diameter and greater density of muscle fiber, and higher content of MyHC I type muscle fiber, while Desert camel muscle fiber has large diameter, small density and high content of MyHC IIb type muscle fiber [4]. Muscle fiber is the basic unit of skeletal muscle and the main component of meat production performance. The development and traits of skeletal muscle fiber determine the meat production performance. Since from the explanation of skeletal muscle traits of double-muscle cattle [7,8] and the application of cloning technology [9,10], genetic modification technology provides a new method for improving meat performance of livestock. In order to further explore the reasons for the differences in meat production performance between Gobi camel and Desert camel, their skeletal muscles were taken as the research objects, and the biceps femoris samples were collected for transcriptome sequencing analysis to obtain gene expression information. We compared and analyzed the biceps femoris samples of Gobi camel and Desert camel in terms of differentially expressed genes, Gene Ontology (GO) function enrichment and Encyclopedia of Genes and Genomes (KEGG) pathway enrichment, in order to reveal the difference of meat production performance between them at molecular level and provide scientific basis for the improvement of meat production performance of Alxa Bactrian camel and the breeding of new strains.

Material and Methods

Ethical Statement

All experimental procedures were approved by the Animal Protection and Use Committee of Inner Mongolia Agricultural University and strictly followed animal welfare and ethical guidelines.

Animal Sample Collection

Five 8-year-old male Alxa Gobi camels and five 8-year-old Desert camels collected 5 g of biceps femoris respectively, washed with alcohol and normal saline once, packed with frozen tubes and marked, temporarily stored in liquid nitrogen, returned to the laboratory and stored in -80ºC refrigerator for a long time. Samples were collected at Western Lifa Slaughterhouse, Alxa League, Inner Mongolia, China.

Extraction of Total RNA and Detection of Total RNA Samples

After the total RNA was extracted from the split liquid of camel biceps femoris by Trizol method (TaKaRa, Kyoto, Japan), the integrity of the total RNA was accurately detected by Agilent 2100 bioanalyzer, the purity of the total RNA (the ratio of OD260/OD280 and OD260/230) was detected by microplate analyzer, and the integrity of the total RNA and the existence of DNA contamination were analyzed by agarose gel electrophoresis (agarose is purchased from Inner Mongolia Chenxin Biology Science and Technology Limited Company, China; markers are purchased from TaKaRa, Kyoto, Japan).

Sample Correlation Analysis

In this study, the square of Pearson correlation coefficient (R^2) was used as an index to judge the similarity of gene expression between samples ($0<R^2<1$, and there is obvious difference when it is higher than 0.8).

Construction, Detection and Computer Sequencing of Library

The sequencing of this experiment was completed by NOVOGENE. The specific situation is shown in *Fig. 1*.

After the construction of the library, the library was initially quantified by using Qubit2.0 Fluorometer, and the library was diluted to 1.5 ng/μL. Then, the insert size of the library was detected by using Agilent 2100 bioanalyzer. After the insert size met expectations, the effective concentration of the library was accurately quantified by qRT-PCR (the effective concentration of the library was higher than 2nM) to ensure the quality of the library.

Data Assembly and Functional Annotation of mRNAs

The original data are stripped of the adapter, which contain N (N means that the base information cannot be determined) and are of low quality (the number of bases with $Q_{phred}<=20$ accounts for more than 50% of the total read length), and filtered to obtain high-quality mRNA clean reads. After analyzing the base mass distribution, CG content and average mass distribution of mRNA clean

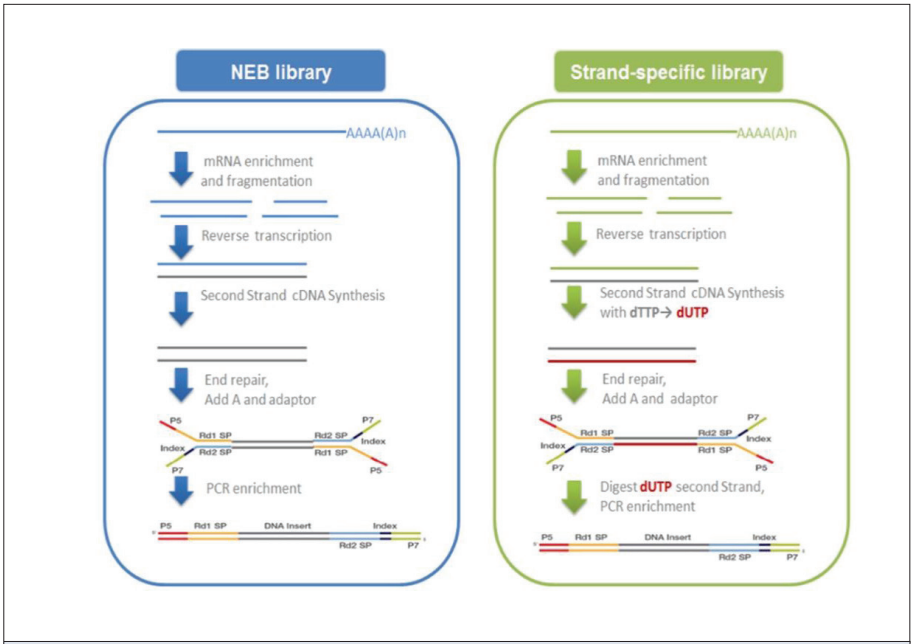

Fig 1. Schematic diagram of library construction

reads, and comparing them with reference genes *(https://www.ncbi.nlm.nih.gov/genome/?term=Camelus+bactrianus)*. HTSeq software was used to study the gene expression level of all the research samples. The selected model was union, and the value of Reads Per Kilo Base of Exon Model Per Million Mapped Reads (FPKM) was determined as 1. Then, the differentially expressed genes were screened by using R language software package DESeq2 (log2(FoldChange)|>0&padj<0.05). Use GOseq software to analyze the GO enrichment of differential genes, calculate the list and number of each term, and calculate the enrichment P-value (hypergeometric distribution, P<0.05 is significant enrichment); In addition, according to KEGG database, the differentially expressed genes were analyzed by KOBAS (2.0) software (hypergeometric distribution, P<0.05 is significant enrichment).

Results

Total RNA Quality Test Results and Integrity Test Electropherogram

The quality test results and integrity test electropherograms of total RNA of 10 samples used in this experiment *(Table 1; Fig. 2)*.

Correlation Between Samples in Quantitative Analysis of Genes

According to the FPKM values calculated for each sample, the correlation coefficients of different samples can

Table 1. *The result of total RNA quality test*

Sample Name	Concentration (ng/μL)	Volume (μL)	Total Quantity(μg)	OD260/280	OD260/230	RIN	Conclusion of Detection
SG_6	43	92	3.956	2	1.33	7.8	A
SG_8	44	92	4.048	1.44	0.9	8	A
SG_10	62	92	5.704	2	1.14	6.6	A
SG_12	35	92	3.22	2.36	0.96	8.4	A
SG_5	192	32	6.144	2	0.76	7.3	A
CG_6	46	92	4.232	1.65	0.94	8.8	A
CG_8	48	92	4.416	2.13	1.39	7.7	A
CG_10	53	92	4.876	1.94	1.55	7.9	A
CG_12	42	92	3.864	1.92	1.09	7.3	A
CG_5	166	32	5.312	2.12	1.09	7	A

SG_6/SG_8/SG_10/SG_12/SG_5 is Alxa Desert camel sample; CG_6/CG_8/CG_10/CG_12/CG_5 is Alxa Gobi camel sample; RIN: RNA integrity number

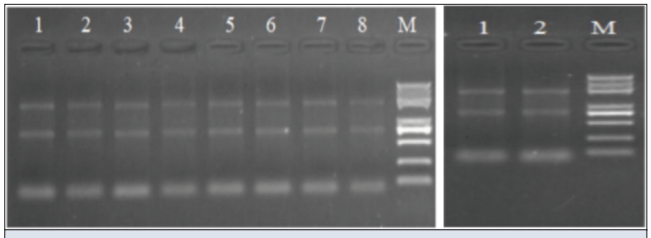

Fig 2. Total RNA extraction electrophoresis results. M: represents Marker (Trans 2K Plus); 1-4 on the left are Alxa Desert camel RNA samples SG_6/SG_8/SG_10/SG_12, 5-8 on the left are Alxa Gobi camel RNA samples CG_6/CG_8/CG_10/CG_12; 1 on the right is Alxa Desert camel SG_5 and 2 on the right is Alxa Gobi camel CG_5

be calculated, so as to obtain the heat map. Analysis of the figure can clarify the differences and repetitions of different samples in each group. If the coefficient is higher, it means that the pattern is closer *(Fig. 3)*.

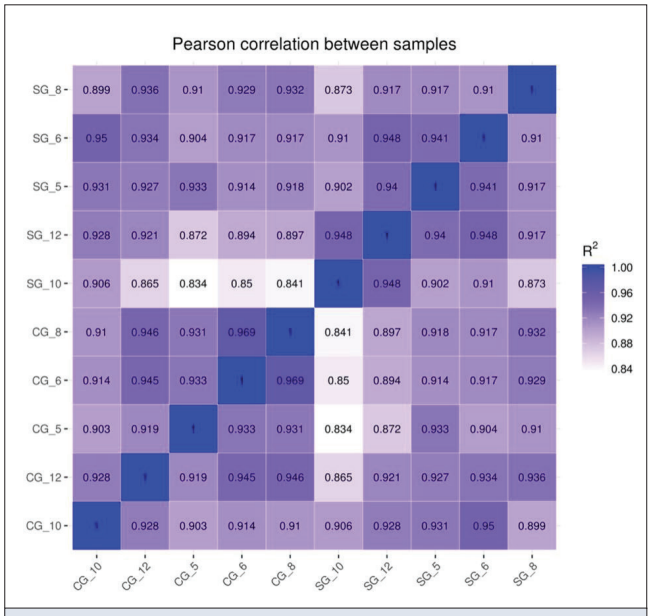

Fig 3. Sample correlation heat map

Gene Expression Distribution in Quantitative Analysis of Genes

Generally, FPKM was selected instead of read count as the expression value of RNA-seq gene. After the FPKM value of all genes is obtained, the gene expression distribution of each sample can be analyzed *(Fig. 4)*.

Differential Gene Statistics in Differential Analysis

The number statistics of differential genes and the criteria for screening differences are shown in *Table 2*.

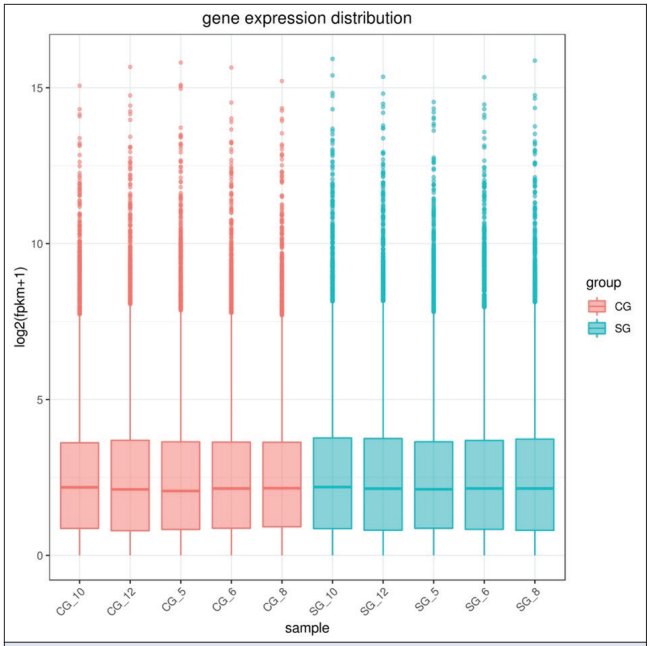

Fig 4. Gene expression level map. The abscissa and ordinate represent the sample name and log2(FPKM+1) respectively. The figure shows a box diagram of each sample, from which the minimum, maximum, median, upper and lower quartile of each sample can be compared and analyzed

The distribution of differentially expressed genes between Alxa Gobi camels (CG) and Alxa Desert camels (SG) is shown in *Fig. 5*.

Venn Diagram of Differential Genes

The sum of all numbers in the Vaine diagram indicated that the total number of differential genes between Alxa Gobi camel and Desert camel was 12592, and the overlap region indicated that the number of common differential genes was 11716, of which 457 genes were unique to Alxa Gobi camel and 419 genes were unique to Alxa Desert camel *(Fig. 6)*.

Differential Gene Clustering

In this experiment, the mainstream hierarchical clustering is used to cluster and analyze the gene expression values, and the genes with similar expression patterns are gathered together to homogenize all the expression data. Genes or samples with similar expression patterns in the heat map are gathered together, and different grid colors indicate the final value after homogenization *(Fig. 7)*.

Table 2. Standard table for number statistics of differential genes and screening differences

Compare	All	Up	Down	Threshold
SG vs CG	489	377	112	DESeq2 padj<0.05 |log2FoldChange|>0.0

Compare: *name of the comparison combination,* ***All:*** *total number of differentially expressed genes in the comparison combination,* ***Up:*** *number of differentially expressed genes up-regulated in the comparison combination,* ***Down:*** *number of differentially expressed genes down-regulated in the comparison combination,* ***Threshold:*** *software and threshold for differential gene screening in the comparison combination. SG is Alxa Desert camel sample; CG is Alxa Gobi camel sample*

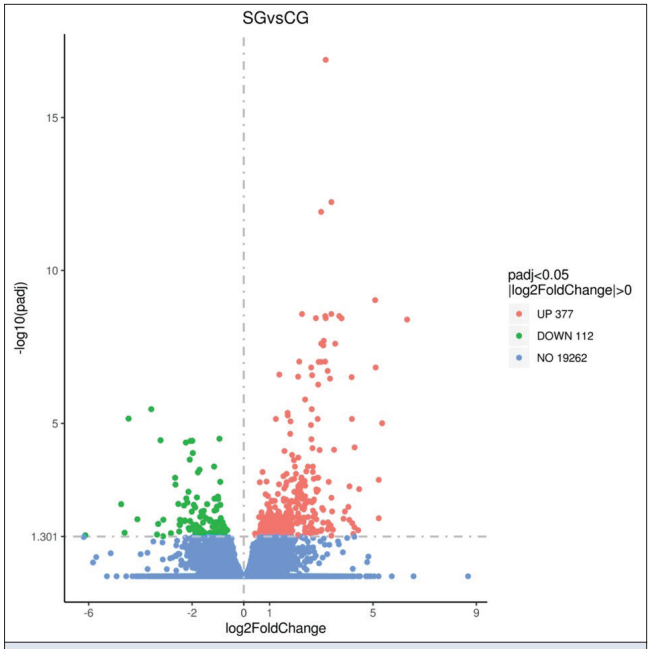

Fig 5. Gene expression level map. The horizontal axis of the figure is the fold change of gene expression (log2FoldChange) between the treatment and control groups, and the vertical axis is the significance level of the difference in gene expression between the treatment and control groups (-log10padj or -log10pvalue). Up-regulated genes are red dots and down-regulated genes are green dots

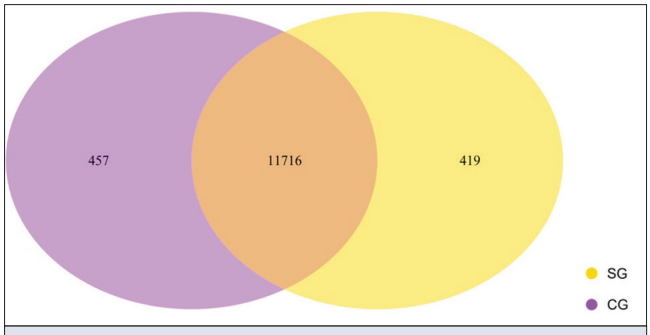

Fig 6. Differential gene Venn map

GO Enrichment Analysis of Differentially Expressed Genes

Among all the genes differentially expressed in the biceps femoris of Alxa Gobi camels and Desert camels, 5695 genes have been annotated, including 148 genes related to biological processes, 66 genes related to cell components and 233 genes related to molecular functions. A total of 484 items were significantly enriched by GO function, among which 246 items were significantly enriched related to biological process, accounting for 50.8%. There were 184 significant enrichment items related to molecular function, accounting for 38.1%. There were 54 significant enrichment items related to cellular component, accounting for 11.1%. The significant enrichment items related to biological process accounted for the largest proportion, but the number of genes involved in biological process was not as large as that involved in molecular function, and

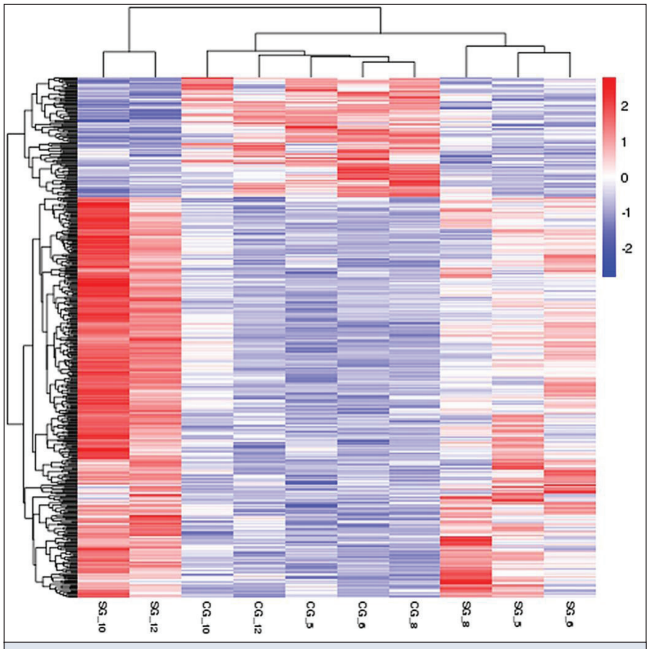

Fig 7. Differential gene cluster map. The colors in the thermogram can only be compared horizontally (the expression of the same gene in different samples), but not vertically (the expression of different genes in the same sample). In horizontal comparison, red indicates high gene expression and blue indicates low gene expression

the genes with extremely significant difference (P<0.01) between Alxa Gobi camel and Desert camel were enriched in molecular function. There were 339 up-regulated genes, including 110 genes related to biological process, 47 genes related to cellular component and 182 genes related to molecular function. A total of 108 genes were down-regulated, among which 38 genes were related to biological process, 19 genes were related to cellular component and 51 genes were related to molecular function.

Analyze the results of GO enrichment analysis, and select the most prominent 30 terms to draw a scatter diagram *(Fig. 8)*.

KEGG Pathway Enrichment Analysis of Differentially Expressed Genes

In this experiment, all differentially expressed genes in biceps femoris of Alxa Gobi camel and Desert camel were enriched by KEGG pathway. The results showed that there were 6116 differentially expressed genes annotated in KEGG database, and the pathway significant enrichment analysis found that these genes participated in 250 biological metabolic pathways, among which 19 pathways were extremely significant differences (P<0.01). There were 37 differential genes involved in skeletal muscle development and 13 differential genes involved in fat metabolism. FoxO signaling pathway, Osteoclast differentiation, and cAMP signaling pathway are involved in skeletal muscle development; in addition, there are two signaling pathways related to lipid metabolism, namely

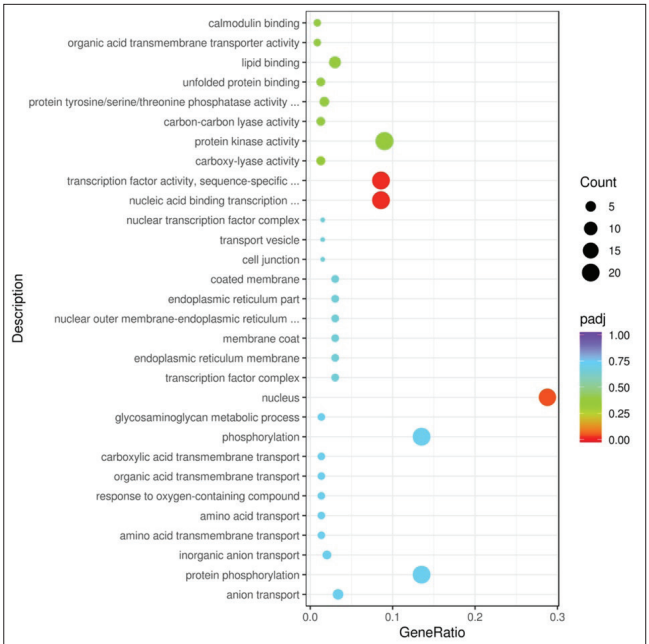

Fig 8. Go enrichment scatter diagram. The ordinate represents Term and the abscissa represents the proportion of the number of differential genes above GO Term in all genes. Each dot represents a specific number of genes, and a different color represents a different significance, ranging from red to purple

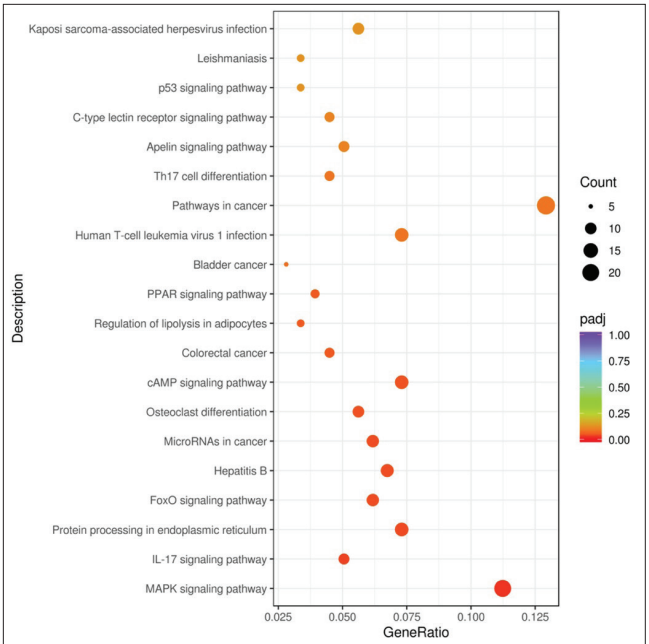

Fig 9. Scatter diagram of KEGG pathway. The abscissa is the ratio of the number of differential genes annotated to the KEGG pathway to the total number of differential genes, the ordinate is the KEGG pathway, the size of the dot represents the number of genes annotated to the KEGG pathway, and the color from red to purple represents the significance of enrichment

Regulation of lipolysis in adipocytes and PPAR signaling pathway.

From the KEGG enrichment results, the most significant 20 KEGG pathways were selected to draw a scatter plot *(Fig. 9)*. Moreover, carefully observing all the signal pathways, MAPK and Pathways in Cancer are rich in the most differentially expressed genes. MAPK and PPAR are rich in up-regulated and down-regulated differentially expressed genes, respectively.

Discussion

In this experiment, the percentage of GC is greater than AT, all of which are over 50%, and the proportion of Q20 of bases in each position is above 97%. Compared with the reference genome, the ratio of the number of reads on the genome is greater than 91.05%, and the number of reads on the gene is greater than 87.75%. At the same time, the FPKM value meets the standard. All these indicate that the sequencing data is reliable and the sequencing quality is high.

In this experiment, the skeletal muscle of Alxa Bactrian camel was analyzed by transcriptome for the first time, and the gene library of Bactrian camel was perfected. A total of 489 differentially expressed genes were screened from the skeletal muscle transcriptome of Alxa Gobi camel and Desert camel, including 377 up-regulated genes and 112 down-regulated genes. Among these genes, candidate genes related to skeletal muscle development and fat deposition were mainly screened.

Among these genes, the genes related to skeletal muscle development are as follows. *FOXO1* gene plays an important role in the transformation of muscle fiber types. *FOXO1* negatively regulates skeletal muscle abundance and is considered to be the key to muscle atrophy [11,12]. *UBE2B* plays an important role in muscle protein homeostasis under catabolism [13]. *EGR1* has been shown to be essential for the expression of key tendon markers and tendine-related *ECM* genes during tendon healing after injury, and *EGR1* controls the balance between bone tissue formation and bone resorption during bone repair [14]. The expression of *SLC25A25* gene is affected by neural pathways, and this gene is a muscle circadian rhythm gene, which is related to muscle thermogenesis and affects the growth and development of skeletal muscle [15]. *ATF4* is a key component of a complex and not fully understood molecular signaling network that leads to muscle atrophy during aging, fasting and imfixation [16]. The normal expression of *AMD1* can improve muscle fibrosis, reduce the overactive pre-fibrotic TGF-b pathway, and lead to improved exercise ability [17]. *ASB5* seems to be essential for muscle recovery after exercise and increases the expression of myogenic hormone, which is a marker of value-added early myogenesis in mammals [18]. *CDKN1A* is involved in the regulation of cell cycle and proliferation of skeletal muscle cells [19,20]. As a muscle-specific regulator,

KLHL30 plays an important role in myoblast proliferation and differentiation [21]. *KLF6* gene can promote the proliferation of skeletal muscle cells, and *miR-148a-3p* can inhibit the proliferation of bovine myoblasts and promote apoptosis through post-transcriptional down-regulation of *KLF6* [22]. *Hspa8* regulates Mef2 protein through chaperonin mediated autophagy to maintain its normal activity, avoid myotonia and mitochondrial function damage, and thus ensure the normal development or regeneration of skeletal muscle [23]. *VGLL2* is a key transcriptional activator of muscle-specific genes, which can be activated by exercise and participate in chronic overload-induced muscle remodeling. It has a great effect on exercise endurance in skeletal muscle, and *VGLL2* is directly or indirectly involved in the specification of mature skeletal muscle fiber characteristics [24-27].

The genes associated with fat deposition are as follows. The *PLIN* gene is involved in lipolysis of neutral lipids and autophagic lipolysis, and regulates lipolysis through the interaction between lipase and lipid droplet protein [28,29]. *LPIN1* plays an important role in adipocyte maturation and adipogenesis [30], and it is essential for de novo synthesis of phospholipids and triglycerides [31].

In terms of signaling pathways, MAPK signaling pathway plays a very important role in adipocyte differentiation [32]. This family controls many important physiological processes, including cell growth, differentiation, proliferation and death, and its pathway is mainly composed of JNK, ERK and p38 pathways [33]. *FOXO1* plays an important role in the regulation of skeletal muscle differentiation and fiber type [34], and is an important transcription factor that determines muscle development and fiber type, but its regulation mode remains controversial. The genes regulated by FoxOs are involved in various pathways, such as metabolic regulation, cell and tissue homeostasis, and immunity [35-38]. Therefore, elucidation of the molecular mechanism of *FOXO1* regulating muscle fiber type and study of FoxO signaling pathway can provide theoretical basis for improving meat quality and new research ideas for genetic improvement and molecular breeding of livestock [39]. The down-regulated genes *PLIN1*, *PLIN4*, *ANGPTL4*, *FABP4* and *PLTP* in the peroxisome proliferator-activated receptor (PPAR) signaling pathway [40] are all genes related to lipid metabolism and lipid deposition.

The results showed that under the influence of living environment and other factors, Alxa Gobi Camel and Desert Camel developed a large number of differential genes in skeletal muscle and fat deposition. These differential genes and their signal pathways changed the phenotypic characteristics of Gobi Camel and improved its meat production performance.

With the development of science and technology, the genetic engineering of livestock [41] is also changing with each passing day. Now, myostatin gene knockout sheep can be efficiently produced by using CRISPR/Cas9 technology and fertilized egg microinjection technology [9]. Compared with the control wild lamb, the weight of these gene knockout sheep is significantly increased. In addition to sheep, livestock genetic engineering has also been used to transform the phenotypic traits of other livestock, such as goats, cattle and pigs [42-45]. Therefore, on the basis of this study, livestock genetic engineering technology can be used to improve the meat production performance of Alashan Bactrian camel and provide scientific basis for cultivating new strains.

Availability of Data and Materials

The datasets during and/or analyzed during the current study available from the corresponding author and can be provided on your request.

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Competing Interests

The authors declared that there is no conflict of interest.

Authors' Contributions

TTF and WCB analyzed the experimental results and consulted a large amount of literature to complete the paper writing. YYF and GD conducted a detailed experimental study and summarized the experimental results. DE planned the experimental research program and the implementation process, and guided the writing process of the paper. DT, SL and TB put forward valuable suggestions for the revision and improvement of the paper.

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SHORT COMMUNICATION

The Genetic Analysis of Broiler-origin H9N2 Influenza Virus with Internal Genes Highly Homologous to the Recent Human H3N8 Virus

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Abstract: The most prevalent subtype of avian influenza worldwide, H9N2, not only threatens human health and causes enormous financial losses in the poultry sector but also has the potential to be transmitted directly or indirectly between other species. This study discovered four H9 subtype avian influenza virus in Fujian in 2022. According to genetic analysis, these four viruses contain several binding sites for mammalian receptors (216 L and 217 M in HA gene). More crucially, the internal genes of the two isolates of XD-L2 and XD-L4 were closely related to those of the human H3N8 virus found this year, which could pose a threat to human health, especially that of poultry producers.

Keywords: *Cross-species transmission, Genetic analysis, H9N2 subtype avian influenza virus, Receptor binding sites*

İnsanlarda Son Dönem Saptanan H3N8 Virüsü İle Yüksek Homologluk Gösteren İnternal Genlere Sahip Broyler Orijinli H9N2 İnfluenza Virüsünün Genetik Analizi

Öz: Dünya genelinde en yaygın kuş gribi alt türü olan H9N2, yalnızca insan sağlığını tehdit etmekle ve kümes hayvancılığı sektöründe büyük mali kayıplara neden olmakla kalmaz, aynı zamanda diğer türler arasında doğrudan veya dolaylı olarak bulaşma potansiyeline de sahiptir. Bu çalışmada, 2022 yılında Fujian'da dört adet H9 alt tipli kuş gribi virüsü saptandı. Genetik araştırmalara göre, bu dört virus, memeli reseptörleri için çeşitli bağlanma bölgeleri içermekteydi (HA geninde 216 L ve 217 M). Daha da önemlisi, XD-L2 ve XD-L4 adlı iki izolatın internal genleri, bu yıl insanlarda saptanan H3N8 virüsünün genlerine çok benziyordu ve bu da insan sağlığı, özellikle de kümes hayvanı üreticileri için bir tehdit oluşturabilir.

Anahtar sözcükler: *Türler arası bulaş, Genetik analiz, H9N2 alt tipli avian influenza virüsü, Reseptör bağlanma bölgeleri*

INTRODUCTION

Except for H5 and H7 subtypes, H9N2 subtype of avian influenza virus (AIV) is considered one of the three major avian influenza (AIV) subtypes that severely endanger public health and poultry industry [1,2]. The single infection of H9 subtype AIV is usually of low pathogenicity to poultry [1], but it can cause immune suppression in poultry [1,3,4], cause secondary infection of pathogens such as infectious bronchitis virus, mycoplasma [3,5], and greatly increase the mortality of poultry. Hemagglutinin (HA) glycoprotein is an important determinant of influenza

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virus pathogenicity, but it is not the only determinant [4]. Whether there is neck deletion of the neuraminidase protein (NA) of AIV, changes in some key loci of its internal genes NS1 (nonstructural protein 1)-149, PA (polymerase acidic protein)-70, PB2 (polymerase basic protein 2)-627, and PB2-701). will affect the pathogenicity of virus [6,7].

Since 1998 [8], a few cases of human H9N2 infection have been documented in China. Research has depicted that H9N2 subtype AIV can cross the species barrier and infect people directly through human-poultry contact without needing an intermediate host [9]. To develop the ability to infect humans, H9N2 AIV can also donate part or even all internal genes and reassort with subtypes such as H5N6 and H7N9 [10,11]. The internal genes of human H3N8 subtype AIV found in the Chinese provinces of Hunan and Henan in 2022 were all derived from H9N2 subtype AIV [12]. Due to their frequent contact with chickens, ducks, and geese, poultry workers are considered to have a significant risk of infecting AIVs; approximately 2.3% of poultry workers have antibodies against H9N2 AIV [13].

The Chinese poultry business is well-established in Fujian. To understand whether the local epidemic H9N2 AIVs can spread across species and threaten the health of local population, four H9N2 subtype AIVs named A/Chicken/Fujian/XD-L1/2022 (H9N2) (XD-L1), A/Chicken/Fujian/XD-L2/2022 (H9N2) (XD-L2), A/Chicken/Fujian/XD-L3/2022 (H9N2) (XD-L3), and A/Chicken/Fujian/XD-L4/2022 (H9N2) (XD-L4) were isolated and identified in the H9N2 positive broiler samples from Fujian province.

Material and Methods

Source of the Virus

The test samples were collected from the farms in Fujian province in January 2022 and the H9N2 viruses isolated in this laboratory.

Genome-Wide Amplification of AIVs

By taking 200 μL of chicken embryo allantoic fluid containing virus, AxyPrep Body Fluid Viral DNA/RNA Miniprep Kit (Axygen, China) was applied, and total viral RNA was extracted according to the manufacturer's instructions. A 50 μL volume of PCR reaction solution was prepared using the Super RT one-step RT-PCR kit (CWBIO, China) for PCR amplification according to the universal primers designed by Hoffmann et al.[14]. PCR product was subjected to 1% agarose gel electrophoresis, and the target band was purified using AxyPrep DNA Gel Extraction Kit (Axygen, China).

Cloning of PCR Products

A 20 μL system was prepared by adding 0.5 μL of pMDTM18-T Vector (TAKARA, China), 9.5 μL of purified DNA, and 10 μL of Solution I to a microcentrifuge tube. The mixture was then incubated at 16°C for 2 h.

According to the instructions of pMDTM18-T Vector Cloning Kit, the linked products were transformed into DH5α competent cells (TIANGEN, China) and coated in LB solid medium containing ampicillin for overnight culturing. A single colony was selected and inoculated into an LB liquid medium for culture. After 6 h, the liquid was identified by PCR.

Statistical Analysis

The bacteria containing the positive plasmids were sent to Tsingke Biotechnology Co., Ltd. for sequencing, and the sequencing results were spliced using SeqMan program in DNAstar software package. In NCBI database *(https://www.ncbi.nlm.nih.gov/),* to download the reference sequence, a phylogenetic tree was constructed using MAGA7 software. MegAlign program in DNAstar package was used for translation and sequence identity analysis.

Results

All isolates were members of h9.4.2.5 branch, according to genetic analysis of the HA genes *(Fig. 1).* The nucleotide sequence identity of isolates was less than 89%, and the amino acid sequence identity was less than 92% with domestic poultry vaccine strains A/chicken/Guangdong/SS/94 (H9N2) (SS), A/chicken/Shandong/6/96 (H9N2) (SD6/96), and A/chicken/Shanghai/F/98 (H9N2) (F/98) *(Table 1),* suggesting that the three vaccine strains may be unable to provide good protection against circulating H9N2 AIVs. Notably, HA of the isolates and mammalian strains share a higher nucleotide sequence identity. In particular, the HA nucleotide sequence identity of XD-L3 isolate and H9N2 subtype swine influenza virus A/swine/Shandong/TA009/2019 (H9N2) (TA009) was 96.6%, and the amino acid sequence identity was 97.5%. This is consistent with the sequence properties of the amino acids of low-pathogenic avian influenza because the cleavage sites of HA genes of XD-L1, XD-L2, XD-L3, and XD-L4 were all PSRSSRGLF, which did not contain multiple continuous basic amino acids [1]. All isolates carried Q216L and Q217M mutations, both of which potentially improve the ability to bind to mammalian receptors [6,15]. The four isolates also carried D148N mutation, which could improve virulence in mice and chickens [5], and N188T mutation, which boosts the virus replication and transmission in ferrets [5] (H9 mature HA numbering used throughout). All NA genes of isolates were closely related to those of avian-origin H9N2 AIV, according to the results of genetic evolution analysis. All four viruses were found to be 274H, 275V, and 294N in NA gene,

Table 1. *Nucleotide and amino acid sequence identities of HA gene*

Virus	Nucleotide and Amino Acid Sequence Identities of HA Gene (%)											
	XD-L1	XD-L2	XD-L3	XD-L4	BJ1/17	BJ1/16	MZ058	201501	TA009	SS	SD6/96	F/98
XD-L1	-	96.2	91.3	95.7	93.5	93.2	94.7	94.9	92.0	87.5	87.3	86.6
XD-L2	96.4	-	91.4	96.7	93.4	93.4	94.4	94.7	91.9	87.6	87.3	86.6
XD-L3	92.3	92.5	-	91.0	93.9	92.9	92.9	93.5	96.6	88.7	88.1	87.9
XD-L4	96.3	96.6	92.0	-	92.7	92.4	93.7	94.1	91.7	87.2	87.0	86.1
BJ1/17	94.1	94.7	93.8	93.6	-	98.8	95.5	96.0	93.7	89.2	89.0	88.5
BJ1/16	93.9	94.5	93.2	93.4	99.3	-	95.2	95.7	92.7	88.6	88.3	87.9
MZ058	95.2	95.0	93.9	94.1	96.3	96.4	-	98.3	93.6	89.3	88.9	88.1
201501	95.4	95.2	94.5	94.3	96.6	96.8	98.4	-	94.0	89.8	89.3	88.7
TA009	92.9	93.0	97.5	92.7	94.3	93.8	95.0	95.2	-	89.4	89.1	88.7
SS	88.9	88.8	91.6	88.6	90.4	90.6	90.7	91.3	91.8	-	97.9	96.5
SD6/96	88.6	88.6	90.9	88.2	90.4	90.6	90.6	90.7	91.1	97.0	-	96.4
F/98	88.8	88.8	90.7	88.6	90.0	90.2	90.0	90.6	91.1	96.6	95.4	-

The upper right corner is nucleotide sequence identities, and the lower left corner is amino acid sequence identities.
Reference strains: BJ1/17: A/Beijing/1/2017 (H9N2), BJ1/16: A/Beijing/1/2016 (H9N2), MZ058: A/Guangdong/MZ058/2016 (H9N2), 201501: A/Zhongshan/201501/2015 (H9N2), TA009: A/swine/Shandong/TA009/2019 (H9N2), SS: A/chicken/Guangdong/SS/94 (H9N2), SD6/96: A/chicken/Shandong/6/96 (H9N2), F/98: A/chicken/Shanghai/F/98 (H9N2)

suggesting that these viruses are sensitive to oseltamivir and enhance their binding ability to mammalian receptors (N2 numbering) [6,16].

Following internal gene analysis, it was found that all isolates had the mutations F103L and M106I in the NS1, which could increase the virulence of AIV in mammals and its capacity to replicate more readily [17]. The matrix protein (M) has 31N, which is amantadine resistant [18]. The three amino acids 70V, 224S, and 400P found in PA may increase the pathogenicity of mammal viruses [6]. The 588V mutation in PB2 increases its pathogenicity in mammals [6]. The fact that the isolates of XD-L2 and XD-L4 clustered closely into the same evolutionary branch as the recent H3N8 human AIV discovered this year raises particular concern based on the genetic evolution analysis of internal genes *(Fig. 1)*. The nucleotide sequence identities of internal genes were analyzed for the isolates, and the recent H3N8 viruses A/Changsha/1000/2022 (H3N8) (1000) and A/Henan/4-10/2022 (H3N8) (4-10) *(Table 2)*. The nucleotide sequence identities of M, nucleoprotein (NP), NS, PA, polymerase basic protein 1 (PB1), and PB2 between XD-L2/XD-L4 and A/Henan/4-10/2022 (H3N8) are 98.5%/98.6%, 98.4%/97.7%, 97.7%/98.1%, 96.8%/96.9%, 98.1%/97.3%, and 97.6%/96.9%, respectively. The amino acid sequence identities of M1, NP, NS1, PA, PB1, and PB2 of XD-L2/XD-L4 and A/Henan/4-10/2022 (H3N8) are 98.8%/99.6%, 100%/99.8%, 97.2%/96.8%, 99.4%/99.0%, 98.9%/98.7%, and 98.7%/98.7%. We must focus on the fact that all isolates contain amino acids of 363 K in HA and 672 L in PA, which play an important role in facilitating airborne transmission of the viruses [19].

Discussion

China is considered as the epicenter of the pandemic influenza virus and is an area where different AIVs co-circulate [20]. The first reported human infection with H9N2 virus in China was in 1998, and a complete genome sequence analysis of the isolates indicated that these human isolates probably originated from local chicken flocks [21]. Human infection with H9N2 virus occurred in Hong Kong in December 2003, in which all eight gene fragments came from poultry [1]. In 2016, there was even a case in which an H9N2 virus infection killed a patient with an underlying disease [1]. This suggests that surveillance of H9N2 virus in poultry should be strengthened to prevent the risk of H9N2 virus infection in humans.

In this study, we isolated four H9N2 subtype low pathogenic AIV from poultry farms in Fujian province. The HA gene sequence analysis demonstrated that the amino acid identity between the isolates and the known vaccine strains (three strains) was less than 92%, suggesting that these vaccine strains could not provide complete protection to the isolates. The results of the genetic study of isolates revealed that each gene has numerous mutations, some of which could enhance the pathogenicity of the virus (e.g., HA-148N, NS-149A, PA-70V, PA-224S, PA-400P) [5,6] and others could enhance its capacity to bind to mammalian receptors (HA-180E/V, HA-216L, HA-217M, NA-275V, and so on) [5-7,15]. N (XD-L1/XD-L3) and S (XD-L2/XD-L4) at position of 375 in PB1 gene plays a key role in adaptation and virulence in mammals [22]. The analysis of key loci of M and NA genes indicated that the isolated strains in this study may

Table 2. *Nucleotide and amino acid sequence identities of the internal genes*

Virus	Nucleotide and Amino Acid Sequence Identities of the Internal Genes (%)											
	M[a]						NP					
	XD-L1	XD-L2	XD-L3	XD-L4	1000	4-10	XD-L1	XD-L2	XD-L3	XD-L4	1000	4-10
XD-L1	-	97.1	95.4	97.8	95.4	97.6	-	95.1	94.8	95.3	96.0	95.3
XD-L2	97.6	-	95.9	98.9	95.4	98.5	99.8	-	95.1	97.5	94.7	98.4
XD-L3	.98.0	99.2	-	95.6	94.8	95.4	99.4	99.6	-	95.1	94.6	95.3
XD-L4	98.4	98.8	99.2	-	96.0	98.6	99.6	99.8	99.4	-	94.6	97.7
1000	98.4	98.4	98.8	99.2	-	95.7	99.4	99.2	99.2	99.0	-	94.8
4-10	98.4	98.8	99.2	99.6	99.2	-	99.8	100	99.6	99.8	99.2	-
	NS[b]						PA					
	XD-L1	XD-L2	XD-L3	XD-L4	1000	4-10	XD-L1	XD-L2	XD-L3	XD-L4	1000	4-10
XD-L1	-	97.0	96.9	97.4	96.4	97.4	-	94.6	95.0	94.7	94.0	94.8
XD-L2	98.2	-	95.6	97.9	97.9	97.7	99.2	-	96.8	96.7	93.8	96.8
XD-L3	97.7	97.2	-	96.2	95.3	96.2	99.0	98.9	-	96.7	94.3	96.8
XD-L4	98.2	98.2	96.8	-	97.4	98.1	98.7	98.6	98.5	-	94.0	96.9
1000	96.8	97.7	96.3	96.8	-	96.9	99.0	98.9	98.7	98.5	-	94.1
4-10	96.8	97.2	95.9	96.8	95.9	-	99.6	99.4	99.3	99.0	99.3	-
	PB1						PB2					
	XD-L1	XD-L2	XD-L3	XD-L4	1000	4-10	XD-L1	XD-L2	XD-L3	XD-L4	1000	4-10
XD-L1	-	94.0	93.6	93.7	93.6	93.8	-	97.4	95.7	96.2	94.2	96.2
XD-L2	98.3	-	92.5	97.6	92.2	98.1	98.7	-	94.1	97.7	94.5	97.6
XD-L3	98.0	97.5	-	92.7	94.8	92.9	97.9	97.5	-	93.7	93.3	93.9
XD-L4	98.0	98.7	97.2	-	91.9	97.3	98.6	98.6	97.5	-	93.7	96.9
1000	98.3	98.0	98.3	98.0	-	92.3	98.2	98.6	97.5	98.4	-	94.1
4-10	98.4	98.9	97.8	98.7	98.2	-	98.7	98.7	97.6	98.7	98.3	-

The upper right corner is nucleotide sequence identities, and the lower left corner is amino acid sequence identities.
[a] *The upper right corner is the nucleotide sequence identities of M gene, the lower left corner is the amino acid sequence identities of M1 protein.* [b] *The upper right corner is the nucleotide sequence identities of NS gene, the lower left corner is the amino acid sequence identities of NS1 protein.*
Reference strains: 1000: A/Changsha/1000/2022 (H3N8), 4-10: A/Henan/4-10/2022 (H3N8)

be amantadine-resistant but sensitive to oseltamivir. Notably, the isolates contain amino acids HA-K363 and PA-L672 [23], which are important for airborne transmission of the virus and may make the isolates more easily transmissible. The isolates XD-L2 and XD-L4 were found to be highly homologous to the most recent human H3N8 isolate based on genetic evolution analysis of their internal genes. This finding suggests that these two isolates may infect humans.

In conclusion, four H9N2 subtypes of low pathogenic AIVs were isolated from the Chinese province of Fujian in 2022. The isolates have mutations that increase virulence and enhance mammalian receptor binding and may pose a threat to humans. The concept that calls for preventing infectious diseases in animals to come before those in people is crucial for preventing and controlling zoonosis. Strengthening surveillance, prevention, and control of H9N2 subtype AIV is crucial, given the widespread prevalence of the virus in poultry in China.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author (C.G. Liu) upon reasonable request.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

Author Contributions

P.L., J.Y., and C.L. planned and designed the research. P.L., H.Z., and X.W. conducted the experiments. H.S., S.C., and L.M. analyzed the data. P.L. wrote the manuscript. T.Y., S.Z., Z.L., and C.L. revised the manuscript.

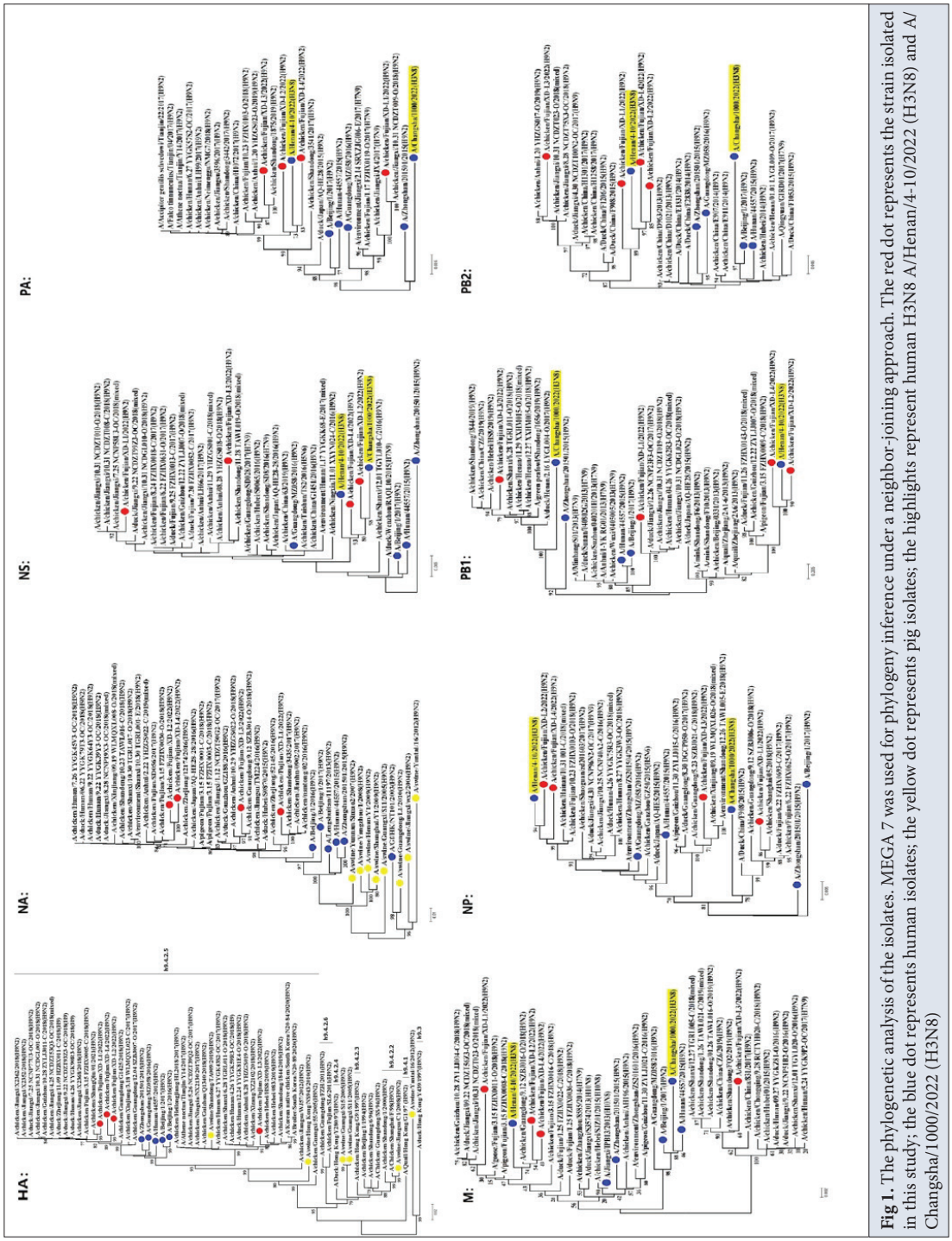

Fig 1. The phylogenetic analysis of the isolates. MEGA 7 was used for phylogeny inference under a neighbor-joining approach. The red dot represents the strain isolated in this study; the blue dot represents human isolates; the yellow dot represents pig isolates; the highlights represent human H3N8 A/Henan/4-10/2022 (H3N8) and A/Changsha/1000/2022 (H3N8)

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Case Report

Characterization of the Nasal Dermoid Sinus in A Rhodesian Ridgeback Dog with Computerized Tomography and Magnetic Resonance Imaging

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Abstract: Dermoid sinus is a congenital defect that develops during embryonic development when the skin cannot be completely separated from the ectodermal neural tube. It extends ventrally at different levels under the skin. It has been reported in many different breeds, but is most common in Rhodesian Ridgeback dogs. In this case, the dermoid sinus in the nasal region of a Rhodesian Ridgeback dog was characterized by computed tomography and magnetic resonance imaging. In this presentation, it is aimed to emphasize the importance of introducing the lesion and race and characterization using advanced imaging techniques in determining the treatment method.

Keywords: *Computed tomography, Dermoid sinus, Dog, Magnetic resonance imaging*

Rhodesian Ridgeback Irkı Bir Köpekte Nazal Dermoid SinüsünBilgisayarlı Tomografi ve Manyetik Rezonans Görüntüleme İle Karakterizasyonu

Öz: Dermoid sinüs embriyonik gelişim aşamasında, derinin ektodermal nöral tüpten tamamen ayrılamamasıyla gelişen bir kongenital kusurdur. Deri altında farklı seviyelerde ventrale doğru uzanır. Bir çok farklı ırkta da bildirilmiştir ancak en çok Rhodesian Ridgeback ırkı köpeklerde görülür. Bu olguda Rhodesian Ridgeback ırkı bir köpekte nazal bölgedeki dermoid kist, bilgisayarlı tomografi ve manyetik rezonans görüntüleme ile karakterize edilmiştir. Bu sunumda, lezyonun ve ırkın tanıtılması ile ileri görüntüleme teknikleri kullanılarak karakterizasyonun tedavi yönteminin belirlenmesindeki önemini vurgulamak amaçlanmıştır.

Anahtar Sözcükler: *Bilgisayarlı tomografi, Dermoid sinüs, Köpek, Manyetik rezonans görüntüleme*

Introduction

The dermoid sinus is a congenital defect in which the skin cannot be completely separated from the ectodermal neural tube during embryonic development [1]. The result of this developmental disruption may also be called a pilonidal sinus, pilonidal cyst, or dermoid cyst [2]. Located along the dorsal midline, these tubular blind sacs extend ventrally to the skin and underlying tissues [1]. Nasal dermoid sinus cysts are extremely rare in dogs and occur when the ectoderm in the prenasal cavity is not completely closed [3]. There may be neurologic problems, ranging from aberrant motor function to posterior paresis and hyperesthesia, if the sinus interacts with the subarachnoid area through a lamina defect. The location of the lesion and the presence or absence of myelitis or encephalitis owing to sinus infection determine the presence and severity of neurologic symptoms. Rarely, spinal abnormalities such hemivertebrae, vertebral body fusions, and lesions that resemble spina bifida may be related to dermoid sinuses [1]. Although many different methods have been proposed to identify dermoid sinuses in dogs, including

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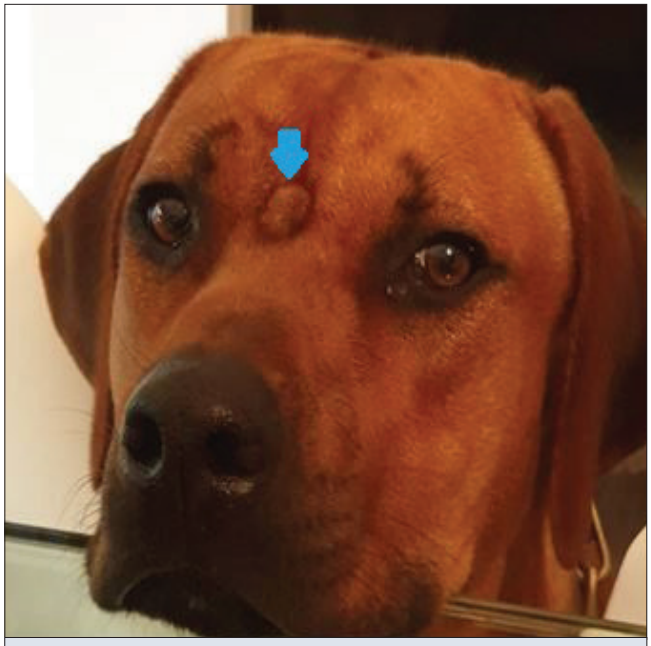

Fig 1. Cystic lesion on the dorsal line between the orbits

computed tomography (CT), magnetic resonance imaging (MRI), fistulography, and myelography, there is no consensus on optimal imaging, and each technique has distinct advantages and disadvantages [4]. If the sinus is not causing symptoms and is not connected to the dura mater, treatment may not be necessary. The lesion should be surgically removed if the sinus becomes infected, is draining or inflamed, or is presenting clinical symptoms. If the sinus extends down to the dura, a dorsal laminectomy or hemilaminectomy may be necessary [1].

The Rhodesian ridgeback is a dog breed of African origin with a characteristic dorsal hair ridge with backward-growing hair [5,6]. The dorsal hair ridge in these dogs has been suggested to be caused by a dominant mutation that predisposes them to dermoid sinus, which is also a congenital developmental disorder [7]. Dermoid sinuses were reported as a prevalent congenital condition in ridged dogs and as a sporadic finding in other dog breeds [2,5,6], exemplary Chow Chow [8], Golden Retriever, Cocker Spaniel, Springer Spaniel [3], German Shepherd [9], Cane Corso [10] and Shiba Inu [11]. For the dermoid sinus in Rhodesian Ridgebacks, various inheritance patterns have been hypothesized, including autosomal recessive, autosomal incomplete dominant, two recessive loci, or complete [6]. No gender predisposition has been previously noted. It has been suggested that dogs with dermoid sinuses be excluded from breeding [7].

This report describes the characterization and diagnostic significance of a dermoid cyst by computed tomography and magnetic resonance imaging in a Rhodesian Ridgeback dog.

Case History

For this case report, informed consent was obtained from the patient owner.

The case is an 8-month-old, intact male Rhodesian Ridgeback dog brought to Istanbul University Cerrahpaşa Veterinary Faculty Research and Practice Animal Hospital Department of Surgery with the complaint of swelling on the nose *(Fig. 1)*. In the anamnesis, it was stated that the patient did not have any complaints other than the difference in his physical appearance. In the clinical examination, a cystic lesion about one cm diameter was palpated under the skin on the dorsal line between the orbits. The patient did not show any sensitivity or pain response in the area. It was also observed that the patient had a dorsal hair ridge *(Fig. 2)* known to cause dermoid sinus predisposition. The patient's mental status and behavior, gait and postural reactions, cranial reflexes and spinal reflexes were evaluated and all neurological examination findings were healthy. Hemogram and biochemistry values (ALP, ALT, BUN, GLU, TP) are within the normal range. Advanced imaging was recommended for the diagnosis of the lesion. After eight hours of fasting, the patient was taken to general anesthesia for MRI and CT. Propofol (Propofol®, Abbott, Turkey) (6-8 mg/kg IV)

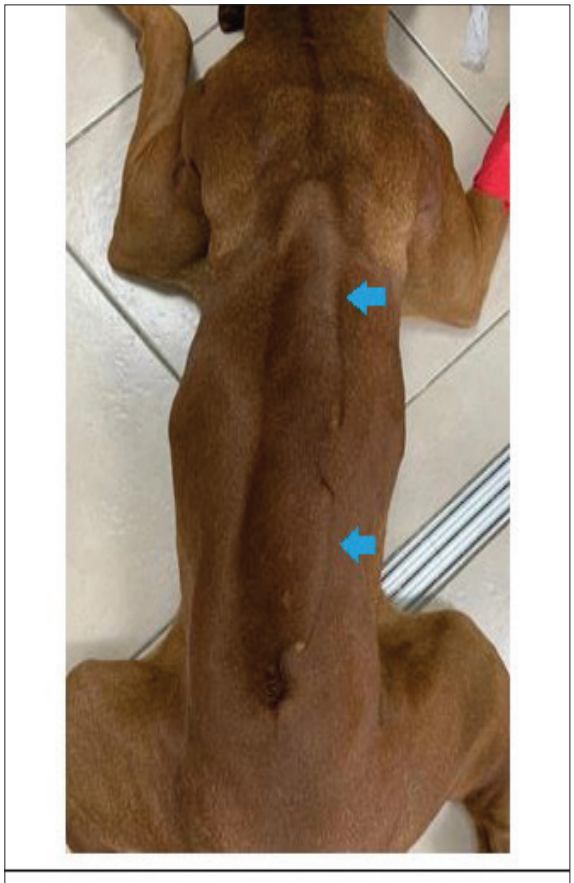

Fig 2. Dorsal hair ridge typical of the Rhodesian Ridgeback breed

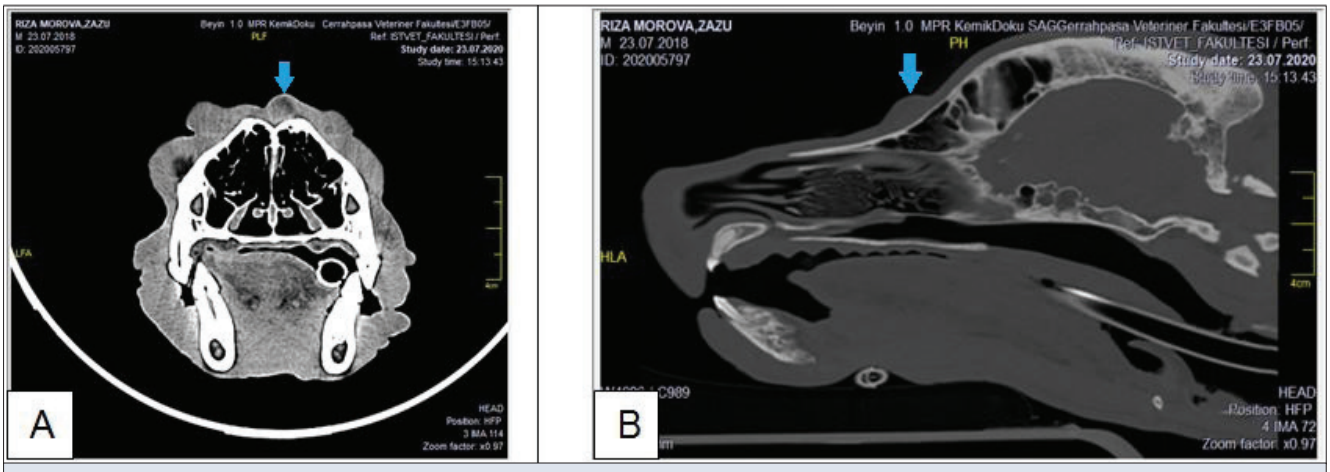

Fig 3. CT image of dermoid cyst. A- transverse plane; B- Sagittal plane. No destruction was observed in the bone tissue adjacent to the dermoid cyst and it was seen that it was not associated with the nasal cavity

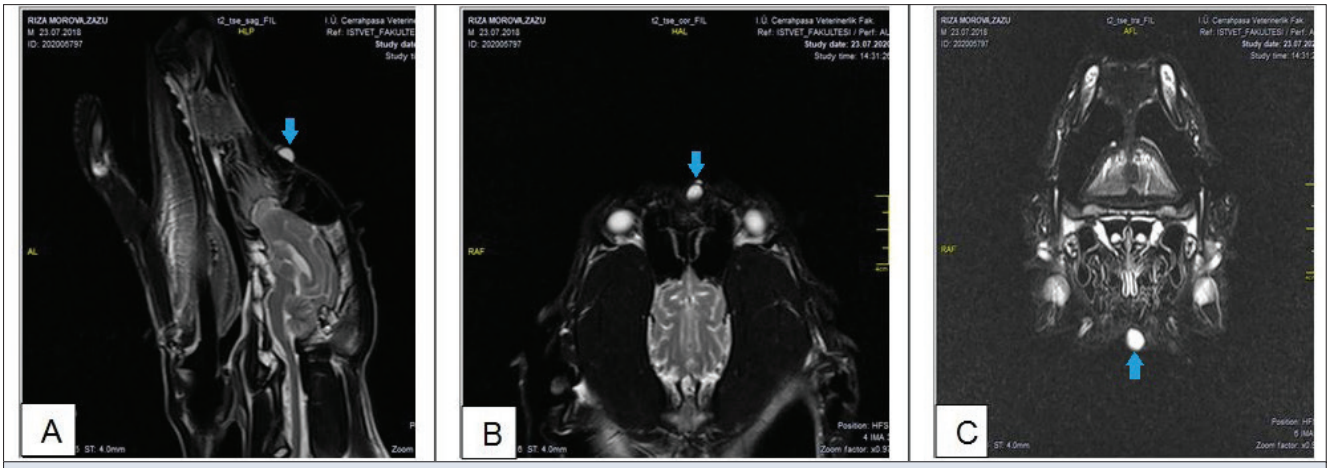

Fig 4. MR images of dermoid cyst. A- T2-weighted sagittal sequence; B- T1-weighted transversal sequence; C- T2-weighted dorsal sequence. An oval-shaped cystic lesion of 12.1x8.2x9.4 mm in size, located on the right of the midline, on the nasal bone adjacent to the frontal bone, was observed hyperintense on T2-weighted images and hypointense on T1-weighted images

was used for induction and anesthesia was continued with isoflurane (Forane®, Abbott, UK) (initially 4%, then 2%) after endotracheal intubation.

Suspecting that the lesion might be dermoid sinus due to the patient's breed predisposition, the paranasal sinuses were evaluated with CT *(Fig. 3)* and MRI *(Fig. 4)*. The line with the dorsal hair ridge was evaluated with MRI to detect the presence of a sinus associated with the nervous system *(Fig. 5)*.

When the patient's images were evaluated, it was determined that the lesion was dermoid sinus due to the structure of the lesion, the patient's breed, and the high predisposition created by the hair ridge on the back. As a result of imaging, the lesion was defined and it was seen that it was not associated with the nervous system. It was determined that it was type V according to the previous typing [1,5,12], and subtype c according to the classification according to the anatomical location [13].

Removal of the cyst by excisional surgery was recommended to the patient's relatives, but it was not accepted because it did not affect the patient's quality of life. It was also notified that breeding the dog was not advised due to the genetic transmission of the disease. The patient has been in our follow-up for 2 years, and there has been no change in his condition during this period. The size, consistency and shape of the cyst are constant and do not cause any discomfort.

Discussion

An animal may have one or more dermoid sinuses. Even if a dermoid sinus is present, soft tissue edema, or vertebral abnormalities are evident, radiographs may still be unremarkable. The extent of the tract has been visualized using myelography or fistulography, although it has been advised against injecting contrast material or probing the tract due to the possibility of causing meningitis or damaging underlying structures. Also, if there is debris

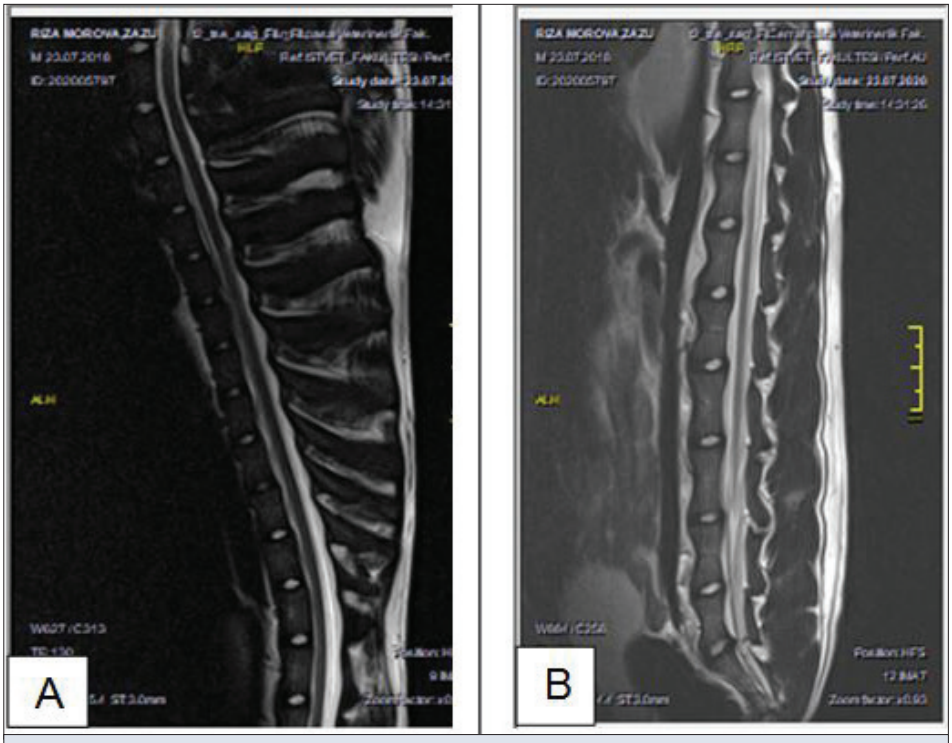

Fig 5. T2-weighted sagittal MR images. A- The thoracic region; B- The lumbar region. It was observed that there was no connection with the skin, subcutaneous soft tissue, spinal canal and medulla spinalis at the borders of the dorsal hair ridge

present, fistulography may not demonstrate the complete depth of the sinus tract. Because they outline the tract and display cross-sectional pictures, MRI and CT are the diagnostics that are most useful in identifying the depth of the dermoid sinus and diagnosing it [5]. Therefore, in our case, the dorsal line was imaged with MRI and no other sinus associated with the nervous system was found. As a result of imaging of the nasal dermoid sinus cyst in the dorsal line between the two eyes with CT, it was determined that it was limited only under the skin and in one region.

According to the penetration into the subcutaneous tissues, primarily 4 types of dermoid sinus have been defined. Type I extends ventrally as a cylindrical sac attached to the supraspinous ligament, Type II consists of a sac-like portion that does not extend to the supraspinous ligament but is attached by a fibrous band, type III does not extend to and is not associated with the supraspinous ligament, type IV extends into the spinal canal and dura mater it is linked with [1,5]. After these definitions, type V; defined as a true cyst consisting of a closed, epithelial-lined sac, and type VI as an open cyst extending to the level of the supraspinous ligament with a distal line of attachment with the dura mater [12] .

The nasal dermoid sinus in our case was defined as type V because it was in the form of a completely closed cyst that is not related to other tissues.

Nasal dermoid sinus cysts in humans have been classified according to the depth of penetration into the craniofacial structures [3], but in dogs the classification is based on anatomical location. These dermoid sinus types are classified into three subtypes: subtype a for dorsal midline, subtype b for head excluding nose and subtype c for nose [13]. In this case, the dermoid sinus was classified as subtype c because it was located in the nasal region.

In Ridgebacks, dermoid sinuses are most commonly encountered in the cervical or craniothoracic regions, but they can be found in the sacrococcygeal region or head, and they usually occur cranially or caudally rather than in the characteristic hair ridge on their back [1,14]. Characterization has previously been reported in the thoracolumbal and dorsal cervical regions of Ridgeback dogs and crosses [15]. Contrary to previous reports, in this case the dermoid sinus was in the nasal region.

Dermoid sinuses may cause different symptoms depending on their location and relationship with the nervous system [10,11]. In this case, no neurological finding was observed as the dermoid sinus was not associated with the nervous system. However, operating without imaging and characterization of the lesion is very risky since the lesion may be associated with the nervous system and may cause various neurological symptoms. In addition, removal of a dermoid sinus cyst that is limited to the subcutaneous tissue is not vital, and follow-up of the lesion may be

recommended instead.

Few cases have been reported of rostral and cervicooccipital dermoid cysts in the veterinary literature [3,13], but dermoid sinuses on the bridge of the nose in humans have been described since 1817 [15]. Embryological analysis of nasal and frontal bones in humans shows that the embryonic origin of nasal dermoid cysts is unique compared to that of the dorsal midline dermoid sinuses [16]. However, lesions at or above the nasofrontal suture have much lower rates of intracranial extension in humans [17].

Removal of the cyst by excisional surgery was recommended to the patient's relatives, but it was not accepted because it did not affect the patient's quality of life. It was also notified that breeding the dog was not advised due to the genetic transmission of the disease. The patient has been in our follow-up for 2 years, and there has been no change in his condition during this period. The size, consistency and shape of the cyst are constant and do not cause any discomfort. In conclusion, this report demonstrates the recognition and characterization of dermoid sinus lesions and the clinical significance of imaging with CT and MRI.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author (E. Eravcı Yalın) upon reasonable request.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

Case examination and evaluation of clinical findings were done by ND and EEY. EEY interpreted MRI and CT images. ND and EEY contributed to the discussion. ND made the article writing and submission of the article.

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The editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi and the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) keep all the articles (electronic and printed) published in the journal in their archives. All articles and their attachment files sent to the journal are kept securely in the archive. In light of the technological developments, the editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi regularly performs electronic processes for the development and updating of materials in digital environment and presents them to its readers on condition of keeping in safe the original documents and information regarding the articles.

Even if the journal ceases to be published for any reason, the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) will continue to protect the journal content in the long term and provide convenient access to users. Electronic services of Kafkas University Information Technologies Department will be used for the journal to maintain this responsibility.

RESPONSIBILITIES OF SUBJECT EDITORS

Subject editors do reviews and evaluations in accordance with the main publication goals and policies of the journal and in line with the criteria that will contribute to the development of the journal.

Author information is kept confidential in articles sent to the subject editor for preliminary evaluation by the editor.

Subject editors thoroughly examine the sections of the introduction, materials and methods, results, discussion and conclusion, in terms of journal publication policies, scope, originality and research ethics. Subject editor submits its decision (rejection, revision or peer-review) after evaluation to the chief editor in a reasoned report.

Subject editor may request additional information and documents related to the study from the authors, when necessary.

In multidisciplinary studies, the article can be submitted for the evaluation of multiple subject editors.

RESPONSIBILITIES OF REFEREES

Double-blinded peer-review procedure is applied in Kafkas Universitesi Veteriner Fakultesi Dergisi in order to evaluate the articles submitted to the journal in accordance with the principle of impartiality and in objective criteria; that is, referees and writers do not know about each other.

The referees submit their opinions and reports to the editor-in-chief to ensure the control and suitability of a submitted article, its scientific content, scientific consistency and compliance with the principles of the journal. When a referee makes a decision "reject" about an article, he/she prepares the reasons for the decision in accordance with the scientific norms and presents it to the editor.

The referee(s) also gives the authors the opportunity to improve the content of the article. Accordingly, the revisions requested from the authors should be of a quality that explains/questions specific issues rather than general statements.

Referees appointed for the evaluation of the articles agree that the articles are confidential documents and will not share any information about these documents with third parties, except for the editors participating in the evaluation.

Referees should place their criticism on scientific infrastructure and write their explanations based on scientific evidence. All comments made by the referees to improve the articles should be clear and direct, and should be written away from disturbing the feelings of the author. Insulting and derogatory statements should be avoided.

If a referee has an interest relationship with the author(s) on one or more issues, he/she must report the situation to the editor and ask his/her to withdraw from the referee position. The same is also applicable when the authors illegally obtain information about the referees of the article and try to influence them.

The editor-in-chief can share the comments and reports from the referees with the editors/associate editors and the relevant subject editor, as necessary, to ensure that the decision on the article is optimal. If necessary, the editor may share the critical decision and its grounds that a referee has sent about the article with the other referee(s) and present them to their attention.

Referee(s) may request revision many times for the article they evaluated.

The content of the referee reports is checked and evaluated by editor-in-chief/editors/associate editors. The final decision belongs to the editorial.

RESPONSIBILITIES OF AUTHOR(S)

It is not tolerable for the author (s) to send an article, which has been already sent to another journal, to Kafkas Universitesi Veteriner Fakultesi Dergisi within the scope of "which accepts" or "which publishes first" approach. If this is detected, the article is rejected at any stage of the evaluation. As a possible result of these actions, in the process following the previous acceptance of the article sent to another journal, the withdrawal request with this excuse that the authors submit for this article, the evaluation process of which is going on in our journal, is evaluated by the editors and associate editors of the journal and disciplinary action on the grounds of ethical violations about those responsible is started. This unethical action is also informed to the journal editor (if known) who accepted the article.

It is essential that the articles to be sent to Kafkas Universitesi Veteriner Fakultesi Dergisi include studies that have up-to-date, original and important clinical/practical results and prepared in accordance with the journal's writing rules.

Authors should choose the references they use during the writing of the article in accordance with the ethical principles and cite them according to the rules.

The authors are obliged to revise the article in line with the issues conveyed to them during the initial evaluation, preliminary evaluation and peer-review phases of the article and to explain the changes they made/did not make sequentially in the "response to editor" and "response to reviewer comments" sections.

If information, documents or data regarding to the study are requested during the evaluation process, the corresponding author is obliged to submit them to the editorial.

Authors should know and take into account the issues listed in the "General Ethical Principles" section regarding scientific research and authors.

The authors do not have the right to simultaneously submit multiple articles to Kafkas Universitesi Veteriner Fakultesi Dergisi. It is more appropriate to submit them with acceptable time intervals for the journal's policy.

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
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Kafkas Univ Vet Fak Derg
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INSTRUCTION FOR AUTHORS

1- Kafkas Universitesi Veteriner Fakultesi Dergisi (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly (ISSN: 1300-6045 and e-ISSN: 1309-2251). We follow a double-blind peer-review process, and therefore the authors should remove their name and any acknowledgment from the manuscript before submission. Author names, affiliations, present/permanent address etc. should be given on the title page only.

The journal publishes full-length research papers, short communications, preliminary scientific reports, case reports, observations, letters to the editor, and reviews. The scope of the journal includes all aspects of veterinary medicine and animal science.

Kafkas Universitesi Veteriner Fakultesi Dergisi is an Open Access journal, which means that all content is freely available without charge to the user or his/her institution. Users are allowed to read, download, copy, distribute, print, search, or link to the full texts of the articles, or use them for any other lawful purpose, without asking prior permission from the publisher or the author. This is in accordance with the BOAI definition of Open Access.

The official language of our journal is English. Additionally, all the manuscripts must also have Turkish title, keywords, and abstract (translation will be provided by our journal office for foreign authors).

2- The manuscripts submitted for publication should be prepared in the format of Times New Roman style, font size 12, A4 paper size, 1.5 line spacing, and 2.5 cm margins of all edges. The legend or caption of all illustrations such as figure and table and their appropriate position should be indicated in the text. Refer to tables and figures in the main text by their numbers. Also figure legends explanations should be given at the end of the text.

The figures should be at least 300 dpi resolution.

The manuscript and supplementary files (figure etc.) should be submitted by using online manuscript submission system at the address of http://vetdergi.kafkas.edu.tr/

During the submission process, the authors should upload the figures of the manuscript to the online manuscript submission system. If the manuscript is accepted for publication, the Copyright Agreement Form signed by all the authors should be sent to the editorial office.

3- The authors should indicate the name of the institute approves the necessary ethical commission report and the serial number of the approval in the material and methods section. If necessary, the editorial board may also request the official document of the ethical commission report. In case reports, a sentence stating that "informed consent" was received from the owner should be added to the main document. If an ethical problem is detected (not reporting project information, lack of ethical committee information, conflict of interest, etc.), the editorial board may reject the manuscript at any stage of the evaluation process.

4- Authors should know and take into account the issues listed in the "Ethical Principles and Publication Policy" section regarding scientific research and authors.

5- Types of Manuscripts

Original (full-length) manuscripts are original and proper scientific papers based on sufficient scientific investigations, observations and experiments.

Manuscripts consist of the title, abstract and keywords, introduction, material and methods, results, discussion, and references and it should not exceed 12 pages including text. The number of references should not exceed 50. The page limit does not include tables and illustrations. Abstract should contain 200±20 words.

Short communication manuscripts contain recent information and findings in the related topics; however, they are written with insufficient length to be a full-length original article. They should be prepared in the format of full-length original article but the abstract should not exceed 100 words, the reference numbers should not exceed 15 and the length of the text should be no longer than 6 pages in total. The page limit does not include tables and illustrations. Additionally, they should not contain more than 4 figures or tables.

Preliminary scientific reports are a short description of partially completed original research findings at an interpretable level. These should be prepared in the format of full-length original articles. The length of the text should be no longer than 4 pages in total.

Case reports describe rare significant findings encountered in the application, clinic, and laboratory of related fields. The title and abstract of these articles should be written in the format of full-length original articles (but the abstract should not exceed 100 words) and the remaining sections should be followed by the Introduction, Case History, Discussion and References. The reference numbers should not exceed 15 and the length of the text should be no longer than 4 pages in total. The page limit does not include tables and illustrations.

Letters to the editor are short and picture-documented presentations of subjects with scientific or practical benefits or interesting cases. The length of the text should be no longer than 3 pages in total. The page limit includes tables and illustrations.

Reviews are original manuscripts that gather the literature on the current and significant subject along with the commentary and findings of the author on a particular subject (It is essential that the author/s have international scientific publications on this subject). The title and summary of this manuscript should be prepared as described for the full-length original articles and the remaining sections should be followed by introduction, text (with appropriate titles), conclusion, and references.

"Invited review" articles requested from authors who have experience and recognition in international publishing in a particular field are primarily published in our journal.

Review articles submitted to our journal must be prepared in accordance with any of the three categories listed below.

Narrative reviews describe current published information on a scientific topic. However, it does not include a specific methodological process.

Systematic reviews include the search for original studies published in that field on a specific topic, the evaluation of validity, synthesis and interpretation within a systematic methodology.

Meta-analysis is a method of evaluating the results of many studies on a subject with the methods defined in this category and statistical analysis of the obtained findings.

6- The necessary descriptive information (thesis, projects, financial supports, etc.) scripted as an italic font style should be explained below the manuscript title after placing a superscript mark at the end of the title.

7- At least 30% of the references of any submitted manuscript (for all article categories) should include references published in the last five years.

References should be listed with numerical order as they appear in the text and the reference number should be indicated inside the parentheses at the cited text place. References should have the order of surnames and initial letters of the authors, title of the article, title of the journal (original abbreviated title), volume and issue numbers, page numbers and the year of publication and the text formatting should be performed as shown in the example below.

Example: Yang L, Liu B, Yan X, Zhang L, Gao F, Liu Z: Expression of ISG15 in bone marrow during early pregnancy in ewes. *Kafkas Univ Vet Fak Derg*, 23 (5): 767-772, 2017. DOI: 10.9775/kvfd.2017.17726

If the reference is a book, it should follow surnames and initial letters of the authors, title of the book, edition number, page numbers, name and location of publisher and year of publication. If a chapter in a book with an editor and several authors is used, names of chapter authors, name of chapter, editors, name of the book, edition number, page numbers, name and location of publisher and year of publication and the formatting should be performed as shown in the example below.

Example: Mcllwraith CW: Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed., 339-447, Lea and Febiger, Philadelphia, 1988.

DOI number should be added to the end of the reference.

In the references can be reached online only, the web address and connection date should be added at the end of the reference information. The generally accepted scientific writing instructions must comply with the other references. Abbreviations, such as "et al" and "and friends" should not be used in the list of the references.

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8- Latin expression such as species names of bacteria, virus, parasite, and fungus and anatomical terms should be written in italic character, keeping their original forms.

9- The editorial board has the right to perform necessary modifications and a reduction in the manuscript submitted for publication and to express recommendations to the authors. The manuscripts sent to authors for correction should be returned to the editorial office within a month. After pre-evaluation and agreement of the submitted manuscripts by the editorial board, the article can only be published after the approval of the field editor and referee/s specialized in the particular field.

10- All responsibilities from published articles merely belong to the authors. According to the ethical policy of our journal, plagiarism/self-plagiarism will not be tolerated. All manuscripts received are checking by plagiarism checker software, which compares the content of the manuscript with a broad database of academic publications.

11- There is no copyright fee for the authors.

12- The authors are charged a fee on acceptance of the manuscript to cover printing costs and other expenses. This payment information can be found at http://vetdergi.kafkas.edu.tr/

SUBMISSION CHECKLIST

Please use below list to carry out a final check of your submission before you send it to the journal for review. Ensure that the following items are present in your submission:

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• Importance and acceptability of the submitted work for the journal have been discussed (Please avoid repeating information that is already present in the abstract and introduction).

• Other information has been added that should be known by the editorial board (e.g.; the manuscript or any part of it has not been published previously or is not under consideration for publication elsewhere.

- Title page

• Title, running title (should be a brief version of the title of your paper, no exceed 50 characters)

• The author's name, institutional affiliation, Open Researcher and Contributor ID (ORCID)

• Congress-symposium, project, thesis etc. information of the manuscript (if any)

• Corresponding author's address, phone, fax, and e-mail information

- Manuscript

• Title, abstract, keywords and main text

• All figures (include relevant captions)

• All tables (including titles, description, footnotes)

• Ensure all figure and table citations in the text match the files provided

• Indicate clearly if color should be used for any figures in print

- Availability of Data and Materials

- Acknowledgements

- Funding Support

- Competing Interests

- Authors' Contributions

Further considerations

• Journal policies detailed in this guide have been reviewed

• The manuscript has been "spell checked" and "grammar checked"

• Relevant declarations of interest have been made

• Statement of Author Contributions added to the text

• Acknowledgment and conflicts of interest statement provided