

## RESEARCH ARTICLE

# The Effects of Fast and Slow Thawing on Spermatological Parameters and Detect of Chromatin Condensation by Toluidine Blue Staining in Frozen-Thawed Bull Sperm

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**Abstract:** The purpose of this study was to observe the effect of different thawing methods on semen parameters such as motility and morphology and sperm chromatin integrity as assessed by toluidine blue (TB) staining. A total of 20 frozen sperm straws from the same Holstein bull were used. While the 30 sec thawing protocol at 37°C, which is used for thawing frozen sperm straws, constitutes our slow thawing group (n=10), the 6 sec thawing protocol at 70°C constitutes our fast-thawing group (n=10). The motility, viability, morphology, plasma membrane integrity, and sperm chromatin condensation parameters of all thawed sperm were investigated. There was a significant difference (P<0.05) in sperm plasma membrane integrity, head defect, and total abnormal sperm morphology. The chromatin decondensation rate detected by TB in bull semen thawed in the slow thaw system, and the decondensation rate in the fast thaw system, differed significantly from each other in line with the literature data (P<0.05). According to the evaluations made in terms of chromatin decondensation rate, the rate obtained in slow thawing (7±0.39) shows an increase up to two times compared to the fast-thawing rate (3.3±0.33) (P<0.05). The TB staining procedure can be used to evaluate infertility and chromatin integrity, especially in cases that are suspicious and require rapid evaluation.

**Keywords:** Diagnostic test, Sperm chromatin, Sperm morphology, Toluidine blue

## Dondurulmuş-Çözdürülmüş Boğa Spermasında Hızlı ve Yavaş Çözdürmenin Spermatolojik Parametreler ve Toluidin Mavisi Boyama ile Kromatin Kondenzasyonunun Belirlenmesi Üzerine Etkisi

**Öz:** Bu çalışmanın amacı, farklı çözündürme yöntemlerinin motilite ve morfoloji gibi sperma parametreleri ve toluidin mavisi boyası kullanılarak sperm kromatin bütünlüğü üzerindeki etkisini gözlemlemektir. Aynı Holstein boğasından toplam 20 adet donmuş sperma payeti kullanıldı. Dondurulmuş sperma payetlerinin çözündürülmesinde kullanılan 37°C'de 30 saniyelik çözündürme protokolü yavaş çözündürme grubumuzu (n=10) oluştururken, 70°C'de 6 saniyelik çözündürme protokolü hızlı çözündürme grubumuzu (n=10) oluşturmaktadır. Tüm çözündürülmüş spermaların motilitesi, canlılığı, morfolojisi, plazma membran bütünlüğü ve sperm kromatin yoğunlaşma parametrelerinin analizleri yapıldı. Karşılaştırılan iki grup arasında spermatozoon plazma membran bütünlüğü, baş ve toplam anormal spermatozoon morfolojisinde önemli bir fark gözlemlendi (P<0.05). Mevcut literatür verileri doğrultusunda TB boyaması ile saptanan kromatin decondenzasyonu yavaş çözündürülen spermalarda hızlı çözündürülen spermalara göre birbirinden önemli ölçüde farklılık göstermiştir (P<0.05). Kromatin yoğunlaşma hızı açısından yapılan değerlendirmelere göre yavaş çözündürme ile elde edilen oran (7±0.39) hızlı çözülme hızına (3.3±0.33) göre iki kata kadar artış göstermektedir (P<0.05). Böylelikle TB boyama prosedürü, özellikle şüpheli ve hızlı değerlendirme gerektiren durumlarda infertilite ve kromatin bütünlüğünü değerlendirmek için kullanılabilir.

**Anahtar sözcükler:** Tamsal test, Sperm kromatin, Sperm morfolojisi, Toluidin mavisi

## INTRODUCTION

The use of frozen-thawed sperm to improve bovine population genetics is widely accepted and used world-

wide<sup>[1]</sup>. Sperm cryopreservation is an effective method for managing and preserving male fertility in animals<sup>[2]</sup>. It is also important for livestock production because it facilitates the storage and transport of germplasm, reducing

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the spread of genetic diversity and increasing the global distribution of genetically superior animals [3]. In terms of spermatozoa survival, the thawing of sperm is as important as the freezing procedure [4]. The thawing rate significantly affects the survival of spermatozoa, and it is well known that the appropriate thawing rate is affected by many factors of cryopreservation procedures, including diluent type, glycerol concentration, and freezing speed [5]. Many studies have been conducted to determine the best thawing temperature and time to obtain the highest percentage of viable sperm after thawing [6,7].

Regardless of extender type and cooling rate, it is recommended to thaw frozen semen in a water bath at 33-35°C for 30-40 sec (slow thawing) in laboratory and farm applications [8]. However, many studies have shown that faster thawing at 60-80°C increases post-thaw motility and preserves acrosome integrity [4,5,9]. Rapid thawing of sperm reduces the harmful effects of recrystallization and hydration, preventing damage to the spermatozoon membrane and cytoplasm. As a result, the thawing rate and temperature of the sperm are critical for improving post-thaw parameters [10]. Motility, concentration, morphology, viability, and membrane function are the spermatological parameters that are routinely tested after thawing frozen sperm. However, DNA analysis has recently gained prominence in routine sperm examination [11]. The nucleus of a spermatozoon has a highly condensed chromatin formed by combining double-stranded DNA with proteins known as protamines and histones [1]. Protamine is an essential protein that replaces histones in the nucleus of mature spermatozoa. Protamine binds to multiple disulphide bonds, allowing chromatin to compact and crystallize [12]. Apoptosis, reactive oxygen species (ROS), and protamine deficiency, on the other hand, cause poor chromatin condensation. It causes spermatozoon DNA damage, infertility, and poor results in assisted reproductive techniques in this case [13].

In the last 20 years, many new techniques have been developed to evaluate sperm chromatin condensation. In the evaluation of sperm chromatin integrity, toluidine blue (TB) [14], Acridine orange [15], aniline blue [16], chromomycin A3 (CMA3) staining, and COMET test, TUNEL test, sperm chromatin structure assay (SCSA), sperm chromatin dispersion test (SCD) [17], DNA breakage detection-fluorescence *in situ* hybridization test (DBD-FISH) are used [18]. However, many of these techniques necessitate the use of equipment that is not readily available in laboratories or animal breeding facilities. In addition to these expensive and more infrastructure-requiring tests, TB is preferable because it is cheaper than the others. Toluidine blue is fast, simple, and inexpensive compared to all these methods [12,14]. Toluidine blue is a basic thiazine metachromatic dye that specifically binds

to acidic cellular components of the tissue. It shows a high affinity for binding to the phosphate residue of immature spermatozoon DNA. The results of sperm chromatin analysis with toluidine blue are also correlated with other methods, indicating that its use is appropriate [19]. For these reasons, our study aimed to show that in the evaluation of thawed bull sperm at different temperatures, in addition to routine semen parameters, DNA damage can be detected by using a staining method (TB), which is easier to find and gives faster results.

## MATERIAL AND METHODS

### Ethical Statement

Ondokuz Mayıs University Animal Experiments Local Ethics Committee provided an ethics report for this study (E-68489742-602.99-104799).

### Sperm Samples and Experimental Design

In the study, a total of 20 frozen sperm straws from the semen of the same Holstein bull were used in order to eliminate the changes that may arise from individual differences. While the 30-sec thawing protocol at 37°C, which is used as the gold standard for thawing frozen sperm straws, constitutes our control group (n=10), the 6 sec thawing protocol at 70°C constitutes our other group (n=10). All thawed sperm were tested for motility, viability, morphology, membrane integrity, and sperm chromatin condensation parameters.

### Sperm Motility

The Computer-Aided Sperm Analyzer (CASA), (SCA®, Microptic, Barcelona, Spain) was used to assess frozen-thawed sperm motility and movement characteristics. Total motility (0-100%), progressive motility (0-100%), VAP (mean path velocity,  $\mu\text{m/s}$ ), VSL (straight-line velocity,  $\mu\text{m/s}$ ), VCL (curvilinear velocity,  $\mu\text{m/s}$ ) and ALH (lateral head change, m), BCF (Crossover frequency rhythm Hertz (Hz) values were measured and recorded in at least 5 microscope fields in the software system.

### Sperm Morphology

Sperm morphology was determined in accordance with the protocol of the SpermBlue® test kit (Microptic, Spain). Ten  $\mu\text{L}$  of the sperm sample was taken and smeared on the slide and left to dry at a 45-degree angle. After drying, the slide was dipped in the jar containing SpermBlue staining. The slide was kept in the dye for 2 min. Then the slide was left to dry at 60-80 degrees. After the drying process, the slide immersed twice into the jar containing distilled water was slowly removed, and the slide was allowed to dry. Following the staining procedure, at least 100 spermatozoa were tested in the CASA system. Morphological disorders of the head, acrosome, middle and tail regions of spermatozoa were evaluated.

## Sperm Viability

According to Gilmore et al.<sup>[20]</sup>, the eosin-nigrosin stain was used to test sperm viability. The slides were stained according to the protocols, dried, and coated with a cover slide before being examined with CASA at 60x magnification. The rates (percentages) of live (white sperm heads) and dead (pink sperm heads) sperm were determined by counting 200 spermatozoa per stained slide.

## Hypoosmotic Swelling Test

The HOS test was used to evaluate the functional membrane integrity of sperm. One mL of the HOST solution (7.35 g sodium citrate and 13.51 g fructose per 1:1, v/v of distilled water) was collected and placed in an eppendorf tube at 37°C<sup>[21]</sup>. It was incubated at 37°C for 30 min after adding 10 µL of the semen sample to the HOST solution. After incubating the mixture, one drop was placed on the slide and a smear was obtained. After drying the slide at a 45-degree angle, this slide was examined at 40x magnification under the microscope, and 200 sperm per slide were counted. The percentage of HOS-positive sperm was calculated with those with a coiling tail.

## Determination of Sperm Chromatin Condensation by TB Staining

According to the study by Beletti and Mello<sup>[22]</sup>, sperm samples were thawed in two ways before being subjected to smear preparation. The smears were first fixed in ethanol acetic acid (3:1, v/v) for 1 min before being fixed in 70%

ethanol (3 min). The smears were then hydrolyzed for 25 min in 4N hydrochloric acid, washed in distilled water and dried at room temperature. The slides were stained with a droplet of TB 0.025% (w/v) in a sodium citric acid-phosphate buffer (McIlvaine buffer) produced at pH 4.0 and then covered with a coverslip. After 3 min, the images were captured using a light microscope at a magnification of 100x (Nikon, Eclipse, Tokyo, Japan) and CASA. A total of 100 spermatozoon were counted in different areas of each slide using oil immersion and 100x magnification under light microscopic examination. Sperm cell heads with good chromatin integrity were light blue, while those with poor chromatin integrity were deep violet (purple). Deep violet sperms were considered abnormal, and the percentage of sperms with this color was calculated.

## Statistical Analyses

Ten semen straws (n=10) were used for each group. Mann Whitney U was used for mean comparisons. The SPSS software (Version 21, SPSS, IBM) was used for all statistical analyses, and differences were considered significant at the P<0.05 level. The results are shown as the Mean±SE.

## RESULTS

### Sperm Characteristics

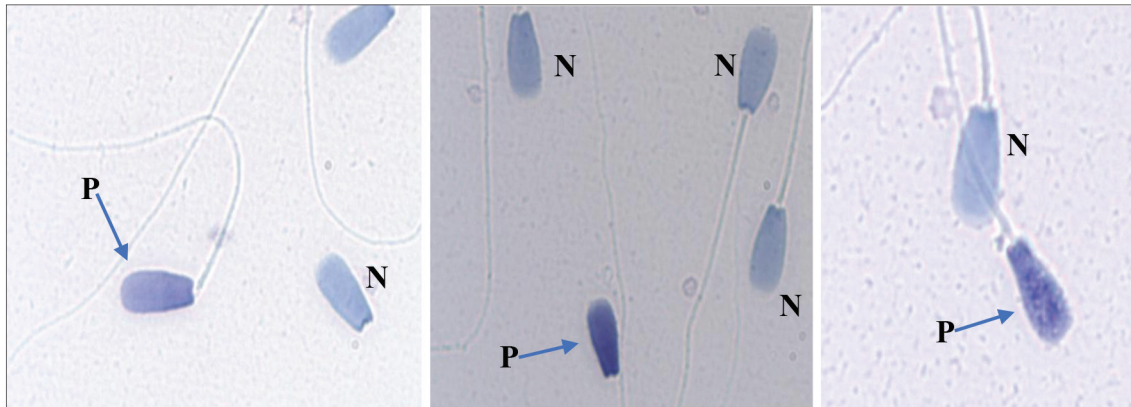
Values of sperm characteristics (motility, progressive motility, kinematic parameters, viability, membrane function, and morphology) can be observed in *Table 1*. There was a significant difference (P<0.05) in sperm plasma

<i>Table 1. Mean and standard error for the post-thaw sperm analysis and the probability values in the slow thawing and fast thawing groups</i>		
Variable	Slow Thawing (n=10)	Fast Thawing (n=10)
Total sperm motility (%)	63.62±3.70	63.49±1.67
Sperm progressive motility (%)	49.68±3.63	48.37±1.35
Sperm curvilinear velocity (µm/s)	111.28±3.37	112.57±2.99
Sperm velocity average pathway (µm/s)	61.91±2.24	58.45±1.86
Sperm velocity straight line (µm/s)	46.17±2.21	42.55±2.03
Sperm straightness (%)	68.09±1.39	66.60±1.55
Sperm linearity (%)	40.42±1.51	36.58±1.89
Wobble (%)	55.82±1.34	52.21±1.64
Amplitude of lateral head displacement (µm)	4.32±0.19	4.63±0.20
Sperm beat cross-frequency (Hz)	9.75±0.38	9.02±0.42
Viability (%)	66.10±2.54	71.40±0.77
Membrane Integrity (%)	63.20±2.78*	74.10±1.71'
Acrosomal defect (%)	1.50±0.26	1.90±0.37
Head defect (%)	5.40±0.30*	3.40±0.37'
Mid-piece Defect (%)	2.50±0.47	2.10±0.23
Tail defect (%)	6.20±0.48	3.70±0.39
Total abnormal sperm morphology (%)	15.60±0.61	11.10±0.40

\* Superscripts within the same line differ significantly at P<0.05

Table 2. Percentages of chromatin alterations identified by toluidine blue in bull sperm from different thawed (Mean±SE)		
Variable	Slow Thawing (n= 10)	Fast Thawing (n= 10)
Normal chromatin condensation	93.0±0.39 <sup>b</sup>	96.7±0.33 <sup>a</sup>
Chromatin decondensation	7.0±0.39 <sup>b</sup>	3.3±0.33 <sup>a</sup>

<sup>a,b</sup> Same letter in the same row indicate that significance ( $P < 0.05$ )



**Fig 1.** Sperm chromatin structure assessed by toluidine blue staining in bull semen. Sperm cell heads with good chromatin structure were light blue (N); those of abnormal chromatin structure were deep violet (P). The photographs were obtained with an image analyzer using the CASA system

membrane integrity, head defect, and total abnormal sperm morphology (Table 1). However, no difference was found in sperm motility, progressive motility, sperm kinematic parameters, viability, tail defect, sperm acrosome defect, and sperm mid-piece defect between the samples for the slow thawing (37°C for 30 sec) and fast thawing (70°C for 6 sec) (Table 1) ( $P > 0.05$ ).

### Changes in Chromatin Condensation

The percentages of abnormal sperm chromatin structure and condensation were compared between the two thawing protocols for sperm. Significant differences ( $P < 0.05$ ) were observed for the TB patterns between the samples stained for slow thawing (37°C for 30 sec) and fast thawing (70°C for 6 sec) when the two thawing protocols were compared (Table 2). The TB staining patterns observed in bull semen were as follows: light blue (TB negative, normal chromatin condensation), and dark blue-violet (TB positive, a high degree of chromatin decondensation; Fig. 1). The rate obtained in slow thawing ( $7 \pm 0.39$ ) is up to two times higher than the rate obtained in fast thawing ( $3.3 \pm 0.33$ ) ( $P < 0.05$ ), according to the evaluations made in terms of chromatin decondensation rate. Rapid thawing, according to the literature, produces a successful outcome in this situation. It is critical to use TB staining to clearly show these changes in sperm chromatin condensation and decondensation.

## DISCUSSION

In this study, besides the analysis of routine semen para-

eters of frozen-thawed bull semen, the effectiveness and success of the Toluidine Blue staining procedure, which is an inexpensive and easily applicable staining method and used to determine the condensation/decondensation change of sperm chromatin, was determined. According to the findings obtained from the study data, membrane integrity, head defect, tail defect, total abnormal sperm morphology, TB negative, and TB positive were statistically significantly different after thawing at 70°C for 6 sec compared with 37°C for 30 sec in cryopreserved bull semen.

Rapid thawing of sperm reduces the negative effects of recrystallization and hydration, preventing damage to the sperm membrane and cytoplasm [23]. Increased thawing rate has been shown to reduce intracellular ice recrystallization, which can result in the formation of larger and more stable ice crystals that can damage mitochondria. However, it should be noted that, while temperatures above 35°C appear to have more positive effects, the thawing time should be shortened and carefully timed. Because protein denaturation causes spermatozoa to lose vitality when exposed to high temperatures for an extended period of time [4]. The plasma membrane is the principal location of freezing injury in spermatozoa and is critical for freeze-thaw survival [24]. HOST has been shown to be effective in detecting minor changes in bull sperm membranes [25]. Membrane proteins denature during the thawing process due to osmotic and mechanical stress, causing membrane integrity to be disrupted. Rapid thawing, according to Mazur [23] and Holt [26], prevents water molecules from recrystallizing, which may be harmful to

cell membranes. The increased osmotic pressure changes are the main disadvantage of the slow thawing method [27]. According to Foote [28], the thawing process must be completed quickly to limit the harm caused by changes in ice crystals during the unfreezing of the sperm and preserve membrane integrity and potential fertility to the greatest extent possible. Confirming this information, our study found that rapid thawing at 70°C resulted in significant reductions in membrane integrity ( $74.10 \pm 1.71$  versus  $63.20 \pm 2.78$ ) and sperm abnormal morphology ( $11.10 \pm 0.40$  versus  $15.60 \pm 0.61$ ), particularly in the rate of chromatin decondensation ( $7 \pm 0.39$  versus  $3.3 \pm 0.33$ ). While previous studies [6,29] found that temperatures above 35°C result in higher motility values, our results revealed no statistically significant difference between motility parameters. This could be due to differences in the composition of the extenders used, changes in the freezing procedure, and, most importantly, differences depending on the semen analysis system used. Our findings show that when the straws were thawed at 70°C for 6 sec, total abnormal sperm morphology, as well as other head defect, were significantly lower than when they were thawed at 37°C for 30 sec. When the methods used and the results obtained are compared, our findings are consistent with those of Nur et al. [4] and Yilmaz et al. [30]. The cause of tail-related abnormalities in spermatozoa is mainly unknown [31]. According to research, coiled and coiled tailed spermatozoa significantly reduce ejaculate motility and may lower male fertility potential [32].

The critical temperature range for thawing sperm is between -60°C and -10°C, and the temperature should be changed as soon as possible during freezing and thawing [30]. Rapid thawing of semen was found to be beneficial in preventing harm during rewarming by Vishwanath and Shannon [33]. It is well known that the cold shock that occurs between these temperatures increases morphological defects [6]. The temperature of the straw in a 37°C water bath reaches 0°C in the first 5 sec and 30°C in the 15<sup>th</sup> sec, but at 70°C, these temperatures are reached in a fraction of the time. While the short transition time minimizes the rate of morphological defects, the exposure period to temperatures must also be attending. If the temperature within the straw rises above 41°C, the spermatozoa will be permanently damaged [30].

The integrity of the paternal genome is linked to reproductive success, which includes fertilization and embryonic development. As a result, research that identifies the chromatin compaction pattern throughout the reproductive tract, as well as its implications for possible sperm aberrations, is critical for understanding the mechanisms underlying male fertility [34]. Several factors can produce chromatin abnormalities in sperm: disturbances during histone to protamine exchange, a lack

of protamines, disturbances at the level of sperm maturation in the epididymis, or maintenance of chromatin stability during ejaculation [35]. Environmental factors such as increased body temperature, toxic chemicals, components of the extender in which semen is stored, storage conditions are known to cause sperm chromatin damage/abnormal structure [36]. For this reason, several methodologies have been used to study abnormal chromatin forms throughout the years [37]. The purpose of this study was to put to the test a TB staining that was simple, inexpensive, and reliable for determining how two different thawing temperatures affect sperm chromatin condensation in the bull. Souza et al. [15] investigated various chromatin assessment methods (TB, 6-diamidino-2-phenylindole (DAPI) and anti-protamine 1 antibody (anti-PR1)). Other methods allow us to see the areas where sperm chromatin condensation occurs in greater detail, but TB cannot distinguish between different types of chromatin changes. Indeed, DAPI and anti-PR1 are not routine procedures because they require immunocytochemistry and fluorescence microscopy. However, the condensation of sperm chromatin as positive and negative in sperm samples could still be detected. Despite being consistent with our findings, we were able to identify positive and negative sperm chromatin condensation differences when comparing two different thawing methods.

Banaszewska et al. [38] compared Acridine orange (AO), Aniline blue (AB) and chromomycin (CMA3) stains to evaluate sperm quality in terms of chromatin abnormalities in the sperm nucleus. Staining with AO identifies the abnormal, single-stranded DNA structure in the sperm cell. The use of AB enables the identification of abnormal histone retention, while CMA3 identifies sperm cells with protamination disorders. TB was not included in the study. However, the examination of nuclear proteins in terms of infertility shows the importance of normal chromatin structure on the functioning of sperm cells [38].

In a study on human sperm, a negative correlation was found between sperm chromatin integrity with TB and AB staining and sperm count, normal morphology, and cut-off value in progressive motility and specificity. AB and TB staining sensitivity were accepted as an indicator of male infertility. Therefore, AB and TB staining has been reported to be helpful for the assessment of male fertility potential. In addition, the study shows a negative relationship between the lack of chromatin material and the fertility potential of spermatozoa [39]. Chromatin integrity assessment using AB and TB is a reliable indicator of pregnancy [38].

Erenpreiss et al. [19] compared toluidine blue (TB), sperm chromatin structure test (SCSA) and Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL) test in a study evaluating sperm DNA integrity.

They reported that the proportion of sperm cells with abnormal DNA integrity obtained from the TB test was strongly correlated with the proportion of abnormal cells detected by the SCSA and TUNEL. They also stated that while AO-based assays (SCSA) are less sensitive to DNA-protein interactions due to limited external staining, TB is sensitive to both DNA strand breaks and chromatin packaging changes. TB has also been evaluated as a cheaper and easier to administer test than the more popular SCSA (sperm chromatin structure test) and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling) tests <sup>[40]</sup>.

Evaluating the presence of chromatin damage in a quick and low-cost manner can make significant contributions to the relevant field, particularly in laboratories that perform routine sperm analysis. Besides, the TB procedures provided have the advantage of not requiring expensive computer-assisted sperm analysis systems, which are not available at most insemination stations or reproduction laboratories <sup>[38]</sup>.

The degree of staining of spermatozoa ranges from light blue to dark blue, and the differentially stained sperm population is thought to represent chromatin changes and to show that this method is capable of identifying alterations in the DNA-protein complex caused by heat exchange in bull spermatozoa <sup>[22]</sup>. The thawing rate influences sperm chromatin condensation rate and previous studies have demonstrated that increasing the thawing rate increases sperm chromatin condensation <sup>[14]</sup>. Furthermore, sperm DNA is compacted during spermatogenesis by replacing histones with protamines <sup>[41]</sup>. Because chromatin is an important component of the sperm head, changes in its compaction process may affect the morphometric features of the sperm head <sup>[42]</sup>. In bulls, there has previously been reported a relationship between sperm head morphometry and chromatin <sup>[14]</sup>. The results of our study's 37°C thawing group are consistent with these findings, and there is a positive relationship between head disorders and primary chromatin decondensation.

In conclusion, increasing the temperature from 37°C to 70°C resulted in a decrease in sperm chromatin condensation, consistent with previous studies. Improved sperm chromatin condensation can be attributed to thawing at 70°C, which preserves the chromatin structure better than thawing at 37°C. In addition, sperm chromatin condensation test was found to be highly correlated with sperm morphology. If CASA and morphology assessments are routinely used to evaluate stud bulls with suspected infertility/subfertility, additional TB and chromatin damage may be determined quickly and inexpensively.

#### AVAILABILITY OF DATA AND MATERIALS

The datasets and analyzed during the current study

available from the corresponding author (M. Çevik) on reasonable request.

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#### CONFLICT OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

#### CONTRIBUTIONS OF AUTHORS

BE, MA, MDT, CK and MÇ are listed according to the determination of the subject, experimental design and writing stages, their contribution rates to laboratory studies and literature review, and corrections. All authors contributed to the revision and final proofreading of the article.

#### ETHICAL STATEMENT

The Local Animal Ethics Committee provided an ethics report for this study (E-68489742-602.99-104799).

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