

Investigation of the Effects of Acupuncture Stimulation on the Size and Blood Flow of Corpus Luteum and Progesterone Levels in Dairy Cows ^{[1][2]}

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Abstract

In this study, effect of acupuncture (AP) on the luteal size (LS), luteal blood flow (LBF) and progesterone (P₄) levels were investigated in the presence of corpus luteum (CL) in cows. Seven days after 14-days interval PGF_{2α} estrus synchronization protocol, CL positive animals were assigned either to a control group (AP-, n=10) or to an AP group (AP+, n=10) stimulated by using B22 and B23 sensitive acupoints. LS and LBF examinations were carried out before the stimulation (0h) and at 1st, 3rd, 6th hour on d7 and thereafter on d9, d10, d11, d12 and d13 following the AP stimulation in each group with a portable color Doppler ultrasonography. Blood samples for P₄ measurement were collected during each examination. There was no significant difference in LS, LBF, or P₄ mean values between groups. However, LBF significantly increased at 6h after stimulation (P<0.05) in AP+ group but it increased at d11 in AP- group (P<0.05). The significant increase in LS was observed earlier in AP+ group (on d9; P<0.01) than AP- (on d11; P<0.05). Serum P₄ concentrations increased at 3h, d9 and d10 in AP+ group (P<0.05), however a significant difference was only observed at 3h in AP- group (P<0.05). In conclusion, AP stimulation induces earlier increases in LS, LBF and P₄ parameters in cows during luteal phase.

Keywords: Acupuncture, Cow, Luteal size, Luteal blood flow, Progesterone

Sütçü İneklerde Akupunktur Stimülasyonlarının Korpus Luteum Büyüklüğü, Kan Akımı ve Progesteron Değerleri Üzerine Etkilerinin İncelenmesi

Özet

Bu çalışmada akupunkturun (AP) ineklerde korpus luteum (CL) varlığında luteal büyüklük (LS), luteal kan akımı (LBF) ve progesteron (P₄) seviyeleri üzerindeki etkisi incelendi. 14 gün aralıklı PGF_{2α} östrus senkronizasyon protokolünden 7 gün sonra, CL tespit edilen hayvanlar kontrol (AP-, n=10) ve duyarlı B22 ve B23 kullanılarak stimüle edilen akupunktur gruplarına ayrıldı (AP+, n=10). LS ve LBF incelemeleri taşınabilir bir renkli Doppler ultrasonografi ile 7. günde stimülasyon öncesi (0. saat), izleyen 1, 3, 6. saatler ve 9, 10, 11, 12 ve 13. günlerde yapıldı. Her muayenede P₄ ölçümleri için kan örnekleri alındı. Gruplar arasında LS, LBF ve P₄ ortalama değerleri farklı değildi. Bununla birlikte, LBF AP+ grubunda stimülasyon sonrası 6. saatte (P<0.05) ancak AP- grubunda 11. günde arttı (P<0.05). LS'deki önemli artış ise AP+ grubunda (9. gün; P<0.01), AP- grubuna göre (11. gün; P<0.05) daha erken gözlemlendi. Serum P₄ konsantrasyonları AP+ grubunda 3. saat, 9. gün ve 10. günde artış gösterdi (P<0.05). Ancak AP- grubunda sadece 3. saatte önemli değişim gözlemlendi (P<0.05). Sonuç olarak, luteal faz sırasında akupunktur stimülasyonu LS, LBF ve P₄ değerlerinde daha erken artışa neden olmaktadır.

Anahtar sözcükler: Akupunktur, İnek, Luteal büyüklük, Luteal kan akımı, Progesteron



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INTRODUCTION

Use of AP for treatment of various illnesses, which is an important part of Traditional Chinese Medicine (TCM), was started around 2200-3000 BC ^[1]. This method became popular in the 1970's, and in 1974, International Veterinary Acupuncture Society (IVAS) was found. Furthermore, in 1979 a symposium was held in Beijing by World Health Organization (WHO) ^[2,3]. The principle of AP was initially based on the balance of Ying and Yang in TCM but today it is mostly thought as sympathetic-parasympathetic interaction ^[4]. According to this method there are meridian systems transporting the life energy which is thought to be regulating the body functions by connecting and communicating specific organs in the body and these meridians intersect in several points on the skin ^[5]. Most diseases and injuries are caused by problems in the flow and balance of the life energy. Therefore these disorders can be influenced by treating AP points. It is well known, that there are lots of neuroreceptors and nerve endings transmitting information, such as pain, heat or pressure, near or close to AP points ^[1,3,6]. By stimulating these points some alterations may occur in the related organs, which may bring out the activation of the natural healing process. These points are named as AP points and anatomical locations of these acupoints have been determined topographically ^[6].

AP can be utilized in large animal medicine both as therapeutics and as a diagnostic tool ^[7]. Specific AP points become sensitive (Ah-Shi points) or painful in case of certain pathological conditions. Applying pressure to these points and eliciting a painful or sensitive response may help to pinpoint areas of concern or disease. The techniques used include manual needle stimulation, electroacupuncture (EAP), moxibustion, injection-AP (liquid needle technique: aquapuncture), suture embedding and laser stimulation ^[8,9]. B meridian is the most used one of Bladder (B), Gallbladder (Gb) and Spleen (SP) meridians, which is used, in veterinary reproduction ^[10,11].

Effect of AP on fertility is explained with three different mechanisms: 1) AP may mediate the release of neurotransmitters to stimulate the secretion of the gonadotropin-releasing hormone (GnRH) 2) AP may stimulate blood flow to the uterus by inhibiting uterine central sympathetic nerve activity and 3) AP may stimulate the production of endogenous opioids, which may inhibit the central nervous system outflow and the biological stress response ^[12]. Thus this method has been used in treating different reproductive disorders such as; pseudopregnancy, silent heat, repeat breeder syndrome, cystic ovaries, uterine prolapse, retained fetal membranes ^[13], polycystic ovaries ^[14,15], induction of cyclicity in anestrus mares ^[16], induction of ovulation ^[17], and increasing the blood flow to uterus and ovaries ^[18].

AP stimulation of certain points historically associated with reproduction significantly alters plasma levels of sex hormones, such as Luteinizing hormone (LH), Follicle-stimulating hormone (FSH), estradiol (E_2) and progesterone (P_4) ^[19-21]. Luteal vascularization is the main factor in the formation and maintenance of pregnancy by the secretion of P_4 . Moreover, there is an intensive molecular process happening during the formation and maintenance of luteal vascularization ^[22] and this process can be observed by colour Doppler ultrasonography that is a non-invasive tool ^[23]. Growth, development, and structural and functional maturity of the CL is completed in the middle of the luteal stage ^[24]. There have been many studies investigating the effects of $PGF_{2\alpha}$ ^[25], GnRH, human chorionic gonadotropin (hCG) ^[26], oxytocin ^[27], estradiol 17β ^[28] and β carotene ^[29] on the blood flow of CL due to the importance of the maintenance of vascularization of this ovarian structure. However, to the best of our knowledge, during the literature search, we did not find any previous study regarding the induction of blood flow of CL with AP stimulation in cows. Hence, this study was designed to investigate the effect of AP stimulation on LBF, LS and the hormone profile during luteal phase in cows. It is aimed to present how AP stimulations form changes in LBF, LS and P_4 according to the AP- group.

MATERIAL and METHODS

This study was conducted on 20 Holstein Friesian cows in good general condition with no reproductive disorders in 50-70 days in milk (DIM), fed with TMR and water provided *ad libitum*. This study was carried out in a dairy herd located in Eregli/KONYA (TR) between November 2012 and November 2013. All cows in the study were synchronized by double injection of $PGF_{2\alpha}$ (Dinoprost, 25 mg/cow, i.m.; Enzaprost[®], CEVA; Turkey) at 14 days interval. Seven days after second $PGF_{2\alpha}$ injection transrectal ultrasonography was performed to confirm the presence of the CL and animals having CL were assigned to either control group (AP-, n=10) or to AP group (AP+, n=10) and were stimulated by using B22 and B23 sensitive acupoints described by Kothbauer ^[9], stainless steel AP needles (Richter Pharma, Wels, Austria) which have silver rounded section at the tip with a 40 mm length were inserted 2 cm caudally at one hand lateral of the median dorsal line in right and left side of the cows between the transversal processus of vertebra lumbalis I-II and II-III, respectively for stimulation of acupoints. In 15 min time period needles were rotated four times around itself without penetrating or withdrawing them. In AP+ group, measurement of LS and LBF were done immediately before the stimulation at hour (h) 0 and at h1, h3 and h6 after the stimulation on day (d) 7 and also on d9, d10, d11, d12, and d13 after the second $PGF_{2\alpha}$ injection using a portable colour Doppler ultrasonography device equipped with a 10 MHz linear transducer. In AP-

group, no stimulation was applied. All other examination procedures were done same as AP+ group.

Ultrasonographic examinations and image collection were performed as described previously [26,29-31]. CL examination and measurement of the LS and LBF were done by transrectal ultrasonography using a portable colour Doppler device (LOGIQ Book XP, General Electric Healthcare, Solingen, Germany) equipped with a 10 MHz linear probe. During each ultrasonographic examination, the maximum cross-sectional area of CL was identified and stored for further off-line measurements. After morphological evaluation, the power mode was activated and LBF mapping in various transverse sections was conducted. To minimize the variations in recording, the settings of the Power Doppler system were fixed and the same was used for all examinations. The entire cross-sectional area of the CL was visible within the Power Doppler sample box. After recording at least six images, six images without flash artefacts and with the maximum number of coloured areas were stored. These images were exported in DICOM format into a computer. CL was measured on B-mode images using a computer-assisted image analysis program (Pixelflux, version 1.0; Chameleon-Software, Leipzig, Germany), and the mean value of six pictures was determined. The same software was also used to assess the total area of colour pixels within the luteal tissue. For this purpose the whole luteal structure and its blood flow area were chosen as the region of interest (ROI) and the coloured area within this ROI was calculated. The averages from four of the six images were used for further evaluation of LBF.

Blood samples for P₄ measurement were collected via coccygeal vein immediately before each examination. Serum samples were stored at -20°C until analysis. Serum P₄ concentrations were measured by Electrochemiluminescence immunoassay method in an accredited laboratory (Düzen Laboratory Group, Ankara, Turkey). Method was performed by autoanalysator (Cobas E601 modular device, Roche Diagnostics GmbH, Mannheim) with the described procedure of the manufacturer. The intra- and inter-assay coefficients of variation averaged 1.4 and 2.9%.

Data were executed by Post hoc procedure in SAS (2014) for distribution of data. Student t test were used

to compare of independent groups with two-tailed distribution. Rank correlation coefficients were executed by Spearman correlation procedure. General linear Model methods with adapted in repeated ANCOVA procedure was using to compare to groups and to investigate for changing data over the exact time (independent regressors in groups). Data were summarized with group of the means and their standard error of means. All calculations and analysis was executed by SAS (2014).

RESULTS

No side effects were observed during the study period in the cows due to the stimulations. LS increased from 3.5 cm² to 4.6 cm² and from 4.4 cm² to 5.4 cm², respectively in AP+ and AP- groups between d0 and d13. When intergroup comparisons were evaluated after the stimulations, no significant difference was found in LS (P>0.05, Table 1). Increase in LS was observed in both groups after the stimulations but significant increase in LS according to h0 was observed earlier in AP+ group (on d9; P<0.01) compared with AP- (on d11; P<0.05) (Table 1).

When AP+ and AP- groups were compared with each other, no significant difference was found in LBF at examination time points (P>0.05). Whereas, the increases in LBF within the different time points were significantly different in each group. In AP+ group, LBF significantly increased at 6h after stimulation (P<0.05). However, LBF remained bumpy and reached its minimal levels at d9 (5.3±0.4) and increased again to the starting levels at d11 in AP- group (P<0.05) (Table 2).

Serum P₄ levels were not statistically different between AP+ and AP- animals both in h0 and also at all examination time points after stimulations (P>0.05). On the other hand serum P₄ concentrations significantly increased at 3h, d9 and d10 in AP+ group (P<0.05), however a significant difference was only observed at 3h in AP- group (Fig. 1).

Correlations between LS and LBF (min: 0.379-max: 0.589, P<0.001) were found in all time points except d11 in AP+ cows. Similarly positive correlations between LS and LBF (min: 0.323-max: 0.594, P<0.001) were found in AP- too (Table 3).

Table 1. LS (cm²) values after the application of AP

Tablo 1. Akupunktur uygulaması sonrası luteal büyüklük (cm²) değerleri

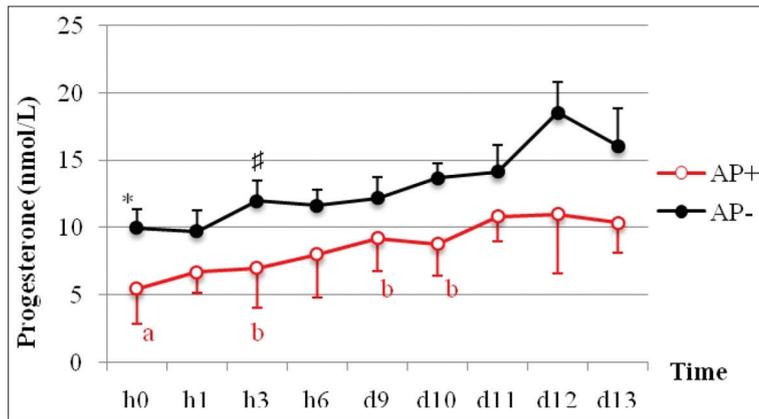
Group	Days									P
	7				9	10	11	12	13	
	h0	h1	h3	h6						
AP+	3.5±0.2 ^a	3.3±0.3	3.7±0.2	3.7±0.2	4.1±0.2 ^b	4.2±0.2	4.4±0.2	4.5±0.2	4.6±0.2	<0.01
AP-	4.4±0.2 [*]	4.6±0.2	4.4±0.2	4.7±0.2	4.5±0.2	5.1±0.2	5.4±0.1 ^a	5.2±0.2	5.4±0.2	<0.05
P	ns	ns	ns	ns	ns	ns	ns	ns	ns	

ns: non-significant (P>0.05), Values with different superscripts (a,b) and asterisk (*, #) in the same line are statistically different

Table 2. LBF (mm²) values after the application of AP**Tablo 2.** Akupunktur uygulaması sonrası luteal kan akımı (mm²) değerleri

Group	Days									P
	7				9	10	11	12	13	
	h0	h1	h3	h6						
AP+	5.7±0.7 ^a	5.9±0.5	6.9±0.5	7.0±0.5 ^b	7.2±0.5	6.5±0.4	7.3±0.4	5.9±0.4	7.8±0.6	<0.05
AP-	7.4±0.7 [*]	6.3±0.6	7.9±0.7	6.9±0.6	5.3±0.4 [#]	7.1±0.5	7.3±0.6	6.7±0.7	7.3±0.6	<0.05
P	ns	ns	ns	ns	ns	ns	ns	ns	ns	

ns: non-significant ($P>0.05$), Values with different superscripts (^{a,b}) and asterisk (^{*},[#]) in the same line are statistically different

**Fig 1.** Mean P₄ (nmol/L) concentrations

Values with different superscripts (^{a,b}) and asterisk (^{*},[#]) are statistically different ($P<0.05$)

Şekil 1. Ortalama P₄ (nmol/L) konsantrasyonları

Farklı harf (^{a,b}) ve (^{*},[#]) işaretleri istatistik olarak farklıdır ($P<0.05$)

Table 3. Correlations between LBF, LS and P₄ levels**Tablo 3.** LBF, LS ve P₄ seviyeleri arasındaki korelasyonlar

r _s	Group	Days								
		7				9	10	11	12	13
		h0	h1	h3	h6					
LBF/LS	AP+	0.435 [*]	0.469 [*]	0.589 [*]	0.423 [*]	0.417 [*]	0.379 [*]	0.061	0.583 [*]	0.542 [*]
	AP-	0.594 [*]	0.459 [*]	0.361 [*]	0.516 [*]	0.349 [*]	0.477 [*]	0.264	0.387 [*]	0.323 [*]
LBF/P ₄	AP+	0.830 [*]	0.333	-0.079	0.127	0.214	0.024	0.617	-0.067	-0.406
	AP-	0.188	-0.535	-0.127	0.345	0.333	0.358	0.224	0.285	-0.126
LS/P ₄	AP+	0.236	0.176	-0.091	0.467	-0.048	0.261	0.617	0.200	0.200
	AP-	0.394	-0.231	-0.418	0.139	0.018	0.406	0.552	0.297	-0.167

* indicates the significance at 0.001 level

DISCUSSION

Fertility/infertility and gynecology are the important topics of the AP that is used for many years in human and veterinary medicine [32]. AP-type stimuli influence reproductive organs and functions, including release of LH, FSH, P₄ and E₂ [33,34]. There are several studies on AP affecting the function of the CL [35,36].

B22 acupoint is used in cases of luteal insufficiencies, ovaritis, and small cystic degenerations of the ovary, anestrus, silent heat and sterility; B23 acupoint is used in cases of ovarian cycle disorders and sterility [9]. These acupoints were chosen and stimulated according to the above-mentioned indications.

As the CL matures, the majority of the steroidogenic cells establish contact with one or more capillaries [37], thus making the CL one of the most highly vascularized organs in the body [38]. Colour Doppler ultrasonography offers a useful, non-invasive approach to evaluate vascular function in the CL, allowing visual observation of local BF within the CL [39].

Maintenance of the pregnancy is dependent on the presence of the CL and continuous P₄ secretion capacity in domestic animals. There are several studies investigating the life cycle, size, blood flow of the CL and correlations between P₄ by using Doppler sonography [31,40,41]. Furthermore, CL grows rapidly during the development phase (ovulation- 5th day), reaches its maximum size in static phase

(5th-16th day) and regresses rapidly during luteolysis (16th-17th day) [40,41]. Life cycle and size of CL can be manipulated by several implementations during these phases. For example Ay et al. [29] reported additional β -carotene injections to the estrus synchronizations increased the size of the CL on day 7. Mann [42] demonstrated an increase in the LS between days 5-8 post ovulation very rapidly, and reported no significant increase after 8th day. In our study, increase in LS according to the stimulation day (h0) occurred earlier in AP+ group (9th day) than AP- group (11th day) and continued until the end of the study.

It is known that CL is one of the most highly vascularized organs in the body [38], therefore maintenance of the luteal function is dependent on the establishment and continuation of angiogenesis and vascularization [22]. LBF is more important than LS in the evaluation of luteal function [23,40]. It was demonstrated that follicular blood flow increased 1 h after hCG administration while it increased 6 h after GnRH injection in a study comparing the effects of GnRH and hCG injections in the presence of ovulatory follicle [26]. By the way, in our study, no significant differences ($P>0.05$) were reported in the LS and LBF between the groups on d9 and d12 after induction of ovulation. This study demonstrates effects of hormonal administrations on blood flow of the ovary in different ways. Although no significant differences were found between AP+ and AP- groups in the investigated parameters on the examination days, increases in these parameters were starting from h6 in AP+ group (except d10 and d12) and on d11 and d13 in AP- group.

Important hemodynamic changes occur during the follicular growth, ovulation and development of CL [22]. Angiogenic and vasoactive factors play vital role in the luteal life cycle in this period. Also it is known that changes in the systemic blood pressure are effective on the ovarian blood flow [43]. AP is effective on the regulation of the systemic blood flow, especially normalizing hypo and hypertension [44]. Studies investigating AP and blood flow of the genital organs were conducted on human or laboratory animals. He et al. [35] demonstrated AP stimulations were increased the ovarian expression of vascular endothelial growth factor (VEGF), VEGF mRNA and luteinizing hormone receptor (LHR) mRNA in rats. Besides EAP increased the blood flow of the target organs in genital tract [18,45].

AP shows this effect by reflecting the ovarian sympathetic nerves, and systemic circulation respectively [46]. Çakmak and Akpınar [18] reported that AP increased the blood flow of the testes in humans. Recurrent EAP stimulation causes increase in the uterine blood flow and it maintains for 10-14 days after the stimulation in women [46]. Although above mentioned studies were carried out in humans and rats they explain how AP stimulations cause earlier increase in LBF in this study. In addition AP stimulations cause not only earlier increase

in LBF than AP- group; they also cause the increase to continue longer. This result supports that AP stimulations are effective on LBF for longer periods.

Angiogenic and vasoactive factors such as VEGF and basic fibroblast growth factor (bFGF) produced by the bovine CL regulates P_4 secretion, cell proliferation and angiogenesis in the developing CL [47]. The findings of our study indicate that AP stimulations can be effective on the activity of the angiogenic and vasoactive factors.

AP applications stimulate several neuropeptides such as serotonin, endogenous opioids and oxytocin in the central nervous system [48,49]. Because pituitary gland is regulated by the hormones originated by the central nervous system, it is thought that AP applications can regulate the endocrine system [49].

Hypothalamic endorphin has a tonic effect on GnRH pulse and the secretion of LH from hypophysis. By this it is in relation with hypothalamo-pituitary-ovarian axis [50,51]. Consequently AP can influence the reproductive hormones and activity by regulating the secretion of β -endorphin effecting the secretion of GnRH and gonadotropins [49].

Studies investigating the relation between AP and reproductive hormones are generally including treatment of reproductive problems or experimental studies. LH, P_4 and prolactin levels are increasing after AP in rats with implantation failure. AP causes this increase by upregulating the expression of the receptors of these hormones [35,52]. Lin and Wu [53] reported pulsatile LH secretion and P_4 levels were increased significantly 4-6 h after AP in gilts with pituitary responsiveness to GnRH. Stimulation of Pai-Hui and Wei-Ken acupoints cause decrease in LH levels within 2 h and increase in P_4 levels within 4-6 h in anestrus sows [21]. Despite no difference was found between the mean P_4 levels in this study conducted on cows, higher levels were achieved until d10 according to h0 in AP stimulated group ($P<0.05$). When increase in P_4 concentrations on d9 and d10 in this study was considered, it could be interpreted that P_4 secretion can be influenced by AP stimulations.

Different correlations between LBF, LS and P_4 were reported in several studies [40,42]. A mild positive correlation was found in between LBF and LS except d11 in this study ($P<0.01$). However, no correlations were found between P_4 and LBF and P_4 and LS.

Studies on AP applications mentioned in this paper were especially carried out in abnormal cases or reproductive disorders. Besides AP whose mechanism of action is still not fully described, is based on the normalization of the defected physiology/function [6,9]. Important points in AP applications are finding the correct acupoint and giving the proper stimulation. This stimulation can be given by electric current, medicine, anesthetic agents or just by

twisting the needles such as in our study. But the frequency and the duration of the stimulation are important too.

In this study, effect of AP stimulations on the function of physiological CL in diestrus cows without reproductive problems was investigated. We could not find any similar study in our literature review. Although no significant difference between AP+ and AP- groups in LS, LBF and P₄ levels were found in this study, we conclude that increasing the frequency and duration of the stimulations may induce significant changes and earlier increases in LBF, LS and P₄ levels by considering the given information in the above mentioned studies. These increases indicate that AP can be effective on the activity of the CL similar to hormones and stimulation of the AP points besides hormonal injections (like GnRH) or injections of the hormones to the selected acupoints may have a positive effect on the life cycle of the CL especially in cows with luteal insufficiency.

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CONFLICTS OF INTEREST

None of the authors have any conflicts of interest to declare.

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