

Comparison of Oxidative/Nitrosative Stress, Leptin and Progesterone Concentrations in Pregnant and Non-pregnant Abaza Goats Synchronized with Controlled Internal Drug Release Application

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Article Code: KVFD-2018-20222 Received: 25.05.2018 Accepted: 28.08.2018 Published Online: 28.08.2018

How to Cite This Article

Kuru M, Öğün M, Kulaksız R, Kükürt A, Oral H: Comparison of oxidative/nitrosative stress, leptin and progesterone concentrations in pregnant and non-pregnant Abaza goats synchronized with controlled internal drug release application. *Kafkas Univ Vet Fak Derg*, 24 (6): 887-892, 2018. DOI: 10.9775/kvfd.2018.20222

Abstract

The aim of this study was to determine the oxidative/nitrosative stress, leptin and progesterone concentrations in pregnant and non-pregnant Abaza goats after synchronization with controlled internal drug release (CIDR) during the breeding season. For this purpose, 40 clinically healthy Abaza goats, aged 2-4 years, were intravaginally exposed to CIDR on day 0, and injected with equine chorionic gonadotropin and prostaglandin F_{2α} on day 9 of the experiment. CIDR was removed on day 11. At the end of the experiment, they were monitored for estrus and exposed to fertile males for mating. Blood samples were collected 8 days before synchronization, then on days 0, 11 of CIDR insertion and on mating day. A pregnancy diagnosis was conducted using transrectal ultrasonography 30 day after mating. The blood serum from 40 goats (30 pregnant + 10 non-pregnant) was used for biochemical analyses. Malondialdehyde (MDA), nitric oxide (NO), total oxidant capacity (TOC), total antioxidant capacity (TAC) and endothelial NO synthase (eNOS) activities were significantly higher on mating day in pregnant goats compared to non-pregnant goats (P<0.05). The eNOS activity and TOC concentrations were significantly higher on day 11 in pregnant goats compared to non-pregnant goats (P<0.001). Serum P4 concentration increased in pregnant group on day 11 and decreased all groups on mating day than day -8, day 0 and day 11 (P<0.001). In conclusion, the administration of CIDR to Abaza goats exacerbated oxidative and nitrosative stress.

Keywords: Abaza goat, Synchronization, eNOS activities, Leptin, Nitric oxide, Progesterone

Controlled Internal Drug Release Uygulaması İle Senkronizasyon Yapılan Gebe ve Gebe Kalmayan Abaza Keçilerinde Oksidatif/Nitrozatif Stres, Leptin ve Progesteron Konsantrasyonlarının Karşılaştırılması

Öz

Bu çalışmanın amacı üreme sezonunda controlled internal drug release (CIDR) ile senkronizasyon sonrasında gebe ve gebe olmayan Abaza keçilerinde oksidatif/nitrosatif stres, leptin ve progesteron konsantrasyonlarının belirlenmesidir. Bu amaçla, klinik olarak sağlıklı 2-4 yaşlı 40 Abaza keçisine 0. gün CIDR intravaginal olarak yerleştirildi ve 9. gün equine chorionic gonadotropin ile prostaglandin F_{2α} enjekte edildi. CIDR 11. gün çıkarıldı. Uygulama sonrasında östrus takibi yapılarak keçiler fertil tekeler ile çiftleştirildi. Senkronizasyona başlamadan 8 gün önce, 0. gün, 11. gün ve çiftleşme günü kan alındı. Çiftleşmeden sonraki 30. gün transrektal ultrasonografi ile gebelik muayenesi yapıldı. Biyokimyasal ölçümler için 40 keçiden (30 gebe + 10 gebe olmayan) elde edilen kan serumu kullanıldı. Gebe olan keçilerle gebe olmayan keçiler karşılaştırıldığında malondialdehid (MDA), nitrik oksit (NO), total oksidan kapasite (TOC), total antioksidan kapasite (TAC) ve endotelial NO sentaz (eNOS) aktiviteleri anlamlı olarak yüksek bulundu (P<0.05). eNOS aktivitesi ve TOC konsantrasyonları gebe olan keçilerde gebe olmayan keçilere göre 11. günde anlamlı olarak daha yüksekti (P<0.001). Serum P4 konsantrasyonu gebe olan grupta 11. günde arttı ve tüm gruplarda çiftleşme günü -8, 0. ve 11. günlere göre azaldı (P<0.001). Sonuç olarak, CIDR uygulaması Abaza keçilerinde oksidatif ve nitrozatif stresi arttırdığı tespit edildi.

Anahtar sözcükler: Abaza keçisi, Senkronizasyon, eNOS aktiviteleri, Leptin, Nitrik oksit, Progesteron



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INTRODUCTION

Vaginal inserts that contain progesterone (P4) [sponge or controlled internal drug release (CIDR)] can be used for estrus synchronization in ruminants [1-4]. These inserts are left in the vagina and can cause tissue damage and inflammation [5]. As a result, such applications can create stress for the animal [6]. A complex relationship has been reported between inflammation and reactive oxygen species (ROS) [7]. Intravaginal inserts in particular are reported to cause oxidative stress in goats [8]. However, there are few studies about the relationship between oxidative stress and CIDR applications [4,8].

Reactive oxygen species (ROS) are eliminated by mechanisms that are known as antioxidants in the organism. Malondialdehyde (MDA), the final product of lipid peroxidation and the most important indicator, is the most important molecule effective in cellular degeneration caused by free radicals [7]. Nitric oxide (NO) plays a role in several physiological events in the body and is produced by nitric oxide synthetase [9]. Endothelium-derived NO is synthesized by eNOS and is an important indicator of basal vascular tonus. Besides protecting vascular integrity and preventing leukocytes from attaching to endothelial cells and the proliferation of smooth muscle cells, endothelium-derived NO also acts to inhibit thrombocyte adhesion and aggregation [10]. A study conducted on dairy heifers reported that NO and MDA levels increased after application of intravaginal inserts and total antioxidant capacity (TAC) was decreased [6].

Leptin plays an important role in reproductive functions and nutritional condition [11,12]. According to Sarraf et al. [13], leptin concentrations and proinflammatory cytokines, such as acute tumor necrosis factor- α and interleukin-1 increased when inflammation is caused by administering substances such as endotoxin or turpentine. However, there is no information about serum leptin concentrations during estrus synchronization using intravaginal devices, especially in goats.

This study aims to determine oxidative and nitrosative stress in pregnant and non-pregnant Abaza goats following estrus synchronization using CIDR during the breeding season and the relationship between oxidative status, progesterone and leptin concentration that may occur after CIDR application.

MATERIAL and METHODS

This study was conducted with the approval of the Ethics Committee of Animal Experiments of Kafkas University, Kars, Turkey (KAÜ-HADYEK - 2016/020).

Location

This study was conducted in Kars province, Turkey. The

research unit is located at 1751 m altitude and 40°34'23"N and 43°02'27"E latitude and longitude, respectively.

Animals and Ration (Diet)

Forty non-lactating Abaza goats aged 2-4 years and weighing 50-60 kg were selected. The animals were fed twice a day with dry clover, dry hay and concentrated feed (12% crude protein, 2600 kcal/kg). Goats were given *ad libitum* access to water.

Estrus Synchronization Protocol

This study began in September (breeding season). The progesterone-releasing device (CIDR, Eazi-Breed CIDR®, Zoetis, Turkey) was inserted into the vagina (day 0) and left there for 11 days. On day 9, all of the goats were injected with 400 IU equine chorionic gonadotropin (i.m., eCG, Chronogest®, MSD-Intervet, Turkey) and prostaglandin F_{2 α} (i.m., 5 mg, dinoprost tromethamine, Dinolytic®, Zoetis, Turkey). The CIDR was removed on day 11, and estrus detection began 12 h later. A buck joined to goat herd every 6 h to test for estrus, and those in estrus were exposed to fertile Abaza bucks for mating. Transrectal ultrasonography with 5-7.5 MHz linear transducer (SonoSite Titan®, SonoSite, USA) was used for pregnancy diagnosis 30 days after mating [14].

Blood Sampling

Blood samples were collected 8 days before the beginning of estrus synchronization protocol (day -8), day 0, day 11 and mating day. The blood was collected from the *vena jugularis* and centrifuged for 15 min (3000 rpm). Serum samples were stored at -20°C until assays were performed.

Biochemical Analysis

Serum MDA concentration was determined using the method described by Yoshioka et al. [15] based on the reaction between MDA and thiobarbituric acid. The optical density was read at 535 nm (Epoch®, Biotek, USA). MDA concentration ($\mu\text{mol/L}$) was calculated from the standard curve obtained using 1,1,3,3-tetraethoxypropane (Sigma).

Nitric oxide measurement was performed according to the method described by Miranda et al. [16], where nitrate is reduced to nitrite by vanadium chloride (VaCl₃), and then in an acidic environment nitrite exposed to sulphanilamide to produce colored diazonium compound, which was read at 540 nm. Nitrite and nitrate concentrations calculated from the standard curve obtained using sodium nitrite (NaNO₂, Sigma) and sodium nitrate (NaNO₃, Sigma), respectively. After nitrate and nitrite concentrations were determined separately, the sum of nitrate and nitrite concentrations shows the amount of NO ($\mu\text{mol/L}$).

Total antioxidant capacity (TAC) was measured by commercial kits (TAC Assay Kit®, Rel Assay Diagnostic, Turkey). Antioxidants in the sample reduce dark blue-green colored

ABTS [2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)] radicals to the colorless reduced ABTS form. The difference in absorbance is related to the total antioxidant concentration in the sample at 660 nm [17]. The reaction rate is calibrated with Trolox, which is widely used as a traditional standard for TAC measurement assays. The results were given in liters per millimolar equivalent of Trolox (mmol Trolox Eq/L).

Total oxidant capacity (TOC) was measured by commercial kits (TOC Assay Kit®, Rel Assay Diagnostic, Turkey). The ferrous ion-o-dianisidine complex is oxidized to the ferric ion by the oxidants present in the sample. It forms a colored complex with xylenol orange. The optical density of the color is related to the total oxidant molecules in the sample at 530 nm [18]. The measurement was calibrated with hydrogen peroxide (H₂O₂), and results were given in liters per micromolar equivalent of H₂O₂ (µmol H₂O₂ Eq/L).

Endothelial NO synthase (eNOS) activity was measured by a commercial goat ELISA kit (Goat eNOS ELISA Kit®, MyBioSource, USA). The assay sensitivity was 1.0 pg/mL. The intensity of color was measured in a microplate reader at 450 nm. A standard curve is plotted relating the optical density of the color to the concentration of standards. The eNOS activity was calculated from this standard curve in each sample.

Leptin assay was made by an ELISA kit purchased from Cusabio (Goat Leptin, LEP ELISA Kit®, Cusabio Biotech, China). The assay procedure was performed as described in the kit instruction manual. The detect range and min detection limit (sensitivity) of the kit were 0.625-40 ng/mL and 0.156 ng/mL, respectively. Determine the optical density of each well within 5 min, using a microplate reader set to 450 nm. The amount of leptin detected in each sample was compared to a leptin standard curve.

A commercial ELISA kit was used (DRG Progesterone ELISA Kit®, DRG Instruments GmbH, Germany) for the quantitative determination of P4. The range of the assay and sensitivity were 0-40 ng/mL and 0.045 ng/mL, respectively. Serum progesterone concentrations were determined according to the manufacturer's instructions. The optical density was determined within 10 min with a spectrophotometer at 450 nm. A standard curve was computed to determine the quantity of progesterone in each sample.

Statistical Analysis

Statistical analysis of the data was performed using the SPSS® 18.0 software (Chicago, IL, USA) program. Groups were compared with nonparametric tests because of the abnormal distribution (Shapiro-Wilk test) of the data. Statistical differences between pregnant and non-pregnant goats were evaluated using the Mann Whitney-U test. The non-parametric Friedman test and Wilcoxon test were also used for time periods (between days). Correlations

between variables were identified with the Spearman correlation test.

RESULTS

When the CIDR was removed vaginitis was detected with speculum examination. Mucopurulent-purulent discharge was also seen on the CIDR when it was removed from the vagina. A pregnancy rate of 75% (30/40) was recorded through transrectal ultrasonography 30 days after mating.

Serum MDA, NO, TOC concentrations and eNOS activity were higher ($P < 0.001$) in pregnant and non-pregnant goats on day 11 and mating day than day -8 and day 0. Leptin concentrations were higher ($P < 0.01$) in the pregnant group on day 11 and mating day than day -8 and day 0. TAC concentrations were lower on day 11 when compare to -8, 0 and mating day in the pregnant group ($P < 0.001$). Serum P4 concentration increased with the insertion of the CIDR in pregnant group on day 11 and decreased all groups on mating day than day -8, day 0 and day 11 ($P < 0.001$, *Table 1*).

Statistically significantly higher in eNOS activity and TOC concentrations on day 11 in pregnant goats compared to non-pregnant goats ($P < 0.001$, *Table 1*). MDA, NO, TOC, TAC and eNOS activity were statistically higher on mating day in pregnant goats compared to non-pregnant goats ($P < 0.05$, *Table 1*).

A positive correlation was found between MDA and TOC ($r = 0.378$, $P < 0.05$), MDA and leptin ($r = 0.384$, $P < 0.05$) on day 11. There was a negative correlation between TAC and TOC ($r = -0.347$, $P < 0.05$) on day 11. There was a strong positive correlation between eNOS activity and TOC ($r = 0.714$, $P < 0.01$) and a strong negative correlation between leptin and TAC ($r = -0.554$, $P < 0.01$).

DISCUSSION

Oxidative stress is a serious problem which is being studied extensively in human and animals, especially in cattle, sheep and goats in conditions of sepsis, mastitis, metritis, retentio secundinarum and genital tract inflammation [6,19]. There are few studies about fertility and the oxidative stress created by P4 sources used intravaginally for estrus synchronization in small ruminants [4,8]. Intravaginal sponge applications are known to be a common cause infection [20]. Results of the present study showed that the use of CIDR caused significantly increases in MDA, NO, TOC concentrations and eNOS activity on day 11. After the CIDR was removed, these concentrations declined but did not reach pre-treatment values. These increases may be the result of stress due to local irritation and inflammation caused by the CIDR in the vagina. Some studies have reported that administering P4 or estrogen can cause an increase in eNOS activity [21]. The present study also showed that eNOS activity increased with CIDR application on day

Table 1. Changes in MDA, NO, TOC, TAC, P4 and leptin concentrations and eNOS activity on days -8, 0, 11 and mating day in pregnant and non-pregnant goats synchronized with CIDR (Mean \pm SE)

Parameters	N	Day -8	Day 0	Day 11	Mating Day	P Value	Pregnancy Status
MDA μ mol/L	10	13.93 \pm 1.22 ^a	13.50 \pm 1.25 ^a	24.41 \pm 1.15 ^b	21.98 \pm 0.92 ^c	<0.001	Not pregnant
	30	14.21 \pm 1.79 ^a	14.14 \pm 2.59 ^a	22.79 \pm 4.87 ^b	22.31 \pm 4.19 ^b	<0.001	Pregnant
	P value	0.659	0.614	0.344	0.013		-
NO μ mol/L	10	12.32 \pm 2.47 ^a	11.61 \pm 1.37 ^a	22.40 \pm 1.24 ^b	18.99 \pm 0.45 ^c	<0.001	Not pregnant
	30	12.24 \pm 2.11 ^a	12.73 \pm 2.34 ^a	22.29 \pm 3.64 ^b	20.95 \pm 3.89 ^b	<0.001	Pregnant
	P value	0.975	0.28	0.571	0.005		-
eNOS U/L	10	5.04 \pm 0.49 ^a	5.08 \pm 0.23 ^a	6.58 \pm 0.38 ^b	6.53 \pm 0.30 ^b	<0.001	Not pregnant
	30	5.13 \pm 0.33 ^a	5.39 \pm 1.48 ^a	8.46 \pm 0.71 ^b	7.58 \pm 0.48 ^c	<0.001	Pregnant
	P value	0.85	0.181	<0.001	<0.001		-
TOC μ mol H ₂ O ₂ Eq/L	10	13.51 \pm 3.09 ^a	13.08 \pm 1.79 ^a	17.55 \pm 1.21 ^b	17.20 \pm 0.24 ^b	<0.001	Not pregnant
	30	13.53 \pm 1.58 ^a	13.68 \pm 2.59 ^a	26.69 \pm 0.48 ^b	23.54 \pm 0.78 ^c	<0.001	Pregnant
	P value	0.91	0.166	<0.001	<0.001		-
TAC mmol Trolox Eq/L	10	1.67 \pm 0.13	1.57 \pm 0.10	1.63 \pm 0.10	1.62 \pm 0.08	0.429	Not pregnant
	30	1.64 \pm 0.12 ^a	1.61 \pm 0.08 ^a	1.58 \pm 0.10 ^b	1.73 \pm 0.11 ^c	<0.001	Pregnant
	P value	0.571	0.18	0.176	0.01		-
P4 ng/mL	10	2.16 \pm 1.20 ^a	3.65 \pm 1.62 ^b	4.73 \pm 1.25 ^b	0.15 \pm 0.07 ^c	<0.001	Not pregnant
	30	2.49 \pm 1.26 ^a	3.10 \pm 0.58 ^a	5.35 \pm 1.27 ^b	0.13 \pm 0.06 ^c	<0.001	Pregnant
	P value	0.571	0.567	0.181	0.850		-
Leptin ng/mL	10	0.71 \pm 0.24	0.75 \pm 0.11	0.84 \pm 0.28	1.09 \pm 0.38	0.237	Not pregnant
	30	0.82 \pm 0.19 ^a	0.73 \pm 0.11 ^b	1.00 \pm 0.24 ^c	1.02 \pm 0.95 ^c	<0.001	Pregnant
	P value	0.101	0.339	0.08	0.570		-

^{a,b,c} The difference between values with different letters in the same row is significant (P<0.05). MDA: Malondialdehyde, NO: Nitric oxide, eNOS: Endothelial NO synthase activities, TAC: Total antioxidant capacity, TOC: Total oxidant capacity, P4: Progesterone, SE: Standard error

11 and mating day than day -8 and day 0 in pregnant goats (Table 1). It is also possible that the physiological increased in the serum estrogen level on estrus day (mating day) affected the increase in eNOS activity.

Oxidative stress can be assessed in terms of certain biological markers. Of the antioxidant parameters, TAC measurement alone can be used to determine the dynamic balance between plasma oxidants and antioxidants [22]. Oral et al. [6] found that TAC declined after the use of an intravaginal progesterone device in heifers, while TOC values remained unchanged. In the present study, however, a decline was assessed in TAC in pregnant goats on day 11, but TOC increased in parallel with MDA, NO and eNOS activity on day 11 and mating day in all groups. It is thought that the rise in TOC occurred in response to the inflammation and stress caused by the application of CIDR.

Hormonal applications are frequently used in sheep and goats to induce and/or synchronize estrus in order to perform artificial insemination easily and to mitigate seasonal effects [1,4]. Plasma P4 concentrations are reported to be above 1 ng/mL 2 h, 4 h, 4 days and 13 days after using CIDR or P4 sponges in sheep and goats [23]. P4 concentrations decreased below 1 ng/mL after CIDR was

removed and PGF_{2 α} injection was given [24]. In the present study, P4 concentrations were increased when the CIDR was inserted in pregnant group on day 11 compared to day -8 and day 0. Serum P4 concentration decreased all groups on mating day than day -8, day 0 and day 11. On estrus day, however, it was found to be 0.13 \pm 0.06 ng/mL in pregnant group (Table 1). P4 concentrations were above 1 ng/mL on application days (except for estrus day) because the study was conducted during breeding season.

Leptin concentrations are elevated in cases of inflammation/immune pathology and leptin can play a role, especially in intestinal inflammation. However, the exact nature of its role is not understood in such cases [25]. The innate immune system plays an important role in regulating leptin production. Leptin concentrations increased sharply when proinflammatories such as tumor necrosis factor- α and interleukin-1 are administered or with inflammation stimulants such as lipopolysaccharides and turpentine [26]. Rises in plasma estrogen concentrations in heifers at puberty also increase leptin concentrations [27]. Estrogen is thought to modulate the expression of leptin and its receptor in rodents as well [28]. Leptin has also been reported to play a role in gonadotropin-releasing hormone (GnRH) synthesis and therefore to raise estrogen

concentrations [29,30]. However, our study showed that leptin concentrations increased with the removal of CIDR (day 11) and mating day in pregnant goats, which was when oxidative stress indicators higher. This could be correlated with the oxidative status markers of leptin, which is associated with several inflammation markers. The correlations between MDA and leptin or TAC and leptin support this theory. Still, leptin concentrations tended to rise on day 11 and mating day when the estrogen concentrations increased. This may be because leptin stimulates GnRH synthesis and therefore increases estrogen synthesis.

It is reported that oxidative stress markers like NO and eNOS may play a role in luteinizing hormone peak or human chorionic gonadotropin synthesis. These mediators may also play a role in the process of oocyte maturation and ovulation [9,19]. Antioxidant system is active against oxidants during this process. In fact, it protects the oocyte from oxidative damage in follicular fluid [31]. Our study, however, showed a statistically significant increase in some oxidative status markers (eNOS activity and TOC) on day 11 and MDA, NO, TAC, TOC, leptin and eNOS activity statistically significantly higher on mating day in pregnant goats compared to non-pregnant goats (Table 1). The exact reason for this difference is not known. But pregnancy status might be different because there was better oocyte maturation and a LH surge in the goats that got pregnant. Mating day oxidative status may have been better compensated for with antioxidant systems in the animals that would be pregnant. This may have affected fertilization.

In conclusion, the administration of CIDR to Abaza goats exacerbated oxidative and nitrosative stress and increased P4 concentrations (pregnant goats). However, serum leptin concentrations were increased on CIDR removal day and mating day in pregnant group. Measuring serum oxidant and antioxidant status markers on mating day may also provide information about the goats' ability to conceive and make fertility projections. However, more comprehensive studies are needed.

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