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Original Research

Levels of Serum Nitric Oxide and Ascorbic Acid in Early Pregnant and Non-pregnant Sheep During Breeding Season

A. A. Malik[1]*, M. R. Fazili[2] and A. Khatun[1]

[1]Division of Animal Reproduction, Gynaecology and Obstetrics, Faculty of Veterinary Sciences and Animal Husbandry, Shere-e-Kashmir, University of Agricultural Sciences and Technology of Kashmir, Shuhama, Alusteng, Srinagar, Jammu & Kashmir, INDIA
[2]Division of Veterinary Clinical Complex, Faculty of Veterinary Sciences and Animal Husbandry, Shere-e-Kashmir, University of Agricultural Sciences and Technology of Kashmir, Shuhama, Alusteng, Srinagar, Jammu & Kashmir, INDIA

*Corresponding Author: malikasloob@gmail.com

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Abstract

The objective of this study was to determine the levels of nitric oxide (NO) and ascorbic acid (AA) in early pregnant and non-pregnant cross bred sheep during breeding season. The cross bred ewes (n=30) were equally allotted to three groups depending upon the length of time the progesterone sponges were kept in the vagina. The groups were designated as short (6 days), medium (10 days) and long-term (14 days) treatment. All the ewes were injected PGF2 α 250μg, intramuscularly 24 hours before sponge removal. After the sponge removal ewes were kept with breeding rams for tupping upto 72 hours. Blood samples were collected on different days for analysis of serum NO and AA concentration. There was progressive significant (P<0.05) increase in serum nitric oxide and ascorbic acid concentrations from day 0 to day 10, 17 and 35 of pregnancy compared to non-pregnant ewes. It was concluded that the levels of the serum nitric oxide and ascorbic acid differ between pregnant and non-pregnant ewes during early pregnancy.

Keywords: Ascorbic Acid, Estrus Synchronization, Ewes, Nitric Oxide, Intra-Vaginal Progesterone Sponge

Introduction

Pregnancy is a unique physiological state associated with the alterations in endocrine and cardiovascular systems leading to dramatic changes in the concentration of various blood biochemical constituents. In animals, the main biological actions of ascorbic acid (AA) include collagen synthesis, hormone secretion and anti-oxidant action (Luck *et al.*, 1995). In females, the AA has an important role in collagen biosynthesis, steroidogenesis and apoptosis during ovarian folliculogenesis (Luck and Zhao, 1993). Nitric Oxide (NO) is an important regulatory agent needed in various female reproductive events (Atakisil *et al.*, 2009). NO is also implicated in peripheral vasodilation during pregnancy and in the control of blood flow in the feto-placental circulation (Xuan and Cheng, 2002). Other studies have shown that NO is involved in maintaining myometrial quiescence during pregnancy (Ali *et al.*, 1997). Considering the significance of NO and AA in the regulation of uterine blood vascularity and luteal tissue functions respectively, the current study was designed to determine the concentration of NO and AA in ewes during early stages of pregnancy following hormonal treatment during breeding season.

Materials and Methods

Study Area

The present study was conducted at Mountain Research Centre for Sheep and Goats (MRCSG), FVSc & AH, Shuhama, SKUAST-Kashmir located in Srinagar, Kashmir, India (34°08′N 74°28′E). The treatment was initiated with the onset of the breeding season; Autumn season (September-November) in this temperate zone.

Selection, Management and Treatment of Animals

Thirty healthy multiparous cross bred ewes (NARI-Swarna ram x non-descript ewes) weighing 36.41 ± 4.25 kg with body condition scores ranging from 2.5-3.5 were used. They were in third or fourth parity. All the ewes were maintained under uniform management conditions. Animals were left outdoor during the day and housed in closed pens at night. They grazed on green natural pastures from 9:00 am to 3:0 pm upto November 15. There after hay (oats + sorghum) daily 1.5 kg and concentrate feed (Agrofeed Industries J&K Ltd) 300gm were provided daily to each ewe upto 31st January. Subsequently the concentrate feed was increased 600g/day/pregnant ewe. Free access to drinking water was ensured throughout the study period.

Cross-bred ewes (n=30) were randomly allotted equally (n=10) to three treatment groups i.e short, medium and long-term. All the ewes were subjected to similar estrus synchronization protocol but the length of time the intravaginal progesterone sponges were retained varied among the groups. Progesterone impregnated intravaginal sponges (AVIKESIL-S, CSWRI, Avikanagar, India) were introduced into the vagina on day 1 with using a speculum and introducer. The vaginal sponges were removed after 6, 10 and 14 days in ewes belonging to the different groups respectively. All the ewes received $PGF_{2\alpha}$ (Cloprostenol Sodium, Pragma™, Intas, Ahmedabad, India) 250µg intramuscularly 24 h prior to the sponge removal**.**

Tupping and Pregnancy Diagnosis

Immediately after removal of sponges, all the ewes were kept with three proven breeding rams at a ewe ram ratio 10:1 upto 72 hours for tupping. Wool colour was applied every evening on the brisket region of the rams in the evenings. The ewes were observed for breeding marks every morning and evening. They were assumed to be in estrus and tupped if more than 60% of the rump was coloured. The pregnancy was confirmed on day 45 post-tupping using a real time B-mode ultrasonography machine (esoate MyLab^{TM}40 VET) equipped with a 3.5 MHz sector array transducer. For USG, the animals were placed on the table and the inguinal region scrubbed. They were then restrained in right lateral recumbency. The visualization of the embryonic vesicle, placentomes and heart beat indicated pregnancy.

Blood Sampling

Samples (8.0 ml) were collected by jugular venipuncture into 15 ml centrifuge tubes without anticoagulant. The tubes were immediately stored in ice and placed in slanting position for 1-2 h. Serum was harvested by centrifugation at 1500 × g at 4 °C for 15 min. Serum was removed from tubes with micropipette and stored in stored 2ml storage

vials in duplicate at -20°C pending further analysis. The blood samples were collected on day of start of the treatment, the day of insemination/tupping (day 0), day 10, day 17 and day 35 post tupping.

Determination of Serum Nitric Oxide and Ascorbic Acid Concentration

The concentration of nitric oxide in the serum samples was determined as per the method described by Sastry *et al.* (2002) and serum ascorbic acid was determined according to Zannoni *et al.* (1974).

Statistical Analysis

The data obtained in the study was analyzed using standard statistical procedures (Snedecor and Cochran, 1994) using statistical software SPSS version 20. In this study, since the number of non-pregnant animals was only one therefore statistical analysis was done by Z-test calculator for a single sample by comparing the single sample mean with the population mean. However, variation in means at different days within pregnant and non-pregnant groups was analyzed by One way-ANOVA. The Post-hoc analysis was performed using Duncan's multiple range test. All the data are presented in the tables as mean ± SEM. The level of significance was set as $P<0.05$.

Results and Discussion

There was non-significant difference in mean NO concentration (µM) between the non-pregnant ewe and the total ewes (pregnant and non-pregnant) at different days of sampling. Although, the mean NO concentration in pregnant ewes varied non-significantly between pre-treatment and day 0, but there was continous significant ($P<0.05$) increase thereafter (Table 1). The mean serum nitric oxide concentration in pregnant ewes showed a significant ($P<0.05$) increase from day pre-treatment to day 35.

Table 1. Serum nitric oxide (nitrite plus nitrate) concentration (Mean ± S.E.M) in pregnant (29/30) cross bred ewes

Stage/Day	Nitric oxide concentration (µM)
Pre-treatment	129.07 ± 3.06^{ab}
Day 0	124.04 ± 4.33^{a}
Day 10	139.33 ± 2.80^{bc}
Day 17	151.56 ± 7.23^{cd}
Day 35	157.79 ± 3.19^{d}

Means bearing different superscript (a, b, c, d) within columns differ significantly (P<0.05)

These results are in agreement with the findings of Malik *et al.* (2018), Rasool *et al.* (2018) and Yang *et al.* (1996). The NO metabolites in blood of pregnant sheep increases as early as day 20 to day 39, with levels increasing upto day 40 to day 69. The values decrease thereafter to non-pregnant level by day 70 to day 100 and again increase until term (Vonnahme *et al.*, 2005). Kwon *et al.* (2004) demonstrated that the ovine placentomes produce NO in a similar biphasic pattern. The highest serum levels of NO have been reported in ewes in third month of pregnancy, followed by second and fifth months. The values remain higher than the cyclic ewes (Kandiel *et al.*, 2016). The establishment and maintenance of pregnancy is regulated by orchestrated action and levels of estrogen and progesterone. During early pregnancy, the uterine endothelium undergoes characteristic changes under the influence of estrogen. They include increased microvascular permeability and stromal edema necessary for successful implantation (Xaun and Cheng, 2002). The production of NO is attributed to the effect of estrogen on uterus (Van Buren *et al.*, 1992). The NO expression is regulated by estrogen and progesterone during pregnancy (Yallampalli and Dong, 2000). NO mediates smooth muscle contractility, spontaneous contraction as well as distension of uterus during pregnancy (Izumi and Garfield, 1995). NO is also responsible for peripheral vasodilation during pregnancy and has role in the fetoplacental circulation. Other studies demonstrated that NO is involved in maintaining myometrial quiescence during pregnancy (Ali *et al.*, 1997). NO also regulates angiogenic factors like vascular epidermal growth factor (VEGF) in the ovary. The primary function of corpus luteum accompanied by the development of an extensive vascular system is mediated by NO (Wiltbank *et al.*, 1990; Reynolds *et al.*, 2000; Augustin, 2001). The quantity of progesterone secretion is highly dependent on the ovarian blood flow (Reynolds *et al.*, 2000; Grazul-Bilska *et al.*, 2001).

Non-significant difference in mean ascorbic acid concentration (µg/ml) were observed between the non-pregnant

ewe and the total ewes (pregnant and non-pregnant) at different days of sampling. In pregnant ewes, mean serum ascorbic acid concentration varied non-significantly (P>0.05) between pre-treatment and day 0, however there was significant (P<0.05) increase on day 10, day 17 and day 35 as compared to day 0 (Table 2).

Table 2: Serum ascorbic acid concentration (Mean±S.E.M) in pregnant (29/30) cross bred ewes

Stage/Day	Ascorbic acid concentration (µg/ml)
Pre-treatment	48.63±3.56[a]
Day 0	47.78±4.01[a]
Day 10	63.10±3.61[b]
Day 17	66.70±5.44[bc]
Day 35	77.16±6.06[c]

Means bearing different superscript (a, b, c, d) within columns differ significantly (P<0.05)

In present study, the results of serum ascorbic acid concentration are in agreement with those of Malik *et al.* (2020), Miszkiel *et al.* (1999) and Petroff *et al.* (1998). Highest level of ascorbic acid is recorded during luteal phase and it continues to remain higher throughout pregnancy but decrease with the regression of the corpus luteum (Petroff *et al.*, 1997). Excessive demands for ascorbic acid are made by the fetus during pregnancy (Wilson and Loh, 1973). The multiple functions of ascorbic acid at ovarian level include collagen synthesis needed for remodelling of gonadal tissue (Luck *et al.*, 1995) and biosynthesis of steroid hormones (Milewich *et al.*, 1981). Ascorbic acid has important role in structure and function of corpus luteum. The protection of corpus luteum from luteolysis is critical for maintenance of pregnancy (Mohebbi-Fani *et al.*, 2011). Vitamin C is necessary for formation of intercellular substances (especially bone matrix) and fibers of the connective tissue in the fetus. The ascorbic acid content in the fully mature corpus luteum reaches maximum level, remains high during pregnancy and decreases as the CL regresses (Petroff *et al.*, 1997).

Conclusion

It was concluded from this study that in levels of serum nitric oxide and ascorbic acid increase with the advancement of pregnancy. In addition, higher levels of serum nitric oxide and ascorbic acid were reported in pregnant sheep than non-pregnant sheep indicating their probable role in maintenance of pregnancy.

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Conflict of Interests

There is no conflict of interest.

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