



Original Research

Do Serum Ascorbic Acid And Nitric Oxide Levels Differ Between Pregnant And Non-Pregnant Crossbred Ewes At Early Stages During Breeding Season?

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Abstract

The main aim of this study was to investigate the serum ascorbic acid and nitric oxide levels in pregnant and non-pregnant ewes during early stages of pregnancy. A total of 24 crossbred ewes were selected randomly and estrus was synchronized by placing progesterone sponges in vagina for 11 days along with an injection of PGF_{2α} 24 hours before sponge removal and GnRH at first fixed time artificial insemination (FTAI) which was done 48 hours after sponge removal and repeated at 12 hours interval. Pregnancy was determined at 45 days by real time B-mode ultrasonography. After obtaining a pregnancy rate of 37.5 %, ewes were grouped in to pregnant and non-pregnant. Jugular blood samples were collected from all animals at day 0 (before treatment), day of AI, day 15 post AI and day 35 post AI. The serum nitric oxide level in pregnant ewes was significantly higher ($P<0.05$) at day 35. The serum ascorbic acid level was significantly higher in pregnant ewes from day 15 to day 35 as compared to non-pregnant ewes. In conclusion, serum ascorbic acid and nitric oxide levels differ between pregnant and non-pregnant ewes during early stages of pregnancy.

Key words: Ascorbic Acid, Breeding Season, Ewe, Nitric Oxide, Pregnant

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Introduction

Ascorbic acid plays important roles in function and structure of corpus luteum (CL), protection of which from luteolysis is vital for pregnancy progression (Mohebbi-Fani *et al.*, 2011). Underproduction of ascorbate has been implicated to impaired steroidogenesis and subfertility in domestic animals (Luck *et al.*, 1995). Large amounts of ascorbic acid are utilized during ovulation and excessive demands for ascorbic acid are made by the fetus during early and later stages of pregnancy (Wilson and Loh, 1973). Its high





concentration in blood during estrus is thought to stimulate development of ascorbic acid rich corpus luteum following ovulation acting as a cofactor in the synthesis of collagen in the luteal extracellular matrix (Hurley and Doane, 1987). In contrary to this, Petroff *et al.* (1997) reported an increase in ascorbic acid content as corpus luteum grows and remains high during pregnancy (Mushtaq, 2014) but decreases as follicle grows in size. Nitric oxide (NO) is an inorganic free radical gas derived from substrate L-arginine by the action of NOS (Palmer and Monacada, 1989). It plays an important role in steroidogenesis, ovulation and luteolysis (Jablonka-sharrif and Olson, 1998, Miyamoto and Shirasuna, 2009, reviewed by Lone, 2013). Moreover, the increased nitric oxide synthesis during pregnancy has been linked to cardiovascular adaptations to normal pregnancy (Yang *et al.*, 1996). Several workers have documented high nitric oxide levels from mid to late gestation in ewes (Vonnahme *et al.*, 2005; Kandiel *et al.*, 2016).

Considering the importance of serum ascorbic acid and nitric oxide levels in corpus luteum dynamics and uterine blood flow, the present trial was performed to investigate whether the serum ascorbic acid and nitric oxide levels differ in pregnant and non-pregnant ewes at early stages and may indicate the health of corpus luteum and pregnancy.

Materials and Methods

Study Location

The study was conducted at Mountain Research Station for Sheep and Goat, FVSc & A.H, Shuhama J&K (India) during September to November, 2016.

Selection and Treatment of Animals

Twenty four (N=24) normal healthy cycling (Progesterone concentration before the start of treatment; 18.78 ± 2.05 ng/ml) crossbred ewes (NARI-Swarna ram X non-descript ewe) 1-3 years of age were selected randomly and estrus was synchronized by placing progesterone sponges (AVIKESIL-S, CSWRI, Avikanagar, India) in vagina for 11 days along with an injection of PGF_{2α} (Intas pharmaceuticals Ltd, Ahmedabad, India) 24 hours before sponge removal. A single shot of GnRH (Receptal® VET MSD Animal Health) was also given at first fixed time artificial insemination (FTAI) which was done 48 hours after sponge removal using 12-24 hour old chilled semen. Insemination was repeated at 12 hours after first FTAI. Pregnancy was determined at day 45 post insemination by real time B-mode ultrasonography. Jugular blood samples were collected from all animals at day 0 (before treatment), day of AI, day 15 post AI and day 35 post AI. The serum harvested was analyzed for nitric oxide and ascorbic acid levels. After determining pregnancy rate, animals were grouped in to pregnant and non-pregnant for analysis of data obtained for ascorbic acid and nitric oxide.



Estimation of Serum Nitric Oxide and Ascorbic Acid

Ascorbic Acid

The ascorbic acid concentration in the serum samples was determined as per the method described by Zannoni *et al.* (1974).

Nitric Oxide

The concentration of nitric oxide in the serum was estimated as per the method described by Sastry *et al.* (2002).

Statistical Analysis

The data obtained in respect of mean serum ascorbic acid and nitric oxide concentration at different stages within non pregnant and pregnant groups was analyzed by One-way ANOVA. Post-hoc analysis was done by DMRT. However, variation in means between pregnant and non-pregnant groups at different stages was analyzed by paired T-test. P-value ≤ 0.05 was considered significant (SPSS, Statistics-21).

Result and Discussion

The mean serum ascorbic acid concentration ($\mu\text{g/ml}$) of pregnant and non-pregnant cross bred ewes following artificial insemination is presented in Table 1.

Table 1: Serum ascorbic acid levels (mean $\pm$ SEM) in non-pregnant and pregnant ewes during breeding season

Ascorbic Acid Concentration ($\mu\text{g/ml}$)	Stage			
	Day 0	Day of AI	Day 15 post AI	Day 35 post AI
Non-Pregnant (N=15)	33.29 $\pm$ 8.86	46.23 $\pm$ 12.02	42.78 $\pm$ 3.87 ^A	47.06 $\pm$ 5.65 ^A
Pregnant (N=9)	39.48 $\pm$ 12.4	45.66 $\pm$ 9.91	65.58 $\pm$ 8.13 ^B	71.15 $\pm$ 2.24 ^B

Means with different superscripts (A, B) within a row differ significantly ($p < 0.05$)

The serum ascorbic acid concentration in pregnant animals were significantly higher in pregnant animals at day 15 and at day 35 as compared to non-pregnant animals. Further, in pregnant ewes, the mean serum ascorbic acid concentration at day of AI (may correspond to estrus) were quite lower although non-significant ($P > 0.05$) as compared to day 15 and Day 35 post AI (correspond to luteal phase in pregnancy). Our results are in agreement with the findings of Mushtaq (2014), Miszkziel *et al.* (1999) and Petroff *et al.* (1998). Highest levels of ascorbic acid are seen during luteal phase and they continue to remain higher in pregnancy, decrease with regression of corpus luteum (Petroff *et al.*, 1997). Ascorbic acid, being an antioxidant vitamin, plays important role in function and structure of corpus luteum (CL) which is crucial for establishment and progression of pregnancy (Mohebbi-Fani *et al.*, 2011). Its depletion from ovarian tissue of rat (Aten *et al.*, 1992; Sato *et al.*, 1974) and pig corpus luteum (Petroff *et al.*, 1998) has been proposed as an early mechanism of luteal regression by rendering luteal cells susceptible to ROS-induced

oxidative stress and apoptosis (Tanaka *et al.*, 2000). As the steroidogenic activity of the corpus luteum increases, the requirement of ascorbic acid also increases (Miszkiet *et al.*, 1999). Ascorbic acid is also required in the development of fetus for formation of intracellular substances, especially the bone matrix and fibers of connective tissue in fetus (Mushtaq, 2014).

The mean nitric oxide concentration (μM) in the serum of pregnant and non-pregnant crossbred ewes is presented in Table 2. The mean nitric oxide concentration in pregnant was significantly ($P < 0.05$) higher than non-pregnant ewes at Day 35 post AI. Within non-pregnant ewes, the mean serum nitric oxide concentration declined significantly from day 0 to day 35 post AI. This indicates that as the corpus luteum regresses in non-pregnant ewes, the NO levels also fall. These results are in close agreement with the findings of other workers (Yang *et al.*, 1996; Vonnahme *et al.*, 2005; Mushtaq, 2014; Kandiel *et al.*, 2016) who also reported higher levels of serum NO in pregnant animals as compared to non-pregnant animals.

Table 2: Serum nitric oxide (nitrite plus nitrate) levels (mean $\pm$ SEM) in in non-pregnant and pregnant ewes during breeding season

Nitric oxide Concentration (μM)	Stage			
	Day 0	Day of AI	Day 15 post AI	Day 35 post AI
Non-Pregnant (N=15)	146.63 $\pm$ 4.69a	136.66 $\pm$ 7.68ab	147.14 $\pm$ 12.97 ^a	121.67 $\pm$ 5.22 ^{bA}
Pregnant (N=9)	148.12 $\pm$ 12.53	139.77 $\pm$ 13.7	149.84 $\pm$ 10.85	152.24 $\pm$ 3.82 ^B

Means with different superscripts (A, B) within a column and (a, b) within rows differ significantly ($p < 0.05$)

For maintenance of normal oxygen and nutrient delivery to fetus, the utero-placental blood flow increases 30-50 fold in pregnancy (Sladek *et al.*, 1997; Magness *et al.*, 2001). This huge increase in blood flow requires vascular bed to proliferate and dilate, in which NO, a key angiogenic factor plays an important role (Rosenfeld *et al.*, 1996). The increased nitric oxide concentration in the maternal circulation during gestation also mediates the low systemic and umbilical vascular resistance in the fetus (Yang *et al.*, 1996). The early development of corpus luteum after ovulation is accompanied by the development of an extensive vascular system, which becomes maximally dilated mediated by NO (Wiltbank *et al.*, 1990; Reynolds *et al.*, 2000; Augustin *et al.*, 2001).

Conclusion

Conclusively in pregnant sheep the ascorbic acid and nitric oxide levels remain higher during pregnancy indicating their role in corpus luteum formation, normal steroidogenesis and increased blood flow to uterus and ovary.

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