Nitric Oxide Profiles In Serum And Follicular Fluid Of Cyclic And Acyclic Sheep

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ABSTRACT

The present study was conducted to estimate nitric oxide metabolites, nitrite plus nitrate, in serum and follicular fluid of cyclic and acyclic sheep. In this study the mean number of medium and large sized follicles differed significantly ($P<0.05$) between cyclic and acyclic sheep; however the small sized follicles were similar ($P>0.05$) in both the groups. The nitrite plus nitrate levels in serum and follicular fluid were significantly higher ($P<0.05$) in cyclic as compared to acyclic sheep. The results indicate higher levels of nitrite plus nitrate in biological fluids (serum and follicular fluid) of cyclic than in acyclic sheep. Presence of higher nitrite plus nitrate, the end products of nitric oxide oxidation suggests higher nitric oxide activity in the serum and follicular fluid of cyclic sheep compared to acyclic sheep.

Key words: Follicular population, nitric oxide, cyclic and acyclic sheep.

INTRODUCTION

"A molecule of the millennium" nitric oxide (NO) has now been found to be an important mediator involved in regulating the reproductive and immune functions in mammals. Nitric oxide is an inorganic, short lived (a few seconds) free radical gas and a versatile signalling molecule regulating a variety of cellular processes. In vitro, it is a stable, colourless gas, moderately soluble in water. It is synthesized from L-arginine via an oxygen- and NADPH- dependent reaction that yields nitric oxide and L-citrulline (Bush *et al*., 1992). Nitric oxide synthesis depends on the action of a NO synthase (NOS) an

enzyme that exists in three isoforms that have been classified depending on the tissue of origin as well as on functional properties. The importance of NO is recognized in several aspects of female reproduction such as ovarian follicular development (Rosselli *et al*., 1998; Jablonka-Shariff and Olson, 2000), preovulatory LH surge (Dhandapani and Brener, 2000), ovulation (Olson *et al*., 1999), release of certain steroids and gonadotropins (Pickard *et al*., 1991), steroidogenesis (Jablonka-Shariff and Olson, 1998) and oocyte meiotic maturation (Schwarz *et al*., 2008). Therefore the present investigation was carried out to quantify NO levels in follicular fluid and serum of cyclic and acyclic sheep.

MATERIAL AND METHODS

The present study was conducted at Division of Animal Reproduction, Gynaecology and Obstetrics. Faculty of Veterinary sciences and Animal Husbandry R.S. Pura, Jammu during the period between March to October, 2011.

Blood (before slaughter) and ovaries of the same animal were collected from a municipal slaughter house. The ovaries collected were classified into cyclic and acyclic.

Ovaries with a corpus hemorrhagicum (CH), a large CL and >5mm follicle(s) in diameter or a regressing CL with follicle(s) >6mm in diameter were classified as active and the animals as cycling.(Fig.1) Whereas ovaries without a CL or CH or the pressure of a regressed CL without <5mm in diameter follicle(s), such ovaries were classified as inactive and the animals as non-cycling (Azawi *et al*., 2008) (Fig.2).

Collection of blood and serum separation

Before slaughter 5ml of blood was collected (without anticoagulant) by jugular venepuncture followed by centrifugation at 2000g for 10minutes to harvest serum followed by storage at -20^{0}C until further use.

Collection of follicular fluid

Ovaries were collected daily from March 2011 to October 2011 from municipal slaughter house. No information regarding identity and history was included in this study. Immediately after slaughter, ovaries of the same animal with no apparent clinical abnormalities were collected and transported to the laboratory in Phosphate Buffered saline in an ice box within 30 minutes. Follicles were divided into three groups

depending on their diameter; small (<2 mm), medium (2-4 mm) and large (>4 mm) (Shailaja and Kumari, 1984). Follicular fluid was aspirated by using a separate hypodermic tuberculin syringe for each ovary and was pooled irrespective of their size. It was collected by applying slight pressure to avoid additional traumatisation of follicles. Aspirated fluid was centrifuged at 3000rpm for 15minutes to remove cellular debris. The sample was kept at -20^{0}C till further use.

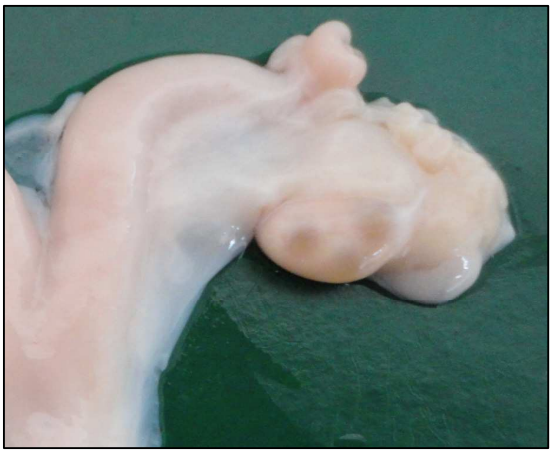

Fig.1. Reproductive tract of cyclic sheep with presence of medium and large sized follicles

Fig.2. Reproductive tract of acyclic sheep with presence of small sized follicles

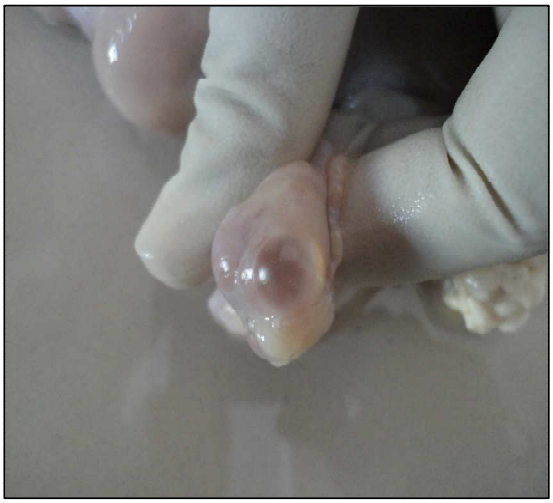

Fig. 3. Presence of large sized follicle on cyclic ovary

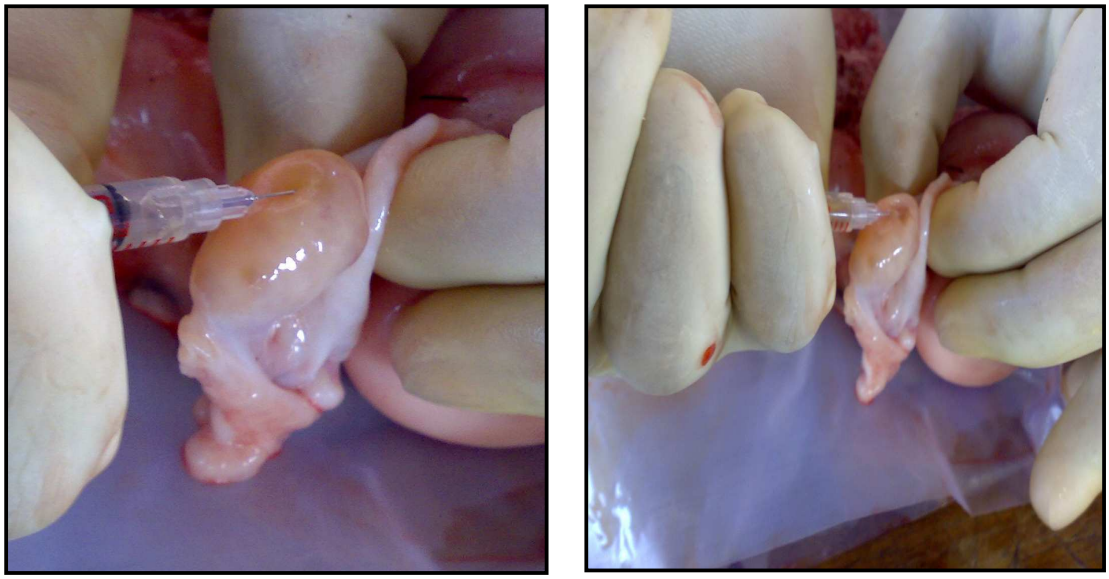

Fig.4. Aspiration of follicular fluid using tuberculin syringe

COPPER-CADMIUM ALLOY

10gms of copper was melted initially in a crucible followed by 90gms of cadmium and were mixed in the molten state. The molten alloy was made into a block immediately. The copper-cadmium alloy thus formed was powdered using a metal file.

Determination of NO_2 and NO_3

The NO_2 and NO_3 concentration in the serum and follicular fluid measured together as described below (Sastry *et al*., 2002).

Copper-cadmium alloy filings were washed twice with 100ml of deionised distilled water in a 150ml Erlenmeyer flask. After discarding the supernatant, the filings were washed with 100ml 0.5N HCl. This washing removed the hydroxide of cadmium ($Cd(OH)_2$), which resulted in a change in texture and color. The filings appeared gray and slightly buoyant. These activated filings are quickly washed twice with 100ml of 0.1N HCl and stored in 50ml of 0.1M HCl at 2-8^{0}C till further use.

100µL of serum and follicular fluid were taken in test tubes and 400µL of 50mM Carbonate buffer was added to each test tube followed by150mg of activated and dried copper-cadmium alloy filings. The tubes were incubated for 1hr at 37^{0}C with thorough shaking. After incubation, 100µL of 0.35M Sodium hydroxide was added in order to halt the reaction; followed by adding 400µL of 120mM Zinc sulphate solution on a vortex mixture. The test tubes were allowed to stand for 10minutes, followed by centrifugation at 4000g for 10minutes at room temperature. Precipitate settled down and 500µL of clear supernatant was transferred to another test tube; 250µL of 1.0% Sulphanilamide (prepared in 3N HCl) and 250µL of 0.1% N-naphthylethylene diamine (prepared in water) were added with shaking. After 10minutes the absorbance was measured at 545nm against a blank containing the same concentration of ingredients without any test sample.

RESULTS AND DISCUSSION

Table 1 shows that the average number of small sized surface follicles was slightly more in cyclic sheep (5.40 ± 0.42) as compared to acyclic sheep (4.5 ± 0.401)(Fig.5). However the difference between cyclic and acyclic sheep for small size surface follicles was non-significant. The average number of medium sized follicles recorded in cyclic

sheep (3.2 ± 0.326) was significantly higher (P<0.05) as compared to (1.0 ± 0.25) follicles in acyclic sheep. Similarly the average number of large size follicles in cyclic sheep (1.8 ± 0.20) were significantly higher (P<0.05) as compared to (0.2 ± 0.13) follicles in acyclic sheep.

Table 1: Average number of small, medium and large size surface follicles in cyclic and acyclic sheep (Mean ± S.E)

S.No.	Follicle size	Cyclic (n=10)	Acyclic (n=10)	"t"-value
1	Small (<2mm)	5.40 ± 0.42	4.5 ± 0.40	1.22^{NS}
2	Medium (2-4mm)	3.2 ± 0.33	1.0 ± 0.25	6.74*
3	Large (>4mm)	1.8 ± 0.20	0.2 ± 0.13	6.00*

* indicates significant values at P<0.05; NS indicates non-significant values at P>0.05

The present study is in agreement with the findings of Andurkar *et al.*, (2004) and Sontakke *et al.*, (2009) who also reported a significant difference in the number of medium and large sized follicles between cyclic and acyclic goats.

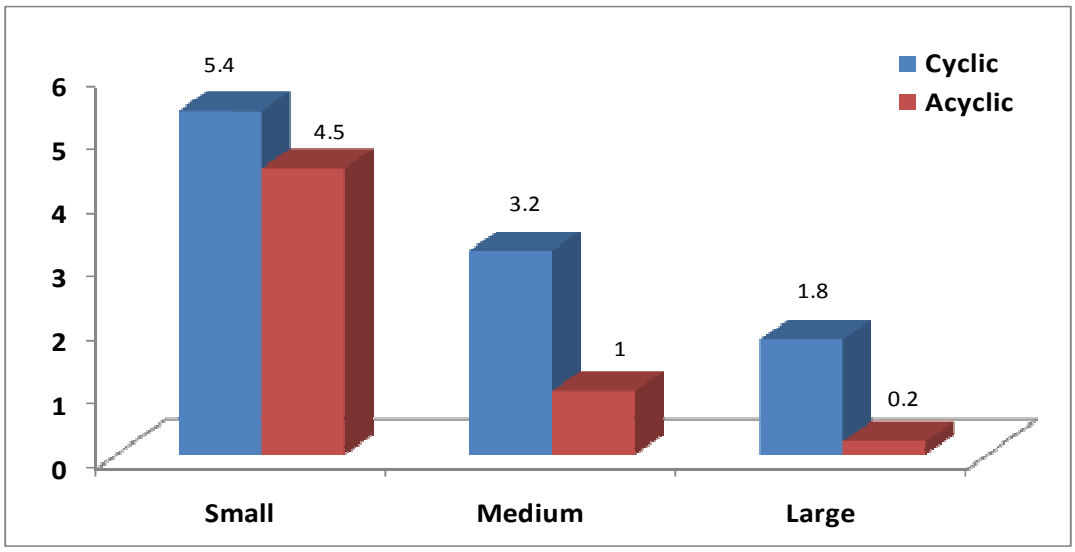

Fig. 5: Average number of small, medium and large sized follicles

Table 2: Nitric oxide metabolites (nitrite plus nitrate) concentration (µM) in follicular fluid and serum in cyclic and acyclic sheep (Mean ± S.E)

S.No.	Variable	Cyclic (n=10)	Acyclic (n=10)	"t"- value
1	Follicular fluid	50.04 ± 2.16	23.32 ± 2.18	7.88*
2	Serum	49.76 ± 1.81	27.24 ± 1.40	8.74*

* indicates significant values at P<0.05

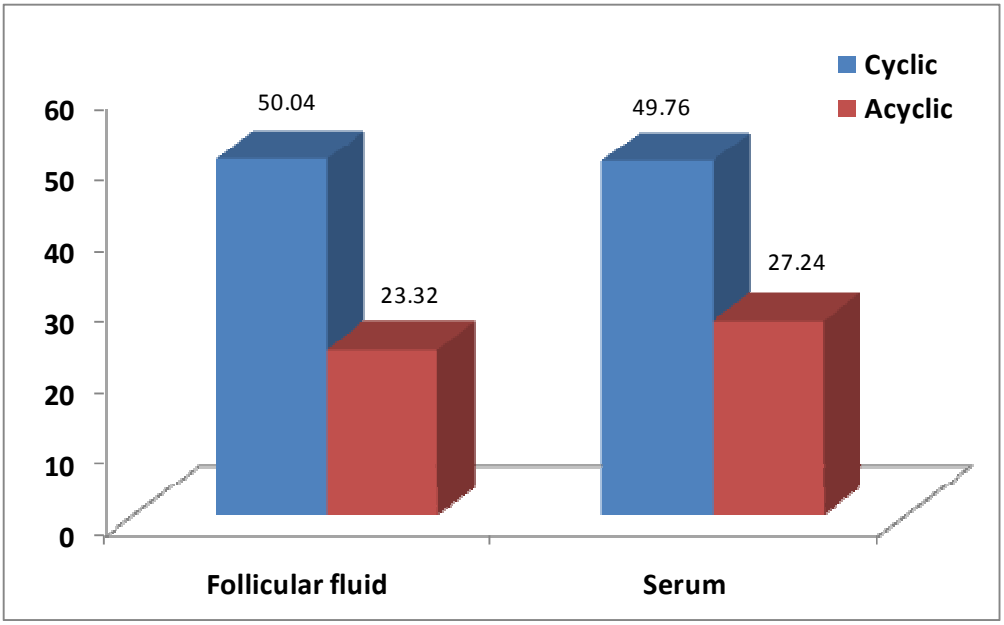

Fig. 6: Nitric oxide metabolites (nitrite plus nitrate) concentration (µM) in follicular fluid and serum

The NO synthesized in tissue is completely metabolized to nitrate and nitrite (NO_2 and NO_3). Therefore, estimation of nitrate/nitrite provides indirect means of estimating endogenous NO concentration (Schulz *et al*., 1999). The present study demonstrates the presence of NO in serum and follicular fluid in sheep. This study revealed a significant difference in concentrations of NO in serum and follicular fluid of cyclic and acyclic sheep.(Table 2; Fig.6).

Increased serum concentrations of NO_2 and NO_3 have been reported to be associated with follicular development in women (Rosselli *et al*., 1994). This higher level indicates that NO may be associated with folliculogenesis and ovulation. The serum NO level observed in the present study is in accordance with Sarath *et al*., (2010) who reported serum nitric oxide concentration in cyclic and acyclic goats ranging from 10.77 ± 0.08 to 11.90 ± 0.37 and 9.64 ± 0.33 to 10.75 ± 0.25 µM, respectively. Sastry *et al*., (2002) who reported 57.53 ± 4.26 and 50.36 ± 6.90 µM/L in sheep and cattle, respectively. Bulbul *et al*., (2008) who reported higher levels of NO during the cyclic stage in Brown Swiss cows. In the present study, the significant higher concentration of NO in cyclic as compared to acyclic sheep might be due to occurrence of oestrus, emergence of follicular wave (Mitchell *et al*., 2004), occurrence of ovulation (Yamauchi *et al*., 1997) and formation of corpus luteum. The low level of NO in acyclic sheep might be due to decreased follicular growth and absence of ovulation.

Follicular fluid environment can be a high quality index of follicular functional status as well as the reproductive state of the animal. In the present study NO (µM) concentration in follicular fluid of cyclic sheep (50.04 ± 2.16) was significantly higher ($P<0.05$) than in acyclic sheep (23.32 ± 2.18). These results are not in accordance with the finding of Khan *et al*., (2012) in buffaloes who reported increased NO levels in follicular fluid of acyclic buffaloes speculating that higher NO triggers the development of acyclicity in buffaloes. The presence of NO in the follicular fluid compartment and its possible role in follicular development has been demonstrated in a number of livestock species (Dixit and Parvizi, 2001). This significant higher ($P<0.05$) level of NO in follicular fluid in cyclic sheep reflects its association with reproductive functions.

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