



Inhibitory effects of nitric oxide on steroidogenesis by buffalo granulosa cells cultured *in vitro*

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ABSTRACT

This study examined the effects of S-nitroso-N-acetyl-penicillamine (SNAP), a nitric oxide (NO) donor and N^w-nitro-L-arginine methyl ester hydrochloride (L-NAME), a nitric oxide synthase inhibitor on estradiol-17 β and progesterone production by buffalo granulosa cells. Granulosa cells (3×10^5) from small (≤ 5 mm diameter) or large (≥ 9 mm diameter) follicles were cultured for 24 h under completely serum-free conditions in DMEM: nutrient mixture F-12 Ham (1:1 ratio) supplemented with 10^{-7} M androstenedione, 5 mg/ml human apo-transferrin, 0.1% BSA, in the presence or absence of FSH (8 ng/ml). Granulosa cells from large follicles produced higher estradiol-17 β and progesterone than those from small follicles. SNAP reduced estradiol-17 β and progesterone production at concentrations of 0.1 and 1 mM when used alone and in the presence of FSH by granulosa cells from follicles of both size categories. L-NAME (0.2, 1 and 5 mM) had, however, no effect on estradiol-17 β or progesterone production, alone or in the presence of FSH. Our results demonstrated strong inhibitory effects of NO on estradiol-17 β and progesterone production by buffalo granulosa cells from small and large follicles and indicated that it may be an autocrine/paracrine regulator of steroidogenesis by granulosa cells in buffalo.

Key words: Buffalo, Granulosa cells, L-NAME, Nitric oxide, SNAP, Steroidogenesis

Delayed puberty, poor expression of estrus, high incidence of early embryonic mortality, long inter-calving period and seasonal variation in fertility compromise the productivity of buffalo (*Bubalus bubalis*). Although some information is now available on the peripheral hormonal profiles of various hormones involved in reproduction (Mondal *et al.* 2007), very few studies have been conducted at the ovarian level in this species (Bhushan *et al.* 2004, 2005).

Granulosa cells are the site of production of steroid hormones namely, estradiol-17 β and progesterone, growth factors like IGF-I, IGF-II, EGF and the inhibin family of hormones, i.e. inhibin, activin and follistatin, which are involved in maintenance and control of ovarian and hypophysial function through autocrine, paracrine and endocrine pathways.

Nitric oxide (NO) is a highly reactive free radical, which is synthesized via the oxidation of L-arginine to NO and citrulline by nitric oxide synthases (NOS). It gained attention as a paracrine/autocrine regulator of granulosa cell

steroidogenesis (Dixit and Parvizi 2001, Tamanini *et al.* 2003). It is reportedly present in follicular fluid in cattle, its concentrations being higher in follicular fluid from small than from large follicles (Basini *et al.* 1998). NO was found involved in various functions in granulosa cells from different species. Effects of NO on steroidogenesis by granulosa cells were studied in several farm animal species including cattle (Basini *et al.* 1998, 2001, Faes *et al.* 2009), pig (Masuda *et al.* 1997, 2001, Ponderato *et al.* 2000, Graselli *et al.* 2001) and horse (Pinto *et al.* 2002). However, there is no information available on the effects of NO on steroidogenesis by buffalo granulosa cells. The present study was, therefore, undertaken to determine if NO affects estradiol-17 β and progesterone production by buffalo granulosa cells.

MATERIALS AND METHODS

Isolation of granulosa cells: Buffalo ovaries were collected immediately after slaughter in an abattoir and washed 3 times with chilled (4°–10°C) isotonic saline (0.9% NaCl) containing 100 IU/ml penicillin and 100 μ g/ml streptomycin. After transport to the laboratory within 5–6 h in chilled saline, the ovaries were washed 3 times with saline at room temperature. Henceforth, the ovaries were kept at room temperature until the collection of granulosa cells was

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completed. Granulosa cells were aspirated from small (≥ 5 mm diameter) and large (≥ 9 mm diameter) follicles with a 24-gauge needle attached to a 10-ml syringe, and were washed 3 times with the culture medium which consisted of DMEM: nutrient mixture F-12 Ham (1:1 ratio) supplemented with L-glutamine (at a concentration recommended by the manufacturer), 10^{-7} M androstenedione, 5 mg/ml human apo-transferrin, 0.1% bovine serum albumin (BSA, w/v), 100 IU/ml penicillin, 100 μ g/ml streptomycin and 0.25 mg/ml amphotericin B. At each wash, the cells were separated by centrifugation at $500 \times g$ for 10 min. The pellet was resuspended in approximately 4 ml of culture medium and was then passed through a 30-gauge needle attached to a 5 ml syringe for breaking up clumps of granulosa cells. The cells were again washed twice with the culture medium. The cells thus obtained were primarily antral (luminal) granulosa cells. The proportion of viable cells in the granulosa cells collected, which was determined by trypan blue exclusion method using a haemocytometer, varied between 40 to 55% among different experiments.

Culture of granulosa cells: Granulosa cells were cultured in 48-well culture plates precoated with fibronectin ($3.3 \mu\text{g}/\text{cm}^2$) at a cell concentration of 3×10^5 viable cells in 400 μ l of culture medium for 24 h at 37°C in a CO_2 incubator (5% CO_2 in air, 90–95% relative humidity, Shanmugam *et al.* 2010). For studying the effects of SNAP, cells were cultured with this NO donor at concentrations of 0.01, 0.1 and 1 mM, either alone or in the presence of FSH (8 ng/ml). To test the effects of L-NAME, in another separate experiment, the cells were cultured with this NOS inhibitor at concentrations of 0.2, 1 and 5 mM, either alone or in the presence of FSH (8 ng/ml). The gonadotropin concentration used was based on our earlier study (Shanmugam *et al.* 2010). The media samples were stored at -20°C until subsequent analysis for estradiol-17 β and progesterone. Direct radioimmunoassays, as described earlier were used for the estimation of progesterone (Bhushan *et al.* 2004) and estradiol-17 β (Palta *et al.* 1996). The intra- and inter-assay coefficients of variation were $<14\%$.

Statistical analysis: Each experiment was repeated 3 times independently and in each experiment, each treatment was performed with 4 replicate culture wells. Data from different experiments are presented as mean $\pm$ SEM hormone production (ng/ml). Using SYSTAT 7.0 software package, data were compared between each treatment and its respective control that is basal and hormone treatments separately by one-way analysis of variance (ANOVA) with Fisher's LSD test, after log transformation of data.

RESULTS AND DISCUSSION

Granulosa cells from large follicles produced significantly higher ($P < 0.01$) estradiol-17 β and progesterone than those from small follicles after 24 h of culture (Table 1).

Table 1. Mean $\pm$ SEM basal estradiol-17 β and progesterone secretion by buffalo granulosa cells after 24 h of culture under completely serum-free conditions

Hormone	Follicle size	
	Small	Large
Estradiol-17 β (ng/ml)	2.18 $\pm$ 0.12 ^a	12.91 $\pm$ 0.91 ^b
Progesterone (ng/ml)	0.40 $\pm$ 0.01 ^a	5.57 $\pm$ 0.39 ^b

Data are from 3 independent experiments. Values with different superscripts in the same row differ significantly ($P < 0.01$).

Following the culture of granulosa cells from small follicles with SNAP, estradiol-17 β production in the absence and presence of FSH was found significantly reduced at concentrations of 0.01 ($P < 0.05$), 0.1 and 1 mM SNAP ($P < 0.01$) in comparison to that in respective controls (Fig. 1a). Similarly, estradiol-17 β production by granulosa cells from large follicles was significantly ($P < 0.05$) reduced by SNAP at concentrations of 0.1 and 1 mM in the absence and presence of FSH (Fig. 2a). Secretion of estradiol-17 β by granulosa cells from follicles of both size categories was not

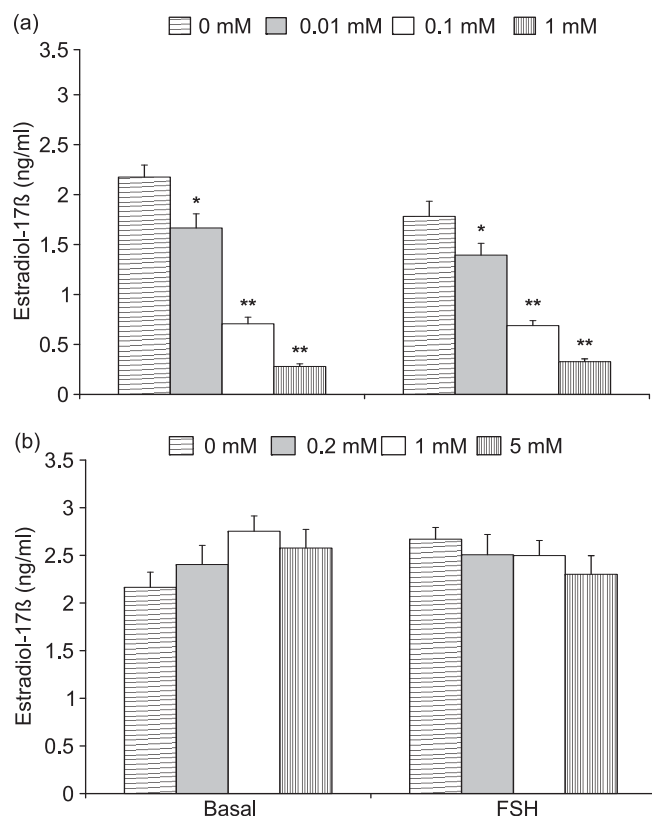


Fig. 1. Effect of (a) NO donor SNAP and (b) NOS inhibitor L-NAME on estradiol-17 β production by buffalo granulosa cells from small follicles cultured under completely serum-free conditions in the absence (basal) or presence of FSH (8 ng/ml). Values marked with asterisks are significantly different from respective controls (* $P < 0.05$; ** $P < 0.01$).

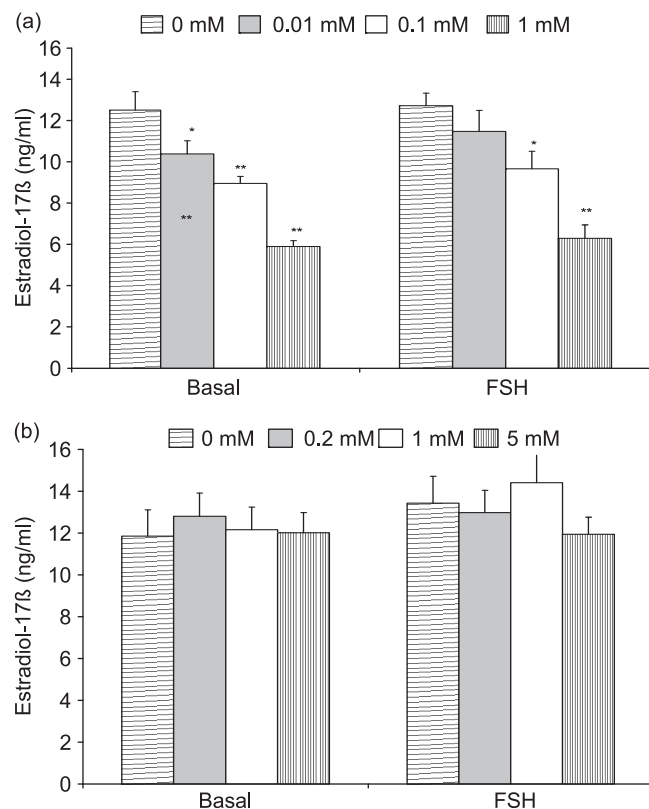


Fig. 2. Effect of (a) NO donor SNAP and (b) NOS inhibitor L-NAME on estradiol-17β production by buffalo granulosa cells from large follicles cultured under completely serum-free conditions in the absence (basal) or presence of FSH (8 ng/ml each). Values marked with asterisks are significantly different from respective controls (* P < 0.05; ** P < 0.01).

affected following treatment with L-NAME at all concentrations tested in the absence and presence of FSH (Fig. 1b, 2b).

In the granulosa cells obtained from small follicles, although SNAP had no effect at the lowest concentration of 0.01 mM, the highest concentration of 1 mM significantly ($P < 0.01$) decreased progesterone production both in the absence and presence of FSH (Fig. 3a). In granulosa cells from large follicles, SNAP inhibited progesterone production at 0.1 and 1 mM concentrations, in the absence and presence of FSH (Fig. 4a). L-NAME had no effect on progesterone production by granulosa cells from follicles of both size categories at all concentrations examined (Fig. 3b, 4b).

A strong inhibitory action of SNAP, a NO donor, was observed on estradiol-17β and progesterone production by granulosa cells obtained from small and large follicles in the presence and absence of FSH. These results are in agreement with those of earlier studies on bovine (Basini *et al.* 1998), and pig (Masuda *et al.* 1997, 2001). The antisteroidogenic effect is unlikely to be due to any deleterious effects of NO on cell viability since in previous studies in cattle, NO showed no effect on cell proliferation (Basini *et al.* 1998), although

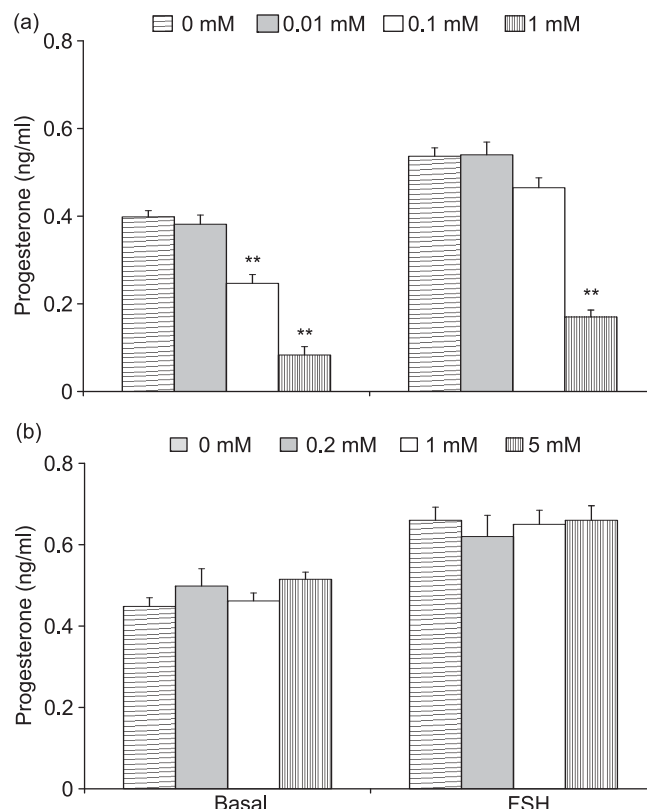


Fig. 3. Effect of (a) NO donor SNAP and (b) NOS inhibitor L-NAME on progesterone production by buffalo granulosa cells from small follicles cultured under completely serum-free conditions in the absence (basal) or presence of FSH (8 ng/ml). Values marked with asterisks are significantly different from respective controls (** P < 0.01).

we cannot rule an effect of NO on cell number in buffalo. However, a recent study reported a stimulatory effect on estradiol secretion at low concentration and inhibitory effect at high concentration and on progesterone secretion by bovine granulosa cells (Faes *et al.* 2009). In this study a different culture system without use of BSA was utilized to study the effect of NO. This may be the reason for stimulatory effect of NO on estradiol secretion. Inhibitory effect of NO on LH stimulated steroidogenesis was also observed similar to that of FSH in large follicle granulosa cell culture (data not shown).

The steroidogenic potential of the granulosa cells is dependent upon the size of the follicles from which they are collected. The higher basal estradiol-17β and progesterone secretion by granulosa cells from large follicles than that by cells from small follicles in this study is in agreement with earlier studies in cattle (Yang and Rajamahendran 1998) and sheep (Gutierrez *et al.* 1997). In terms of follicular fluid concentrations also, large follicles have over 16- and 14-fold higher concentrations of estradiol-17β and progesterone, respectively, than small follicles in buffalo (Palta *et al.* 1998). As the follicle grows in size, the expression of enzyme

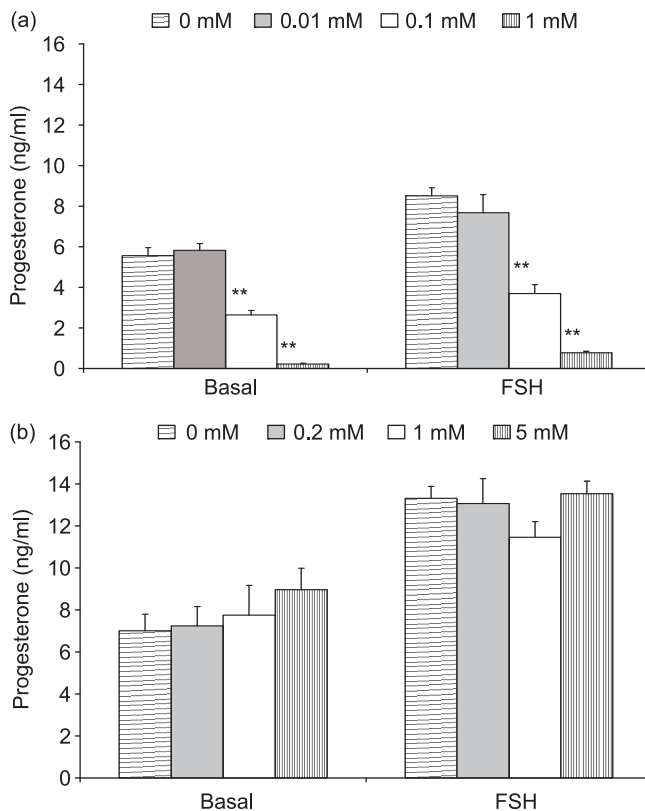


Fig. 4. Effect of (a) NO donor SNAP and (b) NOS inhibitor L-NAME on progesterone production by buffalo granulosa cells from large follicles cultured under completely serum-free conditions in the absence (basal) or presence of FSH (8 ng/ml each). Values marked with asterisks are significantly different from respective controls (** $P < 0.01$).

machinery, P450scc and P450 aromatase, increases, culminates in higher estradiol and progesterone output by large follicle derived granulosa cells (Hamilton *et al.* 1995, Xu *et al.* 1995). Our results suggested that despite the differences in hormonal control and steroidogenic capability between small and large follicles, NO can effectively inhibit estradiol-17 β and progesterone production by granulosa cells from these follicles. This is also indicated by the finding that the magnitude of inhibition by NO was near similar in the absence and presence of FSH.

It was proposed that NO modulates the activity of enzymes by directly reacting with heme- or non-heme iron and iron sulphur complexes located either at the active or allosteric site, making enzymes such as guanylyl cyclase, cyclooxygenase and cytochrome P450 as its possible targets (Stamler *et al.* 1992). NO directly inhibited the granulosa cell cytochrome P450 aromatase activity in pig (Masuda *et al.* 1997) whereas Snyder *et al.* (1996) reported that it inhibited aromatase activity by both direct and indirect actions — with the direct effects attributed to nitrosylation of cysteine at amino acid 437 in the active site of enzyme and the indirect effects involving down regulation of mRNA

levels. In case of bovine granulosa cells the stimulatory effect of sodium nirtopruside on estradiol secretion occurred through cGMP (Faes *et al.* 2009).

In this study, L-NAME, an inhibitor of NOS showed no effect on estradiol-17 β and progesterone production by granulosa cells from small and large follicles, in the absence and presence of FSH. Similar result was obtained when large follicle granulosa cells were cultured in the presence of LH (data not shown). The concentrations at which L-NAME was used in the present study were based on previous reports on pig (Ponderato *et al.* 2000) and horse (Pinto *et al.* 2002) granulosa cells in which it was found to increase estradiol-17 β but not progesterone production. Lack of an effect of L-NAME in the present study could be due to species differences in the efficacy of various NOS inhibitors. Moreover, NO synthesis has a strong dependence on arginine transport from extracellular to intracellular regions, particularly in cell culture models. Whereas other inhibitors, used for reducing NO production by granulosa cells, like L-NMMA (Masuda *et al.* 1997, 2001) can inhibit NO synthesis not only by inhibiting NOS, but also by inhibiting arginine uptake, L-NAME does not affect arginine uptake (Morris *et al.* 1999).

In conclusion, our results showed that NO inhibits estradiol-17 β and progesterone production by granulosa cells from small and large follicles suggesting that NO may be an autocrine/paracrine regulator of steroidogenesis by granulosa cells in buffalo. To confirm that the NO/NOS system is functional in buffalo granulosa cells, the presence of one or more of NOS isoforms in these cells needs to be demonstrated.

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