Asian-Aust. J. Anim. Sci.
Vol. 24, No. 9 : 1204 - 1210
September 2011

www.ajas.info
http://dx.doi.org/10.5713/ajas.2011.10290

Influence of Nitric Oxide on Steroid Synthesis, Growth and Apoptosis of Buffalo (*Bubalus bubalis*) Granulosa Cells *In vitro*

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ABSTRACT : Objective of this study was to examine the effect of sodium nitroprusside (SNP), a nitric oxide (NO) donor on steroid synthesis, growth and apoptosis of buffalo granulosa cells (GCs) *in vitro*. Follicular fluid of antral follicles (3-5 mm diameter) was aspirated and GCs were cultured in 0 (control), 10^{-3}, 10^{-5}, 10^{-7}, 10^{-9} M of SNP for 48 h. To evaluate whether this effect was reversible, GCs were cultured in presence of 10^{-5} M SNP+1.0 mM N^{ω}-nitro-L-arginine methyl ester (L-NAME) a NO synthase (NOS) inhibitor or hemoglobin (Hb, 1.0 μg) as NO scavenger. Nitrate/nitrite concentration was evaluated by Griess method, progesterone and estradiol concentrations by RIA and apoptosis by TUNEL assay. SNP (10^{-3}, 10^{-5}, 10^{-7} M) significantly ($p<0.05$) inhibited estradiol and progesterone synthesis, growth, disorganized GCs aggregates and induced apoptosis in a dose dependent manner. However, 10^{-9} M SNP induced the progesterone synthesis and stimulated GCs to develop into a uniform monolayer. Combination of SNP 10^{-5} M+L-NAME strengthened the inhibitory effect while, SNP+Hb together reversed these inhibitory effects. In conclusion, SNP at greater concentrations (10^{-3}, 10^{-5} and 10^{-7} M) has a cytotoxic effect and it may lead to cell death whereas, at a lower concentration (10^{-9} M) induced progesterone synthesis and growth of GCs. These findings have important implications that NOS derived NO are involved at physiological level during growth and development of buffalo GCs which regulates the steroidogenesis, growth and apoptosis. (**Key Words :** Apoptosis, Buffalo, Granulosa Cell, Nitric Oxide, Steroidogenesis)

INTRODUCTION

Nitric oxide (NO) is a free radical molecule and one of the local factors involved in ovarian folliculogenesis (Jablonka-Shariff and Olson, 2000) and steroidogenesis (Olson et al., 1996). It is synthesized from L-arginine by action of NO synthases (NOSs), an enzyme existing in three isoforms (Moncada et al., 1991). The enzyme responsible for its production has been detected in mouse (Goud et al., 2008), pig (Tao et al., 2004) and cattle (Piers et al., 2009). The presence of endothelial NOS (eNOS) and inducible NOS (iNOS) as a source of NO in ovarian compartments and its function as a steroidogenesis regulator in GC has previously been demonstrated in mice (Janblonka-Shariff and Olson, 1997) and cattle (Basini et al., 1998). However, there are still inconsistencies with regard to the mechanism of NO which affects steroidogenesis and apoptosis in GC. Many studies have shown that NO is a negative regulator of steroids synthesis. NO exerts its effects by binding to the prosthetic heme group of enzymes. Thus, NO may directly bind to the enzyme, P450 aromatase, and inhibit gonadotropin-stimulated steroidogenesis (Van Voorhis et al., 1994). Faes et al. (2009) demonstrated that estradiol (E_2) synthesis by bovine GC was modulated by lesser NO concentrations via the cyclic guanosine monophosphate (cGMP) pathway, but not progesterone (P_4). Whereas, higher concentrations induced mitochondrial damage and the interference on cell cycle progression can led to cell death. Recently, Schwarz et al. (2010) reported that NO deficiency during maturation increases apoptosis in bovine embryos produced *in vitro*. Therefore, the NOS/NO system may have an important role during different developmental stages of follicles but its role in buffalo is not yet clear.

Studies have been shown that NO levels and NOS activities change during follicular growth (Rosselli et al.,

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Received August 3, 2010; Accepted December 28, 2010

1998) and these changes might play an important role in follicle/oocyte development and may also be responsible for follicular atresia in buffalo. Follicular atresia was associated with apoptosis and level of NO in follicular fluid (Hsueh et al., 1994). Chun et al. (1995) reported that NO suppressed follicular apoptosis, suggesting that NO acts as a follicle survival factor. Estimation of NO metabolites such as nitrate/nitrite in follicular fluid/or spent culture medium is used to provide indirect means of estimating endogenous NO and that amounts could be correlate with the apoptosis (Schulz et al., 1999). Although, studies using N^{ω}-nitro-L-arginine methyl ester (L-NAME), an NOS inhibitor to inhibit endogenous and exogenous NO production demonstrated that NO is essential for optimal meiotic maturation both *in vitro* and *in vivo* (Jablonka-Shariff et al., 1999).

The studies available so far provide convincing evidences that NO is involved in many of the ovarian processes but its involvement in controlling reproductive events in buffalo species is not known. The involvement of NO during follicle development may partly be responsible for low reproductive efficiency, low primordial/preantral follicle population and high incidence of follicular atresia in economically important livestock buffalo species especially in Indian context. Using this economically important animal model, the objectives of present study were: i) to verify NO effect using SNP (NO donor) on steroid synthesis, growth and apoptosis in GC ii) to verify whether the effects of SNP are modulated by L-NAME (NOS inhibitor) and heamoglobin (NO scavenger).

MATERIALS AND METHODS

Isolation and *in vitro* culture of GCs

Buffalo ovaries from random stages of the estrous cycle were collected from local abattoir and immediately transported at 25-30°C in 0.9% normal saline to the laboratory within two hour after slaughter. Ovaries were rinsed in pre-warmed phosphate buffer saline (PBS) supplemented with antibiotics (75 mg/L penicillin-G, 50 mg/L streptomycin sulphate). For isolation of GCs, follicular fluid was aspirated from antral follicles (3-5 mm diameter) and placed in plastic tissue culture dish (Nunc, 94 mm) containing 2.0 ml of Dulbecco's Modification of Eagle's Medium (DMEM). Under steriozoom microscope (Olympus SZ 61, Japan), patches of GCs were isolated and washed by centrifugation (800×g/10 min, twice) using 1.0 ml of DMEM. The number of GC was counted with a haemocytometer and the viability was assessed by the dye exclusion method using 0.05% trypan blue. The GC pellet was re-suspended in 500 µl DMEM and 5.0×10^5 GC were seeded in 24-well plates (Nunc) with 1.0 ml of DMEM containing 10% fetal bovine serum (FBS), 2 mM l-glutamine and 0.5 µg/ml follicle stimulating hormone (FSH) (control medium). Addition of SNP at 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} M in control medium was considered as treatment groups. To evaluate the growth inhibitory effect of SNP whether they were reversible or not, GCs were cultured with 10^{-5} M SNP+1.0 mM L-NAME an NO synthase (NOS) inhibitor or 1.0 µg/ml of hemoglobin (Hb) an NO scavenger. These culture plates were placed in a humidified incubation chamber (Thermo Forma, USA) at 38±1°C in a maximum humidified atmosphere having 5% CO_2 in air.

Morphological evaluation of GCs

In vitro cultured GCs from all the aforementioned treatments were evaluated for viability, growth (monolayer formation), adherence to culture plate, steroid (estradiol, progesterone), nitrate/nitrite production and apoptosis. The growth and morphology of GCs from each treatment group was examined using phase contrast microscope (Olympus CKX 41, Japan). At the end of culture, GC viability was evaluated by trypan blue dye exclusion method. GCs were stained with 0.05% (w/v) trypan blue for 5-10 min at the end of culture. At the end of 48 h of culture period, 800 µl cultured medium was collected for steroids and nitrate/nitrite analysis.

Detection of apoptosis in GCs by TUNEL assay

For determination of DNA fragmentation in GCs from different treatment groups, terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end labeling (TUNEL) in-situ detection kit (R&D Systems, Minneapolis, MN) was used. To prevent loss of GCs during assay, poly L-lysin-coated glass slides were used for the analysis of DNA fragmentation. Control and different treatment groups of GCs were immediately transferred on to the slide and fixed in 3.7% formaldehyde in PBS for 15 min at 18-20°C. Slides were washed twice with PBS followed by air drying at room temperature, all the procedures were carried out at 18-20°C unless stated otherwise. The apoptotic signal was recorded as positive when either a diffuse-type or a granular-type dark brown staining of the nucleus appeared. Control slides were run simultaneously during each experiment and all the slides counterstained with methyl green (anti-apoptotic stain). The TUNEL assay was performed as described by Chaube et al. (2007).

Measurement of steroids

To assess the ability of *in vitro* cultured GCs to secrete steroids, estradiol and progesterone were measured in the spent culture medium using commercially available radio-immunoassay kits (Immunotech, Czech Republic). For steroid measurements, replaced medium from each

treatment group was applied in duplicate. The assays were carried out as per the manufacturer protocol. The analytical sensitivity of the progesterone and estradiol kits was 0.02 ng and 4.5 pg respectively.

Measurement of nitrate and nitrite

The NO metabolites (nitrate/nitrite) concentration was measured in spent culture medium of GCs as described by Sastry et al. (2002). Briefly, 100 µl sample or standard (potassium nitrate) was mixed with 400 µl of 0.55 M carbonate buffer (pH 9.0). Approximately, 100 mg activated copper-cadmium alloy filings was added to the samples and the mixture was incubated at 30°C for 1 h with gentle shaking. Then, 100 µl of 0.35 M sodium hydroxide and 400 µl of 0.12 M zinc sulfate were added and incubated for 10 min at room temperature. The tubes were centrifuged at 1,600 g for 10 min and the supernatant (150 µl) was transferred to wells of microtiter plates in duplicate. Then, 75 µl of 1% (w/v) sulfanilamide and 75 µl of 0.1% (w/v) N-naphthalenediamine were gently added and incubated for 10 min. Absorbance was measured at 545 nm in a micro plate reader (Molecular device, India). A dispersion graph was generated with the absorbance values. There was a linear (R^2 = 0.98, $p<0.05$) relation between absorbance and NO concentration.

Statistical analysis

Data of steroids and NO concentrations was repeated twice independently. Statistical significance was determined using SPSS software for Windows, version 7.5 (SPSS GmbH Software, Munich, Germany) by analysis of variance (ANOVA) followed by Duncan's post-hoc multiple comparison test for proportion. The data are presented as the Mean±Standard Error Mean (SEM). A probability of $p<0.05$ were considered to be statistically significant.

RESULTS

At the end of 48 h, GCs cultured with 0 (control) and lower dose (10^{-9} M) of SNP formed a steroidogenically active uniform monolayer (Figure 1a and e), while GCs cultured with medium doses of SNP (10^{-5} and 10^{-7} M) formed disorganized groups with little adherence to the culture plate (Figure 1c and d). The GCs cultured with higher dose (10^{-3} M) of SNP did not form monolayer, had dark color and were flatted in the culture plate (Figure 1b). Co-administration of L-NAME (1.0 mM) with SNP (10^{-5} M) could not overcome the inhibitory effect of SNP, while addition of Hb (1.0 µg/ml) with SNP (10^{-5} M) markedly reduced the SNP induced inhibitory effects (Figure 1f). GCs treated with various doses of SNP (10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} M) significantly ($p<0.05$) decreased the E_2 (Figure 2A) and P_4 (Figure 3A) production in dose dependent manner as compared to control. Although, lower dose (10^{-9} M) of SNP slightly but not significantly stimulated the P_4 production as compared to control (Figure 3A). The addition of L-NAME (1.0 mM) could not overcome the negative effect of SNP while, combination of SNP (10^{-5} M) with Hb (1.0 µg/ml)

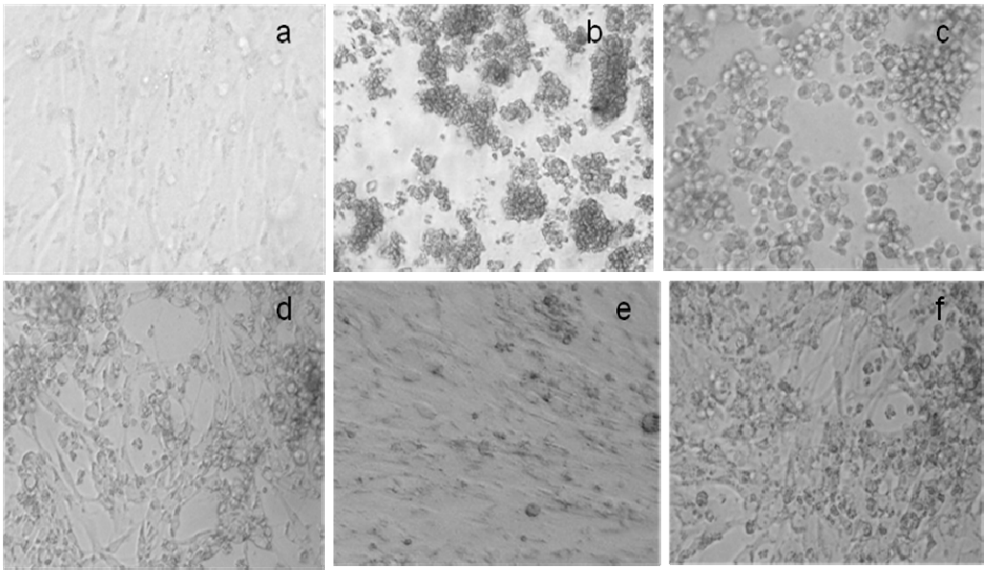

Figure 1. Effect of SNP (0, 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9} M) with or without L-NAME (1.0 mM) and Hb (1.0 µg/ml) on *in vitro* growth of buffalo granulosa cells (GCs). (a) Control group of GCs showing uniform monolayer, (b) 10^{-3} M SNP treated GCs showing dark color, flattening in culture medium and had no monolayer formation, (c) 10^{-5} M SNP treated GCs showing partial attachment of cells to surface of culture plate, (d) partial monolayer formation of GCs with 10^{-7} M SNP, (e) uniform monolayer formation with 10^{-9} M SNP, (f) partial monolayer formed with 10^{-5} M SNP+1.0 µg/ml Hb (Original magnification of photomicrographs was 200×).

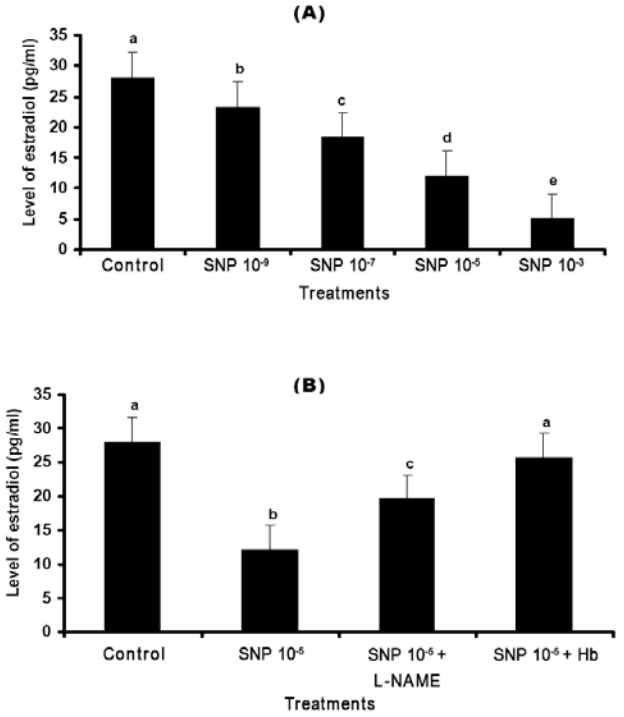

Figure 2. Effect of SNP, L-NAME and Hb on estradiol synthesis in cultured medium of buffalo granulosa cells after 48 h of culture. (A) Level of estradiol after treatment with SNP at various doses (0, 10^{-9}, 10^{-7}, 10^{-5}, 10^{-3} M) and (B) SNP (10^{-5} M) with or without L-NAME (1.0 mM) and Hb (1.0 μg). Each value represents mean±SEM of a total of three determinations from five independents experiments. Bar with different letter of superscripts (a-e) differs significantly at $p<0.05$.

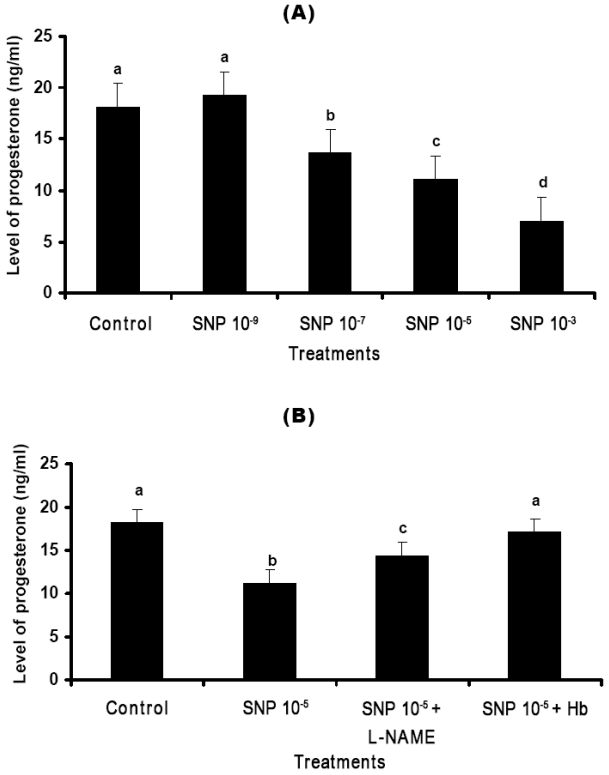

Figure 3. Effect of SNP, L-NAME and Hb on progesterone synthesis in cultured medium of buffalo granulosa cells after 48 h of culture. (A) Level of progesterone after treatment with SNP at various doses (0, 10^{-9}, 10^{-7}, 10^{-5}, 10^{-3} M) and (B) SNP (10^{-5} M) with or without L-NAME (1.0 mM) and Hb (1.0 μg). Each value represents mean±SEM of a total of three determinations from five independents experiments. Bar with different letter of superscripts (a-e) differs significantly at $p<0.05$.

reversed the inhibitory effect on E_2 (Figure 2B) and P_4 (Figure 3B) synthesis. Accumulation of NO metabolites (nitrate/nitrite) in culture medium increased significantly ($p<0.05$) in a dose-dependent manner according to the SNP concentration added (Figure 4A). However, supplementation of L-NAME with SNP 10^{-5} M or Hb (1.0 μg/ml) decreased the level of nitrate/nitrite production in culture medium (Figure 4B). The overall growth and steroidogenesis of GCs were induced by lower dose of SNP (10^{-9} M) while, higher doses of SNP (10^{-3} and 10^{-5} M) induced cell death via apoptosis (Figure 5).

DISCUSSION

Nitric oxide has emerged as a potential regulator of follicular development and implicated in several ovarian processes, including steroidogenesis and ovulation (Tamanini et al., 2003). Several other studies in mammals have provided evidence that NO influences follicular development, apoptosis and participates in the regulation of steroidogenic function (Schwarz et al., 2008; Matta et al., 2009). However, the physiological functions of NO/NOS in buffalo granulosa cells have not been verified previously. GCs treated with higher doses of SNP (10^{-3} and 10^{-5} M) showed disorganized GC aggregates and were not able to adhere over the surface of culture dish to make uniform monolayer. Although GCs treated with lower doses of SNP (10^{-7} and 10^{-9} M) formed a uniform monolayer. These findings suggested that 10^{-3} M SNP kept GCs in the G0/G1 stage and these cells could not progress during the cell cycle and ultimately led to cell death as demonstrated by TUNEL assay. Although these results indicated that an optimum amount of NO (10^{-9} M) is required for physiological regulation of follicular GCs development, but higher concentrations of NO generated in follicles may play a potential role for early death. Inhibitory effects of NO on cell proliferation were demonstrated in vascular smooth muscle cells (VSMCS) (Tanner et al., 2000), and T cells (Yang et al., 1996). Tanner et al. (2000) demonstrated that NO is involved for the over-expression of p21 (cyclin-dependent kinase inhibitors) which induces G1 arrest in VSMCS and this elevated concentration is consistent with

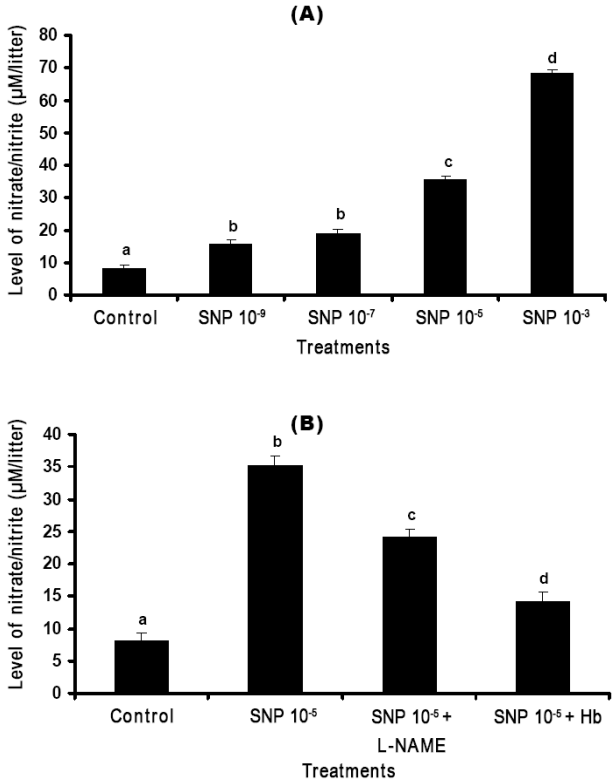

Figure 4. Effect of SNP, L-NAME and Hb on nitrate/nitrite synthesis in cultured medium of buffalo granulosa cells after 48 h of culture. (A) Level of nitrate/nitrite after treatment with SNP at various doses (0, 10^{-9}, 10^{-7}, 10^{-5}, 10^{-3} M) and (B) SNP (10^{-5} M) with or without L-NAME (1.0 mM) and Hb (1.0 µg). Each value represents mean±SEM of a total of three determinations from five independents experiments. Bar with different letter of superscripts (a-e) differs significantly at $p<0.05$.

the anti-proliferative effect of NO.

In this study, effects of SNP were examined on the steroidogenic activity in antral follicular GCs. Results of the present study reveal that SNP (10^{-3}, 10^{-5} 10^{-7} and 10^{-9} M), significantly suppressed E_2 synthesis in a dose dependent manner. Site of NO action and the detailed mechanisms by which NO inhibits E_2 synthesis are not completely understood. A possible explanation is that NO may directly inhibit P-450 aromatase activity in GC cells by binding to a sulfhydryl group near the active site of an enzyme. These results imply that NO has an inhibitory effect on the release of E_2 from GC via suppression of the aromatase activity which is responsible for estrogen biosynthesis. Previous studies have also demonstrated that NO donors inhibited E_2 production in human granulosa-luteal cells and porcine granulosa cells (Snyder et al., 1996; Masuda et al., 1997).

However, it was found that a lower dose of SNP (10^{-9} M) induces P_4 synthesis as compared to control, suggested that a tonic level of NO might be induced aromatase activity in GC. The stimulatory effects of low-NO concentration on P_4 production are possibly mediated through cGMP since cGMP analog mimicked the action of NO donor for steroid production. In general, NO is thought to interact with the iron-containing enzymes, such as soluble guanylyl cyclase (sGC) (Ignarro et al., 1986), cyclo-oxygenase (Salvemini et al., 1993), and a number of the cytochrome P-450 enzymes (Wink et al., 1993). In addition, many authors have demonstrated that the inhibition of NO synthase, enhanced E_2 secretion from cultured ovarian granulosa cells of human (Van Voorhis et al., 1994), rat (Olson et al., 1996) and porcine (Masuda et al., 1997). However, the precise intracellular mechanisms of NO mediating down-regulation of the aromatase gene transcription are not known. These findings suggest that the NO/NOS system may have an important role in the local regulation of ovarian hormonal function, follicle growth and GC differentiation via direct effects on GC. These findings also support the hypothesis that NO is an autocrine/paracrine regulator of GC functions.

L-NAME is a competitive inhibitor of NOS which selectively inhibit NO production by both eNOS and iNOS, but are more potent for eNOS (Moncada et al., 1991). In this study, L-NAME supplemented with 10^{-5} M SNP, enhanced the basal secretion of E_2 and P_4 by GCs in cultured medium. However, L-NAME was not able to

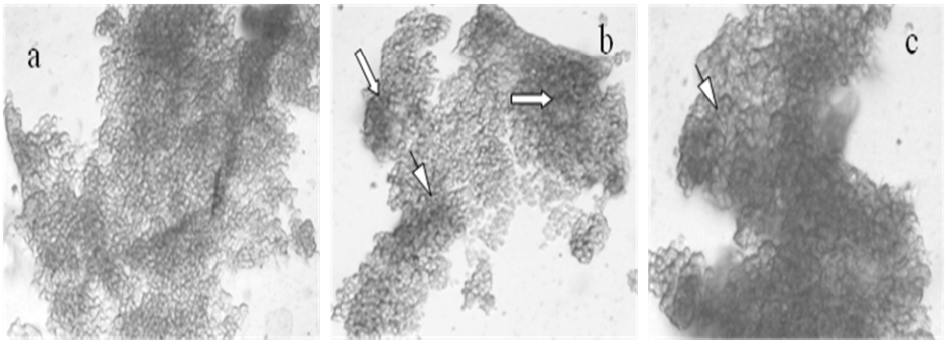

Figure 5. Detection of apoptosis in GCs cultured with 10^{-3} and 10^{-5} M SNP. a) Control group of GCs showing TUNEL negative staining (blue color, counterstained with methyl green), b and c) 10^{-3} and 10^{-5} M SNP treated GCs showing TUNEL positive staining (brown staining ⊣▷). (Original magnification of photomicrographs was 200×).

reverse the inhibitory effect of SNP suggested that other isoforms of NOS might be involved to regulate the steroidogenesis. Thus NOS activity is closely related in the regulation of GCs function and steroidogenesis. The morphological normality, growth and steroid synthesis of GCs are enhanced by the supplementation of Hb with 10^{-5} M SNP hence confirms the reverse effect of Hb. These results suggested that Hb most likely affects the GC development by scavenging excessive GC-derived NO and keeps physiological level of NO in GCs.

NO controls programmed cell death by up-regulation of the tumor suppressor p53 gene, changes in the expression of pro- and anti-apoptotic Bcl-2 family members, activation of caspases, chromatin condensation and DNA fragmentation (Jurisicova et al., 1998). It was found in the present study, higher doses of SNP (10^{-3} and 10^{-5} M) induced cell death in follicular GCs via apoptosis as evidenced by TUNEL positive reaction. These results also showed that L-NAME was not able to inhibit the NO induced apoptosis while, Hb prevents the onset of follicular oocyte apotosis confirming the hypothesis that NO concentration could be a critical factor for cellular survival and function. Although mechanism of apoptosis in GCs treated with SNP was not assessed during this study. However, effects of NO on apoptosis are generally classified as cGMP dependent or independent. NO is able to activate cGMP signaling through the interaction of NO with the heme group of guanylate cyclase. The production of cGMP leads to the activation of cGMP-dependent protein kinases and possibly increased expression of anti-apoptotic proteins (Tilly et al., 1995). NO in greater concentrations also compete with oxygen and binds to heme group enzymes of the respiratory chain, as well as cytochrome oxidase (Sarti et al., 1999). This mechanism triggers calcium release (Richter et al., 1999), disturbances in mitochondrial calcium concentration can kill cells through excessive calcium cycling across the inner mitochondrial membrane which have serious consequences such as disruption of mitochondrial membrane potential, ATP depletion and cell death (Richter et al., 1999).

Measurement of the end products of NO metabolism (nitrate and nitrite) is widely used as an indicator of NO production and NOS activity. The NO synthesized in tissue is completely metabolized to nitrate and nitrite. It was postulated that NO concentration in follicle culture medium could be associated with follicular apoptosis since NO has been reported to induce apoptotic DNA fragmentation depending on the NO concentration in follicular fluid (Kaneto et al., 1995). Results of this study showed that the production of nitrate/nitrite in GCs culture medium is dose dependent and suggested that higher concentrations of NO reacts with other free radical molecules which generates proxynitrate and it interferes the normal proliferative activity of GC led to cell death. Many authors reported that higher concentration of NO induces apoptosis and/or necrosis in rat ovaries (Ellman et al., 1993) and cattle embryos (Orsi, 2006) by competing with oxygen and binds to enzyme heme groups from the respiratory chain, as well as cytochrome oxidase (Sarti et al., 1999).

In conclusion, SNP at higher doses (10^{-3}, 10^{-5} and 10^{-7} M) have a cytotoxic effect which leads to granulosa cell apoptosis and inhibits the steroid production whereas, at lower doses (10^{-9} M) it stimulated survival, growth and progesterone secretion. NO exhibits a dual effect in a dose dependent manner on growth, survival, steroidogenesis and apoptosis of buffalo GCs. These findings have important implications that physiological levels of NO are involved or required during growth and development of buffalo GCs.

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