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Relationship among nitric oxide, progesterone and estradiol-17β concentrations in follicular fluid during follicular development in cattle

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Abstract

The aim of the present study was to investigate changes in nitrate (NO_3^-), progesterone (P_4), and estradiol-17β (E_2) concentrations in follicular fluid (FF) according to the phases of the estrous cycle and to follicle quality and size in cattle. Pairs of ovaries were classified according to the morphological characteristics of the corpus luteum (CL) as mid-luteal or follicular phase. The follicles were classified in three groups according to size: small (<5 mm, n = 120), medium (5 to 8 mm, n = 82), and large (>8 mm, n = 120). Small and medium follicles (n = 2-3) with a clear surface and from the same ovary were punctured and their wall gently scraped. The aspirated FF and granulosa cells (GCs) were pooled separately. Large follicles (high vascularity in theca cells and clear FF) were dissected and FF and GCs collected separately. Follicle integrity was verified by GC-DNA electrophoretic analysis. Follicles were classified as atretic (DNA fragments in multiples of 185 bp) or healthy (without DNA fragments). Concentration of NO_3^- was estimated by chemiluminescence and P_4 and E_2 by radioimmunoassay. The greatest NO_3^- concentration was observed ($P < 0.05$) in medium atretic follicles. Progesterone concentration increased ($P < 0.05$) in atretic follicles according to size ($P < 0.05$) and was greater in atretic than in healthy follicles. Progesterone was also greater ($P < 0.05$) in atretic follicles of the same size collected during the mid-luteal phase compared to the follicular phase. However, 15.5% of follicles that did not show DNA fragmentation had an E_2:P_4 ratio <1. Estradiol-17β concentration increased ($P < 0.05$) in healthy and atretic follicles according to size and was greater ($P < 0.05$) in healthy than in atretic follicles. These results suggest that: 1) NO_3^- did not change with follicular development, except in medium atretic follicles suggesting a possible role in follicular atresia of subordinate follicles and 2) changes in steroids concentrations may precede DNA fragmentation.

Keywords: follicular atresia; bovine; nitric oxide; progesterone; estradiol-17β.

Introduction

Folliculogenesis is a highly selective process during which usually only one follicle assumes dominance while the fate of the rest of the follicles is atresia mediated by apoptosis. The major ovarian cell type undergoing this process is the granulosa cell (GC; Hsueh *et al*., 1994). A biochemical event considered characteristic of apoptosis is the internucleosomal cleavage of genomic DNA into fragments of 180-200 bp in size that separate into a distinctive ladder-like pattern on agarose-gel eletrophoresis (Wyllie, 1980).

The estradiol (E_2) synthesized by GCs is considered one of the major regulators of ovarian follicular development and atresia (Ginther *et al.*, 2000), and the E_2:P_4 ratio has been used to evaluate follicle quality. Other non-steroidal, intra-ovarian factors also synthesized by GCs are involved in ovarian physiology, among these is nitric oxide (NO; Basini *et al*., 1998). This free radical is synthesized by oxidation of L-arginine to NO plus L-citrulline by one of the three isoenzymes of nitric oxide synthase (NOS). The NO in an oxygen-containing aqueous solution has a short half-life that is attributed to rapid oxidation to nitrite (NO_2^-) and nitrate (NO_3^-) and requires the presence of oxyhemoprotein (Ignarro *et al*., 1993). Thus, the measurement of NO_2^- and NO_3^- concentrations in aqueous biological samples is used as an indirect method to estimate NO concentration (Schulz *et al*., 1999).

The majority of studies regarding the role of NO in follicular development (Jablonka-Shariff and Olson, 1997; Matsumi *et al*., 1998, 2000), GC steroidogenesis (van Voorhis *et al*., 1994; Snyder *et al*., 1996; Ishimaru *et al*., 2001), apoptosis (Ellman *et al*., 1993; Endo *et al*., 1993; Chun *et al*., 1995), and oocyte maturation (Jablonka-Shariff *et al*., 1999; Jablonka-Shariff and Olson, 2000) have been realized in the mouse, rat, and human. Basini *et al*. (1998) verified the role of NO in small and large follicles, GC steroidogenesis, and *in vitro* atresia in cows. However, no studies have been done to relate the profile of steroids and NO concentrations in follicular fluid (FF) during follicular development and atresia *in vivo* in cattle. The purpose of the present study was to verify the presence of NO_3^- in FF from healthy and

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atretic follicles during the follicular development and elucidate its relationship with the E_2:P_4 ratio.

Materials and Methods

All the reagents were obtained from Sigma Chemical Company (St. Louis, MO, USA) except those that are specified.

Ovarian collection

Pairs of ovaries were collected from cyclic zebu cows at a local abattoir and transported to the laboratory in a thermos containing physiological solution and antibiotics. Ovaries in the mid-luteal and follicular phase were distinguished and grouped according to corpus luteum (CL) morphology (Ireland *et al*., 1980). The CL from ovaries in the mid-luteal phase was characterized by the change from a red-brown apex into an orange/yellow color and by visible vasculature over the apex. Ovaries in the follicular phase were characterized by CLs that had a light yellow to white color and an internal orange to yellow appearance, which is indicative of regression. The vasculature on the surface of CLs from the follicular stage was no longer visible and one large follicle (>10 mm in diameter) was present in one or both ovaries. According to their diameter, the follicles were classified in 3 groups (Wise, 1987): small (<5 mm, n = 120), medium (5 to 8 mm, n = 82), or large (>8 mm, n = 120).

Follicular fluid and GC collection

Follicles (n = 2-3) with the same size of the same ovary and with 70% its exposed surface clear (Grimes *et al*., 1987) were punctured (18-gauge needle) to obtain FF and their follicle wall scraped to collect the granulosa GC pool. This procedure was done only for small and medium follicles. The FF and GC pool was centrifuged at 184 x *g* for 10 min at 4°C. The supernatant was stored, and the pellet of GCs was washed in 500 µl cold phosphate-buffered saline (PBS, pH = 7.4). Follicular fluid and GCs were pooled to obtain the ideal volume to determine steroid and NO_3^- concentrations and to evaluate follicle quality, respectively. The large follicles (theca cells with high vascularity and clear exposed surface) were dissected carefully from the ovary using a scalpel and thin-tip surgical scissors under a stereomicroscope (20x). The FF was individually collected and processed as mentioned above except for GCs, which were gently scraped from the follicle wall directly into cold PBS (500 µl, pH = 7.4). Granulosa cells of each group were transferred into 1.5-ml microcentrifuge tubes and centrifuged at 184 x *g* for 10 min at 4°C. The pellet was washed with cold PBS (500 µl, pH = 7.4) and centrifuged at 184 x *g* for 10 min once again. The GCs and FF were stored at -20 and -70°C, respectively, until DNA analysis and steroid and NO_3^- quantification, respectively. The collection of FF and GCs occurred within 2 to 3 h. The use of slaughterhouse material and 2 to 3 h elapsing between slaughter and analysis of GCs did not significantly influence the amount apoptosis in cows (Hagemann *et al*., 1999).

Radioimmunoassay for steroids

Steroid concentrations in FF were quantified by radioimmunoassay (RIA) without extraction using free fraction adsorption by the charcoal-dextran method as described by James (1980) and Rosa e Silva *et al*. (1993). The samples were diluted 1/100. The minimum detectable concentrations of P_4 and E_2 were 0.32 ng/ml and 0.043 ng/ml respectively. The average intra- and interassay coefficients of variation for FF samples of the 322 follicles, replicated twice in different assays, were 5.8% and 4.6% for P_4, and 5.6% and 4.5% for E_2, respectively.

Nitrate assay

After the follicular fluid samples were thawed, 100 µl of 95% cold ethanol (Merck S.A. Indústrias, Rio de Janeiro, Brazil) was added to 50 µl of FF to remove protein from the samples. The tubes were vortexed and incubated at 0°C for 30 min. Next, the samples were centrifuged at 9.168 x *g* for 5 min. The supernatant was transferred to a microcentrifuge tube for NO_3^- analysis by chemiluminescent gas phase between NO and ozone, using a highly sensitive detector (NO Analyzer model 280 - NOA™, Sievers Instruments). Before the above procedure, all the tubes were washed with 95% cold ethanol to remove possible contaminants. Since very little or no NO_2^- is normally found in FF, no attempt was made to quantify the amounts of NO_2^-.

Analysis of internucleosomal DNA cleavage

Total cellular DNA was extracted for each GC sample according to Wyllie (1980) with some modifications. The GC pellet (2 x 10^6 cells) was thawed and lysed with 206 µl of lysis buffer containing: 5 µl proteinase K (20 mg/ml); 1 µl 10% SDS; 50 µl TES (40 mM Trizma base, 80 mM EDTA, and 1.6 mM NaCl, pH 7.0-8.0); and 150 µl distilled water for 60 min at 50°C. Next, 1 µl RNAse (10 mg/ml; Merck S.A. Indústrias, Rio de Janeiro, Brazil) was added, and the samples were vigorously mixed and incubated at 50°C for 60 min. The DNA was precipitated by centrifugation (13.4 x *g* for 20 min) after the addition of 2/3 of the sample volume of cold isopropanol and incubation at -20°C for 2 to 3 h. The nucleic acid was reprecipitated with 200 µl of 95% cold ethanol (Merck S. A. Indústrias, Rio de Janeiro, Brazil), pelleted by centrifugation (13.4 x *g* for 5 min), and then air dried. Each sample was resuspended in 25 µl TE

buffer (10 mM Tris, pH 7.5; 1 mM EDTA, pH 8.0) and incubated overnight at room temperature. Then, 5 µl of 6x sample buffer (40% sucrose; 0.25% bromophenol blue in TE buffer) was added to each sample. The samples (30 µl) were submitted to 1.6% agarose-gel electrophoresis, submerged in 1x TBE (Merck S.A. Indústrias, Rio de Janeiro, Brazil) at 80 V for 120 min. The gels were stained with ethidium bromide (0.5 µg/ml) for 15 min and washed in double-distilled H_2O. DNA fluorescence was viewed and photographed with a UV transilluminator (Eagle model, Stratagene). Apoptotic hybridoma cells were used as a positive control and a 100-bp DNA standard (New England Biolabs Ltd, Ontario, Canada) as a molecular-weight marker. The atretic follicles were characterized by the presence of fragmented DNA in GCs (DNA fragments with multiples of 185 bp) visualized as a distinctive ladder pattern in agarose-gel electrophoresis; healthy follicles were characterized by absence of DNA fragments (Fig. 1).

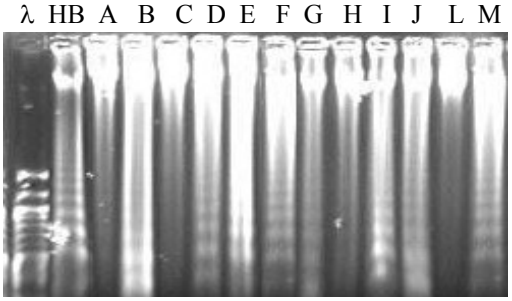

Figure 1. DNA agarose gel electrophoresis analysis of granulosa cells. [100 bp DNA standards (λ); hybridoma cells (HB); without fragmented DNA (A, C, and L); with fragmented DNA (B, D, E, F, G, H, I, J, and M)].

Statistical analyses

In order to test the effects of follicle size and quality, and of the E_2:P_4 ratio (<1 or >1) on NO_3^- concentration ANOVA was used. In addition, the same test was used to evaluate the interactions between phase of the estrous cycle and follicle size and quality on P_4 and E_2 concentrations. Both analyses were conducted using the SAS Computational Program (SAS, 1996). When significant differences were found, means were compared by Turkey's test. Pearson's correlation analysis was used to verify the relationship between NO_3^- concentration and the E_2:P_4 ratio of healthy and atretic follicles according to follicle size. Data were transformed to conform to the assumptions of ANOVA so that error would be normally distributed with constant variance. Data are presented as mean ± SEM. Differences, effects, and interactions were considered significant at $P < 0.05$.

Results

Twenty eight percent of the follicles were healthy (had no DNA fragments). Of these, 13.3% had an E_2:P_4 ratio >1 and 15.5% had E_2:P_4 ratio <1. As for the atretic follicles (71.2 %), 64.2% had an E_2:P_4 ratio >1 and only 6.8% had E_2:P_4 >1.

As ANOVA showed no significant effect of the estrous cycle on NO_3^- concentration, statistical analysis has been restricted to the interaction between follicle size and quality and E_2:P_4 ratio on NO_3^- concentration.

Table 1 shows that 5-8 mm atretic follicles with an E_2:P_4 ratio <1 had a greater ($P < 0.05$) NO_3^- concentration than healthy follicles of the same size and atretic follicles <5 mm and >8 mm.

Table 1. Mean (± SE) NO_3^- (µM) concentration in ovarian follicular fluid according to follicle size and quality and estradiol-17β: progesterone ratio.

Follicle quality	E_2:P_4 ratio	NO_3^- (µM)		
		<5 mm	5 – 8 mm	>8 mm
Healthy	<1	$8.57 \pm 1.50^{a, A}$	$9.70 \pm 1.50^{a, A}$	$9.6 \pm 1.50^{a, A}$
Atretic	<1	$9.89 \pm 0.70^{a, A}$	$12.80 \pm 0.70^{b, B}$	$9.80 \pm 0.70^{a, A}$
Healthy	>1	$10.25 \pm 2.30^{a, x}$	$8.66 \pm 2.30^{a, x}$	$8.54 \pm 2.30^{a, x}$
Atretic	>1	$8.80 \pm 3.10^{a, x}$	$10.30 \pm 3.10^{a, x}$	$10.20 \pm 3.10^{a, x}$

[ab]Means with different letters within the same row are different ($P < 0.05$).

[AB]Means with different letters within the same column are different ($P < 0.05$) among healthy and atretic follicles of the same size with an E_2:P_4 ratio <1..

[x] Means within the same column of healthy and atretic follicles of the same size with an E_2:P_4 ratio > 1 are not different ($P > 0.05$).

Progesterone concentration increased with follicle size only in atretic follicles, and was greater ($P < 0.05$) in 5-8 mm atretic follicles than in healthy follicles of the same size (Table 2). E_2 increased ($P < 0.05$) with follicle size, having greater values ($P < 0.05$) in healthy than in atretic follicles for all size groups (Table 2).

Progesterone concentration was greater ($P < 0.05$) in the mid-luteal phase group for 5 to 8 mm and >8 mm atretic follicles than for healthy ones (Table 3). In addition, only >8 mm atretic follicles had a greater P_4 concentration ($P < 0.05$) during the mid-luteal than follicular phase (Table 3). Estradiol-17β concentration during the mid-luteal phase was greater ($P < 0.05$) in all size groups of healthy follicles than in atretic groups, while in follicular phase, only >8 mm healthy follicles had greater E_2 concentration ($P < 0.05$) than atretic ones.

Table 2. Mean ± SEM Progesterone and estradiol-17β concentrations in ovarian follicular fluid according to size and quality of the follicle.

Follicle size	Follicle quality	Progesterone (ng/ml)	Estradiol (ng/ml)
<5 mm	Healthy	$32.0 \pm 29.0^{a, x}$	$18.4 \pm 9.0^{a, x}$
	Atretic	$68.4 \pm 29.0^{A, x}$	$3.3 \pm 9.0^{A, y}$
5 – 8 mm	Healthy	$106.5 \pm 28.0^{a, x}$	$35.0 \pm 8.0^{a, x}$
	Atretic	$160.5 \pm 28.0^{B, y}$	$106.5 \pm 8.0^{B, y}$
>8 mm	Healthy	$50.6 \pm 21.0^{a, x}$	$55.4 \pm 6.6^{b, x}$
	Atretic	$188.7 \pm 21.0^{B, y}$	$35.3 \pm 6.6^{B, y}$

[ab]Means with different letters within the same column are different ($P < 0.05$) among healthy follicles of different sizes.
[AB]Means with different letters within the same column are different ($P < 0.05$) among atretic follicles of different sizes.
[(x, y)] Means with different letters in parenthesis within the same column are different ($P < 0.05$) among follicles of the same size.

Table 3. Mean ± SEM progesterone and estradiol-17β concentrations (ng/ml) in ovarian follicular fluid according to size and quality of the follicles and estrous cycle phase.

Follicle diameter	Estrous cycle phase	Follicle quality			
		Progesterone (ng/ml)		Estradiol (ng/ml)	
		Healthy	Atretic	Healthy	Atretic
<5 mm	Mid-luteal	$27.7 \pm 34.0^{a, A}$	$76.8 \pm 34.0^{a, A}$	$17.8 \pm 6.0^{a, A}$	$3.7 \pm 6.0^{b, A}$
	Follicular	$43.7 \pm 57.0^{a, A}$	$35.9 \pm 57.0^{a, A}$	$20.1 \pm 17.0^{a, A}$	$2.0 \pm 17.0^{a, A}$
5-8 mm	Mid-luteal	$120.8 \pm 3.0^{a, A}$	$175.0 \pm 3.0^{b, A}$	$41.6 \pm 10.0^{a, A}$	$34.7 \pm 10.0^{b, A}$
	Follicular	$73.9 \pm 52.0^{a, A}$	$119.1 \pm 52.0^{a, A}$	$23.8 \pm 16.0^{a, A}$	$15.5 \pm 16.0^{a, A}$
>8 mm	Mid-luteal	$62.5 \pm 26.0^{a, A}$	$249.0 \pm 26.0^{b, A}$	$47.4 \pm 8.2^{a, A}$	$37.3 \pm 8.2^{b; A}$
	Follicular	$30.0 \pm 30.0^{a, A}$	$120.9 \pm 30.0^{a, B}$	$69.6 \pm 11.0^{a, A}$	$31.2 \pm 11.0^{b; A}$

[ab]Means with different letters within the same row are different ($P < 0.05$).
[AB]Means with different letters within the same column differ for follicles of the same size and quality depending on the estrous cycle phase ($P < 0.05$).

Discussion

The present study demonstrated for the first time the presence of nitrate (NO_3), a major nitric oxide (NO) metabolite in cow FF which has a possible role in follicle atresia. The increase in NO_3^- was only observed in FF of medium atretic follicles (5-8 mm) with an E_2:P_4 ratio <1, suggesting its involvement in atresia of subordinates follicles. However, the negative relationship between NO_3^- and the E_2:P_4 ratio in healthy and atretic follicles of all sizes was not significant. Basini *et al.* (1998) observed that nitrite (NO_2^-) concentration, another stable metabolite of NO, decreases in FF with follicular development and that E_2 and P_4 concentrations increase in cows. However, they did not verify apoptosis of the GCs from the follicles that had FF collected. Nitrate is a stable metabolite of NO, and the samples from this experiment were frozen at -70°C immediately after the collection. In spite of these facts, one cannot exclude the possibility of changes in nitrate levels in follicular fluid have occurred from the time the ovaries were collected until the moment of FF sample collection.

Atresia is mainly induced during the follicle dominance phase and affects follicles of all sizes. Eighty-five percent of the follicles from ovaries taken at any time during the estrous cycle are atretic (Hsueh *et al*., 1994). In addition, subordinate follicles (6-8 mm) showed the greatest level of apoptosis (Hagermann *et al*., 1999). The data from the present study suggest a possible involvement of NO in follicular atresia of medium follicles. However, this data demonstrated a temporal change and not a relation of cause and effect, which demands further investigation.

Several researchers have demonstrated a role for NO in ovarian physiology. An NO-mediated inhibition of E_2 synthesis has been demonstrated *in vitro* in GC cultures from rats (Van Voorhis *et al*., 1994), cows (Basini *et al*., 1998, 2000), pigs (Grasselli *et al*., 2001), and mares (Pinto *et al*., 2003), and it suppresses GC apoptosis in rats (Chun *et al*., 1995) and cows (Basini *et al*., 1998). However, Hurwitz *et al*. (1992) and Ellman *et al*. (1993) suggested that NO has a cytotoxic effect on GCs. Sugino *et al.* (1996) did not verify a significant difference in NO_2^- levels in FF of small and large follicles, whereas Anteby *et al*. (1996) and Tao *et al*. (1997) verified a positive correlation between NO_3^-/NO_2^- and E_2 concentrations in woman that received ovarian stimulation. Additionally, Bonello *et al*. (1996) also demonstrated a decrease in E_2 synthesis in rats treated with eCG/hCG through ovarian perfusion with NOS enzyme inhibitor (L-NAME, Nω-Nitro-L-arginine methyl ester), suggesting that NO could positively influence E_2 synthesis. Thus, the results of research regarding NO in steroidogenesis and apoptosis are controversial and seem to vary with experimental design.

It has been demonstrated that NO in high concentrations induces apoptosis and/or necrosis in rat ovaries (Ellman *et al*., 1993), human epithelial endometrial cells (Li *et al*., 1999), and bovine embryos (Orsi, 2006). Nitric oxide in high concentrations binds to enzyme heme groups in the respiratory chain as well as cytochrome oxidase and inhibits mitochondrial activity causing cell death by hypoxia (Clementi *et al*., 1999; Sarti *et al*., 1999). After several hours of NO exposure, cytochrome oxidase enzyme can be irreversibly inhibited due to NO conversion into reactive nitrogen species (peroxynitrite), which inhibit various cellular respiratory sites. Peroxynitrite is a strong oxidant, and its production can cause lipid peroxidation increasing mitochondrial permeability to protons and other ions, and thus DNA damage.

In this experiment, P_4 concentration was greater than E_2 in follicular fluid of healthy <5 mm and 5-8 mm follicles because 15.5% of follicles that did not show DNA fragmentation had an E_2:P_4 ratio <1, suggesting that changes in steroid concentrations may precede DNA fragmentation. Garret and Guthrie (1996) and Jolly *et al*. (1994) also demonstrated in pigs and cattle, respectively, that an E_2:P_4 ratio <1 occurred before DNA cleavage. Yang and Rajamahendran (2000) demonstrated in cultured bovine GCs that greater P_4 concentrations were not capable of inducing DNA fragmentation directly, but increased expression of genes (Bax) involved in the apoptotic cascade. Thus, it is possible that the change in the steroidogenic pathway lead to apoptosis in GCs.

The results of the current study experiment demonstrated that 6.8% of follicles had DNA fragmentation (atretic) and an E_2:P_4 ratio >1 due the fact that the majority of GCs were not affected by the apoptotic process, thus conserving their steroidogenic activity. Nevertheless, the possibility that DNA fragmentation was still not able to induce a decrease in mRNA of steroidogenic enzymes cannot be excluded. Similar results were already described in cows (Jolly *et al*., 1994) and sheep (Jolly *et al*., 1997) in which 71% and 49% of the atretic follicles had an E_2:P_4 ratio >1, respectively. Alternatively, it is also plausible that a decrease in GC gonadotropin response and follicular estrogen synthesis activates endonucleases culminating with follicular atresia. This theory is supported by previous studies on steroid-sensitive endonuclease activity in the rat mammary gland (Cho-Chung, 1978), thymus (Compton and Cidlowski, 1986) and prostate (Kyprianou and Isaacs, 1988), and ovine GC culture (Lund *et al*., 1999).

Although the mechanisms underlying follicular atresia are not well known, DNA damage, such as that initiated by oxidative free radicals, has been proposed as a possible mechanism leading to the activation of the apoptotic cascade in atretic follicles (Buttker and Sandstrom, 1994). Basini *et al*. (1998) demonstrated that cultured bovine GCs synthesize NO and that NO donors at high concentrations inhibit DNA fragmentation of GCs in small and large follicles while NO donors at low concentrations stimulate this fragmentation in large follicles. The present study verified a high NO_3^- concentration in atretic 5-8 mm follicles. Considering that medium subordinate follicles regressed more in the follicular dominance phase (Hsueh *et al*., 1994), this work suggests that sudden changes in NO concentration could be one of the mechanisms involved in follicular atresia of subordinate follicles.

These results demonstrate that apoptotic events and changes in E_2 and P_4 concentrations and NO could be related to follicle quality. The fact that there was no DNA cleavage does not necessarily mean that it is a healthy follicle since the changes can occur at the level of steroids, which could lead to activation of the apoptotic cascade culminating in follicular atresia. Nitric oxide concentrations together with the FF hormone profile and DNA analysis could be helpful to study atresia. More research is necessary to understand the role of NO in steroidogenesis and follicular atresia in bovine GCs.

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