

JOURNAL OF ANIMAL SCIENCE

The Premier Journal and Leading Source of New Knowledge and Perspective in Animal Science

The endothelial nitric oxide synthase/nitric oxide system is involved in the defective quality of bovine oocytes from low mid-antral follicle count ovaries

I. Tessaro, A. M. Luciano, F. Franciosi, V. Lodde, D. Corbani and S. C. Modena

J ANIM SCI 2011, 89:2389-2396.

doi: 10.2527/jas.2010-3714 originally published online March 18, 2011

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://jas.fass.org/content/89/8/2389>



American Society of Animal Science

www.asas.org

The endothelial nitric oxide synthase/nitric oxide system is involved in the defective quality of bovine oocytes from low mid-antral follicle count ovaries¹

I. Tessaro, A. M. Luciano, F. Franciosi, V. Lodde, D. Corbani, and S. C. Modena²

Dipartimento di Scienze Animali, Università degli Studi di Milano, 20133 Milano, Italy

ABSTRACT: In a previous survey concerning cows of reproductive age, we demonstrated that oocytes isolated from ovaries with <10 medium antral follicles of 2 to 6 mm in diameter (low ovaries; Lo) show less developmental competence than oocytes collected from ovaries with >10 medium antral follicles (high ovaries; Hi). The aim of the present study was to evaluate whether a defective endothelial nitric oxide synthase/nitric oxide (eNOS/NO) system and vasculature in healthy medium antral follicles is likely to reduce oocyte competence from Lo ovaries. Thus, experiments were conducted to 1) immunolocalize eNOS protein during folliculogenesis; 2) quantify eNOS protein/vasculature in the follicle wall; and 3) verify if NO donor, S-nitroso acetyl penicillamine (SNAP) administration during in vitro maturation affects developmental competence of oo-

cytes isolated from Lo ovaries. Endothelial nitric oxide synthase protein was detected in granulosa and theca cells, as well as in blood vessels from primordial to antral follicles. Quantitative analysis indicated that in medium antral follicles from Lo ovaries, eNOS protein expression and vasculature were reduced ($P < 0.05$). The addition of SNAP improved blastocyst and hatching rates of oocytes from Lo ovaries, promoting a percentage similar to oocytes from Hi ovaries, and reduced the percentage of apoptotic nuclei in in vitro-produced blastocysts ($P < 0.05$). Results from our study suggest that in bovine ovaries with small mid antral follicle number, a defective eNOS/NO system is related to a reduced follicle vasculature and may affect oocyte quality, thus inducing a premature decline of fertility.

Key words: cow, endothelial nitric oxide synthase, medium antral follicle, oocyte developmental competence, vasculature

©2011 American Society of Animal Science. All rights reserved.

J. Anim. Sci. 2011. 89:2389–2396
doi:10.2527/jas.2010-3714

INTRODUCTION

During aging, the number of follicles, together with the quantity and quality of oocytes, declines rapidly in women (Coccia and Rizzello, 2008) and cows (Ericsson et al., 1976; Malhi et al., 2005). Even individuals in their reproductive age showing a small number of follicles can undergo suboptimal fertility, diminished ovarian function, and poor oocyte quality (Ireland, J. L., et al., 2008; Alviggi et al., 2009; Ireland, J. J., et al., 2009). In particular, our previous study in cows of reproductive age indicated that in cows whose ovaries showed <10 mid-antral follicles from 2 to 6 mm in diameter and no follicles ≥ 10 mm (low ovaries; Lo),

oocytes had a reduced developmental capability compared with age-matched controls having both ovaries with >10 mid-antral follicles (high ovaries; Hi; Modena et al., 2007). To date, the mechanisms whereby the large variation in follicle numbers may have an impact on ovarian function, oocyte quality, and fertility are poorly understood.

Because follicles are highly vascularized ovarian structures (Van Blerkom, 1998), a reduction in vascularity may affect follicle number, as well as oocyte quality (Borini et al., 2001; Monteleone et al., 2008) and quantity (Tatone et al., 2008). Several angiogenic factors, including endothelial nitric oxide synthase (eNOS), are expressed in ovarian follicles (Grazul-Bilska et al., 2006). Endothelial NOS is involved in follicular and luteal angiogenesis through the production of a free radical gas, nitric oxide (NO; Grazul-Bilska et al., 2006). Importantly, NO also mediates folliculogenesis, steroidogenesis, ovulation, oocyte maturation, and ultimately, embryo development (Rosselli et al., 1998; Thaler and Epel, 2003).

¹This project was supported by Programma dell'Università per la Ricerca (PUR) 2008, grant number 12-1-2884002-27 (SM), University of Milan.

²Corresponding author: silvia.modina@unimi.it

Received November 18, 2010.

Accepted March 16, 2011.

The objective of this study was to assess whether the poor developmental competence of oocytes derived from ovaries with a small mid-antral follicle count (Modina et al., 2007) can be due to the follicular environment and, in particular, to reduced vascularity and a defective eNOS/NO system.

MATERIALS AND METHODS

Bovine ovaries were obtained from a local abattoir (INALCA JBS S.p.A., Ospedaletto Lodigiano, LO, IT 2270M CE, Italy) and derived from pubertal dairy cows in reproductive age, 4 to 8 yr old, subjected to routine veterinary inspection consistent with specific health requirements as stated in EEC Directive 89/556 and following modifications.

All chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO), unless otherwise stated.

Ovary Collection and Classification

Ovaries used for histochemistry and immunohistochemistry were cryopreserved to be transferred to the laboratory, whereas ovaries for oocyte isolation were transported in sterile saline maintained at 26°C.

From each slaughtered animal, ovaries were isolated and classified into the 2 previously described categories, Lo and Hi ovaries (Modina et al., 2007). For this study, ovaries isolated from animals with only Lo ovaries or Hi ovaries were used. The Hi ovaries represent the age-matched control group. In both categories, the presence or the absence of a corpus luteum was not taken into account, as previously stated (Gandolfi et al., 1997; Modina et al., 2007). For each ovary, all visible antral follicles were measured using a ruler, and medium antral follicles with a surface diameter between 2 and 6 mm were counted.

Lectin BS-1 Binding and eNOS Localization and Quantification in Hi and Lo Ovaries

Histochemistry and Immunohistochemistry. Lectin BS-1 (from *Bandeiraea simplicifolia*), a well-known marker of endothelial cells (Grazul-Bilska et al., 2007), was used in the present study to detect amount of vascularity of ovarian tissues. All the procedures were conducted at room temperature, unless otherwise stated. Hi and Lo ovaries were cut transversely into 2 to 4 pieces (cross-sections) and fixed in B5 fixative (Bio-Optica, Milan, Italy) for 12 to 24 h. Then, they were dehydrated by a graded series of ethanol, cleared with xylene, paraffin embedded, and sectioned at 5 μ m to be transferred onto glass slides, previously treated with Vectabond (Vector Laboratories, Burlingame, CA) to enhance the adherence of tissues.

Detection of Lectin BS-1 binding was performed as described previously (Grazul-Bilska et al., 2007). Briefly, ovarian cross-sections were incubated with 3% (vol/

vol) hydrogen peroxide (H_2O_2) in methanol for 30 min to eliminate endogenous peroxidase activity, then incubated with biotin-labeled Lectin BS-1 (10 μ g/mL, Vector Laboratories) for 2 h at 37°C, followed by 2 washes in buffer with HEPES 10 mM and NaCl 0.15 M, and a 30-min incubation with avidin:biotinylated enzyme complex (Vectastain Elite ABC kit, Vector Laboratories). For color development, all sections were incubated with 3,3' Diaminobenzidine (DAB) substrate (DAB substrate kit for peroxidase, Vector Laboratories) for 2 min. After staining, some sections were counterstained with hematoxylin (Hematoxylin QS Nuclear Counterstain, Vector Laboratories). Positive control was performed on a cross-section of bovine aorta; for the negative control, Lectin BS-1 was omitted.

Endothelial NOS expression and localization analysis was performed as described previously (Grazul-Bilska et al., 2007). Antigen retrieval was carried out using a 10 mM citrate buffer solution (pH 6) in a pressure cooker for 1 min. Endogenous peroxidase activity was eliminated as performed for Lectin BS-1; sections were first incubated with 10% (vol/vol) normal goat serum, 0.3% (vol/vol) Triton X-100, and 3% (wt/vol) BSA in PBS for 30 min to block nonspecific binding of secondary antibody. Then, sections were incubated for 30 min with an anti-eNOS antibody developed in rabbit and specific for bovine protein [diluted 1:400 in PBS with 1% (wt/vol) BSA and 0.3% (vol/vol) Triton X-100]. Primary antibody was detected by using a biotinylated anti-rabbit IgG [Vector Laboratories, diluted 1:400 in PBS with 1% (wt/vol) BSA], and the avidin:biotinylated enzyme complex (Vector Laboratories). For color development, DAB substrate (Vector Laboratories) was used as described above. A negative control was performed by omitting the primary antibody. After immunostaining, some tissue sections were counterstained with hematoxylin (Vector Laboratories).

Quantification of Lectin BS-1 Binding and eNOS Protein Expression by Image Analysis.

Lectin BS-1 binding was quantified in 3 follicles from each of 5 animals with Hi ovaries and in 3 follicles from each of 5 animals with Lo ovaries. The abundance of eNOS protein expression was quantified in a total of 3 follicles from each of 6 animals with Hi ovaries and from each of 7 animals with Lo ovaries. Lectin BS-1 binding and eNOS protein expression were evaluated in granulosa and theca layer areas of healthy medium antral follicles. A follicle was considered morphologically healthy when it exhibited intact basal membrane and organized granulosa cell layers with only occasional pyknotic nuclei or atretic bodies in granulosa cells or follicular antrum (Lussier et al., 1987; Yang and Rajamahendran, 2000; Ireland, J. L., et al., 2008; Rodgers and Irving-Rodgers, 2010). After the evaluation of the percentage of healthy follicles, quantitative analysis was supported by images of selected healthy follicle, taken from 9 randomly chosen fields in 3 different slides with no counterstaining. In each field, the area exhibiting positive staining was measured by using an image

analysis system (ImageJ 1.41g, NIH; Abramoff et al., 2004) and expressed as the percentage of the total field area.

Effect of NO Donor Administration During In Vitro Maturation on In Vitro Embryo Development: Oocyte Collection, In Vitro Maturation, Fertilization, and Embryo Culture

A total of 46 and 35 pairs of Hi and Lo ovaries, respectively, were recovered on 5 different days, as described above. All the subsequent procedures were performed at 35 to 38°C.

Cumulus-oocyte complexes (COC) were isolated from 2- to 6-mm antral follicles of Hi and Lo ovaries. In both categories, COC were examined under a stereomicroscope, and only complexes medium-brown in color, with ≥ 5 complete layers of cumulus cells and a finely granulated homogenous ooplasm were used (Luciano et al., 2005). Groups of at most 30 oocytes were in vitro matured (IVM) in 4-well dishes (NUNC, VWR International, Milan, Italy) for 24 h at 38.5°C under 5% CO₂ in humidified air in 500 μ L of TCM-199, supplemented with 0.68 mM L-glutamine, 25 mM NaHCO₃, 0.4% (wt/vol) of BSA fatty acid free, 0.2 mM sodium pyruvate, and 0.1 IU/mL of recombinant human FSH (Gonal-F, Serono, Rome, Italy), as described previously (Luciano et al., 2005).

To evaluate whether the modulation of NO concentration during IVM could affect the rate and the quality of blastocysts obtained in vitro, COC were matured in the presence of a NO donor. For this purpose, COC isolated from Lo or Hi ovaries were matured in the presence or the absence of 100 μ M S-nitroso acetyl penicillamine (SNAP; Goud et al., 2005). The SNAP was dissolved in dimethyl sulfoxide, as indicated in the manufacturer's datasheet. In both SNAP and control IVM medium, final dimethyl sulfoxide concentration was 1 μ L/mL that, as already demonstrated, does not exert a toxic effect on oocyte developmental competence (Lazzari et al., 2008; Luciano et al., 2010). After IVM, the occurrence of cumulus expansion was assessed and COC were fertilized as described previously (Luciano et al., 2005). Briefly, the contents of a straw of cryopreserved bull spermatozoa (CIZ, S. Miniato Pisa, Italy) were thawed and cells separated on a 45 and 90% discontinuous Percoll gradient. Sperm were counted and diluted to a final concentration of 0.75×10^6 spermatozoa/mL in fertilization medium that was a modified Tyrode's solution (TALP) supplemented with 0.6% (wt/vol) BSA fatty acid free, 10 μ g/mL of heparin, 20 μ M penicillamine, 1 μ M epinephrine, and 100 μ M hypotaurine. The COC and sperm were incubated for 18 h at 38.5°C under 5% CO₂ in humidified air.

After in vitro fertilization, presumptive zygotes were washed, and cumulus cells were removed by vortexing for 2 min in 500 μ L of a modified synthetic oviduct fluid (SOF; Tervit et al., 1972) supplemented with 0.3%

(wt/vol) BSA fraction V fatty acid free, MEM essential and nonessential AA, 0.72 mM sodium pyruvate, and buffered with 10 mM HEPES and 5 mM NaHCO₃. All the presumptive zygotes were rinsed and transferred into embryo culture medium, which was SOF buffered with 25 mM NaHCO₃, supplemented with MEM essential and nonessential AA, 0.72 mM sodium pyruvate, 2.74 mM myo-inositol, 0.34 mM sodium citrate, and with 5% (vol/vol) of calf serum (Gibco, Invitrogen, San Giuliano Milanese, Milan, Italy). Incubation was performed at 38.5°C using a tri-gas mixture (5% CO₂, 5% O₂, and 90% N₂), in humidified air as described previously (Luciano et al., 2005). After 168 h of culture, blastocyst rate was calculated on the total number of the oocytes placed in IVM medium. Total cell number per blastocyst, percentage of apoptotic nuclei (as a marker of advanced stage of apoptosis) and rate of caspase-positive cells (as an early indicator of apoptosis) were evaluated using the CaspaTag Pan-Caspase in situ Assay kit, Fluorescein (Millipore Corporation, Billerica, MA; Vandaele et al., 2007) according to the manufacturer's specification sheet. A total of 237 COC from Hi ovaries (119 IVM in presence of SNAP and 118 as control) and 232 COC from Lo ovaries (120 IVM in presence of SNAP and 112 as control) were used in these experiments; all experiments were repeated 5 times on 5 different days. For each replication, about 20 to 25 oocytes from Lo and Hi ovaries were matured in the presence or in the absence of SNAP.

Statistical Analysis

All the experiments were repeated 2 to 5 times, and data are presented as the mean \pm SE. Statistical significance was determined by Student's unpaired *t*-test when means of 2 different groups were being compared (quantification of Lectin BS-1 binding and of eNOS protein expression and percentage of healthy follicles in Hi vs. Lo ovaries). Means of blastocyst rate, hatching rate, blastocyst cell number, percentage of apoptotic nuclei and rate of caspase-positive cells from the different experimental groups were compared using an ANOVA followed by Newman-Keuls multiple comparison test (Prism, GraphPad Software, La Jolla, CA). Regardless of the statistical test, *P*-values of < 0.05 were considered significant.

RESULTS

Lectin BS-1 Binding and eNOS Localization and Quantification in Hi and Lo Ovaries

No difference in percentage of healthy follicles was observed between Hi and Lo ovaries (44.1 ± 6.5 vs. 34.9 ± 8.9 , respectively; *P* > 0.05). In both Hi and Lo ovaries, Lectin BS-1 binding was detected in the endothelial cells of blood vessels of the theca layer from secondary to antral follicles and of the ovarian stromal tissue. Zona pellucida was also positively stained (Fig-

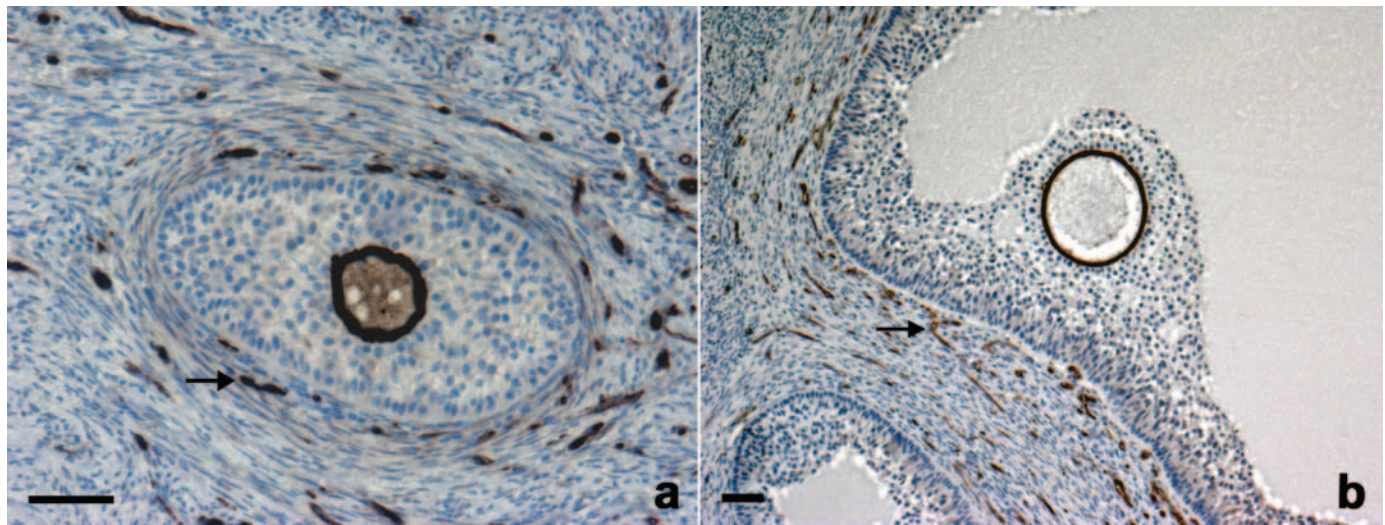


Figure 1. Representative images showing Lectin BS-1 binding in a preantral follicle (panel a) and in a medium antral follicle (panel b) of a bovine ovary. Note the staining of endothelial cells of blood vessels in the theca layer (arrows). Size of bars = 50 μ m. Color version available in the online PDF.

ure 1). Control sections did not exhibit any positive staining. Quantitative analysis of Lectin BS-1 binding in healthy antral follicles revealed that thecal vascularity was greater in follicles of Hi ovaries when compared with follicles of Lo ovaries (13.36 ± 2.68 vs. 5.38 ± 0.37 ; $P < 0.05$; Figure 2).

In both Hi and Lo ovaries, eNOS protein was localized in the cytoplasm of oocytes and of theca and granulosa cells during all the stages of folliculogenesis and, occasionally, in the nucleus of antral follicle oocytes (Figure 3, panels a to d). In all the antral follicles, eNOS protein was mostly concentrated inside the cytoplasm of mural cells, lying on the basal membrane (Figure 3, panels b and c), and in medium antral follicles also in the cumulus cells (Figure 3, panel d).

An intense positive staining was also observed in blood vessels of theca layers and of ovarian stromal tissue, which was demonstrated by colocalization study with Lectin BS-1 (Figure 3, panels e and f). Control sections did not exhibit any positive staining. Quanti-

tative analysis of eNOS expression in theca layers and granulosa cells revealed that eNOS protein was greater in the wall of follicles from Hi ovaries when compared with follicles from Lo ovaries (11.79 ± 1.44 vs. 5.76 ± 1.41 , respectively, $P < 0.05$; Figure 4).

Effect of NO Donor Administration During In Vitro Maturation on In Vitro Embryo Development

Oocytes isolated from Lo ovaries showed a reduced developmental potential compared with oocytes isolated from Hi ovaries (Table 1); however, no significant differences were observed in blastocyst cells number (Table 1), as previously demonstrated (Modina et al., 2007). After IVM, no morphological differences in cumulus expansion were observed between categories or treatments. The addition of SNAP to IVM medium improved the developmental competence of oocytes isolated from Lo ovaries (Table 1). In fact, such oocytes showed a slightly increased percentage of blastocyst and hatching rates, similar to what was noted for oocytes derived from Hi ovaries, whereas the administration of SNAP reduced the percentage of apoptotic nuclei per blastocyst ($P < 0.05$). Neither the blastocyst rate nor the percentage of apoptotic nuclei were further improved by the administration of SNAP during the IVM of oocytes collected from Hi ovaries. Furthermore, SNAP adversely affected the hatching rate of these oocytes ($P < 0.05$). Finally, its presence did not result in a change of cell number or caspase-positive cell percentage in either experimental group (Table 1).

DISCUSSION

In the present study we found that in cows of reproductive age, a reduced number of medium antral fol-

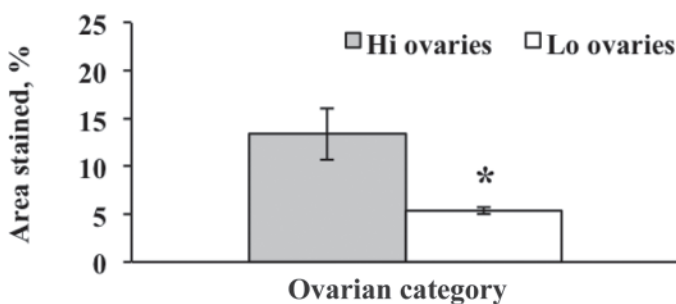


Figure 2. Quantitative analysis of vascularity in the theca layer of follicles of Hi ovaries (with >10 medium antral follicles) and Lo ovaries (with <10 medium antral follicles). Bar charts represent the percentage of area that exhibited positive staining for Lectin BS-1 binding, as vascularity index. Values are expressed as mean \pm SE obtained from 3 follicles from each of 5 animals with Hi ovaries and from 3 follicles from each of 5 animals with Lo ovaries. * $P < 0.05$.

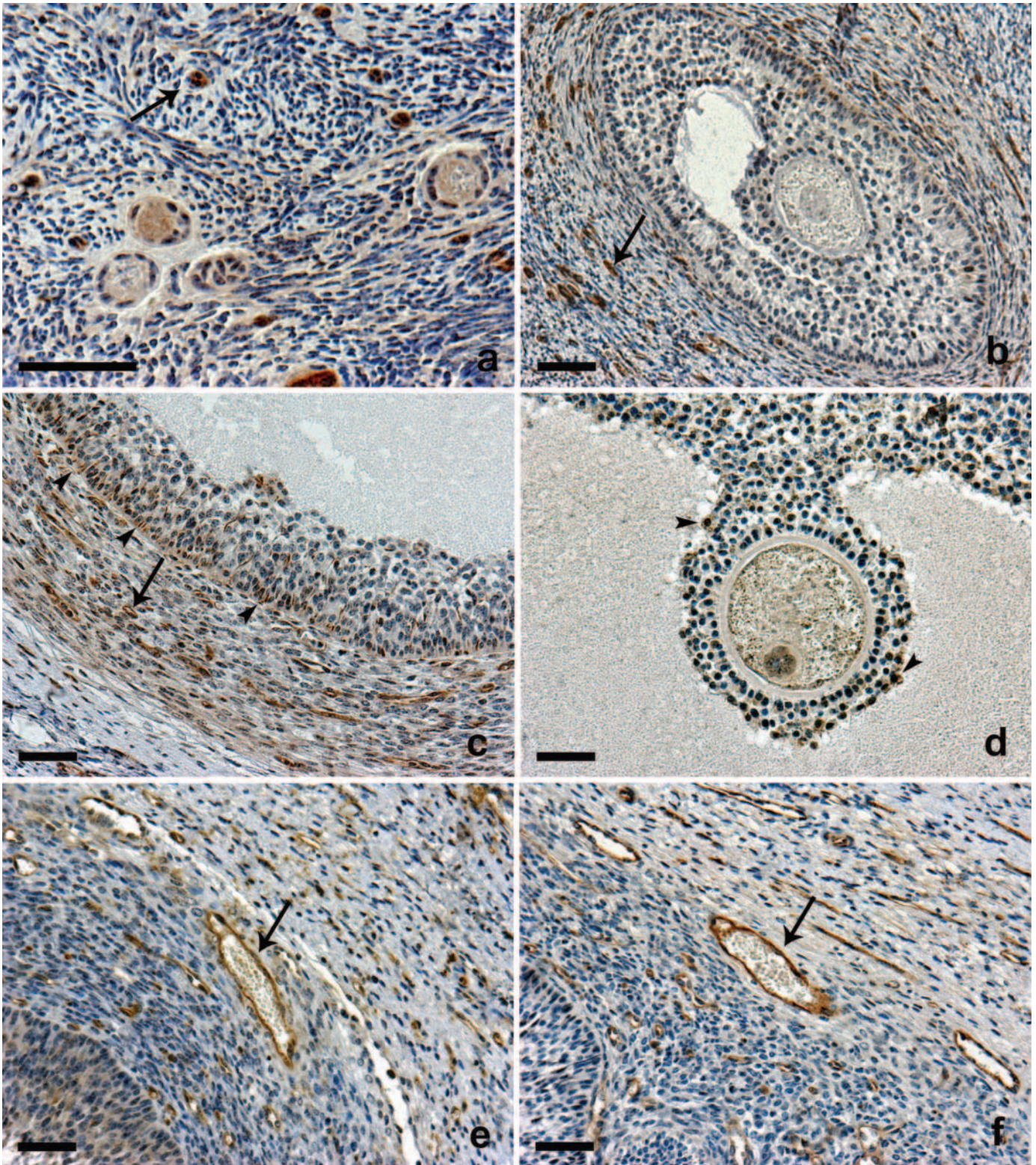


Figure 3. Representative images of endothelial nitric oxide synthase (eNOS) protein localization in primordial and primary follicles (panel a), in an early antral follicle (panel b), in a medium antral follicle wall (panel c), in a cumulus-oocyte complex (panel d), and in a blood vessel (panels e and f). Arrowheads identify the staining in the mural and cumulus cells (panels c and d); arrows indicate the staining in blood vessels (panels a to f). Colocalization of eNOS (panel e) and Lectin BS -1 binding (panel f) in a blood vessel is clearly visible (arrows). Size of bars = 50 μ m. Color version available in the online PDF.

licles is related to a decrease in perifollicular vascularity and eNOS expression and is not related to atresia phenomena evaluated by morphological criteria. Moreover,

it is not influenced by cyclic status of animals or by the presence or absence of a corpus luteum. In the cow, in fact, a corpus luteum or dominant follicle does not

Table 1. Effect of 100 μ M S-nitroso acetyl penicillamine (SNAP) addition during oocyte in vitro maturation on embryo development¹

Ovarian category ²	SNAP	Replicates, n	Blastocysts, %	Hatched blastocysts, %	Cells/blastocyst	Caspase-positive cells, %	Apoptotic cells, %
Hi	–	5	29.8 \pm 5.6 ^a	41.4 \pm 7.2 ^a	109.3 \pm 16.7	7.4 \pm 1.8	7.5 \pm 0.6 ^a
Hi	+	5	27.6 \pm 2.7 ^a	17.8 \pm 7.53 ^b	108.1 \pm 29.9	8.8 \pm 2.2	10.8 \pm 1.3 ^a
Lo	–	5	13.3 \pm 2.7 ^b	17.7 \pm 7.1 ^b	75.5 \pm 26.5	12.1 \pm 4.7	18.0 \pm 1.4 ^b
Lo	+	5	21.9 \pm 4.3 ^{ab}	25.0 \pm 9.9 ^{ab}	105.6 \pm 25.4	8.0 \pm 1.5	9.5 \pm 1.7 ^a

^{a,b}Within a column, means without a common superscript differ ($P < 0.05$).

¹Values are expressed as mean \pm SE.

²Ovarian categories: Hi = ovaries with >10 medium antral follicles; Lo = ovaries with <10 medium antral follicles.

exert a local effect on developmental competence of oocytes derived from subordinate follicles (Vassena et al., 2003). Finally, the antral follicle count is not influenced by the estrous cycle (Cushman et al., 2009).

Ovary of rat (Zackrisson et al., 1996; Jablonka-Shariff and Olson, 1997; Yamagata et al., 2002), mouse (Nishikimi et al., 2001; Mitchell et al., 2004), and pig (Tao et al., 2004; Kim et al., 2005) expresses eNOS. In sheep, it was reported that eNOS mRNA and protein expression changes throughout follicular growth and atresia, and the pattern of eNOS protein expression follows the pattern of vascular development during folliculogenesis (Grazul-Bilska et al., 2006). In fact, whereas eNOS mRNA was detected in both theca and granulosa cell layers, the protein was immunolocalized only in the theca, and exclusively in the blood vessels of developing preovulatory and postovulatory healthy follicles (Grazul-Bilska et al., 2006). As in sheep (Grazul-Bilska et al., 2007; Pires et al., 2009), in cows we found eNOS protein localized in endothelial cells of theca blood vessels. Moreover, we observed a greater eNOS protein expression in follicles from Hi ovaries when compared with follicles from Lo ovaries. The follicles from former ovaries were accompanied by a denser capillary network, which likely represents the main source of NO. A further suggestion for the reduced oocyte developmen-

tal competence of Lo ovaries may be the consequence of vascular defects and of reduced NO availability in follicular fluid.

Interestingly, we localized eNOS protein in granulosa cells during all the stages of folliculogenesis in the cow, confirming a previous study by Pires et al. (2009). The occurrence of eNOS protein in the cytoplasm of granulosa and cumulus cells of antral follicles supports the hypothesis that these cells are directly involved in the synthesis of NO present in the follicular fluid (Basini et al., 1998; Pinto et al., 2003). An entirely new observation, the present study highlights that the decreased perifollicular vascularity and eNOS expression observed in ovaries with a reduced medium antral follicle count may be the reason for a decreased developmental competence of the derived oocytes. This is proven by a decreased eNOS expression in Lo ovarian follicles and by embryonic development of oocytes isolated from Lo ovaries after supplementation of NO donor during IVM, which also prevents the onset of apoptosis.

It is well documented that NO elicits a wide spectrum of intracellular effects (Gross and Wolin, 1995). In fact, the effect of NO on oocyte maturation can be stimulatory or inhibitory depending on its concentration (Bu et al., 2003, 2004; Bilodeau-Goeseels, 2007). Positive effects of SNAP administration during IVM were observed in the mouse (Goud et al., 2005), rat (Nakamura et al., 2002), and pig (Petr et al., 2005, 2006). In our IVM system, the addition of 100 μ M SNAP slightly increased blastocyst and hatching rates of oocytes derived from Lo ovaries to a percentage comparable with that observed in oocytes isolated from Hi ovaries; conversely, it negatively affected the hatching rate of oocytes from Hi ovaries. Therefore, we can presume that SNAP administration is beneficial to oocytes derived from follicles where the eNOS/NO system is defective, but ineffective for oocytes where eNOS/NO system is not compromised and NO concentration presumably optimal; however, further research is needed to resolve this important biological issue.

Nitric oxide is involved in multiple reactions and functions, its effect being mainly dependent on its concentration. For this reason, the specific pathway triggered could be strictly dependent on the milieu and on the quantity synthesized (Hanafy et al., 2001; Schwarz

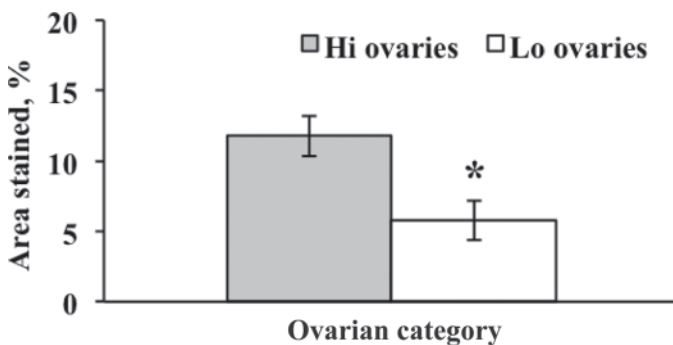


Figure 4. Quantitative analysis of endothelial nitric oxide synthase (eNOS) expression in the theca layer and granulosa cells of follicles of Hi ovaries (with >10 medium antral follicles) and Lo ovaries (with <10 medium antral follicles). Bar charts represent the percentage of area that exhibited positive eNOS staining. Values are expressed as mean \pm SE obtained from 3 follicles from each of 6 animals with Hi ovaries and from each of 7 animals with Lo ovaries. * $P < 0.05$.

et al., 2008). Accordingly, it was reported that the administration of SNAP (10 μ M) during oocyte maturation in bovine oocytes does not improve embryonic developmental competence; on the contrary, meiosis is negatively affected by high supplementation (10 μ M to 1 mM).

Finally, we found that whereas total cell number was not affected by the addition of SNAP, the number of apoptotic nuclei in Lo-derived blastocysts significantly decreased. This confirms that NO may exert a cell-protective function, as recently suggested (Schwarz et al., 2010). Although genetic factors might also contribute to deplete the ovarian oocyte pool and to reduce oocyte quality (Alvigi et al., 2009), the follicular microenvironment of the maturing oocyte is a determining factor for potential implantation of an embryo. Although the intricate mechanisms involving the ovarian environment and oocyte quality demand further study, the present work suggests that a defective eNOS/NO system is likely to be related to a reduced follicle vasculature in ovaries with low mid-antral follicle count that may affect oocyte quality, inducing a premature decline in fertility of mammals.

LITERATURE CITED

- Abramoff, M. D., P. J. Magelhaes, and S. J. Ram. 2004. Image processing with ImageJ. *Biophotonics International* 11:36–42.
- Alvigi, C., P. Humaidan, C. M. Howles, D. Tredway, and S. G. Hillier. 2009. Biological versus chronological ovarian age: Implications for assisted reproductive technology. *Reprod. Biol. Endocrinol.* 7:101.
- Basini, G., M. Baratta, N. Ponderato, S. Bussolati, and C. Tamani. 1998. Is nitric oxide an autocrine modulator of bovine granulosa cell function? *Reprod. Fertil. Dev.* 10:471–478.
- Bilodeau-Goeseels, S. 2007. Effects of manipulating the nitric oxide/cyclic GMP pathway on bovine oocyte meiotic resumption in vitro. *Theriogenology* 68:693–701.
- Borini, A., A. Maccolini, A. Tallarini, M. A. Bonu, R. Sciajno, and C. Flamigni. 2001. Perifollicular vascularity and its relationship with oocyte maturity and IVF outcome. *Ann. N. Y. Acad. Sci.* 943:64–67.
- Bu, S., G. Xia, Y. Tao, L. Lei, and B. Zhou. 2003. Dual effects of nitric oxide on meiotic maturation of mouse cumulus cell-enclosed oocytes in vitro. *Mol. Cell. Endocrinol.* 207:21–30.
- Bu, S., H. Xie, Y. Tao, J. Wang, and G. Xia. 2004. Nitric oxide influences the maturation of cumulus cell-enclosed mouse oocytes cultured in spontaneous maturation medium and hypoxanthine-supplemented medium through different signaling pathways. *Mol. Cell. Endocrinol.* 223:85–93.
- Coccia, M. E., and F. Rizzello. 2008. Ovarian reserve. *Ann. N. Y. Acad. Sci.* 1127:27–30.
- Cushman, R. A., M. F. Allan, L. A. Kuehn, W. M. Snelling, A. S. Cupp, and H. C. Freetly. 2009. Evaluation of antral follicle count and ovarian morphology in crossbred beef cows: Investigation of influence of stage of the estrous cycle, age, and birth weight. *J. Anim. Sci.* 87:1971–1980.
- Erickson, B. H., R. A. Reynolds, and R. L. Murphree. 1976. Ovarian characteristics and reproductive performance of the aged cow. *Biol. Reprod.* 15:555–560.
- Gandolfi, F., A. M. Luciano, S. Modena, A. Ponzini, P. Pocar, D. T. Armstrong, and A. Lauria. 1997. The in vitro developmental competence of bovine oocytes can be related to the morphology of the ovary. *Theriogenology* 48:1153–1160.
- Goud, A. P., P. T. Goud, M. P. Diamond, and H. M. Abu-Soud. 2005. Nitric oxide delays oocyte aging. *Biochemistry* 44:11361–11368.
- Grazul-Bilska, A. T., C. Navanukraw, M. L. Johnson, D. A. Arnold, L. P. Reynolds, and D. A. Redmer. 2006. Expression of endothelial nitric oxide synthase in the ovine ovary throughout the estrous cycle. *Reproduction* 132:579–587.
- Grazul-Bilska, A. T., C. Navanukraw, M. L. Johnson, K. A. Vonnahme, S. P. Ford, L. P. Reynolds, and D. A. Redmer. 2007. Vascularity and expression of angiogenic factors in bovine dominant follicles of the first follicular wave. *J. Anim. Sci.* 85:1914–1922.
- Gross, S. S., and M. S. Wolin. 1995. Nitric oxide: Pathophysiological mechanisms. *Annu. Rev. Physiol.* 57:737–769.
- Hanafy, K. A., J. S. Krumenacker, and F. Murad. 2001. NO, nitrotyrosine, and cyclic GMP in signal transduction. *Med. Sci. Monit.* 7:801–819.
- Ireland, J. J., A. E. Zielak-Steciwko, F. Jimenez-Krassel, J. Folger, A. Bettgowda, D. Scheetz, S. Walsh, F. Mossa, P. G. Knight, G. W. Smith, P. Lonergan, and A. C. Evans. 2009. Variation in the ovarian reserve is linked to alterations in intrafollicular estradiol production and ovarian biomarkers of follicular differentiation and oocyte quality in cattle. *Biol. Reprod.* 80:954–964.
- Ireland, J. L., D. Scheetz, F. Jimenez-Krassel, A. P. Themmen, F. Ward, P. Lonergan, G. W. Smith, G. I. Perez, A. C. Evans, and J. J. Ireland. 2008. Antral follicle count reliably predicts number of morphologically healthy oocytes and follicles in ovaries of young adult cattle. *Biol. Reprod.* 79:1219–1225.
- Jablonka-Shariff, A., and L. M. Olson. 1997. Hormonal regulation of nitric oxide synthases and their cell-specific expression during follicular development in the rat ovary. *Endocrinology* 138:460–468.
- Kim, H., C. Moon, M. Ahn, Y. Lee, H. Kim, S. Kim, T. Ha, Y. Jee, and T. Shin. 2005. Expression of nitric oxide synthase isoforms in the porcine ovary during follicular development. *J. Vet. Sci.* 6:97–101.
- Lazzari, G., I. Tessaro, G. Crotti, C. Galli, S. Hoffmann, S. Bremer, and C. Pellizzer. 2008. Development of an in vitro test battery for assessing chemical effects on bovine germ cells under the ReProTect umbrella. *Toxicol. Appl. Pharmacol.* 233:360–370.
- Luciano, A. M., F. Franciosi, V. Lodde, D. Corbani, G. Lazzari, G. Crotti, C. Galli, C. Pellizzer, S. Bremer, M. Weimer, and S. C. Modena. 2010. Transferability and inter-laboratory variability assessment of the in vitro bovine oocyte maturation (IVM) test within ReProTect. *Reprod. Toxicol.* 30:81–88.
- Luciano, A. M., V. Lodde, M. S. Beretta, S. Colleoni, A. Lauria, and S. Modena. 2005. Developmental capability of denuded bovine oocyte in a co-culture system with intact cumulus-oocyte complexes: Role of cumulus cells, cyclic adenosine 3',5'-monophosphate, and glutathione. *Mol. Reprod. Dev.* 71:389–397.
- Lussier, J. G., P. Matton, and J. J. Dufour. 1987. Growth rates of follicles in the ovary of the cow. *J. Reprod. Fertil.* 81:301–307.
- Malhi, P. S., G. P. Adams, and J. Singh. 2005. Bovine model for the study of reproductive aging in women: Follicular, luteal, and endocrine characteristics. *Biol. Reprod.* 73:45–53.
- Mitchell, L. M., C. R. Kennedy, and G. M. Hartshorne. 2004. Expression of nitric oxide synthase and effect of substrate manipulation of the nitric oxide pathway in mouse ovarian follicles. *Hum. Reprod.* 19:30–40.
- Modena, S., V. Borromeo, A. M. Luciano, V. Lodde, F. Franciosi, and C. Secchi. 2007. Relationship between growth hormone concentrations in bovine oocytes and follicular fluid and oocyte developmental competence. *Eur. J. Histochem.* 51:173–180.
- Monteleone, P., P. Giovanni Artini, G. Simi, E. Casarosa, V. Cela, and A. R. Genazzani. 2008. Follicular fluid VEGF levels directly correlate with perifollicular blood flow in normoresponder patients undergoing IVF. *J. Assist. Reprod. Genet.* 25:183–186.
- Nakamura, Y., Y. Yamagata, N. Sugino, H. Takayama, and H. Kato. 2002. Nitric oxide inhibits oocyte meiotic maturation. *Biol. Reprod.* 67:1588–1592.

- Nishikimi, A., T. Matsukawa, K. Hoshino, S. Ikeda, Y. Kira, E. F. Sato, M. Inoue, and M. Yamada. 2001. Localization of nitric oxide synthase activity in unfertilized oocytes and fertilized embryos during preimplantation development in mice. *Reproduction* 122:957–963.
- Petr, J., R. Rajmon, E. Chmelikova, M. Tomanek, V. Lanska, M. Pribanova, and F. Jilek. 2006. Nitric-oxide-dependent activation of pig oocytes: The role of the cGMP-signalling pathway. *Zygote* 14:9–16.
- Petr, J., R. Rajmon, V. Lanska, M. Sedmikova, and F. Jilek. 2005. Nitric oxide-dependent activation of pig oocytes: Role of calcium. *Mol. Cell. Endocrinol.* 242:16–22.
- Pinto, C. R., D. L. Paccamonti, B. E. Eilts, C. S. Venugopal, C. R. Short, L. R. Gentry, D. L. Jr. Thompson, and R. A. Godke. 2003. Concentrations of nitric oxide in equine preovulatory follicles before and after administration of human chorionic gonadotropin. *Theriogenology* 60:819–827.
- Pires, P. R., N. P. Santos, P. R. Adona, M. M. Natori, K. R. Schwarz, T. H. de Bem, and C. L. Leal. 2009. Endothelial and inducible nitric oxide synthases in oocytes of cattle. *Anim. Reprod. Sci.* 116:233–243.
- Rodgers, R. J., and H. F. Irving-Rodgers. 2010. Morphological classification of bovine ovarian follicles. *Reproduction* 139:309–318.
- Rosselli, M., P. J. Keller, and R. K. Dubey. 1998. Role of nitric oxide in the biology, physiology and pathophysiology of reproduction. *Hum. Reprod. Update* 4:3–24.
- Schwarz, K. R., P. R. Pires, P. R. Adona, T. H. Camara de Bem, and C. L. Leal. 2008. Influence of nitric oxide during maturation on bovine oocyte meiosis and embryo development in vitro. *Reprod. Fertil. Dev.* 20:529–536.
- Schwarz, K. R., P. R. Pires, T. H. de Bem, P. R. Adona, and C. L. Leal. 2010. Consequences of nitric oxide synthase inhibition during bovine oocyte maturation on meiosis and embryo development. *Reprod. Domest. Anim.* 45:75–80.
- Tao, Y., Z. Fu, M. Zhang, G. Xia, J. Yang, and H. Xie. 2004. Immunohistochemical localization of inducible and endothelial nitric oxide synthase in porcine ovaries and effects of no on antrum formation and oocyte meiotic maturation. *Mol. Cell. Endocrinol.* 222:93–103.
- Tatone, C., F. Amicarelli, M. C. Carbone, P. Monteleone, D. Caserta, R. Marci, P. G. Artini, P. Piomboni, and R. Focarelli. 2008. Cellular and molecular aspects of ovarian follicle ageing. *Hum. Reprod. Update* 14:131–142.
- Tervit, H. R., D. Whittingham, and L. Rowson. 1972. Successful culture in vitro of sheep and cattle ova. *J. Reprod. Fertil.* 30:493–497.
- Thaler, C. D., and D. Epel. 2003. Nitric oxide in oocyte maturation, ovulation, fertilization, cleavage and implantation: A little dab'll do ya. *Curr. Pharm. Des.* 9:399–409.
- Van Blerkom, J. 1998. Epigenetic influences on oocyte developmental competence: Perifollicular vascularity and intrafollicular oxygen. *J. Assist. Reprod. Genet.* 15:226–234.
- Vandaele, L., B. Mateusen, D. G. Maes, A. de Kruif, and A. Van Soom. 2007. Temporal detection of caspase-3 and -7 in bovine in vitro produced embryos of different developmental capacity. *Reproduction* 133:709–718.
- Vassena, R., R. J. Mapletoft, S. Allodi, J. Singh, and G. P. Adams. 2003. Morphology and developmental competence of bovine oocytes relative to follicular status. *Theriogenology* 60:923–932.
- Yamagata, Y., Y. Nakamura, N. Sugino, A. Harada, H. Takayama, S. Kashida, and H. Kato. 2002. Alterations in nitrate/nitrite and nitric oxide synthase in preovulatory follicles in gonadotropin-primed immature rat. *Endocr. J.* 49:219–226.
- Yang, M. Y., and R. Rajamahendran. 2000. Morphological and biochemical identification of apoptosis in small, medium, and large bovine follicles and the effects of follicle-stimulating hormone and insulin-like growth factor-I on spontaneous apoptosis in cultured bovine granulosa cells. *Biol. Reprod.* 62:1209–1217.
- Zackrisson, U., M. Mikuni, A. Wallin, D. Delbro, L. Hedin, and M. Brannstrom. 1996. Cell-specific localization of nitric oxide synthases (NOS) in the rat ovary during follicular development, ovulation and luteal formation. *Hum. Reprod.* 11:2667–2673.

References

This article cites 48 articles, 19 of which you can access for free at:
<http://jas.fass.org/content/89/8/2389#BIBL>