Evidence that the effect of angiotensin II on bovine oocyte nuclear maturation is mediated by prostaglandins E₂ and F_{2α}

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Abstract

Angiotensin II (AngII) prevents the inhibitory effect of follicular cells on oocyte maturation, but its involvement in LH-induced meiotic resumption remains unknown. The aim of this study was to assess the involvement of AngII in LH-induced meiotic resumption and of prostaglandins (PGs) in the action of AngII. In the experiment I, seven cows were superovulated, intrafollicularly injected with 10 μM saralasin (a competitive AngII antagonist) or saline when the follicles reached a diameter larger than 12 mm, and challenged with a GnRH agonist to induce an LH surge. Fifteen hours after GnRH, the animals were ovariectomized and the oocytes were recovered to determine the stage of meiosis. The oocytes from follicles that received saline were in germinal vesicle (GV) breakdown (30.8%) or metaphase I (MI; 69.2%) stage while those that received saralasin were in the GV stage (100%; $P < 0.001$) 15 h after GnRH agonist. In another experiment, oocytes were co-cultured with follicular hemisections for 15 h to determine whether PGs mediate the effect of AngII on meiotic resumption. Indomethacin (10 μM) inhibited AngII-induced meiotic resumption (13.4 vs 77.5% MI without indomethacin; $P < 0.001$). Furthermore, the GV oocytes progressed to MI at a similar rate when PGE₂, PGF_{2α} or AngII was present in the co-culture system with follicular cells (PGE₂ 77.4%, PGF_{2α} 70.0%, and AngII 75.0% MI). In conclusion, our results provide strong evidence that AngII mediates the resumption of meiosis induced by an LH surge in bovine oocytes and that this event is dependent on PGE₂ or PGF_{2α} produced by follicular cells.

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Introduction

Oocytes are arrested in the first meiotic prophase during follicular development. Meiotic resumption is dependent on the preovulatory surge of LH, but the bovine cumulus–oocyte complexes (COCs) do not have LH receptors, so the effect must occur through theca and mural granulosa cells (Van Tol *et al.* 1996, Nuttinck *et al.* 2004). The LH surge stimulates the ovarian renin-angiotensin system (RAS), including an increase in the renin, prorenin, and angiotensin II (AngII) concentration in bovine follicular fluid (Nielsen *et al.* 1994, Acosta *et al.* 2000). Moreover, the interactions among LH, AngII, endothelin-1, and atrial natriuretic peptide increased follicular production of prostaglandins (PGs) and modulated steroidogenesis in the bovine preovulatory follicle (Acosta *et al.* 1999). AngII has also been associated with follicular growth (Ferreira *et al.* 2008) and ovulation in cows (Acosta *et al.* 2000, Ferreira *et al.* 2007). Furthermore, AngII stimulated the resumption of meiosis in co-culture systems of cumulus-enclosed bovine oocytes and follicular cells (Giometti *et al.* 2005, Stefanello *et al.* 2006).

AngII is the active octapeptide hormone product of the renin enzymatic cascade, and two receptor subtypes of AngII have been identified (Bottari *et al.* 1993, De Gasparo *et al.* 1995, Gallinat *et al.* 2000). The classic effects of AngII regarding the muscular contraction, aldosterone secretion, and sanguineous pressure regulation are mediated by the AngII receptor type 1. In fact, the AngII receptor type 2 has been demonstrated to be the mediator of several reproductive functions including steroidogenesis, oocyte maturation, and ovulation (Kuji *et al.* 1996, Yoshimura *et al.* 1996, Ferreira *et al.* 2007). In cows, the AngII receptor type 2 is present in the dominant follicle (Schäuser *et al.* 2001), and its mRNA expression was positively correlated with estradiol production (Portela *et al.* 2006). In perfused rabbit ovaries, the ovulation and the meiosis resumption induced by hCG are blocked by saralasin (1-sarcosine-8-alanine-5-isoleucine-AngII, a competitive AngII antagonist; Yoshimura *et al.* 1992). In contrast, Kuo *et al.* (1991) reported that saralasin inhibited ovulation without affecting oocyte nuclear maturation in rabbits. Recently, our group demonstrated that intrafollicular

injection of saralasin in follicles larger than 12 mm, before the LH surge, inhibits ovulation in cows (Ferreira *et al.* 2007). These results suggest that AngII can act as an intrafollicular mediator of LH actions during the periovulatory period. However, the specific role of AngII in the regulatory mechanism of ovulation and oocyte nuclear maturation is unknown.

AngII stimulates PG-endoperoxide synthase 2 (PTGS2) and PG synthesis in the vascular endothelium (Gimbrone & Wayne 1975), renal tissue (Hernández *et al.* 2002), and human monocytes (Kim *et al.* 2005). In rabbit ovaries perfused *in vitro*, AngII was found to stimulate PGE₂ and PGF_{2α} production in the absence of gonadotropins (Yoshimura *et al.* 1993). Recently, our group demonstrated that AngII stimulates the expression of PTGS2 mRNA in cultured bovine granulosa cells, which was potentiated by LH (Portela *et al.* 2008). The involvement of the PTGS2 pathway in oocyte nuclear maturation has been previously demonstrated in sheep (Murdoch 1988, 1996), cattle (Nuttinck *et al.* 2002, Calder *et al.* 2005), mice (Takahashi *et al.* 2006), humans (Ben-Ami *et al.* 2006), and horses (Dell'Aquila *et al.* 2004). In sheep, the systemic administration of indomethacin (nonselective PTGS inhibitor) resulted in suppression of the cumulus cell expansion and oocyte nuclear maturation, which were reversed by PGE₂ intrafollicular injection (Murdoch 1988, 1996). In PTGS2 knockout mice, ovulation and oocyte maturation did not occur normally (Lim *et al.* 1997).

In spite of reports that AngII induces meiotic resumption in oocytes co-cultured with follicular cells (Giometti *et al.* 2005, Stefanello *et al.* 2006), there is no information available regarding the requirement of AngII for oocyte resumption of meiosis after the LH surge. Consequently, our hypotheses were that AngII is essential for meiotic resumption after the LH surge and that it acts through PTGS2-mediated PG synthesis. Therefore, the aim of this study was to verify whether AngII is required for meiotic resumption after the LH surge induced by GnRH agonist and to test the hypothesis that the PGE₂ and F_{2α} mediate this process.

Results

Experiment I: effect of competitive AngII antagonist in LH-induced meiotic resumption

Experiment I was performed to evaluate whether the LH-induced meiotic resumption in bovine oocytes is mediated by AngII. The mean initial diameter of follicles treated with saralasin (12.97 ± 0.92 mm) did not differ from those injected with saline (13.48 ± 1.21 mm; $P > 0.05$). Of the 34 follicles treated with saline ($n = 17$) or saralasin ($n = 17$), two follicles treated with saralasin were eliminated from the experiment because they had a reduction in diameter greater than 2 mm after 2 h of intrafollicular injection. Three oocytes from saralasin

and four from saline groups were lost during stain procedures. The ability of the LH surge (induced by GnRH agonist) to induce resumption of meiosis was impaired when follicles were treated with saralasin (100% of GV; $n = 12$). However, meiotic resumption was observed in all oocytes ($n = 13$) recovered from follicles treated with saline (30.8% in GVBD and 69.2% in MI; $P < 0.001$).

Experiment II: effect of saralasin on oocyte nuclear maturation in the presence of follicular cells

This experiment was performed to evaluate the potential deleterious effect of saralasin on oocyte and follicular cell function. The ability of follicular cells to inhibit meiosis was maintained after treating follicular hemisections with 10 μM saralasin for 15 h (21.4 and 13.3% MI in the saralasin and negative control groups respectively; $P > 0.1$; Fig. 1). Similarly, the follicular cell function was assessed using 100 pM AngII. In oocytes cultured with follicular hemisections, the resumption of meiosis was induced by AngII (80.0% MI), results that did not differ from the positive control group without follicular cells (86.7% MI) in 15 h of culture. The oocytes previously co-cultured for 15 h in the presence of follicular hemisections and saralasin resumed meiosis after co-culture with follicular cells and AngII for an additional 15 h (83.9% MII, $P < 0.001$; Fig. 2). Also, the MII rate observed in oocytes cultured with follicular hemisections and AngII (77.4%) was not different from those cultured in the positive control group (86.7%). Even culturing for 15 h + 15 h with (Sar 15 h + Sar 15 h group; 21.9% MII) or without saralasin (negative control group; 19.4% MII), the oocyte nuclear maturation was inhibited by follicular cells, showing that saralasin did not affect the cell function.

Figure 1 Meiotic resumption after 15 h of *in vitro* co-culture of bovine oocytes and follicular hemisections treated with 100 pM angiotensin II (AngII) or 10 μM saralasin (Sar). The experiment was performed in triplicate and the number of oocytes examined in each treatment is shown at the base of each bar. Different letters indicate statistical difference between groups ($P < 0.001$).

Figure 2 Meiotic resumption after co-culture of bovine oocytes and follicular hemisections treated with 100 pM angiotensin II (AngII) or 10 μ M saralasin (Sar). After 15 h of co-culture, the oocytes were moved to a new maturation medium and those previously cultured with saralasin were divided into two subgroups: cultured with 10 μ M Sar (Sar 15 h + Sar 15 h group) or 100 pM AngII (Sar 15 h + AngII 15 h group). After medium changes, the oocytes were cultured for an additional period of 15 h. The experiment was performed in triplicate and the number of oocytes examined in each treatment is shown at the base of each bar. Different letters indicate statistical difference between groups ($P < 0.001$).

Experiment III: effect of saralasin on bovine oocyte maturation and subsequent blastocyst production

Experiment III was performed to evaluate possible detrimental effects of saralasin on the capacity of oocytes to support embryo development. The rates of cleavage (84.4%), embryo development (44.4%), and hatching (40.0%) of oocytes matured in media containing saralasin (10 μ M; without follicular cells) did not differ from those cultured in the control group (79.1, 48.8, and 38.1% respectively).

Experiment IV: effect of PTGS on AngII-induced meiotic resumption

This experiment was conducted to assess whether AngII-induced oocyte meiotic resumption is mediated by PTGS. The AngII effect in follicular cells was inhibited by indomethacin (10 μ M; nonselective PTGS inhibitor). Oocytes cultured for 15 h with AngII and indomethacin reached 13.4% MI while those cultured with AngII but not indomethacin achieved 77.5% MI ($P < 0.001$; Fig. 3) when follicular cells were present. The inhibition of meiotic resumption rate did not differ between groups of oocytes cultured with or without indomethacin when AngII was absent and follicular cells were present (26.6% MI without indomethacin versus 13% MI with indomethacin). Indomethacin did not affect the meiotic resumption rate (80.7% MI) without follicular hemisections when compared with the positive control group (78.1% MI) and the AngII treated group (77.5% MI; $P > 0.1$).

Figure 3 Meiotic resumption after co-culture of bovine oocytes and follicular hemisections treated with indomethacin and/or angiotensin II (AngII) for 15 h. The experiment was performed in triplicate and the number of oocytes examined in each treatment is shown at the base of each bar. Different letters indicate statistical difference between groups ($P < 0.001$).

Experiment V: effect of PGE₂ and F_{2 α} on bovine oocyte meiosis resumption

Finally, experiment V was designed to determine the role of PGs in mediating the AngII effect on oocyte meiotic resumption. The MI rates of oocytes cultured in the presence of PGE₂ (78.3%) or F_{2 α} (76.1%) did not differ from those cultured with AngII (80.4%) when follicular cells were in the system (Fig. 4).

Discussion

In the present study, we tested the hypothesis that AngII is required for LH-induced bovine oocyte meiotic resumption and that PGs play a key role in this event as a potential mediator of the AngII action. The main new findings are as follows: 1) the intrafollicular injection of saralasin

Figure 4 Effect of PGE₂ and PGF_{2 α} on bovine oocyte meiotic resumption *in vitro*. The cumulus-oocyte complexes were co-cultured for 15 h with follicular cells and angiotensin II (AngII), indomethacin, AngII plus indomethacin, prostaglandins E₂ or F_{2 α} . The experiment was performed in triplicate and the number of oocytes examined in each treatment is shown at the base of each bar. Different letters indicate statistical difference between groups ($P < 0.001$).

inhibited the meiotic resumption induced by LH surge; 2) the nonselective PTGS inhibition blocked AngII-induced meiotic resumption of bovine oocytes co-cultured with follicular hemisections and 3) the addition of PGE₂ or PGF_{2α} into an *in vitro* co-culture system of oocytes and follicular cells resulted in the same pattern of meiotic resumption induced by AngII. Resumption of meiosis in fully grown follicles is triggered by a preovulatory surge of LH (Ayalon *et al.* 1972, Dekel *et al.* 1979), and herein we report the inhibition of these events by a competitive AngII antagonist. These results are complementary to previous findings of our group regarding oocyte meiotic resumption (Giometti *et al.* 2005, Stefanello *et al.* 2006) and ovulation, an event closely related to oocyte maturation (Ferreira *et al.* 2007). Together, these results suggest that AngII is an important intrafollicular mediator of the LH surge to induce meiotic resumption in bovine oocytes.

In this study, an intrafollicular injection was performed when follicles reached a minimal diameter of 12 mm to obtain an efficient response to the GnRH challenge (Sartori *et al.* 2001). The saline intrafollicular injection did not affect meiotic resumption after the GnRH challenge (30.8% GV breakdown (GVBD) and 69.2% MI). These results validate the intrafollicular injection model as a useful tool to study bovine follicular development, ovulation, and oocyte nuclear maturation. Likewise, the intrafollicular injection procedure did not affect the final follicular development and ovulation in previous investigations (Kot *et al.* 1995, Ginther *et al.* 2004, Ferreira *et al.* 2007).

Saralasin was used as a potent AngII antagonist to block all the receptor subtypes (Kuji *et al.* 1996, Yoshimura *et al.* 1996). A saralasin concentration of 10 μM has been used as an effective inhibitor of AngII action (Kuji *et al.* 1996). In experiment II, it was demonstrated that saralasin had no detrimental effects on follicular cells. The follicular cells cultured with saralasin for 15 h responded to AngII, showing that the cell function of inducing the resumption of meiosis was maintained. *In vitro* oocyte maturation in the presence of 10 μM saralasin did not impair the early embryo development and hatching rates (experiment III). The results of experiment II and III together strongly suggest that saralasin has no toxic effect on oocyte nuclear and cytoplasmic maturation. Furthermore, this peptide has been widely used to block the effects of the RAS with high specificity for the AngII receptors (Kuji *et al.* 1996, Yoshimura *et al.* 1996, Camargo & Saad 1999, Ferreira *et al.* 2007). The high rate of embryo development demonstrates that saralasin did not change the kinetics of oocyte nuclear and cytoplasmic maturation. These results show that saralasin does not have a direct effect on bovine oocyte, which are in agreement with those reported previously (Giometti *et al.* 2005, Stefanello *et al.* 2006).

The AngII signaling pathway on follicular cells to promote the meiotic resumption is unknown. In this study, it was demonstrated (experiment IV) that a nonspecific PTGS inhibitor (indomethacin) suppressed

meiotic resumption induced by AngII. Murdoch (1988) demonstrated that systemic administration of indomethacin in ewes inhibited the oocyte nuclear maturation and cumulus cell expansion. Impairment of ovulatory process and oocyte maturation has been observed in PTGS2 knockout mice females (Lim *et al.* 1997). These results indicate the importance of PTGS2 in oocyte meiotic resumption in different species. The cumulus cells of bovine oocytes express PTGS2 mRNA during *in vitro* maturation (Nuttinck *et al.* 2002, Calder *et al.* 2005). However, indomethacin did not affect oocyte nuclear maturation when the follicular hemisections were absent, suggesting that the effect of PTGS and AngII is through follicular cells. Additionally, our laboratory recently demonstrated that AngII stimulated PTGS2 expression in bovine granulosa cells (Portela *et al.* 2008).

In experiment V, PGE₂ and PGF_{2α} induced meiotic resumption in the same way as AngII. In ewes, the intrafollicular injection of PGE₂ was found to reverse the negative effect on oocyte nuclear maturation and cumulus cell expansion caused by systemic administration of indomethacin (Murdoch 1988). In the case of PTGS2 knockout mice, PGE₂ modulated oocyte nuclear maturation and granulosa cell expansion, differentially regulating the intracellular signaling pathways related to MAP kinase, cAMP-dependent protein kinase, phosphatidylinositol 3-kinase/Akt, and nuclear factor kappa B (Takahashi *et al.* 2006). Furthermore, intrafollicular injection of indomethacin decreased PGE₂ secretion in bovine follicular fluid induced by LH (Li *et al.* 2006). Together, these results supply evidence that meiotic resumption induced by AngII is modulated by the PGE₂ and F_{2α}. However, the signaling pathway of PGs in the induction of meiotic resumption needs further investigation. Li *et al.* (2006) demonstrated that indomethacin inhibited the effect of the LH surge on the expression of an enzyme that degrades the preovulatory extracellular matrix in cows, providing evidence for the involvement of the PTGS pathway in the extracellular matrix remodeling of follicular cells during the periovulatory period. Similarly, AngII inhibited the expression of protease nexin-1 in bovine granulosa cells (Portela *et al.* 2006). These results together provide evidence that the signaling pathway of AngII, through PGE₂ and F_{2α}, to induce the meiotic resumption in bovine oocytes involves extracellular matrix remodeling factors. In conclusion, we have demonstrated the involvement of AngII and PGE₂ and F_{2α} in bovine oocyte meiotic resumption induced by LH.

Materials and Methods

Animals and superovulation protocol

All experimental procedures using cattle were reviewed and approved by the Federal University of Santa Maria Animal Care and Use Committee. Seven cycling cows (*Bos taurus*),

multiparous, with body condition scores of 3 and 4 (1 = thin, 5 = obese) were submitted to a superovulation protocol. The animals received 5 mg estradiol benzoate (Genix, Anápolis, Brazil; i.m.) and an intravaginal device with progesterone (DIB, Syntex, Buenos Aires, Argentina) on day 0. On day 5, cloprostenol (synthetic analog of PGF_{2α}; Sincrocio, Ouro Fino, Ribeirão Preto, Brazil; 250 µg; s.c.) was administered and the follicle-stimulating hormone (FSH) treatment was initiated (Pluset, Hertape Calier Saúde Animal, Juatuba, Brazil). Each cow received 437.5 IU FSH, divided into 11 decreasing doses (from 87.5 to 12.5 IU; i.m.) each given 12 h apart. The progesterone intravaginal device was maintained for 10 days.

Ultrasound-guided intrafollicular injection procedure

The intrafollicular injections were guided by ultrasound equipped with a 7.5 MHz vaginal probe (Pie Medical Scanner 200, Maastricht, The Netherlands). A system with two sterile needles was used as previously described by [Ferreira et al. \(2007\)](#). Briefly, the double-channeled needle system consisted of an inner sterile 25-gauge needle attached to a 35 cm long cannula and an outer 20-gauge needle coupled to a needle guide. To the opposite end of the cannula, a Hamilton syringe was attached. Epidural anesthesia was administered and perineal cleaning was performed before injections. The transducer was coupled to a biopsy guide (Pie Medical Equipment BV), coated with lubricant, and placed in the vaginal fornix. The course of the echogenic needle was observed on the ultrasound display, which allowed us to reach the follicle of interest. The ovary was manipulated so that the needle entered the follicle via penetration of the ovarian stroma at the base of the follicle. The needle path to the injected follicle contained ovarian stroma and no additional follicle or corpus luteum.

When the ovary and follicle of interest were in position, the outer needle was advanced until the image of its tip became visible on the screen, ~3–5 mm from the follicle. At this moment, a second operator pushed the inner needle forward until the image of the needle tip was visible within the follicle. Treatments were then injected into the follicle. Swirling of the fluid entering the follicle indicated that the injection was successful. The amount of AngII receptor antagonist for the injection was calculated based on the volume of follicular fluid, to obtain an adequate final concentration inside the follicle. The follicular fluid volume was estimated by the linear regression equation $V = 685.1 + 120.7D$ ($P = 0.0001$), as described by [Ferreira et al. \(2007\)](#), where V corresponds to the estimated follicular volume and D to the diameter of the follicle to be injected. The probe and needles were withdrawn immediately after injection to minimize pressure on the newly punctured follicle. Follicular diameter was measured again 2 h after intrafollicular injection. Follicles were excluded from the experiment if the reduction in the follicular diameter was greater than 2 mm.

Oocyte recovery and nuclear maturation

Bovine ovaries at different stages of the estrous cycle were obtained from an abattoir and transported to the laboratory in saline solution (0.9% NaCl) at 30 °C containing 100 IU/ml

penicillin and 50 µg/ml streptomycin sulfate (All chemicals used were purchased from Sigma Chemical Company, unless otherwise indicated in the text). COCs were aspirated from follicles 3 to 8 mm in diameter. The COCs were recovered under a stereomicroscope and selected according to [Leibfried & First \(1979\)](#). Grade 1 and 2 COCs ($n = 10$ – 30) were randomly distributed into 200 µl maturation medium and cultured in an incubator at 39 °C in a saturated humidity atmosphere containing 5% CO₂ and 95% air, for either 15 or 24 h depending on the experiment. The maturation medium used was TCM 199 containing Earle's salts and L-glutamine (Gibco Labs) supplemented with 25 mM HEPES, 0.2 mM pyruvic acid, 2.2 mg/ml sodium bicarbonate, 5.0 µg/ml LH (Lutropin-V, Bioniche, Ontario, CA, USA), 0.5 µg/ml FSH (Folltropin-V, Bioniche), 0.4% fatty acid-free BSA, 100 IU/ml penicillin, and 50 µg/ml streptomycin sulfate.

At the end of the maturation period, the cumulus cells were removed by vortexing. The oocytes were fixed in glacial acetic acid:methanol (1:3) solution (Merck KG) for 4 h and then stained with 1% lacmoid in 45% glacial acetic acid in PBS. Oocytes were analyzed under a phase contrast microscope (1000×) and classified according to the nuclear maturation stage as germinal vesicle (GV), GVBD, metaphase I (MI), anaphase I (AI), telophase I (TI), and metaphase II (MII).

Sperm preparation, in vitro fertilization, and embryo culture

A tested frozen semen pool of a single batch from two sires (Jersey and Holstein) was used in all experiments. Semen was fractionated on discontinuous Percoll (Amersham Biosciences AB) gradients as described previously ([Parrish et al. 1986](#)). Briefly, 2.7 ml Percoll was mixed with 300 µl 10× sperm-TALP medium (modified Tyrode with albumin, lactate, and pyruvate), yielding a 90% Percoll solution. A 45% Percoll solution was obtained by 1:1 dilution in 1× sperm-TALP medium. A gradient column overlaying 45 and 90% Percoll solutions was then prepared to separate live sperm by centrifugation at 700 *g* for 30 min. The sperm were diluted to a final concentration of 2×10^6 spermatozoa/ml in 250 µl Fert-TALP medium containing 10 mg/ml heparin ([Parrish et al. 1988](#)). *In vitro* fertilization was carried out in drops by co-culture of sperm and oocytes for 18 h in an incubator (same atmospheric conditions as those used for maturation). After gamete co-incubation, cumulus cells were removed by vortexing. Presumptive zygotes and embryos were washed thrice and then cultured at 39 °C in a saturated humidity atmosphere containing 5% CO₂, 5% O₂, and 90% N₂ for 9 days in 200 µl synthetic oviduct fluid medium as previously described by [Holm et al. \(1999\)](#).

Preparation of follicular hemisections

Follicles were isolated from the ovaries and dissected free of stromal tissue ([Richard & Sirard 1996](#)). Transparent follicles measuring 2–5 mm in diameter were selected. The follicles were sectioned into equal halves with a scalpel and the oocytes were removed and discarded. Next, the follicular hemisections were washed ten times in TCM 199 containing 0.4% BSA and incubated for 2 h before adding the COCs.

The dissection and follicle culture procedures have been previously conducted in our laboratory and validated by measuring the progesterone and estradiol ratio and by histological analysis (Giometti *et al.* 2005, Stefanello *et al.* 2006).

Experiment I: effect of competitive AngII antagonist on LH-induced meiotic resumption

As described above, seven cows were submitted to the superovulation protocol. On day 9 of the progesterone treatment, the number of follicles in the ovary was evaluated by ultrasound scanning and all follicles with size from 5 to 11 mm were aspirated using a vacuum pump, remaining only the two to four largest follicles in each ovary. In the afternoon of day 10, after the intravaginal device had been removed, an ultrasound image of each ovary was processed to produce a map of the follicles in the ovary and all follicles greater than 12 mm in diameter were intrafollicularly injected. The follicles of the right ovaries were treated with 10 μ M saralasin (saralasin group; $n=17$) and those of the left ovaries were treated with 0.9% saline (control group; $n=17$). Immediately after the intrafollicular injections, the cows received 100 μ g gonadotropin acetate intramuscularly (GnRH agonist; Profertil, Tortuga, Santo Amaro, Brazil). Fifteen hours after challenging with the GnRH agonist, the animals were ovariectomized for recovery of the oocytes. The oocytes were immediately denuded, fixed, stained, evaluated as described above and classified according to the nuclear maturation stage as GV, GVBD, or MI.

Experiment II: effect of saralasin on oocyte nuclear maturation in the presence of follicular cells

COCs ($n=270$) derived from abattoir ovaries were used to evaluate the saralasin effect on oocyte nuclear maturation with follicular cells. COCs were cultured with follicular hemisections treated with 100 pM AngII (AngII group; $n=60$) or 10 μ M saralasin (saralasin group; $n=90$). As control groups, COCs cultured with (negative control group; $n=60$) or without follicular hemisections (positive control group; $n=60$) were used. After *in vitro* maturation for 15 h, 30 oocytes of each group were denuded, fixed and then stained for evaluation of nuclear maturation. The remaining oocytes and the follicular hemisections were transferred to other culture dishes with the same maturation medium for each group. However, the oocytes that were cultured with saralasin ($n=60$) were divided into two subgroups with follicular hemisections treated with 10 μ M saralasin (Sar 15 h+ Sar 15 h group; $n=30$) or 100 pM AngII (Sar 15 h+ AngII 15 h group; $n=30$). The oocytes were cultured for an additional period of 15 h (totaling 30 h) after changing the medium, excepting those cultured without follicular hemisections, for which the culture period was 22 h. At the end of the maturation period, the oocytes of all groups were denuded, fixed, and then stained for evaluation of meiotic resumption.

Experiment III: effect of saralasin on bovine oocyte maturation and subsequent blastocyst production

This experiment was designed to evaluate possible detrimental effects of saralasin on the capacity of oocytes to support embryo

development. COCs were cultured with (saralasin group; 10 μ M; $n=45$) or without saralasin (control group; $n=43$) with no follicular cells for 24 h. After the maturation period, oocytes were fertilized and the embryos cultured for 7 days to determine the blastocyst rate per oocyte. The hatching blastocyst rate was evaluated on day 9 and calculated as the number of hatching blastocysts divided by the total number of blastocysts in each group.

Experiment IV: effect of PTGS on AngII-induced meiotic resumption

To evaluate whether AngII action on oocyte nuclear maturation is mediated by PTGS from follicular cells, COCs were cultured with follicular hemisections treated with 100 pM AngII ($n=89$), 100 pM AngII, and 10 μ M indomethacin (a PTGS nonselective inhibitor; $n=97$) or 10 μ M indomethacin ($n=100$). The COCs in the control groups were cultured with (negative control group; $n=94$) or without follicular hemisections (positive control group; $n=96$). Eighty-eight COCs were cultured with 10 μ M indomethacin without follicular hemisections to evaluate deleterious effects of indomethacin on bovine oocytes. After 15 h of *in vitro* culture, oocytes of all groups were denuded, fixed, stained, and evaluated for nuclear status.

Experiment V: effect of PGE₂ and F_{2 α} on nuclear maturation

This experiment was designed to assess the role of PGE₂ and F_{2 α} on AngII-induced meiotic resumption. COCs were co-incubated with follicular hemisections treated with 100 pM AngII ($n=92$), 100 pM AngII and 10 μ M indomethacin ($n=92$), and 1 μ M PGE₂ ($n=92$) or 1 μ M PGF_{2 α} ($n=92$). The COCs in the negative control group ($n=95$) were cultured with non-treated follicular hemisections. After 15 h of *in vitro* culture, oocytes of all groups were denuded, fixed, stained, and evaluated for nuclear status.

Statistical analysis

In experiment I, the mean initial follicle diameters of the two groups were compared by ANOVA to validate the results. The progression of nuclear maturation was used as the variable response, grouped into classes (GV, GVBD, and MI), and normalized by PROC RANK. The percentage of meiotic progression among groups was compared by ANOVA (PROC GLM). The results of the experiments II, III, IV, and V were analyzed using the ANOVA test in a statistical model for categorical data, using the PROC CATMOD (Categorical Data Analysis Procedures). All *in vitro* experiments were performed in triplicate. On detecting statistical differences, the independent variables were compared using the contrast test. All the data were analyzed using the statistical analysis software (SAS; SAS Institute Inc., Cary, NC, USA).

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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