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Role of PTGS2-generated PGE₂ during gonadotrophin-induced bovine oocyte maturation and cumulus cell expansion



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Abstract Prostaglandin E₂ (PGE₂) is an autocrine/paracrine factor which mediates gonadotrophin (Gn) stimulation of cumulus expansion and oocyte maturation in rodents. Its role in bovine oocyte maturation is less characterized. This study detected PTGS2 (COX2) and PGE synthases (PTGES1, PTGES2 and PTGES3) in bovine cumulus–oocyte complexes (COC). Only PTGS2 and PTGES1 expression changed during maturation. In Gn-free media, no cumulus expansion and ~45% nuclear maturation was achieved, while Gn-induced maturation showed full cumulus expansion (score 3) and ~87% maturation. PGE₂ supplementation without Gn induced mild cumulus expansion (score 0.5–1) but increased nuclear maturation to levels similar to those obtained with Gn alone. In the presence of Gn, exogenous PGE₂ did not affect expansion or nuclear maturation and subsequent embryo development. Treatment with PTGS2 selective inhibitor (NS398), PTGS2-specific siRNA or PTGER2-receptor antagonist (AH6809) resulted in ~20–25% reduction in nuclear maturation. NS398 and AH6809 did not affect cumulus expansion. Most oocytes not reaching metaphase of second meiosis (MII) following NS398, AH6809 and PTGS2-specific siRNA treatments were at MI. After longer maturation, NS398-treated oocytes had normal MII rate and uncompromised embryo development. PGE₂ has a limited role in cumulus expansion in bovine COC but is important for the timing of Gn-induced nuclear maturation. 

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Introduction

Oocyte competence can be defined as the ability of the oocyte to complete a series of events in order to generate a healthy offspring. These events start with the ability of the oocyte to complete maturation and then to undergo successful fertilization, cleavage and reach the blastocyst stage producing a good-quality embryo (Sirard et al., 2006). Although succeeding in one of these events does not guarantee success in the next, oocyte maturation is considered a critical step that influences the success of fertilization and subsequent embryo development (Lonergan et al., 2003). While ~60% of in-vivo matured bovine oocytes reach the blastocyst stage when fertilized and cultured *in vitro*, less than 40% of oocytes matured *in vitro* do so. This rate is even lower when serum-free conditions are utilized (Korhonen et al., 2010; Marei et al., 2009), which clearly shows that current conditions for in-vitro oocyte maturation are deficient and require improvement. Achieving this will require a better understanding of the mechanisms regulating the process of oocyte maturation.

Oocyte maturation can be defined as the set of nuclear, cytoplasmic and molecular changes that enables the oocyte to be fertilized normally (Gilchrist and Thompson, 2007; Kane, 2003). Immature oocytes are arrested at diplotene of prophase of the first meiotic division (known as the germinal-vesicle stage), while mature oocytes are arrested at metaphase of second meiosis (MII) after extrusion of the first polar body (McGaughey, 1983). During oocyte maturation, cumulus cells undergo a process called mucification or cumulus cell expansion, which is induced by gonadotrophin (Gn) stimulation *in vivo* or *in vitro* and leads to massive production of mucoid extracellular matrix (mainly hyaluronic acid, HA) (Chen et al., 1990). Oocyte maturation also includes biochemical and molecular changes that are important to activate the cell-cycle machinery that regulates oocyte nuclear and cytoplasmic events during maturation. These regulatory molecular mechanisms are complex and determine the intrinsic developmental competence of the oocytes. Resumption of meiosis requires activation of maturation-promoting factor (Masui and Markert, 1971). Many pathways including cAMP and cAMP-dependent protein kinase A are reported to be involved in regulating this process (Downs et al., 2002; Homa, 1988). FSH stimulates germinal-vesicle breakdown (GVBD) and resumption of oocyte maturation both *in vitro* and *in vivo* (Assidi et al., 2008, 2010; Sirard et al., 2007) through binding to FSH receptors on granulosa cells or cumulus cells which are G-protein-coupled receptors (Heitman and Ijzerman, 2008; Segaloff et al., 1990) and results in a signalling cascade involving increasing intracellular cAMP concentrations (Dekel, 1988) and stimulation of prostaglandin E synthesis (Shimada et al., 2006; Yamashita et al., 2011).

Prostaglandins are biologically active lipid mediators that are involved in the regulation of many reproductive events such as ovulation, corpus luteum regression, implantation and establishment of pregnancy (Karim and Hillier, 1979). The key regulatory step in prostaglandin biosynthesis is the enzymic conversion of the fatty acid precursor by prostaglandin endoperoxide synthase PTGS1 and PTGS2 (previously known as cyclo-oxygenase enzymes; COX1 and COX2) into prostaglandin G. This is then reduced to an

unstable endoperoxide intermediate prostaglandin H and sequentially metabolized by cell-specific isomerases (PGE synthases (PTGES) or PGF synthases (PTGFS)) to produce PGE or PGF, respectively (reviewed by Wang and Dey, 2005). Three forms of PGES have been identified (Kudo and Murakami, 2005): PTGES1 (or the microsomal PGES-1) colocalized with PTGS2 (Arosh et al., 2002); PTGES2 (or the microsomal PGES-2) coupled to both PTGS1 and PTGS2 (Murakami et al., 2003); and PTGES3 (or the cytosolic PGES) functionally linked to PTGS1 (Tanioka et al., 2000). On the other hand, PGF synthases are present in multiple isoforms, which belong to C members (AKR1C1, AKR1C3, AKR1C4) or B members (such as AKR1B5) of aldo-keto reductase family 1 (Jez et al., 1997; Madore et al., 2003).

Like Gn, prostaglandins mediate their actions through their G-protein-coupled cell surface receptors. PGE receptors (PTGER) are pharmacologically divided into four subtypes – PTGER1, PTGER2, PTGER3 (A, B, C and D isoforms) and PTGER4 – which are known to activate different intracellular signalling pathways. Activation of PTGER2, PTGER3 (B and C) and PTGER4 results in stimulation of adenylyl cyclase and increased intracellular cAMP, while PTGER1 and PTGER3D activation increases Ca²⁺ and PTGER3A decreases cAMP (reviewed by Hata and Breyer, 2004; Negishi et al., 1995).

In cattle, PTGS2 is found in granulosa cells of pre-ovulatory follicles (Liu and Sirois, 1998; Sirois, 1994) and cumulus cells (Nuttinck et al., 2002). PTGS1 is not observed in isolated bovine cumulus–oocyte complexes (COC) before or after in-vivo and in-vitro maturation and, with the exception of some sparse theca cells, no PTGS1-specific immunostaining is detectable in any compartment of 3–6 mm ovarian antral follicles (Nuttinck et al., 2002). With respect to the PGE₂ receptors, PTGER2 and PTGER3 mRNA are detectable in COC while PTGER4 mRNA concentrations are very low and PTGER1 is not detected (Calder et al., 2001). Since PTGER3-deficient female mice have normal fertility (Ushikubi et al., 1998), it is suggested that PTGER2 plays a main role in mediating PGE₂ functions in COC.

From the foregoing, it is clear that components of PGE synthesis and action are present in COC, suggesting a role for PGE in oocyte maturation and/or cumulus expansion. Several studies have demonstrated that PTGS2-derived PGE₂ plays an important role in cumulus cell expansion and oocyte maturation in rodents (Eppig, 1981; Hizaki et al., 1999; Lim et al., 1997; Takahashi et al., 2006) but with fewer studies in cattle (Nuttinck et al., 2011), the role of PGE₂ in oocyte maturation is less characterized and it is not clear if PGE₂ can be used as an additive to improve oocyte competence. The present study examined the expression of genes involved in the synthesis of PGE₂ and PGF_{2 α} in bovine COC and the role of exogenous PGE₂ supplementation during bovine oocyte maturation in the presence or absence of Gn. The role of endogenous PTGS2-produced PGE₂ in mediating Gn-induced maturation and subsequent early embryo development was studied using a selective inhibitor for PTGS2 (NS398) and a receptor antagonist (AH6809) against PTGER2. Since a low concentration of PGE₂ was still detectable from COC treated with NS398, this study also used specific siRNA for PTGS2 with or without NS398 in a novel attempt to completely block PGE₂ synthesis from COC and to study its role during Gn-induced maturation.

Materials and methods

Chemicals and reagents

All chemicals and reagents were purchased from Sigma Chemical Company (Poole, UK) unless otherwise stated.

Collection of oocytes

Bovine ovaries were collected from a local abattoir and transported to the laboratory in phosphate-buffered saline (PBS) in a thermos container at 37°C within 2 h after slaughter, and they were washed in fresh PBS immediately after arrival. COC were retrieved from antral follicles 3–8 mm in diameter with a 19-gauge needle mounted on a 10-ml syringe containing washing media (M-199 supplemented with 20 mmol HEPES and 0.4% (w/v) bovine serum albumin (BSA)) supplemented with 1 mmol 3-isobutyl-1-methyl xanthine, a cAMP phosphodiesterase inhibitor which has been previously shown to reversibly inhibit spontaneous resumption of meiosis in oocytes (Sirard, 1990; Thomas et al., 2002). Grade-1 COC (characterized by a dark homogenous cytoplasm and more than three compact layers of cumulus cells) were selected under a stereomicroscope and washed twice in inhibitor-free washing medium (Marei et al., 2012b).

In-vitro maturation

Basic Gn-free maturation media was composed of M199 supplemented with 0.6% (w/v) fatty-acid-free BSA and 50 µg/ml gentamycin. In experiments investigating the role of PGE₂ in the presence of Gn, 5 µg/ml FSH (Follitropin; Bioniche Animal Health, Belleville, Canada) and 5 µg/ml LH (Leutropin; Bioniche Animal Health) were also added to maturation media. Grade-1 selected COC were washed twice in maturation media and then randomly allocated among different treatment groups. COC were cultured either in 4-well culture dishes (NUNC; Thermo Fisher Scientific, Loughborough, UK) containing 500 µl maturation media/well (20–30 COC per well).

For experiments investigating the effect of siRNA only, COC were cultured in 96-well plates containing 200 µl/ml (10 COC per well) for 24 h at 38.5°C under 5% CO₂ in humidified air to reduce the amount and cost of the transfection reagent.

In the last experiment, different time points were used. All media including the treatments were equilibrated in the incubator under 5% CO₂ in humidified air for at least 2 h prior to culture. Stock solutions (100 mmol) of NS398 and AH6809 were prepared in dimethylsulphoxide, while stock solution of PGE₂ (100 mg/ml) was prepared in absolute ethanol. Dimethylsulphoxide and ethanol were added to the corresponding control group at the maximum concentration used in the treatments (≤0.05%, v/v).

Assessment of cumulus cell expansion

The degree of cumulus cell expansion was examined under a stereomicroscope after 24 h of maturation and each COC was given a score from 0 to 3 subjectively, where 0 = not

expanded and 3 = fully expanded. A mean score of all COC was then calculated within each treatment group (Marei et al., 2012a).

Oocyte staining and determination of the stage of nuclear maturation

To assess the stage of nuclear maturation at the end of the maturation, oocytes were completely denuded of cumulus cells by vortexing in M-199 containing 300 U/ml hyaluronidase (H3506, from bovine testes) for 2 min. Denuded oocytes were then washed twice and positioned on a grease-free slide and overlaid with a coverslip supported by four droplets of a Vaseline/paraffin mixture (40:1). Oocytes were slightly compressed onto the slide and fixed with acetic acid/methanol (1:3, v/v) for at least 24 h. Oocytes were then stained with aceto-orcein (1% orcein in 45% acetic acid) and examined by phase-contrast microscopy (Leica, Milton Keynes, UK). The stage of nuclear maturation was determined according to the morphology of the nuclear material as previously described (Sirard et al., 1989).

PGE₂ radioimmunoassay

Spent maturation media were collected at 24 h of culture and stored at –20°C awaiting assay. The concentration of PGE₂ was quantified using charcoal–dextran-coated radioimmunoassay as described previously (Cheng et al., 2001). Briefly, standards (0.025–5 ng/ml) or samples were mixed with anti-PGE₂ serum (a kind gift from Dr NL Poyser, University of Edinburgh, Edinburgh, UK) and tritiated tracer ([5,6,8,11,12,14,15(n)-3H]-PGE₂; Amersham International, Amersham, UK) in duplicate. After overnight incubation at 4°C, dextran-coated charcoal suspension containing 0.4% dextran (T-70; Amersham Pharmacia Biotech, Uppsala, Sweden) and 2% neutralized charcoal was added to all tubes except the total count. The tubes were incubated at 4°C for 10 min and centrifuged at 2000g for 10 min. The supernatant was removed into 6-ml scintillation vials containing 4 ml scintillant (Ultima gold; Packard Bioscience, Pangbourne, UK) and counted for 2 min. The concentration of PGE₂ was calculated using a semi-logarithmic plot and was corrected for the number of COC cultured per well. Presented values are equivalent to the amount produced by 10 COC. The intraassay coefficient of variation was 3.3% and the interassay coefficient was 6.2%.

IVF and embryo culture

For experiments designed to assess embryo development of treated COC, in-vitro-matured oocytes were fertilized with frozen semen from a single bull as previously described by Marei et al. (2009). Briefly, motile spermatozoa were selected by swim up for 45 min in calcium-free medium followed by centrifugation at 300g at room temperature and resuspension of the pellet in fertilization medium (TALP supplemented with 0.6% (w/v) fatty acid-free BSA, 1 µg/ml heparin, 50 ng/ml epinephrine and 50 ng/ml hypotaurine). The COC were disaggregated by gentle pipetting. Groups of 20–30 COC were then transferred into 400 µl fertilization

medium containing 1×10^6 spermatozoa /ml and cultured for 18 h at 38.5°C in a humidified incubator of 5% CO₂ in air. Presumptive zygotes were denuded from cumulus cells by gentle pipetting and cultured in 500 µl serum-free SOFaaci (synthetic oviductal fluid medium containing amino acids, sodium citrate and *myo*-inositol; Holm et al., 1999) supplemented with 0.4% (w/v) fatty acid-free BSA and cultured at 38.5°C in a humidified incubator with 5% O₂, 5% CO₂ and 90% N₂. The culture was continued up to day 8 (fertilization = day 0). The number of cleaved embryos was recorded on day 2 and blastocysts were counted on day 8. Blastocysts were stained for quality assessment if required by incubating them in 4% paraformaldehyde containing 30 µg/ml Hoechst for 20 min. Stained blastocysts were examined using an epifluorescent microscope (Leica, Wetzlar, Germany) to count total cell number.

Transfection of siRNA

The siRNA transfection was performed as previously described (Kobayashi et al., 2007) with some modifications. Preliminary experiments showed that Hyal-2 (300 IU/ml) was necessary for transfection so it was added to maturation media used in all treatments in this experiment including controls. Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) was used as the transfection reagent. Scrambled, or PTGS2-specific siRNA, were diluted to final concentration 50 nmol in oocyte maturation media. Lipofectamine (2 µl/100 µl media) was added and gently mixed by pipetting and incubated for 2 h at 38.5°C to allow siRNA–Lipofectamine complexes to form. COC were cultured in 96-well plates containing 200 µl media only (control) or supplemented with Lipofectamine with PTGS2-specific siRNA in the presence or absence of PGE₂. Ten COC were cultured per well.

Assessment of apoptosis in cumulus cells

A proportion of COC treated with or without Lipofectamine and Hyal-2 were fixed in 4% paraformaldehyde in PBS for detection of apoptosis by TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL) labelling using a fluorescein isothiocyanate-conjugated in-situ cell death detection kit (Roche, Penzberg, Germany) according to the manufacturer's instructions. Briefly, COC were fixed in 4% paraformaldehyde containing 30 µg/ml Hoechst for 20 min, then permeabilized using 1% Triton for 5 min and incubated in TUNEL reagent for 1 h at 37°C. Stained COC were then washed, mounted and examined by epifluorescent microscopy.

RNA isolation and reverse transcription

Control (cultured in Gn-containing maturation media) or siRNA-transfected COC (15–20 COC per treatment per repeat) were collected at different time points (according to experimental design), washed twice in oocyte-washing medium (HEPES-M199-BSA) and transferred into a labelled nuclease-free 1.5-ml microcentrifuge tube in 5 µl oocyte-washing medium. Tubes were immediately snap frozen in liquid nitrogen and kept at –20°C.

Total RNA was extracted from COC using an RNeasy Mini Kit (Qiagen, Crawley, UK) following the guidelines supplied by the manufacturer. The concentration and purity of the isolated RNA samples was determined using a NanoDropND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). All samples had an A₂₆₀/A₂₈₀ ratio between 1.9 and 2.1. The integrity of RNA was assessed using 1.5% denaturing formaldehyde agar gel electrophoresis by assessing intact 18S (1874 bp) and 28S (4781 bp) ribosomal RNA.

Total RNA (250 or 500 ng) from each sample was then treated for potential genomic DNA carryover in a single reaction in accordance with guidelines by the manufacturer (Promega, Madison, WI, USA). DNase-treated RNA was reverse transcribed using random hexamer primers and processed accordingly (Reverse Transcription System Kit; Promega). A mastermix of reagents was prepared for the above reaction to minimize potential variation from pipetting. Selected negative control samples were also prepared by including all reagents as above, minus the reverse transcriptase.

Primer design and optimization

Assays were designed for all members of the PTGES isoforms and also for AKR1C3 and PTGS2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also analysed as a house-keeping gene, which was found to be stable among different samples within each experiment (data not shown).

Bovine nucleotide sequences derived from the coding domain sequence were obtained directly from GenBank (NCBI, Bethesda, MD, USA). Exon boundary information was obtained from the Ensembl project database (<http://www.ensembl.org/index.html>). To avoid coamplification of genomic DNA, intron-spanning or intron-flanking gene-specific primers were designed using the Primer3 web software (Rozen and Skaletsky, 2000). Primer alignment specificity was checked using the BLAST search tool (NCBI). Sequence information, accession numbers and expected product lengths are provided in Supplementary Table 1 (available online; all oligonucleotides were commercially synthesized as highly purified salt-free products (MWG-Biotech, London, UK).

For each gene, the primers were tested by conventional PCR amplification using Multiplex PCR kits (Qiagen), 25 ng cDNA and 20 µmol primers. The identity of the PCR products was confirmed by DNA sequence analysis (Geneservice, Cambridge, UK). External standards were prepared from cDNAs identical to real-time PCR products and purified using QIAquick PCR purification columns (Qiagen). The precise concentration of purified cDNA product was determined using spectrophotometry and the presence of a single product was confirmed by electrophoresis on a 2% (w/v) agarose gel. Standards (10^{-1} – 10^{-7} ng/ml) were diluted in nuclease-free water.

Quantitative real-time PCR

Gene transcripts were quantified by real-time PCR as previously described (Wathes et al., 2011). For each assay, a mastermix was prepared that contained a final

concentration of 2× KAPA SYBR Green (Anachem, Bedfordshire, UK), 500 nmol forward and reverse primers and nuclease-free water. For all unknown samples measured, 20- μ l reactions were prepared in 0.2-ml thin-walled tubes with caps (TLS-0851, TCS-0803; Bio-Rad, Hemel Hempstead, UK) containing mastermix plus 50 ng of reverse-transcribed RNA. External standards were run on the same plate in duplicate reactions. No-template controls were included on every plate for each gene product. To minimize variation, all samples included in each analysis were derived from the same reverse-transcription batch, prepared under the same conditions and were analysed on a single plate in duplicate. The optimal annealing temperature (resulting in minimal threshold cycle (C_t)) was determined using the temperature-gradient function of the real-time PCR machine (CFX 96 Real-Time PCR Detection System; Bio-Rad) using a set of eight identical reactions for each primer. A melting curve analysis was performed for each amplicon between 50°C and 95°C and any smaller non-specific products such as dimers were melted (if present) prior to fluorescence acquisition (Supplementary Table 2). Absolute concentrations of the PCR product were calculated by comparing the C values of the unknown samples to the standard curve using the CFX Manager Software version 1.0.1035.131 (Bio-Rad). Efficiency of the assays was $\geq 95\%$, and the standard curve R^2 values were ≥ 0.999 . Data are presented as fg/ μ g reverse-transcribed RNA.

Statistical analysis

In all experiments, the data were from at least three independent repeats using about 20 COC per treatment per repeat. Binominal data from cumulus expansion, oocyte nuclear maturation and development were converted to percentages. All data were then analysed in SPSS version 19 (IBM SPSS Statistics, USA) using a linear mixed model taking the different batches of ovaries into consideration as a random effect. If the main treatment effect was significant, Bonferroni post hoc tests were performed. Differences with P -value < 0.05 were considered significant.

Results

Gene expression of PGE synthase isoforms during COC maturation

Expression of the three isoforms of PGE synthase – PTGES1, PTGES2 and PTGES3 – was detected in COC at 0 h (just after aspiration) (Figure 1A), whereas expression of the PGFS isoform AKR1C3 was detected in bovine endometrium (positive control) but not in COC (Figure 1A and B). After 24 h in maturation media, a 2.6-fold increase was recorded in *PTGES1* expression ($P < 0.05$) whereas *PTGES2* and *PTGES3* expression were not significantly changed (Figure 1C).

In-vitro production of PGE₂

PGE₂ was found in detectable concentrations in spent maturation media collected from COC after 24 h culture.

The concentration of PGE₂ in the control group (mean \pm SEM) was 25 ± 9.2 pg/ml.

Effect of PGE₂ with and without Gn on oocyte maturation

In the absence of Gn, addition of PGE₂ (50, 100, 1000 ng/ml) resulted in a concentration-dependent increase in the percentage of oocytes reaching MII after 24 h in culture (reaching levels similar to the Gn-containing controls and significantly higher than Gn-free controls; $P < 0.05$), associated with only a small but significant increase in cumulus cell expansion ($P < 0.05$; Figure 2). Supplementation of the same concentrations of PGE₂ in the presence of FSH and LH had no effect, where more than 80% of oocytes reached MII after 24 h in all treatment groups (data not shown).

Effect of PGE₂ during oocyte maturation on early embryo development

When COC were cultured in serum-free maturation media containing FSH and LH supplemented with PGE₂ (50, 100, 1000 ng/ml), there was no significant effect on cleavage and blastocyst rates after IVF compared with controls containing FSH and LH only (Table 1).

Effect of PTGS2 selective inhibitor on Gn-mediated oocyte maturation

In Gn-containing maturation medium, treatment of COC with 10 μ mol NS398 decreased the percentage of oocytes reaching MII after 24 h compared with controls ($64 \pm 1.5\%$ versus $84 \pm 1.9\%$; $P < 0.05$). This treatment also resulted in a higher percentage of oocytes at MI stage (27 ± 3.6 versus 6 ± 2.3 ; $P < 0.05$). A lower concentration of NS398 (1 μ mol) did not affect oocyte maturation (Figure 3A). Furthermore, treatment with NS398 (10 μ mol) in the presence of PGE₂ (100 ng/ml) resulted in a maturation rate significantly higher than in oocytes treated only with NS398 ($P < 0.05$) and similar to the Gn-containing control (Figure 3B). Treatment with NS398 (10 μ mol) was associated with a significant decrease ($P < 0.05$) in PGE₂ concentration in the spent maturation media compared with the Gn-containing control (Figure 3C). Cumulus cell expansion was not affected by NS398 treatment (Figure 3D).

Effect of PTGER2-receptor antagonist on Gn-induced oocyte maturation

Treatment of COC with AH6809 had no effect on cumulus cell expansion (Figure 4A) but resulted in a significantly lower percentage of oocytes reaching MII at 24 h of culture (66–70% versus 91% in the control group; $P < 0.01$). Increasing AH6809 concentration did not affect the degree of reduction in maturation. The percentage of oocytes arrested at MI showed an increasing trend with concentration; however, the effect was only significant when 50 μ mol AH6809 was used (26% versus 6.25% in the control group (Figure 4B).

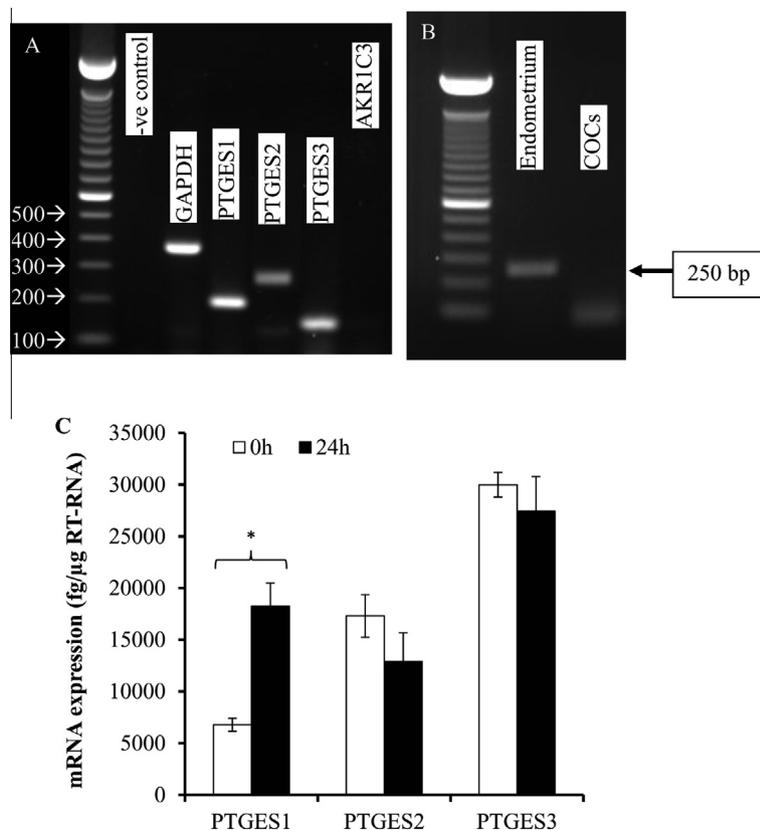


Figure 1 Expression of PGE and PGF synthase in bovine COC. (A and B) Agarose gel electrophoresis showing PCR products of isoforms of PGE synthase (PTGES1, 2 and 3), PGF synthase (AKR1C3) and GAPDH in cumulus–oocyte complexes (COC) at 0 h; B also shows positive control for PGFS in bovine endometrium. (C) Quantitative reverse-transcription PCR analysis of isoforms of PGE synthase expression extracted from immature COC (0 h) and matured COC (24 h). Data are mean \pm SEM from three independent repeats. Asterisk indicates significant difference between the two time points ($P < 0.05$).

Effect of PTGS2-specific siRNA on *PTGS2* expression in COC

PTGS2 expression in COC changed significantly during culture at different time points during maturation ($P < 0.05$). The highest *PTGS2* expression was detected at 6 h in control COC ($P < 0.05$). COC treated with *PTGS2*-specific siRNA had lower *PTGS2* expression at 6, 12 and 24 h ($P < 0.05$) (Figure 5).

Effect of PTGS2-specific siRNA on Gn-mediated oocyte maturation

PGE₂ concentrations in spent media were not affected by scrambled siRNA treatment compared with controls, but decreased to undetectable concentrations in wells treated with *PTGS2*-specific siRNA in the presence or absence of NS398 (Figure 6). The maturation rate was significantly reduced when *PTGS2*-specific siRNA was added to the maturation media for 24 h, with an increased proportion of oocytes at MI ($P < 0.05$). Addition of NS398 to *PTGS2*-specific siRNA-treated media did not have any additional effects on oocyte maturation as compared with oocytes treated with *PTGS2*-specific siRNA only. Addition of PGE₂ (100 ng/ml) to wells containing *PTGS2*-specific siRNA and

NS398 completely abrogated the inhibitory effects on maturation rate. Lipofectamine used for transfection and scrambled siRNA had no significant effect on maturation when added separately (Figure 7). Since Hyal2 was added to facilitate transfection with Lipofectamine, cumulus cell expansion was inhibited in all COC; however, cumulus cell viability was not affected, as shown by TUNEL labelling, compared with control COC, where only a few cells at the peripheral layers of cumulus cells were apoptotic in some COC in both groups (Figure 8).

Effect of PTGS2 selective inhibitor on timing of nuclear maturation and early embryo development

To test whether the NS398-treated oocytes which are observed at MI at 24 h are completely arrested or delayed, nuclear maturation of oocytes cultured with or without NS398 (10 μ mol) at 22 h and 28 h was examined (Figure 9). Similar to results shown in Figure 3B, significantly less oocytes reached MII in NS398-treated group at 22 h where a higher proportion of oocytes was at MI ($P < 0.05$). However, at 28 h, NS398 had no significant effect on nuclear maturation rate compared with the control at the same time point. In addition, treatment of COC with NS398 and AH6809 during maturation had no significant effect on cleavage and blastocyst rates following IVF (Table 2).

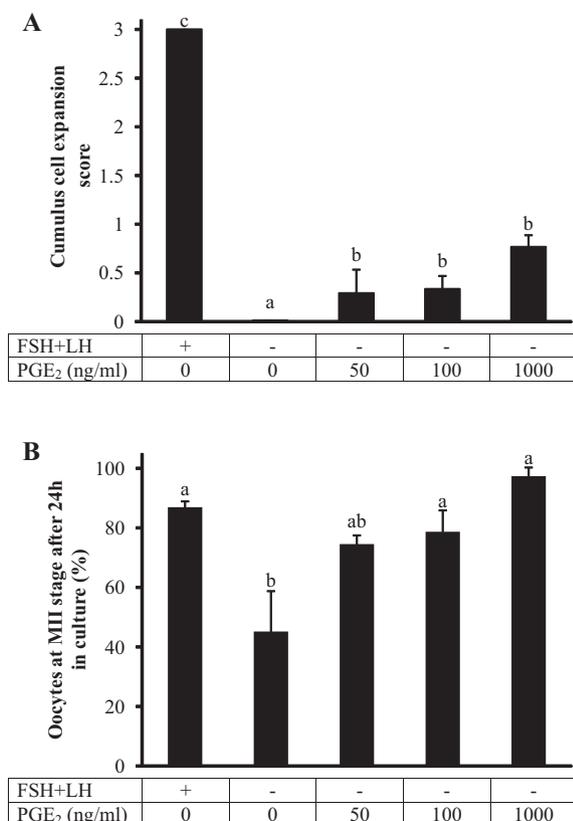


Figure 2 Effect of PGE₂ on cumulus cell expansion (A) and nuclear maturation rate (B). Cumulus–oocyte complexes were treated with different concentrations of PGE₂ in the absence of FSH and LH and stained with aceto-orcein stain at 24 h of culture. Controls with or without FSH and LH are included. Data are mean \pm SEM from three independent repeats. Different superscript letters indicate significant difference ($P < 0.05$).

Discussion

The ability of ovarian follicles to produce prostaglandins is well documented in previous reports. For example, PGE₂ concentration in bovine pre-ovulatory follicles was found to increase after LH stimulation to about 13 ng/ml (Liu and Sirois, 1998). Although, the main source of this PGE₂

production is the granulosa cells, bovine COC were found to produce small amounts of PGE₂ during maturation *in vitro* (Gurevich et al., 1993). The current work has shown that bovine COC produce detectable concentrations of PGE₂ (~20–30 pg/ml) when cultured *in vitro* in media containing Gn.

This study looked at the expression of the three known isoforms of PGE synthase as well as the expression of *PTGS2* in bovine COC. These genes were expressed in freshly aspirated bovine COC and after *in-vitro* maturation. Among the three *PTGES* isoforms, only *PTGES1* was responsive to Gn stimulation during maturation. This finding is in agreement with a previous report where only *PTGES1* of the three isoforms of *PTGES* detected was found to increase during bovine oocyte maturation, paralleling the induction of *PTGS2* enzyme (Nuttinck et al., 2008). This is not only observed in COC, as it was shown that while all *PTGES* isoforms were expressed in the bovine oviduct (Gauvreau et al., 2010) and endometrium (Parent et al., 2002), *PTGES1* was reported to be the main enzyme associated with PGE₂ production (Parent et al., 2002). On the other hand, this study did not detect prostaglandin F synthase (*PGFS*) *AKR1C3* isoform in COC. The primer set used was also able to detect other C members (*AKR1C1* and *AKR1C4*), producing the same PCR product size. Since this study group has previously detected PGF_{2 α} in spent media at 24 h of bovine oocyte maturation (Marei et al., 2010), this suggests that *PGFS* in COC belong to a different isoform. Other enzymes like *AKR1B5* have been identified as functional *PGFS* in bovine endometrium (Madore et al., 2003) and may be functional in COC as well. The *PGFS* isoforms in COC and the role of PGF_{2 α} during oocyte maturation will require further investigation.

The present study also shows the dynamics of *PTGS2* gene expression during oocyte maturation. Basal expression of *PTGS2* increased significantly between 0–6 h, declined between 6–12 h and was unchanged from 12–24 h. The presence of *PTGS2* mRNA and protein has previously been measured in cultured bovine COC during maturation, although their expression pattern was somewhat different to what is reported here. It was reported that *PTGS2* increased at 6 h to reach maximum concentrations at 12 h after incubation in maturation media containing Gn (Calder et al., 2001; Nuttinck et al., 2002), then decreased at 18 and 24 h (Calder et al., 2001) or continued to increase until

Table 1 Early embryo development of bovine COC matured in media containing gonadotrophins with different concentrations of PGE₂.

Treatment	Fertilized oocytes (n)	Cleaved (%)	2-cell (%)	≥ 4 -cell (%)	Blastocyst/total cleaved (%)	Blastocyst/ ≥ 4 -cell (%)
Gn-containing control	64	94 \pm 3.5	8 \pm 1.1	86 \pm 2.5	25 \pm 4.2	28 \pm 4.3
Gn + 50 ng/ml PGE ₂	64	86 \pm 3.8	3 \pm 0.2	83 \pm 3.6	24 \pm 2.2	25 \pm 2.4
Gn + 100 ng/ml PGE ₂	62	92 \pm 1.4	6 \pm 0.2	86 \pm 1.1	21 \pm 0.4	23 \pm 0.4
Gn + 1000 ng/ml PGE ₂	61	92 \pm 1.5	8 \pm 1.5	84 \pm 3.0	23 \pm 1.8	25 \pm 1.5

Values are mean \pm SEM from three independent repeats.

There are no significant differences between control and treatment groups.

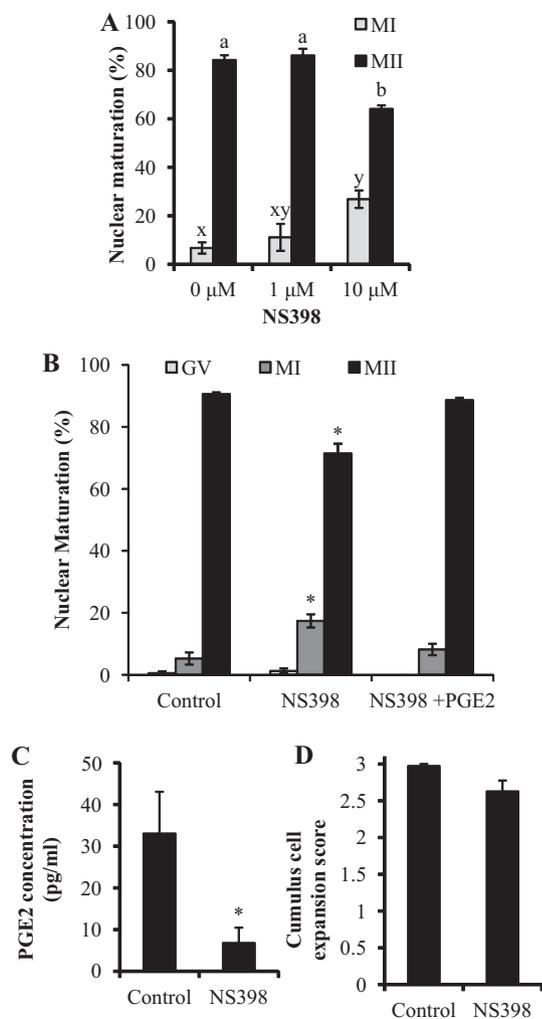


Figure 3 Effect of PTGS2 selective inhibitor (NS398) on oocyte nuclear maturation. Cumulus–oocyte complexes (COC) were supplemented with different concentrations of NS398 (A) or with 10 μmol NS398 with or without 100 ng/ml PGE₂ (B) for 24 h and the stage of nuclear maturation was then assessed; GV = germinal vesicle stage; MI = metaphase of first meiotic division; MII = metaphase of second meiotic division. (C) PGE₂ concentrations in spent maturation media after treatment with 10 μmol NS398. (D) Cumulus cell expansion in COC treated with or without 10 μmol NS398). Data are mean ± SEM from three independent repeats. Different superscript letters indicate significant difference (*P* < 0.05). Asterisks indicate significant difference compared with the control or NS398 with PGE₂ (*P* < 0.05).

24 h (Nuttinck et al., 2002). Notwithstanding the differences in the pattern of PTGS2 expression during the later stages of culture (after 6 h), the increase in PTGS2 expression at 6 h, which occurs just before the time for GVBD (Sirard et al., 1989), supports the idea that prostaglandin production may be important in regulating oocyte maturation and cumulus cell expansion.

To investigate the role of PGE₂ in oocyte maturation, serum-free-maturation media was supplemented with different doses of PGE₂. In the absence of Gn, PGE₂ significantly increased the percentage of oocytes reaching MII

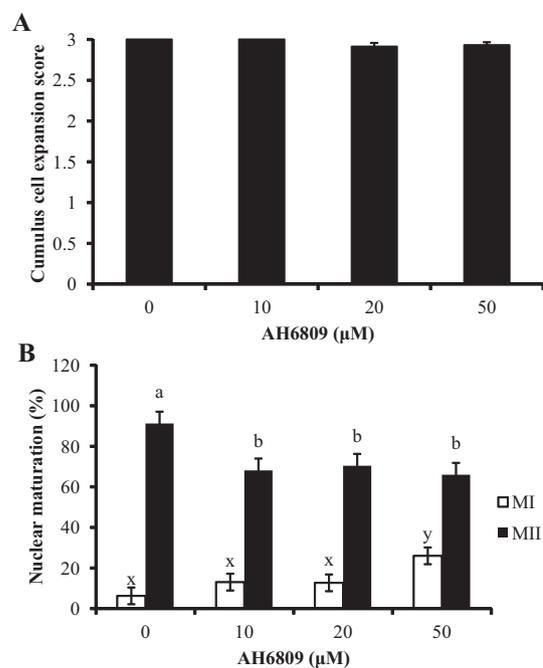


Figure 4 Effect of PTGER2-receptor antagonist (AH6809) on cumulus cell expansion (A) and oocyte nuclear maturation (B). Cumulus–oocyte complexes were treated with different concentrations of AH6809 for 24 h and then expansion and nuclear maturation was assessed. MI = metaphase of first meiotic division; MII = metaphase of second meiotic division. Data are mean ± SEM from three independent repeats. Different superscript letters indicate significant difference (*P* < 0.05).

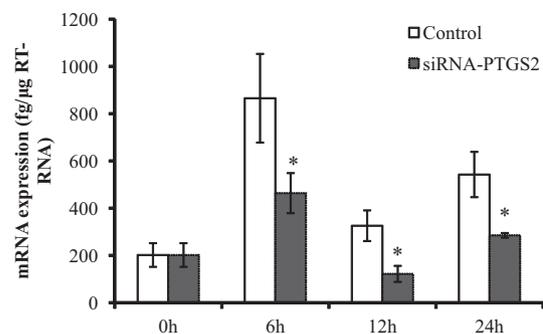


Figure 5 Expression of PTGS2 at different time points of maturation in cumulus–oocyte complexes (COC) treated with or without PTGS2-specific siRNA. RNA was extracted and reverse transcribed from 20 snap-frozen COC at each time point. Data are mean ± SEM from three independent repeats. Asterisk indicates significant difference compared with the control (*P* < 0.05).

after 24 h in culture, achieving maturation rates comparable to those obtained with Gn alone. In the presence of Gn, oocyte nuclear maturation was high and PGE₂ supplementation had no additional effects on oocyte maturation or further embryo development following IVF. This suggests that endogenous PGE₂ produced from the COC in the presence of Gn during culture may be sufficient to mediate, at

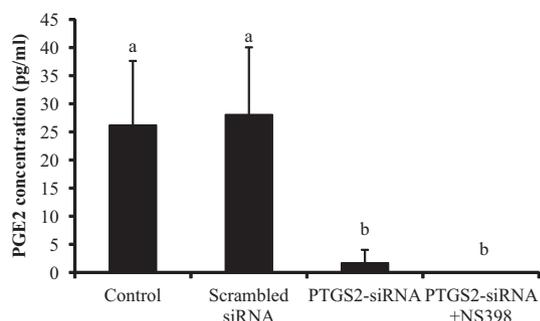


Figure 6 Effect of PTGS2-specific siRNA on PGE₂ concentrations in spent maturation media in presence or absence of PTGS2 selective inhibitor (NS398). Cumulus–oocyte complexes were cultured in media containing FSH and LH (5 µg/ml) supplemented with 50 nM PTGS2-specific siRNA or PTGS2-specific siRNA + 10 µmol NS398 for 24 h. PGE₂ was measured using radioimmunoassay. Data are mean ± SEM from three independent repeats. Different superscript letters indicate significant difference ($P < 0.05$).

least in part, the Gn-induced oocyte maturation. These results are in accordance with data from Cox-2^{-/-} mice, where PGE₂ supplementation significantly increased GVBD and progress to MII only in the absence of Gn, but had no additive effect when supplied in the presence of FSH (Takahashi et al., 2006).

To test this notion further, this work inhibited PGE₂ synthesis or function during Gn-induced oocyte maturation and looked at nuclear maturation rates by inhibiting PTGS2 function using a selective inhibitor (NS398) or inhibiting expression of *PTGS2* using specific siRNA transfection. This work also targeted PGE₂ action using PTGER2-receptor antagonist (AH6809).

Treatment with NS398 resulted in a reduction in PGE₂ concentration associated with limited inhibition (about 20%) of nuclear maturation; however, PGE₂ was still detectable

in the spent maturation media after 24 h. This low concentration of PGE₂ may still be able to support oocyte maturation so this study completely inhibited PGE₂ synthesis to examine the importance of PGE₂ during oocyte maturation. Using a novel approach, COC were successfully transfected with siRNA specific for *PTGS2* using Lipofectamine RNAiMAX transfection reagent in the presence of Hyal2. siRNA has previously been used to silence *PTGS2* expression in bovine granulosa cells, where the significant suppressive effects of siRNA (0.25–0.5 nmol) were observed 6 h after transfection (Kobayashi et al., 2007). COC were rather difficult to transfect, so they needed a higher concentration of siRNA (50 nmol). In addition, COC are known to produce abundant amounts of HA during maturation (Salustri et al., 1989). Cells which develop HA-rich pericellular matrices confer a shielding property against extracellular molecules or pathogens. For example, chondrocytes that express abundant pericellular HA were difficult to transfect with plasmid DNA, and transfection efficiency could be improved by hyaluronidase treatment (Lu Valle et al., 1993). In the present study, transfection of COC with *PTGS2*-specific siRNA was not successful in the absence of Hyal2 (300 U/ml) (data not shown). This study group has recently shown that treatment of COC with Hyal2 during maturation does not affect oocyte maturation, fertilization or further cleavage and development to blastocyst stage (Marei et al., 2012a).

Specific siRNA can significantly reduce *PTGS2* expression (from 6 to 24 h) and is more efficient than NS398 in decreasing PGE₂ concentrations in spent maturation media (down to undetectable concentrations). This effect was not associated with any impact on cell viability as shown by TUNEL labelling and was completely reversible by PGE₂ supplementation. Reduction in PGE₂ concentration using siRNA-*PTGS2* to undetectable concentrations in the presence or absence of NS398 was also associated with a significant but limited reduction in maturation rate (about 20% reduction), similar to results obtained using NS398 only. These results together clearly show that oocyte nuclear maturation is not critically

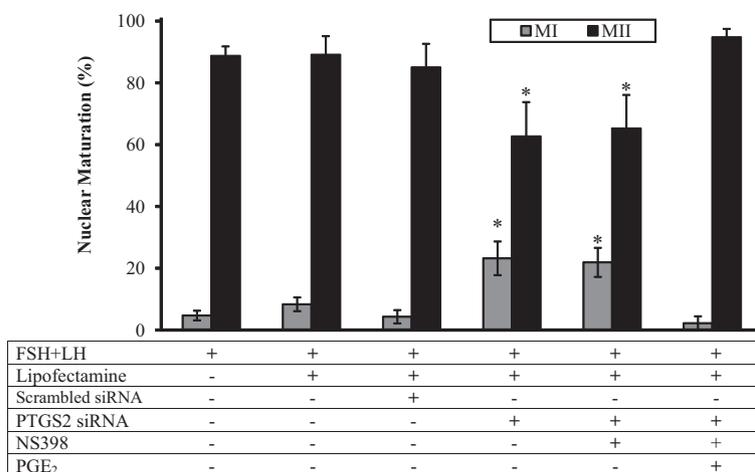


Figure 7 Effect of PTGS2-specific siRNA on oocyte nuclear maturation in presence or absence of PTGS2 selective inhibitor (NS398) and PGE₂. Cumulus–oocyte complexes were cultured in media containing FSH and LH supplemented with different combinations of 50 nM PTGS2-specific siRNA, 10 µmol NS398 and 100 ng/ml PGE₂ for 24 h and the stage of oocyte nuclear maturation was then assessed. MI = metaphase of first meiotic division; MII = metaphase of second meiotic division. Data are mean ± SEM from six independent repeats. Asterisk indicates significant difference with FSH + LH ($P < 0.05$).

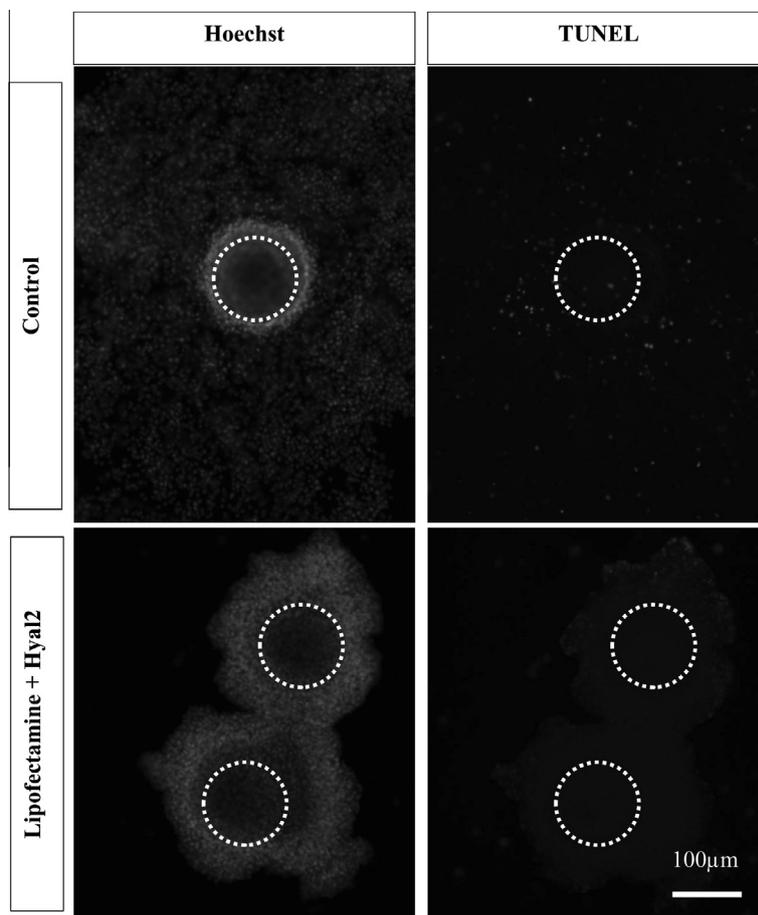


Figure 8 Effect of transfection with Lipofectamine and hyaluronidase on cumulus cell apoptosis. Cumulus–oocyte complexes were cultured in maturation media with or without 20 µl/ml Lipofectamine + 300 IU/ml Hyal-2 for 24 h and then stained by TUNEL to detect apoptosis. Circles indicate the position of the oocytes. Bar = 100 µm.

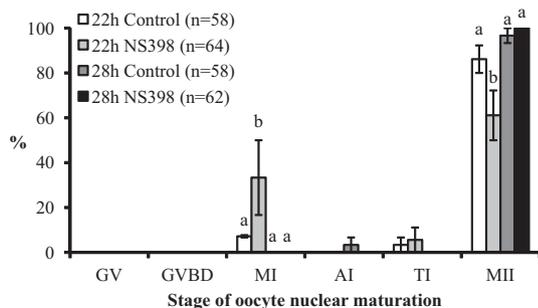


Figure 9 Effect of PTGS2 selective inhibitor (NS389) on oocyte nuclear maturation at different time points. Cumulus–oocyte complexes were cultured in serum-free maturation media supplemented with or without 10 µmol NS398 for 22 h or 28 h and were then denuded and stained with aceto-orcein to determine the stage of nuclear maturation. GV = germinal vesicle; GVBD = germinal vesicle breakdown; MI = metaphase of first meiotic division; AI = anaphase of first meiotic division; T1 = telphase of first meiotic division; MII = metaphase of second meiotic division. Data are mean ± SEM from three independent repeats. Different superscript letters within the same stage of nuclear division indicate significant difference ($P < 0.05$).

dependent on the PGE₂ produced by cumulus cells and that only ~20% of oocytes are affected by the absence of PGE₂.

Regarding PGE receptors, *PTGER2* and *PTGER3* were shown to be expressed in bovine COC, very low levels of *PTGER4* expression were detectable, while *PTGER1* was not detected (Calder et al., 2001). *PTGER3* knockout mice were found to display normal fertility (Ushikubi et al., 1998). The PGE receptor subtype *PTGER2*, however, is more important for fertility. Mice lacking the gene encoding *PTGER2* suffer from decreased ovulation and fertilization (Tilley et al., 1999). The present study shows that blocking *PTGER2* receptors using the selective antagonist AH6809 significantly reduced oocyte maturation rate (about 20% reduction). Collectively, these findings support the notion that PGE₂ acting through *PTGER2* receptors is involved in regulating Gn-induced oocyte maturation in cattle. It is, however, important to note that in all treatments the decrease in maturation rate was only about 20% even when combined treatment of *PTGS2*-specific siRNA and NS398 was used and when no detectable PGE₂ was produced. It is also important to note that most oocytes that failed to reach MII because of inhibition of PGE₂ synthesis (by *PTGS2*-specific siRNA or NS398) or action (by AH6809) were at metaphase of the first meiosis (MI). These observations lead to the suggestions that only a proportion of oocytes (~20%; possibly

Table 2 Early embryo development of bovine COC matured in media containing gonadotrophins with NS398, AH6809 or NS398 + PGE₂.

Treatment	Fertilized oocytes (n)	Cleaved (%)	2-cell (%)	≥4-cell (%)	Blastocyst/total cleaved (%)	Blastocyst/≥4-cell (%)	Total cells in blastocyst
Gn-containing control	84	83 ± 2.6	8 ± 3.4	75 ± 4.5	18 ± 4.1	24 ± 5.0	88 ± 11.8
Gn + 10 μmol NS398	85	85 ± 3.3	12 ± 3.1	73 ± 6.3	24 ± 4.4	32 ± 2.4	87 ± 7.3
Gn + 50 μmol AH6809	85	80 ± 7.6	14 ± 2.2	65 ± 7.8	24 ± 5.0	36 ± 4.0	89 ± 9.6
Gn + 10 μmol NS398 + 100 ng/ml PGE ₂	86	84 ± 6.4	11 ± 3.0	73 ± 6.4	25 ± 5.2	34 ± 5.1	87 ± 5.7

Values are mean ± SEM from three independent repeats.

There are no significant differences between control and treatment groups.

NS398 = PTGS2 selective inhibitor; AH6809 = PTGER2-receptor antagonist.

less competent oocytes) are affected when PGE₂ is compromised, and/or that the PGE₂ is required to accelerate maturation and that those oocytes seen at MI at 24 h when PGE₂ synthesis or action is compromised are able to reach MII if cultured for extended time. Autocrine/paracrine stimulation of PTGER2 by PGE₂ is known to increase intracellular cAMP concentrations which may accelerate the oocyte maturation process (Hata and Breyer, 2004). It was previously shown that good-quality maturation requires the highest concentration of intracellular cAMP, since below-threshold intracellular cAMP in bovine COC caused defective oocyte maturation leading to poor development (Modina et al., 2001).

To test this further, this study looked at nuclear maturation rates at 22 and 28 h in the presence or absence of NS398. NS398 treatment increased the proportion of oocytes at MI at 22 h but had no effect on maturation rate when cultured for longer time (28 h) and had normal development rate following IVF. Therefore, the increase in MI is due to a delay in nuclear maturation without compromising the developmental competence of the oocyte. This is in agreement with the previous data from Nuttinck et al. (2011) who found that PTGS2 inhibition significantly increased oocytes at MI after 22 h and also showed that NS398 decreased embryo output rates on day 6 post fertilization but not on day 7. They also showed that total blastomere number was significantly lower in embryos obtained after PTGS2 inhibition, while no effect on total cell number in the blastocyst on day 7 could be seen in the present study.

Regarding cumulus cell expansion, bovine cumulus cells express *PTGS2* mRNA prior to expansion at a time before *PTGS2* expression was detected in granulosa cells (Calder et al., 2001). This suggests that PGE₂ produced by bovine cumulus cells, not by granulosa cells, may be involved in cumulus cell expansion. The role of PGE₂ in cumulus cell expansion in bovine COC is very limited. PGE₂ supplementation resulted in a very low stimulation of cumulus cell expansion, an effect that was apparent only in the absence of Gn. This agrees with Calder et al. (2001) who showed that in the absence of Gn, PGE₂ supplementation (≥50 ng/ml) of bovine oocytes *in vitro* could stimulate moderate expansion of cumulus cells only in the presence of serum. This suggests that serum factors are required to mediate PGE₂-induced expansion. Furthermore, NS398 and AH6809 had no

significant effect on cumulus cell expansion. The effect of PTGS2-specific siRNA on cumulus cell expansion could not be studied because addition of Hyal2 was required for the transfection process. This depolymerises HA, resulting in complete inhibition of expansion. While PGE₂ appears to play a modest role in bovine cumulus cell expansion, it has been shown to play a very important role in rodents. Immature COC collected from mice lacking the gene encoding *PTGER2* failed to expand *in vitro* in response to FSH and PGE₂ (Hizaki et al., 1999). *PTGS2* null mice exhibited impaired cumulus cell expansion, an effect which was mediated by down-regulation of tumour necrosis factor-induced protein-6 (TSG-6; which has HA-binding functions) and enhanced cumulus cell death (through AKT) (Takahashi et al., 2006).

In conclusion, PGE₂ is produced by bovine COC through the co-ordinated actions of *PTGS2* and *PTGES1* enzymes during Gn-induced maturation. Importantly, inhibition of PGE₂ synthesis slows down oocyte nuclear maturation without any impact on its developmental competence. In the presence of Gn, supplementation with exogenous PGE₂ seems to have no additive effect on oocyte maturation and development in bovine.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.rbmo.2013.11.005>.

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