REPRODUCTION
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Short-term exposure to hydrogen peroxide during oocyte maturation improves bovine embryo development

L Vandaele, M Thys, J Bijttebier, A Van Langendonckt[1], I Donnay[2], D Maes, E Meyer[3] and A Van Soom

Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium, [1]Department of Gynecology, Université Catholique de Louvain, Cliniques Universitaire de St Luc, Avenue Hippocrate 10, B-1200 Brussels, Belgium, [2]Veterinary Unit, Institute of Life Science, Université Catholique de Louvain, Place Croix du Sud 5 Box 10, B-1348 Louvain, Belgium and [3]Department of Pharmacology, Toxicology and Biochemistry, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium

Correspondence should be addressed to L Vandaele; Email: leen.vandaele@ugent.be

Abstract

Recent studies have shown that short-term exposure of oocytes to a stressor such as hydrostatic pressure or osmotic stress might induce stress tolerance in embryos. The aim of the present study was to investigate the consequences of short-term hydrogen peroxide (H_2O_2) exposure to bovine *in vitro* matured cumulus–oocyte complexes (COCs) on subsequent preimplantation embryo development and apoptosis. In the first experiment, mature COCs were incubated in H_2O_2 at concentrations ranging between 0.01 and 100 μmol/l, and subsequently fertilized and cultured. Oocyte incubation with 50–100 μmol/l of H_2O_2 resulted in a significantly higher blastocyst yield (47.3%) in comparison with control medium (31.8%), while apoptotic cell ratio was inversely related with H_2O_2 concentration. In the second experiment, we showed that the stress tolerance after H_2O_2 exposure was not mediated by increased glutathione content in treated oocytes nor by enhanced fertilization or penetration. Further research should concentrate on the potential role of players that have been associated with stress tolerance in somatic cell lines.

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Introduction

In vitro embryo culture conditions never completely mimic the *in vivo* situation which prevails in the oviduct and uterus, and may represent a source of increased oxidative stress for the *in vitro* produced embryo (Nasr-Esfahani *et al*. 1990, Goto *et al*. 1993, Agarwal *et al*. 2006). Especially in human oocytes, oxidative stress has been correlated with problems during meiotic division and with increased fragmentation and aberrant morphology in embryos originating from aging oocytes (Tarin 1996). Although minimum amounts of reactive oxygen species (ROS) are needed in several physiological processes such as sperm capacitation and acrosome reaction (Hsu *et al*. 1999) and maintenance of the balance between inner cell mass and trophectoderm (Gramzinski *et al*. 1990, Pierce *et al*. 1991), increased intracellular levels can be responsible for defective embryo development, apoptosis, and embryonic arrest (Goto *et al*. 1993, Yang *et al*. 1998, Hashimoto *et al*. 2000, Guerin *et al*. 2001). To ensure protection from oxidative damage, cells are endowed with a number of enzymatic and non-enzymatic ROS scavengers such as catalase, superoxide dismutase, and glutathione (GSH) peroxidase (Fridovich 1978, Behl *et al*. 1994, Clement & Stamenkovic 1996), which are also present in the oocyte (Cetica *et al*. 2001).

Consequently, the ultimate oxidative status in an embryo results from the balance between ROS supply and ROS detoxification by the antioxidant mechanisms (Harvey 2007). Incompetent oocytes and embryos at different developmental stages are exposed to increased levels of endogenous superoxide anion radicals and hydrogen peroxide (H_2O_2) production, which are responsible for mitochondrial damage, subsequent caspase activation, and finally apoptosis (Velez-Pardo *et al*. 2007).

Exogenous H_2O_2, if not inactivated, is converted into highly reactive ROS such as hydroxyl radical and reactive nitrogen species such as peroxynitrite (Klaunig & Kamendulis 2004). In previous studies in cattle and mice, H_2O_2 supplementation to zygotes, 9- to 16-cell stage embryos, or blastocysts was shown to be detrimental for embryo development, causing excessive fragmentation and apoptosis (Morales *et al*. 1999,

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Liu & Keefe 2000, Feugang *et al*. 2004). However, data from recent studies indicate that in some cases, a short-term period of stress can be beneficial to oocytes during maturation: 30–120 min of hydrostatic pressure improved porcine embryo development after activation (Pribenszky *et al*. 2008*a*), after vitrification (Du *et al*. 2008*a*, Pribenszky *et al*. 2008*a*) and after handmade cloning (Du *et al*. 2008*b*). Similarly, 1 h of osmotic stress either by sodium chloride, sucrose, or trehalose during oocyte maturation enhanced cleavage and blastocyst production after vitrification and subsequent activation of porcine oocytes (Lin *et al*. 2009). Therefore, the aim of the present study was to test the impact of short-term exposure of mature cumulus–oocyte complexes (COCs) to different concentrations of H_2O_2 on further preimplantation embryo development and apoptosis.

Results

Experiment 1

The results showed that exposure of oocytes to high H_2O_2 produced significantly more blastocysts in comparison with the C+ group (47.3 and 31.8% respectively; Fig. 1). The mean number of blastomeres at the blastocyst stage (7 days post-insemination (dpi)) varied between 100 and 120 and was not different between groups. The mean apoptotic cell ratio (ACR; after TUNEL) was significantly lower after incubation of oocytes in high H_2O_2 in comparison with low H_2O_2 or medium H_2O_2 (Fig. 2).

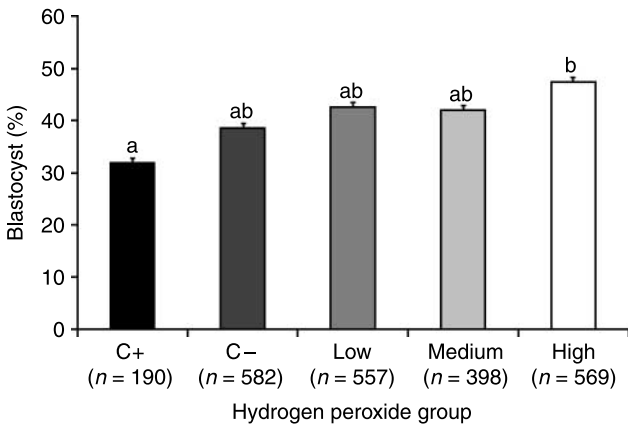

Figure 1 Blastocyst yield (determined on total number of oocytes) at 7 dpi (mean %, S.E.M. in error bars) after incubation of matured cumulus–oocyte complexes in different H_2O_2 concentrations (C+, negative control with pyruvate; C−, negative control without pyruvate; low, 0.01–0.1 μmol/l H_2O_2; medium, 1–10 μmol/l H_2O_2; high, 50–100 μmol/l H_2O_2) (four replicates, number of oocytes is represented under the *X*-axis). [a,b]Bars with different superscript letters differ significantly ($P<0.05$).

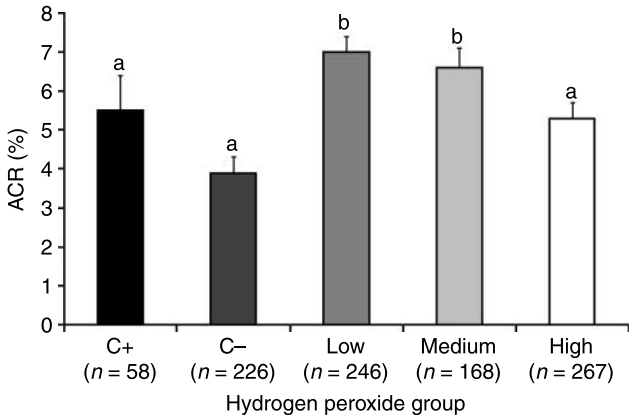

Figure 2 Apoptotic cell ratio (ACR; mean %, S.E.M. in error bars) of day 7 blastocysts, which developed after incubation of matured cumulus–oocyte complexes in different H_2O_2 concentrations (C+, negative control with pyruvate; C−, negative control without pyruvate; low, 0.01–0.1 μmol/l H_2O_2; medium, 1–10 μmol/l H_2O_2; high, 50–100 μmol/l H_2O_2). Number of investigated blastocysts is depicted under the *X*-axis. [a,b]Bars with different superscript letters differ significantly ($P<0.05$).

Experiment 2

GSH content in mature oocytes was not changed following incubation in different H_2O_2 concentrations ($P<0.05$; Table 1).

Fertilization and penetration rates varied around 55 and 72% respectively for all groups (Fig. 3). Fertilization and penetration rates were very comparable with other experiments in the same setting. Only very high concentrations of H_2O_2 (1 mM) resulted in significantly lower fertilization (22.1±5.25%) and penetration rates (34.4±5.22%) in comparison with all other groups.

Discussion

Short-term exposure of mature COCs to high concentrations of H_2O_2 induced stress tolerance during further development visualized by enhanced embryo development, but it did not affect apoptosis in blastocysts. In contrast, low to medium H_2O_2 concentrations significantly increased apoptosis in day 7 blastocysts. The positive effect on embryo development could not be explained by higher GSH levels in mature oocytes or by enhanced fertilization rates.

Increased oxidative stress is considered to be an important cause of defective embryo development *in vitro* (Guerin *et al*. 2001). Most studies investigating the effect of exogenous H_2O_2 supplementation during *in vitro* culture showed a negative effect on embryo development at different stages of development. H_2O_2 incubation of bovine blastocysts (Morales *et al*. 1999), immature rat oocytes (Chaube *et al*. 2005), and murine zygotes (Liu & Keefe 2000, Liu *et al*. 2000) resulted in decreased development and/or increased apoptosis.

Table 1 Mean glutathione content of oocytes exposed to different concentrations of H_2O_2 for 1 h.

H_2O_2 treatment	**Oocytes** (number)	**GSH** (pmol ± S.E.M.)
C+	89	3.7 ± 0.3
C−	58	4.1 ± 0.4
Low	145	4.2 ± 0.3
Medium	142	3.5 ± 0.3
High	115	3.7 ± 0.4
Very high	54	3.7 ± 0.4

C+, negative control with pyruvate; C−, negative control without pyruvate; low, 0.01–0.1 μM H_2O_2; medium, 1–10 μM H_2O_2; high, 50–100 μM H_2O_2.

In contrast, 9- to 16-cell bovine embryos were described to be refractory to H_2O_2 concentrations between 0.1 and 10 μM (Morales *et al.* 1999), and exposure of oocytes to superoxide radicals generated by the hypoxanthine–xanthine system during the first hour of maturation led to improved development of day 6 morulae (Blondin *et al.* 1997).

The most important difference between this study and previous ones is the time at which oocytes or embryos were exposed to H_2O_2, with our study being the only one using H_2O_2 at the end of the maturation period. Oocyte developmental competence or intrinsic oocyte quality is determining further embryo development and quality, e.g. a bovine oocyte diameter above 120 μm is correlated with better embryo development and prevention of apoptosis (Vandaele *et al.* 2007). Critical during maturation is the paracrine communication between oocyte and cumulus cells providing a microenvironment that is necessary for further development (Tanghe *et al.* 2002). Fully grown oocytes are capable of secreting paracrine factors (oocyte-secreted factors), which act on the cumulus cells by inducing expression of several genes and improving cumulus expansion to establish a microenvironment enhancing further embryo development (Elvin *et al.* 1999, Pangas *et al.* 2004, Hussein *et al.* 2006). A very straightforward hypothesis is that incubation with H_2O_2 might increase the level of antioxidants in the oocyte and/or cumulus cells with a positive effect on their future development. Increased levels of antioxidants may improve embryo development to the blastocyst stage as demonstrated for catalase (Li *et al.* 1993, Orsi & Leese 2001), GSH (Luvoni *et al.* 1996, Abeydeera *et al.* 1999, de Matos & Furnus 2000, de Matos *et al.* 2002, Ozawa *et al.* 2006), and superoxide dismutase (Li *et al.* 1993, Chun *et al.* 1994) in different species. GSH content at the end of maturation has been identified as a reliable indicator of oocyte viability (Abeydeera *et al.* 1999). GSH synthesis is based on the presence of glycine, glutamine, and cysteine, of which cysteine is the rate-limiting step (Furnus *et al.* 2008). Therefore, supplementation of cysteine during maturation resulted in improved development to the morula and blastocyst stages (Ali *et al.* 2003). However, in this study, GSH content was similar in all groups of mature oocytes immediately after short-term H_2O_2 incubation. Unfortunately, GSH levels in 9- to 16-cell embryos were under the limit of detection (data not shown). Similar studies in different human cell lines also showed that oxidant resistance after hyperoxia pre-exposure was not mediated by increased antioxidant levels (Wiese *et al.* 1995, Pietarinen-Runtti *et al.* 1998). These results prompted us to investigate other hypotheses in order to explain the improved embryo development after short-term H_2O_2 incubation of mature COCs.

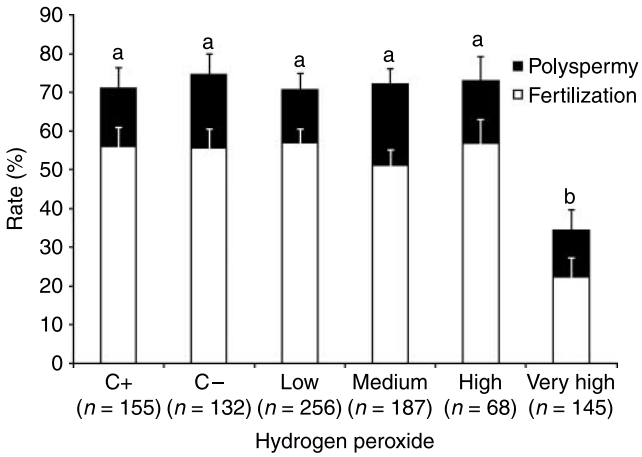

Figure 3 Fertilization rate (%), polyspermy rate (%), and total penetration rate (%) (represented by the sum of both bars) of cumulus–oocyte complexes, which were incubated in different concentrations of H_2O_2 (low, 0.01 μmol/l or 0.1 μmol/l; medium, 1 μmol/l or 10 μmol/l; high, 100 μmol/l; very high, 1 mmol/l) for 1 h before fertilization. Number of analyzed presumed zygotes is depicted under the *X*-axis. [a,b]Bars with different superscript letters differ significantly ($P<0.05$).

Next, neither fertilization nor penetration was stimulated by increased H_2O_2 concentrations in our study. The beneficial effect of H_2O_2 on embryo development in the first experiment should therefore be related to other (uncharacterized) effects of H_2O_2, presumably on the oocyte.

The positive effect of H_2O_2 at the end of the maturation period is not mediated by increased GSH content or improved fertilization, but is an effect on the long-term up to the morula or blastocyst stage more than 4 days later. Previous studies have shown that oocyte quality, *in casu* oocyte diameter, can be related to the incidence of apoptosis up to the morula stage (Vandaele *et al.* 2007). Besides the fact that this study is the first one to apply H_2O_2 at the end of maturation, which likely is a crucial factor, it has previously been demonstrated that H_2O_2 can exert a dual effect upon mammalian somatic cell lines (Davies 1999). It is very speculative to compare data obtained in growing cell lines with oxidant concentrations in embryo culture, especially because embryos seem to be much more sensitive to oxidative stress (Morales *et al.* 1999). Nevertheless, we are convinced that several arguments suggest that

short-term H_2O_2-incubated oocytes might experience a transient stress resistance resulting in increased embryo development and better protection against embryonic apoptosis.

The first argument is that acquisition of stress tolerance after short-term exposure of mature COCs to other stressors such as high hydrostatic pressure has already been observed in murine (Pribenszky *et al*. 2005), bovine (Pribenszky *et al*. 2008*b*), and porcine embryos (Du *et al*. 2008*a*, 2008*b*, Pribenszky *et al*. 2008*a*). Alternatively, osmotic stress in porcine oocytes for 1 h also increased cleavage and blastocyst development after activation of the stressed oocytes (Lin *et al*. 2009). The possible mechanism by which stress tolerance is induced has not been elucidated yet, although the production or enhanced stabilization of heat shock protein (*HSP*) mRNA has been suggested as a possible cause (Kaarniranta *et al*. 1998, Du *et al*. 2008*b*). Likewise, mild heat shock induced phosphorylation of HSP27, which contributes to subsequent heat tolerance (Lavoie *et al*. 1995). HSPs are molecular chaperones assisting in correct folding of proteins which do not only protect against heat shock, but apparently also against toxic effects of H_2O_2 (Jaattela & Wissing 1993). Furthermore, HSPs directly suppress pro-apoptotic signaling events of both the intrinsic and extrinsic apoptotic pathways (Beere 2005). Before the activation of the embryonic genome around the 8- to 16-cell stages in bovine, embryos survive on the maternal mRNA and proteins deposited in the ooplasm during oocyte growth (Braude *et al*. 1988). Until that stage, new transcription of genes is minimal and protein production is determined by mRNA quality and quantity in the oocyte (Vandaele *et al*. 2008). Regulation of protein expression is established through post-translational modifications. The second argument is that in somatic cell culture, adaptation to oxidative stress is mediated through alteration in gene expression and/or mRNA translation of at least 40 gene products (Davies 1999), indicating that *HSPs* might not be the only important candidate genes. Several of those genes are mitogenic genes such as *FOS*, *JUN*, and *MYC*, which might play a role in embryo development (Crawford *et al*. 1988). Until now, no strong evidence is available for a role of proto-oncogenes, e.g. *JUN* and *FOS* during the preimplantation period, although Ozolins & Hales (1997) have suggested that redox-induced aberrations in the activity of the transcription complex AP-1 may either trigger abnormal development or evoke long-term transcriptional changes that protect embryos from oxidative stress.

As far as the effect on embryonic apoptosis is concerned, our setup showed that a high H_2O_2 pulse did not increase ACR in comparison with embryos in the control groups, whereas low H_2O_2 concentrations were able to enhance apoptosis in day 7 blastocysts. In contrast, incubation of immature rat oocytes with low H_2O_2 concentration resulted in increased expression of pro-apoptotic BAX and caspase-3 immediately after incubation (Chaube *et al*. 2005). Altered gene expression and translational or post-translational modification might not only be responsible for enhanced embryo development, but also for H_2O_2-induced apoptosis resistance (Clement & Stamenkovic 1996). Besides the potential role of HSP, the transcription factor, hypoxia-inducible factor 1 (HIF-1), could also be an important player in the stress resistance (Kietzmann & Gorlach 2005, Klimova & Chandel 2008). HIFs are heterodimeric DNA-binding complexes that modulate the expression of several genes and regulate adaptive responses to alterations in O_2 (Semenza 1998). It is certain that bovine blastocysts have the ability to detect and respond to low O_2 conditions by altering the expression levels of glucose transporter (GLUT1, SLC2A1) mediated by alterations in HIF-2α (Harvey *et al*. 2004). Also antioxidant enzymes (superoxide dismutase 1, superoxide dismutase 2, and GSH peroxidase) have been proposed as potential HIF-2 target genes, but their expression was not altered following low-oxygen culture in bovine blastocysts (Harvey 2007). Nevertheless, the response of HIF to hyperoxia or oxidative stress is still a subject of debate (Semenza 2009).

In conclusion, short-term exposure of bovine oocytes to H_2O_2 at the end of the maturation period had no effect on fertilization, although embryo development to the blastocyst stage was stimulated. Secondly, low concentration of H_2O_2 increased the level of apoptosis in day 7 blastocysts, whereas incubation in high levels of H_2O_2 had no effect on the appearance of apoptosis in day 7 blastocysts. Similar effects were reported after hydrostatic pressure and osmotic stress of oocytes, which direct further research to the common molecular background of stress tolerance, with HSP and HIF being the first candidate molecules.

Materials and Methods

Media and reagents

Basal medium Eagle amino acids, minimal essential medium nonessential amino acids (100×), TCM199 medium, and gentamicin were obtained from GIBCO-BRL Life Technologies. FCS was purchased from Biochrom AG (Berlin, Germany), TUNEL reagents were produced by Boehringer, and all other components were purchased from Sigma. All culture media were filtered through a 0.22 μm filter (Millipore Corporation, New Bedford, MA, USA).

In vitro *embryo production*

Bovine embryos were produced by routine *in vitro* methods (Vandaele *et al*. 2006). Briefly, bovine ovaries were collected at the abattoir and processed within 2 h. The ovaries were washed thrice in warm physiological saline supplemented with kanamycin (25 mg/ml, GIBCO-BRL Life Technologies). The COCs were aspirated from follicles between 4 and 8 mm

in diameter and cultured in groups of 100 in 500 µl-modified bicarbonate-buffered TCM199, supplemented with 20% (v/v) heat-inactivated FCS (Biochrom AG); 0.4 mmol/l glutamine; 0.2 mmol/l sodium pyruvate; gentamicin (50 µg/ml) for 20–24 h at 38.5 °C in 5% CO_2 in air. Frozen–thawed bovine semen was separated through a discontinuous Percoll gradient (45 and 90% (v/v); Pharmacia) and washed. The final sperm concentration of 1×10^6 sperm cells/ml was adjusted in IVF-TALP, consisting of bicarbonate-buffered Tyrode solution, supplemented with BSA (6 mg/ml) and heparin (20 µg/ml). The matured oocytes were washed in 500 µl IVF-TALP and incubated with sperm. After 20–24 h of incubation, the presumed zygotes were vortexed to remove excess sperm and cumulus cells. The washed zygotes were transferred to synthetic oviduct fluid (SOF) supplemented with amino acids and 5% (v/v) FCS and were cultured in 50 µl droplets under mineral oil in groups of 25 in 5% CO_2, 5% O_2, and 90% N_2.

Short-term peroxide exposure

For all experiments, immediately before fertilization, cumulus-intact oocytes were incubated in 500 µl of different concentrations of H_2O_2 (H1009, Sigma) for 1 h (0.01, 0.1, 1, 10, 50, and 100 µmol/l). An additional 1 mmol/l group was used for experiment 2. Since pyruvate can neutralize the effect of peroxide (Morales *et al.* 1999), the different concentrations were diluted in maturation medium without pyruvate. Meanwhile, two control groups were kept in maturation medium without pyruvate (C−) and normal maturation medium (C+) respectively. After incubation, COCs were washed twice in HEPES-TALP.

Determination of GSH content

The GSH content was determined as described by Bijttebier *et al.* (2008) with some modifications. Oocytes were washed thrice in polyvinylpyrrolidone (PVP; 1 mg/ml in PBS) and transferred in 5 µl assay buffer (0.2 M sodium phosphate buffer containing 10 mM EDTA, pH 7.2) to which 5 µl of 1.25 mol/l H_3PO_4 was added. Samples were stored at −80 °C until assayed. The intracellular content of GSH was determined using the 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB)–GSH disulfide reductase assay. A 700 µl of 0.29 mg/ml NADPH in assay buffer, 100 µl of 0.75 mmol/l DTNB, and 194 µl of water were added to the sample and mixed. Of a 400 IU/ml GSH reductase solution, 6 µl were added to start the formation of 5-thio-2-nitrobenzoic acid, which has an absorption peak at 412 nm. This was analyzed with a Beckman DU-600 spectrophotometer every 20 s for 2 min. The GSH content in the samples was calculated from a standard curve (0.03–0.5 nmol GSH) and a blank sample.

Evaluation of apoptosis

After fixation in 4% paraformaldehyde, embryos were permeabilized with 0.5% Triton X-100 in PBS for 1 h and washed again in PVP solution (1 mg/ml in PBS, free of Ca^{2+} or Mg^{2+} (PBS)). Positive and negative controls were treated with DNase (50 units/ml in PBS) for 1 h in 37 °C to ensure detection of strand breaks by TUNEL (*In Situ* Cell Detection kit, Boehringer). After washing, positive control and samples were incubated in fluorescein-dUTP and terminal deoxynucleotidyl transferase for 1 h at 37 °C in the dark. Meanwhile, the negative control was incubated in nucleotide mixture only, in the absence of transferase. After a second washing in PVP solution, controls and samples were incubated in RNase A (50 µg/ml in PBS) for 1 h at room temperature. The nuclei were then counterstained with 0.5% (v/v) propidium iodide (PI) for 30 min at room temperature. Subsequently, embryos were quickly washed in PVP solution and mounted in glycerol with 1,4-diazabicyclo (2.2.2) octane (25 mg/ml) on slides with vaseline bridges. Samples were examined by fluorescence microscopy (Leica DMR, Van Hopplynus, Brussels, Belgium). TUNEL-positive nuclei appeared bright yellowish green, whereas the red PI staining allowed to count the total cell number (TCN) and identification, localization, and quantification of normal, fragmented, and condensed nuclei as defined previously by Gjorret *et al.* (2003). Nuclei that appeared TUNEL-positive and condensed or fragmented were defined as apoptotic.

Experimental design

Experiment 1: incubation of mature COCs with different concentrations of H_2O_2

After H_2O_2 exposure, a total of 2460 mature oocytes were fertilized (four replicates, 100 oocytes per group in each replicate). At 24 h post-insemination (hpi), presumed zygotes were denuded and cultured in modified SOF medium with 10% (v/v) FCS at 39.0 °C in 5% O_2, 5% CO_2, and 90% N_2. Embryos were evaluated for blastocyst development and all blastocysts were subsequently fixed in 4% (w/v) paraformaldehyde in PBS at 7 dpi. After TUNEL staining, TCN and ACR were determined in all groups.

Experiment 2: GSH content in mature oocytes and fertilization after H_2O_2 incubation

A total of 2080 oocytes were matured *in vitro* (three replicates, 80–90 oocytes per group per replicate). Immediately after H_2O_2 exposure, cumulus cells of 30 COCs in each group were removed using 0.5% (w/v) hyaluronidase diluted in TCM199 in combination with 4 min vortexing, and the denuded oocytes were used for determination of GSH content. GSH content per oocyte was calculated by dividing the total content per sample by the number of oocytes present in the sample. The remaining 50–60 COCs in each treatment group were fertilized following conventional procedures. To assess fertilization (two pronuclei) and penetration (at least two pronuclei) rates, denuded zygotes were fixed at 20–22 hpi in 2% (w/v) paraformaldehyde and 2% (w/v) glutaraldehyde and subjected to Hoechst 33342 staining (Thys *et al.* 2009).

Statistical analysis

For the statistical analysis, H_2O_2 concentrations were combined to three groups (exp 1) and four groups (exp 2): 0.01 and 0.1 µmol/l (low); 1 and 10 µmol/l (medium); 50 and 100 µmol/l (high); 1 mmol/l (very high). Univariate ANOVA were used

with blastocyst yield and TCN as dependent variables, group as fixed factor, and replicate as random factor (mixed model ANOVA). Pairwise comparisons were performed by the Bonferroni test. Differences in ACR and GSH content were analyzed by a non-parametric Kruskal–Wallis test as the data were not normally distributed. Logistic regression was used to analyze fertilization and penetration rates, with fertilization and penetration as dependent variables and group as independent variable including the effect of replicate.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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