Effects of oxygen concentration on in vitro maturation of canine oocytes in a chemically defined serum-free medium

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Abstract

Canine oocytes require an extended period of culture (72 h) in vitro for nuclear maturation to the metaphase II stage, which also results in high degeneration. Canine cumulus oocyte complexes were isolated by slicing from ovaries collected after ovariohysterectomy and cultured in serum-free synthetic oviductal fluid incubated at low (5%) or high (20%) oxygen levels. Changes in oocyte nuclear maturation rates, H2O2 levels within the oocytes and mRNAs of reactive oxygen species inhibitory genes superoxide dismutase 1 and 2 (SOD1 and 2), glutathione reductase (GSR), glutathione peroxidase (GPX1), and catalase (CAT) were quantified. Higher meiotic resumption from germinal vesicle breakdown up to MII was observed in low O2 (41.8 ± 13.1%) compared to high O2 (15.8 ± 8.2%) (P = 0.014) after 52 h of culture (n = 112). Extension of the culture period up to 84 h at low O2 (n = 457 oocytes) produced the highest meiotic resumption at 72 h (64.1 ± 6.0%; P = 0.008), compared with 52 h. Oocytes (n = 110) cultured in high O2 contained higher levels of peroxidase measured using the 2',7'-dichlorodihydrofluorescein diacetate fluorescence assay after 72 h of culture compared with low O2 (P = 0.004). High O2-cultured oocytes also showed higher amounts of SOD1, SOD2, GSR, GPX1, and CAT mRNA. Vitamin E in high oxygen level was able to decrease degeneration (P = 0.008) but had no improving effect on percentage of oocytes in MII. These results for the first time showed that low oxygen gas composition improves nuclear maturation rates and alleviates the oxidative stress for canine oocytes during in vitro maturation.

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Introduction

Dogs have been the most popular companion animals in the history of mankind and have always been part of the social and personal activities of modern human life. Despite all efforts, due to its unique features, a successful system for in vitro maturation (IVM) and IVF could not be established for canid family (Songsasen & Wildt 2007, Rodrigues & Rodrigues 2010). In contrast to the majority of mammals, canine oocytes are ovulated at the prophase of the first meiotic division (germinal vesicle, GV) and complete the maturation process to metaphase II stage (MII) 48–72 h after ovulation in the oviduct (Hewitt & England 1999, De los Reyes et al. 2011). High degeneration (> 50%) and very low MII maturation rates (16.2 ± 4.2%) (Luvoni et al. 2005, Rodrigues & Rodrigues 2010) are the main features of current IVM in the dog.

Dog oocytes contain abundant lipid droplets that occupy 80–90% of the visible ooplasm surface (Guraya 1965, Tesoriero 1982, Songsasen et al. 2009). This may reflect the importance of energy supply during the prolonged period of oviductal travel and the maternal zygotic transition period (Guraya 1965, Luvoni et al. 2005, Lopes et al. 2010). However, presence of these abundant lipid droplets in ooplasm has an impeccable influence on predisposition of cumulus oocyte complexes (COCs) to oxidative stress by reactive oxygen species (ROS; Wakefield et al. 2008, Whitaker & Knight 2008, Tao et al. 2010). Oxygen concentration during the IVM culture period can contribute to the extent and velocity of this oxidative stress of which oocyte nuclear and cytoplasmic maturation and development pattern may perturb (Kim et al. 2007, Whitaker & Knight 2008).

Glutathione (GSH) is the main non-enzymatic cellular defense mechanisms against ROS and other free radicals (Guerin et al. 2001, Menezo et al. 2010). GSH becomes a substrate of glutathione peroxidase (GPX) in alliance with catalase (CAT), which degrades hydrogen peroxide (H2O2) to water and oxygen (Whitaker & Knight 2008). H2O2 itself is the resulting product in neutralization of ROS by superoxide dismutases (SOD) (cytosolic SOD = Cu-Zn SOD1, mitochondrial SOD = Mn-SOD2) (Guerin et al. 2001). In order to recover GSH, oocytes use...
another enzyme, glutathione reductase (GSR), to reduce the GS-SG (oxidized form of GSH) back to GSH (Guerin et al. 2001). Because of lower levels of GSH within in vitro matured canine oocytes (<8 pMol/oocyte) in comparison with in vivo (19.2 pMol/oocyte) (Kim et al. 2007), high levels of H$_2$O$_2$ and ROS within COCs during IVM can severely impair maturation and increase degeneration rates (Whitaker & Knight 2008). These studies showed that the H$_2$O$_2$ level in oocytes is a good indication of oxidative stress.

High oxygen level has detrimental effects on cumulus cell survival (Silva et al. 2009), which influences expansion during oocyte maturation in vitro. In the majority of IVM techniques in domestic animals, cumulus cell expansion and nuclear maturation are concomitant phenomena during the culture period. In other words, expansion of cumulus cells could be a good indicator of the right conditions for nuclear maturation of oocytes within the IVM process (Chen et al. 1990, Qian et al. 2003). In canine oocytes, the relationship between cumulus expansion and nuclear maturation is quite controversial (Otoi et al. 2007, Chebrout et al. 2009, Reynaud et al. 2009), and there have been counter-intuitive interpretations for cumulus expansion in canine IVM (Reynaud et al. 2005, 2006, Lee et al. 2007a, Chastant-Maillard et al. 2010). In vivo, expansion of cumulus cells may not concomitant with meiotic resumption as the maturation occurs after ovulation while the expansion initiation time is not clear (Reynaud et al. 2009). In vitro, extensive mucification of cumulus cells occurred only in the presence of canine serum in the maturation media (Lopes et al. 2011). The credibility of cumulus expansion as a reliable indicator of oocyte maturation is to be further elucidated. In addition, due to entrapment of villi of the two or three most inner layers of cumulus cells inside the zona pellucida (ZP; Blackmore et al. 2004, De los Reyes et al. 2009), it is difficult to denude the oocytes for assessment of nuclear maturation or manipulation for other assisted reproduction techniques.

Vitamin E (α-tocopherol) as a lipid-soluble antioxidant has been proven to have beneficial effects on oocyte maturation and embryo development in pigs (Tao et al. 2010) and alleviates the degeneration rates of bovine and ovine oocytes (Dalvit et al. 2005, Natarajan et al. 2010). This study investigated the impact of oxidative stress on oocyte nuclear maturation and degeneration. Changes in H$_2$O$_2$ content of oocytes cultured in two oxygen levels were analyzed, and the protective role of an antioxidant (vitamin E) was investigated in pursuit of oxidative stress alleviation and improvement of cumulus cell expansion and oocyte nuclear maturation rates.

**Results**

**Cumulus cell expansion and oxygen concentration**

Expansion of cumulus cells was assessed in COCs cultured under low- or high-oxygen conditions. No full mucification was observed throughout IVM in either group (absence of extracellular matrix cloud) at 52 h (0.0% full expansion in both groups; not expanded oocytes 97.1 $\pm$ 1.6% in low O$_2$, and 95.0 $\pm$ 2.7% in high O$_2$ ($P < 0.05$)). However, after 52 h, limited numbers of cumulus cells were disintegrated from the COCs, with higher disintegration in COCs cultured in the high-oxygen incubator (5.0 $\pm$ 2.7% in high O$_2$ vs 2.9 $\pm$ 1.6% in low O$_2$; ($P > 0.05$)). Moreover, in high-oxygen culture, disintegrated cells were less attached to the culture dish (Fig. 1).

**Figure 1** Effect of oxygen level during oocyte culture on cumulus expansion of canine oocytes at 24 and 52 h. Canine oocytes cultured in low (A) or high (B) levels of oxygen (5 vs 20%). Photographs were taken with light field optics to visualize cumulus expansion pattern (A1 and B1 = oocytes at 24 h of culture (4× objective)), (A2 and B2 = oocytes at 52 h of culture (10× objective)).
Nuclear maturation

A total of 118 oocytes from three different repeats were cultured in low- or high-O2 conditions. After 52 h of culture (Table 1), there was no significant difference in degeneration rates of the oocytes cultured under two gas compositions (P>0.05). However, a higher percentage of oocytes resumed meiosis after GV breakdown (GVBD) toward MII stage in low O2 (41.8 ±13.1%) compared with their high-oxygen counterparts (Table 1). On the other hand, the 84-h group resulted in 4.8% of oocytes (three repeats) were stained for H2O2 levels using 2',7'-dichlorodihydrofluorescein diacetate (DCHFDA) after 72 h culture. Densitometry using ImageJ software showed that the overall intensity of fluorescence was greater for high O2 oocytes higher than low-O2 oocytes (P = 0.004; Fig. 2).

ROS repair enzymes mRNA expression

Analyses of the fold induction of target genes (GPX1, SOD1, SOD2, GSR, and CAT) at 0 and 72 h in culture showed significant end point differences between COCs cultured in low- and high-oxygen incubators after 72 h (P<0.05). The expression of mitochondrial Mn-SOD2 among other enzymes involved in GSH metabolism was more prominent in the high-oxygen group (Fig. 3).

Vitamin E and oxidative stress

COCs cultured in low- and high-oxygen incubators were supplemented with vitamin E during 72 h of IVM. Vitamin E at 100 μM reduced degeneration in the high-oxygen group compared with its control (41.7 ±7.6% down to 30.4 ±2.3%; P = 0.008; Table 3). Meiotic resumption in control (P = 0.0001) and vitamin E (P = 0.007)-treated oocytes in the low-oxygen group was higher than their high-oxygen counterparts (Fig. 4). Also vitamin E has no significant effect on the percentage of MII-matured oocytes in both control groups (low = 13.1 ±3.1% and high = 4.6 ±2.5%; P = 0.072; Table 3). However, in the presence of vitamin E, the MII maturation rate in the low-oxygen group was higher than in high-oxygen group (P = 0.008; Fig. 4).

Table 2 Effect of the duration of culture period from 52 to 84 h on nuclear maturation of canine oocytes (5% O2, 5% CO2, and 90% N2). Table shows distribution of oocytes in different stages of meiotic division.

<table>
<thead>
<tr>
<th>Percentage of oocytes (average±S.E.M.) in 52 vs 72 vs 84 h and low oxygen</th>
<th>Meiotic resumption (GVBD-MII) (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GV</td>
<td>GVBD</td>
</tr>
<tr>
<td>52 h</td>
<td>35.0±13.4</td>
</tr>
<tr>
<td>72 h</td>
<td>4.0±1.1</td>
</tr>
<tr>
<td>84 h</td>
<td>0.7±0.7</td>
</tr>
</tbody>
</table>

*P value <0.05, within the column compared with 52 h. GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; AI, anaphase I; TI, telophase I; MII, metaphase II.
Discussion

Establishment of an efficient IVM system for canine oocytes, which is complicated by unique and complex canine reproductive physiology, is still unsolved. During this study, attempts were made to establish a chemically defined and time course optimally modified IVM protocol for canine oocytes. The majority of IVM protocols are based on serum-enriched culture media (Otoi et al. 1999, Luvoni et al. 2005, Oh et al. 2005) in which the exact beneficial effects of serum are unknown and in most cases unpredictable (Bolamba et al. 2002, Lee et al. 2007b). In addition, results from our preliminary experiments (unpublished data) indicated that presence of fetal bovine serum tends to have detrimental effects on nuclear maturation of canine oocytes and significantly increases degeneration rates. Thus, in this study, all the experiments were carried out in a chemically defined medium and without serum supplementation.

Staining and staging

Nuclear staining and staging of canine oocytes after IVM is one of the most critical issues in canine-assisted reproductive techniques. Therefore, it is worth mentioning that we have optimized a protocol that allows precise assessment of oocyte nuclear stage during meiotic maturation. It includes a procedure for complete denuding of cumulus cells and fixation followed by staining of oocytes and visualization of oocyte chromatin under a fluorescence microscope. Denuding canine oocytes due to their highly inter-digitated cumulus, ZP attachment (Blackmore et al. 2004, De los Reyes et al. 2009) after culture, is challenging (Hewitt et al. 1998). In addition, the lipid droplets inside the oocyte make it very difficult to visualize the chromatin content by simple aceto-orcein staining unless oocytes are fixed for 5–7 days (Song et al. 2010). After trying several denuding buffers with different timetables of incubation and vortexing (Reynaud et al. 2004), incubation in sodium tri-citrate 1% (Hewitt & England 1999) and denuding using an oocyte holding needle with gauge of 135 m (Yellow EZ-Strip Research Instruments Limited, Cornwall, UK) resulted in complete removal of the cumulus cells. For nuclear staining, the combination of 10-min fixation with acetone at −20°C followed by 5 min staining in 10 µg/ml Bisbenzimide (Hoechst 33342) solution provided a quick and reliable solution. Using this method, we were able to provide a panel of images that can be used as guidance for investigators (Fig. 5).

Oxygen level and IVM

Expansion of cumulus cells did not differ after 52 h of culture of COCs in low- (5%) or high (20%)-oxygen
Effects of oxygen level on canine oocyte maturation

Table 3 Effect of vitamin E on the nuclear maturation of canine oocytes in low and high oxygen. Table shows distribution of oocytes in different stages of meiotic division.

<table>
<thead>
<tr>
<th></th>
<th>Percentage of oocytes (average ± s.e.m.) in vitamin E experiment (72 h)</th>
<th>Meiotic resumption (GVBD-MII) (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GV</td>
<td>GVBD</td>
</tr>
<tr>
<td>Ctrl low</td>
<td>14.9 ± 4.1</td>
<td>31.5 ± 5.4</td>
</tr>
<tr>
<td>Vitamin E low</td>
<td>16.9 ± 2.9</td>
<td>24.9 ± 4.7^‡</td>
</tr>
<tr>
<td>Ctrl high</td>
<td>27.2 ± 2.2^†</td>
<td>16.2 ± 3.1</td>
</tr>
<tr>
<td>Vitamin E high</td>
<td>34.7 ± 5.7^‡</td>
<td>16.5 ± 3.6</td>
</tr>
</tbody>
</table>

*P value < 0.05 within the column in low or high oxygen compared with their controls; †,‡P value < 0.05 within the column between control groups (†), between vitamin E groups (‡).
GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; AI, anaphase I; TI, telophase I; MII, metaphase II.

incubators; nevertheless, the disintegration of cumulus cells differed in high oxygen (Fig. 1). The oviductal oxygen level is almost one-forth to one-third of the normal air oxygen level (e.g. 5 vs 20%) (Rodrigues & Rodrigues 2010). Despite the presence of anti-apoptotic intracellular mechanisms acting via GSH against ROS (Silva et al. 2009), having high lipid content in canine COCs can reduce GSH level and predispose them to oxidative damage and induce apoptosis in the cumulus cells (Silva et al. 2009). This can contribute to lowered oocyte maturation rates (Kim et al. 2007, Rodrigues & Rodrigues 2010). Expansion of cumulus cells in vivo or during culture of COCs in vitro occurs through production of hyaluronan by cumulus cells under the influence of LH. Cumulus cells must be viable and express hyaluronan synthase II in the cell membrane (Marei et al. 2012). Induction of apoptosis in COCs through oxidative stress during the extended culture period is expected to hamper production of hyaluronan and cumulus cells expansion. Albeit that cumulus cell expansion is not a convincing marker of canine oocyte maturation (Reynaud et al. 2005, Otoi et al. 2007, Rodrigues & Rodrigues 2010), there was significantly higher number of maturing oocytes (GVBD-MII) after 52 h culture in the low-oxygen (41.8 ± 13.1%) group compared with the high-oxygen group (15.8 ± 8.2%). This was accompanied by lower degeneration rate in the low-oxygen group (23.2 ± 6.6%) compared with the high-oxygen group (31.9 ± 2.2%) (P<0.05), confirming the detrimental effects of high oxygen tension on dog oocytes.

Duration of culture period for IVM of canine oocytes ranges from 48 to 96 h in different studies (Luvoni et al. 2005, Rodrigues & Rodrigues 2010). In vivo, oocytes need 2–4 days for completion of this stage (Concannon 2011). Therefore, it was decided to extend the culture period up to 84 h in order to find the optimal culture period for canine oocytes, which result in highest maturation and lowest degeneration rates in the low-oxygen atmosphere. Extension of culture period to 72 h resulted in increased meiotic resumption to 64.1 ± 5.9% (Table 2). However, further extension of the culture period to 84 h resulted in elevation (7.4%) in the percentage of MII oocytes (Table 2) but increased degeneration rate. Similar findings were reported by other research groups (Otoi et al. 2002, Rodrigues Bde et al. 2004). Therefore, for the remainder of experiments presented here including H2O2 comparison, ROS, and vitamin E experiments, 72-h incubation period was used to avoid high degeneration but achieve highest meiotic resumption.

**Oxidative stress and IVM**

In the H2O2 staining (DCHFDA) experiment, a comparison was done between low and high oxygen levels and the total amount of H2O2 produced inside ooplasm of canine oocytes after 72 h of culture. Oocytes stained with DCHFDA (Fig. 2A) produced significantly higher levels of H2O2 (P=0.004) in the high-oxygen group (Fig. 2B). It is noteworthy that H2O2 is a byproduct of the ROS protection system in which SOD enzymes transform damaging oxygen-free radicals into less aggressive H2O2 molecules (Whitaker & Knight 2008). As oxidative stress is introduced to oocytes immediately after mechanical extraction till the end of the culture period via various sources of free radicals, light and physical trauma (Guerin et al. 2001, Menezes et al. 2010), it is necessary to provide these cells with least amount of...
stressors. Reduction of oxidative stress can profoundly contribute to improved nuclear and cytoplasmic maturation.

Results of the real-time qPCR showed that mRNA of ROS repair enzymes are significantly higher in oocytes cultured in high oxygen (Fig. 3), which is concomitant with high H$_2$O$_2$ level, lower meiotic resumption, and higher degeneration rates. SOD, present in mitochondria (SOD2), showed remarkable increase in mRNA expression in the high-oxygen group, which is in agreement with previous reports (De los Reyes et al. 2011). Involvement of mitochondrial mobilization during cytoplasmic maturation of canine oocytes in terms of perturbation due to oxidative pressure could be the main retarding factor in canine IVM. Moreover, oocytes cultured in high oxygen showed increased GPX1 and GSR expression in response to high oxygen tension (Fig. 3), indicating that the mitochondrial oxidative profile requires further investigation.

Supplementation of vitamin E in the maturation medium did not prevent the detrimental effects of high oxygen tension on degeneration rates of canine oocytes during 72-h culture. Meiotic resumption of oocytes cultured in low oxygen tension was higher in both control and 100 μM vitamin E-treated oocytes than in the high-oxygen group. Vitamin E in the presence of high oxygen was able to decrease degeneration (P=0.008) but did not improve the percentage of oocytes in MII. The beneficial effect of vitamin E on oocyte maturation and embryo development was previously reported in animal species regardless of lipid content, including porcine cells (Tao et al. 2010), which contain high lipid, or in ovine oocytes (Natarajan et al. 2010), which contain relatively low lipid contents.

The studies presented here concerned culture of canine oocytes in a serum-free and chemically defined maturation media. Culture period of 72 h in low oxygen level (5%) was the optimal condition for canine COCs. Oxidative stress and the level of ROS in canine ooplasm affected maturation efficiency, particularly involving mitochondrial activity. To the best of authors’ knowledge, this is the first study that demonstrated the importance of oxygen tension during canine IVM and its possible contributions to maturation and degeneration.

Materials and Methods

Chemicals and reagents

All the materials and chemical reagents were purchased from Sigma–Aldrich Chemical Co. unless otherwise stated.

Collection of ovaries

The sample collection procedure was carried out after routine ovariectomy of bitches by approval of the ethics committee of the Royal Veterinary College from a small animal hospital under supervision and consent of dog owners. Ethical approval was also verified by the University of Bedfordshire Ethical Scrutiny Committee. Due to the nature of this study and also previously published reports (Otoi et al. 2000, 2001, 2002, Songsasen & Wildt 2005), sample collection was blind to reproductive stage of the animal, breed, age, weight, or size.

Collection of COCs

Ovaries were collected immediately after ovariectomy and placed in a 60 ml container (VWR International, Westchester, PA, USA) half full of warm (37 °C) sterile PBS and transferred to the laboratory <2 h after surgery. The container was immersed in a thermos flask containing warm PBS. Before dissection of follicle and oocytes, the ovaries were washed with warm PBS and trimmed of the ovarian bursa and other debris with a scalpel blade. The ovaries were washed with a sterile filtered (0.2 μm microbial filter (Anachem, Bedfordshire, UK)) washing media (TCM199+20 mM HEPES buffer +10% FBS (PAA

Figure 5 Canine oocytes at different stages of meiotic division: oocytes were stained with Hoechst 33342 (10 μg/ml) and visualized by fluorescence microscope with 40× objective. Prophase 1 (germinal vesicle, GV), germinal vesicle breakdown, GVBD accompanied by chromatin condensation, metaphase I (MI), anaphase I (AI), telophase I (TI), metaphase II (MII).

Figure 5 Canine oocytes at different stages of meiotic division: oocytes were stained with Hoechst 33342 (10 μg/ml) and visualized by fluorescence microscope with 40× objective. Prophase 1 (germinal vesicle, GV), germinal vesicle breakdown, GVBD accompanied by chromatin condensation, metaphase I (MI), anaphase I (AI), telophase I (TI), metaphase II (MII).
Laboratories, Dartmouth, MA, USA) and then sliced gently using a set of multiple blades (Fisher Scientific, Loughborough, UK) as described previously (Alhaider & Watson 2009). COCs with an oocyte diameter over 100 μm (surrounded by at least three layers of cumulus cells) having dense and homogenous lipid yolk in the ooplasm were selected and washed twice before culture.

COCs selection criteria for canine IVM protocols used to be quite controversial (Songsasen & Wildt 2005, Concannon et al. 2009). Recently, it has been accepted that age (older than 6 months), breed, weight, and the stage of estrous cycle are less likely to affect the maturation rate of oocytes to MII stage or their degeneration rate (Concannon et al. 2009). Instead cellular criteria have been introduced for oocyte quality including condensed and homogenous lipid yolk, oocyte diameter above 100 μm excluding the ZP, and being surrounded with at least three layers of cumulus cells (Otoi et al. 2000, 2001, 2002). Oocyte diameter above 100 μm is one of cellular criteria during selection that is highly recommended by many reports (Songsasen & Wildt 2005). Nevertheless, large size of the oocyte is not necessarily a guarantee of either meiotic competency or cumulus expansion (Rodrigues & Rodrigues 2010). During all experiments of this study, oocytes were collected from ovaries according to the above cellular criteria to maintain the unity.

In vitro maturation

Oocytes were cultured in 25 oocyte maximum groups according to Hewitt & England (1999) with minor modifications. Selected oocytes were cultured in four-well culture dishes (NUNC, VWR International, Milan, Italy). The base maturation media was modified synthetic oviductal fluid (mSOF; Holm et al. 1999, Marei et al. 2009) supplemented with LH 5 μg/ml (Leutropin; Bioniche Animal Health, Belleville, ON, Canada), FSH 5 μg/ml (Follitropin; Bioniche Animal Health), 17β-estradiol 1 μg/ml, progesterone 1 μg/ml, 50 μg/ml gentamicin sulfate, and 6 mg/ml BSA.

Assessment of cumulus cell expansion

Cumulus expansion (mucification) was described by the partial or complete loosening of cumulus cells into extracellular matrix surrounding the oocyte (Lee et al. 2007a, Marei et al. 2009, Silva et al. 2009). COCs were assessed for expansion of cumulus cells at 24 h after the beginning of the culture and by the end of 52 h under a stereomicroscope.

Denuding oocytes and assessment of nuclear maturation

Oocytes were denuded by incubation in 1% (W/V) Tri-Sodium Citrate in PBS buffer for 3–5 min and vortexed for 2 min at maximum agitation inside a 15 ml conical centrifuge tube (VWR International). Remaining cumulus cells were denuded using an oocyte holding needle with gauge of 135 m (Yellow EZ-Strip Research Instruments Limited). The oocytes were placed in a drop on superfrost slides (VWR International) and air dried in room temperature for 20 min. Afterward, the oocytes were fixed in cold (−20°C) 99% Acetone (Merck) for 10 min. Hoechst 33342 fluorescent DNA dye (excitation/emission = 350/461 nm) was prepared in PBS at 10 μg/ml for simultaneous staining and rehydration of slides for 5 min at 4°C. Hoechst 33342 and PBS were dried with a stripped filter paper as much as possible before mounting the oocytes. Oocytes were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) beneath a coverslip fixed with four paraffin/vaseline drops (1/40; w/w) at the corners of the coverslip. Nuclear stage of the oocytes was assessed with an Olympus BX60 fluorescence microscope (Olympus, UK).

During maturation, oocytes go through different stages between prophase I and MII. By the time they had reached the end of culture period, they were distributed among seven groups according to nuclear stage. The number of oocytes at each stage was recorded (Hewitt et al. 1998). Degenerate oocytes with undetermined, disappeared, or morphologically abnormal nuclear material were categorized as a separate group (Degen) in all experiments. A panel of stained oocytes at different meiotic stages is shown in Fig. 5, which was used as a guide for all experiments.

ROS staining using DCHFIDA

A total number of 130 oocytes from high- and low-O2 groups in three replicates were denuded after 72 h of culture and stained using DCHFDA. This substance hydrolyzes via intracellular esterase to produce 2′,7′-dichlorodihydrofluorescein (DCHF), and the latter metabolite will be oxidized via H2O2 to 2′,7′-dichlorofluorescein (DCF; Nasr-Esfahani et al. 1990, Wakefield et al. 2008). With DCF excitation at 470 nm wavelength, the emission at 522–530 nm could be captured by fluorescence microscopy. Thus, the oocytes were washed twice in 0.04% PVP in PBS and then incubated for 30 min at 38.5°C in the dark with 0.04% PVP-PBS buffer containing 10 μM DCHFDA. Afterward, oocytes were washed two times in the same buffer and mounted on slides using Vectashield mounting media (VectorLabs, Peterborough, UK) under a coverslip. Oocytes were visualized by 470 nm LED lamp Olympus BX60 fluorescence microscope, and photographs were obtained from ten oocytes of the two groups in each repeat. The intensity of fluorescent signal was quantified using ImageJ software particle analysis plug-in (Abramoff et al. 2004).

Real-time PCR and expression profile of ROS repair enzymes

Mechanically dissected COCs (n=340) cultured in high and low oxygen were snap frozen after 72 h of culture in PBS-PVP 0.4% (polyvinylpyrrolidone) using liquid nitrogen and kept at −20°C until analysis. RNA extraction was done using a QIAGEN RNAeasy kit (Qiagen). Briefly, 20 COCs were lysed in 350 μl lysis buffer (RLT), mixed with equal amount of 70% ethanol, and transferred to the RNAeasy spin columns. Washes and centrifugation were carried out according to the manufacturer’s instruction, and RNAs were finally eluted in 30 μl nuclease-free water and the concentration was measured by a TECAN plate reader (TECAN, Switzerland). RNA concentration was normalized among samples by dilution to 50 ng in 8 μl.
volume. DNA digestion was carried out using RQ1 RNAase-free DNase-free DNAse kit (Promega; M6101). RT of RNA samples was conducted by Promega RT kit (Promega) in an equal amount of 50 ng RNA (total 20 μl) and the PCRs were done in 12.5 ng cDNA per reaction in duplicate tubes and four sets of repeats. Conventional PCR (38 cycles) was conducted on every batch of cDNA including the negative control for RT for every primer before temperature gradient optimization. A temperature gradient real-time PCR was done in the range of 50–60 °C and except CAT (57 °C), the rest of primers had an optimal melting temperature at 59 °C. Quantitative PCR was done using KAPA SYBR FAST qPCR Kits (Kapa Biosystems, Bedfordshire, UK) and 20 μM primers in 20 μl volume and 38 cycles via Bio-Rad CFX96 real-time machine (Bio-Rad) using CFX manager 1.7 software (Bio-Rad). Genomic sequences were obtained from PubMed, and primers were designed using Primer3 (Rozen & Skaletsky 2000) web-based software (Table 4).

Relative real-time qPCR was carried out using canine GAPDH as the housekeeping gene, which had a stable expression among three groups (control (0 h) and the two treatments) in four replicates (Levene’s test, P = 0.086; one-way ANOVA (equal variances assumed, LSD post-HOC); P value = 0.227). The fold induction (expression) of target genes was analyzed using Livak method (2−ΔΔCt) (Livak & Schmittgen 2001).

**Experimental design**

Experiments were repeated at least three times. A total of 1249 oocytes presenting homogenous and condensed lipid contents in the cytoplasm surrounded by at least three layers of cumulus cells were used. During the first experiment, 118 COCs were cultured in humidified incubators under two gas compositions for 52 h at 38.5 °C: high O2 ((5% CO2 in air (~20% O2)) or low O2 (5% CO2, 5% O2, and 90% N2) in order to study the pattern of cumulus expansion. COCs were photographed twice during the 52 h of culture (at 24 and 52 h) using a digital camera and an inverted microscope to record any pattern of cumulus expansion within different oxygen levels. At the end of culture (52 h), COCs were denuded fixed and stained to assess nuclear maturation of the oocytes. Each stage of meiotic resumption was reported as a percentage of total number of the oocytes cultured from one animal/replicate.

From results of the nuclear maturation rates, a second experiment was designed to compare nuclear maturation rates of canine oocytes in longer incubation periods. A total number of 460 COCs were cultured in the low oxygen for 72 and 84 h time periods; oocyte maturation and degeneration rates were analyzed after staining.

The third experiment was designed to assess the role of oxidative stress as a detrimental factor, which may contribute to the high degeneration and low maturation rates in canine IVM oocytes. A total number of 130 oocytes were cultured for 72 h in the low or high oxygen gas atmospheres. The H2O2 concentration in oocytes as the product of ROS was quantified after DCHFDA fluorescent staining. Photographs of stained oocytes were analyzed via ImageJ software to quantify the spot density of fluorescence, which is directly correlated with the amount of H2O2 produced inside each oocyte (Nasr-Esfahani et al. 1990, Wakefield et al. 2008). An average of total intensity was compared between the low- and high-oxygen groups.

For investigating the mRNA expression profile of ROS-defensive enzymes, 340 oocytes in four repeats were cultured in low- and high-oxygen incubators (groups of 20). At the beginning of the experiment, 20 COCs were snap frozen as the 0-h control group. At the end of the 72-h culture period, oocytes were snap frozen as low- and high-oxygen treatment groups. PCR reactions were carried out in duplicates and four repeats. In Livak analysis, cycles of threshold (Ct) of the target genes (TG) were first deduced by the Ct of housekeeping gene (HKG; GAPDH: ΔCt = average Ct (TG) − average Ct (HKG)). Resulting values for treatment groups (72 h) were normalized against the 0-h control using the following equation: ΔΔCt (TG) = ΔCt treatment 72 h (TG) − ΔCt Ctrl 0 h (TG); normalized expression ratio = 2−ΔΔCt (TG).

Experiment 4 concerned protective effects of vitamin E over oxidative stress. A total of 201 oocytes were cultured in low- and high-oxygen incubators in two groups in absence (control) and presence of 100 μM vitamin E. Nuclear maturation rates and meiotic resumption of oocytes were analyzed after 72 h of culture using fluorescent staining.

**Statistical analysis**

All experiments in this study were repeated at least three times. The proportional average of oocytes in different stages of meiotic resumption was calculated at the end of the culture

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**Table 4** Sequence of the designed primers and accession numbers of the ROS repairing enzyme genes.

<table>
<thead>
<tr>
<th>Canine genes</th>
<th>Accession number</th>
<th>Oligos (5’→3’)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPX1</td>
<td>NM_001115119.1</td>
<td>F: GACACCACCTCCGCTATGAC</td>
<td>215</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AGCAAGAGGGCTGGTGTTA</td>
<td></td>
</tr>
<tr>
<td>SOD1</td>
<td>NM_001003035.1</td>
<td>F: ACCATTACGGGCTGACTGAAG</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TGGCAGAGTGTTAAAGTGACGA</td>
<td></td>
</tr>
<tr>
<td>SOD2</td>
<td>XM_533463.3</td>
<td>F: AGAAGGGTGACATTACAGCTCA</td>
<td>153</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: AATCAGTTTGTAGGCTTCC</td>
<td></td>
</tr>
<tr>
<td>CAT</td>
<td>NM_001002984.1</td>
<td>F: CCCATTCGCTCTGAGCTTC</td>
<td>179</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CTATGGGATAAAAGCAGGAAACA</td>
<td></td>
</tr>
<tr>
<td>GSR</td>
<td>XM_532813.3</td>
<td>F: CTACGTTGACGGGCTAAATAC</td>
<td>155</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: CTGTGCGAATACGGATGTGAG</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_001003142.1</td>
<td>F: GTGATGTGCTGCTGAGTGATGT</td>
<td>233</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: ATGGATGACCTTTGGCTAGAGGA</td>
<td></td>
</tr>
</tbody>
</table>
period in comparison to the total number. The oocytes were categorized in to seven groups of GV, GVBD, metaphase I, AI, TI, MI, and degenerates (Degen). Statistical analysis was carried out in PAWS statistics 18: Statistical Package for Social Sciences (SPSS, Inc., Chicago, IL, USA) using binary and ordinal logistic regressions via generalized linear model. Analysis of qPCR data was carried out using CFX manager software (Bio-Rad) and one-way ANOVA (LSD PostHoc multiple comparisons) in PAWS statistics 18: Statistical Package for Social Sciences (SPSS, Inc.).

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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