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

Mazdak Salavati^{1,4}, Fataneh Ghafari², Tiantian Zhang³ and Ali A Fouladi-Nashta⁴

¹Institute of Biomedical and Environmental Science and Technology, University of Bedfordshire, Luton, Bedford LU2 8DL, UK, ²Centre for Reproductive Medicine, Walsgrave University Hospital, Coventry CV2 2DX, UK, ³School of Applied Sciences, Bournemouth University, Poole BH12 5BB, UK and ⁴Reproduction, Genes and Development Group, Royal Veterinary College, Hawkshead Campus, Hatfield AL9 7TA, UK

Correspondence should be addressed to A A Fouladi-Nashta; Email: afouladi@rvc.ac.uk

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_2O_2 and ROS within COCs during IVM can severely impair maturation and increase degeneration rates (Whitaker & Knight 2008). These studies showed that the H_2O_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_2O_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)).

Table 1 Effect of oxygen concentration during oocyte culture on nuclear maturation of canine oocytes. Table shows distribution of oocytes in different stages of meiotic division. Meiotic resumption of oocytes (total number of oocytes between GVBD and MII) was significantly higher in low O₂ condition.

	Percentage of oocytes (average $\pm$ S.E.M.) in 52-h oxygen comparison							Meiotic resumption (GVBD-MII)	(n)
	GV	GVBD	MI	AI	TI	MI	Degen		
Low O ₂	35.0 $\pm$ 13.4	27.1 $\pm$ 12.1	4.0 $\pm$ 4.0	4.8 $\pm$ 2.5	3.8 $\pm$ 2.4	2.0 $\pm$ 2.0	23.2 $\pm$ 6.6	41.8 $\pm$ 13.1*	66
High O ₂	52.3 $\pm$ 9.9	12.7 $\pm$ 6.4	3.0 $\pm$ 3.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	31.9 $\pm$ 2.2	15.8 $\pm$ 8.2	52

*P value <0.05, high O₂ compared with low O₂. GV, germinal vesicle; GVBD, germinal vesicle breakdown; MI, metaphase I; AI, anaphase I; TI, telophase I; MII, metaphase II.

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 $\pm$ 13.1%) compared with the oocytes cultured in high-O₂ incubator (15.8 $\pm$ 8.1%) (P value = 0.014; Table 1).

Extended IVM

A total number of 460 oocytes (three repeats) were cultured in base maturation media for 52 h ($n = 66$), 72 h ($n = 231$), and 84 h ($n = 163$). The number of oocytes that remained at GV stage in the 52 h group was higher ($P < 0.05$) than the other two and the lowest MII stage percentage occurred within this group. Highest meiotic resumption (GVBD-MII) was in the 72-h cultured group (64.1 $\pm$ 6.0%), which was higher than 52 h ($P < 0.05$; Table 2). On the other hand, the 84-h group resulted in the highest rate of degeneration with 43.2 $\pm$ 4.5% compared with 52 h ($P < 0.05$; Table 2). Although there were no significant differences in anaphase I (AI) or telophase I (TI) percentages among the three groups, the highest MII matured oocytes (7.5 $\pm$ 4.0%) occurred at 84 h ($P < 0.05$; Table 2).

ROS and oxygen level

To analyze the effects of oxygen level during IVM, oocytes ($n = 130$) 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 μ M reduced degeneration in the high-oxygen group compared with its control (41.7 $\pm$ 7.6% down to 30.4 $\pm$ 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 $\pm$ 3.1% and high = 4.6 $\pm$ 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.

	Percentage of oocytes (average $\pm$ S.E.M.) in 52 vs 72 vs 84 h and low oxygen							Meiotic resumption (GVBD-MII)	(n)
	GV	GVBD	MI	AI	TI	MI	Degen		
52 h	35.0 $\pm$ 13.4	27.1 $\pm$ 12.0	4.0 $\pm$ 4.0	4.8 $\pm$ 2.4	3.8 $\pm$ 2.4	2.0 $\pm$ 2.0	23.2 $\pm$ 6.6	41.8 $\pm$ 13.1	66
72 h	4.0 $\pm$ 1.1*	37.2 $\pm$ 8.4*	16.5 $\pm$ 2.5	2.9 $\pm$ 1.8	3.7 $\pm$ 0.8	3.8 $\pm$ 0.8	31.9 $\pm$ 5.4*	64.1 $\pm$ 5.9*	231
84 h	0.7 $\pm$ 0.7*	22.5 $\pm$ 4.2	20.6 $\pm$ 4.8*	2.8 $\pm$ 1.4	2.5 $\pm$ 1.3	7.5 $\pm$ 3.9*	43.2 $\pm$ 4.4*	56.0 $\pm$ 5.1	163

*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.

Figure 2 Effect of oxygen concentration on H_2O_2 (ROS) levels of canine oocytes after 72 h of culture. (A) Oocytes were stained with DCHFDA for 30 min at 38.5 °C in the dark and then mounted on superfrost slides using Vectashield mounting media. Slides were visualized using fluorescence (470 nm wavelength LED lamp) and photographs taken immediately. (B) The total intensity of DCHFDA fluorescence signal of oocytes cultured in low (5%) and high (20%) oxygen levels in the base maturation media after 72 h. DCHFDA intensity is a direct indicator of H_2O_2 level. **P* value < 0.05.

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 Bisbenzimidazole (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

Figure 3 Effect of oxygen concentration on mRNA expression profile of ROS repairing enzymes in canine oocytes. Graph shows the fold induction of ROS repairing enzymes after 72 h of IVM compared with 0 h and normalized to the GAPDH housekeeping gene. SOD1, cytosolic superoxide dismutase; SOD2, mitochondrial superoxide dismutase; GSR, glutathione reductase; GPX1, glutathione peroxidase; CAT, catalase; LO, low oxygen; HO, high oxygen.

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.

	Percentage of oocytes (average $\pm$ S.E.M.) in vitamin E experiment (72 h)							Meiotic resumption (GVBD-MII)	(n)
	GV	GVBD	MI	AI	TI	MII	Degen		
Ctrl low	14.9 $\pm$ 4.1	31.5 $\pm$ 5.4 [†]	7.6 $\pm$ 2.6	2.3 $\pm$ 2.3	2.8 $\pm$ 2.8	13.1 $\pm$ 3.1	27.9 $\pm$ 4.2	57.2 $\pm$ 5.5 [†]	39
Vitamin E low	16.9 $\pm$ 2.9	24.9 $\pm$ 4.7 [†]	8.0 $\pm$ 4.8	0.0 $\pm$ 0.0	3.6 $\pm$ 2.2	10.5 $\pm$ 2.5 [†]	35.9 $\pm$ 2.3	47.2 $\pm$ 2.7 [†]	51
Ctrl high	27.2 $\pm$ 2.2 [†]	16.2 $\pm$ 3.1	8.6 $\pm$ 3.7*	2.1 $\pm$ 2.1	0.0 $\pm$ 0.0	4.6 $\pm$ 2.5	41.6 $\pm$ 7.5*	31.2 $\pm$ 7.3	45
Vitamin E high	34.7 $\pm$ 5.7 [†]	16.5 $\pm$ 3.6	7.6 $\pm$ 1.5	1.3 $\pm$ 1.3	4.6 $\pm$ 2.9	4.8 $\pm$ 1.6	30.4 $\pm$ 2.3	34.8 $\pm$ 7.3	66

*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-fourth 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 $\pm$ 13.1%) group compared with the high-oxygen group (15.8 $\pm$ 8.2%). This was accompanied by lower degeneration rate in the low-oxygen group (23.2 $\pm$ 6.6%) compared with the high-oxygen group (31.9 $\pm$ 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 $\pm$ 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, Menezo *et al.* 2010), it is necessary to provide these cells with least amount of

Figure 4 Effect of vitamin E on the nuclear maturation of canine oocytes in low and high oxygen. Graph shows the percentages of meiotic resumption (MR; GVBD-MII), metaphase II (MI), and degenerated (Degen) oocytes cultured in the presence or absence of vitamin E. LO, low oxygen; HO, high oxygen.

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 I (germinal vesicle, GV), germinal vesicle breakdown, GVBD accompanied by chromatin condensation, metaphase I (MI), anaphase I (AI), telophase I (TI), metaphase II (MII).

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_2O_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

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 DCHFDA

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 in the 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 (Abràmoff *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 RNeasy kit (Qiagen). Briefly, 20 COCs were lysed in 350 µl lysis buffer (RLT), mixed with equal amount of 70% ethanol, and transferred to the RNeasy 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

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

Canine genes	Accession number	Oligos (5' → 3')	Product size (bp)
GPX1	NM_001115119.1	F: GACACCACTGCGCTAATGAC R: AGGGAAAGGAGGGTTGCCTA	215
SOD1	NM_001003035.1	F: ACCATTACAGGGCTGACTGAAG R: TGGACAGAGGATTAAGTGAGGA	115
SOD2	XM_533463.3	F: AGAAGGGTGACATTACAGCTCA R: AATCACGTTTGATGGCTTCC	153
CAT	NM_001002984.1	F: CCCATTGCAGTTCGATTCTC R: CTATGGATAAAGGACGGAACA	179
GSR	XM_532813.3	F: CTACGTGAGCCGCTAAATAC R: CTGTGGCAATCAGGATGTGAG	155
GAPDH	NM_001003142.1	F: GTGATGCTGGTGCTGAGTATGT R: ATGGATGACTTTGGCTAGAGGA	233

volume. DNA digestion was carried out using RQ1 RNAase-free DNase kit (Promega; M6101). RT of RNA samples was conducted by Promega RT kit (Promega) in a 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 ($2^{-\Delta\Delta C_t}$) (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 different 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): $\Delta C_t = \text{average } C_t(\text{TG}) - \text{average } C_t(\text{HKG})$. Resulting values for treatment groups (72 h) were normalized against the 0-h control using the following equation: $\Delta\Delta C_t(\text{TG}) = \Delta C_t \text{ treatment } 72 \text{ h}(\text{TG}) - \Delta C_t \text{ Ctrl } 0 \text{ h}(\text{TG})$; normalized expression ratio = $2^{-\Delta\Delta C_t(\text{TG})}$.

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

period in comparison to the total number. The oocytes were categorized in to seven groups of GV, GVBD, metaphase I, AI, TI, MII, 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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