The Influence of Ascorbic Acid on *in Vitro* Maturation of Canine Oocytes

Anamaria PERNES*, Ileana MICLEA, Marius ZAHAN, Vasile MICLEA, Delia ORLOVSCHI, Andrei Răzvan CODEA

University of Agricultural Sciences and Veterinary Medicine, Faculty of Animal Science and Biotechnologies, 3-5 Mănăştur Street, 400372 Cluj-Napoca, România
* corresponding author: pernes.jeni@gmail.com

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Abstract

It is known that L-ascorbic acid (vitamin C) can modulate many biochemical processes intracellularly or extracellularly as antioxidant. The aim of the present study was to examine the effects of media supplementation with ascorbic acid on canine oocyte meiotic maturation, viability and the cumulus cell expansion. Various concentrations of ascorbic acid supplemented in *in vitro* maturation (IVM) media were tested. Canine oocyte was exposed to different levels of ascorbic acid (0, 50, 150, 250, 500, 750μM). Cumulus expansion, meiotic maturation and degeneration of oocytes were assessed 72 h after *in vitro* culture. As results, on the group treated with 250μM ascorbic acid was a significant difference compared to the control group on nuclear maturation in stages metaphase I (MI) and metaphase II (MII) (26.98% vs. 6.00%). The groups treated with 50, 150, 250, 500μM had an increase in stage (GVBD), and a significant decrease of degenerate-undefined oocytes compared with the control (23.31%, 18.85%, 13.41% vs 40.80). Concentration 750μM had similar effect to that in the control group. The groups treated with 50, 150, 250, 500μM had an increase in meiosis resumption (GVBD), metaphase I (MI) and metaphase II (MII) with the best result in the group treated with 250 μM ascorbic acid.

Keywords: *ascorbic acid, canine, in vitro maturation, oocytes*

INTRODUCTION

The development of *in vitro* techniques using the canine oocyte as an experimental model would retain powerful tools for gamete rescue programs, which are important to preserve the existence of various endangered canid species (Hewitt and England, 2001; Otoi *et al.*, 2002), as a model for studying human genetic disorders. Domestic dogs exhibit spontaneous occurrence of cancers, and as pets, they are exposed to environmental factors common to humans (Davis and Ostrander, 2014; Switonski, 2014). The 350 traits/disorders identified in the dog with potential to be models for human disease is almost twice that of any other species (Nicholas, 2003).

Development of assisted reproductive technologies (ART) in the dog has resisted progress for decades, due to their unique reproductive physiology. The oocyte of the domestic dog is unique from that of other mammalian species studied to date. Ovulation occurs either once or twice per year, with the oocyte released at the germinal vesicle stage and then completing nuclear and cytoplasmic maturation within the oviduct, and requires 48–72 h in the oviduct post-ovulation to complete nuclear maturation (Reynaud *et al.*, 2005). From 1976 (Mahi and Yanagimaci, 1976) to date, numerous studies have been conducted to improve success of *in vitro* maturation (IVM) of bitch oocytes but an efficient protocol for IVM has not been established. *In vitro* maturation, generally has resulted in low rates of successful resumption of meiosis, with the exception being one study that found that use of oocytes from follicles larger than 2 mm yielded significantly higher rates of metaphase

II development *in vitro* (Songsasen and Wildt, 2005), where 79.5% of oocytes recovered from follicles 2 mm size reached the second metaphase (MII), usually less than 20% of canine oocytes achieve nuclear maturation (Songsasen and Wildt, 2007). Moreover, only a single blastocyst has been produced after *in vitro* fertilization of *in vitro* matured oocytes (Otoi *et al.*, 2000).

Age of the donor bitch (Hewitt and England, 1998; Songsasen *et al.*, 2002), stage of the reproductive cycle (Yamada *et al.*, 1993; Otoi *et al.*, 2001), oocyte diameter (Otoi *et al.*, 2000; Ariu *et al.*, 2011), and the size of the ovarian follicle (Songsasen and Wildt, 2005) are factors influencing meiotic competence of bitch oocytes cultured *in vitro*. In particular, oocyte diameter may be a useful selection parameter. It has been reported (Otoi *et al.*, 2000) that large oocytes (>120 µm diameter) had a higher frequency of maturation than oocytes (<110 µm diameter). This result also suggests the importance of selecting good quality of cumulus-oocyte complexes (COCs) by morphological appearance for IVM of oocytes. Low maturation rates could be due to either suboptimal culture conditions or low meiotic competence of the oocytes (Farstad, 2000). Vitamins are indispensable nutrients involved in a variety of multiple cell functions and they are also essential for mammalian reproduction (Meister and Tate, 1976; Hurley and Doane, 1989). They function not only as cellular antioxidants, but also as modulators of many intracellular or extracellular biochemical processes. Ascorbic acid (vitamin C) is functional in many biological processes such as the biosynthesis of collagen and other components of the extracellular matrix and is the most important antioxidant in extracellular fluids (Buettner, 1993). Predominant in preovulatory follicles in rat ovaries (Guarnaccia *et al.*, 2000), ascorbic acid prevents follicular apoptosis in cultured rat (Tilly and Tilly, 1995) and mouse follicles (Eppig *et al.*, 2000). Ascorbic acid enhances porcine oocyte developmental competence (Tatemoto *et al.*, 2001) and prevents the apoptosis of granulosa cells (Murray et al., 2001) and ovarian follicular cells (Tilly and Tilly, 1995). Few systematic studies have investigated the function of ascorbic acid on oocyte *in vitro* maturation. The aim of the present study was to examine the effects of media supplementation with various concentrations (0, 50, 150, 250, 500, 750µM) of ascorbic acid on canine oocyte meiotic maturation *in vitro.*

MATERIALS AND METHODS

All the chemicals used in this study were purchased from Sigma-Aldrich (USA). To investigate the effect of ascorbic acid for improving oocyte maturation, *in vitro* maturation (IVM) TCM-199 medium was supplemented with five concentrations 50, 150, 250, 500, 750µM acid ascorbic during 72 h *in vitro* culture.

Reproductive tracts from normal bitches greater than 6 months of age of different breeds and at various stages of their estrus cycle, were collected after routine ovariohysterectomy at private clinics, placed immediately into physiological saline solution at 37°C supplemented with penicillin G and streptomycin sulfate, and transported back to the laboratory within 1 h. Ovaries were removed from the tract and washed free from blood in fresh physiological saline solution.

Cumulus–oocyte complexes (COCs) were released by slicing the ovarian cortex with a scalpel blade; oocytes were washed in the bench medium to wash of blood and other debris prior to transfer to maturation medium. Cumulus–oocyte complexes (COCs) which has compact and more than three layers of cumulus cells, dark oocyte cytoplasm were selected (Hewitt and England, 1998) and transferred in IVM medium containing TCM-199 supplemented with sodium pyruvate, fetal bovine serum (FBS) 10 %, Folligon (10 IU/ml), Chorulon (10 IU/ml), β-estradiol and antibiotics (penicillin, streptomycin, gentamicin) under paraffin oil for 72 h at 37°C (Luvoni *et al.*, 2001) in an atmosphere of 5% CO_2 and 95% air humidity. To investigate the effect various concentrations of ascorbic acid were tested 0.0 (control), 50, 150, 250, 500 and 750µM.

At the end of the maturation culture, oocytes were completely denuded by gently pipetting with phosphate buffer saline (PBS) and 0.1% PVA. After 72 h of *in vitro* culture, oocytes were evaluated for viability and nuclear meiotic stage under fluorescence microscopy (Olympus IX 51) with UV light. The effect of ascorbic acid on the percentages of oocytes reaching various stages of nuclear maturation compared to the control and the differences analyzed using the analysis of variance and interpreted using the Tukey test

and the IBM SPSS software. Differences of $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

COCs were obtained by slicing the ovarian cortex from bitches at various stages of their estrus cycle. The progression of meiosis was evaluated after staining with propidium iodide under fluorescence microscopy and the data are summarized in (Table 1). Results demonstrated that incubation with 50, 150, 250 or 500μM ascorbic acid showed a decrease in the proportion of oocytes that remained at the germinal vesicle stage (GV) compared to the control group. The percentage of GVBD oocytes in the 750μM (15.21%) group was similar to that in the control group (18.78%), but a significantly higher proportion was in the 250μM group ($P<0.05$)

The percentage of oocytes reaching MI/MII stage was significantly higher after incubation with 150 or 250μM ascorbic acid ($P<0.001$, 23.61% or 26.98%) compared with the control group (6.00%) and the incubation with 250μM was significantly higher ($p<0.05$) compared with the other groups. The groups treated with 50, 150, 250μM had a significantly decrease of degenerate-undefined oocytes compared with the control (23.31%, 18.85%, 13.41% vs 40.80) and the group treated with the 750μM had similar effect to that in the control group.

Dog oocytes, like those of other species, are able to resume meiosis *in vitro,* but are not fully competent to complete nuclear maturation. From 1976 (Mahi and Yanagimaci, 1976) to date, numerous studies have been conducted to improve success of *in vitro* maturation (IVM) of bitch oocytes but an efficient protocol for IVM has not been established.

The influence of various factors on the *in vitro* maturation of canine oocytes such as age of the donor bitch (Hewitt and England, 1998; Songsasen *et al.*, 2002) the stage of the reproductive cycle (Yamada *et al.*, 1993; Otoi *et al.*, 2001), oocyte diameter (Ariu *et al.*, 2011; Otoi *et al.*, 2000), the size of the ovarian follicle (Songsasen and Wildt, 2005) using hormonal supplementation (Apparicio *et al.*, 2011) have been tested to improve canine IVM system. Nevertheless, efforts to assess various factors have provided a lot of valuable information for the advancement of canine IVM system. Some investigators have indicated no association on the relationship between stage of reproductive cycle and oocyte meiotic competence (Hossein *et al.*, 2007; Otoi *et al.*, 2002; Songsasen and Wildt, 2005) whereas others have demonstrated that reproductive cycle stage significantly impacts developmental capacity of the oocyte (Yamada *et al.*, 1993; Otoi *et al.*, 2001; Luvoni *et al.*, 2001; Kim *et al.*, 2004). In the present study, higher proportion of oocytes reached MI and MII stage in the presence of ascorbic acid at certain concentrations compared to those in the control group. It has been shown that ascorbic acid protects cells from oxidative stress and improve maturation on mouse oocytes (Eppig *et al.*, 2000).

Predominant in preovulatory follicles in rat ovaries (Guarnaccia *et al.*, 2000) ascorbic acid prevents follicular apoptosis in cultured rat (Tilly and Tilly, 1995) enhanced porcine oocyte developmental competence (Tatemoto *et al.*, 2001; Miclea *et al.*, 2012), improved the suboptimal culture conditions for porcine parthenotes and

Tab. 1. Meiotic status of canine oocytes cultured in TCM-199 supplemented with ascorbic acid

Concentrations (μM)	Number of oocytes	%nuclear status of oocytes (mean ± SD)			
		GV	GVBD	MI/MII	DEG/UND
0	53	34.40±9.99	18.79±10.34[a]	6.01±5.97[a]	40.81±9.06[a]
50	54	27.65±6.08	32.89±9.92	16.15±8.86	23.31±8.39[b]
150	55	27.57±6.05	29.96±10.17	23.61±1.96[b]	18.85±8.82[b]
250	57	24.01±3.46	35.59±8.35[b]	26.99±6.79[b]	13.42±8.15[b]
500	55	28.31±9.16	31.16±10.28	13.92±6.90	26.61±13.24
750	52	35.17±9.33	15.22±11.01	8.87±7.26	40.75±13.26

GV, germinal vesicle; GVBD, germinal vesicle break down; MI/MII metaphase I/II; DEG/UND, degenerate/undefined oocytes. Different superscript within the same column indicate a significant difference [a] vs [b] $P< 0.05$.

cloned embryos (Kere *et al.*, 2013) and prevents the apoptosis of granulosa cells (Murray *et al.*, 2001) and ovarian follicular cells (Tilly and Tilly, 1995). The results of this study showed that ascorbic acid increases meiotic resumption of canine oocytes. The highest percentage of maturation to MI/MII stage was in the groups treated with 150 or 250μM ascorbic acid, but it also impose some side effects by decreasing the maturation rate at 750 μM. That may be because ascorbic acid has two different actions: an antioxidant action at lower concentrations, and a pro-oxidant action at higher concentrations (Tatemoto *et al.*, 2001). Our results suggest that ascorbic acid increases meiotic resumption of canine oocytes at certain concentrations. The highest rate of maturation to metaphase I/II stage was obtained after treatment of oocytes with 250μM. This is similar to work of other authors (Tao *et al.*, 2004; Tatemoto *et al.*, 2001; Eppig *et al.*, 2000). In canine species recent reports also showed that addition of β-mercaptoethanol (β-ME) (Kim *et al.*, 2004), cysteine and cysteamine (Hossein *et al.*, 2007), polyether fatty acid, okadaic acid (Ariu *et al.*, 2011), 9-cis retinoic acid (Liang *et al.*, 2012) to IVM media improved meiotic competence of oocytes.

In conclusion, the addition of ascorbic acid at certain concentration (250μM) to the *in vitro* maturation system of canine oocytes increases meiotic resumption and maturation rates to MI/MII.

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