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Full Length Research Paper

Ascorbic acid effects on *in vitro* maturation of mouse oocyte with or without cumulus cell

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Ascorbic acid has long been associated with fertility. This study was designed to determine the effects of ascorbic acid on *in vitro* maturation of mouse oocyte with or without cumulus cells. In this study, 508 denuded oocytes (DOs) and 527 cumulus–oocyte complexes (COCs) from mice stimulated with pregnant mare's serum gonadotrophin (PMSG) were incubated for 24 h in medium containing 0, 80, 250 and 750 µM/ml of ascorbic acid prior to *in vitro* maturation. Maturation rate was compared. A significant decrease in the maturation rate was observed only when the DOs and COCs were exposed to 750 µM/ml of ascorbic acid (P < 0.05). The maturation rate in COCs was significantly higher than DOs in all groups (P < 0.05). These results indicate that exposure ascorbic acid promotes the development of mouse DOs and COCs from germinal vesicle breakdown (GVBD) to metaphase II (MII) and prevents cumulus cell degeneration at certain levels, especially 250 µM/ml of ascorbic acid (P < 0.05). However, further studies on the potential effects of different concentrations of ascorbic acid on oocyte maturation are needed.

Key words: Ascorbic acid, cumulus cell, *in vitro* maturation, mice.

INTRODUCTION

Maturation process in mammalian oocytes includes important nuclear and cytoplasmic changes, which are considered to be the reinitiation and completion of the first meiotic division from prophase I to metaphase II as well as the accompanying cytoplasmic maturation. Several factors, such as high levels of cAMP (Thibault et al., 1987) and hypoxanthine in the compartment surrounding the oocyte, prevent large, meiotically competent oocytes from resuming meiosis spontaneously (Downs et al., 1985; Eppig et al., 1985; To¨rnell and Hillensjo¨ T, 1993). *In vitro*, meiosis activation occurs when the intracellular concentrations of cAMP in oocytes decline; an effect initiated rapidly by removal of the cumulus cells from oocytes in large follicles (Downs, 1995). Importantly, the potential of the oocyte to become fertilized and to support successful pre- and post implantation development not only depends on meiotic/chromosomal maturation events but is also critically influenced by the quality and maturity of the ooplasm and the plasma membrane (Trounson, 1998; Eppig et al., 1985).

In living cells, mitochondria are a major source of ROS (reactive oxygen species) that can damage surrounding macromolecules. ROS are produced continuously as a by-product of aerobic metabolism (Miwa and Brand, 2003). ROS have been recognized to pose a constant threat to cells as they can severely damage DNA, protein and lipids, despite that they are produced endogenously or derived from external sources (Liu et al., 2003). To protect the cells and organs against reactive oxygen species, organisms have evolved a highly sophisticated and complex antioxidant protection system. It involves a variety of components, both endogenous and exogenous in origin, that function interactively and synergistically to neutralize free radicals. These components include: nutrient-derived antioxidants like ascorbic acid (vitamin C), tocopherols and tocotrienols (vitamin E), antioxidant enzymes, e.g., superoxide dismutase and metal binding proteins, such as ferritin, lactoferrin that are capable of catalyzing oxidative reactions (Percival, 1998; Wang et al., 1996).

Vitamin C (L-ascorbic acid, ASC, L-threohex-2-enono-l,

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4-lactone) is a six-carbon keto-lactone, synthesized from glucose via several intermediates. Vitamin C is the most important water soluble antioxidant in plasma (Frei et al., 1988) and its antioxidant properties both *in vitro* and *in vivo* have been documented extensively (Carr and Frei, 1999). However, it also has the ability to reduce transition metal ions such as copper and iron and can therefore potentially act as a pro-oxidant (Halliwell, 1996). The antioxidant properties of ascorbic acid enable it to protect tissues from reactive oxygen species such as O^{2-}, OH^{-}, H_2O_2, $'O_2$, OCl^{-}, NO and metal-oxygen complexes. A range of body tissues accumulate the native vitamin and its fully active, oxidized form, dehydroascorbic acid. Tissues vary widely in content but the highest concentrations occur in the pituitary, adrenal gland and gonads. It has been associated with fertility for many years and may have evolutionary significance (Millar, 1992; Luck et al., 1995). Granulosa cells that surround the mammalian oocyte are known as the cumulus oophorus. The cumulus cells are responsible for providing several trophic or metabolic factors to the preovulatory oocyte and may be extremely important for oocyte metabolism (Gilula et al., 1978). The presence of cumulus cells is required for the transfer of energy to support oocyte maturation in mice (Donahue and Stern, 1968; Pavlok and McLaren, 1972), pigs (McGaughey, 1977; Sato et al., 1978) and cattle (Sato et al., 1977; Fukui and Sakuma, 1980). The aim of this study was to determine whether supplementation with ascorbic acid could promote viability and meiotic maturation of mouse oocytes with or without cumulus cells and the function of cumulus cells.

MATERIALS AND METHODS

Animals

Female NMRI mice (aged 6 - 8 weeks, weighing 22 to ± 2 g) were housed in an environmentally controlled room on a 12 h light: 12 h dark photoperoid. Food and water were available *ad libitum*.

In vitro oocyte maturation

Female NMRI mice 48 h prior to *in vitro* oocyte maturation experiments were injected by the i.p. route with 7.5 IU pregnant mare's serum gonadotrophin (PMSG) (Folligon serum gonadotropin; Intervet, Boxmeer, Holland). On the day of the experiment, the mice were killed by cervical dislocation and dissected. Using sterile technique, the ovaries were dissected and transferred to a Petri dish with a minimum essential medium (MEM) (GIBCO Invitrogen Corporation, 11900-073) supplemented with 5 mg/ml streptomycin (Sigma, S-9137), 6 mg/ml penicillin (Biochrom, A321-42) and 5% fetal bovine serum (FBS) (Hyclone, SH 30070.03). The follicles were punctured using a 28-gauge needle. Immature cumulus-oocyte complexes (COCs) and denuded oocytes (DOs) were collected and randomly transferred to maturation drops of amem supplemented with 5 mg/ml streptomycin, 6 mg/ml penicillin, 5% FBS and 100 mIU/ml recombinant human FSH (Gonal-F; N. V. Organon, Oss, Holand) and 7.5 IU/ml hCG (Chorionic Gonadotrophin; N. V. Organon, Oss, Holand) and different levels of ascorbic acid (Sigma, A-4544) (0, 80, 250 and 750 µM) overlaid with mineral oil (Sigma, 8410-I) in culture dishes. Then, they were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2 in a CO_2 incubator. After incubation for 24 h, the oocytes were assessed for germinal vesicle (GV), germinal vesicle breakdown (GVBD) and progression to metaphase II (MII). In the case of COCs, the cumulus cells were dissected off using a pulled pipette.

Statistical analysis

All data were analyzed using the PROC GLM procedure from SAS (SAS v9.1, Institute, Inc). Significant effects ($p < 0.05$) were further analyzed using LSMEANS to determine significance between the control and treatment groups (65).

RESULTS

In vitro oocyte maturation

A total of 1035 oocytes were incubated in the culture media. Of these, 319 (30.8%) oocytes arrested at GV stage, 360 (34/7%) of them underwent GVBD and also 356 (34/3%) of them progressed to MII in 24 h (Tables 2). COCs achieved a significantly higher maturation rate compared with DOs (42.3 vs. 26.5 %; $P < 0.05$; Tables 2). *In vitro* oocyte maturation in culture media containing ascorbic acid resulted in a significant ($P < 0.05$) increase in the MII formation rate at concentrations of 80 and 250µM/ml. The MII formation rate at a concentration of 750µM/ml, compared with control group, was reduced ($P < 0.05$; Tables 1 and 2).

Denuded oocytes

In this experiment, 508 DOs were incubated for 24 h in the different concentrations of ascorbic acid. Of these, 135 (26.5%) oocytes reached MII (Table 2). Adding the 80 and 250 µM/ml of ascorbic acid significantly increased the MII formation compared to control and 750 µM/ml groups ($P < 0.05$). But in the 750 µM/ml of ascorbic acid the maturation rate significantly decreased compared to 80, 250µM/ml and the COCs of the control groups ($P < 0.05$). Also the DOs achieved a significantly higher maturation rate at the 250 µM/ml concentration of ascorbic acid compared with the other groups ($P < 0.05$), except with the COCs of the 80 µM/ml (Table 1).

Cumulus–oocyte complexes

In this experiment, 527 cumulus–oocyte complexes were incubated for 24 h in the different concentrations of ascorbic acid. Of these, 221 (42.3%) oocytes reached MII (Table 2). The increase in MII formation was statistically

Table 1. The effect of different concentrations of ascorbic acid (vit C) on *in vitro* maturation of mouse oocyte with or without cumulus cells.

Vit C (µm/ml)	No. of oocyte		GV (%)	GVBD (%)	MII (%)
0	DOs	125	54 (43.3 ± 2.4)^{b}	52 (40.1 ± 2.7)^{a}	19 (17.2 ± 4.1)^{de}
	COCs	131	47 (30.6 ± 2.1)^{c}	51 (39.5 ± 2.1)^{a}	33 (29.7 ± 3.3)^{cd}
80	DOs	128	38 (30.7 ± 3)^{c}	47 (33.3 ± 3.2)^{a}	43 (36.7 ± 3.2)^{c}
	COCs	132	24(17.5 ± 2.6)^{d}	44 (30.4 ± 2.7)^{ab}	64 (51.9 ± 2.5)^{b}
250	DOs	127	20 (13.1 ± 2.9)^{d}	49 (35.9 ± 2.4)^{a}	58 (52.2 ± 2.7)^{b}
	COCs	138	15 (8.7 ± 2.5)^{d}	28 (21.2 ± 2.2)^{b}	95 (70.3 ± 1.6)^{a}
750	DOs	128	69 (55.4 ± 2.1)^{ab}	44 (32.5 ± 2.2)^{ab}	15 (13.2 ± 5.4)^{e}
	COCs	126	52 (44.0 ± 2.4)^{ab}	45 (32.2 ± 2.5)^{ab}	29 (23.7 ± 3.7)^{cde}

Values in columns with different superscripts (a, b, c, d and e) differ significantly ($p < 0.05$).
GV, Germinal vesicle; GVBD, germinal vesicle breakdown; M-I, metaphase-I; M-II, metaphase-II; DOs, denuded oocytes; COCs, cumulus–oocyte complexes.

Table 2. Total percentage of the three phases of oocyte maturation.

No. of oocyte		GV (%)	GVBD (%)	MII (%)
DOs	508	181 (35.6)	192 (37.7)	135 (26.5)
COCs	527	138 (26.1)	168 (32.7)	221 (42.3)
Total	1035	139 (30.8)	360 (34.7)	325 (34.3)

GV, Germinal vesicle; GVBD, germinal vesicle breakdown; M-I, metaphase-I; M-II, metaphase-II; DOs, denuded oocytes; COCs, cumulus–oocyte complexes.

significant ($P < 0.05$) at the 80 and 250 µM/ml ascorbic acid concentrations (Table 1). But the reduction in MII formation was significant ($P < 0.05$) at the 750 µM/ml concentration of ascorbic acid compared with the 250 and the COCs of the 80 µM/ml groups (Table 1). Also the COCs achieved a significantly higher maturation rate at the 250 µM/ml concentration of ascorbic acid compared with the other groups ($P < 0.05$) (Table 1).

DISSCUSION

In this prospective study, we investigated the effect of ascorbic acid on oocyte maturation with or without cumulus cell *in vitro* in mice. Significant effects were seen at the 80 and 250 µM/ml ascorbic acid concentrations compared with the control and 750 µM/ml groups ($P < 0.05$). Spontaneous *in vitro* oocyte maturation was adversely affected only when the oocytes were incubated in medium containing 750 µM/ml concentration of ascorbic acid. This effect was seen in both COCs and DOs and was more pronounced in the DOs. These findings would suggest that oocytes exposed to a high concentration of ascorbic acid may not mature adequately *in vitro*. Previous studies are consistent with our findings (Tilly and Tilly, 1995; Tsuji et al., 1985; Ullah et al., 2006).

Early evidence for the role of ascorbic acid has come from the use of scorbutic guinea-pigs (Kramer et al., 1933). These guinea-pigs are infertile due to ascorbic acid deficiency and demonstrate degeneration in the follicle wall that is consistent with loss of basement membrane integrity (Kramer et al., 1933). It is important to define conditions that are conducive to somatic cell survival to maintain health and normal development of the oocyte *in vitro*. The role of ascorbic acid in the neutralization of free radical species has been studied extensively (Halliwell, 1996; Pinnell, 1985; Arrigoni and Tullio, 2002; Asard et al., 2004). Ascorbic acid, which functions as a general free radical scavenger, also suppressed apoptosis (Tilly and Tilly, 1995).

Ascorbic acid is thought to play a similar role in steroidogenesis in the ovary, in which high concentrations of ascorbic acid inhibit steroid biosynthesis through inhibition of hydroxylation systems (Kitabchi, 1967). In this study, it has been suggested that ascorbic acid at high concentration may act as a pro-oxidant (Rehman et al., 1998; Fraga et al., 1991) that can affect deleteriously on oocyte maturation and cumulus cell viability. In fact, the lower concentrations of ascorbic acid (80 and 250 µM/ml) promote cell growth whereas the higher concentrations of ascorbic acid (750µM/ml) appear to have a pro-oxidant effect upon the cells (Asard et al., 2004). In

this experiment, COCs were found to undergo *in vitro* maturation at a significantly higher rate compared with DOs ($P < 0.05$). Previous studies are agreeable with these findings (Boni et al., 2002; Downs et al., 1988; Fukui and Sakuma, 1980). Tao et al. (2004) showed that 250 µM/ml L-ascorbic acid among the four concentrations (0, 50, 250 and 750 µM/ml), promoted the development of porcine DOs from MI to MII and prevented cumulus cell DNA fragmentation. They also indicated that L-ascorbic acid caused lower percentage of DOs arrested at GV stage and higher percentage of DOs undergoing GVBD, especially at MII.

Our findings suggest that cumulus cells produce a positive factor in response to ligand treatment that bypasses their negative influence, to bring about meiotic maturation. Oocyte development may be partly dependent on the cumulus cells via their gap junctions, paracrine communication and interactions with elements of the extracellular matrix (Eppig, 1992; Eppig et al., 2000). Tatemoto et al. (2001) demonstrated that cumulus cells have a critical role in protecting oocytes against oxidative stress-induced apoptosis through the enhancement of glutathione (GSH) content in oocytes. This increase concentration of Glutathione protects cell membranes by providing a reducing environment to prevent cell membrane damage from circulating oxidants (Kosower and Kosower, 1973). Previous studies have shown that high concentration of glutathione is found in COCs compared to DOs (Geshi et al., 2000).

In conclusion, our findings suggest that high concentrations of ascorbic acid may be detrimental to *in vitro* oocyte maturation in mice.

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