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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 9 (12): 2369-2374, 2006
ISSN 1028-8880

Effect of L-ascorbic Acid on Nili Ravi Buffalo Oocytes During *in vitro* Maturation

[1]Ikram Ullah, [1]Samina Jalali, [2]Hamayun Khan, [3]Sajjad A. Shami and [3]Muhammad Mohsin Kiyani
[1]Department of Biological sciences, Reproductive Physiology Laboratory,
Quaid-e-Azam University Islamabad, Pakistan
[2]Department of Animal Health, NWFP, Agriculture University Peshawar, Pakistan
[3]Department of Biological Sciences Quaid-i-Azam University Islamabad, Pakistan

Abstract: The present study was conducted to investigate the effect of L-ascorbic acid (AsA) on Nile Ravi buffalo oocytes. Cumulus Enclosed Oocytes (CEOs) and Cumulus Denuded Oocytes (CDOs) were cultured for 24 h in TCM-199 supplemented with Recombinant Human Follicular Stimulating Hormone (RhFSH), 0.4% Bovine Serum Albumin (BSA), oestradiol and Gentamycin. Nili Ravi buffalo oocytes with or free from cumulus cells were exposed to 250 μM AsA. Meiotic maturation, degeneration of oocytes was assessed at different maturation time (0, 12, 24 h). CDOs treated with L-ascorbic acid resumed meiosis and oocytes were significantly ($p<0.05$) increased to MII stage. Where as CDOs matured in control medium with out L-ascorbic acid supplementation did not show any significant increase in MII oocytes proportion as the maturation time was extended to 24 h ($p<0.05$). In present study it was observed that cumulus cells are involved in protection against degeneration as CEOs treated in control medium the degenerated oocytes high significantly ($p<0.05$) decreased to 7.40% as compared to CDOs in which 21.4% oocytes were found to be degenerated. These results indicate that exposure to L-ascorbic acid promote the development of the Nili Ravi buffalo CDOs and cumulus cells are involved in protection against degeneration of oocytes.

Key words: Oocyte, L-ascorbic acid, *in vitro* maturation

INTRODUCTION

It has been a long-standing objective to identify optimal conditions for the *in vitro* maturation (IVM) of follicular oocytes from domestic animals in order to provide basic agricultural research and biotechnology. The technology of manipulating the *in vitro* system has drastically improved during recent years, there are still many inadequacies. Various approaches have been taken to improve *in vitro* development of embryos. The co culture of bovine embryos with somatic cells (Ellington *et al*., 1990; Rorie *et al*., 1994; Thibodeaux *et al*., 1992), the use of conditioned medium prepared from culture of somatic cells (Eyestone and First, 1989; Eyestone *et al*., 1991; Mermillod *et al*., 1993) and the supplementation of medium with serum (Fukui and One, 1989; Takahashi and First, 1993) or Bovine Serum Albumin (BSA) (Carolan *et al*., 1995; Menino *et al*., 1985; Natsuyama *et al*., 1993) have been used somewhat successfully to overcome the developmental arrest exhibited by embryos that were cultured *in vitro*. 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. Consequently, many chemically defied media systems used for culturing gonadal tissues and gem cells such as oocyte and sperm contain vitamins.

The advantageous effects of vitamins have been confirmed previously in glucose metabolism, oocyte meiotic resumption, blastocyst development and embryo pre-implantation (Arends and Wyllie 1991; Whaley *et al*., 2000). Tatemoto *et al*. (2001) reported that the addition of ascorbic acid 2-α-glucoside increased cytoplasmic maturation and increased developmental competence after IVF.

Ascorbate is a potent direct antioxidant protects against endogenous oxidative DNA damage (Fraga *et al*., 1991) furthermore, ascorbate, at physiological concentrations, induces the release of hypotaurine and taurine by oviduct epithelial cells (Guerin *et al*., 1995). The liquid present in the ampullar section of the oviduct at the time of fertilization is a mixture of tubal and follicular fluids (Hansen *et al*., 1991). It is possible that large amounts of ascorbate, present in follicular fluid (Paszkowski and Clarke, 1999), induce taurine and hypotaurine release in oviductal fluid at the time of ovulation. Reactive oxygen

Corresponding Author: Ikram Ullah, Department of Biological Sciences, Reproductive Physiology Laboratory, Quaid-e-Azam University Islamabad, Pakistan Tel: +923009788988

species can cause granulosa cell death and addition of ascorbic acid has been seen to reduce this (Tilly and Tilly, 1995) and also mediate enhanced repair and replacement of the electron side chains and the mitochondria, thus allowing more sites for hormone synthesis (Byrd *et al*., 1993; Luck *et al*., 1995). Some studies addressed the negative effects of α-tocopherol and L-ascorbic acid on gamete cells or subsequent embryo developmental competence. α-tocopherol had no effect on murine oocyte maturation (Takami *et al*., 1999) and even impaired *in vitro* fertilization in cattle (Dalvit *et al*. 1998). Oral administration of vitamin C and E reduced reproductive fitness and impaired the ovarian and uterine functions of female mice (Tarin *et al*., 2002a). However, dietary supplementation with L-ascorbic acid and α-tocopherol decreased Mitogen-activated Protein Kinase (MAPK) activity and increased the probability of reaching the pronuclear stage after parthenogenetic activation (Tarin *et al*. 2002b). Some studies have addressed the positive effect of L-ascorbic acid on oocyte maturation. Tao *et al*. (2004) reported that exposure to L-ascorbic acid or α-Tocopherol Facilitates the development of porcine denuded oocytes from Metaphase I (MI) to Metaphase II (MII) and prevents cumulus cells from fragmentation. Supplementing the IVM media with the ascorbic acid or cysteine enhance the development competence of cat oocytes during the non-breeding season, especially from the 8-16 cells to the blastocyst stage (Comizzoli *et al*., 2003). In the present study, the object was to determine whether supplementation with L-ascorbic acid could improve viability and meiotic maturation of Nili Ravi buffalo oocytes and the function of cumulus cells.

MATERIALS AND METHODS

Reagents and chemicals: Ascorbic acid was the product of Merck Germany; Estradiol, Glutamine, BSA, as well as other chemical were purchased from Sigma Chemical Co. (St Louis, MO, USA), unless otherwise stated. 250 μM ascorbic acid solution was freshly prepared and the pH value of the medium was maintained just prior to incubation of oocytes.

Oocyte collection: Nili Ravi buffalo ovaries, obtained at a local slaughter house, were transported to the laboratory in 0.9% (w/v) NaCl containing 50 μg mL^{-1} streptomycin and 100 U mL^{-1} penicillin G within 2 h of post-slaughter. The follicular contents were recovered by aspiration the visible small antral follicles on the ovarian surface using a 5 mL syringe equipped with an 18-gauge needle. The mixture was collected in a beaker containing PBS containing 0.4% BSA, 100 U mL^{-1} Penicillin and 0.1 mg mL^{-1} glutamine. After sedimentation for 15 min only the intact CEOs with a compact and multilayer cumulus were used in the study. In experiments requiring denuded oocytes, those CEOs had their cumulus cells removed by gentle pipetting. Subsequently the CEOs and CDOs in PBS were placed in separate 4-well plates by narrow bore pipette. Under a phase contrast microscope (Nikon Japan), the oocytes were three time washed in TCM-199 supplemented with 0.1/mg glutamine, 0.4% BSA, 50 μg mL^{-1} gentamicin. In order to wash the oocytes completely, the oocytes were transferred between the 4-wells by mouth pipette and oocytes were transferred from one well to the next leaving all debris behind.

***In vitro* maturation:** The basic medium for maturation culture of oocytes was TCM-199, supplemented with 0.05 IU mL^{-1} recombinant human FSH (Organon, Oss, The Netherlands), 1 μg mL^{-1} estradiol, 0.4% BSA, 50 μg mL^{-1} gentamycine. The pH value of the medium was adjusted to 7.4 and the solution was sterilized by 0.22 μm filter. After washing the oocytes in basic medium, each group of 15 CEOs and 15 CDOs was individually placed into 100 μL droplets of the basic medium covered with mineral oil that was previously equilibrated in CO_2 incubator. The oocytes were placed into micro drops by micropipette which was adjusted to 2 μL and autoclaved tips were utilized. The micropipette was found to be advantageous as 10-15 oocytes can be placed by one pickup, the size of micro drop is not increasing and also eliminating any chance of debris to be transferred to micro drop. The culture was carried out at 37°C in an atmosphere with 5% CO_2 in air for 24 h.

Examination of the nuclear phase: In all experimental groups the nuclear examination of CDOs was assessed at 0 h or one h after placing into maturation medium. The same observation was repeated at 12 and 24 h of maturation. Nuclear examination of the CEOs was assessed at 24 h of *in vitro* culture after removing the surrounding cumulus cells by pipetting.

Oocytes were observed and evaluated morphologically at X 20 magnification under a phase contrast microscope, for nuclear status. Which often have been accepted to evaluate the meiotic maturation of mammalian oocytes (Nagai *et al*., 1997; Hunter, 2000; Sto *et al*., 2001). Oocyte with a single prominent nucleus was classified as being in the Germinal Vesicle (GV), oocyte that has underwent germinal vesicle breakdown but no polar body were classified as Germinal Vesicle Break Down (GVBD) stage and oocyte with a polar body were classified as being in the meta phase II (MII) stage of maturation process.

Experimental design

Experiment 1: In this experiment, oocytes were cultured in TCM-199 without the supplementation of 250 μM L-ascorbic acid was designated control group.

Experiment 2: In this experiment, Oocytes were matured in TCM-199 supplemented with 250 μM L-ascorbic acid was designated treated group.

Statistical analysis: All percentage values of nuclear status among the treatments in each experiment were analyzed by applying t-test. Statistical package, statistic was used for statistical analysis. Probability level $p<0.05$ was considered as statistically significant.

RESULTS

Meiotic resumption of oocytes matured in control medium: Results of oocytes meiotic maturation are given in Table 1. CDOs matured in control medium showed 97.6% GVBD and 3.38% MI stage oocytes at 0 h. At 12 h GVBD oocytes were 23.8%. The percentage of MI stage oocytes at 12 h was 35.71 and 28.5% CDOs progressed to MII stage, the incidence of degenerated oocytes was found to be 11.9% (Table 1).

After 24 h of maturation, no difference was found regarding GVBD, MI and MII oocytes percentage when compared with GVBD, MI and MII oocytes percentages at 12 h (Table 1).

CEOs matured in control medium showed 29.62% MI, 62.96% MII and 7.40% degenerated oocytes (Table 1). There was no significant difference found between the percentages of MII oocytes in either CEOs or CDOs (62.96 and 40.47%, respectively) in control group after 24 h of maturation ($p = 0.072$). The protective effect of the cumulus cells against degeneration of oocytes was observed when CEOs were matured in control medium (without antioxidants), highly significantly ($p = 0.000$) lower percentage of degenerated oocytes (7.40%) was observed as compared to CDOs (21.4%) matured in control medium after 24 h of maturation (Table 1).

Effect of L-ascorbic acid on meiotic resumption of oocytes: The values given in Table 2 show that at 0 h all the collected CDOs were at GVBD stage.

At 12 h of maturation the GVBD percentage was decreased to 26.38% due to the progression of oocytes to next stages. The percentage of MI stage oocytes at 12 h was 43.00 and 22.22% progressed to MII stage, the incidence of degenerated oocytes was found to be 8.33% (Table 2).

Table 1: Meiotic maturation of CDOs and CEOs cultured in control medium at different incubation time

Type of oocyte	n	Time (h)	GVBD n (%)	MI n (%)	MII n (%)	Degenerated n (%)
CDOs		0	41 (97.6)	1 (3.38)	-	-
	42	12	10 (23.8)	15 (35.71)	12 (28.5)	5 (11.9)
		24	5 (11.90)	11 (26.19)	17 (40.47	9 (21.4)
CEOs	27	24		8 (29.62)	17 (62.96)	2 (7.40)^{b***}

GVBD, germinal vesicle breakdown; MI, metaphase I; MII, metaphase II; CEOs, cumulus-enclosed oocytes; CDOs, cumulus denuded oocytes. b = When 24 h CEOs compared with 24 h CDOs, ***p<0.0

Table 2: Effect of L-ascorbic acid on meiotic resumption and degeneration of Nile Ravi buffalo CEOs and CDOs

Type of oocyte	n	Time (h)	GVBD n (%)	MI n (%)	MII n (%)	Degenerated- n (%)
CDOs		0	72 (100)	-	-	-
	72	12	19 (26.38)	31 (43.00)	16 (22.22)	6 (8.33)
		24	10 (13.88)	17 (23.61)^{a**}	32 (44.44)^{a***}	13 (18.00)^{a***}
CEOs	21	24	-	4 (19.04)	14 (66.66)	3 (14.28)

a = When 24 h CDOs compared with 12 h CDOs, ***p<0.01, **p<0.02

At 24 h of maturation, significantly ($p = 0.014$) lower percentage of MI oocytes at 24 h (23.61%) were observed, as the majority of the oocytes were progressed to next stages, which was significantly different from the percentage of MI stage CDOs at 12 h (43.00%). CDOs treated with L-ascorbic acid resumed meiosis as most of the CDOs reached to MII stage (44.44%) oocytes after 24 h of incubation. This increase was significantly ($p = 0.005$) different from MII stage oocytes at 12 h (22.22%). As depicted in Table 2 supplementation of L-ascorbic acid significantly ($p = 0.0000$) increased degenerated oocytes percentage in CDOs after 24 h of maturation (18.00%) when compared with degenerated oocytes percentage at 12 h of incubation (8.31%).

CEOs treated with AsA showed 19.04% MI, 66.66% MII and 14.28% degenerated oocytes (Table 2). There was no significant ($p = 0.076$) difference observed between the percentages of MII stage oocytes in both CEOs and CDOs (66.66% and 44.44%, respectively) treated with AsA after 24 h of maturation (Table 2). Similarly no significant ($p = 0.6917$) difference was observed between the percentages of degenerative oocytes in CDOs (18.00%) and CEOs (14.28%) after 24 h of maturation (Table 2).

DISCUSSION

The present study revealed that when buffalo CDOs were culture in TCM-199 without supplementation of L-ascorbic acid (control), they resolved their nuclear membrane and resumed meiosis. It is found that when the mouse oocytes were cultured in modified α-MEM initiated GVBD and after 5-6 h nearly all oocytes 96% had resolved their nuclear membrane and resumed maturation (Christ *et al*., 1999). In present study when Nile Ravi

buffalo CDOs were matured in control medium without L-ascorbic acid non-significantly (p = 0.2518) difference was found between the percentage of MII stage CDOs on both 12 h (28.5%) and 24 h of maturation (40.47%).

In contrast when cumulus-enclosed oocytes (CEOs) were matured in TCM-199 medium, most of the CEOs reached to MII stage (62.96%). Present results have some agreement with previous studies that cumulus cells during the maturation period support *in vitro* maturation (IVM) of oocytes to the metaphase-II stage and are involved in the cytoplasmic maturation needed for developmental competence of post fertilization such as male pronucleus formation in porcine oocytes (Nagai *et al*., 1993).

In present study the protective role of the cumulus cells resulted into highly significantly (p = 0.001) lower percentage of degenerative oocytes against CDOs, which is in close accordance with the results of Tatemoto *et al*., (2000) on porcine. This lower percentage of degenerated oocytes might be associated with diminished ROS level and increase concentration of antioxidant enzymes in CEOs. In bovine diminished level of Reactive Oxygen Species (ROS) activity and increase level of glutathione peroxidase, Superoxide dismutase (SOD) and catalase activity were detected in cumulus oocytes, which is lower in denuded oocytes with respect to cumulus (Cetica *et al*., 2001). Tatemoto *et al*. (2000) 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 CEOs compared to CDOs (Geshi *et al*., 2000).

In present study Slightly higher percentage of MII oocytes (62.96%) was observed compared to CDOs (40.47%) but no statistically difference was found between the both groups (p = 0.072). The cumulus cells benefit bovine oocyte development either by secreting soluble factors which induce developmental competence or by removing an embryo development-suppressive component from the medium (Hashimoto *et al*., 1998). The present study indicates that cumulus cells protect Nile Ravi buffalo oocytes from degeneration and increase meiotic maturation which is in agreement with previous studies (Tatemoto *et al*., 2000; Hashimoto *et al*., 1998).

The present study showed that after 24 h of maturation L-ascorbic acid supplementation high significantly (p = 0.005) increased MII oocytes proportion compared to control group in which no significant (p = 0.2518) difference was found between 12 and 24 h of maturation (Table 2). Present results have close agreement with previous study that 250 μM L-ascorbic acid might optimize meiotic maturation when porcine Denuded Oocytes (DOs) are cultured *in vitro* in gonadotropins and hypoxanthine-supplemented medium, especially from MI to MII stage (Tao *et al*., 2004). The present study showed that L-ascorbic acid facilitate meiotic maturation especially from MI to MII as the proportion of MI oocyte significantly (p = 0.148) decreased to 23.61% after 24 h compared to percentage of MI oocytes (43.00%) at 12 h of maturation. In control group there was no such significant (p = 0.3481) effect of time on MI oocytes proportion either at 12 or 24 h (35.71 and 26.19%, respectively). However the proportion of degenerated oocytes high significantly (p = 0.000) increased to 18.00% at 24 h of maturation compared to at 12 h of maturation (8.33%).

Supplementation of L-ascorbic acid increased atypical oocytes percentage in both CEOs and DOs, especially at DOs, especially at 250 μM L-ascorbic acid in CEOs and 750 μM in DOs (Tao *et al*., 2004). This increase percentage of degenerated oocytes might be due to less stability of AsA in medium which is easily oxidized in aqueous solution. (Tolbert *et al*., 1975; Yamamoto *et al*., 1990), it might be therefore its effectiveness does not remained stable for long time in maturation medium.

Previous studies showed that supplementing the diet with a mixture of vitamins C and E prevented the maternal age-associated increase in the percentage of oocytes with abnormal distribution of chromosomes in the metaphase II spindle and abnormal chromosomal segregation during the first meiotic division of mouse oocytes (Tarin *et al*., 1998). Oocytes retrieved from females on the antioxidant diet had decreased mitogen-activated protein kinase (MAPK) activity (Tarin *et al*., 2002b). MII exit is induced by a decrease in Maturation Promoting Factor (MPF) activity while chromosome decondensation and pronuclear formation is associated with a decrease in MAPK (Olson and Seidel, 1995; Murray *et al*., 2001). We postulate that an L-ascorbic acid increases the levels of MPF and MAPK activities of CDOs and consequently improves the meiotic maturation of CDOs from MI to MII stage.

REFERENCES

Arends, M.J. and A.H. Wyllie, 1991. Apoptosis: Mechanisms and roles in pathology. Intl. Rev. Exp. Pathol., 32: 223-254.

Byrd, J.A., S.L. Pardue and B.M. Hargis, 1993. Effect of ascorbate on luteinizing hormone stimulated progesterone biosynthesis in chicken granulosa cells *in vitro*. Comp. Biochem. Physiol., 102A: 279-281.

Carolan, C., P. Lonergan, A. Van Langendonckt and P. Mermillod, 1995. Factors affecting bovine embryo development in synthetic oviduct fluid following oocyte maturation and fertilization *in vitro*. Theriogenol., 43: 1115-1128.

Cetica, P.D., L.N. Pintos, G.C. Dalvit and M.T. Beconi, 2001. Antioxidant enzyme activity and oxidative stress in bovine oocyte *in vitro* maturation. Taylor and Francis, Pub., 51: 57-64.

Christ, H.H., K. Joachim, L. Monika, G. Christian, O. Jan, M.B. Henning, E. Sabine and E.R. Ursula, 1999. Nuclear and cytoplasmic maturation off mouse oocytes after treatment with meiosis-activating sterol *in vitro*. Biol. Reprod., 61: 1362-1372.

Comizzoli, P., D.E. Wildt and B.S. Pukazhenthi, 2003. Overcoming poor *in vitro* nuclear maturation and developmental competence of domestic cat oocytes during the non-breeding season. Reproduction, 126: 809-816.

Dalvit, G.C., P.D. Cetica and M.T. Beconi, 1998. Effect of α-tocopherol and ascorbic acid on bovine *in vitro* fertilization. Theriogenol., 49: 619-627.

Ellington, J.E., E.W. Carney, P.B. Farrel, M.E. Simkin and R.H. Foote, 1990. Bovine 1-2-cell embryo development using a simple medium in three oviduct epithelial cell coculture systems. Biol. Reprod., 43: 97-104.

Eyestone, W.H. and N.L. First, 1989. Co-culture of early cattle embryos blastocyst stage with oviductal tissue or in conditioned medium. J. Reprod. Fertil., 85: 715-720.

Eyestone, W.H., J.M. Jones and N.L. First, 1991. Some factors affecting efficacy of oviduct tissue-conditioned medium for the culture of embryos. J. Reprod. Fertil., 92: 59-64.

Fraga, C.G., P.A. Mmotchnik and M.K. Shigenaga, *et al*., 1991. Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. Proc. Natl. Acad. Sci. USA., 88: 11003-11006.

Fukui, Y. and H. Ono, 1989. Effects of sera, hormones and granulosa cells medium for *in vitro* maturation, fertilization, cleavage and development of bovine oocytes. J. Reprod. Fertil., 86: 501-506.

Geshi, M., N. Takenouchi, N. Yamauchi and T. Nagai, 2000. Effects of sodium pyruvate in nonserum maturation medium on maturation, fertilization and subsequent development of bovine oocytes with or without cumulus cells biol. Reprod., 63: 1730-1734.

Guerin, P., J. Guillaud and Y. Menezo, 1995. Hypotaurine in spermatozoa and genital secretions and its production by oviduct epithelial cells *in vitro*. Hum. Reprod., 10: 866-872.

Hansen, C., A. Srikandakumar and B.R. Downey, 1991. Presence of follicular fluid in the porcine oviduct and its contribution to the acrosome reaction. Mol. Reprod. Dev., 30: 148-153.

Hashimoto, S., K. Saeki, Y. Nagao, N. Minami, M. Yamada and K. Utsumi, 1998. Effects of cumulus cell density during *in vitro* maturation on the developmental competence of bovine oocytes. Theriogenol., 50: 334-335.

Hunter, M.G., 2000. Oocyte maturation and ovum quality in pigs. Rev. Reprod., 5: 122-130.

Hurley, W.L. and R.M. Doane, 1989. Recent developments in the roles of vitamins and minerals in reproduction. J. Dairy. Sci., 72: 784-804.

Kosower, N.S. and E.M. Kosower, 1973. Protection of Membranes by Glutathione. In Glutathione. L., C. Flohe, Benohr, H. Sies, (Eds.), Academic Press, New York, NY, pp: 216-226.

Luck, M.R., I. Jeyaseelan and R.A. Scholes, 1995. Ascorbic acid and fertility. Biol. Reprod., 52: 262-266.

Meister, A. and S. Tate, 1976. Glutathione and related γ-glutamyl compounds: Biosynthesis and utilization. Anal. Biochem., 45: 559-604.

Menino, A.R., K. Cheek and J.L. O'Claray, 1985. The effects of bovine serum albumin, bovine uterine fluid and heat treated bovine serum on *in vitro* mouse embryo development. Theriogenol., 23: 461-472.

Mermillod, P., A. Vansteenbrugge, C. Wils, J.L. Mourmeaux, A. Massip and F. Dessy, 1993. Characterization of the embryotrophic activity of exogenous protein-free oviduct-conditioned medium used in culture of cattle embryos. Biol. Reprod., 49: 582-587.

Murray A.A., M.D. Molinek, S.J. Baker, F.N. Kojima, M.F. Smith, S.G. Hillier and N. Spears, 2001. Role of ascorbic acids in promoting follicle integrity and survival in intact mouse ovarian follicles *in vitro*. Reprod., 121: 89-96.

Nagai, T., J. Ding and R.M. Moor, 1993. Effect of follicle cells and steroidogenesis on maturation and fertilization *in vitro* of pig oocytes. J. Exp. Zool., 266: 146-151.

Nagai, T., M. Ebihara, A. Onishi and M. Kubo, 1997. Germinal vesicle stages in pig follicular oocytes collected by different method. J. Reprod. Dev., 43: 339-343.

Natsuyama, S., Y. Noda, K. Narimoto and T. Mori, 1993. Role of protein supplements in the culture of mouse embryos. Theriogenol., 40: 149-151.

Olson, S.E. and G.E. Jr. Seidel, 1995. Vitamin E improves development of bovine embryos produced *in vitro*. Theriogenol., 43: 289-289.

Paszkowski, T. and R.N. Clarke, 1999. The Graafian follicle is a site of L-ascorbate accumulation. J. Assist. Reprod. Genet., 16: 41-45.

Rorie, R.W., T.D. Lester, G.F. Miller, S.W. Gliedt and R.W. McNew, 1994. Effects of protein source and co-culture on bovine embryo development in synthetic oviductal fluid medium. Theriogenol., 42: 385-395.

Sto, E., N. Kimura, M. Yokoo, H. Sasada and K. Naito, 2001. Molecular basis of oocyte maturation in culture in the pig. J. Reprod. Dev., 47: S63-S69.

Takahashi, Y. and N.L. First, 1993. *In vitro* culture of bovine one-cell embryos fertilized *in vitro* using synthetic oviduct fluid medium with and without glucose and supplemented with fetal calf serum. Anim. Reprod. Sci., 31: 33-47.

Takami, M., S.L. Preston, V.A. Toyloy and H.R. Behrman,. 1999. Antioxidants reversibly inhibit the spontaneous resumption of meiosis. Am. J. Physiol., 276: E684-E688.

Tao, Y., B. Zhou1, G. Xia1, 1.F. Wang, Z. Wu1 and M. Fu1, 2004. Exposure to l-ascorbic acid or a-Tocopherol facilitates the development of porcine denuded oocytes from metaphase 1 to metaphase ii and prevents cumulus cells from fragmentation. Reprod. Dom. Anim., 39: 1-6.

Tarin, J.J., F.J. Vendrell, J. Ten and A. Cano, 1998. Antioxidant therapy counteracts the disturbing effects of diamide and maternal ageing on meiotic division and chromosomal segregation in mouse oocytes. Mol. Hum. Reprod., 4: 281-288.

Tarin, J.J., S. Perez-Albala, J.F. Pertusaand and A. Cano, 2002a. Oral administration of pharmacological doses of vitamins C and E reduces reproductive fitness and impairs the ovarian and uterine functions of female mice. Theriogenol., 57: 1539-1550.

Tarin, J.J., S. Perez-Albala, M.A. Garcia-Perez and A. Cano, 2002b. Effect of dietary supplementation with a mixture of Vitamins C and E on fertilization of tertiary butyl hydroperoxide-treated oocytes and parthenogenetic activation in the mouse. Theriogenology, 57: 869-881.

Tatemoto, H., N. Sakurai and N. Muto, 2000. Protection of porcine oocytes against apoptotic cell death caused by oxidative stress during *in vitro* maturation: Role of cumulus cells. Biol. Reprod., 63: 805-810.

Tatemoto, H., K. Ootaki, K. Shigeta and N. Muto, 2001. Enhancement of developmental competence after *in vitro* fertilization of porcine oocytes by treatment with ascorbic acid 2-α-glucoside during *in vitro* maturation. Biol. Reprod., 65: 1800-1806.

Thibodeaux, J.K., M.W. Myers, L.L. Goodeaux, Y. Me´ne´zo, J.D. Roussel, J.R. Broussard and R.A. Godke, 1992. Evaluating an *in vitro* culture system of bovine uterine and oviduct epithelial cells for subsequent embryo co-culture. Reprod. Fertil. Dev., 4: 573-583.

Tilly, J.L. and K.I. Tilly, 1995. Inhibitors of oxidative stress mimic the ability of follicle stimulating hormone to suppress apoptosis in culture rat ovarian follicles. Endocrinol., 136: 242-252.

Tolbert, B.M., M. Downing, R.W. Carlson, M.K. Knight and E.M. Baker, 1975. Chemistry and metabolism of ascorbic acid and ascorbate sulfate. Ann. NY Acad Sci., 258: 48-69.

Whaley, S.L., V.S. Hedgpeth, C.E. Farin, N.S. Martus, F.C. Jayes and J. H. Britt, 2000. Influence of vitamin A injection before mating on oocyte development, follicular hormones and ovulation in gilts fed high-energy diets. J. Anim. Sci., 78: 1598-1607.

Yamamoto, I., N. Muto, K. Murakami, S. Suga and H. Yamaguchi, 1990. L-Ascorbic acid α-glucoside formed by regioselective transglucosylation with rat intestinal and rice seed α-glucosidases: Its improve stability and structure determination. Chem. Pharm. Bull. (Tokyo), 38: 3020-3023.