Antioxidative Effect of Astaxanthin on *In Vitro* Development of Porcine IVF Embryos

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ABSTRACT

Astaxanthin is a kind of carotenoid compounds, having a antioxidant and anti-inflammatory activities. The antioxidative mechanism by which carotenoid scavenge free radicals has been clearly elucidated, but has not tried for the development of mammalian preimplantation embryo. This study was conducted to investigate the antioxidative effect of astaxanthin on *in vitro* development of porcine *in vitro* fertilized embryos. Porcine embryos derived from *in vitro* fertilization (IVF) were cultured in 5% CO_2 in air at 38.5℃ in PZM-3 medium supplemented with different dosages of astaxanthin (0, 1, 5 and 10 μM) and taurine (0, 1, 2.5 and 5 mM) as a positive control, and execute to compare the effects of various antioxidants such as taurine, melatonin and asculatin on *in vitro* development. The proportions of embryos developed to the blastocyst stage were increased when 1 and 5 μM of astaxanthin (26.6 and 23.4%, respectively) and 1 and 2.5 mM taurine (25.8 and 26.4%, respectively) were supplemented, compared to controls (p<0.05). Also, various antioxidant-treated groups were significantly higher rates of blastocysts (astaxanthin, 27.4%; taurine, 29.1%; melatonin, 26.8%; aesculetin, 27.9%, respectively) than control (18.8%). There was no difference in mean cell number of blastocysts between antioxidants and control. This result indicates that astaxanthin has an antioxidant feature when porcine IVF embryos were cultured *in vitro*.

(Key words : Porcine IVF embryos, Antioxidant, Astaxanthin, Taurine, PZM-3)

INTRODUCTION

One factor believed to impact *in vitro* embryo development is oxidative stress. The production of damage caused by oxidative stress in cells, as well as the protective mechanisms invoked by cells under oxidative stress, have been studied (Mortensen *et al.*, 1997; Liveingston *et al.*, 2004). Some researchers have suggested a highly relationship between embryonic developmental arrest and an increase in free radical formation in embryos culture *in vitro* (El-Hage and Singh, 1990), but the antioxidant mechanisms in embryo culture have been, and still are, a matter for discussion.

Little information is available concerning antioxidative defense for *in vitro* culture of preimplantation embryos. Embryo protection against reactive oxygen species (ROS) depends, in part, upon an endogenous pool of antioxidant enzymes, stored as mRNA in the oocyte during oogenesis (Harvey *et al.*, 1995). It appears that variations in maternal mRNA synthesis or accumulation during oocyte maturation may affect the *in vitro* development of the embryo until zygotic gene activated (Piko and Clegg, 1982; Telford *et al.*, 1990). A drop below a critical threshold may lead to developmental arrest. Embryo culture in media supplemented with ROS scavengers and metal chelators, which are normally present in the genital tract, has been shown to promote bovine embryo development (Johnson and Nasr-Esfahani, 1994; Ranina *et al.*, 2002).

Astaxanthin is one of the carotinoid and found primarily in crustacean. In general, carotinoid have been known to have antioxidant activity and contained in various food sources so used as a dietary supplement (Kobayashi, 1999). Astaxanthin is also known to have antioxidant properties in chicken embryo fibroblasts in *in vitro* systems (Lawlor and O'Brien, 1995; Kobayashi, 1999). However, whether or not astaxanthin exerts its effects on embryo development in pig has not been attempted.

The aim of the present study was to examine the effect of astaxanthin on the *in vitro* development of por-

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cine IVF embryos under 20% O_2 conditions.

MATERIALS AND METHODS

Culture Media

The maturation medium for the first 22 hr of *in vitro* maturation, designated IVM-I, was PZM-3 (Yoshioka *et al.*, 2002) with 10% pFF, 0.57 mM cysteine, 20 mM Hepes, 10 IU/ml eCG and 10 IU/ml hCG. For the second 22 hr of maturation (IVM-II), the same medium without eCG and hCG was used. The fertilization medium (IVF-medium) was modified Tris-buffered medium (mTBM, Abeydera and Day, 1997) containing 2 mM caffeine and 0.1% BSA. Embryo culture medium was PZM-3 medium supplemented with 0.4% BSA. All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

In Vitro Maturation of Oocytes

Porcine ovaries obtained from a local slaughterhouse were transported at 37℃ in 0.9% NaCl solution to the laboratory. Cumulus-oocytes complexes (COCs) were collected from ovarian follicles (2~5 mm in diameter) and then 20~25 oocytes were cultured in IVM- I and IVM-II medium for each 22 hr. After maturation, the COCs were washed and placed in culture medium.

In Vitro Fertilization and Embryo Culture

After maturation, 20 oocytes were introduced into a 50 μl droplet of IVF medium covered with mineral oil. Frozen semen was thawed in water bath at 37℃ and spermatozoa were washed twice by centrifugation at 1,500 rpm for 10 min and resuspended with IVF medium to give a concentration to 2×10^6 spermatozoa/ml, and 50 μl of the sperm suspension was add to 50 μl of the fertilization drops containing oocytes. Six to seven hours after insemination, the spermatozoa bound to the oocytes were removed and cultured in 100 μl of culture medium for 40~44 hr at 38.5℃, 5% CO_2 in air. After culture, 2- to 8-cell embryos were freed of cumulus cells by repeated pipetting and randomly allotted in each experimental culture drop. Embryos were cultured in culture medium with different concentration of astaxhantin (0, 1, 5 and 10 μM) and taurine (0, 1, 2.5 and 5 mM). In anothor experiment, embryos were cultured in culture medium with astaxanthin (1 μM), taurine (2.5 mM), melaronin (1 nM) and asculetin (1 μg/ml). For 6 to 7 days of culture, the embryos were evaluated for appearance and stage of development. Blastocysts were examined the cell number with Hoechst stain.

Statistical Analysis

The SAS mixed linear model program was used to analyze the data. Percentage of developmental stage was based upon the number of premorulae (2- to 8-cell embryos), morulae and blastosysts cultured in each treatment. Treatment means were compared for differences by Duncan's modified multiple range test.

RESULTS

The developmental rates and cell number of porcine embryos generated in PZM-3 medium supplemented with astaxanthin as antioxidant are summarized in Table 1. A significantly higher percentage of blastocysts were obtained in PZM-3 medium supplemented with 1 and 5 μM astaxanthin groups than control and 10 μM astaxanthin groups ($p<0.05$). There was no difference in mean cell number of blastocysts among groups.

The developmental rates and cell numbers of porcine IVF embryos cultured in PZM-3 medium containing different dosage of taurine are shown in Table 2. Developmental rate to the blastocyst stage in PZM-3

Table 1. Effect of astaxanthin on the development of porcine IVF embryos

Astaxanthin (μM)	No. of* cultured embryos	No. (%) of embryos developed to;			Cell no. in blastocysts (mean±SE)
		Premorulae	Morulae	Blastocysts	
0	66	41	13(19.7)[a]	12(18.2)[b]	41.0±2.5
1	64	36	11(17.2)[a]	17(26.6)[a]	42.6±3.3
5	64	39	10(15.6)[a]	15(23.4)[a]	49.6±5.4
10	64	42	10(15.6)[b]	12(18.8)[b]	43.3±4.2

* Two to eight cell stage embryos were cultured.

[a,b] Values with different superscripts within columns differ significantly ($p<0.05$).

Table 2. Effect of taurine on the development of porcine IVF embryos

Taurine (mM)	No. of* cultured embryos	No. (%) of embryos developed to;			Cell no. in blastocysts (mean±SE)
		Premorulae	Morulae	Blastocysts	
0	69	44	12(17.4)[a]	13(18.8)[b]	39.0±1.0
1	66	39	10(15.2)[a]	17(25.8)[a]	42.3±2.1
2.5	68	37	13(19.1)[a]	18(26.5)[a]	41.2±1.4
5	69	46	11(15.9)[b]	12(17.4)[b]	39.8±0.5

* Two to eight cell stage embryos were cultured.
[a,b] Values with different superscripts within columns differ significantly ($p<0.05$).

Table 3. Effects of various antioxidants on the development of porcine IVF embryos

Antioxidants	Dosage	No. of* cultured embryos	No. (%) of embryos developed to;			Cell no. in blastocysts (means±S.E)
			Premorulae	Morulae	Blastocysts	
Control	-	85	50(58.8)	19(22.4)[a]	16(18.8)[b]	44.0±3.2
Astaxanthin	1 μM	84	36(42.9)	25(29.8)[a]	23(27.4)[a]	45.3±5.2
Taurine	2.5 mM	86	40(46.5)	21(24.4)[a]	25(29.1)[a]	46.2±4.1
Melatonin	1 nM	82	49(59.8)	11(13.4)[b]	22(26.8)[a]	42.6±0.6
Aesculetin	1 μg/ml	86	46(53.5)	16(18.6)[ab]	24(27.9)[a]	41.8±5.6

* Two to eight cell stage embryos were cultured.
[a,b] Values with different superscripts within columns differ significantly ($p<0.05$).

medium with 1 and 2.5 mM taurine groups were significantly higher than that of control group ($p<0.05$). There was no difference in mean cell number of blastocysts among groups.

The developmental rates and cell number of blastocyst generated in PZM-3 medium supplemented with astaxanthin, taurine, melatonin and aesculetin as antioxidant are summarized in Table 3. Antioxidant-treated groups were significantly higher developmental rates of blastocysts (astaxanthin, 27.4%; taurine, 29.1%; melatonin, 26.8%; aesculetin, 27.9%) compare to control group (18.8%, $p<0.05$). There was no difference in mean cell number of blastocysts among groups.

DISCUSSION

Mammalian embryos are unable to proceed through preimplantation development by DNA fragmentation, arresting and dying within a few days post fertilization (Hardy *et al.*, 2001). One of important factors in this events may be oxidative stress. Recent attention for mammalian preimplantation embryo culture has been focused on ROS as major causal events for *in vitro* embryonic arrest (Johnson and Nasr-Esfahani, 1994) and also on the protective biochemical function of antioxidants and on the mechanism of their action in biological systems.

ROS are highly reactive with complex cellular molecules such as proteins, lipid and DNA, and cause serious dysfunction such as enzyme inactivation, mitocondrial abnormality or DNA fragmentation (Guerin *et al.*, 2001). Antioxidant and metal chelators has been shown to promote mammalian embryo development (Johnson and Nasr-Esfahani, 1994; Ranina *et al.*, 2002). But little information is available concerning antioxidative defense in preimplantation embryo. ROS generation is controlled by enzymatic and non-enzymatic process. There are two kinds of antioxidants; one is non-enzymatic antioxidants including vitamin E, vitamin C and Taurine (Wright *et al.*, 1989), melatonin (Jang *et al.*, 2004) and esculetin (Jang *et al.*, 2004), and the other is enzymatic antioxidants including superoxide dismutase, catalase and glutathime peroxidase, which are produced by cells and are able to detoxify

the free radicals.

The caroteonids, which are widely distributed in various foods, are considered to play an important role as dietary antioxidants in the prevention of oxidative damage in lining systems (Hertog and Feskens, 1993). The caroteonids, which are lycopene, lutein, zeaxanthin, astaxanthin and canthaxanthin, has characterized antioxidants and anti-imflammatory activities (Kurashige *et al.*, 1990; Bennedsen *et al.*, 1999). Many studies have been demonstrated that astaxanthin *in vitro* culture of mammalian cell have an oxygen free radical scavenging property and activation of cellular antioxidant defense mechanism (Chen and Chuang, 1999; Borlongan *et al.*, 2000).

The aim of the present study was to examine whether or not astaxanthin has a antioxidative feature on development of porcine IVF embryos. To our knowledge, this is the first report in which the effect of astaxanthin exposure has been shown to be beneficial on the *in vitro* development of porcine embryos. We found that astaxanthin improved the development rates to the blastocyst stage when embryos were cultured in PZM-3 medium containing low concentration of astaxanthin (1~5 μM). Antioxidative effect of astaxanthin was similar to that of different antioxidant groups (taurine, melatonin and aesculetin), which have highly antioxidative effect on development porcine IVF embryos (Wright *et al.*, 1986; Huxtable, 1992; Jang *et al.*, 2004). These findings demonstrate that astaxanthin stimulates early embryo development and has a antioxidant property. Thus the molecule action of anstaxanthin may be involved in metabolism at certain for the formation of blastomere compaction and blastocysts in *in vitro* development of preimplantation embryos.

These results suggest that astaxanthin as antioxidant may support the early embryo development through its ROS scavenging action.

REFERENCES

1. Abeydera LR, Day BN (1997): Fertilzation and subsefuent development *in vitro* of pig oocytes insemination in modified tris-buffered medium with frozen-thawed ejaculated spermatozoa. Biol Reprod 57: 729-734.
2. Bennedsen M, Wang X, Willen R, Wadstrom T, Andersen LP (1999): Treatment of *H. pylori* infected mice with antioxidant astaxanthin reduces gastric inflammation, bacterial load and modulates cytokine release bysplenocytes. Immunol Lett 71:185-189.
3. Borlongan CV, Yamamoto M, Takei N, Kumazaki M, Ungsuparkorn C, Hida H, Sanberg PR, Nishino H (2000): Glial cell survival is enhanced during melatonin induced neuroprotection against cerebral ischemia. FASEB 14:1307-1317.
4. Chen ST, Chuang JI(1999): The antioxidant melatonin reduces cortical neuronal death after intrastriatal injection of kainate in the rat. Exp Brain Res 124:241-247.
5. El-Hage SS, Singh M (1990): A 5-fold reduction in sister-chromatid exchange following implantation of mouse embryos is not directly related to the expression of embryonic genes responsible for oxygen radical metabolism. Mutat Res 232:217-226.
6. Guerin P, El Mouatassim S, Menezo Y (2001): Oxidative stress and protection against reactive oxygeen species in the pre-implation embryo and its surrounding. Hum Reprod Update 7:175-189.
7. Hardy K, Spanos S, Becker D, Lannelli P, Wiston RM, Stark J (2001): From cell death to embryo arrest: Mathematical models of human preimplantation embryo development. Proc Natl Aca Sci USA 98:1655-1660.
8. Harvey MB, Arcellana-Panlilio MY, Zhang X, Schultz GA, Watson J (1995): Expression of genes encoding antioxidant enzymes in preimplantation mouse and cow embryos and primary bovine oviduct cultures employed for embryo coculture. Biol Reprod 53:532-540.
9. Hertog MG, Feskens EJ, Hollman PC, Katan MB, Kromhout D (1994): Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet 342:1007-1011.
10. Jang HY, Kong HS, Choi KD, Jeon GJ, Yang BK, Lee CK, Lee HK (2005): Effect of melatonin on gene expression of IVM/IVF porcine embryos. Asian Aust J Anim Sci 18:17-21.
11. Jang HY, Oh JY, Kim JT, Park CK, Cheong HT, Kim CI, Lee HK, Choi KD Yang BK (2004): Effects of antioxidants on porcin IVM/IVF embryos. Reprod Dev Biol 28:77-82.
12. Johnson MH, Nasr-Esfahani MH (1994): Radical solutions and cultural problems: could free oxygen radicals be responsible for the impaired development of preimplantation mammalian embryos *in vitro*? Bioessays 16:31-38.
13. Kobayashi M, Sakamoto Y (1999): Singlet oxygen quenching ability of astaxanthin esters from the green alga *Haematococcus pluvialis*. Biotechnol Lett 21:265- 269.
14. Kurashige M, Okimasu E, Inoue M, Utsuni K (1990): Inhibition of oxidative injury of bilogical membranes by astaxantin. Physiol Chem Phys Med NMR 22:27-38.
15. Lawlor SM, O'Brien NM (1995): Modulation of oxidative stress by beta-carotene in chicken embryo fibroblasts. Br J Nutr 73:841-850.
16. Livingston T, Eberhardt D, Edwards JL, Godin J (2004): Retinol improves bovine embryonic development *in vitro*. Reprod Biol Endo 20:83-90.

17. Mortensen A, Skibsted LH, Sampson J, Rice-Evans C, Everett SA (1997): Comparative mechanism and rates of free radical scavenging by carotenoid antioxidants. FEBS Lett 418:91-97.
18. Piko L, Clegg KB (1982): Quantitative changes in total RNA, total poly(A), and ribosomes in early mouse embryos. Dev Biol 89:362-378.
19. Raina VS, Gupta AK, Singh K (2002): Effect of antioxidant fortification on preservability of buffalo semen. Asian-Aust J Anim Sci 15:16-18.
20. Telford NA, Hogan A, Franz CR, Schultz GA (1990): Expression of genes for insulin and insulin-like growth factors and receptors in early postimplantation mouse embryos and embryonal carcinoma cells. Mol Reprod Dev 27:81-92.
21. Wright CE, Tallan HH, Lin YY, Gaull GE (1986): Taurine: biological update. Ann Rev Biolchem 55: 427-453.
22. Yoshioka K, Suzuki C, Tanaka A, Anas IM, Iwamura S (2002): Birth of piglets derived from porcine zygotes cultured in a chmiclly defined medium. Bio Reprod 66:112-119.

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