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Epinephrine Promotes Development Potential of Vitrified Mouse Oocytes

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Abstract: Cryopreserved oocytes show low developmental ability. To understand the mechanism underlying their development impairment, study was designed to determine the effect of epinephrine on the *in vitro* developmental competence of vitrified mouse oocytes. Mature oocytes were vitrified using Open Pulled Straw (OPS) method. The vitrified oocytes were warmed and introduced into M2 medium which contains epinephrine at different concentrations (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8} mol L^{-1}) in an incubator for 1 h. Then the survival rate of the oocytes was evaluated and the subsequent development of oocytes was assessed through *in vitro* Fertilization (IVF). Furthermore, the levels of intracellular ROS, GSH and the concentration of ATP were determined among 10^{-4} mol L^{-1} epinephrine-treated group, vitrification group and fresh group. Results showed that vitrified oocytes treated with 10^{-4}) mol L^{-1} epinephrine had significant higher rates of cleavage (66.4 vs.45.2%) and blastocyst (47.2 vs. 34.7%) than no epinephrine treated group, as well as more blastocyst cells (54.5 vs. 36.8) and lower ratio of apoptotic cells (5.9 vs. 21.5%; $p<0.05$). Further experiment found that 10^{-4} mol L^{-1} epinephrine treatment could significantly reduce intracellular ROS level and enhance cytoplasmic ATP concentration ($p<0.05$), but there was no different in GSH level compared to vitrification group. In conclusion, epinephrine could promote vitrified oocytes cryosurvival and their subsequent development ability, which maybe related with the changes of intracellular ROS level and ATP content.

Key words: Mouse, oocytes, embryo, epinephrine, cryopreservation

INTRODUCTION

At present, cryopreservation of mammalian oocytes and embryos has been accepted as an important part of assisted reproduction in human and veterinary medicine. Unlike embryos cryopreservation, oocytes cryopreservation is considered as an experimental procedure other than an established technique, which has been recognized by low rates of survival, fertilization and development viability (Trounson and Kirby, 1989; Van Blerkom and Davis, 1994; Wang *et al*., 2011; Katayama *et al*., 2003; Pereira and Marques, 2008). Previous studies found that cryosurvived oocytes may accumulate Reactive Oxygen Species (ROS). Reactive oxygen species was well known for its detrimental effects on cells such as damage of mitochondrial, ATP depletion and abnormal calcium oscillation during fertilization, apoptosis and developmental block (Favetta *et al*., 2007; Takahashi *et al*., 2003; Yoneda *et al*., 2004). Increased ROS activity and decreased the viability and *in vitro* development were found in vitrified porcine oocytes (Gupta *et al*., 2010). Besides, reduction of cytoplasmic ATP content (Zhao *et al*., 2011; Manipalviratn *et al*., 2011) and reduced development potential of oocytes (Van Blerkom *et al*., 1995; Huang *et al*., 2003) were also been noticed. It can be inferred that the reduced oocyte developmental ability associated with vitrification can be, at least, partially enhanced by lowering intracellular ROS level or improving ATP content.

Epinephrine, as well as norepinephrine and dopamine, is a catecholamine, which function as neurotransmitters in the central nervous system and also as hormones in the peripheral endocrine system (Hauser and Longo, 2006). Epinephrine is expected to react with ROS as the catechol structure is found widely distributed in many naturally antioxidants and plays a part in scavenging ROS (Shimizu *et al*., 2010). On the other side, epinephrine plays an essential role in the regulation of the energy metabolism, especially in the case of stress or increased energy needs (Cikos *et al*., 2007).

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Epinephrine increased the activity in mitochondrial complexes, suggesting uncoupling of oxidative phosphorylation and/or increased ATP requirements (Costa *et al*., 2009). Besides, many basic developmental processes such as embryogenesis and morphogenesis, regulating cell proliferation, differentiation and migration are related with epinephrine (Herlenius and Lagercrantz, 2001; Anitole-Misleh and Brown, 2004).

Adrenergic Receptors (AR) which were expressed in mouse oocytes, embryos and embryonic stem cells (ESCs) (Cikos *et al*., 2005, 2007; Kim *et al*., 2008) can mediate the effects of catecholamines (Cikos *et al*., 2005).There were some studies showed that epinephrine could influence the cells development ability, Cikos and associates found that exposure of mouse preimplantation embryos to 10 μmol L^{-1} isoproterenol (a potent β adrenoceptor agonist) or UK 14304 (α2-adrenoceptor agonist) led to significant reduction of the embryo cell number. The researcher explained that epinephrine could affect the embryo development in the oviduct via adrenergic receptors directly (Cikos *et al*., 2005, 2007). Kim *et al*. (2008) showed that epinephrine increased DNA synthesis in a dose- and time-dependent manner; a significant increase in the number of cells was observed after incubation with epinephrine (10^{-6} mol L^{-1}) for 8 h in mouse ESCs (Kim *et al*., 2008). However, there are very few reports involving that whether epinephrine could affect the development potential of vitrified oocytes.

In current study, mouse oocytes were vitrified by OPS (Open Pulled Straw) method and the warmed oocytes were exposed to epinephrine with different concentration. The effect of epinephrine on the development potential of vitrified mouse oocytes after in vitro fertilization and the consequence changes in intracellular ROS, glutathione (GSH) and ATP were also investigated.

MATERIALS AND METHODS

All handling and experimental procedures concerning the animals utilized in this study were in accordance with the Institutional Animal Care and Use Committee of the China Agricultural University. Written informed consent was obtained from the participants of this study.

Reagent: All chemicals and media were obtained from Sigma-Aldrich Chemical (St. Louis, MO, USA) unless otherwise stated.

Source of mouse oocytes: Kunming (KM) mice (closed colony, Academy of Military Medical Science, Beijing, China) were maintained in the room at 20 ~ 22°C under a 14 h (6:00 ~ 20:00) light and 10 h (20:00 ~ 6:00) dark schedule. Female mice aged 6~8 weeks were induced to superovulation by intraperitoneal injection of 10 IU of Pregnant Mare Serum Gonadotropin (PMSG, Ningbo Hormone Products Co.), followed by a second injection of 10 IU human chorionic gonadotropin (hCG, Ningbo Hormone Products Co.) 48 h later. Approximately 14 ~ 16h post hCG injection, the Cumulus-oocyte Complexes (COCs) were collected from oviducts in a pre-warmed (37°C) M2 medium supplemented with 4 mg mL^{-1} BSA (Bovine Serum Albumin fraction V powder, Roche diagnostics GmbH Mannheim, Germany). Cumulus cells were removed by repeating pipetting the COCs in 300 units mL^{-1} hyaluronidase in M2 medium. Only matured MII oocytes with visible polar body were used in the experiments.

Vitrification of oocytes: The vitrification procedure was essentially that described by previous method (Zhou *et al*., 2005) . Briefly, a 0.25 mL plastic straw (I.M.V., L'Aigle, France) was used for making OPS, which had a narrow tip with an inner diameter of 0.10 to 0.15 mm and a wall thickness of 0.05 mm. The experiments were performed on a hot plate (Wenesco, Inc. Chicago, USA) set at 37°C and the ambient temperature was maintained at 25°C. For vitrification, 8 ~ 10 oocytes were pretreated in 10% Ethylene Glycol (EG) (v/v) + 10% DMSO (v/v) for 30 sec and then loaded into the narrow end of OPS with EDFS30 solution for 25 seconds. EDFS30 were composite of 15% EG and 15% DMSO in FS solution, which consisted of DPBS medium with 300 g L^{-1} Ficoll, 0.5M sucrose and 3% bovine serum albumin. Finally, the OPS was plunged into liquid nitrogen. For warming, the OPS narrow tip was immersed in 1 mL of prewarmed 0.5 mol L^{-1} sucrose and then immediately transferred into another drop of 0.5 mol L^{-1} sucrose for 5 min to dilute the cryoprotectants.

Warmed oocytes treated by epinephrine: The warmed oocytes were randomly divided into M2 medium which contains epinephrine at different concentrations (0, 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8} mol L^{-1}) in an incubator (37°C, 5% CO_2 in air) for 1h. Subsequently, oocyte was evaluated under a stereomicroscope and recorded survival rate, respectively.

Oocytes *in vitro* fertilization and embryos culture: Oocytes with normal morphology were placed into a 70 μL drop of HTF medium supplemented with 4 mg mL^{-1} BSA. Sperm from Kunming mice had been capacitated by incubated for 1 ~ 1.5 h in HTF medium supplemented with 4 mg mL^{-1} BSA at 37°C. Capacitation sperm was added to yield a final sperm concentration of approximately 1 106 cells mL^{-1}. The mixture was incubated at 37°C, in an atmosphere of 5% CO_2 in air for 4 ~ 6 h. Oocytes were then washed in HTF medium and transferred into 70 μL drops of HTF medium under mineral oil. The rate of

cleavage and development to blastocyst was recorded 24 and 96 h after *in vitro* fertilization, respectively.

TUNEL assay: Apoptotic cells in blastocysts were evaluated by terminal deoxynucleotidylTtransferase dUTP Nick End Labeling (TUNEL) assay using an in situ cell death detection kit (Roche, Mannheim, Germany). The blastocysts were washed with PBS for 3 times and fixed with 4% paraformaldehyde for 1h. The fixed samples were infiltrated in 0.25% Trition X-100 for 1h and reacted with TUNEL reaction solution at 37°C for 1h and then stained with 10 μg mL^{-1} Hoechst 33342 for 10 min for tablet observation. The fluorescent image of each blastocyst was recorded and cell nuclei were counted. Nuclei with green fluorescence were counted as apoptotic; the apoptotic indexes were recorded including the average number of apoptotic cells per blastocyst and the ratio of apoptosis cells to total cells.

Measurement of ROS and GSH level in oocytes: The intracellular ROS level of different group (vitrification group, 10^{-4} mol L^{-1} epinephrine-treated group and fresh group) were measured by 2',7'-dichlorofluorescein (DCF) fluorescence assay, as previous described (Sakatani *et al*., 2008). In brief, oocytes from each group were incubated in M2 medium supplemented with 0.5 mmol L^{-1} 2', 7'-dichlorodihydrofluorescein diacetate (DCHFDA) for 20 min at 37°C, rinsed and transferred into 100 μL droplet of M2 medium. Fluorescence was measured under the epifluorescence microscope (with filters at 460 nm excitation) and fluorescence images were recorded and the fluorescence intensities of oocytes were quantified using EZ-C1 Free Viewer software (Nikon, Tokyo, Japan). The experiment was replicated at least three times with a group of 15 oocytes or more in each replicate. Intracellular GSH level in different group (vitrification group, 10^{-4} mol L^{-1} epinephrine-treated group and fresh group) were determined by the 4-chloromethyl-6.8-difluoro-7-hydroxycoumarin (Cell Tracker Blue CMF2HC) according to previous described method (Sakatani *et al*., 2008), using 10 μmol L^{-1} Cell Tracker and 370 nm excitation light. The procedure of experimental handling was the same as measurement of ROS levels described above.

ATP assay: The measurement of intracellular ATP content in different group (vitrification group, 10^{-4}) mol L^{-1} epinephrine-treated group and fresh group) was performed using a commercial assay kit (Beyotime Institute of Biotechnology, China) based on the luciferin-luciferase reaction following the manufacturer's recommendations. The experiment was replicated at least three times with a group of 20 oocytes in each replicate. A standard curve was calculated using the formula derived from the linear regression of the standard curve.

Statistical analysis: Each experiment was repeated at least three times. The data were analyzed by one-way ANOVA combined Duncan's test using SPSS 18.0 (SPSS Inc, Chicago, IL, USA). $p<0.05$ was considered statistically significant.

RESULTS

Effects of epinephrine on vitrified oocytes survival rate and subsequent IVF development: As shown in Table 1, the group treated with 10^{-4} mol L^{-1} and 10(-6) mol L^{-1} epinephrine had higher survial rate (88.6, 86.8 vs. 79.3%) and blastocyst rate (47.2, 45.1 vs.34.7%) than that of vitrification group ($p<0.05$). Besides, the blastocyst embyro cells number of 10^{-4} mol L^{-1} epinephrine group was higher than that of 10^{-6} mol L^{-1} epinephrine group (54.5 vs. 45.7, $p<0.05$). However, there was no significant difference in apoptotic cells/blastocyst (3.2 vs.3.0, $p<0.05$) and ratio of apoptotic cells (5.9 vs. 4.9%, $p<0.05$) between 10^{-4} mol L^{-1} epinephrine group and fresh group (Table 2).

Effect of epinephrine on the intracellular level of ROS and GSH in oocytes: As shown in Fig. 1, 10(-4) mol L^{-1} epinephrine-treated group had lower intracellular ROS level than that of vitrification group ($p<0.05$). However there was no difference in GSH level between them ($p>0.05$).

Effect of epinephrine on intracellular ATP concentrations in oocytes: As shown in Fig. 2, the intracellular ATP concentrations in mouse oocytes of 10^{-4} mol L^{-1} epinephrine-treated group was significantly higher than that of vitrification group (0.88 vs. 0.62 pmol L^{-1}; $p<0.05$), but lower than that of the fresh (1.10 pmol L^{-1} $p<0.05$).

Table 1: Oocytes survival rate, *in vitro* fertilization and subsequent development *in vitro* of each group

Groups	Epi 1(mol L^{-1})	No.Oocyte	No. Survival (%, Mean±SD)	No. 2cell (%, Mean±SD) 2	No. Blastocyst (%, Mean±SD) 3
	10(-2)	137	105 (76.6±2.6)^{b}	84(61.2±3.4)^{c}	6 (4.4±1.7)^{d}
	10(-4)	131	116 (88.6±+2.5)^{c}	87(66.4±1.5)^{d}	62 (47.2±3.2)^{e}
Vitrification	10(-6)	122	106 (86.8±2.7)^{c}	78(63.9±3.3)^{cd}	55 (45.1±1.8)^{e}
	10(-8)	133	109 (81.9±1.9)^{b}	77(57.9±2.3)^{c}	47 (35.4±0.9)^{b}
	0	141	112 (79.3±3.4)^{b}	64(45.2±-4.9)^{b}	49 (34.7±1.5)^{b}
Fresh	0	114	114 (100.0)^{a}	100(87.6±2.7)^{a}	77 (67.3±2.9)^{a}

'1'was short for epinephrine. '2'and '3' were both in percentage of No. Oocyte. The value (a-d) in the same column with different superscripts are significantly different, $p<0.05$

Table 2: Effect of epinephrine on blastocyst cells number and apoptotic index

Groups	Epi (mol L^{-1})	No.blastocyst	Cells numbers/blastocyst (Mean±SD)	Apoptic cells /Blastocyst 2 (Mean±SD)	Ratio of apoptic cells 3 (%, Mean±SD)
Vitrification	10^{-2}	4	32.3±1.5^{c}	2.2±0.3^{c}	6.7±0.6^{c}
	10^{-4}	40	54.5±2.3^{d}	3.2±0.3^{a}	5.9±0.7^{ab}
	10^{-6}	38	45.7±2.1^{e}	4.7±0.2^{d}	10.2±1.0^{d}
	10^{-8}	29	42.7±3.1^{e}	5.2±0.3^{e}	12.2±1.3^{e}
	0	28	36.8±1.9^{b}	7.9±0.2^{b}	21.5±1.6^{b}
Fresh	0	43	61.7±2.5^{a}	3.0±0.1^{a}	4.9±0.1^{a}

[1]was short for epinephrine. [2]was the average of apoptic cells per blastocyst. [3]was the ratio of apoptic cells to total cells. The value (a-e) in the same column with different superscripts are significantly different, $p<0.05$

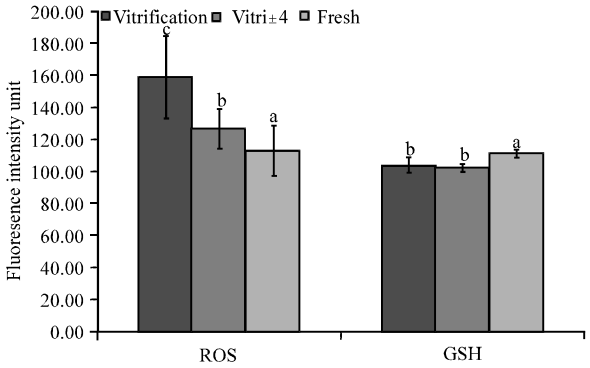

Fig. 1: Intracellular levels of ROS and GSH in mouse oocytes of Vitri±4 group (10^{-4} mol L^{-1} epinephrine-treated group), vitrification group and fresh group, Intracellular levels of ROS and GSH in mouse oocytes of fresh group, Vitri±4 group (10^{-4} mol L^{-1} epinephrine-treated group) and vitrification group. Fluorescence intensities were correlated with intracellular levels of ROS and GSH. Values are mean±SD. Different superscripts (a, b, c) represent a significant difference ($p<0.05$)

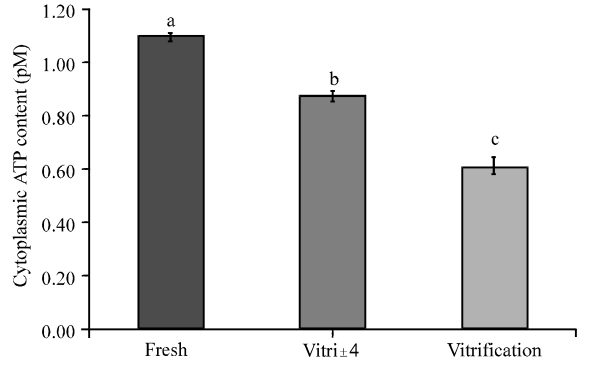

Fig. 2: Intracellular ATP contents in mouse oocytes of Vitri±4 group (10^{-4} mol L^{-1} epinephrine-treated group), vitrification group and fresh group, Intracellular ATP contents in mouse oocytes of group fresh group, Vitri±4 group (10^{-4} mol L^{-1} epinephrine-treated group) and vitrification group. Different superscripts (a, b, c) represent a significant difference ($p<0.05$)

DISCUSSION

In the present study, 10^{-4} mol L^{-1} epinephrine could promote vitrified oocytes subsequent development ability, increase the rates of cleavage and blastocyst (Table 1) as well as cell number (Table 2). This was similar to previous report that when appropriate of epinephrine used, the number of mouse embryo stem cells increased significantly by the effect of increased DNA synthesis (Kim *et al*., 2008). Further experiment proved that the levels of intracellular ROS significantly reduce and the concentration of ATP was increased in 10^{-4} mol L^{-1} epinephrine-treated group. The improved development capacity of vitrified mouse oocytes after addition of epinephrine in vitro culture may be related to the decreased level of intracellular ROS and the increased concentration of ATP.

Previous researches suggested that after cryopreservation survived oocytes may have accumulated ROS (Somfai *et al*., 2007; Ahn *et al*., 2002), which are well known to exert detrimental effects such as mitochondrial damage, ATP depletion, altered calcium oscillation during fertilization, apoptosis and developmental block (Favetta *et al*., 2007; Takahashi *et al*., 2003; Yoneda *et al*., 2004). Recently, porcine MII oocytes were vitrified by Solid Surface Vitrification (SSV) and increased ROS activity and decreased the viability and *in vitro* development were found in those oocytes (Gupta *et al*., 2010). It is showed that cryopreservation significantly increased ROS production in young CD-1 mouse MII oocytes, which decreased its development competence after parthenogenesis activation. In this experiment, the ROS level significantly increased in vitrified mouse oocytes, which led to the decrease of oocytes survival rate and development potential. Epinephrine treatment at 10^{-4} mol L^{-1} could significantly lower ROS level compared with that of the fresh (Fig. 1). Epinephrine can be an antioxidant as a result of its catechol structure. Catecholamines reacted immediately with 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals, their reactivity being stronger than that of ascorbic acid as a reference (Shimizu *et al*., 2010). Adding epinephrine to M2 medium could give the oocytes a low atmospheric oxygen condition by decreasing the oxygen content in solution, which is helpful to lower the ROS level in these oocytes

(Milosevic *et al*., 2005; Halliwell, 1992). In addition, epinephrine treatment resulted in an accentuated increase in mitochondrial complexes activity and the decrease in alanine/lactate ratio, thus reflecting a high cytosolic NADH/NAD^+ ratio, besides an increase in manganese SOD expression and total SOD activity occurred in the epinephrine group to prevent more extensive oxidative damage (Costa *et al*., 2009). The mechanism mentioned above could be the possible reason that epinephrine improve oocytes subsequent development competence (Table 1 and 2).

Intracellular ATP concentration is one of the metabolic determinants of oocyte survival and there was positive relationship between oocytes ATP content and developmental potential of oocytes (Van Blerkom *et al*., 1995; Huang *et al*., 2003). Previous studies showed that cyropreservation of human (Manipalviratn *et al*., 2011), rabbit (Salvetti *et al*., 2010) and bovine (Zhao *et al*., 2011) oocyte could decrease their ATP content and reduced their subsequent development potential. In this study, vitrification decreased oocytes ATP concentration (0.62 vs.1.10 pmol L^{-1}, $p<0.05$) and resulted in a significant decrease of cryosurvival and subsequent blastocyst rate. The decreased ATP concentration in vitrified oocytes in the present study may result from the seriously disturbed mitochondrial distribution and behavior in oocytes due to cryopreservation (Yan *et al*., 2010). As it is reported that the timely and accurate localization of mitochondria in the cytoplasm of oocytes may be involved in concentrating ATP or calcium to the specific regions (Sun *et al*., 2001; Van Blerkom and Runner, 1984).

When vitrified oocytes were treated with 10(-4) mol L^{-1} epinephrine ATP concentration significantly enhanced (Fig. 2). It is because epinephrine could react with ROS to reduce the consumption of ATP at a certain extent, as it is reported high level of ROS would consume ATP (Zhang *et al*., 2006). It needs further study to understand the molecular mechanism involved in increasing ATP content by epinephrine treatment in oocytes.

CONCLUSION

Cryopreserved oocytes treated with 10^{-4} mol L^{-1} Epinephrine could significantly reduce cell intracellular ROS level and enhance ATP content and thereby improve their subsequent development potential.

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