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DEVELOPMENTAL POTENTIAL OF ZONA-MANIPULATED AND IN VITRO MATURED MOUSE OOCYTES UPON ACTIVATION/FERTILIZATION

(With 2 Tables and 2 Figures)

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تطور بويضات الفئران ذات النطاق الشفاف المعالج والناضجة خارجيا بعد التنشيط أو الإخصاب

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يهدف البحث إلي دراسة ١) تطور بويضات الفئران ذات النطاق الشفاف المزال أو المثقوب وكذلك البويضات المكونة من خلايا جسمية بعد الإخصاب ٢) تطور البويضات الناضجة خارجيا بعد التنشيط بالكحول أو الاسترنشيوم. استخدم في الدراسة البويضات غير الناضجة التي جمعت من المبيض (ثم إنضاجها خارجيا) والناضجة التي جمعت من قناة المبيض (ناضجة داخليا). استخدمت البويضات غير الناضجة بعد إزالة أنويتها كمستقبل للخلايا الجسمية لتكوين البويضات المكونة من خلايا جسمية (ثم إنضاجها خارجيا). أزيل النطاق الشفاف أو ثقب لبعض البويضات الناضجة خارجيا. عرضت البويضات الناضجة للإخصاب بالحيوانات المنوية أو التنشيط بالكحول/ الاسترنشيوم ثم زرعت البويضات خارجيا وتم متابعتها إلي طور البلاستوسيست. أظهرت النتائج أن البويضات الناضجة خارجيا ذات النطاق الشفاف السليم لم تخصب علي الإطلاق في حين أن البويضات ذات المنطقة الشفافة المزالة أو المثقوبة وكذلك البويضات المكونة من خلايا جسمية قد أخصبت وانقسمت. ومع أن البويضات المكونة من خلايا جسمية لم تتخطي طور الخليتين إلا أن البويضات ذات النطاق الشفاف المزال أو المثقوب وكذلك البويضات الناضجة في قناة المبيض تخطت طور الخليتين إلي طور البلاستوسيست والتي زادت نسبته في النوع الأخير من البويضات. زادت نسبة تطور البويضات إلي طور البلاستوسيست مع التنشيط بالاسترنشيوم مقارنة بالكحول.

SUMMARY

The aim of the study was to investigate 1) the developmental potential of zona-manipulated oocytes (zona-free and zona-drilled oocytes and reconstituted oocytes) after *in vitro* fertilization (IVF); 2) the developmental potential of *in vitro* matured oocytes activated with either alcohol or strontium chloride ($Srcl_2$). Immature (germinal vesicle

oocytes) and *in vivo* matured (ovulated) oocytes were collected from the ovaries and oviducts respectively and used during the study. Enucleated germinal vesicle (GV) oocytes were used as recipient of fetal fibroblasts for oocytes reconstruction. Immature and reconstructed oocytes were matured *in vitro*. Zona pellucida were either removed or drilled of some in vitro matured oocytes. The results showed that the zona-intact oocytes (*in vitro* matured) were not penetrated by spermatozoa after IVF whereas zona-manipulated oocytes (zona-free, zona-drilled and reconstituted oocytes) and in vivo matured oocytes were fertilized and cleaved. Although the reconstituted oocytes could not exceed two-cell stage embryos, the *in vitro* matured zona-free and zona-drilled oocytes as well as *in vivo* matured oocytes were cleaved further and developed to the blastocyst stage which markedly increased in the latter ones. Developmental potential to the blastocyst stage was higher of oocytes activated with strontium chloride ($Srcl_2$) compared with those activated with alcohol.

Key word: *Activation, fertilization, developmental potential, zona-manipulated*

INTRODUCTION

Germinal vesicle (GV) oocytes were used for *in vitro* production of embryos (Mohammed *et al.*, 2005) and as recipient cells of embryonic and somatic nuclei after enucleation ((Mohammed, 2006 and Mohammed *et al.*, 2008). Immature GV oocytes seem to be an interesting model for studying the mechanisms of meiotic maturation (Mohammed 2008). Also, the future applying these of "artificial" gametes for the treatment of reproductive disorders or for embryonic and somatic cloning can not be excluded (Mohammed *et al.*, 2008).

Cumulus cells are removed from the oocytes before micromanipulation or morphological/cytological studies. Removal of cumulus cells before *in vitro* maturation results in zona hardening and adversely affects the maturation, fertilization and embryo development in rat (Vanderhyden and Armstrong, 1989) and mice (Yamazaki *et al.*, 2001). Penetration of hardened zona pellucida with the sperm required removal or drilling the zona pellucida of oocytes in which their fertilization and developmental competence to the blastocyst stage were investigated primarily during this study.

Artificial activation has become an important component of assisted reproductive technology (ART). The earliest notable event in

oocyte activation, in all studied species, is an increase in the level of intracellular calcium (Swann & Ozil 1994). The methods which were used to induce artificial activation include the direct injection of calcium into the oocyte (Machaty *et al.*, 1996), an electrical pulse that through phospholipid destabilization creates pores for the influx of extracellular calcium (Sasagawa & Yanagimachi 1996), promoting of extracelluar calcium entry via the use of ethanol (Cuthbertson & Cobbold 1985), and inducing calcium oscillations by employing the divalent cation, strontium (Chang *et al.*, 2004). While these methods may be credible for re-enacting oocyte activation, none of them are able to achieve this with the efficiency provided by spermatozoa. Therefore, the main aim of the study was to investigate the developmental potential of zona-manipulated and *in vitro* matured mouse oocytes upon activation/fertilization.

MATERIALS and METHODS

All inorganic and organic compounds used in this study were purchased from Sigma (Sigma Chemical Co., St. Louis, MO), unless otherwise stated. All media were prepared fresh and sterilized by filtering through a 0.22-μm filter (Acrodisc; Pall Gelman Laboratory, Ann Arbor, MI).

Thirty hybrid females (6-8 weeks old) and five males (3-6 months) were used during the study. Mice were kept under a 12h light/12h dark cycle starting at 7 a.m. Food (Labofeed H, Poland) and water were available *ad libitum*. Mice were killed by cervical dislocation.

Collection of germinal vesicle (GV) oocytes: the female mice were injected with 7.5 IU of pregnant serum gonadotrophin (PMSG; Folligon, Intervet, Holland). Ovaries were removed from the donor females 44-48 h after PMSG injection. Antral follicles were punctured by 30-ga needles, and cumulus - GV oocyte complexes were released into Hepes-buffered M2 medium containing 0.2 mM dibutyryl cyclic adenosine 3',5'-monophosphate (dbcAMP) to inhibit GV breakdown. Cumulus cells were removed by gentle, repeated pipetting of oocytes (Grabarek *et al.*, 2004).

For collection of ovulated (*in vivo* matured) oocytes, female mice were injected with 7.5 IU of human chorionic gonadotrophin (hCG, Chorulon, Intervet, Holland) 44-48h after injection with PMSG. Oviducts were removed from the donor females 13-14h after hCG

injection. Oviducts were punctured by 30-ga needles, and ovulated oocytes were released into Hepes-buffered M2 medium (Gao *et al.*, 2002).

Enucleation of germinal vesicle oocytes: Complete enucleation (CE) of GV stage oocytes was performed as described by Grabarek *et al.*, (2004). GV with a smallest possible amount of surrounding cytoplasm was removed by smooth suction.

Experimental design

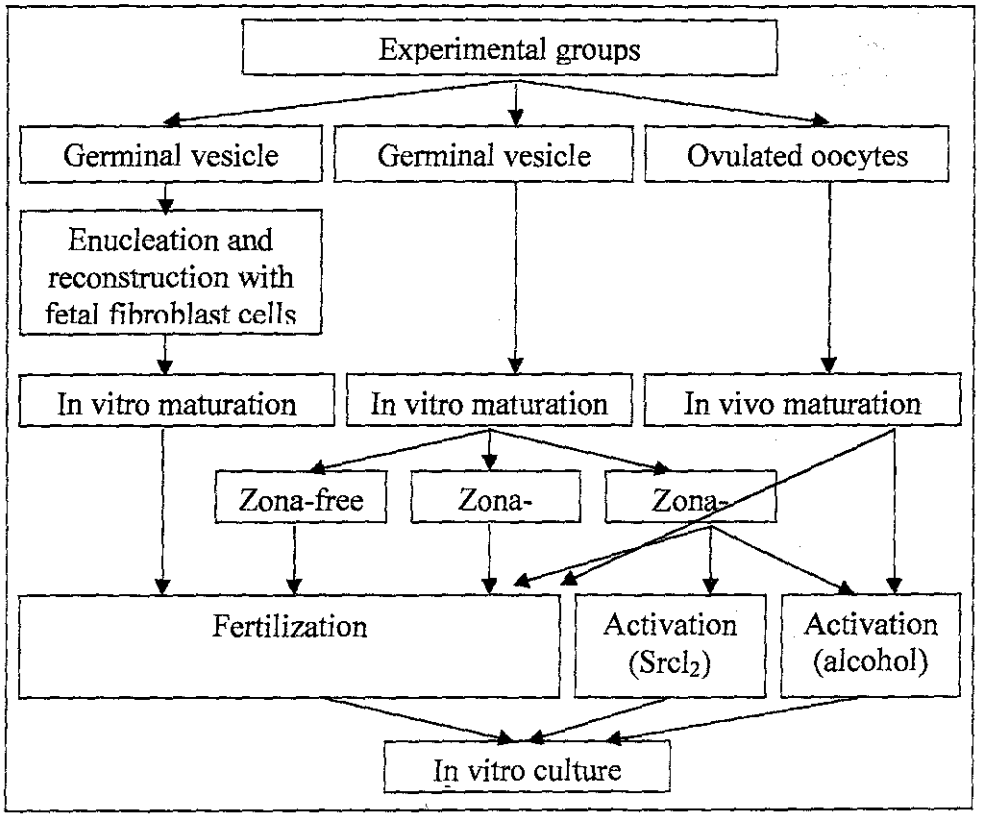

Fig. 1: Schematic diagram indicates the experimental groups used during the study.

All manipulations were performed under inverted Leitz Fluowert microscope (Leitz, Germany) equipped with Nomarski differential-interferential contrast (DIC) and connected with Leitz (Germany) mechanical micromanipulators. Beaudouin Alcatel (France) and Cell Tram Air (Eppendorf, Germany) micropumps were connected with micromanipulation and holding pipettes, respectively. Pipettes were prepared from thin-walled borosilicate glass capillaries of an external

diameter 1 mm (GC 100T-15, Harvard Apparatus Ltd, Kent, Great Britain). Enucleation and nuclear injection pipettes were prepared using PB-7 vertical puller (Narishige, Japan). After pulling, the end of pipettes was broken at the appropriate point using a MF-79 micro-forge (Narishige, Japan) and beveled on an EG-4 grinding wheel (Narishige, Japan) to obtain 45° cut. To facilitate penetration of the *zona pellucida* and to minimize the damage of the oolemma, a spike was formed (by means of the MF-79 Narishige micro-forge) at the tip of enucleation and injection pipettes and the tools were bent with micro-forge (Alcatel, France). The ends of pipettes were bent twice in order to adopt them to the micromanipulation chamber used.

Donor cells: The primary cultures of fetal fibroblasts (FFs) were established from 12 -13 day C57Bl/10 fetuses. Donor females were sacrificed by cervical dislocation and uterine horns were cut out and placed in sterile PBS. After the fetuses were dissected out from the foetal membranes, they were decapitated and eviscerated. The carcasses were washed in PBS, cut into small pieces, and transferred to the 0.25% trypsin/EDTA for 30 min. at 37oC. Digestion by trypsin was stopped by adding Dulbecco's modified Eagle medium (DMEM) supplemented with 10% of foetal calf serum (FCS, Gibco). Cells were centifugated (10 min, 200g), suspended in the same medium and seeded onto 6 cm tissue culture Petri dishes. Upon reaching confluence the cells were passaged and then frozen at passages 1-4 to be used later. For manipulation cultures of FFs, they were trypsinized, centrifuged and suspended in a small amount of M2 medium (200μm) to be taken to the manipulation chamber.

Somatic nuclear transfer: The recipient cytoplasts and donor nuclei were preincubated in M2 medium supplemented with CB (5μg/ml) for 20-30 min before being placed in a micromanipulation chamber filled with the enucleation medium. After insertion of a donor cell into the perivitelline space, the pairs were washed out of enucleation solution and were placed in M2. Then, GV-FF complexes were washed twice in electrofusion solution (0.3 M mannitol supplemented with 0.1 mM $CaCl_2$, and 0.1 mM $MgSO_4$) and were placed between two parallel platinum electrodes in an electrofusion chamber filled with the same solution. Complexes were exposed to 2 DC pulses (50-60 μsec each, 1.8–2.5 $kV.cm^{-1}$) generated by the BTX 2001 ElectroCell Manipulator (Genetronics, Inc., San Diego, CA). Fusion usually occurred within 30 min. Fused pairs were then washed in maturation medium and cultured 17 h till fertilization.

Maturation: The oocytes were transferred into 50 µl droplets (10 oocytes per droplet) of pre-equilibrated IVM medium (TCM199 medium supplemented with 10% fetal calf serum, 75 µg/ml penicillin G potassium salt and 50 µg/ml streptomycin sulfate) overlaid with light mineral oil and were cultured at 37 °C in an atmosphere of 5% CO2 in air with the saturated humidity. Loose cumulus cells stripped previously from the GV oocytes were added to the maturation medium. Upon maturation, the oocytes were either fertilized or activated.

Activation: The matured oocytes were activated either by the culture for 5–6 h in Ca^{2+}-free KSOM medium containing 10 mM Sr^{2+} and 5 µg/ml CB (37°C, 5% CO_2 in air) or by 5 min. exposure to the medium M2 containing 7% ethanol at room temperature and were cultured in KSOM containing 5 µg/ml CB for 5-6 h. After activation, the surviving embryos were collected and cultured in KSOM for further development.

Fertilization: Spermatozoa were collected from mature males of the proven fertility. Spermatozoa were squeezed out from *cauda epididymides* into 1.0 mL of HTF medium. Spermatozoa were allowed to disperse and were capacitated for 1.5 h at 37 °C, 5% CO_2 in air. The matured oocytes (control and reconstructed ones) were inseminated in 50 µl microdroplets (10 oocytes/droplet) of sperm suspension in IVF medium (HTF). Prior to insemination either the *zona pellucida* was completely removed from the oocytes (*zona* – free oocytes) or the small opening was made in the *zona* (*zona* – drilled oocytes). The removal and the drilling of the *zona* was performed by using the acidified Tyrode's solution (pH = 2.5) supplemented with polivinylpyrrolidone (m.w. 40.000). Removal of the *zona pellucida* (Fig. 2A) was achieved by a short pretreatment of oocytes with acidified Tyrode's solution and then, after their transfer into IVF medium, by their gentle pipetting. In order to obtain the small hole in the *zona pellucida* (*zona*-drilled oocytes) the tip of micropipette filled with acidified Tyrode's solution was placed in the vicinity of the *zona* (Fig. 2B) and the solution was slowly blown off until the opening in the *zona* has been made. For the fertilization of *zona* – free and *zona* - drilled oocytes the concentration of about 50 motile sperm/droplet and 0.5×10^6 of motile sperm/ml was used, respectively. Inseminated oocytes were kept, also respectively, 2h and 4h with the sperm suspensions. After insemination, oocytes were washed twice in pre-heated KSOM medium and were transferred into KSOM medium for further culture (37°C, 5% CO_2 in air).

Culture: Upon activation/fertilization, the oocytes were cultured in KSOM (37°C; 5% CO_2 in air). They were monitored for the first

cleavages and further development to the blastocyst stage 120 h of culture (Fig. 2 C-D).

RESULTS

Developmental potential of oocytes was observed after fertilization. In vivo matured oocytes and in vitro matured zona-free and zona-drilled and reconstituted oocytes were fertilized whereas zona-intact oocytes were not penetrated. Fertilization was confirmed with the observation of the second polar body (2^{nd} PB). Extrusions of second polar bodies (PBs) were not different between ovulated oocytes and zona-free and zona-drilled oocytes whereas the extrusions of 2^{nd} PBs of the reconstituted oocytes were lower of the former ones (Table 1). Cleavages (%) were followed the same trend as the extrusions of 2^{nd} PBs. The reconstituted oocytes could not exceed two-cell stage embryos whereas the *in vivo* matured oocytes cleaved further and developed to the blastocyst stage in a percentage higher than zona-free and zona-drilled in vitro matured oocytes.

Table 1: Development potential of mouse oocytes after in vitro fertilization

Experimental design	No. ooctyes	Stages of development % (n)		
		2^{nd} PB	cleavage	Blastocyst
In vivo matured oocytes	55	96.3 (53/55)	94.3 (50/53)	86.0 (43/50)
In vitro matured zona-intact oocytes	100	0.0 (0)	0.0 (0)	0.0 (0)
In vitro matured reconstituted oocytes	78	15.4 (12/78)	33.3 (4/12)	0.0 (0)
In vitro matured zona- drilled oocytes	50	94.0 (47/50)	95.7 (45/47)	50.0 (25/47)
In vitro matured zona-free oocytes	49	91.8 (45/49)	95.5 (43/45)	32.5% (14/43)

n = number of oocytes or embryos PB= Polar body

Developmental potential of oocytes was observed after activation with either alcohol or $SrCl_2$. The percentages of activated oocytes or cleaved embryos were not differed with either maturation of oocytes (*in vivo* vs. *in vitro*) or their activation (alcohol vs. $SrCl_2$). Development to the blastocyst stage after activation was differed between *in vivo* and in vitro matured oocytes (50.7 vs. 24.6%) and between oocytes activated either with $SrCl_2$ or alcohol (37.3 vs. 24.6%), respectively. Therefore, it can be concluded that maturation of oocytes *in vivo* and activation with $SrCl_2$ were markedly increased the developmental potential to the

blastocyst stage compared with maturation *in vitro* and activation with alcohol, respectively (Table 2 & Fig. 2C-D).

Table 2: Development potential of mouse oocytes after activation

Experimental design	No. ooctyes	Stages of development % (n)		
		Activated	cleaved	Blastocyst
In vivo matured oocytes (alcohol)	75	97.3 (73/75)	97.3 (71/73)	50.7 (36/71)
In vitro matured oocytes (alcohol)	60	98.3 (59/60)	96.6 (57/59)	24.6 (14/57)
In vitro matured oocytes (Strontium chloride)	80	95.0 (76/80)	98.7 (75/76)	37.3 (28/75)

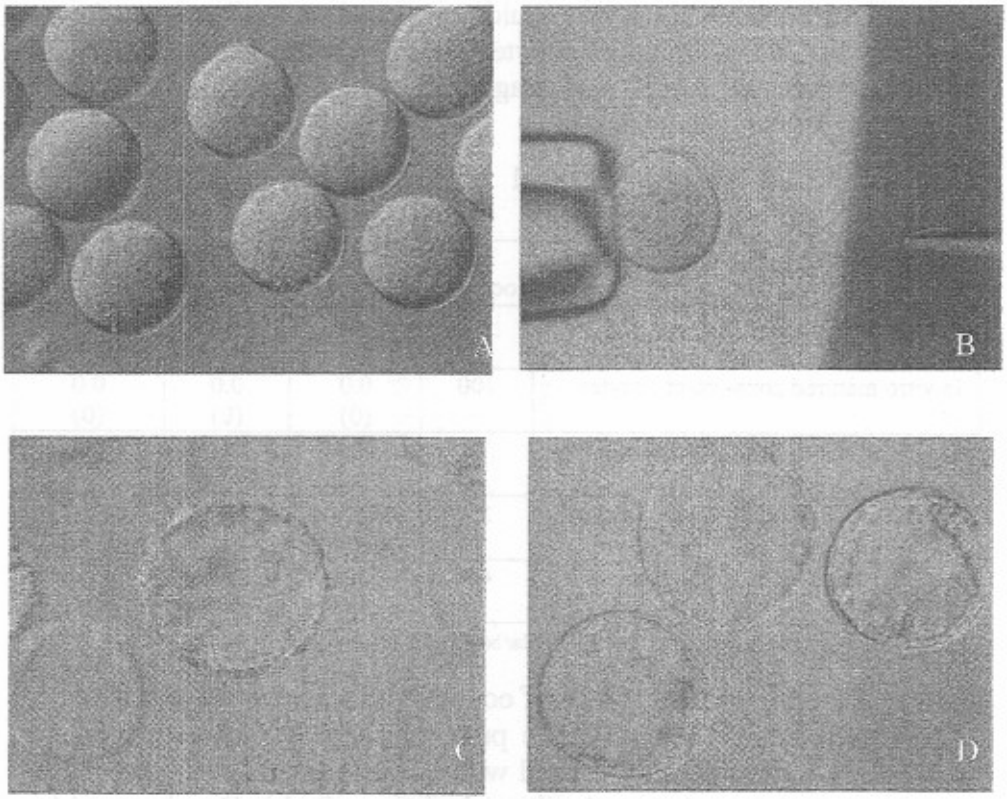

Fig. 2: Developmental potential of in vitro matured and zona-manipulated oocytes; a) zona-free oocytes b) drilling the zona pellucida C) hatched blastocyst of fertilized zona-drilled oocytes D) blastocysts of activated oocytes using strontium.

DISCUSSION

Removal of cumulus cells from the immature oocytes before maturation resulted in zona hardening in mice. Therefore, removal or drilling the zona pellucida resulted in fertilization the oocytes which developed to the blastocyst stage in percentages lower than ovulated oocytes. These results were in agreement with those obtained by (Yamazaki *et al.*, 2001). The zona-intact oocytes were not penetrated whereas reconstituted oocytes (15.4%) were penetrated. This might due to the slit made during reconstruction which persisted till the time of fertilization (personal observation). The reconstituted oocytes could not exceed two-stage embryos whereas the none-reconstituted oocytes developed further to the blastocyst stage. Nuclei transferred into enucleated GV oocytes undergo disassembly which involves nuclear envelope and lamina breakdown and chromatin condensation (Chang *et al.*, 2004; Grabarek *et al.*, 2004; Polanski *et al.*, 2005, Mohammed 2006; Mohammed *et al.*, 2008). Following activation of the reconstructed oocyte, the disassembled nuclei undergo reassembly which involves decondensation of the chromatin, formation of a new nuclear envelope, polymerization of a new lamina, formation of nucleoli and expansion (swelling) of the nuclei (Stice and Robl *et al.*, 1988) in MII oocytes whereas - according to Chang *et al.* (2004) and Mohammed *et al.*, (2008) observations - the disassembled nuclei in GV cytoplasts could not reassemble and characterize by absence of nuclear membrane and nucleoli which might affect the developmental competence. Therefore, factors which are present in GV play an important role (in cooperation with the cytoplasmic factors) in conferring the full competence of oocytes.

Developmental potential to the blastocyst stage of oocytes activated with $SrCl_2$ was higher than those activated with alcohol. These results are in agreement with other studies. This might be due to strontium which is the only parthenogenetic agent for mouse oocytes that induces repetitive intracellular calcium releases in a fashion similar to those following normal fertilization by spermatozoa (Kline 1996; Bos-Mikich *et al.*, 1995). The results lead to conclude that drilling the hardened zona pellucida for fertilization as well as activation with strontium was better than removal the zona pellucida and activation with alcohol respectively in conferring the full competence of mouse oocytes. It can be concluded that drilling the zona pellucida is an applicable technique in animal production for increasing productivity. Further

studies are required for improving *in vitro* maturation and *in vitro* culture conditions.

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