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DEVELOPMENTAL COMPETENCE OF IMMATURE CYTOPLASTS UPON SOMATIC NUCLEAR TRANSFER BEFORE AND AFTER MATURATION

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Abstract: The aim of this study was to investigate the developmental potential of reconstituted germinal vesicle (GV) cytoplasts with fetal fibroblast cells before/after maturation. Immature germinal vesicle oocytes were collected from the ovaries. Complete and selective enucleation techniques were performed for obtaining complete (GV) and selective (GVsel) cytoplasts. Fetal fibroblast cells (FFs) were used as the donor nuclei. Part of both types of cytoplasts (GV & GVsel) was reconstructed with FFs before in vitro maturation whereas the remaining part was reconstructed after in vitro maturation. The matured and reconstructed oocytes were activated using alcohol and cultured in vitro for further development. The results indicated that survival rates of enucleation GV oocytes were similar with selective and complete enucleation techniques. Selective enucleation technique of GV oocytes resulted in obtaining pronuclei containing nucleoli after transfer of fetal fibroblast nuclei into the cytoplasts before/after maturation compared with complete enucleation which resulted in obtaining pronuclei without nucleoli. Reconstruction of cytoplasts after maturation resulted in the cleavage of activated oocytes compared with reconstruction of cytoplasts before maturation which resulted in uncleavage of activated oocytes. In conclusion, transfer of FFs into GVsel cytoplasts after maturation resulted in obtaining the best results.

Key words:cytoplast, maturation, selective enucleation, complete enucleation

Introduction

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 and Mohammed 2009).

Several studies demonstrated that meiotic maturation of GV cytoplasts reconstructed with embryonic/somatic nuclei were associated with abnormalities in

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cytokinesis and chromosome condensation as well as premature division in about 1/3 of the reconstructed oocytes (Chang *et al.,* 2004; Grabarek *et al.,* 2004; Polanski *et al.,* 2005; Mohammed 2006; Nan *et al.,* 2007; Mohammed *et al.,* 2008 and Mohammed 2009).

In order to avoid disruption of reconstituted oocytes during maturation, this experiment aimed to investigate the developmental potential of reconstructed germinal vesicle cytoplasts with fetal fibroblast cells before and after maturation.

Materials and Methods

Experimental animals: Female mice (6-8 week old) originated from the mouse colony which is bred in the Department of Experimental Embryology were used. Mice were kept under a 12h light/12h dark cycle starting at 7 a.m. Food and water were available *ad libitum.* Donor females were killed by cervical dislocation.

Reagents and culture media: 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).

Recipient cells: Fully grown (germinal vesicles – GV) oocytes were used as recipients of donor nuclei. For collection of germinal vesicle (GV) oocytes, 6- 8 week old F1 (C57Bl10xCBA/H) females were injected with 7.5 IU of pregnant mare 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 needle, 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

Donor cells: The primary cultures of fetal fibroblasts (FFs) were established from 12 -13 day C57Bl/10 fetuses. For manipulation, cultures of FFs were trypsinized, centrifuged and suspended in a small amount (200μl) of M2 medium then transferred to the manipulation chamber.

Micromanipulation tools and equipments: All manipulations were performed under inverted Leitz Fluowert microscope (Leitz, Germany) equipped with Nomarski differential-interferential contrast (DIC) and connected with Leitz mechanical micromanipulators (Germany). 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 with an external diameters 1 mm (GC 100T-15, Harvard Apparatus Ltd, Kent, Great Britain). For complete enucleation (CE) and nuclear injection, pipettes were prepared using PB-7 vertical puller (Narishige, Japan). After pulling, the end of pipettes was cut 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). For selective enucleation (SE), the narrow conical micropipettes were pulled out on M-97 micropipette puller (Sutter Instrument Co., USA). The edges of pipettes were bent twice in order to adopt them to the micromanipulation chamber.

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

Selective enucleation of GV oocytes: Selective enucleation (SE) of GV stage oocytes was performed as described by Mohammed *et al.,* (2008). The liquid GV contents and nucleoli remain in the cytoplasm and only the nuclear (GV) envelope with the attached chromatin network was removed from the oocyte.

***In vitro* maturation of oocytes:** For *in vitro* maturation, oocytes and cytoplasts were transferred into 50 µl droplets (10 oocytes/cytoplasts 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 for 17 hours at 37 ^{0}C in an atmosphere of 5% CO2 in air with the saturated humidity

Somatic nuclear transfer: Cells were preincubated in M2 medium supplemented with cytochalasin B (5µg/ml) for 20-30 min before being placed in a micromanipulation chamber filled with the enucleation medium. Cells were introduced into the perivitelline space of ooplasts through the slit made in the *zona pellucida* during enucleation. After insertion of a donor cell into the perivitelline space, the pairs were washed out of enucleation solution, placed in M2 with dbcAMP to inhibit maturation, and then incubated until electrofusion. Thereafter, FF – oocyte 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 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, 1.8–2.0 $kV.cm^{-1}$) generated by the BTX 2001 ElectroCell Manipulator (Genetronics, Inc., San Diego, CA). Fusion usually occurred within 30 min. Fused pairs were then washed twice in maturation medium and cultured.

Oocyte activation: Reconstructed oocytes were activated (after maturation) through exposure to the medium M2 containing 7% ethanol for 5 min. at room temperature and were cultured in KSOM containing 5 µg/ml CB for 5-6 hrs. After activation, the surviving embryos were collected and cultured in KSOM for further development.

Developmental competence of activated oocytes: The reconstructed oocytes were cultured in KSOM (37^0 C; 5% CO_2 in air) and then were monitored for the first cleavage and further development.

Results

1. Survival rate of cytoplasts produced by complete or selective enucleation of oocytes

Cytoplast were obtained from immature ovarian oocytes either by removing the entire GV (complete enucleation, GV cytoplasts) or by selective enucleation, in which liquid contents of GV was left in the cytoplasts (GVsel cytoplasts). The results from this experiment showed that 114 and 106 cytoplasts out of 133 and 115 immature ovarian oocytes were obtained from complete and selective enucleation, respectively. Selective enucleation provided cytoplasts at a similar survival rate as did complete enucleation (92.2 % as opposed to 85.7 %, respectively (**Table 1**). The survival rate (%) and number of survived cytoplasts used for reconstruction with FFs before and after maturation are presented in Table (1).

Table(1): Survival rate of cytoplasts produced by complete or selective enucleation of oocytes

Experimental design	Complete enucleation	Selective enucleation
Total survival rate of enucleated oocytes, % (no)	85.7 (114/133)	92.2 (106/115)
Number of survived cytoplasts used for reconstruction with FFs before maturation	71	65
Number of survived cytoplasts used for reconstruction with FFs after maturation	43	41

2. Developmental competence of cytoplasts reconstructed before maturation

Fusion of the obtained cytoplasts before maturation with fetal fibroblast cells and maturation rates were slightly increased with selective enucleation compared with complete enucleation (Table 2). When the matured oocytes were activated, pronuclei were formed in GVsel cytoplasts with visible nucleoli whereas the pronuclei in GV cytoplasts did not contain nucleoli. The pronucleus diameter was higher in GVsel cytoplasts than the one in GV cytoplasts. Both the GV and GVsel cytoplasts reconstructed with fetal fibroblasts were not cleaved after activation.

Table(2): Developmental competence of cytoplasts reconstructed before maturation

Technique of enucleation	Complete enucleation % (No.)	Selective enucleation % (No.)
Ooplast/fibroblast formation	88.7 (63/71)	92.3 (60/65)
Fusion	63.5 (40/63)	70.0 (42/60)
Maturation	62.5 (25/40)	71.4 (30/42)
Activation	Unkown (25)	90.0 (27/30)
Cleavage	00	00

3. Developmental competence of cytoplasts reconstructed after maturation

Germinal vesicle cytoplasts and fetal fibroblast cells were used to investigate the developmental competence after maturation. When the cytoplasts were activated after reconstruction, the pronuclei of FF-GV oocytes were not visible because of absence of nucleoli and pronuclear membrane whereas the pronuclei of activated FF-GVsel oocytes were visible with clear membrane and nucleoli in 93.3 % of oocytes. In addition, first cleavages were increased significantly with FF-GVsel oocytes compared to FF-GV oocytes. Furthermore, none of FF-GV oocytes was exceeded two cell stage embryos whereas 21.4% developed to three cells stage embryos (Table 3).

Table(3): Developmental competence of cytoplasts reconstructed after maturation

Technique of enucleation	Complete enucleation % (No.)	Selective enucleation % (No.)
Ooplast/fibroblast formation	88.4 (38/43)	92.7 (38/41)
Fusion	68.4 (26/38)	78.9 (30/38)
Activation	Unkown (26)	93.3 (28/30)
Cleavage, 2 cells	38.5 (10/26)	57.1 (16/28)
Cleavage, 3 cells	00	21.4 (6/28)

Discussion

The results indicated for the first time the possibility of cleavage of GV cytoplasts after reconstruction with somatic cells. Reconstruction of cytoplasts after maturation in combination with selective enucleation highly increased the pronuclei formation and the cleavage rate compared to reconstruction of cytoplasts before maturation and/or complete enucleation.

The cytoplasts reconstituted before maturation with fetal fibroblasts were not cleaved upon activation. 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). 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 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 the absence of nuclear membrane and nucleoli which might affect the developmental competence.

On the other hand, the cytoplasts reconstituted after maturation with fetal fibroblasts were cleaved upon activation and highly increased with selective enucleation. Gao *et al.,* (2002) proved that germinal vesicle material is essential for nucleus remodeling after nuclear transfer. When the ProMI cytoplasts were reconstructed with 1/4- and 1/8-embryonic nuclei, the resulting oocytes were cleaved upon maturation and fertilization. The oocytes were cleaved twice (extrusion the second polar bodies and the first cleavage) but they did not cleave further, (Mohammed, unpublished data). The reason of the cleavage and its limitation might be due to the presence of GV material in ProMI cytoslasts and abnormalities in meiotic maturation of reconstructed oocytes.Therefore, unkown factors which are present in GV play an important role in conferring the cleavage of reconstituted oocytes. Blocking the cleavages of reconstituted oocytes after maturation at three cell stage embryos might be either due to; 1) absence of cumulus cells, 2) inability of somatic nuclei to remodel after nuclear transfer and 3) inefficiency of the culture system.

In conclusion, this study indicates for the first time the cleavages of somatic nuclei after transfer into enucleated GV oocytes. However, the presence of GV matarial in oocyte' cytoplasm seems to be conditions *sine qua non*

of successful development. Further researches are required for modification *in vitro* culture system of the reconstituted oocytes and investigating its efficiency through developmental competence of reconstituted oocytes.

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تطور سيتوبلازم البويضات الغير ناضجة عند نقل الانوية الجسمية اليها قبل او بعد الانضاج

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الهدف من البحث هو دراسة إمكانية تطور سيتوبلازم البويضات الغير ناضجة المُعَاد تكوينة بخلايا ال fetal fibroblast (FF) قبل او بعد الانضاج. تم الحصول علي البويضات الغير ناضجة من المبيض. استخدمت تقنيات ازالة الانوية الكاملة Complete enucleation والإنتقائية Selective enucleation لازالة الانوية من البويضات والحصول علي نوعين من السيتوبلازم واستعملا كمستقبل لخلايا ال fetal fibroblast. تم نقل خلايا ال fetal fibroblast الي البعض من نوعي السيتوبلازم قبل الانضاج في حين ان البعض الاخر تم النقل اليه بعد الانضاج. تم تنشيط البويضات المكونة بعد الانضاج باستخدام الكحول. اظهرت النتائج ان نسب بقاء البويضات حية بعد ازالة الانوية كانت مماثلة باستخدام تقنيات ازالة الانوية الكاملة والإنتقائية. بعد نقل خلايا ال FF الي السيتوبلازم ، ادت التقنية الانتقائية لازالة الانوية الي الحصول علي انوية اولية محتوية علي نويات مقارنة بتقنية ازالة الانوية الكاملة التي ادت الي الحصول علي انوية اولية غير محتوية علي نويات. أدي نقل خلايا ال FF بعد انضاج السيتوبلازم الي انقسام البويضات المنشطة مقارنة بالنقل قبل انضاج السيتوبلازم التي لم ينقسم اي منها. نقل خلايا ال FF الي السيتوبلازم الانتقائي بعد الانضاج ادت الي الحصول علي افضل النتائج.