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Influences of Cumulus Cells During in vitro Fertilization of Mouse Oocytes in Different Mouse Strains

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Abstract: In this study, the influences of cumulus cells during in vitro fertilization (IVF) and their subsequent effects on mouse embryo development in vitro were studied in inbred and hybrid strain mice. Embryos at the 2-cell stage obtained after in vitro fertilization were transferred to the oviduct of foster mothers to assess their implantation rate. Mouse oocytes, which were intact at the time of in vitro fertilization, demonstrated less polyspermic fertilization and more blastocyst development than those inseminated without any cumulus cells. The data demonstrate that more oocytes were arrested at the 2-cell stage in groups that contained no cumulus cells. There was no statistical difference between cumulus-enclosed and cumulus-free groups for embryo implantation. Data suggest that although there was no difference in terms of implantation rate, more blastocyst stage embryos were obtained from cumulus cell enclosed oocytes. Therefore, we suggest that cumulus cells play many roles during in vitro fertilization, and they should be intact during in vitro fertilization.

Key Words: Mouse, embryo, in vitro fertilization, cumulus, inbred, hybrid

Farklı Irktan Fare Oositlerin in vitro Fertilizasyonunda Kumulus Hücrelerinin Etkileri

Özet: Bu çalışmada, kumulus hücrelerinin in vitro fertilizasyon (IVF) aşamasında ve fertilizasyondan sonrasında fare embriyolarının in vitro gelişimleri üzerindeki etkileri inbred ve hibrid fare ırklarında incelenmiştir. In vitro fertilizasyondan sonra 2-hücre aşamasına gelen embriyolar, implantasyon oranlarını belirlemek için alıcı farelerin oviduktlarına transfer edilmiştir. İn vitro fertilizasyondan sonra kumuluslu fare oositleri kumulussuz gruba kıyasla daha düşük polispermik fertilizasyon ve daha yüksek blastosist gelişim oranı göstermiştir. Elde edilen veriler, kumulussuz deney grubunun kumuluslu olana göre daha yüksek oranda iki hücre safhasında blok yaptığını göstermiştir. Embriyoların implantasyonu açısından, kumuluslu ve kumulussuz gruplar arasında istatistiksel bir fark bulunamamıştır. Verilere göre, implantasyon oranında bir fark bulunmamasına rağmen, kumuluslu oositlerden elde edilen embriyolar daha yüksek blastosist oranına ulaşmıştır. Sonuç olarak, kumulus hücreleri in vitro fertilizasyonda çok sayıda değişik rol oynadığından, oositlerin kumulus hücreleri uzaklaştırılmadan fertilizasyon yapılmalıdır.

Anahtar Sözcükler: Fare, embriyo, in vitro fertilizasyon, kumulus, inbred, hibrid

Introduction

IVF has been widely used by investigators for studying morphological and molecular details of sperm-egg interactions during fertilization. Normal fertilization results in the formation of a zygote from the union of an egg with a single spermatozoon. Under normal

conditions, sperm decondensation and pronucleus formation occur as a continuum (1,2). Time course studies in both humans and rodents show that the spermatozoon's nuclear decondensation/recondensation and meiotic progression, and formation of both the male and female pronuclei occur in a coordinated fashion (1-4). Malfunction of these events at any stage results in failure of the oocyte to develop normally.

IVF and related techniques are important tools for both maintenance and rederivation of mice lines as well. Such techniques have been extensively evaluated and developed for routine use in the rederivation of rodent species (5,6). Interest in mouse IVF has increased in laboratories utilizing the mouse as a model system to develop new methods for human IVF programs, and for the purpose of investigating genetic diseases by mutations (6). The cumulus cells apparently serve as a suitable milieu to fertile sperm and also to partially immobilized sperm as they interact with the surface of the zona pellucida (7). For this reason, it is recommended that sperm be mixed directly with oocytes with cumulus masses. However, there are conflicting reports regarding the requirement for cumulus-intact oocytes during in vitro fertilization (7-9). In this study, IVF efficiencies using cumulus-intact and cumulus-free oocytes were investigated in inbred and hybrid mice strains (10,11)

Materials and Methods

Animals

The animals used in this study were maintained in accordance with the guidelines of the Committee on Laboratory Animals of the RIGEB and Medical College of Georgia. Oocytes were produced by the superovulating of hybrid CB6F1 and C57BL/6J females (12). Sperm was obtained from the cauda epididymis of hybrid CB6F1 and C57BL/6J males (BALB/C x C57BL/6J). Vasectomized CD-1 males served to prepare foster mothers (CD-1) for embryo transfers. Mice were maintained at 14L:10D cycle (lights on at 05:00 h).

Preparation of media

All components were purchased from Sigma Company. M2 medium (13) was prepared freshly for each trial from four different stock medium solutions weekly. Whittingham's medium for IVF was prepared without BSA and aliquoted as 10 ml volumes in 15 ml centrifuge tubes. They were stored at –20ºC for several weeks. CZB medium (14) for embryo culture was prepared weekly from chemical components. Eighteen-milli-Ω double distilled water was used for preparing all media. All media used in this study were equilibrated at 37ºC in 5% CO_2, and 95% humidity for 24 h before they were used.

Superovulation and oocytes collection

Four-week-old female C57BL/6J and CB6F1 mice were superovulated by intra peritoneal (i.p.) injection of 5 IU PMSG (Sigma G-4877) at 8 pm followed by ip injection of 5 IU hCG (Chorulon, Intervet) 48 h apart. Twelve to 13 h after the hCG injection, the mice were killed by cervical dislocation, and the oviducts were dissected and transferred to M2 medium. Ovulated oocytes surrounded by cumulus cells were collected from the ampulla of the oviduct. Cumulus cells were removed with hyaluronidase (80 IU/ml) (H-3506; Sigma) for 2 min (13). After removal of cumulus masses, all oocytes were washed three times with M2 medium without hyaluronidase before they were transferred into the fertilization medium.

Sperm preparation

Sperm was obtained from the dissected cauda epididymides of 5-month-old F1 hybrid (C57BL/6J x BALB/C) and C57BL/6J mice after 2 weeks of abstinence. Each cauda epididymis was placed into a 500-µl drop of Whittingham's medium with BSA (30mg/ml) (13) under mineral oil. The epididymal contents were squeezed out by the use of watchmaker's forceps. The residual caudal tissue was discarded. The drops containing freshly released spermatozoa were placed in a 37ºC with 5% CO_2 incubator for 20 min to allow dispersal. After 20 min incubation, 5 ml of spermatozoa was diluted in 95 µl of tap water, and sperm number per ml was determined by a hemacytometer (13). Capacitation of spermatozoa was achieved by allowing them to stay at 37ºC in 5% CO_2 and 95% humidity for 1 h.

In Vitro Fertilization

IVF was carried out in 500 µl Whittingham's medium under mineral oil (M-8410, Sigma). Ten to 15 µl of capacitated sperm suspension was added to the fertilization medium to give the final motile sperm concentration of 2 x 106/ml. Oocytes and sperm were incubated for 6 h at 37ºC in 5% CO_2, and then they were removed from the fertilization drops, and rinsed 3 times with M2 medium. They were further cultured in 10 µl of

CZB medium (14) covered with mineral oil at 37ºC in 5% CO_2, and 95% humidity. Twenty-four hours after fertilization, oocytes were examined under a stereomicroscope and those that had not reached the next developmental stage were removed from the culture drops. Some embryos were cultured for 96 h to observe the developmental competence of in vitro fertilized oocytes and some two-cell embryos were transferred into the oviduct of recipient mice. Each experiment was repeated at least three times, and each replicate had three groups cumulus-enclosed (intact), cumulus-free and control. In the control group, dead sperm was used for activation.

Foster mother preparation

In order to examine the potential embryo development to term, foster mothers (CD-1) were prepared by introducing them to vasectomized males 1 day before the transfer (8,12,13). Females were checked for a vaginal plug, and those with a vaginal plug were separated for embryo transfer.

Embryo transfer

Embryos were transferred bilaterally to the oviduct of foster mothers. A small incision was made in left fossa paralumbal area and oviduct was visualized by a small forceps. A small incision was made on the fimbria of the oviduct, and then the transfer pipette (120 mm in diameter) was introduced into the opening of the oviduct. Ten embryos were transferred into each oviduct and the oviduct was placed back inside the body wall. The operation wound was closed by 5-0 chromic guts.

Statistical analysis

The data analysis was carried out by using the SigmaStat Software Program (Version 1.0, Jandel Corporation, San Rafael, CA). Data from the different treatments was compared with Chi-squared analysis using Yates' correction for continuity, and analyses of variance for differences between experimental groups. The data was transformed by the arcsine square method before the statistical analyses. The Bonferroni-t test was used to determine differences among groups.

Results

In the first experiment, we evaluated normal fertilization parameters by eosin staining 6 h after in vitro fertilization. The results are summarized in Table 1.

More oocytes were fertilized by a single sperm in the cumulus cell enclosed group than the cumulus cell free group in the two strains included in this study. We obtained some 2 pronuclei zygotes in the control group. This is because of a development defect in which first polar body occasionally could not be released into perivitelline space, and is seen as second pronuclei. In the next experiment, we tested our results by determining the cleavage percentage into the two-cell stage and beyond. Presumptive zygotes after 6 h fertilization interval were transferred into 10 mL of CZB medium covered with mineral oil. The results, in Table 2, show that more zygotes reached the morula and blastocyst stages in C57BL/6J and CB6F1 in the intact group than in the cumulus free group. Although there is no statistical difference between strains, slightly better development was observed in the hybrid strain (CB6F1) than in the inbred strain (C57BL/6J).

Table 1. Normalcy of in vitro fertilized presumptive zygotes of C57BL/6J and CB6F1 mice.

Experimental Groups	No. of oocytes stained at 6 h	No. of oocytes w/1 PN	No. of oocytes w/2 PN	No. of oocytes w/3 or more PN
C57BL/6J				
Intact	50	10	38	6
Cumulus free	50	4	28	18
Control	50	41	9	0
CB6F1				
Intact	50	5	41	4
Cumulus free	50	12	25	13
Control	50	47	3	0

PN: pronucleus, w/: with, intact: cumulus enclosed

Table 2. Comparative developmental competence of C57BL/6J and CB6F1 presumptive zygotes in CZB medium.

Experimental Groups	No. of zygotes cultured	Developmental Stages (%)			
		2 Cell 24 h	4 Cell 48 h	Morula 72 h	Blastocyst 96 h
C57BL/6J					
Intact	300	215 (71.6) [a]	193 (64.3) [d]	174 (58.0) [h]	174 (58.0) [j]
Cumulus free	300	169 (56.3) [b]	141 (47.0) [e]	128 (42.6) [i]	125(41.6) [k]
Control	100	8 (8.0) c	3 (3.0) f	0	0
CB6F1					
Intact	300	238 (79.3) [a]	224 (74.6) [g]	192 (64.0) [h]	191 (63.6) [j]
Cumulus free	300	173 (57.7) [b]	157 (52.3) [e]	107 (35.7) [i]	103 (34.3) [k]
Control	100	7 (7.0) [c]	2 (2.0) [f]	0	0

[a - k] Different superscripts in the same column demonstrate statistical difference at $p<0.05$.

Table 3. Implantation rate of hybrid and inbred strains of embryos after in vitro fertilization.

Experimental Groups	No. of embryos transferred	No. of fosters used	No. of pregnant mothers	No. of pups obtained	Mean No. of pups/mother
C57BL/6J					
Intact	560	28	19 (67.8) [a]	68 [d]	3.6 [h]
Cumulus free	500	25	16 (64.0) [a]	51 [e]	3.2 [h]
CB6F1					
Intact	560	28	22 (78.6) [b]	115 [f]	5.2 [h]
Cumulus free	500	25	17 (68.0) [a, c]	73 [d, g]	4.3 [h]

[a - h] Different superscripts in the same column demonstrate statistical difference at $p<0.05$.

In the next experiments, we evaluated the implantation rate of 2-cell embryos transferred to the oviduct of 0.5-day-old pregnant foster mothers. Twenty-four hours after fertilization, embryos at the two-cell stage were evaluated for their normalcy by their blastomer size; then, they were transferred to the foster mothers. The results are presented in Table 3.

The data demonstrate that more pregnancies and pups were obtained in the strain and between the strains used for this study. However, mean numbers of pups obtained for each group were not different in and between strains. It has previously been suggested that the inbred strain C57BL/6J mouse line produces fewer pups than hybrid and outbred strains (13). Although we obtained significantly fewer pups from C57BL/6J than from CB6F1 in intact and cumulus-free groups, the mean numbers of pups per mother did not exhibit any significance.

Discussion

Despite extensive trials of culture conditions and media design, embryos produced by IVF have significantly reduced viability compared with corresponding embryos fertilized in the reproductive tract. The causes are likely to be multifactorial but it was shown that lower levels of production or release of autocrine growth factors by IVF zygotes made a significant contribution to the reduction in viability (15,16). A role for autocrine or endogenous growth factors was suggested by observations that embryo development was less successful when embryos were cultured in relatively large volumes of medium (15-18), and evidence shows that this was due to dilution of embryo-derived diffusible autocrine factors (19,20). Our results were similar to these reports since we achieved inferior results for generating live pups compared to the same strains in normal reproduction. In our unit C57BL/6J and CB6F1 strains produced 6-8 and 8-10 pups per litter, respectively.

Mixtures of nonessential amino acids increase the rate and frequency at which blastocysts are formed from one-cell conceptuses in culture (21,22). Moreover, the proportion of such blastocysts that implant in the uterus upon transfer to foster mothers is greater when nonessential amino acids are present during preimplantation development in vitro (22,23). Even brief exposure of approximately 5 min to medium not containing nonessential amino acids significantly reduces the proportion of zygotes that develop into morulae and blastocysts in vitro and the total number of cells in the blastocysts so formed (24). In our culture conditions, glutamine was the only amino acid source included in the CZB medium. However, our embryo development rates to the blastocyst stage in the cumulus-intact group were comparable to earlier results (21,24). We are conductiong further experiments to explore the possibility of improving our culture conditions for improving implantation rates.

The molecular events that occur during the fertilization process in mammals are becoming better understood. Unfortunately, studying the events that take place during gamete fusion and sperm decondensation in the oocyte is more difficult because sperm-oocyte fusion and subsequent sperm decondensation represent a short part of the fertilization process, and their exact timing is difficult to determine. An increase in both glycolysis and pentose-phosphate-pathway (PPP) activity augmented during fertilization with oocytes surrounded with cumulus masses (25-27). An increase in glucose utilization promoted by fertilization has been suggested by studies reporting higher glucose oxidation (25), hexokinase activity (26), and glycolysis rate (27) in fertilized oocytes with intact cumulus cells than in oocytes without cumulus masses in the mouse. The present study showed that embryo development and implantation rates were lower for oocytes that did not have cumulus masses.

In conclusion, our results demonstrated the supremacy of cumulus cells during in vitro fertilization. They possess multiple roles during maturation and fertilization as well as preparing spermatozoon for acrosome reaction. Therefore, they should be intact during in vitro fertilization.

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