Effect of Intracytoplasmic Sperm Injection (ICSI) on Mouse Embryos Preimplantational Development

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Abstract
It is known that the in vitro culture (IVC) of preimplantation embryos is associated with changes in gene expression. It is however, not known if the method of fertilization affects the global pattern of gene expression. We compared the development of mouse blastocysts produced by intracytoplasmic sperm injection (ICSI) versus blastocysts fertilized in vivo and cultured in vitro from the zygote stage (IVC). At the end of cultivation (96 hrs for blastocyst stage embryos), expanded blastocysts of each group were randomly selected, and ICM and total cells number were differentially stained. The total cell number of blastocysts was estimated by counting the total number of nuclei using DAPI staining. Cell number for inner cell mass (ICM) was estimated by counting the OCT4 (POU5FL) positive cells. Digitally recombined, composite images were analyzed using the Zeiss Axion Vision software and Zeiss Apotome. All 5–10 optical sections were divided using a standard grid over each layer to count all. Comparing the total cells and the ICM cells number, it appears that each method of fertilization has a unique pattern development. The developmental rate and the total cell number of the blastocyst were significantly lower in ICSI versus in vivo fertilized embryos which affect the embryonic developmental rate and the total cell number of blastocysts.

Keywords: ICSI, mouse embryo, OCT4, total cell number

1. Introduction

It is estimated that 1% of children in the western world are born with the help of assisted reproductive techniques, in vitro fertilization (IVF), and intracytoplasmic sperm injection (ICSI). While these techniques are thought to be safe [1], studies in animal models have demonstrated that in vitro culture (IVC) is suboptimal and represents an additional stress to gametes and embryos. IVC of preimplantational embryos alters their global gene expression patterns [2] and affects the behavior of mice born after such procedures (Ecker DJ).

Unfortunately, these studies have only examined the effect of IVC on outcome and have not explored the effect of gamete manipulation by IVF or ICSI.

Specifically, morphologic studies regarding total cell number and ICM cell number were performed on blastocysts fertilized in vivo but grown in vitro (IVC group) and those fertilized and grown in vitro (ICSI group). Therefore, any comparisons between the IVC and ICSI groups would tease out the effects of gamete manipulation and the effect of fertilization method.

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2. Materials and methods

Reagents and media
All chemicals and media were purchased from Sigma Chemical Co.

Animals
- Oocytes donor mice: strain B6D2F1, (female C57BL/6 x male DBA/2)
- Sperm donor mice: CD1 males

All micromanipulations were performed by using Narishige micromanipulation system installed on Olympus IX 71 inverted microscope and equipped with a Piezo-driven system (Prime Tech, Japan)

In vivo produced embryos
To get 1-cell stage embryos, females were superovulated with intraperitoneal injection of pregnant mare’s serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) at specific times [3]. After hCG injection, the females were caged individually with the appropriate males. Matting was verified by the presence of a vaginal plug the following morning. We collected the one cell stage embryos by rupturing the oviducts of superovulated females using CZB-HEPES medium.

ICSI procedure. ICSI was performed according to the procedures of Naoko Yoshida & Anthony CF Perry (2007) (Naoko Yoshida) [4]. Briefly, a spermatozoon repeatedly was sucked into an injection pipette from the tail and then ejected. These procedures were repeated to remove the tail at the head-midpiece junction (neck) by piezoelectric pulses PMAS-CT150, PrimeTech LTD, Ibaraki, Japan). The spermatozoon head then was injected into the oocytes. The injection was performed in HEPES-CZB medium.

Embryos cultivation and whole mount immunocytochemistry
After ICSI procedure, or in case of in vivo produced embryos after flushing the zygots from the oviduct, the embryos were cultured for 96 h until they rich the blastocyst stage in KSOM media. Whole-mount immunostaining was carried out as follows: Zona pellucida of ICSI control and ICSI HHP treated blastocyst-stage embryos was removed by brief incubation in acid tyrode solution (Sigma-Aldrich) and the embryos were fixed in 4% paraformaldehyde (15 min), permeabilized with ionic detergent 0.1% Triton X-100 and blocked with 1% BSA/PBS, followed by binding of primary (overnight 4°C) and secondary antibodies (RT 1 h, RT). We used the following primary antibodies: anti-OCT4 antibody (Polyclonal Rabbit Santa Cruz 19081, 1:100 dilutions). We used Alexa Fluor 594 anti-rabbit secondary antibodies (Molecular Probe, 1:2000). After immunostaining for OCT4, blastocysts were mounted on the slides and covered with Vectashield containing DAPI, followed by microscopic examination.

Cells counting method
The assessment for cell number counting was realized using digital images on different focus obtained with a Zeiss optical sectioning apotome microscope equipped with a HBO lamp. The cells presented in different focuses were counted and summarized. The total cells were identified by their blue (DAPI staining) fluorescence; ICM cells were identified by thei red (Alexa fluor 595) fluorescence.

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Figure 1. Different sections of the same embryo. A) ICM cells counting based on OCT4 staining (with black camera); B) total cells counting based on DAPI staining (with black camera)
Figure 2. Differential staining of the same embryo: 1) Bright field; 2) OCT4 staining; 3) OCT4+ DAPI staining; 4) DAPI staining

Statistic analyze
For statistical interpretation was used the Minitab, 16. In order to interpret the results obtained, we used Student test. T test is a difference significance testing procedure between two means. Theoretic t test can be used for whatever small lots, if the distribution for the two lots is normal and if the variance for the two lots is not significantly different. For testing the correlation we used Pearson correlation and Fitted Line Plot regression, which allows the linear and polynomial regression (degree 2 and 3) testing.

3. Results and discussion
Expanded mouse blastocysts are easily recognizable, and therefore offer an excellent endpoint for morphologic assessment. In order to investigate the effect of the method of fertilization and IVC, on cell division and cell allocation, we counted the number of ICM and TE cell in expanded blastocysts. It is important to notice that only late cavitating blastocysts with similar morphology as observed with a stereomicroscope were used for analysis.

Table 1. Results obtained for total cell number and ICM cell number for embryos obtained by in vivo or ICSI fertilization

<table>
<thead>
<tr>
<th>Specification</th>
<th>Embryo No.</th>
<th>Total cells number (DAPI staining)</th>
<th>ICM cells number (OCT4 staining)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( \overline{x} ) St Dev CV</td>
<td>( \overline{x} ) StDev CV</td>
</tr>
<tr>
<td>Fertilization method</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In vivo</td>
<td>24</td>
<td>61.1±1.6a 6.5 10.6</td>
<td>24.9±1.44a 5.9 23.8</td>
</tr>
<tr>
<td>ICSI</td>
<td>17</td>
<td>45.9±2.6c 12.7 27.6</td>
<td>17.2±0.07b 3.7 21.6</td>
</tr>
</tbody>
</table>

Values with different indices are significant at p>0.001 (T Test)

From the data presented in table 1, it can be noticed that total cells number for in vivo produced embryos (61.1±1.6) was higher than the total number for ICSI embryos (45.9±2.6), the differences are statistically significant (p<0.001). For ICM cells the number was greater for embryos fertilized in vivo (24.9±1.44) compared with embryos fertilized by ICSI method (17.2±0.07), the differences are statistically significant (p<0.001). The results suggested that ICSI obtained blastocysts embryos have a lower number of cells compared with in vivo produced embryos.
IVF versus ICSI

This comparison allows us to observe and isolate the effect of the conditions at fertilization (in vitro versus in vivo), since, the culture conditions from the zygote stage to the blastocyst stage were exactly the same in the two groups.

Surprisingly, ICSI embryos developed few hours faster from the two-cell stage to the blastocyst stage than IVC embryos. On the other hand, ICSI embryos had a reduced number of total cells (p<0.001) and number of ICM cells.

We also investigated the correlation between ICM cell number and total cell number of the embryos and applied Pearson correlation. For blastocyst obtained by ICSI method there is a positive and moderate correlation (r=0.585, p=0.003) between ICM cell number (17.2 ± 0.07) and total cell number (45.9±2.6) but, for in vivo obtained embryos there is no correlation (r=0.204, p=0.431) between total cells number (61.1± 1.6) and ICM cells number (24.9± 1.44). For embryos obtained by ICSI it appears that the ICM cells number is correlated with the total cells number, but it is not the only contributing factor, the polynomial regression showed that cubic regression coefficient (R-Sq) is = 49.2%, the regression equation is: y= - 48.84 + 3.694 x**2 + 0.000403 x**3, were y is ICM cells number and x is total cells number.

Our study is similar with other authors which have observed changes in the morphology of preimplantation embryos after culture. Crosier et al.,Z (2001) [5], analyzed the ultrastructural morphometry of bovine blastocysts produced in vivo or in vitro using three different culture media and noted that in vitro blastocysts had a smaller nucleocytoplasmic ratio. They associated this finding with reduced quality of in vitro embryos. However, Petzoldt & Muggleton-Harris (1987) [6] manipulated the nucleocytoplasmic ratio of mouse preimplantation embryos by removing or injecting cytoplasm and concluded that gene stage specific gene activity was independent of this ratio.

Global pattern of gene expression in in vitro versus in vivo produced embryos was also analyzed by Corcoran et al. (2006) [7] in bovine embryos. They found 384 genes differentially regulated in IVF embryos. There was a general down-regulation of genes involved in transcription and translation, as well as genes involved in cellular metabolism.

Other authors have documented a significant change in cell number, mitotic, and apoptotic index in blastocysts cultured in different media [8,9].

4. Conclusions

This study establishes that ICSI procedure has a discrete and distinctive effect on the preimplantational development of mouse in vitro produced embryos.

The differences between ICSI and in vivo embryos are quite remarkable. The most unanticipated findings are the net reduction of ICM cells comparing with the in vivo produced embryos.

In conclusion, the in vitro produced mouse embryos in the specific culture conditions used are at a disadvantage when compared with in vivo embryos.

It remains to be investigated if these changes are only temporary or are associated with long-term consequences.

Another question to be elucidated is whether changes reported in one species might be present in another species.

More studies need to be performed to evaluate what is the effect of other forms of artificial fertilization on embryo development.

The differential immunostaining it is optimal method for cell number assessment and gene expression visualization.

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