Organization of cytoskeleton and chromatin is related to the timing of the first zygotic cleavage and early developmental competence.

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Abstract

Timing of the first zygotic cleavage is a reliable predictor of embryo quality. Embryos that cleave early have higher developmental viability compared to their late counterparts. It is hypothesized that differences in viability is attributed to cytoskeletal and chromatin organizations. This study investigated cytoskeletal and chromatin structures, and distributions in early-cleaving (EC) versus late-cleaving (LC) embryos. Embryos were retrieved from superovulated ICR mice, 28 hours after hCG injection. Two-cell stage embryos were categorized as EC, while zygotes with two pronuclei as LC embryos. After overnight culture in M16 medium, embryos were fixed and immunostained to visualize cytoskeletal and chromatin distributions, and intensities. EC embryos were observed to have significantly higher actin and chromatin fluorescence intensities compared to LC embryos [(14.68 + 14.07) x 10^5 versus (1.50 + 1.20) x 10^5 pixels (p<0.05) and (11.43 + 3.48) x 10^5 versus (7.98 + 3.08) x 10^5 pixels (p<0.05)], respectively. There was no significant difference in tubulin intensity between EC and LC embryos. This suggests that higher densities of actin and chromatin in EC embryos appreciably contributed to more efficient cell division and therefore, greater developmental competence.

Keywords: Early cleavage; mouse embryo; cytoskeleton; chromatin; fluorescence intensity

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Introduction

Timing of the first zygotic cleavage is a reliable indicator for embryo quality [1-3]. It is a better assessment method compared to traditional morphological grading. Embryos that cleaved early were proven to be of better quality compared to their later counterparts. Many animal and clinical studies have shown that early cleaving embryos have not only higher developmental viability and higher blastocyst formation, but also produced higher pregnancy and live birth rates [3-6].

Although the selection of embryos based on the timing of the first zygotic cleavage has now become a common selection practice in many in vitro fertilization (IVF) laboratories, factors that contribute to the superiority of early cleavers are still not clear. It has been hypothesized that it may be related to cytoskeletal ultrastructure of the embryos. This is because the cytoskeleton plays an important role in organelle transport, cell division, motility, and signalling [7].

There are three subclasses of cytoskeleton - microfilaments including actin, intermediate filaments, and microtubules, including tubulin [7]. Actin and tubulin have been demonstrated to play an important role during embryo cleavage. Tubulin assists migration of pronuclei during the fertilization process [8]. It is involved in mitosis and chromosomal spindle formation and movement of organelles such as mitochondria [8]. Actin is also involved in many important cellular processes, such as cell motility, cytokinesis and division, organelle movement, spindle migration, distribution of mito-
chondria [9, 10], polarization of embryos, pronuclear apposi-
tion, cell signalling and maintenance of cell shape [11, 12].
Many of these processes are mediated by the interactions of actin with cell membranes.

Data on the distribution patterns and intensities of actin, tubulin and nuclear chromatin during early stages of em-
bro development is scarce. Understanding the distribution patterns and intensity of those structures will provide
important knowledge of their contribution and association with the viability of early and late cleaving embryos.

**Material and Methods**

**Source of embryos**

All procedures involving animals were approved by Ani-
mal Care and Use Committee (ACUC), UiTM (ACUC-
7/11). Embryos were obtained from female ICR mice, aged 6 – 8 weeks. Mice were superovulated by intraperi-
toneal (i.p.) injection of 5 IU of Pregnant Mare Serum
Gonadotrophin (PMSG; Folligon, Intervet), followed 48
hours later by 5 IU Human Chorionic Gonadotrophin
(i.p.) (hCG; Chorulon, Intervet). The females were subse-
sequently mated with male mice of the same strain, at a ra-
tio of 1:1. The females were checked for the presence of a
vaginal plug the morning after mating. After 28 hours of
hCG administration, the oviducts of the pregnant fe-
male mice were excised and embryos were flushed out into M2
medium (Sigma, USA: M7167). The embryos were ob-
served under an inverted microscope (Leica DMIRB, Germany). One-cell embryos with 2 pronuclei and em-
byos at the 2-cell stage were considered fertilized.

**Timing of the first zygotic cleavage**

Embyros were divided into two groups; early-cleaving and
late-cleaving embryos according to the timing of their first
zygotic cleavage. Embryos at the 2-cell stage at 28 – 30
hours post hCG administration were categorized as early
cleaving (EC) embryos, whereas zygotes with the presence
of the second polar body and two pronuclei were category-
ed as late cleaving (LC) embryos. The embryos were cul-
tured overnight in a CO₂ incubator in 50 μl drops of M16
culture medium (Sigma, USA: M7292) with 3% Bovine
Serum Albumin (BSA) (Sigma, USA: A9418). Droplets
were overlaid with mineral oil (Sigma, USA: M8410).

**Experimental Design**

A total of 50 EC embryos and 50 LC embryos were fixed
at 48 hours post-hCG to study the distributions and densi-
ties of the actin, tubulin and chromatin at the 2-cell stage.
Following immunolabeling with monoclonal anti-α-
tubulin, Alexa Fluor and DAPI to identify tubulin, actin
and nuclear chromatin respectively, embryos were exam-
ined under the Confocal Laser Scanning Microscope
(CLSM) (Leica TCS SP5 AOBS, Germany). The intensi-
ties and distribution of the cytoskeletal components and
nuclear chromatin were recorded.

**Embryo fixation and immunofluorescent staining**

Embryos were fixed in 4% paraformaldehyde at 37°C
until further processing (for a maximum of three weeks).
Following fixation, the embryos were washed in PBS for
10 min, to reduce free aldehydes and to block non-
specific reactions. Embryos were further processed for
immunostaining, through serial incubations in dye.

Nuclear staining was performed by incubation with 4’,
6-diamino-2-phenylindole dihydrochloride (DAPI)
(Molecular Probes, Life Technologies, USA: D3571) for
30 min before being permeabilized with 1% Triton® X-
100 (Sigma, USA: X100) in Phosphate Buffer Saline
(PBS) (Sigma, USA: P4417). After 10 min incubation in
1% Triton® X-100, actin structures were labelled with
Alexa Fluor 635 Phalloidin (Molecular Probes, Life
Technologies, USA: A34054) while tubulin structures
were labelled with Monoclonal anti-α-Tubulin conjugate
with FITC (Sigma, USA: F2168) for 1 hour. The em-
byos were then washed twice with PBS for 10 min and
counterstained with DAPI for 30 min. Finally, the la-
belled samples were mounted on a glass microscope
slide in a droplet of antifade medium (Pro Long Gold
Antifading Agent) (Molecular Probes, Life Technolo-
gies, USA: P36934) to retard photobleaching. All sam-
ple were stored in the dark at 4°C prior to processing
and imaging.

Slides were viewed under the Confocal Laser Scanning
Microscope (CLSM) (Leica TCS SP5 AOBS, Germany),
and the images taken were converted to JPEG format.
Four laser sources from Diode 405nm, Argon 488nm,
HeNe 633nm and HeNe 543nm were used to simultane-
ously excite the fluorescent signals from DAPI, Alexa
Fluor 635 and anti-α-Tubulin. Thresholds settings for in-
tensity and saturation were maintained constant across all
experimental groups. The actin, tubulin and chromatin
intensities in each confocal image of EC embryos and LC
embryos were calculated using the QWin Software.

**Statistical analysis**

Statistical analysis was performed using the SPSS soft-
ware for Windows version 19.0.1 (Statistical Package for
Social Sciences, Inc., USA). Assessment of actin, tubulin
and chromatin distributions was based on the intensity of
fluorescent probes after immunofluorescence staining.
Independent T-test was performed to analyze differences
in intensities among EC and LC embryos. P value of less
than 0.05 was considered statistically significant.

**Results**

A total of 100 embryos at the 2-cell stage (50 EC em-
byos; 50 LC embryos) were stained by immunofluo-
rescence staining for cytoskeleton study. The embryos
were analyzed for distributions and fluorescence intensi-
ties of actin, tubulin and nuclear chromatin using Confo-
Confocal Laser Scanning Microscope (CLSM). The confocal images of embryos were analyzed by using QWin Software V3.

Figures 1 (A) and (B) show actin distributions in EC and LC embryos. The fluorescence images showed that actin of EC and LC embryos were mostly located at the plasma cell membrane. Generally, actin of EC embryos were concentrated at the intercellular space. Actin of LC embryos were seen to have a relatively weak cytoplasmic background. Figures 2 (A) and (B) show tubulin distributions in EC and LC embryos. Tubulin of EC as well as LC embryos were homogenously distributed in the cytoplasmic region of blastomeres. The cytoplasm of EC and LC embryos were strongly stained with anti α-tubulin (green-stain). Figures 3 (A) and (B) show nuclear chromatin distributions in EC and LC embryos. Apart from visual observations, this study also compared the integral intensity of fluorescence in confocal images. Table 1 shows the fluorescence intensities of actin, tubulin and chromatin of EC and LC embryos. Analysis by QWin Software version 3 showed that EC embryos had significantly higher actin fluorescence intensity [(14.68 ± 14.07) x 10^5 pixel] compared to LC embryos [(1.50 ± 1.20) x 10^5 pixel] (p<0.05) (Figure 4).

Figure 5 shows that the mean ± SD of tubulin fluorescence intensity in EC and LC embryos were (108.96 ± 25.30) x 10^5 pixel and (115.89 ± 32.54) x 10^5 pixel respectively (Figure 5). Tubulin intensities of EC and LC embryos were not significantly different.

Analysis of nuclear chromatin intensity showed that EC embryos had significantly higher mean nuclear intensity [(11.43 ± 3.48) x 10^5 pixel] compared to LC embryos [(7.98 ± 3.08) x 10^5 pixel] (Figure 6).

Table 1. Mean fluorescent intensities of actin, tubulin and nucleus in 2-cell EC and LC embryos (expressed as mean ± SD).

<table>
<thead>
<tr>
<th>Structure</th>
<th>No. of embryos</th>
<th>Mean of fluorescent intensity ± SD (x 10^5 pixel)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>50</td>
<td>14.68* ± 14.07 (EC embryos) 1.50 ± 1.20 (LC embryos)</td>
<td>0.000</td>
</tr>
<tr>
<td>Tubulin</td>
<td>50</td>
<td>108.96 ± 25.30 (EC embryos) 115.89 ± 32.54 (LC embryos)</td>
<td>0.290</td>
</tr>
<tr>
<td>Nucleus</td>
<td>50</td>
<td>11.43* ± 3.48 (EC embryos) 7.98 ± 3.08 (LC embryos)</td>
<td>0.000</td>
</tr>
</tbody>
</table>

p< 0.05 versus LC embryo

Figure 1. (1A) EC embryo (2-cell stage) and (1B) LC embryo (2-cell stage) stained with antibody to actin (Alexa Fluor 635-red). Actin of EC and LC embryos were mostly located at the plasma cell membrane. Generally, actin of EC embryos were concentrated at the intercellular space, while actin of LC embryos had a relative weak cytoplasmic background.
Figure 2: (2A) EC embryo (2-cell stage) and (2B) LC embryo (2-cell stage) stained with antibody to tubulin (anti α-Tubulin - green). The distributions of tubulin in EC embryos were similar to LC embryos. They were distributed homogenously in the cytoplasmic region of blastomeres. Confocal images show that the cytoplasm of the EC and LC embryos were strongly stained with anti α-tubulin (green stain).

Figure 3: (3A) EC embryo (2-cell stage) and (3B) LC embryo (2-cell stage) stained with antibody to chromatin (DAPI - blue). Nuclei of EC and LC embryos were centrally located, with no fragmentation detected.
**Figure 4.** Comparison of Actin Fluorescence Intensity in EC vs LC Embryos (Mean±SD)

**Figure 5.** Comparison of Tubulin Fluorescence Intensity in EC vs LC Embryos (Mean±SD)

**Figure 6.** Comparison of Nuclear Fluorescence Intensity in EC vs LC Embryos (Mean±SD)
Discussion

Recent studies in Assisted Reproduction Technology (ART) focused on the selection of the best quality embryo to improve the success of live birth from single embryo transfers (SETs). Many studies showed that embryos which cleaved early are of better quality compared to their later counterparts [3 - 6]. However, factors contributing to this difference are still not clear. This study examined the cytoskeletal organization and chromatin configurations of EC and LC mouse embryos at the 2-cell stage. The visualization of cytoskeletal morphology and quantification of visual data would provide greater understanding of the differences controlling development competency in EC and LC embryos.

Results from the present study showed that actin of EC and LC embryos were mostly located at the plasma membrane. This is in accordance with results from a previous study on mouse embryos, which found that actin was located beneath the cell membrane and was accentuated at the intercellular contact areas [13]. In the present study, EC embryos had their actin concentrated at the pericellular space. A study by Matsumoto et al. [14] examined the distribution of actin in non-blocked and blocked 2-cell stage rat embryos. The authors found that actin was distributed adjacent to nuclei and along the inside of the plasma membrane in non-blocked embryos at the two-cell stage. In embryos blocked at the two-cell stage, however, actin formed granules and dispersed in the cytoplasm.

The distribution of actin is related to its function in providing support and structure to the plasma membrane. Apart from that, actin is also known to functionally interact with integrin proteins in the plasma membrane. Actin-integrin protein interactions are important for maintaining cell growth, survival [7] and intracellular organelle organisation [15].

Quantitative analyses of confocal images from the present study showed that EC embryos had significantly higher actin intensities compared to LC embryos. Increased actin provides benefits to the EC embryos in maintaining cell growth and survivability until the blastocyst stage. The differences in actin abundance, which was reflected by greater intensities, could provide the explanation as to why EC embryos have higher viability compared to LC embryos [3 - 6]. In a study on equine embryos, Tremoleda et al. [16] noted that cell cleavages during early embryonic development were accompanied by complex arrangements of the cytoskeleton. Failure in the series of integrated cytoskeleton-mediated events resulted in developmental arrest. Levy et al. [17] also found that human embryos which were arrested early in their development showed actin abnormalities. Another study by Zijlstra et al. [18] found a correlation between actin quality with embryo viability. Embryos which had typical actin content and distribution underwent normal cell division. The present study suggests that higher intensities of actin in EC embryos were correlated to better actin quality, thus resulting in improved development competence over LC embryos.

Another component of the cytoskeleton which plays an important role in the development of preimplantation embryos is tubulin. Tubulin is involved in the regulation of cell shape and organization during compaction [11]. The present study found that tubulin of EC and LC embryos were distributed homogenously in the cytoplasmic region of blastomeres. In congruence, previous studies on mouse embryos also found that tubulin were distributed homogenously in the cytoplasm in non-blocked embryos at the two-cell stage [14]. However, in embryos exhibiting the two-cell block, thicker fibrous microtubules were formed and distributed as rude meshwork structures in the cytoplasm. The distribution of tubulin in the cytoplasmic region is related to their function in a variety of cellular processes such as cell motility, cell division, intracellular transport and organelle distribution maintenance [19, 20]. In the present study, tubulin intensity was higher than actin. A possible reason is that tubulin are more rigid and have larger diameters (25 nm) compared to actin (5 to 9 nm).

The main function of the nucleus is to control gene expression and mediate the replication of DNA during the cell cycle. Bavister et al. [21] stated that the ultrastructural study of embryo through fluorescence staining might provide a better understanding of the energetic and dynamic relationships between the nucleus and mitochondria. The fluorescence intensity of nucleus in confocal images is directly proportional to the amount of DNA present. The present study showed that nuclear chromatin intensity of EC embryos was significantly higher than LC embryos (p<0.05). This finding implies increased DNA replication in the nucleus of EC embryos. In tandem with this, our previous study also showed that nuclear intensity increased as the number of the cells increased [22]. Although Levy et al. [17] found that most arrested embryos displayed nuclear abnormalities such as chromatin condensation and fragmentation, results from the present study showed that nuclei morphology were normal in both EC and LC embryos, with no fragmentation observed.

Conclusion

It can be concluded that the event of first zygotic cleavage are highly dependent upon the organization of actin and tubulin. Higher densities of actin and chromatin in EC embryos suggest improved functionality of these structures, which appeared to result in more efficient cell division and therefore greater developmental competence.
**Conflict of Interests**
The authors declare that there is no conflict of interests regarding the publication of this paper.

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