Analysis of *In Vitro* Developmental Block of Rat Embryos: Assessment from the View Point of Oxygen Toxicity

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Abstract. We have already reported that the block to development in cultured 1-cell rat embryos was overcome using HECM-1, a protein-free chemically defined medium without glucose nor phosphate. In addition, we showed the beneficial effect of low oxygen tension in rat embryos. In this study, in order to clarify the reason why *in vitro* developmental block of rat embryos is overcome in HECM-1, we examined the effects of phosphate, glucose and L-cysteine on the *in vitro* development of rat embryos. Furthermore, we examined their effects on the production of H\(_2\)O\(_2\) in embryos by the fluorimetric method. Addition of KH\(_2\)PO\(_4\) even at only 1 \(\mu\)M completely inhibited the development of rat (Wistar) embryos either from the 1-cell stage or the 2-cell stage. Addition of glucose at the concentration of 1000 \(\mu\)g/ml significantly decreased the blastulation rate. The production of H\(_2\)O\(_2\) in embryos cultured under 5% O\(_2\) was significantly (P<0.01) lower than that of embryos cultured under 20% O\(_2\) in HECM-1. H\(_2\)O\(_2\) production in embryos cultured in mKRB was significantly (P<0.01) higher than that in embryos cultured in HECM-1, irrespective of the oxygen tension. Addition of neither phosphate nor glucose to HECM-1 affected the H\(_2\)O\(_2\) production at any concentration examined. L-cysteine significantly decreased the H\(_2\)O\(_2\) production in embryos in mKRB, but developmental block could not be overcome by addition of L-cysteine to mKRB. These results revealed that glucose and phosphate independently inhibited the development of rat embryos *in vitro*, and suggest that the mechanism of *in vitro* developmental block of rat embryos could not be explained simply in terms of oxygen toxicity.

Key words: Rat embryo, Developmental block, Phosphate, Glucose, L-cysteine, Hydrogen peroxide.


Rat early embryos are known to be very difficult to culture *in vitro*. Since the first experiments in rat embryo culture by Defrise [1], many investigators have endeavored to overcome this developmental block *in vitro* [2–5], but there had been no reports showing successful *in vitro* development from the 1-cell stage to the blastocyst stage until 1991, when we reported that the block to development in cultured 1-cell rat embryos was overcome for the first time using HECM-1, a protein-free defined medium without glucose nor phosphate [6]. However, the developmental rate of the embryos to the blastocyst stage was comparatively low, about 20%. Recently, Yamada
et al. [7] found that more than 70% of cultured 1-cell embryos developed to blastocyst by lowering the concentration of NaCl in HECM-1 from 98 mM to 85 mM. However, the reason why glucose and/or phosphate inhibit the development of rat embryos in vitro remains to be elucidated.

The block to development in vitro has been similarly observed in a variety of mammalian species [8-13]. Recently, there has been considerable evidence that oxygen radical-induced cellular dysfunction is one of the causes of this phenomenon [14-18]. Lowering the oxygen tension during culture has been shown to enhance embryo development in a number of species [17, 19-23]. In rat embryos, we reported that the blastulation rate under 5% oxygen was significantly higher than that under 20% oxygen [6].

In this study, in order to clarify the reason why in vitro developmental block of rat embryos is overcome in HECM-1, we examined the effects of phosphate, glucose and L-cysteine, mainly from the viewpoint of oxygen toxicity.

Materials and Methods

Animals and chemicals

Female Wistar rats aged 28 days were obtained from Oriental Bio Service (Kyoto, Japan). Sexually mature male rats were of the same strain. They were maintained in air-conditioned quarters with free access to feed and water. The lighting was controlled, with lights on between 0700 h and 2100 h every day. Pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) were purchased from Teikoku Zoki Co. (Tokyo, Japan). Hyaluronidase was from Sigma Co. (St. Louis, MO, USA). 2',7'-dichlorodihydrofluorescin diacetate (DCHFDA) was from Kodak Co. (Rochester, NY, USA). The other chemicals were of reagent grade and obtained from Nakalai Tesque Inc. (Kyoto, Japan).

Embryo collection and handling

Female rats were injected subcutaneously with 20 U of PMSG, and then 48 h later with 20 U of hCG intraperitoneally and mated with male rats. After confirmation of vaginal plug (day 1 of pregnancy), the pregnant rats were sacrificed by cervical dislocation 22 h after hCG injection and the oviducts were dissected in the medium (HECM-1 [24] or mKRB [25]). One-cell embryos were collected from the oviducts by flushing in the medium. Cumulus cells were then removed from 1-cell embryos in the medium in the presence of 0.2% hyaluronidase and they were rinsed three times in the medium. Degenerated ova were excluded from the experiments. Two-cell embryos were also collected on day 2 (48 h after hCG) using the same method described above.

In each experiment, all the embryos from 3 to 4 females were pooled in the medium, and were introduced into each experimental droplet of medium at random. The standard culture medium was HECM-1 or mKRB solution supplemented with 0.1% polyvinyl alcohol (PVA), and the standard culture conditions were 37 C under 5% CO2 in air.

Measurement of hydrogen peroxide within embryos

To quantify the level of H2O2 in embryos, we used the fluorimetric method described by Nasr-Esfahani et al. [15] and Goto et al. [26]. It has been known that 2',7'-dichlorodihydrofluorescein diacetate (DCHFDA), membrane permeant, is hydrolyzed by intracellular esterase activity forming 2',7'-dichlorodihydrofluorescein (DCHF) and trapped within the cell. DCHF itself does not fluoresce, but when it is oxidised by H2O2 or lipid peroxides into 2',7'-dichlorofluorescein (DCF), the dye fluoresces with λex: 460 nm and λem: 535 nm. Bass et al. [27, 28] have already shown that, in polymorphonuclear leukocytes (PMNL) pre-incubated with DCHFDA, a stimulation of the respiratory burst caused an oxidation of the cytosolic DCHF and that the resultant fluorescence of the product, DCF, appeared linearly related to the activity of the respiratory burst, and appeared to be mediated by H2O2 produced by stimulated PMNL. Moreover, Nasr-Esfahani et al. [15] demonstrated that the fluorescence emission from individual embryos was related to the rate of oxidation of DCHF by H2O2 produced within the embryos.

Stock solution of DCHFDA was prepared in acetone at 1 mM just before the start of each experiment and was kept in the dark. The stock solution was diluted in HECM-1 +0.1% PVA to the experimental concentration (10 µM). Droplets (100 µl) of medium with the dye were made in a 4-well multiplate (Nunc Co., Roskilde, Denmark) and covered with mineral oil. Freshly recovered embryos were placed into the droplet and loaded.
with the dye and cultured for a specified time. The embryos were then washed in HECM-1 + 0.1% PVA to remove the traces of the dye and were placed individually in a small droplet of HECM-1 + 0.1% PVA which had been made in a 4-well multiplate (Nunc Co.). The fluorescence emissions of the embryos were measured immediately by using a microscope with stabilized xenon lamp, filter set, photomultiplier and photometer (IMT-2, OSP-MBI, OSP-PMU, OXP-CBI; Olympus Optical Co., Tokyo, Japan). The individual embryo was exposed to the excitation wavelength and the fluorescent emission was recorded. As the measurement system contained a variable measuring window, the background value was excluded.

To decide adequate concentration of DCHFDA and incubation time of embryos with DCHFDA, we performed two series of experiments in advance. According to the results (data not shown), we set the concentration of DCHFDA at 10 μM and the incubation time at 30 min.

Experiment 1: Effect of phosphate on the development of rat embryos in vitro

To examine the effect of phosphate on the development of rat embryo in vitro, we cultured 1-cell and 2-cell embryos in HECM-1 + 0.1% PVA supplemented with various concentrations of KH₂PO₄ (0, 1, 10, 100 μM). The development of embryos was observed under a phase-contrast microscope (IMT-2; Olympus Optical Co.) every 24 h until day 6. The experiments were replicated 4 times.

Experiment 2: Effect of glucose on the development of rat embryos in vitro

To examine the effect of glucose on the development of rat embryo in vitro, we cultured 1-cell embryos in HECM-1 + 0.1% PVA supplemented with various concentrations of glucose (0, 1, 10, 100, 1000 μg/ml). The development of embryos was observed as described above. The experiments were replicated 4 times.

Experiment 3: Effect of L-cysteine on the development of rat embryos in vitro

To examine the effect of L-cysteine on the development of rat embryos in vitro, we cultured 1-cell embryos in mKRB + 0.1% PVA supplemented with various concentrations of L-cysteine (0, 1, 10, 100 μM). The development of embryos was observed as described above. The experiments were replicated three times.

Experiment 4: Effect of culture medium on the production of H₂O₂ in embryos

One-cell embryos were loaded with DCHFDA in either of the following two media: HECM-1 + 0.1% PVA and mKRB + 0.1% PVA. They were cultured under 5% or 20% O₂ (37 C, 5% CO₂) for 30 min. After the cultures, the embryos were washed and placed in other droplets of the medium (HECM-1 or mKRB), and the fluorescence emissions were measured. Eleven embryos were used for each group of the experiment.

Experiment 5: Effect of phosphate on the production of H₂O₂ in embryos

One-cell embryos were placed in HECM-1 + 0.1% PVA + DCHFDA (10 μM) to which KH₂PO₄ was added at various concentrations (0, 1, 10, 100 μM), and cultured under 37 C, 5% CO₂ in air for 30 min. After the culture, the embryos were washed and placed in other droplets of HECM-1 + 0.1% PVA, and the fluorescence emissions were measured. Eleven embryos were used for each group of the experiment.

Experiment 6: Effect of glucose on the production of H₂O₂ in embryos

One-cell embryos were placed in HECM-1 + 0.1% PVA + DCHFDA (10 μM) to which glucose was added at various concentrations (0, 100, 1000 μg/ml), and cultured under 37 C, 5% CO₂ in air for 30 min. After the culture, the embryos were washed and placed in other droplets of HECM-1 + 0.1% PVA, and the fluorescence emissions were measured. Eleven embryos were used for each group of the experiment.

Experiment 7: Effect of L-cysteine on the production of H₂O₂ in embryos

One-cell embryos were placed in mKRB + 0.1% PVA + DCHFDA (10 μM) to which L-cysteine was added at the concentration of 100 μM, and cultured under 37 C, 5% CO₂ in air for 30 min. After the culture, the embryos were washed and placed in other droplets of mKRB + 0.1% PVA (without the chemicals), and the fluorescence emissions were measured. Twelve embryos were used for each group of the experiment.
Statistical analysis

The data of experiments were statistically analyzed using chi-square test (Experiments 1, 2 and 3) and Student’s t-test (Experiments 4, 5, 6 and 7). Analyzed data were of simultaneously performed experiments.

Results

Embryo development

As shown in Table 1, addition of KH₂PO₄ even at only 1 μM completely inhibited the development of embryos either from the 1-cell stage or the 2-cell stage. In contrast, addition of glucose to HECM-1 did not decrease the blastulation rate when the concentration of glucose was 100 mg/ml or less. Addition of glucose at the concentration of 1000 mg/ml significantly decreased the blastulation rate (Table 2). Developmental block of rat embryos could not be overcome by addition of L-cysteine (Table 3).

Production of H₂O₂

The fluorescence emissions of the embryos cultured under 5% O₂ were significantly (P<0.01) lower than those cultured under 20% O₂ in HECM-1 (Table 4). The fluorescence emissions of the embryos cultured in mKRB were significantly (P<0.01) higher than those cultured in HECM-1, irrespective of the oxygen tension (Table 4). Addition of phosphate to HECM-1 did not affect the fluorescence emissions of the embryos at any concentration examined (Table 5). Addition of glucose did not affect the fluorescence emissions (Table 6). L-cysteine significantly decreased the fluorescence emissions of the embryos in mKRB (Table 7).

Discussion

Rat embryos are known to show a complete developmental block at the 2-cell or 4-cell stage.

Table 1. Effect of phosphate on the development of rat embryos in vitro

<table>
<thead>
<tr>
<th>Concentrations (μM)</th>
<th>No. (%)) of embryos developed to blastocysts</th>
<th>from 1-cell</th>
<th>from 2-cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12/64(18.8)</td>
<td>48/65(73.8)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0/68*</td>
<td>0/64*</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0/65*</td>
<td>0/66*</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0/65*</td>
<td>0/61*</td>
<td></td>
</tr>
</tbody>
</table>

Culture medium was HECM-1. Fifteen to 17 embryos were used per experiment. Numbers are total of 4 experiments.

Table 2. Effect of glucose on the development of rat embryos in vitro

<table>
<thead>
<tr>
<th>Concentrations (μM)</th>
<th>No. (%) of embryos developed to blastocysts</th>
<th>from 1-cell</th>
<th>from 2-cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12/64 (18.8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>14/66 (21.2)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>10/66 (15.1)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>12/66 (18.2)*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>2/66 (3.0)*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Culture medium was HECM-1. Fifteen to 17 1-cell embryos were used per experiment. Numbers are total of 4 experiments.

Table 3. Effect of L-cysteine on the development of rat embryos in vitro

<table>
<thead>
<tr>
<th>Concentrations (μM)</th>
<th>No. of embryos developed to blastocysts</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0/45</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0/46</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0/47</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0/45</td>
<td></td>
</tr>
</tbody>
</table>

Culture medium was mKRB. Thirteen to 16 1-cell embryos were used per experiment. Numbers are total of 3 experiments.

Table 4. Effect of culture medium on the production of H₂O₂ in embryos

<table>
<thead>
<tr>
<th>Medium</th>
<th>Fluorescence emissions a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5% O₂</td>
</tr>
<tr>
<td>mKRB</td>
<td>4.537 ± 0.262*</td>
</tr>
<tr>
<td>HECM-1</td>
<td>0.664 ± 0.042*</td>
</tr>
</tbody>
</table>

Values are mean ± s.e. for 11 1-cell embryos.

* Relative values for the value of HECM-1, 20% O₂.

a P<0.01, significantly different from the value of HECM-1 of each O₂ tension.
when they are cultured in mKRB, which is usually used for rat in vitro fertilization [25]. In the previous report, we overcame this developmental block of rat embryos by culturing in HECM-1 [6]. One major difference between mKRB and HECM-1 is that HECM-1 is free of glucose and phosphate. In Experiment 1 of the present study, addition of phosphate completely inhibited the in vitro development of rat embryos. In Experiment 2, addition of glucose at the concentration of 1000 µg/ml inhibited the development of rat embryos. These results suggest that glucose and phosphate may independently inhibit the in vitro development of rat embryos. These results are fundamentally (except for some differences) consistent with the report by Yamada et al. [7], who showed that, in the absence of glucose, decreased percentage, about 30%, of 1-cell rat embryos developed to blastocysts by the addition of 10 to 500 µM of KH₂PO₄ and that, in the presence of 5.5 mM (≈1000 µg/ml) glucose, addition of KH₂PO₄ even at only 0.5 µM completely inhibited the development. Similarly in hamster embryos, Bavister and his colleagues [24, 29] reported that glucose inhibited the development of embryos in the presence of phosphate. However, the reason why and how glucose and/or phosphate exert the detrimental effect on the embryo development has not been fully elucidated.

A growing knowledge of the “darker side” of oxygen tells us that oxygen is both necessary for aerobic life and toxic to all life forms. The detrimental effects of oxygen are attributed to the formation of intermediates of oxygen reduction i.e., superoxide (O₂⁻), hydrogen peroxide (H₂O₂) and hydroxyl radical (·OH) [30]. Recently, the in vitro block phenomenon, which is observed in a variety of mammalian species, has been explained in terms of oxygen toxicity. Noda et al. [14, 16] demonstrated that the culture of mouse pronuclear embryos in the presence of superoxide dismutase (SOD), a scavenger of superoxide radicals, significantly increased the rate of development to the blastocyst stage. Further evidence was provided by Nasr-Esfahani et al. [15], who demonstrated that intracellular H₂O₂ levels are higher in 2-cell mouse embryos cultured in vitro than in vivo counterparts. H₂O₂ is one of the reactive oxygen species and the level of H₂O₂ may reflect the level of enhancement of the oxygen activation system. In this context, we measured the fluorescence emissions of the embryos cultured in mKRB and HECM-1 in Experiment 4 of the present study, and found that generation of H₂O₂ was significantly higher in the embryos cultured in mKRB than in those cultured in HECM-1. In Experiment 5, we examined whether addition of phosphate might affect the production of H₂O₂ in embryos. The result was that addition of phosphate to HECM-1 did not affect the fluorescence emissions of the embryos. Similarly, addition of glucose did not affect the oxygen activation system in embryos in Experiment 6. These results suggest that the inhibitory effects of phosphate and/or glucose on the in vitro development of rat embryos may not be explained simply by oxygen radical-induced toxicity. However, it is known that glucose and phosphate are abundantly present in the oviduct [31-33], and they cannot exert the inhibitory effect on the development of embryos in vivo. Therefore, the
toxic effect by glucose and/or phosphate must be a phenomenon observed only in vitro. It is known that, in the presence of phosphate anion, Fe^{2+} readily autoxidizes producing •OH, the highly reactive species that can attack and destroy almost all known biomolecules [34]. Furthermore, we have already confirmed that Fe^{2+} contaminated in almost all chemicals which were used for preparing culture media [35]. The interaction of phosphate and iron, i.e. the toxic effect of •OH might be a clue for solving this problem.

Another major difference between HECM-1 and mKRB is that HECM-1 contains 20 kinds of amino acids. Among these amino acids the effect of L-cysteine needs to be discussed, because it is known that L-cysteine promotes the in vitro development of mouse embryos [36]. Furthermore, L-cysteine is known to have radical scavenging activity [37]. In fact, L-cysteine significantly decreased the production of H_{2}O_{2} in embryos in the Experiment 7 of the present study. Lower production of H_{2}O_{2} in rat embryos in HECM-1 than those in mKRB, which was observed in Experiment 4, may be due to this radical scavenging activity of L-cysteine. However, as observed in Experiment 3, the more addition of L-cysteine was not sufficient to overcome the developmental block in mKRB.

In conclusion, we demonstrated that addition of glucose and phosphate independently inhibited the development of rat embryos in HECM-1. However, the reason why in vitro developmental block of rat embryos is overcome in HECM-1 could not be explained simply in terms of oxygen toxicity. Oxygen radical-induced cellular dysfunction may be but one of a number of factors which lead to the in vitro developmental block. Another factor contributing to the block may be metabolic regulation, including that of glucose, since there have been several reports linking metabolic regulation and the developmental block [24, 38-42]. Glucose metabolism potentiates the developmental block induced by hypoxanthine [38], and the replacement of glucose with fructose overcame the in vitro block in embryos from random-bred Swiss mice [40]. However, it has been shown that glucose metabolism was altered by low oxygen tension [39]. The reason why glucose and/or phosphate, which are present in the oviduct [31-33], exert the toxic effect only in vitro cannot be elucidated without further study on the effects of oxygen radicals on metabolic regulation in embryos.

References

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