Impact of erythrocytes on mouse embryonal development in vitro

Itsuko Matsuoka[a], Yuji Fujino[a,*], Sachio Ogita[a], Masayasu Inoue[b]

[a]*Departments of Obstetrics and Gynecology, Osaka City University Medical School, 1-5-7 Asahi-machi, Abeno, Osaka 545, Japan*
[b]*Department of Biochemistry, Osaka City University Medical School, 1-5-7 Asahi-machi, Abeno, Osaka 545, Japan*

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Abstract To elucidate the role of reactive oxygen species (ROS) in the development of mouse embryo, effects of superoxide dismutase (SOD), catalase and erythrocytes were studied in vitro. Oocytes were fertilized and 2-cell-cleaved embryos were cultured in the presence or absence of either erythrocytes, SOD or catalase. Under standard culture conditions, the fertilization and cleavage rates were 77.4 and 12.5%, respectively. In the presence of xanthine and xanthine oxidase, those rates decreased to 28.2 and 4.5%, respectively. The hazardous effect of ROS was completely inhibited by erythrocytes. These results suggested that small amounts of erythrocytes might effectively degrade ROS during the development of cultured embryos.

Key words: Superoxide; SOD; Catalase; Embryonal development; Erythrocyte

1. Introduction

Although methods of culture for mammalian embryos from various species have been established for a long time, the process of cell cleavage is often impaired in vitro. The arrest of cell cleavage usually occurs with mouse embryos at their 2-cell stage, which is called as '2-cell block'. The 2-cell block can be inhibited by changing the culture medium [1] or by adding ethylenediaminetetraacetic acid (EDTA) [2]. Since superoxide dismutase (SOD) also inhibits the arrest of embryonal cleavage, oxygen stress has been postulated to underlie the pathogenesis of 2-cell block [3]. Nasr-Esfahani and co-workers [4] reported that reactive oxygen species, formed in the culture medium might be responsible for the occurrence of 2-cell block of mouse oocytes. However, the molecular mechanism and reactive oxygen species involved in 2-cell block remain to be elucidated.

Protection of tissues from oxidative stress is one of the major prerequisite for aerobic life Recently, oxygen toxicity to spermatozoa has been recognized. The fact that pre-implanted embryos are sensitive to high oxygen concentrations suggested that oxygen toxicity might result in developmental arrest of embryos [5–7]. High concentrations of oxygen might affect the balance between synthesis and degradation of glycogen. The superoxide radical and/or its hazardous metabolites have been known to impair cell membranes, DNA and various enzymes.

Hence, living cells must effectively detoxify superoxide radicals. SOD localizes ubiquitously in tissues of most species and catalyzes the dismutation of superoxide radicals [8], and plays important roles in biological defense mechanisms [9]. In fact, favorable effects of SOD on reproductive medicine have been reported. Recently, Noda and co-workers demonstrated the presence of SOD in oviductal fluid and epithelium [10]. Umaoka et al. [3] suggested that oviductal fluid contained low concentrations of oxygen and high concentrations of SOD thus protecting the developing embryos from local oxidative stress. Furthermore, Nonogaki and co-workers reported that SOD added to the culture medium protected embryos from oxidative stress and promoted their development in vitro [11].

Erythrocytes have high levels of SOD and catalase. Predominantly, because of amphipathic nature of H_2O_2, this reactive species occurring extracellularly is readily transferred across membrane/lipid bilayers and degraded intracellular by various cells, such as erythrocytes [12]. To elucidate the pathogenesis of 2-cell block, effects of SOD, catalase and erythrocytes on fertilized oocytes and embryos were studied in vitro.

2. Materials and methods

Female and male ICR mice (7–8 weeks old) were purchased from Clea Breeding (Tokyo, Japan) Female mice were intraperitoneally injected with 25 IU of human menopausal gonadotropin (hMG: Humegon; Japan Organon, Tokyo, Japan). After 48 h, 10 IU of human chorionic gonadotropin (hCG: hCG-Mochida; Mochid, Tokyo, Japan) was injected intraperitoneally. 14 h after hCG injection, ovaries and uterine horns were rapidly removed through a laparotomy and placed in a recovery dish containing Ham's F-10 (GIBCO; Life Technologies, New York, NY) supplemented with 10 mM HEPES. To release oocytes, the fallopian tubes were separated and punctured under light microscope. The oocytes were transferred into organ culture dishes filled with 1 ml of Ham's F-10 medium. Each culture dish contained 15–20 oocytes

Spermatozoa were released from male mice by puncturing the caudal epididymidis with a 27-gauge needle. After incubation for 60–90 min at 37°C under 5% CO_2 in air, 50 70-μl aliquots of the sperm suspension were added to the culture dishes containing oocytes to give a final concentration of $5–10 \times 10^5$ spermatozoa/ml.

In a separate experiment, female mice treated with 25 IU of hMG and 10 IU of hCG were mated with male mice just after hCG injection 44 h after hCG injection to female mice, 2-cell-cleaved embryos were collected from the fallopian tubes and transferred to the culture dishes (15–20 2-cell-cleaved embryos/dish)

Ham's F-10 medium was prepared with bi-distilled water supplemented with 25 mg/l calcium lactate and 211 mg/l sodium bicarbonate; its osmolarity was adjusted to 280 mOsm/kg Prior to use, the medium was placed in a CO_2 incubator (5% CO_2 95% air, pH 7 35–7.45) at 37°C for 24 h

Blood was collected from the caval vein into a heparinized syringe. Erythrocytes were isolated by centrifugation at 3000 rpm for 10 min and removed plasma, white blood cells and platelets. After washing 3 × with 10 volumes of ice-cold phosphate-buffered saline (PBS) by centrifugation at 3000 rpm for 10 min, erythrocytes were resuspended in Ham's F10 and incubated with oocytes and embryos at 0.1 or 0.5% hematocrit. Under light microscopy, no detectable contamination of white blood cells and platelets were found in erythrocytes samples used for the experiments

To investigate the effect of reactive oxygen species on mouse oocytes and embryos, the rates of fertilization and cleavage were determined in the presence or absence of 1 mM hypoxanthine and 1 mU/ml of xanthine oxidase (EC 1.2.3.2; Wako Pure Chemical, Osaka, Japan). The effects of erythrocytes, 50 U/ml SOD (EC 1.15.1.1, Wako Pure Chemical) and 50 U/ml catalase (EC 1.11.1.6; Wako Pure Chemical) on the

*Corresponding author. Fax: (81) (6) 646-5800

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rates of fertilization and cleavage were also determined. Furthermore, catalase was inactivated by incubation with 0.1 M 3-amino-1,2,4-triazole (Wako Pure Chemical) and 100 mM H_2O_2 (Mitsubishi Chemical, Tokyo, Japan) at 37°C for 2 h. SOD activity was inactivated in the water bath at 100°C for 3 min. All reagents used were of analytical grade.

Statistical analysis was performed by the χ^2 test. $P < 0.05$ was considered statistically significant. In the calculation of the percentage of fertilization and cleavage, over 100 oocytes and embryos were analysed. Generally, all experiments were performed 5× with reproducible results.

3. Results

Under standard culture conditions, the rates of fertilization and cleavage were 77.4 and 12.5%, respectively (Table 1). In Ham's F10, the fertilization rate remained unchanged after adding 0.5% erythrocytes (71.4%). However, the cleavage rate was increased significantly (42.4%) by adding erythrocytes.

To know the effect of reactive oxygen species on the fertilization of oocytes, fertilization rate was determined in the presence of xanthine and xanthine oxidase. Xanthine oxidase markedly decreased the rates of fertilization and cleavage to 28.2 and 4.5%, respectively. As shown in Table 1, erythrocytes also increased the rates of fertilization and cleavage in a concentration-dependent manner. The inhibitory effects of the oxidase were completely blocked by adding erythrocytes to Ham's F10 medium. Table 2 shows the effect of erythrocytes on the cleavage of embryonal cells cultured under oxygen stress. In the presence of xanthine and xanthine oxidase, >90% of the embryos remained at their 2-cell stage and, hence, their development was fairly low (8.3%). In the presence of 0.5% erythrocytes, however, the rate of development increased to a level almost equal to that observed with standard culture conditions (57.1%).

4. Discussion

The protective effect of erythrocytes against oxygen toxicity has been discussed with other cells and tissues subjected to oxidative stress [13]. The present study demonstrates that a fairly small amount of erythrocytes effectively protected the cultured oocytes and embryos from oxidative stress and increased the rate of cleavage of the fertilized oocytes. To investigate which of the reactive species responsible for the inhibition, oocytes were cultured in the presence of SOD and/or catalase. Both catalase and SOD partially inhibited the hazardous effect of xanthine oxidase; the protective effect was more marked with catalase than with SOD. In the presence of both SOD and catalase, fertilization and cleavage occurred normally. Inactivated SOD and catalase had no appreciable effect on the cleavage and fertilization. These results suggested that both O_2^- and H_2O_2 might play important roles in the oxidase-induced inhibition of the two processes.

It has been known that the presence of both O_2^- and H_2O_2 generates highly toxic OH particularly in the presence of trace metals. Thus, the protective effect of SOD might reflect the inhibition of ·OH generation from O_2^-.

The toxicity of H_2O_2 was effectively inhibited either by extracellular catalase or erythrocytes. It should be noted that, unlike anionic O_2^-, H_2O_2 is amphipathic in nature and, hence, extracellular H_2O_2 readily enters into erythrocytes which are highly enriched with catalase and glutathione peroxidase [14,15]. Thus, enzymes in erythrocyte might effectively degrade extracellular H_2O_2. Such a metabolic sink mechanism may also function in protecting embryos from oxidative stress [16].

Since intratubal space and endometrial tissue are readily accessible to the circulating erythrocytes, they might also protect oocytes and embryos from the hazardous effects of reactive oxygen species and promote fertilization and cleavage of oocytes and development of embryos.

These results indicate that erythrocytes abolish the hazardous effects of oxygen stress on cultured mouse embryos and that addition of homologous erythrocytes into culture medium might have clinical potential for promoting fertilization of oocytes and development of embryos in culture.

Table 1
Effect of erythrocytes and enzymes on the rates of fertilization and cleavage of mouse oocytes in vitro

Culture condition	(n)	Fertilization rate (%)	Cleavage rate (%)
Control	(5)	77.4	12.5
+RBC (0.5%)	(5)	71.4	42.4
+XO	(5)	28.2	4.5
+XO + SOD	(5)	47.1	11.4
+XO + Cat	(5)	60 0	17.9
+XO + Cat + SOD	(5)	80.0**	31.3*
+inactivated Cat and SOD	(3)	55.0	12.1
+XO + RBC (0.1%)	(3)	37.8	11.8
+XO + RBC (0.5%)	(5)	72.2**	50.8*

$$\text{Fertilization rate (\%)} = \frac{\text{Number of fertilized oocytes}}{\text{Number of investigated oocytes}} \times 100.$$

$$\text{Cleavage rate (\%)} = \frac{\text{Number of cleaved oocytes}}{\text{Number of fertilized oocytes}} \times 100.$$

*vs control value ($P < 0.05$), **vs. +XO value ($P < 0.05$). The parentheses (n) shows the number of experiments performed. XO, 1 mM hypoxanthine and 0.001 U/ml xanthine oxidase; SOD:Cu/Zn-SOD, 50 U/ml; Cat: catalase, 50 U/ml; RBC, 0.1 or 0.5% in hematocrit.

Table 2
Effects of homologous erythrocytes on the development of mouse embryos cultured in presence of hypoxanthine and xanthine oxidase

Culture condition	(n)	Development rate (%)
Control	(5)	58.3
+XO	(5)	8.3*
+XO + RBC	(5)	57.1

$$\text{Development rate (\%)} = \frac{\text{Number of over 3-cell-cleaved embryos}}{\text{Number of 2-cell-cleaved embryos}} \times 100$$

*vs. control value ($P < 0.05$). The parentheses (n) shows the number of experiments performed. XO, 1 mM hypoxanthine and 0.001 U/ml xanthine oxidase Ham's F10 medium. The amount of RBC used was 0.5% in hematocrit.

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