Effects of Heat Stress on the Redox Status in the Oviduct and Early Embryonic Development in Mice

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Abstract. This study examined the association between redox status in the oviduct and early embryonic death in heat-stressed mice. In Experiment 1, non-pregnant mice were heat-stressed at 35°C with 60% relative humidity for 12, 24, or 36 h, and the maternal redox status was verified by measuring the levels of reactive oxygen species (ROS) and free radical scavenging activity (FRSA) in the oviduct, and thiobarbituric acid reactive substances (TBARS) and glutathione peroxidase (GSH-Px) activity in the liver. In Experiment 2, zygotes were collected from mice heat-stressed for 12 h on the day of pregnancy, and their developmental abilities were assessed in vitro, along with the intensity of DNA damage at the 2-cell stage. The TBARS value and GSH-Px activity in the liver, and ROS level in the oviduct were significantly higher in heat-stressed mice, and this increase appeared to depend on the duration of the heat stress. Maternal heat stress significantly reduced the percentage of zygotes that developed to the morula and blastocyst and the total cell number in the blastocyst. In addition, DNA damage at the 2-cell stage was significantly higher in maternally heat-stressed embryos. These results suggest that heat stress induces systemic changes in redox status in the maternal body, and the resultant increase in oxidative stress in the oviduct is possibly involved in heat stress-induced early embryonic death.

Key words: Early embryo death, Heat stress, Oxidative stress, Reactive oxygen species

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through the interaction of the embryo with its maternal environment [5]. Elevated body temperature [11] and high metabolic rate [12, 13] are known to increase the production of reactive oxygen species (ROS), which react with various cellular molecules, such as lipids, proteins, and nucleic acids, and result in cellular injury [14]. Ando et al. [15] have reported that heat stress over 24 h increased the lipid peroxidation levels and glutathione peroxidase (GSH-Px) activity in the livers of rats and guinea pigs. Recently, we observed increased intracellular oxidative stress in maternally heat-stressed embryos, as shown by the increased H$_2$O$_2$ and decreased glutathione (GSH) concentrations within embryos [5, 16], and increased ROS production in the heat-stressed oviduct [17]. Pre-implantation embryos are vulnerable to oxidative stress; exposure of in vitro cultured hamster or bovine embryos to oxidative stress increases DNA damage [18, 19] and arrests the embryo development [20, 21]. These previous findings lead us to speculate that maternal heat stress shifts the redox status of the maternal body toward oxidation, and the resultant oxidative stress damages the normal embryonic development. However, it still remains unclear if maternal heat stress causally increases ROS production in the oviduct. Therefore, the present study was conducted to determine whether ROS production in the oviduct can be changed by maternal heat stress and whether it is associated with hyperthermia-induced early embryonic death in mice.

**Materials and Methods**

**Materials**

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified. Equine chorionic gonadotropin (eCG; Serotropin) and human chorionic gonadotropin (hCG; Gonatropin) were purchased from Teikokuzouki Pharmaceutical Co. (Tokyo, Japan).

**Animals**

Female ICR strain (8 to 12 weeks old) and male BDF$_1$ (9 to 24 weeks old) mice were purchased from Charles River Japan (Yokohama, Japan). All of the mice were raised under controlled conditions of 25 C and 50% relative humidity (RH) with a 12-h light: 12-h dark photoperiodic cycle (light on at 0600 h) throughout the experiments.

Female mice were superovulated by an intraperitoneal (i.p.) injection of 5 IU eCG, followed 48 h later by an i.p. injection of 5 IU hCG. The mice were housed with BDF$_1$ males during the dark period. Mating was verified by the presence of vaginal plugs at 0600 h the next morning (designated as day 1 of pregnancy).

All of the experimental protocols and animal handling procedures were reviewed and approved by the Animal Care and Use Committee of the University of Tsukuba, Japan.

**Heat stress treatment**

Mice in the heat stress groups were exposed to a high ambient temperature of 35 C with 60% RH. The rectal temperature of each mouse was measured with a thermistor instrument (D611; Takara Thermistor Co., Tokyo) in the environmental chamber immediately before the end of the heat stress treatment.

**Experimental design**

In Experiment 1, forty non-pregnant females were allotted randomly to the non-stressed (Control) group or to the 12-h, 24-h, or 36-h heat-stressed groups (10 animals to each group). Each heat stress treatment was scheduled to finish at 1800 h. After the heat stress, mice were sacrificed by decapitation, and the oviducts were removed and flash-frozen in liquid nitrogen. The whole left oviduct was used for ROS level assay, and the right oviduct was used for the free radical scavenging activity (FRSA) assay. The liver tissue was also collected and subjected to determinations of GSH-Px activity and lipid peroxidation levels in order to confirm the increase in oxidative stress induced by heat stress. The lipid peroxidation levels were expressed as the thiobarbituric acid reactive substances (TBARS).

In Experiment 2, superovulated and mated females were heat-stressed for 12 h (from 0600 to 1800 h) on day 1 of pregnancy, or non-stressed (Control). Then, zygotes were collected to assess the developmental ability in vitro (7 animals from each group) and DNA damages at the 2-cell stage by the comet assay (5 animals from each group).

**Assays for ROS level and FRSA**

The oviduct homogenates were prepared
according to the methods of Ozawa et al. [17]. ROS level in the oviduct was assayed according to the method described by Bejma and Ji [22], with some modifications. Briefly, 100 µl of supernatant, 98 µl of assay buffer, and 2 µl of 5 mM 2',7'-dichlorodihydro-fluorescein diacetate (DCFH-DA) in DMSO were added to each well, and incubated at 37°C for 15 min. The oxidation of DCFH-DA was observed for 30 min using a fluorescence microplate reader (Fluoroskan Ascent FL; Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) that was equipped with a filter for the excitation wavelength of 485 nm and emission wavelength of 538 nm. The ROS level was expressed as the average value of relative fluorescence intensity for 30 min, or fluorescence intensity unit (FIU). The FRSA in the oviduct was measured as described by Kojima et al. [23] and Ozawa et al. [17]. The FRSA was expressed as the scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Wako Pure Chemical Industries, Ltd., Osaka, Japan), which is a chemically stable radical with peak absorbance at 517 nm. The absorbance at 517 nm of the mixture of 400 µl of supernatant, 200 µl of assay buffer, and 400 µl of 200 µM DPPH in absolute ethanol was recorded at 1-min intervals for 20 min. The data was expressed as the decrease in absorbance per 1-mg-protein per 1-min. The ratio of ROS level to FRSA was used as an indicator of the redox status in the oviduct as described in our previous study [17].

Assays for GSH-Px activity and the TBARS value

The GSH-Px activity was measured using a commercial kit (GPx-340; Oxis International, Inc., Portland, OR), according to the manufacturer's instructions. The activity of GSH-Px was determined by measuring β-nicotinamide adenine dinucleotide phosphate (NADPH) oxidation as a decrease in absorbance at 340 nm. The TBARS value was measured according to procedures described by Ohkawa et al. [24]. The compound 1,1,3,3-tetramethoxypropane was used as the standard, and the TBARS values are expressed as, moles of malondialdehyde (MDA).

Zygote collection and assessment of the developmental ability

Zygotes were collected by flushing the oviduct with potassium simplex optimised medium (KSOM) [25] at 1800 h on day 1 of pregnancy. The collected zygotes were cultured as a litter in a 50-µl KSOM drop under mineral oil at 37°C in 5% CO2 in humid air for 84 h, to assess their developmental ability. The blastocyst-stage embryos were fixed with acetic alcohol (1:3) and stained with aceto-orcein on a glass slide glass with a coverslip. The blastocysts derived from each treatment were pooled and the stained nuclei in the blastocysts were counted to arrive at the total cell number. Another set of zygotes was cultured for 18 h, and 2-cell stage embryos were subjected to analysis of DNA damage, as described below.

Detection of DNA damage in 2-cell embryos using the comet assay

DNA damage in individual embryos was detected using the method described by Takahashi et al. [18, 19]. Briefly, the embryos were placed with 0.5% (w/v) low melting-point agarose in PBS on a glass slide that was pre-coated with 0.65% (w/v) normal melting-point agarose in PBS. The slides were immersed for 2 h at room temperature in lysing solution (pH 10.0) that contained 10 mM Tris, 250 mM NaCl, 100 mM Na2-EDTA, 1% (w/v) sodium lauryl sarcosine, 1% (v/v) Triton X-100, and 10% (v/v) DMSO. Then, the slides were placed and equilibrated in electrophoresis buffer (1 mM Na2-EDTA, 300 mM NaOH) for 20 min. Electrophoresis was conducted for 40 min at 300 mA and 25–26 V. After electrophoresis, the slides were soaked in 0.4 M Tris-HCl (pH 7.5) for 45 min. DNA was detected by adding SYBR Green I (Takara Bio. Inc., Otsu, Japan), that was diluted 1:10,000 in TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM Na2-EDTA), to the gel. The slides were then observed under a fluorescence microscope (Leica Microsystems, Tokyo, Japan) that was equipped with a 490-nm excitation filter. DNA damage was quantified by measuring the length of the DNA comet tail between the edge of the nucleus and the end of the visible comet tail. The length was calculated by referring to a photograph of a micrometer at the same magnification as the embryos. The average comet length (10 to 15 embryos from each mouse) was calculated, and compared among the treatment groups.

Statistical analysis

The data are expressed as the mean ± SEM. In Experiment 1, the data were analyzed by one-way analysis of variance (ANOVA) and the Duncan’s multiple range test was used to compare among the treatment groups.
ANOVA followed by Fisher’s protected least-significance test. In Experiment 2, the differences between the means were analyzed using Student’s t-test. The percentages of embryos that cleaved to the 2-cell stage or that developed to the morula or blastocyst stage were arcsine transformed and then analyzed using the Student’s t-test.

Results

Rectal temperatures of heat-stressed mice

In Experiment 1, the mice in the heat-stressed group showed significantly higher rectal temperatures (39.4 ± 0.3 C for 12-h heat stress, \( P < 0.001 \); 39.2 ± 0.1 C for 24-h heat stress, \( P < 0.001 \); 38.7 ± 0.8 C for 36-h heat stress, \( P < 0.05 \)) than the mice in the control group (37.8 ± 0.2 C). In Experiment 2, the rectal temperatures of the heat-stressed mice (39.4 ± 0.1 C) had increased significantly \( (P < 0.001) \), as compared to the control group (37.7 ± 0.1 C).

Effects of heat stress on the redox status of the oviduct (Experiment 1.)

As shown in Fig. 1A, heat stress resulted in a linear increase in the ROS level in the oviduct, which was dependent on the duration of the stress, and the differences between the control and heat-stressed groups (24 h and 36 h) were significant \( (P < 0.05) \). On the other hand, heat stress did not affect FRSA in the oviduct (Fig. 1B). As a consequence, the redox status in the oviduct, as indicated by the ratio of ROS level to FRSA, increased with the heat stress in parallel with the ROS level (Fig. 1C).

The GSH-\( \text{Px} \) activity and the TBARS values in the liver of heat-treated mice are shown in Table 1. GSH-\( \text{Px} \) activity tended to increase in a manner that was dependent on the duration of the heat stress, and the difference between the control and each heat-stressed group was statistically significant \( (P < 0.05) \). Similarly, the TBARS values increased significantly in each heat-stressed group, as compared to the control group \( (P < 0.05) \), although there were no significant differences among the heat-stressed groups.

Effects of maternal heat stress on developmental ability of zygotes (Experiment 2.)

The data on the developmental abilities of the heat-stressed embryos are shown in Table 2. The percentage of embryos that cleaved to the 2-cell stage did not differ significantly between the treatments. However, the percentage of embryos that developed to the morula or blastocyst stage decreased significantly in the heat-stressed group \( (P < 0.01) \). In addition, the total cell number in the blastocysts decreased significantly in the heat-stressed group, as compared to the control group.
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DNA damage in the embryos, as defined by the comet tail lengths, was increased significantly ($P<0.01$) by maternal heat stress (446.1 ± 17.4 vs. 358.3 ± 13.5 µm, Fig. 2).

**Discussion**

The present results demonstrate that heat stress enhanced ROS production in the mouse oviduct and shifted the redox status toward oxidation, and that heat stress-induced early embryonic death was accompanied with an increase in DNA damage at the 2-cell stage and a decrease in total cell numbers of the resultant blastocysts. These results support the hypothesis that heat stress-mediated generation of oxidative stress in the oviduct is involved in early embryonic death in heat-stressed animals, in addition to the direct deleterious effects of high temperature.

Although the increase in ROS production and the ROS/FRSA ratio in heat-stressed pregnant mouse oviduct was first demonstrated in our previous study [17], it was not clear whether the increase was a direct consequence of heat stress or merely a downstream response, such as interaction between the oviduct and damaged embryos. In the present study, we confirmed that the TBARS and GSH-Px activity in the liver increase time-dependently in heat-stressed mice. This result is in agreement with previous studies in rats and guinea pigs [15, 26], showing that the heat stress conditions adopted in our study were physiologically relevant and induced systemic changes in oxidative stress in the maternal body. Under these conditions, a time-dependent increase in ROS production in the oviduct during the heat stress was observed in the present study. These results suggest that maternal heat stress, or

<table>
<thead>
<tr>
<th>Table 1. Effects of heat stress on GSH-Px activity and the TBARS values in the liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>12 h-heat</td>
</tr>
<tr>
<td>24 h-heat</td>
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<tr>
<td>36 h-heat</td>
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</tbody>
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a) Glutathione peroxidase
b) Thiobarbituric acid reactive substances
c) Data are expressed as means ± SEM of 10 animals.
d) β-nicotinamide adenine dinucleotide phosphate
e) Malondialdehyde

Values with different superscripts in the same column differ significantly ($P<0.05$).

**Table 2. Developmental ability of zygotes and the total cell number in the blastocyst derived from heat-stressed mice**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of embryos that^a^ Cleaved to the 2-cell stage</th>
<th>Developed to the morula or blastocyst from 2-cell</th>
<th>Total cell number in a blastocyst^b^</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>98.7 ± 1.0</td>
<td>87.5 ± 9.7</td>
<td>105.7 ± 4.7 (48)</td>
</tr>
<tr>
<td>Heat stress</td>
<td>92.3 ± 5.1</td>
<td>32.2 ± 13.1**</td>
<td>71.8 ± 3.6*** (50)</td>
</tr>
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</table>

a) Data are expressed as the mean ± SEM of 7 animals.
b) Data are expressed as the mean ± SEM. Numbers of examined blastocysts are shown in the parenthesis.

**Fig. 2.** Effect of maternal heat stress on DNA damage in mouse 2-cell embryos. Pregnant mice were either heat-stressed for 12 h on day 1 (Heat stress) or were untreated (Control). Zygotes were collected at 1800 h on day 1 and cultured for 18 h. The level of DNA damage at the 2-cell stage was assayed using comet tail length measurements. The data are expressed as the mean ± SEM of 5 animals (each performed in 10–15 embryos). **Significantly different from control at $P<0.01$.**
resultant hyperthermia, enhances oxidative stress in the oviduct regardless of whether the animal is pregnant or not.

Another finding of the present study is the increase in DNA damage at the 2-cell stage and the reduction in the total cell number in blastocysts derived from maternally heat-stressed zygotes. The reduction in the total cell number in blastocysts has also been reported for bovine embryos [27], in which zygotes cultured in vitro or 2-cell embryos were heat-shocked at 41°C for 6 h. Interestingly, in that study, a decrease in developmental ability and cell proliferation in bovine early embryos heat-stressed in vitro was associated with an increase in intracellular ROS levels within embryos. On the other hand, Rivera et al. [28] observed that exposure of 2-cell bovine embryos to heat shock at 41°C for 6 h resulted in disruptions of ultrastructural morphology, such as an increase in the percentage of mitochondria exhibiting swollen morphology and the movement of organelles away from the plasma membrane. Recently, Jousan and Hansen [29] have reported that in vitro heat stress to bovine embryos increased the percentage of apoptotic blastomeres in blastocyst stage embryos. These recent findings demonstrate that heat stress to early embryos, either via the mother or in vitro, causes many types of cell damage similar to those associated with oxidative stress.

Until recently, it has been believed that maternal high body temperature per se is the main cause of heat stress-induced early embryonic death [8, 9], and that this phenomenon has been ascribed, mainly based on in vitro study, to the inability of early embryos (up to the 8-cell stage) to produce molecules, such as heat shock protein 70 and GSH, that protect the cells against heat shock [30]. However, it is unclear why exposure of embryos to moderately high temperatures similar to those experienced by heat-stressed animals does not compromise the normal development [5, 10]. Recently, we have shown that maternal heat stress causes the inactivation of Cdc2 kinase at the 2-cell stage and results in developmental arrest for the majority of heat-stressed mouse embryos [17]. Cdc2 kinase is a key molecule in the cell cycle checkpoint mechanism at the G2/M transition [31]. Furthermore, it is known that Cdc2 kinase remains inactive and leads to cell cycle arrest, when DNA is damaged [32] or oxidative stress is present [33]. Thus, it is possible to speculate that heat stress-induced oxidative stress damages the cell cycles of early embryos, and leads to developmental arrest or early embryonic death.

Recently, Rivera et al. [34] demonstrated that ROS production is not a main cause of the heat shock-induced disruption of development by showing that heat shock at 41°C for 6 h to bovine 2-cell stage embryos did not change intracellular GSH contents, and that low oxygen conditions did not alleviate the deleterious effects of heat shock on the development. These results are inconsistent with those of Sakatani et al. [27], who demonstrated that heat shock at 41°C for 6 h increased ROS production in bovine embryos. These contradictory observations suggest the existence of unknown factors that affect the response of early embryos to severe heat shock at 41°C or more under in vitro culture. Thus, further studies should be directed at resolving the discrepancies existing within in vitro studies, and between in vivo and in vitro studies.

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