Developmental competence and oxidative state of mouse zygotes heat-stressed maternally or *in vitro*

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Mammalian preimplantation embryos are sensitive to maternal and direct heat stress. However, the mechanisms by which heat stress affects early embryonic development *in vivo* or *in vitro* are unknown. This study examined whether heat-stress-induced loss of developmental competence in mouse embryos was mediated by physiological changes in the maternal environment or by high temperatures alone. After fertilization, zygotes at the same stage were heat-stressed at 39.5°C for 12 h either maternally (measured by maternal rectal temperature) or directly in culture. Zygotes in each group were cultured at 37.5°C for a further 84 h to assess their developmental ability. Neither type of heat stress affected the first cleavage rate. However, the proportion of embryos that developed to

morulae or blastocysts was significantly lower in the maternally heat-stressed group, but not in the directly heat-stressed group. Moreover, maternal heat stress significantly reduced intracellular glutathione concentrations and enhanced hydrogen peroxide concentrations in both zygotes and two-cell embryos that were recovered immediately after heat stress or 12 h later, respectively. In contrast, direct heat stress had little effect on concentrations of glutathione or hydrogen peroxide in cultured early embryos. These results demonstrate that maternal heat stress at the zygote stage reduces the developmental ability of mouse embryos via physiological changes in the maternal environment that lead to an increase in intracellular oxidative stress on the embryo.

Introduction

Mammalian preimplantation embryos are sensitive to high temperatures. Maternal heat stress, or the resultant hyperthermia, leads to an increased loss of early stage embryos (Edwards *et al.*, 2001). The heat-stress-induced loss of early embryos has been well documented in farm animals, the susceptibility to heat stress of which has increased together with their productivity (Cavestany *et al.*, 1985; Hansen *et al.*, 2000). Lactating dairy cows with high milk yields are the most sensitive to hot environments because of their high metabolic rates and they become hyperthermic at air temperatures as low as 27°C (Berman *et al.*, 1985). As a result, infertility caused by heat stress is more severe in lactating cows than in non-lactating heifers (Ingraham *et al.*, 1974; Badinga *et al.*, 1985). It is also notable that the effect of hyperthermia on preimplantation embryos appears to be more pronounced at the zygote than at the morula or blastocyst stage (Edwards *et al.*, 2001). Exposing lactating cows to a hot environment on the day of insemination significantly reduces the proportion of embryos that develop into blastocysts, whereas the same heat stress at day 3 or day 5 after insemination has a minimal influence on embryonic development (Putney *et al.*, 1989; Ealy *et al.*, 1993). The vulnerability of early cleavage-stage embryos to maternal hyperthermia has been reported in a wide range of

mammalian species, including sheep (Dutt, 1963), pigs (Wildt *et al.*, 1975) and laboratory animals, such as mice (Baumgartner and Chrisman, 1987) and rats (Narendranath and Kiracofe, 1975).

In vitro culture systems that directly expose embryos to high temperatures have demonstrated a correlation of the vulnerability of early embryos to heat stress with a stage-dependent acquisition of thermotolerance (Edwards *et al.*, 2001). In cultured mouse embryos, thermotolerance can be induced by short-term heat shock at the eight-cell (Ealy and Hansen, 1994) or morula stage (Aréchiga *et al.*, 1998), but not at the one- or two-cell stage. Furthermore, developmental acquisition of thermotolerance is associated with the ability of embryos to produce glutathione. Embryos cannot synthesize glutathione until the morula or blastocyst stage (Gardiner and Reed, 1995), and the inhibition of glutathione synthesis reduces the thermotolerance of heat-shocked embryos at the morula stage (Aréchiga *et al.*, 1995).

High body temperatures (Ikeda *et al.*, 1999) and high metabolic rates (Ji, 1999; Niess *et al.*, 1999) lead to increased production of reactive oxygen species (ROS), which react with various molecules in cells, such as lipids, proteins and nucleic acids, and result in cellular injury (Lin *et al.*, 1991; Wills *et al.*, 1997). Early cleavage-stage embryos are sensitive to oxidative stress and ROS inhibit embryonic development, inducing cellular fragmentation and degeneration (Nasr-Esfahani *et al.*, 1990; Fujitani *et al.*, 1997; Takahashi *et al.*, 1999; Guérin *et al.*, 2001). These

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reports support the hypothesis that oxidative stress is a direct cause of heat-stress-induced early embryonic loss. However, direct evidence has not yet been obtained for embryos that have been maternally heat-stressed *in vivo*. In the present study, the disruption of embryonic development in mice after maternal heat stress at the zygote stage was investigated to determine whether it is associated with changes in embryonic glutathione and H_2O_2 concentrations. In addition, the study examined whether the adverse effects of maternal heat stress could be reproduced by direct exposure of zygotes to a similar high temperature under culture conditions *in vitro*.

Materials and Methods

Animals

Eight- to 12-week-old male and female ICR mice and male BDF₁ mice were purchased from Charles River Japan Inc. (Yokohama). Mice were housed at 25°C and 50% relative humidity with a 12 h light:12 h dark photoperiod (lights on at 06:00 h) until they were used in experiments. All experimental protocols and animal handling procedures were reviewed and approved by the Animal Care and Use Committee of the University of Tsukuba.

Materials

Mineral oil, hyaluronidase, glutathione (reduced form), glutathione disulphide reductase, β -nicotinamide adenine dinucleotide phosphate (reduced form; NADPH), 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), Na₂-EDTA and 2',7'-dichlorodihydro-fluorescein diacetate (DCHFDA) were purchased from Sigma Chemical Co. (St Louis, MO). Equine chorionic gonadotrophin (eCG, serotrophin) and hCG (gonadotrophin) were purchased from Teikokuzouki Pharmaceutical Co. (Tokyo). Potassium simplex optimized medium (KSOM) was purchased from Specialty Media (Phillipsburg, NJ).

Disruption of normal embryonic development by maternal heat stress

Female ICR mice were kept overnight with males of the same strain, and mating was verified at 06:00 h on the next day by the presence of a vaginal plug (designated as day 1 of pregnancy). The mated females were then assigned randomly to one of the following three treatment groups: (i) heat-stressed on day 1 only (day 1 heat), (ii) successively heat-stressed from day 1 to day 3 (successive heat) and (iii) non-stressed (control). Mice in the heat-stressed groups were exposed to a high ambient temperature of 35°C with 60% relative humidity for 12 h during the light period. Subsequently, ten mice per group were killed by cervical dislocation on day 2, day 3 and day 4, and embryos were collected by flushing the oviducts and uteri with PBS. The morphology of collected embryos was noted and their developmental stages were confirmed.

Assessment of developmental ability of maternally and directly heat-stressed zygotes

Female ICR mice were superovulated by i.p. injection of 5 iu eCG at 12:00 h, followed by an i.p. injection of 5 iu hCG 48 h later. Female mice were then mated with BDF₁ males during the dark period. After observation of a vaginal plug on the subsequent day at 06:00 h, ten mated females were exposed to heat stress (35°C and 60% relative humidity) for 12 h during the light period on day 1 (maternal heat stress). Another ten mated female mice were housed at 25°C and 50% relative humidity (control). At 18:00 h on day 1, mice in each group were killed and embryos were collected by flushing oviducts with KSOM (Warner *et al.*, 1998). Subsequently, embryos were washed three times in fresh KSOM and cultured in groups of 20 in 50 μ l KSOM under mineral oil at 37.5°C in 5% CO₂ in humidified air for 84 h.

Another 20 mated mice were killed at 06:00 h on day 1 and zygotes were recovered from the oviducts. Cumulus cells were eliminated by culturing zygotes in KSOM with 0.3 mg hyaluronidase ml⁻¹ for 2 min. Subsequently, zygotes were rinsed three times in fresh KSOM to remove the enzyme and cultured in KSOM under mineral oil. Zygotes from ten mice were cultured at 39.5°C in 5% CO₂ for 12 h on day 1, followed by ordinal culture at 37.5°C in 5% CO₂ for 84 h (*in vitro* heat stress). Zygotes from the other ten mice were cultured at 37.5°C in 5% CO₂ throughout the entire culture period (control).

The developmental ability of zygotes in each treatment group was assessed by determining the proportion of embryos that developed to the two-cell stage (fertilization rate) and the proportion of embryos that reached the morula or blastocyst stage (normal developmental competence).

Monitoring of rectal temperature

In the preliminary experiments, the rectal temperature was measured at 0, 1, 6, 12 and 13 h after the onset of maternal heat stress for 12 h with a thermistor instrument (D611; Takara Thermistor Co., Tokyo) by inserting a probe 1 cm in length into the rectum for 20 s. The results indicated that the rectal temperature during heat stress varied among animals but did not differ during the period of heat stress. Average rectal temperatures at 1, 6 and 12 h after the onset of heat stress ranged from 39.5°C to 39.6°C, and returned to normal (37.7°C at 0 h) within 1 h after heat stress (data not shown). Therefore, in the present study, rectal temperature was monitored inside an environment chamber for each animal once a day at 18:00 h (immediately before the end of heat stress).

Assay for intracellular glutathione and H_2O_2 concentrations

Embryos were exposed to maternal or direct heat stress for 12 h as described above. In the maternally heat-stressed group, zygotes were recovered soon after treatment (at 18:00 h on day 1), and two-cell embryos were recovered at 06:00 h on day 2. In the *in vitro* group, zygotes were prepared at 18:00 h on day 1 of culture, and two-cell embryos

Table 1. Percentage of normal embryos recovered from intact mice exposed to maternal heat stress on day 1 or from day 1 to day 3 of pregnancy

Group	Day of pregnancy at embryo recovery (normal developmental stages)		
	Day 2 (two-cell stage)	Day 3 (four- to eight-cell stage)	Day 4 (morula or blastocyst)
Control	90.4 ± 3.8	78.5 ± 4.3 ^a	97.8 ± 1.4 ^a
Heat stress on day 1	77.2 ± 6.4	4.7 ± 2.5 ^c	12.5 ± 7.6 ^b
Heat stress from day 1 to day 3	–	12.3 ± 3.8 ^b	3.7 ± 1.9 ^c

Mice in the heat-stressed groups were exposed to a high ambient temperature of 35°C with 60% relative humidity for 12 h during the light period (06:00 h–18:00 h) on specific days, and were kept under control conditions (25°C and 50% relative humidity) for the rest of the experimental period.

Values are expressed as the mean ± SEM of ten replicates.

^{abc}Values within columns with different letters are significantly different ($P < 0.01$, ANOVA).

at 06:00 h on day 2 of culture. Glutathione and H₂O₂ concentrations were then determined. Total glutathione concentrations were measured by the DTNB–glutathione disulphide reductase recycling assay as described by Anderson (1985). In brief, embryos were stored in groups of 20 in 5 µl of 0.2 mol sodium phosphate buffer l⁻¹ with 10 mmol Na₂-EDTA l⁻¹ and 5 µl of 1.25 mol phosphoric acid l⁻¹ in a 1.5 ml microfuge tube. Assay samples were warmed at room temperature for 15 min after the addition and mixing of 350 µl sodium phosphate containing 0.33 mg NADPH ml⁻¹, 50 µl of 6 mmol DTNB l⁻¹ and 90 µl water. Subsequently, 5 µl of 250 U glutathione disulphide reductase ml⁻¹ was added to the microfuge tube to initiate the re-action. Absorbance was recorded five times by spectrophotometer at 30 s intervals at 412 nm. A glutathione standard and a sample blank were also assayed ($n = 7$ in each group).

The intracellular H₂O₂ concentration in the embryos was quantified by measuring 2',7'-dichlorofluorescein fluorescence (Nasr-Esfahani *et al.*, 1990; Nasr-Esfahani and Johnson, 1991; Yang *et al.*, 1998; Hashimoto *et al.*, 2000). A stock solution of DCHFDA, prepared in DMSO at 1×10^{-3} mol l⁻¹, was diluted in KSOM at 1×10^{-5} mol l⁻¹ immediately before the start of the assay. Embryos were incubated for 15 min in KSOM containing DCHFDA. Subsequently, embryos were rinsed three times in fresh KSOM to remove traces of DCHFDA, and were placed on a glass slide with KSOM and covered with a cover slip. The relative concentrations of intracellular H₂O₂ were measured using Analytical Imaging Station (Imaging Research Co., St Catharines, Ontario) after excitation at 480 nm and emission at 510 nm under a fluorescent microscope (BX 50; Olympus, Tokyo). The possible influence of assay protocol, which may increase intracellular H₂O₂ generation, was minimised by handling embryos under the lowest level of room light and fluorescence intensity was recorded 5 s after excitation of embryos.

Statistical analysis

Data were expressed as the mean ± SEM. The percentages of embryos that were fertilized or which developed into mor-

ulae or blastocysts were subjected to arc-sin-transformation and then analysed by one-way ANOVA followed by the Fisher's protected least significant difference test. The statistical significance of intracellular concentrations of glutathione and H₂O₂ in embryos was determined by Student's *t* test. Differences were considered significant at $P < 0.05$.

Results

Effect of maternal heat stress on embryonic development in vivo

The rectal temperature increased significantly in the maternally heat-stressed group ($39.8 \pm 0.2^\circ\text{C}$) compared with the control group ($37.8 \pm 0.1^\circ\text{C}$) ($P < 0.001$). The percentages of normal developing embryos after maternal heat stress are presented (Table 1). The majority of recovered embryos in the control group were at the two-cell stage on day 2, the four- to eight-cell stage on day 3 and at the morula or blastocyst stage on day 4. Maternal heat stress on day 1 did not significantly affect the percentage of two-cell embryos on day 2, but resulted in a marked reduction in normal embryos after day 3. Successive heat stress from day 1 to day 3 produced similar results to those of day 1 heat stress. Most of the abnormal embryos in the heat-stressed groups were arrested at the two-cell stage (34.7–53.4% of total embryos) or had degenerated (11.6–16.9%) on days 3–4. Although the rectal temperature in heat-stressed animals varied from 39.4°C to 40.3°C , the heat-stress-induced embryonic loss did not correlate with the level of hyperthermia in individual animals.

Effect of maternal heat stress and heat stress in vitro on first cleavage and subsequent developmental ability of zygotes

The first cleavage rate of embryos was not affected by either maternal heat stress or *in vitro* heat stress (Fig. 1a). The percentage of embryos that reached the morula or blastocyst stage was significantly ($P < 0.01$) lower in the maternally heat-stressed group ($44.0 \pm 2.5\%$) than in the

Fig. 1. Effects of maternal heat stress and *in vitro* heat stress on subsequent developmental ability of cultured mouse zygotes. (a) Percentage of embryos that cleaved. (b) Percentage of embryos that developed from the first cleavage stage to the morula or blastocyst stage. In the maternally heat-stressed groups, pregnant female mice were exposed to heat stress for 12 h on day 1 (HS, ■) or were untreated (C, □). Subsequently, embryos were recovered and cultured for 84 h. In the *in vitro* heat-stressed groups, zygotes were recovered at 06:00 h on day 1 and cultured at 39.5°C in 5% CO₂ for 12 h, followed by ordinal culture at 37.5°C in 5% CO₂ (HS), or were cultured at 37.5°C in 5% CO₂ throughout the culture period (C). Values are expressed as mean $\pm$ SEM of ten replicates. ***Significantly different from control ($P < 0.001$).

control group ($95.0 \pm 1.9\%$) (Fig. 1b). However, there was no significant change in embryonic development between zygotes that were directly heat-stressed *in vitro* ($87.5 \pm 3.6\%$) and control zygotes ($89.5 \pm 2.7\%$) (Fig. 1b). In this experiment, the rectal temperature in the maternally heat-stressed group ($39.7 \pm 0.2^\circ\text{C}$) was significantly higher than that of the control group ($37.5 \pm 0.1^\circ\text{C}$). However, there was no apparent correlation between the rectal temperatures (39.2 – 40.4°C) of individual heat-stressed animals, and the corresponding percentage of zygotes normally developed (30–55%).

Effect of heat stress on intracellular glutathione and H₂O₂ concentrations in embryos

Intracellular glutathione concentrations were significantly lower in maternally heat-stressed embryos than in

Fig. 2. Effect of (a) maternal heat stress or (b) *in vitro* heat stress on intracellular glutathione (GSH) concentrations of zygotes and two-cell-stage mouse embryos. (a) Pregnant female mice were exposed to heat stress for 12 h on day 1 (HS, ■) or were untreated (C, □). Zygotes used for GSH assay were recovered soon after treatment (at 18:00 h on day 1), and two-cell embryos were recovered at 06:00 h on day 2. (b) Zygotes were recovered at 06:00 h on day 1 and cultured at 39.5°C in 5% CO₂ for 12 h, followed by ordinal culture at 37.5°C in 5% CO₂ (HS), or were cultured at 37.5°C in 5% CO₂ throughout the entire culture period (C). Zygotes used for GSH assay were prepared at 18:00 h on day 1 of culture, and two-cell embryos were prepared at 06:00 h on day 2 of culture. GSH concentrations were measured by 5,5'-dithio-bis-2-nitrobenzoic acid–glutathione reductase recycling assay. Values are expressed as mean $\pm$ SEM of seven replicates. Values are significantly different from control (* $P < 0.05$ and ** $P < 0.01$).

control embryos at both the zygote stage (0.99 ± 0.06 versus 1.36 ± 0.08 pmol glutathione per embryo, respectively, $P < 0.01$) and the two-cell stage (0.45 ± 0.09 versus 0.76 ± 0.13 pmol glutathione per embryo, respectively, $P < 0.01$) (Fig. 2a). In contrast, there were no significant differences in glutathione concentrations between embryos that were directly heat-stressed *in vitro* and control embryos at the zygote stage (0.86 ± 0.13 versus 0.93 ± 0.10 pmol glutathione per embryo, respectively) and the two-cell stage (0.64 ± 0.10 versus 0.68 ± 0.13 pmol glutathione per embryo, respectively) (Fig. 2b).

Maternal heat stress enhanced H₂O₂ concentrations in

Fig. 3. Pseudocolour images of control and maternally heat-stressed mouse embryos after incubation for 15 min with 2',7'-dichlorodihydro-fluorescein diacetate. Fluorescence intensity of 2',7'-dichlorofluorescein, corresponding to intracellular H_2O_2 concentrations, is expressed in pseudocolour using Analytical Imaging Station according to the colour bar. (a) Maternally heat-stressed zygotes, (b) untreated zygotes, (c) maternally heat-stressed two-cell embryos and (d) untreated two-cell embryos.

embryos, as expressed by the fluorescence intensity of DCF (Fig. 3). Hydrogen peroxide concentrations were significantly higher in maternally heat-stressed embryos compared with control embryos at both the zygote and two-cell stages (0.22 ± 0.01 versus 0.17 ± 0.01 fluorescence intensity units (FIU) per embryo for zygote, $P < 0.01$ and 0.25 ± 0.03 versus 0.18 ± 0.01 FIU per embryo for two-cell stage, $P < 0.05$, respectively, Fig. 4a). In contrast, direct heat stress did not affect intracellular H_2O_2 levels of embryos at either the zygote or the two-cell stage (Fig. 4b). Maternal heat stress resulted in a significant increase ($P < 0.001$) in rectal temperatures ($39.7 \pm 0.2^\circ\text{C}$ for glutathione and $39.4 \pm 0.1^\circ\text{C}$ for H_2O_2), but there were no consistent correlations between the degree of hyperthermia in individual animals and glutathione or H_2O_2 concentrations in the corresponding embryos.

Discussion

The results of the present study demonstrated that the detrimental effects of maternal heat stress on zygotes are not necessarily related to high body temperatures, but are mediated through physiological changes in the maternal environment or interaction between the zygote and its surroundings. Results from this study also provide direct evidence that the developmental arrest of maternally heat-

stressed zygotes is associated with an increase of intracellular oxidative stress, as shown by the increase in intracellular H_2O_2 concentrations and the reduction of glutathione content within the embryos.

It is known that mammalian preimplantation embryos, particularly zygotes, are sensitive to heat stress, and that exposure of animals to hot environments soon after fertilization leads to an increase in the loss of early stage embryos (Dutt, 1963; Badinga *et al.*, 1985; Ealy *et al.*, 1993). It has also been demonstrated that direct exposure of cultured early embryos to temperatures of $41\text{--}43^\circ\text{C}$ results in developmental arrest and an increase in embryonic death (Aréchiga *et al.*, 1995; Ealy *et al.*, 1995). The similarity between maternal heat stress and *in vitro* heat stress, that is, the vulnerability of zygotes to high temperatures, was considered to be a main cause for heat-stress-induced early embryonic death. However, the rectal temperatures of milking cows that are heat-stressed in the field are about $40\text{--}40.5^\circ\text{C}$ (Rivera and Hansen, 2001), which are similar to those observed in the present study (rectal temperatures of heat-stressed mice were on average $39.4\text{--}39.7^\circ\text{C}$). Thus, the high temperatures previously adopted for several *in vitro* experimental models of early embryonic death might be higher than those experienced in heat-stressed animals under field conditions. Ryan *et al.* (1992) reported that culturing bovine zygotes at 40°C did not compromise

Fig. 4. Effects of (a) maternal heat stress and (b) *in vitro* heat stress on intracellular H₂O₂ concentrations of zygotes and two-cell mouse embryos. Embryos were heat-stressed maternally or *in vitro* and cultured at 39.5°C in 5% CO₂ for 12 h, followed by ordinal culture at 37.5°C in 5% CO₂ (HS, ■), or were cultured at 37.5°C in 5% CO₂ throughout the entire culturing period (C, □). H₂O₂ concentrations were quantified by measuring 2',7'-dichlorofluorescein fluorescence. Values represent arbitrary fluorescence intensity units (FIU). Values are expressed as mean ± SEM of seven replicates (each performed in 6–9 embryos). Values are significantly different from controls (**P* < 0.05 and ***P* < 0.01).

subsequent development, and Rivera and Hansen (2001) revealed that exposure of bovine zygotes to a fluctuating high temperature that carefully mimicked the rectal temperature of heat-stressed cows for 12 h did not affect first cleavage and development to the blastocyst stage. These reports support findings of the present study, which demonstrate that developmental arrest or preimplantation embryonic death in hyperthermic animals might be caused by an interaction with the maternal reproductive tracts (for example, oviducts), and not by high temperature alone. It is possible that maternal heat stress changes the physiological conditions of the oviducts. Fallopian tubal fluid normally contains many ions and macromolecules, such as growth factors and anti-oxidants (Kane *et al.*, 1997; Guérin *et al.*, 2001; Leese *et al.*, 2001), that are necessary for embryo survival and subsequent development. Further studies to analyse the effect of maternal heat stress on the reproductive tracts are required.

The data obtained in the present study revealed that maternal heat stress for 12 h, which induced hyperthermia at about 39.5°C as determined by rectal temperatures, reduced intracellular glutathione concentrations and increased H₂O₂ concentrations in zygotes recovered immediately after heat stress and in subsequent two-cell embryos. High temperatures enhance the production of free radicals in both somatic cells (Lin *et al.*, 1991; Ji, 1999) and germ cells (Ikeda *et al.*, 1999). In testicular germ cells, heat stress increases the intracellular H₂O₂ concentration and induces apoptosis (Ikeda *et al.*, 1999). An increase in free radicals is critical for cultured embryos and often leads to developmental arrest (Fujitani *et al.*, 1997; Takahashi *et al.*, 1999) or embryo degeneration (Nasr-Esfahani *et al.*, 1990; Yang *et al.*, 1998; Guérin *et al.*, 2001). Glutathione, an antioxidant tripeptide, scavenges H₂O₂, and plays a critical role in the normal development of preimplantation embryos. Supplementation of culture medium with glutathione increases the resistance of bovine embryos to heat shock at 42°C *in vitro* (Ealy *et al.*, 1992). Aréchiga *et al.* (1995) reported that in mouse embryos, glutathione-dependent mechanisms limit the deleterious effects of heat stress.

Unlike somatic cells, preimplantation embryos cannot synthesize glutathione *de novo* (Gardiner and Reed, 1995). Therefore, the early embryos may be very sensitive to oxygen stress, even at low concentrations. The results from the present study show that intracellular H₂O₂ concentrations were consistently higher in zygotes and in two-cell embryos recovered from heat-stressed animals compared with H₂O₂ concentrations in zygotes and two-cell embryos from non-stressed animals or those directly heat-stressed *in vitro*. This enhanced ROS generation was apparently correlated with a more severe reduction in glutathione concentration in two-cell embryos obtained from heat-stressed animals. This finding indicates the possible involvement of oxidative stress in maternal heat-stress-induced early embryonic death. It is also important to note that an increase in ROS in early embryos occurs at a lower temperature in the maternal environment compared with that observed *in vitro*. Further studies are required to identify factor(s) influencing the intracellular oxidative status of early embryos in the maternal environment.

Embryo genome activation, which occurs during the late one-cell and two-cell stages (Latham, 1999), is essential for normal embryonic development. When oxidative stress occurs before and during embryo genome activation, embryonic development is arrested at the two-cell stage (Nasr-Esfahani *et al.*, 1990). A two-cell block and an increased oxidative state in maternally heat-stressed zygotes were observed in the present study. Haraguchi *et al.* (1999) reported that initiation of embryo genome activation was delayed in phosphate-induced two-cell-blocked embryos and speculated that the time lag for the onset of embryo genome activation may be the cause of the two-cell block. Therefore, it is important to determine whether the developmental arrest caused by maternal heat stress is associated with a disturbance in embryo genome activation.

The authors thank I. Ohshima for her kind assistance, M. Kajihara for her generous guidance with embryo culture and N. Minami for technical advice concerning GSH and H₂O₂ assays. This work was supported by the Grant-in-Aid for Exploratory Research from Japanese Society for the Promotion of Science to Y. Kanai and in part by grant from Morinaga Hoshikai to M. Hirabayashi.

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Received 2 April 2002.

First decision 4 June 2002.

Revised manuscript received 5 July 2002.

Accepted 8 August 2002.