DL-\(\alpha\)-Tocopherol acetate mitigates maternal hyperthermia-induced pre-implantation embryonic death accompanied by a reduction of physiological oxidative stress in mice

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Abstract

Maternal hyperthermia induces pre-implantation embryo death, which is accompanied by enhanced physiological oxidative stress. We evaluated whether the administration of DL-\(\alpha\)-tocopherol acetate (TA) to hyperthermic mothers mitigated pre-implantation embryo death. Mice were exposed to heat stress (35\(^\circ\)C, 60% relative humidity) for 12 h or not heated (25\(^\circ\)C) on the day of mating. Twelve hours before the beginning of temperature treatment, TA was injected intraperitoneally at a dose of 1 g/kg body weight. After the treatment, zygotes were recovered and the developmental abilities and intracellular glutathione (GSH) levels were evaluated. Another set of mice, with or without TA treatment, was exposed to heat stress for 12, 24 and 36 h, and the urinary levels of the oxidative stress marker 8-hydroxy-2'-deoxyguanosine (8-OHdG) were measured. Heat stress significantly decreased the blastocyst development rate and the GSH content in zygotes, as compared with the non-heat-stressed embryos, while TA administration significantly mitigated the deleterious effects of heat stress with regard to both parameters. Moreover, although the urinary levels of 8-OHdG gradually increased according to the duration of heat exposure, with or without TA administration, the levels were lower in the TA-administered group than in the placebo-injected mice. These results suggest that heat stress enhances physiological oxidative stress, and that TA administration alleviates the hyperthermia-induced death of pre-implantation embryos by reducing physiological oxidative stress.

Reproduction (2008) 135 489–496

Introduction

The mammalian pre-implantation embryo is susceptible to stressors and its development is easily disturbed. Maternal hyperthermia, induced by either high ambient temperature or heightened metabolism, is known to be a major cause of serious damage to human embryos (reviewed by Edwards et al. 1997). This phenomenon has been reported in mammals, including large farm animals, such as cows (Ingraham et al. 1974), sheep (Woody & Ulberg 1964) and pigs (Wildt et al. 2002), and in experimental animals, such as mice (Ozawa et al. 2002). Although previous studies using in vitro models have shown that the early developing embryo is itself vulnerable to high temperature and readily degenerates when cultured at temperatures above 41\(^\circ\)C (Aréchiga & Hansen 1998, Rivera & Hansen 2001), recent studies using in vivo models have suggested that early embryonic death caused by maternal hyperthermia is the result not only of elevated temperature but also of interactions between the embryo and the maternal reproductive tract. For example, maternal hyperthermia on the day of artificial insemination causes a significant reduction in blastocyst development in vivo in cows (Ealy et al. 1993), whereas in vitro exposure to high temperatures, as mimicked by a fluctuating pattern of rectal temperature in a hyperthermic mother, of zygotes for 24 h does not compromise developmental ability to the blastocyst stage (Rivera & Hansen 2001). Similarly, we have reported that the exposure of murine zygotes in vitro to 39.5\(^\circ\)C, which is the average rectal temperature of heat-stressed mice, does not affect their development to the blastocyst stage, compared with the development of unstressed embryos, whereas maternal heat exposure on the day of mating for 12 h significantly compromises subsequent embryonic development (Ozawa et al. 2002). Furthermore, oxidative stress in the embryo is heightened in relation to maternal hyperthermia; for example, higher levels of reactive oxygen species (ROS) and decreased concentrations of glutathione (GSH), a major antioxidant in the embryo, have been observed in maternally heat-exposed embryos in mice (Ozawa et al. 2002).
Hyperthermia has been reported to increase oxidative stress in some tissues. For example, Salo et al. (1991) have reported that hyperthermia caused by exercise increases the generation of superoxide anion radicals and hydrogen peroxide in skeletal muscles and the heart. Similarly, heat stress-induced maternal hyperthermia enhances ROS production in the oviduct (Ozawa et al. 2004, Matsuzuka et al. 2005a, 2005b) and lipid peroxidation in the liver (Matsuzuka et al. 2005b). ROS are strongly reactive with cellular molecules and can cause serious dysfunction, such as enzyme inactivation (Halliwell & Gutteridge 1984), mitochondrial abnormalities (Kowaltowski & Vercesi 1999) and DNA fragmentation (Lopes et al. 1998, Takahashi et al. 1999).

Early developing embryos are susceptible to oxidative stress, and the exposure of cultured embryos to exogenous oxidative stress compromises their pre-implantation development (reviewed by Guérin et al. 2001). In contrast, the detrimental effects of in vitro oxidative stress on pre-implantation embryos can be reduced by the addition of antioxidant molecules to the culture medium. The addition of triiodothyronine to the culture medium relieves the stage-dependent developmental arrest of mice embryos, known as ‘cell block’, which is triggered by oxidative stress (Natsuyama et al. 1993). Iwata et al. (1998) have also reported that superoxide dismutase administration improves the blastocyst development rate of porcine embryos under conditions of high glucose concentration in the culture medium, which are known to enhance oxidative stress (Catherwood et al. 2002, Karja et al. 2006). In addition to the in vitro studies, we have recently reported that in an in vivo model, administration of melatonin, which has antioxidant properties and is a free radical scavenger, to pregnant mice partially alleviates either hyperthermia-enhanced lipid peroxidation in the liver or reduction in intracellular GSH levels in zygotes, resulting in an improved rate of embryonic development to the blastocyst stage, as compared with embryos exposed to heat stress plus placebo (Matsuzuka et al. 2005b).

Taken together, these previous studies have led us to hypothesise that maternal hyperthermia enhances physiological oxidative stress at the whole-body level, and that heightened oxidative stress may be a factor in the induction of pre-implantation fatalities. DL-α-tocopherol acetate (TA), which is generally known as vitamin E, is a common lipid-soluble antioxidant that has been administered to reduce oxidative stress in cancer patients (Valko et al. 2006) and pregnant women with pre-eclampsia (Holmes & McCance 2005). In the present study, we investigated the degree of physiological oxidative stress in heat-stressed mice by measuring the urinary levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG), which is a predominant form of ROS-induced lesion in DNA and is widely used as a sensitive biomarker of oxidative stress (de Zwart et al. 1999, Wu et al. 2004). We also evaluated the effect of maternal administration of TA on hyperthermia-induced death of pre-implantation embryos by monitoring embryonic development and physiological and embryonic oxidative stress statuses after heat and TA treatments.

Results

Embryo developmental competence

The average rectal temperatures and parameters related to pre-implantation embryo development in each treatment group are shown in Table 1. The heat-stressed mice experienced a significant (P<0.01) increase in rectal temperature, as compared with the controls. Almost all zygotes underwent a first cleavage; no significant difference was detected between the treatment groups in this respect. In contrast, although the percentage of embryos that developed to the blastocyst stage decreased in both heat-stressed groups (P<0.01), as compared with the control, the percentage was significantly (P<0.05) improved, from 36.9 ± 6.7 to 59.4 ± 6.7%, by TA administration. In addition, the percentage of two-cell arrested embryos under heat stress conditions decreased significantly (P<0.01), from 27.0 ± 5.2 to 11.7 ± 3.7%, with TA administration. The mean cell numbers in the blastocysts recovered from heat-stressed mice treated with TA were not different from those in the control group, while the blastocyst cell numbers in the heat-with-placebo-treated group were significantly (P<0.01) lower than in the other two groups.

Intracellular GSH levels in zygotes

The GSH levels in the zygotes are presented in Fig. 1. The zygotes from TA-administered mice had significantly higher GSH levels than those from heat-stressed mice. The average rectal temperatures and parameters related to pre-implantation embryo development in each treatment group are shown in Table 1. The heat-stressed mice experienced a significant (P<0.01) increase in rectal temperature, as compared with the controls. Almost all zygotes underwent a first cleavage; no significant difference was detected between the treatment groups in this respect. In contrast, although the percentage of embryos that developed to the blastocyst stage decreased in both heat-stressed groups (P<0.01), as compared with the control, the percentage was significantly (P<0.05) increased, from 36.9 ± 6.7 to 59.4 ± 6.7%, by TA administration. In addition, the percentage of two-cell arrested embryos under heat stress conditions decreased significantly (P<0.01), from 27.0 ± 5.2 to 11.7 ± 3.7%, with TA administration. The mean cell numbers in the blastocysts recovered from heat-stressed mice treated with TA were not different from those in the control group, while the blastocyst cell numbers in the heat-with-placebo-treated group were significantly (P<0.01) lower than in the other two groups.

In Table 1, the effect of heat stress and DL-α-tocopherol acetate (TA) administration on embryo developmental competence is shown.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Replication</th>
<th>Rectal temperature</th>
<th>Cleaved</th>
<th>Arrested at two-cell</th>
<th>Developed to blastocysts</th>
<th>Cell numbers in blastocyst</th>
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<tr>
<td>Control</td>
<td>10</td>
<td>37.6 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>99.3 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.2 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>97.7 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>74.2 ± 2.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Heat stress</td>
<td>9</td>
<td>39.6 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>98.8 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.0 ± 5.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36.9 ± 6.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.4 ± 3.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Heat + TA</td>
<td>10</td>
<td>39.5 ± 0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>97.9 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.7 ± 3.7&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>59.4 ± 6.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>66.9 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
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Data are the mean ± S.E.M. of animals per group. Different superscript letters indicate significantly different values (P<0.05, one-way ANOVA with Tukey’s multiple comparisons test).
higher GSH levels (2.28 ± 0.14 pmol/zygote, P < 0.05) than the heat-stressed mice treated with placebo (1.89 ± 0.08 pmol/zygote).

**TA concentrations in plasma and liver**

The TA concentrations in the plasma and liver samples from each treatment group are presented in Fig. 2. The TA concentrations in heat-stressed mice (2.6 ± 0.2 µg/ml in the plasma and 4.7 ± 0.1 µg/g wet tissue in the liver) were significantly lower (P < 0.01) than those in the control mice (4.6 ± 0.2 µg/ml in the plasma and 26.9 ± 1.3 µg/g wet tissue in the liver). In contrast, no significant difference was detected for plasma TA concentration between TA-administered, heat-stressed mice (5.0 ± 0.2 µg/ml) and the controls. Moreover, the degree of TA content reduction in the liver was lower in the heat-stressed mice administered TA than in those administered the placebo, although the TA levels in both groups were significantly lower (P < 0.01) than in the controls.

**Urinary 8-OHdG levels**

The patterns of urinary 8-OHdG levels for each treatment group during heat treatment are shown in Fig. 3. Significant differences were detected in the patterns of the urinary 8-OHdG concentrations either among the treatments, or time of sampling (P < 0.05). The 8-OHdG level increased in relation to the duration of high-temperature treatment in the heat-stressed mice that were administered the placebo, and this level was significantly higher (1.14 ± 0.09 µg/mg creatinine, P < 0.05) at 48 h compared with that in the controls (0.70 ± 0.12 µg/mg creatinine). In contrast, the 8-OHdG level in the TA-administered group after 12 h of heat treatment was far lower, less than one-third (0.18 ± 0.08 µg/mg creatinine) of that in the controls (P < 0.05). In addition, the 8-OHdG levels did not differ significantly from the controls, even though the TA-administered mice were exposed to heat stress for...
48 h (0.70 ± 0.12 μg/mg creatinine in controls versus 0.87 ± 0.18 μg/mg creatinine in the TA-administered group), although the levels in the TA-administered group were also increased in line with the duration of heat exposure.

Discussion

We investigated the degree of physiological oxidative stress during heat stress and the effects of maternal TA administration on physiological oxidative stress status and hyperthermia-induced pre-implantation embryonic death. To our knowledge, the results indicate for the first time that physiological oxidative stress in the whole body is increased according to the duration of maternal hyperthermia, as determined by urinary 8-OHdG levels. Moreover, TA administration to the mated female improved the developmental success of maternally heat-stressed embryos, and this was accompanied by a significant reduction in the physiological oxidative damage induced by hyperthermia.

Mortality and developmental malformations in pre-implantation embryos under conditions of maternal hyperthermia are well known in mammalian species (Mirkes 1997, Hansen et al. 2004). For example, heat exposure of cattle on the day of artificial insemination compromises pre-implantation embryo development in vivo (Ealy et al. 1993). Previous reports from our group have shown that maternal hyperthermia for 12 h soon after mating strongly disturbs normal embryonic development both in vitro (Ozawa et al. 2002, Matsuzuka et al. 2005a) and in vivo (Ozawa et al. 2003). The present results are consistent with those of previous studies, in that heat exposure of mated mice for 12 h severely compromised normal embryonic development, as determined by the blastocyst formation rate and the cell numbers in the blastocysts (Table 1). It is noteworthy that females with heightened metabolism were reported to have an increased risk of hyperthermia-induced pre-implantation embryo death. For example, exercise produces excess heat and increases at least a theoretical risk of embryo malformations in humans (Edwards 1986, Weissgerber & Wolfe 2006). Similarly, embryo mortality in dairy cattle has been reported to be pronounced, not only in the summer, but also into the fall in hot regions, such as Florida (Hansen et al. 2001), whereas the embryo mortality was less evident in heifers (Ingraham et al. 1974).

Although mammalian pre-implantation embryos are susceptible to exogenous stresses, such as heat stress, high temperature does not seem to be the only factor that compromises embryonic development during maternal hyperthermia; enhanced physiological oxidative stress is also implicated. In fact, recent reports have demonstrated that culturing zygotes at high temperatures, within the physiological range, in hyperthermic cows (40.5 °C) for 12 h did not alter subsequent development to the blastocyst stage (Ryan et al. 1992, Rivera & Hansen 2001). Our previous study has also indicated that in vitro exposure of zygotes to high temperature, mimicking the average rectal temperature of hyperthermic mice (39.5 °C), does not alter the blastocyst development rate, whereas maternal heat exposure for 12 h soon after mating drastically arrests subsequent embryonic development and is accompanied by increased H₂O₂ levels, decreased GSH levels (Ozawa et al. 2002) and damaged genomic DNA in the embryos, as assessed by elongation of the ladder tail in a comet assay, which is typical of ROS-mediated injury (Matsuzuka et al. 2005a).

It has been reported that hyperthermia and enhanced metabolic rate can cause increased oxidative stress in some tissues. For example, hyperthermia-enhanced ROS production in testicular germ cells (Ikeda et al. 1999) and excessive exercise produce H₂O₂ in rat vastus lateralis and cardiac muscle (Ji 1999). We have also observed that the TA concentration is decreased by maternal hyperthermia. Decreased liver TA levels have been reported to be induced by oxidative stress. For example, the administration of polychlorinated biphenyls (PCBs, oxidative stress inducers) decreases the TA concentration in the liver (Banudevi et al. 2006). In addition, in the present study, we showed that the urinary 8-OHdG levels were elevated, paralleling the duration of heat exposure, with or without TA administration (Fig. 2). The oxidised DNA damage product 8-OHdG is a predominant and stable form of ROS-induced DNA lesion. The level of urinary 8-OHdG should reflect whole-body formation of oxidised nucleobases (Pilger et al. 2002), and it has been used as a biomarker of the degree of oxidative stress (Cheng et al. 1992, Saito et al. 2000). Taken together, our results suggest that maternal hyperthermia increases oxidative stress, not only in certain tissue(s) but also at whole-body physiological levels.

In contrast, TA administration reduced the increased oxidative stress induced by hyperthermia, as determined by decreases in the 8-OHdG levels (Fig. 3) and increased liver TA concentrations (Fig. 2). In addition, the GSH levels in heat-stressed zygotes were significantly decreased by maternal heat stress, while they recovered to the unstressed levels after TA administration (Fig. 1). TA, which is a well-known lipid-soluble antioxidant, has been reported to inhibit NADPH oxidase-mediated superoxide anion generation and to protect cellular membranes against oxidative stress (Pascoe et al. 1987). In general, pre-implantation embryos are vulnerable to oxidative stress, and their development is easy to arrest or disturb by exposure to excessive oxidative stress (Guérin et al. 2001). GSH is synthesised and accumulated during the oocyte maturation period (Yoshida et al. 1993), and acts as an important antioxidant tripeptide, to maintain the balance of reduce-oxidative environment in the embryo (Guérin et al. 2001). The mouse embryo cannot synthesise GSH de novo until the blastocyst stage (Gardiner & Reed 1994, 1995). Therefore, the
intracellular GSH level in the embryo reflects the environmental oxidative stress status and the developmental ability of the embryo. In fact, intracellular GSH levels in embryos are significantly decreased when the embryos are exposed to exogenous oxidative stress, and subsequent development is compromised (Nasr-Esfahani & Johnson 1991, Gardiner & Reed 1994). In contrast, the addition of the antioxidant thioredoxin to the culture medium has been shown to maintain intracellular GSH at higher levels and to increase the blastocyst development rate, as compared with embryos cultured without the antioxidant, in pigs (Ozawa et al. 2006). The addition of TA to a culture in which oxidative stress has been enhanced by the ROS inducer 12-phorbol-13-myristate acetate can restore the blastocyst development rate (Wang et al. 2002). Thus, it seems that TA administration protects the embryo against the excessive oxidative stress induced by hyperthermia, and alleviates the decline in developmental abilities of heat-stressed embryos, although further study is needed to clarify whether TA acts directly on the embryo or indirectly via the enhanced reducing status of the mother.

On the other hand, Ealy et al. (1994) have reported that i.m. administration of vitamin E to dairy cows at the time of AI during summer did not have a consistent beneficial effect on pregnancy rates in Florida. This conflict between the previous study and the present result may be attributed to the duration of the heat period; the cows were kept in hot environments before and after the TA administration (Ealy et al. 1994), whereas the female mice in the present study were exposed to heat stress for only 12 hours after mating. In addition, the embryos in the dairy cows were developed in vivo in the hyperthermic mother, while the embryos in the present study were cultured in vitro. Thus, the beneficial effect of TA administration on embryo development under hyperthermia may be less evident in the previous study using cows (Ealy et al. 1994).

Recently, we have reported that s.c. administration of melatonin, which acts as a free radical scavenger in vivo (Tan et al. 1993) and in vitro (Allegra et al. 2003), to mated female mice alleviates heat stress-induced pre-implantation embryo death, and this is accompanied by the maintenance of the reducing status in the oviduct (Matsuzuka et al. 2005b). On the other hand, melatonin is a water-soluble hormone, and it was necessary to inject it six times at 2-h interval to achieve a beneficial effect (Matsuzuka et al. 2005b). In addition, several studies have pointed out adverse effects of exogenous melatonin administration; melatonin injections in adult females disrupt the normal oestrous cycle by disturbing the secretion of LH-releasing hormone, resulting in low fertility in rats (Walker et al. 1982, Rivest, 1987). In contrast, since TA is a lipid-soluble vitamin, the effects of TA administration are longer lasting and even a single dose can be effective. For example, an injection of TA in dairy cattle increases the TA concentration in the milk for 6 days (Hidiroglou 1989). Furthermore, there are many results concerning TA administration in mammals, including human patients, with the aim of reducing physiological damage induced by oxidative stress, for example, in cancer (Valko et al. 2006), pre-eclampsia (Holmes & McCance 2005) and diabetic (Elliott et al. 1993) patients. Therefore, TA may be a beneficial and practical countermeasure against maternal hyperthermia-induced oxidation syndrome, including pre-implantation embryo mortality.

In conclusion, maternal heat exposure enhances physiological oxidative stress, and TA administration to hyperthermic mice can alleviate hyperthermia-induced pre-implantation embryo death by reducing the elevated physiological oxidative stress. These findings may help in the development of a new and practical strategy to overcome embryo mortality in pregnant females exposed to hyperthermia.

Materials and Methods

Animals

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

Female ICR (8–12 weeks old) and male BDF1 (>8 weeks old) mice were purchased from Charles River Japan (Yokohama, Japan). All animals were raised at 25 °C and 50% relative humidity (RH) with a 12 h light/12 h darkness photoperiod (lights on at 0600 h).

Chemicals

All reagents were purchased from Sigma Chemical Co., unless otherwise specified. Equine chorionic gonadotrophin (eCG; Serotrophin) and human chorionic gonadotrophin (hCG; Gonatrophin) were purchased from Teikokuzouki Pharmaceutical Co. (Tokyo, Japan).

Superovulation, TA administration and heat exposure

Females were superovulated by i.p. injection of eCG (5 IU), followed by hCG (5 IU), 48 h apart. Then, each female was mated with a male during the dark phase of the light/darkness cycle. At the time of start of mating, females were injected intraperitoneally with TA dissolved in peanut oil (0.01% w/v) at 1 g/kg body weight) or with the oil alone (placebo control). Mating was evaluated by the presence of a vaginal plug at 0600 h the next morning (day 1 of pregnancy). The mated mice in each group were then heat stressed (35 °C, 60% RH) between 0600 and 1800 h on day 1 (heat stress) or were kept at 25 °C and 50% RH (Control). The rectal temperature of each mouse was measured in vivo (Matsuzuka et al. 2005b). On the other hand, melatonin is a water-soluble hormone, and it was necessary to inject it six times at 2-h interval to achieve a beneficial effect (Matsuzuka et al. 2005b). In addition, several studies have pointed out adverse effects of exogenous melatonin administration; melatonin injections in adult females disrupt the normal oestrous cycle by disturbing the secretion of LH-releasing hormone, resulting in low fertility in rats (Walker et al. 1982, Rivest, 1987). In contrast, since TA is a lipid-soluble vitamin, the effects of TA administration are longer lasting and even a single dose can be effective. For example, an injection of TA in dairy cattle increases the TA concentration in the milk for 6 days (Hidiroglou 1989). Furthermore, there are many results concerning TA administration in mammals, including human patients, with the aim of reducing physiological damage induced by oxidative stress, for example, in cancer (Valko et al. 2006), pre-eclampsia (Holmes & McCance 2005) and diabetic (Elliott et al. 1993) patients. Therefore, TA may be a beneficial and practical countermeasure against maternal hyperthermia-induced oxidation syndrome, including pre-implantation embryo mortality.

In conclusion, maternal heat exposure enhances physiological oxidative stress, and TA administration to hyperthermic mice can alleviate hyperthermia-induced pre-implantation embryo death by reducing the elevated physiological oxidative stress. These findings may help in the development of a new and practical strategy to overcome embryo mortality in pregnant females exposed to hyperthermia.
Embryo collection and evaluation of developmental ability in vitro

Mated females were killed by diethyl ether anaesthesia, followed by decapitation at the end of the temperature treatment (1800 h). Embryos were immediately recovered from the killed mother by flushing the oviducts with potassium simplex optimised medium (KSOM) and cultured as a group from each female in 50 µl KSOM under mineral oil at 37 °C in 5% CO₂, in a humidified incubator for 84 h, and the blastocyst developmental rate was observed. In addition, blastocyst-stage embryos were fixed with acetic alcohol (1:3) in whole-mount preparations for more than 48 h. Then, the fixed blastocysts were stained with 1% (w/v) aceto-orcein and the total cell number in each blastocyst was scored. The mean blastocyst development rate or cell number of the blastocysts in a group from each female was designated as one replicate, and nine (Control or Heat+ TA group) or ten (heat group) replicate trials were carried out.

Assay for glutathione in zygotes

Another set of zygotes from each treatment group was used for GSH measurements. Immediately after recovery from the oviduct, zygotes were washed five times with GSH assay buffer (0.2 M phosphate buffer (pH 7.4), 10 mM Na₂-EDTA). Then, the zygotes were sampled in groups of 15–20 in 5 µl GSH assay buffer plus 5 µl of 1.25 M phosphoric acid, and stored at −80 °C until assayed. Intracellular GSH concentrations in embryos were measured according to Anderson (1985), with some modifications. Briefly, 350 µl assay buffer that contained 0.33 mg/ml β-NAD (reduced form, NADPH), 50 µl of 6 mM 5,5′-dithio-bis-2-nitrobenzoic acid and 90 µl distilled water were added to the sample tube and mixed. After warming at room temperature for 15 min, 5 µl of 125 U/ml glutathione reductase were added to the reaction mix to start the reaction. Absorbance at 412 nm was recorded six times at 30-s interval using a spectrophotometer (u.v.-160A; Shimadzu Co., Kyoto, Japan). The GSH standards and a blank were also assayed under the same conditions. Eight replicate trials were conducted.

Measurements of plasma and liver TA contents by HPLC

Quantification of TA in plasma and liver was performed by HPLC methods using a u.v. detector (Et al 1991) with some modifications. Plasma and livers were collected from the killed mice. Collected plasma (0.5 ml) was mixed with 0.5 ml internal standard solution (2,2,5,7,8-pentamethyl-6-chromanol in isopropyl alcohol) and 1 ml absolute ethyl alcohol, and was then used for the following TA extraction procedure. Approximately, 0.1 g liver was homogenised on ice in 0.5 ml internal standard solution. Then, 1 ml ethyl alcohol that contained 3% (w/v) pyrogallol and 50 µl of 1% (w/v) NaCl were added into the homogenate and heated at 70 °C for 2 min. Subsequently, 100 µl of 60% (w/v) KOH were added to the sample and heated at 70 °C for 30 min. Liver samples were then snap-cooled on ice and 2 ml of 1% (w/v) NaCl were added, and this mixture was used in the following TA extraction procedure.

Extraction of TA from plasma or liver samples was carried out by adding 1.5 ml ethyl acetate:n-hexane (1:9) to each sample, vortexing for 5 min and centrifuging at 910 g for 5 min. After centrifugation, 1.3 ml supernatant that contained the TA were collected in another tube, 1 ml isopropanol was added and then the solvent was removed by vacuum extraction at 35 °C. The sample was reconstituted in 100 µl of 100% methanol and injected into the HPLC. The HPLC system was equipped with dual pumps (DP-8020; Tosoh Corp., Tokyo, Japan), a reverse-phase column (4.6×250 mm, TSK gel ODS-80Ts; Tosoh), and a u.v. detector (u.v.-8020; Tosoh). TA was detected at a wavelength of 290 nm and the peak heights were evaluated using a computer integrator (n=7 for each treatment).

Urinary 8-OHdG and creatinine measurements

The other sets of mice were used for measurement of urinary 8-OHdG levels. Each female mouse was housed in a metabolic cage (CM-105; CLEA Japan, Tokyo, Japan) without superovulation treatment or mating, and exposed to heat stress for 36 h or not subjected to heat stress. During temperature treatment, mice were fed Lab Animal Diet (0.75 g/animal per day; MF, Oriental Yeast Co., Tokyo, Japan) and urine was collected three times at 12-h interval. Water was available ad libitum. TA was administered once to the females at the same dose and for the same time as in the earlier experiment in the present study. Collected urine was centrifuged (1000 g for 15 min at room temperature) and the supernatants were stored at −80 °C until analysis. The 8-OHdG levels in the collected urine were measured with a commercial 8-OHdG ELISA kit (Japan Institute for the Control of Aging, Shizuoka, Japan), in accordance with manufacturer’s protocol, using a microplate reader (Model 680; Bio-Rad Laboratories) at 450 nm. Urinary creatinine was also measured by Jaffe’s reaction (Bartels et al. 1972). Briefly, 100 µl deproteinised urine sample were added to 700 µl alkaline picric solution (saturated picric acid solution, distilled water and 2.5 M NaOH; v:v:v=5:5:2), mixed well, and incubated for 15 min at room temperature. The absorbance at 500 nm of each sample was measured using a spectrophotometer. Creatinine standards and a blank were also assayed under the same conditions. The urinary 8-OHdG levels were standardised by the creatinine content of each sample. Six replicate trials were conducted.

Statistical analysis

Data are expressed as means± S.E.M. All percentage data were initially arcsin-transformed. The rectal temperature, embryo developmental status, intra-embryo GSH and TA content were analysed using one-way ANOVA, followed by Tukey’s multiple comparisons test. Changes in urinary 8-OHdG concentrations were analysed using the ANOVA for repeated measures, followed by Tukey’s multiple comparisons test.

Acknowledgements

The authors thank Dr H Honda for general advice concerning the use of the fluorescence microplate reader, and Ms K Neath for proofreading. Part of this study was supported by
grant-in-aid (no. 16658102) from The Japan Society for the Promotion of Science to Y K. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Vitamin E mitigates embryo death 495

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