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Effects of heat stress on bovine preimplantation embryos produced *in vitro*

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Abstract. Summer heat stress decreases the pregnancy rate in cattle and has been thought to be associated with the early embryonic death caused by the elevation of maternal body temperature. *In vitro* cultures have been widely used for the evaluation of effects of heat stress on oocytes, fertilization, preimplantation, and embryonic development. Susceptibility to heat stress is present in developmental stages from oocytes to cleavage-stage (before embryonic gene activation, EGA) embryos, leading to a consequent decrease in developmental competence. On the other hand, advanced-stage embryos such as morula or blastocysts have acquired thermotolerance. The mechanism for the developmental stage-dependent change in thermotolerance is considered to be the accumulation of antioxidants in embryos in response to heat-inducible production of reactive oxygen species. The supplementation of antioxidants to the culture media has been known to neutralize the detrimental effects of heat stress. Besides, EGA could be involved in acquisition of thermotolerance in later stages of embryos. Morulae or blastocysts can repair heat-induced unfolded proteins or prevent DNA damage occurring in processes such as apoptosis. Therefore, embryo transfer (ET) that can bypass the heat-sensitive stage could be a good solution to improve the pregnancy rate under heat stress. However, frozen-thawed ET could not improve the pregnancy rate as expected. Frozen-thawed blastocysts were more sensitive to heat stress and showed less proliferation upon heat exposure, compared to fresh blastocysts. Therefore, further research is required to improve the reduction in pregnancy rates due to summer heat stress.

Key words: Bovine, Heat stress, *In vitro* culture, Oxidative stress, Preimplantation embryo

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Recently, global warming has had an impact worldwide. The elevation of ambient temperature, especially in summer, is a critical issue in the agricultural industry. Livestock productivity is also affected by summer heat stress, indicated by reduction in milk yield, dietary intake, daily gain, and reproductivity. One of the most important effects of heat stress in the livestock industry is the decrease in cattle reproductivity. It has been reported since the 1990s that the pregnancy rate by artificial insemination (AI) in dairy cattle dramatically decreased in the summers [1–5]. A major reason for this adverse effect is considered the elevation of maternal body temperature, which disrupts the ovarian and uterine functions [4, 6–12]. Besides, it is well known that heat stress induces embryonic mortality [13–21]. Elevation of maternal body temperature affects various aspects related to pregnancy such as secretion of reproductive hormones, oocyte quality, success of fertilization, and embryo development [5, 7–9, 12, 16, 21]. *In vitro* embryo production technique enables us to artificially culture oocytes and embryos at any temperature and evaluate the mechanism of heat stress-induced early embryonic death in detail. Gendelman *et al.* had reported that the *in vitro* culture model is reliable and would be relevant for

evaluating the effect of heat stress on oocytes and embryos [22]. Therefore, many *in vitro* studies have been performed, through the period from oocyte maturation to preimplantation development, by mimicking the maternal body temperature in summer.

Moreover, it has been suggested that heat stress not only affects cell function directly, but also disrupts the DNA or organellar functions by inducing oxidative stress [12, 23, 24]. Oxidative stress agents like reactive oxygen species (ROS) induce DNA damage (leading to apoptosis), lipid peroxidation, and disrupt the mitochondrial function, causing abnormal gene expression and protein synthesis and finally resulting in cell death [23, 24]. Therefore, one factor that adversely affects oocytes and embryos has been considered to be the increase of oxidative stress originating from heat stress [25–27]. Besides, the change from maternal gene expression to embryonic gene expression could be associated with thermotolerance in oocytes and embryos. Therefore, this review discusses the stage-dependent change in thermotolerance in relation to oxidative stress and gene expression.

Effect of Heat Stress on Preimplantation Embryos

Effect of heat stress on oocyte maturation

It has been suggested that exposing cows to heat stress reduces the oocyte quality and affects subsequent embryo development [12, 22, 28–30]. To understand the mechanism underlying the effect of heat stress on germinal vesicle (GV)-stage cumulus-oocyte complexes (COCs), *in vitro* studies were widely performed. GV-stage COCs exposed to high temperatures from 40.0°C to 41.0°C show impaired oocyte nuclear and cytoplasmic maturation, increased abnormal

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spindle formation, and decreased developmental competence after IVF [12, 31–38]. Thus, heat-stressed oocytes fail to mature and are arrested at the metaphase I stage [12, 32]. Some reports suggest that oocytes are more susceptible to the elevation of temperature during the first 12 h of maturation because the exposure of oocytes to heat stress would hasten the progress of cytoplasmic and nuclear maturation [33–35, 39].

Heat stress in GV-stage COCs compromises oocyte functions and induces apoptosis [34, 35, 40, 41], disrupts cytoskeleton components [34], and alters maternal transcription [12, 22, 42], ATP content [43], and mitochondrial functions. Heat stress changes not only oocyte function, but also cumulus cell functions such as matrix metalloproteinase 9 (MMP9) and progesterone secretion [42]. These changes affect the oocyte's protein synthesis and disrupt the oocyte maturation and further development [12, 42, 43].

Heat stress also induces oxidative stress leading to elevation in ROS levels in oocytes [12, 25, 40, 44]. ROS damage the DNA and induce apoptosis or dysfunction of cellular organelles such as the mitochondria [12]. Thus, ROS production in heat-stressed oocytes would disrupt oocyte quality and viability. However, researchers showed that supplementation of antioxidants during *in vitro* maturation at high temperature improved oocyte maturation and developmental competence of embryos [12, 25, 40]. Glutathione (GSH), a thiol-containing peptide, is a major antioxidant component in the oocyte stage that scavenges ROS [25, 45]. Some antioxidants increased the glutathione levels and decreased ROS levels of oocytes exposed to heat stress. This indicates that the administration of antioxidants in heat stress conditions could decrease thermal-oxidative stress in oocytes and improve fertility.

Effect of heat stress on in vitro fertilization

In vivo studies suggest that there is a correlation between the ambient temperature on the day of AI and the conception rate [4, 7, 18, 46]. In the case of higher body temperature or ambient temperature on the day of insemination, lower pregnancy rates were observed [4, 18, 46]. *In vitro* studies have also reported that high temperature during fertilization reduced embryonic competence [37, 44, 47]. However, it is difficult to reveal how heat stress affects the success of fertilization, which involves two factors (oocyte and sperm) that could both be damaged by the high temperature.

As mentioned, oocyte quality is reduced by heat stress. It has also been reported that sperm motility, integrity, and function are reduced by the elevation of temperature [48–51]. Pre-incubation of sperm at 40.0°C to 42.0°C for 4 h decreased sperm motility and integrity, and increased sperm damage [47, 48, 51]. However, there was no adverse effect on developmental competence when oocytes were fertilized at normal temperature (38.5°C) with sperm pre-incubated at 40.0°C to 42.0°C for 4 h [48, 51].

On the other hand, *in vitro* fertilization performed at 40.0°C or 41.0°C for 6 h decreased both cleavage and blastocysts rates significantly [47]. There was no difference observed in sperm penetration rate but polyspermy was induced by heat stress [47]. These results indicate that the anti-polyspermy mechanism of oocytes could have been disrupted by heat stress and increase of polyspermy could be part of the reason for the disturbed embryonic development. Zona pellucida (ZP) and cortical granules have important roles in inhibit-

ing polyspermy in mammalian oocytes [52–55]. Zygotes exposed to high temperatures demonstrated changes in factors associated with prevention of polyspermy by the cortical granule migration by showing shorter ZP digestion time (weaker ZP-hardening) and lower *UCHL1* (Ubiquitin C-terminal hydrolase-L1) gene expression [47]. Thus, it can be suggested that heat stress could disrupt the anti-polyspermy mechanism, increase polyspermy, and lead to decreased developmental competence.

Besides the effects on the anti-polyspermy mechanism, direct damage of zygotes can also suppress fertilization success and developmental competence. Mouse zygotes exposed to heat stress showed higher ROS levels and reduced developmental competence [44, 56]. We also evaluated ROS levels and expression levels of genes related to heat stress (heat shock protein 70 (HSP70); *HSPA1*) in zygotes exposed to 40.0°C for 6 h during *in vitro* fertilization. It has been reported that the GSH content at the zygote stage is much lower than that at the oocyte stage, which suggests that zygotes would be more sensitive to heat stress. Therefore, the intensity of H₂O₂ emission, which reflects ROS content in zygotes, was higher in heat stress-fertilized zygotes [47]. Besides, *HSPA1* gene expression was induced by heat treatment.

During fertilization, it is likely that oocytes are more sensitive to heat-induced damage than sperm. Low fertilization rates might be due to sperm damage, whereas low embryonic competence could be due to the increase in polyspermy, zygote DNA damage, and changes in maternal transcript abundance associated with oxidative stress originating from heat stress.

Effect of heat stress on embryo development

Experimental exposure to heat stress during preimplantation development in maternal body revealed the reduction of the proportion of embryos developing to blastocyst stage [16, 18]. Preimplantation stage embryos have been recognized as stress-sensitive, and the early embryonic loss happens during preimplantation stages. The heat sensitivity of embryos is considered stage dependent [21, 26, 57]. Thus, early stage embryos such as the 1-to 8-cell stage embryo are more susceptible to elevated temperatures than advanced stage embryos such as morulae or blastocysts [26, 27, 58–60]. To a varying degree, heat exposure of 40.0°C to 42.0°C significantly decreased developmental competence of 1-to 8-cell stage embryos, but showed little to no effect at morula and blastocyst stages (Fig. 1) [26, 58, 59, 61]. Besides, even if they survived the initial heat stress, early stage embryos exposed to elevated temperatures showed a lower total cell number, especially a reduced trophectoderm cell number in blastocysts [26, 59, 62, 63]. Heat exposure of the early stage embryos causes microfilament and microtubule disruption and mitochondrial swelling, which results in organellar damage [64, 65]. Moreover, the elevation of temperature increases the number of apoptotic cells in 2-cell-stage embryos [66, 67]. Thus, this evidence indicates that heat stress directly damages early stage embryos and leads to decreased developmental competence.

On the other hand, oxidative stress originating from the elevation of temperature is another factor. ROS plays an important role in mediating deleterious effects of elevated temperature on *in vivo* embryonic development in mouse [44, 56]. Therefore, we exposed embryos at various stages (Days 0, 2, 4 and 6, Day 0 = the day of

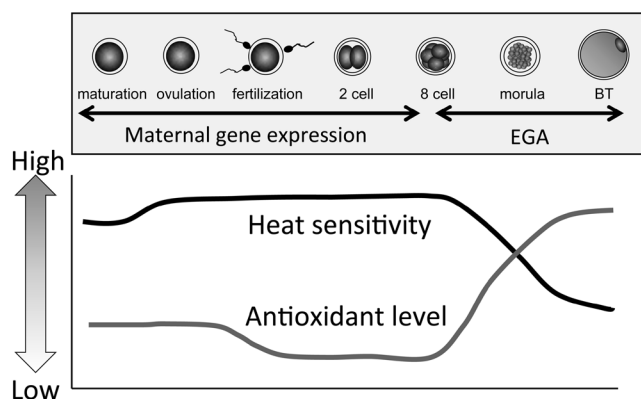


Fig. 1. Schematic illustration of thermotolerance dynamics during bovine preimplantation development (EGA, embryonic gene activation; BT, blastocysts).

fertilization) to a temperature of 41.0°C for 6 h *in vitro* and evaluated the embryonic development and oxidative stress level [26]. Embryos from heat-sensitive stage derived from Days 0 and 2 showed higher ROS levels and lower GSH levels by heat stress, but embryos in Days 4 and 6 had neither their ROS nor GSH levels affected by heat stress (Fig. 1). The amount of the antioxidant glutathione is low during early development and does not increase until the 9- to 16-cell stage [25, 45, 68]. Besides, 2- to 8-cell stage embryos exposed to heat stress in the presence of antioxidants did not show decreased developmental competence and their ROS level was similar to that of embryos not exposed to heat stress [62, 63, 69]. These results indicate that oxidative stress originating from the elevation of temperature could be the main factor inhibiting normal embryonic development and prevention of this increase in oxidative stress would result in the survival of embryos under heat stress.

Based on the aforementioned, the nature of the mechanisms involved in the stage-dependent thermotolerance and antioxidant function can be questioned. One possibility for the high susceptibility of early stage embryos to heat stress is that transcription is limited in this stage [70]. From the zygote to the 8-cell stage embryo, gene expression is of maternal (oocyte) origin; embryonic gene activation (EGA) starts after the 8-cell stage and increases dramatically after the morula stage (Fig. 1) [70]. HSP70, encoded by *HSPA1*, plays an important role as a molecular chaperone and in the prevention of apoptosis due to various stress conditions including heat stress [71]. Interestingly, HSP70 synthesis and *HSPA1* transcription increase in early stage embryos even though EGA does not occur by then [39, 72, 73]. Moreover, *HSPA1* transcription level is much higher in 2-cell embryos than in thermotolerant morula stage embryos [59, 67]. However, the fold change in the *HSPA1* mRNA level, of heat-stressed morulae is much higher (9.72-fold over morula control) than that of heat-stressed 2-cell stage embryos (1.83-fold over 2-cell control) [59]. An important point here is that HSPs can be divided into two categories: constitutive and inducible [74]. Constitutive HSPs are essential for normal proliferation and are expressed stably under unstressed conditions. On the other hand, expression of inducible HSPs is activated by stress conditions such as heat stress. Thus,

the higher *HSPA1* expression level in early stage embryos could be that of constitutive HSPs and might contribute less to the heat stress response. Inducible HSPs could be activated by heat stress and mediate the detrimental effect of temperature elevation in advanced stages such as the morula stage [75]. However, this interpretation of HSP70 expression in preimplantation embryos could be controversial. The precise function of HSPs in the heat stress response in embryos is still unclear and needs further consideration.

Global gene expression analysis in thermotolerant morula stage embryos revealed that the expression of some genes related to HSPs was induced by heat stress [75]. Antioxidant genes were less affected by heat stress in the morula stage as this heat stress did not change the ROS production in Days 4 and 6 embryos [26] and antioxidant glutathione levels increased in advanced stage embryos [25, 45]. In addition, expression of genes involved in ubiquitin pathways, which are critical for removal of protein damaged by external stress, was induced by heat stress [75]. Thus, thermotolerance of advanced stage embryos is due in part to developmental mechanisms that prevent accumulation of denatured proteins and oxidative stress damage.

Effect of heat stress on blastocyst

According to previous reports, advanced preimplantation embryos and blastocyst stage embryos acquire thermotolerance and thus would be less damaged on exposure to heat stress [25–27, 58, 76]. Therefore, an embryo transfer (ET) that can bypass the heat-sensitive stage (the oocyte maturation, fertilization, and early embryonic stage) is the most promising technology to improve the low pregnancy rate in summer [1, 27, 57, 77–80]. It has been reported that fresh superovulation or IVF-derived ETs improved the summer pregnancy rate in dairy cattle [17, 19, 79, 81–83]. Moreover, ET following AI improved the pregnancy rate of dairy cattle [81, 84]. On the other hand, the quality and the number of embryos derived from superovulation decreased under heat stress conditions [15, 16, 18]. To produce embryos for fresh ET in summer would be inefficient and the utilization of fresh ET must be limited. Therefore, some researchers have evaluated cryopreserved ETs derived from superovulation or IVF. However, the efficacy of cryopreserved ETs in improving summer conception rate in dairy cattle could be controversial or may not be observed [20, 81, 82]. These aspects can be an obstacle to the dissemination of ET techniques to improve the low pregnancy rate in summer.

In consideration of these findings, we hypothesized that the cryopreserved blastocysts will be more susceptible to heat stress than fresh blastocysts, possibly owing to thermotolerance. Therefore, the effect of heat stress treatment on viability and gene expression of frozen-thawed cryopreserved blastocysts was evaluated. Firstly, the morphology and gene expression of Day 7 blastocysts exposed to 41.0°C for 6 h were evaluated. Even though *HSPA1* expression was induced, expression of blastocyst-specific genes *POU5F1* and *IFNT* and survival rates of embryos were not affected by heat exposure (Fig. 2) [85]. However, the second experiment showed that the viability and the diameter of embryos highly decreased during heat exposure to 41.0°C for 6 h after thawing cryopreserved blastocysts (Fig. 2). Besides, *HSPA1* expression was dramatically induced (Fig. 2) and *IFNT* expression decreased in heat-exposed cryopreserved blastocysts [85]. Thus, these results indicate that the thermotolerance of cryopreserved blastocysts reduces in the process of freezing.

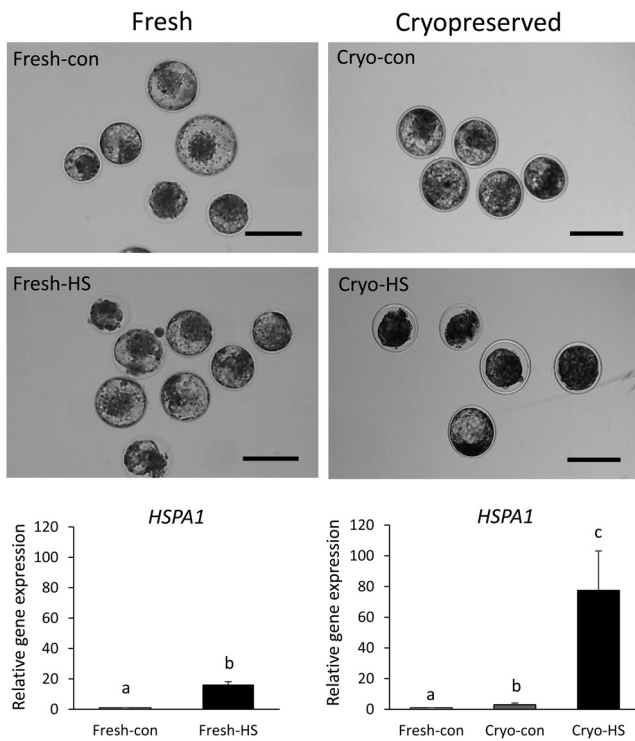


Fig. 2. Representative images and *HSPA1* gene expression of blastocysts exposed to heat stress with or without cryopreservation. Fresh-con: blastocysts incubated at 38.5°C for 6 h from 174 hpi (hours post insemination); Fresh-HS: blastocysts incubated at 41.0°C for 6 h from 174 hpi; Cryo-con: blastocysts incubated at 38.5°C for 6 h after thawing; Cryo-HS: blastocysts incubated at 41.0°C for 6 h after thawing. Scale bar, 200 μ m. Relative gene expression (Fresh-con = 1) of *HSPA1* gene (mean \pm SE). Different letters above bars indicated $P < 0.05$. Modified from [85].

Heat stress damage after ET could cause a low conception rate in blastocysts subjected to cryopreserved ET during the summer. Vasques *et al.* suggested that heat stress after cryopreserved ET retarded trophoblastic function, leading to embryo death [81]. This report agreed with our result that heat-exposed frozen-thawed blastocysts showed lower *IFNT* expression. Therefore, it is necessary to improve cattle pregnancy in summer by utilizing the ET technique to produce more thermotolerant cryopreserved embryos.

On the other hand, vitrification has been considered a less cryo-damaging technique than the slow-freezing cryopreservation techniques, because vitrification can eradicate the damage due to ice crystal formation [86]. The viability of embryos after thawing is higher when derived from vitrification than from slow-freezing [86]. Therefore, some researchers applied vitrified ET for improving low summer conception rate in dairy cattle. The vitrified ET improved summer conception rate compared to AI [87]. However, it is still not comparable to fresh ET technique, and the vitrification technique has not become widespread for domestic animals due to challenges in handling observed during ET. Therefore, further studies on such culture methods or freezing processes are needed to apply the cryopreserved (slow-freezing and vitrified) ET to improve the

low summer conception rate.

Conclusion and Future Perspectives

Previous studies showed that increase of antioxidant capacity by the supplementation of various antioxidants or chemicals improved oocyte and embryo quality and improved developmental competence under heat stress conditions [12, 25, 40, 41, 45, 60, 62, 63, 69, 88]. Chemical treatments could induce thermotolerance in oocytes or embryos by increasing HSP70 synthesis or anti-apoptosis functions [12, 88]. Modification of embryo cryopreservation could improve the pregnancy rate by employing *in vitro* frozen ET. It is likely that culturing blastocysts with some cryoprotectants before freezing or using vitrification could increase the cryotolerance, improve embryo survival after thawing [89–91], and increase the ET pregnancy rate in summer [87]. Moreover, the genetic differences affecting thermotolerance in oocytes or embryos have been reported between breeds of cattle [38, 80, 92]. Recently, global gene analysis has been applied for domestic animals to reveal differences in single nucleotide polymorphisms (SNPs) and expression of specific genes associated with thermotolerance or fertility in breeds [74, 80]. Studies like these could provide more information on the physiological impacts of heat stress on embryonic developmental competence and could utilize cattle selection or new techniques to improve fertility under heat stress conditions.

In conclusion, heat stress shows its adverse effects through the period from oocyte maturation to preimplantation development. Most of the detrimental effects of heat stress could be due to the oxidative stress originating from the elevation of temperature (Fig. 1). Heat stress and oxidative stress could damage organelles directly or induce DNA damage (leading to apoptosis), alter gene expression, and reduce developmental competence in preimplantation embryos. Oocytes, zygotes, and early stage embryos have low potential for scavenging ROS emissions and cannot mediate heat stress. On the other hand, it is likely that the advanced stage embryos after EGA would have higher ROS scavenging ability and higher ability for the repair of DNA damage or unfolded proteins. Although a number of reports provide proof of the effects of heat stress on preimplantation embryos, further studies would be required to reveal the mechanism underlying thermotolerance and develop techniques to improve the low pregnancy rate due to heat stress.

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