Journal of Reproduction and Development, Vol. 53, No. 3, 2007

—Full Paper—

Effects of Purple Sweet Potato Anthocyanins on Development and Intracellular Redox Status of Bovine Preimplantation Embryos Exposed to Heat Shock

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Abstract. The development of cleavage stage preimplantation embryos is disrupted by exposure to heat shock, such as high temperatures in the summer season. In this study, we investigated whether addition of anthocyanins, which are strong scavengers of reactive oxygen species (ROS), improves development and intracellular redox status of heat-exposed bovine preimplantation embryos by reduction of heat shock-derived oxidative stress. After *in vitro* fertilization (IVF), embryos were cultured at 38.5 C through Day 8 (Day 0=day of IVF) with 0, 0.1, 1 and 10 μg/ml anthocyanins (non-heat-shocked group). On Day 2, embryos were cultured at 41.5 C for 6 h with 0, 0.1, 1 and 10 μg/ml anthocyanins followed by culture at 38.5 C until Day 8 (HS group). After exposure to heat shock, the intracellular ROS and glutathione (GSH) contents of individual embryos were measured in the non-heat-shocked and HS groups using fluorescent probes. On Day 8, the blastocysts formation rates of the embryos and total cell numbers of blastocysts were evaluated. Embryos exposed to heat shock without anthocyanins showed a significant decrease in blastocyst formation rate and GSH content ($P<0.05$) and an increase in intracellular ROS ($P<0.05$) compared with non-heat-shocked embryos. In contrast, addition of 0.1 μg/ml anthocyanins significantly ($P<0.05$) improved the blastocyst formation rate of the heat-shocked embryos. Addition of any dose of anthocyanins produced a significant decrease in the ROS levels ($P<0.05$) and tended to increase the GSH levels under heat-shock conditions. However, addition of higher concentrations (1 and 10 μg/ml) of anthocyanins to the culture media under heat shock did not improve the development of embryos. These results indicate that anthocyanins maintain the intracellular redox balance of heat-shocked bovine embryos by reducing intracellular oxidative stress and increasing the GSH levels. Thus, alterations of the redox state using natural antioxidative polyphenols is a useful approach for reducing heat shock-derived oxidative stress.

Key words: Anthocyanins, Bovine *in vitro* culture (IVC), Glutathione (GSH), Heat shock, Reactive oxygen species (ROS)

(**J. Reprod. Dev. 53:** 605–614, 2007)

Fertility of cows is highly influenced by environmental conditions, especially high temperatures in the summer season [1, 2]. Body temperature increases to more than 41 C when cows are exposed to a hot environment [3, 4]. This high body temperature also affects the temperatures of reproductive organs such as the oviduct and uterus, in which development of embryos is achieved. Heat exposure to the maternal body of early pregnant animals significantly reduces the viability of embryos in

Accepted for publication: January 22, 2007
Published online: February 27, 2007
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mice [5, 6] and cows [2, 4, 7]. In particular, early stage embryos around Day 1 after fertilization are most sensitive to heat stress [4, 8] compared with later stage embryos. Therefore, an increase of body temperature in the early stages of embryos is detrimental to their development. *In vitro* studies have also confirmed the stage-specific sensitivity of development of embryos in mice [6] and cows [8–12]. Heat shock to early (1- to 8-cell) stage embryos significantly reduces the rate of blastocyst formation and total cell numbers of blastocysts [12] compared with later stage embryos. These *in vivo* and *in vitro* studies indicate the importance of study of the effects of heat stress responses on development of embryos and protection of embryos from heat stress. Evaluation of the effects of heat stress on embryos including the intracellular molecular and biochemical environments, is important for improvement of the fertility of cattle in the hot season.

Recent studies have revealed that intracellular reactive oxygen species (ROS) are responsible for the deleterious effects of heat shock on development of early stage embryos [6, 12]. Accordingly, it is important to reduce the mortality of early-stage embryos caused by heat shock by reducing intracellular ROS levels. Glutathione (GSH), a low molecular weight thiol, is well known to protect cells against ROS and maintain the intracellular redox balance. Addition of GSH reduces the toxic effects of heat shock on development of mouse morulae by scavenging free radicals [13]. Inhibition of the GSH synthesis of embryos decreases their development [14, 15]. Therefore, embryos may cease development due to excess ROS that might be a result of redox imbalance by depletion of intracellular GSH. Supplementation of embryo culture medium with extracellular antioxidants has been shown to reduce the generation of intracellular ROS due to heat shock. Furthermore, addition of antioxidants other than GSH to culture medium also improves development of embryos under normal temperature [16–19] or oxidative stress conditions [20].

Anthocyanins are vacuolar flavonoids that are widely contained in vegetables and fruits as natural pigments. Anthocyanins have also been reported to have various biological activities, including antioxidant activities, antimutagenicity and α-glucosidase inhibition [21–25]. Because of their electron deficiency, anthocyanins react very easily with ROS. Purple sweet potato, *Ipomoea batatas*, contains high concentrations of anthocyanins and is widely cultivated in the southwestern area of Japan for food, processed foods and drinks. Purple sweet potato anthocyanins are also known to be strong free radical scavengers [26, 27] that reduce high blood pressure and liver injuries [28].

The characteristics of purple sweet potato anthocyanins are highly resistant to changes in temperature and pH [28–31]. Moreover, anthocyanins are water soluble and easily incorporated into cells [31, 32]. For these reasons, anthocyanins may become one of the candidates for protection of cells from oxidative damage by addition to culture media.

In the present study, we investigated the effects of sweet potato anthocyanins on the development and intracellular redox status of *in vitro*-produced bovine embryos exposed to heat shock on Day 2, which is when they are most sensitive to heat stress [8, 12].

Materials and Methods

In vitro maturation

Bovine ovaries were collected from a local abattoir and washed in a sterile solution of saline containing 100 U/ml of penicillin and 100 μg/ml streptomycin (Nacalai Tesque, Kyoto, Japan). Cumulus-oocyte complexes (COCs) were aspirated from follicles (2–6 mm in diameter) using a 5-ml syringe attached to an 18-gauge needle and washed 3 times in TCM-199 (Gibco, Grand Island, NY, USA) containing 5% (v/v) fetal calf serum (FCS, Gibco). Fifty COCs were matured in a 500 μl drop of TCM-199 containing FSH (0.02 IU/ml; Denka, Kawasaki, Japan), estradiol-17β (1 μg/ml; Sigma-Aldrich, St. Louis, MO, USA), and gentamicin (10 μg/ml, Sigma-Aldrich) covered with mineral oil (Sigma-Aldrich) for 22 h at 38.5 C in a humidified atmosphere of 5% CO_2 in air.

In vitro fertilization

Frozen semen was thawed by immersing it in warm water (37 C) for 20 sec. Sperm were washed twice by centrifugation (700 g for 5 min) with Brackett and Oliphant's (BO) solution [33] containing 10 mM caffeine (Sigma-Aldrich) and 4 U/ml heparin (Nacalai tesque) without bovine

serum albumin (BSA; Nacalai tesque). After removing the supernatant, the sperm pellet was diluted with BO solution to prepare the final concentration of 5 mM caffeine, 2 U/ml heparin, and 5 mg/ml BSA at a concentration of 1×10^7 cells/ml.

After maturation, COCs were washed 3 times with BO solution containing 5 mM caffeine and 10 mg/ml BSA and then transferred into drops of sperm suspension (20 oocytes/100 μl drop). Fertilization was carried out for 6 h at 38.5 C in a humidified atmosphere of 5% CO_2 in air.

Preparation of anthocyanins

Preparation of anthocyanins was achieved by following the methods of Suda *et al*. [34]. Purple sweet potato (*Ipomoea batatas* cv. Ayamurasaki) tubers were washed with tap water, peeled and heated at 90 C for 10 min. The heated flesh was ground in the same weight of water using a Super Masscolloider (Masuko Sangyo, Saitama, Japan) and centrifuged. The resultant sweet potato juice was treated with a enzyme solution containing amylase, cellulase, hemicellulase and pectinase at 50 C for 60 min and centrifuged. The supernatant was concentrated by a continuous evaporator and stored at –20 C until use. The concentrated sweet potato extract was acidified in 4 volumes of 1% acetic acid solution and put directly onto a Diaion HP-20 (Nippon Rensui, Tokyo, Japan) column (10 cm I.D. × 100 cm) directly. After washing with 15 l of distilled water, the anthocyanin fraction was eluted with 5 l of a 70% ethanol solution. The ethanolic elute was concentrated to 18° Brix under reduced pressure at 35 C and then freeze-dried. The extract of purple sweet potato contained anthocyanin at a concentration of 124.4 mg peonidin 3-caffeoylsophoroside-5-glycoside (Pn 3-Caf·sop-5-glc) equivalent/g, calculated with a molar extinction coefficient of 2.76×10^4/M/cm in 1% trifluoroacetic acid solution. Eight acylated anthocyanins have been identified in the Ayamurasaki cultivar; Pn 3-Caf·sop-5-glc has been identified as the major anthocyanin [26].

For embryo culture, anthocyanins stock solution was diluted with CR1aa [35] containing 5% (v/v) FCS to final concentrations of 0.1, 1 and 10 μg/ml.

Embryo culture and heat shock treatment

After insemination, the cumulus cells surrounding putative zygotes were removed by pipetting. The putative zygotes were cultured at 38.5 C until Day 8 (Day 0=day of IVF) in CR1aa supplemented with 5% FCS and 0, 0.1, 1 and 10 μg/ml of anthocyanins. The embryos of heat-shocked group were exposed to heat shock on Day 2, 42 h postinsemination (hpi), which is the stage most sensitive to heat stress [8, 12]. Heat shock treatment was effected by culturing the embryos at 41.5 C with or without anthocyanins which mimicks the body temperature of cows [3, 4]. Embryos cultured at 41.5 C were returned to 38.5 C after 6 h. The rate of cleavage was evaluated on Day 2 before heat shock treatment. The rates of blastocyst formation and the total cell numbers of blastocysts were evaluated on Day 8, respectively. A humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 was used for all treatments and cultures.

Differential cell staining

The quality of blastocysts was determined by differential staining of the inner cell mass (ICM) and trophectoderm (TE) according to the method described by Thouas *et al*. [36]. In brief, blastocysts were treated with 0.1 mg/ml propidium iodide (Molecular Probes, Eugene, OR, USA) in 2% (v/v) Triton X-100 for 1 min and then stained with 25 μg/ml bisbenzimide (Sigma-Aldrich) in 99.5% EtOH at 4 C for 3 h. After rinsing in glycerol, the stained blastocysts were mounted onto a glass slide, flattened with a cover slip, and observed with a fluorescence microscope. The nuclei of the ICM were stained blue with bisbenzimide, and the nuclei of TE cells were stained pink with both bisbenzimide and propidium iodide.

Measurement of ROS and GSH content

On 48 hpi, non-heat-shocked and heat-shocked 8- to 16-cell stage embryos were immediately sampled to determine the intracellular ROS and GSH contents. To measure the ROS content, embryos were transferred to a drop containing 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Molecular Probes) in CR1aa with 5% FCS for 30 min at 38 C in the dark [37]. After incubation, the embryos were washed with PBS(–) and transferred to an 8-well chamber slide glass (Nunc, Rochester, NY, USA) with PBS(–). Green fluorescence was detected for oxidized H_2DCFDA using a fluorescence microscope (Nikon, Tokyo, Japan) with an FITC filter (460 nm). Fluorescence intensity was measured using image analysis software (Atto

Table 1. Effects of heat shock and anthocyanins on development of embryos

Concentration of anthocyanins		No. of replicates	No. of oocytes	% of embryos developed (mean ± SEM)		
				Cleaved	Blastocysts	>Expanded
38.5 C	0 μg/ml	6	136	71.7 ± 4.1	37.7 ± 4.1 [a]	21.7 ± 5.8 [a]
	0.1 μg/ml	5	120	72.9 ± 5.0	33.3 ± 7.9 [a, b]	16.2 ± 6.2 [a, b]
	1 μg/ml	5	119	71.0 ± 3.6	21.5 ± 6.2 [b, c, d]	16.2 ± 6.1 [a, b]
	10 μg/ml	5	115	71.5 ± 3.6	25.6 ± 4.4 [a, b, c, d]	13.6 ± 3.9 [a, b]
41.5 C	0 μg/ml	6	91	66.1 ± 6.8	12.2 ± 3.7 [d]	3.3 ± 1.7 [b]
	0.1 μg/ml	6	89	80.5 ± 4.5	28.7 ± 4.7 [a, b, c]	10.0 ± 3.8 [a, b]
	1 μg/ml	6	97	71.3 ± 5.2	18.8 ± 3.6 [b, c, d]	10.0 ± 3.8 [a, b]
	10 μg/ml	6	87	74.1 ± 4.6	17.1 ± 4.1 [c, d]	10.0 ± 3.8 [a, b]

[a, b, c, d] Different superscripts denote significant differences ($P<0.05$).

Table 2. Effect of anthocyanins and heat shock on cell numbers of blastocysts

Concentration of anthocyanins		No. of replicates (blastocysts)	No. of cells (mean ± SEM)			TE/ICM Ratio (mean ± SEM)
			ICM	TE	Total	
38.5 C	0 μg/ml	4 (19)	23.3 ± 1.4	88.9 ± 8.2	112.2 ± 9.4	3.77 ± 0.23
	0.1 μg/ml	4 (11)	27.2 ± 3.4	78.3 ± 4.6	108.0 ± 4.7	3.39 ± 0.42
	1 μg/ml	4 (19)	31.3 ± 2.9	79.9 ± 7.5	111.3 ± 9.3	2.70 ± 0.23
	10 μg/ml	4 (19)	26.2 ± 1.8	89.7 ± 8.7	115.9 ± 9.6	3.51 ± 0.28
41.5 C	0 μg/ml	4 (13)	23.4 ± 2.8	67.2 ± 8.7	89.7 ± 10.8	3.01 ± 0.25
	0.1 μg/ml	4 (12)	34.9 ± 2.7	74.9 ± 5.2	109.8 ± 5.7	2.30 ± 0.22
	1 μg/ml	4 (18)	30.0 ± 3.2	74.4 ± 7.1	104.4 ± 9.2	2.67 ± 0.25
	10 μg/ml	4 (18)	33.1 ± 3.3	67.6 ± 6.0	100.6 ± 8.1	2.20 ± 0.20

Densitograph; Atto, Tokyo, Japan). The fluorescence emission of each experimental group was calculated and normalized to that of non-heat-shocked embryos.

CellTracker Blue CMF_2HC (4-chloromethyl-6,8-difluoro-7-hydroxycoumarin; Molecular Probes) was used to detect the intracellular GSH levels of living cells as a blue fluorescence [38]. Embryos were incubated for 30 min with CR1aa without FCS and were supplemented with 10 μM CellTracker at 38 C in the dark. The embryos were then incubated for 30 min with CR1aa supplemented 5% FCS at 38 C in the dark. After rinsing with PBS(–), the embryos were placed onto an 8-well chamber slide glass (Nunc) and their fluorescence was observed by fluorescent microscope with a UV filter (370 nm). Fluorescence density was measured as described above.

Statistical analysis

Data were expressed as means ± SEM. Differences among the treatments were analyzed by ANOVA using StatView (version 5.0; Abacus Concepts, Berkeley, CA, USA). Percentage data were subjected to arcsine and logarithmic transformation, respectively, before statistical analysis. When ANOVA indicated a treatment effect with a probability of less than 0.05, the data were analyzed by Fisher's protected least-significant difference test.

Results

Effect of anthocyanins on development of heat-shocked embryos

A concentration of anthocyanins of less than 0.1 μg/ml was evaluated in a previous experiment. However, there was no effect on the development of embryos in both non-heat-shocked and heat-shocked embryos. Therefore, we excluded that concentration in this study and evaluated concentrations of more than 0.1 μg/ml anthocyanins. Treatment with various concentrations of anthocyanins had no effect on cleavage of the embryo (Table 1). Compared with

embryos cultured at 38.5 C without anthocyanins, the blastocyst formation rates were significantly decreased in the heat-shocked embryos ($P<0.05$; Table 1). However, addition of 0.1 μg/ml anthocyanins to heat-shocked embryos significantly improved the blastocyst formation rate compared with heat-shocked embryos without anthocyanins ($P<0.05$). Blastocyst formation rates were also decreased by supplementation with high concentrations (1 and 10 μg/ml) of anthocyanins in non-heat-shocked embryos cultured at 38.5 C (Table 1).

Effects of heat shock and anthocyanins supplementation on the total cell number and TE/ICM ratio of blastocysts

Although there was no significant difference, heat shock tended to decrease the total cell numbers of blastocysts without anthocyanins (Table 2). However, there were no differences in total cell number among the embryos cultured at 41.5 C with anthocyanins. Neither heat shock nor anthocyanins affected the TE/ICM ratio.

Effects of anthocyanins on the intracellular ROS and GSH of heat-shocked embryos

The relative fluorescence emission of ROS was significantly increased ($P<0.05$) by 6 h of heat shock treatment without anthocyanins (Table 3). In contrast, addition of any concentration of anthocyanins in the study significantly ($P<0.05$) decreased the ROS levels of the heat-shocked embryos (Table 3 and Fig. 1). Moreover, the embryos cultured with 10 μg/ml anthocyanins at 38.5 C had significantly decreased ROS levels compared with the non-heat-shocked embryos cultured without anthocyanins.

Compared with embryos cultured at 38.5 C, the GSH level was significantly ($P<0.05$) decreased in the heat-shocked embryos without anthocyanins.

Table 3. Effect of anthocyanins on the intracellular ROS levels of embryos exposed to heat shock on day 2 after fertilization

Concentration of anthocyanins		No. of replicates	No. of embryos	Relative fluorescence intensity (mean ± SEM)
38.5 C	0 μg/ml	4	16	1.00 ± 0.00 [a]
	0.1 μg/ml	4	14	0.77 ± 0.20 [a, b]
	1 μg/ml	4	12	0.81 ± 0.15 [a, b]
	10 μg/ml	4	14	0.67 ± 0.05 [b]
41.5 C	0 μg/ml	4	12	1.56 ± 0.11 [c]
	0.1 μg/ml	4	16	0.69 ± 0.10 [a, b]
	1 μg/ml	4	14	0.92 ± 0.13 [a, b]
	10 μg/ml	4	13	0.70 ± 0.12 [a, b]

[a, b, c] Different superscripts denote significant differences ($P<0.05$).

Table 4. Effect of anthocyanins on the intracellular GSH levels of embryos exposed to heat shock on day 2 after fertilization

Concentration of anthocyanins		No. of replicates	No. of embryos	Relative fluorescence intensity (mean ± SEM)
38.5 C	0 μg/ml	6	31	1.00 ± 0.00 [a]
	0.1 μg/ml	6	25	1.08 ± 0.10 [a]
	11 μg/ml	6	24	1.24 ± 0.13 [a]
	10 μg/ml	6	27	1.12 ± 0.10 [a]
41.5 C	0 μg/ml	6	31	0.78 ± 0.05 [b]
	0.1 μg/ml	6	25	1.01 ± 0.11 [a, b]
	1 μg/ml	6	23	0.92 ± 0.05 [a, b]
	10 μg/ml	6	25	0.96 ± 0.07 [a, b]

[a, b] Different superscripts denote significant differences ($P<0.05$).

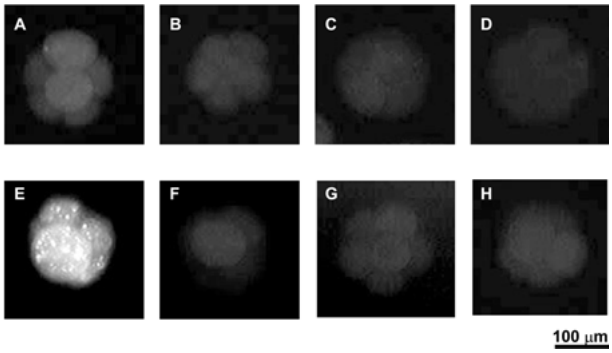

Fig. 1. Effect of anthocyanins on generation of intracellular ROS in 8–16 cell stage embryos after heat shock treatment. Fluorescent photomicrographs of 8–16 cell stage embryos detected with 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA). Non-heat-shocked 8–16-cell stage embryos cultured at 38.5 C with 0 (A), 0.1 (B), 1 (C) or 10 μg/ml anthocyanins (D). Heat-shocked 8–16-cell stage embryos exposed to 41.5 C for 6 h on day 2 after fertilization with 0 (E), 0.1 (F), 1 (G) or 10 μg/ml anthocyanins (H).

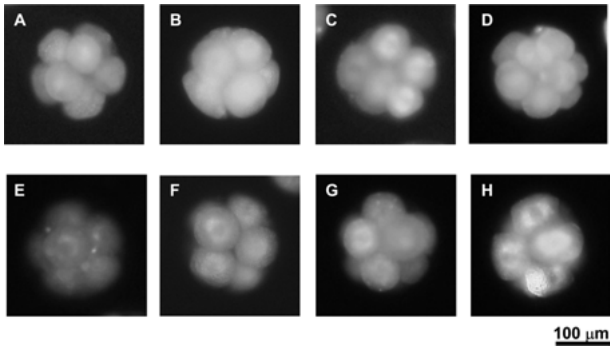

Fig. 2. Effect of anthocyanins on the intracellular GSH levels of 8–16 cell stage embryos after heat shock treatment. Fluorescent photomicrographs of 8-cell stage embryos detected with CellTracker Blue. Non heat-shocked 8–16-cell stage embryos cultured at 38.5 C with 0 (A), 0.1 (B), 1 (C) or 10 μg/ml anthocyanins (D). Heat-shocked 8–16-cell stage embryos exposed to 41.5 C for 6 h on day 2 after fertilization with 0 (E), 0.1 (F), 1 (G) or 10 μg/ml anthocyanins (H).

However, there were no significant differences in the GSH levels of the embryos cultured with 0.1, 1 and 10 μg/ml anthocyanins under heat shock conditions (Table 4 and Fig. 2).

Discussion

Cattle, especially dairy cows, are susceptible to heat shock, and their body temperatures elevate dramatically in the summer season [3]. This hyperthermia affects not only food intake and milk production but also reproduction [1]. During the summer season, the pregnancy rates of cattle decrease significantly [2]. These detrimental effects to reproduction are correlated with embryo mortality under high temperature conditions. Therefore, various studies regarding heat stress and blastocyst formation rates have been performed under both *in vivo* and *in vitro* conditions. In *in vivo* experiments, maternal heat stress decreases the viability of early stage embryos [4, 6, 7]. Ozawa *et al*. [6] reported that maternal heat stress increases hydrogen peroxide and decreases the intracellular glutathione concentration, which have been correlated with 2-cell block, in two cell embryos. Similarly, high temperatures also decrease embryonic viability and ability to develop *in vitro* [8, 10, 39]. Furthermore, heat shock also increases the generation of intracellular ROS [12] associated with DNA fragmentation in embryos [40, 41], suggesting the involvement of free radicals. These studies support the hypothesis that heat shock strongly reduces the developmental potential of embryos and increases the intracellular ROS level under both *in vivo* and *in vitro* conditions. Moreover, heat shock significantly reduces intracellular GSH levels, and GSH is known as an antioxidant that protects cells from free radicals. These results indicate that heat-shocked embryos suffer redox imbalance by accumulating intracellular ROS. These ROS are significantly responsible for damage to the DNA strand, RNA transcriptions and protein synthesis, which is important for development to blastocyst stage. Therefore, development of embryos is prevented or stopped by heat shock exposure. In the present study, supplementation with anthocyanins improved blastocyst formation rates under heat shock conditions with decreasing ROS and increasing GSH levels.

Anthocyanins are contained in plants and provide color in leaves, stems, roots, flowers, and fruits [42]. In plants, anthocyanins have been shown to act as a "sunscreen" by protecting cells from photo-damage by absorbing UV and blue-green light and

protecting tissues from photoinhibition or light stress [42]. Thus anthocyanins act as a strong antioxidant that protects plants from radicals generated by UV light and metabolic processes. Supplementation of extracted anthocyanins to cells or animals is reported to improve various types of physiological damage and is effective in preventing cardiovascular diseases [43] and increasing dark adaptation [44]. They have also been shown to improve the viability of cultured mammalian cells by preventing cell damage due to oxidative stress [45–48]. Anthocyanins even show their antioxidant function in mammalian tissues [22]. Therefore, we used anthocyanins in this study that were extracted from purple sweet potato, which contains various molecules acting as scavengers of ROS induced by heat shock. Supplementation with the anthocyanins decreased the intracellular ROS of the heat-shocked embryos. Supplementation with anthocyanins produced no differences in the ROS levels of the embryos cultured at 38.5 C. This result indicates that excess ROS generation due to heat shock was reduced in the embryos by supplementation with anthocyanins.

In this study, the intracellular content of GSH was significantly decreased by heat shock compared with embryos cultured at 38.5 C. However, the GSH levels of embryos cultured with anthocyanins did not decrease under heat shock conditions. GSH reduces ROS by reacting with electrons directly or indirectly via an enzymatic reaction [49]. By scavenging ROS, GSH protects preimplantation embryos against heat shock-mediated ROS generation [8]. A decrease in intracellular GSH levels reduces the thermotolerance of murine morulae [13]. GSH depletion also decreases thermotolerance in cultured mammalian cells by impairing transcription of heat shock proteins [50–52].

Phenolic compounds of anthocyanins are reported to increase GSH levels by activating γ-glutamylcysteine synthetase (γGCS) [53]. Oral intake of anthocyanins also increases total blood GSH levels and decreases oxidative DNA fragmentation in mice and rats [54]. Thus, supplementation of culture medium with anthocyanins might strongly activate γGCS, increase intracellular GSH levels, and reduce the intracellular ROS. There are several studies regarding the mechanisms of anthocyanins, but most of these studies are related to intestinal cells. However, the uptake and metabolism of undifferentiated cells such as embryonic cells is not yet fully understood. Therefore, it is interesting to consider whether the beneficial effects of anthocyanins in mammalian embryos are related to the same mechanisms in differentiated cells.

Heat shock tended to decrease the total cell number of blastocysts without anthocyanins. This result indicates a similar tendency to the percentage of embryos developed to expanded blastocysts ($r = 0.611$, $P<0.1$). This indicates that heat shock not only increases embryonic mortality but also delays development, even after removal from heat-shock conditions.

Moreover, lower concentrations of anthocyanins (0.1 μg/ml) improved the development of embryos, probably by directly scavenging heat shock-induced free radicals. This result indicates that a lower concentration of anthocyanins improves the development of heat-shocked embryos. On the other hand, supplementation with more than 1 μg/ml anthocyanins had a toxic effect on development in both non-heat-shocked and heat-shocked embryos. High concentrations of anthocyanins decrease cell viability and proliferation in human cultured cells [55]. In addition, high concentrations of antioxidants, such as Trolox (a hydrosoluble analogue of Vitamin E), Vitamin C, Vitamin E and epicatechin conjugates, also have toxic effects on embryos [18, 56, 57]. The cytotoxicity of these antioxidants has a direct correlation with their levels of antioxidant effects [57]. Antioxidants that have strong antioxidant effects show high cytotoxity. In addition, excess anthocyanins change cell membrane structure, impair cell polarization, and induce intracellular hypoxia [58, 59]. Thus, excess ROS is detrimental to embryos and cells, but a certain level of ROS might be necessary for their viability, signal transduction, or transcription. In this study, the level of intracellular ROS in the embryos cultured with 10 μg/ml anthocyanins at 38.5 C decreased compared with the non-heat-shocked embryos. This result supports previous reports that high concentrations of antioxidants have cytotoxic effects by depletion of ROS. Furthermore, it is possible that high concentrations of anthocyanins affect embryonic viability by altering their cell structure before depletion of ROS by their antioxidant effects.

In conclusion, the addition of anthocyanins enhances the intracellular redox status and

improves the development of heat-shocked embryos. Our results suggest that use of antioxidants is beneficial in reducing the effects of maternal hyperthermia on embryonic development.

Acknowledgement

The authors thank E. Kawano and S. Ohyama for assistance with the experiments. Special thanks are extended to Kumamoto Meat Trading Center and the Kumamoto Prefecture Meat Inspection Office for providing ovaries.

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