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N, N-Dimethylglycine decreases oxidative stress and improves *in vitro* development of bovine embryos

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Abstract. The antioxidant effect of N, N-dimethylglycine (DMG) on *in vitro*-produced (IVP) bovine embryos was examined. After *in vitro* fertilization, presumptive zygotes were cultured with or without 0.1 μM DMG under different oxygen tensions. The percentage of embryos developing to the blastocyst stage was lowest under a 20% oxygen concentration without DMG, and it was significantly increased ($P < 0.05$) by applying a 5% oxygen concentration. Under the 20% oxygen concentration, supplementation of the medium with DMG significantly improved blastocyst development, which was nearly equal to that achieved under 5% oxygen without DMG. Furthermore, a tendentious increase ($P = 0.06$) in blastocyst cell numbers was observed when DMG was applied. In the second experiment, addition of H_2O_2 (0.5 mM) to the culture medium significantly ($P < 0.01$) reduced the percentage of embryos developing to the blastocyst stage. However, DMG supplementation prevented this reduction. In conclusion, DMG enhanced the *in vitro* development of IVP bovine embryos by acting as an antioxidant.
Key words: Antioxidant, Bovine embryo, DMG, *In vitro* fertilization, N, N-Dimethylglycine

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In vitro-produced (IVP) bovine embryos at early developmental stages are known to be sensitive to reactive oxygen species (ROS) [1]. Under oxidative stress, ROS can accumulate in embryos, causing DNA damage and developmental arrest [2, 3]. Oxygen in air contributes to oxidative stress in embryos during culture. Reducing the oxygen concentration in the incubator from 20% to approximately 5% during culture has been reported to improve the development of IVP embryos in several mammal species including cattle [4–7].

N, N-Dimethylglycine (DMG) (also known as vitamin B15 or pangamic acid) acts as an antioxidant, extending the lifespan of animal cells through protection from oxidation [8–10]. DMG is presumably involved in the betaine pathway, the metabolism of homocysteine and glutathione synthesis [9, 10]. In a previous report, addition of 0.1 μM DMG to a culture medium prompted the development of early IVP bovine embryos [11].

However, to clarify whether or not DMG promotes *in vitro* development of bovine embryos by acting as an antioxidant, the embryos must be exposed to oxidized *in vitro* culture (IVC) conditions. This study was conducted to demonstrate the antioxidant effect of DMG on bovine embryos for the first time. For this purpose, two experiments were performed.

In Experiment 1, the effect of addition of 0.1 μM DMG and no DMG (+DMG and –DMG, respectively) to a culture medium on *in vitro* development of IVP bovine embryos was examined under both 20% ($20O_2$) an oxygen concentration of and 5% ($5O_2$) oxygen concentrations. Embryos were cultured in the presence or absence of DMG under 20% (5% CO_2 in air) or 5% (5% CO_2, 5% O_2 and 90% N_2) as described below (+DMG/$20O_2$, +DMG/$5O_2$, –DMG/$20O_2$ and –DMG/$5O_2$ groups, respectively). Without DMG addition to the medium, blastocyst development was significantly lower under the 20% oxygen concentration conditions (–DMG/$20O_2$ group) than that obtained under 5% oxygen (–DMG/$5O_2$ group); however, they had similar cleavage rates (Table 1). When DMG was added to the culture medium under the 20% oxygen concentration conditions (+DMG/$20O_2$ group), embryo development to the blastocyst stage increased significantly to a level similar to that of embryos cultured without DMG under 5% oxygen (–DMG/$5O_2$ group) (Table 1). Moreover, when DMG was added to the medium and 5% oxygen was used (+DMG/$5O_2$ group), the highest blastocyst formation rate was obtained among all the groups ($P < 0.05$) (Table 1). Also, when cultured under the 20% oxygen concentration conditions, a trend ($P = 0.06$) was found for higher numbers of total cells per blastocyst in favor of DMG-treated embryos (+DMG/$20O_2$ group) compared with non-treated embryos (–DMG/$20O_2$ group) (Table 2).

Experiment 2 demonstrated the antioxidant actions of DMG. *In vitro* culture of IVP bovine embryos at the 8- to 16-cell stage was

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Table 1. Effect of N, N-Dimethylglycine (DMG) addition to the IVC medium* on development of bovine IVP embryos cultured under different oxygen concentrations

Groups	No. of oocytes examined	Oxygen concentration	DMG	No. (%, mean ± SEM) of embryos developed to **	
				Cleaved stage	Blastocyst stage
–DMG/20O_2	308	20%	–	237 (76.9 ± 3.3 [a])	75 (24.4 ± 1.1 [a])
+DMG/20O_2	302	20%	+	254 (84.1 ± 1.9 [ab])	91 (30.1 ± 1.0 [b])
–DMG/5O_2	305	5%	–	257 (84.3 ± 3.3 [ab])	96 (31.5 ± 0.6 [b])
+DMG/5O_2	354	5%	+	302 (85.3 ± 1.0 [b])	134 (37.9 ± 0.6 [c])

Values with different superscripts are significant ($P < 0.05$). * SOF1: mSOFaa supplemented with 0.3% BSA, 10 µl/ml ITS, 1 ng/ml TGFb1 and 10 ng/ml FGF was used during 0–96 h of IVC. SOF2: SOF1 supplemented with 1.5 mM glucose (BSA was replaced with 5% FBS) was used after 96 h of IVC. ** Rates of cleavage and development to the blastocyst stage were assessed 48 h and 8 days, respectively, after insemination. Five replicates were performed for experiments on embryo development.

Table 2. Effect of N, N-Dimethylglycine (DMG) addition to the IVC medium* on cell number of bovine IVP embryos cultured under different oxygen concentrations

Groups	No. of blastocysts examined	No. of cells per blastocysts		
		No. of ICM cells	No. of TE cells	Total no. of cells
–DMG/20O_2	10	35.5 ± 4.1	74.0 ± 8.4	109.5 ± 10.7
+DMG/20O_2	10	37.1 ± 3.3	90.5 ± 5.1	127.6 ± 5.8
–DMG/5O_2	10	40.0 ± 2.2	89.1 ± 5.5	129.1 ± 6.8
+DMG/5O_2	10	39.2 ± 2.8	94.8 ± 5.1	134.0 ± 6.0

* SOF1: mSOFaa supplemented with 0.3% BSA, 10 µl/ml ITS, 1 ng/ml TGFb1 and 10 ng/ml FGF was used during 0–96 h of IVC. SOF2: SOF1 supplemented with 1.5 mM glucose (BSA was replaced with 5% FBS) was used after 96 h of IVC. The experiment was replicated three times. ICM, inner cell mass; TE, trophectoderm.

Table 3. *In vitro* development of 8- to 16-cell stage bovine embryos to the blastocyst stage in the presence of H_2O_2 and DMG

Treatment	No. of embryos examined	No. of embryos developed to the blastocyst stage (% ± SEM)
Control	20	13 (65.0 ± 5.5 [a])
H_2O_2 (0.5 mM)	21	3 (14.3 ± 8.3 [b])
H_2O_2 (0.5 mM) + DMG (0.1 µM)	21	12 (57.1 ± 8.2 [a])

H_2O_2: hydrogen peroxide, DMG: N, N-Dimethylglycine. Base medium: SOF1/SOF2. SOF1: mSOFaa supplemented with 0.3% BSA, 10 µl/ml ITS, 1 ng/ml TGFb1 and 10 ng/ml FGF was used during 0–96 h of IVC. SOF2: SOF1 supplemented with 1.5 mM glucose (BSA was replaced with 5% FBS) was used after 96 h of IVC. The experiment was replicated three times. SOF2: SOF1 supplemented with 1.5 mM glucose (BSA was replaced with 5% FBS) was used after 96 h of IVC. Values with different superscripts are significant ($P < 0.01$).

conducted using the culture medium either in the absence (control group) or presence of 0.5 mM hydrogen peroxide (H_2O_2). In another group, both H_2O_2 and 0.1 µM DMG were added to the culture medium. As described below, IVP embryos were cultured to the blastocyst stage. Table 3 shows that the addition of H_2O_2 significantly ($P < 0.01$) reduced blastocyst development compared with the control group (65.0% and 14.3%, respectively). However, when DMG was added to the H_2O_2-containing medium, the blastocyst formation rate improved significantly ($P < 0.01$) to a level (57.1%) equivalent to that obtained in the control group (Table 3), demonstrating that DMG exerted a strong antioxidant effect by counteracting exogenous oxidative stress in embryos.

The concentration of oxygen in the body is lower than that in the atmosphere. Previous studies have demonstrated that the oxygen concentration in the oviduct and uterus is approximately one-fourth of that in the atmosphere [12]. Embryo development has reportedly been improved by adjusting the oxygen concentration to approximately 5% in *in vitro* culture of early embryos [5]. Our results confirm that a 5% oxygen concentration, which is presumed to approximate the oxygen concentration in the body, is effective as a culture condition for early embryos and that the level of oxidative stress is involved in determining the quality of embryos, such as the number of cells. Oxygen present in the culture medium changes into free radical or nonradical molecular species. Previous reports have described that ROS affects fertilization of *in vitro* maturation (IVM) oocytes and their subsequent cleavage [13, 14]. Differences attributable to

the concentration of oxygen have also been demonstrated in the *in vitro* production of embryos in cattle, including the IVM and *in vitro* fertilization (IVF) of oocytes and IVC of early embryos [15]. Results of previous studies have shown that decreasing the oxygen concentration to 5% during maturation improved embryo development and changed the expression of genes related to oocyte competence and glucose metabolism [16] and that the addition of antioxidants to the culture medium for IVF oocytes affects their pronucleus formation rate in cattle [17]. However, exceedingly low oxygen concentrations might adversely affect development, as the proliferative capacity was decreased and apoptosis was increased under a markedly low oxygen concentration of 1% in human and mouse stem cells [18].

Although the blastocyst formation rate of embryos cultured under an oxygen concentration of 5% was found to be significantly higher even without DMG (–DMG/5O_2 group) than that of embryos cultured without DMG under the 20% oxygen concentration conditions (–DMG/20O_2 group), the development rate improved significantly when DMG was added to the culture medium and embryos were cultured, even under the 5% oxygen concentration conditions (+DMG/5O_2 group); the highest blastocyst formation rate was obtained in the +DMG/5O_2 group among all the group. Furthermore, cells in the prepared embryos were slightly more numerous with the addition of DMG. There are two possible explanations for these phenomena; it is possible that (1) even under 5% oxygen the embryos still suffer from oxidative stress to some degree or (2) DMG might improve embryo development not only by neutralizing oxidative stress but also via another, currently unknown, way. Further research will be necessary to clarify this point. Based on the findings presented above, we conclude that 0.1 μM DMG apparently exerted an antioxidant effect on bovine embryos produced *in vitro*, increasing blastocyst formation rates and tending to increase the number of cells in resultant embryos.

Methods

Reagents were obtained from Sigma Chemical (St. Louis, MO, USA) unless otherwise specified.

The protocol was approved by the Ethics Committee for Experimentation with Animals of the Akita Prefectural Experiment Station.

Oocytes were collected from follicles (2–5 mm in diameter) of bovine ovaries collected at a meat processing plant by aspirating follicular fluid containing cumulus–oocyte complexes (COCs) with a 5-ml syringe equipped with an 18G needle. The IVM medium was 25 mM HEPES-buffered TCM-199 (TCM-199; Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 0.4% (w/v) bovine serum albumin (BSA, Fraction V), 100 μM cysteamine, 50 ng/ml epidermal growth factor, 100 ng/ml transforming growth factor α, 0.05 IU/ml human menopausal gonadotropin (Serono Laboratories, Tokyo, Japan) and 10 μg/ml gentamicin (Gibco) [11]. The COCs with two or more layers of adhered cumulus cells were washed three times with IVM medium. They were then placed in IVM medium covered with paraffin oil at a ratio of 5 μl/COCs (15–20 COCs/culture drop) using Repro C-1 plates (Research Institute for the Functional Peptides, Yamagata, Japan). They were incubated for 20 h under gaseous-phase conditions of 38.5°C, 5% CO_2, 95% air and saturated humidity.

Frozen semen from one Japanese black bull was thawed in hot water heated at 38°C and was washed twice with the Brackett and Oliphant (BO) solution [19] containing 10 mM caffeine (Caff-BO solution) by centrifugation (500 × *g* for 5 min). The supernatant was removed from the sperm pellet. The Caff-BO solution was then added to achieve a final sperm concentration of 1×10^7/ml. Then the same volume of BO solution supplemented with 20 mg/ml BSA (Fraction V) (BSA-BO solution) and 10 IU/ml heparin (Novo-Heparin; Mochida Pharmaceutical, Tokyo, Japan) was added to achieve a final sperm concentration of 5×10^6/ml. This suspension was used as the IVF medium. After IVM of COCs, oocytes with adhered cumulus cells were washed three times with the BSA-BO solution. They were then transferred into IVF medium covered with paraffin oil at a ratio of 5 μl/oocyte using Repro C-1 plates and were incubated under gaseous-phase conditions of 38.5°C, 5% CO_2, 95% air and saturated humidity.

After 6 h of IVF, cumulus cells and spermatozoa were removed from the surface of the zona pellucida by gentle pipetting with a fine glass pipette. The putative zygotes were then washed three times in SOF1 medium, which was SOFaa medium supplemented with 3 mg/ml BSA (Fraction V), 10 μl/ml insulin-transferrin-selenium (Gibco), 1 ng/ml transforming growth factor β1 and 10 ng/ml fibroblast growth factor [20]. Twenty zygotes per Repro C-1 plate were placed in 100 μl of SOF1 medium covered with paraffin oil and cultured with or without 0.1 μM DMG at 38.5°C under gaseous conditions of 5% CO_2, 5% O_2 and 90% N_2 or 5% CO_2, 95% air and saturated humidity. After 96 h of culture, the embryos were then transferred to SOF2 medium, which was SOF1 medium supplemented with 1.5 mM glucose and 5% (v/v) fetal bovine serum in place of BSA [21], and cultured with or without 0.1 μM DMG under the same gaseous conditions. We changed the IVC medium based on a report by Matsumoto *et al*. [21] and performed IVC using the culture media supplemented with glucose thereafter because it was shown that the addition of glucose to the IVC medium immediately after IVF until 96 h of IVC is detrimental to bovine embryo development, although it is necessary later for embryo hatching [22].

The embryonic cell number was assessed using double staining. Embryos were treated with 0.2% Triton X-100-PBS supplemented with 0.1 mg/ml propidium iodide for 1 min. They were then transferred into 99.5% ethanol supplemented with 25 μg/ml Bis-benzimide (33342; Hoechst). Embryos were stained at 4°C for 3 h and protected from light. They were then washed with Vectashield (Vector Laboratories, Burlingame, CA, USA). The treatment solution containing embryos was dropped onto a slide glass. A cover slip was added to flatten the embryos. Observations were conducted using a fluorescence microscope (IMT-2, Olympus, Tokyo, Japan). The inner cell mass (blue) and trophectoderm (pink) cells were counted.

In one experiment, embryos at the 8-cell to 16-cell stage were used to examine the antioxidant effect of DMG. The test was conducted with three groups: a group cultured with H_2O_2 (0.5 mM), a group cultured with H_2O_2 (0.5 mM) and DMG (0.1 μM) and a control group cultured without H_2O_2 and DMG. The embryos were cultured in SOF1 medium and then in SOF2 medium until Day 8 (Day 0 = the day of IVF), and the development rate was examined.

The StatView software was used for statistical analyses. After all percentage data were subjected to arcsine transformation, significance was tested using a one-way analysis of variance (ANOVA) and the Tukey-Kramer test. Differences with $P < 0.05$ were regarded as significant.

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