Journal of Reproduction and Development, Vol. 55, No. 3, 2009

—Technical Note—

Effects of N, N-Dimethylglycine on the Development of *In Vitro* Produced Bovine Embryos

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Abstract. This study investigated the effects of N, N-Dimethylglycine (DMG) on the development of *in vitro* produced (IVP) bovine embryos. IVP embryos were obtained by *in vitro* fertilization of *in vitro* matured oocytes for 6 h. In Experiment 1, IVP embryos were cultured in mSOFaa supplemented with bovine serum albumin but without glucose (SOF1) for 4 days, transferred to mSOFaa (with 5% fetal bovine serum and 1.5 mM glucose; SOF2) supplemented with 0 (control), 0.1,1 or 10 μM DMG and cultured for an additional 7 days (11 days in total) to assess their development *in vitro*. When cultured in the medium with 0.1 μM DMG, a significantly higher number of IVP embryos developed to the blastocyst and hatched blastocyst stages (40.3 and 40.8%, respectively) compared with the other groups (18.7–31.0% and 15.0–28.7%, respectively; $P<0.05$, analysis of variance). In Experiment 2, IVP embryos were cultured in SOF1 with or without 0.1 μM DMG for 4 days, transferred to SOF2 with or without 0.1 μM DMG and further cultured as in Experiment 1; DMG was added to either SOF1 or SOF2 and to both of them to assess its exposure effects on embryo development. When cultured continuously with DMG for 11 days, significantly higher rates of IVP embryos developed into blastocyst and hatched blastocyst stages (39.0 and 47.7%, respectively) compared with the other groups (31.0–32.2% and 29.5–31.0%, respectively; $P<0.05$). In Experiment 3, we examined developmental speed of IVP embryos cultured with or without addition of 0.1 μM DMG to IVC medium after 7 days of IVC. When DMG was added to IVC medium, the ratio of embryos developed to advanced developmental stages (No. of embryos developed to the blastocyst and expanded blastocyst stages/No. of embryos developed to the morula stage) was 28.7% (86/3) and 7 times higher than that of those cultured without DMG, 4.0% (52/13). These results suggest that addition of 0.1 μM DMG to mSOFaa during IVC of IVP bovine embryos has a promoting effect on their development.

Key words: Bovine embryo, Culture medium, *In vitro* production, N, N-Dimethylglycine

(**J. Reprod. Dev. 55:** 339–342, 2009)

Previous studies have reported that various additives in the *in vitro* culture (IVC) medium for development of *in vitro*-produced (IVP) bovine embryos are effective for embryonic development [1, 2]. Addition of β-mercaptoethanol into IVC medium has been reported to have a protective effect against oxidative stress during embryo development, resulting in a high developmental rate to the blastocyst stage in cattle [3]. Also, for the protection of mouse and bovine oocytes or embryos in culture from oxidative stress, ascorbic acid (Vitamin C) and α-tocopherol (Vitamin E) have been added to the medium, resulting in high developmental rates to the blastocyst stage [4, 5].

N, N-Dimethylglycine (DMG) was previously called vitamin B15, and it is now known as a vitamin-like substance called pangamic acid; it acts as an antioxidant, helping to lengthen cell life through its protection from oxidation [6]. This natural substance is contained in beer yeast, brown rice and sesame, and it may play a role in strengthening the immune system involving antibodies, lymphocytes and cytokine production by directly acting on cells *in vivo* [7, 8]. In addition, DMG could be mediated by its free radical scavenging activity and cytoprotection of human gastric mucosa, and its therapeutic efficacy for gastric ulcer has been reported [9].

However, no study has reported the influence of addition of DMG to IVC and IVC medium on embryonic development in cattle. In this study, we examined the effects of DMG on the *in vitro* development of IVP bovine embryos.

Accepted for publication: February 11, 2009
Published online in J-STAGE: March 19, 2009
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Materials and Methods

Oocyte collection and in vitro maturation (IVM) culture

Using a 5-ml syringe with an 18G injection needle, we aspirated follicular fluid containing cumulus-oocyte complexes (COCs) from ovarian follicles measuring 2 to 5 mm in diameter in bovine ovaries obtained from a slaughterhouse. As the *in vitro* maturation (IVM) medium, we used 25 mM HEPES buffered TCM-199 (TCM-199, Gibco Laboratories, Grand Island, NY, USA) containing inactivated fetal bovine serum (FBS, 10%, Gibco), cysteamine (100 μM, Sigma Chemical, St. Louis, MO, USA), epithelial growth factor (EGF, 50 ng/ml, Sigma), transforming growth factor α (TGF α, 100 ng/ml, Sigma), human menopausal gonadotrophin (0.05 IU/ml, Serono Japan, Tokyo, Japan) and gentamycin (10 μg/ml, Gibco). COCs, to which 2 or more layers of cumulus cells were adhered, were washed in the IVM medium 3 times. Using a Repro C-1 plate (Research Institute for the Functional Peptides, Yamagata, Japan) as a culture dish, 20 COCs were placed in 100 μl of the IVM medium covered with paraffin oil (Sigma) and cultured at 38.5 C for 22 h under gaseous-phase conditions of 5% CO_2, 95% air and 100% humidity.

Table 1. The effect of N, N-Dimethylglycine (DMG) in the *in vitro* culture medium* on development of *in vitro* produced bovine embryos

DMG (μM)	No. of embryos examined	No. (%) of embryos developed to the stage** of		
		Cleaved	Blastocyst	Hatched blastocyst***
0	210	157 (74.8)[a]	65 (31.0)[c]	45 (28.7)[h]
0.1	295	233 (79.0)[a]	119 (40.3)[d]	95 (40.8)[g]
1	133	76 (57.1)[b]	29 (21.8)[e]	12 (15.8)[h]
10	107	60 (56.1)[b]	20 (18.7)[f]	9 (15.0)[h]

Values with different superscripts in the same column are significantly different (P<0.05). *mSOFaa. **Rates of embryos cleaved, developed to the blastocyst and hatched blastocyst stages were assessed at 48 h and 8 and 11 days after insemination, respectively. ***The number of cleaved embryos was used as the denominator for the hatched blastocyst rate.

Sperm treatment and in vitro fertilization (IVF)

Frozen semen from one Japanese black bull was thawed in hot water heated at 38 C and washed twice by centrifugation in BO solution [10] (Caff-BO solution) containing 10 mM caffeine (Sigma). The supernatant was removed, Caff-BO solution was added to the sperm suspension and the sperm concentration was then adjusted to 1×10^7. In addition, an equivalent volume of BO solution (BSA-BO solution) containing bovine serum albumin (BSA, Fraction V, 20 mg/ml, Sigma) and heparin (10 IU/ml, Novo Heparin, Mochida Pharmaceutical, Tokyo, Japan) was added to prepare a final *in vitro* fertilization (IVF) medium; the final sperm concentration was adjusted to 5×10^6/ml in IVF medium. All the solutions were pre-incubated for 15 min before use.

After IVM culture, COCs were washed in BSA-BO solution 3 times. Twenty oocytes each were plated in Repro C-1 plates in 100 μl of IVF medium containing spermatozoa, covered with paraffin oil and cultured at 38.5 C for 6 h under gaseous-phase conditions of 5% CO_2, 95% air and 100% humidity.

IVC of IVP embryos

After IVF, cumulus cells and spermatozoa were removed from the surface of the zona pellucida by gentle pipetting with a fine glass pipette. The oocytes (putative zygotes) were then washed in IVC medium 3 times. Twenty zygotes per Repro C-1 plate were placed in 100 μl of IVC medium covered with paraffin oil and cultured at 38.5 C under gaseous-phase conditions of 5% CO_2, 5% O_2, 90% N_2 and 100% humidity. For the first IVC of the zygotes (0–4 days after IVF), we employed mSOFaa [11] containing BSA (3 mg/ml, Sigma), insulin-transferrin-selenium (ITS, 10 μl/ml; Gibco), transforming growth factor β1 (TGF β1, 1 ng/ml; Sigma) and fibroblast growth factor (bFGF, 10 ng/ml; Sigma) as IVC medium (SOF1). After 4 days of IVC, the embryos were transferred to SOF2; BSA in SOF1 was substituted for 5% FBS and supplemented with glucose (1.5 mM, Sigma) [12]. We changed IVC medium after 96 h of culture based on a report by Matsumoto *et al*. [12] and performed IVC using the culture media supplemented with glucose thereafter because it has been reported that addition of glucose to the IVC medium just after IVF until 96 h of IVC is detrimental to the development of bovine embryos but is needed for their hatching [13].

We investigated the rates of embryos developed to the blastocyst and hatched blastocyst stages on Day 8 (the day of IVF was defined as Day 0) and Day11 (to obtain the complete rates of embryos developed to the hatched blastocyst stage), respectively.

Experimental design

Experiment 1: We added 0.1, 1 or 10 μM DMG (Sigma) for IVC medium (SOF1 and SOF2) of the IVP embryos (0.1, 1 and 10 μM-treated groups, respectively) and examined its influence on their *in vitro* development. DMG-free medium was used for the control group.

Experiment 2: We added 0.1 μM DMG to either SOF1 for IVC of the IVP embryos from Day 0 to Day 4 or SOF2 for IVC from Day 4 to Day 11 and both of them (+–, –+ and ++ groups, respectively) to assess the effect and duration of its exposure on their *in vitro* development.

Experiment 3: We added 0.1 μM DMG to SOF1 for IVC of IVP embryos throughout the culture period and examined their development to different stages on Day 7 to assess the effect of DMG on the developmental speed of IVP embryos.

Statistical analysis

For statistical analysis, we employed the StatView program. After all the percentage data were subjected to arcsine transformation, and significance was analyzed using ANOVA and Fisher's PLSD software (ANOVA).

Results

Experiment 1

As shown in Table 1, the rate of cultured embryos developed to the blastocyst stage in the 0.1 μM-treated group (40.3%) was significantly higher than those in the control and 1 μM- and 10 μM-treated groups (31.0, 21.8 and 18.7%, respectively; P<0.05). Also, the rate of cleaved embryos developed to the hatched blastocyst stage in the 0.1 μM-treated group (40.8%) was significantly higher than those in the control, 1 μM- and 10 μM-treated groups (28.7, 15.8, and 15.0%, respectively; P<0.05).

Experiment 2

As shown in Table 2, the blastocyst and hatched blastocyst rates in the ++ group cultured with DMG over the entire IVC (39.0 and 47.7%, respectively) were significantly higher than those in the +– group cultured with DMG between 0 and 96 h of IVC (31.0 and

Table 2. The effect of N, N-Dimethylglycine (DMG) added to the *in vitro* culture medium* before and/or after 96 h of *in vitro* culture on development of *in vitro* produced bovine embryos

Addition of 0.1 μM DMG		No. of embryos	No. (%) of embryos developed to the stage** of		
Before 96 h	After 96 h	examined	Cleaved	Blastocyst	Hatched***
+	–	58	42 (72.4)	18 (31.0)[a]	13 (31.0)[c]
–	+	59	44 (74.6)	19 (32.2)[a]	13 (29.5)[c]
+	+	59	44 (74.6)	24 (39.0)[b]	21 (47.7)[d]

Values with different superscripts in the same column are significantly different (P<0.05). *mSOFaa. **Rates of embryos cleaved, developed to the blastocyst and hatched blastocyst stages were assessed 48 h and 8 and 11 days after insemination, respectively. *** The number of cleaved embryos was used as the denominator for the hatched blastocyst rate.

Table 3. The effect of N, N-Dimethylglycine (DMG) added to the *in vitro* culture medium* on the developmental speed of *in vitro* produced bovine embryos on Day 7

Addition of 0.1 μM DMG	No. of embryos examined	Ratios of embryos developed to advanced stages***
–	213	4.0 (52/13)
+	210	28.7 (86/3)

*mSOFaa. ** Day 0 was defined as the day of IVF. ***No. of embryos developed to the blastocyst and expanded blastocyst stages/No. of embryos developed to the morula stage.

31.0%, respectively) or the –+ group cultured with DMG after 96 h of IVC (32.2 and 29.5%, respectively; P < 0.05).

Experiment 3

As shown in Table 3, when 0.1 μM DMG was added to IVC medium, the ratio of embryos developed to advanced developmental stages (No. of embryos developed to the blastocyst and expanded blastocyst stages/ No. of embryos developed to the morula stage) was 28.7 (86/3) and 7 times higher than that of those cultured without DMG, 4.0 (52/13).

Discussion

Our results demonstrate that the rates of IVM/IVF bovine oocytes (putative zygotes) that developed to the blastocyst and hatched blastocyst stages were significantly higher for zygotes cultured in IVC medium supplemented with 0.1 μM DMG for the last 7 days of an 11-day IVC period than that of the control group cultured without DMG. Furthermore, its promoting effect on the development of the zygotes was found to be enhanced when the zygotes were cultured throughout IVC for 11 days compared with those cultured with 0.1 μM DMG for the first 4 days of IVC or the last 7 days of IVC. Thus, it is suggested for the first time, that addition of 0.1 μM DMG to IVC medium for IVP bovine embryos has a promoting effect on their development. Also, when cultured with DMG, development of embryos to the blastocyst stage was faster than those cultured without DMG. It is possible that embryonic growth was promoted by antioxidation action of DMG. However, when embryos were cultured at high concentrations, such as 1 and 10 μM, in this study, the promoting effects of DMG on embryonic development were lost, and significantly lower rates of cleavage and development to the blastocyst and hatched blastocyst stages were obtained compared with that of embryos cultured without DMG. Thus, it can be suggested that high concentrations of DMG have detrimental effects on the development of IVP bovine embryos.

The reason for the promoting effect of DMG, a natural substance, on the development of bovine IVP embryos in this study is not clear. However, it has been reported that choline and DMG contribute glycine for GSH synthesis in human development [14]. Furthermore, low choline, betaine and DMG status in children with CF, a kind of hereditary disease, has been reported, suggesting that DMG is essential for normal development in humans [15]. In addition, human immunodeficiency virus (HIV)-infected patients are reported to have significantly elevated DMG serum concentrations [16], suggesting a connection between normalization of the pathologic immune activation state and DMG in HIV disease. Taken together, this natural substance is important for normal development in humans by strengthening the immune system and its free radical scavenging activity.

In mammals it has been reported that active oxygen inhibits embryonic growth [17–19]. To suppress the detrimental effects of oxidative stress such as reactive oxygen species (ROS) on embryonic development, addition of peptides and enzymes, such as glutathione and catalase [18], or reducers, such as β-mercaptoethanol and cysteamine, to IVC medium have been reported to be effective [3, 20, 21]. Furthermore, it has been reported that anthocyanin, a scavenger of ROS in bovine embryonic culture medium, is able to protect the embryo from oxidation and heat stress [22].

In addition, it is well known that vitamin addition promotes embryonic development [4, 5] and that vitamin E addition inhibits oxidation in IVC media for mouse embryos [5]. In cattle, it has been reported that vitamin E protects embryos from oxidative stress in IVC medium [4].

Thus, DMG, as a vitamin-like substance, may promote the development of bovine IVP embryos by protecting them from the

oxidative stress caused by active oxygen. However, the influence of DMG on such embryos remains to be clarified; this issue needs further investigation.

This study suggests that the addition of 0.1 μM DMG in mSOFaa during IVC of IVP bovine embryos has a promoting effect on their development.

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