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Effect of the Addition of β-Hydroxybutyrate to Chemically Defined Maturation Medium on the Nuclear Maturation, Sperm Penetration and Embryonic Development of Porcine Oocytes *In vitro*

R. Endo[1], A. Ishii[1], A. Nakanishi[1], H. Nabenishi[1,2], K. Ashizawa[1] and Y. Tsuzuki[1,] *

[1] Animal Reproduction Laboratory, Faculty of Agriculture, University of Miyazaki, Miyazaki 889-2192, Japan

ABSTRACT : We investigated the effects of various concentrations of β-hydroxybutyrate (BHB, 0, 0.1, 1 and 10 mM), a ketone body, added to chemically-defined maturation medium with or without energy substrates (glucose, pyruvate and lactate) on nuclear maturation rates up to the metaphase stage of the second meiotic division (M-II stage). In addition, we also assessed the influence of BHB on glutathione content, sperm penetration rate and embryonic development up to the blastocyst stage of oocytes matured under the presence of these energy substrates. Nuclear maturation rates up to the M-II stage of oocytes matured with BHB in each concentration group did not show a significant increase compared with the control (0 mM) groups in both the presence and absence of energy substrates. Although glutathione contents were not significantly different in each BHB concentration group, the sperm penetration rate in the 1 mM BHB group was significantly higher ($p<0.05$) and the embryonic development rate of oocytes up to the blastocyst stage was significantly lower ($p<0.05$) than the respective values of the control groups. These results suggest that BHB added to a chemically-defined maturation medium may stimulate sperm penetration while inhibiting embryonic development of porcine oocytes. (**Key Words :** Porcine oocytes, β-Hydroxybutyrate, Nuclear Maturation, Embryonic Development)

INTRODUCTION

β-Hydroxybutyrate (BHB) is a ketone body synthesized *in vivo* and used as an energy substrate during nutritional starvation such as suckling, fasting and hibernation (Kabine et al., 2003; Haces et al., 2008). BHB is contained in healthy follicular fluids of sheep, pigs and cattle at maximum concentrations of 0.31, 4.20 and 0.69 mM, respectively (Gosden et al., 1990).

In an *in vitro* assay, BHB can stimulate the expansion and hatching rate of bovine blastyocysts produced in the absence of energy substrates (Gómez et al., 2002). Otherwise, BHB in the IVM medium of bovine oocytes has a detrimental effect, which is variable according to the glucose concentration, on embryonic development after IVF in the presence of glucose as an energy substrate (Leroy et al., 2006). However, BHB stimulated the *in vitro* nuclear maturation of porcine oocytes without changing their glutathione (GSH) content in the presence of glucose in our laboratory (Tsuzuki et al., 2009). From these reports, it is postulated that the effects of BHB may differ by cell type and animal species.

Glutathione (GSH), a tripeptide thiol (γ-glutamyl-cysteinylglycine), is the major non-protein sulphydryl compound in mammalian cells. In both male and female gametes, GSH is involved in the protection of these cells against oxidative damage. GSH has been implicated in maintaining the meiotic spindle morphology of oocytes (Luberda, 2005). BHB increased GSH in rat hippocampal mitochondria, but not in human erythrocytes (Jain and McVie, 1999; Jarrett et al., 2008). We reported that BHB did not influence the GSH content of oocytes matured with modified NCSU37 that contained only 5.5 mM glucose as an energy substrate (Tsuzuki et al., 2009). However, the GSH content of oocytes and embryos varied according to the amino acid or energy substrates such as glucose, pyruvate and lactate (Karja et al., 2006; Furnus et al., 2008). Therefore, it is thought that the GSH content of oocytes matured with BHB may differ according to media

* Corresponding Author : Yasuhiro Tsuzuki. Tel: +81-985-58-7195, Fax: +81-985-58-7195, E-mail: a01207u@cc.miyazaki-u.ac.jp

[2] Domestic Animal Biotechnology Department, Miyazaki Livestock Research Institute, Takaharu-Cho, Nishi-Morokata-Gun, Miyazaki 889-4411, Japan.

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composition of different energy substrates and amino acids.

In this study, we assessed the effect(s) of BHB added to a chemically defined medium, POM (Yoshioka et al., 2008), which contained 4 mM glucose, 0.2 mM pryruvate and 2 mM lactate as energy substrates, on the nuclear maturation, glutathione content, sperm penetration and embryonic development of porcine oocytes.

MATERIALS AND METHODS

In vitro maturation and nuclear maturation assay

Porcine ovaries were obtained from a local slaughterhouse and immersed in physiological saline (0.9% NaCl, 25-32°C) supplemented with 100 U/ml penicillin G, potassium salts (No. 26239-42, Nacalai Tesque, Kyoto, Japan) and 50 μg/ml streptomycin sulfate (No. 32237-72, Nacalai Tesque). The oocytes were aspirated from superficial follicles (2 to 6 mm in diameter) with an 18-gauge needle (No. NN-1838R, Terumo, Tokyo, Japan) attached to a 5-ml syringe (No.SS-05Lz, Terumo). The syringe contained a small amount of TCM-199 (Hank's salt, No. M-0393, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2% (v/v) heat-inactivated (56°C for 30 min) calf serum (CS, No.16170-086, Gibco BRL Products, Grand Island, NY, USA). Oocytes that were enclosed with multi-layers of cumulus cells (COCs), were selected and washed four times with TCM-199 without CS. Some of these oocytes were randomly selected and fixed by the method described below to observe the nuclear phase before maturation. The remaining COCs were matured for 21±1 h in POM containing 0.3% (w/v) polyvinyl alcohol (No.P-8139, Sigma-Aldrich) in the presence or absence of three energy substrates (4 mM glucose, 0.2 mM pyruvate and 2 mM lactate) (Yoshioka et al., 2008). These media also contained 10 iu/ml hCG (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan), 10 iu/ml PMSG (Aska Pharmaceutical Co., Ltd., Tokyo, Japan), 1 mM dibutyryl cAMP (No.D-0627, Sigma-Aldrich) and various concentrations of BHB (No.29836-0, Sigma-Aldrich, D-form, 0, 0.1, 1.0 and 10 mM). Furthermore, additional culturing (22±1 h) was done in the same medium without these hormones and dibutyryl cAMP under 5% CO_2, 95% room air and 100% humidity at 38.5°C. During IVM, the medium volume was adjusted to 10 μl/COC.

After IVM, the COCs were exposed to 0.1% hyarulonidase (No.H-3506, Sigma-Aldrich) for 3 min on a warm plate (set at 39°C) and cumulus cells were denuded by pipetting and using a vortex mixer for less than 10 s at room temperature. Some of the COCs were removed and fixed with acetic alcohol (alcohol:acetic acid = 3:1, v/v) for at least 2 days at room temperature and stained with 1% acetic orcein (No. 7100, Merck, Darmstädt, Germany) for 20-30 min at room temperature.

As BHB can change medium pH because of its strong acidity, the pH of the medium containing each concentration of BHB, hormones and dibutyryl cAMP and energy substrates was measured by a pH meter (No.AS-212, As One, Tokyo) under the same gas conditions in an incubator.

GSH content measurement

COCs matured in the medium containing energy substrates and various concentrations of BHB were denuded, and washed with a buffer (10 mM EDTA-Na_4, No.15114-02, Nacalai Tesuque, in 0.2 M Na_2HPO_4, pH 7.2). To measure the GSH content, the oocytes derived from COCs by removing the cumulus cells were loaded into a 1.5-ml Eppendorf tube with 10 μl buffer and stored at -20°C until measurement. Then, 5 μl of 1.25 M H_3PO_4 (No. 27618-55, Nacalai Tesque) was added to the thawed samples together with 695 μl buffer (pH 7.2) supplemented with 0.33 mg/ml NADPH (No. 309-50471, Oriental Yeast Co., Osaka), 100 μl of 5,5'-dithibis (2-nitrobenzoic acid) (DTNB, No. 047-16401, Wako Pure Chemical Industries, Osaka), 190 μl of water and 10 μl GSSG-reductase (No. G-3664, 250 U/ml, Sigma-Aldrich). The formation of TNB in samples was followed at 412 nm on a spectrophotometer (UV-150-02, Shimazu Co., Tokyo, Japan) every 30 seconds for five minutes at room temperature. The GSH content of the immature oocytes was also assessed. Standards were prepared for each assay and GSH content per sample was determined from a standard curve.

IVF and culture for sperm penetration and embryonic development up to the blastocyst stage

One frozen sperm pellet (approximately 100 μl), derived from one Landrace boar, was thawed in 0.5 ml PGM tac4 (Yamanaka et al., 2009) without theophylline within 10 s, and the sperm concentration was adjusted to 40×10^6 sperm/ml. Fifty μl of the spermatozoa was introduced into a 50-μl PGM tac4 drop which contained 5 mM theophylline and 20 cumulus cell-denuded oocytes (DOs) matured in the presence of energy substrates. With this treatment, the final concentrations of the theophylline and sperm were 2.5 mM and 20×10^6/ml, respectively, while the medium volume was 5 μl/DO. Co-incubation with sperm and oocytes was carried out at 38.5°C under 5% CO_2, 95% air for 5 h.

After fertilization, the DOs were washed three times without further selection and transferred into mPZM-5 (Yamanaka et al., 2009) at a 5 μl/DO volume. At 24±1 h, some DOs in each group were fixed with acetic alcohol and stained with 1% acetic orcein in a manner similar to that used for matured oocytes to assess the rate of sperm penetration. The oocytes were considered to be penetrated

Table 1. Effects of various concentrations of BHB added to the maturation medium in the presence of energy substrates on the nuclear maturation of porcine oocytes

BHB concentration (mM)	N	Percentage of oocytes reaching the M-II stage
0	50	77.9±1.89
0.1	51	82.9±3.80
1	50	73.6±1.81
10	51	82.0±4.37

Values are mean±SE of four replicates.

Table 2. Effects of various concentrations of BHB added to the maturation medium in the absence of energy substrates on the nuclear maturation of oocytes

BHB concentartion (mM)	Energy substrates	N	Percentage of oocytes reaching the M-II stage
0	+	121	86.1±1.52[a]
0	-	127	2.3±1.75[b]
0.1	-	121	0.0±0.00[b]
1	-	139	0.8±0.78[b]
10	-	118	0.0±0.00[b]

[a, b] Values with different superscripts in a column are significantly different ($p<0.05$).

Values are mean±SE of four replicates. +; Presence, -; Absence.

when they contained one or more swollen sperm heads or male pronuclei with their corresponding sperm tails (Gil et al., 2007; Hong and Lee, 2007). The remaining DOs were cultured further and, at a cleavage rate beyond 2-cells on Day 2 and embryonic development up to the blastocyst stage on Day 7 after fertilization, were observed using an inverted microscope.

Statistical analysis

The percentage of the oocytes that developed to the metaphase stage of the second meiotic division (M-II stage) in each group, sperm penetration, and embryonic development rates were subjected to arcsine transformation, and all data were analyzed with ANOVA followed by Duncan's Multiple Range Test.

RESULTS

All 57 oocytes before maturation (100%) collected in 4 replicates (14-15 oocytes per replicate) were found to be at the germinal vesicle stage.

There were no significant differences in the percentage of the COCs (12-13 COCs in each group per replicate) that developed to the M-II stage in the IVM medium supplemented with various concentrations of BHB in the presence of the energy substrates (Table 1).

The percentages of the COCs (30-35 COCs in each group per replicate) matured with various concentrations of BHB in the absence of the energy substrates were significantly lower ($p<0.05$) than when matured in medium containing energy substrates and without BHB (Table 2).

Table 3. Effects of various concentrations of BHB added to the maturation medium in the presence of energy substrates on the pH of the medium

BHB concentration (mM)	pH of the medium
0	7.6±0.06
0.1	7.5±0.06
1	7.6±0.05
10	7.6±0.09

Values are mean±SE in four replicates.

Table 4. Glutathione content of oocytes matured with various concentrations of BHB in the presence of energy substrates

BHB concentration (mM)	Glutathione content (p mol/oocyte)
Immature	2.1±0.26[a]
0	3.5±0.29[b]
0.1	3.7±0.40[b]
1	3.2±0.42[b]
10	3.0±0.24[ab]

[a, b] Values with different superscripts in a column are significantly different ($p<0.05$).

Values are mean±SE of twelve replicates.

The pH of the media containing each concentration of BHB (Table 3) was similar for all groups, and there were no significant differences.

The GSH contents of the oocytes matured with each BHB group are presented in Table 4. The GSH contents of the group before IVM were significantly lower ($p<0.05$) than for each BHB group after IVM except for the 10 mM group. However, when the GSH contents of the groups matured with BHB were compared, no significant difference was observed.

Sperm penetration rates for all groups (28-35 in each oocytes per replicate) are presented in Table 5. The sperm penetration of the 1 mM group was significantly higher ($p<0.05$) than that of the control (0 mM) group.

The embryonic development rates on Day 2 and 7 in each BHB group (38-40 oocytes in each group per replicate) are presented in Table 6. Although there was no significant difference among the groups in cleavage rates on Day 2, the development rate up to the blastocyst stage in the 0 mM group was significantly higher ($p<0.05$) than in the other groups which contained BHB.

DISCUSSION

In the present study, the nuclear maturation rates of

Table 5. Sperm penetration rate of oocytes matured with various concentrations of BHB

BHB concentration (mM)	N	Percentage of oocytes penetrated	N*	Percentage of oocytes with monospermy
0	113	26.8±1.84[a]	30	45.1±7.44[a]
0.1	140	36.4±0.64[b]	51	46.9±5.12[a]
1	127	46.4±1.27[c]	59	44.1±6.58[a]
10	136	16.6±3.39[d]	23	48.2±7.03[a]

[a, b, c, d] Values with different superscripts in a column are significantly different ($p<0.05$).

Values are mean±SE of four replicates. N* = Number of oocytes showing monospermy per number penetrated.

Table 6. Development rate of oocytes matured with various concentartions of BHB up to the blastocyst stage

BHB concentration (mM)	N	Cleavage rate of the oocytes on Day 2 after fertilization	Development rate up to the blastocyst stage on Day 7
0	158	24.6±2.59[a]	1.9±0.63[a]
0.1	157	21.7±2.51[a]	0.0±0.00[b]
1	154	30.3±7.70[a]	0.0±0.00[b]
10	155	18.7±1.88[a]	0.0±0.00[b]

[a, b] Values with different superscripts in a column are significantly different ($p<0.05$). Values are mean±SE of four replicates.

oocytes up to the M-II stage in all BHB groups matured without energy substrates were significantly lower ($p<0.05$) than those of the non-BHB groups matured with energy substrates. This indicates that porcine oocytes may not use BHB in the absence of the energy substrates. In general, ketone bodies such as BHB and acetoacetate are metabolized in nutritional starvation states such as suckling, fasting and hibernation (Kabine et al., 2003; Haces et al., 2008). Furthermore, BHB can be used by bovine early embryos in the absence of energy substrates (Gómez et al., 2002). However, in the present study, all concentrations of BHB seemed not to be metabolized during nutritional starvation. It is well known that mammalian oocyte glucose is predominantly metabolized in the pentose phosphate pathway to support DNA and RNA synthesis and to serve NADPH (Sutton et al., 2003), in which BHB cannot enter. The decrease in the nuclear maturation of BHB addition groups in the present study may have been caused by the absence of glucose in the medium.

However, when BHB was added to the maturation medium with energy substrates, the nuclear maturation rates up to the M-II stage in all BHB addition groups were almost the same as in the control group. In our previous study, the nuclear maturation rates of oocytes matured with BHB were significantly increased compared with the control (0 mM) group (Tsuzuki et al., 2009). In that study, modified NCSU37 medium, which contained only 5.5 mM glucose as an energy substrate (Petters and Wells, 1993), was used for IVM. However, the POM used in this study contained 4 mM glucose, 0.2 mM pruvate and 2 mM lactate (Yoshioka et al., 2008). Like glucose, pyruvate can also stimulate the nuclear maturation of porcine oocytes when it is added to NCSU37 medium (Funahashi et al., 2008). Therefore, the discrepancy in the present study that addition of BHB to the IVM medium did not increase nuclear maturation of oocytes may have been caused by the different energy substrates in the IVM medium.

Here, BHB did not influence the GSH content of the oocytes. This is consistent with the results of our previous study (Tsuzuki et al., 2009) in which oocytes were matured in a modified NCSU37 medium. It was reported that the glutathione content of pig oocytes varied according to the type of medium used for IVM (Wang et al., 1997), and may be induced by additives such as γ-glutamyl cycle compounds, cysteine, cystine and β-mercaptoethanol (Whitaker and Knight, 2004), amino acids or energy substrates such as glucose, pyruvate and lactate (Karja et al., 2006; Furnus et al., 2008). On the other hand, Brad et al. (2003) reported no difference in the glutathione content of pig oocytes matured with NCSU23 and TCM-199. Considering these reports, it is postulated that the effects of BHB on the GSH content of porcine oocytes did not vary in the different media we used for IVM.

The pH of each media with various concentrations of BHB was almost the same (approximately 7.6 in each group) as in a previous report in which the pH of porcine follicular fluids was 7.4 just after collection from the ovaries (Sakamoto et al., 2006). Therefore, it is considered that the addition of BHB to the medium up to 10 mM maintained normal physiological conditions, and had no negative effect on oocyte maturation. This is similar to a report in dogs that blood pH was not influenced by the presence of ketone bodies over 20 mM (Durocher et al., 2008).

The 1 mM BHB group had a significantly higher penetration rate than the control group, although the glutathione content of the ooctytes matured at this BHB concentration was not increased compared with the non-

BHB (control) group. It is well known that glutathione participates in sperm nucleus de-condensation and male pronuclear formation in mammals (Rodríguez-González et al., 2003; Luberda, 2005). However, our results were different from previous reports.

Although the mechanism is not known, one possibility may be considered. Intracellular Ca^{2+} spikes in oocytes are one of the important events to induce sperm chromatin de-condensation and male pronuclear formation in mammals (Jones, 2005). Compared with *in vivo* oocytes, *in vitro* matured oocytes showed reduced ability to generate Ca^{2+} transition (Cheung et al., 2000). BHB stimulated the increase in intracellular calcium in murine fibroblast L929 cells (Cheng et al., 2005). In view of these observations, it can be postulated that BHB increases the intracellular calcium level of oocytes, which can result in a higher penetration rate.

In the present study, although the GSH contents of all groups matured with various concentrations of BHB were similar, no groups containing BHB had embryos at the blastosyst stage. This suggests that the addition of BHB during maturation may suppress embryonic development after fertilization without influencing the GSH production of the oocytes during IVM. This negative effect of BHB in the IVM medium for the development of embryos after fertilization was also reported in cattle (Leroy et al., 2006). Although the cause(s) for decreased rates of embryonic development are unknown, polyploidy of the oocytes and/or cumulus cells during IVM with BHB is postulated because BHB plus glucose in the medium induced polyploidy of mouse embryonic cells (Tatewaki et al., 2006). Further study will be needed to clarify this point.

It is considered that measurement of the GSH concentration in oocytes after IVM may be a valuable indicator of oocyte cytoplasmic maturation which correlates well with embryonic development up to the blastocyst stage (Eppig, 1996; Abeydeera et al., 2000; Luberda, 2005). However, no relationship between the GSH content of oocytes and blastocyst formation rate was reported in pigs (Viet Linh et al., 2009). Considering these reports, it is clear that further study will be needed to clarify the relationship between addition of BHB during IVM and GSH content.

In conclusion, 1 mM BHB added to the IVM medium may inhibit embryonic development and increase sperm penetration without influencing synthesis of glutathione by oocytes.

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