Effect of β-hydroxybutyrate Added to Maturation Medium on Nuclear Maturation of Pig Oocytes

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Abstract: *The effects of β-hydroxybutyrate (BHB) added to a modified NCSU37 medium during in vitro maturation (IVM) on nuclear maturation, and ATP and glutathione (GSH) contents of porcine oocytes were investigated. Nuclear maturation up to the metaphase stage of the second meiotic division (the M-II stage) of cumulus cell-enclosed oocytes (COs) matured with various concentrations (0.0, 2.3, 3.1, 3.9 and 4.7 mM) of BHB was observed. The nuclear maturation rate of the oocytes of the 3.1 mM group was significantly higher (P < 0.05) than that of the control (0.0 mM) group. Both the ATP and GSH contents of the oocytes matured with or without 3.1 mM BHB and removed from cumulus cells after IVM were not significantly different. These results indicate that 3.1 mM BHB added to the maturation medium may stimulate the nuclear maturation of porcine oocytes without changing their ATP and GSH contents.*
Key words: β-hydroxybutyrate, Porcine oocytes, ATP, Glutathione

Introduction

β-Hydroxybutyrate (BHB), a major ketone body found *in vivo*, increases in plasma levels under nutrient starvation (i.e. fasting or the early lactation period) and in diabetics [1–4]. In general, it is thought that ketone bodies such as acetoacetate or BHB must be converted back into acetyl-CoA before their complete combustion to CO_2 and water via the reactions of the TCA cycle [5, 6] to produce ATP. In healthy animals, BHB was detected at around 0.4 mM, 0.5 mM and 0.25 mM in sheep, cattle and pig serum, respectively [1, 7, 8]. In Graafian follicular fluid, BHB was detected at a maximum 0.3 and 0.7 mM in sheep and cattle, respectively. However, the BHB level of pig Graafian follicular fluid was detected at 4.2 mM which is higher than that of sheep [9] and cattle [9, 10]. It is known that BHB can be metabolized by early bovine embryos during *in vitro* development, allowing lipid mobilization within the embryonic cells and resulting in an improvement in blastocyst hatching [2, 11] at 3.6 mM. This is considered to be subclinical and clinical ketosis [4], because the physiological limit of BHB in cattle is considered to be 1.0 mM [2]. However, when bovine oocytes were matured *in vitro* with BHB at concentrations over 3.6 mM, embryonic development after *in vitro* fertilization was significantly decreased [4]. Although a concentration of BHB in porcine follicular fluid higher than in other animals has been reported [9], there are no reports about the effect of BHB on porcine oocyte maturation.

Intracellular concentrations of ATP and glutathione (GSH) are indicative of metabolic activity and the ability of the oocytes to form a male pronucleus and cope with cellular stress, respectively. They have been used as markers of cytoplasmic maturation *in vitro*, and the degree of *in vitro* cytoplasmic maturation is considered to influence the embryonic development after *in vitro* fertilization [12–14]. The addition of BHB to IVM medium may influence the ATP content of the oocytes, because BHB can enter the TCA cycle to produce ATP, as mentioned above. BHB inhibited the decline of ATP in rat hippocampal neurons stressed by reactive oxygen species [15]. In addition, BHB increased GSH in rat

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hippocampal mitochondria otherwise did not increase in human erythrocytes [16, 17]. However, there have been no reports about the effects of BHB on the ATP and GSH contents of mammalian oocytes. Therefore, in the present study, we investigated the effects of various concentrations of BHB added to an IVM medium on the nuclear maturation, ATP and GSH contents of porcine oocytes.

Materials and Methods

In vitro maturation (IVM)

Oocytes were collected from the ovaries of slaughtered prepubertal gilts and matured *in vitro* by a previously described method with some modifications [18, 19]. Briefly, intact cumulus cell-enclosed oocytes (COs) were matured in NCSU37 medium for 42 to 46 hours under 5% CO_2, 95% air, and 100% humidity at 39°C. The NCSU37 medium [18] used for IVM in this study was modified with 51.5 mM taurine (No. T-7146; Sigma-Aldrich Co.), 1.2 mM cysteine (No. 10309-12; Nacalai Tesque), 1% MEM essential amino acid (No. 11130-051; Invitrogen Co.), 0.5% non-essential amino acid (No. 11140-050; Invitrogen Co.), 15 ng/ml epidermal growth factor (No. E-4127, Sigma-Aldrich Co.), 5 μg/ml insulin (No. I-6634, Sigma-Aldrich Co.), 0.23 μg/ml LH (No. L-9773, Sigma-Aldrich Co.), 0.63 μg/ml FSH (No. F-2293, Sigma-Aldrich Co.), 150 μM cysteamine (No. M-6500, Sigma-Aldrich), 5% (v/v) fetal calf serum (FBS, 56°C, 30 min heat-inactivated, No. 16140-063; Invitrogen Co.) and antibiotics (100 U/ml penicillin G potassium, No. P-4687, Sigma-Aldrich Co; 100 μg/ml streptomycin sulfate, No. S-1277, Sigma-Aldrich Co; and 100 μg/ml dibekacin sulfate, No. DBK, Meiji Seika Co., Tokyo Japan) [20].

Nuclear maturation test

Before IVM, some COs aspirated from the ovaries were denuded of their cumulus cells using a vortex mixture, fixed with acetic-alcohol (alcohol: acetic acid = 3:1, v/v) for at least 2 days at room temperature and stained with 1% orcein (No. 7100, Merck, Darmstädt, Germany) in 45% (v/v) acetic acid at room temperature.

The remaining COs were randomly allocated to BHB (DL type, No. 6501, Sigma-Aldrich) treatment groups and their nuclear maturation rates up to the M-II stage were observed. The concentrations of BHB for IVM were 0.0, 2.3, 3.1, 3.9 and 4.7 mM, since in a preliminary study, the nuclear maturation rate up to the M-II stage of the oocytes matured with 3.9 mM BHB was significantly higher ($P < 0.05$) than those of the 0.0, 2.0 and 7.8 and 15.6 mM BHB groups. The culture volume of IVM was adjusted to 7.5 μl/CO for all treatment groups. After IVM, the COs of each group were fixed and stained by the same method used for the immature oocytes described above.

ATP and GSH assay

The ATP and GSH contents of the oocytes were measured by methods described in previous reports [19, 20]. In brief, COs matured with or without 3.1 mM BHB in the same culture volume (7.5 μl/CO) in the nuclear maturation test were randomly divided into two groups without further selection. One group (30 to 40 COs/replicate) was used to measure the ATP contents of the oocytes and the other group (28 to 30 COs/replicate) was used to measure the GSH contents of the oocytes.

For the ATP assay, the COs matured with or without 3.1 mM BHB had their cumulus cells removed by the method described above. Then, the oocytes were loaded into a 1.5-ml Eppendorf tube (No. A-150, Assist Co., Tokyo, Japan) with 50 μl physiological saline supplemented with 2% CS (2% CS-PS). After washing with the same solution, and boiling at 100°C for 4 min after the addition of 100 μl pure water, they were stored at –20°C until assayed. A 2 mM ATP stock solution was thawed and diluted with pure water to concentrations of 50 to 100 p mol/ml. To measure the ATP contents of the oocytes, 100 μl of sample and the ATP stock solutions were placed in a lumicounter (No. A-237, Advantec Co., Tokyo, Japan), and pure luciferase (No. 60311, Kikkoman, Noda, Chiba, Japan) was added at room temperature (22–25°C). The total amount of ATP measured was divided by the number of oocytes in the sample to obtain the mean content per oocytes (p mol/oocyte).

To measure the GSH contents, denuded oocytes derived from matured COs were transferred to 1.5-ml Eppendorf tubes containing 5 μl 2% CS-PS, and stored at –20°C until assayed. GSH (G-6013, Sigma-Aldrich) was dissolved and diluted with pure water to concentrations of 0, 10, 100, 500 and 1,000 p mol/5 μl. After thawing of the samples, 1.25 M H_3PO_4 (No. 27618-55, Nacalai Tesque), 700 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 pure water and 10 μl GSSG-reductase (No. G-3664, 250 U/ml, Sigma-Aldrich) were added. The formation of TNB in samples was measured at 412 nm on a spectrophotometer (UV-

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

Concentration of BHB (mM)	N	Nuclear maturation rate to the M-II stage (%)
0.0	208	68.7 ± 2054^{b}
2.3	198	68.3 ± 3.26^{b}
3.1	213	83.0 ± 2.07^{a}
3.9	212	81.1 ± 2.39^{a}
4.7	205	73.2 ± 2.78^{ab}

a, b: Values with different superscripts in a column are significantly different ($P < 0.05$). Values are mean ± SE of 5 replicates.

Table 2. Effect of BHB added to the maturation medium on the ATP content of porcine oocytes

Treatment	ATP contents (pmol/oocyte)
Immature	0.8 ± 0.18
Matured –	1.3 ± 0.16
Matured +	1.2 ± 0.19

Values are mean ± SE of 4 replicates. +: presence of 3.1 mM BHB, –: absence of BHB (0 mM BHB).

Table 3. Effect of BHB added to the maturation medium on the GSH content of porcine oocytes

Treatment	GSH contents (pmol/oocyte)
Immature	2.6 ± 0.37^{b}
Matured –	5.0 ± 0.82^{a}
Matured +	4.1 ± 0.73^{ab}

a, b: Values with different superscripts in a column are significantly different ($P < 0.05$). Values are mean ± SE of 4 replicates. +: presence of 3.1 mM BHB, –: absence of BHB (0 mM BHB).

150-02, Shimadzu Co., Tokyo, Japan) every 30 sec for three minutes at room temperature (22–25°C). Standards were prepared for each assay and the GSH content per oocyte was calculated.

The ATP and GSH contents of immature oocytes before IVM were also measured using the same method used for the matured ones described above.

Statistical analysis

All percentage data of the nuclear maturation assays were subjected to arc-sine transformation before statistical analysis. All data were analyzed with ANOVA followed by Duncan's Multiple Range Test.

Results

Before IVM, 66 (88.0%) of the 75 retrieved immature oocytes were at the germinal vesicle stage, and the remainder were at the prometaphase I stage. The nuclear maturation rates up to the M-II stage of the oocytes matured with the various concentrations of BHB are presented in Table 1. The nuclear maturation rate of the 3.1 mM group was the highest, and this value was significantly higher ($P < 0.05$) than that of the control (0.0 mM) group.

The ATP contents of the oocytes in each group are presented in Table 2. The ATP contents of the oocytes derived from the COs matured with and without 3.1 mM BHB were similar to those of the immature group. The GSH contents of the oocytes in each group are presented in Table 3. The GSH content of the oocytes derived from the COs matured without BHB was significantly higher ($P < 0.05$) than that of the immature group. However, the GSH content of the oocytes derived from the COs matured with 3.1 mM BHB was not significantly different from those of the COs matured with other BHB concentrations and the immature group.

Discussion

In the present study, the nuclear maturation rate up to the M-II stage of the oocytes matured in the 3.1 mM group was the highest, and this rate was significantly higher ($P < 0.05$) than that of the control (0.0 mM) group. This suggests that 3.1 mM BHB may stimulate the nuclear maturation of porcine oocytes.

It is well known that BHB is converted back into acetyl-CoA, a precursor of citric acid in the TCA cycle, before its complete combustion to CO_2 and water via the reactions of the TCA cycle [5, 6]. Cetica *et al.* [21] reported that both bovine cumulus cells and oocytes maintained the activity of some TCA cycle enzymes during IVM and helped progress the nuclear maturation

of the oocytes up to the M-II stage.

Surprisingly, in this study, 3.1 mM BHB stimulated the nuclear maturation of the oocytes regardless of the presence of 5.5 mM glucose, a concentration usually used for porcine IVM [18]. As mentioned above, increases in BHB plasma levels are well-documented in nutrient starvation (i.e. fasting or the early lactation period) and diabetics [1–4]. In general, BHB can be used for cells under hypoglycaemic conditions or for the deletion of energy substrates such as glucose, pyruvate and lactate [2, 4]. It is well known that the glucose pentose phosphate pathway which produces NADPH, a key regulator of glutathione, and/or glycolysis which provides pyruvate, and the TCA cycle play key roles in the control of both nuclear and cytoplasmic maturation of porcine oocytes and in the production of energy, such as ATP [14, 22]. In the mouse, oocytes prefer to consume pyruvate rather than glucose, but cumulus cells prefer glucose [23]. In addition, Funahashi *et al.* [24] reported that both glucose and pyruvate play critical roles in the release of porcine oocytes from arrest at the GV-I stage (immature stage). It is well known that pyruvate is converted to acetyl-CoA when it is transported into mitochondria. The medium we used for IVM contained no pyruvate and 5.55 mM glucose [18], which appeared to be enough for the porcine oocytes because the glucose concentration in porcine follicular fluid *in vivo* is speculated to be over 4 mM [25]. This led us to infer that the energy substrate for nuclear or cytoplasmic maturation of the oocytes and production of ATP was sufficient for the porcine oocytes in the present study.

In the present study, if BHB (3.1 mM) had entered the TCA cycle of the oocytes, the ATP content of the oocytes matured with BHB would have increased. However, no increase of the ATP contents of the oocytes was seen. He *et al.* [26] reported that calcium release may participate in the progression of meiosis of bovine oocytes. Also BHB had a stimulatory effect on cell cycle progression that is mediated by a signaling pathway dependent upon increases in intracellular Ca^{2+} [3]. Taken together with our present results, we postulate that BHB in the IVM medium may stimulate intracellular Ca^{2+} to progress the nuclear maturation of the oocytes.

When the ATP contents of the oocytes matured with and without BHB were compared, no significant differences were observed in the present study. This indicates that 3.1 mM BHB added to the IVM medium does not influence the ATP content of porcine oocytes. Haces *et al.* [15] reported that both BHB and acetoacetate induced recovery of the ATP content of rat hippocampal neurons after their glycolysis was inhibited by iodoacetate, a glycolysis inhibitor. In the present study, the glycolysis of the oocytes in the IVM medium was not inhibited since the medium we used contained 5.55 mM glucose [18]. This may have negated the influence of BHB on the ATP content of the oocytes.

The ATP contents of the oocytes matured without BHB were not increased compared with those of the immature oocytes before IVM. Brevini *et al.* [27] reported that the ATP content of porcine oocytes was increased after IVM. However, the addition of 150 μM cysteamine, a low molecular thiol compound, to the IVM medium did not increase the ATP content after IVM regardless of the nuclear maturation rate up to the M-II stage [20]. Therefore, in this study, the lack of increase in the ATP contents of the oocytes in the groups matured with or without BHB may have been caused by the addition of cysteamine to the IVM medium.

In the present study, although the GSH content of the oocytes matured with BHB was a little lower than that of the oocytes matured without BHB, no significant difference was seen between these groups. This suggests that the addition of 3.1 mM BHB to the IVM medium did not influence the oocyte's GSH content. In this study, we did not select the oocytes which had reached the M-II stage for GSH measurement, because it was difficult to identify the oocytes with a first polar body under a conventional microscope. Consequently, this may be one reason why we could not clarify the effect(s) of BHB on the GSH content of the oocytes. In contrast, the lack of change in ATP content of the oocytes in both groups after IVM in this study would not have been caused by our lack of selection of M-II stage oocytes, because the ATP content of the porcine oocytes is not influenced by the nuclear maturation rate after IVM [19].

The GSH content of the oocytes matured with BHB was significantly increased ($P < 0.05$) compared to that of the immature group. It is well known that the concentration of GSH within the oocyte increases throughout maturation [14, 28]. However, it is not known whether this increase is caused by meiotic progression or the associated changes in cellular metabolism [14]. Furthermore, the addition of cysteamine to the medium increased oocytic GSH contents in canine [29], porcine [20, 30], goat [31], bovine [32] and equine oocytes [33] by preventing the oxidation of cysteine to cystine and by promoting the conversion of cystine to cysteine [30]. The significant increase in the GSH content of the oocytes matured

without BHB compared to that of the immature group in this study may have been caused by the addition of cysteamine to the IVM medium. However, although the GSH contents of the oocytes matured with 3.1 mM BHB was higher than that of the immature group, no significant difference was observed. Also, when the GSH contents of the oocytes matured with or without BHB were compared, no difference was observed even if the nuclear maturation rates of the oocytes matured with BHB were significantly higher ($P < 0.05$) than those of the oocyes matured without BHB. These results indicate that the increase in the GSH content of the oocytes during IVM was not caused by the progression of nuclear maturation in this study. This is in agreement with the study by Abeydeera *et al.* [34], who reported that no relation was observed between the increase of GSH content and progression of nuclear maturation of porcine oocytes matured *in vitro*.

In conclusion, 3.1 mM BHB added to the IVM medium may stimulate the nuclear maturation of porcine oocytes without influencing ATP and GSH metabolism.

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