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Effect of β-hydroxybutyrate acid on gene expression levels of antioxidant biomarkers and growth hormone–related genes in liver cell culture

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Abstract

Introduction: In dairy cattle, oxidative stress is a predominant problem associated with diseases and reproductive health issues. This study aimed to detect the variation in the antioxidant biomarkers by adding different concentrations of β-hydroxybutyric acid (BHBA) and sought to elucidate its effects on the gene expression levels of growth hormone (GH) and antioxidant biomarkers in bovine hepatocytes. **Material and Methods:** Four antioxidant biomarkers, namely malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH Px) were evaluated using commercially available bovine ELISA kits. The expression levels of the bovine GH, its receptor (GHR), insulin-like growth factor (IGF), IGF-1, IGF-1 receptor, CAT, SOD, GSH-Px and β-actin (as a reference) genes in liver cell culture were determined by reverse transcriptase-PCR assay. **Results:** With the increase of BHBA concentration and culture time, the activities of SOD, CAT, and GSH Px biomarkers in hepatocytes decreased. However, the content of MDA in hepatocytes increased gradually with the increase of hepatocyte culture time and BHBA concentration. The qPCR results revealed that after adding BHBA, gene expression levels of GSH-Px, SOD and IGF biomarkers in hepatocytes began to differ in the culture groups at 12 h, whereas the gene expression level of the CAT and GHR biomarkers in hepatocytes began to differ at 6 h. **Conclusion:** Quantitative PCR results showed that the BHBA significantly downregulated the expression levels of the GHR gene and CAT, GSH Px and SOD antioxidant biomarker genes.

Keywords: enzymatic antioxidant, β-hydroxybutyric acid, growth hormone, biomarkers, hepatocytes.

Introduction

In dairy cattle, oxidative stress has been associated with diseases and reproductive problems (35, 38). During the stage of pregnancy and lactation, physiological changes are considered to induce metabolic and oxidative stress, which may be associated with metabolic diseases affecting the peripartum period (6, 9, 14). In general, polyunsaturated lipids are more prone to oxidation. Lipids are one of the most susceptible substrates to free-radical damage, and this lipid peroxidation resulting from oxidative stress is not indicated better by many biomarkers than by malondialdehyde (MDA). It is one of several low-molecular-weight end products formed during the radical induced decomposition of polyunsaturated fatty acid (27). Malondialdehyde is an active aldehyde and electrophilic substance that causes cytotoxic stress and forms covalent proteins. By detecting the level of MDA, the level of lipid oxidation can be detected, so the determination of MDA is widely used to evaluate lipid oxidation. In dairy cattle, MDA assays (8, 9) show significant changes in plasma MDA concentrations during the peripartum and calving period (7, 46). In our study, we utilised a commercial enzyme-linked immunosorbent assay (ELISA)-based method, which is considered to be the most reliable measurement of lipid oxidation among assays (39).

Endogenous antioxidants have been traditionally classified into three different groups (38), the first being enzymatic antioxidants, of which glutathione peroxidase (GSH-Px; EC 1.11.1.9) and superoxide dismutase (SOD; EC 1.15.1.1) are the most widely known; the second being non-enzymatic protein antioxidants; and the third being non-enzymatic low molecular weight antioxidants (10). The general term GSH-Px is for the enzyme family with peroxidase activity, which can protect the body from oxidative damage. As a secreted protein, GSH-Px is considered to be a scavenger for reactive oxygen species (ROS) and lipid hydroperoxides (LOOH) in extracellular tissues and blood circulation (49). It can reduce LOOH to their corresponding alcohol and H_2O_2 to H_2O, thereby clearing peroxides in living cells and protecting cells from free-radical damage. There are literature reports that the short-term increase in GSH-Px activity after parturition and the slight decrease in GSH-Px activity during lactation seem to be protective mechanisms that can prevent lipid and free radicals transported in the plasma from peroxidation to produce LOOH, protecting albumin from entering tissues and the breast. Glutathione peroxidase, which is distributed in almost all tissues, can utilise GSH to reduce LOOH, thereby eliminating the toxic effects of ROS (49). Some diseases can lead to an upregulation or downregulation of GSH-Px activity; therefore, when evaluating oxidative stress in cows, the measurement of cell or plasma GSH-Px activity is usually used as a diagnostic tool. Enzymatic antioxidant SOD can alternately catalyse the disproportionation of O_2^- groups, generating H_2O_2 and O_2. Therefore, SOD is considered the first line of defence in conversion to O_2 (10, 29). According to protein folding and metal cofactors, SOD is currently mainly divided into three families: the Cu/Zn type, Fe/Mn type and Ni type, all of which are present in mammals. The superoxide anion O_2^- is a byproduct of oxygen metabolism, which, if not regulated, can cause many types of cell damage. Hydrogen peroxide is also destructive, but can be degraded by other enzymes such as catalase (CAT). The CAT antioxidant is a tetramer containing four polypeptide chains, each consisting of over 500 amino acids and containing four iron containing haem groups (29). It is widely distributed and is abundant in the liver, kidneys, and red blood cells. It catalyses H_2O_2 and decomposes it into H_2O and O_2, making it a very important enzyme that protects cells from oxidative damage. Catalase has the efficient catalytic properties of all enzymes: a single CAT molecule can convert millions of H_2O_2 molecules into H_2O and O_2 per second, and within a certain range, CAT levels increase as ROS production increases. Therefore, CAT is also an important indicator of oxidative stress.

The endogenous antioxidants SOD and GSH-Px represent the main form of intracellular antioxidant defence. As an indicator of oxidative stress, plasma GSH-Px activity contributes to the oxidative defence of animal tissues by catalysing the reduction of hydrogen and lipid peroxides (24, 50). This antioxidant's function in cellular oxidation-reduction reactions helps to protect the cell membrane from oxidative damage caused by free radicals (19). Its counterpart SOD catalyses the dismutation of superoxide to hydrogen peroxide and it is considered the first defence against pro-oxidants (24). Since SOD activity increases hydrogen peroxide production, the protection from ROS would only be given by a simultaneous increase in CAT, GSH-Px activities and availability of glutathione (20, 28).

Studies carried out in dairy goats have shown that blood GSH-Px activity is decreased during the postpartum period, suggesting that the does may have experienced some degree of oxidative stress and lipid peroxidation (11, 12, 31). Since GSH-Px is directly targeted at removing hydrogen peroxide generated during the dismutation of free radicals (15), it would be reasonable to see a parallel decrease in reactive oxygen metabolite (ROM) levels. These levels did indeed decrease in week 4 postpartum; however, their concentrations in week 2 were significantly higher (11), which further indicated oxidative stress in goats during the early postpartum period. Even if blood GSH-Px activity is inhibited, the body could have been defended against oxidative stress by alternative means (11, 12). For example, CAT is another antioxidant biomarker that can catabolise hydrogen peroxide (15). Oxidative stress results from increased exposure to or production of oxidants, or from decreased dietary intake, *de novo* synthesis or increased turnover of antioxidants. The understanding of the roles of oxidants and antioxidants in physiological and pathological conditions is rapidly

expanding. In ruminants, oxidative stress is implicated in numerous disease processes including sepsis, mastitis, acidosis, sub-clinical ketosis (SCK), enteritis, pneumonia, and respiratory and joint diseases. The pathological basis of SCK in dairy cows is based on the negative energy balance (NEB) and β-hydroxybutyric acid (BHBA) concentration (2, 21). The concentrations of NEB and BHBA in SCK cows were significantly higher than those in healthy cows. Beta hydroxybutyric acid is an important metabolite in β-oxidation and the main component of ketone bodies, therefore the determination of BHBA is employed as an indicator in the diagnosis of SCK (5). In cattle, BHBA is considered a gold standard for diagnosing SCK because of its stability in blood. A cut-off value of 1.2 to 1.4 mmol L^{-1} of BHBA in blood samples is used to distinguish between cows with and without SCK (17, 26, 41). Compared with healthy cows, SCK cows showed severe oxidative stress and liver injury, which were positively correlated with BHBA concentration (1). The BHBA concentration also regulates the synthesis and secretion of growth hormone (GH), and is modulated by various metabolic influences. The study of Meier *et al.* (37) found that during the period of NEB, the somatotropic axis responds by increasing plasma GH and decreasing plasma interferon growth factor 1 (IGF-1) levels. In another study, Laeger *et al.* (31) reported that high blood BHBA concentrations inhibit the secretion of GH in humans and rhesus monkeys. In cattle, serum growth hormone (GH) concentrations were elevated during SCK, and several studies described uncoupling of the GH–IGF-1 axis after delivery (16). The hypothesis was followed for the present research that lower liver growth hormone receptor (GHR) during SCK may further affect liver IGF function. Overall, these results led to interest in exploring the relationship between BHBA, GH and antioxidant biomarker–related factor mRNA expression in bovine liver cells. Therefore, the aim of this study was to analyse the effect of BHBA concentration on the gene expression levels of GH and antioxidant biomarker–related factor in bovine hepatocytes *in vitro*, and to explore the pathogenesis of ketosis in postpartum dairy cows.

Material and Methods

Ethical statement. All animals used in this study were treated according to the international guiding principles for biomedical research involving animals and under animal welfare ethical protocol No. GXU-2018-203, Guangxi University, Nanning, Guangxi, China.

Cell culture experiment. The bovine hepatocytes used in this study were donated by the Internal Medicine Laboratory of Nanjing Agricultural University but had originally been obtained from Professor Juan J. Loor of the Department of Animal Science and Nutritional Sciences, University of Illinois at Urbana. The primary liver cells of Holstein cows (1–3 g) were isolated from biopsied liver tissue by non-perfusion technique (0–160 days postpartum). The liver tissue was immersed in 70% ethanol for 30 s to reduce surface contamination and then placed in a 10 mL sterile test tube containing 5 mL of a previously prepared 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid solution containing 0.05 mM ethylenediaminetetraacetic acid. The sample in solution was delivered to the laboratory within 30 min. The tissue was finely chopped with a scalpel blade, put into Hanks' balanced salt solution without Ca^{2+} or Mg^{2+} (Mediatech/Corning, Manassas, VA, USA) and centrifuged at 50 × *g* for 3 min for washing of tissue. Then the tissue was suspended in 50 mL of type I collagenase (1mmol L^{-1} calcium chloride and 150 U mL^{-1}; Sigma-Aldrich, St. Louis, MO, USA). The culture conditions were 5% CO_2, 37°C, at the end of incubation, the collagenase was neutralised by 25 mL of medium containing 10% foetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA). The cell suspension was filtered through a 200-mesh cell filter and washed with phosphate-buffered saline (PBS) three times. Then the filtrate was resuspended in a medium supplemented with 10% FBS, 1 mmol·L^{-1} glucagon, 10 nmol·L^{-1} dexamethasone, 10 ng·L^{-1} epidermal growth factor, 10 nmol/L insulin and 1% penicillin/streptomyces solution. The cell suspension was placed in a culture chamber to be covered with cells, and then digested and frozen for further use.

Cell culture. The liver cells of the Holstein cows were inoculated into 25 cm^3 culture flasks for cultivation. In the control group, the same amount of sample was obtained from healthy cows' livers. After the cells reached a confluent monolayer, they were digested and suspended at predetermined time points. After counting the cells, 5×10^5 cells in suspension were pipetted into a sterile six-well plate and cultured at 37°C in 5% CO_2 overnight to adhere the cells. After discarding the original culture medium, cell culture was washed twice, first with preheated 37°C PBS, and then the cells were divided into four experimental groups by adding a different volume of BHBA stock solution and low sugar Dulbecco's modified Eagle's medium. A concentration gradient of BHBA in the medium was set with 0.0 mmol L^{-1}, 0.6 mmol L^{-1}, 1.2 mmol L^{-1} and 3.0 mmol L^{-1}. For the differentiation of each experimental group, the culture time was 0 h, 6 h, 12 h, 24 h and 48 h. After the culture period ended, the cells were collected for storage, and were kept for future analysis.

Configuration of BHBA standard solutions. A BHBA standard solution was prepared by accurately weighing 104.1 mg of BHBA powder, adding it into 10 mL of distilled water and then configuring the solution into 100 mmol·L^{-1} of BHBA under aseptic conditions. After passing it through a small filter screen of 0.22 μm, the BHBA solution was separated into sterile EP tubes and stored at −20°C for further use.

Table 1. Primer sequences utilised in a reverse-transcriptase-PCR

Gene	GenBank accession No.	Primer sequences (5′–3′)	Base pairs
GSH-Px	NM_001101113.2	Fwd GCGGGAGCAGGACTTCTACGA Rev CCCGATAGTGCTGGTCTGTGAA	137
SOD	NM_201527.2	Fwd TTCAATAAGGAGCAGGGACG Rev CAGTGTAAGGCTGACGGTTT	234
CAT	NM_001035386.1	Fwd AGATACTCCAAGGCGAAGGTG Rev AAAGCCACGAGGGTCACGAAC	120
IGF-1	NM_001077828.1	Fwd TCGCATCTCTTCTATCTGGCCCTGT Rev GCAGTACATCTCCAGCCTCCTCAGA	101
IGF-1R	NM_001244612.1	Fwd TTAAAATGGCCAGAACCTGAG Rev ATTATAACCAAGCCTCCCAC	240
GHR	NM_176608.1	Fwd CCAGTTTCCATGGTTCTTAATTAT Rev TTCCTTTAATCTTTGGAACTGG	138
β-actin	NM_173979.3	Fwd CTCTTCCAGCCTTCCTTCCT Rev GGGCAGTGATCTCTTTCTGC	233

GSH-Px – glutathione peroxidase; SOD – superoxide dismutase; CAT – catalase; IGF-1 – insulin-like growth factor 1; IGF-1R – insulin-like growth factor 1 receptor; GHR – growth hormone receptor; Fwd – forward primer; Rev – reverse primer

Cell antioxidant index experiment: cell protein extraction procedure. After cleaning the cells 2–3 times with pre-cooled PBS, cells were lysed by adding 200 μL of cell lysate and an appropriate amount of phenylmethylsulfonyl fluoride to keep its final concentration at 1 mmol L^{-1}. The cell lysis was performed at 4°C for 30 min, during which the cells were blown repeatedly with a pipette gun to ensure complete lysis. After centrifugation at 14,000 × *g* for 5 min, the supernatant cell protein extracts were collected and stored at −80°C. Antioxidant tests for MDA, SOD, GSH-Px and CAT were performed using commercially available bovine ELISA kits (Baoman Biological, Shanghai, China) according to the manufacturer's protocols.

Gene expression experiment: total RNA extraction from hepatocytes. After discarding the cell culture medium, 1mL of Trizol reagent (Thermo Fisher Scientific) was added directly to the lysis buffer with cell culture medium. The total RNA was isolated using a single extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture (13).

Gene expression of targeted genes using RT-PCR. The RNA quality and quantity were measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific), and RNA integrity was determined by gel electrophoresis. The total cDNA was reverse-transcribed using a kit (HiScript II One Step RT-PCR Kit P611; Vazyme Biotech, Nanjing, China). The relative mRNA expression of target genes (Table 1) was detected using the FastStart Universal SYBR Green Master (ROX) (Roche, Norwalk, CT, USA) on the Roche LightCycler 96 real-time PCR system (Roche, Mannheim, Germany). The primers of target genes (GH, GHR, IGF-1, β-actin, CAT, SOD and GSH-Px) were designed by Primer Express software (Applied Biosystems Inc., Carlsbad, CA, USA) using the gene sequences published in GenBank (Table 1). The β-actin gene was used as an endogenous reference gene (53). The reaction conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The relative quantitation values were normalised to the geometric mean of each reference gene's threshold cycle (Ct). The quantification cycle values were extrapolated using a relative quantitative 2–ΔΔCt method, where △△Ct = (Ct.Target − Ct.Actin) of target gene − (Ct.Target − Ct.Actin) of control set of genes (44).

Statistical Analysis. Statistical analysis of the antioxidant tests was performed using three replications of each sample and one-way analysis of variance (ANOVA) in SPSS software, version 20.0 for Windows (IBM, Armonk, NY, USA). The results were expressed as mean ± standard error of the mean. The experimental data of the RT-PCR assay was also analysed using SPSS. The significance of differences between groups was analysed by ANOVA. The significance of differences was observed as significant (P-value < 0.01), but not very significant (P-value < 0.05).

Results

Effects of BHBA on hepatocyte oxidation. The effects of different concentrations of BHBA on antioxidant biomarkers and oxidation indices of hepatocytes are shown in Table 2. The results revealed that the overall levels of SOD, CAT and GSH-Px decreased with the increase in the concentration of BHBA in the culture medium, and the difference was very significant (P-value < 0.05). However, the level of MDA increased with the decrease in the concentration of BHBA in the culture medium, and this difference was also very significant (P-value < 0.05) (Table 2). The level of SOD decreased about twofold from its highest in the 0.0 mmol L^{-1} BHBA concentration to its lowest in the 3.0 mmol L^{-1} concentration. The level of CAT changed 1.5-fold, whereas MDA showed 0.91-fold changes.

Table 2. Effect of different concentrations of β-hydroxybutyrate acid (BHBA) on the oxidase index of hepatocytes *in vitro*

	SOD (U·mL^{-1})	CAT (U·mL^{-1})	MDA (mmol·mL^{-1})	GSH-Px (U·mL^{-1})
0.0	7.43 ± 0.44^{a}	13.15 ± 0.48^{a}	11.94 ± 0.35^{b}	1.65 ± 0.05^{a}
0.6	5.77 ± 0.69^{a}	12.44 ± 0.50^{a}	12.19 ± 0.32^{a}	1.52 ± 0.06^{a}
1.2	4.02 ± 0.73^{b}	11.64 ± 0.72^{a}	12.86 ± 0.38^{a}	1.37 ± 0.08^{b}
3.0	3.87 ± 0.84^{b}	9.15 ± 1.06^{b}	13.16 ± 0.38^{a}	1.39 ± 0.06^{b}

The same letters in the same column mean no significant difference. Different letters in the same column indicate significant difference (P-value < 0.05)
SOD – superoxide dismutase; CAT – catalase; MDA – malondialdehyde; GSH-Px – glutathione peroxidase

The results of multiple comparison showed that the levels of SOD, GSH-Px and CAT in the groups with BHBA concentrations of 0.0 and 0.6 were significantly higher than those in the group with BHBA concentration of 3.0 (P-value < 0.05), while the level of MDA in the group with BHBA concentration of 3.0 was significantly higher than that in the groups with lower concentrations (P-value < 0.05).

Effects of BHBA on MDA in hepatocytes. The effects of BHBA on MDA in hepatocytes are shown in Fig. 1. The results revealed that with the increase of hepatocyte culture time and concentration, the content of MDA increases gradually. Comparing the initial (0 h) MDA concentration to the concentration at the longest culture time of 48 h, it increases about five-fold. However, comparing the control group (BHBA 0.0 mmol L^{-1}), there was no significant difference in MDA concentration until the cessation of hepatocyte culture. After 6 h of hepatocyte culture, the MDA concentration in the 0.6 mmol L^{-1} BHBA group was significantly different (P-value < 0.05) to that of the control group, and there was no significant difference between the MDA concentrations in the other experimental groups and the concentration in the control group. After 12 h of hepatocyte culture, the 1.2 and 3.0 mmol L^{-1} BHBA concentration groups were upregulated highly significantly in MDA concentration (P-value < 0.01) compared to the control group. After 24 h of hepatocyte culture, the 3.0 mmol L^{-1} BHBA group had a significantly different concentration of the aldehyde (P-value < 0.05) than did the control group. At the end, after 48 h of hepatocyte culture, the 1.2 and 3.0 mmol L^{-1} BHBA groups were different highly significantly (P-value < 0.01) in this parameter to the control group (Fig. 1). It was evident that MDA expression was upregulated with increasing BHBA concentration and with the lengthening of the hepatocyte culture time past 12 h.

Effects of BHBA on CAT in hepatocytes. The effects of BHBA on CAT in hepatocytes are shown in Fig. 2. The results revealed that with the increase of hepatocyte culture time and BHBA concentration, the expression of CAT showed a decreasing trend. When cells had been cultured for 0 h, there was no significant difference in the CAT levels between the control group and each experimental group, and the difference was about 16 U m L^{-1}. After 6 h and 12 h of hepatocyte culture, the CAT concentration in the control group was highly significantly different (P-value < 0.01) to that of the 3.0 mmol L^{-1} BHBA group. After 24 h of culture, the CAT concentration in the 1.2 mmol L^{-1} BHBA group differed significantly (P-value < 0.05) from that of the control group, and the CAT concentration of the 3.0 mmol L^{-1} BHBA group differed highly significantly (P-value < 0.01) from it. After 48 h culture, this parameter in the 1.2 and 3.0 mmol L^{-1} BHBA groups was different to a highly significant degree (P-value < 0.01) to that of the control group (Fig. 2). The CAT expression showed downregulation with increasing BHBA concentration that became more overt with increasing hepatocyte culture time past 12 h.

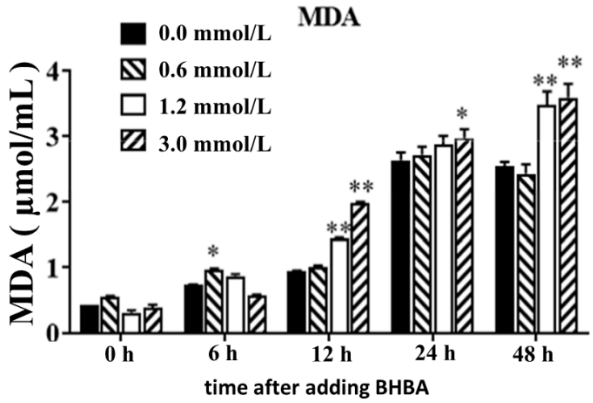

Fig. 1. Effect of different concentrations of β-hydroxybutyrate acid (BHBA) on malondialdehyde (MDA) in hepatocytes at different time points
* – significant MDA concentration difference between BHBA concentration groups and the 0.0 mmol·L^{-1} BHBA concentration group (P-value < 0.05) at the same time point; ** – highly significant difference (P-value < 0.01)

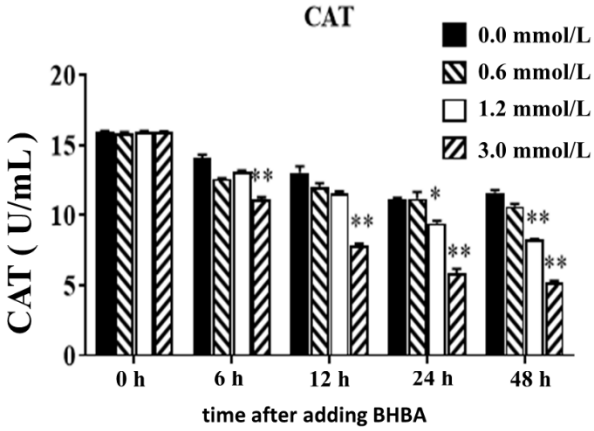

Fig. 2. Effect of different concentrations of β-hydroxybutyrate acid (BHBA) on catalase (CAT) in hepatocytes at different time points
* – significant CAT concentration difference between BHBA concentration groups and the 0.0 mmol·L^{-1} BHBA concentration group (P-value <0.05) at the same time point; ** – highly significant difference (P-value <0.01)

Effects of BHBA on GSH-Px in hepatocytes. The effects of BHBA on GSH-Px in hepatocytes are shown

in Fig. 3. The results revealed that GSH-Px concentrations in hepatocytes decreased with increasing culture time. At 0 h cell culture time, the GSH-Px concentrations were not significantly different, and the enzyme activity was about 2.0 U mL^{-1}. The GSH-Px level decreased with the increase of incubation time of hepatocyte culture (6 h, 12 h, and 24 h) in the groups to which BHBA was added, and the 1.2 and 3.0 mmol L^{-1} BHBA groups' GSH-Px concentrations differed highly significantly (P-value < 0.01) from that of the control group at 0 h and 6 h. When hepatocytes were cultured for 48 h, the difference between the GSH-Px concentrations in the 0.6 mmol L^{-1} BHBA group and the control group was not significant; however, the GSH-Px concentration in the 1.2 and 3.0 mmol L^{-1} BHBA groups did differ significantly (P-value < 0.05) from that of control group (Fig. 3).

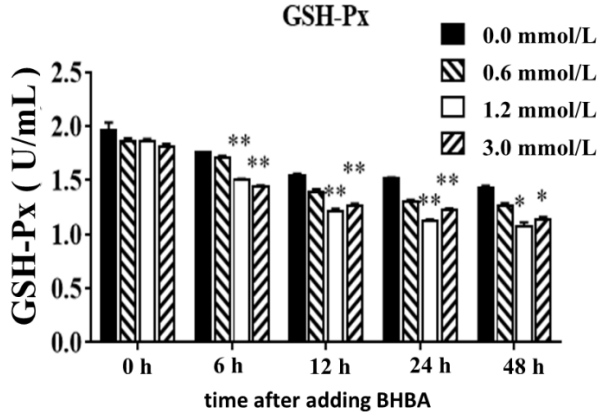

Fig. 3. Effect of different concentrations of β-hydroxybutyrate acid (BHBA) on glutathione peroxidase (GSH-Px) in hepatocytes at different time points
* –significant GSH-Px concentration difference between BHBA concentration groups and the 0.0 mmol·L^{-1} BHBA concentration group (P-value < 0.05) at the same time point; ** – highly significant difference (P-value < 0.01)

Effects of BHBA on SOD in hepatocytes. The effects of BHBA on SOD in hepatocytes are shown in Fig. 4. Results revealed that the SOD concentration in the hepatocytes decreased with the increase of BHBA concentration and hepatocyte culture time. When hepatocytes were cultured for 6 h, the SOD concentration in the 1.2 and 3.0 mmol L^{-1} BHBA groups differed significantly (P-value < 0.05) from that of the control group. When the hepatocytes were cultured for 12 h, the SOD concentration in the 3.0 mmol L^{-1} BHBA group had diminished to a highly significantly different extent (P-value < 0.01) from this concentration in the control group, but the differences in this parameter between other groups were insignificant. When the hepatocytes were cultured for 24 h, the SOD activity in the 1.2 and 3.0 mmol L^{-1} BHBA experimental groups were highly significantly different (P-value < 0.01) to that in the control group. When the hepatocytes were cultured for 48 h, the SOD activity in the 0.6, 1.2 and 3.0 mmol L^{-1} BHBA experimental groups differed highly significantly (P-value < 0.01) from that in the control group (Fig. 4).

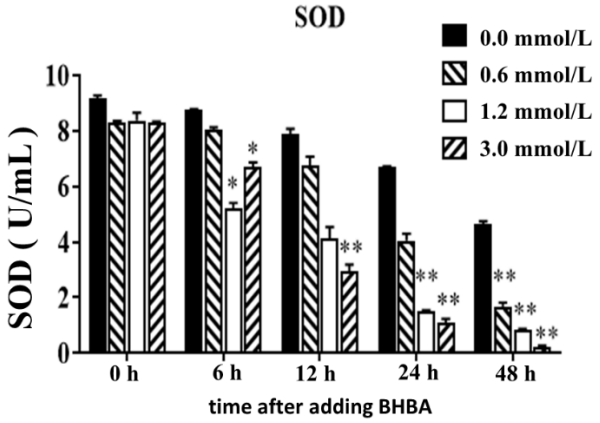

Fig. 4. Effect of different concentrations of β-hydroxybutyrate acid (BHBA) on superoxide dismutase (SOD) in hepatocytes at different time points
* – significant SOD concentration difference between BHBA concentration groups and the 0.0 mmol·L^{-1} BHBA concentration group (P-value < 0.05) at the same time point; ** – highly significant difference (P-value < 0.01)

Effect of BHBA on gene expression levels of CAT. The gene expression levels of CAT were measured by a relative fluorescence quantitative PCR assay and analysed with one-way ANOVA. Results revealed that the gene expression level of the CAT antioxidant biomarker in hepatocytes decreased with the time of culture and the concentration of BHBA (Fig. 5). There was no significant difference in the gene expression level of CAT at the initial hepatocyte culture time of 0 h. After 6 h of hepatocyte culture, the gene expression level of the CAT antioxidant biomarker in each group began to decrease at different degrees, which differed significantly in the 1.2 mmol L^{-1} BHBA group (P-value < 0.05) in comparison to the control group. After 12 h and 24 h of culture, the expression of CAT in the 3.0 mmol L^{-1} BHBA group was significantly less (P-value < 0.05) than it was in the control group, and after 48 h of culture, the expression in the 1.2 and 3.0 mmol L^{-1} BHBA groups was significantly downregulated (P-value < 0.05) from that in the control group (Fig. 5).

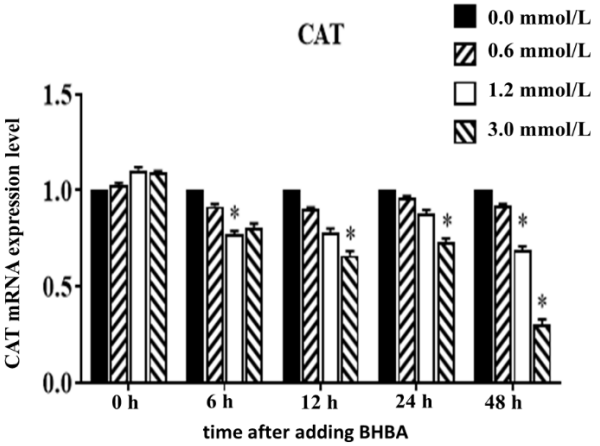

Fig. 5. Effect of different concentrations of β-hydroxybutyrate acid (BHBA) on the gene expression level of catalase (CAT) messenger RNA (mRNA) in hepatocytes at different time points
* – significant CAT expression difference between BHBA concentration groups and the 0.0 mmol L^{-1} BHBA concentration group (P-value < 0.05) at the same time point; ** – highly significant difference (P-value < 0.01)

Effect of BHBA on the expression of GSH-Px messenger RNA (mRNA). The results revealed that the differences in gene expression level of the GSH-Px antioxidant biomarker in hepatocytes began to appear between groups after 12 h culture time with BHBA. There was no significant difference in the gene expression level of GSH-Px at the initial hepatocytes culture time of 0 h or at 6 h. After 12 h of hepatocyte culture, the expression of GSH-Px differed significantly in the 1.2 mmol L^{-1} BHBA group (P-value < 0.05) and highly significant in the 3.0 mmol L^{-1} BHBA group (P-value < 0.01) in comparison to the expression in the control group. After 24 h of hepatocyte culture, the expression continued to be highly significantly weaker in the 3.0 mmol L^{-1} BHBA group (P-value < 0.01) in comparison to the control group. After 48 h of culture, the parameter also differed significantly in the 0.6 mmol L^{-1} BHBA group, did so again in the 1.2 mmol L^{-1} BHBA group (P-value < 0.05) and continued to differ highly significantly in the 3.0 mmol L^{-1} group (P-value < 0.01) from its control group value (Fig. 6).

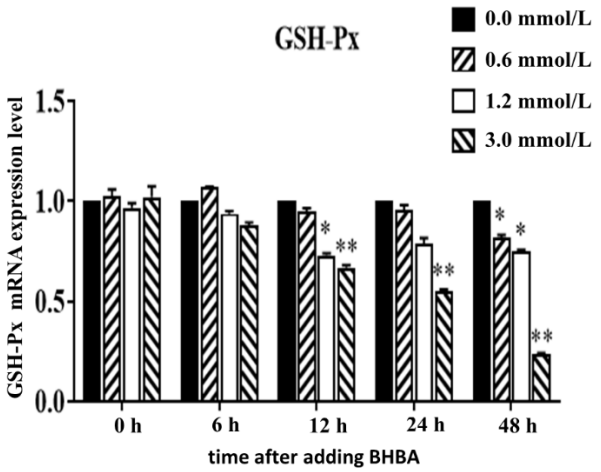

Fig. 6. Effect of different concentrations of β-hydroxybutyrate acid (BHBA) on the expression of glutathione peroxidase (GSH-Px) messenger RNA (mRNA) in hepatocytes at different time points
* – significant GSH-Px expression difference between BHBA concentration groups and the 0.0 mmol·L^{-1} BHBA concentration group (P-value < 0.05) at the same time point; ** – highly significant difference (P-value < 0.01)

Effect of BHBA on the expression of SOD mRNA. The results revealed that the differences in the gene expression level of the SOD antioxidant biomarker in hepatocytes began to appear between groups after 12 h culture time with BHBA. There was no significant difference in the gene expression level of SOD at the initial hepatocytes culture time of 0 h or at 6 h. After 12 h of hepatocyte culture, the expression of SOD was different significantly in the 0.6 and 1.2 mmol L^{-1} BHBA groups (P-value < 0.05), and different highly significant in the 3.0 mmol L^{-1} BHBA group (P-value < 0.01) in comparison to its expression in the control group. After 24 h and 48 h of culture, the gene expression level of the SOD antioxidant biomarker was downregulated significantly in the 0.6 and 1.2 mmol L^{-1} BHBA groups (P-value < 0.05) and highly significantly in the 3.0 mmol L^{-1} group (P-value < 0.01) from that in the control group (Fig. 7).

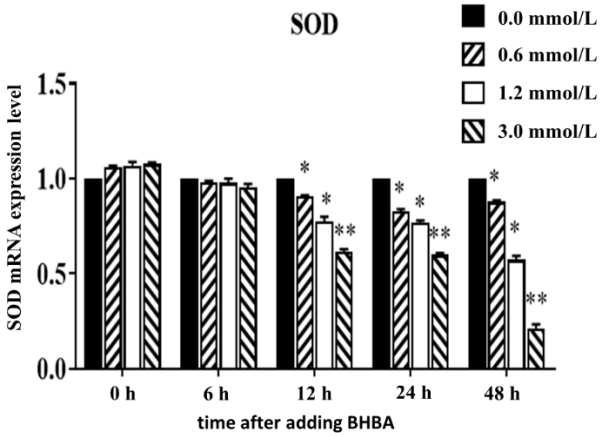

Fig. 7. Effect of different concentrations of β-hydroxybutyrate acid (BHBA) on the expression of superoxide dismutase (SOD) messenger RNA (mRNA) in hepatocytes at different time points
* – significant SOD expression difference between BHBA concentration groups and the 0.0 mmol·L^{-1} BHBA concentration group (P-value < 0.05) at the same time point; ** – highly significant difference (P-value < 0.01)

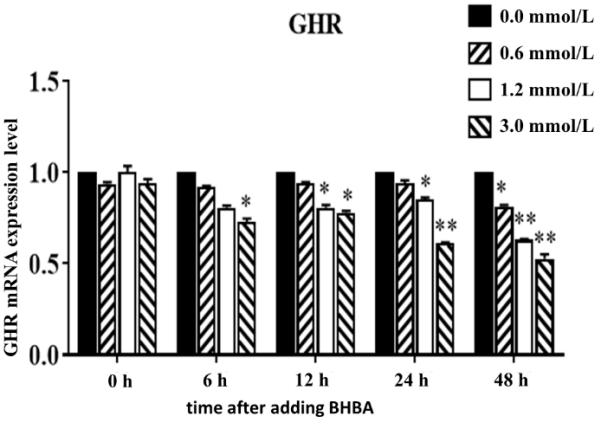

Fig. 8. Effect of different concentrations of β-hydroxybutyrate acid (BHBA) on the expression of growth hormone receptor (GHR) messenger RNA (mRNA) in hepatocytes at different time points
* – significant GHR expression difference between BHBA concentration groups and the 0.0 mmol·L^{-1} BHBA concentration group (P-value < 0.05) at the same time point; ** – highly significant difference (P-value < 0.01)

Effect of BHBA on the expression of GHR mRNA. The gene expression level of GHR in hepatocytes decreased with lengthening culture time and rising concentrations of BHBA in the culture medium. The results revealed that the differences in the gene expression level of GHR in hepatocytes began to appear between groups after 6 h culture time with BHBA. There was no significant difference in the expression of GHR at the initial hepatocyte culture time at 0 h. After 6 h of hepatocyte culture, the expression of GHR was less by a significantly different margin in the 3.0 mmol L^{-1} BHBA group (P-value < 0.05) than it was in the control group. After 12 h of hepatocyte culture, it differed significantly in the 1.2 and 3.0 mmol L^{-1} BHBA groups (P-value < 0.05) from the control group's expression level. After 24 h of hepatocyte culture, the gene expression level of GHR had declined significantly in the 1.2 mmol L^{-1} BHBA group (P-value < 0.05) and highly significantly in the 3.0 mmol L^{-1} BHBA group (P-value < 0.01) in comparison to the control group.

When culture ended after 48 h, the parameter differed significantly (0.6 mmol L^{-1} BHBA group (P-value < 0.05)) or highly significantly (other BHBA groups (P-value < 0.01)) from its value in the control group (Fig. 8).

Effect of BHBA on the expression of IGF-1 mRNA. The results revealed that the differences in gene expression level of IGF-1 in hepatocytes began to appear between groups after 12 h culture time with BHBA. There was no significant difference in the gene expression level of IGF-1 at the initial hepatocytes culture time of 0 h or at 6 h. The fallen gene expression level of IGF-1 differed significantly in the 1.2 and 3.0 mmol L^{-1} BHBA groups (P-value < 0.05) after 12 h of culture from its expression level in the control group. After 24 h of hepatocytes culture, there was significantly less expression of IGF-1 in the 1.2 mmol L^{-1} BHBA group (P-value < 0.05) and highly significantly less in the 3.0 mmol L^{-1} BHBA group (P-value < 0.01) than in the control group. After 48 h of culture, this marker's express in was downregulated highly significantly in the 1.2 and 3.0 mmol L^{-1} BHBA groups (P-value < 0.01) from that of the control group (Fig. 9).

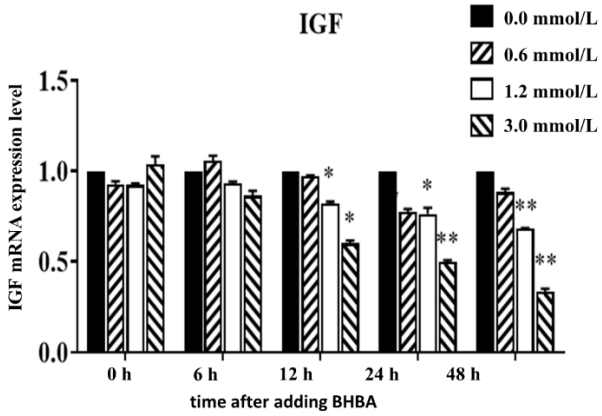

Fig. 9. Effect of different concentrations of β-hydroxybutyrate acid (BHBA) on the expression of insulin-like growth factor (IGF) messenger RNA (mRNA) in hepatocytes at different time points
* – significant IGF expression difference between BHBA concentration groups and the 0.0 mmol L^{-1} BHBA concentration group (P-value < 0.05) at the same time point; ** – highly significant difference (P-value < 0.01)

Effect of BHBA on the expression of IGF-1R mRNA. The results revealed that the differences in gene expression level of IGF in hepatocytes began to appear between groups after 6 h culture time with BHBA. There was no significant difference in the gene expression level of IGF-1R in the initial hepatocyte culture time of 0 h. After 6 h of hepatocyte culture, the gene expression level of IGF-1R had dropped significantly in the experimental group containing 3.0 mmol L^{-1} BHBA (P-value < 0.05) in comparison to the control group. After 12 h of hepatocyte culture, its expression highly significantly weaker in the 1.2 and 3.0 mmol L^{-1} BHBA groups (P-value < 0.01) than it was in the control group. After 24 h of hepatocyte culture, IGF-1R was expressed to a highly significantly smaller extent in the 3.0 mmol L^{-1} BHBA group (P-value < 0.01) in comparison to the control group. At the experiment's end after 48 h of culture, the gene expression level of IGF-1R differed significantly in the 1.2 mmol L^{-1} BHBA group (P-value < 0.05) and highly significantly in the 3.0 mmol L^{-1} BHBA group (P-value < 0.01) from that of the control group (Fig. 10).

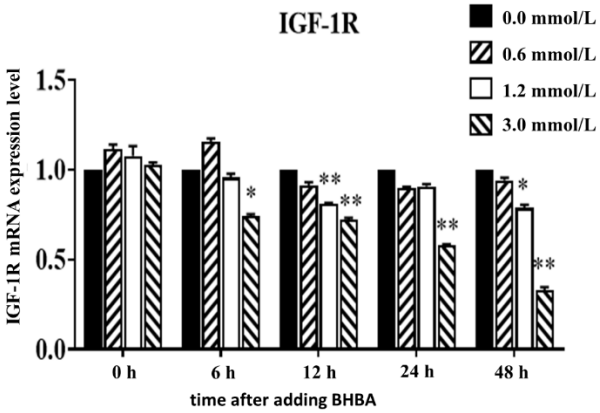

Fig. 10. Effect of different concentrations of β-hydroxybutyrate acid (BHBA) on the expression of insulin-like growth factor 1 receptor (IGF-1R) messenger RNA (mRNA) in hepatocytes at different time points
* – significant IGF-1R expression difference between BHBA concentration groups and the 0.0 mmol·L^{-1} BHBA concentration group (P value < 0.05) at the same time point; ** – highly significant difference (P-value <0.01)

Discussion

The transition period for cows is defined as the period from three weeks before calving to three weeks after calving (23), when significant hormonal, physiological and energy balance–related changes occur because of calving and the onset of lactation. A number of reviews have reported important physiological changes when dairy cows change from non-lactating to lactating states (14). The transition period is a time of high incidence rates of diseases in dairy cattle, and the impact of these diseases on the cow's health and productivity usually extends into the next lactation period (40). Therefore, successfully adapting cows to this stage of change is critical to the efficiency of the dairy industry. Although there are some ways to prevent and mitigate NEB through dietary management and dietary supplements (43, 52), ketosis among dairy cattle continues to have a significant negative economic impact on dairy cattle production, and researchers have sought to elucidate the causes of ketosis at the level of genes and their expression (33). It is well known that various physiological and biochemical changes in organisms may be the result of changes in the expression of a large number of genes (36). As with most metabolic states, changes in gene expression in the liver are central to the development of ketosis.

In this study, the effects of BHBA at different concentrations on the oxidative metabolism of MDA, CAT, SOD and GSH-Px in the liver cells of ketosis-mimicking dairy cows were explored *in vitro* with 0 mmol·L^{-1} as a blank control, 0.6 mmol·L^{-1} simulating healthy cows; 1.2 mmol·L^{-1} simulating SK (subclinical

ketosis) cows, and 3.0 mmol·L^{-1} simulating clinical ketosis (CK) cows. The results showed that MDA content of the highest-concentration BHBA group was significantly higher, but the activities of CAT, SOD and GSH-Px were significantly lower in the ketosis cows than those in control cows. These data and previous studies showed that ketosis cows exhibited oxidative stress and liver damage. *In vitro* data further showed that BHBA treatment significantly increased MDA content and downregulated hepatocyte antioxidant biomarker mRNA expression. Beta hydroxybutyric acid treatment decreased cell antioxidant capacity and induced oxidative and antioxidant imbalance in hepatocytes. There was no difference in the mRNA level and enzyme activity of CAT between the control group and the BHBA 0.6 mmol L^{-1} group although the level and activity in both groups decreased over time. The activities of SOD and GSH-Px in the 0.6 mmol L^{-1} group, which were downregulated at different time points, decreased only at 48 h (SOD) or did not decrease (GSH-Px). The obtained results indicated that BHBA at physiological concentration had little effect on the antioxidant capacity. It can generally maintain the dynamic balance of antioxidant system, which is consistent with other reports. However, with the increase of BHBA concentration ≥1.2 mmol L^{-1}), the activities and expression levels of these antioxidant biomarkers were inhibited and downregulated significantly. The study suggested that high concentration of BHBA can destroy the physiological functions of these key enzymes, lead to the imbalance of the oxidation and antioxidant system, and then induce oxidative stress. This suggests that high BHBA in severe ketosis cows may lead to oxidative stress in the body (48). High levels of ROS alter the integrity and function of cell membranes and induce lipid peroxidation, structural alteration of proteins and strand breaks of nucleic acids, thereby destroying cells and organelles and promoting and inducing apoptosis of cells (42).

In general, our results showed that there was significant oxidative stress in ketosis cows, and the oxidative stress in ketosis was closely related to high plasma GH levels. Therefore, in order to further confirm the relationship between ketosis and oxidative stress, we isolated and cultured cow liver cells. Hepatocytes were collected at different time points after adding different concentrations of BHBA to evaluate oxidative stress and the activity and expression of some antioxidant biomarkers in hepatocytes. Negative energy balance is known to occur in dairy cows following parturition because in a situation of low dry matter intake and increased energy requirements to support milk production (25), lipids are mobilised to release non-esterified fatty acids (NEFA) into the bloodstream and serve as an energy source. Excess lipid mobilisation causes ketosis in cows (47), and increased blood NEFA concentrations affect postpartum cow health. Oxidative stress can be caused by fat breakdown that increases ROS production during beta-oxidation of NEFA and ketone body production in cows (3, 4). It is widely accepted that ketosis in dairy cows is caused by a lack of energy that alters the expression of key metabolic hormones and leads to tissue-specific changes in hormonal reactivity (51). Growth hormone is a peptide hormone secreted by the anterior pituitary gland into the circulation which acts on GHR. Growth hormone receptor is a type I cytokine receptor, activated through GH binding on the JAK2-STAT5 signal pathway. It increases the synthesis and secretion of IGF-1 in hepatocytes, thereby initiating a variety of signal cascades, leading to a variety of physiological responses. The GH secreted by the pituitary gland induces hepatic IGF-1 synthesis, while IGF-1 negatively regulates GH production (18). Insulin-like growth factor 1 plays a key role in the anabolism of various tissues and circulates from the liver as its main source (34). The biological role of IGF-1 is mediated by activating IGF-1R, and the central role of the IGF family in regulating physiological and pathological processes has been established. Insulin-like growth factor 1 receptor is a transmembrane heterotetrametric, which promotes mitotic activity and protects cells from apoptosis.

It has been reported that a qPCR can detect the changes of liver transcription level in cows with ketosis (36). The qPCR method was used to quantify gene expression, to explore the role of GH in the pathogenesis of ketosis, to better understand the expression of GHR and its related molecules and antioxidant genes in the liver, and to elucidate the role of GH in the pathogenesis of ketosis. Our qPCR identified that the differentiation of gene expression levels of GSH-Px, SOD and IGF in hepatocytes began to appear at 12 h in culture groups, whereas the differentiation of gene expression levels of IGF and GHR in hepatocytes began to appear at 6 h. The expression GHR, IGF-1 and IGF-1R mRNA in group 3.0 mmol·L^{-1} was lower than that in the 1.2 mmol·L^{-1} BHBA concentration group. Our results indicated that the mRNA levels were correlated with BHBA concentrations, *i.e*. with ketosis. This finding also suggested that the IGF-1 transcriptional mechanism in the liver is controlled by GHR signalling transduction (18). The GH–IGF axis in the liver during ketosis is altered to mediate the mobilisation of fat stores to provide adequate nutrition for lactation. Energy deficiency decreased serum IGF-1 concentration in dairy cows; Fenwick *et al.* (18) showed that severe ketosis reduced liver IGF-I synthesis and that GH concentration increased in dairy cows. Observing the results of this test, the levels of GHR mRNA, IGF-1 and IGF-1R in the 3.0 mmol·L^{-1} group were lower than those in the 1.2 mmol·L^{-1} group. The decrease in plasma IGF-I reflects a decrease in the activity of the promoter responsible for GHR synthesis, resulting in reduced production of GHR and IGF-I in the liver. The plasma IGF-1 concentrations decreased with decreasing IGF-1 and GHR concentrations during hypoglycaemia, leading to some degree of uncoupling of the GH–IGF axis (34). It has been reported that GHR can coordinate the

distribution of nutrients in early lactation after calving. The net effect of reduced GHR in ketotic cows is reduced GHR signalling and reduced IGF-I synthesis. It has been reported that there is an overall decrease in GHR in the liver of high-yielding cows in the early postpartum period. The results showed that the expression of GHR decreased with the increase in BHBA concentration and culture time. The reasons are as follows: GHR synthesis lacks the stimulation of GH secreted by pituitary gland there is a certain inverse correlation with BHBA concentration; and it is affected by the culture time of hepatocytes (34, 45). Because of low blood IGF-1 concentrations (a negative feedback reduction to GH), there is an increase in blood GH concentrations in the first week after delivery. An increase in blood GH concentration promotes lipolysis, thereby releasing NEFA into the blood. Non-esterified fatty acids can be oxidised in the liver or extra-hepatic tissues. Therefore, the internal concentration of GH and NEFA in the SK group was higher than that in the healthy cows group. Growth hormone promotes the breakdown of adipose tissue during ketosis, and a correlation between GH and adipose mobilisation and metabolites has been observed, indicating that GH production has an important effect on NEB status (34, 45).

Overall, the gene expression results showed that BHBA significantly downregulates the expression levels of GHR, IGF1 and IGF-IR and also lowers the activities of antioxidant biomarker such as CAT, GSH-Px and SOD. In a previous study, a strong correlation between the expression levels of GHR and IGF-1 was reported (30). The experimental results showed that with the increase in culture time and BHBA concentration, the decrease in GHR expression was accompanied by one in mRNA levels of IGF-1 and IGF-1R in the experimental groups, and these decreases were to levels of significant difference to the control group level. The results showed that GHR was correlated with IGF gene level, which was consistent with the literature conclusion (30). In addition, it has been reported that postpartum GHR and IGF-1 liver transcription decreases may be the result of NEB status (32). Thus, the loss of GHR activity in the liver may be an endocrine mechanism that ensures the production of milk by lipid mobilisation during ketosis.

Conclusion

The study concludes that the imbalance of energy budget in postpartum dairy cows leads to NEB and ketosis. It suggests that over mobilisation of fat can produce a large amount of NEFA, enhancing the production of ROS and MDA during its β-oxidation, resulting in oxidative stress. Thus, in the case of severe ketosis, GH coordinates and processes nutrient distribution through the liver; this can be associated with the transcription of GHR and IGF genes, as well as with significant changes in many IGF-related and antioxidant biomarker genes. The research concludes that the decreased GHR synthesis mediates the decrease in IGF-I synthesis in order to achieve energy balance and to meet the lactation needs of dairy cows. In this experiment, the hepatocytes were cultured with BHBA at different concentrations *in vitro* to simulate the environment of healthy, SCK and CK cow hepatocytes. We found that the decrease in GHR mRNA level led to others in IGF-1 and IGF-1R mRNA expression. The decrease in CAT, SOD and GSH Px mRNA expression indicated that GHR could be mediated by target hepatocytes. The expression of genes related to glycolipid mobilisation can help to mobilise the fat and protein reserves. Large-scale body fat mobilisation can cause elevated ketone body numbers in the blood leading to ketosis. On the other hand, the increase of metabolism will increase the risk of oxidative stress to dairy cows. It can be suggested that GH is an internal basis for the pathogenesis of ketosis.

Conflict of Interests Statement: The authors declare there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: All animals used in this study were treated according to the international guiding principles for biomedical research involving animals and under animal welfare ethical protocol No. GXU-2018-203, Guangxi University, Nanning, Guangxi, China.

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