Total Glutathione and Glutathione Reductase in Bovine Erythrocytes and Liver Biopsy

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ABSTRACT. The goal of the present study was to measure the total glutathione level and glutathione reductase activity in bovine erythrocytes and liver biopsy. Five cows were injected intraperitoneally with DL-ethionine (12.5 mg/kg B.W.), and two control cows were injected with normal saline (0.9% NaCl). Ultrasonography guided liver biopsy, and blood samples were collected at 0, 4, 7 and 10 days after injection. The hepatic total glutathione level was significantly increased on Days 7 ($p<0.05$) and 10 ($p<0.01$), and hepatic glutathione reductase activity was significantly increased on Days 4 ($p<0.05$), 7 ($p<0.01$) and 10 ($p<0.01$). There were insignificant changes in the erythrocytic total glutathione level. The present study demonstrated that liver biopsy is a valuable tool for detecting oxidative stress and for diagnosing hepatic dysfunction in cattle from the viewpoint of the status of glutathione and glutathione reductase.

KEY WORDS: bovine liver biopsy, glutathione, glutathione reductase.

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Oxidative stress is a process based upon the action of free radicals formed by oxygen or other molecules and fragments. Free radicals are highly reactive substances produced continuously during metabolic processes and participate to a great extent in physiological events such as immune response, metabolism of unsaturated fatty acids and inflammatory reaction. However, their excess results in the impairment of DNA, enzymes and membranes [22]. Oxidative stress results when reactive forms of oxygen are produced faster than they can be safely neutralized by antioxidant mechanisms. The most important sources of free radical stress in the liver are hepatic disease, inflammation or exposure of the liver to a toxic agent [4].

Glutathione occurs mainly intracellularly primarily in the reduced form. Reduced glutathione fulfills several essential functions such as detoxification of oxygen-derived free radicals and storage and transfer of cysteine [12]. Cellular glutathione and related enzymes such as glutathione peroxidase, glutathione S-transferase and glutathione reductase are among the principal protective mechanisms against endogenous and exogenous toxic substances and free radicals-mediated damage in liver tissue as well as in other tissues [5, 8, 9, 13, 14]. Increased oxidative stress in bovine liver diseases was established in fatty liver [3] and liver abscesses [2]. In addition, underfeeding in cattle was reported to induce changes in the antioxidant systems in the liver [16]. The liver glutathione level in hepatic dysfunction induced by ethionine was extensively studied in rats [10, 19, 24, 25]. It was reported that erythrocytic oxidative stress increased in human patients with severe hepatic disease [17,

26]. In the current study, the erythrocytic glutathione level was measured to study the effect of hepatic dysfunction induced by ethionine on the erythrocytic oxidative status. The goal of the present study is to measure the total glutathione level and glutathione reductase activity in bovine erythrocytes and liver biopsy as indicators for increased oxidative stress and also to evaluate the effect of hepatic dysfunction on antioxidant levels in the liver and blood and their value in diagnosis from the viewpoint of the status of glutathione and glutathione reductase.

Seven Holstein cows (7–9 years old, 515 to 775 kg) were used. No cows were pregnant, and none were lactating. Five cows were treated with DL-ethionine, and the remaining two cows were treated with normal saline (0.9% NaCl) and kept as the control. Animal handling and procedures were conducted in strict accordance with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals and approved by the Animal Experiment Committee in Iwate University, Japan. DL-ethionine (Kanto Chemical, Tokyo, Japan) dissolved in 0.9% NaCl (20 mg/ml) was injected once intraperitoneally at a dose of 12.5 mg/kg body weight. Control cows were injected once intraperitoneally with 500 ml normal saline (0.9% NaCl). Liver biopsy and whole blood samples were collected at 0, 4, 7 and 10 days after ethionine injection. Liver biopsy for Day 0 was performed directly before DL-ethionine administration. On Day 0, the biopsy site was selected on the right side of the cow in the 11th intercostal space, biopsy specimens for Days 4, 7 and 10 were selected in areas near the Day 0 specimen's site, and the biopsy areas were selected precisely with the aid of ultrasonography. Liver biopsy specimens were collected with the cows under procaine local anesthesia using a Bard® Magnum biopsy instrument and core 14G

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tissue biopsy needle (C.R. Bard, Covinton, GA, U.S.A.). Two liver biopsy specimens were used for the current experiment; one specimen was used to measure the hepatic total glutathione level and glutathione reductase activity, the other specimen was kept in neutral buffered formalin 10% for histopathological examination.

The liver biopsy specimen was washed twice in cold 0.9% NaCl, and then homogenized in 20 volumes (per weight tissues) of cold buffer (50 mM TRIS-HCL, pH 7.5) using a mechanical homogenizer surrounded by ice. After centrifugation of the homogenized samples at $5,000 \times g$ for 30 min at 4°C , the supernatant fluid containing the enzyme was removed and used to measure the hepatic total glutathione level ($\mu\text{mol/g}$ protein), glutathione reductase activity (U/g protein) and total protein. Blood samples were collected in EDTA and then centrifuged at 3,500 rpm for 15 min at 4°C . The plasma and buffy coat were drawn off directly after centrifugation, and the packed cells were washed twice with ten volumes of cold saline. The washed cells were hemolysed with 4 volumes of cold deionized water and used to measure the erythrocytes total glutathione level ($\mu\text{mol/g}$ Hb) and hemoglobin (Hb) concentration. The hepatic and erythrocytic total glutathione level and hepatic glutathione reductase activity were measured using commercial test kits (OXIS International, Foster, CA, U.S.A.). Bioxytech® GSH-420 assay was used for the total glutathione level, and Bioxytech® GR-340 assay was used for glutathione reductase activity. Assays were performed using a spectrophotometer (UV-1200 Spectrophotometer, Shimadzu, Kyoto, Japan). Total protein in the supernatant fluid of the liver homogenate was measured using an Autoanalyzer (Automatic Autoanalyzer 7060, Hitachi, Japan). Hb concentration of the hemolysate was measured using an automatic cell counter (Celltac alpha, MEK-6258, Nihon Kohden, Tokyo, Japan). Hepatic biopsies for histopathological examination were fixed in 10% neutral buffered formalin, sectioned, and then stained with hematoxylin and eosin (HE) and PAS stain [6]. Statistical analysis was performed by comparing data from Days 4, 7 and 10 with those from Day 0 using one-way ANOVA and Dunnett test

Fig. 1. Cattle liver in Days 0, 4, 7 and 10 before and after DL-ethionine administration showing glycogen degeneration and cell swelling, which varied from slight in days 4 and 10 to moderate in day 7.

and was performed with Statistical Package for the Social Sciences for Windows (SPSS, version 10.0, Chicago, Ill, U.S.A.).

No apparent clinical signs were observed in animals during the experiment except that the weight of the animals decreased on Days 4, 7 and 10 compared with Day 0. Different degrees of cell swelling and glycogen degeneration were developed in the liver tissues, which varied in intensity from slight on Days 4 and 10 to moderate on Day 7 (Fig. 1). The hepatic total glutathione level ($\mu\text{mol/g}$ protein) showed significant increase on Day 7 ($p < 0.05$) and Day 10 ($p < 0.01$), and hepatic glutathione reductase activity (U/g protein) showed significant increase on Days 4 ($p < 0.05$), 7 ($p < 0.01$) and 10 ($p < 0.01$). There was insignificant change in the erythrocytic total glutathione level ($\mu\text{mol/g}$ Hb) on Days 4, 7 and 10 after DL-ethionine administration (Table 1).

Studies on the action of ethionine in cattle liver have shown that this ethyl analog of methionine produces a number of adverse effects including decrease in cholesterol ester, apolipoproteins A1, B-100 concentrations [15],

Table 1. Value of total glutathione and glutathione reductase in cattle erythrocytes and liver before and after DL-ethionine administration

	Animals	Day 0	Day 4	Day 7	Day 10
Hepatic total glutathione ($\mu\text{mol/g}$ protein)	Control (n=2)	12.64	12.03	9.40	9.05
	Ethionine group (n=5)	11.67 $\pm$ 1.79	12.54 $\pm$ 1.18 ^{NS}	15.84 $\pm$ 3.30*	20.24 $\pm$ 4.96**
Hepatic glutathione reductase (U/g protein)	Control (n=2)	9.94	8.73	10.27	8.86
	Ethionine group (n=5)	8.20 $\pm$ 1.71	11.55 $\pm$ 2.13*	20.24 $\pm$ 5.23**	14.45 $\pm$ 1.83**
Erythrocytes total glutathione ($\mu\text{mol/g}$ Hb)	Control (n=2)	9.75	9.46	11.83	8.91
	Ethionine group (n=5)	11.20 $\pm$ 0.91	10.95 $\pm$ 1.68 ^{NS}	11.25 $\pm$ 1.99 ^{NS}	11.14 $\pm$ 1.30 ^{NS}

Data for control animals were presented as Mean value.

Data for ethionine treated group were presented as Mean $\pm$ SD.

* Significant $p < 0.05$, ** Highly significant $p < 0.01$.

Fig. 2. Cattle liver from control animals in Days 0, 4, 7 and 10 before and after injection of normal saline 0.9%.

reduced hepatic protein kinase C activity [11] and haptoglobin induction [20] and decreased serum lecithin:cholesterol acyltransferase activity [21]. Previous studies aimed to study different biochemical parameters in severe fatty degeneration in cattle, and hence DL-ethionine was injected at a dose of 25 mg/kg B.W. [15, 20, 21]. However, in the current study, DL-ethionine was administered at a dose of 12.5 mg/kg B.W., which is sufficient to produce hepatic dysfunction and not essentially fatty liver. According to the histopathological examination (Fig. 1), using ethionine at a dose of 12.5 mg/kg B.W. produced hepatic dysfunction in the form of glycogen degeneration and cell swelling, which varied in intensity from slight on Days 4 and 10 to moderate on Day 7 (Fig. 1). The sites of liver biopsy for the 4th, 7th, and 10th day were selected precisely using ultrasonography near the site of the Day 0 biopsy to ensure that the biopsy samples were collected from the liver, and also to avoid the collection of multiple biopsy specimens from the same site, which may affect the measured parameters. The absence of hepatic histopathological changes in control cows (Fig. 2) indicated that the histopathological changes in the ethionine group were attributed to the toxic effect of ethionine on the liver.

In the present study, the changes in the hepatic total glutathione level and glutathione reductase activity may be attributed to a combination of hepatic dysfunction and the effect of ethionine. The absence of oxidative stress in natural cases of hepatic glycogen degeneration [3] indicated that the effect on hepatic antioxidant parameters in the current study may be attributed to ethionine. Ethionine was reported to affect the glutathione metabolism in rat liver a short period after injection, which varied from 4 hr [25] to 24 hr [10] followed by significant increase in 2 days [19] or 3 days [24]. Furthermore, the administration of ethionine induced an increase in the synthesis of the H_2O_2 -generating peroxisomal β -oxidation enzyme system in the rat liver. An imbalance between production and degradation due to high rates of the H_2O_2 generating peroxisomal β -oxida-

tion system can cause an intrahepatic oxidation stress [1]. H_2O_2 is a cytotoxic and can be converted to hydroxyl radical ($\cdot OH$) via Fenton like reaction [18]. Catalase together with glutathione peroxidase (GSH-Px) plays an important role in the removal of H_2O_2 from the cell, where H_2O_2 is reduced to water [7]. Ethionine exposure was reported to inhibit hepatic catalase activity in rats [1], which activates the GSH-Px pathway to remove the excess H_2O_2 . The endogenously produced H_2O_2 is reduced by reduced glutathione (GSH) in the presence of selenium dependent GSH-Px; as a consequence, GSH is oxidized to glutathione disulfide, which is rapidly reduced back to GSH by glutathione reductase in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) [23]. This may explain the significant increase in the hepatic GSH level on Day 7 and Day 10 and in hepatic glutathione reductase activity on Days 4, 7 and 10 after ethionine injection. The insignificant changes in the erythrocytes total glutathione level (Table 1) indicate the absence of oxidative stress in the erythrocytes of cows suffering from glycogen degeneration and also indicate that DL-ethionine has no effect on the erythrocytes glutathione level when used at a dose of 12.5 mg/kg B.W., and the absence of oxidative stress in erythrocytes of cattle suffering from hepatic dysfunction was reported previously in natural cases of glycogen degeneration and fatty liver [2]. The present study demonstrated that liver biopsy is a valuable tool for detecting oxidative stress and for diagnosing hepatic dysfunction in cattle from the viewpoint of the status of glutathione and glutathione reductase. It is recommended to measure the glutathione level and glutathione reductase activity in liver biopsy from natural cases with glycogen degeneration to elucidate whether the increased oxidative stress was due to glycogen degeneration or due to the direct effect of ethionine on antioxidant parameters.

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