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Research Article

The Red Blood Cell Membrane Proteins in Rabbits with Experimental Ketosis*

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Abstract: This study investigated the effects of experimentally induced ketosis on erythrocyte membrane proteins in rabbits by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE). The animals were allocated to 2 groups; ketosis and control. The experiment was lasted 13 days. Food was withheld for 5 days in the ketosis group, and then the animals were fed for 8 days. The control group was fed standard rabbit rations. Erythrocyte membrane proteins were isolated from the blood, and analyzed by SDS-PAGE using 10% (w/v) acrylamide monomer. Erythrocyte membrane proteins separated on the polyacrylamide gel were quantified by densitometry. Band 3 and actin on day 1, and spectrin on day 5 were significantly increased ($P < 0.05$), while glyceraldehyde-3-phosphate dehydrogenase (G3PD, E.C. 1.2.1.12) was significantly decreased ($P < 0.01$) on day 5 in the ketosis group. On the other hand, ankyrin increased ($P < 0.05$) when compared to the controls on day 6 of the trial. The results show that ketosis may lead to quantitative changes in erythrocyte membrane proteins including band 3, actin, spectrin, G3PD and ankyrin.

Key Words: Rabbit, ketosis, erythrocyte membrane protein, SDS-PAGE.

Tavşanlarda Deneysel Olarak Oluşturulan Ketozis Olgularında Eritrosit Membran Proteinlerinin İncelenmesi

Özet: Bu çalışmada, deneysel ketozis oluşturulan tavşanlarda eritrosit zar proteinlerinin sodyum dodesil sülfat/poliakrilamid jel elektroforez tekniği (SDS-PAGE) ile incelenmesi amaçlandı. Hayvanlar, ketozis ve kontrol olmak üzere iki gruba ayrıldı. Deneme 13 gün sürdürüldü. Ketozis grubu hayvanlara 5 gün boyunca yem verilmedi ve sonrasında 8 gün besleme uygulandı. Kontrol grubu hayvanlar standart tavşan yemi ile beslendi. Kan örneklerinden izole edilen eritrosit zar proteinleri % 10 akrilamid monomerin kullanıldığı SDS-PAGE tekniği ile analiz edildi. Poliakrilamid jelde ayrımları gerçekleşen eritrosit zar proteinleri dansitometre ile değerlendirildi. Ketozis grubunda, band 3 ile aktin'in 1. günde ve spektrin'in 5. günde önemli düzeyde arttıkları ($P < 0,05$); G3PD'nin ise 5. günde önemli seviyede azaldığı ($P < 0,01$) saptandı. Diğer taraftan denemenin 6. günde ankirin'in kontrole gore anlamlı olarak arttığı ($P < 0,05$) belirlendi. Bu çalışmadan elde edilen sonuçlar, ketozisin, eritrosit zar proteinlerinden band 3, aktin, spektrin, G3PD ve ankirin'de kantitatif değişikliklere yol açabileceğini göstermektedir.

Anahtar Sözcükler: Tavşan, ketozis, eritrosit membran protein, SDS-PAGE

Introduction

Ketosis, which is characterized by hypoglycemia, ketonemia, ketonuria, inappetence, either lethargy or excitability, weight loss, depressed milk production and low levels of hepatic glycogen (1-3), is a metabolic disease of lactating cows that occurs within a few days to a few weeks of calving. As the milk performance of dairy cows is generally highest after 5 to 6 lactation periods, primarily older cows are affected (4). Ketosis commonly occurs secondary due to metritis, mastitis, or abomasal displacement (2).

The ruminant's principle sources of energy are acetic, propionic, and butyric acids, which are produced by microbial fermentation in the rumen. Propionic acid is generally considered the major carbohydrate precursor and the only one that has antiketogenic properties. Lactating cows receive little or no carbohydrate beyond that required for synthesis of the lactose secreted in the

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milk. Caloric intake can be inadequate when the food is insufficient or unpalatable, or when the balance of ketogenic and antiketogenic substances in the diet is disturbed, e.g., by feeding on certain silages with high butyric acid content. The composition of the diet similarly can modify the microbial population of the rumen, and thus influence the relative proportions of the volatile fatty acids (VFAs), which lead to the predisposition for ketosis (3,5). In cattle there is no difference between clinical ketosis and starvation ketosis in biochemical terms, but starvation ketosis can be a model for clinical ketosis events (6).

In ketosis, antioxidative systems are affected to a high degree and free radicals can result in damage to living cells (7); likewise, the keton body metabolism is an important source of free oxygen radicals (8). Oxidative injury to hemoglobin (Hb) is associated with the formation of methemoglobin (MetHb) and degradation to a group of pigments collectively called reversible (rHCRs) and irreversible hemichromes (iHCRs). In intact red cells, this denaturated Hb precipitates in the form of Heinz bodies that are attached to the red cell membrane. This process is associated with striking membrane damage as evidenced by an increased permeability to potassium, lipid peroxidation and cross linking of membrane proteins, decreased deformability and destabilization of protein interactions (9).

In this study, we investigated the effects of experimentally induced ketosis on erythrocyte membrane proteins in rabbits by SDS-PAGE.

Materials and Methods

Forty New Zealand rabbits, aged 6 months, were allocated to 2 equal groups; the ketosis and control groups. Before the experimental procedure, all the rabbits were adapted to the experimental conditions for 45 days. The animals were fed a pelleted concentrate ration with ad libitum access to fresh water, and kept in stainless steel cages. Food was withheld for 5 days in the ketosis group until the keton bodies were determined with keton sticks (Bayer), and then the animals was fed for 8 days. Standard feeding was applied to the control group during the experiment. Blood samples were collected from the *v. auricularis* (1.5 ml in tubes containing 0.2 M EDTA) of the rabbits on days 0, 1, 2, 3, 5, 6, 7 and 13 for each group. Erythrocyte membranes (ghost) were isolated from the blood using the methods reported by Dodge et al. (10) and Steck et al. (11), and were mixed with an equal volume of 10% SDS solution and stored at -70 °C until analysis (12). Total protein (TP) concentration of erythrocyte membrane preparations was measured by the method described by Lowry et al. (13). Erythrocyte membrane proteins, isolated from the blood specimens, were analyzed by SDS-PAGE based on the Laemmli method (14) using a 10% (w/v) acrylamide monomer gradient in a Hoefer mini slab gel vertical electrophoresis unit. The Coomassie Blue-stained gels were scanned at 590 nm in a Junior 24 Helena densitometer.

Statistical analysis

The numerical results are given as mean ± standard deviation (SD). Comparisons of the results between the 2 groups were analyzed with Student's t test. Variance analysis of repeated measures was used for analyzing the changes in the same groups according to time. All statements of significance are based on the 0.05 level of probability.

Results

The rabbit erythrocyte membrane contained 6 proteins, spectrin (α and β subunits), ankyrin (2.1 and 2.2 subunits), anion channel (band 3), band 4 (4.1 and 4.2 subunits), actin (band 5) and G3PD, which could be detected by Coomassie Blue staining after sodium-polyacrylamide gel electrophoresis (Figure 1), and their percentages were determined (Figure 2) from densitometric measurements. In addition, spectrin and band 4 proteins were separated with their subunits α- and β- spectrin, and band 4.1 and 4.2, respectively, in some erythrocyte membrane preparations (Figure 1).

TP concentration of ghosts

The TP concentration of ghosts did not vary between the control and ketosis groups (Tables 1 and 2).

The level of spectrin in erythrocyte membranes

On day 5, the amount of spectrin in the ketosis group (28.68% ± 1.94%) was significantly ($P < 0.05$) higher than that in the control group (26.52% ± 0.77%) (Table 1). No significant differences were detected in the level of spectrin between the control and ketosis groups on the other days of the trial (Tables 1 and 2).

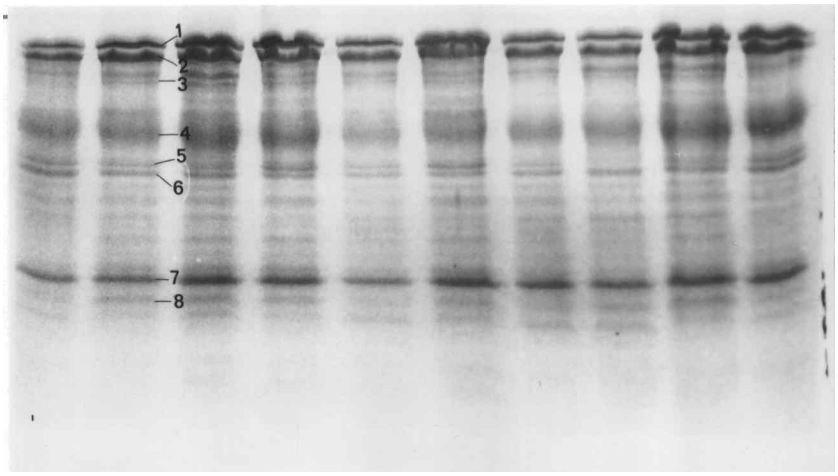

Figure 1. Erythrocyte membrane protein electrophoregram of a control rabbit (1: α-spectrin, 2: β-spectrin, 3: Band 2, 4: Band 3, 5: Band 4.1, 6: Band 4.2, 7: Actin, 8: G3PD).

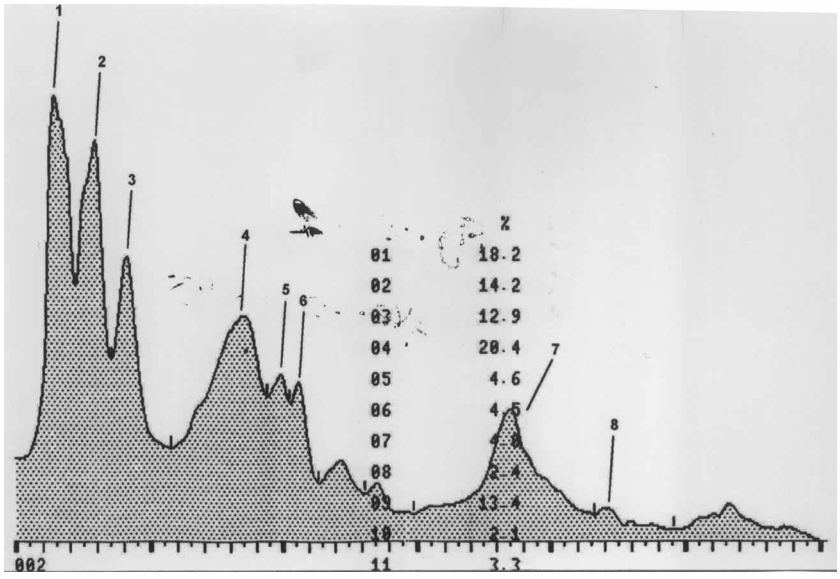

Figure 2. Densitometric analysis of erythrocyte membrane proteins of a control rabbit (α-sp: α-spectrin; β-sp: β-spectrin; 2: Ankyrin, 3: Band 3; 4.1: Band 4.1; 4.2: Band 4.2).

The level of ankyrin in erythrocyte membranes

On day 6, the erythrocyte membrane ankyrin level in the ketosis group was significantly higher ($P < 0.05$) (Table 2) than that in the control group (Table 1).

The level of band 3 in erythrocyte membranes

The band 3 level of erythrocyte membranes in the control and ketosis groups were 24.68% ± 0.36% and 25.78% ± 0.55% on day 1, respectively. The difference between these values was significant ($P < 0.05$) (Tables 1 and 2).

The level of band 4 in erythrocyte membranes

Band 4 quantitation showed no difference between the control and ketosis groups (Tables 1 and 2).

The level of actin (band 5) in erythrocyte membranes

On day 1, the erythrocyte membrane actin level in the ketosis (12.33% ± 0.52%) group was higher (Table 2) than that the control (11.90% ± 0.32%) group (Table 1).

Table 1. Total protein (TP) concentrations (mg/dl) and membrane protein patterns (%) of erythrocyte membranes in the control group.

		Protein patterns of erythrocyte membranes					
Day	TP	Spectrin	Ankyrin	Band 3	Band 4	Actin	G3PD
0*	418.69 ± 9.6	26.22 ± 1.6	8.23 ± 0.4	26.00 ± 0.6	9.57 ± 0.3	12.63 ± 0.9	2.58 ± 0.2
1†	378.16 ± 13.2	29.62 ± 0.8	10.46 ± 0.6	24.68 ± 0.4	8.75 ± 0.3	11.90 ± 0.3	3.05 ± 0.4
2†	439.16 ± 20.2	27.46 ± 0.6	10.85 ± 0.5	24.02 ± 0.4	8.23 ± 0.2	10.67 ± 0.3	2.96 ± 0.3
3†	366.97 ± 15.3	27.19 ± 1.2	13.84 ± 1.0	22.27 ± 0.8	8.63 ± 0.3	10.29 ± 0.4	3.76 ± 0.3
5†	382.07 ± 15.8	26.52 ± 0.8	10.20 ± 0.4	22.87 ± 0.4	8.82 ± 0.2	11.51 ± 0.2	4.50 ± 0.3
6‡	389.14 ± 16.6	23.92 ± 0.6	9.87 ± 0.5	24.94 ± 0.5	8.82 ± 0.2	11.92 ± 0.3	4.51 ± 0.3
7‡	429.67 ± 25.1	28.62 ± 1.4	7.82 ± 0.6	23.73 ± 0.4	9.08 ± 0.3	11.94 ± 0.4	3.43 ± 0.5
13‡	411.41 ± 17.0	26.15 ± 0.6	6.40 ± 0.5	24.17 ± 0.4	9.82 ± 0.2	12.59 ± 0.3	3.61 ± 0.2

† Days of starvation period
‡ Days of re-feeding period after starvation
* Analyses were performed on only 10 rabbits of each group on day 0.

Table 2. Total protein (TP) concentrations (mg/dl) and membrane protein patterns (%) of erythrocyte membranes in the ketosis group.

		Protein patterns of erythrocyte membranes					
Day	TP	Spectrin	Ankyrin	Band 3	Band 4	Actin	G3PD
0#	372.64 ± 16.7	28.28 ± 1.3	9.27 ± 1.0	24.28 ± 0.7	9.21 ± 0.4	11.86 ± 0.8	2.75 ± 0.3
1†	384.94 ± 22.7	28.41 ± 0.8	10.60 ± 0.8	25.78 ± 0.6*	9.05 ± 0.3	12.33 ± 0.5*	2.81 ± 0.5
2†	461.17 ± 26.2	27.33 ± 0.7	9.99 ± 0.4	24.48 ± 0.3	8.45 ± 0.2	10.79 ± 0.3	2.60 ± 0.2
3†	331.12 ± 16.6	26.67 ± 1.2	13.38 ± 0.9	22.07 ± 0.9	8.50 ± 0.4	10.48 ± 0.4	3.57 ± 0.3
5†	379.80 ± 14.5	28.68 ± 1.9*	9.71 ± 0.4	24.03 ± 0.5	8.62 ± 0.2	10.87 ± 0.3	3.82 ± 0.4**
6‡	393.26 ± 17.5	24.96 ± 0.8	10.30 ± 0.4*	24.01 ± 0.6	8.12 ± 0.3	11.34 ± 0.3	4.62 ± 0.3
7‡	386.78 ± 18.8	27.70 ± 1.2	8.20 ± 0.6	22.90 ± 0.9	9.23 ± 0.3	10.04 ± 0.4	3.64 ± 0.5
13‡	421.48 ± 13.3	25.74 ± 0.9	6.24 ± 0.4	24.41 ± 0.3	9.35 ± 0.2	13.43 ± 0.6	3.62 ± 0.2

* Significantly different from control group ($P \leq 0.05$)
** Significantly different from control group ($P \leq 0.01$)
† Days of starvation period
‡ Days of re-feeding period after starvation
# Analyses were performed on only 10 rabbits of each group on day 0.

The level of G3PD (band 6) in erythrocyte membranes

The erythrocyte membrane G3PD level in the ketosis group was significantly ($P \leq 0.01$) higher (Table 2) than that in the control group on day 5 (Table 1).

Discussion

In the study presented here erythrocyte membrane proteins (spectrin, ankyrin, band 3, band 4, band 5 and G3PD) were investigated quantitatively in rabbits with experimentally induced ketosis by SDS-PAGE. Erythrocyte membrane proteins including spectrin and band 6 on day 5, band 3 and band 5 on day 1 and ankyrin on day 6 were significantly changed in the rabbits with experimentally induced ketosis. These results may be attributed to antioxidative metabolism disorders and the negative effects of oxidant radicals that occurred in ketosis. It was reported that antioxidative defense systems were affected negatively and oxidant agents caused important damage in rabbits with ketosis (7).

Concentrations of malondialdehyde (MDA), and activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) enzymes, which are signs of

oxidative damage in mammalian cells (15), are higher in rabbits with ketosis (7). The metabolism of ketone bodies is an important source of the free oxygen radicals (8) that caused the oxidative injury of hemoglobin leading to the formation of methemoglobin, rHCRs and iHCRs. iHCRs precipitate and form Heinz bodies that attach to the red cell membrane causing injury in ketosis (9). Furthermore, Heinz bodies damage membrane proteins by attaching to the inner surface of the erythrocyte membrane, which results in aggregation of proteins (16). In the present study, increasing concentrations of membrane proteins including spectrin, ankyrin, band 3 and band 5, might be related to membrane protein aggregations and oxidation events, which is consistent with the studies reported above.

On day 5, the G3PD enzyme concentration was significantly lower ($P \leq 0.01$) in the ketosis group (Table 2). We think that this enzyme is very sensitive to oxidation because it has a number of –SH groups (17).

Ankyrin protein plays a role as a linkage between the membrane skeleton and the membrane surface (18). In our study, on day 6 (first day of re-feeding), the concentration of ankyrin protein was significantly ($P \leq 0.05$) higher in the ketosis group (Table 2) than that in the control group (Table 1). The increases in ankyrin show that the oxidative effects of ketosis on membrane proteins might continue after starvation.

In conclusion, it was determined that experimental ketosis could cause quantitative changes in the erythrocyte membrane proteins of rabbits and these changes could be related to disorders of the antioxidative defense systems in ketosis.

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