Relation between Erythrocyte Reduced Glutathione and Glutamate Concentrations in Korean Jindo Dogs with Erythrocytes Possessing Hereditary High Activity of Na-K-ATPase and a High Concentration of Potassium

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(Received 4 May 1999/Accepted 22 June 1999)

ABSTRACT. The concentrations of sodium, potassium, reduced glutathione (GSH) and free amino acids and Na-K-ATPase activity in erythrocytes were examined in 35 purebred Jindo dogs in Korea. The incidence of Jindo dogs with a high potassium concentration and high activity of Na-K-ATPase in erythrocytes (HK phenotype) was 25.7%. The erythrocyte GSH concentration in HK Jindo dogs varied widely, from 2.45 to 12.38 mmol/*l* of RBCs, and was positively correlated with the erythrocyte glutamate concentration. These results indicate that HK Jindo dogs have normal to very high levels of erythrocyte GSH, which might result from the varying quantity of Na-dependent glutamate influx in the erythrocytes.—KEY WORDS: canine erythrocyte, Jindo dog, reduced glutathione.
J. Vet. Med. Sci. 61(10): 1179–1182, 1999

Normal canine and feline erythrocytes completely lack Na-K-ATPase activity, resulting in intracellular low potassium (K) and high sodium (Na) concentrations (LK) [3]. In contrast, there are certain Japanese mixed-breed dogs that have erythrocytes characterized by inherited high Na-K-ATPase activity, with high K and low Na concentrations (HK) [11, 13, 14, 16]. The HK phenotype is inherited in an autosomal recessive mode [13]. HK dogs are also found among Japanese purebred dogs such as Shiba [8, 12] and Akita dogs [4, 18], but not in European breeds. Therefore, it has been thought that the gene for the HK phenotype might be inherent in dogs indigenous to Japan [12]. However, Fujise *et al.* [7] reported recently that the HK phenotype is distributed not only in Japan but also in Korea.

The HK phenotype was originally found as an inherent high concentration of reduced glutathione (GSH) in erythrocytes (HK/HG) [11]. The Na-dependent L-glutamate and L-aspartate transport in erythrocytes was greatly increased by the steep gradient of Na and K across the cell membrane induced by the Na-K-ATPase, resulting in a high concentration of these amino acids in the erythrocytes [10]. Consequently, the feedback inhibition of a rate-limiting enzyme, γ-glutamyl cysteine synthetase, in GSH synthesis by GSH was overcome by the high level of glutamate in HK/HG erythrocytes, resulting in an accumulation of GSH to approximately five times the normal concentration [13]. HK/HG dogs usually show no clinical manifestations, but they are greatly susceptible to onion-induced hemolytic anemia compared with normal LK dogs [20]. In addition, a variant dog possessing erythrocytes with a high K concentration, but a normal GSH concentration (HK/LG), was found by Fujise *et al.* [9]. The HK/LG erythrocytes failed to accumulate GSH because of a low influx of L-glutamate [6, 9]. The two variant characteristics of remaining Na-K-ATPase and low transport of glutamate appear to be defined by at least two genes [8].

Jindo dogs, the most popular breed of dogs in Korea, had the highest incidence (42%) of the HK phenotype of all dog groups in Japan and East Asia surveyed by Fujise *et al.* [7]. However, there was no description of the two phenotypic variants of HK, i.e., HK/HG and HK/LG dogs in that report. The objective of the present study was to determine the incidences of the two variants of the HK phenotype in Jindo dogs, with reference to the potentiality of certain diseases induced by onions or other Allium plants in Korea.

Thirty-five purebred Jindo dogs (14 males and 21 females, 4 months to 11 years old) were examined. Thirty Jindo dogs belonged to five different breeding colonies in Taegu city and the suburbs of Taegu. The remaining five Jindo dogs were owned privately in Taegu city. Venous blood was drawn and divided into tubes containing ethylenediaminetetraacetic acid disodium salt (EDTA; 2 mg/m*l* of blood) or heparin (20 U/m*l* of blood) as an anticoagulant. Using blood treated with EDTA, the erythrocyte count and hemoglobin (Hb) concentration were determined using an automatic cell counter (HEMA VET; CDC Technologies, Oxford, CT, USA), and the hematocrit (Hct) value was determined by a microhematocrit method. Heparinized blood was used for determinations of all other parameters. The concentrations of cellular Na and K were calculated from the analytical results of hemolysates, which were measured using an electrolyte analyzer (AVL9180; AVL, Graz, Austria). The concentration of erythrocyte GSH was determined by measurement of the 5,5'-dithiobis-(2-nitrobenzoate) derivative [1]. The concentrations of free amino acids in erythrocytes were measured with an automatic amino acid analyzer as reported previously [13]. The osmotic fragility of erythrocytes was measured by the method of Parpart *et al.* [17], and expressed as the percent of sodium chloride in the incubation medium inducing 50% hemolysis. The activity of total ATPase in hemoglobin-free erythrocyte membranes was assayed essentially by the method of Nakao *et al.* [15]. One hundred microliters of the membrane suspension containing about 0.12 mg of protein was incubated for 1 hr at 37°C in 1.0 m*l* of a reaction

mixture consisting of 5 mM ATP, 5 mM $MgCl_2$, 140 mM NaCl, 14 mM KCl, 0.5 mM EDTA, and 50 mM imidazole buffer (pH 7.2). The activity of Na-K-ATPase was measured by adding 0.5 mM ouabain to the reaction mixture. The released inorganic phosphorus (Pi) in the reaction mixture was measured by the method of Fiske and SubbaRow [5]. The activity was expressed as micromoles of Pi released per hour per milligram of protein. The protein concentration of the membrane suspension was measured by the method of Bradford [2]. Statistical analysis was carried out using Student's *t*-test. Linear regression analysis of the data was performed to obtain the Pearson product moment correlation coefficient and level of significance.

Hematological data from LK and HK phenotypes in Jindo dogs in Korea are shown in Table 1. The incidence of the HK phenotype in Jindo dogs was 25.7% (9 of 35 Jindo dogs). The erythrocyte count, Hb concentration and Hct value in HK Jindo dogs were significantly lower than those in LK Jindo dogs. The MCV and MCH in HK Jindo dogs were significantly higher and the MCHC in HK Jindo dogs was significantly lower than those in LK Jindo dogs. There was no significant difference in osmotic fragility between the two groups. The concentrations of glutamate, aspartate and glutamine in erythrocytes in HK Jindo dogs were markedly increased and significantly higher than in LK Jindo dogs. In addition, the Na-K-ATPase activity in the erythrocyte membrane from the HK Jindo dog was markedly increased, although that from the LK phenotypic dog was negligible. The concentration of erythrocyte GSH was 7.0 ± 3.8 (mean ± standard deviation) mmol/*l* of RBCs in HK Jindo dogs, which was significantly higher than that (2.2 ± 0.5 mmol/*l* of RBCs) in LK Jindo dogs (Fig. 1). The erythrocyte GSH concentration in HK Jindo dogs varied widely, from 2.45 to 12.38 mmol/*l* of RBCs, and a highly positive correlation between the erythrocyte GSH and glutamate concentrations was observed in HK Jindo dogs (Fig. 2).

The present study confirmed that the Jindo breed has a high incidence of the HK phenotype, as reported by Fujise *et al.* [7]. The hematological variables, except osmotic

Table 1. Comparison of hematological data from LK and HK phenotype in Jindo dogs in Korea

	LK phenotype	HK phenotype
Erythrocyte Na (mEq/*l* of RBCs)	118.6 ± 5.6[a)] (n=26)	9.1 ± 5.7† (n=9)
Erythrocyte K (mEq/*l* of RBCs)	8.8 ± 2.0 (n=26)	129.5 ± 4.9† (n=9)
Erythrocyte count ($\times 10^6/\mu l$)	8.4 ± 1.0 (n=25)	6.4 ± 1.0† (n=8)
Hb concentration (g/d*l*)	15.9 ± 2.0 (n=25)	12.6 ± 2.0† (n=8)
Hct value (%)	48.8 ± 7.1 (n=26)	41.9 ± 6.4* (n=9)
MCV (f*l*)	59.0 ± 2.4 (n=25)	65.6 ± 4.6† (n=8)
MCH (pg)	18.8 ± 0.8 (n=25)	19.6 ± 1.1* (n=8)
MCHC (%)	31.9 ± 1.0 (n=25)	30.0 ± 1.6† (n=8)
Osmotic fragility[b)]	0.447 ± 0.023 (n=6)	0.448 ± 0.041 (n=8)
Free amino acid concentration (μmol/*l* of RBCs)		
Glutamate	51 ± 24	1687 ± 1870*
Aspartate	900 ± 759	3462 ± 1131†
Glutamine	660 ± 119 (n=8)	4618 ± 1285† (n=8)
ATPase activity (μmol Pi/hr/mg protein)		
Total ATPase	0.16	2.04
Na-K-ATPase	0.00 (n=1)	1.50 (n=1)

a) Data were represented as mean ± standard deviation.
b) The osmotic fragility of erythrocytes was expressed as the percent of sodium chloride in the incubation medium inducing 50% hemolysis.
* $P<0.05$ and † $P<0.001$, *t*-test compared with the value obtained in LK Jindo dogs.

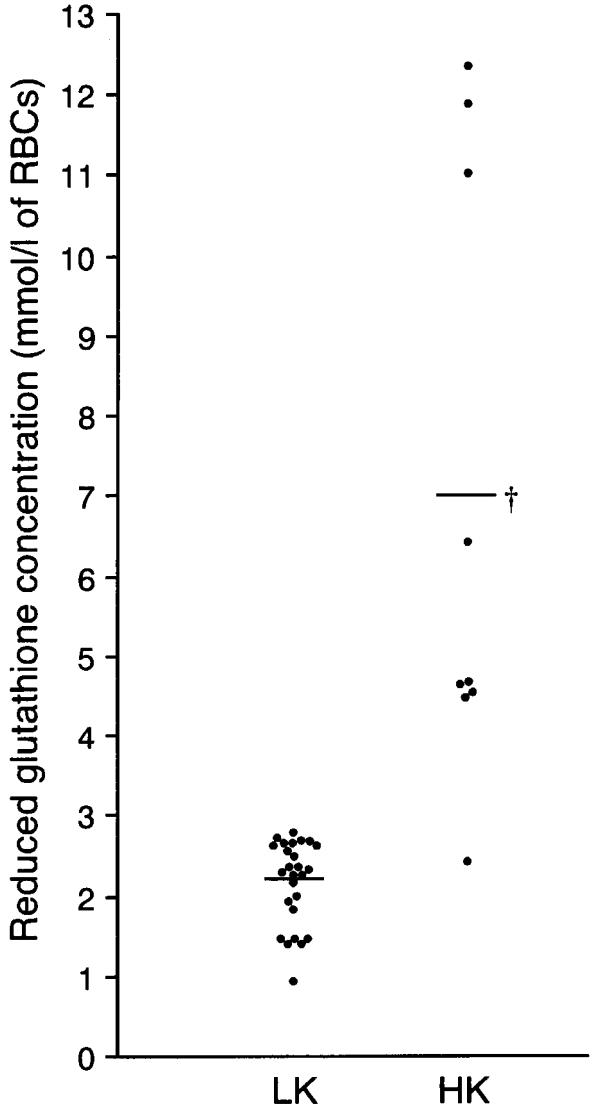

Fig. 1. Erythrocyte reduced glutathione concentration in LK and HK phenotype Jindo dogs in Korea. Horizontal bars represent mean values. †$P<0.001$, *t*-test compared with the value obtained in LK Jindo dogs.

fragility, in HK Jindo dogs resembled those previously reported for HK phenotypic dogs [9, 13, 14, 16]. Furthermore, some HK/HG dogs, with a markedly high level of erythrocyte GSH, and some HK/LG dogs, with a normal erythrocyte GSH concentration, were found among HK Jindo dogs in this survey. However, the erythrocyte GSH concentration in HK Jindo dogs varied widely, from normal to very high levels, making it impossible to divide HK Jindo dogs into two groups. In addition, there was a significant positive correlation between erythrocyte GSH and glutamate concentrations in HK Jindo dogs. Although the concentration of GSH in normal mammalian erythrocytes is maintained fairly constant by the inhibitory action of GSH

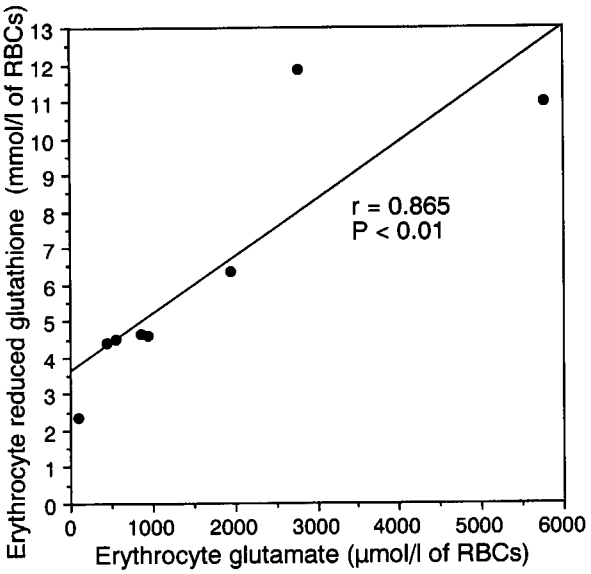

Fig. 2. Correlation between reduced glutathione and glutamate concentrations in erythrocytes in HK Jindo dogs.

itself on γ-glutamyl cysteine synthetase [19], the enzyme is activated by an increased concentration of glutamate, releasing feedback inhibition of the enzyme by GSH [13]. In HK Jindo dogs in the present study, the varying concentration of erythrocyte GSH may result from the varying concentration of erythrocyte glutamate resulting from the activation of γ-glutamyl cysteine synthetase. It was reported that in HK phenotypic Shiba dogs, glutamate influx varied widely, ranging from 3.4 to 22 μmol/hr/*l* of cells in HK/LG erythrocytes and from 40 to 225 μmol/hr/*l* of cells in HK/HG erythrocytes [8]. The varying concentration of erythrocyte glutamate in Jindo dogs might result from a varying quantity of glutamate transporter [6], and consequently affect the concentration of erythrocyte GSH in HK phenotypic dogs. To verify this mechanism, however, further studies to determine the glutamate influx and the quantity of glutamate transporter would be required.

The high susceptibility to onion-induced hemolytic anemia in HK/HG dogs is due to the high concentration of erythrocyte GSH, which accelerates the oxidative damage produced by sodium *n*-propylthiosulfate and its derivatives found in onions [21–23]. Korean people commonly consume onions, garlic and other Allium plants, and may feed them to their dogs. The HK Jindo dogs with erythrocytes containing a high GSH concentration might suffer from oxidative stress induced by Allium plants such as onions and garlic. In the present study, the significant decrease in erythrocyte count, Hb concentration and Hct value in HK Jindo dogs compared with those in LK Jindo dogs might be due to the exposure of their erythrocytes to the toxic constituents of Allium plants.

ACKNOWLEDGMENTS. The authors wish to thank Drs.

Jae-Hyun Lee, In-Ho Jang and Jae-Chan Song, College of Veterinary Medicine, Kyungpook National University for useful advice and technical help, and Mr. Yukichi Abe, the Center for Instrumental Analysis in Hokkaido University, for the measurement of amino acids. This study was supported in part by a grant-in-aid from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. Beutler, E., Duron, O. and Kelly, B.M. 1963. *J. Lab. Clin. Med.* 61: 882–888.
2. Bradford, M.M. 1976. *Anal. Biochem.* 72: 248–254.
3. Chan, P.C., Calabrese, V. and Theil, L.S. 1964. *Biochim. Biophys. Acta* 79: 424–426.
4. Degen, M. 1987. *J. Am. Vet. Med. Assoc.* 190: 541–543.
5. Fiske, C.H. and SubbaRow, Y. 1925. *J. Biol. Chem.* 66: 375–400.
6. Fujise, H., Hamada, Y., Mori, M. and Ochiai, H. 1995. *Biochim. Biophys. Acta* 1239: 22–26.
7. Fujise, H., Higa, K., Nakayama, T., Wada, K., Ochiai, H. and Tanabe, Y. 1997. *J. Vet. Med. Sci.* 59: 495–497.
8. Fujise, H., Hishiyama, N. and Ochiai, H. 1997. *Exp. Anim.* (*Tokyo*) 46: 41–46.
9. Fujise, H., Mori, M., Ogawa, E. and Maede, Y. 1993. *Am. J. Vet. Res.* 54: 602–606.
10. Inaba, M. and Maede, Y. 1984. *J. Biol. Chem.* 259: 312–317.
11. Maede, Y. 1977. *Jpn. J. Vet. Sci.* 39: 187–189.
12. Maede, Y., Amano, Y., Nishida, A., Murase, T., Sasaki, A.and Inaba, M. 1990. *Res. Vet. Sci.* 50: 123–125.
13. Maede, Y., Kasai, N., and Taniguchi, N. 1982. *Blood* 59: 883–889.
14. Maede, Y., Inaba, M. and Taniguchi, N. 1983. *Blood* 61: 493–499.
15. Nakao, T., Nagano, K., Adachi, K. and Nakao, M. 1963. *Biochem. Biophys. Res. Commun.* 13: 444–448.
16. Ogawa, E., Fujise, H. and Kobayashi, K. 1988. *Exp. Anim.* (*Tokyo*) 37: 187–190.
17. Parpart, A.K., Lorenz, P.B., Parpart, E.R., Gregg, J.R. and Chase, A.M. 1947. *J. Clin. Invest.* 26: 636–641.
18. Rich, L.J., Bernreuter, D.C. and Cowell, R.L. 1986. *Vet. Clin. Pathol.* 15: 12–14.
19. Richman, P.G. and Meister, A. 1975. *J. Biol. Chem.* 250: 1422–1426.
20. Yamato, O. and Maede, Y. 1992. *Am. J. Vet. Res.* 53: 134–137.
21. Yamato, O., Hayashi, M., Kasai, E., Tajima, M., Yamasaki, M. and Maede, Y. 1999. *Biochim. Biophys. Acta* 1427: 175–182.
22. Yamato, O., Hayashi, M., Yamasaki, M. and Maede, Y. 1998. *Vet. Rec.* 142: 216–219.
23. Yamato, O., Yoshihara, T., Ichihara, A. and Maede, Y. 1994. *Biosci. Biotech. Biochem.* 58: 221–222.