Rumen Bacteria are Involved in the Onset of Onion-induced Hemolytic Anemia in Sheep

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ABSTRACT. The mechanism of onion-induced hemolytic anemia in ruminants was investigated. The ether-extract obtained from the mixture of rumen fluid and onion juice incubated at 38.5°C for 9 hr induced oxidative damage in sheep erythrocytes *in vitro*, indicating the production of certain oxidants in the mixture. The increase of the oxidative effect in the mixture was inhibited completely by the removal of rumen microorganisms and partly by treatment with antibiotics and by oxygen gas. The sheep fed onions (50 g/kg body weight/day) for 15 days developed more severe Heinz body hemolytic anemia than did the sheep fed the equivalent amount of onions with 5 g/day ampicillin sodium salt. The results indicated that certain rumen bacteria appear to be involved in the onset of onion-induced hemolytic anemia in sheep. —KEY WORDS: Heinz body, hemolytic anemia, onion, rumen microorganism, ruminant.

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Onions (*Allium cepa*) are known to induce methemoglobinemia and Heinz body hemolytic anemia in many domestic animals. In the affected animals, heme iron in erythrocytes is oxidized to the ferric state producing methemoglobin, which is unable to transport oxygen and denaturization of hemoglobin with oxidative injury to the globin chains results in Heinz body formation. Therefore, some oxidants contained in onions have been considered the causative agents of onion-induced hemolytic anemia. One of the major flavor components of onions, *n*-propyl disulfide, is generally held to be responsible [16]. Furthermore, three alk(en)yl thiosulfates extracted from boiled onions may also be involved in onion-induced hemolytic anemia [23, 24].

Attempts to use cultivated onions (*A. cepa*) or wild onions (*A. validum*) as a feed for cattle and sheep have induced toxicosis [10, 12–14, 21, 22]. Symptoms including hemoglobinuria and anemia may appear as early as the 5th or 6th day of onion feeding [13, 21, 22]. Onion-induced hemolytic anemia has also been reported in dogs, cats and horses in which symptons seem to appear earlier than in ruminants [5, 15, 17, 20]. The delay of the onset of the disease in ruminants may be responsible for the function of ruminal stomach.

In domestic ruminants such as cattle, sheep and goats, kale (*Brassica oleracea*) is also known to cause Heinz body hemolytic anemia, the so-called kale poisoning or Brassica anemia [6, 7]. It has been thought that the primary toxic factor in kale poisoning is *S*-methylcysteine sulfoxide, which is hydrolyzed by the rumen fermentation to dimethyl disulfide, the secondary hemolysin [19]. Onions also contain a large amount of *S*-methyl- and *S*-prop(en)ylcysteine sulfoxides. When the plant tissue is disrupted, these substances are hydrolyzed by the catalysis of alliinase to thiosulfinates and disulfides, which are the primary flavor compounds of onions [4]. Farm animals have developed clinical manifestations after eating fresh onions, and dogs and cats have developed Heinz body hemolytic anemia following the intake of cooked onions such as in onion soufflé [17] or onion powder in baby food [11]. Rumen fermentation might therefore be involved with the onset of onion-induced hemolytic anemia if ruminants ingest native compounds such as *S*-alk(en)ylcysteine sulfoxides from fresh onions.

The experiments described in the present study were performed to clarify the mechanism of onion-induced hemolytic anemia in ruminants.

MATERIALS AND METHODS

Bioassay for the oxidation of sheep erythrocytes: The oxidation of sheep erythrocytes was assayed by determining the methemoglobin formation *in vitro*. Whole blood from clinically normal sheep was drawn into a heparinized tubes and centrifuged at 1,250 g for 5 min at 4°C. After removal of the buffy coat, the erythrocytes were washed three times with 10 mM phosphate-buffered saline (PBS, pH 7.4) with 0.9% sodium chloride, and resuspended in PBS with a packed cell volume of 25%. One m*l* of the erythrocyte suspension was incubated for 2 hr at 38.5°C with each sample derived from onions, and the methemoglobin concentration was then measured as described by Hegesh *et al.* [9].

In vitro experiment using fresh rumen fluid of sheep: Rumen fluid was collected from four clinically healthy sheep by means of a stomach tube. The rumen fluid collected was filtrated through gauze. Peeled onion bulbs were

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homogenized and filtrated through gauze. Four m*l* of the rumen fluid and 1 m*l* of the onion juice were mixed in glass test tubes and then incubated at 38.5°C for 0, 3, 6 or 9 hr. After incubation, the mixture was centrifuged at 2,800 g for 10 min. Two m*l* of the supernatant was partitioned with 2 m*l* of diethylether twice. The ether was removed with a stream of nitrogen to obtain the ether-soluble dry materials (ether-extract). The residual aqueous fraction of the supernatant was evaporated to dryness. Each of the ether-extract and water-soluble materials was mixed with the sheep erythrocyte suspension, and the bioassay described above was performed. As a control, the ether-extract obtained from the incubated mixture of the rumen fluid and 0.9% sodium chloride was also assayed.

In vitro experiments using sterilized rumen fluid, antibiotics and antiprotozoal agent: As a positive control, 3.8 m*l* of fresh rumen fluid, 1.0 m*l* of onion juice and 0.2 m*l* of 0.9% sodium chloride were mixed, and the mixture was incubated at 38.5°C for 9 hr. The ether-extract of the mixture was then incubated with sheep erythrocytes at 38.5°C for 2 hr, and then the methemoglobin concentration was measured as described above. For the negative control, 0.9% sodium chloride was used instead of fresh rumen fluid or onion juice. To investigate the role of rumen microorganisms in onion-induced hemolytic anemia, the rumen fluid was sterilized by filtration (pore size 0.22 μm; Millipore Co., Bedford, U.S.A.) and used instead of fresh rumen fluid. As an antibacterial or antiprotozoal agent, ampicillin sodium salt (Amipenix; Asahi Chemical Industry Co., Ltd., Osaka, Japan), penicillin G (penicillin G Meiji; Meiji Seika Kaisha, Ltd., Tokyo, Japan) and sodium di(2-ethylhexyl) sulfosuccinate (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) [1] were added to the mixture of fresh rumen fluid and onion juice, respectively. These reagents were dissolved in 0.9% sodium chloride in advance. The final concentrations of ampicillin sodium salt, penicillin G and sodium di(2-ethylhexyl) sulfosuccinate were 1 mg/m*l*, 1,000 IU/m*l* and 2 mM, respectively in the mixture of rumen fluid and onion juice.

In vitro experiments under aerobic or anaerobic conditions: Four m*l* of the fresh rumen fluid filtrated through gauze was mixed with 1 m*l* of fresh onion juice. The mixed solutions were incubated at 38.5°C for 6 hr under 100% oxygen gas or 100% nitrogen gas. As a control, a mixture of fresh rumen fluid and onion juice was used in ambient air. After each sample was incubated, the ether-extract of each sample was prepared, and the bioassay was performed as described above.

Animals of in vivo experiments: Six clinically normal sheep were divided into 2 groups. Three sheep, 2 males and 1 female, served as controls (control group), and the remaining three (2 males and 1 female) were treated with ampicillin sodium salt (antibiotic group). The mean body weight of the sheep in the control group was 70.7 ± 9.3 kg (mean ± standard deviation) and 73.7 ± 5.5 kg in the antibiotic group. All of the sheep were given timothy hay (2 kg/head/day) and wheat bran (0.1 kg/head/day) during the experimental period. Water and salt were provided *ad libitum*.

Procedures of in vivo experiments: Peeled onion bulbs were homogenized, and onion juice was obtained from the homogenate through gauze. The onion juice, equivalent to 50 g of fresh onions/kg body weight, was administered to each sheep using a stomach tube daily for 15 days. Five g of ampicillin sodium salt together with the onion juice was administered daily to each sheep in the antibiotic group for 15 days. Blood samples were collected using heparin sodium salt as an anticoagulant on day 0 (before the onion feeding) and every other day during the experimental period of 30 days.

Hematological examinations: The methemoglobin concentration was measured as described by Hegesh *et al.* [9]. Heinz bodies in erythrocytes were detected by vital stain with brilliant green (0.5% brilliant green in 0.85% sodium chloride). The Heinz body count was determined as the percentage of cells that had Heinz bodies by the examination of more than 500 erythrocytes. The erythrocyte count and hemoglobin concentration were measured using an automatic cell counter (System 9,000; Baker Instruments Co., Allentown, U.S.A). The hematocrit value was determined by a microhematocrit method. The hemoglobin concentration in plasma was measured by the cyanmethemoglobin method, and the intravascular hemolysis was calculated as the percentage of plasma hemoglobin in whole blood hemoglobin. Reticulocytes were detected by vital stain with new methylene blue [3], and expressed as a percentage of the cells by the examination of more than 500 erythrocytes. The concentration of reduced glutathione in the erythrocytes was determined by the measurement of the 5,5'-dithiobis-(2-nitrobenzoate) derivative [2].

Analysis of data: Statistical analysis of results was done utilizing Student's *t* test. The significance level was set at $P<0.05$.

RESULTS

In vitro studies: The methemoglobin concentraiton of the erythrocytes incubated with the ether-extract from the mixture of fresh rumen fluid and onion juice was slightly higher than that of the control before incubation, and there was a significant difference ($P<0.05$) between them (Fig. 1). The methemoglobin concentration of the erythrocytes incubated with the ether-extract from the mixture of fresh rumen fluid and onion juice increased markedly after 3 hr of incubation, and was significantly higher ($P<0.001$) than that of the water-soluble materials at 6 and 9 hr, while that of the control did not change during the incubation period (Fig. 1). In contrast, the methemoglobin concentration did not increase when the extract from the mixture of sterilized rumen fluid and onion juice was used (No. 4 in Table 1). Both ampicillin sodium salt and penicillin G significantly ($P<0.001$) inhibited the increase of methemoglobin in the mixture of rumen fluid and onion juice (Nos. 5 and 6 in

Table 1). In contrast, the antiprotozoal agent, sodium di(2-ethylhexyl) sulfosuccinate had no inhibitory effect on the formation of methemoglobin in the mixture (No. 7 in Table 1).

The methemoglobin formation in the mixture was significantly ($P<0.001$) decreased when the mixture was incubated under 100% oxygen gas, while it was slightly increased when incubated under 100% nitrogen gas, compared with that under ambient air (Fig. 2).

In vivo studies: The methemoglobin concentration in the blood of the control group began to increase after the onion feeding began, reached its highest level on day 14, and then returned to the initial level on day 22 (Fig. 3A). The methemoglobin concentration of the antibiotic group remained at a low level during the experimental period. The Heinz body count of the control group also increased, with a maximum of 59.8 ± 21.1% on day 14, and decreased gradually after the end of the onion feeding (Fig. 3B). The Heinz body counts of the antibiotic group on days 10 and 12 were significantly lower ($P<0.05$) than those of the control group.

The erythrocyte count in the control group decreased and reached 59 ± 12% of the initial level on the 20th day (Fig. 4A), while the decrease in the erythrocyte count of the antibiotic group was slower than that of the control group. A significant difference ($P<0.05$) in the erythrocyte count between the control and antibiotic groups was found on days 16 and 20. The hemoglobin concentration and hematocrit value of both groups also showed changes similar to those of the erythrocyte count (Fig. 4B and 4C).

Intravascular hemolysis was detected in the control group on the 10th day of the onion feeding, and remained at a higher level than that of the antibiotic group (Fig. 5A). The intravascular hemolysis of the antibiotic group was significantly lower ($P<0.05$) than that of the control group on days 12 and 26. The reticulocyte count began to increase on day 12 in the control group and on day 16 in the antibiotic

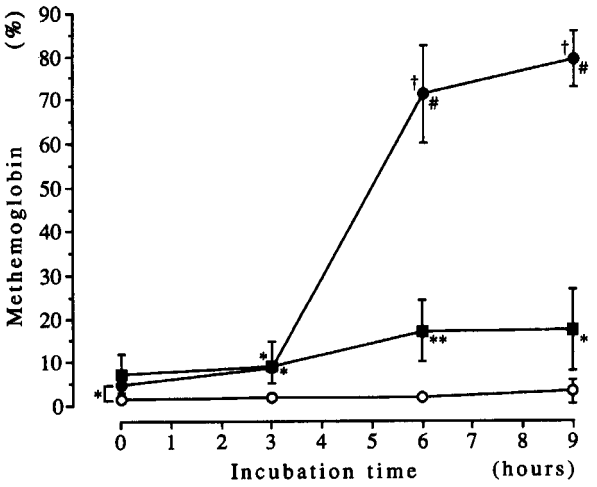

Fig. 1. The effect on methemoglobin formation of onions incubated with fresh rumen fluid on sheep erythrocytes. Four m*l* of fresh sheep rumen fluid and 1 m*l* of onion juice were mixed and incubated at 38.5°C. After incubation, the ether-extract (●) from 2 m*l* of the supernatant of the mixture, and the residual water-soluble materials (■) were mixed with 1 m*l* of sheep erythrocyte suspension, and then incubated for 2 hr prior to the measurement of the methemoglobin concentration. As a control (○), the ether-extract obtained from the incubated mixture of rumen fluid and 0.9% sodium chloride was assayed. Data are mean ± standard deviation (n=4). *$P<0.05$, **$P<0.01$ and †$P<0.001$, compared with the value of the control. #$P<0.001$, compared with the value of the water-soluble materials.

Table 1. Methemoglobin formation in sheep erythrocytes by ether-extracts from onion juice incubated with rumen fluid under various conditions

No.	Composition of incubated fluid[a)]			Methemoglobin[b)] (%)
	A (3.8 m*l*)	B (1.0 m*l*)	C (0.2 m*l*)	
1	Rumen fluid	Onion juice	0.9% NaCl	68.3 ± 5.0[c)]
2	0.9% NaCl	Onion juice	0.9% NaCl	3.0 ± 0.5†
3	Rumen fluid	0.9% NaCl	0.9% NaCl	3.1 ± 2.6†
4	Sterilized rumen[d)]	Onion juice	0.9% NaCl	2.7 ± 1.6†
5	Rumen fluid	Onion juice	Ampicillin Na[e)]	14.1 ± 6.8†
6	Rumen fluid	Onion juice	Penicillin G[f)]	9.8 ± 5.8†
7	Rumen fluid	Onion juice	Antiprotozoal agent[g)]	67.9 ± 6.9

a) A, B and C solutions were mixed, and the mixture was incubated at 38.5°C for 9 hr.
b) After the mixture was incubated, the ether-extract from the mixture was incubated with sheep erythrocytes at 38.5°C for 2 hr, and then methemoglobin concentration was measured.
c) Data were represented as mean ± standard deviation (n=4).
d) The rumen fluid was sterilized by means of filter (pore size 0.22 μm).
e) The final concentration of ampicillin sodium salt in the incubated fluid was 1 mg/m*l*.
f) The final concentration of penicillin G in the incubated fluid was 1,000 IU/m*l*.
g) The final concentration of antiprotozoal agent, sodium di(2-ethylhexyl) sulfosuccinate, in the incubated fluid was 2 mM.
† $P<0.001$, compared with methemoglobin concentration of No. 1.

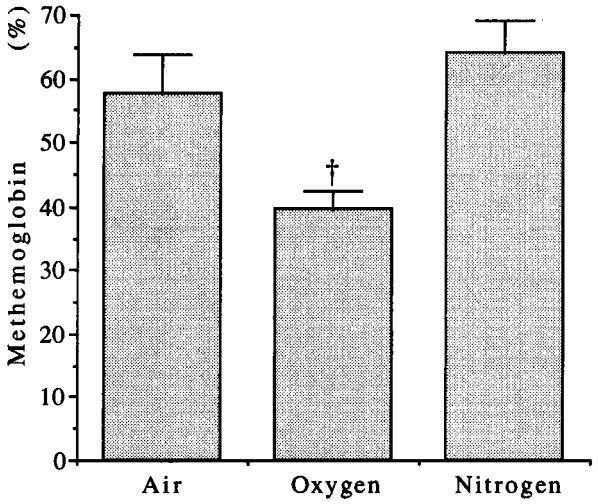

Fig. 2. The effect of oxygen and nitrogen on the formation of methemoglobin induced by onions incubated with fresh rumen fluid. Four m*l* of fresh sheep rumen fluid and 1 m*l* of onion juice were mixed and incubated at 38.5°C for 6 hr. Each sample was incubated under ambient air (Air), 100% oxygen gas (Oxygen) and 100% nitrogen gas (Nitrogen). After incubation, the ether-extract from 2 m*l* of the supernatant of the incubated solution was mixed with 1 m*l* of sheep erythrocyte suspension, and then incubated for 2 hr prior to the measurement of the methemoglobin concentration. Data are mean ± standard deviation (n=5). †P<0.001, compared with the value of Air.

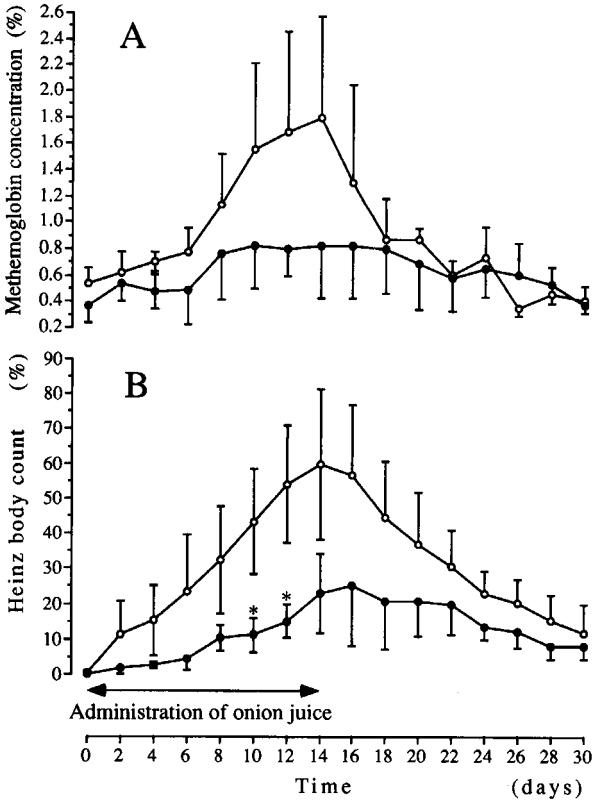

Fig. 3. Changes in methemoglobin concentration (A) and Heinz body count (B) in the control (○) and antibiotic (●) groups. Data are mean ± standard deviation (n=3). *P<0.05, compared with the control group.

group, and the reticulocyte count of the antibiotic group was significantly lower (P<0.05) than that of the control group on day 28 (Fig. 5B).

The concentration of reduced glutathione in the erythrocytes of the sheep used in this study was 11.31 ± 1.77 μmol/g of hemoglobin in the control group and 11.84 ± 2.15 μmol/g of hemoglobin in the antibiotic group. The reduced glutathione concentration of both groups did not change significantly during the experimental period (Data not shown).

DISCUSSION

The results of the *in vitro* experiments indicated that certain ether-soluble oxidants were produced from onion constituents by rumen fermentation. The increase of the oxidative effect on erythrocytes in the mixture of rumen fluid and onion juice was markedly inhibited by the removal of rumen microorganisms, and by the addition of antibiotics such as ampicillin sodium salt or penicillin G to the rumen fluid. Since an antiprotozoal agent had no inhibitory effect on the formation of methemoglobin by the mixture, the increase of the oxidative effect on erythrocytes seems to be involved in rumen bacteria. In addition, it is considered that the bacteria may be anaerobic because the production of the oxidants was inhibited when the mixture was incubated in the presence of 100% oxygen.

The *in vivo* experiments also showed that the degree of anemia induced by onions was more mild in sheep administered ampicillin sodium salt than in sheep without the treatment. This result suggests that ampicillin sodium salt inhibits the proliferation of the causative rumen bacteria, resulting in the decrease of oxidant production in rumen treated with the antibiotic. However, the administration of the antibiotics did not result in the complete inhibition of the onset of Heinz body hemolytic anemia in sheep. The reason may be that onions contain some native oxidants.

As described elsewhere, the primary causative agent of kale poisoning in ruminants is thought to be *S*-methylcysteine sulfoxide, which is converted enzymatically by rumen organisms into dimethyl disulfide, an oxidant [18, 19]. Non-ruminant animals, i.e., pigs, rats, rabbits, guinea pigs, hamsters and mice did not become anemic when fed kale, suggesting that kale poisoning may be restricted to ruminants [6]. The primary causative agents of onion-induced hemolytic anemia in ruminants might also be *S*-methylcysteine sulfoxide and its derivatives, and the secondary agent may be alk(en)yl disulfides such as dimethy disulfide, since onions contain a large amount of *S*-alk(en)ylcysteine sulfoxides. However, fresh onions possess an enzyme which can convert *S*-alk(en)ylcysteine sulfoxide to disulfide [4]. Some oxidants (i.e., *n*-propyl disulfide and

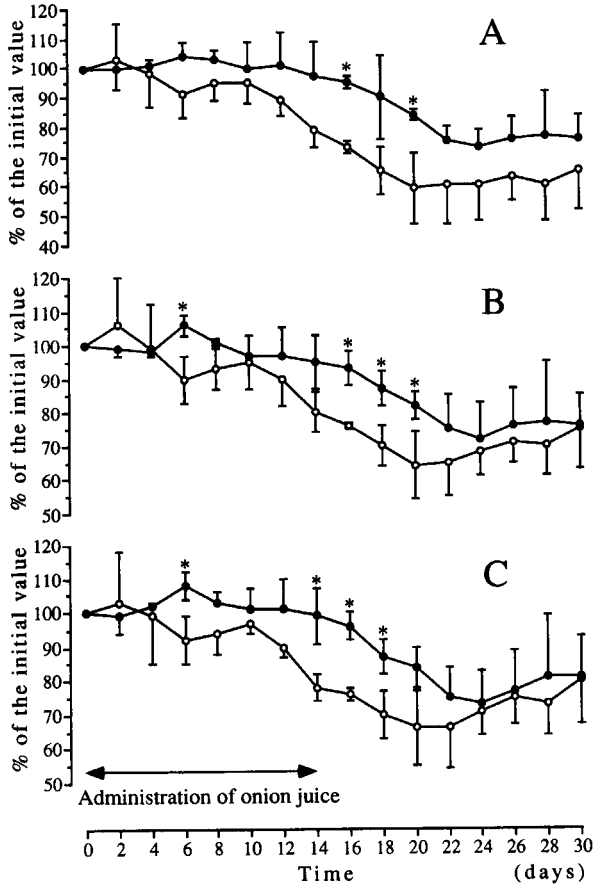

Fig. 4. Changes in erythrocyte count (A), hemoglobin concentration (B) and hematocrit value (C) in the control (○) and antibiotic (●) groups. The erythrocyte count, hemoglobin concentration and hematocrit value are expressed as the percentage relative to the initial values. Data are mean ± standard deviation (n=3). *P<0.05, compared with the control group.

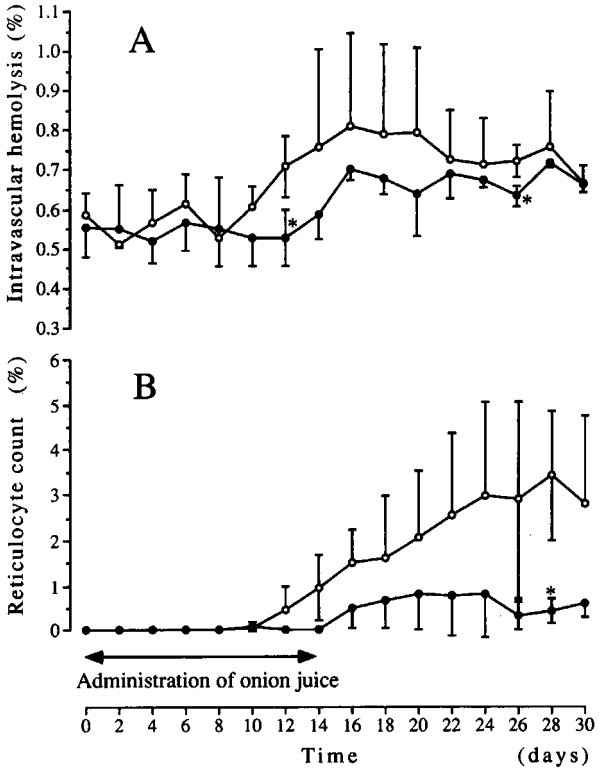

Fig. 5. Changes in intravascular hemolysis (A) and reticulocyte count (B) in the control (○) and antibiotic (●) groups. Data are mean ± standard deviation (n=3). *P<0.05, compared with the control group.

sodium alk(en)yl thiosulfates) which are contained in onions have been reported to induce Heinz body hemolytic anemia in simple-stomach animals such as dogs [8, 24]. Therefore, the oxidants produced from onions by rumen fermentation in the present study seem to be different from native compounds such as the disulfides which are contained in onions.

In conclusion, the present results suggest that onion-induced hemolytic anemia in sheep is attributable partly to certain oxidants produced from onions by a function of rumen bacteria. The results also suggest that the oral administration of some antibiotics may be effective for the treatment of onion-induced hemolytic anemia in sheep.

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