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ORIGINAL ARTICLE

Oxidative effects of long-term onion (*Allium cepa*) feeding on goat erythrocytes

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Abstract Toxic compounds such as disulfides of onions cause oxidative-induced haemolysis in several animal species. In order to study the outcomes of long-term onion consumption on some oxidative haemolysis markers, 12 adult female goats were allocated to three groups, receiving 0% (served as control), 30% (dry matter basis) and 60% spring-grown onion for 60 days. Blood samples were obtained before feeding the onion and every 10 days up to 80 days for measuring malonyldialdehyde (MDA), methaemoglobin (MetHb), mean corpuscular fragility (MCF), serum-free haemoglobin and serum lactate dehydrogenase (LDH) and for evaluating their relation to packed cell volume (PCV) and haemoglobin (Hb) concentrations. PCV and Hb concentrations reduced, however, remained within reference ranges in onion-fed goats. MetHb showed a significant negative correlation with both PCV and Hb ($P<0.05$) in onion-fed goats and a significant positive correlation with MCF and serum-free haemoglobin in goats receiving 30% onion. MetHb showed a significant positive correlation with MCF in goats fed with 60% onion. MDA showed a positive correlation with LDH and serum-free haemoglobin concentrations. These results suggest a role for oxidative damage in destructing red cells in goats feeding onions. However, it seems that up to 60% onions in diet can be consumed by goats without noticeable clinical anaemia.

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Introduction

In areas where onions are grown commercially, it is a common practice to use culled onions as a source of feed for livestock (Crespo and Chin 2004). Naturally occurring as well as experimentally induced onion poisoning has been reported in different animal species (Hutchison 1977; Verhoeff et al. 1985; Carbery 1999; Rae 1999; Van Der Kolk 2000; Van Kampen et al. 1970; Kirk and Bulgin 1979; Aslani et al. 2005; Pierce et al. 1972; Stallbaumer 1981; Solter and Scott 1987; Kobayashi 1981; Crespo and Chin 2004; Harvey and Rackear 1985; Fredrickson et al. 1995; Robertson et al. 1998; Selim et al. 1999; Fighera et al. 2002).

Plants of the Allium family such as onions contain methyl- and prop-(en)ylcysteine sulfoxides. When the plant tissue is disrupted, these substances are degraded to thiosulfinates. After eating, thiosulfinates break up to mono-, di-, tri- and tetrasulfides (Munday and Manns 1994). Many of these compounds are responsible for the toxic and pharmacologic effects of these plants.

Onion toxicosis has been associated with oxidative haemolytic anaemia and formation of Heinz bodies in the erythrocytes, eccentrocytosis and methemoglobinemia (Borelli et al. 2009). Heinz bodies and eccentrocytes increase erythrocyte fragility and extravascular haemolysis. Direct oxidative damage to the erythrocyte cell membrane or the oxidative production of hemin also contributes to cell lysis (Harvey 2008). Thus, the result of the oxidative haemolysis induced by onion consumption is development of anaemia, Heinz bodies, eccentrocytosis, haemoglobinemia, haemoglobinuria, hyperbilirubinemia, methemoglobinemia, increased LDH in blood serum and increased osmotic fragility of erythrocytes. In addition,

lipids, especially polyunsaturated fatty acids, are sensitive to oxidation, leading to the term lipid peroxidation or the thiobarbituric acid reactive substances, of which, malondialdehyde (MDA) is the most abundant (Janero 1990).

Among animal species, cats and dogs are relatively susceptible to onion-induced oxidative damage, followed, in order of increasing resistance, by cattle, horses, sheep, goats, rats and mice (Rae 1999). Differences in the structure of haemoglobin and the activities of some enzymes involved in protecting biological systems from oxidative stress in erythrocytes might be the cause of the species difference in the susceptibility of their erythrocytes to oxidative damage induced by onions (Kasai 1996). It has been shown that feeding up to 25% cull onions on a dry matter basis in cattle resulted in mild decreases in RBCs, haemoglobin (Hb) and packed cell volume (PCV) without obvious clinical anaemia (Lincoln et al. 1992). Sheep can also be maintained on diets up to 50% (DM) of onion bulbs with no clinical abnormalities and weight gains comparable to those from whole sorghum grain (Fredrickson et al. 1995). Although sheep and goats seem most resistant animal species to onion toxicosis, unlimited access to onions led to notable haematological pathology in sheep (Aslani et al. 2005). Studies examining onion toxicosis have focused on cases in which animal death occurred; however, few studies of the chronic effect of onion consumption on animal health are available (Fredrickson et al. 1995; Kirk and Bulgin 1979).

According to the authors' knowledge, data on experimental onions feeding and safety in goats are not available. In the present study, the effects of long-term onion consumption on PCV and Hb concentrations, as well as oxidative damage to erythrocytes in goats are reported.

Materials and methods

Animals and diets

Twelve adult goats aged 2–3 years and weighed 35–40 kg were dewormed by subcutaneous injection of ivermectin (0.22 mg/kg) and oral administration of rafoxanide (7.5 mg/kg) and kept for 2 weeks to be acclimatised. Then goats were assigned randomly to one of the three treatment diets: control, test 1 and test 2. Animals of test 1 and test 2 groups were received a diet containing 30% and 60% spring onions (DM basis) for 60 days, respectively. Goats of group 3 served as control and were fed whole alfalfa hay. Spring onions were obtained every other day from a local market to ensure freshness. Their DM, ash, crude protein and crude fibre contents were determined using the standard methods of proximate analysis (Cullison and Lowery 1987). DM, ash, crude protein and crude fibre contents were 7.5%, 20.2%, 22.8% and 10.8%, respectively. Freshwater was available ad libitum. The experiment was approved by the Animal Welfare Committee of the School of the Ferdowsi University of Mashhad.

Blood sample collection

Blood samples were collected from all animals by the jugular vein puncture into sterile tubes with or without anticoagulant on day 0 and every 10 days intervals until the 80th day (corresponding to the 20th day after cessation of onion feeding). Large bore needles were used to minimise iatrogenic haemolysis. Blood samples were transferred to tubes containing EDTA for routine haematological analysis and heparin for preparation of erythrocyte haemolysate and determination of MDA concentration. Plain tubes supplied serum for analysis. The serum was separated after centrifugation at 1,800g for 10 min and stored at −20°C until analysis. For preparation of erythrocyte haemolysate, blood samples were centrifuged at 800g for 15 min at 4°C. The plasma and buffy coats were removed by aspiration. The sediment containing blood cells was washed three times by resuspending in isotonic phosphate-buffered saline, followed by recentrifugation and removal of the supernatant fluid and the buffy coats. One volume of the crude red cells was lysed in nine volumes of ice-cold distilled water to prepare a 10% erythrocyte haemolysate.

The blood anticoagulated with EDTA was analysed shortly after collection for haemoglobin (Hb), PCV, methaemoglobin (MetHb) and MCF. Free Hb and LDH were measured in serum samples, while erythrocyte haemolysate was used to determine MDA concentration.

Clinical chemistry and haematology

Serum LDH was measured by commercial kit (Pars Azmoon, Tehran, Iran) using an autoanalyser (Biotecnica, Targa 3000, Rome, Italy). Control serum (Randox control sera, Antrim, UK) was used for controlling measurement accuracy. PCV and Hb concentrations were determined by microhaematocrit and cyanomethaemoglobin methods, respectively (Jain 1986).

Serum-free haemoglobin and MetHb concentrations

Concentrations of serum-free Hb and MetHb (as percentage of total Hb) were measured spectrophotometrically according to the methods described by Noe et al. (1984) and Evelyn and Malloy (1938), respectively.

Median corpuscular fragility

Median corpuscular fragility (MCF) was determined by the methods of Luzzatto and Roper (1995). Briefly, 50 μl of well-mixed fresh blood was added into tubes with increasing concentration of buffered saline ranging from 0% (distilled

water) to 0.9% NaCl at pH 7.4. The tubes were well-mixed and incubated at 25°C for 30 min. Then, the samples were centrifuged at 1,000 rpm for 15 min. Absorbance was measured at 540 nm. The 0.9% NaCl was used as a negative control and the distilled water as a positive control. Haemolysis in each NaCl concentration was expressed as percentage of the absorbance in distilled water. The percentage of haemolysis at each concentration of NaCl was calculated and a graph of haemolysis percent against concentration of NaCl was plotted. The results were expressed as the concentration of NaCl causing 50% haemolysis, i.e. MCF.

Malonyldialdehyde (MDA) concentration

The concentration of MDA was estimated in RBC haemolysate (10%) according to the method of Placer et al. (1966). The reaction mixture consisted of 0.2 ml of haemolysate, 1.3 ml of 0.2 M Tris–0.16 MKCl buffer (pH 7.4) and 1.5 ml of thiobarbituric acid reagent. The mixture was heated in a boiling water bath for 10 min. After cooling, 3 ml of pyridine/n-butanol (3:1, *v*/*v*) and 1 ml of 1 N sodium hydroxide were added and mixed by vigorous shaking. A blank was run simultaneously by incorporating 0.2 ml distilled water instead of the RBC haemolysate. The absorbance of the test sample was determined at 548 nm. MDA concentration (in nanomoles per millilitres) was calculated using 1.56×10^5 as extinction coefficient. Lipid peroxides levels in erythrocytes were expressed as nanomoles MDA per gram of haemoglobin.

Statistical analysis

Statistical analysis was conducted using SPSS for Windows (release 16, SPSS Inc, Chicago, IL, USA). Repeated measure ANOVA was used for comparison of measured factors in trial groups. For parameters with significant interaction of time and group, one way ANOVA with bonferroni *t* test was used for comparison between groups at each sampling time. Pearson's method was used for determination of the correlation between different measured parameters in the test groups. All values were expressed as mean and standard error (SE), and $P<0.05$ was considered as statistically significant.

Results

The values (mean ± SE) of Hb, PCV, MetHb, MCF, MDA, serum-free Hb and activity of LDH in different groups (control, 30% and 60%) are presented in Table 1. Group had significant effect on the MCF, MetHb, LDH and serum-free Hb ($P<0.05$). In addition, significant differences were detected between control and test 2 (60%) groups for Hb at day 30 ($P<0.05$) and between test 2 (60%) and test 1 (30 %) and control groups for MDA at day 40 ($P<0.05$). Time had significant effects on the amounts of PCV, Hb, MCF, MetHb, LDH, serum Hb and MDA ($P<0.05$). Significant interactions between time and group were seen for PCV, Hb, MCF, MetHb, LDH, serum Hb and MDA ($P<0.05$).

Table 1 Effects of onion consumption on measured parameters

Parameters	Control	Test 1	Test 2	SE
PCV (%)*	29.85	30.31	30.63	2.904
Hgb (g/L)*	107.2	104.1	106.1	0.876
MetHb (%)**,*	3.104a	4.658ab	7.941b	0.906
MDA (nmol/g HgB)*	82.91	90.29	139.55	15.06
LDH (U/L)**,*	399.92a	442.44b	467.81b	7.174
Serum HgB (mg/L) **,*	0.139 a	0.341 b	0.403 c	0.11
MCF (g/dl) **, *	0.601a	0.613a	0.655b	0.007

Means within rows lacking a common lowercase letter differ ($P<0.05$)

*$P<0.05$, significant group and time interaction; *$P<0.05$,significant effect of group

Onion consumption reduced both PCV and Hb concentrations, but the values were within the normal range and no clinical anaemia was observed. PCV and Hb showed a similar pattern of changes in the onion groups over time and decreased slowly during the first 40 days (Figs. 1 and 2). The lowest values were observed on day 40 of onion consumption, which were about 70% of the baseline values. After day 40, a slow and gradual increase was seen in the onion groups.

The effective concentration of NaCl solution inducing 50% haemolysis (MCF) of RBCs was significantly higher in the test 2 (60%) group compared to the control and test 1 (30%) groups ($P<0.05$). MCF was also higher in the test 1 (30 %) group compared to the control group but there was

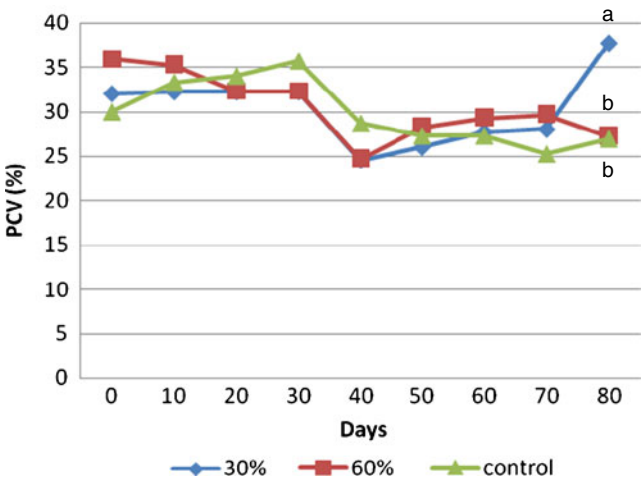

Fig. 1 Changes of PCV in the goats given 30% and 60% onion in diet. Means lacking a common superscript differ ($P<0.05$)

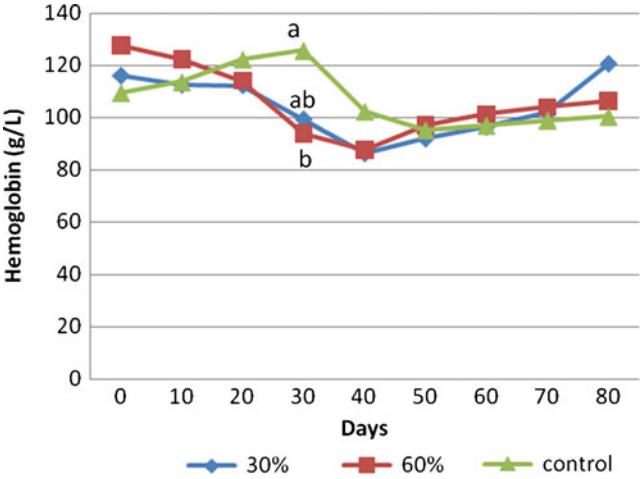

Fig. 2 Changes of Hb in the goats given 30% and 60% onion in diet. Means lacking a common superscript differ ($P<0.05$)

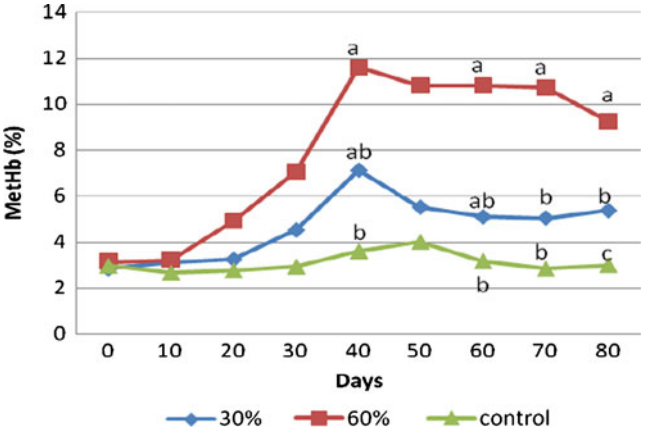

Fig. 4 Changes of MetHb in the goats given 30% and 60% onion in diet. Means lacking a common superscript differ ($P<0.05$)

no significant difference (Table 1). A gradual increase in MCF concentrations was observed during the first weeks of onion feeding in test groups and reached around 110 % of its pre-dosing value on days 40 and 50 for 60% onion group and on day 40 for the 30% onion group, followed by a gradual decline toward the normal (Fig. 3).

A significant elevation of MetHb was detected in the test 2 group compared with the control group ($P<0.05$), while there was no significant difference between the test groups (Table 1). The MetHb concentration increased during the first 40 days of onion consumption and reached 250% and 370% of its baseline values on day 40 for the 30% and 60% onion groups, respectively, followed by a gradual return to the pre-dosing values (Fig. 4).

The serum LDH in the test groups was higher than it in the control group ($P<0.05$), and showed an increasing trend from the first week of onion feeding and peaked to a level about 1255 and 135% of pre-dosing values on day 40 for the 30% and 60% onion groups, respectively, then, returned to baseline values on day 80 (Fig. 5).

Serum-free Hb was significantly higher in the test groups compared to the control group ($P<0.05$). Serum-free Hb concentration increased during the first 40 days of onion consumption and reached 453% and 600% of its baseline values on day 40 for the 30% and 60% onion groups, respectively, followed by a decline toward the pre-dosing values (Fig. 6).

MDA was increased on first 40 days and reached the peak value on day 40 (172% and 552% of its baseline values for the 30% and 60% onion groups, respectively), followed by recovering to the pre-feeding baseline value (Fig. 7). The correlations between different measured parameters in the onion groups have been shown in Tables 2 and 3.

Discussion

The primary toxicologic mechanism of *Allium* species-derived organosulfur compounds is oxidative haemolysis,

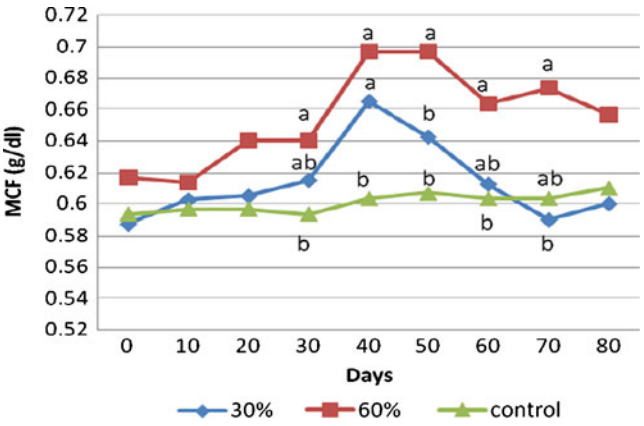

Fig. 3 Changes of MCF in the goats given 30% and 60% onion in diet. Means lacking a common superscript differ ($P<0.05$)

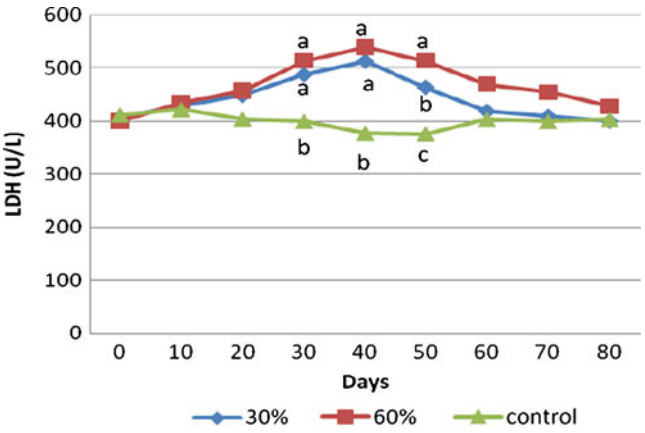

Fig. 5 Changes of LDH in the goats given 30% and 60% onion in diet. Means lacking a common superscript differ ($P<0.05$)

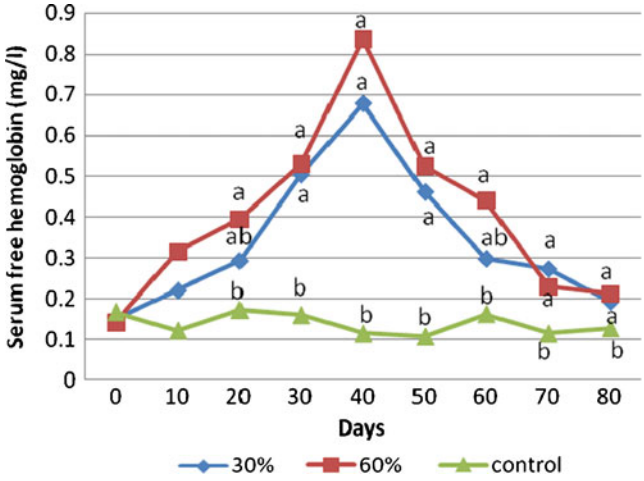

Fig. 6 Changes of serum-free haemoglobin in the goats given 30% and 60% onion in diet. Means lacking a common superscript differ ($P<0.05$)

which occurs when the concentration of oxidants in the erythrocyte exceeds the capacity of the antioxidant metabolic pathways (Cope 2005). Disulfides are the most important compounds in onions which have been implicated as the oxidative agents (Densoyers 2000). The disulfides (R-SS-R) are first reduced by glutathione and the enzyme glutathione peroxidase to the corresponding thiols (Rae 1999). Oxyhaemoglobin then mediates one-electron oxidation of thiols, forming the thiyl radical, methaemoglobin and hydrogen peroxide. The thiyl radical re-forms the disulphide either through dimerisation or via the disulphide radical anion. Superoxide formed in the latter reaction may begin a radical chain reaction through oxidation of the thiolate anion, again with formation of hydrogen peroxide. In this way, haemoglobin promotes a redox cycle with formation of 'active oxygen' species, which directly damage red blood cell

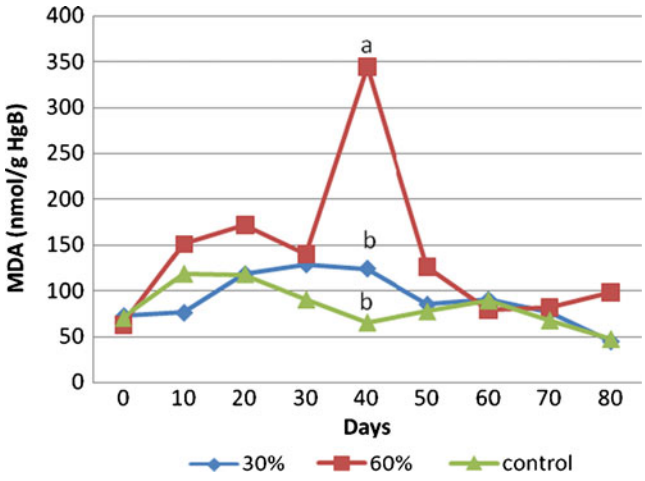

Fig. 7 Changes of MDA in the goats given 30% and 60% onion in diet. Means lacking a common superscript differ ($P<0.05$)

membranes and metabolic machinery, with consequent haemolytic anaemia (Munday 1989; Munday and Munday 2003).

A notable feature of onion-induced haemolysis is its species selectivity. Various causes are implicated in the wide variation in species susceptibility, including differences in haemoglobin structure and protective enzymes. Sheep have detoxification enzymes that allow them to adapt to a diet high in onions. High dietary protein prevents death by providing a substrate for protective enzyme and cofactor synthesis. In addition, species of onion, time of year and growing conditions affect on the amounts of disulfide and susceptibility to onion toxicity (Rae 1999).

Up to the authors' knowledge, this is the first study that evaluated the effects of long-term onion consumption on oxidative damage of erythrocytes in goats. In this study, elevation of oxidative damage markers (MetHb and MDA) and MCF, LDH and serum-free haemoglobin was observed during the first weeks of onion ingestion (the highest values were observed on day 40), while PCV and Hb concentrations significantly dropped in this period. After day 40 and especially after onion withdrawal at day- 60, these parameters gradually returned to baseline values.

Although onion consumption reduced both PCV and Hb concentrations (Figs. 1 and 2), but clinical anaemia was not obvious and the values were within the normal range for goats (Kramer 2000). No significant difference was seen for PCV, Hb, MetHb, LDH and MDA concentrations between two onion groups. However, the amounts of MCF and serum-free haemoglobin were higher in 60% onion group than those in the 30% onion group. These results implicated red blood cell membrane disruption which elevated with increasing onion consumption. However, the injury was not high enough to cause anaemia in goats and this species could stand up to 60% onions on a dry matter basis without overt clinical anaemia. Similarly, Fredrickson et al. (1995) reported that after 6 weeks of onion consumption (50% DM) by sheep, except for two sheep, the PCV values in all animals were within the normal range. In addition it has been indicated that clinical anaemia was not observed in cattle feeding diets containing up to 25% onions (Lincoln et al. 1992). The increased erythrocytic MetHb and MDA, as well as the negative correlation between MetHb concentration and PCV and Hb values in onion-fed goats suggests a role for oxidative damage in destructing the red cells in onion poisoning.

When ferrous iron (Fe^{2+}) is oxidised to ferric iron (Fe^{3+}), the resultant brownish pigment is MetHb, a non-oxygen binding form of haemoglobin that binds with a water molecule instead of oxygen (Harvey 2008). MetHb is unable to bind to oxygen, but its presence alone does not result in shortened erythrocyte lifespan (Harvey 2010). Nearly 3% of haemoglobin within erythrocytes is oxidised to MetHb in

Table 2 Correlation between oxidative stress markers and haematological parameters in goat feeding 30% onion

Parameter	PCV	HgB	MCF	Met Hb	LDH	Serum HgB	MDA
PCV							
PC	1.000	0.903	−0.0657	−0.0526	-0.521	−0.649	−0.451
P value	–	0.001	0.054	0.146	0.150	0.059	0.223
HgB							
PC		1.000	−0.807	−0.711	−0.718	−0.859	−0.589
P value		–	0.009	0.032	0.029	0.003	0.095
MCF							
PC			1.000	0.787	0.810	0.894	0.478
P value			–	0.012	0.008	0.001	0.193
Met Hb							
PC				1.000	0.474	0.715	0.165
P value				–	0.197	0.030	0.671
LDH							
PC					1.000	0.948	0.845
P value					–	0.000	0.004
Serum HgB							
PC						1.000	0.745
P value						–	0.021
MDA							
PC							1.000
P value							–

normal animals. However MetHb usually accounts for less than 1% of total haemoglobin because it is constantly reduced back to haemoglobin by an intraerythrocytic NADH-dependent methemoglobin reductase (cytochrome-

Table 3 Correlation between oxidative stress markers and haematological parameters in goat feeding 60% onion

Parameter	PCV	HgB	MCF	Met Hb	LDH	Serum HgB	MDA
PCV							
PC	1.000	0.828	−0.901	−0.915	−0.624	−0.592	−0.484
P value	–	0.006	0.001	0.001	0.072	0.093	0.187
HgB							
PC			−0.848	−0.847	−0.899	−0.785	−0.499
P value			0.004	0.004	0.001	0.012	0.172
MCF							
PC			1.000	0.935	0.741	0.629	0.404
P value			–	0.000	0.022	0.069	0.281
Met Hb							
PC				1.000	0.618	0.496	0.232
P value				–	0.076	0.175	0.549
LDH							
PC					1.000	0.935	0.670
P value					–	0.000	0.048
Serum HgB							
PC						1.000	0.840
P value						–	0.005
MDA							
PC							1.000
P value							–

b_5-reductase) (Harvey 2010). There is a balance between MetHb and Hb in the normal erythrocytes which is affected by oxygen-derived free radicals produced and released in the oxygen–iron binding process (Tang et al. 2007). Similar to our results, increased MetHb concentration was reported in beef cows (Rae 1999), cats (Fighera et al. 2002) and dogs (Harvey and Rackear 1985) feeding onions.

The presence of MetHb in the erythrocytes induces the release of superoxide radical and H_2O_2 from haemoglobin (Weiss 1982) and may aggravate oxidative damage to erythrocyte leading to disruption of the cell (Bolchoz et al. 2002). In the present study, the increased erythrocytic MetHb in the test groups correlated well with the increased MCF. This means that formation of MetHb and resulting reactive oxygen species in erythrocytes of the goats feeding onion disintegrated erythrocytes' membrane and increased permeability.

The erythrocytes membrane is rich in polyunsaturated fatty acids, a primary target for reactions involving free radicals, and is susceptible to lipid peroxidation (May et al. 1998). Increased MDA concentration, as a reliable marker of lipid peroxidation, was observed in the goats receiving 60% onion in diet. Similar finding was observed by Tang et al. (2007) in dogs after onion feeding. Although there was no significant correlation between MDA and MCF in the goats feeding onions; however, the positive correlation of MDA with LDH and serum-free haemoglobin concentrations may suggest contributory role of lipid peroxidation in erythrocyte destruction. Extensive lipid peroxidation in RBC membrane leads to rupture of the membrane and release of haemoglobin and enzymes including LDH (Saleh 2009).

The present study proved that onion feeding causes oxidative haemolysis in goats. However, goat could stand onions up to 60% of the diet (DM basis) without noticeable clinical anaemia.

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Conflict of interest statement The authors declare there is no conflict of interests.

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