

## Dextran iron in anemic lambs: effects on reticulocytosis and free radical production

### Ferro dextrano em cordeiros anêmicos: efeitos na reticulocitose e produção de radicais livres

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#### ABSTRACT

Anemia due to worm infection is a major cause of loss in the sheep industry, due to deaths, drop in average daily gains and long recovery time following treatment. The present experiment was aimed at evaluating the oxidative status and the recovery of red blood cell (RBC) profile in lambs with induced anemia by bleeding, treated or not with dextran iron. Ten ram lambs 5 to 7 months old were used. Blood samples were drawn every other day and when reached packed cell volume (PCV) of 15% were randomly allocated (day zero) to one of the experimental groups. Treated group received a single dose of 25mg per kg body weight of a commercial formulation of dextran iron, the control group received no treatment. Blood samples were taken on days 0, 7, 14, and 21 after treatment. On days 7 and 21 treated animals presented higher thiobarbituric acid reactive species (TBARS) values, reduced non-protein thiol groups (NPTH) levels were found in the treated group on days 7, 14 and 21. Erythrocyte membrane resistance to osmotic challenge was improved on day 7 in treated animals. Recovery to normal values for the RBC profile was faster in the treated group with significant differences starting on day 7. It was concluded that although the iron treatment increased the oxidative stress, it also accelerated recovery of the hematological profile. Moreover, it did not increase hemolysis in anemic blood by the action of oxygen reactive species upon biological membranes.

**Key words:** TBARS, NPTH, lambs, free-radicals, iron.

#### RESUMO

A anemia verminótica é a principal causa de perdas na ovinocultura, incluindo diminuição no ganho médio diário, demora no tempo de recuperação pós-tratamento e óbito. O objetivo deste trabalho foi avaliar o status oxidativo e a recuperação do quadro hematológico de cordeiros com anemia induzida por flebotomia e suplementados ou não com ferro dextrano. Foram utilizados dez cordeiros machos entre cinco e sete meses de idade. O dia zero do experimento foi

considerado quando cada animal atingiu o hematócrito 15% após a indução da anemia, sendo alocado em um dos grupos experimentais. O grupo tratado recebeu uma dose única de 25mg<sup>-1</sup> kg<sup>-1</sup> de peso vivo de uma formulação comercial de ferro dextrano intramuscular e o grupo controle não foi tratado. As coletas de sangue foram feitas nos dias zero, 7, 14 e 21 pós-tratamento. Nos dias sete e 21, os animais do grupo tratado tiveram aumento nos valores de espécies reativas ao ácido tiobarbitúrico (TBARS) e diminuição nos valores dos grupamentos tióis não-protéicos (NPTH) nos dias 7, 14 e 21. No dia sete, o teste de fragilidade osmótica eritrocitária indicou um aumento na resistência da membrana dos eritrócitos dos animais do grupo tratado quando comparado aos animais do grupo controle. A recuperação da série vermelha do sangue (número de eritrócitos, hematócrito e hemoglobina) foi mais rápida no grupo tratado. A suplementação de ferro, apesar de aumentar o estresse oxidativo, acelerou a recuperação do quadro hemático, não aumentando a porcentagem de hemólise pela ação das espécies reativas de oxigênio sobre a membrana eritrocitária.

**Palavras-chave:** NPTH, TBARS, radicais livres, cordeiros, ferro.

#### INTRODUCTION

In most countries with tropical or sub-tropical climate gastrointestinal parasites are one of the most limiting factors in the lamb industry (RIBEIRO, 1989). Parasites are responsible for large losses due to a drop in fleece weight and grade, reduction in weight gain and death (ECHEVARRIA, 1988). Weaned lambs are the most affected individuals (ECHEVARRIA et al., 1989).

Economically, *Haemochus* sp. is the most important parasite infection in the Brazilian herds

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(BORBA, 1996) this hematophagous nematode causes anaemia, hypoproteinemia and reduced weight gain (ANDERSON, 1982). A state of anaemia is attained when one or more parameters of the red cell line (hematocrit, hemoglobin, erythrocyte count) are below normal levels for age, sex and specie. Rarely anaemia is a primary disease, but rather a result of a systemic process (JAIN, 1993).

DALLMAN (1991) reported that iron is recommended for the treatment of anemia, since around 80% of this transition metal is captured by the bone marrow for the production of hemoglobin. The soil, and thus forage in southern Brazil are quite rich in iron (TOKARNIA, 2000), non supplemented cattle and sheep grazing such pastures are not usually iron deficient nor iron is used in sheep treated for hemochromatosis. Iron can readily accept and donate electrons, interconverting between the ferric ( $\text{Fe}^{+3}$ ) and ferrous ( $\text{Fe}^{+2}$ ) forms. Such property makes iron very useful for cytochrome systems, oxygen carrying molecules (hemoglobin and myoglobin) as well as different redox enzymes that act as electron carriers. Iron can also induce tissue damage catalyzing the conversion of hydrogen peroxide into free radicals that will attack cell membranes, protein and DNA. (SCHIMMEL & BAUER, 2002) When the free radicals act upon the red blood cell membrane there is oxidation of lipids and proteins leading to hemolysis. (SIES, 1993)

In order to avoid damage by lipoperoxidation the cell has an antioxidant defense system that can act in two different ways. First, enzymes like reduced glutathione (GSH) superoxide dismutase (SOD), catalase, glutathione peroxidase (GSHpx) and vitamin E (ROSS & MOLDEUS, 1991; MEISTER & ANDERSON, 1983). Glutathione (GSH, L-g-glutamyl-L-cysteinyl-glycine) is present in most cells and is the most abundant intracellular thiol (-SH). (GALLEANO & PUNTARULO, 1995) Glutathione's major function is to break hydrogen peroxide into two water molecules using electrons from the pentose cycle (RIEGEL, 2002).

This research was aimed at evaluating iron supplementation in anemic lambs upon the oxidative status and the recovery of red blood cell profile.

## MATERIAL AND METHODS

Ten Texel cross ram lambs ranging between 5 and 7 months were used. Animals were kept in indoor stalls and fed a variety of napier grass (*Pennisetum purpureum*). Anaemia was induced by drawing 8 to 10% of total blood volume (8% body weight) until each animal attained a packed cell volume (PCV) of 15%. Then animals were randomly allocated into two

experimental groups (control and treated). Five animals in each group. The day each animal attained PCV of 15% was considered day zero, when the treated group received a single injection of dextran iron (25mg per kg body weight) intramuscular, control group was not treated. Blood samples were collected on days zero, 7, 14, and 21. The evaluation of red blood cell profile (erythrocyte count, hemoglobin, reticulocyte, MCV) were performed according to JAIN et al. (2006).

Thyobarbituric acid reactive species (TBARS) were determined according to OHKAWA et al. (1979). Briefly, blood was centrifuged for 10 min. at 1000 g and red blood cells were washed 3 times with saline solution 0.9%. Then RBC were rediluted with saline solution 0.9% to a 50% hematocrit. This solution was precipitated with 40% trichloroacetic acid (TCA) and the supernatant removed and kept in ice for 30 min. TBARS was quantified by addition of 1ml of the supernatant to 0.5 ml of 0.8% thyobarbituric acid (TBA). The amount TBARS produced was measured at 532 nm using malondialdehyde (MDA) for the standard curve.

Non-protein thiol groups (NPTH) present in RBC were determined using Ellman's reagent, 5,5-dithiobis 2-nitrobenzoate (DTNB). Blood was precipitated with an equal volume of 40% TCA. Then samples were centrifuged at 2000g for 10min. NPTH were quantified after the addition of 200µL of the supernatant in 800mL of 1mmol L<sup>-1</sup> potassium phosphate buffer, pH 7.0 and 0.5mmol of DTNB pH 7.0. The thiol groups concentration was calculated using a glutathione reduction standard curve according to ELLMAN (1959).

RBC analysis of osmotic fragility was carried according to method described by JAIN et al. (2006). In short, it measures erythrocyte stability in a sodium chloride solution in concentrations ranging from 0 to 0.85%. Serum iron levels were determined with commercial kit (Labtest, Minas Gerais, Brasil). Plasma copper levels were determined by atomic absorption spectrophotometry according to FICK et al. (1980). Statistical analyses was performed using ANOVA to compare averages within groups among different experimental days. Student's "t" test was used to compare averages among groups in each experimental day.

## RESULTS AND DISCUSSION

The low blood values for iron found in day zero associated with a prompt recuperation in the control group demonstrate a loss of iron due to external bleeding and not to nutritional deficiency (Table 1). There were no changes in parameters of oxidative stress

Table 1 - Average  $\pm$  standard deviation of erythrocyte count, serum iron levels (Fe), packed cell volume (PCV), hemoglobin (Hb), mean corpuscular volume (MCV), plasma copper (Cu), reticulocytes, non-protein thiol groups (NPTH) and thyobabutaric acid reactive species (TBARS) for control (GC) and treated (GT) animals in each experimental day

Parameters	Day zero		Day 7		Day 14		Day 21	
	GC	GT	GC	GT	GC	GT	GC	GT
PCV (%)	15 $\pm$ 0.81	15.8 $\pm$ 0.48	18 $\pm$ 0.9	21.4 $\pm$ 1.0*	22.5 $\pm$ 0.8	26.8 $\pm$ 0.8**	26.5 $\pm$ 1.1	34 $\pm$ 1.3***
Erythrocytes (n <sup>a</sup> x 10 <sup>6</sup> $\mu$ L)	3.67 $\pm$ 0.11	3.70 $\pm$ 0.09	4.03 $\pm$ 0.17	4.80 $\pm$ 0.17**	4.92 $\pm$ 0.16	5.73 $\pm$ 0.21**	5.78 $\pm$ 0.42	8.51 $\pm$ 0.51***
Hb (g dl <sup>-1</sup> )	5.0 $\pm$ 0.28	5.2 $\pm$ 0.17	6.0 $\pm$ 0.3	7.1 $\pm$ 0.3*	7.5 $\pm$ 0.2	8.9 $\pm$ 0.2**	8.8 $\pm$ 0.4	11.3 $\pm$ 0.4***
MCV(fl)	43.0 $\pm$ 0.46	42.9 $\pm$ 0.76	44.6 $\pm$ 0.9	44.5 $\pm$ 1.5	45.6 $\pm$ 0.2	46.7 $\pm$ 0.6	46.2 $\pm$ 1.4	39.6 $\pm$ 0.4***
Reticulocytes (%)	0.1 $\pm$ 0.07	0.2 $\pm$ 0.16	0.37 $\pm$ 0.16	1.6 $\pm$ 0.2***	0.25 $\pm$ 0.1	0.76 $\pm$ 0.2	0.31 $\pm$ 0.14	0.57 $\pm$ 0.17
Fe <sup>+2</sup> ( $\mu$ g dl <sup>-1</sup> )	71.5 $\pm$ 14.4	78 $\pm$ 13.1	111 $\pm$ 18.6*****	410.2 $\pm$ 27	166 $\pm$ 20.8***	355.8 $\pm$ 29	220.50 $\pm$ 25	266.60 $\pm$ 15.8
Cu <sup>+</sup> ( $\mu$ mol L <sup>-1</sup> )	13.4 $\pm$ 1.0	11.06 $\pm$ 0.5	14 $\pm$ 1.5	15.3 $\pm$ 1.3	14.3 $\pm$ 1.6	13.9 $\pm$ 1.0	15.1 $\pm$ 0.7	12.7 $\pm$ 0.3**
TBARS(nmol MDA g <sup>-1</sup> Hb)	1.74 $\pm$ 0.07	1.76 $\pm$ 0.08	1.66 $\pm$ 0.11	2.57 $\pm$ 0.16***	1.60 $\pm$ 0.16	1.99 $\pm$ 0.09	1.63 $\pm$ 0.08	2.16 $\pm$ 0.11***
NPTH(nmol L <sup>-1</sup> sg total)	1.62 $\pm$ 0.08	1.56 $\pm$ 0.05	1.3 $\pm$ 0.07	0.60 $\pm$ 0.06*****	1.4 $\pm$ 0.05	0.92 $\pm$ 0.05*****	1.4 $\pm$ 0.06	1.02 $\pm$ 0.05***

Averages with different superscripts denote significant differences between groups in each experimental day. \* P = 0.05

\*\* P < 0.05

\*\*\* P < 0.01

\*\*\*\* P < 0.001

\*\*\*\*\* P < 0.0001

at day zero. This might be explained by the fact that anemia was a consequence of external bleeding and not hemolysis which would liberate in the circulation iron and hemoglobin increasing oxidation reactions (HALLIWEL & GUTTERIDGE, 1990). Or, the drop in serum iron levels could have made this transition metal less available for the Fenton reaction.

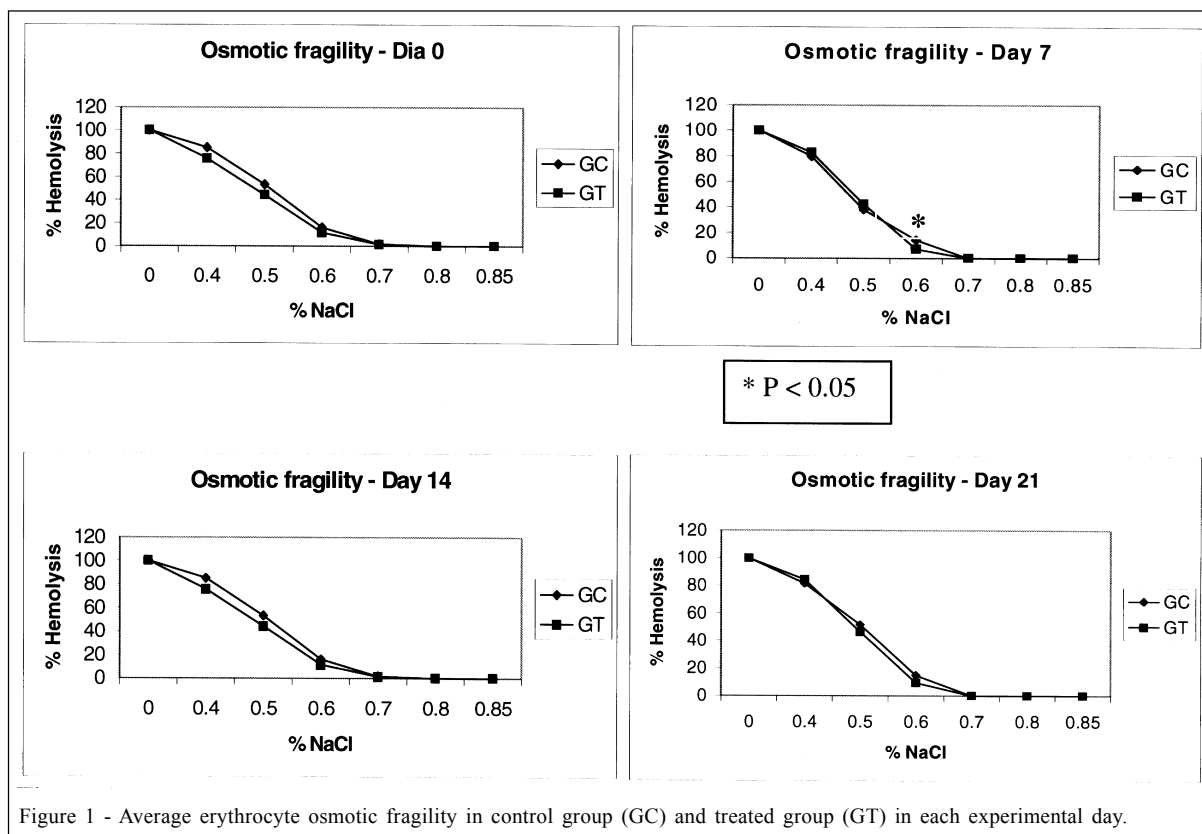
SCHOLL et al. (2000) reported that with reduced hemoglobin, tissues receive less oxygen which stimulates the production of erythropoietin that also stimulates the bone marrow to increase, depending on available iron, the production of erythrocytes. Plasma iron concentration showed a significant increase on days 7 and 14 in the treated group (Table 1). HILLMAN (1998), describes that about 60% of injected dextran iron is absorbed 72 hrs after administration, and, the rest of it, between 1 to 4 weeks.

At days 7, 14 and 21 after treatment was observed a significant increase in packed cell value, erythrocytes and hemoglobin in treated animals as compared to control group (Table 1). Evidence of an increase in the production of RBC (polycromasia, reticulocytosis) appears after 48 – 72 hrs and attain a peak around 7 days after hemorrhagy (DALLMAN, 1991). Moreover, the magnitude of the reticulocytosis is determined by levels of available iron (DUNCAN & PRASSE, 1982). Normally, around 70 to 90% of circulating iron can be captured by the bone marrow

and used in hemoglobin production (DALLMAN, 1991).

The treated group showed increased TBARS values on days 7 and 21 and reduced NPTH values on on days 7, 14 and 21 (Table 1). According IMAI et al. (1991), increased TBARS associated with reduced NPTH values are a common figure in elevated oxidative stress situations. Iron supplementation can induce increases in TBARS and reduction in NPTH (SEYMEN et al., 2004). TROOST et al. (2003), have reported that a single clinical dosis of iron sulphate can induce oxidative damage in healthy human subjects. The production of the hidroxy radical, is catalized by iron in the reactions of Fenton and Haber-Weiss (SCHIMMEL & BAUER, 2002).

The erythrocyte oxidation model has been used to evaluate oxidative damage on biomembranes. Free radicals attack RBC membranes inducing lipid oxidation and hemolysis (SIES, 1993). Osmotic fragility can be influenced by factors like shape, volume and size of the erythrocyte as well as amount of hemoglobin and chemical composition of the membrane. Apparently, smaller cells have a reduced capacity to expand, thus, attain the critical volume earlier (PERK et al, 1964). This can explain the reduction in hemolysis in samples from treated animals on day 7 (P < 0.05, Figure 1) since this group at this moment showed an increased number of reticulocytes (Table 1). Serum copper values were within



physiological range for the specie (FLOREZ, 1997) eliminating the possibility of anemia due to copper deficiency and also indicating that the production of hydroxyl radical from hydrogen peroxide was not catalyzed by excessive copper levels.

## CONCLUSIONS

The results indicate that iron supplementation accelerate the recovery of the hematological profile in anemic lambs, eventhough it increases momentarily lipoperoxidation and the oxidative stress, without any harmful consequence.

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