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PROTEIN CARBONYL, OXIDATIVE STRESS, ANEMIA, TOTAL FREE AMINO ACIDS AND SHEEP HAEMONCHOSIS RELATIONSHIP

By

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Abstract

The present study estimated oxidant/antioxidant status in blood of sheep naturally infested with *Haemonchus contortus*. Blood samples from infected and healthy (control) sheep were used to determine hematological, some antioxidants and biochemical parameters. The sheep showed microcytic hypochromic anemia. Haemonchosis in sheep resulted in a significant stimulation in MDA & PC. By contrast, a significant inhibition of the antioxidants activity of both SOD and GSH coupled with a significant inhibition of both ceruloplasmin (CP), Total Iron (Fe) and Copper (Cu) in infected sheep. In turn, a significant stimulation in CAT activity and in TFAA in infected sheep compared to control values was detected. The results showed a significant positive correlation between the increasing level of PC with the stimulating levels of MDA *($r=0.47$, $R^2=0.22$, $P=0.02$)*, CAT activity *($r=0.60$, $R^2=0.37$, $P=0.001$)*, TFAA in plasma *($r=0.46$, $R^2=0.21$, $P=0.03$)* and EP *($r=0.43$, $R^2=0.19$, $P=0.04$)*. By contrast, stimulated level of PC was inversely correlated with diminished values of SOD *($r=-0.55$, $R^2=0.31$, $P=0.03$)*, Hb *($r=-0.43$, $R^2=0.19$, $P=0.04$)* and PCV% *($r=-0.65$, $R^2=0.42$, $P=0.003$)*. Haemonchosis was accompanied by disturbances in protein synthesis and a general oxidative damage.

Keywords: Sheep, *Haemonchus contortus,;* Protein carbonyl, Eosinophils; Anemia; Oxidative stress.

Introduction

Sheep are an important source of high quality food products for humans (milk and meat). Gastrointestinal nematodes are among the most destructive pathologies affecting ovine production, given the high prevalence in flocks and the serious consequences of infection. In lambs, the acute form of gastrointestinal nematode infection causes death; the more frequent, chronic form leads to notable reductions in productivity. Ruminant gastrointestinal nematode infestations are common and widespread, and their immunopathology closely resembles that of human gastrointestinal infestation. Amongst gastrointestinal nematodes of sheep, *Haemonchus contortus* is the predominant species (Bordoloi *et al*, 2012). Haemonchosis caused by *H. contortus* is the most widely distributed of the pathogenic sheep gastrointestinal nematodiasis in Egypt. Both adult and fourth stage larvae suck blood and their migration caused haemorrhages into abomasum (Soulsby, 1982). The average blood loss due to *H. contortus* infection is 0.03 ml/parasite/day. Blood loss reduced feed intake, decreased body weight and wool growth (Hayat *et al*, 1996).

Parasitosis is a causative source of oxidative stress, indeed, several studies have reported on the presence of oxidative stress in humans and animals infected with parasites (Upcroft and Upcroft, 2001). Parasites cause inflammation when imbedding and penetrating tissues. Inflammation reactions are accompanied by generation of reactive oxygen species (Pal *et al,* 2005). Reactive oxygen species (ROS) include the superoxide radical (O_2), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2). Oxidative stress occurs due to excessive production of ROS and/or impotence of enzyme and non-enzyme systems

(Ridnour *et al*, 2005). Increased exposure to ROS led to cellular oxidative stress and damages of biomacromolecules (Lykkesfeldt and Svendsen, 2007).

Free radicals acted as cytotoxic agents (Gutteridge, 1995). The erythrocyte, due its role as O_2 and CO_2 transporter, is under constant exposure of free radicals (Harvey, 1997). However, red cells have a potent antioxidant protection that modifies free radicals into substantially less reactive intermediates (Cimen, 2008). The RBC antioxidant system primarily consists of superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), glutathione-peroxidase, and vitamins A, E & C (Kurata *et al*, 1993). Synergistic and co-operative interactions of antioxidants rely on sequential degradation of peroxides, free radicals and mutual protections of enzymes (Gutteridge, 1995).
Lipids especially polyunsaturated fatty acids are sensitive to oxidation, leading to the term lipid peroxidation which, malondialdehyde (MDA) being abundant (Halliwell and Chirico, 1993). The accumulation of MDA in tissues or biological fluids is indicative of the extent of free radical generation, oxidative stress and tissue damage (Gutteridge, 1995). Dimitrijevi´c *et al.* (2012) reported that lipid peroxidation increased in sheep infected with *Strongyloides papillosus*.

Protein carbonyl (PC) content is actually the most general indicator and by far the most commonly used marker of protein oxidation (Beal, 2002). In mammalian system including humans that direct damage to proteins or chemical modification of amino acids in proteins during oxidative stress can give rise to protein carbonyls (Stadtman and Berlett, 1998). Oxidation of protein (protein carbonyl) included all enzymes particularly antioxidants (Stadtman *et al*, 1991). Excessive protein carbonyl combined with inhibitions in superoxide dismutase (SOD) and glutathione (GSH) depended on enzymatic antioxidants (Reznick and Packer, 1994). Rare data are available on protein oxidation in *H. contortus* infected sheep.

The study aimed the efficacy of sheep natural infected with haemonchosis on ROS production and resulted oxidative damages to proteins and lipids, defensive mechanism of some antioxidants on hematological and biochemical parameters. Also, it focused on correlation of PC with other parameters.

Materials and Methods

Study area: Beni-Sanad farm (El-qusya, Assiut Governorate) is located in mountainous area where the climate is continental with cold winter and hot summers. Sheep reared under natural pasture resources that grown in the ground with supplementation of concentrates and some minerals. They were identified by their name and fire tattoo.

Animals: Sixty female sheep (4–8 months, weight about 15-20 kg b.w.) were included in this study. These animals were selected from Beni-Sanad farm (El-Qusya, Assiut Governorate) during summer season (June-September 2014). The animals were reared under similar feeding systems, management and environmental conditions. All sheep were subjected to careful clinical and laboratory investigations. Sheep were divided into 2 groups (30 each); GI naturally infected with *H contortus* and GII healthy control.

Positive *Haemonchus* signs were observed and recorded. Signs included body condition, abnormal behavior, and status of mucus membranes, lymph nodes, gastrointestinal disorders, rectal temperature and feces consistency. No clinical signs were in the control group.

Sampling: From each sheep two blood samples were collected by the jugular vein puncture, in a tube containing EDTA. One was used for hematological studies; and second was centrifuged at 3000rpm for 10 min. Plasma was collected and stored at -20˚C until processing and preparation of erythrocyte hemolysate. Fecal samples were collected from the rectum of infected and control sheep.

Parasitological examination: Standard flotation sedimentation technique and modified Berman's technique (Coles, 1980) was used for fecal samples to ensure that GII was parasites free and to prove that GI infected with *H. contortus* eggs and 3rd stage larvae.

Hematological investigations: Hemogram were determined automatically by automatic blood cell counter (Telldyn 3700, Germany).

Biochemical analysis: Plasma values of Total Free Amino Acids (TFAA), Ceruloplasmin (CP), Copper (Cu) and Iron (Fe) were determined (Rosen, 1957; Houchin, 1958; Kolmer *et al,* 1951; Bauer, 1984) respectively.

Preparation of erythrocyte hemolysate: Plasma and buffy coats were aspirated; sediment containing blood cells was washed three times by re-suspending in isotonic phosphate-buffered saline, followed by recentrifugation and removal of supernatant fluid and buffy coats. Crude red cells were lysed in nine volumes of ice-cold distilled water to have a 10% erythrocyte hemolysate. Erythrocyte hemolysate was used to determine protein carbonyl (PC), lipid peroxidation, reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT) (Levine *et al,* 1990; Placer *et al,* 1966; Beutler *et al,* 1963; Misra and Fridovich, 1972; Beers and Sizer, 1952) respectively.

Statistical analysis was performed using Graph Pad Prism 5.0 Software, CA, USA. Parameters were analyzed by ANOVA, and Newman-Keuls test. Data were expressed as means ± standard and differences between groups by Student t-test. Pearson's correlation (r) and linear regression (R^2) were done on paired data by GI, significant p< 0.05.

Clinically: GI were pale, mucous membranes, accompanied by debilitation, poor body condition (loss weight, wool and projection of ribs) with or without diarrhea.

Hematological findings: A significant reduction (P < 0.001) was in Hb, RBCs count & PCV% in GI as compared with GII, nonsignificant leucocytopenia in both GI & GII. eosinophils, lymphocytes and monocytes values were significant (P<0.05), while neutrophil value decreased (P <0.05) in infected sheep. MCV & MCHC values significantly decreased (P <0.05) in infected sheep, indicating a microcytic hypochromic anemia in infested sheep (Tab. 1).

Biochemical changes: infection caused significant increase in erythrocytic lipid peroxidation eMDA and erythrocytic Protein Carbonyl ePC (P <0.01) compared to normal ones. By contrast, a significant inhibition of the erythrocytic antioxidant activity of SOD (P <0.01), GSH (P <0.01), ceruloplasmin (CP) (P <0.01), Total Iron (Fe) (P <0.001) and Copper (Cu) (P <0.05) in plasma was in GII. Significant increase was in erythrocytic catalase activity CAT) & total free amino acids TFAA (P <0.001) in GI (Tab. 2).

Pearson's correlation and regression analysis showed a significant positive correlation between ePC value and eMDA (r=0.470, R^2=0.221, P=0.02, Fig. 1A*)*, eCAT *(*r=0.608, R^2=0.370, P=0.001, Fig. 1B*)*, TFAA in plasma (r=0.460, R^2=0.211, P=0.031, Fig. 1C*)* & Eosinophils% (r=0.436, R^2=0.190, P=0.047, Fig. 1D*)*. Concentration of ePC was inversely correlated with values of eSOD (r=-0.554, R^2=0.307, P=0.039, Fig. 2A), Hb (r=-0.438, R^2=0.191, P=0.041, Fig. 2B) and PCV% (r=-0.651, R^2=0.423, P=0.003, Fig. 2C).

Table 1: Mean values of hematological parameters in healthy and infected sheep:

Parameter	Normal (healthy)	*H. contortus* infected
Hb g/dl	12.76±0.28[a]	7.39±0.54[b]
RBCs count (10^{12}/l)	7.96±0.16[a]	7.76±0.42[b]
PCV%	35.99±2.19[a]	24.41±0.95[b]
WBCs count K/ul	7.37±0.50[a]	6.83±0.43[a]
Eosinophils K/ul	0.078±0.02[a]	0.31±0.05[b]
Lymphocytes K/ul	1.72±0.12[a]	2.30±0.18[b]
Neutrophil K/ul	4.80±0.33[a]	2.95±0.37[b]
Monocytes	0.73±0.08[a]	1.29±0.11[b]
MCV fl	39.85±1.2[a]	32.11±1.9[b]
MCHCg/dl	35.92±0.81[a]	29.99±1.25[b]

Table 2: Mean values of biochemical parameters in healthy and infested sheep:

Parameter	Normal (healthy)	*H. contortus* infected
MDA nmole/mg Hb	31.45±0.17[a]	43.90±5.51 [b]
PC µmole/mg Hb	0.67 ±0.04[a]	1.12 ±0.13[b]
Catalase U/mg Hb	7.99±0.39 [a]	10.65±0.53[b]
Ceruloplasmin mg/g protein	4.96±0.25[a]	4.16±0.14[b]
SOD U/mg Hb	2.46±0.17[a]	1.80±0.11[b]
GSH mg/mg Hb	1.01±0.05[a]	0.77±0.05[b]
TFAA µg/mg protein	4.50±0.15[a]	6.24±0.31[b]
Fe µg/dl	228.8±10.7[a]	110.3±21.4[b]
Cu µg /dl	50.08±9.00[a]	23.80±2.61[b]

[a,b] Values in same row with unlike descriptive superscript letters =significantly different (*P<0.05*).

Discussion

Few literatures dealt with gastrointestinal nematodes effect on the oxidative stress parameters and antioxidant defenses in sheep (Dede *et al,* 2002) in spite of the exceeded exposure of sheep to *H. contortus* parasite now and later which considered amongst the main parasitic infestations in Egypt. Anemia is a characteristic feature of nematodiasis in sheep. The reported microcytic hypochromic (Iron deficiency) anemia in infected sheep agreed with Qamar and Maqbool (2012). Reduction of serum iron level in infected sheep could be attributed to expanded erythropoiesis to compensate for blood loss leading to depletion of iron stores. These findings agreed with Albers *et al* (1990).

Oxidative stress arises when there is an imbalance between radical-generating and radical-scavenging activity; it may therefore cause an increase in the formation of oxidation products (Gutteridge, 1995). The excessive production of reactive oxygen species (ROS) resulted from the excitation of O_2 to form singlet oxygen or from the transfer of one, two or three electrons to O_2 to form a superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) or a hydroxyl radical (HO), respectively (Urban-Chmiel *et al,* 2009). The most sensitive part of the cell towards the action of ROS, primarily the hydroxyl radical, was polyunsaturated fatty acids (PUFA) of cell membranes. The end product of PUFA destruction during lipid peroxidation was malondialdehyde, so the increase of its concentration in tissues or biological fluids was considered as an indicator of increased production of free radicals and oxidative stress (Marnett, 1999). Dede *et al.* (2000) determined that the MDA level increased significantly in Akkaraman sheep infested with *Fasciola* spp., Trichostrongylidae, and *Eimeria spp*. Also, Simsek*et al.* (2006) reported MDA level increased with *Dicrocoelium dendriticum* and *Strongyloides papillosus* infection of sheep respectively. The data showed thatpronounced increase in MDA values in infected sheep with *H. contortus*. This might be due to oxidative damage in the cell membrane which possibly resulted from overproduction of free radicals.

Protein oxidation by ROS was associated with the formation of many different kinds of inter- and intra-protein cross-linkages, including those formed by addition of lysine amino groups to the carbonyl group of an oxidized protein (Valko *et al,* 2006). In mammalian system direct damage to proteins or chemical modification of amino acids in proteins during oxidative stress gave rise to protein carbonyls (PC) (Stadtman and Berlett, 1998). The usage of PC as bio-markers of oxidative stress has some advantages in comparison with the measurement of other oxidation products because of the relative early formation and the relative stability of carbonylated proteins (Dalle-Donne *et al,* 2003). The highly reactive hydroxyl radical (OH•) was generated by high concentration of H_2O_2 to be responsible for the formation of PC (Oliver, 1987). There were no reports available about protein oxidation in gastrointestinal nematodiasis of sheep. However, Dimitrijevi´c *et al.* (2012) reported that the level of PC was increased with the intensity of the *Strongyloides papillosus* infection in sheep, reaching the maximum value in the group with the highest degree of infestation. In the present study, the increased erythrocytic PC (ePC) in GI suggested a rise in the oxidative damage of both cell membrane protein and hemoglobin confirming an enhancement of erythrocytic free radical overproduction in parasitized sheep. So, nematodiasis in sheep caused excessive release of hydroxyl free radical rather than hydrogen peroxide. The present data showed a significant decrease of erythrocytic reduced glutathione (GSH) acted as a substrate in detoxification of peroxides such as hydrogen peroxide: H_2O_2 (Rahman and MacNee, 1999).

The presence of parasites in the host spurs defense mechanisms. The first, unspecific line of defense represented activated macrophages (Saleh, 2008). Catalase (CAT) facilitated the removal of H_2O_2, metabolized to molecular oxygen (O_2) and water (Van der Oost *et al,* 2003). Baghshani *et al.* (2011) assigned that increased activity of CAT caused by parasitic infestation of sheep was due to overproduction of H_2O_2. The current data, in agreement with the previous results, showed increased activity of CAT and may point out to a moderate not severe intensity of parasitic infestation or that the stage of infestation was early which counteracted by the host defense mechanism to overcome the overproduction of H_2O_2. This concept was in harmony with the depletion of erythrocytic superoxide dismutase activity (eSOD) shown in the present study. eSOD activity had been proposed as the main reductant of oxygen in mitochondrial membranes (Inoue *et al,* 2003) and its activity increased in the case of a larger production of O_2^-or inhibited in the case of an increased production of H_2O_2, which raised by enzymatic oxidation of the radical superoxide anion (Halliwell and Gutteridge, 1999). Reduced glutathione (GSH), a tripeptidethiol, is an important antioxidant protein enzyme, as well as a cofactor for various antioxidant enzymes (Kidd, 1997). Reznick and Packer (1994) found that Oxidative modification of proteins may lead to the structural alteration and functional inactivation of many enzyme proteins including GSH and SOD. The current data exhibited that both GSH and SOD depletions may be at least in part to the excess protein carbonyl accumulation which was probably concomitant with increased production of H_2O_2.

Ceruloplasmin (CP) is the main cupremic determinant in plasma and acts as an extracellular scavenger of free radicals, thus it may protect the cells against ROS (Saenko *et al,* 1994). Ceruloplasmin activity and the serum or plasma copper concentration decreased with nutritional copper depletion of ruminants (Blakley and Hamilton, 1985). Frandsen (1982) reported that blood copper levels were depressed in ruminants infested with nematodes. Most of the works reported on the relationship between endoparasitism and copper deficiency had been based on the oral copper supplementation (Adogwa *et al,* 2005). The previous authors added that gastrointestinal parasites affected copper metabolism probably by interference with Cu absorption from gastrointestinal tract. The present data explained that both plasma CP and Cu ions were significantly decreased. Mulcahy *et al.* (2004) reported that *H. contortus* increased pH value of abomasums. So, it could be suggested that *H. contortus* affected copper metabolism perhaps due to interference with copper absorption from gastrointestinal tract by increasing pH of the abomasal environment. So, infection caused Cu deficiency in blood and led to reduction in plasma ceruloplasmin.

A reduction of plasma protein concentrations (Wallace *et al,* 1996) was due to blood loss, hemorrhagic gastritis and leakage of proteins to gastric lumen caused disruption of intercellular unions and increased gut permeability (Baker *et al,* 2003). Proteases facilitate the invasion of host tissues, aid in the digestion of host proteins and help parasites evade the host immune response. Proteases encompass a broad class of hydrolytic enzymes that play essential roles in digestive processes of proteins (Williamson *et al*, 2003) leading to protein degradation and increased formation of plasma total free amino acids (TFAA). Determination of TFAA provided useful marker of total pool of each free amino acid and protein metabolism (Canepa *et al,* 2002). Infected sheep had lower food intake (Vervelde *et al,* 2001). Their malnutrition caused increase in protein breakdown and increased plasma TFAA level to support gluconeogenesis (Almeida *et al,* 2006). The present study showed a significant increase in plasma TFAA in infected sheep, such finding perhaps due to increased protein degradation through the action of proteases enzyme and muscle breakdown which probably due to activation of gluconeogenesis as a compensatory mechanism and/or due to decreased both uptake and absorption of nutritional amino acids caused by malnutrition and increased abomasal pH respectively. The significant rise of plasma TFAA might be attributed to an increase in free hemoglobin released probably from the exploded RBCs after filling with H_2O_2 due to severe depletion of eGSH. Anemia led to hypoxia (Chang and Stevenson, 2004) and hypoxia, increased Hb autoxidation augments superoxide production in RBCs led to RBCs release of H_2O_2 (Kiefmann *et al,* 2008). RBCs contain a large proportion of free amino acids in total blood (Felig *et al,* 1979).

In this study, non-significant decrease in total leucocytes with marked eosinophilia, monocytosis and lymphocytosis in blood of infected sheep. Rahman and Collins (1990) went with the present results, stated that infection with *H. contortus* did not lead to significant changes in total white cell counts with marked eosinophilia. Eosinophils produce and store many secondary granule proteins prior to their exit from the bone marrow, after maturation, eosinophils circulate in blood and migrate to inflammatory sites in tissues, or to sites of helminthes infection in response to chemokines and certain leukotriens (Wagner *et al,* 2007). Nebiat *et al.* (2012) found that Intestinal infection with, *Trichinella spiralis*, induced a pronounced eosinophilia and revealed benefit and protective role of eosinophils in the host by preserving the antigenic stimulus for a Th2 response that prevents reinfection of the intestine. The authors added that eosinophils may support parasite growth and survival by promoting accumulation of Th2 cells and preventing induction of iNOS in macrophages and neutrophils. However, eosinophil peroxidase forms reactive oxygen species that promote oxidative stress in the target, causing cell death by apoptosis and necrosis (Rothenberg and Hogan, 2006). Eosinophil activation in vivo resulted in eosinophil peroxidase (EPO) release and oxidative damage to proteins through bromination of tyrosine residues (Mitra *et al,* 2000). Meeusen and Balic (2000) found that eosinophils killed only infective larval stages of most helminthes. Hohenhaus *et al.* (1999) in *H. contortus* infected sheep, found a strong association between high eosinophil leucocyte counts and retention of normal levels of circulating eosinophil leucocytes with resistance to stress. The present result assumed that eosinophilia responded to haemonchosis was an immunological regulator against any excessive harmful parasitic effect on the host. Furthermore, the obtained data showed that there was a positive correlation of ePC with eosinophilia suggesting a recruiting role of eosinophils to curtail magnification of oxidative damage extent *in H. contortus* infected sheep through their resisting effect against stress due to parasitosis.

The current study showed negative correlation of ePC with eSOD, Hb and PCV due to protein oxidation that led to injury of functional proteins in hemoglobin, erythrocytic membrane and enzymatic antioxidant SOD led to drop in them. Reznick and Packer (1994) reported that oxidative modification of proteins led to structural alteration and functional inactivation of many enzyme proteins. Another cause of anemia in infected sheep rose in ePC and protein degradation. There was positive correlation of erythrocytic PC with plasma TFAA and eMDA.

Conclusion

The outcome results showed that sheep haemonchosis was accompanied by protein oxidation and a state of oxidative stress that led to disturbances in protein synthesis and to the pathogenesis of the disease and due to inappetance, gastrointestinal losses of protein and increased plasma TFAA. Infection decreased availability for growth, milk, and meat and wool production in sheep.

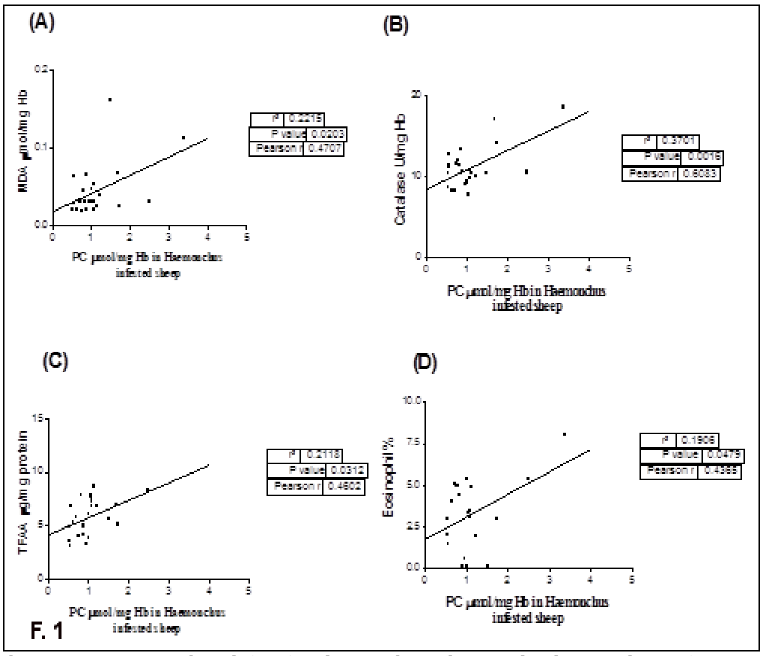

Fig. 1: Correlation of erythrocytic Protein Carbonyl (ePC) values with erythocytic lipid peroxidation (eMDA; A), erythrocytic Catalase (eCAT; B), plasma Total Free Amino Acids (TFAA; C) and Eosinophil % D in *Haemonchus contortus* infected sheep.

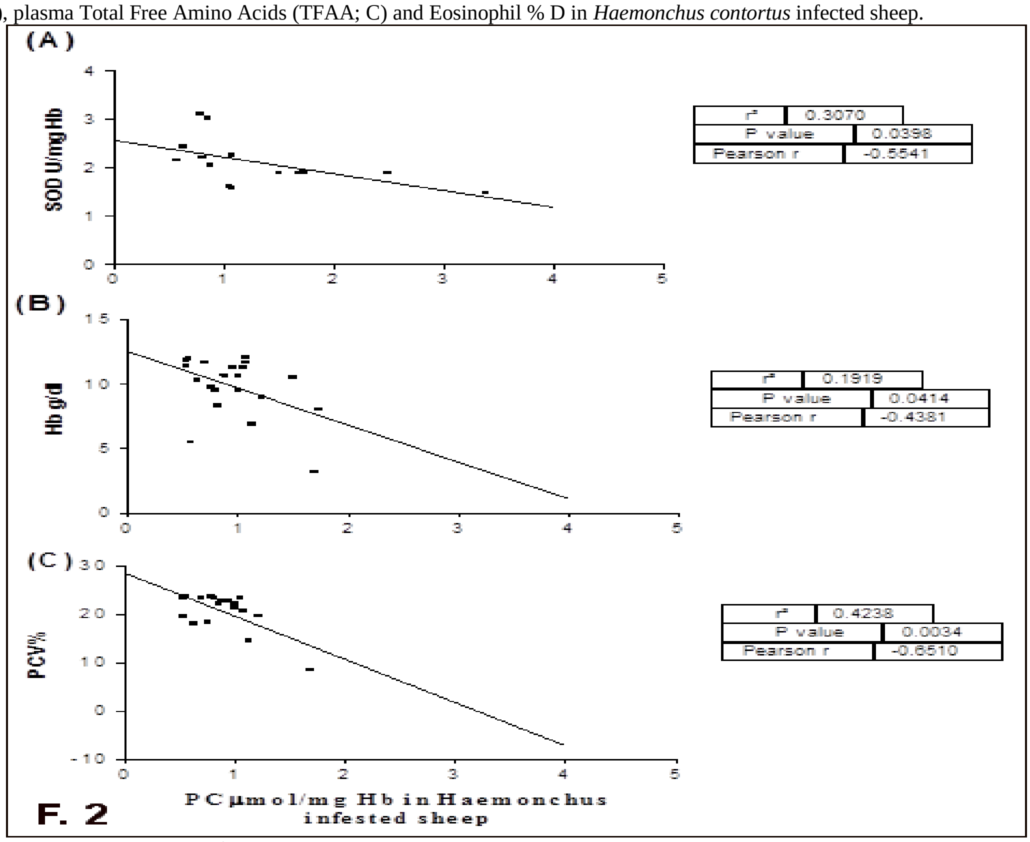

Fig. 2: Correlation of erythrocytic Protein Carbonyl (ePC) values with erythrocytic Superoxide dismutase(eSOD; A), Blood Haemoglobin (Hb; B) and Blood PCV% (PCV%; C). in *Haemonchus contortus* infected sheep.

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