The Role of Oxidative Stress in *Brachiaria decumbens* Toxicity in Sheep

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ABSTRACT

In an attempt to elucidate potential time-dependent oxidative stress mechanisms associated with *Brachiaria decumbens* toxicity in sheep, selected blood malondialdehyde (MD), as peroxidation tissue function biomarker and tissue morphology, were assessed. Six young adult Wiltshire cross bred ram were acclimatized for 3 weeks, where ivermectin injection and liver and kidney function tests were done. Blood samples were collected during the pre-treatment, namely on 3rd, 5th, 7th, 9th, and 11th days of continuous feeding of *Brachiaria*. Meanwhile, clinical signs were monitored and sheep were euthanised on the 7th, 9th, and 11th day, where a post-mortem was performed and relevant tissues were sampled. Results revealed that plasma thiobarbituric acid reactive substances (TBARS), serum (bilirubin total and conjugated, AST, GGT, urea, and creatinine) were significantly ($p < 0.05$) increased, whereas total antioxidant potential was decreased ($p < 0.05$) in a time dependant manner. Continuous *B. decumbens* feeding induced a time dependent appearance of jaundice, photosensitization, and subcutaneous oedema. Unique intracytoplasmic accumulation of Schmorl's positive greenish lipofuscin granules were observed primarily within the centrilobular hepatocytes and Kupffer cells. This may serve as a histochemical oxidative biomarker in the liver, kidney, brain, and skin. Taken together, this study shows that oxidative stress plays a major role in *B. decumbens* toxicity.

Keywords: *Brachiaria decumbens*, lipid peroxidation, oxidative stress, sheep

INTRODUCTION

Hepatogenous photosensitization (HPS) of sheep, associated with grazing plants containing steroidal saponins, is both economically important and an animal welfare problem (Flaoyen, 1996). *Brachiaria decumbens* (signal grass) intoxication, a disease similar to HPS in small ruminant, has been reported globally (Noordin, 1988; Amaral-de-lemos *et al.,* 1996; Brum *et al.,* 2007). Grasses are high yielding, stoloniferous, and very well adapted to tropical climatic conditions (Loch, 1977) and much preferred by sheep and goats. It has been postulated that *B. decumben*, *per se,* is not toxic but it is converted into hepatotoxic compounds in the rumen of sheep and goats (Noordin, 1988). It was found that diosgenin and its metabolites in *B. decumbens* are hepatotoxic and nephrotoxic (Zhang, 2000).

Oxidative stress is otherwise referred to as "oxidoreductive stress" is an imbalance between the production of oxidizing molecular species (pro-oxidants) and the presence of cellular antioxidants in favour of the pro-oxidants leading to potential damage. Lipid peroxidation is a well-established mechanism of cellular injury and it has been used as an indicator of oxidative stress in cell and tissues. In particular, lipid peroxides

derived from polyunsaturated fatty acids are unstable and can decompose to form a complex series of compounds. These include reactive carbonyl compounds of which MDA is the most abundant. Therefore, measurement of MDA is widely used as an indicator of lipid peroxidation. Increased levels of lipid peroxidation products have been associated with several models of liver injury (Panozzo *et al.*, 1995; Sergent *et al*., 1995). There are considerable pathologic evidences which indicate the involvement of intrahepatic oxidative stress and subsequently lipid peroxidation in the pathogenesis of this intoxication. There is a dearth of knowledge on the development of pro-oxidant and anti-oxidant status during continuous feeding of *B. decumbens*. Therefore, the objective of this study was to explore the role of oxidative stress in *B. decumbens* toxicity in sheep by measuring or assessing the oxidative parameters and pathological changes in the liver, kidneys, brain, and skin.

MATERIALS AND METHODS

The experimental protocols and ethics were approved by the Animal Care and Use Committee (UPM/FPV/3.2.1.551/AUP-R42). Six clinically healthy young adult (12-14 months) cross-bred Malin rams (20-22kg) were purchased from a local commercial farm in Malaysia. The acquired sheep were kept for an acclimatization period of three weeks, during which they fed on concentrate and non *B. decumbens* fodder with *ad libitum* provision of drinking water. The sheep were dewormed with Ivermectin and both the liver and kidney functions were assessed. Two groups of three animals each were used as a control and *B. decumbens* fed sheep. The treated group was continuously fed with only *B. decumbens* grass (only the green leafy and stem portions), whereas the control was given non *B. decumbens* fodder. Daily monitoring of the development of clinical signs was done while blood samples were collected on day 0, 3, 5, 7, 9, and 11 post feeding. Assays of serum aspartate aminotransferase (AST) and

γ-glutamyl transferase (GGT), serum conjugated and total bilirubin, blood urea nitrogen (BUN), and creatinine were done using diagnostic kits (Roche Diagnostic).

The end product of peroxidative decomposition MDA of polyenic fatty acids in the lipid peroxidation process was measured in the serum using the double heating method as described by Placer *et al*. (1966). The SOD activity was measured by pyrogallol oxidation inhibition assay of Marklund and Marklund (1974) using UV/visible spectrophotometer at 330 nm, whereas the activity of GSH-Px in erythrocytes was measured using the DTNB direct method at 422 nm.

Sheep were euthanised on the 7th, 9th, and 11th days (one from each group) and post-mortem was also performed. Samples of the liver, kidney, brain, and skin were collected at necropsy and fixed in 10% neutral buffered formalin, and this was followed by embedding in paraffin wax, sectioning at 5µm and staining with haematoxylin and eosin (H&E). Sections of liver and kidneys were also stained with Schmorl's reaction for lipofuscin, while some sections from photosensitized skin were stained with Masson trichrome for collagen. Data were stated as mean ± standard deviation (SD) and subjected to statistical analysis using the SPSS software package (version 11.0 for windows).

RESULTS AND DISCUSSION

While animals in the control group showed no clinical and pathological changes, variations in the sequences of appearance and severity of clinical signs were observed in the treated group during multiple periods of this study. Signs of photosensitization, which included facial and submandibular oedema and jaundice, were observed as early as the 7th day of post feeding and this conformed to earlier findings (Abas *et al*., 1983). The prominence of jaundice in this study could be due to intra-hepatic cholestasis as indicated by the marked elevation in GGT and unconjugated hyperbilirubinemia. The submandibular, facial, and subcutaneous oedema

was likely to be the result of two factors, namely hypoalbuminaemia (i.e. due to hepatic damage which is strongly related to the severe elevation in AST) and systemic hypertension and congestion (i.e. well-related to the prominence of macula densa of the distal convoluted tubules in the kidney, as shown in *Fig. 1A*). During the late stage of *B. decumbens* sheep
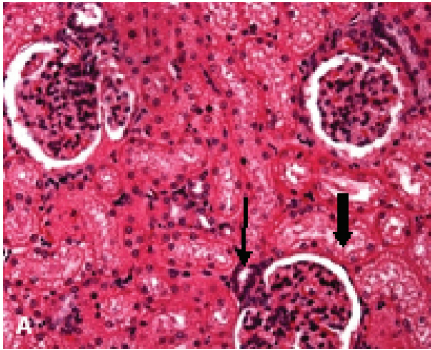

Fig. 1A: Photomicrograph of the kidney of an intoxicated sheep at seven days of post-feeding showing prominence of macula densa of the distal convoluted tubules (thin arrow) with associated glomerular congestion. The renal tubules appear either necrotic or degenerated (thick arrow) [H&E]

toxicity, there were consistent developments of nervous signs such as stamping of forelimbs, head shaking, ataxia, circling movement, and reverse locomotion. These clinical signs were confirmed histopathologically by the presence of status spongiosus in the cerebellum (*Fig. 1B*), a lesion which may have developed as a consequence of hepatic encephalopathy induced by hyperammonianaemia resulting from hepatic failure (Salam *et al.,* 1989).

Liver, being the target organ of *B. decumbens* toxicity, appeared swollen, bronze-yellow with accentuated lobular pattern, distended gall bladder, greyish pinpoint necrotic areas, and slight thickening of bile duct. Histopathologically, mild bile duct hyperplasia and centrilobular hepatocytes degeneration and necrosis (*Fig. 1C*). The centrilobular region in the liver appeared to be the most vulnerable area for lipid peroxidation as indicated by the gradual aggregations of greenish blue granules of lipofuscin. This may serve as a vital histochemical biomarker of oxidoreductive stress during time dependent *B. decumbens* toxicity. Two criteria render the centrilobular hepatocytes more susceptible to the oxidoreductive stress, namely the minimal oxygen supply and the normally high concentration of detoxifying enzyme content
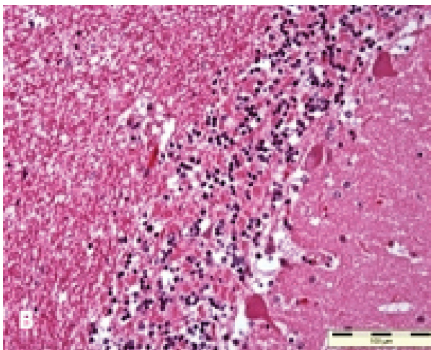

Fig. 1B: Photomicrograph of the cerebellum of an intoxicated sheep at eleven days post-feeding showing vacuolation of the white matter of the cerebellar folium (thick arrow). In the grey matter, the Purkinje cell appears decreased in size and number (thin arrows) [H&E]
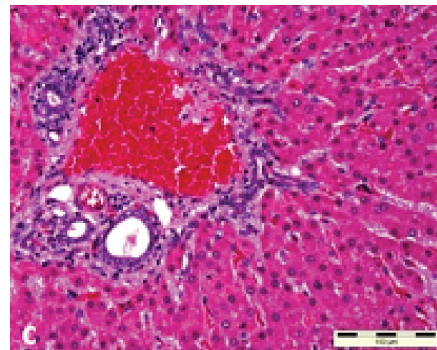

Fig. 1C: Photomicrograph of the liver of an intoxicated sheep, nine days post-feeding showing bile duct hyperplasia (thin arrows), periductal lymphocytic infiltration and severe congestion of the portal vein, hepatic artery and sinusoidal spaces [H&E]

(mixed function oxidase and P-450) (Cheville, 1983). Biochemical and morphological findings were found to be correlated well with evidence of oxidative stress. There is a significant and strong relationship (P>0.001) between the values of AST and that of MDA (*Fig. 2*), and this reflects the role of oxidoreductive stress in the development of hepatotoxic damage in a time dependant manner. These findings quite agree with that of Zhang (2000) who described the AST as a better indicator of the liver function than GGT in cases of *B. decumbens* toxicity in sheep.

As a part of systemic oxidative stress phenomenon, necrotizing dermatitis (*Fig. 1D*) observed in this study may be a consequence of interaction of photoactivated molecules (phylloerythrin) with oxygen, where singlet oxygen or oxygen radicals are generated and leading to disruptions of protein and DNA synthesis, mitochondrial damage, lysosomal damage, and cell death (Allen and Balin, 1989).

The almost parallel increase and decrease of MDA and GSH-Px activities respectively over time indicate a graduation of the severity of tissue damage which is induced by oxidative stress in *B. decumbens* intoxication. Furthermore, this is supported by the variable degree of lipofuscinosis in the liver of the affected sheep (*Fig. 1E*). Likewise, the change in GSH-Px activities (*Fig. 3*) during *B. decumbens* intoxication seems to be important and reflects the glutathione depletion leading to disruption in redox balance. This acts in the normal state to prevent or repair the oxidation of lipids and keep the cellular thiol redox status of liver in the reduced form. Low activity of this enzyme is one of the early consequences of a disturbance of the prooxidant/ antioxidant balance in favour of the former during *B. decumbens* toxicity in this study. During multiple periods of this experiment, insignificant fluctuation in the erythrocyte SOD activites was the overwhelming criterion and it is believed that SOD plays a minimal or negligible role in this intoxication (*Fig. 4*).
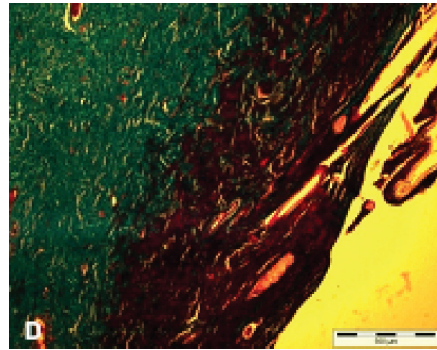

Fig. 1D: Photomicrograph of the skin of an intoxicated sheep at seven days of post-feeding showing red masses of necrotized collagenous tissue which was negatively (purplish) stained while viable parts appeared greenish [Masson trichrome]
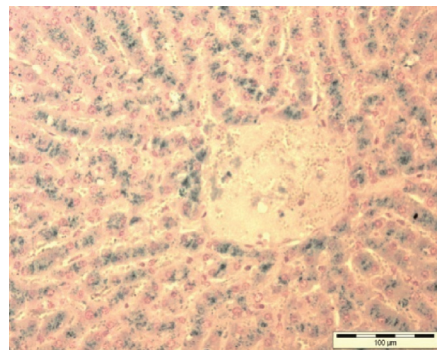

Fig. 1E: Photomicrograph of liver of an intoxicated sheep at eleven days post-feeding showing intra-cytoplasmic dark greenish lipofuscin granules within exhausted hepatocytes, especially in the centrilobular zone [Schmorl]

CONCLUSIONS

These data revealed that the experimental *B. decumbens* sheep toxicity is associated with increased lipid peroxidation in a time dependant manner. We concluded that the oxidative stress in this toxicity is a systemic phenomenon which probably encompasses other tissues and organs.
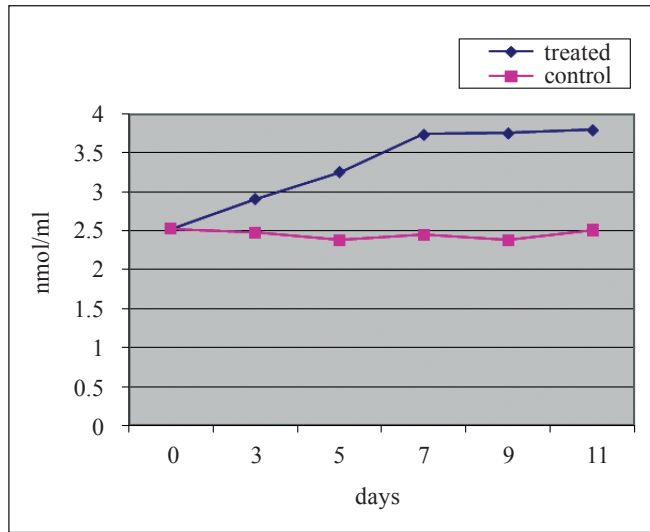
Fig. 2:. Malondialdehyde levels in the serum of sheep during the experimental period
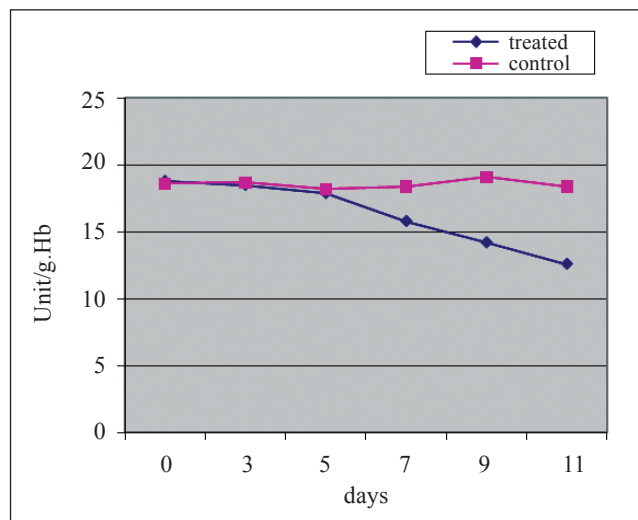

Fig. 3: The activity of erythrocytic GSH-Px in sheep during the experimental period

More importantly, diosgenin the toxic principle of *B. decumbens* can cause not only oxidoreductive stress and subsequently cell injury due to the production of strong intermediate free radicals, but it may also involved in signal transduction and the regulation of gene expression via redox-sensitive mechanisms.
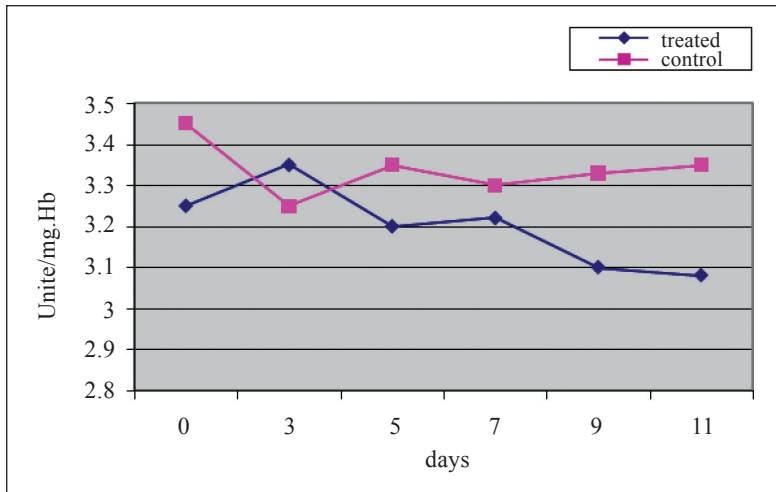
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Fig. 4:. Dynamics of erythrocyte superoxide dismutase of sheep during the study period

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