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THE ROLE OF FREE RADICALS IN BRASSICA-INDUCED ANAEMIA OF SHEEP: AN ESR SPIN TRAPPING STUDY

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The formation of reactive free radical species in sheep erythrocytes challenged with dimethyldisulphide, a brassica-derived haemolysin, has been investigated by electron spin resonance spectroscopy using the spin trap α-(4-pyridyl 1-oxide)-N-*tert*-butylnitrone. Erythrocytes exposed to this agent undergo a burst of free radical activity as demonstrated by the appearance of a spin adduct. The results suggest that haemolytic anaemia which can occur in sheep grazing forage brassicas is a consequence of oxidative stress.

KEY WORDS: Brassica anaemia, sheep, free radicals, ESR, spin trapping.

INTRODUCTION

Sheep, in common with other ruminants, may develop haemolytic anaemia when brassica crops comprise a significant proportion of their diet. Onset of the condition is characterized by the formation of denatured haemoglobin aggregates, known as Heinz bodies, on the erythrocyte cell wall. Other manifestations include growth retardation in lambs. The principal haemolytic factor present in such crops has been attributed to S-methylcysteine sulphoxide (SMCO) its effects being mediated through formation of dimethyldisulphide (DMDS) in the rumen by micro-organisms exhibiting SMCO-lyase activity.[1] In cattle grazing kale (*brassica olaracea*), reduction in erythrocyte glutathione (GSH) and copper levels have been reported[2] as well as increased levels of lipid peroxidation, increased erythrocyte osmotic fragility and a decrease in lipid packing density.[3] It has also been reported that sheep with an inherited GSH deficiency (which may compromise their antioxidant capacity) become more anaemic when fed kale and exhibit a higher Heinz body count (HBC) compared with GSH-sufficient animals.[4] Such observations indicate that the haemolytic activity of DMDS may be mediated through the formation of oxidative free radical species within the red cell.

The effect of rumen infusion of DMDS in cattle and goats on packed cell volume (PCV) and HBC has been reported previously,[1] and in the present study the effects of such infusion in sheep has been investigated. In an attempt to evaluate the possible role of free radicals in the aetiology of brassica-induced anaemia, electron spin

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resonance (ESR) spin trapping studies have been carried out on sheep erythrocytes challenged with low concentrations of DMDS.

MATERIALS AND METHODS

Rumen Infusion of DMDS

Six rumen-cannulated Greyface ewes of age range 8–10 years and mean live weight 69 ± 1.8 kg, were used in the study; three animals serving as controls, three being treated with DMDS. They were offered a daily diet of dried grass pellets (1 kg) and hay (0.5 kg) to which they were accustomed. The treated ewes were injected with DMDS (3 ml) *via* the cannula into the centre of the rumen at 08.30 and 18.00 h daily for between 8–11 days, the treatment being halted when the PCV levels fell to ca. 18%. Prior to the morning injections, a 10 ml sample of blood was collected by heparinised vacutainer (Beckton Dickinson, Cowley, Oxford, UK) from the jugular vein of each animal and measurements made of PCV using a Hawksley micro-haematocrit system. HBC levels were determined by staining films of whole blood with brilliant cresyl blue.[5] Those red cells which contained Heinz bodies were expressed as a percentage of the total number of cells counted. After treatment had been terminated measurements were taken at regular intervals from animals in both groups until the PCV and HBC parameters in the treated animals had returned to control levels.

Spin Trapping

Approximately 8 ml of blood was collected by heparinised vacutainer from the jugular vein of 4–5 year old Greyface ewes which had been offered, *ad libitum*, a hay and concentrate diet. Erythrocytes were recovered by centrifugation (10 min, 2300 *g*) and a wash/centrifuge cycle carried out three times using isotonic (0.9% NaCl), potassium phosphate buffer (pH 7.4). After the final cycle 1 ml of packed cells was removed and added to 4 ml of the buffer to give a 20% cell suspension. A solution of the spin trapping agent α-(4-pyridyl 1-oxide)-N-*tert*-butylnitrone (4-POBN (Aldrich, Gillingham, UK), 3.2 ml, 200 mM) was prepared in buffer. Incubation of a combined red cell suspension (1.5 ml) and 4-POBN solution (1.5 ml) was carried out, with stirring, in the presence or absence of DMDS (Aldrich "Gold Label", Gillingham, UK), at 37°C in Reactivials (Pierce, Rockford, Ill., USA) open to the atmosphere. DMDS (2.7 μl) was introduced to one of the incubations using a 5.0 μl Hamilton syringe (Hamilton Co., Reno, Nevada, USA) giving an effective DMDS concentration of 10 mM. The preparation of the two incubations was offset by 10 min to enable spectral examination at the same period of time. Aliquots (0.3 ml) were removed from the incubations after 10, 30, 50, 70, 90 and 110 min, placed in an aqueous quartz cell and examined by ESR. The central doublet of the resulting spin adduct spectrum was recorded each time, its intensity being used as an indicator of the arbitrary spin adduct concentration. Spectra were run on a Varian E104 spectrometer operating at ca. 9.5 GHz (X-band) frequency. A microwave power level of 10 mW and modulation amplitude of 0.2 mT were used throughout having established that power saturation and modulation broadening would not occur at these values. The experiments were replicated on blood from four sheep. An additional control incubation was prepared containing buffer (1.5 ml), 4-POBN solution (1.5 ml) and DMDS (2.7 μl), in the absence of erythrocytes, and was examined in the same way.

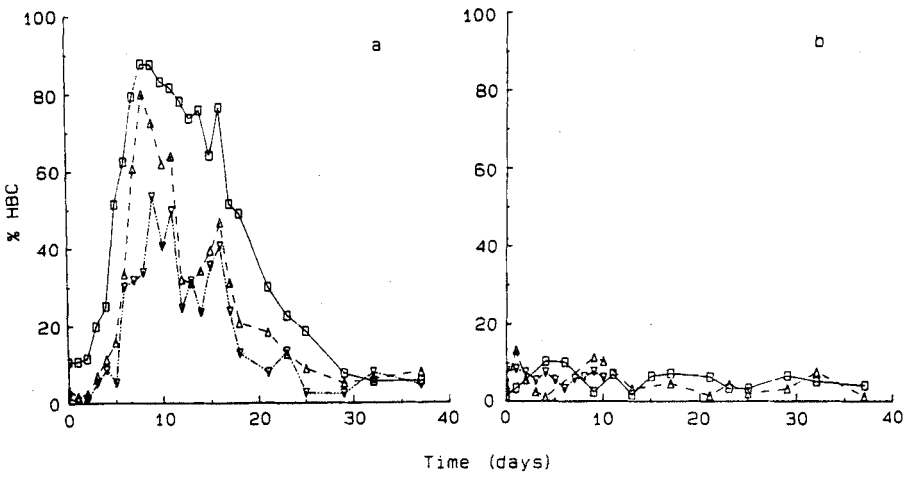

FIGURE 1 Heinz body count (HBC) of (a) DMDS-treated and (b) control ewes. Each set of points refers to an individual animal, one control ewe was monitored for a 10-day period only. Dosing period of treated animals: □, 11 days; ▽, 10 days; △, 8 days.

To monitor the variation in spin adduct intensity as a function of DMDS concentration, blood was sampled from four Greyface ewes under the conditions previously described. Incubations of spin trap and cell suspensions were prepared as outlined above but containing 0, 0.25, 0.5, 1.0, 2.0 μl DMDS. The ESR spectrum of the central doublet was obtained for each solution after an incubation period of 140 min.

A final experiment was conducted to establish if cell lysing in the absence of DMDS could result in the formation of a 4-POBN spin adduct. Blood was collected from a Greyface ewe maintained on a hay and concentrate diet, the erythrocytes being washed and retrieved as described previously. Three cell and spin trap incubations were prepared as before except that the 20% cell suspensions and 4-POBN solutions were formulated in either isotonic (0.9% NaCl) potassium phosphate buffer (pH 7.4) or hypotonic (0.6% or 0% NaCl) buffer. An additional isotonic incubation containing DMDS (2.7 μl) was also prepared. The incubation starting times were offset by 15 min and the ESR spectrum of the central doublet from each sample obtained after 10 and 110 min had elapsed.

RESULTS

Rumen Infusion of DMDS

Blood parameters for the treated and control animals are shown in Figures 1 and 2. The HBC of the three treated animals increased rapidly 3–4 days after the infusion regime commenced (Figure 1a). A gradual return to pretreatment values of less than 6% occurred after infusion had ceased. Throughout the experimental period the HBC of the control animals remained low (Figure 1b) with a mean value of 5.5 $\pm$ 0.5%. In the treated animals a marked decrease in PCV was observed which lagged behind the rise in HBC. The recovery rate after termination of treatment was considerably

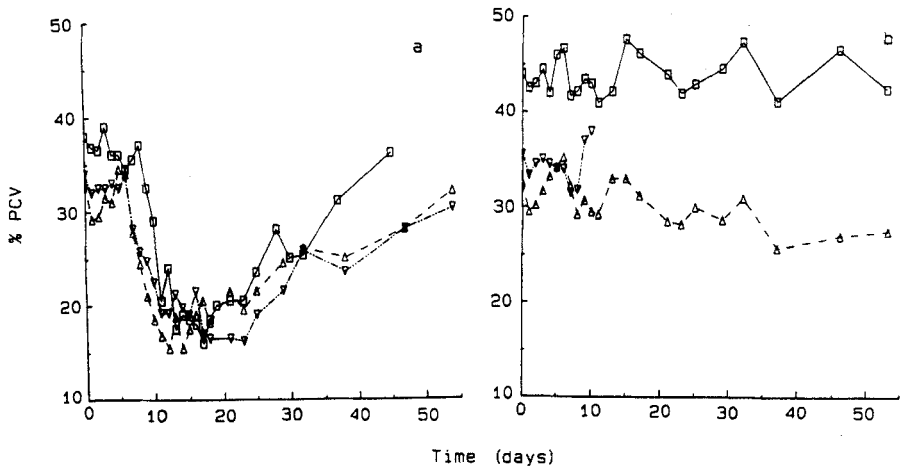

FIGURE 2 Packed cell volume (PCV) of (a) DMDS-treated and (b) control ewes. Each set of points refers to an individual animal, one control ewe was monitored for a 10-day period only. Dosing period of treated animals: □, 11 days; ▽, 10 days; △, 8 days.

longer than that observed for the HBC. The PCV of the control animals remained relatively stable (Figure 2b) for the duration of the experiment.

Throughout the experiment all the other animals ate normally with no reduction in feed intake or any other signs of stress being observed in the treated ewes.

Spin Trapping Studies

ESR spectra representative of those obtained from DMDS treated and untreated incubations are shown in Figure 3. In the treated sample a triplet of doublets originates from a 4-POBN spin adduct with a g-value of 2.006 ± 0.001 and parameters $A(N) = 1.575 \pm 0.010$ mT and $A(H) = 0.262 \pm 0.010$ mT where $A(N)$ and $A(H)$ refer to the isotropic hyperfine coupling constants arising from interaction of the unpaired electron with the nitrogen and β-hydrogen nuclei of the trap (nuclear spin $(I) = 1$ and 1/2 respectively). In the untreated incubation a weak signal arising from a spin adduct of similar parameters is only just detected.

The spectral intensity of the central doublet of the 4-POBN spin adduct is shown as a function of time in Figure 4. It can be seen that in the DMDS-treated incubations a rapid increase occurs in spin adduct concentration, the ESR signal intensity at 110 min being approximately 22-fold greater than in the untreated incubation. Little intensity increase was observed in the untreated incubations and indeed the intensities were very similar to those obtained from an incubation containing buffer, trap and DMDS but no red cells (results not shown).

The variation in spin adduct intensity as a function of DMDS concentration after an incubation period of 140 min (Figure 5) indicates that the lower limit at which an effect attributable to DMDS would be observed in this system is ca. 0.3 mM.

With respect to the effects of cell lysing, incubation of cells lysed in hypotonic buffer in the absence of DMDS did not show any increase in spin adduct signal intensity when compared to equivalent non-lysed incubations in isotonic buffer. The response

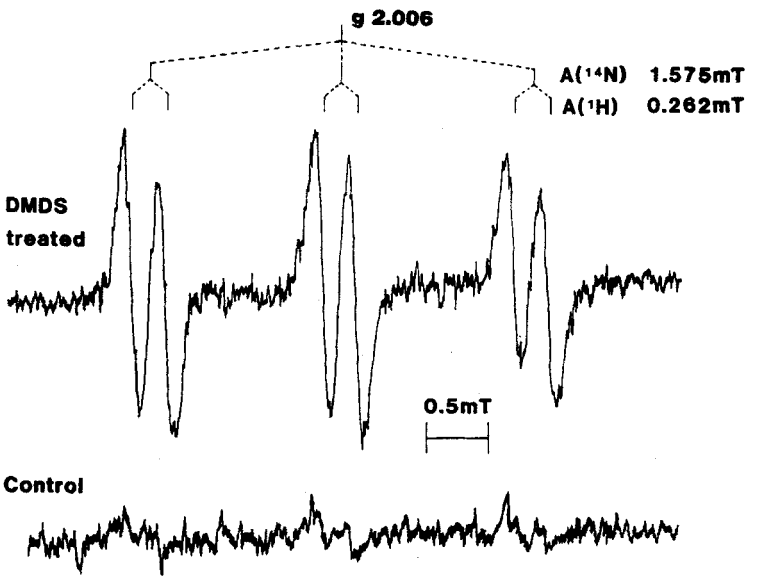

FIGURE 3 ESR spectra of 4-POBN spin adduct obtained after 70 min incubation of erythrocyte suspensions at 37°C.

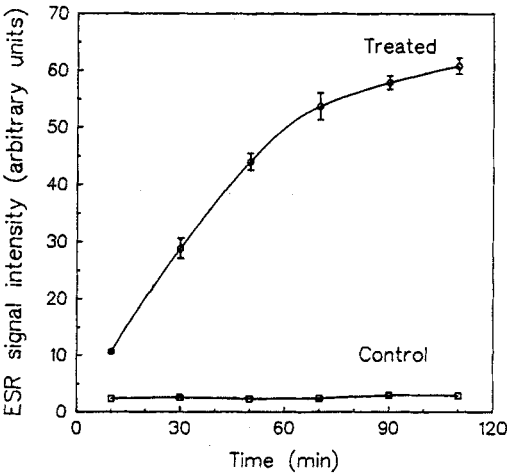

FIGURE 4 Variation with time of 4-POBN spin adduct concentration in cell incubations. (n = 4, SEM displayed where greater than symbol size.)

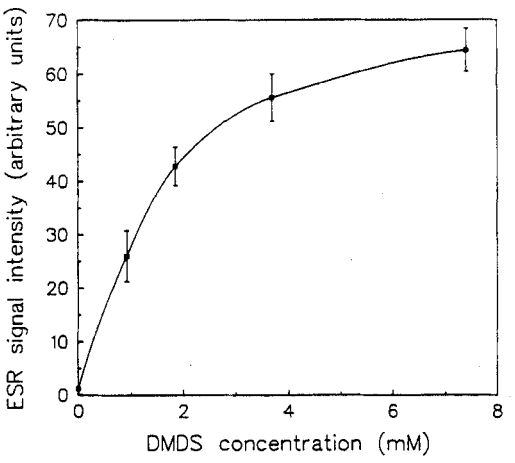

FIGURE 5 Variation in 4-POBN spin adduct concentration as a function of DMDS concentration in cell incubations. (n = 4, SEM displayed.)

of the intact cell preparation to DMDS treatment was again demonstrated (results not shown).

DISCUSSION

The rapid rise in HBC and fall in PCV as a result of DMDS infusion into the sheep rumen demonstrates the animal's susceptibility to the haemolytic effects of this brassica-derived agent and parallels closely the effects observed in goats by Smith.[1]

The ESR spin trapping studies indicate that exposure of sheep erythrocytes to DMDS results in a burst of free radical activity within the cell leading to the formation and build-up in concentration of a 4-POBN spin adduct. The possibility that the observed radical burst was solely a consequence of the eventual lysing effect of DMDS has been discounted as a result of studies carried out on cells lysed in hypotonic buffer in the absence of DMDS which failed to show the formation of the spin adduct.

The isotropic hyperfine coupling constants (A-values) of the spin adduct are very similar to those reported by various workers[6] in microsomal and other systems undergoing peroxidation and have often been ascribed to trapping of a lipid-based peroxyl species, LOO·. However, work by Connor *et al.*[7] in which $^{17}O_2$ isotopic substitution was utilized in a linoleic acid-lipoxygenase generating system casts considerable doubt on the validity of this assignment and instead supports the idea of the formation of a carbon-centred, fatty acid radical adduct such as a pentadienyl breakdown product of linoleic acid.

On the basis of the spectroscopic parameters reported here it is possible to exclude assignment of the adduct to the hydroxyl (HO·) hydroperoxyl (HOO·) or superoxide (O_2^-) species.[6] However, lack of an ESR-detectable adduct from these species does not in itself imply their non-involvement in the system. From the isotropic nature of the adduct spectrum it can also be inferred that the trapped radical is of relatively low molecular weight and that the adduct does not have any significant motional restrictions

placed upon it which would be expected to occur if, for example, the trap had attached itself to a long chain fatty acid radical situated within the membrane bilayer.

The mechanism by which DMDS interacts with cellular components and thus gives rise to increased free radical activity has not been fully investigated. Smith *et al.*[3] have postulated that disulphide exchange reactions may occur with thiol groups and in particular with glutathione:

$$2GSH + CH_3SSCH_3 \rightarrow GSSG + 2CH_3SH$$

This is consistent with the reduction in GSH levels observed in kale-fed cattle.[2] A resulting impairment of the glutathione peroxidase (GSH_{px}) pathway could therefore lead to a build-up of cellular peroxides which in turn would provide a source of HO· through the occurrence of Fenton-type reactions.[8] The radical trapped in the current work may be formed at some stage in a reaction chain which has been initiated by this highly-reactive species. However, the observed reduction in GSH levels could be the result of, rather than the cause of, increased oxidative free radical activity. The possibility that other mechanisms may operate cannot be discounted. Indeed, Steven *et al.*[9] have demonstrated *in vitro* the ability of DMDS to alter enzymic activity of trypsin and papain. Inhibition of enzymes associated with the functioning of the cellular antioxidant pathways could also result in increased free radical activity within the cell.

The extent to which DMDS-mediated radical stress demonstrated in this paper can be linked to symptoms such as reduced weight gain and growth retardation which occur in the field situation has yet to be ascertained. However, an ESR spin trapping approach may provide a sensitive, *in vitro* method for assessing the effectiveness of a range of strategies designed to reduce the oxidative radical burden placed on the cell by DMDS exposure. Ultimately this may prove a basis for affording protection at the whole-animal level.

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