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EFFECT OF ANTIOXIDANTS ON AMMONIA INDUCED CNS-RENAL PATHOBIOLOGY IN SHEEP

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The effect of antioxidants on ammonia induced CNS and renal pathobiology in 10 sheep infested by *L. Cuprina* larvae was investigated. The condition produces severe dermatitis, proliferation of macrophages and hyperammonaemia, and free radicals may therefore be involved in the pathogenesis. Five of the sheep (treated group) were given daily intramuscular (im) injections of 2 g sodium ascorbate, 5.9 g dl-alpha tocopherol (11 days) and 3 g desferrioxamine mesylate (6 days) with 70 mg oral butylated-hydroxyanisole (11 days). The treatment prevented rises in jugular ammonia, creatinine, urea, sodium and pH, and decreases in water intake, urine output and glucose. The findings showed that antioxidants prevented ammonia induced CNS and renal pathobiology and suggest that free radicals contribute to the pathogenesis of the condition.

KEY WORDS: Antioxidants, ammonia, pathobiology, sheep.

INTRODUCTION

There is a lack of information concerning the interaction of free radicals and anti-oxidants on pathobiology *in vivo*. It is becoming apparent that chemical exposures, stress or disease deplete antioxidants in tissues.[1-3] Lipid tissues are susceptible to peroxidation by free radicals[2] and are highly sensitive to even slight rises in blood ammonia levels.[4-5] The capacity of high levels of ammonia to generate free radicals is presently unknown as is the interaction between antioxidants and ammonia.

Infestation of sheep by the ammonia producing larvae *L. cuprina* provides a suitable model for testing the effects of anti-oxidants on ammonia induced brain and renal pathobiology. This condition is associated with extensive skin wounds, severe dermatitis and necrosis,[7] a marked neutrophilia,[8] generalised infiltration of tissues by macrophages and progressive degeneration of white cells.[7-8] Infested sheep suffer hyperammonaemia compounded by alkalosis[9] which together with severe dermatitis[6] and brain lesions,[9] results in anorexia, hypodypsia and oliguria, hypernatraemia, and hypoglycaemia.[9-11] Elevated levels of ammonia in the CNS may affect circulation in peripheral tissues such as the kidneys.[12]

Large amounts of reactive oxygen species may be generated in sheep infested by *L. cuprina* because there is severe dermatitis[13] and leucocytosis.[14] Increased activity of xanthine oxidase in skin wounds could generate superoxide and hydrogen peroxide

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leading to release of free iron and formation of toxic hydroxyl radical.[14] Superoxide generated by activated neutrophils and macrophages could release iron from ferritin to catalyze lipid peroxidation.[14]

In the infestation, toxic ammonia is readily absorbed through the skin[9] and reaches the brain, by-passing liver detoxification.[10] Initially, exhalation of peripheral ammonia and detoxification in the CNS by glutamic acid[15] prevent detectable rises in jugular ammonia concentrations. However, jugular levels will eventually rise as glutamic acid is depleted and detoxification is inhibited.[4]

Oxygen free radicals may be involved in the inflammation caused by *L. cuprina*. Vitamins C and E, and butylated hydroxyanisole could prevent antioxidant depletion and peroxidation.[1-3] Desferrioxamine mesylate may reduce iron-catalyzed free radical production.[4,14] The purpose of this report is to describe for the first time, the effects of an antioxidant treatment on ammonia induced CNS and renal pathobiology in sheep.

MATERIALS AND METHODS

The procedures used were approved by The Animal Ethics Committee of The Animal Research Institute, Department of Primary Industries, Yeerongpilly, Australia. The utmost care was taken to avoid distress and suffering to the animals. The main objective of the experiment was to find a means to reduce suffering and death in sheep. Of the 12 sheep originally used, it was found that one (treated) had meningitis and one (not-treated) was implanted with *L. cuprina* eggs instead of larvae. The data from these sheep were therefore not considered representative and were not included in any analysis.

Ten long wooled adult merino sheep aged 2 to 3 years were used. The sheep were placed in a room with natural light and ventilation 4 weeks before larval implant for acclimatisation. The sheep were kept in metabolism cages and each fed 700 g lucerne pellets and provided with 9 l of drinking water daily between 0700 and 0900 h daily.

The method of larval infestation, blood sampling, post mortem tests and symptoms of infestation have been described previously.[9-10] The mean weight of the 10 sheep at the day of larval implant was 41 ± 3 (SE) Kg. Pre-treatment physiological and blood measurements were taken from day −6 to day 0 and post-treatment data from Day 0 to Day 7. Pre-loading with Vitamin C and E, and butylated hydroxyanisole (BHA) administrations began at day −5. Intramuscular injections of Desferal (Ciba-Geigy, Sydney, Australia) and larval implants began at day 0 and continued each day until day 6.

Five of the sheep (treated group) were each injected with 1 g (4 mls of a 250 mg/ml sodium ascorbate and 5 mg/ml phenol solution) vitamin C at 0900 and 1200, 5.9 g vitamin E (6 ml 99% dl-alpha tocopherol oil) at 0900 (EVP, United Veterinary Supplies, Brisbane, Australia), by deep intramuscular gluteal injection and orally drenched with 70 mg BHA (10 ml of sunflower oil containing 3.58 g 99% BHA dissolved in 500 ml sunflower oil; Gowlings, Archifield, Qld, Australia) each day from day −5 to 6 at 0900 h. One gram Desferal was injected by deep gluteal intramuscular injection at 0800, 1300 and 2000 hr in each sheep from day 0 to 6. Controls consisted of five sheep implanted with larvae at Day 0 but not treated with antioxidants (Treatment controls). Values at Day −6 and Day −2 were taken from both groups of sheep which were not infested by *L. cuprina* (Pre-infestation controls).

Blood samples were collected from the jugular vein at days −6, −2, and Days 1

to 7 for jugular pH, ammonium ion (NH_4^+), electrolyte, glucose, creatinine and urea determinations. At Day 3, larvae were counted on each sheep for 2 hours to provide an estimate of severity of the infestation on that day. Jugular pH was determined with a blood gas analyser (IL 1312 Gilford). Serum was used to determine creatinine, urea, glucose, sodium and potassium according to the manufacturer's instructions (Gilford Autoanalyser) Jugular blood was analysed for NH_4^+ a using a kit (Ammonia Mono-test, Boehringer Mannheim). The concentration of lipid soluble ammonia (NH_3^+) was calculated by methods described previously.[16]

Water intake and urine output were determined at 0830–0900 hrs each day. The percentage changes from pre-infestation levels were calculated and analysed by the t-test paired method. The significance of the difference between values measured in treated and non-treated sheep was determined using ANOVA. Linear regression analysis was used to test the significance of the correlation between data in both groups.[17]

RESULTS

Brain Pathology, Time of Death and Number of Larvae

Two non-treated sheep died at 130 h, and 3 treated sheep died at 144, 156 and 166 h, after the first larval implants commenced. Mild status spongiosus of the white matter tracts was found in all the sheep but more severe vacuolation of the tracts was also found in 3 of the non-treated sheep. At Day 3, 312 ± 144 (SE) and 80 ± 15 (SE) larvae were counted on treated and non-treated sheep, respectively. The mean ratio of blood ammonia concentration to larvae on Day 3 was 0.005 ± 0.0009 (SE) umol/L/larvae in the treated group and 0.025 ± 0.003 (SE) umol/L/larvae in the non-treated group.

Blood Ammonia Concentrations

Mean venous ammonia concentration in non-treated sheep increased ($P < 0.01$) from 1.97 ± 0.20 (SE) umol/L at Day 2 to 6.36 ± 0.26 (SE) umol/L at Day 3, representing an increase of 230% above the control values of 1.96 ± 0.25 (SE) umol/L found at Day −6 (Figure 1). At Day 3, ammonia levels in non-treated sheep were 160% higher ($P < 0.02$) than ammonia levels in treated sheep (3.29 ± 0.54 (SE) umol/L). In fact, blood ammonia levels in treated sheep did not become elevated until Day 4.

Creatinine and Urea

In the non-treated group, creatinine levels increased 25% ($P < 0.05$) to 146 ± 8 (SE) mmol/L at Day 3, and 50% (175 ± 29 (SE) mmol/L) at Day 6 (Figure 1). In 2 of these sheep creatinine concentrations reached 233 and 267 mmol/L at day 6. Creatinine concentrations in non-treated sheep were higher ($P < 0.05$) than creatinine levels in treated sheep at Days 3 and 6. In treated sheep, mean creatinine concentrations did not rise significantly above control values at any day (Figure 1).

In non-treated sheep mean urea concentrations increased ($P < 0.01$) to 12.8 ± 2.4 (SE) mmol/L at Day 4 representing an increase of 85% above control values. Compared with values in treated sheep, urea levels were higher ($P < 0.05$) in this group at Day 4. In contrast, in treated sheep urea did not increase significantly.

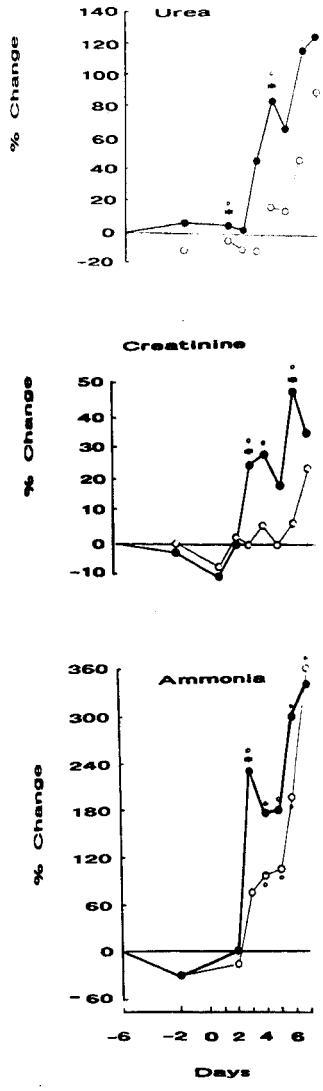

FIGURE 1 Mean percentage changes from pre-infestation (Day −6) in 10 sheep infested by *L. cuprina* larvae from day 0. Five sheep were treated with 2 g sodium ascorbate, 5.9 g dl-alpha tocopherol, 70 mg butylated hydroxyanisole and 3 g desferrioxamine mesylate daily for 6 to 11 days (0). Five sheep were infested but not-treated (●). • significantly ($P < 0.05$) different from pre-implant values, * Significant difference ($P < 0.05$) between treated and non-treated groups.

Increased ammonia levels were correlated ($P < 0.0001$) with higher creatinine and urea concentrations in the non-treated group.

Blood pH, Water Intake and Urine Output

In non-treated sheep, blood pH increased ($P < 0.001$) from 7.38 ± 0.02 (SE) in controls at Day −6 to 7.52 ± 0.02 (SE) from days 2 to 7 resulting in alkalosis (Figure 2).

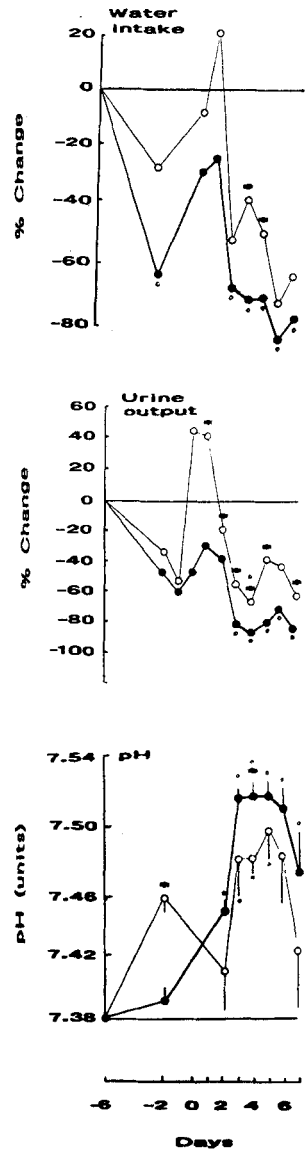

FIGURE 2 Mean percentage changes from pre-infestation (Day −6) in 10 sheep infested by *L. cuprina* larvae from day 0. Five sheep were treated with 2 g sodium ascorbate, 5.9 g dl-alpha tocopherol, 70 mg butylated hydroxyanisole and 3 g desferrioxamine mesylate daily for 6 to 11 days (0). Five sheep were infested but not-treated (●). • significantly ($P < 0.05$) different from pre-implant values, * Significant difference ($P < 0.05$) between treated and non-treated groups.

In contrast, in treated sheep, pH increased from 7.38 ± 0.01 (SE) to only 7.48 ± 0.01 (SE) from Day 3 to 5. At day 4, pH was significantly higher ($P < 0.04$) in non-treated sheep.

In non-treated sheep from Day 3 to 7, water intake declined ($P < 0.05$) between 25 ± 18 (SE) and 53 ± 19 (SE) ml/hr representing a decrease of 65 to 83% compared

with control levels of 159 ± 19 (SE) ml/hr at Day −6. Water intake was significantly ($P < 0.01$) lower in non-treated sheep at days 4 and 5. In contrast, water intake in treated sheep did not decline significantly below control values.

In non-treated sheep, urine output declined ($P < 0.01$) between 19 ± 5 and 23 ± 14 (SE) ml/hr from Day 3 to 7 representing a decline of 70 to 85% compared with control levels of 84 ± 37 (SE) ml/hr. Urine output did not change significantly from control levels (140 ± 61 (SE) ml/hr) in treated sheep except at Day 4 when it declined ($P < 0.01$) 65%.

Glucose, Sodium and Potassium

In non-treated sheep, glucose levels decreased ($P < 0.04$) 19% from 3.2 ± 0.1 (SE) mmol/L in controls to 2.7 ± 0.1 (SE) mmol/L ($P < 0.01$) at Days 4 and 5 (Figure 3). In treated sheep glucose values did not change significantly but were higher ($P < 0.02$) compared with the same values found in non-treated sheep at days −2, 2 and 5.

Compared with values found in treated sheep, sodium concentrations in non-treated sheep fluctuated between 153 ± 2 (SE) mmol/L at day 2 and hypernatraemic levels (182 ± 12 (SE) mmol/L), at Days 4 and 7. Sodium values were higher ($P <$ 0.03 to 0.05) in non-treated sheep at Days 2, 3 and 7. Potassium changes in non-treated sheep correlated with sodium levels but were higher ($P < 0.05$) compared with those values found in treated sheep at days 4 and 6 (5.2–5.9 mmol/L). Chloride concentrations fell 8–10% in both groups by Day 5–7.

DISCUSSION

The sheep were given Vitamins C and E and BHA to prevent tissue depletions and peroxidation reactions especially in lipid tissues such as the adrenals and brain.[1-3] Desferal was given mainly to prevent iron catalysed hydroxyl radical production and lipid peroxidation.[14] The results showed that there was no difference in mean NH_3^+, creatinine, urine output, sodium or potassium between treated and non-treated uninfested sheep from Day −6 to Day −2. Antioxidants therefore did not affect those values in normal sheep. However, pH and glucose values were significantly different but normal at Day −2. The injections could have activated short term pituitary adrenocortical hormone related stress effects on glucose and acid-base balance.[4]

Antioxidants appeared to increased survival time as well as reduce the severity of brain lesions in treated sheep. However, this finding may merely reflect individual responses[6-7] and should therefore be interpreted with caution.

The anti-oxidant treatment clearly prevented early rises in jugular lipid soluble ammonia. Elevated levels of ammonia in the brain originating from infested skin sites[9] may have depleted glutamic acid pools and increased the utilization of 2-ketoglutaric acid leading to a depletion of the citric acid cycle and its intermediates.[4] Repletion can only be accomplished by CO_2 fixation involving pyruvate to form oxaloacetic acid.[4] The anti-oxidant treatment may have; a) protected detoxification of ammonia in the brain; b) maintained glutamic acid and glucose levels[4] or; c) enhanced lung and renal clearance of ammonia[4].

The greater number of larvae leaving treated sheep on Day 3 was associated with lower ammonia concentrations whereas the lower number of larvae on non-treated sheep was associated with a large rise in blood ammonia. The treatment may have

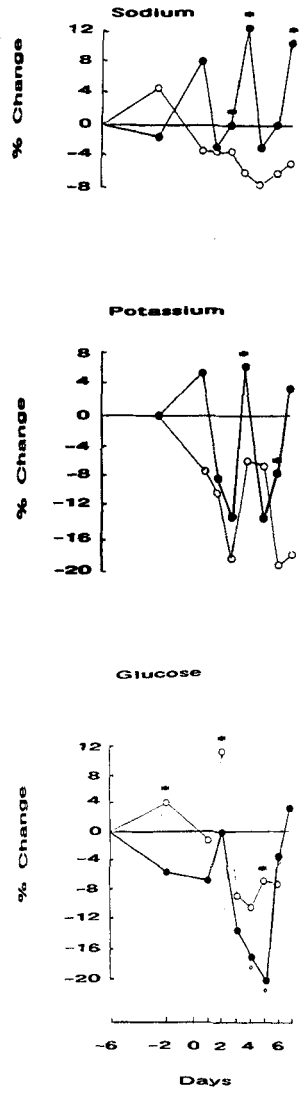

FIGURE 3 Mean percentage changes from pre-infestation (Day −6) in 10 sheep infested by *L. cuprina* larvae from day 0. Five sheep were treated with 2 g sodium ascorbate, 5.9 g dl-alpha tocopherol, 70 mg butylated hydroxyanisole and 3 g desferrioxamine mesylate daily for 6 to 11 days (0). Five sheep were infested but not-treated (●). • = significantly ($P < 0.05$) different from pre-implant values, * Significant difference ($P < 0.05$) between treated and non-treated groups.

inhibited the absorption or production of larval ammonium but this finding needs further clarification.

Antioxidants not only maintained glucose levels in treated sheep but also prevented hypoglycaemia. Glucose provides virtually the sole source of energy for brain metabolism.[4] This finding suggests that antioxidants prevented ammonia induced CNS damage by maintaining blood glucose levels.

As previously reported,[9] the concentration of ammonia in veins draining infested sites was up to 9 times greater (516 umol/L) than systemic jugular levels (58 umol/L). Early rises in peripheral ammonia from infested sites could affect CNS function.

The sharp early rises in jugular toxic ammonia produced rises in creatinine and urea levels suggesting that renal filtration was reduced by the elevated ammonia levels.[18-19] In contrast, in sheep treated with anti-oxidants, creatinine and urea levels did not rise significantly. Antioxidants therefore prevented a reduction in renal function as determined by creatinine and urea values.

Water intake and urine output were higher throughout infestation in treated sheep. Evidently treated sheep were more predisposed to drink supporting the finding that antioxidants protected brain function. Further, this may have protected vasomotor control of blood flow in the hind brain maintaining glomerular filtration.[18] Increased water intake in treated sheep prevented hypernatraemia and dehydration in the non-treated group and enhanced the renal excretion of ammonia and other toxins.[18]

Alkalosis compounded the toxicity of ammonia[16] and antioxidants prevented alkalosis. The findings suggest that antioxidants may protect buffering in hyperammonaemic sheep. The protective effect may have been mediated via brain, hepatic or renal mechanisms and requires further investigation. In conclusion, the results of this report suggest that antioxidants prevent ammonia induced CNS-renal pathobiology.

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