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EFFECT OF PARENTERAL ANTIOXIDANTS ON ADRENAL PATHOBIOLOGY AND LEUKOCYTES IN HYPERAMMONAEMIC TOXAEMIA

VINCENT H. GUERRINI

Pestsearch International Pty. Ltd., 173 Chatswood Road, Daisy Hill 4127, Queensland, Australia and Queensland Department of Primary Industries, Animal Research Institute, Yeroongpilly, Queensland, Australia.

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Infestation of sheep by *L. cuprina* larvae produces extensive skin wounds, severe dermatitis, hyperammonaemia and stress with adrenal necrosis and haemmorhage. In infested sheep, intramuscular (im) injections of Dl-Alpha tocopherol induced wool shedding and Desferrioxamine im prevented declines in white blood cells (WBC). In further trials, daily im injections of sodium ascorbate with Dl-alpha tocopherol, desferrioxamine and oral butylated-hydroxyanisole prevented adrenal damage and induced adrenocortical hypertrophy of the zona fasciculata. The treatment boosted the levels of mature and juvenile neutrophils, and blood glucose. Increases in toxic ammonia levels were correlated with increased toxic and band neutrophils, and globulin levels in treated sheep and toxic neutrophils in non-treated sheep. Decreases in serum zinc were correlated with declining lymphocytes and globulin levels. The results suggested that antioxidants protect and enhance adrenal activation in hyperammonaemic toxaemia. The changes in WBC, globulins and glucose were consistent with protected adrenocortical activation.

KEY WORDS: Antioxidants, adrenals, immunity, toxaemia.

INTRODUCTION

There is a plethora of *In Vitro* mechanistic information about antioxidants but little data on whether parenteral antioxidants have useful effects *In Vivo*. Further, in severely stressed or diseased animals, oral antioxidants may not elicit effects because the digestive tract may not absorb the level of antioxidants required in activated organs such as the adrenal cortex.[1]

Infestation of sheep by the ammonia producing larvae *L. Cuprina* could be a suitable pathobiological model for demonstrating antioxidant effects *In Vivo*.[2] The infestation is associated with extensive skin wounds, severe dermatitis, necrosis and hyperammonaemia,[2,3] a marked neutrophilia, generalized macrophage infiltration and progressive degeneration of leucocytes.[4,5] The severe inflammatory changes with activation of leucocytes in infested sheep suggest that large oxidant pools may be generated and therefore free radicals could be involved in the pathobiology.[6-7]

In sheep, toxaemia is often associated with lesions of the anterior pituitary and degeneration of the adrenal gland.[8] Large rises in serum cortisol levels are a common finding in stressed sheep.[9] Increased synthesis or release of glucocorticoids and

Address correspondence to Vincent Guerrini, Pestsearch International Pty. Ltd., 173 Chatswood Road, Daisy Hill 4127, Queensland, Australia.

catecholamines may be favorable in toxaemia because adrenal hormones have a protective and curative action.[10]

It is now generally accepted that chemical exposures, skin burns, toxaemia and even cigarette smoke generate oxidants which could deplete the brain, adrenals, lungs or testes of antioxidants.[11] The stress and hyperammonemia of infested sheep may stimulate glucocorticoid and catecholamine release which could deplete the adrenals of antioxidants.[8] In fact, adrenal ascorbate is rapidly depleted in toxaemia and a deficiency of vitamin E in the adrenal cortex is associated with a twentyfold increase in peroxidation.[12,13]

Activation of the adrenal gland in stress or disease produces rises in cortisol[14] associated with neutrophilia, lymphopenia and eosinopenia.[15] Hepatic globulin levels are boosted and gluconeogenesis increases blood glucose.[16] The concentration of zinc in leukocytes is high (3.2×10 μg/million cells) and Zinc-containing superoxide dismutase regulates superoxide production in leukocytes.[17] About 20% of the total body zinc is present in the skin. Zinc controls the release of insulin form the pancreas.[18] Zinc therefore could be involved in the inflammatory changes induced by *L cuprina* larvae on the skin of sheep.

The primary aim of this study had been to evaluate the effects of antioxidants on immunological, physiological, biochemical and histological parameters in infested sheep to determine whether free radicals contributed to its pathobiology.[19] However, in pilot studies, infested sheep given Vitamin E shed their wool, a process induced by adrenal activation; and a further aim became to evaluate the adrenal morphology. Vitamin E and ascorbate were given to prevent adrenal depletions and desferrioxamine was given to prevent metal catalized superoxide radicals in activated white blood cells and tissues subjected to free radical damage.[19] The purpose of this report is to describe the effects of (parenteral) antioxidants on adrenal pathobiology, WBC and serum zinc levels in hyperammonaemic toxaemic sheep.

MATERIALS AND METHODS

The procedures used were approved by The Animal Ethics Committee of The Animal Research Institute, Department of Primary Industries, Yeeroongpilly, Australia. The utmost care was take to avoid distress and suffering to the animals. The main objective of the principal experiment was to find means to reduce suffering and death in sheep.[2,3]

Pilot Experiments

These experiments were conducted to determine if selected antioxidants could alter physiological and blood parameters in normal or infested sheep. Dl-alphatocopherol (3.9g oil) was injected im daily 4 days before and during infestation to 6 sheep. Three sheep were also infested but not treated (infested controls) and 3 sheep were treated but not infested (treatment controls). Blood samples were taken at Day 5 of infestation from each group. Another group of 6 infested sheep were given 1.0g desferrioxamine im each day for 5 days. Controls were 6 non-treated infested sheep. Venous blood samples were taken before and during the infestations.

Principal Experiment

Animals The sheep were placed in a room with natural light and ventilation 4 weeks before larval implant for acclimatization. The animals were kept in metabolism cages and each fed 700g lucerne pellets and provided with 9L drinking water daily between 0700 and 0900 h daily. The mean weight of the 11 sheep at first larval implant was 42 + 5SD kg. The method of larval implant, blood sampling and symptoms of infestation have been described previously.[2,3,19] Larval implants began at Day 0 and continued daily to day 5. Of the 12 sheep originally used, one (not-treated) was implanted with *L. cuprina* eggs instead of larvae and therefore was not used in the analysis.

Treatments The six treated sheep were each injected im with 4 ml of a 250 mg/ml aqueous sodium ascorbate solution at 0900 and 1200 hrs, 5.9g Dl-alpha tocopherol oil (UVS, Brisbane, Australia), at 0920 hrs, and orally drenched with 70 mg Butylated-Hydroxyanisole (Gowlings, Archefield, Australia) between 0800 and 0900 each Day from Day −5 to Day 6. One gram Desferal (Desferrioxamine mesylate, Ciba-Geigi, Sydney, Australia) was injected by deep gluteal im injection at about 0900, 1200 and 1800 hrs daily from Days 0 to 6.

Controls Base line control samples were taken at Day −6 from both groups (n = 11). Treatment controls were taken at Day −2 from treated sheep (n = 6) and non-treated sheep (n = 5). Six sheep infested from Day 1 were treated with antioxidants (treated sheep) daily from Day −5 (n = 6 × 6 days). Another five sheep were infested from Day 1 but not treated (non-treated infested controls, n = 5 × 6 days).

Pathology Post mortem examinations were carried out immediately after sheep died or were euthanased at Days 6 and 7. At Post Mortem both adrenals were excised, transected laterally and examined macroscopically for congestion, haemorrhage, necrosis, atrophy or hypertrophy. The vertical width was measured by placing a graduated caliper mm metric ruler between the pelvis and shortest medial surface. Adrenal slices were preserved in 10% formaldehyde for microscopical examination.

White cell counts Blood samples were collected from the jugular vein at days −6, −2 and Days 1 to 7 for differential white cells counts using standard techniques[20] and white cells counts using an electronic cell counter.

Statistical analysis Base line data from each group was compared with treatment control and infested sheep from each group by the paired t-test. Daily data from both groups was compared by the un-paired t test. The mean width of 2 adrenal(s) measurements from each treated (n = 6) and nontreated (n = 5) animal were compared by the un-paired t test (Table 1). Linear regression was used to test the significance of the correlation between data in each group.[21]

RESULTS

Pilot experiments: Of six sheep given vitamin E, five completely shed their fleece and one had a tender fleece 21 days after the infestation. Of the three treated non-infested sheep all had tender fleeces. In the three infested non-treated sheep none of the fleeces were tender or shedding. Microscopical examination of the shed or tender fibres

TABLE 1

Width (cm) of transected adrenal glands taken at Post mortem from sheep infested for 5–7 days with *L. cuprina* larvae. Six sheep were injected im daily with 2g ascorbate, 5.9g Dl-alpha tocopherol, 3g desferrioxamine and 70 mg oral butylated hydroxyanisole for 5 days before infestation and/or 6 days during infestations. Five control sheep were infested but non-treated (NT).

	Treated					
Sheep No.	1	2	3	4	5	6
Right adrenal	2.50	3.20	3.00	3.50	4.00	3.20
Left adrenal	3.00	3.20	3.00	3.25	4.00	3.80
	Not-Treated					
Sheep No.	7	8	9	10	11*	12
Right adrenal	2.00	2.50	2.50	2.00	2.50	2.90
Left adrenal	3.50	2.45	2.50	2.80	3.25	2.90

* Sheep No. 11 was implanted with *L. Cuprina* eggs *in lieu* of larvae and was not used in the analysis.

revealed thinning or complete breaks with blunt enlarged ends and brown dark picnotic nuclei. At day 4 of the infestation, total white cell counts in Vitamin E treated-infested sheep were 4.1 + 1.33SD, in non-infested treated sheep (A), 6.6 + 1.02SD, and in infested non-treated sheep (B) 3.5 + 0.65SD 10^9/L. The B value was lower ($P < 0.05$) than the A values.

None of the infested sheep given Desferrioxamine or their infested controls shed wool. In the treated group base line white cell counts were 7.83 + 1.8(SD) and 5.48 + 1.2(SD) and 7.35 + 3.2(SD) 10^9/L after 5 and 9 days of infestation. In infested controls base line values were 6.51 + 1.8(SD) 10^9/L but declined ($P < 0.01$) to 3.86 + 0.6(SD) 10^9/L after 5 days of infestation and 5.51 + 1.0(SD) 10^9/L at day 9. The levels found at Day 5 were lower ($P < 0.005$) in the non-treated group.

Principal experiment Adrenal measurements are shown on Table 1. The mean width of the adrenal glands in treated sheep was 3.30 + 0.17(SE) cm which exceeded ($P < 0.007$) the width in non-treated (2.60 + 0.19(SE) cm) sheep by 20%. In treated sheep, eleven adrenals measured between 3.0 and 4.0 cm. In non-treated sheep, one adrenal (3.50 cm) was enlarged and nine measured between 2 and 2.9 cm.

At post-mortem the cortex in the treated group was mildly congested in 2 adrenals (2 sheep) and mildly haemmorhagic in one adrenal. The medullae were normal except in one adrenal which was infarcted and necrotic. Microscopical examination revealed hypertrophy of the zona fasciculata in the adrenals of 4 of the 6 treated sheep. In the non-treated group, the cortex was congested in 8 adrenals (4 sheep) heammorhagic in 4, (2 sheep) and necrotic in 1. The adrenal medullae appeared congested in 8 (4 sheep), haemorrhagic in 6 (3 sheep), and atrophied and liquefied in 1. Microscopical examination did not reveal adrenocortical hypertrophy in the non-treated sheep.

Mean white blood cell counts and total neutrophils are shown on Table 2. One day after the first larval implant, mean neutrophil counts increased 190% above base line values in treated sheep and 106% in non-treated animals. However, between Days 3 and 4 neutrophils had declined 27 to 47% further in the non-treated group. Glucose levels declined 7 to 12% further in the non-treated group between Days 4 and 5.

Zinc levels declined 38 to 59% in both groups from Days 2–3 to 6 but at Day 2 were 43% lower in treated sheep.

Differential white cell counts are shown in Table 3. In treated sheep, mature neutrophils increased by 186% and 50% at Days 1 and 2 and then declined 75 and 97% between Days 3 and 6. In non-treated sheep, mature neutrophils increased 127% at Day

TABLE 2
Mean + Standard error (SE) values in 6 sheep infested by *L. cuprina* larvae and treated (T) daily with antioxidants from Day −5. Five control sheep were infested but non-treated (NT). Larval implants commenced at day 0 (not shown), continued daily until Day 5.

	Controls Baseline Treatment		Infestation					
Days	−6	−2	1	2	3	4	5	6
Treatment		CEB	CEBD	CEBD	CEBD	CEBD	CEBD	CEBD
White cells (10^9/L)					*	**	**	*
T	5.45	5.58	8.22+£	5.61	3.23+£	3.38+£	3.93+	6.10
SE	0.45	0.85	0.95	0.57	0.30	0.18	0.39	1.21
NT	4.77	5.63	7.12+£	4.54	$2.44+£	$2.42+£	$2.92+£	3.68£
SE	0.35	0.17	0.55	0.70	0.20	0.17	0.23	0.53
Neutrophils (10^9/L)					**	**		
T	1.84	2.40	5.34+£	3.14+	1.18+	1.72	2.13	3.46
SE	0.22	0.41	0.92	0.47	0.10	0.22	0.35	0.98
NT	1.80	2.65	3.72+£	2.40	$0.86+£	$0.92+£	1.48£	2.10
SE	0.24	0.44	0.66	0.48	0.06	0.13	0.24	0.46
Toxic Neutrophils (0 3)					*			
T	0.00	0.00	0.00	$0.33	1.67+	2.08+	2.66+	2.91+
SE	0.00	0.00	0.00	0.19	0.46	0.24	0.09	0.07
NT	0.00	0.00	0.00	$0.60+	2.60+	2.10+	2.70+	3.00+
SE	0.00	0.00	0.00	0.21	0.19	0.08	0.11	0.00
Globulin (g/L)								
T	47.5	48.3	46.6	41.3+£	39.3+£	42.8	39.1£	44.2
SE	2.40	1.63	3.20	3.55	2.57	3.06	2.82	2.94
NT	44.2	45.2	45.2	36.8+£	$34.8+£	37.6+£	40.0+	44.4
SE	1.00	1.13	2.58	1.90	1.73	3.15	3.08	0.11
Glucose (mmol/L)		*		•		**	*	
T	3.31	3.41	3.18	3.70+	2.97	2.93+£	2.95+£	3.28
SE	0.11	0.06	0.15	0.14	0.15	0.08	0.08	0.34
NT	3.22	3.12	3.04	3.24	2.84	2.70+£	$2.56+£	3.30
SE	0.10	0.11	0.05	0.08	0.15	0.06	0.12	0.39
Zinc (μg/dl)			*					
T	72.3	68.8	$44.5+£	37.2+£	31.0+£	$26.3+£	$30.4+£	37.0+£
SE	3.64	2.21	3.27	3.55	3.71	2.91	2.46	3.37
NT	71.1	72.6	64.5	36.5+£	$29.2+£	37.1+£	32.4+£	41.9+£
SE	1.02	1.10	0.53	0.80	1.57	0.64	1.23	3.91

C = 2.0g sodium ascorbate (im), E = 5.9g Dl-alpha tocopherol (im), B = 70mg Butylated hydroxyanisole (oral), D = 3g Desferrioxamine (im), + = Significantly ($P < 0.05$) different from base line values, £ = Significantly ($P < 0.05$) different from treatment controls, * = Significance ($P < 0.05$ ** 0.01) of difference between treated (T) and non-treated (NT) groups, $ = Abnormal values[8]

1 but declined 90 and 99% between Days 3 and 6. In the treated animals, the levels of mature neutrophils were 45 to 88% higher between Days 3 and 6.

Inmature (band) neutrophils appeared 1 to 2 days after the first implant in both groups but increased 85 to 100% further in treated sheep between days 4 and 6 (Table 3). In contrast, toxic neutrophils appeared 2 to 3 days after the first implant in both groups but increased a further 50 to 73% in the non-treated animals (Table 2). In treated sheep, one day after the first implant, lymphocytes declined 22% and eosinophils 55%. In both groups lymphocytes declined 29 to 53% between Days 2 and 5. Monocytes increased 1 to 1.5 times in treated sheep at Days 2 and 3 but thereafter decreased 55 to 140% in both groups.

TABLE 3

Mean + Standard error (SE) differential white blood cell counts, in 6 sheep infested by *L. cuprina* larvae and treated (T) daily with antioxidants from Day −5. Five control sheep were infested but non-treated (NT). Larval implants commenced on Day 0 (not shown) continued daily until Day 5.

	Controls Baseline Treatment		Infestation					
Day	−6	−2	1	2	3	4	5	6
Treatment		CEB	CEBD	CEBD	CEBD	CEBD	CEBD	CEBD
Mature neutrophils (10^9/L)					*	*		**
T	1.84	2.40	5.26+£	2.76+	0.46+£	$0.09+£	$0.05+£	0.26
SE	0.21	0.49	0.99	0.57	0.14	0.04	0.02	0.07
NT	1.77	2.23	4.02+£	1.99	$0.18+£	$0.05+£	$0.06+£	$0.03+£
SE	0.24	0.23	0.43	0.42	0.06	0.03	0.04	0.02
Inmature neutrophils (10^9/L)						**	**	*
T	0.00	0.00	0.07	0.37+£	0.78+£	$1.61+£	$2.08+£	$3.26+£
SE	0.00	0.00	0.03	0.11	0.08	0.26	0.38	1.14
NT	0.01	0.01	0.02	0.40+£	0.68+£	0.87+£	$1.43+£	$1.59+£
SE	0.01	0.01	0.01	0.11	0.10	0.12	0.20	0.48
Lymphocytes (10^9/L)			*					
T	3.27	2.92	2.56+	2.31+	$1.77+£	$1.55+£	1.96+	2.16
SE	0.25	0.53	0.19	0.18	0.20	0.12	0.30	0.23
NT	2.65	2.65	2.85	1.86+£	$1.40+£	$1.44+£	$1.41+£	1.56+£
SE	0.12	0.17	0.16	0.24	0.21	0.13	0.21	0.14
Monocytes (10^9/L)					*			
T	0.09	0.07	0.11	0.23+£	0.18+	0.04+	0.03+£	0.04+£
SE	0.02	0.02	0.02	0.03	0.06	0.01	0.01	0.02
NT	0.14	0.17	0.11	0.25	0.08£	0.03+£	0.01+£	0.01+£
SE	0.02	0.06	0.02	0.06	0.02	0.01	0.01	0.01
Eosinophils (10^9/L)					*			
T	0.20	0.25	0.11+	0.02+	0.02+	0.02+	0.02+	0.03+
SE	0.05	0.09	0.05	0.01	0.01	0.01	0.01	0.02
NT	0.13	0.15	0.13	0.02+£	0.00+£	0.01+£	0.01+	0.03+
SE	0.04	0.03	0.05	0.01	0.00	0.01	0.00	0.01

C = 2.0g sodium ascorbate (im), E = 5.9g Dl-alpha tocopherol (im), B = 70mg Butylated hydroxyanisole (oral), D = 3g Desferrioxamine (im), + = Significantly ($P < 0.05$) different from base line values, £ = Significantly ($P < 0.05$) different from treatment controls, * = Significance ($P < 0.05$ ** 0.01) of difference between treated (T) and non-treated (NT) groups, $ = Abnormal values[8]

There were no significant differences between base line data (Tables 2–3) from treated and non-treated groups. However, glucose levels were 9% higher at Day −2 (treatment controls) in treated sheep (Table 2). A highly positive correlation ($r = 0.72$ to 0.98 for $n = 106$ pairs) was found between serum zinc with lymphocytes and serum globulin levels ($P < 0.0001$). In treated sheep, increases in toxic ammonia were highly correlated ($r = 0.98$; $P < 0.0001$) with increased levels of inmature and toxic neutrophils. In non-treated sheep the rises in ammonia were correlated ($r = 0.94$ $P < 0.0001$) with rises in toxic neutrophils but not with the rises in band neutrophils.

DISCUSSION

The results suggest that antioxidant(s) may protect and enhance adrenal activation in

severely hyperammonaemic toxaemic sheep. The shedding of wool[22] together with adrenal enlargement, hypertrophy of the zona fasciculata[23] and adrenal pathobiology provide the physical evidence to support the finding. The changes in white blood cells[17] and glucose levels[18] provide indirect evidence of enhanced adrenocortical activation. Previously published data[19] further supports the findings. With the exception of one sheep which died with meningitis at 96 h, the survival time in treated sheep was extended by 14 to 26 h compared with the non-treated sheep.

Necrosis and haemorrhage of the adrenal cortex and in particular the medulla occurs in toxaemic sheep.[8] The adrenal lesions found mostly in the non-treated group were consistent with previous findings in toxaemic ruminants.[8,24] Even non-toxic infusions of ammonia in sheep are known to activate the adrenal glands.[25] In the present experiment toxic ammonia levels rose up to 8 times above normal and under these circumstances the adrenals could have been rapidly depleted of antioxidants through increased synthesis of glucocorticoids and catecholamines.[26,27] Increased hormone synthesis requires molecular oxygen and NADPH, reactants which produce toxic superoxides and peroxides in the presence of free iron or copper.[27] Although free radicals were not measured, the effects of the antioxidants suggest that free radicals could have contributed to the adrenal pathobiology. The treatments may have partly repleted antioxidants depleted by oxidants.[11,13]

The results suggest that daily injections of alpha tocopherol activated the adrenal cortex even in non-infested sheep. In stress, wool shedding is induced by cortisol[28] and non-infested treated sheep had tender fibres. Large rises in cortisol may have inhibited mitosis in follicular cells.[28]

Daily alpha-tocopherol or desferrioxamine injections boosted neutrophils in infested sheep but did not produce significant white blood cell changes in normal sheep. The rise in mature neutrophils after implant in both groups was probably stimulated by toxic ammonia,[25] skin and larval toxin[4] or adrenal activation. Significantly, the decline in the levels of mature neutrophils was significantly less, and the rise in new neutrophils occurred sooner and was significantly greater in sheep given antioxidants. The antioxidant treatment may have; directly: a) inhibited dermal inflammation and reduced migration of neutrophils to the site of infestation; b) Prevented free radical damage in mature neutrophils; c) enhanced the production of young neutrophils or indirectly; d) activated the adrenal gland. Alternatively, higher rises in toxic ammonia in non-treated sheep may have destroyed more mature cells.[29] Rises in ammonia were highly correlated with increased inmature and toxic neutrophils. However, in non-treated sheep, the rises were not correlated with increased band neutrophils levels. This finding suggests that the neutrophil response to ammonia was more efficient in the treated group.

Zinc levels dropped well below normal values in both groups and were highly correlated ($P < 0.0001$ $n = 53$ pairs) with the declines in lymphocytes and globulin. However, losses in zinc from infested skin may have merely coincided with the changes in lymphocytes and globulins. Further research is needed to determine why there were large changes in zinc levels in the present experiment.

The results of the present experiments should be interpreted with caution; adrenal activation should be confirmed by cortisol and catecholamine assays.[30] Free radicals in the adrenals should be measured. In summary, the results suggest that parenteral antioxidants may have significant effects on the adrenal gland (Vitamin E) and immunity (Desferrioxamine/Vitamin E) in toxaemia and/or stress.

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