The clinical effects of dimethyl sulfoxide in sheep suffering from experimentally induced heartwater.

by

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To

my wife Kim Tutt

and my parents Leslie and Rona Tutt

Thanks be to God

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SUMMARY

The objective of this research project was to evaluate the clinical effect of Dimethyl sulphoxide in the symptomatic treatment of sheep suffering from heartwater caused by *Ehrlichia ruminantium* (formerly *Cowdria ruminantium*). 32 Merino crossbred sheep were used of which, 16 were infected with heartwater and 16 were control animals. Of the 16 sheep infected with heartwater, 8 were treated with a 10% solution of DMSO in polyionic fluid at the dose rate of 1g/kg twice daily for three consecutive days. Treatment was initiated two days after the onset of clinical disease. Eight of the control sheep were treated with DMSO following the same protocol. The remaining 8 infected and 8 not infected sheep were given similar volumes of polyionic fluid as placebo treatment. Arterial and venous blood samples for blood gas, haematocrit and total plasma protein measurement were collected daily from 5 days before, to 7 days following the onset of clinical disease. Gross pathological findings and cytological confirmation of the disease were recorded for the 16 infected sheep.

The infected sheep treated with DMSO were able to maintain pulmonary gas exchange, had reduced pleural effusion and plasma protein loss compared to the untreated infected sheep that became hypoxic, developed severe pleural effusion and plasma protein loss. However, the infected sheep treated with DMSO developed a mild uncompensated metabolic acidosis. Non-infected sheep treated with DMSO showed reduced appetite while non-infected untreated sheep remained normal.

The reduction in pleural effusion, maintenance of gaseous exchange and plasma protein levels, as a result of the use of DMSO in the symptomatic treatment of sheep suffering from heartwater, are considered beneficial.

CHAPTER ONE

INTRODUCTION

Heartwater is a tick-borne[1] rickettsial[2] disease of livestock, which, despite prophylactic immunization programmes and specific therapeutic treatment, results in severe stock losses amongst susceptible livestock in sub-Saharan Africa[3]. Mortalities due to heartwater are three times greater than those attributed to anaplasmosis and babesiosis in endemic areas[4]. The disease may present in peracute, acute, subacute or clinically unapparent forms[4,5]. There are numerous strains of *Ehrlichia ruminantium* (formerly *Cowdria ruminantium*), most of which differ in their pathogenicity, but cross-immunity between them exists[6].

Amblyomma hebraeum is the primary vector responsible for the transmission of *Ehrlichia ruminantium* in South Africa[1]. This tick has a widespread distribution[7], which makes its eradication a difficult task. *Amblyomma variegatum*, an important vector in the transmission of heartwater, has a widespread distribution within and outside of Africa[8], while *Amblyomma maculatum*, *A. cajennense* and *A. dissimile* (a reptilian tick) are American *Amblyomma* ticks able to transmit the disease experimentally[9 10 11]. *Amblyomma maculatum* has been shown to have acquisition and transmission abilities similar to those of *A. variegatum*, highlighting the importance of this tick as a potential vector in the spread of heartwater on the mainland of the United Sates of America[12]. The introduction of *Amblyomma hebraeum* and *Amblyomma variegatum*, both efficient vectors of *Ehrlichia ruminantium*[8], to the American mainland, either via migrating cattle egrets or the importation of infested zoo animals, pose a real threat to the naïve, susceptible white-tailed deer and the domestic livestock populations[13]. Eradication of the disease from North America, should it become established there, would be very difficult considering the subclinical nature of the disease in some animals and also the increase in wildlife ranching that results in direct contact between domestic livestock and the ranched deer that are translocated between properties[13].

Attempts at strategic control of the vectors[14], breeding of resistant animals[15], routine immunisation of young susceptible stock and the specific and supportive treatment of diseased animals remain the only options when farming livestock in endemic and unstable areas.

Although work has been published on the specific treatment of heartwater, as reviewed by van Amstel and Oberem (1987)[16], very little is known about the symptomatic treatment of the disease. The aim of this trial was to evaluate the clinical effect of dimethyl sulfoxide in the treatment of sheep suffering from heartwater.

CHAPTER TWO

LITERATURE REVIEW

2.1 Clinical signs

Clinical heartwater, an often fatal disease, is characterised by: pyrexia (rectal temperature exceeding 40 ^{o}C), listlessness and depression, inappetence (complete inappetence occurs from the third day after the body temperature exceeds 40 ^{o}C[17]) and fore stomach atony, hyperaesthesia (with a severe blinking response to mild stimulation of the head), a high-stepping gait, ataxia, lateral recumbency and paddling movements of all four limbs agonally. Many animals display licking of the lips and continuous chewing movements without rumination, prehension or ingestion of food. Animals may experience convulsions and die shortly thereafter. Nystagmus is a common sign. When in lateral recumbency, many animals display opisthotonus and extension or paddling motions of all four limbs[5]. Foam (as a result of lung oedema) may flow from the nostrils agonally. In severe cases, increased lung sounds and expiratory wheezes can be auscultated bilaterally over the lung fields. Hyperpnoea, a common clinical finding, may be as a result of the pyrexia[17], while dyspnoea is probably due to the formation of lung oedema[5]. Petechiation of the vulva mucosa and conjunctivae[18] is a common finding as well as chemosis of the conjunctivae (personal communication, Van Amstel, Dept Medicine, Faculty of Veterinary Science, University of Pretoria, 1995). Some animals may present with diarrhoea, a clinical sign considered by some authors to be consistent in cattle suffering from heartwater[5,19,20], while Van de Pypenkamp (1987) considered this an inconsistent clinical finding[18]. Head pressing may be associated with ataxia and hypermetria[18]. Sheep usually succumb to the acute form of heartwater, with the majority of animals showing milder central nervous signs than those observed in cattle. Other signs in sheep include a progressive unsteady gait, a wide-based stance, head held low, drooping ears and listlessness. These animals eventually go into lateral recumbency, show continuous galloping motions, chewing movements, continuous licking of the lips and nystagmus, and die shortly thereafter[5].

Morbidity and mortality rates are influenced by: species, age, breed and immune status of the animal, virulence of the heartwater strain[5], season, tick control programmes on the specific farm and the specific and supportive therapeutic regimen used[21]. Inter-species susceptibility varies widely as does the

susceptibility within species. In a large field trial in which 2743 cattle of various breeds were immunised against heartwater, breed, sex and age of the animals affected the severity of the reaction[22]. Persian sheep and Africander cattle (*Bos indicus*) appear to be more resistant than Merino sheep and imported cattle[5]. Mortality figures cited by early investigators revealed that about 60% of susceptible cattle and up to 95% of susceptible sheep introduced to an endemic area succumbed to the disease[5,23].

2.2 Pathophysiology and Pathogenesis

The pathogenesis of heartwater is poorly understood[24] and hypotheses on this topic are often contradictory[21]. It is agreed that the effusion of fluid into the body cavities is as a result of increased permeability of the smaller blood vessels[17,25-27]. This results in mild to severe hydrothorax, hydropericardium and ascites[28]. The causal organism has an affinity for the endothelial cells of different tissues in different species. In the goat[29], sheep and cattle the predilection site is the endothelial cell of the microvasculature of the brain, while in mice the predilection site is the lung microvasculature endothelial cells[30]. In mice there is no correlation between the extent of the pathology within the lungs and the number of *Ehrlichia ruminantium* colonies present within the endothelial cells[30,31]. In sheep and goats there appears to be no correlation between the mild morphologic changes to the alveolar walls and the severe lung oedema seen. Light microscopy revealed that the intracytoplasmic growth of *Ehrlichia ruminantium* has little visible deleterious effect on the parasitized cell[32]. Occasionally, however, cytopathic changes were observed in parasitized and unparasitized cells[30,32,33]. This would suggest that either the parasites have vacated the damaged cells, or damage to unparasitized cells may be due to the body's inflammatory or immune response to parasitization of adjoining cells[33]. Electron microscopy of endothelial cells of sheep suffering from heartwater revealed that the parasite was contained within a vacuole within the cytoplasm. Although severe distension of the cytoplasm was often seen, signs of cell injury were rarely noted. More commonly, endothelial cells that did not contain parasites showed marked signs of cell injury. These cells were swollen and contained cytoplasm of decreased density and appeared to be devoid of normal organelles. It would appear therefore, that the damage to the endothelial cells resulting in increased permeability might be due to immune mediated responses[33]. The mechanism

involved in the effusion of a modified transudate (a protein rich, low cellular transudate[34]) into the body cavities remains unexplained. The fact that the plasma protein concentration remained the same even though there was a marked decrease in plasma volume, lead some investigators to believe that the plasma proteins leave the vascular system at the same rate as the fluid component[17]. This was also supported by the fact that the effusions coagulated on exposure to air, an indication that the large fibrinogen molecule (molecular mass = 450 000 Dalton) also passed through the endothelial membrane[17]. It has since been shown that there is a marked decrease in the plasma protein concentration agonally[35]. The increase in vascular permeability has been ascribed to a toxin[4,28,31,33,36]. The presence of endotoxin in sheep and calves suffering from experimentally induced heartwater has been demonstrated but it was concluded that this endotoxin was unlikely to be the main cause of the severe increase in vascular permeability. This conclusion was reached when an endotoxin peak was measured in only three of seven animals in a trial, but all of the animals displayed similar volumes of effusion within their body cavities at necropsy examination[37,38]. It has also been hypothesised that vaso-active substances released by degranulating mast cells may cause the increase in vascular permeability[25]. This was, however, not substantiated in a trial in which mice were treated using histamine and serotonin antagonists and mast cell stabilisers (to prevent mast cell degranulation) prior to them being infected with the Kumm strain of *Ehrlichia ruminantium*[24]. The role of complement in the pathogenesis of heartwater was also investigated and it was found that calves that had higher levels of circulating complement prior to infection with heartwater, displayed less severe reactions. Du Plessis *et al* (1987), quoting Roit (1977), stated that complement had been shown to have bactericidal effects *in vitro* and concluded that this may be the reason for milder clinical reactions in calves with higher levels of circulating complement at the time of infection[24]. During the course of the following three weeks, these animals showed very little fluctuation in complement levels whereas the animals that had had severe reactions showed a marked increase in complement during that period. It therefore appears that the complement system is activated in animals infected with heartwater[24]. In most of the affected animals a substantial rise in immunoconglutinin (auto-antibodies to complement fixed to immune-complexes) occurred during the progression of the disease. This indicates the formation and presence of immune-complexes suggesting the possible presence of a Type III hypersensitivity reaction[24].

Laboratory evidence of the development of disseminated intra-vascular coagulation (DIC) in sheep suffering from experimentally induced heartwater has been demonstrated[35]. Evidence for this was provided by prolonged prothrombin time and partial thromboplastin time and a thrombocytopaenia. Macropathological changes in cadavers of animals that had succumbed to heartwater, that can be ascribed to DIC include: lung oedema and petechial haemorrhages in the conjunctivae, lymph nodes, heart, intestine and rumen, the central nervous system, and vagina and urinary bladder[35].

The main functional disturbance in the terminal stages of heartwater, resulting in death, is sympatholysis, evidenced by peripheral vasodilatation, increased capillary permeability and a drastic reduction in blood volume, which precipitates general circulatory failure. Increased capillary permeability leads to loss of plasma proteins from the vascular system resulting in decreased plasma volume[17].

With regards to central nervous system pathology, once again there are conflicting reports. In a study on the pathogenesis of heartwater in goats, the investigators reported that there was no evidence of inflammatory changes in brain or spinal cord sections[29]. A fairly widespread and prominent meningo-encephalitis has, however, been described in cattle that have succumbed to the disease[31].
The central nervous signs often seen during the acute and agonal stages of the disease have been ascribed to brain oedema and increases in cerebrospinal fluid pressure[5,18,29,39]. Electro-encephalography of cattle suffering from heartwater revealed the presence of a high voltage theta or delta rhythm, which was suggestive of diffuse brain involvement. This was considered to be associated with the presence of cerebral congestion and oedema[40].

During most inflammatory reactions, the three major changes in the capillary bed and post-capillary venules consist of increased vascular permeability, enhanced leukocyte adherence to the endothelial cells and promotion of intra-vascular coagulation. Activated neutrophils and monocytes in close association with activated endothelium may lead to leukocyte-dependent endothelial cell damage. In a rat lung injury model, the leukocyte-mediated damage appears to be dependent on tumour necrosis factor, platelet activating factor and an intact complement system. Toxic oxygen metabolites, hydrogen peroxide,

phospholipase products, proteases and a xanthine dehydrogenase "converting factor", all released from neutrophils, have been found to be mediators of endothelial cell damage[41].

2.3 Clinical Pathology

2.3.1 Haematology

Van Amstel *et al* (1987) reviewed the literature concerning the haematology of animals suffering from heartwater[42]. Except for Graf (1933) (as reported by Van Amstel and others 1987), other researchers concurred that there was a progressive drop in haemoglobin during the course of the disease[42]. A decrease in haematocrit was found in all studies while mean corpuscular haemoglobin content (MCHC) and mean corpuscular volume (MCV) were found to be variable. During the acute phase of the disease, neutropaenia, eosinopaenia and lymphocytosis are the most marked and consistent changes seen in the haemogram associated with heartwater. Both neutrophils and eosinophils are granulocytes formed in the bone marrow and the above finding may be evidence of bone marrow suppression[42] Fred = how does this relate to Steck (28)?.

2.3.2 Blood biochemistry and blood-gas status

A terminal glycosuria reported by Clark (1962)[17] was not confirmed by Van Amstel *et al* (1988)[43]. A progressive decline in total serum calcium during the acute phase of the disease has been demonstrated[17,44]. This decline mirrored the drop in serum albumin. Ionised calcium, however, peaked during the acute phase of the disease and then declined terminally[35]. Calcium exists in the bound (to albumen) and unbound form (ionised calcium) in the blood. When albumin decreases, the amount of bound calcium also decreases while the ionised calcium levels increase proportionately. During inflammation the rolling of leukocytes along the inflamed venule wall precedes adhesion[45]. Intracellular free calcium concentration increases in these cells prior to adhesion[45].

In contrast to the finding of a respiratory acidosis by Owen *et al* (1973)[26], Van Amstel *et al* (1994) found a marked decline in the partial pressure of CO_2 in arterial blood ($PaCO_2$) with a corresponding respiratory alkalosis that persisted until death[46]. It should be pointed out that the respiratory acidosis found by Owen *et al* (1973) was only present on one day (about two days after the onset of the febrile reaction). The $PaCO_2$, pH and respiratory rate had all returned to within normal limits the following day and remained as such until death[26]. A decrease in the partial pressure of O_2 in arterial blood (PaO_2) during the acute phase of the disease that persisted until death was demonstrated by Owen *et al* (1973) and Van Amstel *et al* (1994)[26,46]. This is in contrast to the work reported in calves where variable results on $PaCO_2$ and PaO_2 levels were found[44]. Van Amstel *et al* (1994) demonstrated a precipitous decline in total serum protein due to a drop in both albumin and globulin during the acute phase of the disease. Plasma fibrinogen levels, however, rose during the same period[35]. The drop in albumen would result in decreased intravascular oncotic pressure leading to hypovolaemia. Owen *et al* (1973) demonstrated a marked drop in the systolic and diastolic blood pressures of sheep in the acute and agonal stages of the disease[26].

2.4 Pathology

2.4.1 Macro pathology

A tentative diagnosis of heartwater can often be made on clinical signs and macroscopic pathology noted during necropsy examination, but a definitive diagnosis requires demonstration of the *Ehrlichia ruminantium* colonies or morulae within the cytoplasm of endothelial cells in stained brain squash smears[2,47]. Immunohistochemical staining of formalin fixed tissues has been shown to be an accurate means of demonstrating *Ehrlichia ruminantium* and can be used in cases where demonstration of the organism in brain squash smears has not been possible[48].

As the name suggests, animals that succumb to heartwater inevitably have mild to severe pericardial effusion[5]. Owen *et al* (1973) suggested that there were two distinct forms of the disease present in the sheep in their trial. Two of the four sheep had copious amounts of fluid within the pericardial sack while the other two sheep had a large volume (about 775ml) of fluid in the pleural cavity with only a few millilitres of fluid in their pericardial sacks[26]. Lung oedema characterised by widening of the interlobar septae, free foam in the trachea and on cut surface of the lung as well as hydrothorax, mesenteric oedema and ascites are common findings[5]. Body cavity effusions are usually straw coloured and exposure thereof to air results in coagulation of fibrin[28]. The volumes of fluid within the pleural cavity measured at necropsy examination by Steck (1928) ranged from several litres in cattle, to about half a litre in sheep and about 20 ml in goats[28]. Steck (1928) also pointed out that about half of the goats and two thirds of the cattle and sheep that died from heartwater had increased volumes of fluid within their body cavities[28]. Oedema and petechiation of the superficial lymph nodes and abomasal mucosa are common findings, while catarrhal enteritis is present in a small percentage of domestic ruminants[27]. Splenomegaly is present in the majority of animals[27,28].

2.4.2 Histopathology

Steck (1928) described the presence of perivascular infiltration of cells – mainly plasma cells associated with the microvasculature of the kidneys, liver and lungs. Intralobar hepatic capillary vessels were found to contain increased numbers of lymphocytes, neutrophils, macrophages and to a lesser extent plasma cells and eosinophils[28]. Steck (1928) also suggested that the perivascular cellular infiltration may arise as a result of a toxic ("noxe") assault[28]. In the lung, the most marked changes seen were alveolar and interstitial oedema and a leukostasis characterised by macrophages, neutrophils, lymphocytes and to a lesser extent eosinophils[28]. Brown and Skowronek (1990) described a mononuclear cell infiltrate within the widened interstitial septa of the lung[29]. Prozesky and Du Plessis (1985) demonstrated interstitial pulmonary oedema and scattered mononuclear cellular infiltrates. The alveolar spaces were found to be filled with serum while some contained considerable amounts of fibrin[30]. Cowdry (1925) and Steck (1928) demonstrated the presence of *Ehrlichia ruminantium* organisms within the endothelial cells of renal capillaries[2,28]. Leukostasis was also present in these vessels and consisted of macrophages, lymphocytes and neutrophils. An interstitial nephritis was also described but this was not attributable to a local reaction as a result of parasitized cells[28]. Perivascular cellular infiltrates consisting mainly of macrophages, lymphocytes, and plasma cells and to a lesser extent neutrophils were found in the cardiac muscle of some animals. Leukostasis was also demonstrated in the central nervous system. In some instances where severe parasitism occurred there was no evidence of local damage. Steck (1928) summarised his work by stating that the characteristic changes in heartwater are leukostasis and a perivascular "cellularity" consisting mainly of macrophages and to a lesser extent lymphocytes and neutrophils. These changes were most likely attributable to a toxin[28].

Histologically the following has been found on brain sections: micocavitation, swollen axis-cylinders and necrosis of the granular layer of the cerebellar cortex. A Periodic Acid-Schiff stain positive granular substance, proportional in amount to the severity of the oedema, was observed within the cells, perivascular spaces and the brain substance[31].

2.5 Approaches To Treatment

2.5.1 Specific treatment

Van Amstel and Oberem (1987) reviewed the available literature addressing the treatment of heartwater[16]. Drugs, which have been used in the specific treatment of heartwater, include: aldehydes, antibiotics, antimicrobials, heavy metals, biological products (e.g. hyperimmune serum) and antiviral drugs[16]. Tetracyclines (chlortetracycline) was shown to be very effective in the specific treatment of heartwater by Weiss *et al* (1952)[49]. Doxycycline, a semi-synthetic derivative of oxytetracycline that is more lipid soluble, penetrates body fluids including cerebrospinal fluid better than oxytetracycline[50]. Doxycycline, injected at 2mg/kg body mass, was found to be as effective a treatment for heartwater as was oxytetracycline[51].

2.5.2 Symptomatic treatment

Numerous drugs have been used in the symptomatic treatment of animals suffering from clinical heartwater[16]. Clark (1962) recommended the use of sympathomimetics to overcome the sympatholysis that he suggested is caused by the disease[17]. In a trial reported by Gummow *et al* (1988), it was found that 40% of mice suffering from experimentally induced heartwater survived after being treated with betamethazone while 56% of infected mice survived the disease after being treated with Dimethyl sulfoxide (DMSO). Only 10% of the untreated control mice survived[52]. This would suggest that inflammatory mediators are implicated in the pathogenesis of heartwater and that anti-inflammatory drugs are indicated in the symptomatic treatment of this disease.

DMSO is an aprotic (unable to donate its protons in chemical reactions) solvent that readily penetrates intact skin, crosses biological membranes and acts as a carrier of its solutes[53]. The latter is best demonstrated by the combination of DMSO and corticosteroids. DMSO has a strong affinity for water (is hygroscopic) and undergoes an exothermic reaction when the two are mixed. This substance or its metabolites occur in fresh water, rain and the oceans. Dimethyl sulphone occurs in bovine blood and contributes to the flavour of milk[53]. DMSO is a hydroxyl radical scavenger while its metabolite, dimethyl sulphide, traps free oxygen radicals. Free oxygen radicals are known to cause damage to endothelial cells

resulting in increased vascular permeability. DMSO, by its radical scavenging effect, prevents damage to the endothelium and aids in membrane stabilisation[53].

DMSO is a potent osmotic diuretic that is rapidly excreted by the kidney and, due to its hygroscopic nature, draws water into the urine. Diuresis is evident following oral, parenteral and topical administration[54]. DMSO has also been shown to have positive inotropic and chronotropic effects[55].

Sloughing of skin, in areas where inadvertent peri-vascular injection of irritant substances has occurred, can be prevented by the topical application of DMSO[56,57]. DMSO will also reduce pain and swelling associated with peri-vascular injection of irritant substances[57].

In the treatment of acute central nervous system trauma (resulting in inflammation, oedema and ischaemia), DMSO, when administered intravenously, resulted in improved neurological status and increased survival rates[53]. Intra-cranial pressure is decreased, cerebral blood flow is improved and tissue damage minimised in a number of species including man, horses, dogs and laboratory animals[54]. It has been shown that xylazine-induced pulmonary oedema and acute lung injury may be prevented by pre-treatment with DMSO[58]. Kimura *et al* (1988) showed that the lung lymph flow in sheep suffering from smoke-induced pulmonary injury was markedly reduced in the sheep treated with DMSO in combination with heparin. The reduction in lung lymph flow was, however, not statistically lower than in those sheep treated with heparin alone[59].

DMSO has anticholinesterase activity as well as anticoagulation effects due to inhibition of platelet aggregation[53,60]. This drug should, therefore, not be used in conjunction with other cholinesterase inhibitors (for example organophosphates and diminazene aceturate).

DMSO has been found to be teratogenic in chickens and mammals[53]. However, there are conflicting reports in the literature[61] with DMSO sometimes used as a protectant against known teratogens[62]. DMSO is used as a cryoprotectant in the cryopreservation of rabbit embryos[63], bovine oocytes[64], and semen and also the production of heartwater vaccine[65,66]. Ashwood-Smith (1985) has reviewed some of the literature

pertaining to the use of DMSO in cryopreservation. It was concluded that DMSO, because of its gene activation properties, should be used with caution in the cryopreservation of human embryos[67].

DMSO has been reported by Appell *et al* (1992) to cause intravascular haemolysis after rapid intravenous injection of high (40% solution) concentrations[68]. It should, however, be pointed out that the dose rate and concentration of DMSO used by Appell *et al* exceeds that which is recommended[50,56].

On the grounds of macropathology , seen at necropsy of animals that had succumbed to heartwater, diuretics were considered necessary in an attempt to reduce the oedema[16]. Furosemide, a loop diuretic[50], has been used in clinical cases of heartwater, although its effect has not been evaluated[16]. Shakespeare *et al* (1998) showed that although furosemide is an effective diuretic in sheep it causes major disruption to the electrolyte status of healthy sheep and they concluded that its use in sheep suffering from clinical heartwater may be contraindicated as it would exacerbate the electrolyte imbalances in these animals[69].

The work by Gummow et al, (1986), using a mouse model[52], appeared to hold great promise but the applicability of this treatment to ruminants had not been established. Consequently, this trial was designed to assess the effectiveness of DMSO in the symptomatic treatment of heartwater in sheep.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Model system and justification of the model

Although 56% of mice suffering from experimentally induced heartwater survived after being treated with DMSO[52], direct extrapolation to ruminant production animals is inappropriate and hence the necessity to confirm this work in sheep.

The model system chosen was a group of, on average, one year old Merino-type, heartwater-susceptible sheep. Sheep were chosen as the model rather than cattle because of the high cost of the latter. The sheep were sourced from the eastern Free State Province of the Republic of South Africa, an area known to be free of *Amblyomma hebraeum*, the South African tick vector of *Ehrlichia ruminantium*[7]. This ensured that the sheep would be heartwater antibody free and therefore susceptible to the disease. These sheep were not tested for heartwater antibody titres because it had been found that animals tested from similar areas, known to be free of heartwater and its vectors, were found to have positive *Ehrlichia* titres. It was therefore decided that the false positive results associated with this test did not warrant it being performed.

3.2 Experimental design

3.2.1 Treatment groups:

Group 1 infected with heartwater, not-treated with DMSO (positive control)

Group 2 infected with heartwater, treated with DMSO

Group 3 not-infected with heartwater, treated with DMSO

Group 4 not-infected with heartwater, not-treated with DMSO (negative control)

The calculation of the number of replicates in each treatment group was based on the variation in the partial pressure of oxygen (PaO_2) in the arterial blood of sheep suffering from experimentally induced heartwater, as reported by van Amstel *et al* 1994[46].

Using these data the number of replicates was calculated as follows: The variance (S^2) was 175. Applied to the formula; ($n=4S^2/L^2$)[70] where L=10, the number of replicates required was 700/100=7 sheep. The detection limit, L, is the magnitude of difference between sample means considered clinically significant. In this case an error to the magnitude of 10mm mercury (Hg) from the mean PaO_2 was considered tolerable.

The minimum number of replicates as calculated above was 7 sheep, which meant that 28 sheep (4 treatment groups X 7 replicates) would be required for the trial. The research facility could however only house a maximum of 18 sheep at one time and, therefore, on the advice of the project statistician (Prof H. Groeneveld, Department of Statistics, University of Pretoria, 1995 personal communication) the trial was divided into two blocks of 16 sheep each, which resulted in the number of replicates being increased to 8 and the total to 32 sheep.

3.2.2 Randomisation

On arrival at the research facility, the ewes and wethers were numbered separately as they came off of the truck for identification and randomisation purposes. The animals were then each randomly allocated numbers and on the basis of these, randomly allocated to the two blocks and subsequently to the four treatment groups. Each animal was then randomly allocated to a specific research pen due to the fact that some of the pens were north-facing, which may have made them warmer than those facing south. All randomisation performed was based on random number tables.

The sheep were ear tagged using coloured tags based on their group and the individual sheep were numbered consecutively from 1 to 32.

3.2.3 Immunization

All sheep were vaccinated against enterotoxaemia using the alum precipitated enterotoxaemia vaccine 25 days prior to the beginning of the trial (Onderstepoort Biological Products Factory).

3.2.4 Anthelmintics

All sheep were dosed with Ranide Super[a] (Closantel®) at the recommended dose of 1 ml/10 kg live mass 19 days prior to the commencement of the trial.

3.2.5 Shearing

All sheep were shorn 20 days prior to the trial to facilitate auscultation of the heart, lungs and rumen and facilitate location of the blood vessels used for sample collection.

3.2.6 Housing

Whilst the first 16 sheep were in the research facility the other 16 sheep were housed in an adjacent building in group pens and fed coarsely milled hay *ad lib*. Clinical observations of these sheep were carried out on a daily basis.

[a] Logos Pharmaceuticals (Pty) Ltd, 16th Rd, Halfway House, 1685

3.3 Experimental procedure

To facilitate the daily collection of arterial blood samples, the right common carotid artery was translocated sub-cutaneously in each sheep using a modification of the procedure described by Butler (1962)[71], at least ten days prior to the collection of the initial blood samples. Instead of the carotid artery being placed into a plastic tube sub-cutaneously (as performed by Butler 1962[71]), it was supported medially by the closure of the access site through the sternocephalic muscle. The skin incision was closed in a routine manner. The pulsation of the carotid artery was easily palpated subcutaneously, which made location of the carotid artery and sample collection easy.

Sheep that have been raised under semi-extensive conditions are unaccustomed to being handled and housed. It was found that the sheep became distressed when handled, resulting in an elevation of heart and respiratory rates. It was, therefore, decided to institute an introductory phase during which the sheep were handled and blood samples collected to make them accustomed to these procedures. After about a week of being handled on a twice daily basis the sheep became less distressed, evidenced by the fact that the heart rate would return to its normal resting level within about 30 seconds of the beginning of auscultation.

Arterial blood samples were aseptically collected from the translocated carotids using a heparinized 5ml syringe and a 25 mm X 0.15 mm hypodermic needle. Indwelling intravenous catheters were installed into the left external jugular of each sheep for the purpose of treatment administration. The catheters used were 16g Vygon[a] catheters that were securely sutured to the skin.

Each of the "infected" sheep, in groups one and two, were given a 5ml LD_{100} dose of heartwater inoculum intravenously. This dose of inoculum contained sufficient of the Welgevonden strain of *Ehrlichia ruminantium* to induce disease and death if not treated (Personal communication with Heartwater Research Section, Onderstepoort Veterinary Institute). The concentration of the inoculum used in block one was the same as the LD_{100} used by van Amstel *et al* 1994[46], which caused disease

[a] Viking, PO Box 8100, Edenglen, JNB, 1613

resulting in death in all seven sheep. The inoculum used in the second block appeared to be more virulent as the temperature reaction occurred on average one day earlier in these animals. Both batches of inoculum were kindly provided by the Onderstepoort Veterinary Research Institute. The Welgevonden strain was chosen for this trial because it is known to be virulent and cause fatal infections in sheep. This ensured that any effects seen in the infected, treated sheep (group two) could be ascribed to the use of the DMSO, as sheep do not usually recover spontaneously after being infected with this strain. No placebo was given to the control sheep in place of the heartwater inoculum

3.3.1 Data collection

The following clinical parameters were measured and recorded on a daily basis by the investigator: heart rate, respiratory rate, appearance of mucous membranes, appetite, habitus and rumen function. The body temperature, measured as the rectal temperature, was recorded in the morning between 07h00 and 10h00 and in the afternoon between 16h00 and 17h00. Ambient maximum and minimum temperatures were recorded each morning between 07h00 and 08h00.

Blood samples for the determination of serum biochemistry and blood gas parameters were collected from 4 days prior to until about 15 days after infection.

3.3.2 Description Of Procedures Performed

A Heart rate

The heart was auscultated over the point of maximal intensity on the left thoracic wall, for a period of one minute, and the rate recorded as beats per minute. Counting the heartbeat commenced a couple of minutes after the sheep was restrained to allow the heart rate to return to its resting rate. Any abnormalities heard on auscultation e.g. arrhythmias, murmurs and fluid splashing sounds were recorded.

B Respiratory rate

Respiration was monitored for one minute by means of auscultation of the lung fields and the rate recorded as breaths per minute. The left and right lung fields were auscultated and any abnormalities were recorded.

C Mucous membranes

These were examined for colour, moisture status and visual appearance. They were recorded as normal, congested or petechiated.

D Appetite

A measured mass of coarsely milled *Eragrostis teff* hay was fed to each animal beginning at the introductory phase and continued throughout the trial. On subsequent days, the food remaining in the trough each morning was removed and weighed to calculate the mass eaten and recorded as grams of hay eaten per sheep per day. The hay was coarsely milled to prevent selective eating and minimise spillage and wastage. Prior to the introductory phase, it was noticed that the sheep would selectively eat the long hay resulting in spillage and wastage that would affect the calculation of the amount of food consumed.

E Habitus

A sheep that was alert and responsive was judged as having a habitus score of 3^{+}, while one that was depressed but ambulatory was assigned a score of 2^{+}. Any sheep, which was severely depressed and unable to rise or stand immediately when stimulated or assisted, was assigned a score of 1^{+}.

F Rumen motility

Rumen motility was determined by auscultation and palpation of the rumen in the left para-lumbar fossa, the motility was recorded as normal (about two cycles per minute), weak (less than one cycle per minute) or absent (no cycles heard or palpated during about two minutes of auscultation).

G Faecal appearance

Faeces were recorded as normal (pelleted), pasty or diarrhoea. The presence of blood and mucous was also recorded.

H Body temperature

The body temperature (T °C) of each sheep was measured using a mercury rectal thermometer inserted into the rectum and maintained there for about one minute. This was performed each morning and evening. A temperature reaction was deemed to have occurred when the morning body temperature exceeded 40 °C, or the afternoon body temperature exceeded 41 °C and was followed by a morning temperature of at least 40 °C. In the latter case treatment was commenced 48 hours after the confirmed rise in afternoon temperature.

I Ambient maximum and minimum temperatures

These were read from two (max and min) thermometers that were strategically placed within the stabling facility. The temperatures were recorded as T (max) °C and T (min) °C. These temperatures were recorded on a daily basis, as a reference in the event of there being marked changes in environmental temperature, which may have resulted in unexpected abnormal variations in the body temperature of the sheep.

J Blood sample collection for biochemistry

Samples were collected from the jugular vein using 5 ml evacuated plain tubes and a 21-gauge needle (Vacutainer® and Vacutainer Precision Glide™)[a]. These samples were allowed to stand at room temperature for approximately two hours prior to centrifugation to allow adequate time for clotting. The serum was pipetted off, frozen to and stored at –20°C.

K Blood sample collection for blood-gas determination

Samples were collected from the translocated carotid arteries using 5ml heparinized syringes and 23 gauge needles. The samples were anaerobically collected and stored on ice for about an hour before processing.

[a] Becton Dickinson Vacutainer Systems, SA Scientific, PO Box 4261, Randburg, 2125

3.3.3 Treatment

The infected treated and the non-infected treated sheep (groups 2 and 3) were treated with 2ml of DMSO[a] (94% concentration) per kg body mass (BM) per day (equivalent to 2g DMSO per kg BM per day) as a 10% solution in polyionic fluid[b] and administered intravenously via the indwelling jugular catheter. For example, a sheep weighing 60 kg was given 60ml of DMSO (94%) in 600 ml of polyionic fluid twice daily. The treatment was given in two divided doses administered over a 30-minute period on three consecutive days commencing 48 hours after the increase in body temperature. The morning treatment was administered between 10h00 and 12h00 and the afternoon treatment between 19h00 and 21h00. Sample collection was performed between 08h00 and 10h00 on the mornings of each sampling day and the treatment administered thereafter. Treatment was continued until the sheep died or were euthanized.

The infected, not-treated sheep (group 1) and the not-infected, not-treated sheep (group 4) were given similar volumes of polyionic fluid[b] (10ml per kg body mass), at the same dosage intervals and duration and via the same route.

3.3.4 Termination of the trial

3.3.4.1 Criteria indicating euthanasia

When an animal displayed three (3) of the following clinical signs or blood-gas parameters it was considered moribund and euthanized by the intra-venous administration of barbiturates.

A. Clinical signs

[a] Kyron Laboratories (Pty) Ltd, 29 Barney Rd, Benrose, Johannesburg, 2094
[b] "Ringers Lactate" Adcock Ingram Critical Care, PO Box 688, Johannesburg, 2000

(i). Respiratory rate exceeding 90 breaths per minute.

(ii). Involuntary recumbency and severe depression characterised by an inability to rise or stand immediately, even when stimulated and assisted.

(iii). Anorexia characterised by the consumption of less than 15% of normal daily food intake, with fewer, weak fore-stomach mixing cycles (compared to the uninfected animals).

(iv). Central nervous signs characterized by hypermetria, ataxia, repetitive chewing motions or recurrent protrusion of the tongue.

B. Blood-gas changes

(i) Partial pressure of arterial oxygen (PaO_2) less than 70 mm mercury.

The presence of any three of the above was considered sufficient grounds for euthanasia on ethical grounds.

In the protocol, provision was made for the removal from the trial of any animal that showed signs or symptoms of disease, unrelated to heartwater. Close to the end of the trial one of the negative control sheep (in group 4) developed pneumonia, was pyrexic and anorexic. The sheep was treated with oxytetracycline hydrochloride at 10 mg per kg twice daily and responded without further incident. The data from this sheep corresponding to these days were not used in the statistical analysis.

All cadavers, whether the animal was euthanized or died as a result of the disease, were submitted to the Department of Pathology, Faculty of Veterinary Science, University of Pretoria for routine necropsy examination. The presence of heartwater organisms in these sheep was investigated by light microscopic examination of brain squash smears.

All of the sheep that survived the trail, i.e. the not-infected, treated and not-infected, not-treated groups (groups 3 and 4) were sold to help defray expenses.

3.4 Analytical procedures

3.4.1 Blood-gas determination was performed using an ABL300 Acid Base Laboratory[a].

3.4.2 Haematocrit determination was performed on heparinized samples using a microhaematocrit centrifuge[b].

3.4.3 Plasma protein was measured using a refractometer[c].

3.5 Data analysis

Statistical analysis was performed using SAS®[72] (SAS Institute Inc, Box 8000, Cary, North Carolina, 27511, Version 8.1).

The data were checked for conformity to the assumptions for linear model analysis. Analysis of variance techniques together with multiple comparison techniques were used to compare the four treatment groups for each of the variables.

Depending on the variables processed, PROC ANOVA was performed, on balanced data, while PROC GLM (LSMEANS) was performed on unbalanced data. The PROC ANOVA produced means, standard deviations and Tukey's multiple comparison tests for each level of main effect variables. Tukey groupings indicate which group means are statistically significantly different. LSMEANS performs multiple comparisons on the main effect variables as well as on the interactions between such variables. LSMEANS produces means, standard error and the probability

[a] Radiometer, Copenhagen
[b] Jouan Haema-C, SA Scientific, 387 Surrey Avenue, Randburg, SA
[c] AO Handheld, temperature-compensated refractometer.
American Optical Corporation (Instrument Division, Buffalo, NY, USA)

level for the hypothesis that the difference between LS means of the effect variables is zero. The probability level that the hypothesis that the LS means of pairs of variables are equal is also provided. All probabilities of $P<0.05$ are considered significant.

Dr MJ van de Linde of the Division of Academic Computing, Department of Information Technology, University of Pretoria processed the data. Prof H Groeneveld of the Department of Statistics, University of Pretoria, was consulted regarding the statistical analyses.

CHAPTER FOUR

RESULTS and DISCUSSION

4.1 RESULTS

All sixteen sheep in Groups 1 and 2 (infected groups) either died acutely (n=13) or were euthanized (n=3), 3-6 days (mean 4.8) after the rise in body temperature and an incubation period, which ranged from 8-12 days (mean 9.4). There was no significant difference in the survival rates of the infected, treated and untreated sheep (Groups 1 and 2).

During the first block of the experiment, it was noted that there were changes in the packed red cell volume of the blood samples that had been collected and centrifuged. It was therefore decided that the haematocrits and plasma protein concentrations would be recorded for the second block. This data therefore represents replicates of four rather than eight (i.e. one block rather than two).

To enable future investigators to compare their results with those obtained here, the blood gas parameters and bicarbonate measurements were performed at body temperature and then standardised to 38°C. The tables containing these values may be found in Appendix 1, Tables 6-13.

4.1.1 Clinical parameters

It should be noted that initially DAY = 0 was the day on which the infected sheep (groups 1 and 2) were infected with the heartwater inoculum. Due to individual sheep resistance, some sheep reacted earlier than others and therefore it was necessary to adjust the DAY label so that all sheep would be at the same stage of the disease on each DAY for comparative purposes. The corrected DAY = 0 became the day prior to the temperature reaction, DAY = 1 became the day on which the temperature reaction occurred (see Materials and Methods). The not-infected sheep (groups 3 and 4) were each paired with an infected sheep using random number tables so that treatment and sampling periods coincided.

4.1.1.1 Body temperature (see Figures 1 and 2)

The majority of the infected sheep (groups 1 and 2) reacted in the morning . The pyrexia was maintained throughout the course of the disease until about two days prior to death when there was a decrease of less than one degree centigrade agonally. The mean morning body temperature of the infected sheep (groups 1 and 2) remained significantly higher (P= 0.001) than the not-infected sheep (groups 3 and 4) to the extent that the mean final temperature to be measured, prior to death, exceeded 40 °C. The mean body temperature of the not-infected, treated sheep (group 3) was raised between days four and six due to one

sheep that had developed respiratory disease. By day seven the mean body temperature of this group had returned to within normal limits following the treatment of the affected sheep. The mean afternoon body temperature of the infected sheep (groups 1 and 2) was significantly higher than that of the not-infected sheep (groups 3 and 4) from day zero until day five. On day six there was a drop of about one degree centigrade, with the mean body temperature of the infected sheep (groups 1 and 2) still being above 40 °C agonally. The mean afternoon body temperature of the not-infected, treated sheep (group 3) rose by about one degree centigrade to 40 °C as a result of an animal that had developed respiratory disease associated with pyrexia of 42 °C. The difference between the mean afternoon body temperatures of the not-infected, treated sheep (group 3) and the not-infected, not-treated sheep (group 4) was however not significant. On day seven the mean body temperature of the not-infected, treated sheep (group 3) had returned to within normal limits.

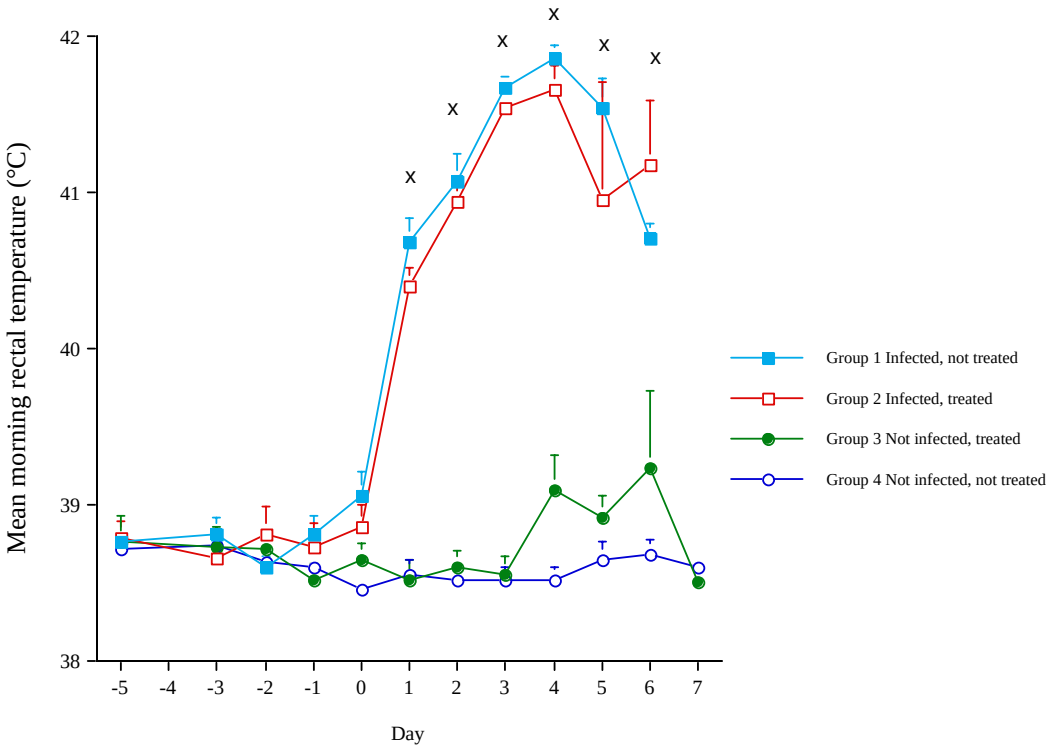

Figure 1. Mean morning rectal temperature, in degrees centigrade, of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The error bars represent +1.0 standard error of the mean. Days on which significant differences ($P<0.05$) occur between the infected sheep (Grp 1 and Grp 2) and not-infected sheep (Grp 3 and Grp 4), attributable to the disease, are indicated by "x". PROC ANOVA was performed on balanced data and PROC GLM (LSMEANS) was performed on unbalanced data. All probabilities of $P<0.05$ are considered significant.

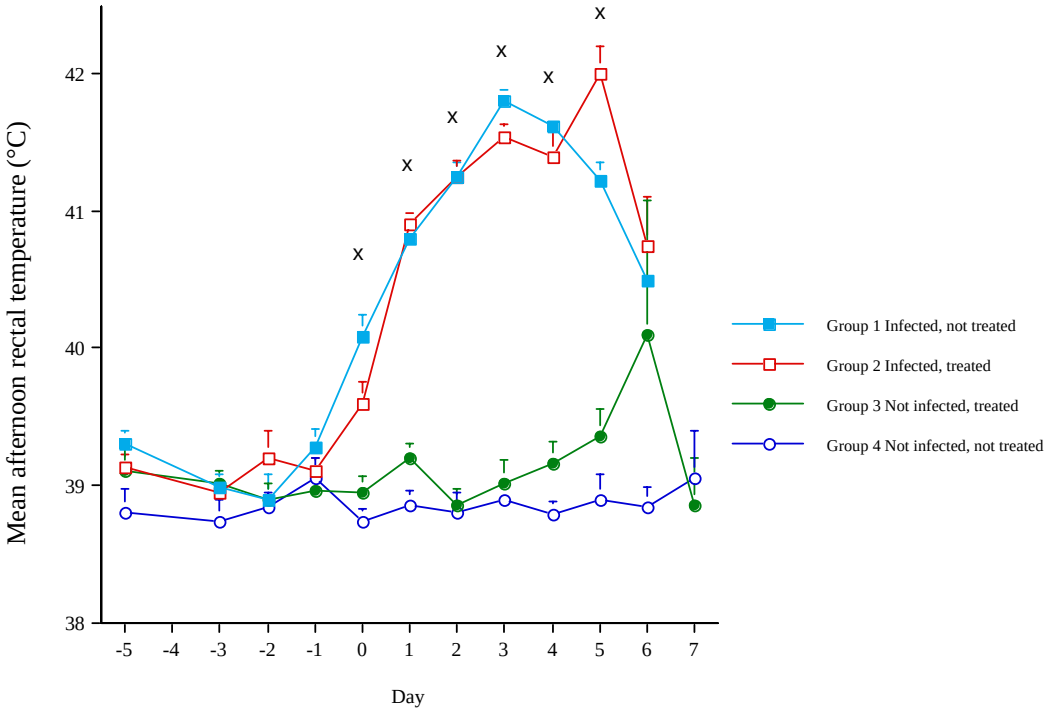

Figure 2. Mean afternoon rectal temperature, in degrees centigrade, of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The error bars represent +1.0 standard error of the mean. Days on which significant differences ($P<0.05$) occur between the infected sheep (Grp 1 and Grp 2) and not-infected sheep (Grp 3 and Grp 4), attributable to the disease, are indicated by "x". PROC ANOVA was performed on balanced data and PROC GLM (LSMEANS) was performed on unbalanced data. All probabilities of $P<0.05$ are considered significant.

4.1.1.2 Heart rate (see Figure 3)

The mean heart rate of the infected, treated sheep (group 2) was raised on day minus five due to the introduction of a replacement sheep that had not been handled regularly. This was necessary because one of the original sheep in the group had lambed and was considered an inappropriate candidate.

From day three the mean heart rate of the infected sheep (groups 1 and 2) was significantly higher (P=0.02) than the not-infected sheep (groups 3 and 4). On days five and six the mean heart rate of the infected, treated sheep (group 2) was significantly higher (P=0.02) than that of the infected, not-treated sheep (group 1). The mean heart rate of the not-infected, treated sheep (group 3) was significantly higher (P=0.005) than that of the not-infected, not-treated sheep (group 4) on days four to six (from the day following the initiation of treatment until the day following the cessation of treatment). On day seven the mean heart rate of the not-infected, treated sheep (group 3) had returned to within normal limits. DMSO is known to have a positive inotropic and positive chronotropic effect as well.

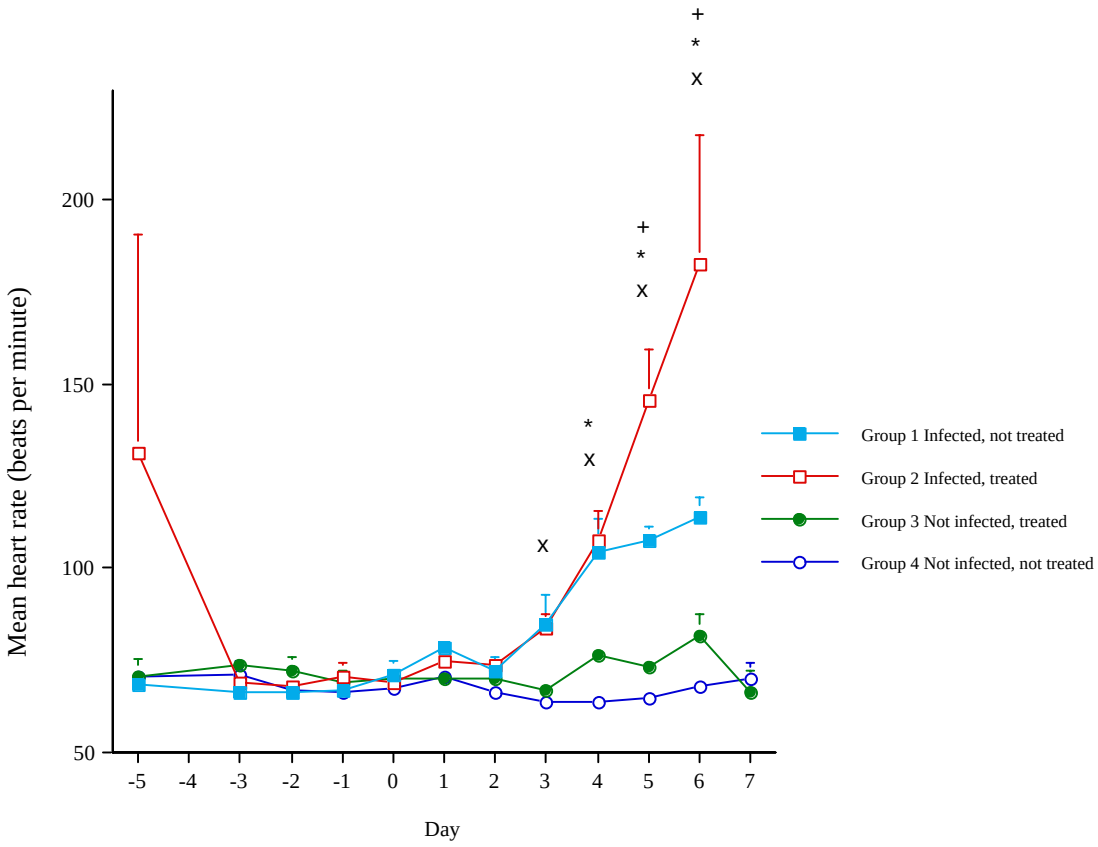

Figure 3. Mean heart rate, in beats per minute, of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The error bars represent +1.0 standard error of the mean. Days on which significant differences ($P<0.05$) occur between the infected sheep (Grp 1 and Grp 2) and not-infected sheep (Grp 3 and Grp 4), attributable to the disease, are indicated by "x". Days on which significant differences ($P<0.05$) occur between the infected, not-treated sheep (Grp 1) and the infected, treated sheep (Grp 2), attributable to the DMSO treatment are indicated by "+". Days on which significant differences ($P<0.05$) occur between the not-infected, treated sheep (Grp 3) and the not-infected, not-treated sheep (Grp 4), attributable to the DMSO treatment are indicated by "*". PROC ANOVA was performed on balanced data and PROC GLM (LSMEANS) was performed on unbalanced data. All probabilities of $P<0.05$ are considered significant.

4.1.1.3 Respiratory rate (see Figure 4)

The mean respiratory rate of the infected, treated sheep (group 2), although markedly increased on days five and six, was not significantly higher than the infected, not-treated sheep (group 1). The mean respiratory rate of the not-infected, treated sheep (group 3) was raised from days four to six but this increased rate was not significantly higher than the mean respiratory rate of the not-infected, not-treated sheep (group 4) during the same period. The mean respiratory rates of the treated sheep (groups 2 and 3) were raised compared to the not-treated sheep (groups 1 and 4) respectively leading to the assumption that this increase, although not significant, may be as a result of the DMSO treatment.

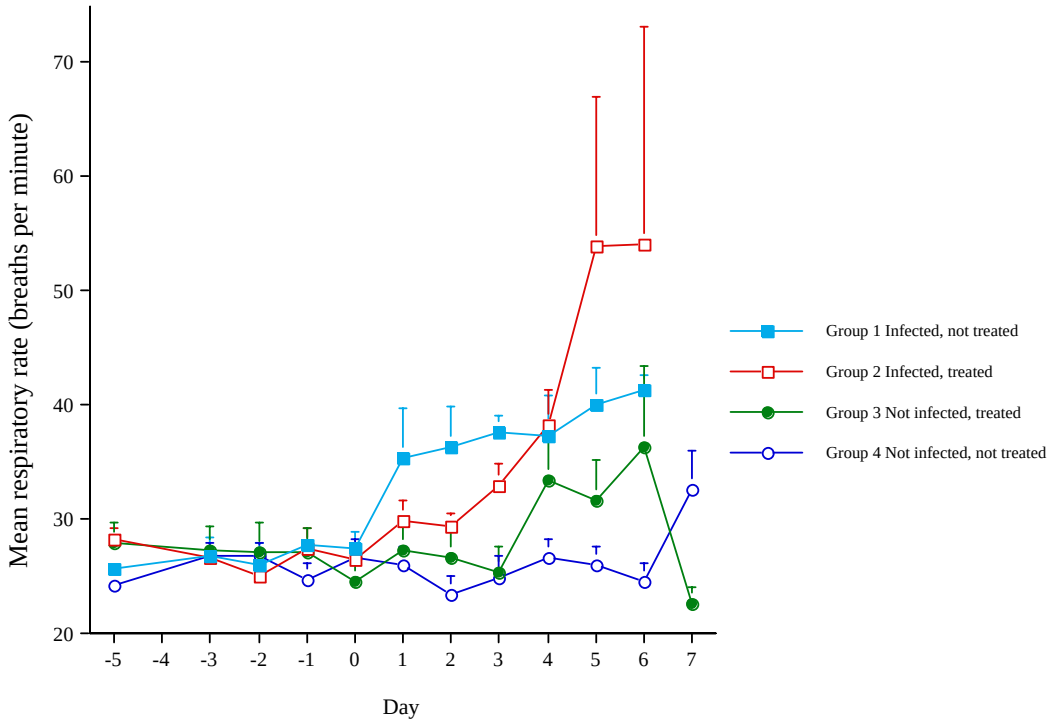

Figure 4. Mean respiratory rate, in breaths per minute, of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The error bars represent +1.0 standard error of the mean. PROC ANOVA was performed on balanced data and PROC GLM (LSMEANS) was performed on unbalanced data. All probabilities of $P<0.05$ are considered significant.

4.1.1.4 Other clinical parameters

When comparing the infected sheep (groups 1 and 2), there was no clinically apparent difference in habitus, appetite, and the appearance of the conjunctival mucous membranes or rumen motility between the treated (group 2) and untreated (group 1) groups. During the last two days of life, some of the infected sheep developed pasty to diarrhoeic faeces but there was no significant difference between the treated and untreated groups.

4.1.2 Mean mass of food consumed (see Figure 5)

The mean mass of food consumed by the infected sheep (groups 1 and 2) began to decline on the first day of pyrexia and by day five the mean mass of food consumed by each of these groups was negligible. This was significantly less (P=0.001) than the not-infected sheep. From day four (the second day of treatment) to day six (the day following the cessation of treatment), the mean mass of food consumed by the not-infected, treated sheep (group 3) was significantly less (P=0.05) than that consumed by the not-infected, not-treated sheep (group 4). This severe suppression of food intake is probably associated with the DMSO treatment. DMSO is partially excreted via exhalation (a pungent garlic-like odour is evident in facilities housing animals being treated with DMSO) and it is assumed that it is also secreted in the saliva. This would probably affect the palatability of the food consumed and lead to decreased consumption.

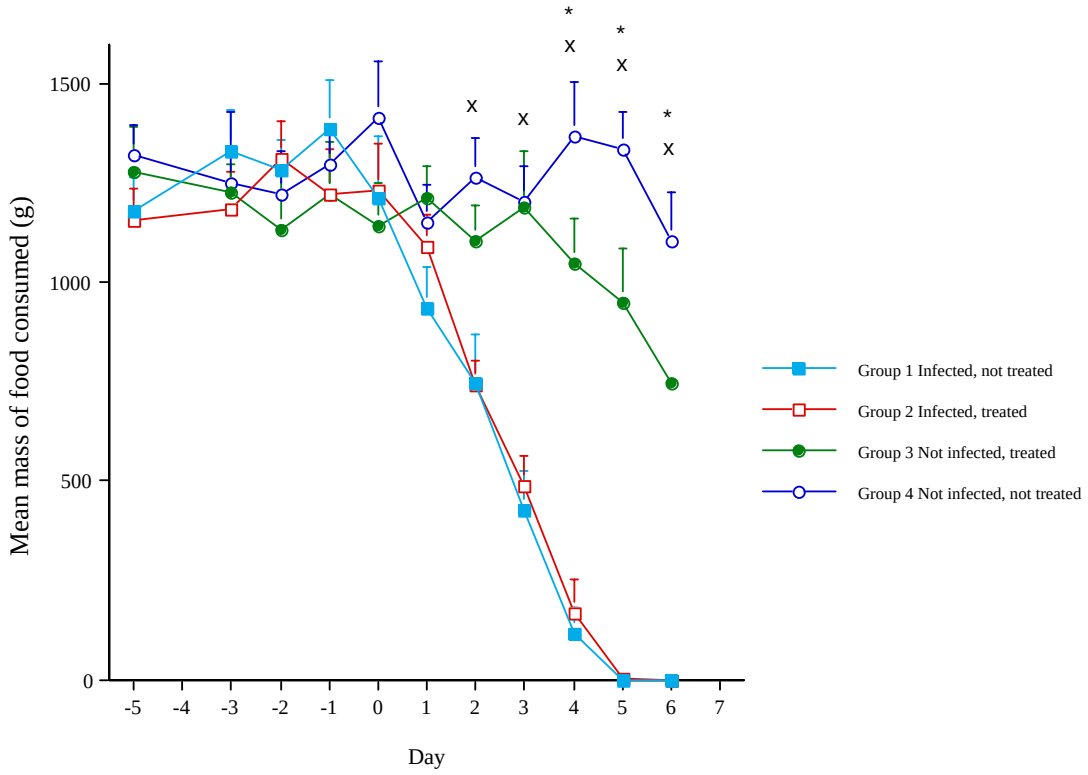

Figure 5. Mean mass of food consumed per sheep, in grams (g) per day, of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The error bars represent +1.0 standard error of the mean. Days on which significant differences ($P<0.05$) occur between the infected sheep (Grp 1 and Grp 2) and not-infected sheep (Grp 3 and Grp 4), attributable to the disease, are indicated by "x". Days on which significant differences ($P<0.05$) occur between the not-infected, treated sheep (Grp 3) and the not-infected, not-treated sheep (Grp 4), attributable to the DMSO treatment are indicated by "*". PROC ANOVA was performed on balanced data and PROC GLM (LSMEANS) was performed on unbalanced data. All probabilities of $P<0.05$ are considered significant.

4.1.3 Laboratory measurements

4.1.3.1 Mean arterial blood pH (see Figure 6)

Although the infected, not-treated sheep (group 1) developed a moderate alkalosis agonally (day six), the infected, treated sheep (group 2) developed an acidosis, beginning on day four, that had become very severe agonally (by day six). The mean arterial blood pH of the not-infected, treated sheep (group 3) was significantly lower than the not-infected, not-treated sheep (group 4) on days six and seven. The fact that the not-infected, not-treated sheep (group 4) had an unexplained mild increase in mean arterial blood pH should be born in mind. An agonal respiratory alkalosis is usually found in animals suffering from heartwater and therefore the development of an acidosis in the treated sheep (groups 2 and 3) is significant. The mild increase in the mean arterial blood pH of the not-infected, not-treated sheep (group 4) may be ascribed to a mild respiratory alkalosis due to a mild increase in respiratory rate (see Figure 4) of undetermined cause.

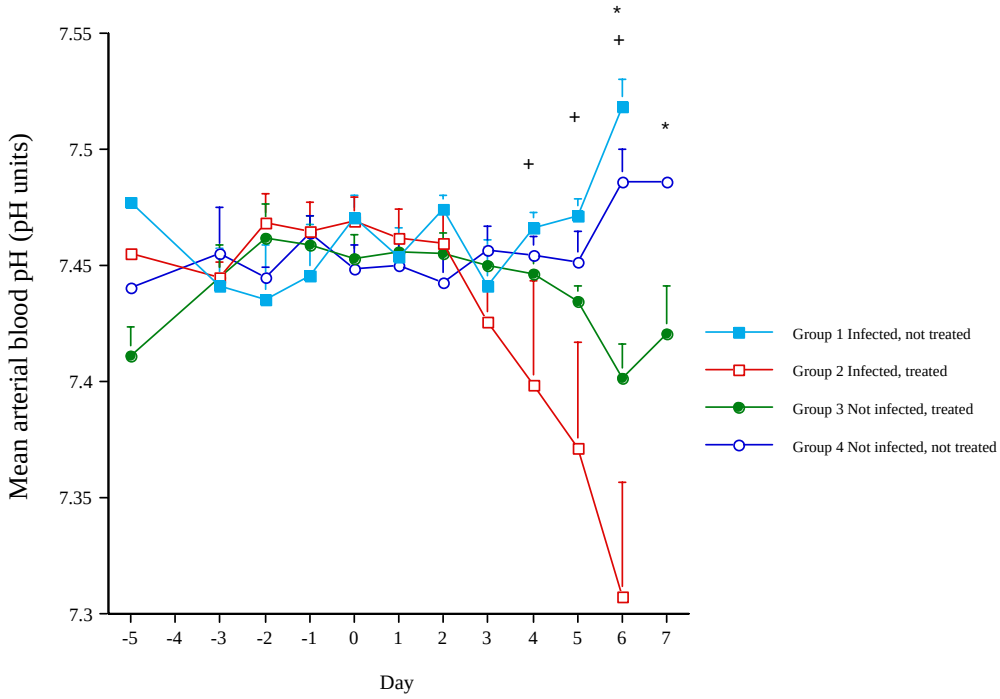

Figure 6. Mean arterial blood pH, in pH units, of four groups of sheep (Grp 1; infected, not-treated,Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The error bars represent +1.0 standard error of the mean. Days on which significant differences ($P<0.05$) occur between the infected, not-treated sheep (Grp 1) and infected, treated sheep (Grp 2), attributable to the DMSO treatment, are indicated by "+". Days on which significant differences ($P<0.05$) occur between the not-infected, treated sheep (Grp 3) and the not-infected, not-treated sheep (Grp 4), attributable to the DMSO treatment are indicated by "*". PROC ANOVA was performed on balanced data and PROC GLM (LSMEANS) was performed on unbalanced data. All probabilities of $P<0.05$ are considered significant.

4.1.3.2 Mean arterial blood partial pressure of oxygen (PaO_2) (see Figure 7)

The mean PaO_2 of the infected, not-treated sheep (group 1) was significantly lower than the infected, treated sheep (group 2) on days five and six. By day six the mean PaO_2 of the infected, not-treated sheep (group 1) was so low as to be incompatible with life (below 50 mm mercury), and these animals were suffering from severe hypoxia. The infected, treated sheep (group 2) however, were able to maintain their PaO_2 to within about 10 mm mercury of the means of the not-infected sheep (groups 3 and 4).

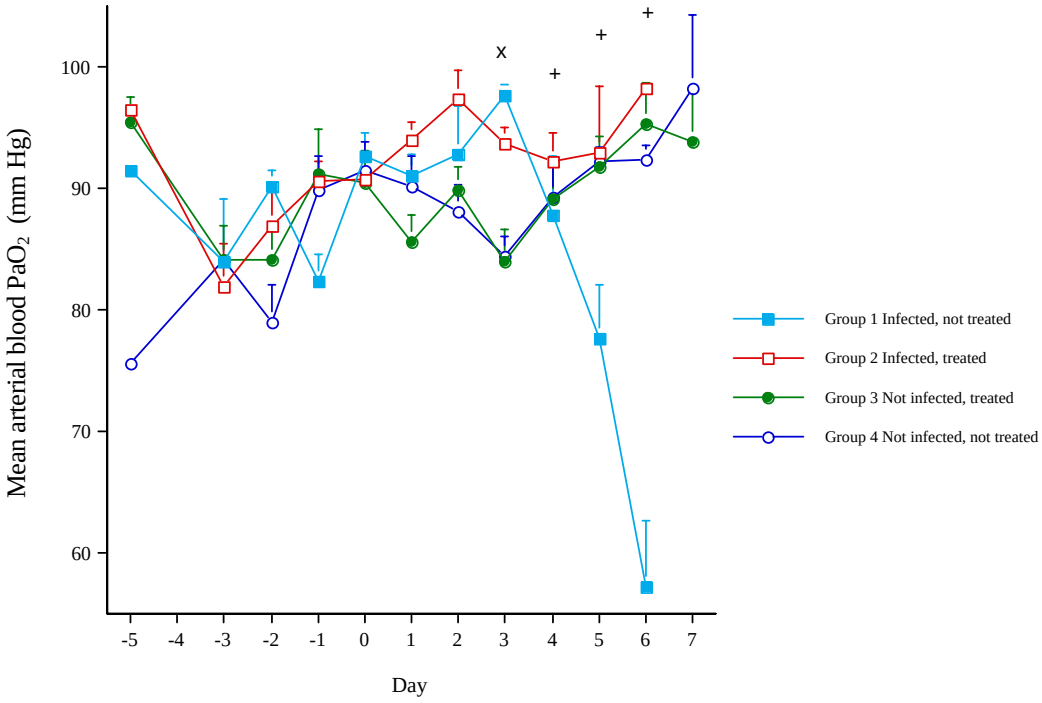

Figure 7. Mean arterial blood partial pressure of oxygen (PaO_2), in mm Hg, at body temperature, of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The error bars represent +1.0 standard error of the mean. Day on which significant differences ($P<0.05$) occur between the infected sheep (Grp 1 and Grp 2) and not-infected sheep (Grp 3 and Grp 4), attributable to the disease, is indicated by "x". Days on which significant differences ($P<0.05$) occur between the infected, not-treated sheep (Grp 1) and infected, treated sheep (Grp 2), attributable to the DMSO treatment, are indicated by "+". PROC ANOVA was performed on balanced data and PROC GLM (LSMEANS) was performed on unbalanced data. All probabilities of $P<0.05$ are considered significant.

4.1.3.3 Mean arterial blood partial pressure of carbon dioxide ($PaCO_2$) (see Figure 8)

The mean arterial blood partial pressure of carbon dioxide ($PaCO_2$) of the infected sheep (groups 1 and 2) began to decrease on day zero and the decline continued until day four. On day five the $PaCO_2$ of the infected, not-treated sheep (group 1) rose slightly before dropping again agonally, although still within normal limits. The $PaCO_2$ of the infected treated sheep (group 2) dropped again dramatically agonally on day six and this is probably due to the increase in respiratory rate seen in these sheep (see Figure 4). The $PaCO_2$ of the not-infected, treated sheep (group 3) was significantly lower than the not-infected, not-treated sheep (group 4) on days four and five. Although this decrease in $PaCO_2$ may be as a result of a mild increase in respiratory rate in these sheep (group 3) there is no concomitant increase in PaO_2 as may have been anticipated. This then may mean that gaseous exchange of carbon dioxide is improved by DMSO.

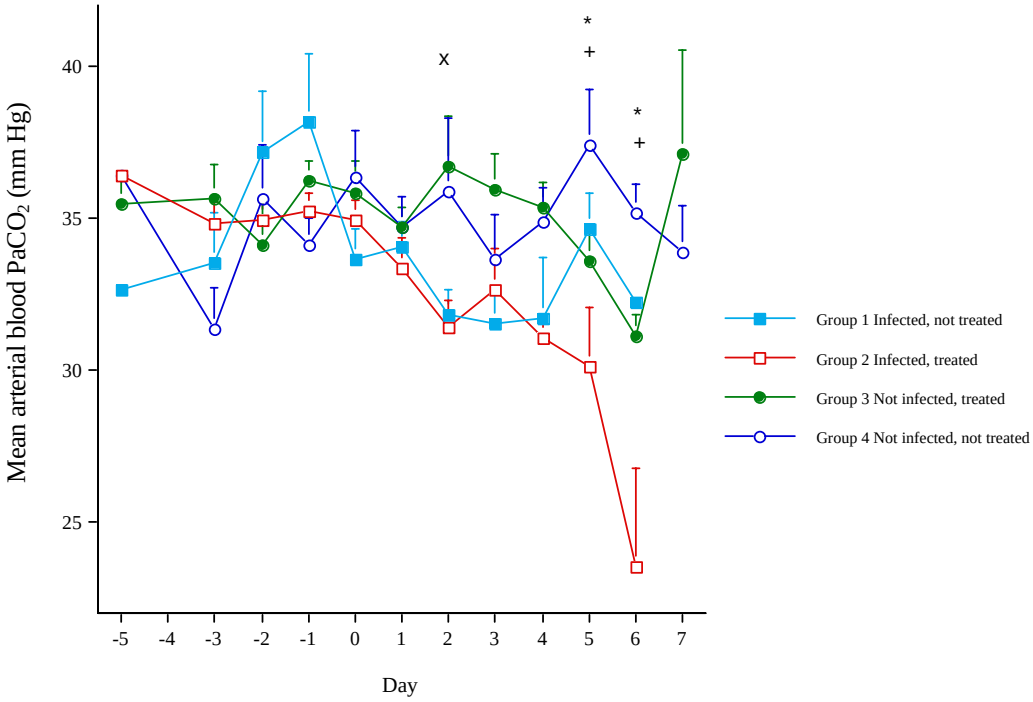

Figure 8. Mean arterial blood partial pressure of carbon dioxide ($PaCO_2$), in mm Hg, of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The error bars represent +1.0 standard error of the mean. Day on which significant differences ($P<0.05$) occur between the infected sheep (Grp 1 and Grp 2) and not-infected sheep (Grp 3 and Grp 4), attributable to the disease, is indicated by "x". Days on which significant differences ($P<0.05$) occur between the infected, not-treated sheep (Grp 1) and infected, treated sheep (Grp 2), attributable to the DMSO treatment, are indicated by "+". Days on which significant differences ($P<0.05$) occur between the not-infected, treated sheep (Grp 3) and the not-infected, not-treated sheep (Grp 4), attributable to the DMSO treatment are indicated by "*". PROC ANOVA was performed on balanced data and PROC GLM (LSMEANS) was performed on unbalanced data. All probabilities of $P<0.05$ are considered significant.

4.1.3.4 Mean arterial blood bicarbonate (HCO_3^-) (see Figure 9)

Mean arterial blood bicarbonate levels of the infected sheep were significantly lower than the not-infected sheep on day 3. The levels in the infected, treated sheep continued to drop with the final level being less than 18 mmol/l. There was a significant difference between the not-infected groups of sheep on day 5 but both groups were still within normal limits.

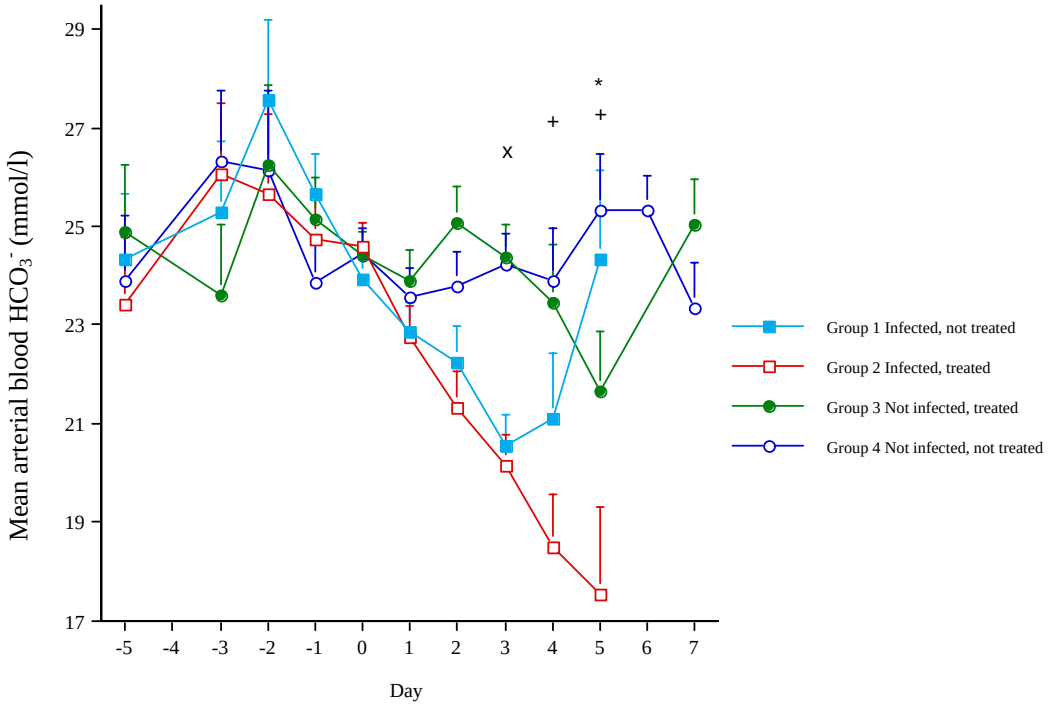

Figure 9. Mean arterial blood bicarbonate (HCO_3^-), in mmol/_, of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The error bars represent +1.0 standard error of the mean. Day on which significant differences ($P<0.05$) occur between the infected sheep (Grp 1 and Grp 2) and not-infected sheep (Grp 3 and Grp 4), attributable to the disease, is indicated by "x". Day on which significant differences ($P<0.05$) occur between the infected not-treated sheep (Grp 1) and infected treated sheep (Grp 2) and between not-infected treated sheep (Grp 3) and the not-infected not-treated sheep (Grp 4), attributable to the DMSO treatment, are indicated by "+". Days on which significant differences ($P<0.05$) occur between the not-infected, treated sheep (Grp 3) and the not-infected, not-treated sheep (Grp 4), attributable to the DMSO treatment are indicated by "*". PROC ANOVA was performed on balanced data and PROC GLM (LSMEANS) was performed on unbalanced data. All probabilities of $P<0.05$ are considered significant.

Summary of acid:base changes

The infected not-treated sheep developed a mild alkalosis of undetermined cause while the infected treated sheep developed an uncompensated metabolic acidosis.

To enable future investigators to compare their results with those obtained here, the blood gas parameters and bicarbonate measurements were performed at body temperature and then standardised to 38°C. The tables containing these values may be found in Appendix 1, Tables 6-13.

4.1.3.5 Mean haematocrit (see Figure 10)

There is a significant difference between the mean haematocrits of the infected, not-treated sheep (group 1) and the infected, treated sheep (group 2) on day four, but on days five and six both of these groups had mean haematocrits that were almost the same. The infected sheep (groups 1 and 2) had mean haematocrits that were significantly higher than the not-infected sheep (groups 3 and 4) on day six even though the haematocrits attained were still within the normal limits for the species (24-40 %). The differences between the means of the haematocrits of the four groups of sheep prior to day 0 are an indication of within species variation.

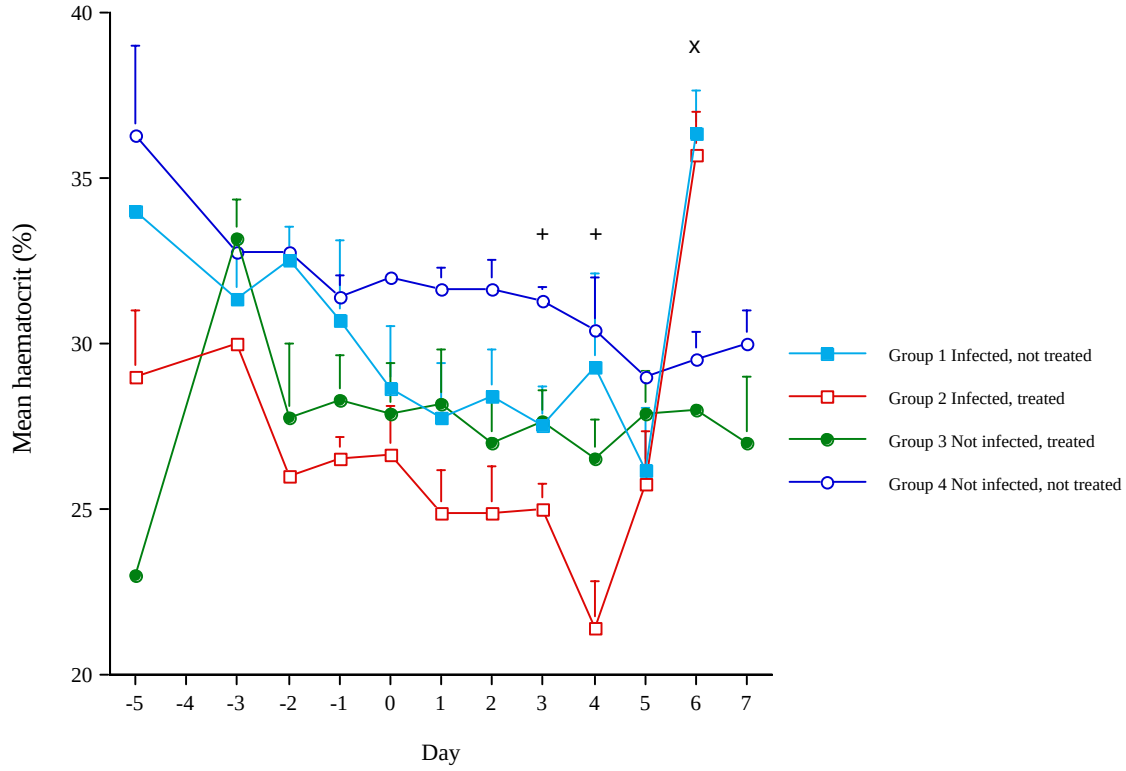

Figure 10. Mean haematocrit, in %, of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The error bars represent +1.0 standard error of the mean. Day on which significant differences ($P<0.05$) occur between the infected sheep (Grp 1 and Grp 2) and not-infected sheep (Grp 3 and Grp 4), attributable to the disease, is indicated by "x". Days on which significant differences ($P<0.05$) occur between the infected, not-treated sheep (Grp 1) and infected, treated sheep (Grp 2), attributable to the DMSO treatment, are indicated by "+". PROC ANOVA was performed on balanced data and PROC GLM (LSMEANS) was performed on unbalanced data. All probabilities of $P<0.05$ are considered significant.

4.1.3.6 Mean plasma protein concentration (see Figure 11)

Although the mean total plasma protein concentration of the infected sheep (groups 1 and 2) began to decline on day four, only on days five and six the total plasma protein concentration for these groups were significantly lower than those of the not-infected sheep (groups 3 and 4). On day six, the mean total plasma protein concentration of the infected, not-treated sheep (group 1) declined severely and was significantly lower than that of the infected, treated sheep (group 2) indicating that there is possibly some membrane stabilization attributable to the DMSO treatment resulting in improved retention of the plasma proteins within the vascular compartment. As the plasma protein concentration was measured using refractometry it is not possible to determine which of the proteins were lost by the infected, not-treated sheep (group 1) to the extra-vascular effusions.

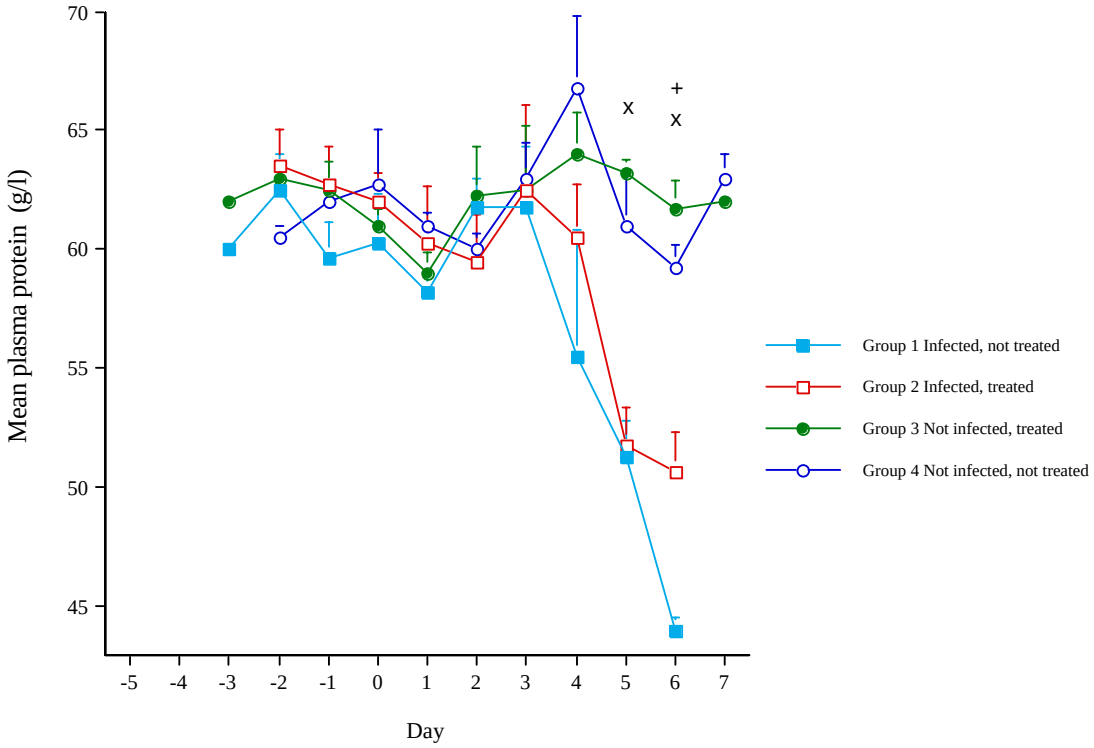

Figure 11. Mean plasma protein, in g/l, of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The error bars represent +1.0 standard error of the mean. Days on which significant differences ($P<0.05$) occur between the infected sheep (Grp 1 and Grp 2) and not-infected sheep (Grp 3 and Grp 4), attributable to the disease, are indicated by "x". Day on which significant differences ($P<0.05$) occur between the infected, not-treated sheep (Grp 1) and infected, treated sheep (Grp 2), attributable to the DMSO treatment, is indicated by "+". PROC ANOVA was performed on balanced data and PROC GLM (LSMEANS) was performed on unbalanced data. All probabilities of $P<0.05$ are considered significant.

4.1.3.7 Haematocrit and plasma protein

During the first block of the experiment, it was noted that there were changes in the packed red cell volume of the blood samples that had been collected and centrifuged. It was therefore decided that the haematocrits and plasma protein concentrations would be recorded for the second block. This data therefore represents replicates of four rather than eight (i.e. one block rather than two).

4.1.4 Necropsy findings

4.1.4.1 Mean body cavity effusions (see Figure 12)

There was significantly less mean pleural effusion found on necropsy examination in the infected, treated sheep (group 2) compared to the infected, not-treated sheep (group 1). There was however no significant difference in mean pericardial and mean peritoneal fluid volumes between the infected, not-treated sheep (group 1) and the infected treated sheep (group 2).

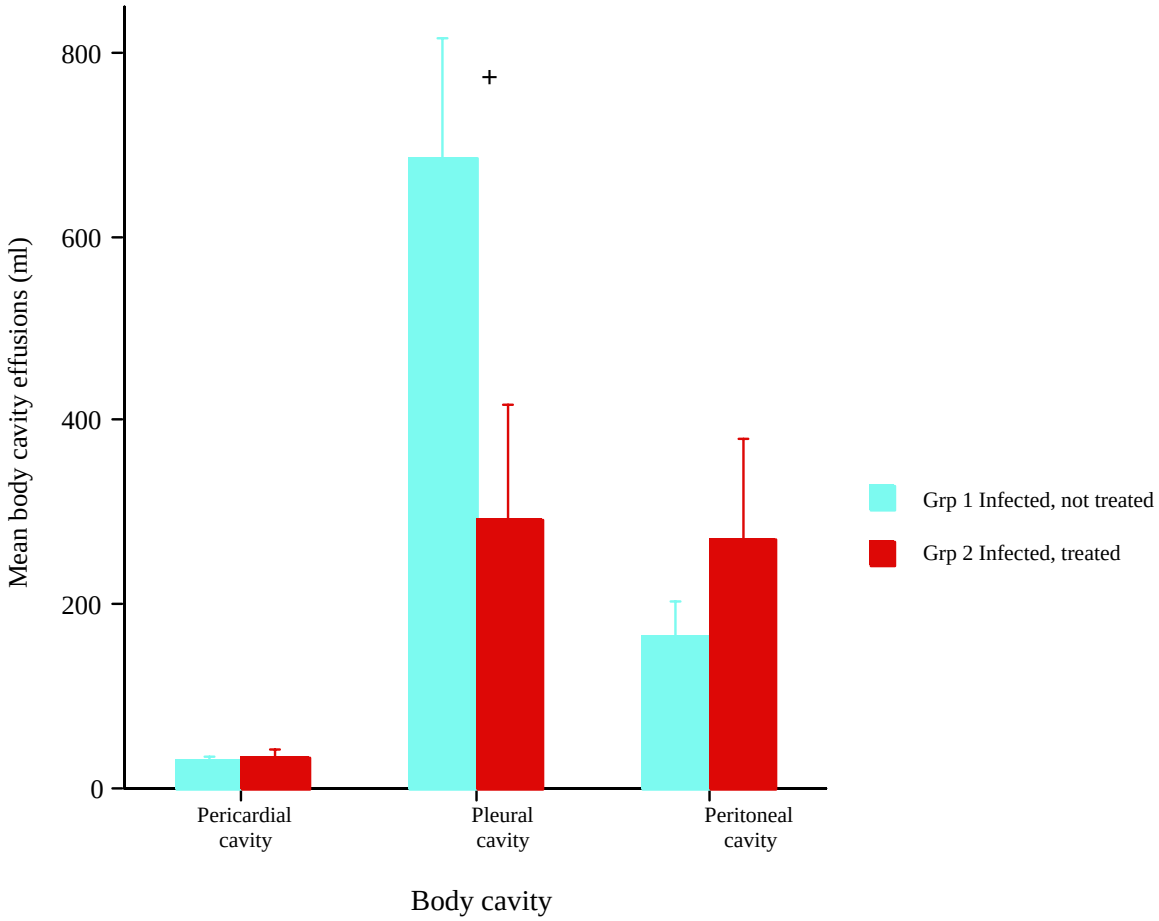

Figure 12. Mean body cavity effusions, in ml, of two groups of sheep (Grp 1; infected, not-treated and Grp 2; infected, treated). The error bars represent +1.0 standard error of the mean. "+" indicates a significant difference between the infected, not-treated sheep (Grp 1) and infected, treated sheep (Grp 2). PROC ANOVA was performed on balanced data. All probabilities of $P<0.05$ are considered significant.

4.2 DISCUSSION

The clinical course, clinical pathological and pathological changes recorded in this study are very similar to other reports of acute cases of naturally occurring or experimentally induced heartwater[18, 35]. Typical clinical pathological changes, as found in this study, include hypoxia associated with inappropriate oxygen exchange due to lung oedema, a precipitous drop in plasma protein levels and vascular volume due to increased capillary permeability, leading to fluid and protein effusions into particularly the lungs, brain and body cavities [17,18, 35,46].

DMSO is used as a cryoprotectant in the manufacture of "heartwater blood vaccine" and has been shown not to be detrimental to *E. ruminantium* organisms[65,66]. On the basis of this, the assumption has been made that DMSO has no therapeutic effect in the specific treatment of animals infected with *E. ruminantium*. The use of DMSO is purely as supportive therapy.

The infected not-treated sheep (Group 1) showed a marked correlation between pleural effusion and hypoxia, however this was not confirmed in a previous study[46]. DMSO treatment of the infected sheep not only reduced the volume of pleural effusion but also enabled the treated animals to maintain their PaO_2 within the normal range. Direct correlation between the severity of lung oedema and PaO_2 in this study was not possible because oedema fluid is washed out during histopathological preparation of the tissue sections (personal communication with Department of Pathology, Faculty of Veterinary Science, University of Pretoria).

One of the marked necropsy findings in animals suffering from heartwater is lung oedema, sometimes manifested as foam flowing from the nostrils agonally. Fox et al (1983) were able to prevent lung oedema in their trial by pre-treating some animals with DMSO prior to treatment with thiourea. Untreated animals developed severe lung oedema. The animals treated with DMSO were also shown to have produced significantly less lung lavage albumin than the untreated control animals[75]. The prevention of lung oedema and lung lavage albumin formation was ascribed to the hydroxyl scavenging effect of DMSO[75]. In our study, pleural effusion (a product of lung oedema) was decreased while plasma

protein levels were maintained in the treated animals compared to those that were not treated. These data can be explained by the scavenging of hydroxyl-radicals (probably derived from polymorpho-nuclear cells infiltrates in the lungs) and membrane stabilization preventing protein leakage, by DMSO.

Increased heart rate in animals with heartwater is associated with pyrexia and decreased vascular volume[17, 46]. However, both infected and not-infected treated groups in this study showed elevated heart rates, which coincided with DMSO treatment. DMSO is reported to have a positive chronotropic and inotropic effect[76]. In dogs DMSO administered intravenously at 2g/kg resulted in increased heart rate, pulmonary arterial pressure and cardiac index (cardiac output per minute square meter of body surface)[76]. These effects were ascribed to hyperosmotic expansion of plasma volume. This increased total blood volume increases end-diastolic volume resulting in stronger ventricular contractions[76].

Infected, not-treated sheep in this study developed a progressive alkalosis, which had neither a clear metabolic nor respiratory component. In a previous study a similar alkalosis was demonstrated to be of respiratory origin[46]. The basis for the mild uncompensated metabolic acidosis in infected treated sheep is not clear. A similar but lesser drop in blood pH, $PaCO_2$ as well as bicarbonate was also observed in not-infected treated sheep. This suggests that DMSO *per se* may have been responsible for this tendency towards metabolic acidosis in not-infected treated sheep. The drop in blood pH observed in both groups coincided with initiation of DMSO treatment. It is possible that one or more of the chemical properties of DMSO may play a role in the changes in blood pH observed in this study.

DMSO can complex / associate with a number of compounds and ions including; Na^+, K^+ and Ca^{2+} and other body components including; tissue, blood, plasma, cerebrospinal fluid and proteins[73]. Complexing with minerals and electrolytes may affect the blood pH but since these were not measured in this study, this was not confirmed.

The pharmacological actions of DMSO also include; anti-inflammatory effects reducing oedema and leukocyte chemotaxis, protection of tissues against ischaemia by stabilization of biological membranes, increasing blood levels of prostaglandin I_2, improving tissue oxygen uptake and preventing thrombocyte aggregation[73]. The inhibition of thrombocyte aggregation in animals suffering from heartwater is probably important, as it has been shown that these animals develop DIC[35]. DMSO is an effective hydroxyl radical scavenger, which provides the basis for the efficient treatment of cerebral oedema in experimental and clinical cases[77,78]. In man, DMSO at 1g/kg has been found to be more effective than mannitol, urea, cortisone, or barbiturates in treating severe head trauma[77].

Because of its dipolar nature, DMSO can act either as an oxidant or a reducing agent. Acting as an oxidant, DMSO can be reduced to dimethyl sulphide (DMS), while acting as a reducing agent it can accept oxygen and be oxidised to dimethyl sulphone ($DMSO_2$)[73]. In cattle, DMSO has been shown to be metabolised into both of its main metabolites, namely dimethyl sulphone and dimethyl sulphide and excreted mainly via the lungs as DMS and kidneys as DMSO and $DMSO_2$[74]. Excretion of DMSO via urine reaches a peak in 6-12 hours and is negligible soon thereafter, while the excretion of $DMSO_2$ via urine continues for a few days. The excretion of DMS via exhalation follows a similar pattern to that of the DMSO (DMS is only derived from DMSO and not from $DMSO_2$)[74]. As mentioned before, the metabolites of endogenous DMSO are found in milk and parenteral sources of the substance are metabolised and excreted along already established metabolic pathways[74]. Excretion of unmetabolised DMSO via the kidneys is, therefore, probably the mechanism of bulk diuresis.

CHAPTER FIVE

CONCLUSION

It is evident from the results of this trial that DMSO does have beneficial effects in sheep suffering from experimentally induced heartwater. The beneficial effects of DMSO were: the infected, treated sheep (group 2) were able to maintain higher concentrations of oxygen and plasma protein and they produced significantly less pleural effusion. It is concluded that these sheep were also able to maintain cardiac output, DMSO causing an increase in heart rate and improved cardiac contractility.

The clinical effect of DMSO may be dependent upon the time of administration relative to onset of disease, dosage and treatment interval[53,77]. It is feasible that earlier onset of treatment in this study may have resulted in a better clinical response.

Based on the findings in this study, extrapolation of treatment results between mice and sheep does not appear feasible. Further studies on the clinical effects of DMSO in experimentally induced heartwater will have to be combined with appropriate antibiotic or anti-microbial treatment. Other practical problems related to the use of DMSO are route of administration and tissue and milk residues. Oral, subcutaneous, intra-mammary, topical and intravenous routes have been used for DMSO administration in cattle, [30] although it is usually given intravenously as a 10% solution. In terms of practical application, this method of treatment administration may present problems under field conditions. In terms of tissue residues, urine values in cows returned to normal within 3-5 days after administration of DMSO and excretion via milk was found to be minimal[74].

Inclusion of DMSO in a treatment protocol for animals infected with *E. ruminantium* may be beneficial since treatment of infected sheep in this study resulted in maintenance of oxygen exchange, which could be attributed to less oedema, and pleural effusion formation. In addition, reduction in plasma protein loss helped preserve osmotic pressure difference between the blood and lymph or tissue fluid thus reducing fluid losses and maintaining intra-vascular volume.

DMSO treatment of clinical cases of heartwater in the absence of concurrent appropriate antibiotic / anti-microbial treatment is not recommended.

Symptomatic treatment of sheep suffering from heartwater has included corticosteroids and loop diuretics. Shakespeare et al 1998, showed that furosemide (a loop diuretic), although effective in diuresis of healthy sheep also caused severe electrolyte imbalances including loss of potassium ions. Hypokalaemia occurs in animals suffering from heartwater and therefore the use of furosemide in sheep suffering from heartwater is contraindicated[69].

The use of other pharmacological agents in the supportive treatment of heartwater such as non-steroidal inflammatory agents also requires investigation.

Appendix 1 Tabulated results

	Days											
Group	-5	-3	-2	-1	0 x	1 x	2 x	3 x	4 x	5 x	6 x	7
1	38.76 (0.04)	38.81 (0.1)	38.59 (0.08)	38.81 (0.12)	39.06 (0.15)	40.68 (0.15)	41.06 (0.18)	41.66 (0.08)	41.85 (0.09)	41.53 (0.19)	40.7 (0.1)	
2	38.78 (0.11)	38.65 (0.09)	38.81 (0.17)	38.73 (0.15)	38.85 (0.14)	40.39 (0.12)	40.93 (0.17)	41.53 (0.06)	41.65 (0.16)	40.95 (0.75)	41.17 (0.41)	
3	38.76 (0.16)	38.73 (0.13)	38.71 (0.11)	38.51 (0.11)	38.64 (0.11)	38.51 (0.13)	38.59 (0.11)	38.55 (0.12)	39.09 (0.22)	38.91 (0.15)	39.23 (0.49)	38.5 (0)
4	38.71 (0.1)	38.74 (0.08)	38.63 (0.08)	38.59 (0.05)	38.46 (0.07)	38.55 (0.09)	38.51 (0.1)	38.51 (0.09	38.51 (0.09)	38.64 (0.12)	38.68 (0.09)	38.6 (0)

Table 1. Mean morning rectal temperature, in degrees centigrade, of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The numbers in brackets represents +/- 1.0 standard error of the mean. Days on which significant differences ($P<0.05$) occur between the infected sheep (Grp 1 and Grp 2) and not-infected sheep (Grp 3 and Grp 4), attributable to the disease, are indicated by "x".

	Days											
Group	-5	-3	-2	-1	0 x	1 x	2 x	3 x	4 x	5 x	6	7
1	39.3 (0.1)	38.99 (0.09)	38.9 (0.18)	39.28 (0.13)	40.08 (0.17)	40.8 (0.13)	41.25 (0.1)	41.8 (0.08)	41.62 (0.05)	41.23 (0.13)	40.5 (0)	. (.)
2	39.13 (0.09)	38.95 (0.07)	39.2 (0.2)	39.11 (0.09)	39.6 (0.16)	40.9 (0.09)	41.25 (0.12)	41.54 (0.09)	41.4 (0.19)	42 (0.2)	40.75 (0.35)	. (.)
3	39.11 (0.15)	39.01 (0.1)	38.89 (0.12)	38.96 (0.1)	38.95 (0.12)	39.2 (0.11)	38.86 (0.12)	39.01 (0.17)	39.16 (0.16)	39.36 (0.19)	40.1 (0.98)	38.85 (0.35)
4	38.8 (0.17)	38.73 (0.17)	38.84 (0.11)	39.05 (0.15)	38.73 (0.1)	38.86 (0.1)	38.8 (0.15)	38.9 (0.07)	38.79 (0.09)	38.89 (0.19)	38.84 (0.15)	39.05 (0.35)

Table 2. Mean afternoon rectal temperature, in degrees centigrade, of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The numbers in brackets represents +/-1.0 standard error of the mean. Days on which significant differences ($P<0.05$) occur between the infected sheep (Grp 1 and Grp 2) and not-infected sheep (Grp 3 and Grp 4), attributable to the disease, are indicated by "x".

	Days											
Group	-5	-3	-2	-1	0	1	2	3 x	4 x *	5 x+*	6 x+*	7
1	68.38 (2.58)	66 (2.65)	66.13 (2.03)	66.63 (2.79)	70.75 (4.06)	78.38 (2.24)	72.13 (3.53)	84.86 (7.7)	104.5 (8.88)	107.5 (3.48)	114 (5)	0 (0)
2	131.5 (59.09)	68.88 (3.35)	67.75 (2.62)	70.5 (3.91)	68.63 (3.1)	74.63 (3.95)	73.88 (2.87)	83.5 (3.83)	107.43 (8.25)	145.5 (13.75)	182.67 (34.74)	0 (0)
3	70.25 (5.03)	73.5 (3.08)	72.25 (3.62)	68.63 (3.65)	69.88 (1.85)	70 (3.13)	70.13 (3.1)	66.63 (2.28)	76.43 (3.01)	73.14 (2.31)	81.67 (5.78)	66 (6)
4	70.25 (2.84)	70.75 (3.84)	66.75 (2.46)	66.38 (3.06)	67.38 (2.08)	70.5 (2.23)	66.38 (3.17)	63.63 (1.78)	63.75 (1.83)	64.38 (2.41)	68 (1.61)	70 (4)

Table 3. Mean heart rate, in beats per minute, of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The numbers in brackets represent +/- 1.0 standard error of the mean. Days on which significant differences ($P<0.05$) occur between the infected sheep (Grp 1 and Grp 2) and not-infected sheep (Grp 3 and Grp 4), attributable to the disease, are indicated by "x". Days on which significant differences ($P<0.05$) occur between the infected, not-treated sheep (Grp 1) and the infected, treated sheep (Grp 2), attributable to the DMSO treatment are indicated by "+". Days on which significant differences ($P<0.05$) occur between the not-infected, treated sheep (Grp 3) and the not-infected, not-treated sheep (Grp 4), attributable to the DMSO treatment are indicated by "*".

	Days											
Group	-5	-3	-2	-1	0	1	2	3	4	5	6	7
1	25.63 (0.96)	26.75 (1.64)	25.88 (1.95)	27.63 (0.96)	27.38 (1.53)	35.25 (4.36)	36.25 (3.55)	37.57 (1.43)	37.17 (3.65)	40 (3.16)	41.33 (1.33)	. (.)
2	28.13 (1.04)	26.63 (0.94)	25 (0.91)	27.38 (1.72)	26.38 (1.28)	29.88 (1.65)	29.38 (1.12)	32.88 (1.97)	38.14 (3.17)	53.83 (13.09)	54 (19.08)	. (.)
3	27.88 (1.83)	27.25 (2.11)	27.13 (2.5)	27 (2.19)	24.5 (2.58)	27.25 (2.65)	26.63 (2.81)	25.25 (2.33)	33.43 (4.86)	31.57 (3.62)	36.33 (7.13)	22.5 (1.5)
4	24.13 (1.72)	26.75 (1.19)	26.75 (1.18)	24.63 (1.41)	26.63 (1.64)	25.88 (1.6)	23.38 (1.55)	24.75 (1.94)	26.5 (1.65)	25.88 (1.62)	24.4 (1.72)	32.5 (3.5)

Table 4. Mean respiratory rate, in breaths per minute, of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The numbers in brackets represent +/- 1.0 standard error of the mean.

	Days										
Group	-5	-3	-2	-1	0	1	2 x	3 x	4 x*	5 x*	6 x*
1	1179.25 (83.49)	1328.13 (104.69)	1280.75 (78)	1385.13 (123.87)	1213.75 (151.71)	934.75 (101.89)	748.25 (118.93)	425.29 (97.72)	116.5 (66.91)	0 (0)	0 (0)
2	1154.88 (78.87)	1181.88 (94.57)	1310.25 (96.05)	1223.38 (109.13)	1228.75 (119.5)	1088.75 (80.67)	741 (62.63)	488.13 (75.52)	166.86 (86.05)	3.25 (3.25)	0 (0)
3	1280.13 (108.85)	1226.38 (71.99)	1130.75 (139.6)	1220.88 (130.11)	1140.13 (108.26)	1211.88 (79.69)	1106 (89.03)	1186.75 (144.33)	1048.86 (109.38)	948.83 (136.58)	746 (22)
4	1318.38 (74.79)	1247.43 (182.16)	1221.5 (105.97)	1296.88 (82.39)	1412 (143.11)	1151 (95.49)	1265.75 (95.34)	1203.63 (87.93)	1367.88 (134.5)	1336.5 (92.04)	1101.67 (122.99)

Table 5. Mean mass of food consumed per sheep, in grams per day, of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The numbers in brackets represent +/- 1.0 standard error of the mean. Days on which significant differences ($P<0.05$) occur between the infected sheep (Grp 1 and Grp 2) and not-infected sheep (Grp 3 and Grp 4), attributable to the disease, are indicated by "x". Days on which significant differences ($P<0.05$) occur between the not-infected, treated sheep (Grp 3) and the not-infected, not-treated sheep (Grp 4), attributable to the DMSO treatment are indicated by "*".

	Days											
Group	-5	-3	-2	-1	0	1	2	3	4 +	5 +	6 +*	7 *
1	7.477 (0)	7.441 (0.0162)	7.4353 (0.0229)	7.4454 (0.0218)	7.4701 (0.01)	7.4535 (0.0121)	7.4739 (0.0057)	7.441 (0.0194)	7.4662 (0.0064)	7.471 (0.0076)	7.5177 (0.0118)	0 (0)
2	7.455 (0)	7.4448 (0.0067)	7.4682 (0.012)	7.4645 (0.0121)	7.4686 (0.0103)	7.4618 (0.0122)	7.4595 (0.015)	7.4251 (0.0153)	7.3979 (0.045)	7.371 (0.0453)	7.307 (0.0494)	0 (0)
3	7.411 (0.012)	7.4443 (0.0139)	7.4612 (0.0147)	7.4583 (0.0129)	7.4525 (0.0107)	7.4555 (0.007)	7.4545 (0.0091)	7.4494 (0.0084)	7.446 (0.0083)	7.4346 (0.0066)	7.4013 (0.0149)	7.4205 (0.0205)
4	7.44 (0)	7.4547 (0.0203)	7.4447 (0.0045)	7.4633 (0.0077)	7.4483 (0.0105)	7.4498 (0.0095)	7.4425 (0.0125)	7.456 (0.0109)	7.4538 (0.0083)	7.4513 (0.0131)	7.486 (0.0134)	7.486 (0.004)

Table 6. Mean arterial blood pH, in pH units, (at body temperature) of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The numbers in brackets represent +/- 1.0 standard error of the mean. Days on which significant differences ($P<0.05$) occur between the infected, not-treated sheep (Grp 1) and infected, treated sheep (Grp 2), attributable to the DMSO treatment, are indicated by "+". Days on which significant differences ($P<0.05$) occur between the not-infected, treated sheep (Grp 3) and the not-infected, not-treated sheep (Grp 4), attributable to the DMSO treatment are indicated by "*".

	Days											
Group	-5	-3	-2	-1	0	1	2	3	4	5	6	7
1	7.486 (0)	7.4558 (0.0166)	7.4482 (0.0236)	7.4597 (0.0219)	7.4865 (0.0093)	7.4945 (0.0122)	7.5214 (0.0055)	7.497 (0.0196)	7.5255 (0.0072)	7.525 (0.0062)	7.5583 (0.0116)	0 (0)
2	7.475 (0)	7.454 (0.0062)	7.4843 (0.0151)	7.477 (0.0121)	7.4816 (0.0117)	7.4984 (0.0112)	7.5046 (0.0154)	7.4788 (0.0161)	7.4531 (0.0475)	7.4138 (0.0526)	7.3527 (0.0453)	0 (0)
3	7.42 (0.016)	7.4569 (0.0138)	7.4742 (0.0155)	7.468 (0.0128)	7.4567 (0.0096)	7.4633 (0.0077)	7.4634 (0.0092)	7.4578 (0.0087)	7.4624 (0.0086)	7.4484 (0.0058)	7.4197 (0.0181)	7.428 (0.021)
4	7.458 (0)	7.4643 (0.0206)	7.4572 (0.006)	7.4731 (0.0082)	7.4551 (0.0105)	7.4578 (0.0102)	7.4501 (0.0118)	7.4635 (0.0108)	7.4613 (0.0079)	7.461 (0.0122)	7.4962 (0.0142)	7.4955 (0.0045)

Table 7. Mean arterial blood pH in pH units, (standardized to 38°C) of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The numbers in brackets represent +/- 1.0 standard error of the mean. Days on which significant differences ($P<0.05$) occur between the infected, not-treated sheep (Grp 1) and infected, treated sheep (Grp 2), attributable to the DMSO treatment, are indicated by "+". Days on which significant differences ($P<0.05$) occur between the not-infected, treated sheep (Grp 3) and the not-infected, not-treated sheep (Grp 4), attributable to the DMSO treatment are indicated by "*".

	Days											
Group	-5	-3	-2	-1	0	1	2	3	4 x	5 x+	6 x+	7
1	91.4 (.)	83.98 (5.07)	90.12 (1.29)	82.3 (2.25)	92.6 (1.95)	91.01 (1.66)	92.7 (4.04)	97.51 (0.9)	87.78 (4.76)	77.65 (4.36)	57.13 (5.41)	. (.)
2	96.4 (.)	81.78 (3.67)	86.92 (2.65)	90.53 (1.63)	90.68 (1.52)	93.83 (1.51)	97.24 (2.38)	93.61 (1.28)	92.15 (2.41)	92.9 (5.36)	98.13 (0.67)	. (.)
3	95.4 (2.1)	84.01 (2.86)	84.07 (3.05)	91.15 (3.64)	90.4 (2.69)	85.51 (2.28)	89.85 (1.92)	83.89 (2.6)	89.05 (2.86)	91.63 (2.52)	95.2 (3.37)	93.7 (4.3)
4	75.5 (.)	84.1 (.)	78.98 (3.01)	89.78 (2.78)	91.45 (2.37)	90.08 (2.46)	88.06 (2.21)	84.29 (1.67)	89.18 (2.61)	92.1 (1.25)	92.24 (1.22)	98.2 (6)

Table 8. Mean arterial blood partial pressure of oxygen (PaO_2) in mm Hg, (at body temperature) of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The numbers in brackets represent +/- 1.0 standard error of the mean. Days on which significant differences ($P<0.05$) occur between the infected sheep (Grp 1 and Grp 2) and not infected sheep (Grp 3 and Grp 4), attributable to the disease, are indicated by "x". Days on which significant differences ($P<0.05$) occur between the infected, not-treated sheep (Grp 1) and infected, treated sheep (Grp 2), attributable to the DMSO treatment, are indicated by "+".

	Days											
Group	-5	-3	-2	-1	0	1	2	3	4	5	6	7

1	87.9 (.)	78.66 (4.78)	85.32 (1.38)	81.77 (4.91)	86.34 (1.62)	76.01 (1.65)	77.06 (3.82)	76.23 (1.03)	73.28 (4.31)	61 (3.35)	47.53 (4.21)	. (.)
2	88.6 (.)	78.63 (2.95)	81.1 (3.04)	87.99 (3.03)	85.76 (1.59)	80 (1.3)	82.46 (2.69)	76.99 (3.3)	74.93 (2.16)	76.2 (2.17)	79.47 (2.83)	. (.)
3	91.85 (3.85)	79.56 (3.35)	88.6 (5.37)	87.44 (3.56)	86.7 (2.56)	85.58 (3.43)	86.5 (2.14)	80.93 (2.61)	82.53 (3.39)	86.3 (2.31)	87.8 (2.65)	90.75 (4.25)
4	69.6 (.)	80.3 (.)	74.86 (2.94)	85.87 (2.39)	88.74 (2.21)	86.88 (2.24)	85.28 (2.48)	81.5 (1.9)	86.2 (2.23)	88.41 (1.62)	88.26 (1.16)	94.6 (5.9)

Table 9. Mean arterial blood partial pressure of oxygen (PaO_2) in mm Hg, (standardized to 38°C) of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The numbers in brackets represent +/- 1.0 standard error of the mean. Days on which significant differences occur between the infected sheep (Grp 1 and Grp 2) and not infected sheep (Grp 3 and Grp 4), attributable to the disease, are indicated by "x". Days on which significant differences occur between the infected, not-treated sheep (Grp 1) and infected, treated sheep (Grp 2), attributable to the DMSO treatment, are indicated by "+".

	Days											
Group	-5	-3	-2	-1	0	1 x	2 x	3 x	4 x	5 x*	6 +*	7
1	32.6 (0)	33.52 (1.61)	37.12 (2.01)	38.13 (2.28)	33.65 (0.98)	34.06 (0.82)	31.79 (0.86)	31.53 (0.91)	31.7 (2)	34.65 (1.13)	32.23 (0.34)	0 (0)
2	36.4 (0)	34.78 (0.73)	34.92 (0.76)	35.19 (0.63)	34.91 (0.63)	33.3 (1.01)	31.39 (0.86)	32.64 (1.33)	31.03 (0.73)	30.08 (1.97)	23.53 (3.2)	0 (0)
3	35.45 (0.75)	35.64 (1.07)	34.1 (1.47)	36.2 (0.65)	35.78 (1.05)	34.7 (0.63)	36.66 (1.69)	35.91 (1.18)	35.34 (0.82)	33.56 (0.99)	31.07 (0.74)	37.1 (3.4)
4	36.4 (0)	31.3 (1.36)	35.6 (1.81)	34.08 (0.92)	36.3 (1.54)	34.66 (1.01)	35.88 (2.4)	35.11 (1.43)	34.86 (1.13)	37.39 (1.84)	35.16 (0.94)	33.85 (1.55)

Table 10. Mean arterial blood partial pressure of carbon dioxide ($PaCO_2$) in mm Hg, (at body temperature) of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The numbers in brackets represent +/- 1.0 standard error of the mean. Days on which significant differences ($P<0.05$) occur between the infected sheep (Grp 1 and Grp 2) and not infected sheep (Grp 3 and Grp 4), attributable to the disease, are indicated by "x". Days on which significant differences ($P<0.05$) occur between the infected, not-treated sheep (Grp 1) and infected, treated sheep (Grp 2), attributable to the DMSO treatment, are indicated by "+". Days on which significant differences ($P<0.05$) occur between the not-infected, treated sheep (Grp 3) and the not-infected, not-treated sheep (Grp 4), attributable to the DMSO treatment are indicated by "*".

	Days											
Group	-5	-3	-2	-1	0	1	2	3	4	5	6	7
1	31.6 (0)	31.96 (1.58)	35.67 (2.01)	36.41 (2.11)	31.95 (0.92)	29.91 (0.73)	27.38 (0.58)	26.43 (0.71)	26.32 (1.71)	29.25 (0.86)	28.4 (0.35)	0 (0)
2	34.2 (0)	33.75 (0.78)	33.17 (0.55)	33.81 (0.48)	33.5 (0.71)	29.64 (0.84)	27.25 (0.72)	27.5 (1.13)	26.01 (0.7)	26.48 (2.68)	20.07 (2.38)	0 (0)
3	34.45 (1.25)	34.19 (0.89)	32.73 (1.45)	35.11 (0.66)	34.69 (0.89)	33.85 (0.49)	35.65 (1.65)	34.99 (1.17)	33.57 (0.87)	32.07 (0.89)	29.27 (1.31)	36.2 (3.3)
4	34.3 (0)	30.37 (1.33)	34.2 (1.59)	33.01 (0.92)	35.45 (1.42)	33.74 (0.91)	34.98 (2.23)	34.24 (1.37)	34 (1.09)	36.24 (1.67)	34.02 (1.05)	32.9 (1.5)

Table 11. Mean arterial blood partial pressure of carbon dioxide ($PaCO_2$) in mm Hg, (standardized to 38°C) of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The numbers in brackets represent +1.0 standard error of the mean. Days on which significant differences occur between the infected sheep (Grp 1 and Grp 2) and not-infected sheep (Grp 3 and Grp 4), attributable to the disease, are indicated by "x". Days on which significant differences occur between the infected, not-treated sheep (Grp 1) and infected, treated sheep (Grp 2), attributable to the DMSO treatment, are indicated by "+". Days on which significant differences occur between the not-infected, treated sheep (Grp 3) and the not-infected, not-treated sheep (Grp 4), attributable to the DMSO treatment are indicated by "*".

	Days											
Group	-5	-3	-2	-1	0	1	2	3 x	4	5 +	6	7
1	24.34 1.33	25.29 1.44	27.56 1.62	26.65 0.83	23.95 0.49	22.85 0.61	22.26 0.72	20.55 0.63	21.1 1.07	24.32 1.77		
2	23.43 1.33	26.06 1.44	25.65 1.62	24.75 0.83	24.6 0.49	22.76 0.61	21.34 0.72	20.14 0.63	18.48 1.07	17.55 1.77		
3	24.9 1.33	23.6 1.44	26.23 1.62	25.15 0.83	24.4 0.49	23.9 0.61	25.08 0.72	24.39 0.63	23.47 1.16	21.65 1.21		25.05 1.12
4	23.9 1.33	26.31 1.44	26.15 1.62	23.86 0.83	24.46 0.49	23.56 0.61	23.78 0.72	24.23 0.63	23.9 1.07	25.34 1.12	25.34 0.69	23.35 0.09

Table 12. Mean arterial blood bicarbonate (HCO_3^-) in mmol/_, (at body temperature) of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The numbers in brackets represent +/- 1.0 standard error of the mean. Day on which significant differences ($P<0.05$) occur between the infected sheep (Grp 1 and Grp 2) and not-infected sheep (Grp 3 and Grp 4), attributable to the disease, indicated by "x". Day on which significant differences ($P<0.05$) occur between the infected not-treated sheep (Grp 1) and infected treated sheep (Grp 2) and between not-infected treated sheep (Grp 3) and the not-infected not-treated sheep (Grp 4), attributable to the DMSO treatment, is indicated by "+".

	Days											
Group	-5	-3	-2	-1	0	1	2	3 x	4	5 +	6	7
1	25.61 1.02	26.34 1.04	27.94 1.27	26.67 0.82	25.85 0.47	25.05 0.52	25.05 0.59	23.21 0.56	24.35 1.22	26.75 1.65		
2	24.99 1.02	26.75 1.04	26.88 1.27	26.21 0.76	26.19 0.47	25.03 0.52	24.1 0.59	22.91 0.56	21.35 0.99	19.66 1.6		
3	25.73 1.02	25.03 1.04	27.26 1.27	26.38 0.76	25.69 0.47	25.38 0.52	26.16 0.59	25.59 0.56	24.94 1.07	23.45 1.09		24.30 0.51
4	25.33 1.02	27.11 1.04	26.78 1.27	25.55 0.76	25.69 0.47	25.09 0.52	25.06 0.59	25.63 0.56	25.36 0.99	26.33 1.01	26.85 0.74	26.70 1.01

Table 13. Mean arterial blood standard bicarbonate (HCO_3^-) in mmol/_ of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The numbers in brackets represent +/- 1.0 standard error of the mean. Day on which significant differences ($P<0.05$) occur between the infected sheep (Grp 1 and Grp 2) and not-infected sheep (Grp 3 and Grp 4), attributable to the disease, indicated by "x". Day on which significant difference ($P<0.05$) occurs between the infected not-treated sheep (Grp 1) and infected treated sheep (Grp 2), attributable to the DMSO treatment, is indicated by "+".

	Days											
Group	-5	-3	-2	-1	0	1	2	3	4 +*	5	6 x	7
1	34 (.)	31.33 (1.69)	32.5 (1)	30.67 (2.4)	28.63 (1.85)	27.75 (1.61)	28.38 (1.39)	27.5 (1.19)	29.25 (2.82)	26.17 (1.88)	36.33 (1.3)	. (.)
2	29 (2)	30 (0)	26 (0)	26.5 (0.68)	26.63 (1.46)	24.88 (1.26)	24.88 (1.42)	25 (0.74)	21.38 (1.45)	25.75 (1.56)	35.67 (1.33)	. (.)
3	23 (.)	33.17 (1.17)	27.75 (2.25)	28.25 (1.39)	27.88 (1.51)	28.13 (1.64)	27 (1.51)	27.63 (0.94)	26.5 (1.17)	27.88 (1.26)	28 (0.29)	27 (2)
4	36.25 (2.75)	32.75 (0.25)	32.75 (0.25)	31.38 (0.66)	32 (0.35)	31.63 (0.63)	31.63 (0.85)	31.25 (0.43)	30.38 (1.6)	29 (0.2)	29.5 (0.84)	30 (1)

Table 14. Mean haematocrit in % of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The numbers in brackets represent +/- 1.0 standard error of the mean. Days on which significant differences ($P<0.05$) occur between the infected sheep (Grp 1 and Grp 2) and not-infected sheep (Grp 3 and Grp 4), attributable to the disease, are indicated by "x". Days on which significant differences ($P<0.05$) occur between the infected, not-treated sheep (Grp 1) and infected, treated sheep (Grp 2), attributable to the DMSO treatment, are indicated by "+". Days on which significant differences ($P<0.05$) occur between the not-infected, treated sheep (Grp 3) and the not-infected, not-treated sheep (Grp 4), attributable to the DMSO treatment are indicated by "*".

	Days											
Group	-5	-3	-2	-1	0	1	2	3	4	5 x	6 x+	7
1	. (.)	60 (.)	62.5 (1.5)	59.67 (1.45)	60.25 (2.06)	58.25 (0.75)	61.75 (1.25)	61.75 (2.59)	55.5 (5.3)	51.33 (1.45)	44 (0.58)	. (.)
2	. (.)	. (.)	63.5 (1.5)	62.75 (1.55)	62 (1.22)	60.25 (2.39)	59.5 (1.94)	62.5 (3.57)	60.5 (2.25)	51.75 (1.65)	50.67 (1.67)	. (.)
3	. (.)	62 (.)	63 (0)	62.5 (1.19)	61 (0.71)	59 (0.91)	62.25 (2.1)	62.5 (2.72)	64 (1.73)	63.25 (0.48)	61.67 (1.2)	62 (0)
4	. (.)	. (.)	60.5 (0.5)	62 (0.82)	62.75 (2.25)	61 (0.58)	60 (0.71)	63 (1.47)	66.75 (3.09)	61 (2.27)	59.25 (0.95)	63 (1)

Table 15. Mean total plasma protein in g/_ of four groups of sheep (Grp 1; infected, not-treated, Grp 2; infected, treated, Grp 3; not-infected, treated and Grp 4; not-infected, not-treated). Day 0 represents the day prior to the increase in rectal temperature. All sheep receiving treatment (Grp 2 and Grp 3) were treated twice daily on days three, four and five. The numbers in brackets represent +/- 1.0 standard error of the mean. Days on which significant differences ($P<0.05$) occur between the infected sheep (Grp 1 and Grp 2) and not-infected sheep (Grp 3 and Grp 4), attributable to the disease, are indicated by "x". Days on which significant differences ($P<0.05$) occur between the infected, not-treated sheep (Grp 1) and infected, treated sheep (Grp 2), attributable to the DMSO treatment, are indicated by "+".

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