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CANINE ANTI-ENDOTOXIN IMMUNOTHERAPY IN CRANIAL MESENTERIC ARTERIAL  
OCCLUSION SHOCK AND CANINE PARVOVIRUS DISEASE ENDOTOXAEMIA

by

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## PREFACE

The experimental work described in this thesis was carried out in the Department of Physiology, Faculty of Medicine, University of Natal, Durban, from January 1984 to June 1986. The clinical trial was performed at the Highway Veterinary Hospital, Union Lane, Pinetown, and in the Department of Physiology, Faculty of Medicine, University of Natal, Durban, from June 1984 to June 1986. In both instances, this work was carried out under the supervision of Professor S.L.Gaffin, Head, Department of Biochemistry, Department of Physiology, Faculty of Medicine, University of Natal, Durban.

These studies represent original work by the author and have not been submitted in any form to another University. Where use was made of the work of others it has been acknowledged in the text.

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(iii)

### ABSTRACT

Endotoxin (LPS, lipopolysaccharide) forms an integral part of the outer cellular membrane of gram negative bacteria (GNB). The canines' intestine always contains large amounts of GNB, and hence LPS. If these GNB with their LPS, remain within the intestinal lumen, they are not harmful to the host. When GNB do gain entry into a hosts' circulation a bacteraemia will occur with a concurrent endotoxaemia.

In the past, it had been accepted that GNB were, themselves, primarily responsible for the mortality and morbidity of bacteraemic and septicaemic patients. Evidence has emerged to indicate that this is not altogether true as isolated LPS, without the presence of GNB, can also lead to fatalities.

Circulating LPS is exceptionally chemically stable and highly toxic to host cells. Antimicrobial chemotherapy can destroy GNB, but this therapy does not reduce the toxicity of LPS, nor does it clear LPS from the circulation. Destruction of the GNB by certain antibiotics can, in fact, increase the concentration of circulating plasma LPS in a host.

The functional integrity of the intestinal wall is highly dependent upon an adequate blood supply, and the mucosal cells acts as the primary defence against the potentially pathogenic, endogenous and exogenous GNB and LPS. Once these pathogens become intravascular then the liver is the next most important organ of defence.

(iv)

Shock, irrespective of its aetiology, without adequate therapy, leads to reduced micro-vascular circulation, and thus a state of either localised or generalised hypoxia occurs.

Partial or complete intestinal vascular ischaemia will produce a state of regional hypoxia, and lead to damage of the intestinal wall allowing GNB, with their LPS, or LPS by itself, to enter into the hosts' blood circulation. Therefore, an aetiology that gives rise to any type of "classified shock," may eventually give rise to concurrent endotoxaemia.

In clinical practice there are numerous different diseases, physical onslaughts, and either acquired or congenital anatomical defects, that can give rise to intestinal vascular ischaemia, and hence, endotoxaemia. Many treatment regimens to combat the effects of an endotoxaemia have been advocated over the years, but this problem still has an unacceptably high mortality and morbidity index, probably because almost all such therapeutic regimens fail to destroy the LPS molecule.

Recent clinical studies have shown that immunotherapy is effective in combating gram negative bacteraemia and septicaemia in humans and animals. Research workers have been able to produce a "broad-spectrum" or "polyvalent" equine, hyperimmune, anti-endotoxin antibody-enriched plasma (ANTI-LPS), with favourable responses recorded when this plasma was used to treat a variety of experimentally-induced endotoxin-shocked subjects. ANTI-LPS significantly reduced the mortality in experimentally produced superior mesenteric arterial occlusion endotoxaemia in rabbits, presumably by neutralizing and opsonizing the circulating plasma LPS.

Equine practitioners have reported successful results when ANTI-LPS was incorporated into the treatment of certain medical and surgical equine endotoxic related problems.

A very recent, independent, Canadian study showed the effectiveness of ANTI-LPS, where this preparation was tested against other anti-LPS products, to treat experimentally-induced sepsis in rats. The polyvalent equine ANTI-LPS was the most effective, in that its use resulted in the longest survival.

In order to establish the generality of the use of equine ANTI-LPS plasma, I have extended these studies to the canine, since an abdominal vascular ischaemia carries a serious, high-risk, surgical emergency with unsatisfactorily high mortality rates, despite successful surgical intervention with concurrent supportive medical therapy.

Twenty healthy dogs were divided into four groups; a control group (n=5) and three experimentally treated groups (n=5 in each group). All twenty dogs were subjected to the well-documented cranial (superior) mesenteric arterial occlusion (CMAO) shock model. The three experimental groups received the polyvalent equine, ANTI-LPS at different times and by two different routes, with no side effects being observed in any of these dogs. One group (n=5) received ANTI-LPS s.c. before CMAO was performed, a second group (n=5) received their dosage of ANTI-LPS i.v. during the three-hour occlusion period, and a third group (n=5) received their dose s.c., within three minutes after the CMAO was released. Survival was recorded when any dog lived for a minimum of 14 days after the occluded

vessel was released. All 5/5 (100%) controls died within 17 hours after the release of the occluded vessel, whereas only one of the 15 (6,5%) experimentally ANTI-LPS treated dogs died ( $P < 0,001$ ) during a fourteen-day period following the release of the vessel. It was concluded that the polyvalent equine ANTI-LPS produced the significant difference in the survival rate that was recorded in this research.

The experimental therapeutic importance of this immunotherapy was then applied to the clinical environment of routine veterinary practice. Patients with Canine Parvovirus Disease (CPV) are known to die from the combined effects of the viraemia, a septicaemia, and an endotoxaemia. In this study, CPV patients admitted to a private veterinary hospital were randomly divided into two groups, where one group ( $n = 89$ ) received ANTI-LPS whilst the control group ( $n = 36$ ) did not. The ANTI-LPS-treated group had a significantly greater survival rate (83% vs 16% :  $p < 0,001$ ).

In summary, in this work I found that equine ANTI-LPS hyperimmune plasma significantly reduced mortality in endotoxin shock caused by both intestinal ischaemia and Canine Parvovirus Disease.



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"Errors have a way of becoming established by a kind of law of mass action. If something is said or written often enough, regardless of whether it is wrong to start with, it becomes accepted. As far as I know nothing can be done about it. Thus a reasonable error, soon mimicked and often repeated, may lead to the establishment of the error in acceptable form.....Thus we have the interesting phenomenon of an infinite number of wrongs making a right."

William B. Bean. (1975).\*

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\*W.B.Bean : Medical Writing : Discrepancies between theory and practice; in S.B.Day (ed) Communications of Scientific Information, Barsel S. Karger, 1975, Chapter 1.

Shock can be likened to a mysterious footprint on the early morning beach, after the high tide has receded. Who made it? It does not really matter, as, at the next high tide, a wave will rise to wipe it out, as easily as shock will claim its next victim.

Shock is a serious medical crisis, so often a prelude to death, and a recurring problem in every-day emergencies.

Shock is a mysterious malady which has no respect for breed, age, sex, weight, colour, general health status, or medical speciality.

Shock is usually recognised from behind, while the clinician surveys the widespread destruction in its wake. Attempts are being made to recognise shock as it approaches, and to try and divert its often fatal course.

Shock has many and varied aetiological agents. It is a syndrome of responses to cellular injury, and at some point these responses lead to a common pathway, which is recognised as the patient in shock.

Shock, whatever its initiating cause, eventually leads to a generally agreed-upon common pathophysiological injury, namely, a reduced microvascular perfusion and hence, a decreased oxygen supply to the critical cells.

## CHAPTER ONE

### INTRODUCTION

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## 1.1. HISTORY OF ENDOTOXIN SHOCK

Endotoxin shock is a pathophysiological phenomenon resulting from the release of LPS from dead GNB,<sup>1</sup> or following experimental parenteral and per os administration of LPS.<sup>2-4</sup> The pathophysiology of endotoxin shock has been a major subject of research for many years, and has resulted in enormous bibliographies in reviews of this vast subject.<sup>5,6</sup>

It has been said that the history of endotoxin shock research goes back many years, and could have begun with observations by physicians who were attending patients ill with the manifestations of fever, observations as old as the study of medicine itself.<sup>7</sup> This universal interest in LPS could possibly have been due to the fact that:

1. bacterial endotoxins evoke a variety of biological responses which are reported to have adverse effects on almost all host organ systems,<sup>8</sup>
2. GNB infections, sepsis and endotoxaemia are important medical and surgical problems, because patients with this type of infection may be life-threatening, despite the use of antibiotics in conjunction with other life-supporting therapy.<sup>6,9</sup>

Brill and Libman, cited by Hinshaw, described the first case of gram negative bacteraemia in 1899.<sup>5</sup> In the same year Crile, cited by Agawal, pointed out that the maintenance of an adequate blood volume and central venous pressure, with an i.v. infusion of warm saline, led to reduced mortality in experimental haemorrhagic shock.<sup>10</sup> However, Westphal states that it was left to Richard Pfeiffer who, in 1904, introduced the term "endotoxin",<sup>11</sup> a heat-stabile and toxic chemical released from

disintegrating bacteria. He used this term to distinguish the chemical from "exotoxin", which is a heat-labile chemical released from living bacteria.

The modern understanding of shock was reported by Thal to have been based on field studies conducted behind the fighting lines during the First World War by several workers including Cannon, Bayliss and Mclean.<sup>12</sup> Cannon pointed out that acute circulatory failure was produced by hypovolaemia and from this work stemmed the belief that shock was the result of tissue hypoxia.

Spink and co-workers gave one of the first indications that LPS participates in the genesis of shock.<sup>13</sup> This arose from their clinical observations following the use of an antibiotic, aureomycin, to treat patients with Brucella melitensis. In 1951, Waisbren described how patients with a bacteraemia exhibited the "typical shock picture" of peripheral vascular collapse.<sup>14</sup> During the same year, Borden and Hall described a fatal case of shock as the result of bacteria-contaminated blood transfusions.<sup>15</sup> In 1953, Braude and co-workers experienced and described the same condition by using blood contaminated with GNB.<sup>16</sup> Three years later, Studdiford and Douglas described shock as a sequel to septic abortion.<sup>17</sup>

These early, and extremely important observations and recordings opened the door to a vastly expanded, and still expanding, area of medical and veterinary research, in mans' endeavour to gain knowledge that will be beneficial to humans and animals alike.

Initially, endotoxin shock was thought to be due to GNB with its LPS,

where the two pathogens were exerting their effects in combination with one another. The combination of live GNB and LPS was shown to elicit an accelerated Schwartzman reaction or an acute allergic reaction, either of which could lead to shock.<sup>18</sup> This supported the concept that the GNB and LPS were acting together to produce their fatal consequences. Later, researchers were able to isolate LPS and administer it into experimental animals where fatalities were recorded.<sup>19</sup> This work was to form the basis of further in-depth projects, which showed that LPS is the mediator of the endotoxin-shocked patient. It was then recorded that LPS alone could cause shock, and that a concurrent bacterial infection produced other toxic problems in the same patient.<sup>20,21</sup> Irrespective of whether the GNB and LPS are producing their effects singularly or in combination with each other, it has been reported that these GNB account for the majority of "septic" shock cases.<sup>19</sup>

The source of the GNB now came into being and in 1974 Goldstein<sup>22</sup> recorded that dogs always have GNB present in their gut in health and in sickness, which supported the work of earlier researchers.<sup>23</sup> Therefore these intestinal commensals should be described as the endogenous source of GNB and LPS. However, GNB and LPS, singularly or together, may gain entry into a host via a variety of sources (through the skin,<sup>24-26</sup> respiratory system,<sup>27</sup> per os<sup>28,29</sup>, etc) and these sources of infection must therefore be exogenous.

Initially the toxic effects of bacterial LPS under experimental conditions could only be demonstrated when they were given parenterally.<sup>30,31</sup> However, researchers were able to demonstrate the effects of transmural migration of the LPS by itself, and the lethal effects produced by this migration of LPS in the host.<sup>28</sup>

The symptoms of various types of shock generally resemble endotoxin-induced experimental shock. Because of this fact, it was suggested that LPS played an important role in the pathogenesis of shock. In this respect, endotoxin shock has become a popular model in shock research.<sup>31,32</sup> It has also been recorded that the limulus amoebocyte lysate test with its various modifications, had become popular for the demonstration of LPS in a host's circulating plasma.<sup>33-37</sup>

Further research has shown that the endotoxin shock process appeared to have an immunological basis which involved the release of vasoactive substances, and neuro-endocrine agents, with the activation of the coagulation and complement pathways.<sup>6,38-40</sup> It has also been documented that LPS is a potent immunosuppressor<sup>41</sup> even though they are known to be immunogenic.<sup>11,42</sup>

Current research on the problem of shock is no longer concerned with the initiation of a shock syndrome, but with the mechanisms responsible for the progressive, and seemingly inevitable, decompensatory deterioration during profound and protracted shock. There are from 15 to 20 different ways of producing circulatory collapse in as many as 8 to 10 different animal species, and in 1982 Hinshaw said " We are now able to produce shock, but we are still a long way from recognising its development in the natural case, as well as in the prevention of it."<sup>5</sup> The therapy of the shock syndrome has advanced, and the research towards its elimination in practice has gained momentum world-wide. Our ultimate goal must be to recognise endotoxin shock in its developmental stages, and then to eliminate its often fatal course.

## 1.2. MEDICAL SIGNIFICANCE OF ENDOTOXIN SHOCK

In American surveys, it was reported that approximately 300,000 persons develop GNB-sepsis in hospitals annually, and about 100,000 of them die.<sup>13,43</sup> Another survey estimated that out of 333,000 bacteraemic patients in the United States, 132,000 deaths occurred.<sup>44</sup> Yet another report, this time from Japan, stated, "despite the judicious use of antibiotics, mortality due to endotoxin shock is still very high, and this type of shock produces substantial mortality in hospitalized patients."<sup>1</sup> It has been estimated that between 1 and 5% of all hospitalised patients develop secondary GNB infections and, in a significant number of these cases, bacteraemic sequelae are the primary cause of morbidity and mortality.<sup>44,45</sup>

The extent to which modern health care practices or nosocomial agents contribute to an incidence of secondary infections remains conjectural. Mortality associated with established gram negative bacterial sepsis remains distressingly high, and current evidence indicates that the frequency of bacteraemic complications are on the rise.<sup>46</sup>

During a ten-year period from 1972 to 1982, surgical patients were assayed for the presence or absence of circulating plasma LPS.<sup>47</sup> Of 487 patients tested for the presence of LPS, 222 were shown to be positive. The mortality rate in these 222 patients was 52.2% and the authors stated that circulating plasma LPS was the lethal factor. Hinshaw, in his address at the annual congress of the Physiology Society of South Africa in Pretoria (1984), stated that the mortality rate in endotoxin

shock was 30 to 80% world-wide.<sup>43</sup>

Despite aggressive surgical treatment, specific antibiotic therapy and other pharmacological support, the mortality rate of human endotoxaemia is still between 30 to 70%.<sup>48</sup> These percentages differ from each other, which is possibly due to the fact that the work was undertaken at different centres in different countries. Absolute figures are generally not forthcoming in veterinary literature, but a report did state that endotoxin shock is often an unrecognised clinical entity that is associated with a high rate of mortality.<sup>49</sup> A recent report has recorded that during a ten-year period from January 1974 to December 1983, the overall mortality rate of dogs treated at a veterinary faculty, for sepsis, was 71%.<sup>50</sup>

There has been a tendency for the veterinarian to record the death of a patient by the anatomical aetiology, or by the disease aetiological diagnosis. For example, Canine Parvovirus Disease has had reports of up to 80% mortality yet death in these patients is actually due to secondary endotoxaemia.<sup>51-55</sup> Similarly, gastro-splenic torsion is associated with over a 50% mortality in spite of successful surgery.<sup>56</sup> It has, however, been established that the latter cases have high concentrations of plasma LPS just prior to death.<sup>57</sup> With all the above facts in mind, the overall mortality rate of the veterinary endotoxin shock patient probably has similar percentages to those recorded for the human, or even higher.

GNB and LPS are known to exert profound multiple biological effects in the host. Thomas, in 1974, made the following very significant comment, "Our arsenal for fighting off bacteria are so powerful, and involve so

many different defence mechanisms, that we are in more danger from them than from the invaders. These macromolecules (LPS), are read by our tissues as the very worst of bad news. When we sense lipopolysaccharide (LPS), we are likely to turn on every defence at our disposal."<sup>5</sup> It is known that the LPS have serious adverse effects on coagulation and complement pathways, on the platelets and the leucocytes, on endothelial cells, and a wide variety of other host cells which are doing their best to preserve life.<sup>6,58</sup> These reactions will be discussed in a section to follow (see Chapter 2). Body defence mechanisms usually destroy or reduce the accumulation of circulating plasma LPS slowly, thus LPS is capable of remaining in circulation for a long time after the parent GNB have been destroyed by antibiotics or by the host's natural defence processes.<sup>13,59,60</sup>

Lethal dosages of live E.coli have been administered to different species of experimental animals with resultant death being reported as being due to the E.coli.<sup>61-63</sup> In the practise of medicine this is an important statement, as theoretically, if the bacteria are destroyed, then survival should improve. These statements may not be entirely true, as recent ongoing research has demonstrated that when primates were given a LD<sub>100</sub> dose of live E.coli i.v., no bacteria were cultured from the systemic circulation at the time of the death.<sup>64</sup> On the other hand, circulating LPS concentrations at death were extremely high when compared to baseline concentrations in the same animal. This could indicate that although the bacteria were destroyed by host defence mechanisms, the LPS released from such destroyed bacteria was not eliminated or neutralized. Another explanation could be that the E.coli could have produced intestinal hypoxia, with a resultant entry of

endogenous LPS into the vascular system, resulting in mortality.

Endotoxaemia is now considered to be an important contributory factor towards the mortality and morbidity associated with septic and non-septic shock in man and animals.<sup>19,20,49</sup> There is a critical need for research in clinical endotoxin shock to circumvent the unacceptably high mortality and morbidity rates that are still recorded. There is also a serious need for research regarding the basic mechanisms responsible for the all too often fatal consequences of the circulating plasma LPS.

### 1.3. ANIMAL MODEL FOR ENDOTOXIN SHOCK RESEARCH

Many questions have arisen concerning the adequacy of animal shock models with regard to their application in human endotoxin shock. However, as these questions were being raised, our knowledge of endotoxaemia increased in veterinary medicine. In 1964, Waisbren,<sup>14</sup> made what was then a very important statement, "in the last analysis, the true explanation of the pathophysiology of gram-negative shock will come from more intensive studies of patients while they are suffering from the condition and, perhaps more practically, from intensive study of an animal model .... These further studies .... may prove that endotoxin has a role in gram-negative shock." Later, Weil<sup>65</sup> commented, "the extent to which the reactions to endotoxin in one type of experimental animal serves as an applicable model of bacterial shock in another type of animal is controversial."

In order to develop more realistic animal models, recent emphasis has been placed on the administration of live organisms rather than



endotoxin, and results from these two means of eliciting experimental septic shock have been compared.<sup>19</sup> The use of live E.coli organisms introduced into the normal animal seems to possess the more clinically relevant characteristics of a slow release of endotoxin within the host's circulation following endogenous destruction of the E.coli.

Other models include:

1. the canine "septic leg" procedure,<sup>66</sup>
2. the injection of live E.coli into the canine gallbladder following division of the cystic artery and duct,<sup>67</sup>
3. the placing of human faecal material within gelatin capsules which are then introduced into the abdominal cavity of the experimental subject,<sup>68</sup>
4. the performing of an appendectomy in dogs without suturing the caecal stump to allow the contents of the caecum to escape directly into the peritoneal cavity,<sup>69</sup> and,
5. caecal ligation in the dog.<sup>70</sup>

Researchers showed that occluding the CMA in dogs produced endotoxaemic death.<sup>71</sup> What was more fascinating was that they recorded the fact that in those dogs where the occlusion was not released, these dogs took longer to die compared to those who had the occlusion released. This is a very typical and recorded observation in the clinically presented case of the gastric-dilatation-volvulus syndrome with or without splenic and/or intestinal involvement. Similar personal observations have been made with regards to the traumatically produced diaphragmatic hernia with herniation of the small intestine into the thoracic cavity. The occlusion of the CMA has been found to be a very reliable method of producing endotoxaemia in the dog.<sup>72,73</sup>

Papa et al were able to demonstrate that LPS from intra-intestinal bacteria were capable of entering the general circulation transmurally from the caecum.<sup>28</sup> This mode of entero-endotoxaemia is further supported by researchers who found that patients with liver cirrhosis showed positive endotoxaemia especially if an ascites was present.<sup>74</sup>

#### 1.4. DEFINITION OF SHOCK

Three common misconceptions about shock are perpetuated in most textbooks and have obscured the understanding of its essential nature.

These are:

1. that shock is a single disease entity,
2. that the physiologic defect of shock is hypotension and low cardiac output, with high peripheral resistance, and,
3. that this physiologic picture characterises all stages of all aetiologic types of shock.

These misconceptions most probably arose because of a failure to recognise that physiologic derangements start with the onset of the precipitating aetiological event, and not when the patient is first recognised as being in shock, nor at the terminal stage when they become refractory to therapy.

A definition of shock which is satisfactory to all researchers and clinicians is difficult to produce. However, based on present day knowledge it should include the facts that:

1. it is more of an acute process than a chronic one,
2. it is not a single entity but a syndrome of manifested problems,

3. the hallmark of all types of shock is the altered micro-vascular circulation, so that whether the total blood flow is low, normal or high, critical organs in the patient are inadequately perfused.

Therefore, shock may be defined as an acute syndrome of cardiovascular failure, in which many vital organs are inadequately provided with nutrients and oxygen, which in turn leads to their inadequate function and prolonged recovery or possible death.

Inadequate blood perfusion indicates not only inadequate nourishment to, but also inadequate waste removal from cells, organs and tissues. This inadequate perfusion is capable of leading to a state of localised or generalised hypoxaemia with resultant ischaemia. In 1976, Dr. Leena Mela<sup>75</sup> cited two researchers by stating that "an adult mammal can withstand total lack of oxygen for only a couple of minutes, before vital intracellular functions are destroyed, resulting in the ultimate loss of organ function."

### 1.5. AETIOLOGICAL CLASSIFICATION OF SHOCK

Aetiological classification has contributed to the apparent confusion encountered when shock is discussed. Terminology such as "surgical" "cardiogenic", "neurogenic", "traumatic", "ice pick", "Chevrolet", "anaphylactic", "oligaemic", "haemorrhagic", "hypovolaemic", "septic", "bacteraemic" "septicaemic" and "toxic", should be removed from the vocabulary of shock as they unfortunately cloud the happenings during an episode of shock.

Four general forms of shock, according to a recent classification, are

hypovolaemic, cardiogenic, distributive, and obstructive shock.<sup>76</sup> Endotoxin shock has been placed in the category of distributive shock by this author, who terms it "bacterial shock". As such, it possesses a defect in blood volume distribution in as much as alterations in capacitance account for sequestration of blood in the venous circuit.

#### 1.6. CLASSIFICATION OF ENDOTOXAEMIAS

The liver has been shown to be the main organ of defence in the endotoxaemic patient.<sup>37,59,77</sup> A recent study has suggested a means of actually classifying endotoxaemic patients according to the presence or absence of circulating LPS in various blood vessels.<sup>47</sup> Of 237 patients with digestive disorders, 83 (35%) were positive for circulating plasma LPS. Oesophageal varices comprised 59 of these 237 patients, and of these 59 cases, 29 (49.2%) were positive for LPS. Blood was collected from the portal and hepatic veins and from a systemic artery in 23 of these 29 cases. These 23 cases were then classified according to where the LPS was found (see table 1, page 14).

It is interesting to note that these researchers did not record a value for the normal concentration of circulating plasma LPS. Therefore are these determinants of value, in as much as they could be "normal"?

However this type of classification could be a means of obtaining a prognostic evaluation of the endotoxaemic patient and assist in the type and intensity of therapy, where and when it is practically possible to catheterise the hepatic-portal blood vessel.

**Table 1. THE PRESENCE OF LPS IN VARIOUS BLOOD VESSELS**

TYPE*	PORTAL VEIN	HEPATIC VEIN	SYSTEMIC ARTERY	NO. OF CASES
1	+	+	+	6
2	+	+	-	3
3	+	-	-	4
4	+	-	+	6
5	-	-	-	4

\*Type 1: All the blood samples were positive for LPS.

Type 2: LPS was only detected in the portal and hepatic veins.

Type 3: Only the portal vein had detectable LPS concentrations.

Type 4: LPS was only present in the portal vein and systemic artery.

Type 5: LPS was not detected in any of the blood sampled.

### 1.7. THE STRUCTURE OF THE GRAM NEGATIVE BACTERIAL CELL WALL

Bacteria are divided into two major groups, gram positive and gram negative, depending on their reaction to a Gram staining procedure.<sup>78</sup> The reaction of the bacterium to the stain, which differentiates these two groups, will depend upon the chemistry of the relevant bacteria's outer cell membranes.

Gram negative bacteria have a thin peptidoglycan layer adjacent to the outer cell membrane surface.<sup>79,80</sup> Lipoprotein serves to cross-link this substance with the outer cell membrane. Lipopolysaccharide is associated

with the outer cellular membrane, and it is this biochemical molecule which contributes most of the uniqueness to the GNB outer cellular membrane. A diagrammatic representation of the GNB outer membrane is shown in figure 1.<sup>78</sup>

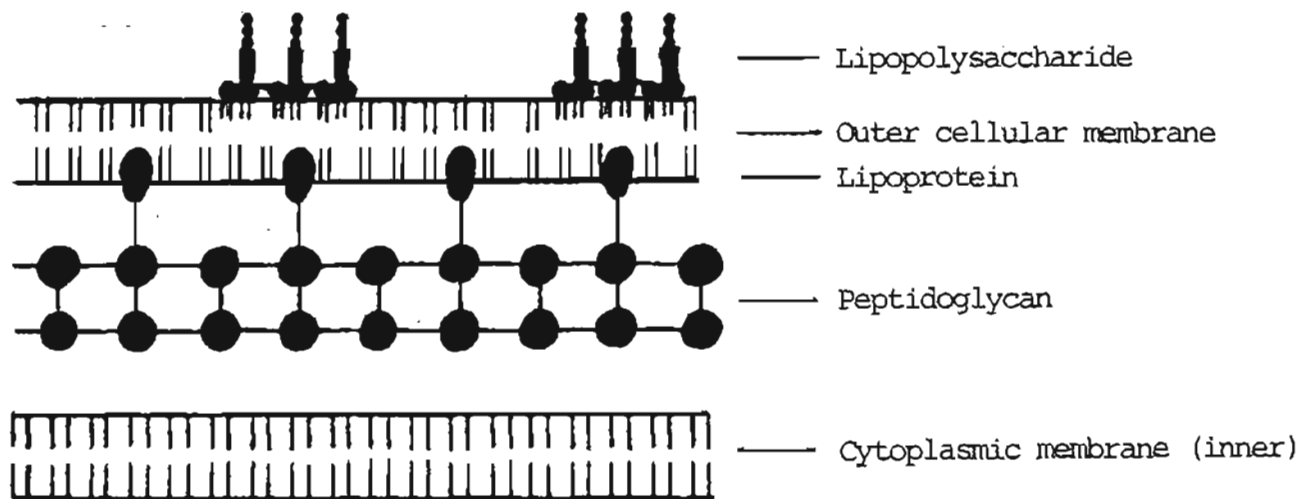


Fig. 1. Diagrammatic illustration of the structure of the outer cell membrane of gram negative bacteria.

### 1.8. BIOCHEMICAL STRUCTURE OF ENDOTOXIN

Lipopolysaccharides (endotoxin, LPS) are pathogenic agents of GNB, responsible for many pathological alterations seen during gram-negative infections.<sup>5,6,8</sup> Robert Pfeiffer (1904) introduced the term endotoxin to distinguish this heat-stable and toxic chemical from exotoxin, which is the heat-labile chemical that is released from living bacteria during an infection.<sup>11</sup> Endotoxin is mainly released when the bacteria disintegrate, by lysis<sup>49,59</sup> or by chemotherapeutic agents,<sup>81,82</sup> and possibly when they undergo active replication.<sup>49</sup>

LPS is not an exogenous product of GNB. After their biosynthesis in the cytoplasmic membrane, they are transported to the surface where they are retained as a vital constituent of the outer membrane of the cell. In this exposed location they also function as the characteristic "O" antigens of these bacteria.

LPS conform to a common biochemical structural principle, consisting of a polysaccharide, an oligosaccharide and a covalently bound lipid component. The oligosaccharide consists of two sub-components which are under separate genetic control.<sup>83,84</sup> The molecular weights of LPS from different GNB species vary from between 400 000 to about 4 000 000 daltons, and these can be further modified by varying the nature of the solvent.<sup>85</sup> In summary, figure 2 schematically shows the structure of a typical Salmonella LPS.<sup>83</sup>

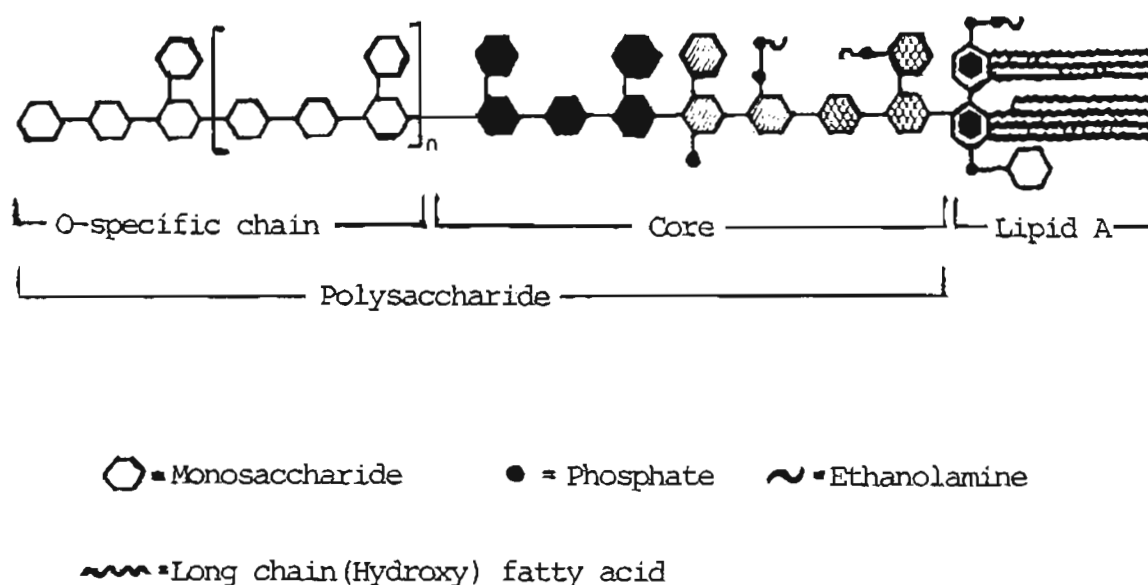


Fig.2 : Schematic structure of the Salmonella LPS.

### 1.8.1. POLYSACCHARIDE COMPONENT

The "O" antigen specific side chain is the hydrophilic portion of LPS and is a polymer of saccharide molecules, designated the "repeating unit". Many types of sugars have been found as constituents of the O-chain and include neutral sugars with 5 - 7 carbon atoms, deoxy and amino sugars, uronic and aminuronic acids, O-methyl, O-acetyl, and phosphate substituted sugars.<sup>86</sup> This variability of constituents and their linkages produces an immense diversity of O-specific chains. The nature, sequence, and type of linkage of the O-chain, is characteristic and unique for a given LPS from a given parental GNB strain. Serological and biological investigations revealed that the O-chain produces the O-antigenicity which is used to classify GNB.<sup>84-86</sup>

The O-antigenic groups may affect the expressed pathogenicity which is contained totally in the toxic lipid-A region. These O-chain groups do not possess any toxic effects themselves, and are thought to be engaged in the protection of the bacteria from host enzymes and phagocytic cells.<sup>87</sup> Anti-"O" antibodies are relatively specific for the GNB serotype and are protective against homologous bacterial strains only, cross protection in the host being limited.<sup>84</sup>

### 1.8.2. OLIGOSACCHARIDE COMPONENT

The oligosaccharide component covalently binds the lipid-A to the "O" polysaccharide. It is composed of branched hetero-oligosaccharides which show some structural variability.<sup>87</sup> The oligosaccharide and lipid-A, together, are known as "core glycolipid". At least six individual



oligosaccharide types have been identified and they show only minor differences in their composition.<sup>11</sup> In general, two core types are now recognised, an inner core of 3-deoxy-D-manno-octulosonic acid (KDO), and an outer core made up of different glucose molecules. The core is immunogenic but it does not express as much diversity as the O-antigen. It has been shown that anti-core antibodies bind to a variety of LPS, since there are few core types.<sup>88-90</sup>

### 1.8.3. LIPID-A COMPONENT

Lipid-A is the hydrophobic portion of LPS and consists of a phosphorylated hexosamine disaccharide to which long chain fatty acids are bound.<sup>88-90</sup> Isolated Lipid-A has been recorded as being weakly immunogenic and some lipid-A specific antibodies may be detected in host blood in response to a suitable challenge.<sup>84</sup> In more recent studies it would appear that this is not entirely true because when LPS was bound to a GNB, then both the GNB and the LPS act as a stronger antigen in the host.<sup>91</sup> This lipid-A contains large amounts of beta-hydroxymyristic acid which is incorporated into its toxic site.<sup>84</sup> In this respect, the lipid-A demonstrates a wide diversity of biological activity, as has been shown by researchers using soluble forms of free lipid-A.<sup>91</sup> Table 2 (page 19) summarizes the various activities that have so far been demonstrated with soluble forms of lipid-A.<sup>92</sup>

A synthetic analogue of lipid-A has been prepared which is almost fully active in all lipid-A toxic properties.<sup>93</sup> Investigations have shown that the lipid-A's of many, but not all, GNB strains are structurally closely related.<sup>86,87,92</sup> and the lipid-A represents the least variable part of the LPS molecule.

TABLE 2. BIOLOGICAL ACTIVITIES OF FREE LIPID-A.

Pyrogenicity	Lethal toxicity in mice
Leukopaenia	Leukocytosis
Local Schwartzman reaction	Bone marrow necrosis
Embryonic bone reabsorption	Complement activation
Depression of blood pressure	Platelet aggregation
Hageman factor activation	Limulus lysate gelation
Induction of plasminogen activator	Toxicity enhanced by BCG
Tumor necrotic activity	Toxicity enhanced by adrenalectomy
Adjuvance activity	Mitogenic activity for cells
Macrophage activation	Hypothermia in mice
Induction of non-specific resistance to infection	
Induction of early refractory state to temperature change	
Enhanced dermal reactivity to epinephrine	
Induction of IgG synthesis in newborn mice	
Induction of prostaglandin and interferon synthesis	
Induction of mouse liver pyruvate kinase	
-----	

Anti-lipid-A immunoglobulins occur naturally in the serum of normal animals and humans, and significantly more frequently in sera of patients with chronic urinary tract infections.<sup>91</sup> Anti-lipid-A IgG's exhibit biological activities that may be protective or damaging. Under defined experimental conditions, anti-lipid-A antisera suppressed the pyrogenic activity of LPS,<sup>94</sup> it protected mice from the abortive effect of LPS<sup>95</sup> and from infection with Salmonella typhimurium.<sup>96</sup> On the other hand, anti-lipid-A antibodies were shown to mediate the development of

kidney damage induced by lipid-A.<sup>97</sup>

### 1.9. AETIOLOGY OF CIRCULATING PLASMA ENDOTOXIN

LPS may gain access into the host's circulation as the result of very wide and diversified aetiological onslaughts from both physical and biological conditions. Once in the host, this LPS will interact with numerous host-defence mechanisms, which in turn will activate other harmful pathways, causing cellular injury and possible depletion of essential elements which are needed to sustain life. These interactions are discussed in more detail in a later chapter (refer chapter 2).

When GNB gain access to the host's circulation, LPS in the outer membrane is accessible to the blood where it will begin to exert its toxic effects. There are numerous GNB infectious and contagious diseases of man and animals, which are therefore essentially endotoxaemias. Zoonotic GNB infections may also be an important source of a potential endotoxaemia which is often overlooked. Traumatic wounds that become contaminated with faecal GNB or from the external environment, may also provide aetiology. All these causes are possible exogenous sources of an eventual endotoxaemia.

As mentioned earlier in this work, the gastro-intestinal tract plays host to a myriad of different GNB. Normally these GNB with their LPS are not harmful to the host if they remain within the lumen of the gut. The gut wall acts as the prime defence against the invasion of these potential pathogens.<sup>29</sup> Therefore, almost any damage to the gut mucosa will allow the entry of these bacteria and LPS into the general circulation of the host. These represent the possible endogenous source

of endotoxaemia.

It has been shown that pathological events within and outside the gut lumen are capable of producing an often irreversible damage to the defence mechanism of the gut mucosa. For example, peritoneal dialysis for kidney disease can produce a transmural migration of intestinal bacteria into the host's circulation.<sup>31</sup> In fact, early studies on pathology caused by various dialysing fluids, led Jacob Fine and others to postulate that intestinal damage was probably the most important cause of irreversible shock.<sup>31,98</sup> Later it was shown that CMAO, when released produced fatality, but the actual pathogen was not isolated.<sup>71</sup> In addition, it was demonstrated that transmural migration of intestinal endotoxin occurred after a period of colonic ischaemia without the migration of their gram negative bacteria.<sup>28</sup>

Non-infected or aseptic wounds are also known to evoke an uptake of endogenous LPS. A study was undertaken in rats which had been subjected to dermal burning, using hot water.<sup>24</sup> An endotoxaemia was detected within 30-40 mins in venous blood draining the burnt area and at 90-120 mins in arterial blood after the experimental hyperthermia had been completed. E.coli and Klebsiella were recovered from the plasma and livers of these experimental animals, but not in sufficient amounts to produce detectable LPS. When blood draining the burnt area without any detectable LPS was injected i.v. into other normal rabbits, a fatal endotoxaemia was produced in such recipients within 12-18 hours. On the other hand if the second rabbit's intestine had been sterilised with a non-absorbable antibiotic then the animal survived the subsequent challenge with the "burnt" blood. On this basis it appeared that a

vasoactive substance was released from the burn into the circulation, producing a generalised increase in vascular permeability. This in turn allowed the transmural migration of endotoxin from the intestinal lumen.

Catecholamines and/or other vasoactive agents are released from damaged tissues. These chemicals circulate and are capable of producing regions of localised intestinal ischaemia. Thus, secondary intestinal mucosal damage results, causing an elevated escape of LPS from the gut to the hepatic-portal circulation.<sup>24</sup>

Once LPS has entered the circulation from the gut, its accumulation in the blood and tissues is in part largely due to the failure of the host's hepatic or systemic antibacterial and detoxifying defences, as well as the concentration of circulating plasma high-density lipoproteins.<sup>59,99,100</sup> Furthermore, experiments have shown that the prolonged infusion of epinephrine, norepinephrine, bradykinin or serotonin can lead to fatal endotoxaemia.<sup>6</sup>

#### 1.10. MEASUREMENT OF CIRCULATING PLASMA ENDOTOXIN

*Limulus polyphemus* is commonly known as the horseshoe crab. This crab has amoebocytes contained in its haemolymph, which can lyse to form a protein solution. This limulus amoebocyte lysate (LAL) is particularly sensitive to the presence of LPS and forms a firm gel or clot in the presence of less than  $10^{-9}$  g/ml LPS. This finding created a new horizon with regard to the diagnosis of the endotoxaemic patient.<sup>101,102</sup>

The crab's haemolymph is collected by inserting a needle into a joint between the cephalothorax and the abdominal region. About 50 to 250 ml

haemolymph can be collected from each crab and this haemolymph is then centrifuged to separate the amoebocytes.<sup>102</sup> Normally, these amoebocytes have a coagulation system which participates in both haemostasis and defence against invading micro-organisms in the crab. This coagulation system consists of several protein components, some of which are highly sensitive to LPS.<sup>101,102</sup>

The LAL-gel test is used to detect the presence of circulating plasma LPS qualitatively, because the test lacks a degree of reliability and reproducibility, with both false positives and false negatives reported,<sup>35,103</sup> for example, synthetic dextran derivatives can produce positive LAL assays.<sup>104</sup> These and more recent findings question whether a positive LAL test proves that a plasma sample actually contains LPS.<sup>105,106</sup> However this test has been used for the diagnosis of endotoxaemias and meningitis and for the detection of LPS in blood samples.<sup>107</sup>

To date several modifications of the conventional LAL gel test have been reported to improve the sensitivity and accuracy of the test.<sup>108</sup> These tests have been based on, for example:

1. nephelometry,<sup>109</sup>
2. spectro-photometric assay for use with seawater,<sup>110</sup>
3. a turbidometric method,<sup>111</sup> and,
4. the measurement of the amount of protein gelled during the clot reaction.<sup>112</sup>

The recent introduction of the chromogenic substrate method provides the greatest sensitivity and reproducibility required for the quantitation of circulating plasma LPS.<sup>36,102</sup> These newer methods

incorporate a chromogenic substrate (Boc-Leu-Gly-Arg-p-nitroanilide) or fluorogenic substrate (Boc-Leu-Gly-Arg-4-methylcoumarin-7-amide) for the amoebocyte clotting enzyme, which had been activated by LPS. In these techniques the activated LAL enzyme splits a peptide bond, resulting in a marked increase in the absorbance quantum yield of terminal chromophore or fluorophore. The chromogenic substrate method allows determination of LPS concentrations in circulating plasma as low as 20 pg/ml, with accurate and replicable results.<sup>37</sup>

#### 1.11. NORMAL CONCENTRATION OF CIRCULATING PLASMA ENDOTOXIN

A study has recently been completed wherein it was established that the circulating blood of normal healthy dogs contains LPS at a concentration of  $0.053 \pm 0.004$  ng/ml.<sup>113</sup> These circulating plasma LPS molecules, could have either beneficial or no effects, or continuous harmful effects, as yet unknown in the normal healthy individual. Certain experiments in man have been performed, in which beneficial effects of LPS were recorded,<sup>11</sup> and it may be possible to extrapolate these findings to the canine.

## CHAPTER TWO

### PATHOPHYSIOLOGICAL ASPECTS OF CIRCULATING PLASMA ENDOTOXIN

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## 2.1. MITOCHONDRIAL ENERGY-LINKED FUNCTIONS

Mitochondrial energy-linked transfer reactions fulfill almost 95% of the body's energy needs<sup>75</sup>. To support these vital activities these organelles utilise over 90% of the available cellular oxygen under normal circumstances. Therefore adequate cellular oxygenation is essential for the continuation of mitochondrial metabolism.

Intracellular oxidative glucose metabolism proceeds through the citric acid cycle (Krebs cycle) for adenosine triphosphate (ATP) production. In this process the energy provided by the oxidation of one molecule of glucose is used to form 36 ATP molecules.<sup>75</sup> ATP synthesis is not possible without the correct function of the adenine nucleotide translocase and the phosphate carriers. For these mitochondrial electron-transfer reactions to proceed uninhibited, adequate amounts of oxygen are necessary for the essential oxidative phosphorylation.

Given substrates and oxygen, in the presence or absence of adenosine diphosphate (ADP), the mitochondria is capable of assuming four states of respiratory activity.<sup>114</sup> State 3, which is the active state of respiration, is achieved during the synthesis of ATP when adequate amounts of ADP are available. State 4 is said to be a resting state when no ATP is synthesised, due to a high ATP : ADP ratio. This ratio, expressing the phosphorylation potential, controls the mitochondrial respiratory activity in the cell, that is, respiration is determined by the cell energy demand.<sup>115</sup>

An equally important function of the mitochondrion, in addition to oxidative phosphorylation, is its capacity to transport or "pump"

calcium internally from the cytoplasm. By doing so, mitochondria are partly responsible for the control of the concentration of intracellular unbound calcium. Therefore it is significant that this mechanism is energy dependent, is sensitive to ischaemia, and is more sensitive than the respiratory activity.<sup>116,117</sup> The processes by which the mitochondria regulate intracellular calcium consist of two separate pathways; one is responsible for the uptake of calcium from the intracellular fluid, whilst the other is for the release of the cation back into the intracellular fluid. The uptake pathway transports calcium inside the organelle, utilising ATP. The release pathway returns the calcium to the environment by simple diffusion. Consequently, calcium ions become available again to the uptake pathway, thus establishing the "calcium cycle".<sup>118</sup>

A lack of oxygen transport to a cell is a most damaging insult to its mitochondria.<sup>116</sup> After a long-term cessation of blood, and hence oxygen perfusion to an organ, the mitochondrial membranes become irreversibly damaged. Mitochondrial enzyme reactions involved in oxidative phosphorylation are inhibited concurrently with an increase in anaerobic respiratory activity.<sup>119</sup> However, hypoxia in vital organs does not fully explain mitochondrial damage in shock. Possibly, the reduction of blood flow to, and hence nutrients into, and waste products out of cells, may also constitute a key factor in mitochondrial injury.

In a shocked patient, it has been shown that anaerobic glycolysis plays an important role in providing the energy requirements. However, in this process a nett of only two ATP molecules are formed from the oxidation of one glucose molecule, which is only 5.6% of the total of 36 molecules

of ATP synthesized during oxidative glucose metabolism.<sup>75</sup>

In rats, cats and dogs in shock, at the same time that hypoxaemia causes a fall in ATP concentrations, tissue lactate concentrations and lactate/pyruvate ratios increase, and tissue pH falls.<sup>120</sup> Similar metabolic alterations have been demonstrated in experimental animals as a result of endotoxin shock.<sup>121</sup> Significantly, lowered tissue ATP concentrations and increased lactate concentrations are found after several hours of endotoxaemia in the brain, liver and kidney, but not in the cardiac or skeletal muscle of the animals studied.<sup>121,122</sup>

These alterations in tissue metabolite levels indicate metabolic failure in the vital organs of animals in endotoxin shock. In the liver, brain and kidney, mitochondria have been shown to become functionally damaged during an episode of endotoxaemia.<sup>123</sup> Dog's brain mitochondria are particularly sensitive to the presence of live E.coli in the blood, and at the time of the dog's death 60% of brain mitochondrial ATP synthetic capacity is inhibited, compared to only 30% in the kidney of the same dog.<sup>124</sup> It has been demonstrated that cerebral, hepatic, and renal blood flows are significantly diminished in endotoxin shock whereas, only slight alterations occur in the coronary flow of dogs and sub-human primates.<sup>123</sup>

Within the cell itself, there is a 60-70% reduction of mitochondrial ATP content during the late stages of an endotoxaemia. At the same time, adenine nucleotide translocase can no longer transport ADP or ATP across mitochondrial membranes, thereby further inhibiting ATP synthesis.<sup>123</sup> This can result in a reduction in the activity of membrane ionic pumps, which in turn causes reductions in intracellular  $K^+$  and  $Mg^{2+}$  and an

increase in  $\text{Na}^+$ ,  $\text{Cl}^-$  and free  $\text{Ca}^{2+}$ .

## 2.2. THE CLEARANCE OF CIRCULATING PLASMA ENDOTOXIN

The rate of LPS uptake into the vascular circulation would appear to be partly dependent on the site and the source of aetiological entry, the degree of concurrent tissue injury and the health status of host defence mechanisms. The uptake from secondary wound infection depends upon the size of the wound, the degree of contamination, and the speed of bacterial replication. The latter is in turn influenced by the type of organism involved and the medical attention that the infected area has or has not received.

Experimental administration of either LPS or live E.coli has produced elevated concentrations of plasma LPS.<sup>69,70,125</sup> In these experiments blood samples were taken simultaneously from the hepatic-portal and a systemic vein. In view of the fact that the hepatic-portal vein usually had the greater LPS concentration, this suggested that the liver was mainly responsible for the primary clearance of circulating LPS. A number of other researchers hypothesized the same thoughts.

Later research indicated that the rate of clearance of LPS from the vascular circulation appeared to be fairly slow.<sup>59</sup>  $^{14}\text{C}$ -labelled LPS, prepared from a smooth and rough variety of Salmonella abortus equi and Salmonella minnesota respectively, were administered i.v. into experimental subjects, and their respective rates of removal from the vascular circulation was then determined. It was demonstrated that the rate of removal of the rough mutant form of LPS was incomplete compared to the smooth wild type, and that their respective rates were slow.

Furthermore, it was reported that the rate of clearance was not dose-dependent.

Other investigators' observations on the fate of the LPS molecule after i.v. administration into experimental animals, contradicts some of the earlier observations made on the speed with which LPS is cleared from the circulating plasma.<sup>100,126</sup> In the latter studies, general agreement seems to have been reached in that the LPS leaves the plasma in two phases. The initial phase is very rapid (within a few minutes) where unbound LPS appears to be taken up mainly by the liver and spleen, and, to a lesser extent the lungs.<sup>127</sup> This appears to represent the removal of LPS which is not complexed to high-density lipoproteins (HDL). During this rapid phase of LPS removal from the plasma, some LPS becomes attached to platelets which is then removed by tissue blood vessels (liver sinusoids) or phagocytosed by fixed tissue macrophages (kupffer cells).<sup>100,128</sup>

More recent research has reported that the rate of removal of circulating LPS depends on, firstly, the concentration of circulating HDL, and secondly, factors affecting the binding of LPS to HDL.<sup>99</sup> One third of LPS in circulation rapidly binds to circulating HDL to form a complex. This interaction prevents some LPS from attaching to platelets and decreases its uptake by macrophages because LPS is sterically unavailable in this complex.<sup>125</sup> The LPS-HDL complex is cleared slowly from the plasma compared to the LPS which remains unbound, the process takes up to a few hours and the LPS is mainly taken up by the liver, adrenals and ovaries. These workers also demonstrated that the presence of antibodies to LPS inhibited the binding of LPS to the HDL.<sup>99</sup>

Therefore, the presence of specific circulating anti-LPS antibodies would speed the clearance of LPS which was not bound to HDL from the circulation.

Two thirds of the injected LPS remains unbound and is removed very rapidly from the plasma by the liver and spleen.<sup>99</sup> It is of great interest to note that pre-treatment of the experimental animals with an adrenoglucocorticoid, dexamethasone, decreased the uptake of LPS-HDL complex by the adrenals, but at the same time increased the uptake of "free" LPS by the same glands, thus allowing bound LPS to continue to circulate for much longer. Other research workers reported that anti-LPS antibodies circulating in the plasma were able to modify the binding of LPS to HDL. Firstly, in vitro experiments suggest that homologous antibodies inhibit LPS-HDL binding, presumably by blocking LPS binding sites. A similar mechanism has been proposed for an acute-phase protein that may also inhibit the LPS-HDL binding.<sup>129</sup> Secondly, homologous IgG antibodies to LPS were able to opsonize both LPS and preformed LPS-HDL complexes and increase their uptake by tissues rich in phagocytic cells.<sup>130</sup>

### 2.3. FATE OF ENDOTOXIN IN TISSUES

Shock was induced by injecting experimental animals with radio-labelled LPS and the fate of this <sup>14</sup>C-LPS was followed with immunohistochemistry.<sup>59</sup> The removal of the circulating LPS was primarily dependent upon the amount of circulating plasma HDL and this elimination was primarily the responsibility of the cells of the macrophage-phagocytic system, and to a lesser extent, the granulocytes.<sup>100</sup> Some LPS is also

taken up by epithelial cells of the adrenal cortical cells,<sup>99</sup> and some endothelial cells of the pulmonary capillaries and alveolar epithelium.<sup>59</sup> If the LPS is derived from a rough mutant GNB, then this LPS can be readily phagocytosed by the hepatocytes.<sup>131</sup>

The immunohistochemical detectability of LPS in most tissues increased continuously during the first 48 hours after the administration of the <sup>14</sup>C-LPS, showing the strongest LPS staining in the liver and adrenals.<sup>59</sup> Macrophages were found to be the most important cellular site of primary LPS uptake in all organs except the adrenals, where LPS was mainly present in phagocytic vacuoles of the cortical epithelium.<sup>99</sup> In all the organs studied it was found that maximal detectable levels of LPS was noted four to five hours after the <sup>14</sup>C-LPS was injected. These high levels persisted for three to four days, with detectable levels still present fourteen days after the initial challenge.

Immunoperoxidase methodology and radioactivity measurements for LPS revealed that 14 days after the administration of the <sup>14</sup>C-LPS, the persisting LPS in the liver and spleen had lost its antigenic properties.<sup>59</sup> However the loss of LPS toxicity was not reported.

#### 2.4. EXCRETION OF ENDOTOXIN FROM THE HOST

Experimental animals were injected with a dose of biosynthetically radiolabelled LPS from Salmonella abortus equi and the route of excretion of this LPS was observed.<sup>132</sup> It was found that excretion was slow and that a large part of the radioactivity was still present in the liver fourteen days after the initial injection. Further to this, it was established that the excretion of the radioactivity occurred mainly in



the faeces and to a smaller extent, in the urine.

It is interesting to note that the rate of excretion of LPS appeared to be dependent on the route of administration of the LPS, and on whether or not the subject had been given an anaesthetic. Under an anaesthetic, when LPS was administered by either the i.v. or i.p. route, its subsequent excretion from a host was slow. In contrast, i.p. administration of LPS without an anaesthetic led to the rapid excretion of the radioactivity.<sup>132</sup>

## 2.5. BIOLOGICAL ACTIVITY OF ENDOTOXIN

LPS is known to be an exceptionally potent toxin which is capable of producing clinical symptoms at plasma concentrations as low as  $1,10^{-9}$  g/ml, which, due to its high molecular weight, results in a molar concentration of about  $10^{-12}$  M.<sup>133</sup> The potent nerve poison tetrodotoxin, found in the Japanese puffer fish and said to be responsible for 150-200 gourmets' deaths per year, is not active at concentrations less than  $10^{-9}$  M. Therefore LPS is at least 1,000 times more potent than tetrodotoxin.<sup>133</sup> Another clinically important feature of LPS is the fact that it is extremely chemically stable and it does not lose its potency even when boiled.<sup>134</sup> Further to this, LPS is not destroyed by antibiotics, except possibly by polymyxin B,<sup>135</sup> or other known antimicrobial chemotherapeutic agents.

When the bacteria which produced it have been destroyed either by antibiotics<sup>13,81</sup> or by some of the host's normal defence mechanisms, the LPS may continue to circulate or may remain fixed in host tissues for up

to 14 days,<sup>59,131</sup> presumably continuing its toxic effects. Furthermore, a recent report has demonstrated that gentamicin destroyed GNB so rapidly in experimental E.coli sepsis, that there was a 2000-fold increase in plasma LPS concentrations.<sup>82</sup>

#### 2.5.1. INTRACELLULAR CHEMICAL MEDIATION BY ENDOTOXIN

LPS interacts with numerous host-defence components which in turn have the ability to either activate or inhibit endogenous inflammatory mechanisms, that themselves are capable of causing host injury. These interactions are also capable of depleting the host of essential elements required by the various defence systems.

2.5.1.1. LYSOSOMAL ENZYMES have been identified in virtually all animal cells that have been studied. They are described as an intracellular membrane-delimited body consisting of acid-hydrolase activity, and they are capable of digesting most biological macromolecules by unknown mechanisms. LPS causes the release of these lysosomal enzymes from most cells, but especially from those in the the liver, kidney, spleen and pancreas.<sup>60</sup> Subsequently, these enzymes may disrupt the cellular membranes of nearby normal cells which in turn release their lysosomal enzymes. When they enter the circulation the lysosomal enzymes may cleave proteins, and cause a depression of heart contractility with coronary vasoconstriction.<sup>136</sup>

2.5.1.2. KININS are normally formed by the action of certain proteolytic enzymes on kallikreins and kininogens. LPS produces an increase in kinins as a result of the action of lysosomal enzyme action on alpha-2-globulin. Bradykinin, a nonapeptide, is then produced in the plasma,

whilst decapeptide lysylbradykinin forms in the tissues.<sup>137</sup> These kinins are thought to play a major role in causing damage to the micro-vascular circulatory system, producing vasodilation with an increase in capillary permeability leading to hypotension. They may also initiate disseminated intravascular coagulopathy (DIC) by their interaction with the haemostatic coagulation factor XII (Hageman factor).<sup>60</sup>

2.5.1.3. HISTAMINE normally behaves as a co-transmitter in the regulation of the micro-vascular circulation when hypoxaemic conditions are prevalent in the host.<sup>138</sup> Histamine is released from a variety of damaged cells and is able to produce a local increase in capillary permeability and vasodilation. Since some of the histamine released by the presence of LPS may be produced by the disruptions of cells other than endogenous mast cells, all the histamine is not effectively blocked by antihistamines.<sup>139</sup> Histamine produces local dilatation of the gastric mucosal blood vessels and oedema in rats<sup>140</sup> and dogs.<sup>141</sup> This, in turn, increases gastric mucosal blood flow, mainly to the body of the stomach and to a lesser extent, the antrum. Mast cells from canine fundic mucosa can synthesise histamine which will increase the production of cyclic-AMP and acid secretion.<sup>142</sup> In dogs, auto-regulation occurs as an adjustment of vascular resistance, to maintain a constant perfusion. Gastric blood flow during hypoxia shows this phenomenon where an increased flow occurs with decreased resistance and an increased oxygen uptake during histamine stimulation.<sup>143</sup>

2.5.1.4. SEROTONIN (5-hydroxytryptamine) normally occurs in a high concentration in platelets. An equally high concentration is normally present in the chromaffin cells of the gut. When these cells are damaged by LPS, serotonin is released and exerts a potent, local and sustained

vasoconstrictor effect on arteries and arterioles.<sup>137</sup> In large doses these chemicals are capable of producing renal vasoconstriction which will reduce the renal blood flow and glomerular filtration rate.

2.5.1.5. FIBRONECTIN is an important adhesive glycoprotein, and an organiser of tissue topography in the interstitium. It binds avidly to denatured collagen, and to fibrin at an inflammatory site.<sup>24</sup> Through surface receptors this fibronectin is capable of binding to monocytes and macrophages and by so doing, enhances the activity of these cells. This increases their phagocytotic activity and induces the secretion of proteases and plasminogen activator into the blood which is necessary for the scavenger function of the macrophages. Fibronectin becomes depleted during an episode of sepsis and DIC, thus allowing the fibrin degradation products to accumulate and circulate for prolonged periods.<sup>144</sup>

2.5.1.6. LACTIC ACID increases intracellularly with increased anaerobic glycolysis.<sup>75</sup> This may lead to a ten-fold increase in the concentration of circulating lactate which, in turn, could reduce cardiac performance and reduce receptor sensitivity to catecholamines which are also elevated in concentration.<sup>145</sup>

2.5.1.7. PEROXISOMES are a group of intracellular membrane-delimited bodies involved in peroxide metabolism. These peroxisomes contain enzymes which both produce and destroy peroxide, namely, D-amino acid oxidase and urate oxidase. Peroxisomes are always present in kidney and liver cells, and most other cells of the body. LPS is capable of producing hyperpolarization of the mitochondrial membrane with an increase in calcium ion concentration in the cytosol.<sup>8</sup> When LPS is

phagocytosed, reactions involving membrane phospholipids cause the release and oxidation of arachidonic acid as well as a burst in cellular respiration together with the generation of the super-oxide anion. Furthermore, free oxygen radicals and super-oxides are released by aggregating cells into the vascular circulation. LPS may initiate this aggregation which can give rise to an excess production of free radicals. If this occurs in aggregating leukocytes within the lungs, pulmonary endothelial cells are damaged and capillary leakage occurs, which is so often seen in the terminal endotoxin-shocked patient.<sup>146</sup>

In intestinal ischaemia, additional significant tissue damage may occur after restitution of the intestinal blood flow, and data indicating that this additional tissue damage is caused by oxygen-derived free radicals is accumulating.<sup>147,148</sup> The generation of free radicals takes place during the re-oxygenation period of the hypoxic tissues, whereas super-oxide radicals have been proposed to be generated by the oxygen-dependent xanthine oxidase.<sup>149</sup> It was suggested that the catabolism of hypoxanthine to uric acid, catalysed by xanthine oxidase, produces super-oxide radicals in the post-ischaemic intestine. During hypoxia, xanthine dehydrogenase is converted to xanthine oxidase, therefore, re-oxygenation during reperfusion, which allows this enzyme to be active, generates the super-oxide radicals.<sup>148</sup>

2.5.1.8. PROTEASES are released from neutrophils accumulating in response to the presence of LPS in the pulmonary circulation. These proteases can destroy both structural proteins (collagen and elastin) and circulating proteins plasma proteins, fibrinogen, fibronectin, Hageman factor and complement.<sup>150</sup>

2.5.1.9. FIBRIN DEGRADATION PRODUCTS (FDP) accumulate as a result of LPS mediation. Normally, fibrinolysis is a continuous process and if it does not occur, then significant anti-coagulative effects may be experienced by the host. Fibrinolysin lyses fibrin and fibrinogen forming a degradation product, which, in turn, inhibits thrombin's ability to split fibrinogen if it is not removed by the macrophage-phagocyte system.<sup>137</sup> Excessive accumulation of these FDP's are able to block the functions of the reticulo-endothelial system, are anticoagulant, and are hypotensive in action.

2.5.1.10. MYOCARDIAL DEPRESSANT SUBSTANCES have been reported to be released by fragmentation of cellular proteins. At least nine such substances are suggested to be formed with molecular weights between 250 and 1000 daltons.<sup>60</sup> The depressant effect on the myocardium has been demonstrated in both in vitro and in vivo experiments. This effect is independent of electrolyte concentrations and pH, and changes in coronary blood flow or preload. Some groups however, do not agree that such factors are present in significant amounts (L.B.Hinshaw, personal communication, 1985).

2.5.1.11. PASSIVE TRANSFERABLE LETHAL FACTOR has a molecular weight of 16,000 daltons and is found in the circulation following increased LPS concentrations and after cardiopulmonary bypass.<sup>60</sup> It acts by producing myocardial depression via the macrophage-phagocyte system (RES).

### 2.5.2. HOMEOSTATIC RESPONSES TO CIRCULATING PLASMA ENDOTOXIN

2.5.2.1. SYMPATHETIC ACTIVITY is mediated by baro- and chemoreceptors which results in an increased rate and force of myocardial contraction. Widespread arteriolar and venoconstriction occurs which diverts available blood to the heart and brain.<sup>151</sup>

2.5.2.2. CIRCULATING CATECHOLAMINES are increased between ten- and forty-fold (adrenaline = up to 3 ug/ml; noradrenaline = up to 7 ug/ml). Noradrenaline arises from the synaptic clefts, whereas adrenaline is released from the adrenal medulla.<sup>152</sup> These increased concentrations of circulating catecholamines increase the effects of the sympathetic activity, but the specific effects of these hormones on any particular end-organ is modulated locally by at least 20 different mechanisms. These effects vary from species to species, from individual to individual and from organ to organ.

2.5.2.3. RENIN-ANGIOTENSIN The reduction in the glomerular blood flow increases renin secretion by the juxta-glomerular apparatus. Consequential decrease of sodium delivery to the macula densa of the distal tubule occurs with a reduced afferent arteriolar pressure. The resultant increase in circulating angiotensin will lead to vasoconstriction which also produces the release of aldosterone.<sup>153</sup>

2.5.2.4. ANTIDIURETIC HORMONE Increased blood fluid osmolarity stimulates the osmoreceptors situated near the supra-optic nucleus of the hypothalamus. Antidiuretic hormone is released into the neurohypophysis from where it will be rapidly dispensed into the

vascular system. This hormone is thought to play a role in the maintenance of blood pressure by contributing to splanchnic vasoconstriction.<sup>137</sup> Intestinal hypoxia results, which will add to the insult of vascular ischaemia causing extensive damage to the intestinal defence mechanisms. This will allow the escape of more endogenous LPS into the host's circulation.

2.5.2.5. ALDOSTERONE secretion is increased during an episode of endotoxaemia with a resultant increase in the distal tubular resorption of sodium in the kidney. This was brought about by increased renin-angiotensin production. Water is also resorbed with the sodium, whilst there is an increased potassium excretion by the kidney tubules.<sup>137</sup>

2.5.2.6. PROSTAGLANDINS are normally present in the cellular membrane of all mammalian tissues. Their production is increased as a result of the activation of the phospholipase  $A_2$  enzyme. This enzyme is activated when cellular membranes are damaged by LPS.<sup>154</sup> When this insult occurs, arachidonic acid (AA) is released from the cell with its subsequent metabolism to unstable prostaglandins. Inter-reactions between prostaglandin and platelets have been the subject of intense and recent research.<sup>154-156</sup> In addition to the mediation of various inflammatory responses, these metabolic end-products are the mediators of the interaction between platelets and the vascular endothelium during normal coagulative homeostasis. Thromboxane  $A_2$  ( $TxA_2$ ) and prostacyclin ( $PGI_2$ ) have received a great deal of attention and it is recognised that they are the most important physiological products of AA metabolism in the pathogenesis of septic and endotoxin-mediated shock.<sup>155,157</sup>

Metabolism to intermediate unstable endoperoxides is mediated through



cyclo-oxygenase, an enzyme present in all cells. These products are converted to  $\text{PGI}_2$  and  $\text{TxA}_2$  by prostacyclin synthetase and thromboxane synthetase or they may undergo spontaneous degeneration to  $\text{PGE}_2$ ,  $\text{PGF}_{2a}$  or  $\text{PGD}_2$ . Another pathway of AA metabolism is via an enzyme, lipo-oxygenase to produce leukotrienes which have pathologic effects.<sup>158</sup>  $\text{PGI}_2$  is an arterial smooth muscle relaxant and a potent inhibitor of platelet aggregation.  $\text{TxA}_2$  is a potent vasoconstrictor and it has been suggested that  $\text{PGI}_2$  and  $\text{TxA}_2$  are normally present in low "balanced" quantities. During shock  $\text{TxA}_2$  accumulates which leads to some of the vasoconstriction observed.

LPS produces direct endothelial damage by as yet unknown mechanisms.<sup>159</sup> The endothelial damage allows the metabolism of AA to be dominated by the production of  $\text{TxA}_2$ .<sup>155-159</sup> The overall net effect is that widespread platelet aggregation and adhesion occurs with vasoconstriction. These reactions will add to micro-vascular circulatory stagnation, ischaemia, and DIC, and eventual organ failure may result.

2.5.2.7. COMPLEMENT ACTIVATION The complement system consists of a series of circulating inactive plasma proteins which are activated in a sequential manner to bring about the destruction of micro-organisms and various toxins.<sup>160</sup> This cascade of events can be triggered at two different levels. "Classical" activation involves IgG and IgM immunoglobulins and occurs at the first complement component ( $\text{C}_1$ ) and the "alternative" pathway can be activated without involving any immunoglobulins. This second pathway is triggered at the third level ( $\text{C}_3$ ) of the cascade by various substances including, LPS.<sup>161</sup> It would appear that cell membranes are the primary target of the complement enzymes, which are then irreversibly damaged.<sup>102</sup>

When  $C_3$  is activated by LPS, the circulating and previously inactive plasma complement proteins, will culminate in histamine release, cellular lysis, leukocytic chemotaxis and opsonization, among other functions. Excessive complement is capable of producing disordered leukocytic function.<sup>144</sup> It has been established that the activation of complement results in the generation of a factor that can stimulate polymorphonuclear leukocytes to selectively release lysosomal enzymes.<sup>162</sup> This lysosomal enzyme-releasing factor shares many of the properties of complement  $C_{5a}$  which displays chemotactic and anaphylatoxin activities. Evidence suggests that some of the lethal effects of LPS are related to the presence of polymorphonuclear leukocytes. When these circulating cells were depleted in experimental animals, then the animal's response to an i.v. dose of LPS was profoundly detrimentally, altered.<sup>144</sup>

Complement activation is not always beneficial to the host.<sup>58,160</sup> Experiments have been conducted in dogs to suggest that the complement components may be involved in the lethal effects of LPS.<sup>65</sup> When dogs received transfusions of decompemented blood ( $56^{\circ}\text{C}$ , 30 mins), they were protected against the lethal effects of experimentally administered LPS. In many cases of GNB infections, there appears to be an overproduction of circulating complement. Consequently, there is an exaggerated inflammatory response, producing increased endothelial permeability. This permits blood leakage into the interstitial spaces and the release of lysosomes from the leukocytes. Lethal dosages of LPS injected into rabbits produced a rapid and sustained fall in circulating plasma concentrations of complement.<sup>163</sup> In a similar study it was found that

serum complement activity declined after adjuvant amounts of LPS was introduced into rabbits.<sup>164</sup>

Alterations in complement concentrations in gram negative bacteraemic patients were studied.<sup>165</sup> Patients with uncomplicated gram negative bacteraemia had mean  $C_3$  values similar to those of the controls, whereas there was a significant decrease in the  $C_3$  values in those patients who were in shock and in those who died.

2.5.2.8. VASCULAR CELLULAR ELEMENTS can be altered during a homeostatic response to circulating plasma LPS. These responses include:

a. Erythrocyte response -- when increased concentrations of plasma LPS are present in the vascular circulation, the erythrocytes may undergo stagnation and margination, with concurrent separation of the plasma and the vascular elements in small blood vessels.<sup>166</sup> Decreased blood flow through the micro-vascular circulation occurs producing the local hypoxia which is damaging to the mitochondria. In addition, consequential haemoconcentration occurs resulting in increased blood viscosity, and the stagnation of erythrocytes will increase the incidence of DIC during an episode of endotoxaemia.

b. Platelet response -- a thrombocytopenia occurs at the onset of LPS administration to rabbits, dogs and guinea pigs<sup>58</sup> as well as at the onset of induced sepsis.<sup>167</sup> The drop in the platelet count is ascribed to their widespread destruction, adhesion and aggregation. It has also been suggested that this thrombocytopenia may be an aid to the positive diagnosis of an endotoxaemia, if other aetiology of thrombocytopenias can be excluded.<sup>168</sup>

The aggregation of blood cells in the presence of antigen, antibody and the first four complement components has been termed immune adherence. Receptor mediated immune adherence is present on the platelets of non-primate species.<sup>168</sup> One of the mechanisms of the platelet-endotoxin interaction depends on the presence or absence of immune adherence receptor sites on the platelet membrane.<sup>58</sup>

c. Neutrophils -- also show a response after the administration of LPS. An initial neutropenia has been observed which is then followed by a neutrophilia within a few hours. This is usually a regenerative response with a shift to the left.<sup>49</sup> There is a relocation of the neutrophils from the circulation to the marginal pools, especially in the pulmonary capillary bed, where they degenerate and produce lysosomal-induced micro-vascular damage.<sup>58</sup>

LPS exposure causes an increase in both the consumption of oxygen and the production of carbon dioxide by the neutrophils. LPS destruction of the neutrophils will bring about a release of kininase, lysozyme and other enzymes, mimicking the changes occurring in the process of phagocytosis.<sup>58</sup> These events indicate that LPS causes toxic damage to circulating neutrophils and fewer neutrophils are available to migrate to inflamed tissue sites. Both neutrophils and the cells of the macrophage-phagocyte system have the ability to detoxify LPS.<sup>169</sup>

d. Monocytes and macrophages -- these will also show a response to circulating plasma LPS. Circulating monocytes and tissue macrophages provide defence against microbial invaders because of their phagocytic properties. At the same time, they are stimulated to release prostaglandins and collagenase.<sup>58</sup> It has been reported that LPS can

actually stimulate the monocyte to increase its ability to phagocytose bacteria and produce lysosomal hydrolases.<sup>105</sup>

Peritoneal macrophages are activated, particularly by the lipid-A component of LPS to produce collagenase.<sup>169</sup> This collagenase production was significantly inhibited by indomethacin, an anti-prostaglandin, suggesting that prostaglandins mediate this effect.<sup>83</sup> Macrophages are stimulated to produce a plasminogen activator when exposed to LPS. Purified populations of peripheral blood monocytes and peritoneal macrophages have been shown to synthesise and release prostaglandin-E which is enhanced when these cells are exposed to LPS and suppressed by the anti-prostaglandin, indomethacin.<sup>169</sup>

e. Lymphocytes -- will show their response when LPS stimulates the proliferation of large populations of lymphoreticular cells. It is thought that the lipid-A, and to a lesser extent, the "O"-antigen component of the LPS evokes this mitogenic effect, which was shown to be thymus cell independent.<sup>93</sup>

2.5.2.9. COAGULATIVE ACTIVITY Endotoxin-shocked patients exhibit DIC which is thought to be brought about by the activation of the intrinsic pathway of the haemostatic system, as it is known that factor XII is activated by LPS.<sup>170</sup> Extrinsic activation is also thought to be involved since leukocytes are required to produce both the local and generalised Schwartzman reactions. In addition, monocytes produce a tissue factor which can initiate clotting via the extrinsic pathway.<sup>58</sup> Furthermore, activated factor XII is capable of activating plasma prekallikrein to form kallikrein, which can generate bradykinin to activate plasminogen.

Two injections of culture filtrates from GNB demonstrated the ability of

this filtrate to produce tissue injury via coagulation changes.<sup>157</sup> These observations were extended and resulted in two widely used test models, namely, the local and the generalised Schwartzman reactions. It is now known that the culture filtrates contained LPS and that LPS by itself can produce these reactions. In the local reaction, dermal necrosis is seen after the second dose of LPS is administered, whilst in the generalised reaction, bilateral cortical necrosis of the kidneys is observed.<sup>171</sup>

DIC eventually leads to a depletion of the clotting factors and enhanced fibrinolysis. This will also result in the accumulation of FDP in the blood where they act as anticoagulants. These factors contribute to the extremely serious bleeding disorders associated with GNB infections and endotoxaemia.<sup>91</sup>

### 2.5.3. ENDOTOXIN EFFECTS ON METABOLIC PRODUCTS

2.5.3.1. EFFECT ON CARBOHYDRATE METABOLISM. In muscle tissues, LPS disturbs the carbohydrate metabolism and decreases the utilisation of glucose and ketone bodies, with increased catabolism of branched chain amino acids.<sup>172</sup> Different severities of endotoxaemia are capable of affecting the glucose and insulin concentrations in circulation.<sup>173</sup> With early endotoxaemia the cardiac output is still high, the blood glucose and plasma insulin concentration are elevated. As the cardiac output decreases and blood lactate rises, a progressive hypoglycaemia occurs with insulin concentrations dropping rapidly. This hypoglycaemia results from the depletion of glycogen stores and depressed gluconeogenesis with increased tissue usage of glucose.<sup>172,174</sup> LPS is also known to increase

adrenalin secretions which, in turn, inhibits insulin secretions.<sup>172,173</sup>

2.5.3.2. EFFECT ON LIPID METABOLISM. Marked increases in the serum concentrations of either or both free fatty acids and triglycerides, occur in patients that have a gram negative bacteraemia.<sup>175</sup> LPS can significantly elevate the serum triglyceride concentration leading to impairment of lipid disposal mechanisms and it can enhance lipolysis due to the release of adrenaline and noradrenaline. It can also increase the adenylate activity in fat cells as a direct effect of the lipid-A component of the LPS<sup>176</sup>.

2.5.3.3. OTHER METABOLIC EFFECTS. Serum iron concentrations decrease as a result of LPS and the drop in the concentration is so replicable that it has been suggested that serum iron concentrations could be used as a diagnostic screen for an endotoxaemia.<sup>46,91</sup> LPS depresses cellular respiration producing irreversible mitochondrial damage,<sup>119,174</sup> it can produce damage to the surface phospholipids of lung alveoli cells,<sup>172</sup> cause a cholestasis,<sup>177</sup> and produce hypophosphataemia.<sup>178</sup>

## 2.6. SUMMARY OF CIRCULATING PLASMA ENDOTOXIN EFFECTS

It has been demonstrated that high concentrations of circulating plasma LPS produces a series of haemodynamic changes which in turn will lead to hypotension. These changes can be summarised as follows:

- a. Vasoactive substances are released by LPS, these include catecholamines, histamine, kinin, prostaglandin and serotonin.<sup>179,180</sup>
- b. These vasoactive agents produce a local "contraction" away from each neighbouring endothelial cell, and damage each cell, thus increasing

capillary permeability.<sup>181</sup>

c. Oedema and hypovolaemia result from this increased permeability of the capillaries. In the thoracic cavity this phenomenon gives rise to the well described "shocked lung syndrome".<sup>182,183</sup> Similarly, this endothelial damage gives rise to the intra-intestinal haemorrhage so often described as a post mortem finding or as a clinical symptom of the endotoxin shock patient.<sup>184</sup>

d. The plasma loss that occurs with circulating plasma LPS, leads to hemoconcentration and increased blood viscosity.<sup>133</sup>

e. LPS evoke mechanisms which give rise to vasoconstriction of the central and hepatic veins allowing the blood to "pool" in the splanchnic region.<sup>133</sup> This vasoconstriction together with the above described hypovolaemia, will lead to a reduced venous return to the heart.

The above described events will allow a reduced cardiac output and sequential hypotension. This will in turn give rise to a reduced oxygen delivery to the tissues (localised ischaemia) and a reduced removal of waste from these tissues. The hypoxia so produced will give rise to an increased plasma lactate concentration and acidosis.<sup>185</sup>

The total effect of the hypotension is to instigate and contribute to the deterioration of lysosomal membranes with subsequent release of proteinases and many other hydrolytic enzymes and harmful chemicals.



## CHAPTER THREE

### ENDOTOXIN SHOCK TREATMENT

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The patient in shock, irrespective of the aetiology, is diagnosed when life-threatening changes to the micro-vascular circulation have already occurred. A reduced flow of the micro-vascular circulation gives rise to either a generalised or localised hypoxia. It is known that intestinal defences are adversely affected by a reduced blood flow to and from itself, and this event occurs during hypoxia or ischaemia. As a result LPS leaks into the vasculature from the intestinal lumen, which adds to the pathology of the patient in shock, even if the aetiology has a non-septic origin.

Many treatment regimens have been advocated, with varying success, where the objectives have centred around the:

1. Restoration of the reduced micro-vascular circulation.
2. Counteracting pathologic disturbances which have occurred to homeostasis.
3. Destruction of any bacteria that are concurrently present.

Unfortunately, the neutralization and elimination of circulating plasma LPS has not been well recognised until fairly recently. Therefore, a fourth objective should be the elimination of these pathogens as early as possible.

Whilst a patient is being treated for endotoxin shock, physiological monitoring should provide a continuous assessment of fundamental defects, to guide the therapeutic management of the patient. Unfortunately, this monitoring requires extremely expensive equipment, which is often beyond the realms of the private veterinary practitioner. Furthermore, the variables that are most often measured are those that

are easiest to monitor, rather than those that are relevant to the crucial question of survival or death.

### 3.1. RESTORATION OF REDUCED MICRO-VASCULAR CIRCULATION

The haemodynamic and metabolic effects of treatment with various types of fluids have been reported extensively,<sup>185</sup> however the micro-vascular circulatory effects are not known in detail. Most indirect studies indicate that disturbances in tissue perfusion may persist in tissues for prolonged periods of time following treatment.<sup>186</sup>

Infusion of hypertonic solutions prevented tissue damage during shock and ischaemia. Recovery from haemorrhagic shock occurred more rapidly in dogs treated with 1.8% saline, 2.74% sodium bicarbonate, or 10% glucose, than in dogs treated with isotonic solutions.<sup>186</sup> In this report it was postulated that the hypertonicity reduced post-ischaemic cellular swelling. Since then, the use of hypertonic solutions for situations such as tissue preservation has become commonly accepted.<sup>187</sup>

Reduced micro-vascular circulation may be restored by using any of the agents described below, either singularly or in combination.

#### 3.1.1. VOLUME REPLACEMENT

Volume replacement is standard therapy for hypovolaemic and traumatic shock, but it is not as well recognized as being beneficial for septic or cardiogenic shock. In septic shock, large volumes of blood are sequestered in the micro-vascular circulation of the viscerocutaneous areas, and further volume is lost through damaged vascular membranes.

Therefore, this fluid is lost from the effective circulating volume. Large volumes of plasma, plasma substitutes and electrolyte solutions are often required by a patient in endotoxin shock.<sup>188</sup> Unless there is pre-existing anaemia or intravascular haemolysis, the need for erythrocyte transfusions is not urgent. The goal in the therapy for endotoxin shock, specifically with regards to fluid volume, is to restore blood pressure by an increase in cardiac output, rather than by an increase in peripheral resistance where central venous pressure can be used to monitor blood pressure. Fluids which may be used include;

3.1.1.1. BLOOD TRANSFUSIONS This is instituted only if there has been haemorrhage, haemolysis or pre-existing anaemia. Generally, if the haematocrit is below 30%, then whole blood is necessary, at a dosage of 20-40 ml/kg body mass.

3.1.1.2. PLASMA TRANSFUSIONS Plasma, lyophilized plasma, serum or albumin solutions may be used. When plasma is used, it must be remembered that antiplasma-antibodies may be present in the recipient and the necessary precautions must be taken. Initially, the plasma can be administered at a rate of 5 to 10 ml/min, and then more slowly until a total dosage of about 20 to 40 ml/kg body mass is given.

3.1.1.3. PLASMA EXPANDERS Several types have been used with success e.g., dextran 6%, low molecular weight dextran and gelatin polymer 3.5%. The dosage rate of these substitutes is in the region of 10 to 20 ml/kg body mass.

3.1.1.4. ELECTROLYTE SOLUTIONS Lactated Ringer's solution is a balanced electrolyte solution which replaces extracellular fluid. If energy is required, then 5% dextrose or 5% levulose can be added. Plasmalyte B, a

bicarbonated solution, can also be used with satisfaction.<sup>49</sup>

### 3.1.2. DIRECT CARDIAC STIMULANTS

When volume replacement alone fails to increase cardiac output, additional therapy is required in the treatment of some shocked patients, in order to reduce vasoconstriction and improve tissue perfusion. Cardiac stimulants that are inotropic agents, would be an extremely helpful form of therapy, but there are only a few available in veterinary medicine and these have not yet proved to be successful when used alone.

3.1.2.1. DIGITALIS This pharmacological agent can increase cardiac tone and may be beneficial in the sub-acute or chronic patient, but in the acute situation it will often fail.

3.1.2.2 ISOPRENALINE. This is a beta stimulating drug which increases the rate and force of myocardial contractions. It has the added advantage of being a peripheral vasodilator and can increase cardiac output and reduce vasoconstriction in beta beds, principally in the voluntary muscles.

3.1.2.3. HEPTAMINOL This drug can be used, because it is an indirect-acting sympathomimetic, which will thus augment the force of myocardial contractions.

### 3.1.3. VASODILATORS

In shock, cardiac performance can be increased through reduction of peripheral resistance and stagnation. There are three general types of

drugs that induce vasodilatation. These are; i. those that produce direct vasodilatation, such as isoprenaline, ii. those that produce vasodilatation by blocking the vasoconstrictive effects of adrenaline and noradrenaline on alpha receptors, thereby reducing vasoconstriction produced by increased sympathetic tone. An example being the alpha adrenergic blocking agents, and iii. those that produce vasodilatation by unknown mechanisms, such as the corticosteroids.

#### 3.1.4. BETA ADRENERGIC BLOCKERS

It has been suggested that excessive beta adrenergic stimulation with the opening of multiple arterio-venous shunts in the pulmonary and splanchnic areas is of primary importance in the pathogenesis of late shock.<sup>186,188</sup> Propranolol administration significantly increases the survival rate of dogs in states of haemorrhagic and endotoxin shock.

#### 3.1.5. ANTICOAGULANT THERAPY

The complication of DIC which often accompanies endotoxin shock is particularly evident in the dog. Anticoagulant agents may play an important role in the survival of the dog. Their mechanism of action is simply based on the clearing of sludged blood clots and thrombi from damaged peripheral vessels.

#### 3.1.6. OXYGEN THERAPY

The patient in shock has either a generalized or localized hypoxaemia due to an altered micro-vascular circulation. An adequate oxygen supply should be administered to the patient in shock, especially that produced

by haemorrhage or endotoxaemia.<sup>189</sup>

### 3.2. COUNTERACTION OF PATHOLOGIC DISTURBANCES

#### 3.2.1. ANTI-PROSTAGLANDIN THERAPY

Some phospholipids in cell membranes are prostaglandin precursors and convert to active prostaglandins prior to their release by a variety of physiological, pharmacological and pathological stimuli.<sup>190,191</sup> It now appears that prostaglandins have been implicated in the pathogenesis of the symptomatology associated with an endotoxaemia.<sup>192</sup> These prostaglandins released by the LPS participate in a variety of pathophysiological processes including inflammation, thermal injury, hypertension, peptic ulcer disease, diarrhoea, skin conditions, vasomotor regulation, derangement in platelet function, gynaecological disorders, and fever.<sup>180,190,193-196</sup>

The use of anti-prostaglandin therapy could therefore be beneficial to a patient with endotoxin shock. Non-steroidal anti-inflammatory drugs have been shown to attenuate the haemodynamic events in canine endotoxin shock, but results have been conflicting.<sup>180</sup>

Pre- and post-treatment with aspirin or indomethacin were employed in experimentally-induced canine shock models.<sup>197</sup> Both of these drugs were equally effective in improving survival without any apparent alteration to coagulation pathways. However, indomethacin, whether used prophylactically or therapeutically, in pharmacological or physiological doses, did not improve survival in an overwhelmingly lethal model of endotoxin-induced shock.<sup>180</sup>



Studies in a number of species, including dog<sup>192</sup>, calves<sup>198</sup> and sub-human primates<sup>199</sup> have demonstrated a relationship between the concentrations of prostaglandins in the blood and the severity of LPS shock. The pulmonary vasculature has been shown to be quite sensitive to the vasoconstrictive properties of sublethal doses of administered LPS, and this response was associated with significant changes in the levels of prostaglandin in the blood<sup>196</sup>. Inhibitors of prostaglandin synthetase prevented this undesirable response.

It would appear that foetuses do not produce large amounts of prostaglandins in response to circulating LPS.<sup>196</sup> Prostaglandin blood concentrations in maternal and foetal circulations were measured after each individual had received a separate experimental infusion of E.coli LPS. Five hours after LPS administration, the concentration of prostaglandin in maternal blood rose. In contrast, prostaglandin in foetal blood showed no significant change even after foetal administration of LPS, at ten times the dose required for maternal response. In the same study, indomethacin was administered to a group of maternal subjects after the experimental infusion of LPS. Prostaglandin blood values were decreased by one third, presumably by the indomethacin.

### 3.2.2. OPIATE ANTAGONISTS

The discovery of enkephalins as natural opiates within the central nervous system led to the discovery of other endogenous opiate substances, for example, beta-endorphin.<sup>49</sup> This chemical is produced by the anterior pituitary gland in response to stress and it is released

systemically in conjunction with ACTH. Target cells for beta-endorphin are situated both centrally and in the peripheral tissues.<sup>200</sup>

Endogenous opioid peptides have been shown to participate in the pathophysiology of experimentally-induced LPS shock and is accompanied by a rise of enkephalins in the cerebrospinal fluid.<sup>200</sup> Experimental use of a pure opiate antagonist, naloxone, has shown beneficial effects in the reversal of degenerative haemodynamic changes seen in septic shock.<sup>201</sup> Naloxone attenuates the decrease in cardiac output seen with experimentally-induced endotoxaemia and it reverses the myocardial depression by unknown mechanisms. A direct positive inotropic effect of naloxone is thought to be responsible for these changes.<sup>200</sup>

The anti-opiate effects appear to last for only 2-10 minutes and therefore, its therapeutic benefit is questionable. Furthermore, high doses of naloxone must be administered frequently, and the high cost of this chemical probably excludes its usage in most settings.

### 3.2.3. SUPER-OXIDE ANTAGONISTS

Various experimental shock protocols produce intestinal ischaemia, resulting in haemorrhagic mucosal lesions in the small intestine. These mucosal lesions cause the release of cardio-depressive substances, from damaged cells, into the vascular circulation which further compounds the damage that has already occurred.<sup>202</sup>

Models of localized intestinal ischaemia have been reported to produce additional tissue damage after the restoration of the intestinal blood flow.<sup>147,202,203</sup> Data indicating that this additional damage is caused

by oxygen-derived free radicals, and that the generation of these free radicals takes place during the re-oxygenation of the ischaemic and hypoxic tissues is accumulating.<sup>147,148,204</sup> Recently, it was suggested that the catabolism of hypoxanthine to uric acid, catalysed by oxygen-dependent xanthine oxidase, produced the super-oxide radicals,<sup>147,149</sup> which in turn causes peroxidation of lipid components of the cellular membranes.<sup>148</sup> During hypoxaemia, xanthine dehydrogenase is converted to xanthine oxidase and consequently, re-oxygenation during reperfusion allows this enzyme to become active, and to generate more super-oxide radicals.<sup>148</sup>

Competitive inhibition of xanthine oxidase might provide protection to the body during a shock phase. Allopurinol is a competitive inhibitor of xanthine oxidase. When experimental cats were given allopurinol prior to an intestinal ischaemia, then cats were protected. They showed no signs of small intestinal mucosal damage at 10 and 60 mins following restoration of perfusion pressure to the small intestine.<sup>148</sup> In a haemorrhagic shock model in the dog, pre-treatment with allopurinol also increased survival time.<sup>205</sup>

#### 3.2.4. CORTICOSTEROID THERAPY

An aspect that should be emphasized, and already has been mentioned, is that haemorrhagic shock causes regional hypoxia of the gut which, in turn, gives rise to intestinal mucosal damage. This allows endogenous GNB with their LPS, or endogenous LPS by itself, to enter the host's blood circulation. Therefore, much of the early work performed on haemorrhagic shock models in different species of experimental animals, can be interpreted as work performed on special cases of endotoxin

shock.

Scientific investigations into the effects of corticosteroid (CTS) therapy in canine haemorrhagic shock began in 1942<sup>206</sup> when researchers concluded that the administration of CTS alone, did not produce significant favourable effects on homeostatic mechanisms in the experimental animals. However, the combination of blood transfusions or i.v. fluids with CTS did produce beneficial effects.

Howard et al., combined CTS and vitamin B<sub>12</sub> treatment for experimentally-induced haemorrhagic shock in dogs<sup>207</sup> and reported that this therapy had no beneficial effect. However, upon closer examination of their data, it was reported that it was necessary to withdraw 25% more blood from the treated animals than from the control group, to produce the same degree of shock.<sup>208</sup>

Prior to the gradual withdrawal of 50% of adrenalectomised rats' blood volume, they were treated with either cortisone or desoxycorticosterone.<sup>209</sup> Rats receiving desoxycorticosterone maintained a higher blood pressure than the control rats, but not as high as those that received cortisone. It was concluded that the former steroid provided better protection in this experimental model. Researchers were later to report on a successful regimen of using a "cocktail" of 11 adrenal cortical hormones in patients with post-operative shock, where the shock had not been reversed by large amounts of blood or i.v. fluids. All 24 human patients showed a definite response to this "cocktail".<sup>210</sup>

The effects of administered CTS during an endotoxic episode varied from

negligible<sup>211</sup> to nearly complete protection.<sup>63</sup> Therefore, the value of CTS alone in these shock states still remained controversial.<sup>212,213</sup>

Facts that did emerge from these conflicting conclusions were, firstly, that once haemorrhagic hypotension in the dog had persisted for longer than 45 minutes, administration of hydrocortisone would not affect the eventual outcome of the shock process. Secondly, for hydrocortisone to be effective it had to be administered i.v., and finally, although the hypertensive effect of hydrocortisone occurred in the absence of fluid administration, blood transfusion was still the most appropriate initial response in clinical haemorrhagic shock.<sup>208</sup>

Later it was postulated that beneficial effects following hydrocortisone administration were due to modifications by CTS on the vascular response to endogenous catecholamines.<sup>214</sup> These workers showed a different response in experimental subjects to cortisone, a glucocorticoid, and desoxycorticosterone, a potent mineralocorticoid. In view of these differences, the effects of several adrenal cortical compounds on the course of haemorrhagic shock in the dog were studied.<sup>215</sup> It was concluded that CTS, with predominantly glucocorticoid activity, were the more effective agents than were the CTS, which were primarily mineralocorticoid in their action.

It appeared that the dosage of CTS administered to the shocked experimental subject was of importance. Normal physiologic doses of CTS indicated that experimentally-induced shock mortality decreased. When high physiologic doses of CTS were employed in similar experimental models, an improved survival rate was recorded in the latter animals compared to those animals which received the normal physiological dose

of CTS.<sup>216</sup> Ultra-physiologic (pharmacologic) doses demonstrated the greatest improvement in the survival rates of experimentally-induced endotoxaemia in the same type of experimental shock.<sup>217</sup> These pharmacologic doses then became accepted by those treating these shock cases, however the actual timing of the administered CTS appeared to be of paramount clinical significance.

A 100% lethal model of canine haemorrhagic shock was used to investigate the effects of a prophylactic dose of 60 mg/kg body mass of methylprednisolone sodium succinate (MPSS), administered 1 hour prior to the onset of bleeding.<sup>218</sup> Despite a significant reduction in the total peripheral resistance and a transient improvement in the cardiac output in the MPSS treated dogs, there was no significant improvement in the mean survival time. It thus appeared that neither physiologic nor pharmacologic doses of CTS could improve survival in models of haemorrhagic shock that are severe enough to cause 100% mortality in dogs receiving only fluid supportive therapy.

This work was modified by other researchers who evaluated the influence of the timing of the administration of the glucocorticoid on the course of canine haemorrhagic shock.<sup>219</sup> It was found that the haemodynamic and oxygenation indices in dogs receiving CTS at the beginning of hypotension or 1 hour later, were significantly better than in those of untreated controls. When CTS administration was delayed until the end of the hypotensive phase, then haemodynamic indices were identical to those of the untreated dogs. Also, CTS treated dogs required less fluid during the recovery phase to achieve normovolaemia. In addition, following restoration to normovolaemia, the haemodynamic indices remained closer to pre-shock values in the CTS treated group of dogs.

Pre-treatment using MPSS in E.coli LPS-induced porcine shock was studied.<sup>220</sup> This preparation was administered 25 minutes before the infusion of E.coli LPS. Control pigs received the LPS alone. Within 210 minutes of the i.v. infusion of LPS, all 17 controls were dead. In contrast, of 7 pigs which received a pre-LPS infusion dosage of MPSS, 6 were still alive 210 minutes after the LPS infusion. These results were consistent with those recorded in similar experiments in dogs<sup>221</sup>, rats<sup>222</sup>, cats<sup>223</sup> and monkeys.<sup>48</sup>

The ability of MPSS to protect gut mucosa during a regional hypotensive phase was investigated.<sup>224</sup> Cats given MPSS at the conclusion of a 2-hour abdominal vascular ischaemic period, demonstrated significantly fewer small intestinal mucosal lesions one hour later, than did the untreated controls. In addition, it was reported that in the presence of MPSS there was a reduction of cardiotoxic enzymes released from the ischaemic small intestine into the general vascular circulation. Furthermore, the MPSS-treated cats did not manifest systemic hypotension following release of the occluded intestinal artery, as occurred in the control cats.

In another study, experimental animals were given MPSS with and without an infusion of E.coli LPS, and the effectiveness of MPSS in preventing or reversing tissue damage caused by the administered LPS was studied.<sup>225</sup> It was concluded that the early administration of CTS to LPS-treated animals prevented deleterious effects of LPS in the lungs, kidneys, haematological system and lysosomal enzyme liberation, but not in the liver. However, late treatment with MPSS did not prevent LPS-induced changes in the kidneys, and detrimental effects on lung

metabolism were observed. The administration of CTS to patients in the pre-terminal stages of endotoxin shock may therefore be dangerous.

Several mechanisms resulting in beneficial changes in haemodynamic and metabolic parameters by glucocorticoid therapy for endotoxin shock have been proposed.<sup>208,216,217</sup> Corticosteroids have been suggested to function by:

- a. Elevating the cardiac output,<sup>226</sup>
- b. Augmenting venous return by diminishing peripheral pooling,<sup>221</sup>
- c. Increasing the coronary blood flow and elevating regional blood flow,<sup>221</sup>
- d. Preventing aggregation of platelets,<sup>227</sup>
- e. Protecting against activation of the coagulation pathway,<sup>227</sup>
- f. Inhibiting the activation of complement,<sup>227</sup>
- g. Maintaining the RES function,<sup>5</sup>
- h. Inhibiting the generation and release of vasoactive substances such as kinins, prostaglandins, histamine and beta-endorphin,<sup>5</sup>
- i. Stimulating hepatic gluconeogenesis and maintenance of oxidative phosphorylation,<sup>228</sup>
- j. Supporting liver carbohydrate metabolism,<sup>229</sup>
- k. Stabilizing hepatic lysosomal membranes,<sup>230</sup>
- l. Protecting endothelial integrity, particularly of the capillaries.<sup>227</sup>

#### 3.2.5. CORTICOSTEROID-ANTIBIOTIC COMBINATION THERAPY

Several animal models have shown that the early administration of CTS in combination with an antibiotic for the treatment of a lethal infusion of GNB, markedly reduced the mortality seen in the antibiotic-only treated



controls.<sup>227,231</sup> It has also been reported that CTS alone protected against lethality during the first 15 hours better than did gentamicin alone, although ultimately, both displayed the same mortality rate.<sup>227</sup> More recently, it has been shown that CTS plus antibiotic treatment of the same animal had a lower mortality rate than mortality after treatment with either of the two drugs individually.<sup>232</sup>

Different steroids have been employed in combination with certain antibiotics to treat experimentally-induced endotoxin shock animals.<sup>231</sup> Experimental dogs were i.v. infused with live E.coli organisms ( $1.1 \times 10^{10}$  organisms/kg body mass) over an one-hour period. These dogs were divided into various groups and received either a combination of a steroid and an antibiotic or an antibiotic by itself. An 80% mortality was observed in the groups that received dexamethasone and gentamicin together or gentamicin alone. In contrast, the group that received combined MPSS and gentamicin therapy had a 100% survival. However, it should be stressed that the treatments were always administered prior to or with the infusion of the pathogenic organisms.

This combined CTS-antibiotic therapy holds promise, but unfortunately the time lapse from GNB infusion to the beginning of the combined treatment does not coincide with the typically presented clinical case of endotoxin shock.

### 3.2.6. ANTIBIOTIC THERAPY

During an episode of shock the eradication of exogenous and endogenous GNB are important aspects, and the early administration of non-absorbable antibiotics to destroy endogenous GNB in the gut has been

advocated.<sup>233</sup> When circulating GNB are destroyed by antibiotics, increased circulating plasma levels of LPS can occur and thus the administration of antibiotics should be carried out with caution in late endotoxin shock.<sup>81,82</sup> Antibiotics with broad spectra should preferably be administered, orally and parenterally.<sup>234</sup> If the patient in shock has concurrent wounds then basic traumatic wound therapy is essential to eliminate septic foci.

Basic wound treatment is absolutely necessary if this was part of the aetiology or if concurrent wounds are present.<sup>26</sup> Sterile gauze sponges should be placed into the wound to protect it while hair is shaved from a generous area around the wound. The shaved area should then be prepared with surgical soap and warm saline. Surgical debridement is necessary to remove dirt, necrotic tissues, and to make the contaminated wound as aseptic as possible. Atraumatic handling of tissues must be performed since additional trauma will increase the likelihood of bacterial invasion. Bleeding blood vessels must be closed and care taken to preserve nerves. Suturing of open wounds is mandatory and is determined by the physiological status of the patient in shock. It is not unreasonable to leave wounds unsutured initially, but then adequate coverage and use of local antibacterial chemotherapeutic agents must be incorporated into the regimen of secondary wound closure.

For most GNB, gentamicin has been reported to be effective at a dose rate of 4 mg/kg body mass, every 12 hours. Clindamycin, 4 mg/kg body mass every 6 hours or chloramphenicol, 20 to 30 mg/kg body mass every 8 hours have also been suggested as initial therapeutics.<sup>235</sup> Specific treatment will depend upon the results of wound culture and

antimicrobial susceptibility testing.

The outcome of septic shock appears to be more dependent upon factors involved in host prognosis, than whether appropriate antibiotics were used. Antibiotic therapeutic regimens have been focused on those patients with the worst prognosis and in whom many attempts have been made to improve their outcome. A summary of such antibiotic treatment is as follows;

- a. Empiric therapy - therapy is started when the first signs of infection is appreciated. Combinations should be used that have a broad spectrum of activity designed to be effective against the bacteria likely to be aetiologically implicated.<sup>188</sup>
- b. Synergistic combinations of antibiotics - the combinations of two drugs often displays more activity against a bacteria than can be expected from the sum of the activities of each individual antibiotic.<sup>235</sup>
- c. Development of new antibiotics - determinations of which antibiotics are more effective because of increasing antibiotic resistance.<sup>234</sup>
- d. Prophylactic use of antibiotics - reduction of the opportunities for nosocomial infection and for resistance to develop by the bacteria against the antibiotic being used.

The combination of carbenicillin and gentamicin has been reported to give better overall results (83%) than either drug used alone (50% and 57% respectively).<sup>234</sup> When a combination of cephalosporin plus carbenicillin or ticarcillin were used together, then this treatment was equally as successful as an aminoglycoside plus carbenicillin or ticarcillin.<sup>188</sup>

Polymyxin B is a cyclic peptide antibiotic which is bactericidal for most GNB species. In addition, it is known to bind avidly to the lipid A region of LPS<sup>236</sup> and this reduces some of the biological activities of LPS. However, these researchers had to use polymyxin B at 100-fold excess concentrations and then only 30-50% inhibition of LPS binding to the amoebocyte of the horseshoe crab was demonstrated. Also, polymyxin B should not be used as a systemic antibiotic because of its known toxic effects within the host.

A very important consequence of antibiotic therapy against GNB infection was recently described.<sup>82</sup> The rapid destruction of E.coli by gentamicin lead to a 2000-fold increase in circulating plasma LPS concentration, from their "sloughing off" killed bacteria. Therefore it must always be remembered that although the antibiotics may destroy bacteria, they appear to have no harmful effects on circulating LPS, and in fact, are liable to increase LPS concentration.

### 3.2.7. ENDOTOXIN SHOCK IMMUNOTHERAPY

LPS can stimulate host defence mechanisms to produce specific antibodies which in turn will eventually destroy the circulating plasma LPS. Immunotherapy directed specifically against LPS has recently received increased attention<sup>133</sup> as this form of treatment reduced the mortality associated with septic shock in several experimental animals, clinical veterinary patients and human studies.<sup>37,51,77,113,134,237,238</sup>

Serum obtained from persons who had recently recovered from various bacterial infections was used successfully to treat Pseudomonas infections.<sup>239</sup> The presence of high titres of antibodies specific to

LPS or other gram negative bacterial antigens in the host's circulating plasma, was protective in patients with gram negative septicaemia and reduced their mortality rate from 64% to less than 10%.<sup>240</sup> Specific antibody-rich plasma fractions are already used therapeutically in suspected exposure to Varicella and tetanus.<sup>171</sup>

Success was obtained when anti-Pseudomonas antibodies were used for the treatment of pseudomonas-induced sepsis in burn patients.<sup>239</sup> The effectiveness of this therapy was limited mainly to Pseudomonas spp. This work was followed by a complicated process of actively immunizing healthy human volunteers with a boiled E.coli mutant to obtain antiserum.<sup>133</sup> This preparation reduced morbidity in patients suffering from neutropenia following treatment for leukaemia or lymphoma.

Another study identified an important immunological marker, which correlates with protective immunity in a common form of gram negative septicaemia, by studying the relationship between serum antibody to the cross-reactive LPS "core" of E.coli, and survival following Pseudomonas aeruginosa septicaemia.<sup>241</sup> A total of 43 patients with Pseudomonas septicaemia, among whom there was a mortality of 42%, were evaluated. "Core"-specific antibodies of both isotypes were higher in patients who survived compared with those who succumbed to their septicaemias. Stepwise linear discriminant analysis indicated that type-specific antibody levels were the best predictor of outcome, among those antibodies examined. It was also noted that prior steroid therapy was associated with low levels of both "core"-specific IgG and IgM. These data suggested a heterologous protection against Pseudomonas aeruginosa septicaemia by naturally occurring antibodies to the "core" of E.coli.

Until 1975 it appeared that only mono- or bivalent sera were investigated. At this point Gaffin and co-workers in Israel produced a polyvalent anti-LPS serum from human blood.<sup>242</sup> Units of human blood that had been donated to blood banks were screened for high concentrations of LPS-specific antibodies. The naturally occurring IgG antibodies to LPS which were found in this donated blood, had high affinities to bind LPS prepared from E.coli, Klebsiella, Salmonella, Shigella and Pseudomonas. Also, these IgG's had a longer in vivo half-life than did the IgM preparation of previous workers.

Commencing 2 hours after the onset of an experimental haemorrhagic shock, a group of cats were experimentally treated with a polyvalent human hyperimmune plasma rich in anti-LPS antibodies, whilst control group received normal plasma.<sup>242</sup> Survival was improved in the treated group compared to the controls. These workers thus showed that antibodies specific to LPS may be therapeutically useful in "non-septic" shock aetiology. This polyvalent anti-LPS was found to bind to and, presumably, opsonize the LPS released from antibiotic-killed GNB. Another very important biological activity of this polyvalent anti-LPS is that, in the presence of complement, the anti-LPS antibodies can destroy a range of GNB themselves.<sup>243-245</sup> Since several antibodies could bind to a single LPS molecule, the likelihood of developing resistant strains of GNB is lower with anti-LPS, than with conventional antibiotic therapy acting at one site.

Human patients in septic shock were treated with conventional therapy and they also received an intramuscular gamma globulin preparation of anti-LPS.<sup>246</sup> Twenty of twenty-two patients responded by either

recovering completely or showing a temporary improvement in their clinical signs.

In another clinical trial, patients were admitted to a study after they had satisfied certain stringent criteria for shock.<sup>247</sup> Control patients received conventional therapy whilst an experimental group received the same conventional therapy plus an intravenous human freeze-dried plasma rich in anti-LPS IgG. This was also polyvalent in nature as the anti-LPS contained IgG which bound to a wide range of GNB, including E.coli, Pseudomonas, Klebsiella, Salmonella and Proteus. Mortality in control patients was 40% compared with 4.7% in the patients who received the anti-LPS. Moreover, the mean hospital stay of survivors was 28,2 days in control patients and 14,2 days for those patients who received anti-LPS. Unfortunately, the excellent results obtained in this clinical trial are open to subjective bias, as this was not a double-blind study.

Polyvalent anti-LPS hyperimmune plasma was produced in equines (ANTI-LPS) and this plasma has been used in experimental animal models of endotoxin shock as well as in clinical veterinary practice.<sup>37,51,57,113,134,237,238</sup> Equines with Colitis"X" responded very rapidly to their ailment after they were given the equine anti-LPS plasma despite the previous failure of conventional therapy. Equine post-operative peritonitis, foals with periacute enteritis and epidemic gastroenteritis were found to respond very favourably to the addition of equine ANTI-LPS into their treatment regimens compared to those that had only received the conventional therapy.<sup>134</sup> Flushing GNB infected joints with ANTI-LPS was also reported to produce beneficial results.<sup>244</sup>

Experimental rabbits were subjected to a superior mesenteric artery occlusion shock for 60 mins.<sup>77</sup> Twelve animals were pre-treated with a single dose of human anti-LPS serum 2 days prior to the arterial occlusion shock. Eight animals acted as controls and survival was recorded when each animal was still apparently normal 10 days post-release of the occlusion. One of eight controls survived the experimentally-induced shock, compared to 10 out of 12 treated animals.

In a preliminary report, equine ANTI-LPS antibodies were shown to bind with LPS present on the surface of living bacteria (Wells, Gaffin, Gregory, et al., 1986, submitted). During this binding process, it was shown that complement was activated and the Klebsiella bacteria were destroyed. This property of the equine hyperimmune plasma was successfully exploited in the treatment of Pseudomonas keratitis in rabbits,<sup>245</sup> and intra-uterine Klebsiella infection in equines.<sup>134</sup>

When equine ANTI-LPS was mixed with various GNB for 30 minutes there was a very rapid (seconds) reduction in the number of viable organisms detected by plate counts of 21 different isolates, including various multi-resistant forms (Wells, Gaffin, Gregory, et al., 1986, submitted). In the various controls, normal growth of these same 21 bacterial isolates were obtained. In this work, it was also shown that various Pseudomonas species, as well as Proteus and Providencia were the most susceptible to the equine ANTI-LPS.

In summary, ANTI-LPS has three effects;

1. it destroys GNB,
2. it binds to the LPS released from killed GNB,
3. it speeds the removal of GNB and LPS from circulating plasma.



## CHAPTER FOUR

### SUMMARY

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#### 4.1. SUMMARY

GNB alone were generally considered to be the cause of the mortality and morbidity of septic and gram negative bacteraemic patients.<sup>45,240</sup> Later it was reported that GNB were also responsible for the death of non-septic patients.<sup>5</sup> German and other workers showed that GNB possess a biochemically structured molecule consistent with that of a lipopolysaccharide (LPS) situated in, and forming an integral part of, the outer cellular membrane of these bacteria.<sup>83,84</sup> These and other workers showed that LPS was potently toxic and chemically very stable.<sup>248</sup>

This work led to the isolation of LPS which was then parenterally administered into experimental animals.<sup>5</sup> Death occurred in these experimental animals as a result of the administered LPS, and the symptomatology and pathology were described as being identical to those of septic, non-septic and gram negative bacteraemic shock. LPS was also termed "pyrogen", since it could cause a rapid biphasic increase in body temperature, and "endotoxin" because it was not secreted by living bacteria.<sup>11</sup> It is now generally accepted, without direct proof, that it is the LPS which is primarily responsible for the mortality and morbidity associated with septic, non-septic, and endotoxin shock.<sup>5</sup>

The gastric microflora of most monogastric mammals consist of acidophilic gram positive bacteria and yeasts.<sup>22</sup> Likewise, the normal bacterial flora of the small bowel includes many genera and dozens to thousands of species of GNB.<sup>23,249</sup> It is thought that carnivorous mammals have more GNB present normally, than do omnivorous mammals.

There appears to be variations in the concentration of different GNB species from individual to individual of the same species of mammal. Because LPS forms an integral part of the outer cellular membrane of GNB, the dog always has LPS present in its gut.

The LPS molecule of GNB has three distinct regions, which are recognised as: i. the lipid-A, ii. the core oligosaccharide and iii. the outer polysaccharide region which contains the "O"-antigen.<sup>83,84</sup> Lipid-A displays the least variability from one GNB species to another, but contains the exceptionally potent toxic region. In the outer polysaccharide region, the "O"-antigens exhibit specificity for a particular GNB species, and are thought to be weakly immunogenic in a host.<sup>84</sup> Certain mutant GNB lack the "O"-antigen and some core oligosaccharides.<sup>250</sup>

LPS is released or "sloughed off" from the parent bacterium when it dies or it is killed by chemotherapy.<sup>91</sup> Others have suggested that LPS may also be released when the parent bacteria undergo active replication.<sup>49</sup> However, if LPS remains within the intestinal lumen, it does not exert any obvious toxic effects within the host.<sup>72</sup>

GNB with their LPS, or isolated LPS, can gain entrance into a host's vascular circulation, and produce the clinical signs of endotoxaemia.<sup>5</sup> These GNB, with their LPS, circulating in the host's vasculature during an episode of bacteraemia and endotoxaemia, can have either an exogenous or an endogenous (or both) source. It has also been established that endogenous LPS from the intestines, gains entrance to the circulation without their parent bacteria,<sup>28</sup> and in non-septic conditions.<sup>24</sup>

The intact intestinal mucosa acts as the host's primary defence against endogenous and exogenous LPS which infect the host per os.<sup>29</sup> These findings were supported when animals with normal intestinal function, were dosed per os with between 500-3000 times the minimal lethal parenteral dose of LPS, and they displayed no clinical signs to this experimentally administered LPS.<sup>30</sup> In addition, no evidence has emerged to indicate that an intestinal enzyme is present to destroy or inactivate LPS when it is present in a dog's intestine. Furthermore, orally administered LPS can be recovered intact from the normal intestine.<sup>72</sup>

Shock has many and varied aetiology and many reviewers of this subject have tunnelled their treatment regimens, sometimes with tragic results. Today our understanding of shock indicates that irrespective of the aetiology of the shock, an altered micro-vascular circulation results, which will provide inadequate nutrition to, and waste removal from, tissues.<sup>5,251</sup>

The oxygen-reliant intestinal mucosal cells require an adequately perfused vasculature to prevent LPS from escaping into the host's vascular circulation.<sup>75</sup> During intestinal ischaemia, LPS with or without their GNB, enter the hepatic-portal circulation<sup>1</sup> and when these toxic molecules are taken up by the liver, they can then be rendered harmless by the macrophage-phagocyte system.<sup>77,97</sup> If the liver is overwhelmed or if the liver is itself not functioning adequately, LPS can enter the systemic circulation, where it will exert its toxic effects for up to two weeks.<sup>59</sup>

Circulating plasma LPS is removed by various tissues at different times

and in different sequences.<sup>59,99</sup> It has been shown that a reduction in total plasma LPS concentration appears to be related to the concentration of circulating plasma HDL.<sup>99</sup> LPS that are not bound to HDL are removed rapidly from the plasma by tissues that are rich in phagocytic cells, mainly the liver and spleen. The LPS-HDL bound complex has a prolonged half-life in the plasma and accumulates in tissues by a mechanism that probably involves receptors for HDL, these tissues being primarily the liver, adrenal glands and the ovaries. After the LPS (bound and unbound) is taken up by the different organs, it is destroyed by the macrophage-phagocyte system in those organs and finally excreted in the bile and faeces.

The haemolymph of horseshoe crabs (*Limulus polyphemus*) contain amoebocytes that are particularly sensitive to LPS, and combine with it to form a firm gel.<sup>33,252</sup> This discovery brought about the limulus amoebocyte lysate (LAL) gel test for LPS. Unfortunately, this test was found to be not entirely reliable, and today it is mainly used to detect LPS in pharmaceutical products.<sup>34,35</sup> Later work established that the chromogenic substrate modification of the LAL gel test was both a reliable and reproducible method to measure, quantitatively, circulating LPS in plasma.<sup>36,37</sup>

In order to determine whether there is an abnormal concentration of LPS in the circulating plasma of the dog, it is necessary to first establish normal values. This value has been recorded and in view of this achievement it should now be possible to accurately diagnose a developing endotoxaemia, and a developed endotoxin shock status.<sup>113</sup>

When LPS gains entry to various host tissues it can exert fatal effects

on the cells within these tissues. These include the activation of certain host systems whose end products can exert further serious and life-threatening effects.<sup>5</sup> LPS is capable of stimulating host homeostatic systems, and once these pathways have been activated, the toxic end products of their pathways may add to the destruction of more cells and tissues<sup>253</sup>. Eventual organ collapse will occur, resulting in the possible death of the host.

"Free" LPS has been reported to be immunogenic, albeit weakly.<sup>91</sup> Given sufficient time, anti-LPS antibodies can be synthesised by a host, which could then render the circulating plasma LPS harmless. If the host has not succumbed to the initial effects of circulating LPS, the recovery period is invariably prolonged, compared to the patient who has adequate antibodies to negate circulating plasma LPS. During the recovery phase from endotoxaemia, a patient must be fully supported with treatment aimed at neutralizing the harmful effects of the different toxic metabolites that were produced by damaged host cells and LPS circulating in the plasma. In addition, corrective surgery, if the aetiology indicated this approach, must be performed on the high-risk, emergency patient. Yet, in spite of successful surgical intervention, mortality due to the effects of circulating plasma LPS remains high.<sup>49</sup>

It is known that the surgical correction of a partially or completely obstructed intestinal vascular bed produces a further rise in circulating plasma LPS.<sup>57,238</sup> Rapid, passive immunotherapy, using antibody-enriched serum or plasma, prior to removal of the obstructing anatomical feature, should be able to improve the patient's chances of survival. Preferably, a broad-spectrum or polyvalent anti-LPS antibody enriched plasma would be of greatest value, as invariably, several

species and strains of GNB are involved during an endotoxaemic episode.

Endotoxin shock can occur in any patient as a result of septic or non-septic aetiological onslaughts.<sup>24,28,37,51,113,134,237,238,254</sup> In the United States, the frequency of gram negative bacteraemia has been estimated to be as high as 1 per 100 hospital patients, with fatality rates as high as 30-80%.<sup>44</sup> This figure may be even higher in cases of "pure" endotoxaemia, since endogenous LPS is able to enter the circulation without GNB during an onslaught of exogenous GNB septicaemia or endotoxaemia. This high mortality is confirmed by other studies in America and Japan.<sup>5,47</sup> In veterinary medicine mortality rates due to endotoxin shock are known to be the same or possibly even higher.<sup>49,50</sup>

Despite the advancements made in the development and use of broad-spectrum antimicrobial chemotherapeutic agents, the judicious use of the corticosteroids, anti-prostaglandin chemicals, adjuvant supportive electrolyte and nutritional fluids, other supportive therapy to combat released activated metabolites, and expensive and elaborate life-supporting and monitoring equipment, the incidence of gram negative bacteraemia, and hence endotoxaemia, continues to increase with sustained high mortality rates.<sup>133</sup> The treatment for endotoxin shock has focused on the killing of the GNB, and supporting the patient until there was recovery, whilst, little or no attention was given to the neutralization or abrogation of the concurrent endotoxaemia.

Antibiotics can destroy GNB which are sensitive to the antibiotic used.<sup>187,188</sup> Once killed, GNB will release their potent, toxic LPS which are known to be refractory to these antimicrobial chemotherapeutic agents.<sup>31</sup> Recent studies have shown that the concentration of

circulating plasma LPS can increase up to 2000-fold after an antibiotic was used to destroy the GNB that produced them.<sup>82</sup>

Tolerance to LPS can be induced by vaccinating animals with very small doses of either LPS or killed GNB.<sup>43,46,255</sup> The harvesting of the plasma from sensitised human donors, and the subsequent administration of this harvested plasma into human recipients, is known to protect the recipient against the lethal effects of LPS.<sup>256,257</sup> It was thought that specific anti-LPS antibodies in the donor's plasma provided this protection. Braude<sup>171</sup> reviewed most of this work and in 1973, he and co-workers demonstrated that human antiserum to an incompletely synthesized form of LPS conferred passive immunity to LPS effects in animals.<sup>18</sup> Later, Gaffin et al., produced the first "broad-spectrum" or "polyvalent" anti-LPS plasma from human blood donors.<sup>242</sup> This anti-LPS improved the survival rate of cats subjected to endotoxin shock, secondary to an experimentally-induced haemorrhagic shock.

A polyvalent, anti-LPS, antibody-enriched, plasma (ANTI-LPS) is now made in equines by immunizing horses with an undisclosed vaccine.\* Successful treatment of a wide variety of equine endotoxin-mediated disorders have been reported, when this equine ANTI-LPS was incorporated into the treatment regimens of these conditions.<sup>134</sup> Similar successes have been achieved when this ANTI-LPS was used in conjunction with routine therapy for diagnosed endotoxaemic conditions in the cat and dog.<sup>57,113,237</sup>

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\* "Atoxin", Atox Pharmaceuticals, Gillitts, Rep.So.Afr.



The success of this particular ANTI-LPS antibody preparation probably lies in the fact that it is broad-spectrum in activity against a wide range of LPS structures, as well as the parent bacterium.<sup>51,113,133,134</sup> It is therefore unnecessary for the clinician to isolate and identify either the species of GNB or the LPS when gram negative bacteraemia or endotoxaemia have been diagnosed.

Values for the concentration of circulating plasma LPS in healthy dogs have been established.<sup>113</sup> With this knowledge the clinician is now able to positively diagnose a developing endotoxaemia. The administration of this equine polyvalent ANTI-LPS will lead to the early administration of an LPS specific therapy with safety, as to date, no side effects of this equine origin ANTI-LPS have been reported in dogs.

Dogs are popular household pets throughout most of the Western world and they have also been used as experimental subjects in veterinary medical, and surgical research, in the investigation of the problems associated with abdominal ischaemia. Partial and complete abdominal vascular ischaemic problems are encountered in clinical veterinary practice and when diagnosed, these patients represent immediate surgical intervention on a high-risk emergency.

#### 4.2. PURPOSE OF INVESTIGATION

Zanotti and Gaffin (1983) were able to reduce the mortality and morbidity of rabbits subjected to an experimental superior mesenteric arterial occlusion shock for one hour, when they used equine origin ANTI-LPS plasma.<sup>77</sup> Rabbits are omnivorous animals and therefore it is

possible that the concentration of circulating endogenous plasma LPS would not be as high as that which could arise in a carnivorous animal experiencing the same type of experimental endotoxin shock.

The purpose of this study was, first, to investigate whether the polyvalent equine ANTI-LPS hyperimmune plasma was capable of reducing the mortality rate of experimental, abdominal vascular obstruction in a carnivorous species, the dog. This model would simulate similar intestinal vascular ischaemic canine problems which are frequently diagnosed in veterinary practice. In order to establish which were the most representative canine subjects to use as the experimental subjects, the hospitalisation records of all hospitalised patients admitted to a private veterinary hospital\* over a two-year period were examined.

Secondly, I investigated whether the polyvalent equine ANTI-LPS plasma could be beneficial in the treatment regimen of Canine Parvovirus Disease patients. This recently described canine disease has been well documented, is known to occur epidemically, and produce an endotoxaemia with exceptionally high mortality rates and prolonged morbidity periods.<sup>51-55</sup>

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\* Highway Veterinary Hospital, Pinetown, Rep.of So. Afr.

## CHAPTER FIVE

### MATERIALS AND METHODS

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## 5.1. MATERIALS FOR CRANIAL MESENTERIC ARTERIAL OCCLUSION SHOCK MODEL

### 5.1.1. SELECTION OF SUBJECTS

The aetiology of partial and/or complete abdominal vascular occlusions may be congenital or acquired. When diagnosed, these patients are invariably high-risk, surgical emergencies. Yet, despite successful surgical intervention, the mortality and morbidity rate in these cases, remains high.

Standardisation of animal species, and biological subjects for experimental work should always be attempted. Because I was to evaluate the validity of a specific anti-endotoxin immunotherapy for dogs, only canine subjects were used in this study. In this respect all the donated dogs were subjected to definite standardised selection criteria. Initially the records of all patients admitted to a private veterinary hospital (refer page 83) were scrutinised under strict confidence in an attempt to find the "type" of dog most frequently hospitalised, with regards to age, sex, breed, body mass, and colour. After these records were examined it was decided to select donated healthy dogs based on body mass and sex. These potential subjects then had to fulfill other criteria based on their vaccinal status and clinical examination.

5.1.1.1. VACCINATION STATUS Certain highly infectious and contagious canine diseases are known to produce endotoxaemia and some of these diseases can be prevented by vaccination. The first criterion applied to each potential subject, was to ensure that it was accompanied by a valid vaccination certificate, indicating that the vaccinal status was up

to date against ;

- |                                |                              |
|--------------------------------|------------------------------|
| a. Canine Distemper Virus      | b. Leptospirosis             |
| c. Infectious Canine Hepatitis | d. Canine Parvovirus Disease |
| e. Parainfluenza               | f. Rabies                    |

5.1.1.2. CLINICAL EXAMINATION Once the validity of the vaccination status was satisfied, each potential subject was provisionally admitted to this study. Each dog was kenneled separately at the private veterinary hospital where it was kept under observation for a forty-eight hour period. Food and water were provided, and where undesirable behavioural patterns (e.g., viciousness, timidness ) emerged, the dog was excluded from the study. When this initial observation period had lapsed, each potential subject underwent a clinical examination, performed by myself, to ensure that a standardised procedure was followed. This examination included ;

a. The visible mucous membranes of the eyes, mouth, anus and where applicable, either the penis sheath or the vulva, were examined macroscopically. Attention to their colour, vascular network and capillary refill time was noted.

b. Abdominal palpation of the organs was undertaken to detect abnormalities of the liver, spleen, kidneys, urinary bladder, the small and large intestines, and where applicable, the prostate gland.

c. Thoracic oscultation of the heart and lung sounds were performed using a clinical stethoscope.

d. The integumentum was examined to exclude pruritic conditions which may have been treated with corticosteroids or steroid sex hormones

during the three months prior to this initial examination.

e. The superficial lymph glands, prescapular, popliteal, inguinal and maxillary, were examined for abnormalities in size and shape.

f. The locomotory system was scrutinised by carefully examining the dog at rest and during exercise. Any condition that may have been under treatment with corticosteroids excluded the dog from the study.

If each potential subject fell within the normal parameters of each of the systems described it was then subjected to further laboratory examinations as follows;

g. Urine examination, an examination of the urine from each potential subject was undertaken for quantitative evaluation of pH, protein, glucose, ketones, bilirubin, urobilinogen and blood. A sterile catheter of appropriate size was used to collect a sterile urine sample which was then examined using a reagent strip.\* If the specimen fell within the accepted canine normals then the next examination was performed.

h. Faecal examination, a fresh faecal sample was collected from each dog and a microscopic examination was performed on this collected material, for the presence of helminths and coccidia. Appropriate treatment was instigated where necessary after which routine follow-up faecal examinations were performed to ensure that the potential subject remained free of helminths and coccidia.

When each potential subject passed the above described clinical and laboratory examinations, they were then admitted to the final stages of their selection.

---

\*"Multistix", Ames Division, Miles Labs., England.

5.1.1.3. KENNELING OF POTENTIAL SUBJECTS Potential subjects that passed the above described criteria were transferred to the animal colony of the University of Natal's Medical School. Pre-experimental acclimatisation kenneling followed for a minimum of two weeks, where each dog was housed separately and observed at least four times every day. During this period they were given a commercial dry and wet dog food,<sup>\*</sup> and water was provided ad lib.

Between 08.00h and 09.00h on the day prior to the operation each potential subject was again subjected to the same examinations as previously described above. Those dogs that passed this examination were then fed and left with water. During the course of this day each dog had its ventral abdominal area electrically shaved and then washed with a povidone-iodine solution.<sup>+</sup> All food was removed at 18.00h. but each dog was left with water overnight.

5.1.1.4. FINAL SELECTION CRITERIA At 06.30h on the experimental day, each dog had its water dish removed. Temperatures were recorded after the thermometer had been in the rectum for one minute and those dogs that were within the normal range ( $38,0^{\circ}\text{C}$  to  $38,4^{\circ}\text{C}$ ) were weighed. These dogs had now satisfied all the requirements and they were the finally selected subjects. Each dog was randomly designated to a specific group as will be discussed in the methods.

---

<sup>\*</sup> "Vitagen" and "Chow", Epol Pty.Ltd., Johannesburg, Rep.So.Afr.

<sup>+</sup> "Betadine", Adock-Ingram Labs., Industria, Rep.So.Afr.



### 5.1.2. EQUINE ANTI-ENDOTOXIN HYPERIMMUNE PLASMA (ANTI-LPS)

A commercially prepared product\* was used to treat some of the experimental dogs (see methods). Certain details of procedures of production are confidential. Healthy horses were suitably immunized to produce a plasma containing high concentrations of LPS specific IgG's. Each horse was plasmapheresed into sterile, apyrogen, plastic bottles, which contained sodium citrate as the preservative. After centrifugation this plasma was bottled in a laminar flow hood, and then stored at -20°C until used. Just before administration the plasma was thawed in a container of cold tap water at room temperature.

The concentration of antibodies specific to LPS was determined in the pooled, sterile equine plasma. The final product contained anti-LPS IgG at a concentration of 1200 ug/ml according to an ELISA calibrated by an immunoprecipitation procedure.<sup>242</sup> This anti-LPS plasma contained IgG that could bind to 26 strains of E.coli, Shigella flexneri, Salmonella, Klebsiella pneumoniae, Pseudomonas, Proteus and Enterobacter (Wells, Gaffin, Gregory et al., 1986, submitted).

### 5.2. MATERIALS FOR CANINE PARVOVIRUS DISEASE CLINICAL STUDY

CPV is a very recently described, highly infectious, canine disease, which can assume epidemic proportions.<sup>52-55</sup> It is known to have high mortality indices, and death of the patient is due to the combined effects of a rapidly developing viraemia, bacteraemia and endotoxaemia.

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\* "Atoxin", Atox Pharmaceutical Co., Gillitts, Rep.So.Afr.

During a recent outbreak of CPV, patients were brought to the private veterinary hospital and diagnosed by myself as having this disease. Only those patients that exhibited seven out of eight of the following signs were admitted to this study:

1. A non-vaccinated individual,
2. Acute onset of vomiting, especially after fluids had been taken,
3. Acute onset of the characteristic watery, very foetid, grey to haemorrhagic diarrhoea,
4. Rapid onset of lethargy and depression,
5. Rapid onset of dehydration,
6. Initial pyrexia or subnormal rectal temperature,
7. Marked congestion of the visible mucous membranes, which were also slightly cyanotic,
8. A leukopenia.

Because CPV is a "new" canine disease, the first ten consecutive patients that were admitted to this study, based on the above criteria, had their diagnosis confirmed by the demonstration of the virus in their faeces. A fresh faecal sample of approximately 2 grams, was collected from each dog admitted to this study. This was placed into a sterile container which held 50 ml of a dextrose saline solution. To this mixture, 2 ml of a penicillin-streptomycin antibiotic was added. The sample was then sealed, placed on ice and airfreighted to the Veterinary Research Institute, Onderstepoort, Pretoria, for electron microscopy (Prof.P.Howell, personal communication).

In addition to the above, patients that succumbed to CPV, underwent a post mortem examination and histopathological specimens were collected,

and sent to a private veterinary specialist pathologist for further confirmation of diagnosis (Dr.W.Botha, Pretoria, personal communication). After ten of ten CPV patients had been correctly diagnosed, and the diagnosis confirmed by either or both of the methods described above, the faecal and histopathological examinations were terminated.

These admitted CPV patients were randomly placed into a control group (Group 1 dogs, n=36) which received conventional therapy according to the attending veterinarian's discretion. A second, experimental group (Group 2 dogs, n=89) received the same treatment as the control group but in addition they were also administered two doses of equine ANTI-LPS plasma. Breed, age, weight and sex of each patient was disregarded.

After a total of 72 cases had entered this study, a significantly increased survival rate was recorded in the group that were receiving the ANTI-LPS plasma. At this stage the alternate selection into two groups ceased and all subsequent diagnosed CPV patients were allocated to the experimental group.

### 5.3. METHOD OF CRANIAL MESENTERIC ARTERIAL OCCLUSION SHOCK MODEL

#### 5.3.1. PREMEDICATION OF SUBJECTS

The dogs were premedicated with a mixture of 10 mg/ml atropine<sup>\*</sup> and 10 mg/ml acetylpromazine maleate<sup>+</sup> as is customary in veterinary practice. This mixture was injected subcutaneously at a dosage of 1 ml/10kg

---

<sup>\*</sup>"Atropine 10", Centaur Lab.Pty.Ltd., Johannesburg, Rep.So.Afr.

<sup>+</sup>"Panvet ACP", Panvet Pty.Ltd., Kempton Park, Rep.So.Afr.

body mass. Each dog received the premedication at 07.30h and was then left undisturbed in a kennel until approximately 09.00h.

### 5.3.2. ANAESTHETIC OF SUBJECTS

Each dog was anaesthetised using sterile, pyrogen-free equipment. The upper third of the anterior aspect of the right radial area was clipped short of hair, and then swabbed with alcohol. Pressure was applied to the cephalic vein just below the elbow, and this vein was entered using an i/v catheter<sup>\*</sup> of appropriate size. Half of the calculated dose of pentobarbitone sodium<sup>+</sup> (10 mg/kg) was injected rapidly, using a syringe attached to the i.v. catheter. When each dog had lapsed into the first stage of anaesthesia it was placed in a left lateral recumbent position. An appropriate size endotracheal tube was then positioned in the trachea and the cuff inflated. The remainder of the anaesthetic was injected slowly to the desired plane of surgical anaesthesia.

### 5.3.3. PRE-OPERATIVE PERIOD

When the syringe which was used to administer the anaesthetic was removed and the i.v. catheter suitably stoppered, each dog was then placed in a dorsal recumbent position and the endotracheal tube secured in the trachea by taping it to the mandible. A Starling pump was set to deliver positive respiration at 70 ml/min, and this pump was then attached to the end of the endotracheal tube. A sterile, pyrogen-free,

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<sup>\*</sup>"Surflow", Terumo Corp., Tokyo, Japan.

<sup>+</sup>"Sagatal", Maybaker, Port Elizabeth, Rep.So.Afr.

fluid administration set<sup>\*</sup> was then attached and secured to the i.v.catheter so that a Ringer's lactate solution<sup>+</sup> could be administered during the operative period.

The hind legs were stretched to a comfortable extended position and secured to the operating table. Both front legs were extended to a position so that the elbow joint was just straight enough to ensure that the cephalic vein remained fully patent during the operation. These legs were also secured by taping them to the table.

The shaved ventral abdominal area was thoroughly washed again using a hexachlorophene soap.<sup>\*\*</sup> Alcohol soaked swabs were used to remove this soap and finally, povidone-iodine was applied to this area and left there. A rectal thermometer was inserted and secured to the tail and at the same time the heating mechanism of the operating table was switched on to maintain a constant body temperature.

#### 5.3.4. SURGICAL PROCEDURE

Autoclaved drapes were positioned so that the mid-ventral abdominal area was left exposed from the xiphoid region to about 3-5 cm posterior to the umbilicus. The left-hand margin of the exposed area was about 2 cm lateral to the midline and the right-hand margin 2,5 cm. At this stage the Ringer's lactate solution was started and regulated to deliver approximately 45 drops/min. The surgeon always operated from the right

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\*"I/V Adminset", Terumo Corp., Tokyo, Japan.

+ "Viaflex", Sabex Pty.Ltd., Aerton, Rep.So.Afr.

\*\* "Sumanol Soap", Lever Bros., Durban, Rep.So.Afr.

side of the dog with an assistant standing on the dog's left. Sterile surgical techniques were strictly adhered to throughout the operation. The abdominal cavity was entered via a linea alba incision after adequate haemostasis had been applied to any small bleeding blood vessels. This incision started approximately 3 cm posterior to the xiphoid cartilage and ended approximately 2 cm posterior to the umbilicus.

The superior or cranial mesenteric artery (CMA) is located in the dorsal, anterior abdominal region where it arises posterior to the splenic artery. It is the fourth artery of the abdominal aorta and it gives off a common trunk for two colic arteries and the ileo-caeco-colic artery. These arteries supply arterial blood to the right part of the colon, the jejunum, the ileum, and the caecum.

The duodenum was deflected to the left side of the abdominal cavity and the origin of the CMA was located by blunt dissection. A sterile, pyrogen-free, synthetic, vascular occlusion tape\* (Vesselloop) was applied loosely around the exposed CMA. A stab incision was made through the right lateral abdominal musculature approximately 2 cm lateral to the midline incision. The occlusion apparatus\* (Vesselclade) to take the occlusion tape was positioned through this right lateral stab incision.

A section of jejunum was dislocated out of the abdominal cavity so that the jejunal arterial pulsations could be observed. The presence of these palpations indicated a viable arterial blood supply to the organ. The "Vesselloop" around the CMA was then temporarily tightened to ensure that

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\*"Vesselloop" & "Vesselclade", Neuromedics, Texas, USA.

the pulsations of the jejunal arteries ceased, which indicated that the loop was correctly positioned, after which it was then relaxed. The jejunum was returned to the abdominal cavity, and the dog allowed to acclimatise for 15 min. During this short acclimatisation period, nylon sutures\* of appropriate size, were positioned through the lateral stab incision and around the "Vesselclode". These sutures were left untied and a sterile damp swab was placed over this area.

After this acclimatisation period had lapsed, the "Vesseloop" was positioned through the "Vesselclode", and drawn tight to occlude the CMA. The time was noted and recorded as the beginning of the occlusion period. A loop of jejunum was again dislocated out of the abdominal cavity and observed to ensure that no arterial pulsations were observed by the surgeon. Once this factor was satisfied, the jejunal loops were immediately returned to the abdominal cavity. This was followed by an inspection of the abdominal cavity to ensure that there was no bleeding. Abdominal surgical closure was then performed using appropriate size surgical catgut\* in a simple interrupted pattern through the linea alba, followed by routine closure of the skin using nylon sutures of appropriate size in a horizontal mattress pattern.

The surgical drapes were removed and the surgical site cleaned with an alcohol-soaked swab. Povidone-iodine was applied to the sutured areas and after the leg ties were removed, each dog was placed in a left lateral recumbent position on the operating table. The i.v. Ringer's lactate solution, which had been maintained throughout the operation period, was continued whilst the subject remained in lateral recumbency.

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\* "Ethicon", Johnson & Johnson, Halfway House, Rep So.Afr.

#### 5.3.5. OCCLUSION PERIOD

The occlusion period began with the tightening of the "Vesselloop" and securing it into the "Vesselclade". During this period all the dogs (except no.5) remained in the lateral recumbent position on the operating table. The Starling pump remained attached to the endotracheal tube and the drip was in operation. The dog's rectal temperature was still being maintained between  $38,0^{\circ}\text{C}$  and  $38,4^{\circ}\text{C}$  possibly aided by the heating mechanism of the operating table.

After an occlusion period of three hours, the "Vesselloop" was released and it, together with the "Vesselclade", was removed from the subject. The preplaced nylon sutures were then knotted, the operated area cleaned, and swabbed with the povidone-iodine solution.

In dog no.5 the above-described procedure was varied very slightly. In this dog the abdominal cavity was not closed until 10 min after the release of the "Vesselloop". During the three hour occlusion period, the abdominal cavity was entered and very gently, a loop of jejunum was handled so that photographs could be taken every thirty minutes. Other than this variation, the procedure was as described.

#### 5.3.6. POST-OCCLUSION PERIOD

The subjects remained in the lateral recumbent position on the operating table, with the Starling pump on. When each dog began to shiver, indicating that the effects of the anaesthetic was becoming less, the Starling pump was gradually withdrawn until respiratory activity was



voluntary. At this stage of the procedure the i.v. drip was usually finished, and it was then removed from the catheter. Each dog then received a 10 mg/kg i.v. dose of tetracycline,\* and then the i.v. catheter was withdrawn. When each subject displayed mouth, tongue or swallowing movements, the endotracheal tube was removed, after the cuff had been deflated. Each dog was then transferred from the operating table to a recovery kennel until it could stand unassisted. At this stage each dog was then returned to a kennel where food and water was made available.

#### 5.3.7. CONTROL DOGS

The procedures described above were performed on 5 dogs which were then known as the controls ( no. 1 - 5 ).

#### 5.3.8. EXPERIMENTAL DOGS

A further 15 dogs ( no. 6 - 20 ) were subjected to the identical procedure as the controls. However, in addition, each of the experimental subjects received a dosage of equine anti-endotoxin hyperimmune plasma (ANTI-LPS) at different times as set out below;

GROUP A (n=5); This group received a dose ANTI-LPS s.c. during the pre-occlusion acclimatisation period.

GROUP B (n=5); In this group the dogs received their ANTI-LPS during the three-hour occlusion period incorporating it into the i.v. Ringer's drip.

-----

\*"Liquamycin", Pfizer Labs., Pietermaritzburg, Rep.So.Afr.

GROUP C (n=5); In these dogs, ANTI-LPS was administered s.c. within 3 minutes after the release of the occluded CMA.

Each dog in each of group A, B, and C, received a total dose of 1 ml/kg ANTI-LPS equivalent to 1200 ug/ml of LPS precipitable IgG.

#### 5.3.9. RECOVERY EVALUATION

Subjects that were alive 14 days after the release of the cranial mesenteric arterial occlusion (CMAO) were considered recovered.

### 5.4. METHOD OF CANINE PARVOVIRUS DISEASE CLINICAL STUDY

As previously stated, CPV patients were randomly allocated to a control and an experimental group. Individuals in both of these groups received the same conventional treatment as set out below.

#### 5.4.1. CONVENTIONAL THERAPY

CPV is a virus disease of dogs and, as such, no specific virocidal chemotherapeutic agent is available to combat this disease. Treatment of these patients is based on the severity of day to day symptoms and all therapeutic regimens are therefore symptomatic. In this study patients received the following daily therapy based on presenting symptoms and the veterinarian's discretion. Dosages and frequency of administration of the different drugs varied according to the severity of prevailing symptoms, the progress of each patient, and the manufacturers' instructions.

5.4.1.1. CONTROL GROUP This group received;

1. Anti-emetics and anti-spasmodics; "Maxolon" (metoclopramide mono-hydrochloride, Beechams), "Valoid" (cyclizine lactate, Coopers).
2. Anti-diarrhoea mixtures; "Kantrexil" (kanamycin, Bristol).
3. Appropriate cardiovascular therapy when necessary which included; "Frecardyl" (heptaminol and dihydroxyphenyl theophyllin, Panvet).
4. Prednisolone sodium succinate; "Solu-Delta-Cortef", Upjohn.
5. Broad spectrum antimicrobial agents, including; "Clamoxyl" (amoxycillin, Beechams), "Genta-50" (gentamicin, Phenix), "Synulox" (amoxycillin with clavulanic acid, Beechams).
6. A non-narcotic analgesic with anti-inflammatory and anti-prostaglandin activity; "Finadyne" (flunixin meglumine, Schering).
7. Electrolyte and nutritional supportive therapy consisting of; Ringer's lactate, dextrose saline, "Haemo-15" (multivitamins, Sterivet), "Tioctan" (thioctic acid, Panvet), "Catasol" (metabolic stimulant with vitamins, Bayer).

5.4.2.2. EXPERIMENTAL GROUPS These three groups of dogs received the same conventional therapy as set out for the control group. However, these dogs also received two dosages of equine ANTI-LPS either subcutaneously or mixed with their intravenous fluid therapy. The calculated dosage of ANTI-LPS (0,5 ml/kg body mass) was always administered at the same time as the initial conventional therapy. The second dose was always administered 24 hours after the first.

#### 5.4.3. RECOVERY EVALUATION

CPV patients were considered recovered when they had not vomited for a consecutive 24 hour period after they had voluntarily eaten or had taken fluids. At this stage their stools were porridge-like and they were then discharged from the hospital. Furthermore, none of the dogs in either the control or experimentally treated groups were re-admitted for the same problem.

#### 5.5. STATISTICS

The student "t" test and the paired "t" test were applied to statistical analysis.

## CHAPTER SIX

### RESULTS

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## 6.1. DEMOGRAPHIC DATA

### 6.1.1. SEX SELECTION

The medical hospitalisation records of all patients (n = 1405) which were admitted to and hospitalised at the private veterinary hospital were examined for their recorded sex. During the period 01/01/1983 to 31/12/1983, 612 patients were admitted for treatment and for the corresponding period during 1984 a total of 793 patients were attended to. There were 315 (58%) male dogs, 590 (42%) females.

The practising South African veterinarian is requested to perform sterilizing operations on dogs. In the case of males, a bilateral orchidectomy is performed whilst bitches receive a panhysterectomy, with or without an ovarian autograph. Sex records at this hospital were subdivided as shown in table 3.

TABLE 3: THE RECORD OF STERILIZED CANINE PATIENTS

Total number of male dogs -----	315
Number of entire male dogs -----	704 (36.4%)
Number of orchidectomised dogs -----	111 (13.6%)
Entire to sterilized males -----	$P < 0,0001$
-----	
Total number of female dogs -----	590
Number of entire female dogs -----	48 (8.1%)
Number of panhysterectomised dogs -----	542 (91.9%)
Entire to sterilized females -----	$P < 0,0001$
-----	

These statistics show that in this practice, more entire male and sterilized female dogs were hospitalised than their individual corresponding sex type. Because of the predominances seen at this private veterinary hospital, only donated, healthy, entire males or healthy, panhysterectomised females were used as subjects in this study.

#### 6.1.2. WEIGHT SELECTION

The body weight of every hospitalized patient was not recorded. However, the following facts do show significance:

Total number of dogs admitted for hospitalisation ----- 1405

Total number of patient's weights recorded ----- 934

Group 1, total number of patients weights 15Kg ----- 198 (21.2%)

Group 2, total number of patients weights 15Kg to 35Kg - 635 (68.0%)\*

Group 3, total number of patients weights 35Kg ----- 101 (10.81%)+

$$* \chi^2 (1 - 2) = 413,76; \quad P < 0,0001$$

$$+ \chi^2 (2 - 3) = 539,74; \quad P < 0,0001$$

As a result of these findings, only donated, healthy dogs, that were either entire males or panhysterectomised females, and which weighed between 15 and 35 Kg were admitted to this study.

#### 6.1.3. AGE SELECTION

Patients ages ranged from birth date to 21 years of age. However, ages were not substantiated by birth certificates and frequently only an approximate age was recorded. Table 4 below shows the number of patients in different age groups where the age of the dog was recorded



by a single figure.

TABLE 4: RECORDED AGE GROUPS OF HOSPITALIZED PATIENTS

Age (years)	Number	Percentage
1 -----	38 -----	5,0
2 -----	44 -----	7,0
3 -----	47 -----	7,5
4 -----	46 -----	7,3
5 -----	49 -----	7,8
6 -----	45 -----	7,2
7 -----	37 -----	5,9
8 -----	47 -----	7,1
9 -----	52 -----	8,3
10 -----	44 -----	7,0
11 -----	38 -----	6,0
12 -----	41 -----	6,5
13 -----	35 -----	5,6
14 -----	33 -----	5,3
15 -----	17 -----	2,7
15 -----	15 -----	2,4

Vascular ischaemic conditions were recorded as early as day old (congenital diaphragmatic hernia with herniation of the abdominal organs into the thoracic cavity) to 21 years ( splenic haemangioma with splenic torsion and rupture). In this study no selection was made with regards to the age of the healthy donated subject.

## 6.2. CRANIAL MESENTERIC ARTERIAL OCCLUSION SHOCK MODEL RESULTS

### 6.2.1. ANTE-MORTAL OBSERVATIONS

6.2.1.1. CONTROL GROUP The description set out below refers to the control dogs collectively. Slight variations would naturally have occurred as the subjects were alive and biological, and natural

differences would have existed. These slight variations are not mentioned, but where obvious variations occurred, these are noted.

Each dog was considered recovered from the operation and general anaesthetic when it could stand and balance unassisted. This occurred between 1 to 1 1/2 hours after the release of the occluded CMA. At this stage each dog was in a separate kennel where it remained subdued and lethargic for up to 3 hours. Dogs no. 2 and 3 drank small quantities of water but no control dog attempted to eat during this 3-hour period.

Rectal temperatures were recorded and averaged  $40^{\circ}\text{C}$  (range=  $39,6^{\circ}\text{C}$  to  $40,2^{\circ}\text{C}$ ; normal =  $38,4^{\circ}\text{C}$  ) as each dog was returned to its kennel. At 6 hours post-CMAO release (post-CMAO-r) all these dogs were exhibiting subnormal rectal temperatures with a mean of  $37,0^{\circ}\text{C}$  and a range of  $36,8^{\circ}\text{C}$  to  $37,4^{\circ}\text{C}$ . Palpation of the abdominal cavity was accompanied by a tensing of the abdominal musculature. A sterile abdominal paracentesis was performed and a watery, bloody-coloured fluid was withdrawn. All visible mucous membranes were congested and slightly cyanotic. Oscultation of the thoracic cavity revealed a tachycardia and normal lung sounds. One dog (no.3) had urinated a small quantity of yellow urine.

During the third to fifth hour post-CMAO-r each dog showed varying degrees of diarrhoea. The faeces were large in quantity, had an offensive smell, were watery to porridge-like in consistency and appeared to be arising mainly from the small intestine because of the lack of mucus with the faeces and the absence of straining when they voided the diarrhoea. At first the faeces were normal in colour but they then changed to a dark purple-red colour. Dogs 3, 4, and 5 began passing

a bloody-coloured, watery fluid shortly before each of them succumbed.

The first control dog died at 6h30min, and the last one succumbed at 16h05min post-CMAO-r. Just prior to the eventual death of each dog, rectal temperatures remained subnormal (mean=37,2<sup>0</sup>C). Bloody-watery faeces and abdominal discomfort were noted and their visible mucous membranes had become exceptionally congested and cyanosed. Two dogs (no.2 and 4) vomited an offensive smelling, almost black, tar-like vomitus about one hour prior to their individual deaths.

Each dog exhibited an increasing tachycardia and one dog (no.2) developed a heart murmur. Lung sounds remained fairly normal although each dog showed an increase in its respiratory rate. Post mortem examinations were performed on the abdominal and thoracic cavities of these control dogs.

6.2.1.2. EXPERIMENTAL GROUPS Although the experimental group of dogs were divided into three groups (see page 98), their symptomatology and recovery followed similar patterns, except that dog number 16 died. This dog will be dealt with separately (see page 110). When each dog recovered from the anaesthetic and operation it remained quiet and lethargic for up to 6 hours post-CMAO-r. Twelve of the fifteen dogs drank water within 6 hours of being returned to their kennel (dogs 10, 11 and 16 did not drink water). Their rectal temperatures were subnormal upon recovery from the anaesthetic, but gradually rose to become normal. At 20 hours post-CMAO-r all the dogs, except number 6, exhibited normal rectal temperatures and continued to do so for the next 14 days. Dog 6 had a rectal temperature of 40<sup>0</sup>C at 20 hours post-CMAO-r which returned to normal 10 hours later.

These dogs also exhibited an initial abdominal tension and discomfort similar to that described in the control dogs but at 20 hours post-CMAO-r each dog allowed its abdomen to be palpated without showing discomfort. Initially, a sterile paracentesis of the abdominal cavity revealed the same type of bloody-coloured fluid as that seen in the controls. This bloody-coloured fluid was still evident at 20 hours post-CMAO-r but had cleared 72 hours later. The following subjective observations were also noted;

a. Mucous membranes of the buccal cavity and eye were initially cyanotic and congested as was observed in the controls. However, by 20 hours post-CMAO-r the mucous membranes of the experimental dogs had returned to a normal colour and remained normal for the next 14 days.

b. Urine. Up to 20 hours post-CMAO-r all the dogs of these groups had passed only very small quantities of urine infrequently. During the next 24 hours the frequency of urination and the quantity of urine returned to normal in all dogs and they were drinking normally.

c. Appetite. Only one dog (no.7) ate food during the first 12 hours after the release of the occluded CMA. After this period, every dog in the experimental groups ate food, and normal appetites were maintained during the next two weeks. Two dogs, 6 and 20, vomited during the first 6 hours post-CMAO-r which was of the same nature as that seen in the control dogs. Only one dog in this group (no. 16) vomited after this time had lapsed.

d. Diarrhoea exhibited by the controls was also present in all experimental dogs. However, by 20 hours post-CMAO-r all but dog number

12, were voiding melanic, foetid, porridge-like faeces. At this stage dog number 12 still showed watery, slightly bloody faeces. During the next 24-hour period the faeces of all the dogs of this group returned to normal.

e. Heart and lungs. Tachycardia and increased respiratory rate as seen in the control group were also a feature of all of the dogs in this group. As with the control group the lung sounds remained normal. When these parameters were examined at 20 hours post-CMAO-r they had returned to normal pre-operative baseline values in every dog in this group.

6.2.1.3. DOG NUMBER 16 This dog stopped eating approximately 48 hours post-CMAO-r. A clinical examination was undertaken and a differential diagnosis of either acute pancreatitis or acute peritonitis or both, was made. Symptomatic pain relieving drugs\* were administered but no specific diagnostic treatment was instigated. The dog died the following day and a post mortem examination of the abdominal cavity revealed an acute pancreatitis. Streptococcal bacteria were isolated from the pancreas and it was thought that the bacteria most probably were introduced at the time of the experimental operation by either the surgeon or his assistant.

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\* 1. "Pethidine", Centaur Lab., Johannesburg, Rep. So. Afr.

2. "Buscopan", Boehr. Ingel Janssen. Johannesburg, Rep. So. Afr.

3. "Isaverin", Bayer, Isando, Rep. So. Afr.

### 6.2.2. POST MORTEM EXAMINATIONS

Post mortems were performed within one hour of the death of each of the control individuals and the experimental dog no. 16. It must be emphasised that only the abdominal and thoracic cavities were examined in an attempt to establish a diagnosis of fatal endotoxaemia. Except for minor macroscopic variations, all these dogs exhibited the same post mortal changes. Dog number 16 has already been described and the description that follows concerns the dead control dogs only.

#### 6.2.2.1. ABDOMINAL CAVITY

a. General signs of hyperaemia, vascular congestion and cyanosis of all abdominal organs and parietal pleura was very evident. These changes were noticed by the increased filling of vascular networks with a deep red to almost purple-coloured blood. The intestinal regions supplied by the CIA were the most severely affected organs. The area where the "Vesselloop" was placed around the CIA did not show any evidence of a thrombosis nor were thrombo-emboli detected in any of the blood vessels leading to or coming from the small intestine or first third of the colon.

Ascites was a positive feature in all the post mortems. On average some 500 ml (range = 375 to 900 ml) of a watery, bloody-coloured fluid containing red clots, was present.

Haemorrhages were present throughout the cavity, which ranged from small petechial areas to frank extravasations.

b. Individual organs such as the intestines were flaccid and the contents consisted of a foul-smelling, watery, bloody fluid.

Hepatomegaly was marked, and the livers were deep port wine in colour and very friable. Vascular and bile stasis was evident as seen by the slow escape of these fluids from the cut surfaces. Each gall bladder was very distended and contained a thick, dark olive-green bile.

The spleens were friable, and slightly shrunken with sharp edges. Each spleen was paler than normal in colour and the pulp was not prominent.

The kidneys were enlarged with the cortex and medulla well-demarcated. Urinary bladders contained a small quantity (average = 2 ml, range 0,5 to 5,0 ml) of concentrated urine, and the bladder walls were of normal thickness.

The pancreas glands were involved with the general signs of hyperaemia and congestion. Two dogs (1 and 5) had evidence of pancreatitis in that the surrounding tissues were showing evidence of autodigestion.

The adrenal glands were enlarged and exhibited haemorrhagic necrosis of the medulla.

#### 6.2.2.2. THE THORACIC CAVITY

Each heart had a flaccid musculature which showed extensive petechial haemorrhages. In one dog (no. 2), a hydropericardium of 30 ml of a bloody-coloured fluid was present.

The lungs were all slightly oedematous and when cut into, a small quantity of a bloody-coloured froth exuded from the cut surfaces.

### 6.2.3. MORTALITY AND RECOVERY RESULTS

6.2.3.1. CONTROL GROUP All (5/5) the control dogs died within the first 24 hours after the CMAO had been released.

6.2.3.2. EXPERIMENTAL GROUPS One dog (no.16) died 72 hours after the release of the CMAO. The remaining 14 dogs recovered and remained alive for the next fourteen days.

Table 5 (page 114) records the results of the control and experimental groups.

### 6.3. CANINE PARVOVIRUS DISEASE CLINICAL STUDY RESULTS

#### 6.3.1. DURING ALLOCATION PERIOD

During the period when patients were randomly allocated to either of the control or experimental group, it became apparent that the the ANTI-LPS treated group were experiencing a better survival rate than the controls. At this stage a total of 72 CPV patients had been entered into the study, 36 in each of the two groups. In the control group the mortality was 66,6% (24 dogs) whilst that of the experimental group was only 19,4% (7 dogs;  $p < 0,02$ ).

#### 6.3.2. AFTER THE ALLOCATION PERIOD

The ANTI-LPS treated dogs showed a significant reduction in their overall mortality rate, and therefore the random allocation into the two groups ceased, for ethical reasons. All further admitted cases were allocated only into the experimental group and were given ANTI-LPS with



TABLE 5: THE RESULTS OF THE EXPERIMENTAL AND CONTROL DOGS (CMAO-SHOCK)

Dog no.	Breed	Sex*	Age(years)	Weight(kgs)	Result <sup>+</sup>
<u>Control group</u>					
1	GSD	E M	1.0	20	D
2	xGSD	E M	1.2	20	D
3	Dobe	S F	1.0	20	D
4	xGSD	S F	3.0	35	D
5	Mix	S F	5.0	15	D
<u>Experimental groups</u>					
6	Lab	S F	11.0	30	R
7	Mast	E M	2.0	35	R
8	Mix	E M	0.6	17	R
9	Mix	E M	4.0	18	R
10	xLab	S F	1.0	22	R
11	xLab	S F	0.6	15	R
12	xCol	E M	6.0	34	R
13	xBox	S F	13.0	30	R
14	Box	E M	10.0	28	R
15	Dobe	E M	1.0	27	R
16	Rott	S F	0.9	31	D
17	Lab	E M	2.0	26	R
18	Mix	E M	3.0	17	R
19	Mix	E M	1.0	15	R
20	Mix	S F	1.5	22	R

\* S F = panhysterectomised female

E M = entire male

<sup>+</sup> D = died

R = recovered

GSD = German Shepherd

Mast = Bull Mastiff

xGSD = crossbred GSD

xCol = crossbred Collie

Dobe = Doberman

Box = Boxer

Mix = mixed breed

xBox = crossbred Boxer

Lab = Labrador

Rott = Rottweiler

xLab = crossbred Lab

their conventional therapy as has already been described. During this stage of the study, a total of 53 patients was allocated to this ANTI-LPS group. Of the 53 dogs admitted to this second part of the study, the mortality rate was 15% (8 dogs), which was an improvement on the first results obtained during the allocation period.

### 6.3.3. OVERALL RESULTS

A total of 125 CPV patients was admitted to this study. The control group receiving conventional therapy experienced an overall mortality rate of 66,6% (24/36). Those patients that received equine ANTI-LPS in addition to conventional therapy had a substantially elevated survival rate of 83,1% (74/89;  $P < 0,001$ ).

## CHAPTER SEVEN

### DISCUSSION AND CONCLUSIONS

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## 7.1. DISCUSSION

The potential for developing an endotoxaemia is always present in the canine gut,<sup>22,23,249</sup> (endogenous source) and in its total environment (exogenous source). LPS is known to be a potent, chemically stable toxin, consisting of three biochemically active components, which form an integral part of the outer cellular membrane of GNB.<sup>83,84,248</sup> The LPS components, which possess immunogenic activity in a host, remain harmless to a host until released from dying and dead GNB, and it becomes intravascular.<sup>30,72</sup>

Non-specific defence barriers include intact skin and mucous membranes, and the intact mucosal linings of the respiratory, urogenital and gastro-enteric systems. These are the host's initial defences against the invasion of either or both exogenous and endogenous LPS, with or without their parent bacteria.<sup>28</sup> An extremely wide diversification of aetiology, both septic and non-septic, are capable of producing the necessary pathological changes which can damage and disrupt these natural defences. Once this undesirable phenomenon has occurred and LPS becomes intravascular, various life-threatening pathophysiological processes are set in motion. These morbid effects are instigated either by the damaged cells themselves, or by LPS, or by the combined effects of both.<sup>5,6</sup> Besides being a cause of early cellular death, LPS can itself initiate the activation of different defence cascades which are themselves capable of adding to the already developed pathological processes.<sup>5,6</sup>

Damaged cells release vasoactive chemicals and neuro-endocrine substances into the vascular circulation where they are capable of producing both cellular and systemic pathological disarray.<sup>5,6</sup> In this manner more host cells are irreparably damaged, thus adding to the pathology of the patient. The continuation of this chain of events are capable of producing the necessary changes seen in the shocked patient. A patient is diagnosed as being in shock when serious, potentially fatal, decompensatory, reductions to the micro-vascular circulation have already occurred by the cycle described above. It is now recognised that a patient in shock has an extremely high potential of becoming endotoxic if the diagnosed shock is not immediately reversed, irrespective of whether the original onslaught had a septic or non-septic origin.

Decreased oxygen supply to cells will adversely affect mitochondrial energy-linked membrane transport systems. In addition, decreased nutritional delivery to, and waste metabolite removal from these oxygen-deprived cells, adds to the intracellular pathological processes. If the hypoxic state persists then premature death of cells will occur.<sup>75</sup> When this happens, organ failure may take place which can result in either a protracted recovery or death of the patient.

As a compensatory mechanism to this shock-induced ischaemia, it would appear that the intestinal region is among the first to be affected by the reduced micro-vascular perfusion.<sup>75,123</sup> Thus, cellular functions of the more vital areas (brain, heart and skeletal muscles), are maintained during the early stages of a shock episode. Intestinal mucosal cells, however, experience premature death during the same shock onslaught.

During this ischaemic period vital oxygen-reliant intestinal mucosal

defences, which act against endogenous LPS and GNB, are damaged. As a result of the breakdown of these defences, intestinal LPS, with or without GNB, enter the host's hepatic-portal vascular network.<sup>1</sup> These intravascular LPS molecules can either be rendered harmless by both the host's macrophage-phagocyte system and naturally-occurring specific immunoglobulins, or it will continue to circulate in the plasma.<sup>77,97</sup>

The persistence of circulating plasma LPS will depend upon the host's concentration of circulating plasma HDL and the total efficiency of the immune mediated defence processes.<sup>99</sup> If these vital fighting mechanisms are malfunctioning or if they have been overwhelmed, then LPS is capable of circulating for up to two weeks, and will still maintain its destructive nature on host cells.<sup>131</sup> At the end of two weeks, LPS has lost its immunogenicity but no reports mention if its toxicity has either decreased or has been lost altogether.<sup>59,125</sup> If the host has not succumbed to the effects of LPS, then LPS will eventually be excreted via the bile into the faeces.<sup>59,99</sup> Therefore, a therapeutic regimen that incorporates a specific anti-LPS substance should be of benefit to these patients.

Antimicrobial chemotherapeutic agents can destroy GNB, but they do not reduce the toxicity of LPS.<sup>133</sup> Research has shown that certain broad spectrum antibiotics, either by themselves or in combination with certain corticosteroids, do not improve survival rates when given to dogs that have received a severe experimentally-induced endotoxin shock.<sup>231</sup> Furthermore, antibiotics are known to produce an increase in the concentration of circulating plasma LPS of up to 2000 times that present prior to these agents being administered.<sup>82</sup> Worse, alarm is spreading due to the fact that more and more GNB are becoming resistant

to the widely used antimicrobial agents.<sup>234</sup> Therefore, in addition to conventional antimicrobial chemotherapeutics, it would appear that a specific anti-endotoxin treatment is indicated in all developing and developed shocked states.

LPS is known to instigate a potentially fatal chain of events through chemical mediation once it enters the plasma.<sup>5,6</sup> To be able to recognise shock and to successfully treat the endotoxaemia would be an achievement for all veterinarians. Our ultimate goal must be to diagnose the shock, and hence endotoxaemia, as it develops, and then to neutralize the effects of and destroy the circulating plasma LPS. In order to achieve this we must appreciate the fact that an endotoxaemia occurs with many and varied septic and non-septic conditions.

In addition, a normal healthy individual could have low concentrations of circulating plasma LPS.<sup>11,113</sup> This normal concentration, due to as yet unknown factors, but which do not appear to have harmful effects in a host, has been determined in 50 healthy dogs.<sup>113</sup> Therefore, we are now in a position to recognise canine shock (endotoxaemia) in its developmental stages.

Specific anti-LPS antibodies have been harvested from human blood and these antibodies protected cats from the effects of an experimentally-induced endotoxaemia resulting from haemorrhagic shock.<sup>242</sup> Other researchers have reported similar results when they used a specific anti-LPS against a known specific LPS.<sup>233,239,243,247,257</sup> Later, Gaffin and co-workers developed a polyvalent equine anti-LPS (ANTI-LPS) which successfully reduced the endotoxaemia produced by diversified aetiology.<sup>134</sup>

E.coli septic abortion in experimental animals was reduced when they were given ANTI-LPS.<sup>258</sup> Whole body ionizing radiation is known to produce an endotoxaemia in animals and when ANTI-LPS was administered to these experimental animals, increased survival resulted.<sup>259</sup> Experimental rabbits which received a dose of ANTI-LPS prior to a one-hour occlusion of their CMA, had significantly greater survival than controls.<sup>77</sup> Experimentally-induced acute hypoxia in primates was reported to be followed by an increase in circulating plasma LPS.<sup>37</sup> This increase was prevented when the primates were pre-treated with ANTI-LPS.

In yet another project, anaesthetised cats were subjected to a one-hour occlusion of their CMA.<sup>238</sup> During the occlusion period there was a rise in circulating plasma LPS concentration, which increased even further after this vessel was released. Shortly thereafter, the concentration of LPS dropped. In some cats a second and significantly higher increase in plasma LPS occurred, and all these cats died. This work indicated that a "biphasic pattern" of increased LPS concentration occurred in cats following CMAO. In the same experiment, a group of cats were pre-treated with ANTI-LPS before CMAO-induced shock. This group of experimentally-treated cats showed no rise in their plasma LPS concentration during both the occlusion period and the post-occlusion phase. It was stated that the ANTI-LPS completely prevented the increase in LPS concentrations that was observed in the control group.

Endotoxin shock has high mortality rates and prolonged morbidity periods among survivors.<sup>5</sup> A recent survey conducted over a 10-year period from January 1974 reported an overall mortality rate of 71% in canine



patients who developed post-surgical septicaemia.<sup>50</sup> In this survey GNB made up 60% of all culture isolates and a further 20% were due to a mixture of gram positive and GNB. E.coli was the primary organism involved and accounted for 38% of all the septicaemic patients. Intestinal surgery was responsible for 34% of all these post-surgical septicaemic patients and furthermore, in 49% of cases, the source of infection originated from the gastrointestinal tract. 45% of the patients received antibiotic and steroid therapy prior to or during surgery, and yet this high mortality rate was still experienced.

Peritonitis often accompanies abdominal vascular ischaemic conditions. Although an animal may die from a chemical peritonitis without a bacterial component, the most rapidly developing and dangerous form of peritonitis involves bacteria, the most common being E.coli.<sup>250</sup> This is most probably when an adynamic, dilated, irritated and ischaemic intestinal tract loses its ability to retain endogenous bacteria and LPS within its lumen.

There are few conditions, other than trauma, which are as acutely devastating to the canine patient as the gastric-dilatation-volvulus/splenic-torsion complex. This condition will produce both partial and complete occlusion of intestinal vasculature. These patients always represent high-risk, surgical emergencies and regardless of the successful outcome of the surgical procedure performed, a high mortality rate can be expected due to the severe nature of the disease process and associated surgical stress.<sup>56,261</sup> The veterinarian's surgical correction may well be adequate in these patients but the medical supportive therapy is lacking in its entirety. Endotoxin shock is the recorded

aetiology of death in these cases.

Research work has shown that when an occluded intestinal vascular network is released in experimental dogs then these dogs die sooner than those who did not have the occlusion released.<sup>71</sup> Furthermore, it has been demonstrated that circulating plasma LPS concentrations increase substantially after the occluded CMA was released.<sup>238</sup> At the same time, there is also a significant rise in the concentration of harmful super-oxides after restoration of the poorly perfused intestinal vasculature.<sup>147,148</sup> These super-radicals and LPS molecules are now known to add to the pathological processes of the patient in shock.

The observations of many veterinarians coincide with my own experiences in canine practice, with regard to the time of death in intestinal ischaemic patients. Most cases died after successful surgical intervention with what was thought to be adequate supportive medical treatment. The experimental and clinical findings of these patients, with regard to their time of death, support each other.

Misleading information may be extrapolated from the results obtained in one species, when this data is applied to a different species.<sup>65</sup> Hence, it is more reliable to record the findings from one species and then to apply this knowledge to the same species. In my research I was attempting to find out if ANTI-LPS could be beneficial in the canine endotoxin shock case. I therefore chose the canine as my experimental subject.

It has also been noted that differences within a species exists from individual to individual. Rats from two different laboratories behaved differently after they had been subjected to the same endotoxin shock

onslaught.<sup>252</sup> It was thought that variations in their respective diets and environments could explain these discrepancies. Riser has recorded major differences in canine zootechnology, with special reference to body mass.<sup>263</sup> In my canine experimental subjects, variables were reduced by the standardisation of sex and weight selection (page 104). Furthermore, environmental and dietary factors were partly eliminated by the choice of the same diet and kenneling for all the subjects, both pre- and post experimentally (page 89). The result of my standardisation is further supported by the few differences that existed in the ante- and post mortal findings in the CMAO shock subjects (page 106).

Researchers recorded that the administration of live GNB organisms, rather than LPS, appeared to produce the more relevant clinical characteristics of a patient in endotoxin shock.<sup>19</sup> This was thought to be due to the slow release of LPS from the administered GNB. These facts would hold some truth if one is simulating an exogenous GNB infection which is capable of producing endotoxin shock. Ongoing research has demonstrated that the experimental administration of live GNB into a canine will allow endogenous LPS to become intravascular.<sup>64</sup> Other researchers have demonstrated that endotoxin shock can occur in the absence of a bacteraemia.<sup>24,28</sup>

It has been demonstrated that although both GNB and LPS have detrimental effects in a host, their individual actions can be different.<sup>20,21</sup> Since I was simulating the non-septic clinical endotoxin shock patient where I was to evaluate a specific therapy, I chose to utilize the host's endogenous LPS in order to eliminate other possible influences by

administered GIB.

The occlusion of the CIA has been documented to produce reliable clinical characteristics of endotoxin shock,<sup>72,73,77</sup> and this model does simulate the diagnosis of many similar canine problems. Traumatic injury to soft tissue will allow the release of vaso-active and neuro-endocrine chemicals, which are known to produce intestinal mucosal damage. This in turn will allow intestinal LPS to "leak" into the micro-vascular circulation thus adding to the pathology. Therefore, the operative procedure to occlude, and then release the occluded CIA, must be performed with the minimal amount of soft tissue damage in order to simulate the clinical case more realistically.

Previous researchers have used varying techniques to occlude the CIA which necessitated either keeping the abdominal cavity open during the occlusion period or re-opening the cavity so that the CIA could be released. In my surgical technique I made use of a specialised vascular clamp (page 94) which enabled me to enter the abdominal cavity once only. This minimized handling of soft tissues, thereby decreasing the effects of surgical trauma. Therefore, in my surgical technique I believe that I did not exacerbate the pathophysiological processes which were responsible for the "manufactured" endotoxin shock, thus further simulating the clinical entity.

In my experimental CIAO study, five dogs acted as control subjects and all died within 17 hours after the release of their occluded CIA, death being due to endotoxin shock. Fifteen experimentally-treated dogs were divided into three groups of five, depending on when they received a dose of ANTI-LPS. Group I dogs received their ANTI-LPS just prior to

the beginning of the occlusion period, and 100% survived for 14 days after the occluded CMA was released. Group II dogs received their dose of ANTI-LPS during the occlusion period, and a survival rate of 80% was obtained. The non-surviving subject died as a result of the gram-positive pancreatitis introduced at the time of surgery. Group III dogs received their ANTI-LPS dosage within three minutes of the release of the CMAO, and 100% survived for a further 14 days.

The post mortem examinations performed on the non-survival (controls) subjects exhibited the general signs consistent with recorded endotoxin shock death (page 111). Both the control and experimental groups received supportive i.v. fluid, and a single antibiotic injection following the experimental procedure. As described above, the experimental groups also received a dose of ANTI-LPS. This was the only difference in the treatment received by the subjects in this experimental study. Therefore, the ANTI-LPS plasma most probably made the significant difference ( $p < 0.001$ ) recorded between the survival of the experimental subjects and the non-survival of the control group.

Corticosteroid (CTS) administration in experimentally-induced endotoxin shock subjects has received considerable attention amongst researchers (page 60). The addition of CTS therapy appears to have achieved, on average, the highest recorded survival rates when used in experimental endotoxin shock models. Some important deductions that can be made from this CTS-endotoxin shock research are:

1. CTS therapy could not improve survival numbers if canine endotoxin shock had persisted for more than 45 minutes,<sup>208</sup>
2. for CTS to be effective it must be administered i.v.,<sup>62,63,208</sup>
3. pharmacological doses are more effective than physiological doses if

- administered early during the shock episode,<sup>217</sup>
4. that CTS, in either pharmacological or physiological doses, could not improve survival in a LD<sub>100</sub> canine endotoxin shock model,<sup>219</sup>
  5. the i.v. administration of CTS has shown to have detrimental effects when utilized in the late stages of experimentally-induced endotoxin shock.<sup>225</sup>

The canine endotoxin shock model utilized in this study produced a LD<sub>100</sub> in the control subjects. Yet, when ANTI-LPS was administered to the individuals in the experimental groups, either early (before CMAO was performed), during the occlusion period, or late (three hours after the induction of endotoxin shock), an overall survival rate of 93,3% (14/15) was recorded. This immunotherapy contains naturally occurring IgG's and therefore has neither a pharmacological or physiological dose. Also, this high survival index was recorded after the administration of ANTI-LPS by both the i.v. and s.c. routes. The ANTI-LPS was easy to administer and it mixed with the i.v. fluid without any detectable reaction. Furthermore, no side effects were noted in any of the subjects that received the ANTI-LPS (n=104). These results indicate a definite improvement on other recorded experimental treatments for an LD<sub>100</sub> endotoxin shock.

CPV is a recently described disease of the canine having been recognised only since 1979.<sup>52-55</sup> It occurs both endemically and epidemically in South Africa and many other countries. This virus is thought to be a mutation of the virus producing feline panleukopenia and is known to produce an endotoxaemia with very high mortality rates world-wide. CPV appears to be particularly fatal in young and very old dogs, most

probably due in part to the immune suppression that accompanies this disease. Most CPV fatalities are recorded as being the result of acute endotoxin shock.

For the clinical study of this research I used naturally infected CPV cases that were brought to my veterinary practice. Initially, I randomly placed dogs into one of two groups. Both groups received the same conventional therapy but only one group received additional treatment with ANTI-LPS (page 99). After 72 CPV patients had been treated, the results were analysed and it was found that the survival rate of those dogs which received ANTI-LPS was significantly greater than those which did not get ANTI-LPS (17% vs 67%). At this stage the random selection of CPV patients ceased, for ethical reasons, and every patient received the ANTI-LPS in addition to their conventional medical support. Survival in this group of dogs (n = 53) was 66%, which supported the findings of the original 72 randomly selected cases. The overall survival rate of the group that received ANTI-LPS (n = 89) was finally recorded at 83%. This significant increase in survival rate can be attributed to the addition of ANTI-LPS to the total treatment regimen that was used in this clinical study ( $p < 0,001$ ).

Researchers are now able to produce endotoxin shock in many different species, and in many and varied ways. Numerous different aspects of endotoxin shock have been studied, but we have been a long way from recognising endotoxin shock in its developmental stages, in the natural case, and even more so, from preventing the many paths of destruction produced by LPS. Circulating plasma LPS can be detected by using a reliable method,<sup>37</sup> but even so, normal values have not been recorded for all species. Without a normal baseline value, it is almost impossible to

recognise an abnormal value. Japanese workers were able to detect circulating plasma LPS concentrations in various blood vessels and they then attempted to classify endotoxaemia on these findings. These researchers did not establish a normal value in their work and therefore the values that they did record could have been normal.

During the course of my experimental work we established the normal value for circulating plasma LPS in healthy dogs.<sup>113</sup> Knowing this normal value enabled us to recognise a developing endotoxaemia and instigate a anti-LPS specific therapy early in the course of the naturally developing endotoxin shock. Furthermore, we were successful in this treatment, and were able to eliminate the higher than normal concentration of circulating plasma LPS.<sup>113</sup>

In the CPV clinical study it is thought that the main reasons for the significantly higher survival in the ANTI-LPS-treated group of dogs as compared to those that did not receive this ANTI-LPS was:

1. a specific, broad-spectrum anti-LPS therapy was used to eliminate the greater than normal concentration of circulating plasma LPS,
2. this specific therapy was administered during the developing endotoxin shock stages.



## 7.2. CONCLUSIONS

A. EXPERIMENTAL STUDY: A LD<sub>100</sub> C11A0-induced endotoxin shock model was utilized. The control group which did not receive equine ANTI-LPS had a mortality rate of 100%. Three experimentally-treated groups received this ANTI-LPS and 80% of these canines survived for a minimum of 2 weeks after receiving their dose of ANTI-LPS. Therefore, the significant survival rate ( $p < 0,001$ ) in the experimentally-treated groups can be attributed to the administration of equine hyperimmune anti-LPS plasma.

B. CLINICAL STUDY: The addition of equine ANTI-LPS to the conventional therapeutic regimen for CPV patients produced a significant survival rate during the allocation period ( $p < 0,02$ ) and an even more significant survival rate over the entire study period ( $p < 0,001$ ).

From both the experimental and clinical study the following recommendations are made:

1. A polyvalent, equine ANTI-LPS hyperimmune plasma should be used in preference to either a monovalent or bivalent ANTI-LPS plasma.
2. This equine ANTI-LPS should be incorporated into the surgical and medical management of the clinical conditions which are known to produce either a partial or complete occlusion of the intestinal vasculature.
3. This ANTI-LPS should be preferably administered prior to or during the surgical correction of the underlying anatomical abnormality that is producing the partial or complete obstruction.
4. ANTI-LPS immunotherapy should be incorporated into the medical management of canine enteric disorders, other than CPV, which are capable of damaging the intestinal mucosa.

5. Equine ANTI-LPS can be given with safety to a canine patient because no side effects were recorded in any (n=104) of the dogs that received this ANTI-LPS.
6. Any canine patient that is either developing or has developed shock from any aetiological agent, should be treated, in part, and as early as possible, with this ANTI-LPS hyperimmune plasma.
7. Either the s.c. or i.v. routes should be followed and either one, or at the most two, injections of ANTI-LPS need to be administered.
8. The use of this equine ANTI-LPS plasma in the therapeutic regimen of endotoxin-producing problems in species other than the equine and canine should be investigated.

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ANTI-ENDOTOXIN IMMUNOTHERAPY FOR CANINE PARVOVIRUS ENDOTOXAEMIA

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**ABSTRACT**

The morbidity and mortality associated with canine parvovirus disease (CPV) is caused, in part, by endotoxin (LPS). Equine anti-endotoxin hyperimmune plasma (Anti-LPS) was administered to 89 CPV patients in addition to conventional therapy. In Anti-LPS treated CPV patients mortality was lower (16.8 per cent, 15/89) than in controls who received conventional therapy alone (66.7 per cent, 24/36,  $p < 0.0005$ ). The hospitalization period of survivors was reduced from  $8.5 \pm 4.0$  days (controls) to  $5.2 \pm 2.0$  days (Anti-LPS treated group). These results suggest that an anti-endotoxin specific therapy should be incorporated into the treatment regimen of CPV, and possibly, other canine enteric disorders, known to produce endotoxaemia.

## INTRODUCTION

Endotoxins (lipopolysaccharides, LPS) are contained in the outer cellular membrane of gram negative bacteria, the latter of which are always present in the intestines of dogs (Goldstein, 1974). During an episode of canine parvovirus (CPV), the intestinal defense mechanism is extensively damaged which allows gram negative bacteria and LPS to enter into the general circulation. This produces mortality and morbidity as a result of the combined effects of the viraemia, the gram negative bacteraemia, and endotoxaemia. Any circulating plasma LPS can remain toxic to host cells for as long as 2 weeks after the gram negative bacteria which produced them, have been destroyed by host immune systems or by chemotherapy (Freudenberg et al., 1985). This endotoxaemia itself, is thought to be a major cause of death in gram negative bacteraemia (Zinner & McCabe, 1976). Furthermore, in many cases such LPS alone may be absorbed from the intestinal tract (Papa et al., 1983; Bertok, 1983).

Treatment regimes for CPV have been directed towards the symptomatic relief of the potentially fatal consequences of vomiting, diarrhoea and dehydration (Krakowka et al., 1982). An LPS-directed therapy would appear, in principle, to be of benefit to CPV patients. This study investigated whether such an approach could reduce the mortality rate and morbidity period associated with clinically presented CPV cases. The results showed that a polyvalent, equine anti-endotoxin hyperimmune plasma was a successful adjuvant to the total therapy for CPV patients.

## MATERIALS AND METHODS

### A. ENTRY INTO THE STUDY:

Patients that were exhibiting seven out of eight of the following signs were admitted to this study:

1. A susceptible (non-vaccinated) individual,
2. Acute onset of vomiting, and vomiting after food or liquids had been taken,
3. Acute onset of a watery, foetid, grey to haemorrhagic diarrhoea,
4. Rapid onset of depression,
5. Rapid onset of dehydration,
6. Initial pyrexia or subnormal rectal temperature,
7. Slight cyanosis and marked congestion of the visible mucous membranes, with an increased capillary refill time,
8. A Leukopenia.

Initially, patients admitted to this study had their CPV diagnosis confirmed by demonstrating the virus in faecal samples by electron microscopy (Veterinary Research Institute, Onderstepoort, Pretoria). In addition, patients in both groups that died were autopsied and histopathological specimens were examined and the diagnosis again confirmed by a specialist veterinary pathologist (Botha, Pretoria). After ten of ten dogs had been correctly diagnosed, the faecal and histopathological examinations were terminated.

CPV patients were diagnosed at a veterinary hospital where no selection was made with regard to breed, age, sex or body mass. Initially, selected dogs were placed alternately into the control or experimental group. After a total of 72 cases had entered this study, a

significantly increased survival rate and decreased morbidity period in the Anti-LPS treated group was observed, and the alternate selection was abandoned. Subsequently, all diagnosed CPV patients were allocated to the experimental group.

## B. THERAPY

Control Group. This group received:

1. Anti-emetics and anti-spasmodics; "Maxolon" (Metoclopramide monohydrochloride, Beechams); "Valoid" (Cyclizine lactate, Coopers).
2. Anti-diarrhoea mixtures; "Kantrexil" (Kanamycin, Bristol).
3. Appropriate cardiovascular therapy when necessary; "Frecardyl" (Heptaminol and dihydroxyphenyl theophyllin, Panvet).
4. Prednisolone sodium succinate; "Solu-Delta-Cortef", (Upjohn). 30 mg/kg, i.v. as a single injection.
5. Broad spectrum antimicrobial agents; "Clamoxyl" (Amoxycillin, Beechams), "Genta-50". (Gentamycin, Phenix), "Synulox" (Amoxycillin with clavulanic acid, Beechams).
6. A non-narcotic analgesic with anti-inflammatory and anti-prostaglandin activity; "Finadyne" (Flunixin meglumine, Schering).
7. Electrolyte (Ringer's lactate, dextrose saline), nutritional and vitamin ("Haemo-15" (Sterivet), "Tioctan" (Panvet), "Catasol" (Bayer)), supportive therapy.

Dosages and frequency of administration varied according to the severity of clinical symptoms, the progress of each patient, and manufacturers' instructions.

Experimental Group. In addition to that treatment which was given to the control dogs, this group received two dosages (0.5 ml/kg each) of polyvalent, equine anti-endotoxin hyperimmune plasma (Anti-LPS). The first injection was given either subcutaneously, undiluted, or intravenously, diluted with approximately 300 ml electrolyte fluid depending upon the severity of the patient's condition when admitted. In all the subjects of this group, the second injection was administered subcutaneously, 24 hours after the first.

C. CRITERIA FOR RECOVERY:

Patients were considered recovered when their diarrhoea was reduced to a porridge-like stool, they had taken fluids and food per os and had not vomited for a continuous 24 hour period, and they were discharged from hospital.

D. ANTI-ENDOTOXIN HYPERIMMUNE PLASMA (ANTI-LPS):\*

Healthy horses were suitably immunized with an appropriate proprietary vaccine and their plasma was collected under sterile conditions by plasmapheresis. The final plasma product contained 1500 µg/ml of LPS-precipitable IgG antibodies.

\* "DETOXIN" Liga Pharmaceutical Co., Vienna, Austria).

These antibodies could bind to LPS prepared from Shigella flexneri, five strains of E. coli, five species of Salmonella, plus Klebsiella spp., Proteus spp., and Pseudomonas spp. (Wells et. al. 1986. Submitted). This plasma was bottled under a laminar flow hood and stored at  $-20^{\circ}\text{C}$  until used. The experimentally treated group then received 0.5 ml/kg body weight per injection either i.v. or s.c. as described.

#### F. STATISTICAL ANALYSES

The Fisher exact probability test was used to examine significant differences in survival rates of the two groups of CPV patients.

## RESULTS

After examining the results of 36 patients in each group, those dogs which had received Anti-LPS showed a significantly reduced mortality compared to those which received only conventional therapy (7/36 vs 24/36,  $p < 0.02$ ). At this stage of the field trial selection into the control group ceased. The results for the entire study are recorded in Table 1. Mortality in the group which received Anti-LPS continued to be less than those that did not receive Anti-LPS. Furthermore, Anti-LPS reduced the mean hospitalization (morbidity period) of survivors from  $8.5 \pm 4.0$  days to  $5.2 \pm 2.0$  days ( $p < 0.05$ ).

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## DISCUSSION

LPS present in the outer cellular membranes of normal gram negative bacterial gut flora are harmless to the host if they remain within the gut lumen. Biologically, LPS is a chemically stable, potent, bacterial toxin, whose biological structure and role in sepsis have been described (Spink et al., 1948; Rietschel et al., 1982). When LPS enters into the circulation of normal individuals, the liver detoxifies and/or opsonizes these pathogens, in part, utilizing specific antibodies and the macrophage-phagocyte system (Jacob et al., 1977; Zanotti & Gaffin, 1985).

CPV produces massive and rapid destruction of intestinal villi which allows virus, gram negative bacteria and their LPS to gain entry into the host's general circulation (Appel, Scott & Carmichael, 1979; Hayes, Russell & Baibur, 1979; Nelson, Eustis & McAdaragh, 1979; Carpenter et al., 1980; Evermann et al., 1980; Mulvey et al., 1980; Janssen et al., 1982). Because of the rapid entry of these pathogens, the liver's immunological defenses become depleted and a viraemia, gram-negative bacteraemia and endotoxaemia result. Their combined effects produce the symptomatology of CPV.

To date, no specific anti-endotoxin therapy for the CPV patient has been documented. A recent report has shown that in gram negative bacteraemia, circulating plasma LPS concentrations can increase 2000 fold after conventional antibiotic therapy destroyed the parent bacteria, thus permitting bound LPS to "slough off" into the blood. There they remain in circulation producing a toxic effect on host's cells (Sheneb & Morgan, 1984). An anti-LPS specific therapy would appear



to be an advancement towards the treatment of a CPV patient.

Human Anti-LPS was successful in the management of septic shock in humans (Lachman, Pitsoe & Gaffin, 1984) as well as in protecting cats against endotoxin shock induced by haemorrhage (Gaffin et al., 1981). Similarly, equine Anti-LPS was therapeutic in the management of gastroenteritis and endotoxaemia in equines (Gaffin et al., 1983) and reduced mortality as a result of endotoxic shock following cranial mesenteric arterial occlusion in dogs (Wessels, Gaffin & Brock-Utne, 1985) and rabbits (Zanotti & Gaffin, 1985). Current information shows that Anti-LPS preparations function by three mechanisms: toxin neutralization, opsonization and complement mediated destruction of gram negative bacteria (Gaffin, Badsha & Vorster, 1985; Pudifin et al. 1985; Wells et al., 1986. Submitted).

The results of this clinical trial show a reduction in hospitalization and mortality rate in Anti-LPS treated patients. It must be noted that when the Anti-LPS was administered intravenously, it was always diluted in several volumes of electrolyte solution and given slowly. On the other hand, subcutaneous injections were given at full strength. The same total dosage of 0.5 ml/kg (750 µg/kg of LPS specific IgG) was utilized for both routes. No side effects were observed in any patient that received one or more injections of the Anti-LPS. This was in agreement with our previous clinical experience with the Anti-LPS. However, only three patients have received Anti-LPS again on a second occasion, more than 6 months from the time of original therapy, and all without side effects. Furthermore, it was observed that when a highly moribund animal received Anti-LPS by the intravenous route, recovery in

clinical signs was faster than by the subcutaneous route.

It should be noted that this was not a double blind study and therefore open to subjective bias. However, similar results were obtained from participating veterinary surgeons in their own, unrelated, private practices.

Recently, this Anti-LPS preparation was shown to be the most effective antibody preparation to treat a lethal model of abdominal sepsis (Shennib et al., 1985). When used in combination with antibiotics, Anti-LPS provided even longer survival. In summary, we conclude that the polyvalent, equine anti-endotoxin hyperimmune plasma can be of benefit when treating the clinical CPV patient and probably would advantageously affect the clinical course of many LPS mediated disorders.

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TABLE 1  
MORTALITY RATES DURING THE ENTIRE STUDY PERIOD  
(n = 125)

	NUMBER	DEATHS	MORTALITY
CONTROL DOGS	36	24	66.7%
ANTI-LPS TREATED DOGS	89	15	16.8%

(p = < 0.0005)



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CIRCULATING PLASMA ENDOTOXIN (LPS) CONCENTRATIONS IN HEALTHY  
AND HEMORRHAGIC ENTERIC DOGS: ANTI-ENDOTOXIN IMMUNOTHERAPY  
IN HEMORRHAGIC ENTERIC ENDOTOXEMIA

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## ABSTRACT

Circulating plasma endotoxin (LPS, lipopolysaccharide) concentrations were determined in fifty healthy dogs (25 of each sex) by means of the chromogenic substrate modification of the Limulus amoebocyte lysate assay. The mean value in 50 dogs was  $0.053 \pm 0.004$  ng/ml. In contrast, two groups of canine patients with hemorrhagic enteritis (Group 1,  $n = 15$ ; Group 2,  $n = 20$ ) had significantly elevated plasma LPS concentrations upon admission (Group 1 =  $0.216 \pm 0.024$  ng/ml; Group 2 =  $0.368 \pm 0.082$  ng/ml;  $p < 0.001$ ). Both groups of hemorrhagic enteric dogs received conventional enteric therapy after being admitted to hospital. In addition, Group 2 dogs received a subcutaneous dose of 0.5 ml/kg of polyvalent equine anti-endotoxin hyperimmune plasma (Anti-LPS) on admission, whilst Group 1 dogs received the same dosage of Anti-LPS 24 hours after being admitted. Anti-LPS treatment significantly reduced the endotoxemia in both groups of hemorrhagic enteric patients. Circulating plasma LPS concentrations in Group 2 dogs declined rapidly to normal levels ( $0.058$  ng/ml,  $p < 0.001$ ) after 24 hours. Group 1 dogs exhibited only a small reduction in plasma LPS concentrations after 24 hours ( $0.179$  ng/ml,  $p < 0.05$ ) following conventional therapy. When this group received Anti-LPS their plasma LPS concentrations were reduced to normal values 24 hours later ( $0.049 \pm 0.006$  ng/ml).

An earlier and more accurate diagnosis of canine endotoxemia can be made now that normal values of circulating plasma LPS concentrations have been established. Furthermore, this endotoxemia was rapidly reversed by using a commercial polyvalent equine Anti-LPS hyperimmune plasma.

## INTRODUCTION

Gram-negative bacteria and, hence, their endotoxins (lipopolysaccharides, LPS, pyrogens) are normally present in the intestines of healthy dogs<sup>1</sup>. A wide variety of diseases and physical onslaughts can damage the intestinal defenses leading to a gram-negative bacteremia and endotoxemia<sup>2-4,14-17</sup>. Conventional antimicrobial chemotherapy may kill the parent gram-negative bacteria but these agents have no detrimental effect on LPS<sup>5,6</sup>. Moreover, a rapid destruction of the parent bacteria, such as, by certain antibiotics, can cause a 2000 fold increase in plasma LPS concentrations<sup>6</sup>.

Endotoxemia is invariably diagnosed from the patient's history and symptomatology. Despite the advances made in diagnostic and treatment regimens, the mortality rate of endotoxemia still remains high<sup>7-12</sup>. The veterinarian would advance in scientific knowledge if the endotoxemia was accurately diagnosed, confirmed and successfully treated as it was developing.

Confirmation of the diagnosed canine endotoxic patient can now be made by measuring the concentration of circulating plasma LPS. Recently an accurate, reliable and replicable test to determine plasma LPS was reported<sup>13,18</sup>. This chromogenic substrate modification of the Limulus amoebocyte lysate assay can be performed in most properly equipped laboratories by competent technicians. With this test, it should be possible to make an earlier and more accurate diagnosis. However, in order to evaluate the clinical significance of any particular LPS concentration determined, a knowledge of the normal range of concentrations of circulating plasma LPS in healthy dogs must be established. To our knowledge such data has not been recorded in a

canine population. .

If a patient's plasma LPS concentration is recognized to be elevated, then an earlier treatment could lead to a reduction in mortality rates and morbidity periods, especially if the recently reported anti-LPS hyperimmune equine plasma (Anti-LPS) is incorporated into the total treatment regimen<sup>14-17</sup>. Anti-LPS contains specific IgG's which bind to the circulating LPS in vivo<sup>16,17</sup>, opsonize it, and at the same time can rapidly destroy a wide range of circulating gram-negative bacteria by means of complement activation<sup>19</sup>.

In this study we determined the circulating plasma LPS concentrations in the blood of 50 healthy dogs (25 of each sex). We compared these baseline values with the plasma LPS concentrations of hemorrhagic enteric patients and the efficacy of Anti-LPS treatment was evaluated.

## MATERIALS AND METHODS

### The Subjects For Normal Determinations

Dogs were taken by their owners to a veterinary hospital for primary vaccination or routine annual revaccination. Each dog was subjected to a routine physical examination and if found to be clinically normal and healthy, a blood sample was withdrawn prior to vaccination. The appropriate vaccines were then administered according to manufacturer's directions for use. Owners were contacted by telephone two weeks later and if they reported any unusual behavioural pattern, (e.g., vomiting, diarrhea, "skipped" meal) then the dog was eliminated from this survey. A selection of an equal number of males and females was made, (total number = 25 of each sex) without regard to age, breed or weight.

### The Blood Samples:

Sterile procedures were strictly adhered to for blood collections in both healthy and sick dogs. Only sterile, pyrogen-free, equipment was utilized.

The anterior, mid-radial, aspect of the right foreleg was clipped and sterilized with alcohol and povidone-iodine. A 3 ml disposable syringe was "wet" internally with a 5000 i.u./ml solution of heparin. Using this syringe, a 2 ml blood sample was withdrawn from the cephalic vein and immediately placed into a disposable plastic test tube which was centrifuged in a clinical centrifuge for 10 mins. This plasma was transferred to another labelled test tube and then stored in the freezer compartment of a household refrigerator (approx.  $-14^{\circ}\text{C}$ ). The time interval from withdrawal to being placed in the freezer was never longer

than 20 minutes. These samples were later delivered, in their frozen state, to a laboratory in the University of Natal Medical School, Physiology Department, where all LPS determinations were made by the same technician.

#### Endotoxin Determinations

The chromogenic substrate modification of the Limulus amoebocyte lysate (LAL) test, as previously described<sup>13,18</sup>, was performed on each sample.

#### Hemorrhagic Enteric Patients

Thirty-five unselected, consecutive canine patients routinely admitted to a veterinary hospital, with the following signs were included in this study:

- a) Vomit or diarrhea or both, noticed by owner during 48 hour period prior to admission.
- b) No food taken during 24 hours prior to admission.
- c) Lethargy,
- d) Congested mucous membranes with increased capillary refill time and showing slight to moderate cyanosis.
- e) Slightly tense to tense abdomen, when palpated.
- f) Tachycardia.
- g) Presence of melana or blood on a rectal thermometer at initial examination. Rectal temperature not critical, although 33 of 35 patients exhibited either a subnormal (n=11) or above normal temperature.

These admitted patients were randomly split into two groups which

received the following therapy:

Group 1 (n = 15) received conventional enteric treatment upon admission which included gentamycin, prednisolone and routine supportive intravenous fluid treatment, as determined by the attending veterinarian. Twenty four hours after admission these patients then received Anti-LPS (0.5 ml/kg, s.c.). Group 2 (n = 20) received similar enteric therapy but they also received, on admission Anti-LPS as above, with their conventional therapy.

Blood samples for LPS determinations, were taken from both groups on admission and prior to any treatment, and daily thereafter.

#### Anti-endotoxin Hyperimmune Plasma.

Polyvalent equine hyperimmune plasma was obtained by the plasmapheresis of suitably immunized healthy equines, and contained 1200 µg/ml of anti-LPS precipitable IgG ("ATOXIN", ATOX Pharmaceutical Co. 14 Old Main Road, Gillitts 3600, South Africa). These specific antibodies were previously found to bind to LPS obtained from a wide range of gram-negative bacteria, to opsonize the bacteria by polymorphonuclear leukocytes<sup>20</sup>, and by complement activation, destroy a wide range of gram-negative bacteria within seconds to minutes<sup>19</sup>. Furthermore, it reduced elevated plasma LPS levels within 5 - 10 minutes of i.v. administration in cats<sup>16</sup>.

#### STATISTICS

Statistical analyses were carried out using the student "t" test or paired "t" test.

## RESULTS

Table 1 shows that, while there was a small difference in the mean concentrations of circulating plasma LPS, between healthy male and female dogs, this difference was not statistically significant ( $p < 0.1$ ). Therefore, all results were pooled to record a normal value for the concentration of circulating plasma LPS in dogs, of  $0.053 \pm 0.004$  ng/ml with a range of 0.000 to 0.101 ng/ml.

Hemorrhagic enteritis in canines, irrespective of etiology, produced a significant elevation in the concentration of circulating plasma LPS (Group 1 dogs =  $0.216 \pm 0.024$  ng/ml; Group 2 dogs =  $0.368 \pm 0.082$  ng/ml;  $p < 0.001$ ) as shown in Figure 1. In Group 1 dogs, routine therapy alone slightly reduced the concentration of circulating plasma LPS (0.216 to 0.179 ng/ml,  $p < 0.05$ ). However, these LPS concentrations were still significantly higher than normal baseline values ( $p < 0.001$ ). When Anti-LPS was administered, to this group, the plasma LPS concentration returned to normal values of  $0.049 \pm 0.006$  ng/ml, twenty four hours later. In Group 2 dogs, the plasma LPS concentration returned to normal values (0.058 ng/ml) twenty four hours after receiving Anti-LPS therapy in conjunction with conventional therapy.



## DISCUSSION

Until recently, the value of the conventional LAL gel test was treated with scepticism because of an insensitivity of the original qualitative gelation test and false positive results<sup>21</sup>. The recently reported chromogenic substrate modification of the LAL test, however, is both reliable and replicable<sup>13,17</sup>.

No significant difference in plasma LPS concentrations in the 50 healthy dogs was observed when analyzed by age, mass and sex groups. We therefore conclude that our findings should be considered the normal value for circulating plasma LPS concentrations in a healthy canine population. This value is 0.053 ng/ml, with a range of 0 to 0.10 ng/ml. Any value above 0.10 ng/ml should be regarded as abnormally high.

It has been demonstrated that a single injection of 0.5 ng/kg LPS produces granulocytosis of 200-300% within 4 hours, without other reactions in healthy adult human volunteers<sup>22</sup>. A slightly higher dose of 0.8 ng/kg LPS can be pyrogenic<sup>22</sup> while 3.0 ng/ml LPS appeared to cause death in shocked cats<sup>17</sup>. It is generally accepted that LPS is responsible for most of the mortality and morbidity associated with gram-negative bacteraemia<sup>23</sup>.

Normal canine gut flora consists of large amounts of gram-negative bacteria and hence endotoxins (LPS)<sup>1</sup> which form an integral part of the outer cellular membrane of these bacteria<sup>24</sup>. Circulating LPS is extremely toxic to host cells, it is both chemical and heat-stable, and its toxicity is not reduced by conventional chemotherapeutic agents<sup>5,6,24</sup>. Provided that LPS remains in the intestinal lumen, no life-threatening effects have been reported<sup>25</sup>. The intact intestinal

wall acts as the primary defence mechanism which appears to be highly dependent upon an adequate oxygenated blood supply<sup>17,26</sup>.

Small amounts of gram-negative bacteria "leak" by unknown mechanisms, into the portal system, which are then transported to the liver. In the liver, these bacteria are rendered harmless, in part by specific antibodies and the macrophage-phagocyte system<sup>6,27</sup>. In the systemic circulation some gram-negative bacteria may be destroyed by host defence mechanisms with the release of free LPS<sup>28</sup>. This LPS may then be bound and neutralized by specific Anti-LPS antibodies if these antibodies are present in sufficient concentration<sup>29</sup>. If this neutralization action is functioning weakly, then LPS may exert its toxic effects causing depression, fever, and anorexia for up to 2 weeks before it is excreted, mainly in the feces<sup>30</sup>.

Rising concentrations of LPS above those normally present in circulating plasma may indicate that a clinically significant endotoxemia is developing. It is doubtful that a developing endotoxemia could be accurately diagnosed, on clinical grounds alone. If treatment was initiated at this early stage, in particular by incorporating Anti-LPS, then the often fatal consequences of an endotoxemia may well be reduced or eliminated altogether<sup>14-17</sup>.

Four dogs were eliminated from the study group of healthy dogs because they developed gastroenteritis or depression 1-2 days after vaccination. Although they appeared normal at the time of vaccination, all four had significantly elevated plasma LPS concentrations (mean = 0.300 ng/ml) compared to healthy dogs. While these numbers are small, it does suggest that it should be possible to detect developing endotoxemia by measuring

the circulating plasma LPS concentration.

Hemorrhagic enteritis is a symptomatic disease of canines with many and varied etiology. Whatever the cause of hemorrhagic enteritis, an endotoxemia may develop because of the breakdown of the host's intestinal defence mechanisms. In clinical practice, priority must be given to saving a patient's life, without the absolute necessity of obtaining an etiological agent. In this series of hemorrhagic enteric canines the etiological agent was unconfirmed in most cases, yet all of the patients survived.

We consider the normal plasma LPS concentration in healthy canines to be established for our laboratory and that hemorrhagic enteritis patients have elevated LPS concentrations which are easily recognizable. Furthermore, these elevated plasma LPS concentrations can be effectively reduced by using polyvalent equine Anti-LPS plasma. Patients with rising plasma LPS concentrations can now be recognised earlier and LPS specific therapy can be instigated earlier, thus reducing mortality and morbidity.

#### ACKNOWLEDGEMENTS

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Table 1. CIRCULATING PLASMA LPS CONCENTRATION IN  
HEALTHY DOGS.

	Number	Mean LPS conc. ng/ml.	S.D. ng/ml.	Range ng/ml.
Males	25	0.046	0.026	0.000 - 0.097
Females	25	0.060 <sup>*</sup>	0.029	0.000 - 0.101
Combined	50	0.053	0.029	0.000 - 0.101

<sup>\*</sup>Males to females p 0.1 (not significant).

LEGEND

Figure 1. CIRCULATING PLASMA LPS CONCENTRATIONS IN DOGS

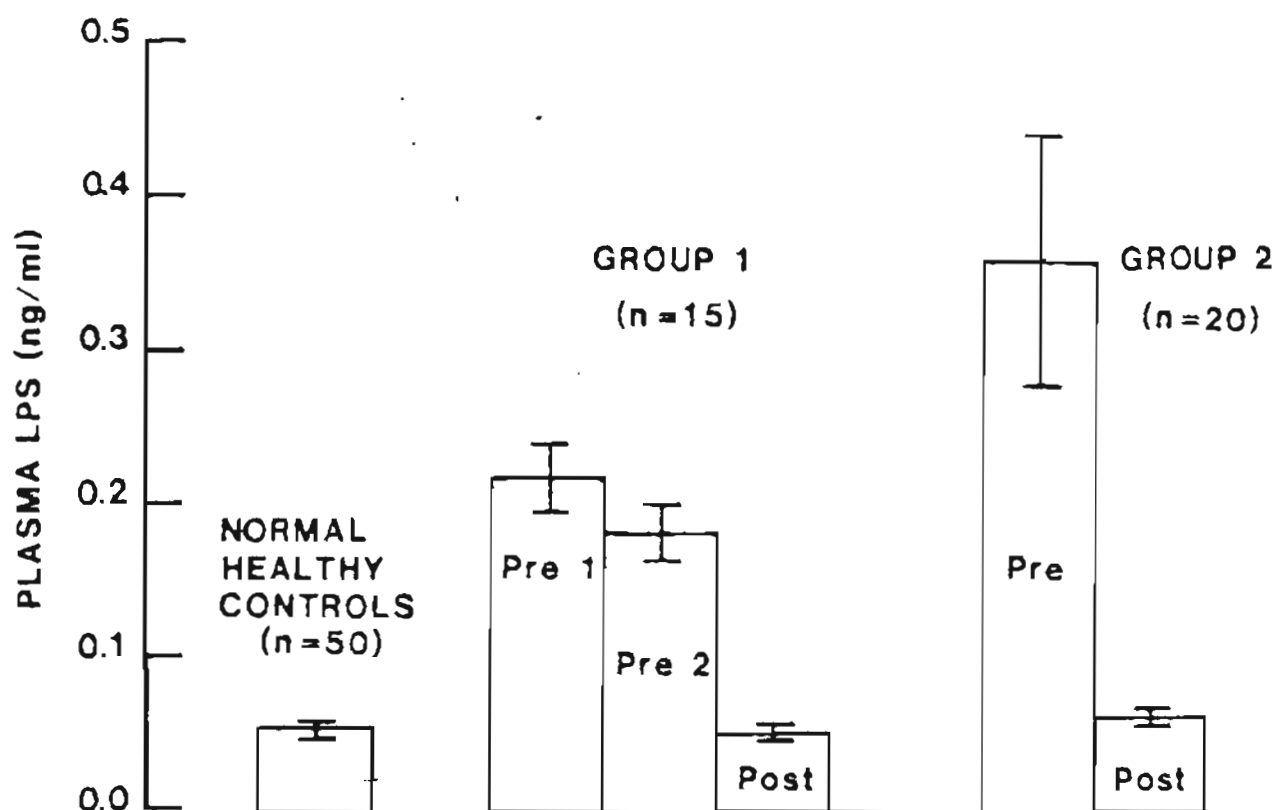
Group 1. = Dogs which received Anti-LPS 48 hours after admission.

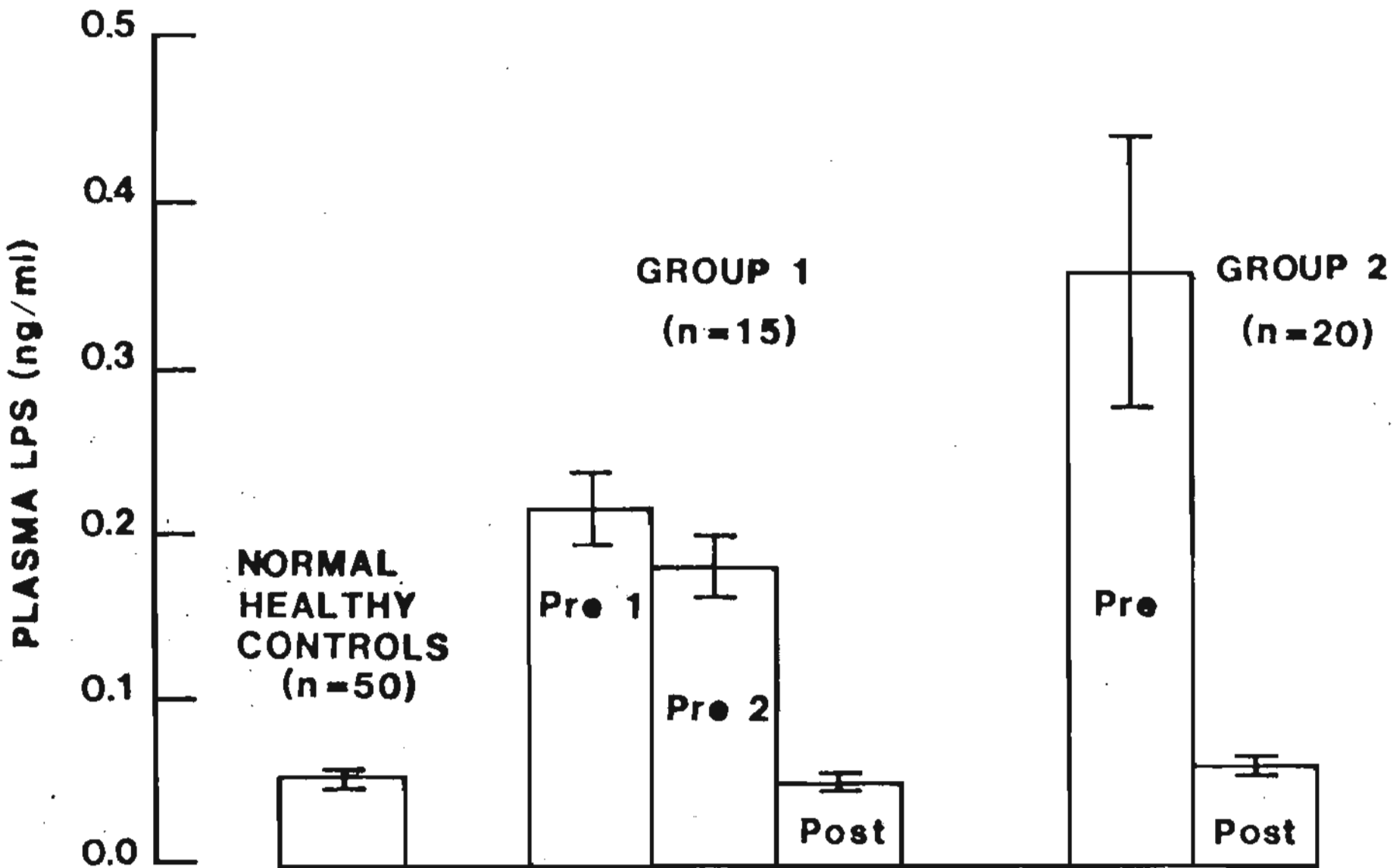
Group 2. = Dogs which received Anti-LPS 24 hours after admission.

Pre 1 and Pre = Sample taken on admission before any treatment  
was administered.

Pre 2 = Sample taken 24 hours after admission and before Anti-LPS  
was administered.

Post = Sample taken 24 hours after Anti-LPS administration.





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REDUCTION IN CANINE MORTALITY WITH ANTI-LPS IMMUNOTHERAPY  
FOLLOWING INTESTINAL ISCHEMIC SHOCK

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RUNNING HEAD: Anti-LPS Therapy for Shock

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# ABSTRACT

Intestinal ischemia is known to produce endotoxemia. Twenty-two dogs were subjected to a cranial mesenteric arterial occlusion for three hours. Equine anti-endotoxin hyperimmune plasma (Anti-LPS) was administered to fifteen dogs. Fourteen of these fifteen Anti-LPS treated dogs recovered for a minimum period of two weeks after the occlusion was released. Seven out of seven controls died within sixteen hours of the released occlusion. ( $p < 0.001$ ). Anti-LPS thus significantly reduced mortality in this experimental endotoxic shock model. This would indicate that Anti-LPS may be of considerable benefit in the therapy of other canine endotoxic mediated disorders.

## INTRODUCTION

Endotoxins (lipopolysaccharide, LPS, pyrogen) are highly toxic and extremely chemically stable breakdown products from the walls of gram negative bacteria<sup>(1,2)</sup>. During bacteremia the concentration of LPS in plasma can increase during the active growth phase of bacterial replication or after these bacteria are destroyed<sup>(3)</sup>. Endotoxemia is now considered an important contributory factor towards the mortality and morbidity associated with septic and non-septic shock in man and animals<sup>(4,5)</sup>. In man, the mortality associated with endotoxemia still remains at an unacceptable rate of 30-80% worldwide<sup>(6-10)</sup>.

Antimicrobials have an important role in the therapy of gram negative bacteremia but they do not decrease the potent toxic effects of the LPS. Thus, the LPS may persist in the circulation, producing toxic effects long after the bacteria have been destroyed by antibiotics<sup>(1)</sup>. A recent work has shown that rapid destruction of E.coli by gentamycin may produce a 2000 fold increase in plasma LPS concentration<sup>(11)</sup>.

Normally, large amounts of gram negative bacteria and, hence, LPS, are present within the intestines of most animals<sup>(12)</sup>. The intestinal wall acts as an effective barrier to prevent the entry of LPS into the blood circulation, but, in order to function as such, it requires an adequate oxygenated blood supply. Temporary occlusion of the cranial mesenteric artery in experimental animals is known to result in damage to the small intestinal mucosa, leading to endotoxemia, shock and death<sup>(13,14)</sup>. Previous studies have shown that treatment with Anti-LPS hyperimmune equine plasma (Anti-LPS) can neutralize and opsonize LPS and lead to reduced mortalities in a variety of animal models exhibiting

endotoxemia<sup>(15-18)</sup>.

The object of this study was to evaluate the therapeutic value of Anti-LPS in dogs when given prior to, during, and after a normally lethal three-hour cranial mesenteric artery occlusion insult.

#### MATERIALS AND METHODS

Twenty-two dogs were chosen as available. They were a mixture of males and panhysterectomized females, crossbreeds and pure breeds, ranging in age from one to ten years, from 15 to 35 kg mass. They were all clinically normal before the experiment and had been housed separately in the University's experimental animal colony for a minimum period of two weeks prior to use in this study. All the dogs received the same diet of commercial dry cubes\*, canned meat\* and water ad lib.

Anti-LPS<sup>+</sup> was obtained by suitably immunizing healthy horses and collecting their plasma under sterile conditions by plasmapheresis. This plasma contained IgG antibodies capable of binding LPS prepared from a wide range of different gram negative bacteria, including E.coli, Salmonella spp., Proteus spp., Pseudomonas spp. and Klebsiella spp. (15,19). The concentration of LPS precipitable IgG in Anti-LPS was 1500 µg/ml. The plasma was bottled under sterile conditions and stored frozen at -20°C until used.

\* "VITAGEN" and "CHOW" = Epol (Pty) Ltd., Johannesburg, South Africa.

+ "ATOXIN" ATOX Pharmaceutical Co., 14 Old Main Rd, Gillitts 3600, South Africa.



**EXPERIMENTAL SURGERY** The dogs were anesthetized with pentobarbital sodium (20 mg/kg; i.v.) An endotracheal tube was placed in position and the cuff inflated. The cephalic vein was catheterized to accept a drip administration set. Ventilation of the lungs was performed using a Starling pump with a tidal volume of 70 ml and at a rate of 12/min. A sterile, pyrogen free, catheter of appropriate size was inserted into each dog's bladder. The abdominal cavity was then entered through a linea alba incision using routine sterile surgical techniques. The cranial mesenteric artery was located, isolated and the loop of a Vesselclade (Neuromedics Inc., Texas) was loosely applied around this artery. The Vesselclade was exteriorized through a small lateral abdominal incision where preplaced sutures were inserted for later closure. The bladder was emptied just before the cranial mesenteric artery was occluded and this urine was discarded. During the occlusion period and up to the time the dogs were placed in recovery cages, the amount of urine produced was measured. This was done every hour by applying a syringe to the end of the catheter and draining the bladder.

Following a short acclimatization period, the cranial mesenteric artery was occluded by pulling the handle of the Vesselclade which tightened the loop around it. Routine sterile surgical closure of the linea alba incision was performed. The lateral incision was kept moist and sterile. Ringer's lactate was administered via the cephalic vein at a rate of approximately 45 drops/min.

Three hours after the beginning of the occlusion period, the Vesselclade was released and withdrawn via the small lateral abdominal incision. The preplaced sutures were then knotted. At this stage all the dogs received an i.v. injection of tetracycline (20 mg/kg). The positive

pressure ventilation was gradually withdrawn until spontaneous respiration was adequate. Fluid therapy was discontinued and i.v. catheters were then removed. The dogs were transferred to a recovery kennel where they regained consciousness within 1 to 1-1/2 hours.

**CONTROL DOGS:** Seven dogs constituted the control group and were not given Anti-LPS at any stage. However, their other treatments (surgical and medical) were identical to the treated dogs.

**TREATED DOGS:** Fifteen dogs were divided into three groups, each of which received Anti-LPS (1.0 ml/kg) at different times. Group I received Anti-LPS subcutaneously within one minute of occlusion of the artery; Group II dogs were given Anti-LPS mixed with 350 ml Ringer's lactate solution during the three-hour occlusion period; Group III dogs were given Anti-LPS subcutaneously, within three minutes after release of the occlusion. - These treated dogs then received a subcutaneous injection of Anti-LPS (1.0 ml/kg) 24 hours post occlusion.

**STATISTICS:** The Fisher exact probability test was used to examine significant differences in survival rates of the different groups.

## RESULTS

### CONTROL DOGS:

Seven of seven (100%) control dogs died within 6 to 16 hours after the release of the cranial mesenteric artery occlusion.

All these dogs exhibited hypothermia ( $35-38^{\circ}\text{C}$ ); extreme congestion of all mucous membranes with obvious cyanosis; passage of melenic stools giving way to frank blood within four to five hours and abdominal muscle rigidity following palpation. Oliguria was evident (less than 1 ml of urine/kg/hour) and abdominal paracentesis revealed a bloody fluid. Postmortal examinations performed on the controls showed extreme hyperemia and cyanosis of the small intestine with obvious extravasation of the blood. Multiple pinpoint to large petechial hemorrhages were visible on the internal organs, including the heart. The lungs were edematous and ascites, averaging 500 ml of a bloody fluid was present in all controls. Their bladders contained on average, 2 ml of concentrated urine. In some controls gangrenous lesions were visible in parts of the small intestine. In two dogs extreme hemorrhages of the pancreas were present with signs of autodigestion of the surrounding tissues. Hepatomegaly was obvious and the livers were friable. The kidneys were congested and swollen. In all seven controls, the adrenal cortexes showed hemorrhagic necrosis and mild to moderate splenomegally. No arterial thrombi were noted either at the occlusion site or at any other sites pertaining to the vascular system of the small intestine.

### ANTI-LPS TREATED DOGS:

As seen in Table 1, 5/5 each of group I and of group II survived and 4/5

of group III survived for more than 14 days. This one death occurred at 72 hours after the release of the occlusion. On postmortem a streptococcal pancreatitis and peritonitis was evident. This infection may have been introduced at the time of surgery.

All the dogs in the Anti-LPS treated group were eating and drinking normally at 24 hours post occlusion release, including the one which died. This latter dog stopped eating approximately 48 hours after the release of the occlusion, when the classical symptoms of acute pancreatitis commenced. No specific treatment was given for this pancreatitis.

No Anti-LPS treated dog was euthanased for postmortal examination as they were all clinically normal (except for the one above) two weeks after the release of the cranial mesenteric arterial occlusion. Histopathology was not performed as the postmortem features of the control group were thought to comply with the findings of endotoxemia.

In both control and Anti-LPS treated groups of dogs, the bladder output of urine showed a steady decrease during the occlusion period. After the occlusion was released, however, the bladder output almost ceased. Their catheters were removed when they were returned to their recovery kennel when measurements stopped. However, all Anti-LPS treated dogs were urinating normally 24 hours post occlusion release and continued to do so for the next two weeks.

## DISCUSSION

In veterinary practice a patient with intestinal vascular occlusion usually has a guarded prognosis despite the advances made in the surgical and medical approach to treatment<sup>(20)</sup>. Research utilizing the cranial mesenteric arterial occlusion (CMAO) shock model has shown that death occurs faster in those dogs that had the CMAO released than in those in which this occlusion was not released<sup>(21)</sup>. This is an important concept to remember when corrective surgery is to be performed on any patient with an abdominal vascular occlusion diagnosis. Recent evidence suggests that endotoxemia is a major cause of morbidity and mortality associated with gram negative bacteremia<sup>(5,22)</sup>.

The intestines of dogs always contain gram negative bacteria and hence LPS in large amounts<sup>(12)</sup>. Normally the intestinal defense mechanisms may allow small amounts of these bacteria and LPS entry into the portal circulation. In the liver they are detoxified and opsonized by specific antibodies and the monocyte-macrophage system<sup>(13,23)</sup>. During septicemia the liver becomes overwhelmed and their bacteria and LPS enter the general circulation where they now exert their often fatal consequences.

A variety of diseases and traumatic insults can produce endotoxemia which may then become part of the common pathway leading to shock and death. In this study the endotoxemia producing death of these dogs was brought on by CMAO. Similarly, gastric dilation-volvulus with or without compromise of splenic vasculature, intestinal volvulus, diaphragmatic hernia with hepatic, gastric, splenic or intestinal herniation into the thoracic cavity are all known to be associated with vascular occlusion or ischemia, high mortality and high morbidity rates.

Radioactively labelled LPS and viable gram negative bacteria placed in the small intestine of dogs can be found in the systemic circulation within a few minutes of intestinal ischemia<sup>(14)</sup>. Macroscopic lesions in the intestinal wall seen at postmortem have been well reported in both man and animals following intestinal ischemia<sup>(24,25)</sup>. One would expect that the mucosal damage would be greatest during the ischemic period, but these and previous studies have shown that intestinal mucosal damage is greatest some time after reperfusion of the small intestine with oxygenated blood<sup>(21)</sup>. Other researchers have suggested that reperfusion with oxygenated blood leads to the formation of high levels of toxic tissue peroxides, which, in turn, form free radicals, thus damaging the intestinal barrier even more than that already caused by the ischemia<sup>(25)</sup>.

In earlier studies related to CHAO, pretreatment with antibiotics did provide some protection<sup>(21)</sup>. However, antimicrobial therapy should not be initiated in gram negative bacterial infections restricted to the intestines unless there is evidence for, or a grave likelihood of bacteremia, due to the development of increased bacterial resistance and adverse effects in specific organ systems<sup>(26,27)</sup>. Antibiotics, except for polymyxin, do not alter the toxicity of the LPS and it has been shown that certain antibiotics are actually capable of producing a 2000 fold increase in the plasma LPS levels<sup>(11)</sup>. The LPS can remain in circulation long after the bacteria that produced them have been destroyed.

In principle, the host is capable of synthesizing specific antibodies against specific LPS. All too often, however, the patient dies before this immunological process is complete. Therefore a specific anti-

endotoxin antibody preparation would appear to be a logical therapeutic.

The Anti-LPS preparation used in this study contained IgGs which bind to LPS found in a wide range of gram negative bacteria. Some IgGs in Anti-LPS are highly cross reactive among various genera of bacteria; some are specific; some bind to the interior Lipid A region of the LPS while others bind to the outer "O" antigens on the LPS molecule. Furthermore, the presence of complement in plasma in addition to specific IgG, leads to the destruction of a wide variety of gram negative bacteria, including Klebsiella, Pseudomonas, E.coli and Proteus, Salmonella, Shigella and Enterobacter<sup>(28)</sup>. Thus Anti-LPS has a combined effect: it destroys gram negative bacteria and also neutralizes the LPS released from these bacteria.

LPS specific antibodies (Anti-LPS) were previously shown effective in reducing morbidity and mortality of LPS related disorders in experimental animals and in clinical veterinary practice<sup>(15)</sup>. This included septic abortion, peritonitis, Pseudomonas otitis externa, septicemia and endotoxemia following canine parvovirus infection<sup>(29)</sup>. Anti-LPS appears to be a practical mixture for clinical and prophylactic use since it is broad spectrum in character, so that identification of the infecting bacteria or LPS is not necessary.

In the studies reported here we found that Anti-LPS, irrespective of mode of administration and timing, reduced mortality in CMAO shock in dogs. This is in accordance with similar studies in rabbits using Anti-LPS of equine origin, and endotoxic shock following prolonged hemorrhage in cats using human Anti-LPS<sup>(30)</sup>. Hence Anti-LPS may be beneficially administered during intestinal vascular occlusion conditions or after

the surgical correction has been undertaken, an important consideration in clinical veterinary practice. Furthermore, Anti-LPS could be considered beneficial as a prophylaxis in animals undergoing abdominal surgery. Other studies in this laboratory showed that Anti-LPS administration could lower the concentration of plasma LPS to baseline levels within five minutes in endotoxic cats<sup>(31)</sup>. The possibility that Anti-LPS may reduce the morbidity and mortality following septic shock deserves further clinical assessment.

Recently in an independent study this Anti-LPS successfully treated a lethal model of abdominal sepsis. It was found that Anti-LPS, in combination with selective antibiotic therapy, allowed further prolongation of survival<sup>(32)</sup>.

During the course of this and other studies, not a single side effect has been noted. However, time has not yet allowed for this Anti-LPS to be challenged after a long period has lapsed. The authors find that this Anti-LPS is safe and extremely beneficial in the treatment of vascular ischemic endotoxic canine conditions.



#### ACKNOWLEDGEMENT

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TABLE 1

ANTI-LPS TREATMENT OF DOGS IN CRANIAL MESENTERIC ARTERY OCCLUSION SHOCK

TREATMENT*	N	DEATHS	DEATHS (%)	SIGNIFICANCE <sup>+</sup>
CONTROLS	7	7	100	-
Anti-LPS Group I	5	0	0	p 0,005
Anti-LPS Group II	5	0	0	p 0,005
Anti-LPS Group III	5	1	20	p 0,05
TOTAL ANTI-LPS TREATED	15	1	6,7	p 0,001

\* Group I received Anti-LPS s.c. at initiation of ischemia

Group II received Anti-LPS as a 3-hour drip during ischemic period

Group III received Anti-LPS s.c. after release of occlusion

+ Fisher exact probability test.

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