# The effect of endotoxin and anti-endotoxin serum on synovial fluid parameters in the horse

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## ABSTRACT

The effects of a commercially available equine hyperimmune anti-endotoxin serum on synovial fluid parameters were evaluated in an induced synovitis model in normal horses. Four groups of 3 horses each received lipopolysaccharide (LPS) plus hyperimmune anti-endotoxin (anti-LPS), LPS, anti-LPS, and Ringers lactate (control) respectively injected into the left intercarpal joint. Synovial fluid parameters were measured at 4, 8, 24 and 72 h. It was found that anti-LPS had no attenuating effect on the LPS and that it induced a synovitis almost equivalent to that induced by LPS alone. The introduction of sterile Ringers lactate solution into the carpal joint together with repeated aseptic arthrocentesis induces a mild inflammatory response.

**Key words**: endotoxin, horses, hyperimmune anti-endotoxin, synovitis.

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## INTRODUCTION

Septic arthritis in horses of all ages remains a difficult therapeutic challenge. Despite early diagnosis and appropriate vigorous therapy, the prognosis for return to athletic function is gaurded[1]. The trend appears to be towards increased aggressiveness in the treatment of septic arthritis[6,23]. As this frustrating disease often defies treatment, veterinarians may resort to ill-defined therapeutic methods in the hope of achieving a better response.

Literature on the use of equine hyperimmune anti-endotoxin (anti-LPS) serum in the treatment of septic arthritis in horses is based on reports of its use in clinical cases[27]. Anti-LPS plasma has also been used for the treatment of gastroenteritis and endotoxaemia in horses[8], in cases of haemorrhagic enteric endotoxaemia in dogs[31,32] and *Pseudomonas* keratitis in rabbits[30]. However, the conclusions based on clinical studies may be confounded by various factors, including the bacterial agent involved, the method and dose of inoculation, the duration of infection, host immunological defence competence, concurrent systemic disease, pre-existing joint pathology and the treatment protocol employed[22].

The main purpose of this study was to evaluate the use of a commercially available equine hyperimmune anti-endotoxin preparation[27] in an induced synovitis model[7], as well as to define its effects in normal equine joints.

## MATERIALS AND METHODS

### Subjects

Twelve clinically normal horses (8 mares, a stallion and 3 geldings), ranging in age from 3–18 years, with a mass ranging from 320–505 kg (average 407 kg) were used in the study. The predominant breed was Thoroughbred with 1 American saddle horse and 3 horses of mixed breed. The animals were stabled and *Eragrostis teff* and *Medicago sativa* hay as well as water were available *ad libitum*. Clean and plentiful wheaten straw was used for bedding.

### Experimental design

The 12 horses were randomly allocated to 1 of 4 groups. The treatment groups, each comprising 3 horses, were:

Group I: lipopolysaccharide (LPS) and anti-LPS treatments. LPS was injected into the left intercarpal joint of the horses at a dosage rate of 0.1 µg/kg body weight[7].

The dose was reconstituted in 5 m$\ell$ of sterile Ringers lactate (Ringer-Lactate, Sabax). Immediately after injection of LPS, 5 m$\ell$ equine anti-endotoxin hyperimmune serum was injected through the same needle used to inject the LPS.

Group II: treatment with LPS alone. A dose of 0.1 µg LPS/kg body mass, reconstituted in 5 m$\ell$ Ringers lactate (as above), was injected into the left intercarpal joint of each horse in this group.

Group III: treatment with anti-LPS alone. Five m$\ell$ anti-LPS was injected into the left intercarpal joint of each horse.

Group IV: Ringers lactate control group. Five m$\ell$ Ringers lactate (pH 6.5) was injected into the left intercarpal joint of each horse.

The source of the LPS (*E. coli*, 055:B5, lot 17F4019, Sigma Chemical Corporation, USA) was a freeze-dried lipopolysaccharide preparation derived from *Escherichia coli*, serotype 055:B5. The anti-LPS used was a commercial preparation of equine anti-endotoxin hyperimmune serum, Atoxin® (Atoxin, Atox Pharmaceutical Company) containing 1200 µg/m$\ell$ anti-LPS precipitable IgG obtained by plasmapheresis of suitably immunised healthy horses[9]. The anti-LPS has an affinity for LPS prepared from *Escherichia coli* 055:B5, as well as *Salmonella typhimurium*, *S. typhi*, *S. abortus equi*, *Shigella flexneri*, *E. coli* 0127:B8 and *S. enteritides*[28].

### Duration

The duration of the experimental procedure in each animal was 72 h. Synovial fluid was collected before injection of substances and repeated 6 times (*vide infra*).

### Experimental procedure

During the 72-h experimental period all the horses were stabled. Clinical evaluations were performed in the stable, with the exception of lameness assessments, for which the horses were walked and trotted on a grass lawn adjacent to the stables. Horses that were unwilling to take full weight on the affected leg were evaluated in the stable only.

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Most of the horses tolerated the arthrocentesis procedure without resistance. In the isolated cases where the horses were fractious and/or resisted the procedure, a twitch, applied to the upper lip, was used for restraint.

### Intra-articular injection of substances and synovial fluid collection

On the day preceding the trial the skin over the left carpus of each horse was prepared by shaving, thorough scrubbing for at least 2 min with chlorhexidine disinfectant soap (Hibiscrub, chlorhexidine gluconate 4 g/100 m$\ell$, ICI Pharmaceuticals, Woodmead) and sprayed with a 1 % Povidone iodine solution (Adcock Ingram Laboratories, Johannesburg). The substances were injected using a strictly aseptic technique into the left intercarpal joint of each horse at time 0 h.

Immediately before intra-articular injection or arthrocentesis, the skin over the carpal joints was again scrubbed with chlorhexidine and sprayed with 1 % povidone iodine. The povidone iodine was removed by swabbing with clean cotton wool, starting at the proposed site of injection (or synovial fluid collection) and working outwards. The povidone iodine spraying procedure was repeated 3 times, and the last application was not swabbed off. Strictly aseptic technique was observed by scrubbing hands with a chlorhexidine surgical scrub and gloving with disposable sterile latex surgical gloves.

After skin preparation, a 0.7 mm diameter by 38 mm long sterile hypodermic needle (Terumo single-use needle, Terumo Corporation, Tokyo, Japan) was introduced into the intercarpal joint between the tendon of the *M. extensor carpi radialis* and the *M. extensor digitorum* muscles. The correct placement of the needle was confirmed by the presence of synovial fluid in the hub of the needle. The syringe was attached to the hub of the needle only after correct placement was ascertained. A 5 m$\ell$ syringe was used to inject the specific substance or to draw off approximately 3 m$\ell$ of synovial fluid for analysis.

When synovial fluid was collected it was immediately transferred to a 5 m$\ell$ capacity, evacuated tube (B D Vacutainer, Becton Dickenson Co., USA) containing 57 $\mu\ell$ of a 15 % solution of tri-potassium ethylene diamine tetra-acetic acid (EDTA). The tube was agitated to ensure even distribution of EDTA in the synovial fluid sample. Samples for synovial fluid analysis were delivered to the laboratory within 30 min of collection and processed within 60 min.

### Bacterial sampling

To ensure that no bacterial infection was introduced into the joints, and that all effects seen could be attributed to the substances used, bacteriological examinations were conducted on the LPS and anti-LPS solutions before their introduction into the joints, as well as on synovial fluid collected at 72 h.

The synovial fluid samples were collected aseptically and transferred into blood culture bottles (Oxoid Signal Blood Culture Medium, BC102, Oxoid Limited). These samples were delivered to the laboratory within 20 min of collection.

### Laboratory data

Synovial fluid was collected at 0, 2, 4, 8, 24, 32, 48 and 72 h. Analysis of the fluid was performed by an experienced technologist in the section of Clinical Pathology, Faculty of Veterinary Science, University of Pretoria. Total synovial fluid protein was determined using the biuret reaction on an automated chemical analyser (Technicon Instruments Corporation). A nucleated cell count (NCC) was conducted on a 1:1000 dilution treated with Zappoglobin (Coulter Electronics), to lyse the erythrocytes, on a Coulter model Fn (Coulter Electronics). A differential count was performed to determine the number of each cell type present on a thin smear of synovial fluid stained with CAMS Quick Stain (C A Milsch). The number of each cell type encountered during the classification of 100 nucleated cells was counted and multiplied by the NCC[4].

### Clinical data

Rectal temperature and heart rate were recorded every hour for the 1st 24 h and thereafter every 6 h.

### Clinical observations

These were made every 6 h for the 1st 24 h and thereafter every 12 h. Lameness was evaluated according to a system modified from Stashak[25] and graded from Grade 0 (no lameness) to Grade IV (non-weight-bearing lameness). Discomfort at rest in the stall was graded from Grade 0 (no obvious discomfort) to Grade IV (recumbent, or sweating with colic-like symptoms). Pain on palpation and flexion of the joint was graded from Grade 0 (no pain on digital palpation of the carpal joint, no resistance to flexion) to Grade III where digital palpation was not tolerated and flexion of the joint was resisted. All these subjective clinical observations were made by the same person.

### Statistical analysis

For all analyses, the SAS program for analysis of general linear models, univariate and multivariate analysis of variance (MANOVA) (SAS Institute) was used. Wilks' criterion was computed using the MANOVA test for the hypothesis of no overall group effect. *F*-tests were conducted to test the hypothesis of no group differences in the case of univariate analysis.

Pearson's correlation coefficients were computed amongst the synovial fluid parameters at specific times of collection.

As a measure of comparison of the 4 groups in the experiment, the least square means were computed. Since multiple comparisons between groups were carried out, the Bonferroni inequality was calculated to provide an overall probability of error smaller than usually demanded. This was achieved by multiplying the least square means probabilities by a factor of 6 because pairwise comparisons were made[18]. This test was performed on all parameters at 4, 8, 24 and 72 h, as these were considered to be the times that would indicate the differences between the groups most effectively. The level of significance for all tests was set at $p < 0.05$.

The clinical parameters, although statistically analysed, are not reported in this paper as they were subjective in nature and the treatment groups were relatively small. Their relationship to the synovial fluid findings, however, is alluded to in the discussion.

The number of horses allocated per treatment group was based on the formula by Steel & Torrie[26], the variance recorded in data published by Firth[7] and literature on equine synovial protein and neutrophil changes in septic arthritis[12,15].

The protocol was approved by the institutional Ethics and Research Committees. A clause was included allowing for the treatment or euthanasia of animals showing excessive pain during the trial. Animals showing any signs of pain or discomfort at the end of the trial were treated appropriately.

## RESULTS

### Synovial fluid total protein

The protein values peaked dramatically, from a mean base line of 14.9 g/$\ell$, in Groups I, II and III at 12 h, after which they gradually decreased (Fig. 1). At 72 h the mean values were still substantially higher than baseline values. The mean values of 67 g/$\ell$ in Group I and 66.7 g/$\ell$ in Group II at 8 h are as high as the total serum protein values of 65.85 ± 6.64 g/$\ell$ in

normal horse blood[16]. The highest recorded mean total synovial fluid protein values in Groups III and IV were 51.8 g/ℓ and 38 g/ℓ at 12 and 24 h respectively.

Statistically, the overall group mean total synovial total protein values differed significantly (Wilks' criterion $p = 0.0183$). Using the Bonferroni test, there was no difference between the mean values of Groups I, II and III at any time during the trial. Group IV (control) was markedly different from all other groups at all time intervals after time = 0 h.

### Nucleated cell count (NCC)

This parameter showed dramatic, statistically significant, increases in Groups I and II (Fig. 2). Eight hours post-injection the mean value of NCC in these groups had increased 325 times their baseline values. The Bonferroni test showed no differences between these 2 groups at any time during the trial period. The anti-LPS group (Group III) also demonstrated a marked increase in the first 8 h, but not to the same extent as Groups I and II, with a mean increase of 178 times baseline values. There was no significant difference between Group III and Groups I and II. Group IV (control) increased to a mean of 40 times the baseline value. However, this was significantly less than the other Groups during the 3–12 h post-injection period. A high correlation was found between NCC and synovial fluid protein at 4 and 8 h ($r = 0.9321$ and $0.9668$ respectively).

### Neutrophil count

Neutrophils were the predominant cell type observed in the joint effusion of all groups. Mean values of 152 000 cells/µℓ were found in Group I and 154 000 cells/µℓ in Group II at 12 h (Fig. 3). These values dropped almost as dramatically as they rose to means of 19 900 and 13 500 cells/µℓ respectively at 72 h. Group III reached a lower peak than Groups I and II at a mean value of 90 000 cells/µℓ at 12 h. There was, however, no significant difference between Groups I, II and III, while Group IV values were significantly lower, than these groups throughout the trial after time = 0 h.

### DISCUSSION

The significant increase in protein values (Fig. 1) in the joint fluid can be attributed to increased vascular permeability[3], which may result from damage to arterioles, venules and capillaries inflicted by the agents injected into the joints, or indirectly from the stimulus of released chemical mediators
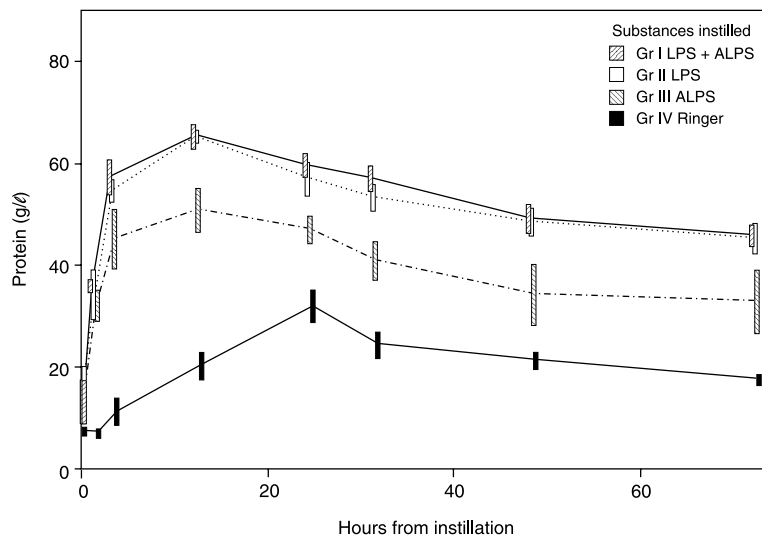
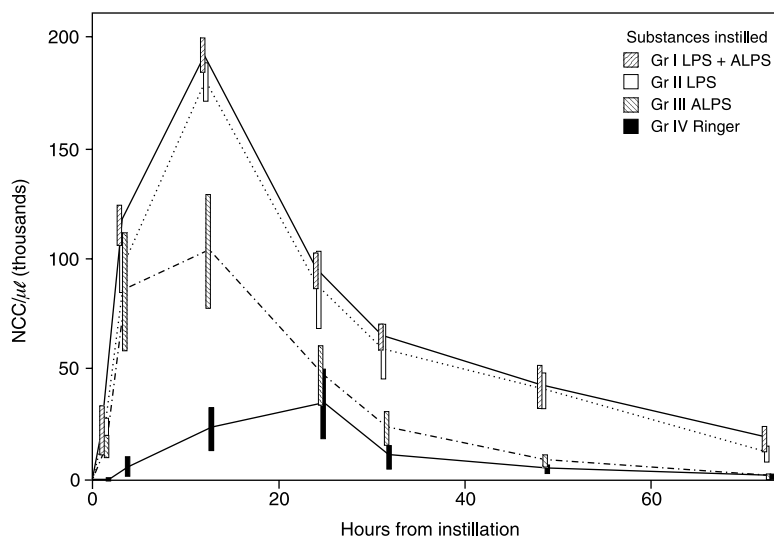

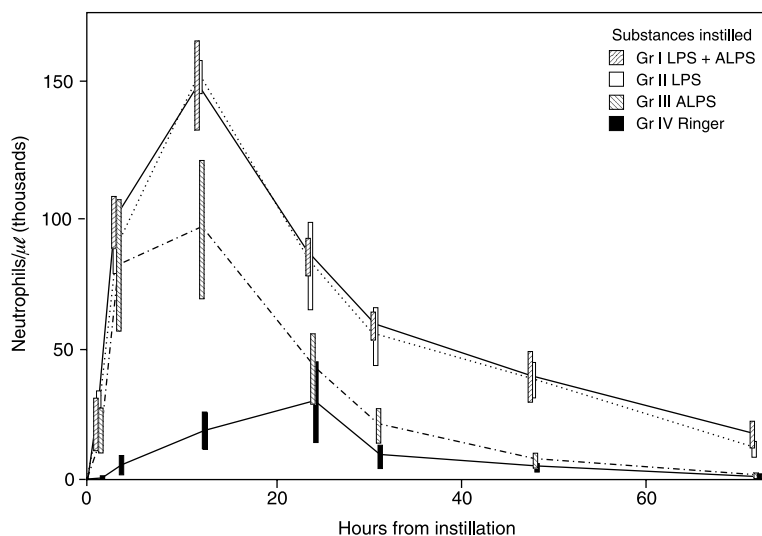Fig. 1: **Synovial fluid total protein.**



Fig. 2: **Nucleated cell count** (NCC).



Fig. 3: **Neutrophil count.**

acting primarily on the venules[10]. The protein values obtained in the synovial fluid samples are considered to be an indicator of the amount of inflammation that occurred in the joints. The synovial membrane, as well as blood vessels, could have been damaged during the arthrocentesis procedure. The inflammation caused by this procedure should be similar in all groups, as an identical number of needle punctures were performed in all horses. It is therefore the difference in inflammation between the groups that should be considered.

Using the total synovial protein values as an index of the inflammatory reaction in the joint, it can be deduced that anti-LPS had no ameliorating effect on the inflammatory reaction caused by LPS and, in fact, caused a marked inflammatory reaction on its own. Repeated arthrocentesis and injection of Ringers lactate into the joint also caused an inflammatory reaction, but this was much milder than that caused by the other treatments. This concurs with the findings of Lloyd et al.[14], who found a short-lived increase in synovial white blood cells, including neutrophils, after intra-articular injection of a sterile, polyionic physiological solution into catheterised equine antebrachiocarpal joints.

The anti-LPS injected into the joints had a protein concentration of 62.5 g/$\ell$. This would have affected the protein concentration of the synovial sample collected. As the synovial fluid volume of the intercarpal joint varied depending on the amount of effusion present in the joint, it is not possible to determine the contribution of the protein injected into the joint. However, considering the rapid increase in protein values in samples collected between 4 and 12 h, it is unlikely that the contribution of the protein in the anti-LPS was significant. Furthermore, as no additional anti-LPS was administered, any increase in protein after 2 h cannot be attributed to this source.

The relatively high correlation between synovial leukocyte counts and protein concentration could result either from cellular debris due to cell breakdown in the synovial fluid, or the fact that that leukocytes were present when synovial inflammation was maximal and therefore the synovial membrane more permeable. The predominant leukocyte type present in the synovial fluid was the neutrophil, and this tends to support the latter alternative. It is widely accepted that endotoxins can exert profound effects on host mediation systems and 'turn on every defence' at the host's disposal[21]. The polysaccharide portion of the LPS is responsible for the activation of the alternate complement pathway[2], activating complement in the absence of antibodies. Endotoxins are not particularly good antigens[20], although they may activate the classic complement pathway by means of complexes formed by antigen and immunoglobulin IgG or IgM[21]. Complement ($C_{3a}$) is a chemotactic substance, attracting neutrophils, eosinophils and basophils, which form part of the NCC[10]. Other mediators of the inflammatory response initiated by LPS include products of arachidonic acid metabolism[19]. Lipoxygenase enzymes metabolise arachidonic acid to a group of noncyclised eicosanoids, the leukotrienes. These compounds are of particular importance in leukocyte-mediated aspects of inflammation[10] and probably help to account for the massive increase in NCC in Groups I and II, where LPS was injected into the joints. The increase in NCC in Group I indicated that anti-LPS appeared to have no ameliorating effects on the inflammation caused by the LPS. In fact, the anti-LPS appeared to have an inflammatory effect of its own, as evidenced by the results obtained in Group III. One could speculate that the protein present in the anti-LPS initiated an antibody response in the joint. The resultant antibody/antigen complexes could have, in turn, activated the classic complement pathway and its leukotactic effects. However, as there was no evidence to suggest prior sensitisation to the donor horse serum protein and the reaction was virtually immediate, this mechanism seems very unlikely. It is interesting to note that repeated arthrocentesis and joint distension with Ringers lactate (Group IV) was itself responsible for a relatively small, though significant, increase in NCC. The NCC also includes desquamated synovial cells. However, these made a very small contribution to the totals (0.00017 % at 8 h).

Leitch[13] described the alteration of the predominating cell type from the mononuclear cell in normal equine synovial fluid to the segmented neutrophil, as the diagnostic hallmark of septic arthritis. The chemical synovitis caused by injection of foreign materials into joints in this trial resulted in a similar phenomenon. The findings of this trial also concur with the view of McIlwraith[17], that any noxious substance injected into a joint can cause an equally dramatic neutrophil response. The differentiating feature between true septic arthritis and chemical synovitis is that, in chemical synovitis, the leukocytosis drops rapidly, while in septic arthritis it does not[17].

As the principal function of neutrophils is phagocytosis of foreign matter, including bacteria and cellular debris[24], it is not surprising that large numbers are found in naturally-occurring septic arthritis. In this trial, the high numbers of neutrophils were probably a direct response to the inflammatory reaction caused by injection of noxious substances into the joints, as no bacteria were noted in the neutrophils, and none were cultured from the synovial fluid.

The aim of this study was to evaluate the reported beneficial effect of anti-LPS in infectious arthritis[27]. The reported action of anti-LPS is to neutralise exogenous LPS and thereby abolish its deleterious effects on the joint[8]. In this trial, using an induced synovitis model according to Firth et al.[7], anti-LPS appeared to have no neutralising effect on simultaneously injected LPS. It is acknowledged that anti-LPS has demonstrable antibacterial properties[9,28,29]. Perhaps the reported beneficial effect of anti-LPS in clinical cases[27] was due to the antibacterial rather than the anti-LPS activity of the product, or to other medication administered concurrently.

A possible inadequacy in the experimental design is that the level of naturally-occurring anti-LPS antibodies in the subjects was not ascertained. However, in the light of the findings of Hollingsworth & Atkins[11], namely that highly immunised rabbits retained their synovial reactivity to endotoxin, this inadequacy is unlikely to be important. The LPS may have had an immediate effect on synoviocytes. By injecting anti-LPS after the LPS had already caused an inflammatory reaction, the interpretation of results could have been affected. Further studies should determine whether anti-LPS injected into the joint prior to the LPS would attenuate the inflammatory response. The amount of anti-LPS (6.000 µg) used to neutralise the injected LPS (mean 470 µg) may have been insufficient. However, it is expected that at least some attenuation of the inflammatory response would have occurred. It is suggested that the beneficial effects reported when this product has been used in field cases may have been due to its known antibacterial effects.

The following conclusions can be drawn from the results of this trial: the induced synovitis model described by Firth et al.[7] in ponies is valid in horses and was suitable for this trial. It appears that intra-articular injection of anti-LPS into the intercarpal joint of normal horses causes a transient synovitis. This synovitis was

most marked at 8 h and decreased to near base-line levels at 72 h. It is evident that anti-LPS had no effect in attenuating the clinico-pathological deviations of the LPS-induced synovitis model. The effects of introducing a sterile, balanced electrolyte solution into the intercarpal joint of normal horses, together with repeated arthrocentesis, indicate that this is not an innocuous procedure. This is in agreement with the findings of other authors[5,14,33].

## ACKNOWLEDGEMENTS

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