

Horses with clinical endotoxaemia

“Effects of hyperimmune equine plasma on clinical and cellular responses in a low-dose endotoxaemia model in horses”

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Summary of the scientific paper is written by Ross Wilson BVSc, Chief Scientific Officer, Plavacc Pty Ltd, and Plavacc USA Inc.



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BACKGROUND

Plavacc Pty Ltd in Australia produces a hyperimmune equine plasma from its closed, quarantined herd of donor horses in South-East Queensland, Australia. The donor horses are hyperimmunised with Plavacc’s own killed *E. coli* vaccine, and the antibodies subsequently produced in the plasma have been found by veterinarians in Australia to be beneficial in the treatment of horses with clinical endotoxaemia. Plavacc have named this product “Equiplas E Equine Immunoglobulin to endotoxin” (Equiplas E¹).

Plavacc have recently been successful in gaining the regulatory approval from the Australian Pesticides and Veterinary Medicines Authority for Equiplas E, and marketing of it to veterinarians in Australia will commence in July 2013.

SUMMARY

This was a randomised cross-over study, where each horse acted as its own control, and the data analysed in a pair-wise manner. Six horses were subjected to a previously published model of intravenous infusion of a low-dose of purified endotoxin (*E. coli* LPS 055:B5; 1.2 million endotoxin units/mg, Sigma Aldrich) at a dose of 1ng/kg/minute over 30 minutes – total dose 30ng/kg. Pre-treatment (i.e. 30 minutes before the endotoxin infusion) of two litres of Equiplas E was administered to three horses selected at random (these were the “treatment horses”), and the other three horses received pre-treatment with 2L sterile normal saline (the “control” horses). Three weeks later the six horses were subjected to the opposite pre-treatments to the one they received initially. Thus it can be seen that each horse acted as its own control. This research was conducted at the University of Melbourne and was approved by its Animal Ethics Committee. Three weeks is regarded as a suitable “wash-out” period after endotoxin administration, after which its effects will no longer affect the endotoxin challenge in the second experiment.

RESULTS

The low-dose endotoxin administration was very well tolerated by all of the horses, and the clinical signs were mild and transient, as expected. There were no significant differences between various clinical parameters measured (body temperature, respiratory rate, white cell counts, and hoof temperatures), however there was a significant difference in the levels of bio-available (unbound) Tumor Necrosis Factor alpha (TNF α) in blood samples – 1373.92 ± 107.63 pg/mL in the control horses Vs. 1044.44 ± 193.93 pg/mL in the treatment horses ($P = 0.05$).

TNF α is one the most important mediators of the systemic inflammatory response. Down-regulation of its levels is the sole, important purpose of many existing monoclonal antibody treatments² currently approved to be used to treat cases of human sepsis.

¹ Plavacc intends to apply for an Unconditional License for this product in the USA and it will be called Equiplas J.

² INFLIXIMAB and ETANERCEPT.

DISCUSSION

The point is made that this model is not ideal for the application of Equiplas E as a pre-treatment, specifically with the administration of a purified endotoxin as the challenge. The killed *E. coli* vaccine used on Plasvacc's donor horses utilises autoclaving of the bacteria as the means of its inactivation. This process produces LPS molecules of various and widely varying molecular weights - it literally "smashes" the *E. coli* cells. Therefore the antibody response in the donor horses is "polyclonal" in nature. Measurement of the activity of Equiplas E against a purified LPS, as was the case in this paper, is a measurement of only a small part of its activity against only one endotoxin.

Similarly, clinical endotoxaemia in horses involves an antigenic challenge with much greater quantities of a similar wide variety of endotoxin molecules of differing molecular weights, therefore it could be argued that the treatment of these cases with Equiplas E would show greater and more readily measurable benefits than those which were observed in this paper.

CONCLUSION

Nevertheless, it can be seen from this paper that the administration of Equiplas E had a statistically significant and beneficial effect on the treatment horses, by down-regulating the levels of unbound TNF α in the blood stream.

The conclusion can therefore be made from this paper that the administration of Equiplas E, given the limitations of the experimental design used here, and detailed above, may be beneficial in the treatment of clinical endotoxaemia in horses.

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Effects of hyperimmune equine plasma on clinical and cellular responses in a low-dose endotoxaemia model in horses

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ABSTRACT

Endotoxaemia is a major cause of equine morbidity, and plasma from horses immunised against *Escherichia coli* is used in its treatment. The aim of this study was to determine the effects of hyperimmune plasma on the clinical and leukocyte responses, including production and activity of TNF α , in an in vivo endotoxin challenge model. Pre-treatment with hyperimmune equine plasma had no significant effect on peak total plasma TNF α concentration (occurring 90 min after the administration of 30 ng/kg LPS). However, the bioavailable (unbound) TNF α measured by bioassay was significantly reduced in plasma-treated horses (1044.44 ± 193.93 pg/ml at 90 min) compared to saline treated controls (1373.92 ± 107.63 pg/ml; $P = 0.05$). Therefore, although pre-treatment with hyperimmune equine plasma did not significantly modify the clinical signs of endotoxaemia in this model, there was some evidence of reduced TNF bioactivity, which may be due to factors in the plasma which bind and reduce the activity of this cytokine.

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1. Introduction

Endotoxaemia remains a major cause of equine morbidity and mortality. Horses are particularly sensitive to the effects of endotoxin, which plays a key role in a number of serious equine conditions including acute abdominal disease, colitis, post operative ileus, laminitis, peritonitis, pleuropneumonia, metritis, exertion, neonatal septicemia, recurrent airway obstruction and inflammatory airway disease (Moore, 1988; Tóth et al., 2009; Beeler-Marfisi et al., 2010). Many current pharmacological therapies, such as non-steroidal anti-inflammatory drugs (NSAIDs), may only affect part of the inflammatory cascade (Baskett et al., 1997), therefore making this a difficult condition to manage effectively (Moore and Barton, 2003).

Lipopolysaccharide (LPS) induces a cytokine mediated systemic inflammatory response syndrome (SIRS) that may lead to hypovolaemic shock, coagulopathy and catastrophic multiple organ failure (Werners et al., 2005). Activated mononuclear leukocytes and other cells secrete inflammatory cytokines, including tumor necrosis factor alpha (TNF α) and interleukin one beta (IL-1 β) which initiate an inflammatory cascade leading to the attachment and migration of leukocytes through the vascular endothelium and resulting in tissue damage (Werners et al., 2005).

Hyperimmune plasma or serum, from horses immunised against Gram negative bacteria and their endotoxins, has been used in the treatment of endotoxaemia in horses, and its clinical effects have been evaluated. Some studies have shown beneficial effects of the administration of anti-endotoxin antibodies in experimental endotoxaemia, horses with colic and critically ill and septic neonatal foals (Garner et al., 1988; Peek et al., 2006; Spier et al., 1989). In contrast, the results of other studies failed to reveal beneficial effects (Durando et al., 1994; Morris et al., 1986; Morris and Whitlock, 1987). The mechanism of action of hyperimmune plasma may be multi-factorial. Firstly, it may contain antibodies that bind to LPS, either reducing induction of the pro-inflammatory response by leukocytes or accelerating the clearance of LPS (Gaffin and Wells, 1987; Wells et al., 1987). In addition, it may contain proteins such as the soluble TNF α receptor (sTNF α R), which bind to TNF α and render it biologically inactive (Kotiw et al., 2006).

While previous studies have investigated the use of hyperimmune plasma or serum on the clinical signs of endotoxaemia in clinical and experimental conditions, its precise mechanism of action, and the extent to which it prevents leukocyte activation, have yet to be fully determined. This project investigated the extent to which hyperimmune equine plasma is able to prevent key parts of the inflammatory response in a low-dose endotoxin challenge model using highly purified *E. coli* LPS. The aims of the study were to measure the effect of hyperimmune plasma on (1) clinical parameters and (2) leukocyte activation as measured by blood leukocyte counts and production of the important cytokine TNF α .

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Furthermore, we aimed to investigate the potential presence of binding proteins (such as soluble TNF α receptor) in hyperimmune plasma, which may reduce bioavailability of this cytokine, by comparing the total and bioactive form of TNF α .

2. Materials and methods

2.1. Animals

Six healthy adult standardbred horses (6 mares; mean age 7 years, range 5–15 years; weight 437.8 ± 14.3 kg) were used. This work was approved by the Animal Ethics Committee of the University of Melbourne (Ethics approval number: 0808347.3).

2.2. Experimental design and pre-treatments

The study was conducted as a crossover design, with each horse acting as its own control. Prior to the first low-dose endotoxin administration, three horses (randomly assigned by simple lottery) received hyperimmune equine plasma (2 L of Equiplas E; Plasvacc Pty. Ltd., Queensland, Australia) and the other three received the equivalent volume of saline (0.9% NaCl), during the 60 min immediately prior to the LPS infusion. After a washout period of 21 days between experiments, the treatments were crossed over so that each of the six horses received both pre-treatments.

2.3. Low-dose endotoxin challenge

Jugular vein catheters were placed under local anaesthesia (2 ml of 2% lignocaine; Ilium Lignocaine 20, Troy Laboratories Pty. Ltd., Smithfield, Australia). Endotoxin (*E. coli* LPS O55:B5; 1.2 million endotoxin units/mg; Sigma–Aldrich Pty. Ltd., Sydney, Australia) was sterile filtered (0.22 μ m syringe filters; Millex GP; Millipore Ltd., Cork, Ireland) into 500 ml saline, and was administered intravenously at a dose of 1 ng/kg/min, over 30 min (Time 0–30 min; total dose 30 ng/kg), controlled by an infusion pump. The LPS used was purchased in a purified form (purification by gel-filtration chromatography) and was low in protein and DNA content.

2.4. Clinical parameters and sampling procedure

Physical examination variables including rectal temperature, heart rate, respiratory rate, and demeanour were recorded immediately prior to pre-treatment (Time –60 min), and before LPS infusion (Time 0) and then every 15 min for the first 2 h, then every 30 min for the following 4 h. Jugular venous blood samples were collected at the same time points into tubes containing anticoagulant EDTA or heparin.

Horses were held in stocks (two horses side by side in separate stocks) in a covered building for the first 4 h, with ad libitum hay and water, after which they were allowed into a small concrete yard for a further two hours. At the 6 h time point, the experiment was completed; all horses then received flunixin meglumine (1.1 mg/kg intravenously) and were allowed back into their paddock. Additional observations were conducted in the paddock after 24 h.

2.5. Analytical methods

Leukocyte counts were performed on whole blood samples using a Coulter Counter (model Z1; Coulter Electronics Inc.).

Total plasma TNF α concentration was measured using an equine specific ELISA assay (Endogen Equine TNF α screening set; Thermo Fisher Scientific Inc., Rockford, IL, USA), as previously de-

scribed and validated by Vick et al. (2007). Equine recombinant TNF α (Thermo Fisher Scientific) was used for the standard curve. Measurements were made in duplicate.

Bioavailable (unbound) plasma TNF α was measured using a cell survival bioassay (L929 mouse fibroblast cell line) as described previously with equine samples by Armstrong and Lees (2002) and Kotiw et al. (2006). This cell line is sensitive to TNF-induced cell death, which was assayed using a MTT assay kit (TACS MTT cell proliferation assay; R&D Systems Inc., Minneapolis MN). The yellow tetrazole dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), is reduced to a purple formazan compound in living cells, which is then quantified using a colourimetric plate reader (absorbance read at 570 nm with reference wavelength of 650 nm). Recombinant equine TNF α , diluted in cell culture medium and an equivalent volume of blank equine plasma containing no detectable TNF α , was used to produce the standard curve.

2.6. Statistical analysis

In this randomised cross-over study, each horse acted as its own control, and therefore data were analysed in a pair-wise manner. The effect of hyperimmune equine plasma was compared with saline treatment at each time point by a two-way repeated measures analysis of variance with Bonferroni's post hoc test. In addition, the peak values for plasma TNF α were compared between saline and hyperimmune equine plasma treatments using a paired *t*-test. In all cases, significance was accepted at $P \leq 0.05$, rounded to two decimal places.

3. Results

3.1. Clinical data

The low-dose endotoxin administration was very well tolerated by all of the horses, and the clinical signs were mild and transient, as expected. There were no significant differences in heart rate or respiratory rate between saline treatment and hyperimmune equine plasma treatments, although the changes from baseline values were not very marked (Figs. 1 and 2).

With saline treatment prior to LPS challenge, the rectal temperature increased from 37.58 ± 0.17 °C at time 0 to a maximum of 39.05 ± 0.18 °C at 210 min; this is consistent with the increases observed in previous studies using this model. Following pre-treatment with the hyperimmune equine plasma, the peak rectal temperature was not significantly reduced compared with saline controls, reaching a maximum of 38.92 ± 0.19 °C (Fig. 3).

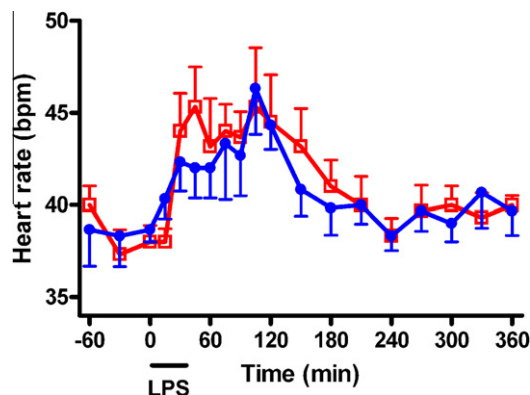


Fig. 1. Effect of pre-treatment with Equiplas hyperimmune plasma (solid circles) or saline (open squares) on the heart rate of horses challenged with 30 ng/kg *E. coli* endotoxin (0–30 min). Each point represents the mean \pm sem value from six horses.

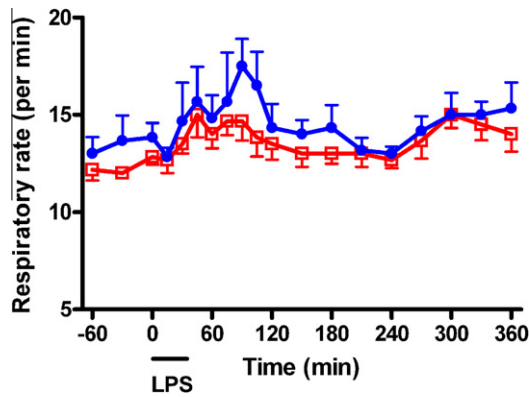


Fig. 2. Effect of pre-treatment with Equiplas hyperimmune plasma (solid circles) or saline (open squares) on the respiratory rate of horses challenged with 30 ng/kg *E. coli* endotoxin (0–30 min). Each point represents the mean \pm sem value from six horses.

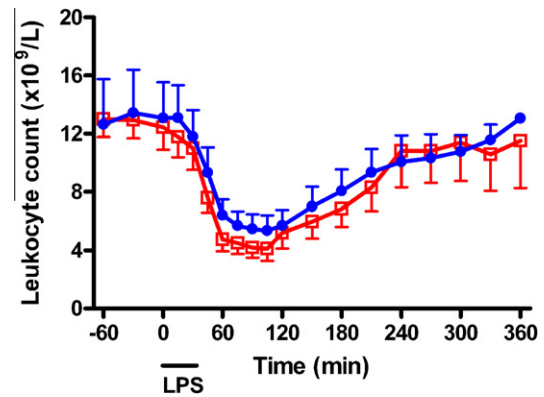


Fig. 4. Effect of pre-treatment with Equiplas hyperimmune plasma (solid circles) or saline (open squares) on peripheral blood leukocyte counts in horses challenged with 30 ng/kg *E. coli* endotoxin (0–30 min). Each point represents the mean \pm sem value from six horses.

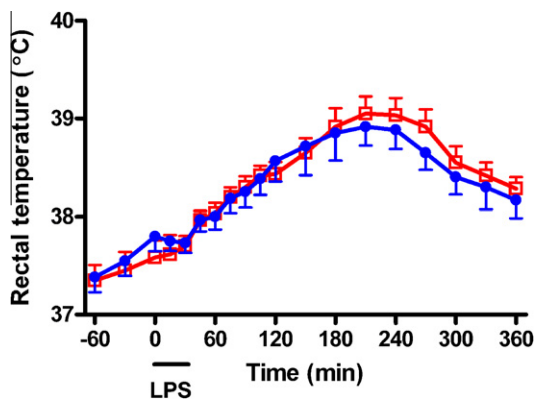


Fig. 3. Effect of pre-treatment with Equiplas hyperimmune plasma (solid circles) or saline (open squares) on the rectal temperature of horses challenged with 30 ng/kg *E. coli* endotoxin (0–30 min). Each point represents the mean \pm sem value from six horses.

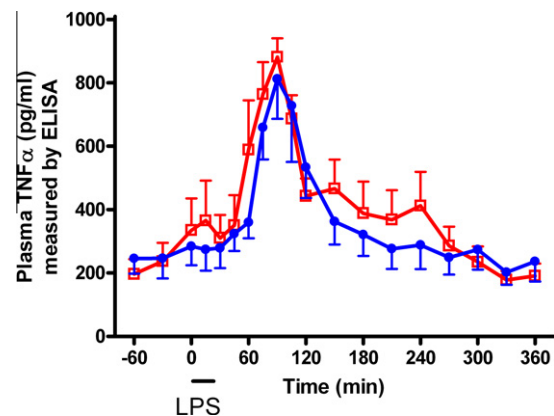


Fig. 5. Effect of pre-treatment with Equiplas hyperimmune plasma (solid circles) or saline (open squares) on total plasma TNF α concentration, as measured by ELISA, in horses challenged with 30 ng/kg *E. coli* endotoxin (0–30 min). Each point represents the mean \pm sem value from six horses.

3.2. Leukocyte counts

With saline pre-treatments (controls) blood leukocyte counts decreased from $12.43 \pm 1.55 \times 10^9$ leukocytes per litre at time 0 to a nadir of $4.12 \pm 0.82 \times 10^9/L$ at 105 min post endotoxin, before recovering to baseline values by 6 h. LPS-induced changes in blood leukocytes after hyperimmune plasma treatment were not significantly different from the controls, reaching a nadir of $5.35 \pm 1.06 \times 10^9$ leukocytes per litre at 105 min, and recovering to $13.07 \pm 0.24 \times 10^9/L$ at 6 h (Fig. 4).

3.3. Tumor necrosis factor – ELISA

Pre-treatment with hyperimmune equine plasma had no significant effect on the peak plasma TNF α concentration, which occurred at 90 min (881.93 ± 58.93 pg/ml with saline treatment compared with 812.36 ± 126.05 pg/ml following hyperimmune equine plasma administration; Fig. 5).

3.4. Tumor necrosis factor – bioassay

There was a significant difference ($P = 0.05$) observed between the bioavailable (unbound) TNF α measured by bioassay in the saline treated controls, which peaked at 1373.92 ± 107.63 pg/ml (90 min), and the hyperimmune equine plasma pre-treatment, which peaked at 1044.44 ± 193.93 pg/ml (Fig. 6).

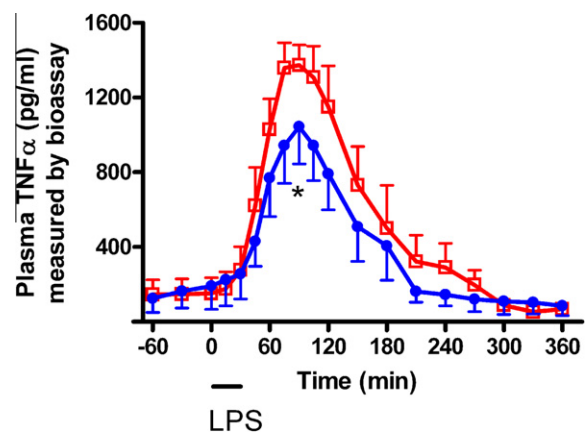


Fig. 6. Effect of pre-treatment with Equiplas hyperimmune plasma (solid circles) or saline (open squares) on plasma TNF α in horses challenged with 30 ng/kg *E. coli* endotoxin (0–30 min), as measured by L929 bioassay. Each point represents the mean \pm sem value from six horses. *Significant difference in peak value (90 min) between plasma and saline pre-treatments; paired t -test ($P = 0.05$).

3.5. Effect of time on clinical signs and cytokine responses

The data from the control experiments (saline pre-treatment before LPS) were compared to confirm that there were no signifi-

cant differences in the measured parameters between the first and second endotoxin challenges, that might indicate endotoxin tolerance. There were no significant differences between the peak rectal temperatures in the three horses given saline then LPS as their first challenge, compared with saline plus LPS as their second challenge (39.17 ± 0.17 °C and 39.13 ± 0.32 °C, respectively). Similarly, the changes in peak TNF α concentrations were not significantly different between first and second challenges (peak TNF α [measured by ELISA] 1008.0 ± 199.8 pg/ml and 971.3 ± 420.6 pg/ml, respectively). Ensuring three plasma treatments and three saline treatments in each of the first and second challenge periods would also have negated any effects of time on the results.

4. Discussion

This study indicates that although hyperimmune equine plasma had no measurable effect on clinical signs in the low-dose endotoxin challenge model (which induces only mild systemic inflammation), there is some evidence to suggest that hyperimmune plasma may inhibit the bioactivity of TNF α .

The experimental model of endotoxaemia used in this study is well established and previously published by a number of groups (Menzies-Gow et al., 2004; Poulin Braim et al., 2009). The low-dose challenge, using 30 ng/kg bodyweight, is preferred by the investigators in the present study on ethical grounds because of the very mild signs of toxæmia shown by the horses. Typically, body temperature is raised by approximately 1.5 °C, and minimal changes in heart rate and respiratory rate are observed. This model does however produce a predictable effect on leukocyte activation, indicative of systemic inflammation, as evidenced by the increase in plasma TNF α activity and the decrease in leukocyte count.

Although endotoxin tolerance has been described in horses given consecutive doses 24 h apart (Allen et al., 1996), this phenomenon is not a significant problem when one adheres to an adequate washout period. The mechanism of short-term tolerance is unknown, but may be associated with reduced leukocyte responsiveness and/or production of anti-inflammatory cytokines (Flohé et al., 1999; Frelstedt and Furr, 2010). Where a sufficient washout period is observed, each horse can be used as its own control in endotoxin challenge experiments (Menzies-Gow et al., 2008). In the present study, with a 21 day washout period, none of the responses caused by endotoxin was significantly altered over time.

Our hypothesis was that the antibodies raised to the *E. coli* J5 rough mutant in hyperimmune equine plasma would include antibodies against the polysaccharide core antigen, and therefore the activity of the administered endotoxin would be partially neutralised. However, the clinical effects of endotoxin (heart rate, respiratory rate, rectal temperature) were not significantly improved by pre-treatment with hyperimmune equine plasma in our model. Furthermore, the fact that the decrease in leukocyte count was similarly unaffected suggests that leukocyte activation was not significantly moderated.

There are several possible explanations for these findings. Firstly, antibody specificity for the different regions of the LPS molecule should be considered. It is the lipid A portion of the LPS molecule which activates the innate immune response binding to CD14 and TLR4 receptors on leukocytes (Bryant et al., 2010; Figueiredo et al., 2008). Rapid neutralisation of LPS would require antibodies binding to the lipid A moiety. Studies in cattle have indicated that antibody isotypes isolated from the plasma of animals immunised with the J5 mutant showed no appreciable binding to purified lipid A, or indeed to purified LPS (Chaiyotwittayakun et al., 2004). The fact that studies have shown protection with hyperimmune J5 plasma in clinical endotoxaemia (Spier et al., 1989; Ziegler et al., 1982) means that the antibodies

in this plasma could be binding to other surface antigens of bacteria, perhaps in conjunction with LPS. One study has shown that antiserum to J5 may bind to whole bacteria with LPS on their surface, at particular growth stages when the core LPS epitopes are most exposed (McCallus and Norcross, 1987). Further studies have suggested that much of the antibody binding may actually be to LPS-outer membrane protein (OMP) complexes, released by bacterial cell walls (Freudenberg et al., 1989).

Antibody binding to the polysaccharide regions may not prevent leukocyte activation in the first instance, although they may accelerate clearance of LPS from the circulation. Effects on LPS clearance may not be appreciated in the present acute model (administering LPS for only 30 min). Furthermore, the *E. coli* LPS used in the present study (and commonly used in this model by other researchers) was O55:B5 LPS, highly purified by phenol extraction, and this was different to the O111:B4 LPS on the *E. coli* used to immunise the horses from which the hyperimmune equine plasma was obtained. Some studies, such as Siber et al. (1985) suggest that immunisation (of rabbits) with whole *E. coli* J5 induces high concentrations of IgG antibody that recognise the homologous LPS (O111:B4), but lower concentrations of antibody that bind to heterologous LPS.

To determine the form(s) of LPS to which the antibodies bind most effectively in horses would require many further laboratory investigations. However it is very likely that the present model using pure LPS does not entirely represent the situation of naturally occurring endotoxaemia, where there will be various forms of LPS released into the circulation, including LPS–OMP complexes. Horses with clinical endotoxaemia have been found to have plasma endotoxin levels up to 469 pg/ml (although great variability exists; Menzies-Gow et al., 2005). The present low-dose challenge model produces plasma concentrations in the range of 10–15 pg/ml (Menzies-Gow et al., 2004).

The fact that the bioassay for TNF α (using L929 cells) showed a reduced bioactivity in horses pretreated with hyperimmune equine plasma (but not reduced total levels vs saline controls), suggests that the hyperimmune plasma contains substances which may bind and inactivate circulating TNF α at high concentrations. This is most likely attributable to soluble TNF α receptor, although there are other circulating protein factors which may also bind TNF α , such as alpha-2 macroglobulin (Coté et al., 1996). Therefore a specific test would be required to confirm this supposition, but the findings are certainly consistent with the previous *in vitro* experiments conducted by Kotiw et al. (2006), which found that the same type of hyperimmune plasma that was used in the present study could reduce the direct effects of recombinant TNF α in the L929 cell bioassay. Soluble TNF α receptor protein was not measured in the present study because there is no equine specific test currently available, although it has been found in canine hyperimmune plasma (Kotiw et al., 2010).

There are several possible reasons for the discrepancy between the TNF α values given by the ELISA assay and the bioassay in the present study. The ELISA assay may underestimate the TNF α concentration at higher concentrations, even though samples with values greater than 500 pg/ml were further diluted and re-assayed, to ensure that they were on the straight part of the (sigmoidal) calibration curve. Also, since the bioassay relies on quantifying the death of L929 murine fibroblasts, this assay may be influenced to some degree by other factors in the plasma, even though appropriate controls were used (TNF α standards diluted in low-TNF equine plasma). The effects of a number of factors in plasma samples may potentially contribute towards increased variability and error in any cell-based bioassay (the most likely being endotoxin); however, it has previously been determined in our laboratory (and others) that LPS and other pro-inflammatory cytokines do not contribute to cell death in this bioassay (data not shown).

TNF α is an important pro-inflammatory cytokine released in endotoxaemia and therefore reducing its activity would be expected to have beneficial effects on the outcome of the condition (by reducing further leukocyte activation, tissue damage and organ dysfunction). Apart from cytokine effects, it is important to be aware of other beneficial effects of plasma therapy in endotoxaemia, such as the restoration of clotting factors which may be depleted if disseminated intravascular coagulation (DIC) occurs. There was no clinical evidence of DIC in the low-dose endotoxin model, and measurements of coagulation cascades and clotting factors were not undertaken in the current study.

In summary, pre-treatment with hyperimmune equine plasma was not associated with significant improvements in the clinical signs of mild, transient endotoxaemia in the low-dose endotoxin challenge model. However there was some evidence of reduced TNF α bioactivity. The effects of other active compounds in hyperimmune plasma which may also be beneficial in the treatment of equine endotoxaemia, such as clotting factors and other plasma proteins, were not evaluated in this model.

5. Conflict of interest statement

None declared.

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