

Rapid infusion of a phospholipid emulsion attenuates the effects of endotoxaemia in horses

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Summary

Reasons for performing study: Endotoxaemia currently is associated with a poor prognosis in horses. The results of recent trials in other species indicate that phospholipid emulsions reduce the deleterious effects of endotoxin (LPS). However, in a previous study in horses, a 2 h infusion of emulsion caused an unacceptable degree of haemolysis.

Hypothesis: Rapid administration of a lower total dose of emulsion would reduce the effects of LPS and induce less haemolysis; the emulsion would reduce inflammatory effects of LPS *in vitro*.

Methods: Twelve healthy horses received an i.v. infusion either of saline or a phospholipid emulsion (100 mg/kg), followed immediately by *E. coli* O55:B5 LPS (30 ng/kg). Clinical parameters, haematological profiles, serum tumour necrosis factor (TNF) activity, serum lipid profiles, urine analyses and severity of haemolysis were monitored before and at selected times after LPS. Monocytes were also incubated *in vitro* with LPS in the presence or absence of emulsion, after which TNF and tissue factor activities were determined.

Results: Clinical signs of endotoxaemia were reduced in horses receiving the emulsion, including clinical score, heart rate, rectal temperature, serum TNF activity, and the characteristic leucopenic response to LPS, when compared to horses not receiving the emulsion. Three horses receiving the emulsion had none, 2 had mild and one had moderate haemolysis. There were no differences in urinalysis results and creatinine concentrations, either within the groups over time or between the groups. Serum concentrations of phosphatidylcholine, bile acids and triglycerides peaked immediately after the infusion; there were no significant changes in concentrations of nonesterified fatty acids or cholesterol. Incubation of equine monocytes with emulsion prevented LPS-induced TNF and tissue factor activities.

Conclusions: Rapid administration of emulsion significantly reduced inflammatory effects of LPS *in vivo* and caused a clinically insignificant degree of haemolysis. The results of the *in vitro* studies indicate that emulsion prevents not only LPS-induced synthesis of cytokines, but also expression of membrane-associated mediators (i.e. tissue factor).

Potential relevance: Rapid i.v. administration of emulsions containing phospholipids that bind endotoxin may provide a clinically useful method of treating endotoxaemia in horses.

Introduction

Endotoxaemia is a clinically important complication of many equine diseases, most notably those associated with the gastrointestinal tract in mature horses and septicaemia in neonatal foals (Werners *et al.* 2005). Based on the results of clinical studies in which the Limulus amoebocyte lysate test was used to detect endotoxin (lipopolysaccharide; LPS), approximately 25% (King and Gerring 1988; Fessler *et al.* 1989) to 41% (Steverink *et al.* 1994) of horses presented to university hospitals with gastrointestinal diseases causing colic had LPS in their plasma at the time of admission. Endotoxaemia in such cases results in development of a systemic inflammatory response syndrome, characterised by increased production and release of inflammatory mediators, alterations in tissue perfusion, initiation of intravascular coagulation and, eventually, death (Barton *et al.* 1998).

The cellular pathways responsible for initiating the deleterious effects of endotoxaemia have been well documented, and involve interaction of LPS with an acute phase protein called LPS-binding protein (Wright *et al.* 1990). This interaction then facilitates the interaction of LPS with its specific receptors on the surface of inflammatory cells, such as mononuclear phagocytes. Interaction of LPS with these cell surface receptors, CD14 and Toll-like receptor 4, and a coreceptor protein called MD2, results in activation of intracellular signalling pathways that lead to the up-regulation of inflammatory genes (Werners *et al.* 2005). Inflammatory mediators, such as tumour necrosis factor (TNF), interleukins, eicosanoids and tissue factor, are responsible for the adverse clinical effects of endotoxaemia.

Based on the fact that high-density lipoproteins neutralise LPS (Levine *et al.* 1993; Wurfel *et al.* 1997), that human patients with low serum concentrations of lipoproteins have an increased risk for infection and reduced prognosis for survival (Gordon *et al.* 1996) and that human patients with systemic inflammatory response syndrome have low serum lipoprotein concentrations (Levels *et al.* 2003), several studies have been performed to evaluate the effects of a protein-free, phospholipid emulsion in sepsis and endotoxaemia. The results of these studies have included improved survival rate of pigs in an experimental model of peritonitis (Goldfarb *et al.* 2003) and significant attenuation of the effects of LPS in healthy human volunteers receiving 210 mg/kg of the emulsion (Gordon *et al.* 2005).

In a recent study performed on horses, administration of this phospholipid emulsion (total dose of 200 mg/kg administered over

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a 2 h period) significantly reduced many of the clinical and cardiopulmonary effects of LPS; however, administration caused gross haemolysis within 1 or 2 h (Winchell *et al.* 2002). In that study, infusion of the emulsion was initiated 1 h before administration of LPS and continued through the 1 h LPS infusion. The current study, in which horses were administered 100 mg/kg over a 30 min period followed by a 30 min infusion of LPS, was undertaken in order to determine whether rapid i.v. infusion of a lower total dose of the phospholipid emulsion would provide protection against challenge with LPS and reduce the degree of haemolysis reported previously (Winchell *et al.* 2002). To determine whether the effects of the emulsion on LPS-induced synthesis of inflammatory mediators were limited to substances secreted by the cell, *in vitro* experiments were performed in which the effects of the emulsion on synthesis and release of TNF were compared to the effects on the expression of tissue factor, an inflammatory mediator that remains associated with the cell membrane. Dilutions of the emulsion bracketing the effects of haemodilution after i.v. administration were used in the *in vitro* experiments.

Materials and methods

This study was approved by the University of Georgia Institutional Animal Care and Use Committee. Twelve healthy mature horses of mixed breeds (8 geldings and 4 mares, mean age 9 years, bodyweight 446 kg) were assigned randomly to one of 2 treatment groups: saline-endotoxin ($n = 6$) or phospholipid emulsion-endotoxin ($n = 6$). Serum, creatinine and GGT concentrations, and urine analysis were performed on the afternoon prior to the day of study. Each horse had a baseline clinical examination performed for evaluation of attitude, heart rate, respiratory rate and rectal temperature; and blood samples collected for haematology serum tumour necrosis factor (TNF) and lipid profiles (phosphatidylcholine, triglycerides, nonesterified fatty acids, cholesterol and bile acids). Objective assessment of the degree of haemolysis was achieved by measurement of absorbance at 570 nm.

Serum TNF activity was determined in a bioassay, using WEHI cells (Morris *et al.* 1991). Briefly, serum samples were diluted 1:10 with tissue culture media prior to use in the assay. Human recombinant TNF was used as the assay standard. One unit of TNF activity was defined as the serum dilution that produced death in 50% of the cells.

The emulsion to be tested contained a patented combination of soy phosphatidylcholine (~92%) and soy triglyceride (~7%) emulsified in 18 mmol/l sodium cholate containing 2.6% glycerol (w/v) by repeated passes through an APV Gaulin homogeniser at 10,000 PSI. The final mixture, containing 100 mg/ml phosphatidylchoine, was sterilised by passage through a 0.45 μm membrane. The emulsion, referenced herein as the phospholipid emulsion, was manufactured by Fresenius Kabi¹.

Horses in the emulsion-LPS group received an infusion of phospholipid emulsion (100 mg/kg) over 30 min (mean 430 ml) and in the saline-LPS group an equal volume of saline over the same period. The dosage of emulsion used was based on the results of a previous study (Winchell *et al.* 2002) performed in horses in which a total dosage of 200 mg/kg was administered as a continuous infusion, with half of that amount being administered 1 h before initiation of the LPS infusion. It was hypothesised that the beneficial effects of the emulsion might be achieved by giving the same 100 mg/kg dosage prior to the LPS infusion, and potentially reduce or eliminate the degree of haemolysis. The

dosage used in this study is similar to priming doses used in a porcine sepsis study (Goldfarb *et al.* 2003) and approximately half the dosage used in a study on endotoxaemia in healthy human volunteers (Gordon *et al.* 2005). In an effort to simulate clinical conditions, the emulsion was administered as rapidly as possible. At the end of these infusions, designated time 0, clinical and blood examinations were repeated, and all horses received an infusion of *E. coli* O55:B5 endotoxin² (30 ng/kg over 30 min). Examinations were repeated at 0.5 (i.e. end of LPS infusion), 1, 1.5, 2, 3, 4, 6, 12 and 24 h later. Clinical scores (0 = normal, 1 = mild, 2 = moderate, 3 = severe) were assigned from the observations of attitude, appetite, evidence of pain and depression, recorded at each sample time. Analysis of urine, including assessments of protein, glucose, bilirubin, white blood cells, creatinine and GGT, and serum creatinine concentrations were repeated 3 days later.

In vitro studies were performed to determine the ability of different dilutions of the phospholipid emulsion to alter LPS-induced synthesis of TNF and membrane expression of tissue factor by peripheral blood monocytes. After careful disinfection of the skin over the jugular furrow, blood samples were collected from 7 healthy mature horses by venipuncture into 60 ml pyrogen-free syringes containing 1.5 ml 100 mmol/l EDTA. Syringes were then positioned vertically, standing on the flat part of the syringe plunger, for approximately 25 min during which period the erythrocytes settled by gravity. The leucocyte-rich plasma was then transferred to a sterile 50 ml conical centrifuge tube and diluted with an equal volume of PBS. The plasma was then layered over Histopaque 1077³ and centrifuged at 800 g in a swinging bucket rotor for 30 min. The plasma/PBS layer above the Histopaque interface was removed using a 25 ml pipette and discarded. The interface containing the mononuclear cells was then collected, transferred to a 50 ml conical centrifuge tube, diluted with an equal volume of PBS, and centrifuged at 800 g for 10 min. The cells were suspended in 20 ml PBS, an aliquot counted on a haemocytometer and viability assessed by trypan blue exclusion (0.04% in PBS). The cells were washed an additional time with PBS, and then suspended in the volume of RPMI 1640⁴ media calculated to yield 1×10^7 cells/ml.

Monocytes were isolated by incubating the mononuclear cell suspensions (4×10^6 cells/tube) for 2 h in RPMI 1640 media in sterile polystyrene tubes at 37°C in 5% CO₂. The nonadherent cells were removed by gently rinsing the tubes with warm PBS and the medium covering the adherent monocytes then replaced with RPMI 1640 media containing 1% heat-inactivated fetal bovine serum⁵ (media/HIFBS) as a source of LPS-binding protein. In previous studies performed in our laboratory, >85% of the adherent cells were identified as monocytes by a nonspecific esterase stain (Henry and Moore 1988). Triplicate tubes were prepared containing either media/HIFBS alone (control) or media/HIFBS containing *E. coli* O55:B5 LPS (100 pg/ml), different dilutions (1:20, 1:40, 1:80) of the phospholipid emulsion in media/HIFBS, or combinations of these dilutions of the emulsion and 100 pg/ml LPS. All tubes were incubated for 6 h at 37°C in 5% CO₂, after which the tubes were centrifuged for 10 min at 800 g and the supernatant removed by decanting into microfuge tubes. The microfuge tubes were stored frozen at -80°C until assayed for TNF by ELISA. After the supernatants were removed from the polystyrene tubes, 0.5 ml PBS-0.1% Tween was added and the tubes were frozen at -80°C until assayed for tissue factor activity.

Immediately prior to determining tissue factor activity, the tubes were thawed and sonicated to lyse the cells. Tissue factor activity was determined by the ability of cellular lysates to shorten

the recalcification time of pooled equine plasma in a one-stage clotting time. Clotting times were determined in triplicate photoelectronically⁶. Tissue factor activities of the cell lysates were determined by reference to a standard curve generated by known dilutions (0.09–100%) of equine brain thromboplastin prepared in our laboratory.

Concentrations of TNF in monocyte supernatants were determined using an ELISA developed and validated in our laboratory. Components in serum and plasma interfere with the linearity of this assay when added to recombinant standards; its use has therefore been limited to cell culture supernatants. In contrast, the WEHI bioassay has been proven to measure TNF activity in serum and plasma of horses (Barton *et al.* 1994). Briefly, 96-well plates are coated with polyclonal antibody⁷ against equine TNF- α . After incubation with a BSA blocking buffer for 1 h, the plates are washed 3 times with PBS containing 0.05% Tween 20. Dilutions of recombinant equine TNF standards

or samples are then added to appropriate wells, the plates incubated for 2 h at 37°C and then washed 3 times. Biotin-labelled polyclonal antibody⁷ against equine TNF- α is then added to each well, incubated for 90 min at 37°C, and then avidin-HRP is added. After an additional incubation and 5 washes, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) HRP substrate is added to the wells, and plates read at 405 nm.

Phosphatidylcholine and nonesterified fatty acids were measured using enzymatic kits⁸ on a Roche COBAS Fara II analyser⁹. Bile acids were measured by an enzymatic method¹⁰ also on a FARA II analyser. Cholesterol and triglycerides were measured using enzymatic methods on a Roche COBAS Integra 700⁹.

All data are expressed as means \pm s.d. Serum TNF data were log transformed prior to analysis. Statistical analysis of data was performed using one-way analysis of variance (ANOVA) followed by a Tukey-Kramer *post hoc* test. $P < 0.05$ was considered significant.

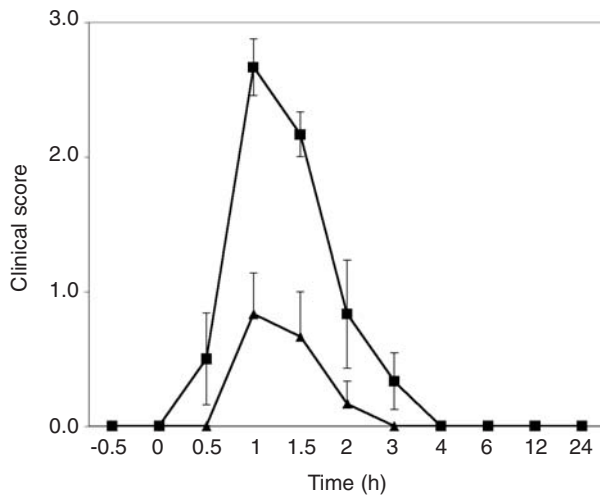


Fig 1: Alterations in clinical scores for horses receiving either saline-LPS (■) or emulsion-LPS (▲). Values for the saline-LPS group at 0.5, 1, 1.5 and 2 h are significantly greater than values at the same times for the emulsion-LPS group.

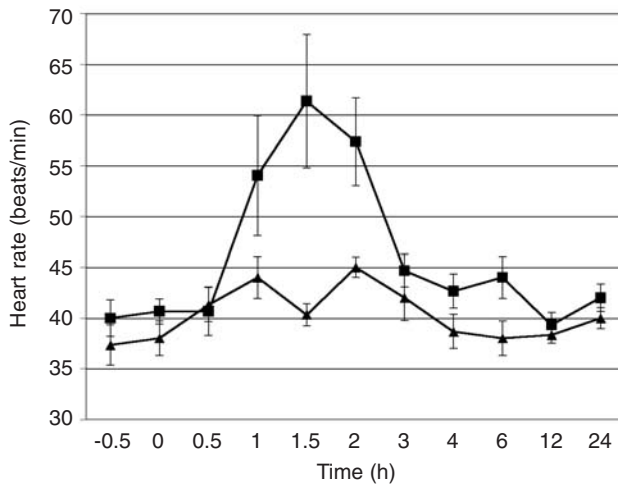


Fig 2: Alterations in heart rate for receiving either saline-LPS (■) or emulsion-LPS (▲). Values for the saline-LPS group at 1, 1.5 and 2 h are significantly greater than values at the same times for the emulsion-LPS group.

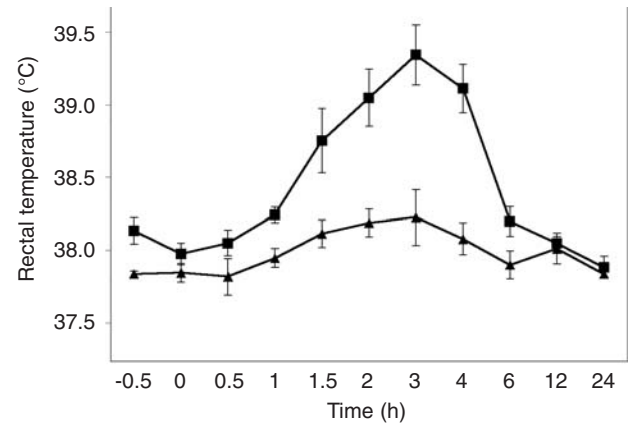


Fig 3: Alterations in rectal temperature for horses receiving either saline-LPS (■) or emulsion-LPS (▲). Values for the saline-LPS group at 1.5, 2, 3 and 4 h are significantly greater than values at the same times for the emulsion-LPS group.

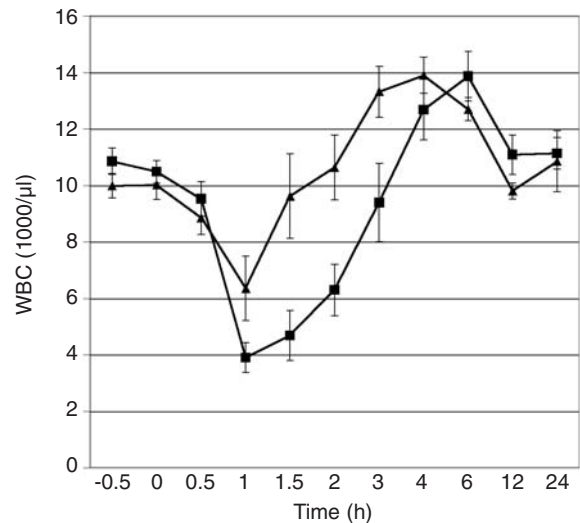


Fig 4: Alterations in circulating white blood cells (WBC) for horses receiving either saline-LPS (■) or emulsion-LPS (▲). Values for the saline-LPS group at 1, 1.5, 2 and 3 h are significantly less than values at the same times for the emulsion-LPS group.

Results

Clinical signs, haematology and urine analysis

Horses in the saline-LPS group exhibited clinical signs consistent with endotoxaemia, including abdominal pain (e.g. pawing, flank watching, stretching out, kicking at abdomen, lying down, yawning, inappetence). These signs began towards the end of LPS infusion, were more obvious over the ensuing hour and were gone by the time of the 4 h sample. In contrast, the clinical signs exhibited by the horses in the emulsion-LPS group were much less pronounced and occurred over a shorter period. These differences are reflected in the significant differences in mean clinical scores at times 0.5, 1, 1.5 and 2 h (Fig 1). Horses in the saline-LPS group had significantly higher heart rates and rectal temperatures, and significantly lower total leucocyte counts than horses in the emulsion-LPS group (Figs 2–4). As a measure of haemolysis, absorbance of light at 570 nm was significantly greater in plasma samples obtained at times 6, 12 and 24 h from horses in the emulsion-LPS group, compared to the saline-LPS group (Fig 5). Subjective assessments of the degree of haemolysis ranged from no haemolysis in 2 horses, trace haemolysis in 2 horses and 4+ haemolysis in one horse in the emulsion-LPS group. There were no significant differences in serum creatinine concentrations or urine analysis results, including GGT/creatinine ratios, either within groups over time or between groups (data not shown).

Serum TNF

Serum TNF values were significantly higher in horses in the

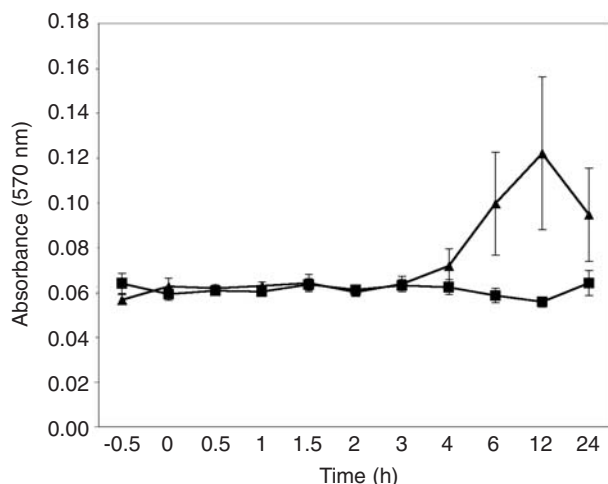


Fig 5: Alterations in plasma light absorbance at 570 nm for horses receiving either saline-LPS (■) or emulsion-LPS (▲). Values for the emulsion-LPS group at 6, 12 and 24 h are significantly greater than values at the same times for the saline-LPS group.

saline-LPS group than in the emulsion-LPS group at times 1, 1.5, 2 and 3 h (Fig 6).

In vitro results

Supernatant concentrations of TNF were significantly greater in monocytes incubated in media/HIFBS containing LPS, when compared with media/HIFBS alone and media/HIFBS containing any of the 3 dilutions of the emulsion (Table 1). Similarly, cellular expression of tissue factor was significantly greater for the monocytes incubated with LPS, compared with those incubated with media/HIFBS alone or media/HIFBS containing any of the 3 dilutions of the emulsion (Table 1).

Serum lipid profiles

Serum concentrations of phosphatidylcholine, bile acids, nonesterified fatty acids, triglycerides and cholesterol for the 2 groups are summarised in Table 2. Serum concentrations of phosphatidylcholine and triglycerides increased from baseline by approximately 3-fold, and bile acids initially by 13-fold, after the completion of the infusion of the phospholipid emulsion (Fig 7). Serum concentrations of phosphatidylcholine and bile acids remained significantly increased for 12 and 6 h, respectively. Serum concentrations of triglycerides were significantly less in the horses administered the phospholipid emulsion from 1.5–12 h; and serum cholesterol concentrations were significantly higher in horses administered the phospholipid emulsion at 4 and 12 h, compared with values for the saline-LPS group.

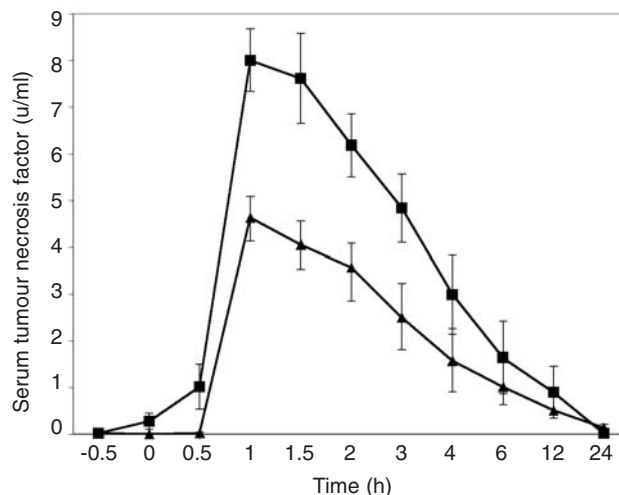


Fig 6: Alterations in serum tumour necrosis factor activity for horses receiving either saline-LPS (■) or emulsion-LPS (▲). Values for the saline-LPS group at 1, 1.5, 2 and 3 h are significantly greater than values at the same times for the emulsion-LPS group.

TABLE 1: Inhibition of LPS-induced production of TNF (n = 6) and membrane expression of tissue factor (n = 7) by equine monocytes incubated with 3 dilutions of the phospholipid emulsion

	Media	LPS (100 pg/ml)	Emulsion 1:20 + LPS	Emulsion 1:40 + LPS	Emulsion 1:80 + LPS
TNF (pg/ml)	18.6 ± 25.2	448.5 ± 302.9 ^a	9.9 ± 22.7 ^b	7.6 ± 12.2 ^b	3.7 ± 8.8 ^b
Tissue factor (%)	7.8 ± 7.9	139.8 ± 140.5 ^a	1.8 ± 1.1 ^b	2.5 ± 1.7 ^b	2.8 ± 2.1 ^b

Values are presented as means ± s.d. ^aSignificantly different from media; ^bSignificantly different from LPS (100 pg/ml).

Discussion

The aims of this study were to determine whether rapid infusion of a phospholipid emulsion at half the dosage evaluated previously would provide significant protection against the deleterious effects of experimental endotoxaemia, without causing the degree of haemolysis noted in the previous study (Winchell *et al.* 2002). In addition *in vitro* studies were performed to determine whether emulsion could reduce proinflammatory responses of isolated equine monocytes to LPS. The results indicate that rapid i.v. administration of 100 mg/kg of emulsion significantly reduced the magnitude of the febrile, tachycardic and leucopenic responses initiated by administration of LPS, improved clinical scores and reduced serum concentrations of TNF. These *in vivo* findings corroborate the results of the previous study in which horses were administered 200 mg/kg of emulsion starting before and continuing throughout administration of LPS (Winchell *et al.* 2002). Furthermore, the results obtained in the present study indicate that the components of the emulsion (primarily, phospholipid and bile acids) persisted sufficiently long in the circulation after rapid i.v. infusion to modulate significantly the deleterious effects of LPS challenge. Serum concentrations of these components of the emulsion persisted in circulation to a similar extent, as has been reported for human individuals administered similar dosages (Gordon *et al.* 2003). In addition, the *in vitro* studies indicate that a direct effect of lipid emulsion on LPS reponse by monocytes was, at least in part, responsible for the effects seen *in vivo*.

Administration of the emulsion in the present study resulted in rapid increases in serum concentrations of phosphatidylcholine and bile acids, and delayed increases in serum cholesterol. The latter change has been reported in other species (Gordon *et al.* 2003) and, presumably, represents efflux from tissues. The lower serum concentrations of triglyceride noted in the present study may be due to differences in cytokine production in the horses in the saline-LPS and emulsion-LPS groups (Fig 6). Although the differences in serum concentrations of triglyceride between the 2 groups were statistically significant, the values were within normal limits for horses and of questionable clinical significance.

In the previous study (Winchell *et al.* 2002), gross haemolysis was identified visually, which may fail to detect mild haemolysis. Consequently, to provide quantifiable evidence for the severity of haemolysis occurring after administration of the phospholipid emulsion, in the present study light absorbance of plasma was

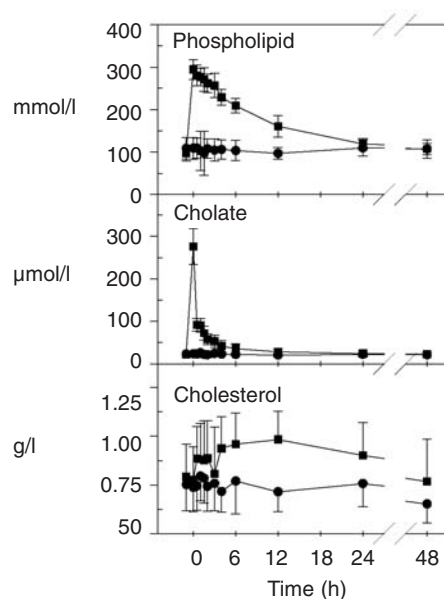


Fig 7: Alterations in serum concentrations of phospholipids (mmol/l), cholate ($\mu\text{mol/l}$) and cholesterol (g/l) for horses receiving either saline-LPS (\bullet) or emulsion-LPS (\blacksquare). Values for phospholipids and cholate for the emulsion-LPS group from 1.5 to 12 h are significantly greater than values at the same times for the saline-LPS group. Serum concentrations of cholesterol are significantly greater in the emulsion-LPS group at 4 and 12 h than values at the same times for the saline-LPS group.

measured using a wavelength at which haemoglobin absorbs light. The results obtained in the present study indicated that haemolysis was first evident 6 h after completion of the infusion and persisted through the 24 h monitoring period. Although haemolysis has been reported in pigs when dosage of the emulsion was sufficient to produce serum phospholipid levels exceeding 20 g/l, the results of safety and pharmacokinetic studies performed in healthy human volunteers provided no evidence for overt haemolysis when peak phospholipid concentrations achieved 7 g/l (Gordon *et al.* 2003). In the present study it was not possible to identify an association between the degree of haemolysis and age, gender or breed of the horses utilised. Therefore, the reason for the selective increased fragility of equine erythrocytes after administration of relatively small dosages of the emulsion remains to be identified.

TABLE 2: Serum concentrations of lipid components in horses administered saline-LPS (Control) or a phospholipid emulsion-LPS (Emulsion)

Time (h)	Phosphatidylcholine (mmol/l)		Bile Acids ($\mu\text{mol/l}$)		Nonesterified fatty acids (meq/l)		Triglyceride (mg/l)		Cholesterol (mg/l)	
	Control	Emulsion	Control	Emulsion	Control	Emulsion	Control	Emulsion	Control	Emulsion
-1	108.6 \pm 25.1	96.8 \pm 17.7	23.1 \pm 2.5	21.4 \pm 2.9	0.4 \pm 0.3	0.3 \pm 0.2	247 \pm 90	187 \pm 57	752 \pm 135	792 \pm 167
0	109.1 \pm 25.4	294.9 \pm 23.5	23.6 \pm 2.5	276.3 \pm 42.6	0.3 \pm 0.1	0.1 \pm 0.1	220 \pm 78	578 \pm 166	737 \pm 124	777 \pm 169
0.5	109.2 \pm 23.9	280.0 \pm 24.3	23.1 \pm 1.7	91.9 \pm 14.7	0.2 \pm 0.2	0.2 \pm 0.1	235 \pm 95	222 \pm 47	745 \pm 124	886 \pm 163
1	102.9 \pm 46.5	276.6 \pm 20.6	26.1 \pm 4.9	90.7 \pm 16.0	0.2 \pm 0.1	0.2 \pm 0.1	252 \pm 103	173 \pm 33	795 \pm 128	882 \pm 183
1.5	97.2 \pm 51.9	270.5 \pm 27.7	22.2 \pm 7.4	72.0 \pm 16.6	0.1 \pm 0.1	0.2 \pm 0.0	262 \pm 98	153 \pm 36	785 \pm 129	877 \pm 204
2	107.6 \pm 22.6	261.8 \pm 22.1	21.6 \pm 6.8	57.9 \pm 12.1	0.2 \pm 0.1	0.2 \pm 0.0	253 \pm 72	163 \pm 37	742 \pm 126	887 \pm 191
3	104.6 \pm 25.7	256.7 \pm 28.6	25.0 \pm 5.2	53.1 \pm 14.7	0.2 \pm 0.1	0.2 \pm 0.1	273 \pm 116	127 \pm 49	758 \pm 140	807 \pm 240
4	106.1 \pm 23.0	229.1 \pm 18.6	22.9 \pm 4.2	41.6 \pm 12.9	0.2 \pm 0.1	0.2 \pm 0.1	242 \pm 87	138 \pm 25	717 \pm 108	938 \pm 162
6	103.5 \pm 24.1	209.1 \pm 16.9	22.2 \pm 4.0	36.1 \pm 11.0	0.2 \pm 0.1	0.2 \pm 0.1	245 \pm 127	123 \pm 29	770 \pm 169	958 \pm 160
12	96.8 \pm 13.7	160.5 \pm 25.6	20.8 \pm 4.2	27.7 \pm 8.3	0.2 \pm 0.2	0.2 \pm 0.2	197 \pm 39	132 \pm 28	715 \pm 103	983 \pm 146
24	109.5 \pm 19.4	118.9 \pm 12.7	22.4 \pm 4.3	24.4 \pm 7.6	0.2 \pm 0.1	0.2 \pm 0.1	388 \pm 181	140 \pm 57	757 \pm 117	902 \pm 168
48	107.4 \pm 22.1	107.1 \pm 13.3	21.4 \pm 6.0	23.1 \pm 5.6	0.2 \pm 0.1	0.1 \pm 0.1	255 \pm 128	202 \pm 70	653 \pm 99	767 \pm 218

Values in **bold** are significantly different from values for the corresponding time points in the Control group.

In an effort to ascertain whether or not the degree of haemolysis observed in the present study adversely affected renal function, serum concentrations of creatinine were measured and urine analysis performed before initiation of the study and 3 days after its completion. There were no alterations in serum creatinine nor any abnormal findings in urine, suggesting that the mild to moderate degree of haemolysis was clinically inconsequential. It should be noted, however, that these findings represent a single point in time after administration of the emulsion and do not provide conclusive evidence that there may have been subclinical nephrotoxicity. Furthermore, it is important to note that these studies were performed in otherwise healthy horses and, as is common in studies such as this, caution must be exercised before extrapolations are made to horses with pre-existing disease.

In the present study, incubation of equine monocytes, with dilutions of the emulsion bracketing the effects of haemodilution, significantly reduced cellular expression of tissue factor and synthesis of TNF induced by LPS. This provides additional evidence for the ability of the phospholipid emulsion to interfere with interactions among LPS, soluble proteins in serum and cell surface receptor proteins for LPS. However, it could not be determined whether the reduced cellular response to LPS was due to binding of LPS monomers to emulsion phospholipid and therefore impaired presentation to either LPS-binding protein in the media or to the CD14, TLR4 and MD2 cell surface receptor complex for LPS.

The effects of the emulsion on tissue factor activity were monitored for 2 reasons. Firstly, unlike many inflammatory mediators, tissue factor predominantly remains associated with the surface of LPS-stimulated mononuclear phagocytes (Henry and Moore 1988), and exposure of these cells to plasma markedly accelerates coagulation. Secondly, a clinical study showed that monocytes isolated from horses with colic expressed significantly more tissue factor than monocytes from healthy horses; and that monocytes from horses with colic incubated *in vitro* with LPS expressed significantly more tissue factor than monocytes handled identically from healthy horses (Henry and Moore 1991).

Based on the results of the present study, many of the adverse effects of LPS can be prevented or significantly ameliorated by rapid administration of a phospholipid emulsion with the production of only mild to moderate haemolysis. Additional *in vivo* and *in vitro* studies with phospholipid emulsions are warranted more fully to characterise their beneficial effects in endotoxaemic horses, to identify the underlying cause for the haemolysis and, potentially, to alter the composition of the emulsion to eliminate any side effects.

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Manufacturers' addresses

¹Fresenius Kabi, Clayton, North Carolina, USA.

²List Biological Laboratories, Campbell, California, USA.

³Sigma-Aldrich, St. Louis, Missouri, USA.

⁴Mediatech, Inc., Herndon, Virginia, USA.

⁵HyClone, Logan, Utah, USA.

⁶ACL 1000, Instrumentation Laboratory, Lexington, Massachusetts, USA.

⁷Endogen, Rockford, Illinois, USA.

⁸Wako Chemicals, Richmond, Virginia, USA.

⁹Trinity Biotech, Bray, Ireland.

¹⁰Roche Diagnostics, Indianapolis, Indiana, USA.

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