The influence of experimentally-induced endotoxaemia on clinical variables and markers of systemic inflammation in donkeys (*Equus asinus*)

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ABSTRACT: In view of the frequent involvement of endotoxins in the pathogenesis of equine diseases, the present study set out to gain preliminary insight into the challenge caused by lipopolysaccharide (LPS) exposure in donkeys and into the responses of animals to LPS challenge. To the best of our knowledge, this is the first study to determine the susceptibility and response of donkeys to LPS administration and the first to describe the extent to which donkeys can tolerate a state of endotoxaemia. For this purpose, 18 clinically healthy, native breed donkeys were randomly allocated into three groups of equal size. The first and second groups received *E. coli* O55:B5 endotoxin at a dose rate of 20 ng/kg (Low dose group), and 5.0 µg/kg (High dose group), respectively, after dilution in 500 ml of 0.9% normal saline, while the third group (Control) received 500 ml of 0.9% isotonic saline solution. Blood samples were drawn from each animal before exposure to LPS and hourly for 6 h subsequently to measure the circulating levels of inflammatory cytokines as well as the cellular response. All animals were clinically monitored throughout the study period. Following LPS exposure, donkeys in both treated groups had quite different temporal patterns of clinical manifestations. The high dose of LPS yielded a statistically significant ($P < 0.001$ and $P < 0.001$, respectively) increase in heart rate, and respiratory rate, as well as hypothermia and poor outcome compared with animals receiving the low dose. The severity of colic was, in general, mild in donkeys receiving the low dose of LPS, while the signs were overt in those receiving the high dose. Donkeys of both treated groups exhibited marked cellular alterations and up-regulation of tumour necrosis factor alpha, interleukin (IL)-12 and IL-10 with a marginal increase in the values of serum amyloid A compared with controls ($P < 0.05$). The results described herein demonstrate that donkeys can respond to even a physiological dose of *E. coli* O55:B5 endotoxin, while a high dose can elicit overt clinical alterations and marked inflammatory responses. Further studies with an extended follow-up time are needed to verify and generalise the obtained findings and to evaluate novel medications to minimise the deleterious consequences of endotoxaemia in equine patients.

Keywords: donkeys; lipopolysaccharide; *E. coli* O55:B5; cytokine response; haematology; heart rate; respiratory rate; colic

The FAO estimates that there are 43.5 million donkeys worldwide (FAOSTAT 2013); the majority of these animals are working animals which support people living in the world's poorest communities by transporting goods and materials by pack or cart. As a consequence of their living conditions and handling, donkeys have various health problems (Pritchard et al. 2005). Among the common clinical entities affecting equine species is endotoxaemia, which represents one of the most commonly encountered life-threatening conditions (Moore 2001). Endotoxaemia is caused by bacterial endotoxin (LPS), a component of the cell wall of Gram-negative bacteria, which is responsible for many of the widespread inflammatory processes observed in this condition. Administration of LPS to animals

causes systemic hypotension and multisystem organ failure similar to that which occurs in sepsis (Bone 1991).

Administration of LPS constitutes a well-established experimental approach to appraise the effects of acute and transient immune activation on the physiological and behavioural aspects of endotoxaemia in animals. In the past decade, there has been a virtual explosion of new information about the mechanisms responsible for the deleterious effects of endotoxins (Moore 2001). Endotoxaemia is now considered as a complex biological event involving the Janus-faced interaction of numerous inflammatory mediators; it can be a protective response as well as a serious threat to the animal patient.

Although numerous mediators are involved in the course of endotoxaemia, the multitude of their effects, and their conflicting roles make research difficult. Endotoxaemia has recently been included in a new disease concept called systemic inflammatory response syndrome, because the overall deleterious effect caused by endotoxaemia results from the host's response to endogenous inflammatory substances (King and Gerring 1988). The immune response to sepsis is, however, very diverse, and includes non-specific innate immune reactions to common components of pathogens as well as adaptive immune responses to specific pathogenic antigens. As a consequence, multiple potential targets exist for the modulation of the immune system (Corley et al. 2000). Endogenous host defence reactions can also be modified therapeutically to promote pathogen killing in a manner which avoids or reduces host injury (Parnham 2011).

One of the most challenging problems faced by equine veterinarians is the management of equine endotoxaemia. Despite the substantial advances that have been made in the medical management of these cases, the mortality rates remain high (Freeman and Paradis 1992; McKenzie and Furr 2001). This is due to the systemic inflammatory response which can rapidly progress to endotoxic shock and death despite aggressive medical management.

Although most veterinarians are aware of the clinical signs of endotoxaemia in horses, there remains little known about the susceptibility and response of donkeys to LPS administration and the extent to which donkeys can tolerate a state of endotoxaemia. The purpose of the present study was to compare the clinical and biological responses in donkeys after intravenous (*i.v.*) administration of various doses of *E. coli* O55:B5 endotoxin. We hypothesised that the clinical and inflammatory response of donkeys would be more severe in donkeys receiving a higher dose of endotoxin.

MATERIAL AND METHODS

Animals. The minimum number of animals that allowed the obtaining of reliable data for evaluation and the generation of valid statistics, was used in the experiment. Eighteen apparently healthy, native breed donkeys (*Equus asinus*) with an average age of seven years (5–10 years) and weighing approximately 180 kg body weight (150–200 kg) were used in this study. In addition to clinical examination, a complete blood count and biochemical renal panel (i.e. blood urea nitrogen and serum creatinine; Biodiagnostic Company, Egypt) were performed on all donkeys prior to the study, according to the manufacturers' instructions. All donkeys had values within the normal reference range and were considered healthy. All procedures and practices were carried out in accordance with the guidelines laid down by the National Institutes of Health in the USA regarding the care and use of animals for experimental procedures or with the European Communities Council Directive of 24 November 1986 (86/609/EEC), as well as in accordance with guidelines for the Care and Use of Agricultural Animals in Research and Teaching, 3rd edn. (http://www.fass.org/). All experiments were, in addition, approved by the local Ethical Committee for animal care and welfare.

Experimental induction of endotoxaemia. Animals were randomly allocated into three groups of equal size. The first and second groups received *E. coli* endotoxin (*E. coli* O55:B5, Sigma-Aldrich) at a dose rate of 20 ng/kg (Low dose group), and 5.0 µg/kg (High dose group), respectively, after dilution in 500 ml of 0.9% normal saline, while the third (Control) group received 500 ml of isotonic saline solution 0.9%. The calculated dose of LPS for each animal was administered slowly over the course of 30 min and was given through an *i.v.* catheter.

Data collection. Heart rate, respiratory rate, rectal temperature, mucous membrane colour, appetite, general demeanour, posture, urination and

defecation patterns, caecal and colon motility were evaluated hourly for 6 h after LPS or saline administration using the standard practical methods described by Radostits et al. (2000). Detailed clinical signs of endotoxaemia, such as restlessness, muscle fasciculation, depression and abdominal pain were also recorded. Signs of abdominal pain were further categorised into mild, moderate and severe, as follows. Mild colic: when donkeys exhibited signs of dullness or anxiety, unwillingness to eat, stretching of the abdomen and cutaneous shivering. Moderate colic: when the animal demonstrated pawing, kicking at the belly, frequent getting up and lying down, prolonged periods of sternal recumbency. Severe colic denoted animals that exhibited the abovementioned signs but, in addition, presented with profuse sweating, rolling, self-inflicted trauma and prolonged periods of lateral recumbency.

Sampling and measurements. After aseptic preparation, *i.v.* catheters (16 G) were placed into both jugular veins. The left catheter was used for LPS infusion, while the right catheter was used for blood collection. Blood samples were placed in commercial blood tubes containing Ethylenediaminetetraacetic acid dipotassium and blood tubes without anticoagulant before (T0) and hourly for a subsequent 6 h (T1–T6) after administration of LPS. The whole blood was used for haematological examinations including total (TLC) and differential leucocyte counts, while blood in the plain tubes was left at 4 °C to clot and then centrifuged at $5000 \times g$ for 10 min to obtain serum. Only clear sera were harvested, aliquoted and kept frozen for estimation of the levels of tumour necrosis factor alpha (TNF-α), interleukin (IL)-12 and IL-10. The levels of these cytokines were measured in undiluted serum according to the manufacturer's instructions using commercially available equine ELISA kits (Genorise Scientific, Inc, Paoli, USA; Cat: 106004, 106140 and 106003, respectively). The plates were read at 450 nm and a correction wave length of 550 nm using an automated microplate reader (Bio TEC; USA). Values were expressed in pg/ml. Samples were run in duplicates for all examined cytokines. Values of serum amyloid A were also determined using the horse serum amyloid A (SAA) ELISA test kit supplied by Life Diagnostics, Inc., West Chester, USA (Cat: SAA-14).

Statistical analysis. Data analyses were performed using a commercial statistical software program (SPSS for Windows, version 16.0; SPSS Inc., Chicago, USA). As data followed a Gaussian distribution and were hence normally distributed, the mean and standard deviation for each variable at each time point was calculated. Repeated measures ANOVA was used to assess the changes occurring in the tested parameters with treatment and time. Wilks' lambda test was selected for within-group assessment and to describe the interaction of time × treatment. Where Wilks' lambda test showed a statistically significant difference between groups, one-way analysis of variance with post-hoc Bonferroni multiple-comparison tests were used. For all statistical analyses, differences of $P < 0.05$ were considered significant.

RESULTS

Clinical findings

Before LPS administration, all donkeys were clinically healthy and all variables were within the normal reference ranges. Following LPS exposure, temporal patterns of clinical manifestations were observed to differ markedly between the two treated groups of donkeys. The mean values of heart rate were significantly affected by the time ($P < 0.0001$) and were affected by the dose of LPS being used ($P < 0.0001$). The high dose of LPS yielded a statistically significant increase in heart rate compared with the low dose, especially at T1 ($P < 0.01$), T2 ($P < 0.001$), T4 ($P < 0.001$) and T5 ($P < 0.001$). Heart rate showed a statistically significant variation in both treated groups compared with controls from T1 to T6 ($P < 0.001$; Figure 1). The respiratory rate was also significantly affected by time ($P < 0.0001$) and by the doses of LPS ($P < 0.0001$). The post-hoc tests revealed that the high dose of LPS evoked a statistically significant increase in respiratory rate at T1, which continued to T6 ($P < 0.001$), in comparison with the low dose and control, while the low dose of LPS elicited a statistically significant elevation at T1 ($P < 0.05$) when compared with controls (Figure 2). On the other hand, the means of rectal temperature were significantly affected by the time ($P < 0.0001$) and the dose of LPS ($P < 0.0001$). The low dose of LPS caused a statistically significant increase in rectal temperature at T3 ($P < 0.01$), T4–T6 ($P < 0.001$) compared with controls, while the high dose elicited a statistically significant decrease in rectal temperature at T2 ($P < 0.01$),
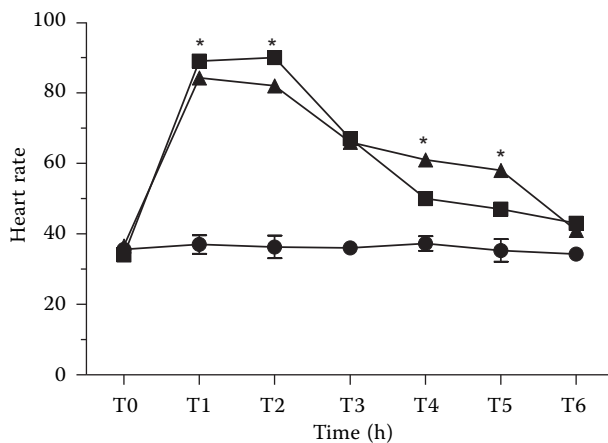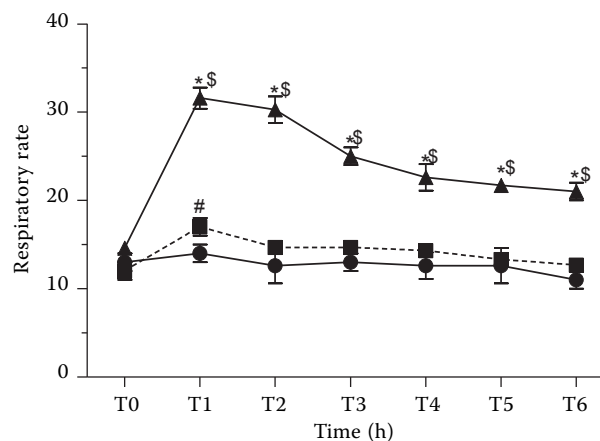
Figure 1. Means ± SD of heart rate in donkeys that received bacterial endotoxin (Low dose versus (■) High dose group (▲) compared with Controls (●))

*Values within the same time point are significantly different at $P < 0.05$ when comparing the High dose with the Low dose group

T3–T6 ($P < 0.001$) compared with the low dose and controls (Figure 3).

Signs of colic were, in general, mild in donkeys of the Low dose group and overt in those of the High dose group. Animals receiving a low dose of LPS showed minimal clinical manifestations through-

Figure 2. Means ± SD of respiratory rate in donkeys that received bacterial endotoxin (Low dose (■) versus High dose group (▲) compared with Controls (●))

*,$Values within the same time point are significantly different at $P < 0.05$ when comparing the High dose group with those of Controls and Low dose group

#Values within the same time point are significantly different at $P < 0.05$ when comparing the High dose with the Low dose group
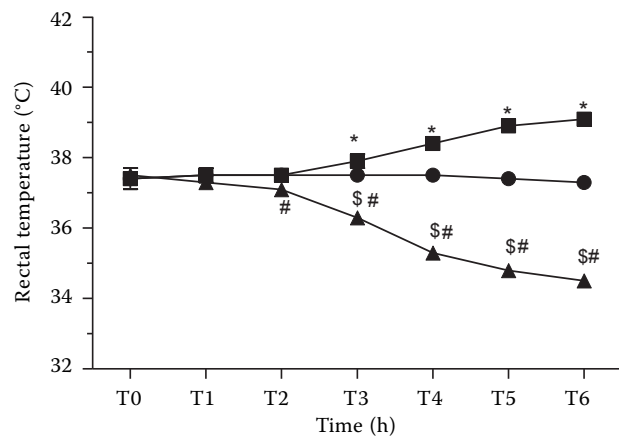

Figure 3. Means ± SD of rectal temperature in donkeys that received bacterial endotoxin (Low dose (■) versus High dose group (▲) compared with Controls (●))

$,#Values within the same time point are significantly different at $P < 0.05$ when comparing the values of High dose group with those of Controls and those of Low dose group

*Values within the same time point are significantly different at $P < 0.05$ when comparing the Low dose group with the Controls

out the experiment, which included the following: muscle fasciculation, congestion of visible mucous membranes, cessation of caecal and colon motility, depression, a brief period of sternal recumbency, and inappetence. These symptoms gradually improved at T3 and became normal from T4 onwards. The observed clinical signs exhibited full abatement after 6 h of LPS administration and all donkeys fully recovered without additional treatments.

Donkey that received the high dose of LPS were severely depressed after approximately 30 min and showed muscle fasciculation with recumbent posture. The donkeys exhibited congested mucosa by the first hour; while profuse salivation with increased capillary refill time and lateral recumbent position prevailed from the second hour and continued in the same manner until the last sampling. After collection of the last blood sample, an *i.v.* isotonic fluid containing Flunixin Meglumine (1.1 mg/kg) was given to these animals. The animals were closely monitored at all times and every attempt was made to prevent their suffering. However, considering that the animals responded slowly to the provided therapy, a humane euthanasia was performed, in which the animals were sedated by xylazine followed by *i.v.* administration of sodium pentobarbital.

Markers of systemic inflammation

Baseline values of all laboratory variables were within the normal reference limits. The baseline values (T0) from the donkeys also served as their own controls. In general, the mean values of TNF-α were significantly changed over time ($P < 0.0001$). The high dose of LPS elicited a statistically significant increase in TNF-α concentrations compared with the low dose, especially at T2 ($P < 0.01$) and T3 ($P < 0.05$). Values of TNF-α also showed a statistically significant variation between the Control and Low group at T3 ($P < 0.001$), T4 ($P < 0.001$), T5 ($P < 0.01$) and T6 ($P < 0.01$), and the High group at the same time points ($P < 0.001$; Figure 4).

The mean values of IL-10 and IL-12 showed a statistically significant variation throughout the time points ($P < 0.0001$ and $P < 0.0011$, respectively). The high dose of LPS elicited a statistically significant increase in IL-10 and IL-12 concentrations compared with the low dose, especially at T2 ($P < 0.001$ and $P < 0.002$, respectively). The levels of these cytokines were significantly increased in the High dose group compared with the Controls at the following time

points: T1 ($P < 0.001$), T2 ($P < 0.001$), T3 ($P < 0.001$) and T4 ($P < 0.001$) for both cytokines (Figures 5 and 6). Values of SAA differed significantly between the time points ($P < 0.0003$). High doses of LPS evoked a statistically significant increase in SAA concentrations in both treated groups compared with Controls in particular at T3–T6 ($P < 0.05$) for High dose groups, and T2, T3 ($P < 0.01$), T4–T6 ($P < 0.0001$) for the Low dose group; however, SAA values showed no significant differences between the High and Low dose groups ($P > 0.05$; Figure 7).

Haematological alterations

The mean values of TLC differed significantly between the time points ($P < 0.0001$). Both doses of LPS elicited a statistically significant decrease in TLC compared with the controls; the lowest observed value was at T2 ($P < 0.001$). Values of TLC also showed a statistically significant difference between the High dose group and the Low dose group at all time points ($P < 0.001$; Figure 8). Neutropenia, lymphopenia and monocytopenia,
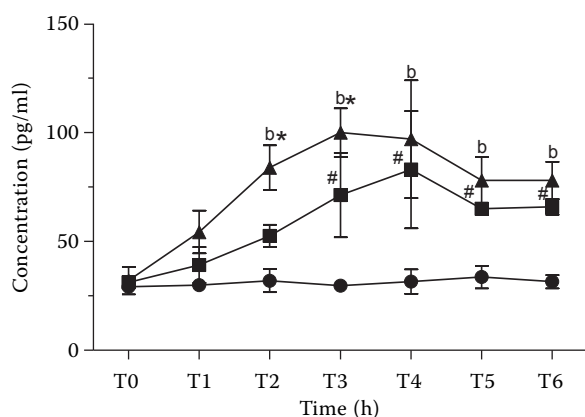

Figure 4. Means ± SD of TNF-α (pg/ml) in donkeys that received bacterial endotoxin (Low dose versus (■) High dose group (▲) compared with Controls (●))

#Values within the same time point are significantly different at $P < 0.05$ when comparing the Low dose group with Controls

bValues within the same time point are significantly different at $P < 0.001$ when comparing Controls with the High dose group

*Values within the same time point are significantly different at $P < 0.05$ when comparing the High dose with the Low dose group
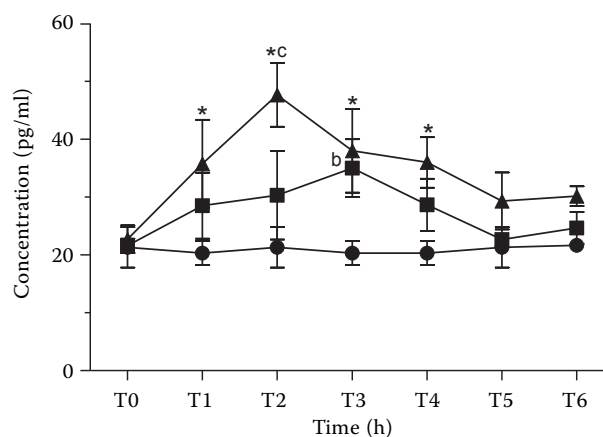

Figure 5. Means ± SD of IL-10 (pg/ml) in donkeys that received bacterial endotoxin (Low dose versus (■) High dose group (▲) compared with Controls (●))

*Values within the same time point are significantly different at $P < 0.0001$ when comparing the High dose group with Controls

bValues within the same time point are significantly different at $P < 0.01$ when comparing the Low dose group with Controls

cValues within the same time point are significantly different at $P < 0.001$ when comparing the High dose with Low dose group
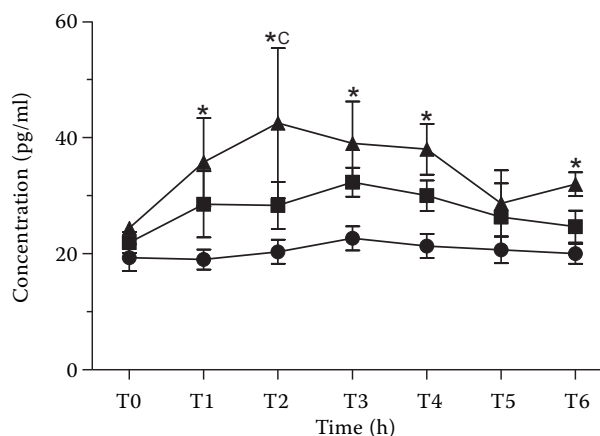
Figure 6. Means ± SD of IL-12 (pg/ml) in donkeys that received bacterial endotoxin (Low dose (■) and High dose group (▲) compared with Controls (●))

*Values within the same time point are significantly different at $P < 0.01$ when comparing the Controls with the High dose group

cValues within the same time point are significantly different at $P < 0.01$ when comparing the High dose with the Low dose group

which occurred with the same pattern and duration as for TLC values (Table 1), were also observed in all animals of both treated groups.
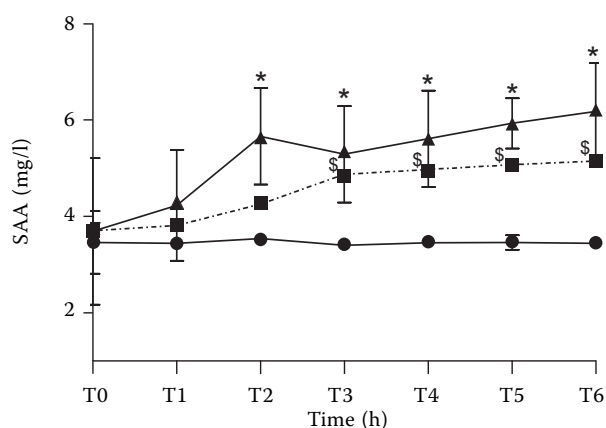

Figure 7. Means ± SD of serum amyloid A (mg/l) in donkeys that received bacterial endotoxin (Low dose (■) and High dose group (▲) compared with Controls (●))

*Values within the same time point are significantly different at $P < 0.00001$ when comparing Controls with High dose group

$Values within the same time point are significantly different at $P < 0.05$ when comparing Controls with Low dose group
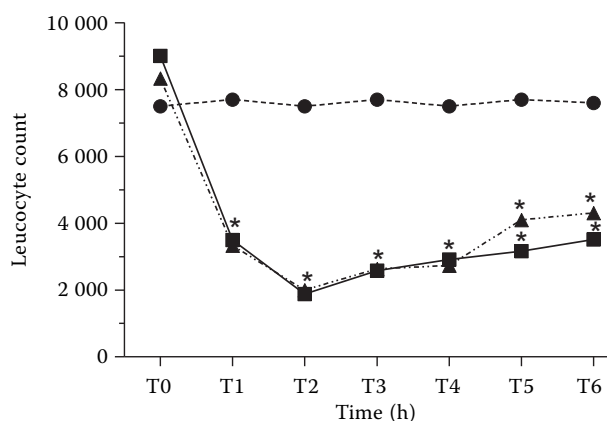

Figure 8. Means ± SD of total leucocyte counts in donkeys that received *E. coli* endotoxin (Low dose (■) and High dose group (▲) compared with Controls (●))

*Values within the same time point are significantly different at $P < 0.05$ when compared with Controls

DISCUSSION

Reference to human medicine has allowed the elucidation of the major pathways involved in LPS signal transduction in laboratory animal species. However, the exact roles of these mechanisms in equine species remain unclear and thus warrant further investigations. In view of the frequent involvement of endotoxin in the pathogenesis of equine diseases, this study was aimed at providing the first insights into the challenge caused by LPS exposure in donkeys as well as the responses of these animals. The clinical data describing the exact course of these responses will allow equine veterinarians to adopt optimal preventive strategies in the future. However, this is not possible without an experimental approach using a minimal number of animals. The subjects in this study showed a characteristic time course of clinical signs in response to the administered LPS. Based on the responses of donkeys in this study, endotoxaemia, by itself, caused donkeys to exhibit signs of abdominal pain which varied from mild (in the Low dose group) to depression and recumbency (in the High dose group). This effect is presumably caused by the synthesis and release of pro-inflammatory substances, which could inhibit the normal gastrointestinal muscular activity, reduce the threshold for painful stimuli, and/or reduce gastrointestinal blood flow. As a consequence of these possible effects of LPS, it may be surmised that endotoxaemia can increase the likelihood that a donkey

Table 1. Means ± SD of differential leucocytes counts in donkeys that received bacterial endotoxin (Low dose (L) versus High dose group (H) compared with Controls (C))

Variables	Group	T0	T1	T2	T3	T4	T5	T6
Neutrophils × 10³	L	5.76 ± 0.30[a]	0.78 ± 0.01[a]	0.30 ± 0.01[b]	0.51 ± 0.030[b]	1.32 ± 0.29[a]	1.82 ± 0.02[a]	2.18 ± 0.047[a]
	H	5.39 ± 0.45[a]	0.22 ± 0.08[b]	0.40 ± 0.09[b]	0.56 ± 0.015[b]	0.63 ± 0.18[b]	0.82 ± 0.02[b]	0.86 ± 0.02[b]
	C	5.27 ± 0.06[a]	5.76 ± 0.08[c]	5.39 ± 0.09[a]	5.29 ± 0.006[a]	5.82 ± 0.002[c]	5.19 ± 0.005[c]	5.70 ± 2.002[c]
Lymphocytes × 10³	L	2.88 ± 0.14[a]	2.68 ± 0.05[b]	1.50 ± 0.06[a]	1.96 ± 0.11[b]	1.16 ± 0.03[a]	1.07 ± 0.012[a]	1.12 ± 0.024[a]
	H	2.93 ± 0.49[a]	2.46 ± 0.08[a]	2.80 ± 0.06[b]	1.68 ± 0.048[b]	1.85 ± 0.051[b]	2.95 ± 0.072[b]	3.18 ± 0.074[b]
	C	2.86 ± 0.01[a]	2.88 ± 0.14[b]	2.93 ± 0.04[c]	2.90 ± 0.20[a]	2.90 ± 0.038[c]	2.92 ± 0.058[b]	2.97 ± 0.112[c]
Monocytes × 10³	L	0.180 ± 0.09[a]	0.07 ± 0.01[a]	0.01 ± 0.01[b]	0.10 ± 0.006[b]	0.24 ± 0.015[b]	0.19 ± 0.002[a]	0.14 ± 0.003[a]
	H	0.244 ± 0.04[a]	0.01 ± 0.01[b]	0.01 ± 0.01[b]	0.12 ± 0.003[b]	0.13 ± 0.028[a]	0.16 ± 0.004[a]	0.10 ± 0.09[a]
	C	0.225 ± 0.03[a]	0.18 ± 0.09[c]	0.24 ± 0.04[a]	0.19 ± 0.004[a]	0.25 ± 0.004[b]	0.19 ± 0.004[a]	0.18 ± 0.009[a]
Eosinophils × 10³	L	0.180 ± 0.09[a]	0.02 ± 0.01[a]	0.75 ± 0.03[c]	0.02 ± 0.014[a]	0.02 ± 0.016[a]	0.06 ± 0.001[a]	0.07 ± 0.015[a]
	H	0.244 ± 0.04[a]	0.11 ± 0.04[b]	0.13 ± 0.03[b]	0.21 ± 0.006[b]	0.15 ± 0.064[b]	0.16 ± 0.004[b]	0.17 ± 0.004[b]
	C	0.225 ± 0.03[a]	0.18 ± 0.09[c]	0.24 ± 0.04[a]	0.18 ± 0.009[c]	0.24 ± 0.036[b]	0.22 ± 0.036[c]	0.20 ± 0.036[b]

[a,b,c]Variables with different superscripts in the different groups within the same time point are significantly different at $P < 0.05$

T1–T6 = time every one hour

with gastrointestinal disease will experience signs of acute abdominal pain, as often occurs in cases of intestinal strangulation or even acute colitis.

Some *in vivo* experiments have been conducted in horses and ponies using physiological amounts of *E. coli* O55:B5 endotoxin at doses of 20–30 ng/kg (Moore et al. 2007; Toth et al. 2008; McGovern et al. 2013; Watts et al. 2014) or 0.1 μg/kg (Rosa et al. 2003), which were given via slow *i.v.* infusion. In those studies, all horses exhibited mild-to-moderate abdominal pain following endotoxin exposure along with marked cellular and inflammatory alterations. In the present study, use of 5 μg/kg was associated with poor outcome. The obtained findings were somewhat in line with those reported by other researchers who used much higher doses of the same *E. coli* serotype (50 μg/kg) (Pantaleon et al. 2007). In that study, horses developed endotoxic shock while being anaesthetised. However, in a study conducted on 16 conscious ponies that received 40 μg/kg of purified *E. coli* O111:B4 endotoxin via the *i.v.* route (Patrick 1981), eleven horses survived after exhibiting signs of endotoxic shock, whereas five ponies became comatose and died. The dramatic collective response of donkeys to the high dose of LPS needs further verification and may be related to the serotype of bacterial endotoxin used or to the sensitivity of donkeys to the administered LPS. The difference in potency among various serotypes of bacterial LPS should be taken into account when experiments are designed to examine the effect of LPS on endothelial cell function.

Our findings clearly demonstrate that donkeys of both treated groups had marked cellular alterations and exhibited up-regulation of pro-inflammatory markers (TNF-α, and IL-12) with marginally increased values of SAA in animals receiving the high dose of LPS. These findings were in part similar to those obtained by other researchers (Tadros and Frank 2012). It was previously stated that the release of TNF-α from macrophages begins at 30 min after the initial exposure, suggesting that this mediator can be an early regulator of the immune response with subsequent release of an array of downstream immunoregulatory mediators. This marked pro-inflammatory state is counteracted by the release of IL-10, as shown in this publication. The synthesis of interleukin-10 appears to be regulated by TNF-α, interleukin-1, transforming growth factor-b, and interferon gamma (Fiorentino et al. 1991). The primary anti-inflammatory effect of interleukin-10 appears to involve deactivation of mononuclear phagocytes and inhibition of proinflammatory cytokine synthesis (Fiorentino et al. 1991). In human patients with sepsis, several studies have shown that TNF-α is present, but at much lower levels than typically found after endotoxin exposure (Waage et al. 1987). These data indicate that TNF-α can be present during sepsis, but it is not clear whether the levels of TNF-α during real sepsis reach a sufficiently high level to induce cellular damage, organ injury, or death. It was also suggested that high systemic levels of TNF-α can be injurious to the host and may be responsible for the organ injury and death in septic patients (Waage et al. 1987).

In conclusion, the results described herein demonstrated that donkeys can respond to even a physiological dose of *E. coli* O55:B5 endotoxin, while a high dose of endotoxin can elicit overt clinical alterations and marked inflammatory responses. Further studies with an extended follow-up time are needed to verify and generalise the obtained findings and to evaluate novel medications to minimise the deleterious consequences of endotoxaemia in equine patients.

Acknowledgement

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