Research in Veterinary Science 94 (2013) 682–686

Contents lists available at SciVerse ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Effects of oral clenbuterol on the clinical and inflammatory response to endotoxaemia in the horse

L.A. Cudmore [a], T. Muurlink [a], T. Whittem [b], S.R. Bailey [b,*]

[a] University of Melbourne Equine Centre, 250 Princes Highway, Werribee, Victoria 3030, Australia
[b] Faculty of Veterinary Science, University of Melbourne, 250 Princes Highway, Werribee, Victoria 3030, Australia

ARTICLE INFO

Article history:
Received 12 March 2012
Accepted 12 January 2013

Keywords:
Equine
Endotoxaemia
Clenbuterol
Inflammation
TNFα
Sepsis

ABSTRACT

Pro-inflammatory cytokines, such as IL-1β and TNFα, play a major role in activating leukocytes and endothelial cells during the systemic inflammatory response to endotoxin in the horse. β_2 agonist drugs, such as clenbuterol, inhibit leukocyte activation. This study aimed to determine the effects of oral clenbuterol on clinical and leukocyte responses, including production of TNFα, in an *in vivo* endotoxin challenge model. In a randomised crossover design, horses received either clenbuterol or a placebo product prior to the administration of low dose endotoxin (30 ng/kg over 30 min). Clinical signs were measured and leukocyte counts and serial blood samples were obtained over 6 h. Pre-treatment with oral clenbuterol (0.8 μg/kg) significantly reduced ($P = 0.046$) the peak rectal temperature and the peak plasma TNFα concentration ($P = 0.026$) following endotoxin challenge. These data suggest that oral clenbuterol at the therapeutic dose has anti-inflammatory effects in horses challenged with a low dose of endotoxin.

1. Introduction

Endotoxaemia remains a major cause of equine morbidity and mortality. Horses are particularly sensitive to the effects of endotoxin, and it plays a key role in a number of serious equine conditions including acute abdominal disease, colitis, laminitis, post operative ileus, metritis, neonatal septicaemia, peritonitis and pleuropneumonia (Shuster et al., 1997).

The pathogenesis of endotoxaemia includes a range of disturbances that ultimately result in a severe systemic inflammatory response syndrome (SIRS), generating hypovolaemia, possible coagulopathies and multiple organ dysfunctions (Werners, 2005). A cytokine mediated response is produced following the activation of blood leukocytes, their adherence to the vascular endothelium and migration into tissues. This process is accompanied by the release of destructive factors such as reactive oxygen species, as well as matrix metalloproteinases and other enzymes. Pro-inflammatory cytokines, such as interleukin-1 (IL-1) and tumour necrosis factor alpha (TNFα) produced from macrophages, monocytes and dendritic cells, play a major role in activating leukocytes and endothelial cells (Werners, 2005).

Non-steroidal anti-inflammatory drugs are the mainstay of anti-inflammatory treatment in horses with endotoxaemia (Shuster et al., 1997). Although these drugs improve some of the clinical signs of inflammation, they are not very effective in preventing activation of leukocytes and the production of pro-inflammatory cytokines (Baskett et al., 1997; Barton et al., 1997; Forbes et al., 2009). Preventing activation of leukocytes may be beneficial in reducing tissue damage, organ dysfunction and improving morbidity and mortality rates.

Activation of β_2-adrenergic receptors on the cell membrane of leukocytes, leads to an increase in the anti-inflammatory intracellular signalling molecule, cyclic adenosine monophosphate (cAMP) (Van Miert, 2002). The β_2-agonist drugs, such as clenbuterol and fenoterol, may inhibit leukocyte activation via this pathway (Yoshimura et al., 1997). Clenbuterol is recognized to have anti-inflammatory effects on equine leukocytes *in vitro* (Chilcoat, 2002) and has also been shown to prevent airway leukocyte activation in horses following bronchial challenge (Laan, 2006). In other species clenbuterol inhibited the release of TNFα and other inflammatory cytokines in models of endotoxaemia (Izeboud et al., 1999). Although used as a bronchodilator in horses, clenbuterol has not previously been evaluated as a treatment for equine endotoxaemia.

The aim of this study was to determine whether oral clenbuterol reduced the production of inflammatory mediators in an experimental endotoxin challenge in horses. We hypothesised that oral clenbuterol would inhibit leukocyte activation and inflammatory cytokine TNFα release in the low dose endotoxin model. Since

Abbreviations: cAMP, cyclic adenosine monophosphate; IL-1β, interleukin-1β; TNFα, tumour necrosis factor alpha; SIRS, systemic inflammatory response syndrome.

* Corresponding author. Tel.: +61 (0) 38344 6315; fax: +61 (0) 38344 7374.
E-mail address: bais@unimelb.edu.au (S.R. Bailey).

0034-5288/$ - see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.rvsc.2013.01.003

TNFα plays a key role in equine endotoxaemia and is the cytokine whose plasma concentration correlates best with the clinical signs of this condition (Morris et al., 1990), this was the primary outcome measured in the study. However, rectal temperature, leukocyte count and heart rate were also used as important clinical indicators.

2. Materials and methods

2.1. Horses

Eight adult healthy Standardbred horses (6 geldings and 2 mares) were used for the study. The horses ranged in age from 5 to 12 years, and body weight from 389 to 520 kg. All horses underwent a complete veterinary examination prior to the commencement of the study including clinical biochemistry and haematology and no abnormalities were detected.

2.2. Study design

The study was conducted as a randomised, blinded crossover design, with each horse acting as its own control. The horses were pair matched by body weight: one horse was selected at random (by simple lottery) to receive the test product in the first experimental period, and the other received the placebo. The treatments were then crossed over for the second experimental period, so that each horse received both the treatment and placebo product. There was a six week washout period between challenges.

The study was approved by the Animal Ethics Committee of the University of Melbourne.

2.3. Test compound and placebo

The test compound used in the study was oral clenbuterol hydrochloride (Claire Gel®, Virbac Animal Health Australia, Sydney, Australia). The oral clenbuterol was given at the labelled dose of 2 mL/100 kg body weight (0.8 μg/kg of clenbuterol hydrochloride). It was administered 2 h prior to the endotoxin infusion, so that maximum plasma concentrations were achieved by that time (Kallings, 1991). The placebo compound was the Claire Gel® vehicle (containing no active ingredient), manufactured to the same specifications as the test compound, and was administered orally at the same dose as the test compound (2 mL/100 kg). The drug was administered by a different clinician so that the investigators remained blinded during the study.

2.4. Low-dose endotoxin challenge

A low-dose endotoxin challenge was conducted as previously described (Menzies-Gow et al., 2004). Jugular venous catheters were placed, under local anaesthesia (2 mL of 2% lignocaine; Ilium Lignocaine 20, Troy Laboratories Pty. Ltd., Smithfield, Australia). Sterile, filtered endotoxin (*Escherichia Coli* LPS 055:B5; 1.2 million endotoxin units/mg) was infused at a dose of 1 ng/kg/min, over 30 min (total dose 30 ng/kg; dissolved in 500 mL sterile saline), controlled by infusion pump (Baxter International Colleague CX, Sydney, Australia). The LPS used (Sigma–Aldrich Pty. Ltd., Castle Hill, Australia) was purified by phenol extraction and was low in protein and DNA contamination.

2.5. Clinical outcome measures

Clinical outcomes including rectal temperature, heart rate, respiratory rate and demeanour were recorded immediately prior to pre-treatment and before LPS infusion, then every 15 min for 2 h, then every 30 min for the following 4 h. Blood was collected via the intravenous catheter at the same time points into tubes containing anticoagulant ethylenediaminetetraacetic acid (EDTA) and lithium heparin. Blood pressure was measured non-invasively using an oscillometric (tail cuff) technique, at 0 min, then at the 2 h and 4 h time points (BpTRU Model BPM-300; VSM Medtech Ltd., Vancouver, Canada).

Horses were held in stocks (two horses side by side in separate stocks) in a covered building for the first 4 h, after which they were placed into a small concrete yard for a further 2 h and allowed hay and water *ad libitum*. 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 checks were conducted in the paddock after 24 h.

2.6. Blood sample analysis

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

Bioavailable (unbound) plasma TNFα was measured using a cell survival bioassay (L929 murine fibroblast cells). Samples were assayed in duplicate, diluted 1:1 in serum-free cell culture medium. This assay has been validated for use in horses (Menzies-Gow et al., 2004). 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 TNFα, diluted in cell culture medium and an equivalent volume of blank equine plasma containing no detectable TNFα, was used to produce the standard curve.

2.7. Statistics and data analysis

The data were analysed in a pair-wise manner. The effect of oral clenbuterol pre-treatment was compared with placebo pre-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 heart rate, rectal temperature and plasma TNFα were compared between oral clenbuterol and placebo treatments using a paired Student's *t*-test. The low point of the leukocyte response (nadir) was also analysed in this manner. Data are presented as mean ± SEM. For all cases, significance was defined as $P \leqslant 0.05$. GraphPad Prism version 4.0 for Windows was used to perform the analysis (GraphPad software, California, USA).

3. Results

3.1. Clinical data

After LPS challenge to the placebo group, heart rate increased from 37.5 ± 1.2 beats per minute (bpm) at time 0 to 57.1 ± 3.4 bpm at 75 min (Fig. 1). Similarly, the heart rate in the clenbuterol pre-treated group increased from 38.6 ± 1.1 bpm to 51.7 ± 4.5 at 105 min after LPS challenge. No significant differences were identified between the heart rates in the two groups at any time point ($P = 0.20$; two-way repeated measures analysis of variance) (Fig. 1). Pre-treatment of horses with oral clenbuterol had no significant effect on the respiratory rate compared to those horses administered the placebo product ($P = 0.24$).

Following administration of LPS to placebo treated horses the rectal temperature increased from 37.4 ± 0.2 °C at time 0 to a peak