# Anti-inflammatory effects of clenbuterol on equine leukocytes stimulated *ex vivo* with bacterial toxins.

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## Abstract

**Objectives**: To determine whether the β-adrenergic agonist, clenbuterol hydrochloride, was able to inhibit leukocyte production of Interleukin-1β (IL-1β) and Tumor Necrosis Factor α (TNFα) after *ex vivo* stimulation of equine blood leukocytes with bacterial toxins from Gram-positive as well as Gram-negative bacteria.

**Hypotheses***:* Clenbuterol hydrochloride would inhibit the production of TNFα following the ex vivo stimulation of whole blood with each of the three bacterial toxins; peptidoglycan (PG), lipoteichoic acid (LTA), lipopolysaccharide (LPS); and would also reduce the production of IL-1β from LPS-stimulated whole blood.

**Materials and methods***:* Whole blood samples from seven adult healthy Standardbred horses were collected into citrate anti-coagulant blood tubes. The blood was diluted 1:1 with Dulbecco's modified Eagle's medium. LPS, PG and LTA were added to all samples to give a final concentration of 10 μg/ml. Varying concentrations of clenbuterol hydrochloride, ranging from $10^{-9}$ to $10^{-5}$ M were added to the samples. All procedures were performed in a bio-cabinet. Bioavailable (unbound) plasma TNFα was measured using a cell survival bioassay (L929 murine fibroblast cells). Plasma IL-1β concentrations were measured using an equine specific enzyme linked radioimmunoassay.

**Results***:* A significant concentration dependent effect of clenbuterol was identified on plasma TNFα production with all three toxins. A marked reduction in IL-1β production by equine leukocytes is evident following *ex vivo* whole blood stimulation with LPS and treatment with clenbuterol hydrochloride.

**Conclusions***:* Clenbuterol has significant, concentration dependent anti-inflammatory effects following *ex vivo* stimulation of whole blood with Gram-negative as well as Gram-positive bacterial toxins.

## Abbreviations

cyclic adenosine monophosphate (cAMP), enzyme linked radioimmunoassay (ELISA), interleukin-1β (IL-1β), lipopolysaccharide (LPS), lipoteichoic acid (LTA), peptidoglycan (PG), tumour necrosis factor alpha (TNFα), toll-like receptor 4 (TLR-4), molar (M).

## Introduction

Systemic inflammatory response syndrome (SIRS) is a common and detrimental clinical condition affecting horses.[1] SIRS is an inflammatory response that occurs in response to the synthesis of inflammatory mediators stimulated by infection, ischaemia reperfusion injury or trauma. Horses are particularly sensitive to the effects of bacterial toxins. Lipopolysaccharide (LPS) is considered the most important bacterial endotoxin causing systemic inflammation in the horse, and is liberated from the outer cell wall membrane of Gram-negative bacteria. Horses are exquisitely sensitive to even very low doses of LPS. However, it is still important to consider the effects of other bacterial toxins in equine disease.[1] Peptidoglycans (PG) from Gram-positive bacteria and lipoteichoic acid (LTA) liberated from both Gram-positive and Gram-negative bacteria have been identified in human sepsis[2] and therefore should also be considered in the development of systemic inflammation and sepsis in the horse.

Therapeutic agents targeting leukocyte activation may have actions complimentary to those of non-steroidal anti-inflammatory drugs in the treatment of systemic inflammation in horses. The β2-adrenoreceptor agonist drug clenbuterol hydrochloride may be effective in reducing equine leukocyte activation and cytokine production, via increased production of the anti-inflammatory intracellular signaling molecule cyclic adenosine monophosphate (cAMP).[3] Cyclic adenosine monophosphate inhibits the activation and subsequent nuclear translocation of the inflammatory transcription factor, Nuclear Factor κB.[4] Increases in intracellular cAMP also stimulate other anti-inflammatory mechanisms through the activation of Mitogen-associated Protein Kinase, Protein Kinase A and the Ras-related Protein 1 pathways, resulting in a reduction in the expression of pro-inflammatory cytokines and cellular activation, proliferation, differentiation and apoptosis.[5-7]

Previous studies have recognised a significant anti-inflammatory effect of clenbuterol against Gram-negative bacteria in models of endotoxaemia.[8,9] Treatment with clenbuterol hydrochloride reduced pro-inflammatory cytokine production; tumor necrosis factor α (TNFα), IL-6;[8] and neutrophil margination[9] following LPS stimulation in human and equine *in vitro* studies. In an equine low-dose endotoxin challenge model, clenbuterol has been shown to reduce the pyrexic response as well as blunting the spike in TNFα release.[10] For the other bacterial cell wall components that may contribute to sepsis, currently there is little scientific evidence supporting the anti-inflammatory potential of novel agents against these molecules, in particular PGs and LTA. This information will be useful as it is likely that these bacterial toxins also play a role in equine sepsis.

The aim of the study was to determine whether clenbuterol hydrochloride was able to inhibit leukocyte production of IL-1β and TNFα after *ex vivo* stimulation of equine blood leukocytes with bacterial toxins from Gram-positive as well as Gram-negative bacteria. It was hypothesized that clenbuterol hydrochloride would inhibit the production of TNFα following the *ex vivo* stimulation of whole blood with each of the three bacterial toxins, and would also reduce the production of IL-1β from LPS-stimulated whole blood.

## Materials and methods

### *Horses*

Seven adult healthy Standardbred horses were used for the study. Horses ranged in age from 5 to 12 years and weighed 389 to 520 kg. These horses were involved in an unrelated diet study. All horses underwent a complete veterinary examination prior to inclusion in the study; no abnormalities were detected.

### *Study design*

Whole blood samples were collected from each horse into citrate anti-coagulant blood tubes. The blood was diluted 1:1 with Dulbecco's modified Eagle's medium and 0.5 mL aliquots were separated into Eppendorf tubes. LPS (Sigma Aldrich Pty Ltd, Castle Hill, Australia, Coli LPS 055:B5; 1.2 million endotoxin units/mg), PG and LTA were added individually to samples to give a final concentration of 10 µg/mL. Varying concentrations of clenbuterol hydrochloride[a], ranging from $10^{-9}$ to $10^{-5}$ molar (M) were added to the samples. Samples with no clenbuterol added were used as controls. The samples were incubated for 24 hours at 37ºC (Ratek orbital shaking incubator, Boronia, Australia), then centrifuged (Heraeus Pico17 centrifuge; Thermo Scientific Ltd; 2000 g for 5 minutes) and the plasma removed. All procedures were performed in a bio-cabinet. The samples were then stored at -80°C until TNFα and IL-1β assays could be performed. The production of IL-1β was assessed in samples stimulated with LPS only.

### *Inflammatory mediator assays*

Bioavailable (unbound) plasma TNFα was measured using a cell survival bioassay (L929 murine fibroblast cells) that has been validated for use in horses.[11] Samples were assayed in duplicate, diluted 1:1 in serum-free cell culture medium. TNFα induces cell death in this cell line, which is assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (TACS MTT cell proliferation assay; R&D Systems Inc., Minneapolis MN). The yellow tetrazole dye, 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. The blank plasma was made by incubation of normal plasma with activated charcoal for six hours.

Plasma IL-1β concentrations were measured using an equine-specific enzyme linked immunosorbent assay (ELISA; Horse IL-1β ELISA kit, Bethyl Laboratories, Montgomery, TX, USA). A standard curve was constructed using a known concentration of equine recombinant IL-1β. The ELISA kits were stored and used according to the manufacturer's instructions. The optical density (OD) values of samples were determined at 450 nm using an ELISA plate reader (BioTek Synergy H1) and concentrations determined using GEN5 Software (version 2.00.17). The IL-1β ELISA limit of detection was calculated to be 2.4 ng/mL (2 standard deviations greater than background) and the intra-assay coefficient of variation was determined to be 8.9%.

### *Statistical and data analysis*

Plasma clenbuterol concentration was plotted against percentage inhibition of TNFα and IL-1β production using GraphPad Prism (Version 4.0; GraphPad software). Most curves were fitted using a single site sigmoidal concentration-response curve with variable slope using the following equation:

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Y=Baseline + (Emax-Baseline)/(1+10^((LogIC50-X)*HillSlope))
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where X is the logarithm of concentration and Y is the response. The IC50 values (the concentration of clenbuterol causing 50% inhibition of TNFα production) were calculated and are represented as $pIC_{50}$ (-log of $IC_{50}$ value; log M) representing the concentration of clenbuterol hydrochloride causing the maximal reduction in TNFα production. The 95% confidence intervals are also reported and the coefficient of determination ($r^2$) calculated for each curve. The maximum response was expressed as the percentage reduction in cytokine production from the control samples. Values for $pIC_{50}$ and maximum response obtained for each bacterial toxin were compared using one-way ANOVA with Fisher's post hoc test, for which $P<0.05$ was accepted as a statistically significant difference.
Where the inhibitory response to clenbuterol was biphasic, a two-site model was used:

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Section1=Span*Frac/(1+10^((LogIC50_1-X)*Hill slope 1))
Section2=Span* (1-Frac)/(1+10^((LogIC50_2-X)*Hill slope 2))
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Where Y = Bottom + Section1 + Section2, and Span = Top - Bottom. Frac is the proportion of maximal response due to the more potent phase.

## Results

### *Inhibition of TNFα*

Treatment with clenbuterol hydrochloride was associated with a marked reduction in TNFα production by equine leukocytes following *ex vivo* whole blood stimulation with LPS, LTA and PG. A significant concentration dependent effect of clenbuterol was identified on plasma TNFα production with all three bacterial toxins (Figure 1). The concentration-response relationship was best characterised by fitting the data to a single-site pharmacological dose-response curve. The coefficients of determination ($r^2$) values for the curves were 0.68, 0.68, and 0.75 for LPS, LTA and PG, respectively.

The $IC_{50}$, $pIC_{50}$ and maximum response values were calculated from the fitted curves and the later are shown in Table 1. These results suggest that clenbuterol hydrochloride had a more potent anti-inflammatory effect on LPS-stimulated cells. However, at very high clenbuterol hydrochloride concentrations approximately 100% inhibition of TNFα production occurred for all individual bacterial toxins.

### *Inhibition of IL-1β*

Treatment with clenbuterol hydrochloride resulted in marked reduction in IL-1β production by equine leukocytes following *ex vivo* whole blood stimulation with LPS (Figure 2). At clenbuterol hydrochloride concentration of $10^{-5}$ M there was complete inhibition of IL-1β.

A concentration dependent decrease in IL-1β was identified; however the data did not conform to a standard concentration-response curve. It could however be fitted to a biphasic concentration-response curve, with an $r^2$ of 0.52. The first $IC_{50}$ value was $10^{-8.8}$ M ($pIC_{50}$ 8.8), with a maximum response of 39.7% inhibition.

## Discussion

Clenbuterol hydrochloride was most potent at inhibiting TNFα production in response to LPS, with the effects of PG and LTA being inhibited only at much higher clenbuterol hydrochloride concentrations (>$10^{-6}$ M). The reason for these differences is unknown but may be associated with the different mechanisms by which LTA and PG initiate inflammation.

PGs and LTA are components of Gram-positive bacterial cell walls and are released into the circulation initiating sepsis.[2,12,13] Once released from the bacterial cell wall these molecules (pathogen associated molecular patterns or PAMPs) are recognised by Toll-like receptors (TLRs) on cells of the innate immune system.[14] The cascade of events leading to leukocyte activation following Gram-negative and Gram-positive bacterial toxin exposure is similar, although differences do occur in the macrophage phagosome recognition by TLRs. Evidence suggests LPS macrophage phagosomes bind to TLR-4[15] although Gram-positive bacterial toxin recognition is demonstrated to be associated with TLR-2 activation.[2] However, there may be some functional cooperation between different TLR receptors.[2,14] Following the binding of these ligands, the intracellular signaling pathway for leukocyte activation leads to the production of the inflammatory cytokines IL-1β, IL-6 and TNFα.[16]

Lipoteichoic acid and PG are also able to initiate leukocyte activation and inflammatory cytokine production via the activation of nucleotide oligomerization domain-like receptors.[17] This mechanism of action is complementary to the role of TLR activation and causes up-regulation of nitric oxide synthase gene expression resulting in Nuclear Factor κB activation and therefore production of pro-inflammatory mediators. These additional pro-inflammatory pathways and the recognised differences in TLR activation may explain the observed results in TNFα inhibition recognised between LTA, PG and LPS in this study.

The anti-inflammatory effects of clenbuterol hydrochloride are achieved by an increase in the intracellular signaling molecule, cAMP.[18] This increase in cAMP is the result of activation of the β2-adrenoreceptors on the surface of equine leukocytes and anti-inflammatory effects have been

| Inflammatory stimulus (type of bacterial toxin) | n | $pIC_{50}$ value (log M) (mean ± 95% Confidence intervals) | Maximum response (mean ± SEM) |
|---|---|---|---|
| LPS | 7 | 7.3 (8.2 to 6.4) | 100 ± 15.19% |
| LTA | 7 | 6.3* (6.6 to 6.1) | 91.17 ± 8.19% |
| PG | 7 | 6.1* (6.5 to 5.9) | 100 ± 11.31% |

**Table 1.** Curve fitting values for the concentration-dependent effects of clenbuterol on TNFα production by equine leukocytes in the whole blood assay. * indicates significant difference compared with LPS, one-way ANOVA with Fisher's post hoc test (P<0.05).

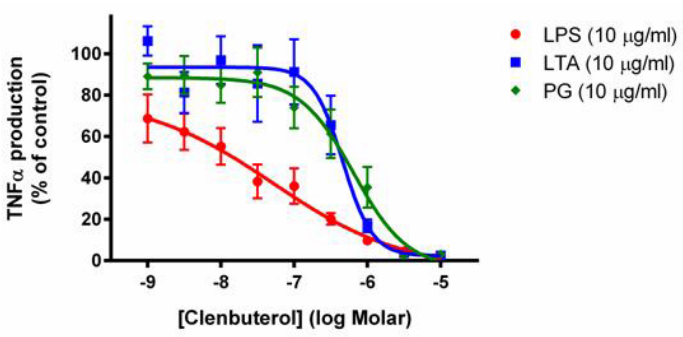


**Figure 1.** Effect of clenbuterol hydrochloride on TNFα production from equine blood leukocytes stimulated with the bacterial toxins: LPS, LTA or PG. Values are expressed as the percentage of the cytokine production caused by the toxin in the absence of clenbuterol. Each point represents the mean ± SEM. LPS $r^2$ = 0.68, LTA r2 = 0.68, PG $r^2$ = 0.75.

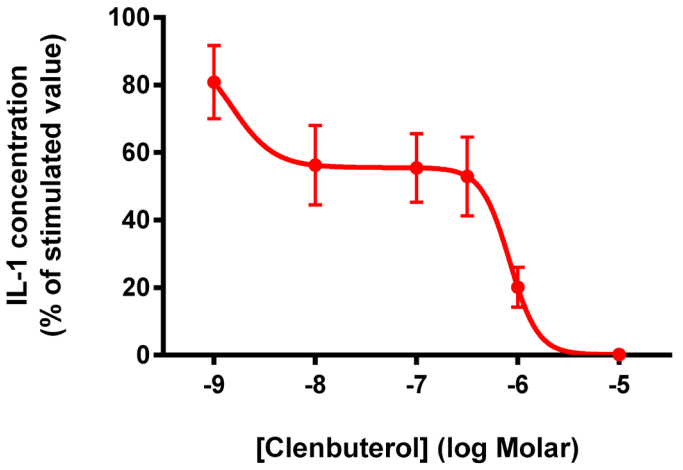


**Figure 2.** Effect of clenbuterol hydrochloride on IL1β production from equine blood leukocytes stimulated with bacterial LPS. Values are expressed as the percentage of the cytokine production caused by the toxin in the absence of clenbuterol. Each point represents the mean ± SEM. The curve was fitted to a two site model.

recognised in a number of *in vitro* and *in vivo* studies.[8,19,20] In this study differences were identified in TNFα percentage inhibition between PG, LTA and LPS bacterial toxins treated with clenbuterol hydrochloride. These differences may result due to diversity in the mechanisms by which clenbuterol hydrochloride stimulates β2-adrenoreceptor activation on equine leukocytes in response to Gram-negative and Gram-positive bacterial toxins. As described above differences in Gram-positive and Gram-negative intracellular inflammatory signaling pathways may explain the observed differences,[15] although it is also possible that other non-specific anti-inflammatory effects are occurring. An increase in intracellular cAMP has been shown to result in an up regulation of cyclo-oxygenase 2 and PGE-2, and it is possible these anti-inflammatory effects are evident in the results of this study.[21] In this study it may have been beneficial to assess and compare the anti-inflammatory potential of the phosphodiesterase inhibitors in response to stimulation with Gram-positive and Gram-negative bacterial toxins. Like clenbuterol hydrochloride, the phosphodiesterase inhibitors exhibit anti-inflammatory effects by increasing intracellular cAMP concentrations.[22] Comparing the anti-inflammatory activity of these two treatments may have provided further evidence that the activity observed in this study was due solely to an increase in cAMP concentrations.

The anti-inflammatory action of clenbuterol hydrochloride is evidenced by the marked reduction in IL-1β production following stimulation of whole blood leukocytes with LPS. Clenbuterol hydrochloride caused a biphasic reduction in IL-1β production following *ex vivo* stimulation with LPS. The reason and/or cause of these results are unknown. The initial anti-inflammatory effects may be associated with β2-adrenergic receptor stimulation and the secondary anti-inflammatory response observed may be associated with differences in the production and structure of IL-1β. Prostaglandins influence the regulation of IL-1β therefore acting as an intrinsic control mechanism in the cytokine synthesis;[23] in this case prostaglandin release may explain the results observed.[21] IL-1β production following stimulation with bacterial toxins is extremely variable, and due to assay kit availability for the present study it was only measured following LPS stimulation. Previously, DeClue et al[24] identified that LTA and PG did stimulate IL-1β production from equine whole blood, but the variability in response to LPS meant that a consistent dose-dependent response to this mediator could not be determined.

It is acknowledged that the concentrations of toxins used in this *in vitro* study, as with other similar studies, are considerably greater than the concentrations that are likely to be present in the plasma of horses with sepsis; however high concentrations were necessary in order to achieve a large enough stimulus to be able to clearly detect the anti-inflammatory effects of clenbuterol, especially at low concentrations. This is partly due to the sensitivity of the analysis methods (using cytokine protein production as opposed to more sensitive methods such as PCR for gene expression changes) utilized in this study. Pilot studies previously indicated that 10 ug/mL of PG and LTA was necessary to induce a robust response in whole blood *in vitro* (although much lower concentrations would be likely to have severe effects *in vivo*). For LPS, 1 ug/mL and 10 ug/mL are commonly used in similar equine whole blood experiments;[25,26] there is little difference in cytokine response between 1 and 10 ug/mL LPS, and we used 10 ug/mL in these studies to be consistent with the other two toxins.

The anti-inflammatory potential of the β2-adrenergic agonist drugs has been demonstrated in *in vitro* models of sepsis attributable to Gram-negative bacteria, with an increase in the anti-inflammatory cytokine IL-10 being identified.[20,27] Assessment of IL-10 in this study would have been beneficial in order to confirm and provide further evidence for the anti-inflammatory potential of clenbuterol hydrochloride. However, a validated assay for equine IL-10 is not currently available and evaluation by RT-PCR for gene expression was outside the scope of the current study.

A further question regarding the extrapolation of the results of this study to the *in vivo* situation is how the typical plasma concentrations of this drug, administered at the licensed dose, might relate to the concentration response curves demonstrated in the present study. We have previously reported the plasma concentrations of clenbuterol in these same horses following a standard intravenous dose of clenbuterol (0.8 µg/kg),[28] and it is in the range of between 0.3–0.4 x $10^{-8}$ M. The plasma concentrations of clenbuterol hydrochloride achieved in this study was sufficient to produce a significant reduction in TNFα release from blood samples stimulated with LPS *ex vivo*. Furthermore, a similar concentration was achieved following oral dosing with clenbuterol, and this was sufficient to inhibit the maximum TNFα production observed in an *in vivo* endotoxin challenge.[9] Given the fact that the concentration of clenbuterol required to inhibit the effects of PG and LTA were at least 10-fold greater than for LPS, the plasma concentrations of clenbuterol hydrochloride following the current licensed dose of this drug would be likely to have much less of an anti-inflammatory effect against these other two toxins *in vivo*. However, if these toxins do play a clinically significant role in sepsis, there may well be a synergistic interaction between PG, LTA and LPS; in which case, small effects on multiple arms of the inflammatory pathway may contribute to a greater beneficial effect overall.

In summary, clenbuterol hydrochloride causes a significant concentration dependent reduction in pro-inflammatory cytokine (TNFα) release following stimulation of blood leukocytes with three different bacterial toxins. A marked reduction in IL-1β pro-inflammatory cytokine production was caused by clenbuterol hydrochloride following stimulation with LPS. The results of this study and another study[10] provide evidence in support of clenbuterol hydrochloride being further investigated as a novel anti-inflammatory agent in the treatment of equine systemic inflammation.

## Trade names

[a]Ventipulmin® Boehringer Ingelheim

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