- CHAPTER IX -

INVESTIGATION OF THE ANTI-INFLAMMATORY EFFECTS OF KETAMINE IN A TISSUE CHAMBER MODEL IN SHETLAND PONIES

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SUMMARY

Ketamine is frequently used in equine anaesthesia. Besides its anaesthetic and analgesic effects in vivo, ketamine has previously been reported to possess cytokine-modulating properties in an equine macrophage cell line.[1] These results suggest that ketamine might also have appreciable anti-inflammatory effects in vivo.

In the present study, the influence of parenterally administered ketamine on a lipopolysaccharide (LPS)-induced, local inflammatory response in tissue chambers implanted in Shetland ponies was investigated. Two dose regimens were tested: a single bolus injection of ketamine (SI group) and a ketamine CRI protocol, comprising a loading dose followed by a continuous rate infusion of ketamine for 6 h (CRI group). For comparison, a saline infusion protocol was used (C group). White blood cell count, tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and prostaglandin E_2 (PGE_2) concentrations in tissue chamber fluid were measured to assess the influence of ketamine on the local inflammatory response. Simultaneously, plasma concentrations of ketamine and its metabolites were determined.

Administration of ketamine, either as bolus or as CRI, did not significantly affect any of the investigated parameters. This lack of effect is thought to be attributable to the fact that only a small fraction (0.5 - 0.6 μM) of the circulating ketamine entered the tissue chambers. These fractions might be too low to generate anti-inflammatory effects since the minimal effective dose in in vitro experiments was 1.8 μM.[1]

However, in equine patients, suffering from systemic infections or reperfusion injury, ketamine could still be effective as the measured serum concentrations exceed the minimal effective concentration determined in the e-CAS model.

1. INTRODUCTION

Ketamine, a dissociative anaesthetic agent, is commonly used in a variety of clinical settings in equine anaesthesia. Besides the induction of anaesthesia,[2] ketamine can be used as an adjunct to inhalant anaesthesia,[3-5] in total intravenous anaesthesia (TIVA) protocols[6-8] and for systemic and local analgesia.[9,10] Recent studies focus on the antinociceptive effects of subanaesthetic ketamine infusions administered to conscious horses.[11] As ketamine preserves respiratory and cardiovascular functions, its use is recommended for anaesthesia of cardiovascular depressed patients.[12]

More recently, accumulated evidence suggests that beside its anaesthetic and analgesic effects, ketamine suppresses the production of inflammatory mediators like tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and prostaglandin E_2 (PGE_2), as demonstrated in in vitro and in vivo studies in rodents[13-16] and humans.[17-19] Previously we demonstrated that in an equine macrophage cell line (e-CAS cells) ketamine also inhibited the lipopolysaccharide (LPS)-induced TNF-α and IL-6 production in a dose-dependent manner, even at subanaesthetic concentrations.[1] These results suggest that ketamine has also appreciable anti-inflammatory effects in equine patients.

The purpose of the present study was to investigate the influence of parenterally administered ketamine on an LPS-induced local inflammatory response in tissue chambers implanted in Shetland ponies. This model is commonly used to assess the effect of anti-inflammatory agents since the localised inflammatory response does not impair the animal's health and well-being.[20]

Both the effects of a single intravenous ketamine injection and a long-term ketamine infusion protocol, comprising a loading dose followed by a ketamine continuous rate infusion (CRI) for 6 h, were investigated. Besides studying the influence of ketamine per se, the results of both dose regimens were compared to differentiate between both short-term as well as long-term ketamine treatments. White blood cell count, TNF-α, IL-6 and PGE_2 concentrations were determined in tissue chamber fluid to assess the influence of ketamine on the local inflammatory response. Concentrations of ketamine and its metabolites in both tissue chamber fluid and plasma samples were determined to study the kinetics and rate of drug penetration into tissue chambers.

The ponies were assigned to three treatment groups in a Latin square cross-over design. This implies that all animals were treated three times with LPS. Since

repeated LPS administration might evoke endotoxin tolerance, individual LPS responses were subjected to statistical analysis.

2. MATERIALS AND METHODS

The experiments described in this study were approved by the Ethical Committee on Animal Experimentation of the Utrecht University and conducted in compliance with the Dutch Act on Animal Experiments.

2.1. PONIES

Eight Shetland ponies (all geldings, age 7 ± 2.4 years, bodyweight 151 ± 25.5 kg) were used. All ponies were considered to be clinically healthy on the basis of routine physical examination and hematologic screening. Ponies were vaccinated and treated with anthelmintics prior to the experiments. During each experiment, ponies were stabled in individual boxes and fed a diet of haylage or fresh grass.

2.2. TISSUE CHAMBER IMPLANTATION

The implantation and use of tissue chambers in Shetland ponies has been previously described by Ensink et al.[21] Briefly, under general anesthesia, custom made round tissue chambers (inner diameter 4.4 cm, depth 1.8 cm, total volume 27 mL) were aseptically implanted in the subcutaneous tissue of the mid-neck region on both sides of each pony. Peri-operative antimicrobial treatment included 6.6 mg/kg gentamycin (Gentamycin®, Eurovet Animal Health BV, Bladel, The Netherlands) and 20.000 IU/kg sodium penicillin (Benzylpenicilline Natrium®, Eurovet Animal Health BV, Bladel, The Netherlands) intravenously prior to the start of surgery, and 20.000 IU/kg procain penicillin (Depocilline®, Intervet, Boxmeer, The Netherlands) intramuscularly at the end of the surgical procedure. Peri-operative analgesia consisted of 0.1 mg/kg methadone IV (Methadon HCl® 10 mg/mL, Eurovet Animal Health BV, Bladel, The Netherlands) and 1.1 mg/kg flunixine meglumine IV (Bedozane®, Eurovet Animal Health BV, Bladel, The Netherlands) at the time of pre-anaesthesic medication. To ensure the proper embedding of the tissue chambers, ponies were allowed to recover from implantation for at least 6 weeks prior to the start of the experiments.

2.3. EXPERIMENTAL DESIGN

To test all dose regimens of ketamine in each pony, the ponies were assigned to three treatment groups in a Latin square cross-over design with an 8-week recovery period between each individual experiment.

2.4. LPS STIMULATION AND TREATMENT PROTOCOLS

Two hours before experimentation, an 8 cm 14-gauge polytetrafluoroethylene catheter (Intraflon 2®, Vygon Nederland BV, Veenendaal, The Netherlands) was inserted under aseptic conditions into the right and left jugular veins to facilitate simultaneous treatment and blood sampling, respectively. Thereafter, ponies were allowed to adapt to the experimental environment for at least 1 hour.

At t = 0 h, an inflammatory response was provoked by injecting 3 mL LPS (20 μg/mL; Escherichia coli, O111:B4) in both tissue chambers. Immediately after inoculation, intravenous treatment with ketamine was started. The treatment consisted of a bolus injection of 2.2 mg/kg ketamine (Narketan® 100 mg/mL, Vétoquinol BV, 's-Hertogenbosch, The Netherlands) IV, directly followed by a ketamine continuous rate infusion (CRI) of 1.5 mg/kg/h for 6 h (CRI-group). Alternatively, only a single bolus injection of 2.2 mg/kg ketamine IV directly followed by a 0.9% NaCl CRI for 6 h (SI-group) was given. In control experiments, a bolus injection of 0.9% NaCl IV was directly followed by a 0.9% NaCl CRI for 6 h (C-group). When using 0.9% NaCl, equal volumes compared to ketamine volumes used, were administered.

Between t=-10 min and t=20 min, a sling attached to an overhead hoist was used to support ponies during the induction phase. To standardise experimental conditions, the sling was used for the control experiments as well. After removing the sling, ponies were restrained in stocks for the entire experimental period and allowed to consume haylage and water.

2.5. SAMPLING

Before each experiment, the area around all tissue chambers was examined for the presence of abnormalities, such as wounds and signs of inflammation.

At specific time points prior to and after LPS stimulation, 1 mL tissue chamber fluid was aspirated aseptically from both tissue chambers. Samples of the left tissue chamber were collected for measurement of ketamine concentrations and its metabolites. Tissue chamber fluid from the right tissue chamber was collected for white blood cell (WBC) and differential cell counts, and for TNF-α, IL-6 and PGE_2 analyses. Samples were aliquoted in either micro tubes containing 16 IU heparin (ketamine, TNF-α, IL-6 and total leukocyte and differential count) or tubes containing 50 IU heparin and 10.73 μg indomethacin (PGE_2 analysis). Immediately after sampling the tissue chambers, venous blood samples were collected to determine plasma WBC and its differentiation and plasma concentrations of

ketamine and its metabolites. Total WBC and differential cell count were determined within 30 min after sampling. Venous blood samples for determining the ketamine concentrations were centrifuged at 400 rpm for 10 min at 0°C and supernatants as well as tissue chamber fluids were stored at -70°C until analysis.

2.6. CLINICAL PARAMETERS

Heart rate and rectal temperature were recorded one hour before and at preselected intervals following LPS inoculation. Simultaneously, the area around the tissue chambers was checked for swelling. Behaviour and appetite were noted during the entire experiment.

2.7. TOTAL WHITE BLOOD CELL AND DIFFERENTIAL COUNTS

The total number of WBC per mL tissue chamber fluid was counted by using the Coulter Counter® (Beckman-Coulter, Mijdrecht, The Netherlands). For counting WBCs in venous blood samples the Medonic CA 530® (A. Menarini Diagnostics, Valkenswaard, The Netherlands) was used. The relative number of PMNs and macrophages were determined microscopically after May-Grünwald Giemsa staining.

2.8. TNF-α ELISA

Concentrations of TNF-α were measured by enzyme-linked immuno-absorbent assay (ELISA) using an equine TNF-α ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, USA). In brief, 96-well plates coated with goat anti-equine TNF-α antibody were incubated with samples or TNF-α standards for 2h at room temperature (RT). A standard curve was established using recombinant equine TNF-α from 0-2000 pg/mL. Plates were washed 3 times followed by incubation with biotinylated goat anti-equine TNF-α antibody for 2 h at RT. Following 3 washes and 20 min incubation with streptavidin-HRP at RT, plates were incubated with substrate solution for 20 min at RT. The reaction was stopped by adding 2 N H_2SO_4 and absorbance was read at 540 nm using a plate reader. TNF-α concentrations were expressed as pg/mL.

2.9. IL-6 BIOASSAY

The relative IL-6 concentrations were measured by using a bioassay utilising the murine hybridoma cell line B13.29 clone B.9 according to the method of Helle et al.[22] The assay is based on the rate of proliferation of IL-6-dependent B.9 cells,

which is quantified by means of the MTT assay. By comparison of the mean inhibitory concentration of serially diluted IL-6 and serial dilutions of the sample, relative IL-6 levels of the samples were determined.

2.10. PGE_2 RADIO-IMMUNOASSAY

Concentrations of PGE_2 were measured by radio-immunoassay (RIA). In brief, anti-PGE_2 antiserum (Sigma Chemical Co., St Louis, MO, USA) and [^{3}H]PGE_2 (Amersham-Pharmacia Biotech, Veenendaal, The Netherlands) were added to each standard or sample. The tubes were incubated for 18-24 h at 4^{o}C. After incubation, dextran-coated charcoal suspension (20 mg/mL charcoal and 4 mg/mL dextran) was added and tubes were centrifuged (3000 rpm, 4^{o}C, 15 min) to separate bound and unbound fractions. Supernatants, containing the bound fraction, were transferred into scintillation-vials and scintillation fluid (Ultima Gold, Perkin Elmer, Wellesley, MA, USA) was added. After mixing, samples were analysed using a liquid scintillation counter (Tri-carb 2900TR®, Packard). Concentrations of PGE_2 were determined by comparing the values of the samples to the standards.

2.11. KETAMINE AND METABOLITES

Concentrations of ketamine, norketamine, hydroxynorketamine and 5,6-dehydronorketamine in both tissue chamber fluid and plasma samples were measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described in detail elsewhere.[23]

2.12. STATISTICAL ANALYSES

Statistical analysis was performed using the SPSS© 12.01 statistical package (SPSS© Inc., Chicago, IL, USA). To study the influence of ketamine on LPS-induced inflammatory responses, data of different ketamine groups (i.e., CRI, SI and C) were analysed using a repeated measures analysis of variance and the Huynh-Feld test statistics to adjust for sphericity, and were expressed as mean ± standard deviation (S.D.). When significant, a paired samples t-test was used to compare the differences with baseline values. Differences were considered to be significant when $p<0.05$.

3. RESULTS

3.1. INFLUENCE OF KETAMINE ON TOTAL WHITE BLOOD CELL COUNT AND DIFFERENTIAL COUNTS

Compared to baseline values, the influx of WBC into the tissue chambers was significantly increased in all treatment groups from 8 h following LPS exposure until the end of the experiment. Administration of ketamine, either as bolus or as CRI, did not significantly influence the LPS-induced WBC influx into the tissue chambers when compared to the saline-treated control group (Fig. 1A). Differential counts of tissue chamber WBC revealed a significant increase in polymorphonuclear neutrophils towards the end of each experiment.

In plasma, the number of WBC in the SI and C-group remained within physiological limits throughout the entire experiment. In the CRI-group, WBC slightly increased to 11.2 ± 1.6 G/L at 8 h following LPS exposure with a subsequent decrease to baseline values after 24 h of exposure (Fig. 1B). No differences in the number of WBC were observed between control and ketamine-treated animals. Differential counts of WBC were not significantly affected in any of the experiments.

3.2. INFLUENCE OF KETAMINE ON LPS-INDUCED TNF-α, IL-6 AND PGE_2 PRODUCTION

In all groups, TNF-α, IL-6 and PGE_2 significantly increased upon inoculation of LPS into the tissue chambers (Fig. 2). Peak concentrations of TNF-α, IL-6 and PGE_2 were reached at 6, 12 and 8 h, respectively. Again, no significant influence of ketamine administration on LPS-induced TNF-α, IL-6 and PGE_2 production could be demonstrated (Fig. 2).

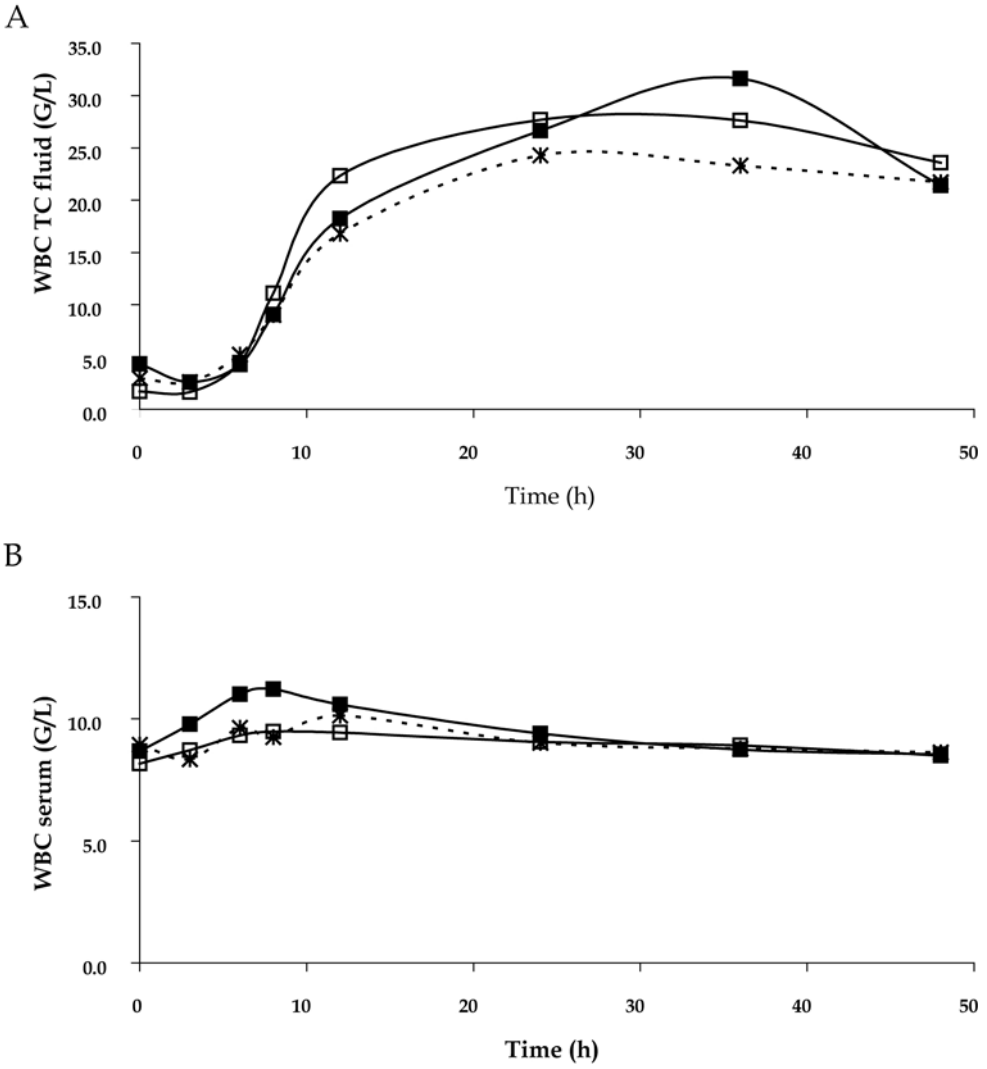

Figure 1. The effect of ketamine CRI (■), ketamine SI (□) and saline (∗) on total white blood cell count in (A) tissue chamber fluid and (B) serum.

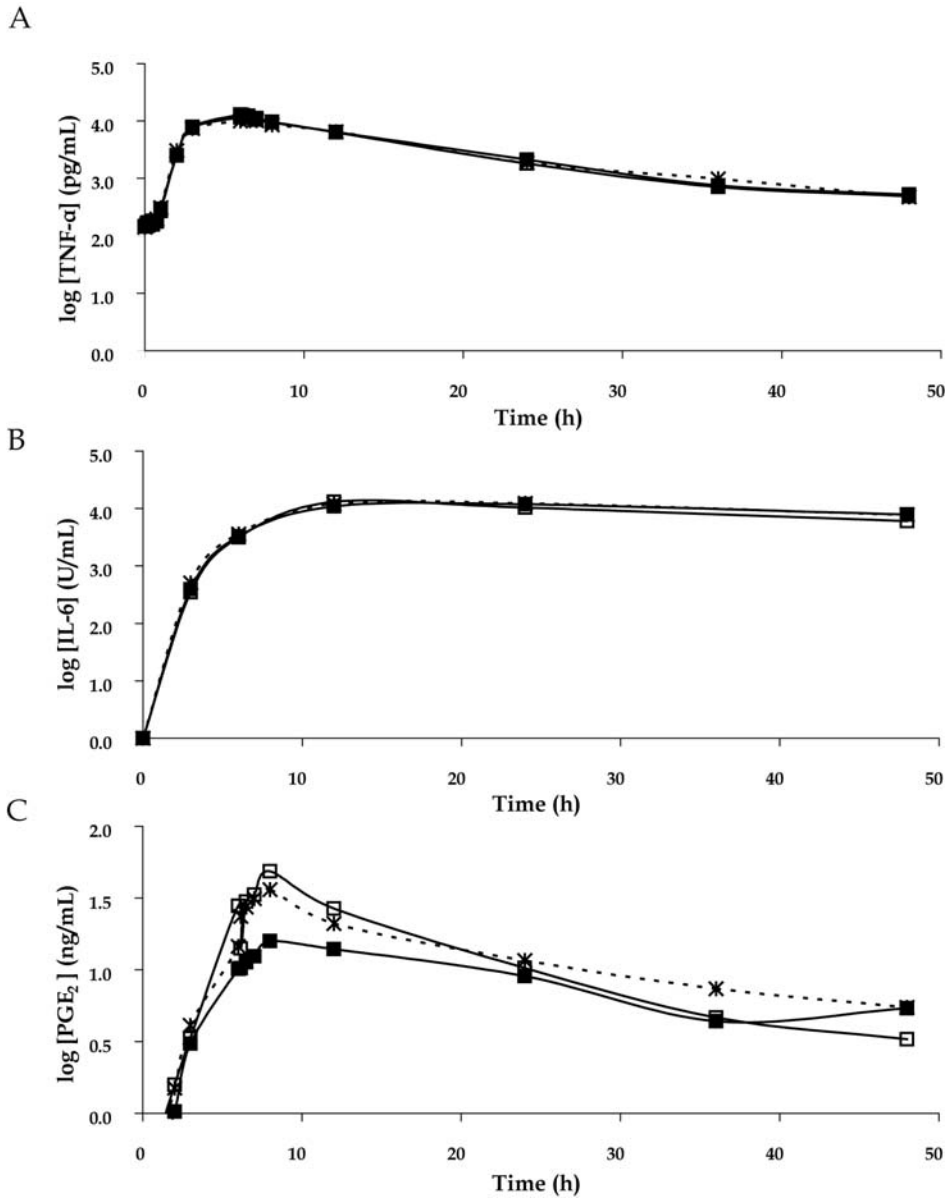

Figure 2. The effect of ketamine CRI (■), ketamine SI (□) and saline (∗) on (A) TNF-α, (B) IL-6 and (C) PGE_2 concentrations in the tissue chamber fluid.

3.3. INFLUENCE OF KETAMINE ON CLINICAL PARAMETERS

Following the bolus injection of ketamine, induction was achieved within 30 s and lasted for 3 to 5 min. Overall, in three individual cases catatonia was observed, one in the SI and two in the CRI group.

In the control group, heart rate remained stable and within the physiological range (28-40 beats per min) throughout the entire experiment. In contrast, in both the CRI and SI-group, heart rate significantly increased to a peak value of 51 ± 6 bpm at 15 min following ketamine injection. Thereafter, rates returned to physiological values in the SI group and remained stable for the remainder of the experiment. In the CRI group, heart rate also decreased following 15 min of the bolus injection, but remained slightly above the physiological range until the end of the ketamine CRI. Then, baseline values were reached within 15 min post-infusion.

Rectal temperature did not significantly differ between the studied groups.

Before each experiment, the area around the tissue chambers did not show any abnormalities. Swelling was first seen approximately 3 h after inoculation and remained visible till the end of the experiment. However, none of the ponies was reluctant to move the neck and abscess formation of the tissue chambers did not occur.

3.4. KETAMINE CONCENTRATIONS IN PLASMA AND TISSUE CHAMBER FLUID

In the CRI group, the peak plasma concentration of 576 ± 305 ng/mL at 5 min following ketamine bolus injection decreased to a minimum of 101 ± 64 ng/mL at 2 h during ketamine CRI (Fig. 3A). Thereafter, the plasma ketamine concentration slightly increased again towards the end of infusion. During ketamine CRI, no obvious steady state level was reached. The average plasma ketamine concentration was 155 ± 54 ng/mL. Once the ketamine CRI was discontinued, the plasma ketamine concentration decreased rapidly to approximately one-tenth of the initial value within 2 h. In tissue chamber fluid, a peak concentration (139 ± 67 ng/mL) was reached at 30 min following ketamine injection. Subsequently, an apparent steady state level at 126 ± 9 ng/mL was observed till the end of the ketamine CRI. After discontinuing the ketamine CRI, the ketamine concentration in the tissue chamber fluid gradually decreased to baseline values until the end of the experiment.

In the SI group, the peak plasma concentration of 685 ± 147 ng/mL progressively decreased to 14 ± 5 ng/mL within 2 h (Fig. 3B). In tissue chamber fluid, a maximum concentration of 177 ± 41 ng/mL was reached between 45 min and 2 h

following ketamine injection, which gradually decreased until the end of the experiment.

3.5. KETAMINE METABOLITES IN PLASMA AND TISSUE CHAMBER FLUID

In the CRI group, the concentration of norketamine remained high above the concentration of the parent compound in both plasma and tissue chamber fluid (Figs 4A and 4B). Plasma concentrations of hydroxynorketamine and 5,6-dehydronorketamine nearly equalled the plasma concentrations of ketamine. Although plasma concentrations of all metabolites reached an apparent steady state during ketamine CRI, concentrations in tissue chamber fluid still increased till the end of ketamine CRI (Figs 4A and 4B).

In the SI group, plasma concentrations of the metabolites remained below or slightly above the plasma concentration of ketamine (Fig. 4C). In the tissue chamber fluid, the concentration of all metabolites remained below the concentration of ketamine (Fig. 4D).

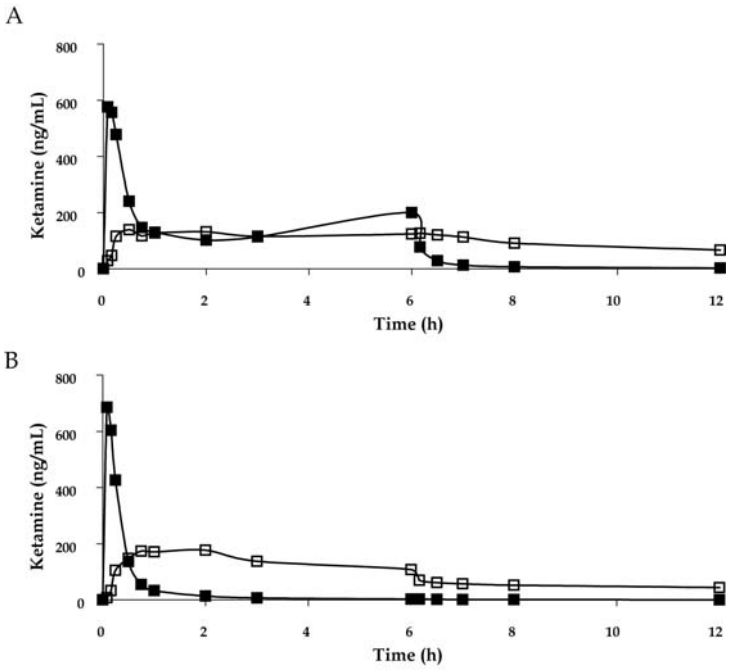

Figure 3. The mean ketamine concentration in plasma (■) and tissue chamber fluid (□) following (A) ketamine CRI and (B) a single injection of ketamine.

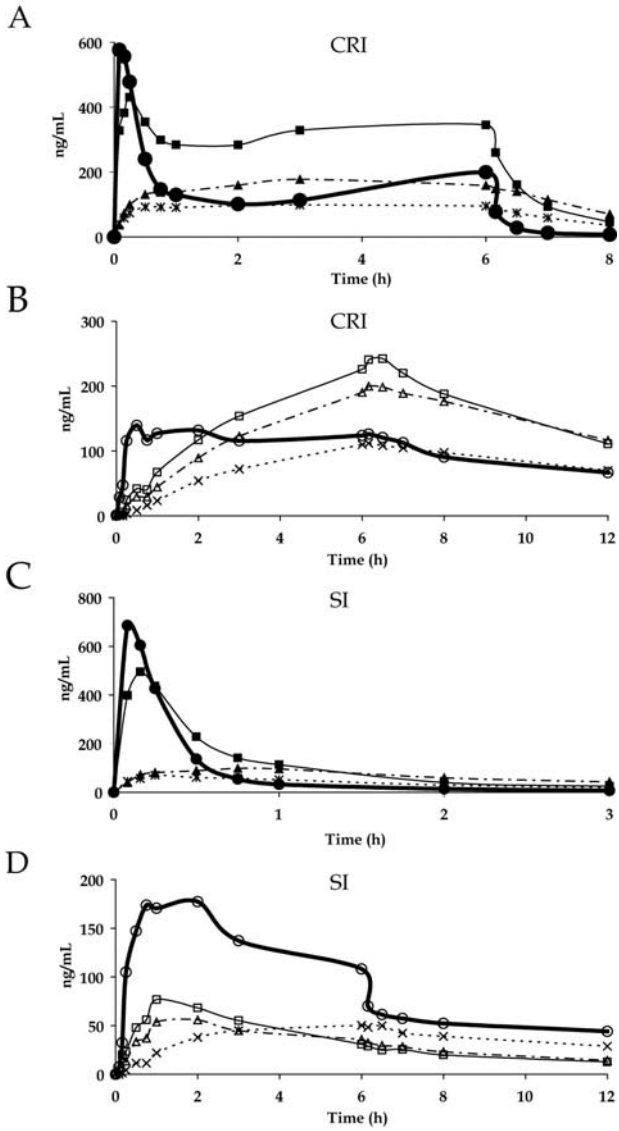

Figure 4. The mean concentrations of ketamine (●/○), norketamine (■/□), hydroxynorketamine (▲/△) and 5,6-dehydronorketamine (∗/×) in plasma (closed symbols in A and C) and tissue chamber fluid (open symbols in B and D) following ketamine CRI (A and B) and a single ketamine injection (C and D).

4. DISCUSSION

Previously, we demonstrated that ketamine exerts anti-inflammatory effects in an equine macrophage cell line (e-CAS cells) by reducing LPS-induced TNF-α and IL-6 production in a concentration-dependent manner.[1] The aim of the present study was to evaluate these anti-inflammatory effects of ketamine in vivo by using an LPS-inoculated equine tissue chamber model.

Considering the concentration-dependent influence and the fact that elevated plasma cytokine levels have been detected in horses for several hours after onset of inflammation, it was hypothesised that ketamine needed to be administered over extended periods of time and at relatively high doses to be effective. To verify the hypothesis regarding long-term ketamine treatment, a bolus injection followed by a ketamine CRI was compared to a bolus injection of ketamine only. For the bolus injection the commonly used induction dose of 2.2 mg/kg body weight was selected. The dose rate of the ketamine CRI tested is based on the premise that it should allow its administration to conscious horses in the post-operative intensive care. The risk of hypnotic and excitatory side effects limits the maximal dose rate of the ketamine CRI in conscious horses. Recently, it has been demonstrated that a ketamine CRI of 1.5 mg/kg/h could be administered safely to healthy conscious horses for at least 6 h.[23]

In contrast to the results obtained in vitro, ketamine did not influence LPS-induced TNF-α, IL-6 and PGE_2 production in the equine tissue chamber model. Moreover, no effect of ketamine on WBC count in tissue chamber fluid could be detected. This discrepancy between the in vitro findings and the present results might be due to the fact that only a limited fraction of ketamine entered the tissue chamber. Following intravenous injection, ketamine rapidly entered the tissue chamber, corresponding well with the rapid distribution ($t_{1/2\alpha}$ = 2-3 min)[23-25] and the low protein binding of ketamine (50%).[24] However, the peak concentrations in tissue chamber fluid remained below 25% of the peak concentrations measured in plasma, despite the generally large volume of distribution.[23-25] This low tissue chamber concentration is associated with a delayed diffusion of ketamine into the tissue chambers and the extremely rapid elimination ($t_{1/2\beta}$ = 65-67 min).[23,25] Moreover, due to its low protein binding and as a weak base, ketamine is presumably not being trapped in the tissue chambers but rapidly redistributed. Hence, the final ketamine concentration in the tissue chamber fluid (range 126-177 ng/mL; 0.5-0.6 μM) was considered to be too low to generate a measurable anti-inflammatory activity. In e-CAS cells, TNF-α concentrations were only

significantly suppressed by ketamine concentrations exceeding 1.8 μM. However, higher dose rates of ketamine could not be tested since a previous pharmacokinetic study on a long-term ketamine CRI in conscious healthy horses showed that at concentrations exceeding 1.5 mg/kg/h considerable undesirable side effects, mainly related to the central nervous system (excitement / cardiovascular stimulation) and increasing skeletal muscle tone (catatonia), will occur.[23]

Ketamine is metabolised extensively by the hepatic cytochrome P_{450} system. The major pathway involves N-demethylation to form norketamine, which is then hydroxylated and oxidated to form hydroxynorketamine and 5,6-dehydronorketamine, respectively.[26] In the present study, all three metabolites were measured in both plasma samples and tissue chamber fluid. However, the pharmacodynamical effects of these metabolites have not been fully elucidated yet.

The tissue chamber model has been proven to be a valid model for simulating inflammatory reactions at soft tissue level following LPS stimulation.[27] Applying the tissue chamber model, LPS-induced inflammatory responses will be restricted mainly to the tissue chamber, thereby minimising discomfort to experimental animals.[28] Similarly, in the present study, the LPS-induced acute inflammatory responses remained largely confined to the tissue chambers. Upon stimulation by LPS, a significant increase in WBC count and TNF-α, IL-6 and PGE_2 concentrations in the tissue chamber fluid was measured, whereas WBC in plasma and rectal temperature remained within or slightly above the physiological range. Furthermore, none of the ponies gave any impression of malaise.

In the present study, substantial inter- and intra-individual differences in the response to LPS were observed. In humans and rodents, this variability in LPS response is ascribed to changes in the coding sequence of the LPS receptor complex.[29-31] In addition, differences in plasma concentrations of LPS binding protein, which presents LPS to the LPS receptor complex, might explain the variability in LPS response.[32] In horses, inter-individual differences in the LPS response have previously been described.[33] However, the altered response to LPS could not be related to mutations observed in parts of the LPS receptor complex.[34] Finally, development of endotoxin tolerance might also be responsible for differences in the LPS response. However, the statistical analysis of the three independent experiments performed in this study showed no significant difference between the three experiments for any of the studied parameters.

In conclusion, following the administration of clinically tolerable dosages, ketamine does not reach tissue chamber concentrations which are effective in

reducing LPS-induced TNF-α, IL-6 and PGE_2 concentrations in tissue chamber fluid. Further experiments should be conducted to measure the systemic effect of ketamine, preferentially in equine patients suffering from inflammatory diseases that receive ketamine as part of the general anaesthesia protocol or in the intensive care.

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