- CHAPTER VII -

KETAMINE REDUCES PRODUCTION OF REACTIVE OXYGEN SPECIES IN AN EQUINE MACROPHAGE CELL LINE FOLLOWING STIMULATION WITH LPS AND PMA

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SUMMARY

Ketamine is commonly used in equine anaesthesia. Next to its anaesthetic and analgesic effects, ketamine has also been found to exhibit potent cytokine-modulating and antioxidative properties in rodents and humans. In addition, in an equine macrophage cell line (e-CAS cells), ketamine reduced lipopolysaccharide (LPS)-induced tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) production in a concentration-dependent manner.[1] In the present study, the antioxidative effects of ketamine in e-CAS cells were studied. Initially, two different ROS-inducers, LPS (1 μg/mL), phorbol myristate acetate (PMA; 1 μM) and the combination LPS (1 μg/mL)/PMA (1 μM) were tested. The capacity of ketamine (0-36 μM) to suppress reactive oxygen species (ROS) production in e-CAS cells was investigated by using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) as a molecular probe. Furthermore, to determine the mechanism underlying the effect of ketamine on stimulated ROS production, the influence of ketamine on intracellular glutathione (GSH) concentrations was measured.

In e-CAS cells, a significant increase in ROS formation was documented only following exposure to the combination LPS/PMA. Ketamine significantly and dose-dependently reduced ROS formation in LPS/PMA-stimulated e-CAS cells. In addition, ketamine significantly increased total intracellular GSH concentrations in LPS/PMA-treated e-CAS cells in a dose-dependent manner. These results suggest that the antioxidative properties of ketamine in e-CAS cells are most likely related to an indirect, GSH-sparing effect of ketamine.

1. INTRODUCTION

Reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH), are produced during normal cellular metabolism. Phagocytic cells like neutrophils, monocytes and macrophages respond to infectious agents with an increased ROS production, often referred to as respiratory burst, which plays a pivotal role in the innate host defence.[2,3] Under physiological conditions, ROS production is counterbalanced by various enzymes, such as superoxide dismutase, catalase and glutathione peroxidase, that equilibrate the intracellular redox potential.[2-5] However, following infection or toxic cell injury, an excessive production of ROS occur, which interacts with cellular macromolecules resulting in cell function impairments, DNA damage and finally apoptosis and necrosis.[2,3]

In rodents and humans, ROS have been associated with inflammatory diseases like arthritis,[6] asthma,[7] cardiac injury[8] and sepsis.[9] Moreover, numerous toxins have been found to induce ROS production. In horses, ROS production has been primarily implicated in the pathogenesis of gastrointestinal ischaemia-reperfusion injury.[4,10] The application of ROS scavengers is considered to be of therapeutic advantage in many diseases, particularly as part of a multimodal therapy concept.

Ketamine, a dissociative anaesthetic agent, is commonly used in equine anaesthesia. Besides the induction of anaesthesia,[11] ketamine is used as an adjunct to inhalant anaesthesia,[12-14] in total intravenous anaesthesia (TIVA) protocols[15-17] and for local analgesia.[18,19] More recently, studies focussed on the antinociceptive effects of low dose ketamine infusions administered to conscious horses[20] and its anti-inflammatory properties.[21] In the evaluation of these anti-inflammatory effects, ketamine was found to reduce the production of inflammatory mediators like cytokines (i.e., tumour necrosis factor-alpha and interleukin-6 (IL-6), nitric oxide (NO) and ROS in rodents[22-24] and humans.[25-28]

In the present study, the effect of ketamine on stimulated ROS production in an equine macrophage cell line and the mechanisms underlying the effect of ketamine on stimulated ROS production were investigated.

2. MATERIALS AND METHOD

2.1. CHEMICALS

Ketamine, lipopolysaccharide (LPS; Escherichia coli, O111:B4), phorbol 12-myristate 13-acetate (PMA), L-buthionine-(S,R)-sulfoximine (BSO), glutathione (GSH) standard, glutathione reductase, dimethylthiazol diphenyl tetrazolium

bromide (MTT), triethanolamine (T-1377), Triton-X, sulfosalicylic acid, nicotinamide adenine dinucleotide phosphate (NADPH) and 5-5'dithio-bis (2-2)-nitrobenzoic acid (DTNB) were purchased from Sigma chemicals (St. Louis, MO, USA). Roswell Park Memorial Institute (RPMI) 1640 medium, penicillin, streptomycin, glutamine and phosphate buffered saline (PBS) were obtained from Biocambrex (Verviers, Belgium). Horse serum (HS), non-essential amino acids and sodium pyruvate were from Gibco Invitrogen (Breda, The Netherlands). The fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) was obtained from Invitrogen Paisley, UK). Isopropanol was from Boom B.V. (Meppel, The Netherlands).

2.2. CELL CULTURE

The equine bone-marrow-derived macrophage cell line (e-CAS cells), described by Werners et al.,[29] was grown in RPMI 1640 medium supplemented with 10% HS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM glutamine, 1 mM sodium pyruvate and 1% non-essential amino acids. Cells were maintained at 37 °C, in a humidified atmosphere of 5% CO_2/ 95 % air.

2.3. ROS INDUCERS AND DETERMINATION OF ROS PRODUCTION

Since ROS production in equine mononuclear cells is highly dependent on the inducer used, initially two inducers, i.e., LPS, PMA and the combination LPS/PMA were tested, before the effect of ketamine on stimulated ROS production was investigated. The intracellular production of ROS was measured by using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) as a molecular probe. After diffusion into cells, intracellular esterases hydrolyse H_2DCF-DA molecules into H_2DCF, which can react with a broad range of oxidants. Upon oxidation, the probe becomes fluorescent and this effect can be quantified by using a fluorescence reader. The determination of ROS was based on the method described by Trayner et al.[30] with minor modifications. In brief, cells were seeded in 96-well plates at a density of $2.5x10^4$ cells per well (100 μL). Following 48 h of culturing, cells were incubated in serum- and phenol red-free RPMI medium containing H_2DCF-DA (10 μM) and LPS (1 μg/mL), PMA (1 μM) or the combination LPS (1 μg/mL)/PMA (1 μM). Following 1 h of incubation, H_2DCF-DA and inducers were removed by washing the cells once with PBS (200 μL). Subsequently, cells were stimulated with one of the inducers tested for an additional 4 h. Fluorescence was then measured with a Cytofluor model 2300 microplate fluorimeter (Millopore, Bedford, MA,

USA) using an excitation wavelength of 485 nm and an emission wavelength of 538 nm. Production of reactive oxygen species (ROS) was expressed as the ratio of fluorescence of the treated samples over the response in the appropriate controls [($fluorescence_{treatment}/fluorescence_{control}$) x 100%, after correction for background absorbance].

2.4. EXPOSURE TO LPS/PMA AND KETAMINE

Based on the results of the experiments described above, the influence of ketamine on ROS production was tested in LPS/PMA-treated e-CAS cells. Briefly, cells were incubated in serum- and phenol red-free RPMI medium containing H_2DCF-DA (10 μM), LPS (1 μg/mL), PMA (1 μM) and ketamine (0-36 μM) for 1 h. Subsequently, cells were washed once with PBS (200 μL) to remove the probe and again exposed to LPS (1 μg/mL), PMA (1 μM) and ketamine (0-36 μM) in fresh serum- and phenol red-free RPMI medium. After 4 h of incubation, fluorescence was measured as described above.

2.5. DETERMINATION OF INTRACELLULAR GLUTATHIONE LEVELS

To determine whether ketamine reduces ROS production in LPS/PMA-treated e-CAS cells by increasing intracellular glutathione (GSH) concentrations, total intracellular GSH was measured based on the method described by Griffith.[31] In brief, cells were seeded in 6 well-plates at a density of 1.0 x 10^6 cells per well (2 mL). Following 24 h of culturing, cells were incubated in serum- and phenol red-free RPMI medium containing LPS (1 μg/mL)/PMA (1 μM) and ketamine (0-36 μM) for 4 h. Subsequently, cell lysates were prepared in lysis buffer [Triton-X 0.05% (w/v), EDTA 0.05 mM in PBS] and total cellular protein amount was determined using the technique described by Lowry et al.[32] Remaining samples were mixed with sulfosalicylic acid (2.5%), incubated on ice for 5 min and centrifuged at 12,000 x g for 5 min at 4 °C. Next, supernatants were neutralised by adding triethanolamine (8 %).

GSH levels were determined in a mixture containing 0.24 mM NADPH and 6 mM 5-5'dithio-bis (2-2)-nitrobenzoic acid (DTNB). After incubation for 5 min at 30 °C, the cell lysates or GSH standards (0, 0.5, 1, 2 and 3 μM) were added and the reaction was initiated by addition of glutathione reductase (0.5 U/sample). Increase in absorption was measured at 412 nm and 30 °C. Specific activity was calculated by comparison to the standard curve and expressed as μmol GSH/mg total cellular protein.

To investigate whether ketamine induces GSH synthesis, the influence of ketamine on intracellular GSH content was measured in GSH-depleted cells following pre-incubation with buthionine sulfoximine (BSO). In brief, following 24 h of culturing in 6-well plates at a density of 1.0 x 10^6 cells per well, cells were pre-incubated with serum- and phenol red-free RPMI medium containing BSO (25 µM) for 4 h. Thereafter, cells were washed and exposed to fresh medium containing ketamine (0-36 µM) only for an additional period of 4 h. Subsequently, cell lysates were prepared and the intracellular GSH production was measured as described above.

2.6. CELL VIABILITY

The mitochondrial-dependent reduction dimethylthiazol diphenyl tetrazolium bromide (MTT) to formazan was used to measure cell respiration as an indicator of cell viability.[33] After measuring ROS production, 3 mg/mL MTT solution was added and plates incubated for 30 min at 37 ºC in a humidified atmosphere. The reaction was terminated by discarding the incubation solutions, followed by dissolving the formed formazan product in 100 µL acidic isopropanol (containing 0.5% SDS and 0.04 M HCl). Absorbance was measured with a Biorad 3550 microplate reader (Biorad, Veenendaal, The Netherlands). Cell viability was expressed as the relative formazan formation in treated samples as percentage of the formazan formation in solvent-treated controls [(A595treated cells/A595 of appropriate control) x 100%, after correction for background absorbance].

2.7. STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS® 12.01 statistical package (SPSS© Inc., Chicago, IL, USA). Data are presented as mean ± standard deviation (S.D.) obtained from at least two independent experiments carried out in quadruplicate. One-way analysis of variance (ANOVA) was used to test for significant differences between group means. When appropriate, post hoc multiple comparisons were performed to test for significant differences between experimental groups (Dunnett's). Data were considered to be statistically significant when $p<0.05$.

3. RESULTS

3.1. ROS INDUCERS

A significant increase in ROS production was only determined following co-stimulation with LPS (1 µg/mL) and PMA (1 µM), compared to cells exposed to medium only (Fig. 1A). Data obtained with the MTT assay demonstrated that cell

viability was not significantly influenced following exposure to any of the inducers tested (Fig. 1B). Based on these results, the effect of ketamine on ROS production was studied in LPS/PMA-treated e-CAS cells.

3.2. KETAMINE INHIBITS ROS PRODUCTION IN LPS/PMA-TREATED E-CAS CELLS

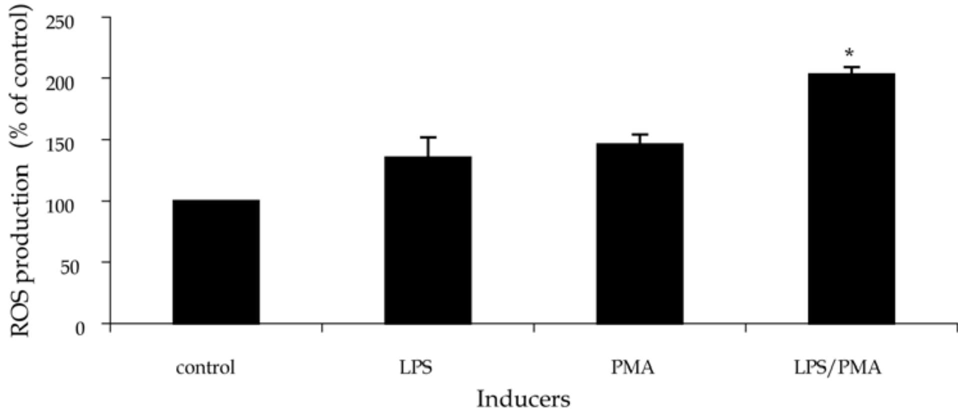
Data indicate that ketamine significantly reduces ROS production in LPS/PMA-stimulated e-CAS cells in a concentration-dependent manner (0-36 μM), compared to LPS/PMA alone (Fig. 2A). Again, no loss of cell viability was observed (Fig. 2B). Control experiments revealed that ketamine alone, in absence of LPS, did not affect ROS production (data not shown).

3.3. EFFECT OF KETAMINE ON INTRACELLULAR GSH CONCENTRATION

To investigate whether ketamine reduces ROS production in LPS/PMA-treated e-CAS cells by increasing intracellular GSH concentrations, total intracellular GSH content was measured following exposure to LPS/PMA and ketamine. In LPS/PMA-treated e-CAS cells, ketamine significantly increased intracellular GSH content in a concentration-dependent manner (Fig. 3A).

To investigate whether ketamine increases the intracellular GSH content by inducing GSH synthesis, the influence of ketamine on total intracellular GSH concentrations was measured in GSH-depleted cells following pre-incubation with BSO. As shown in Fig. 3B, BSO significantly reduced the intracellular GSH production with 54% in the concentration tested. However, ketamine did not affect GSH synthesis at any of the tested concentrations. Ketamine alone, in absence or presence of BSO did not affect the intracellular GSH concentration (data not shown).

A



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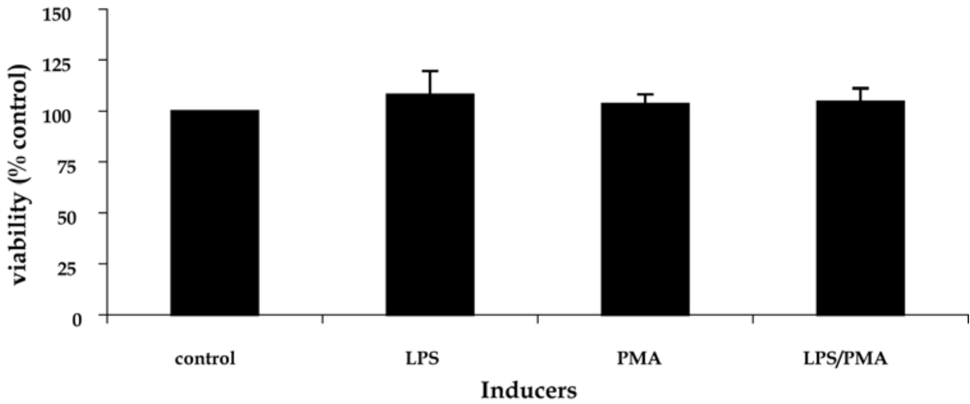
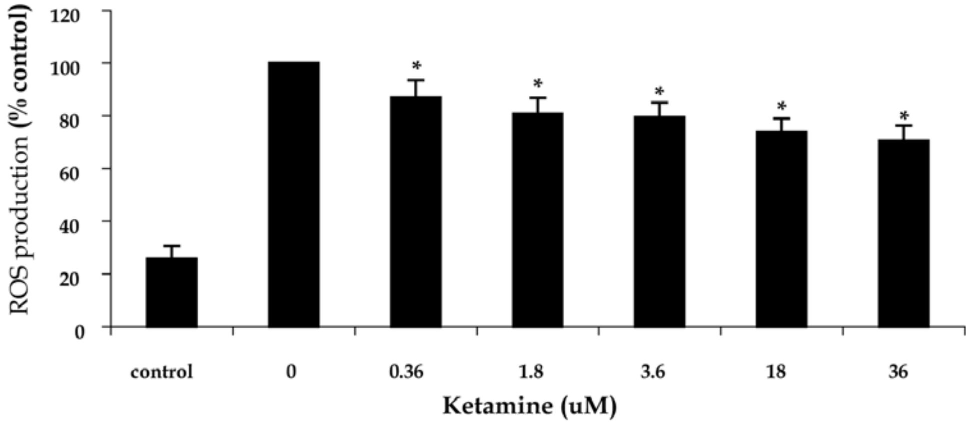


Figure 1. The effect of LPS, PMA and the combination LPS/PMA on (A) ROS production and (B) viability of e-CAS cells following 4 h of exposure. Data are expressed as mean ± S.D. of three independent experiments carried out in quadruplicate. * statistically different from non-stimulated cells (control; $p<0.05$).

A



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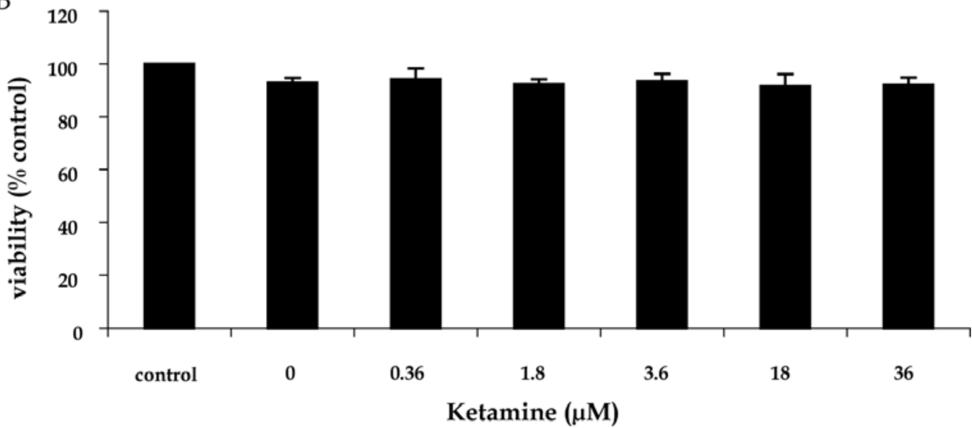

Figure 2. The effect of ketamine on LPS/PMA-induced (A) ROS production and (B) viability of e-CAS cells following 4 h of exposure. Data are expressed as mean ± S.D. of four independent experiments carried out in quadruplicate. * statistically significant differences ($p<0.05$) between ketamine-treated cells and cells treated only with LPS/PMA (controls).

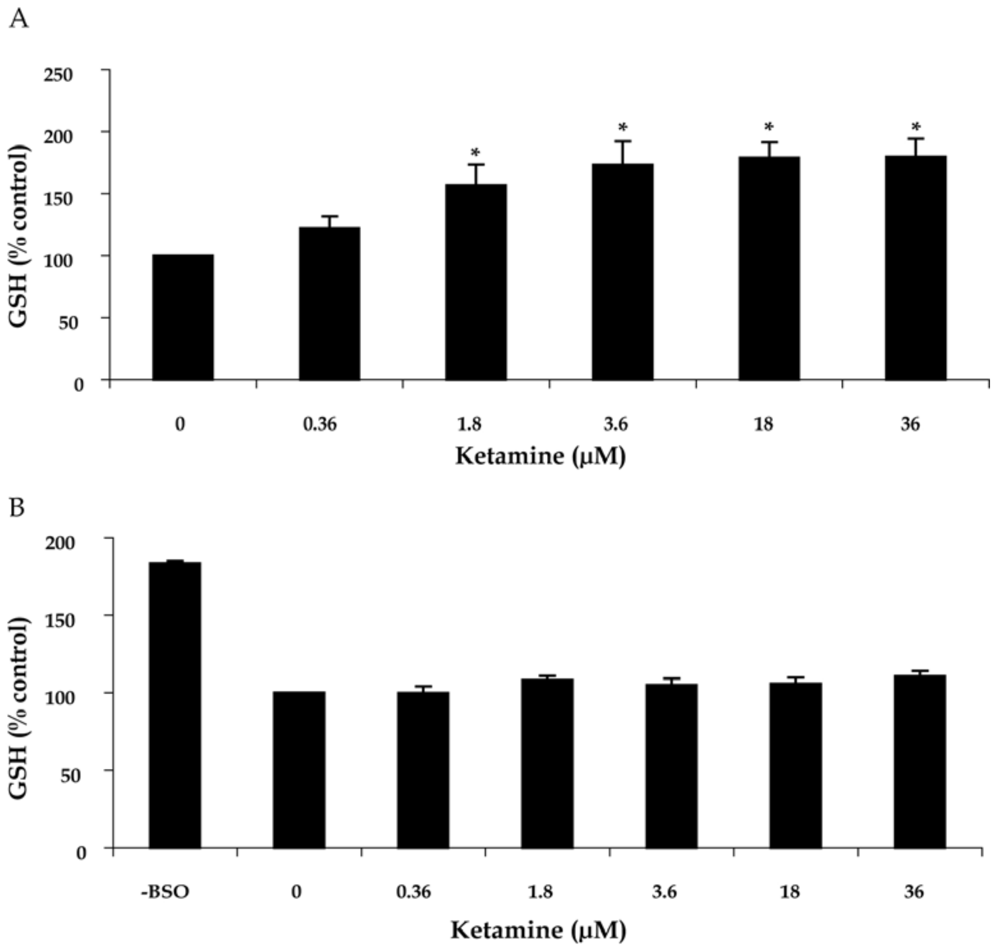

Figure 3. The effect of ketamine on (A) total intracellular GSH in LPS/PMA-treated e-CAS cells and on (B) GSH synthesis in GSH-depleted e-CAS cells following pre-incubation with BSO. Data are expressed as mean ± S.D. of two independent experiments carried out in duplicate. * statistically significant differences ($p<0.05$) between ketamine-treated and non-stimulated cell (controls).

4. DISCUSSION

Phagocytic cells like neutrophils, monocytes and macrophages, produce and release ROS upon stimulation by either endogenous factors (e.g., cytokines, lipid mediators and various breakdown products) or exogenous influences (e.g., LPS, UV light irradiation, toxic chemical and drugs).[2,3,8] This 'respiratory burst' is catalysed by the multicomponent nicotinamide adenine dinucleotide phosphate (NADPH) reduced oxidase system.[2,3,5,34,35] Alternatively, ROS can be generated by the mitochondrial electron transport chain, by xanthine oxidase, lipoxygenase, cyclooxygenase and cytochrome P_{450}s, as well as by auto-oxidation of various substances, particularly catecholamines.[2-5,8]

Mammalian cells possess several endogenous mechanisms, including superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione, ubiquinone and vitamins C and E, to protect themselves against ROS formed during the normal cellular metabolism.[2-5,8] When ROS formation exceeds the antioxidant capacity of a cell, excessive ROS formation will lead to cellular destruction and tissue injury due to lipid peroxidation, inactivation of enzymes and breaking of DNA strands.[2,3] Ultimately, the ROS-induced tissue injury may lead to multiple organ dysfunction and death. Hence, inhibition of exaggerated ROS formation will be beneficial in limiting ROS-induced cellular injury and mutational events.

In contrast to the numerous studies on ROS production in human or rodent cell lines (or isolated primary cell cultures), data on ROS production in equine cells are scarce. One of the few examples are the investigations of Benbarek et al.,[36] who describe LPS-induced ROS production in a whole blood assay, but failed to observe ROS production in isolated equine neutrophils stimulated with LPS. In line with these results, we also found that LPS alone did not stimulate ROS formation in e-CAS cells.

Macrophages produce ROS at a lower rate than neutrophils. Hence, in vitro stimulation of ROS production generally requires application of additional stimulators like N-formyl-methionyl-leucyl-phenylalanine (FMLP), opsonised zymosan or phorbol myristate acetate (PMA) next to LPS.[37-40] In the present study, PMA was chosen as an additive to LPS, since it has been reported to be the most effective activator.[37] Similar to LPS, PMA alone did not induce ROS formation, but a significant increase in ROS formation was observed only when cells were co-stimulated with LPS and PMA. Similar results have been found by Bochsler et al.,[38] as pre-incubation of equine polymorphonuclear leukocytes with LPS followed by PMA significantly increased the secretion of superoxide anions. In consideration of

these findings, the effect of ketamine on ROS production was studied in e-CAS cells co-stimulated with LPS and PMA.
The ketamine concentrations used in this study were selected on the basis of the peak plasma level of 5 μg/ml (18 μM) following the IV induction dose of ketamine of 2.2 mg/kg body weight commonly used in horses.[41] Ketamine significantly reduced LPS/PMA-induced ROS formation in a concentration-dependent manner in e-CAS cells. Similarly, ketamine was found to significantly reduce FMLP/opsonised zymosan[39] or FMLP/PMA-induced[26] ROS production in human polymorphonuclear leukocytes (PMNs) in a dose-dependent manner, whereas no influence of ketamine on ROS synthesis was found in other studies including PMA-treated,[42] FMLP-treated[43] and opsonised zymosan-treated[44,45] human PMNs. The contrasting results from these latter studies may be due to the fact that only one single stimulator was used.
Reduction of ROS concentration in stimulated cells can be achieved either by application of direct radical scavengers or by stimulation of intracellular defence mechanisms. According to the results of the present study, ketamine reduces ROS formation in e-CAS cells by modulation of the intracellular GSH concentration. Cellular GSH content can increase via either a direct or indirect mechanism. The direct mechanism includes the induction of GSH synthesis, whereas the indirect mechanism is based on a rapid GSSG reduction (redox cycling). In the presence of ROS, GSH is converted into its reduced form GSSG by glutathione peroxidase to neutralise ROS.[46,47] Since ketamine did not induce GSH synthesis in GSH-depleted e-CAS cells, it can be assumed that ketamine indirectly increases the total intracellular GSH content by scavenging ROS, thus stabilising the intracellular redox potential. Indeed, ketamine has been described to be a weak radical scavenger, due to its phenol-moiety in early studies.[48] The effect of ketamine on other cellular enzymes involved in ROS production remains to be elucidated.
In conclusion, we demonstrate here that ketamine reduces ROS formation in stimulated e-CAS cells by indirectly increasing the intracellular GSH content. This effect is clinically desirable as it prevents tissue injury following infections or during tissue reperfusion after hypoxia.

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