# - CHAPTER X -

# SUMMARISING DISCUSSION AND CONCLUSIONS

## KETAMINE: AN OLD DRUG WITH NEW TRICKS?

Ketamine, first described in 1966 by Corssen and Domino,[1] is used in human and veterinary anaesthesia for inducing amnesia, loss of consciousness, analgesia and immobility.[2-5] In contrast to other anaesthetics, such as hypnotics and volatile anaesthetics, ketamine produces somatic and visceral analgesia even at subanaesthetic concentrations.[6-12] Since ketamine preserves cardiovascular function, its use is recommended for anaesthesia of critically ill patients in which there is a risk of cardiac depression and hypotension.[13-15]

In equine anaesthesia and analgesia, ketamine is commonly used in a variety of clinical procedures. Besides its application for induction of anaesthesia,[16] ketamine has also been used as an adjunct to inhalant anaesthesia,[17-19] in different total intravenous anaesthesia (TIVA) protocols,[20-22] and for epidural analgesia[23] and peripheral nerve blocks.[24] More recently, studies focus on the antinociceptive effects of a subanaesthetic ketamine continuous rate infusion (CRI) administered to conscious horses.[25]

Since the mid 90s, potential anti-inflammatory effects of ketamine, such as cytokine-modulating effects and anti-oxidative properties, have attracted attention and have been studied intensively in rodents[26-31] and humans.[32-37] However, no data are available on the anti-inflammatory effects of ketamine in horses suffering from inflammatory diseases. This thesis summarises experimental work conducted into the anti-inflammatory effects of ketamine in different in vitro and in vivo equine models.

## CYTOKINE-MODULATING EFFECTS OF KETAMINE IN AN EQUINE MACROPHAGE CELL LINE

For the in vitro experiments, an equine bone-marrow-derived macrophage cell line, referred to as e-CAS cells, was used to investigate the potential anti-inflammatory effects of ketamine in horses in vitro (**Chapter III-VII**). Although primary equine cells like PBMCs,[38] peritoneal macrophages,[39-41] alveolar macrophages,[42,43] vascular smooth muscle cells[44] and chondrocytes[45] can easily be isolated, and have frequently been used to study several anti-inflammatory therapeutic approaches, a cell line over primary cells was chosen, since the former has the advantage of a more uniform response pattern.[46]

Previously, e-CAS cells have intensively been studied by Werners et al.[47] In particular, the equine-specific signalling pathways have been characterised in detail in these cells. However, since the e-CAS model is a rather new model, both a human monoblastoid tumour cell line (U937 cells) (**Chapter III**) and a murine

macrophage cell line (RAW 264.7 cells) (**Chapter V-VI**) were used to confirm the outcome observed with the e-CAS model.

In all experiments, lipopolysaccharide (LPS) was applied as the primary stimulator of the inflammatory response, since LPS is a homogeneous reagent with uniform properties[48] and has been implicated in the pathogenesis of various inflammatory diseases in several species, including horses.[49-54] Upon stimulation with LPS, cells of the innate immune system produce and release inflammatory mediators, such as cytokines, acute phase proteins and prostanoids, which can potentially be used as markers for the early detection of inflammation. Evidence suggests that tumour necrosis factor-alpha (TNF-α) is the primary mediator of the LPS-induced effects,[55] whereas the interleukin-6 (IL-6) concentration predominantly has a prognostic value in horses suffering from inflammatory disorders.[56,57] Hence, both cytokines were used as markers when studying the anti-inflammatory effects of ketamine in equine models.

In all in vitro studies in this thesis, ketamine concentrations ranging between 0-36 μM were tested. This range was selected in consideration of the peak plasma level of 5 μg/mL (18 μM) resulting from the standard IV induction dose in horses of 2.2 mg/kg body weight.[58] Lower concentrations were included to determine the minimal effective concentration that exerts anti-inflammatory effects in vitro. This is of possible clinical relevance since lower plasma concentrations are desirable in the post-operative period to circumvent the hypnotic and excitatory side effects of ketamine in conscious horses. In some cases, high experimental concentrations up to 1000 μM were tested as well to investigate whether ketamine would be able to affect the inflammatory parameters at all.

In the LPS-stimulated e-CAS cells, ketamine significantly suppressed the TNF-α and IL-6 concentrations in a concentration-dependent manner (**Chapter III**). This initial finding prompted us to start with a series of experiments aiming at the understanding of the molecular mechanisms involved in the cytokine-modulating effects of ketamine.

### MOLECULAR MECHANISM UNDERLYING THE CYTOKINE-MODULATING EFFECTS OF KETAMINE

Following LPS exposure, multiple signalling pathways are known to be involved in upregulating gene expression and synthesis of pro-inflammatory cytokines.[59-62] Following binding to and activation of the cell surface receptor Toll-like receptor 4 (TLR4), LPS initiates a complex intracellular signalling cascade.[63-65] The most

prominent signalling pathways encompass the phosphorylation of mitogen-activated protein kinases (MAPKs) and the activation of nuclear factor-kappa B (NF-κB).[66,67] MAPKs represent a highly conserved family of serine/threonine/tyrosine kinases comprising three major subfamilies: c-Jun N-terminal kinase (JNK), mitogen-activated protein kinase p38 (p38) and extracellular signal-regulated kinase (ERK). Upon phosphorylation, MAPKs translocate to the nucleus and activate transcription factors like activating protein-1 (AP-1), Elk1, c-Fos, c-Jun and NF-κB.[67,68]

NF-κB, representing a group of structurally related transcriptional proteins that form dimers of distinct composition, is considered to be a key regulatory transcription factor, which is involved in numerous cell functions, including the cell differentiation and maturation, and control of cell proliferation and apoptosis (for review see 67). The most prominent NF-κB protein is the p50/p65 heterodimer, which has been found to bind to a distinct DNA sequence, regulating the transcription of more than 200 genes, including those related to the expression of inflammatory mediators. In resting cells, NF-κB is sequestered in the cytoplasm in an inactive form by association with the inhibitory proteins of the inhibitor of kappa B (IκB) family, such as IκBα, IκBβ, IκBγ, IκBζ and molecule possessing ankyrin-repeats induced by LPS (MAIL). Of these isoforms, IκBα is most abundant and constitutively expressed, whereas IκBζ and MAIL are induced by LPS and pro-inflammatory cytokines. Exposure to a variety of stimuli, including LPS, results in activation of the IκB kinase complex (IKK), which in turn causes rapid phosphorylation and degradation of IκBs. Subsequently, released NF-κB translocates into the nucleus, where it binds to its cognate DNA sequence, resulting in the transcription of multiple inflammatory genes.[69]

The finding that ketamine suppresses the LPS-induced cytokine production in e-CAS cells could, in principle, be attributed to the impairment of either extracellular, intracellular or intranuclear signalling molecules or any combination thereof. Hence, we studied in a serial approach the different phases of LPS signalling, including the effect of ketamine on LPS-binding to TLR4 (extracellular), on the MAPK pathway (intracellular) and on NF-κB (intranuclear) activation (**Chapter IV**). Results show that ketamine significantly inhibited the LPS-induced signal transduction cascade only at the level of NF-κB. Activation of NF-κB can be divided grossly into two phases: a first phase comprising the dissociation of NF-κB from IκB, and a second phase that involves various additional regulatory mechanisms, such as nuclear uptake and export, and affinity to the DNA

responsive element that determines the final strength and duration of the NF-κB transcriptional response.[69-72] Western blot analysis indicated that, in contrast to previous in vitro studies in rodents,[73,74] from our e-CAS cells experiments no influence of ketamine on IκB could be detected. Hence, the results described in Chapter IV suggest that ketamine inhibits NF-κB expression by affecting the regulatory events occurring in the second phase. This is supported by the finding that ketamine also inhibited NF-κB expression when added directly to the nuclear extract. At present, this is the first report in which reduced NF-κB expression following ketamine exposure could be ascribed to a direct interaction between NF-κB and ketamine.

**EFFECT OF KETAMINE ON INDUCIBLE ENZYMES**

NF-κB is known to be involved in the transcription of enzymes such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS),[67,75] that account for many of the clinical symptoms associated with the inflammatory response. Since ketamine directly inhibits NF-κB expression, it was hypothesised that ketamine might also reduce COX-2 and iNOS expression and subsequently inhibit the production of prostaglandin $E_2$ ($PGE_2$) and nitric oxide (NO).

However, in e-CAS cells ketamine did not reduce COX-2 expression, not even following exposure to concentrations up to 1000 μM (**Chapter VI**). Since these results were unexpected to some extent, we additionally investigated the molecular mechanism underlying LPS-induced COX-2 expression by selectively inhibiting the JNK, p38 and ERK, as well as the NF-κB pathway. We found that COX-2 expression was only regulated by the three MAPKs, as their inhibition resulted also in the inhibition of COX-2 expression. Inhibition of NF-κB did not reduce COX-2 expression. This latter finding correlates with findings by Wadleigh et al.,[76] who described that the transcriptional activation of COX-2 in LPS-stimulated RAW 264.7 cells is independent of NF-κB. Hence, the fact that ketamine inhibits the LPS-induced signalling pathway only at the level of NF-κB explains why ketamine did not reduce COX-2 expression and the subsequent $PGE_2$ production in LPS-treated e-CAS cells.

Similarly, ketamine did not reduce iNOS expression, neither at clinically relevant nor at high experimental concentrations (**Chapter V**). Corresponding to these results, no effect on LPS-induced NO production was found. However, the inhibition experiments in this study revealed that iNOS expression is regulated by the MAPKs as well as the NF-κB pathway. These results and the fact that ketamine

inhibits the LPS-induced signal transduction pathway only at the level of NF-κB suggests that MAPKs are more important in regulating iNOS expression than NF-κB. Moreover, these results show that MAPKs can regulate iNOS expression independently of NF-κB.

#### ANTI-OXIDATIVE EFFECTS OF KETAMINE

Next to LPS, NF-κB is known to be activated by various other stimuli, including reactive oxygen species (ROS).[77] This implies that blocking ROS formation should, theoretically, result in reduced activation of NF-κB and, consequently, reduced cytokine production. In stimulated e-CAS cells, ketamine significantly reduced ROS formation (**Chapter VII**), NF-κB expression (**Chapter IV**) and cytokine production (**Chapter III**) in a concentration-dependent manner. Although no data are available regarding the direct interaction between ROS formation and NF-κB expression in e-CAS cells, it is considered unlikely that the reduced NF-κB expression in e-CAS cells is caused by reduced ROS formation. LPS alone significantly induced NF-κB expression, but resulted only in a marginal increase in ROS formation. Only when e-CAS cells were co-stimulated with LPS and phorbol myristate acetate (PMA), a significant increased ROS formation was observed. However, the significant increase in NF-κB expression was not preceded by nor accompanied with an increased ROS formation. Expression of NF-κB activity was most significantly at 15 min of LPS exposure, whereas ROS formation only significantly increased following 2 h of stimulation. Hence, in e-CAS cells, ketamine reduced the LPS-induced NF-κB expression through a ROS-independent pathway.

#### LONG-TERM KETAMINE CRI AND ITS NON-ANAESTHETIC EFFECTS IN VIVO

The finding that ketamine reduced TNF-α and IL-6 production in LPS-treated e-CAS cells indicates that ketamine has cytokine-modulating effects in vivo. Considering the concentration-dependency 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 may need to be administered for a prolonged period of time and at relatively high dose rates to be clinically effective in modulating inflammatory mediators. During general anaesthesia relatively high dose rates can be used. However in the intensive care, when ketamine is administered to conscious horses, the risk of hypnotic and excitatory side effects limits the dose rate of the ketamine CRI. The results of **Chapter VIII**

show that a ketamine CRI of 1.5 mg/kg/h can safely be administered to conscious healthy horses for at least 6 h. The pharmacokinetic profile of this CRI was not very different from those previously reported in horses after single IV bolus administration.[58,78,79] In equine patients, in which pathophysiological conditions may lead to a different pharmacokinetic profile, delayed total body clearance of ketamine may result in increasing plasma concentrations and, subsequently, in ketamine-associated undesirable side effects. Administration of the ketamine CRI studied over more than six hours could potentially be associated with side effects, since in neither of the studies described in Chapter VIII and IX steady-state plasma levels were reached at the time the infusions were stopped.
Ketamine is metabolised extensively by the hepatic cytochrome $P_{450}$ enzyme system.[80,81] The major pathways involve N-demethylation to produce norketamine, which is then hydroxylated and oxidated to hydroxynorketamine and 5,6-dehydronorketamine, respectively. In both in vivo studies, all three metabolites were identified by using liquid chromatography-tandem mass spectrometry. This newly developed technique has been shown to be more accurate in detecting ketamine metabolites, in particular for 5,6-dehydronorketamine, when compared to the former technique of gas-liquid chromatography-mass-spectrometry.[82]

To evaluate the cytokine-modulating effects of ketamine in vivo, a tissue chamber model was used (**Chapter IX**). In this model the LPS-induced inflammatory response is confined to the tissue chamber and does not affect the overall clinical health status of the animal, thus meeting the modern ethical standards of animal experiments. As anticipated, LPS induced an inflammatory response characterised by an invasion of white blood cells and an increased concentration of pro-inflammatory mediators. However, when the tissue chamber fluid was analysed, ketamine has been shown not to reduce the LPS-induced production of TNF-α, IL-6 and $PGE_2$. This most likely is due to the low ketamine concentrations in the tissue chamber fluid. Following bolus injection and CRI of ketamine, only a limited fraction of ketamine entered the tissue chamber. This was considered to be associated with a very rapid elimination of ketamine ($t_{1/2\beta}$ = 65-67 min) in combination with a limited diffusion of ketamine into the tissue chambers. In addition, due to its low protein binding and as a weak base, ketamine has presumably not been trapped in the tissue chambers but rapidly redistributed. The low tissue fluid concentrations (0.5 μM) might explain the discrepancy found between the cytokine-modulating effects found in vitro (**Chapter III**) and in vivo

(**Chapter IX**), since in vitro LPS-induced TNF-α concentrations were only significantly suppressed by ketamine concentrations exceeding 1.8 μM.
Horses (Chapter VIII) and ponies (Chapter IX) did not significantly differ in the level of plasma concentrations of ketamine or norketamine measured during CRI. However, with respect to both hydroxynorketamine and dehydronorketamine plasma concentrations, significantly higher levels were measured in ponies than in horses during ketamine CRI. These findings most likely reflect the known species- and breed-differences in the activity of biotransformation enzymes found in other animal species.[83,84]

### CLINICAL RELEVANCE OF KETAMINE ADMINISTRATION TO HORSES SUFFERING FROM INFLAMMATORY DISEASES

The finding that ketamine significantly reduces TNF-α, IL-6 and ROS formation in stimulated e-CAS cells suggests that ketamine might be beneficial in horses suffering from inflammatory disorders. The rapid biotransformation and elimination of single ketamine injections as well as the limited affinity to proteins, preventing accumulation of the active compound in the inflamed tissue, indicate that ketamine does not have the favourable pharmacokinetic profile to suppress a systemic inflammatory response, as seen in patients with gastrointestinal disorders and endotoxaemia. However, in these patients, the observed anti-inflammatory properties of ketamine can be regarded as a favourable side effect following its application as anaesthetic and analgesic agent during surgery and in the post-operative intensive care.

### MAIN CONCLUSIONS

The major conclusions of this thesis can be summarised as follows:

- Ketamine significantly inhibits the LPS-induced TNF-α and IL-6 production in an equine macrophage cell line in a dose-dependent manner (Chapter III).
- Ketamine inhibits the LPS-induced intracellular signal transduction pathways only at the level of NF-κB (Chapter IV).
- Ketamine inhibits NF-κB expression through a direct interaction between ketamine and NF-κB (Chapter IV).
- Ketamine does not reduce the MAPK and NF-κB-mediated iNOS expression and the subsequent NO production in an equine macrophage cell line following LPS exposure (Chapter V).

- Expression of COX-2 in an equine macrophage cell line is predominantly regulated via the three classical MAPK pathways without a major contribution of NF-κB (Chapter VI).
- Ketamine does not reduce the MAPK-mediated COX-2 expression and the subsequent $PGE_2$ production in an equine macrophage cell line following LPS exposure, which implies also that the analgesic effects of ketamine are not mediated by COX-2 expression (Chapter VI).
- Ketamine reduces ROS formation in an equine macrophage cell line (Chapter VII).
- Ketamine increases the intracellular GSH concentrations, but does not induce GSH synthesis (Chapter VII).
- A ketamine CRI of 1.5 mg/kg/h can safely be administered to healthy conscious horses for at least 6 hours (Chapter VIII).
- Ketamine is rapidly metabolised into norketamine, hydroxynorketamine and 5,6-dehydronorketamine (Chapter VIII).
- Ketamine does not exhibit cytokine-modulating or antioxidative properties in LPS-inoculated tissue chambers in Shetland ponies (Chapter IX).

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