Mechanisms of neuro- and cytotoxicity of local anesthetics and their adjuvants
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Ketamine induces apoptosis via the mitochondrial pathway in human lymphocytes and neuronal cells

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Abstract

Background: Ketamine has been shown to have neurotoxic properties, when administered neuraxially. The mechanism of this local toxicity is still unknown. Therefore, we investigated the mechanism of cytotoxicity in different human cell lines in vitro.

Methods: We incubated the following cell types for 24 h with increasing concentrations of S(+)-ketamine and racemic ketamine: (1) human Jurkat T-lymphoma cells overexpressing the antiapoptotic B-cell lymphoma 2 protein, (2) cells deficient of caspase 9, caspase 8, or Fas-associated protein with death domain and parental cells, as well as (3) neuroblastoma cells (SHEP). N-methyl-D-aspartate receptors and caspase 3 cleavage were identified by immunoblotting. Cell viability and apoptotic cell death were evaluated flowcytometrically by annexin V and 7-AAD double staining. Mitochondrial metabolic activity and caspase 3 activation were measured.

Results: Ketamine concentration-dependently induced apoptosis in lymphocytes and neuroblastoma cell lines. Cell lines with alterations of the mitochondrial pathway of apoptosis were protected against ketamine-induced apoptosis, whereas alterations of the death receptor pathway did not reduce apoptosis. S(+)-ketamine and racemic ketamine induced the same percentage of cell death in Jurkat cells, whereas in neuroblastoma cells S(+)-ketamine was slightly less toxic.

Conclusions: Ketamine at millimolar concentrations induces apoptosis via the mitochondrial pathway, independent of death receptor signaling. At higher concentrations necrosis is the predominant mechanism. Less toxicity of S(+)-ketamine was observed in neuroblastoma cells, but this difference was minor and therefore unlikely to be mediated via the NMDA receptor.
Ketamine, a noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist, is administered epidurally and intrathecally for the treatment of postoperative, chronic cancer pain and neuropathic pain, respectively.\textsuperscript{1,2} Despite this considerable and promising clinical experience there is concern about the possible toxicity of ketamine when applied next to neural structures. Ketamine, S\textsuperscript{(+)}-ketamine and other NMDA antagonists have been shown to induce neurotoxicity when applied intrathecally over days and weeks.\textsuperscript{3-5} Applied intrathecally, ketamine damages white and gray matter of the spinal cord, most lesions being found subpial and around the spinal canal in animal models and patients. Surprisingly, neither animals nor patients had any detectable loss of function. Histopathologically, signs of chromatolysis were detected after long-term application of preservative-free ketamine.\textsuperscript{5,6} These lesions were recognized as possible retrograde degeneration after a distal axonal lesion or widespread demyelination. Chromatolysis can possibly be a late sign of apoptosis, i.e. programmed cell death, but so far the mechanism of local toxicity induced by ketamine is unknown.

Apoptosis is regulated by a cascade of specialized proteases called caspases. Caspase 3 is activated late in the apoptosis cascade, whereas caspase 8 and 9 are activated at an early stage of apoptosis. Caspase 9 is the central caspase of the mitochondrial signaling pathway, while caspase 8 is essential for death receptor-induced apoptosis. Both pathways converge at the activation of caspase 3 which cleaves more downstream effectors, finally leading to the typical morphological alterations of apoptosis.\textsuperscript{7}

The pathways of apoptosis are delineated in a simplified scheme in figure 1. The mitochondrial pathway of apoptosis is activated by permeabilization of the outer mitochondrial membrane. After permeabilization several apoptogenic factors are released from the mitochondrial intermembrane space, e.g. cytochrome c. Cytochrome c together with caspase 9 induces the formation of the apoptosome, a high-molecular weight complex, which then activates caspase 3 and therewith the common pathway of apoptosis.\textsuperscript{7}

The extrinsic pathway is activated by death receptors. These are specialized cell-surface receptors including Fas/CD95 and tumor necrosis factor-\textalpha related apoptosis-inducing ligand receptors. Activation of this pathway induces the formation of the death inducing signal complex including FADD which then leads via activation of
caspase 8 to cleavage of caspase 3 and induction of the common pathway of apoptosis.\textsuperscript{7}

Figure 1:

**Fig 1: The two major pathways of apoptosis.** The intrinsic or mitochondrial pathway of apoptosis (left side) involves mitochondrial dysfunction, release of cytochrome c (cyt c) and the subsequent activation of caspase-9 (casp-9) at the apoptosome. The antiapoptotic protein Bcl-2 inhibits the release of cytochrome c from the mitochondrion.

The extrinsic or death receptor pathway (right side) is initiated by binding of death ligands to the death receptor and subsequent recruitment of the adapter protein Fas-associated protein with death domain (FADD) and caspase-8 (casp-8) into the death-inducing signaling complex (DISC). Both apoptosis pathways converge at the activation of effector caspase-3 (casp-3), which cleaves several cellular proteins, finally leading to the typical alterations of apoptosis such as DNA fragmentation in the nucleus.
To study the mechanism of ketamine toxicity on a cellular and subcellular level, nonneuronal and neuronal cell lines expressing the NMDA-receptor were investigated. We first evaluated, employing different methods, whether ketamine induces apoptosis. In a lymphoma cell line with genetically modified pathways of apoptosis we determined whether ketamine-induced apoptosis is mediated via the mitochondrial or the death receptor pathway. In neuroblastoma cells the effect of a caspase-inhibitor on ketamine-induced cell death was studied. In neuronal cells the stereospecific effects of ketamine were elucidated by comparing the apoptosis induced by S(+)-ketamine as compared with its racemate. Finally, we analysed the apoptosis induced by incubation (five days) with ketamine in neuroblastoma cells.

**Material and Methods**

All experiments were performed at the laboratory of the Department of Anaesthesiology of the University of Düsseldorf.

**Reagents**

Ketamine and S(+)-ketamine were purchased from Sigma Aldrich (St. Louis, MO) as a pure hydrochloride salt without preservatives. The pancaspase inhibitor Q-VD was purchased from Calbiochem (San Diego, CA). The fluorescent probe annexin V–FITC conjugate and the FITC-labeled anti-caspase 3-antibody were obtained from BD Biosciences (San Diego, CA). The NMDA receptor 1-antibody and the rabbit polyclonal anti-caspase 3 antibody were purchased from Cell Signaling (Cell Signaling Technology/New England Biolabs GmbH, Frankfurt am Main, Germany). A goat anti-rabbit IgG conjugated to horseradish peroxidise was used as secondary antibody (Dianova, Hamburg, Germany). XTT sodium salt was purchased from Sigma Aldrich (St. Louis, MO). Phosphate buffered saline (PBS) without calcium and magnesium was obtained from Gibco, Invitrogen (Carlsbad, CA). Trypsin/EDTA was purchased from Biochrom AG (Berlin, Germany). Reagents not mentioned above were purchased from Sigma Aldrich (St. Louis, MO).

**Cell Culture**

Jurkat cells stably overexpressing Bcl-2 and the corresponding wild-type cells (clone J16) have been described before. Caspase 9 deficient (clone JMR) and proficient Jurkat cells have been characterized before. John Blenis Ph.D. (Department of Cell Biology, Harvard Medical School, Boston, Massachusetts) kindly provided
FADD– and caspase 8 deficient Jurkat cells and the parental cell line (clone A3). The characteristics and origin of human SHEP neuroblastoma cells have been described before. 7, 11 Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine and 50 U ml⁻¹ penicillin and 50 µg ml⁻¹ streptomycin, was used as culture medium for all cell lines. Cells were cultured in a humidified atmosphere containing 5% carbon dioxide at 37°C.

**Exposure to Ketamine and S(+) -Ketamine and Experimental Protocol**

In order to allow logarithmic growth, Jurkat and neuroblastoma (SHEP) cells were cultured overnight in complete medium at a concentration of 4 x 10⁵ cells/ml. Subsequently, cells were cultured with fresh medium alone (as negative control), with the proapoptotic kinase inhibitor staurosporine (as positive control), or with indicated concentrations of racemic ketamine or S(+) -ketamine. When indicated, the pancaspase inhibitor Q-VD (10 µM) was added to cell cultures 1 h before the addition of ketamine.

**Detection of Early Apoptosis**

Double staining of cells with annexin V and 7-aminoactinomycin D (7-AAD) allows estimating the percentage of cells in the early phase of apoptosis and the fraction of cells already in a late apoptotic or necrotic state in the same sample. Detection of intracellular 7-AAD indicates late apoptosis or necrosis in stained cells, while cells only staining positive for annexin V, but not for 7-AAD are defined as early apoptotic. Cells were washed twice with cold PBS. Adherent neuroblastoma cells were detached by 5 min incubation with trypsin/EDTA 0.05% at 37°C. Then, samples were resuspended at a concentration of 1 x 10⁶ cells/ml in annexin V binding buffer. After 15 min incubation at room temperature with 5 µl annexin V and 5 µl 7-AAD (5 µg ml⁻¹) additional 200µl annexin V binding buffer was added and samples were measured with a FACScalibur flowcytometer (Becton Dickinson, Heidelberg, Germany) using CellQuest Pro software. Every measurement includes 10.000 cells.

**Detection of Caspase 3 Activity**

Caspase 3 is one of the key proteases in early apoptosis. To determine caspase 3 activity after incubation with ketamine, the cells were fixed with 4% paraformaldehyde, washed twice with PBS and incubated with 3% BSA, 0.05% Saponin in PBS and 20 µl of the FITC-labeled anti-caspase 3-antibody for 1 h protected against light. Next, cells were washed once with PBS supplemented with 2% BSA, resuspended in PBS and analyzed flowcytometrically.
**Statistical Analysis**

All experiments were performed at least in triplicates. Results are expressed as mean ± SD. All calculations were performed with the SPSS program version 16.0 (SPSS Inc., Chicago, IL). Comparisons between groups were made by Mann–Whitney U test. \( P < 0.05 \) was considered significant.

**Results**

Western blot analysis revealed that T-lymphoma cells (Jurkat) and human neuroblastoma cells (SHEP) expressed the NMDA receptor 1 (see online version). Jurkat T-lymphoma cells were investigated for percentage of early apoptotic and late apoptotic or necrotic cell death after 24 h treatment with ketamine. Flowcytometric analysis revealed a concentration-dependent toxicity of ketamine at millimolar concentrations leading to early apoptotic (annexin V positive/7-AAD negative) and late apoptotic or necrotic (annexin V positive/7-AAD positive) cell death (fig. 2A). Thus, 3 mM ketamine induced 65.9 ± 5.1% cell death, whereas the negative control only had a percentage 5.9 ± 1.3% and the positive control (STS) a percentage of 86.9 ± 2.6% cell death (p<0.05).

In order to determine possible involvement of the mitochondrial pathway of apoptosis in ketamine induced apoptosis, Jurkat cell clones either overexpressing B-cell-lymphoma 2 protein (Bcl-2) or deficient for caspase 9 were exposed to ketamine. These genetically engineered cell lines with an altered mitochondrial pathway of apoptosis were protected against the apoptosis-inducing effects of ketamine (fig. 2B). 2 mM ketamine induced 43.2 ± 1.9% cell death in wild type cells but led only to 11.0 ± 2.9% cell death in cells overexpressing Bcl-2. Interestingly, cells deficient in caspase 9 were completely protected (4.1 ± 0.6% cell death) against the apoptosis-inducing effect of ketamine at the same concentration (fig. 2B). Thus, inhibition of the mitochondrial pathway of apoptosis protected to a considerable extent against ketamine-induced apoptosis. The influence of the death receptor pathway of apoptosis was investigated in Jurkat cell lines deficient of caspase 8 and FADD, an intracellular adapter molecule of the death receptor. Caspase 8 deficient cells and FADD deficient cells were not significantly protected against ketamine toxicity (fig. 2C). Thus, ketamine induces cell death via the mitochondrial pathway without significant death receptor signaling.
Figure 2: Cell survival of Jurkat T-lymphoma cells after 24 h exposure was measured by flowcytometry revealing the percentages of overall cell death as well as early apoptotic cells (annexin V/7-AAD +/-) and late apoptotic or necrotic cells (annexin V/7-AAD +/+). (A) Concentration-dependent toxicity and apoptosis induction by ketamine in Jurkat T-lymphoma cells. Staurosporine (sts) was used as positive control. (B) Wilde-type (wt), Bcl-2 overexpressing (Bcl2+) and caspase 9 deficient (cas9-) cells were exposed to control medium (left) or 2 mM ketamine (right). (C) Wilde-type (wt), caspase 8 deficient (cas8-) and FADD deficient (FADD-) cells were exposed to control medium (left) or 2 mM ketamine (right).

Data are presented as mean ± standard deviation. * denotes p<0.05 compared to negative control, n.s. = not significant (n = 3).
As a model of cells of neuroectodermal origin, we investigated neuroblastoma cells. As in Jurkat cells, apoptosis and overall cell death were analyzed by flowcytometry in SHEP neuroblastoma cells exposed 24 h to increasing concentrations of ketamine. Ketamine induced concentration-dependent toxicity between 2 and 8 mM (fig. 3A). At 2 and 4 mM the percentage of cells undergoing early apoptosis (annexinV positive/7-AAD negative) was 9.4 ± 3.0 and 14.2 ± 0.1%, respectively, whereas with 6 and 8 mM ketamine induced only 8.0 ± 2.2% and 2.6 ± 0.3%, respectively.

**Figure 3:**

![Graph A](image)

**Fig 3:** Apoptosis induction in neuroblastoma cells (SHEP) after 24 h exposure to negative control, staurosporine (sts) as positive control and increasing concentrations of ketamine. In (A) apoptosis induction was measured by flowcytometry revealing the percentages of overall cell death as well as early apoptotic cells (annexin V/7-AAD +/-) and late apoptotic or necrotic cells (annexin V/7-AAD +/ +). In panel (B) activation of caspase 3 was measured as a marker for apoptosis by flowcytometry. Data are presented as mean ± standard deviation. * denotes p<0.05 compared to the negative control (n = 3).
These results were verified by analyzing caspase 3 activity after treatment of cells with different ketamine concentrations (fig. 3B). Caspase 3 activity increased after treatment with 2 mM (4.6 ± 0.8%) to 8 mM ketamine (35.2 ± 7.4%), whereas 12 mM led to a decrease in caspase 3 activity (11.1 ± 3.3%). These results were confirmed by immunoblotting (see online version).

The pancaspase inhibitor (Q-VD) reduced the percentage of cell death induced by 4 mM ketamine from 45.9 ± 2.4% to 8.2 ± 1.9% (p<0.05). The relative effect size of Q-VD was less at greater concentrations (fig. 4). Thus, 8 mM ketamine led to 90.1 ± 1.2% cell death and addition of Q-VD reduced this percentage to only 59.4 ± 3.3% (p<0.05). Thus, ketamine at low concentrations predominantly induced apoptosis whereas at higher concentrations necrosis predominated.

**Figure 4:**

**Figure 4:** Apoptosis induction in neuroblastoma cells (SHEP) after 24 h exposure to negative control, staurosporine (sts) as positive control and increasing concentrations of ketamine with or without the pancaspase-inhibitor Q-VD (10 µM). Flowcytometry revealing the percentages of overall cell death as well as early apoptotic cells (annexin V/7-AAD +/-) and late apoptotic or necrotic cells (annexin V/7-AAD +/+). Data are presented as mean ± standard deviation. * denotes p<0.05.

Next, we investigated whether the same toxic effects occur with lower concentrations and longer exposure times. Thus, neuroblastoma cells were exposed for up to 120 h to various concentrations of ketamine. The lowest concentration inducing a significant reduction in mitochondrial activity and thus viability of the cells was 400 µM, reducing...
mitochondrial activity by $14.9 \pm 4.3\%$ (p<0.01). This effect was concentration dependent, with a maximum reduction of mitochondrial activity of $39.4 \pm 6.5\%$ at $1600 \mu M$ (p<0.001, for detail see online version). Thus, the toxicity of ketamine depends not only on the concentration, but also on exposure time.

In order to investigate whether the observed toxicity may be related to a stereospecific effect of ketamine on the NMDA receptor, we compared racemic ketamine with its enantiomer S(+)-ketamine in Jurkat cells, as well as in neuroblastoma cells, by means of Annexin V/7-AAD flowcytometry. In Jurkat cell S(+)-ketamine and the racemate induced exactly the same toxicity (fig. 5A), whereas in neuroblastoma cells S(+)-ketamine was slightly less toxic than the racemate (fig. 5B). The difference in effect of the enantiomers was rather small. The maximum difference in cell death was observed at a concentration of 2 mM. At this concentration S(+)-ketamine induced $9.1 \pm 0.6\%$ cell death, whereas racemic ketamine induce $18.3 \pm 12.4\%$ cell death (p<0.05).

**Figure 5:**

![Figure 5](image.png)

**Fig 5:** Comparison of the neurotoxicity of S(+) -ketamine and its racemate in equimolar concentrations after 24h exposure in Jurkat T-lymphoma cells (A) and SHEP neuroblastoma cells (B) measured by flowcytometry revealing the percentages of overall cell death as well as early apoptotic cells (annexin V/7-AAD +/-) and late apoptotic or necrotic cells (annexin V/7-AAD +/+). Data are presented as mean ± standard deviation. * denotes p<0.05 (n=3).
Discussion

Our results indicate that ketamine at low millimolar concentrations induces apoptosis in non-neuronal and neuronal cells, whereas higher concentrations predominantly lead to necrosis. Apoptosis induction by ketamine is mediated via the mitochondrial pathway and independent of death receptor signaling. Ketamine-induced apoptosis is concentration- as well as time-dependent and can be almost completely prevented by caspase inhibition. Finally, the apoptosis-inducing effect of ketamine is not or marginally stereospecific, making an involvement of the NMDA receptor unlikely.

Although the potential neurotoxicity of ketamine after application close to neural structures has been discussed, the preservative chlorbutanol was identified as the main toxic agent after single application. Nevertheless, after repeated applications even preservative-free ketamine displayed at least morphological damage, whereas alterations in nerve function were not investigated systematically. Recently, it has been demonstrated that preservative-free S(+)-ketamine applied intrathecally in rabbits also leads to severe histopathologic damage without any functional deficits. Similar histopathologic results, but without functional neurological deficit, have been described after long-term intrathecal application of ketamine in case reports of patients with otherwise unbearable pain.

The locations of the lesions induced by ketamine have been described in detail, and are predominantly subependymal and around the central canal, where presumably the neural structures were exposed to the highest concentrations of ketamine. The morphological features are most frequently reported in terms of demyelination and necrosis. Recently, chromatolysis has been found after intrathecal application of ketamine. Chromatolysis is a late, but not pathognomonic, morphologic sign of apoptosis. In our in vitro model, we demonstrated that ketamine concentration- and time-dependently induces apoptosis and, at higher concentrations, necrosis. Thus, the caspase 3 activity firstly increased concentration-dependently and decreased with the highest concentrations tested (fig. 3B), although the overall toxicity increased further with higher concentrations (fig. 3A and 4). Furthermore, the protective effect of a caspase inhibitor diminished also with higher concentrations (fig. 4). So the mechanism of cell death changes with increasing concentrations from apoptosis to necrosis. This phenomenon is not unusual, e.g. the same effect is also seen with the local anaesthetic lidocaine. Similar to findings using lidocaine our results indicate that apoptosis induced by ketamine is mediated via the mitochondrial pathway and is
independent of death receptor signaling. Yon and colleagues showed that apoptosis induced by systemic application of ketamine in neonatal rats is mediated via the mitochondrial as well as the death receptor pathway.\textsuperscript{15} That appears to contradict the results presented here, but the presumable plasma concentrations of ketamine reached in neonatal animals are certainly far below those occurring after local application. Furthermore, in the neonatal animals ketamine when given systemically induces neuroapoptosis via the NMDA-receptor\textsuperscript{16} whereas in our model it seems independent of NMDA-receptor. But the neurotoxic effects of ketamine observed in neonatal neurons have another pathomechanism, since they are mediated via the NMDA receptor and are induced with concentration about a hundred times lower than in our model. Our results were observed in dedifferentiated tumor cells and not during the physiologic spurt of apoptosis and synaptogenesis as in neonatal animal models. Thus, the pathway of toxicity revealed here is presumably different from the apoptosis seen in the brain after systemic application of general anaesthetics.

Interestingly, Lee et al. found that in human hepatoma cell cultures ketamine also in millimolar concentrations induced incorporation of Bax proteins into the mitochondrial membrane, cytochrome C release, caspase activation and finally apoptosis.\textsuperscript{17} Thus also in this non-neuronal cells ketamine induced apoptosis via the mitochondrial pathway.

The difference in toxicity of the ketamine enantiomers in neuroblastoma cells was - although significant - fairly minor. Presumably, this does not reflect the several-fold difference in potency at the phencyclidine binding site of the NMDA receptor.\textsuperscript{18} Thus, the local toxicity of ketamine observed here is unlikely to be mediated via the NMDA receptor.

Clinically, ketamine is used epidurally in combination with an opioid or a local anaesthetic at concentrations around 0.4 mg/ml (\approx 1.5 mM) over several days.\textsuperscript{19-24} In patients with chronic pain it has been used intrathecally at concentrations up to 25 mg/ml (\approx 93 mM) over weeks and months.\textsuperscript{6,25,26} Unfortunately, ketamine concentrations occurring epidurally or intrathecally during these applications are not known. Nevertheless, in clinical practice ketamine is administered intrathecally at concentrations about a hundred times greater than those already inducing significant toxicity in our \textit{in vitro} model.

However, the results presented should not be extrapolated to the clinical situation. Nevertheless, it is important to know that ketamine and lidocaine (and other local
anaesthetics) induce apoptosis via the same mitochondrial pathway.\textsuperscript{7,11} Thus, an additive effect might be presumed when they are used in combination. However, all available evidence from \textit{in vitro}, \textit{in vivo} and patient studies suggests that caution and good clinical judgment is warranted when applying high concentrations of ketamine intrathecally or epidurally over a long period of time. More safety studies should be performed so that the margin of safety for use of neuraxially administered ketamine can be definitively determined.

In conclusion, ketamine concentration-dependently and time-dependently induces apoptosis and necrosis \textit{in vitro}. Apoptosis is induced via the mitochondrial pathway, independent of death receptor signaling. This toxicity is not or only minimally stereoselective and therefore unlikely to be mediated via the NMDA receptor.

\textbf{Declaration of interests:}

No one of the authors had any conflict of interest in the last five years related to the present investigation.

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