Ketamine and thiopental sodium: individual and combined neuroprotective effects on cortical cultures exposed to NMDA or nitric oxide

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Background. An N-methyl-D-aspartate (NMDA) blocker, ketamine, has been shown to be neuroprotective both in vivo and in vitro. However, ketamine is not commonly recommended for use in patients suffering from cerebral ischaemia because of its adverse neurological effects. We hypothesized that combined administration of ketamine and thiopental sodium (TPS) would be highly effective in protecting cerebral cortical neurones from ischaemia, with possibly reduced dosages.

Methods. We examined the degree of neuroprotection provided by various concentrations of ketamine and TPS, alone and in combination, in cortical cultures exposed to NMDA or a nitric oxide-releasing compound (NOC-5) for 24 h. The survival rate (SR) of E16 Wistar rat cortical neurones was evaluated using photomicrographs before and after exposure to these compounds.

Results. The SRs of cortical neurones exposed to 30 μM NMDA or NOC-5 were 15.0 (3.8)%, 12.8 (3.1)%, respectively. Higher doses (5, 10 and 50 μM) but not lower doses (<1 μM) of ketamine improved SRs [57.9 (2.2)%, 61.1 (5.4)%, 76.7 (3.0)%, respectively] against NMDA but not NOC. Enhanced survival was observed with combined administration of 5 or 10 μM ketamine and 50 μM TPS [SR 71.3 (4.8)%, 74.7 (3.7)%, respectively, P<0.05 if ketamine alone, P<0.01 if TPS alone], against NMDA-induced neurotoxicity in vitro. Only the highest dose of TPS (50 μM) improved survival after NOC exposure. This neuroprotection was not influenced by ketamine.

Conclusions. These data indicate that a low, clinically relevant dose of ketamine offer significant neuroprotection during prolonged exposure to NMDA but not to NOC. Combinations of reduced doses of ketamine and TPS exhibited enhanced neuroprotection against NMDA-induced neurotoxicity. Hence, combinations of these two common i.v. anaesthetics agents could be developed to protect the brain from ischaemia.

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nitrous oxide. However, ketamine is not commonly recommended for use in critically ill patients as it is still believed that ketamine exhibits undesirable effects on cerebral blood flow (CBF), cerebral metabolic rate (CMR) and intracranial pressure (ICP). 20-22

On the other hand, thiopental sodium (TPS) is widely used in neurosurgical and cardiac operations for cerebral protection. As we reported previously, a high dose of TPS exhibited significant neuroprotection against an NO donor or prolonged hypoxic episode through the free radical scavenging effects of the TPS sulphydryl group (SH). 7-9 TPS also decreases both CMR and ICP and inhibits both voltage-gated calcium channels and NMDA receptors. 23-25 However, in order to exert this neuroprotective effect, extremely high doses of the barbiturate are necessary, which can elicit undesirable effects, such as circulatory and respiratory depression. 26 In contrast, at doses used to induce anaesthesia, ketamine can increase arterial pressure, heart rate and cardiac output. We hypothesized that combined administration of ketamine and TPS, even at minimal dose regimens would be highly effective in protecting the brain from cerebral ischaemia.

The aim of this study was to compare the neuroprotective effects of various concentrations of ketamine and TPS, individually and in combination, against NMDA- or NO-mediated cytotoxicity in primary cultures of rat cerebral cortical neurones.

Methods

Chemical reagents

The chemicals used in this study were obtained from the following sources: Dulbecco’s modified Eagle’s medium (DMEM) from Nissui Pharmaceutical (Tokyo, Japan); NMDA, ketamine, 5-fluoro-2-deoxyuridine (5-FU), poly-L-lysine, streptomycin and penicillin, anti-microtubule-associated protein 2 (MAP2) were obtained from Sigma-Aldrich (St Louis, MO, USA); trypsin from Difco Lab (Detroit, MI); fetal calf serum (FCS) from ICN Biochemicals (Costa Mesa, CA, USA); horse serum (HS) from Gibco BRL (Carlsbad, CA, USA); TPS from Tanabe Seiyaku Co Ltd (Osaka, Japan); NOC-5 (1-hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene) from Dojindo (Kumamoto, Japan); anti-glial fibrillary acidic protein (GFAP) antibody from Dako (Carpinteria, CA, USA).

Cell culture

All animals were treated in strict accordance with the institutional and NIH guidelines for the care and treatment of laboratory animals. The study protocol was approved by the Animal Care Committee at the Osaka University Graduate School of Medicine.

Primary cultures of cortical neurones were prepared as described previously. 27 In brief, rat fetuses were removed at embryonic day 16 from anaesthetized pregnant Wistar rats, which were obtained from Nihon SLC (Hamamatsu, Japan). The brains were isolated under a microscope. Cerebral cortical neurones were treated with 0.25% trypsin at 37°C for 20 min, and triturated with a Pasteur pipette. Dispersed cells were diluted to a concentration of 1 × 10⁶ cells ml⁻¹ in DMEM with 8% FCS and 4% HS, 50 μg ml⁻¹ streptomycin and 50 μg ml⁻¹ penicillin. This suspension was put onto poly-L-lysine coated 35 mm diameter/2 mm grid tissue culture dishes (1.5 ml per well) (Nunc, Naperville, IL, USA). We used the grid tissue culture dishes to observe the same neurones in a given area over time, as described previously. 27

After 4 days in culture, the cells were treated with 5 μg ml⁻¹ of 5-FU for 3 days to prevent proliferation of non-neuronal cells. The neurones were maintained in DMEM containing 8% FCS and 4% HS in 5% CO₂, 95% air and under 100% humidity at 37°C. The medium was changed twice weekly. All subsequent experiments were carried out after 13–14 days in culture.

Immunohistochemical assessment

In order to confirm the purity of the neuronal culture, cells were immunostained with anti-MAP2 or anti-GFAP antibody, before and after the experiment. Greater than 97% and less than 2% of the cells expressed MAP2 and GFAP, respectively, irrespective of the duration of the experiments, demonstrating that most of the cells in our cultures were neurones. 28

Cytotoxicity

Neurotoxicity was investigated using Shibuta’s model, as described previously. 8, 27 NMDA and NOC were used as neurotoxic agents. In particular, NOC is considered one of the best of the currently available NO-releasing compounds that has distinct advantages over other NO donors. NOC constantly releases large quantities of NO through a simple mechanism without the production of any other metabolites. 7, 8, 29-31 According to the manufacturer’s instructions, the half-life of this NO donor in phosphate-buffer saline (PBS) at pH 7.4 and 37°C is 25 min. One mol of NOC-5 releases 2 mol of NO. NOC-5, 30 μM, elicited approximately 90% neuronal cell death in cortical neurones, 24 h after administration, in our previous study. 7

To evaluate the neuroprotective effects of the i.v. anaesthetics, three to four photomicrographs were taken of each well shortly before the administration of NMDA or NOC-5, and at the end of each experiment. Cells in all treatment groups were stained with 0.4% trypan blue in PBS to assess cell viability, 24 h after the administration of NMDA or NOC-5. Photomicrographs were taken in the same areas before and after the administration of drugs, as identified by the grid arrangement of the dish. The stained and non-stained cells were counted by an observer who was blinded to the arrangement of photographs, study design or treatment protocol, with approximately 500–1000 cells counted per well. Survival rates (SR) were calculated according to the following formula: 100 × (the number of non-stained...
cells at the end of the experiment)/(the number of whole cells shortly before the administration of the drugs). The cells were exposed to the drugs, as follows: (i) to evaluate the role of ketamine on NMDA or NO-induced neurotoxicity, neurones were exposed to various concentrations of ketamine (from 50 nM to 50 µM) with 30 µM NMDA or NOC-5. In control experiments, 50 µM ketamine was administered without NMDA or NOC-5. (ii) To assess the influence of TPS on NMDA-induced neurotoxicity, neurones were exposed to various concentrations of TPS (from 50 nM to 200 µM) with 30 µM NMDA or NOC-5. In control experiments, we exposed the neurones to 50 µM TPS without NMDA or NOC-5. (iii) To evaluate the effect of combined administration of ketamine and TPS against the cytotoxicity induced by NMDA or NOC-5, these two i.v. anaesthetic agents were administered at various concentrations along with 30 µM NMDA or NOC-5. In our previous study, approximately 90% neuronal cell death was induced in cortical neurones after 24 h by 30 µM NMDA or NOC-5. Therefore, in this study, we induced neurotoxicity using the same concentration of NMDA or NOC-5.

**Statistical analysis**

The results are expressed as the mean (SEM)%. The differences between the means were assessed using analysis of variance (ANOVA) followed by Fisher’s PLSD test with P-values <0.05 considered significant. Regression analysis was used to calculate the slope and 95% confidence intervals of concentration-response curves for TK and ketamine, respectively, using Statcel (OMS Publishing Inc., Saitama, Japan). We assessed the interaction using a classic isobolographic analysis. The effective dose values for the neuroprotective effects of the i.v. anaesthetics were calculated from the dose–response curves.

**Results**

The neuroprotective effect of ketamine on NMDA- or NO-induced cytotoxicity

The SR of neurones exposed to ketamine, to protect against NMDA or NOC-induced cytotoxicity, were assessed in 10 groups, comprising a total of 102 cortical culture dishes. The mean SR of the neurones was 15.0 (3.8)%. When neurones were exposed to lower concentrations of ketamine (50, 500 nM and 1 µM), the SRs of the neurones did not improve. However, significant neuroprotection was achieved with higher concentrations of ketamine (5, 10 and 50 µM) [SRs of 57.9 (2.2)%, 61.1 (5.4)%, 76.7 (3.0)%; respectively; P<0.01 to NMDA alone] as shown in Figures 1A and 2. Ketamine did not attenuate the neurotoxicity that was elicited by 30 µM NOC-5, as shown in Figure 1B. While the SR of cortical neurones exposed to 30 µM NOC-5 was 12.8 (3.1)%,
SR of those exposed to 50 μM ketamine was 19.1 (4.0)%, as shown in Figure 4.

As a control, cortical neurones were exposed to 50 μM ketamine, without NMDA or NOC. There was no effect of ketamine on the SR of the neurones compared with unexposed neurones.

The neuroprotective effect of TPS on NMDA- or NO-induced cytotoxicity

The SRs of neurones exposed to TPS, to protect against NMDA or NOC-induced cytotoxicity, were assessed in 9 groups, comprising a total of 94 cortical culture dishes.

The SRs of neurones exposed to three lower concentrations of TPS, shortly before exposure to 30 μM NMDA, did not change. However, the higher concentration of TPS (50 μM) attenuated NMDA-elicited neurotoxicity [SR 36.7 (5.9)%], as shown in Figure 1A. Likewise, the highest dose of TPS (200 μM) attenuated NMDA-induced neurotoxicity [SR 51.0 (3.0)%]. Only the highest dose of TPS (50 μM), but not the three lower doses, exhibited a significant neuroprotective effect against 30 μM NOC-5, as shown in Figure 1B. The SR of the cortical neurones increased to 33.6 (5.8)% with TPS.

As a control, cortical neurones were exposed to 50 μM TPS, without NMDA or NOC. There was no effect of TPS on the SR of the neurones compared with unexposed neurones.

The SR of those exposed to 50 μM ketamine was 19.1 (4.0)%, as shown in Figure 4.

As a control, cortical neurones were exposed to 50 μM ketamine, without NMDA or NOC. There was no effect of ketamine on the SR of the neurones compared with unexposed neurones.

The neuroprotective effects of combined administration of ketamine and TPS on NMDA-cytotoxicity

The SRs of neurones exposed to combinations of TPS and ketamine, to protect against NMDA-induced cytotoxicity, were analysed in 24 groups, comprising a total of 216 cortical culture dishes.

Lower doses of ketamine (50, 500 nM and 1 μM) did not influence the neuroprotective effect of TPS against NMDA. Only the highest dose of TPS (50 μM), the SRs of the neurones were almost equivalent (approximately 75%) and were independent of the TPS concentrations.
Enhanced survival was observed with the combined administration of 5 \( \mu \text{M} \) ketamine and 50 \( \mu \text{M} \) TPS [SR 71.3 (4.8)%; \( P < 0.05 \) compared with 5 \( \mu \text{M} \) ketamine combined with 0, 50, 500 nM TPS and 50 \( \mu \text{M} \) TPS alone; which exhibited SRs of 57.9 (2.2)%%, 59.7 (3.9)%%, 56.7 (9.7)%%, 36.7 (5.9)%%, respectively] and 10 \( \mu \text{M} \) ketamine with 50 \( \mu \text{M} \) TPS [SR 74.7 (3.1)%; \( P < 0.05 \) compared with 10 \( \mu \text{M} \) ketamine combined with 0, 50, 500 nM TPS and 50 \( \mu \text{M} \) TPS alone; SRs of 61.1 (5.4)%%, 60.7 (5.8)%%, 61.2 (3.9)%%, 36.7 (5.9)%%, respectively], as shown in Figures 1A and 5.

We calculated the SR 71.3% (SR 71.3) and 74.7% (SR 74.7) for each drug. The SR 71.3 concentration for TPS and ketamine were 322 \( \mu \text{M} \) and 36.7 \( \mu \text{M} \), respectively. The SR 74.7 concentration for TPS and ketamine were 344 and 40.1 \( \mu \text{M} \), respectively. The isobolographic representations of the i.v. anaesthetics are shown in Figure 6.

**The neuroprotective effects of combined administration of ketamine and TPS on NOC-cytotoxicity**

The SRs of neurones exposed to combinations of TPS and ketamine, to protect against NOC-induced cytotoxicity, were analysed in 16 groups comprising a total of 152 cortical culture dishes.

Ketamine did not influence the neuroprotective effect of TPS against the NO donor. Only the highest dose of TPS (50 \( \mu \text{M} \)) exhibited significant neuroprotection against NOC-5, which was independent of the ketamine concentration. The SRs of neurones exposed to a combination of various concentrations of ketamine and 50 \( \mu \text{M} \) TPS were ~35% in all groups, and no significant intergroup differences were observed.

**Discussion**

In this study, the combined application of ketamine and TPS at low clinical concentrations exhibited enhanced protection of neurones against NMDA-induced neurotoxicity in vitro, compared with each of these agents alone. We successfully confirmed the optimal combinations of these two common i.v. anaesthetics to provide maximal neuroprotection.

Our study, using a cultured neurone system, provides direct evidence that ketamine is neuroprotective for CNS neurones against excess NMDA. Ketamine, a PCP receptor ligand, is considered a potential neuroprotective agent, as it

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**Fig 5** A clinical concentration of ketamine (5 \( \mu \text{M} \)) with a moderate concentration (50 \( \mu \text{M} \)) of TPS (a possible therapeutic combination) showed an enhanced SR [71.3 (4.8)%] against NMDA-induced neurotoxicity as the same concentration as the highest doses of these two i.v. anaesthetics [SR 79.7 (3.1)%; \( P = 0.2 \)]. Photomicrographs of neurones in cortical cultures shortly before the experiment with phase-contrast microscopy (A) and with transmitted light (B). After the experiment, dead neurones had disappeared or were stained with trypan blue at the same areas in the photomicrographs with phase-contrast microscopy (C) and with transmitted light (D). Arrows indicate the same viable neurone before and after the experiment. Scale bar = 100 \( \mu \text{m} \).

**Fig 6** Isobolographic representation of ketamine and TPS effect in combination. The line joining the SR 71.3 (A) or 74.7 (B) of each drug (ketamine on the x-axis and TPS on the y-axis) is the isobole and defined as representing pure additivity of the combination. SR 71.3s or SR 74.7s are represented with their respective 95% confidence intervals (thick lines). The SR 71.3 (A) or 74.7 (B) of the combination of both drugs is represented by a closed circle with its confidence intervals.
antagonizes NMDA receptor activation by inhibiting the influx of Na\(^+\) and Ca\(^{2+}\) through this channel.\(^{11–13}\) The addition of 1 mM ketamine before exposure to hypoxia or cyanide has been shown to reduce neuronal loss in murine neocortical cell cultures.\(^{33}\) Rats treated with ketamine (180 mg kg\(^{-1}\)) after head injury or complete cerebral ischaemia also exhibit improved neurological outcomes.\(^{34}\) However, several reports have prevented the use of this agent in neuroanaesthetic practice, because of undesirable effects on the brain, such as an increase in CMR, CBF and ICP.\(^{21–23}\)\(^{35–37}\)

It has been reported that ketamine blocks the re-uptake of excitatory amino acid neurotransmitters into synaptosomes and induces deceased GABA release, which could lead to a perturbation of the balance of excitatory and inhibitory factors.\(^{38}\) Furthermore, exposure to ketamine can also cause histopathologic damage. In fact, examination of normal rat brains after 4 h of ketamine administration revealed vacuole formation in the cingulate and retrosplenial cerebrocortical neurones.\(^{39}\) Olney and colleagues\(^{40}\) demonstrated prevention of these NMDA antagonist-induced changes by pre-treatment with barbiturates, and anticholinergics and benzodiazepines.

Barbiturates are currently considered by many clinicians to be the best protective pharmacological agent against cerebral ischaemia. We utilized TPS in the current investigation, because it is believed to be the best neuroprotective barbiturate. The primary neuroprotective mechanism of TPS \(in\in{\text{vivo}}\) has been considered to be through the reduction of the CMR. Reduction in CMR is directly attributable to the effects of TPS on synaptic neurotransmission, while maintaining the energy required for basic cellular functions. The redistribution of CBF to ischaemic tissues and a reduction in ICP may occur secondary to the metabolic effect.\(^{23–41}\) TPS has been demonstrated to reduce ischaemia-induced glutamate release,\(^{32}\) enhance GABAAergic currents\(^{33,44}\) and attenuate the ischaemia-induced increases of intracellular calcium in the cortex, through inhibition of both voltage-gated calcium channels and NMDA receptors.\(^{34}\) Apart from the protective mechanisms of barbiturates described above, TPS possesses a SH that acts as a scavenger of membrane-damaging free radicals providing an additional benefit.\(^{7,9,45,46}\) This beneficial effect was shown in a cell culture study that demonstrated significant neuroprotection with TPS compared with pentobarbital, which does not possess a SH group.\(^{7}\) However, a coma dose of TPS, which is considered to cause circulatory depression, might be necessary to provide these neuroprotective effects. By contrast, at clinical concentrations, ketamine can increase arterial pressure, heart rate and cardiac output, in addition to the CMR, CBF and ICP. Thus, we assessed the effectiveness of various concentrations of ketamine and TPS in combination against NMDA- or NOC-induced neurotoxicity to identify the relevant combinations that could counteract the haemodynamic effects of each drug. Determining the degree of neuroprotection provided against cerebral ischaemia by combinations of these two common i.v. anaesthetics is important for clinical practice and supports the notion that combinations of pharmacological agents can be developed to protect the brain during hypoxic episodes, without serious side-effects.

The concentrations of TPS and ketamine used in this study were between 50 nM and 50 \(\mu\)M. These concentrations were consistent with the free plasma concentrations (median effective dose: \(EC_{50}\)) of these two i.v. anaesthetics for general anaesthesia (approximately 7 and 25 \(\mu\)M, respectively).\(^{47,48}\) In our study, the concentration–response curve for ketamine appeared to be steep. However, these findings are consistent with several experiments that have investigated the pharmacological actions of ketamine. Ketamine, at concentrations between 10 and 30 \(\mu\)M, prevented the NMDA and quisqualate-stimulated production of cyclic GMP \(in\in{\text{vivo}}\).\(^{49}\) NMDA-activated postsynaptic responses were susceptible to ketamine at concentrations between 4 and 50 \(\mu\)M.\(^{50}\) In rat brain slices, ketamine inhibited nicotine-evoked inward current in a dose-dependent manner, and this inhibition was statistically significant at a concentration of 10 \(\mu\)M, but not 1 \(\mu\)M.\(^{51}\) MacDonald and colleagues\(^{52}\) also reported that ketamine inhibited the NMDA receptor at concentrations between 2 and 50 \(\mu\)M. These observations are consistent with our results in which 5 and 50 \(\mu\)M ketamine, but not lower doses, provided neuroprotection against NMDA-induced neurotoxicity. In a separate experiment, we exposed neurones to combinations of the highest concentrations of ketamine and TPS to determine whether deleterious effects could be triggered by ketamine or TPS \(per\ in{\text{se}}\). The SR of these neurones was almost maximal compared with neurones exposed to placebo conditions (approximately 94%), and no significant intergroup differences were observed.

In recent years, there has been a growing interest in the potential neuroprotective effects of various doses of i.v. anaesthetics. Hence, we determined the extent of neuroprotection that could be produced by these two anaesthetic agents in different combinations. In this investigation, low clinical concentrations of ketamine (5 and 10 \(\mu\)M) with a moderate concentration of TPS (50 \(\mu\)M), a potential therapeutic combination, exhibited an enhanced SR [71.3 (4.8)\% and 74.7 (3.1)\%, respectively] against NMDA-induced neurotoxicity, which was almost equivalent to the SRs [SR 79.7 (3.1)\%; \(P>0.2\) observed with the highest doses of these two i.v. anaesthetic agents. In addition, the protection achieved with these combinations was considerably higher than that which could be achieved with each agent alone [SR for ketamine alone 57.9 (2.2)\% and 61.1 (5.4)\% at 5 and 10 \(\mu\)M, respectively; TPS alone: 36.7 (5.9)\%]. In the current study, we calculated the SR 71.3 and 74.7 concentrations, respectively. The combinations of two i.v. anaesthetics were considered supra-additive, as the coordinates (TPS 50 \(\mu\)M plus ketamine 5 and 10 \(\mu\)M) were beneath the isobole, as shown in Figure 6. Furthermore, a clinical benefit was likely, as some of the side-effects would be
reduced. Several investigations have demonstrated that TPS was able to prevent the unwanted side-effects of NMDA antagonists in both histological and behavioural evaluations. Moreover, Irmaten and colleagues reported that concentrations of ketamine greater than 10 μM can inhibit Na⁺ channel function in the brainstem resulting in parasympathetic effects on cardiac activity. Collectively, it is considered likely that combined administration of these two i.v. anaesthetics is the best method for neuroprotection currently available.

It is understood that an in vitro investigation such as ours differs in many ways from the intact brain. However, in this experimental model the SR of viable neurones is calculated more precisely, by counting viable neurones manually after staining with trypan blue. Using this technique, we were able to quantitatively assess neuroprotection in each experimental group.

In conclusion, our observations demonstrate that a clinically relevant dose of ketamine, which is the only NMDA antagonist presently used in clinical practice, offers significant neuroprotection during prolonged exposure of neurones to NMDA but not NOC. These combinations of ketamine and TPS at low clinical concentrations were effective in providing better neuroprotection against NMDA-induced neurotoxicity.

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