Effects of ketamine, midazolam, thiopental, and propofol on brain ischemia injury in rat cerebral cortical slices

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ABSTRACT

AIM: To compare the effects of ketamine, midazolam, thiopental, and propofol on brain ischemia by the model of oxygen-glucose deprivation (OGD) in rat cerebral cortical slices. **METHODS:** Cerebral cortical slices were incubated in 2 % 2,3,5-triphenyltetrazolium chloride (TTC) solution after OGD, the damages and effects of ketamine, midazolam, thiopental, and propofol were quantitativlye evaluated by ELISA reader of absorbance (*A*) at 490 nm, which indicated the red formazan extracted from slices, lactic dehydrogenase (LDH) releases in the incubated supernate were also measured. **RESULTS:** Progressive prolongation of OGD resulted in decreases of TTC staining. The percentage of tissue injury had a positive correlation with LDH releases, r=0.9609, P<0.01. Two hours of reincubation aggravated the decrease of TTC staining compared with those slices stained immediately after OGD (P<0.01). These four anesthetics had no effects on the TTC staining of slices. Ketamine completely inhibited the decrease of *A* value induced by 10 min of OGD injury. High concentrations of midazolam (10 μmol/L) and thiopental (400 μmol/L) partly attenuated this decrease. Propofol at high concentration (100 μmol/L) enhanced the decrease of *A* value induced by 10 min of OGD injury (P<0.01). **CONCLUSION:** Ketamine, high concentration of midazolam and thiopental have neuroprotective effects against OGD injury in rat cerebral cortical slices, while high concentration of propofol augments OGD injury in rat cerebral cortical slices.

INTRODUCTION

Brain ischemia and reperfusion (I/R) injury is a subject of intense interest in clinical research, and involved in various complicated pathophysiological processes. Many events of brain I/R injury are happened in the operation. Intravenous anesthetics are widely used for surgical procedure, more and more researchers are now paying close attention to the neuroprotective effects of these drugs against brain I/R injury, however, many previous experimental results remain controversial, especially *in vivo* and *in vitro* studies. Experimental models may be responsible to these controversial results. The studies with suitable models are able to detect the real effects of those intravenous anesthetics on brain ischemia.

Being an *in vitro* model of brain ischemia, oxygen-glucose deprivation (OGD) injury in rat brain slices has the advantages *of in vivo* and *in vitro*, and can imitate damages induced by brain I/R injury in intact animals[1,2]. 2,3,5-triphenyltetrazolium chloride (TTC) has been longly employed in the assessment of brain ischemia, a new method as quantitative measurement

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of extracted red formazan by the solvent extraction and colorimetry on the brain slices incubated with TTC solution has been introduced recently, which can be used as a simple, objective, and sensitive method in the assessment of brain ischemic *in vitro*[3,4]. Ketamine, midazolam, thiopental, and propofol are 4 iv anesthetics commonly used in the clinical practices. They have different mechanisms in general anesthesia, so they may have different effects on the damage induced by brain I/R injury. Investigation and comparison of these effects may contribute to the understanding of their mechanisms of general anesthesia. The aim of the present study was to compare the effects of ketamine, midazolam, thiopental, and propofol on brain I/R injury by the model of OGD injury in rat cerebral cortical slices.

MATERIALS AND METHODS

Animals Male Sprague-Dawley (SD) rats (Grade II, Certificate No 152) weighing 90-120 g were purchased from Shanghai Experimental Animal Center, Chinese Academy of Sciences.

Reagents and drugs TTC (Shanghai Chemical Reagent Co Ltd, China), kits of lactic dehydrogenase (LDH, Nanjing Jiancheng Bioengineering Institution, China), ketamine (Shanghai Zhongxi Pharmaceutical Co Ltd, China), midazolam (Roche Pharmaceutical Co Ltd, China), thiopental sodium (Shanghai New Asiatic Pharmaceutical Co Ltd, China), propofol (AstraZeneca, UK), and all other reagents were of analytical grade. TTC and all drugs were prepared as stock solution and then dissolved in artificial cerebrospinal fluid (aCSF) solution to achieve final concentrations.

Cerebral cortical slice preparation The slices were made as described[5,6] with several modifications. SD rats were decapitated, the brain were quickly removed and immersed in ice-cold oxygenated (95 % O_2/ 5 % CO_2) normal aCSF, which had the following composition (in mmol/L): NaCl 119, KCl 2.5, $CaCl_2$ 2, $MgSO_4$ 1, NaH_2PO_4 1.25, $NaHCO_3$ 26.2, glucose 10, pH 7.4. Cortical slices (400 μm thick) were prepared using a vibrating tissue slicer (ZQP-86, Xiangshan, Zhejiang, China), then transferred to a 'slice saver' containing continuously-oxygenated normal aCSF at room temperature (24 ºC) for 90 min of incubation to allow recovery from the trauma induced by sectioning.

Oxygen-glucose deprivation (OGD) injury After 90-min recovery, slices were transferred to the glass vials containing oxygenated normal aCSF at 37 ºC for 30 min of preincubation, then were transferred to other vials containing glucose-free aCSF (glucose was substituted by equimolar sucrose) bubbled with 95 % N_2+5 % CO_2 as OGD injury for 5, 10, 15, 30, and 60 min, respectively, and incubated again in the oxygenated normal aCSF for 2 h of reincubation. LDH releases in the incubated supernate of the same period of OGD were also measured. To investigate the effect of reincubation for 2 h, groups of slices were incubated in TTC solution immediately after different periods of OGD. Slices of control groups were briefly removed from and then returned to the same vials without OGD to control for the effects of 'stirring'.

Drugs administration Different concentrations of ketamine (5, 10, and 50 μmol/L), midazolam (0.5, 1, and 10 μmol/L), thiopental (25, 100, and 400 μmol/L), and propofol (5, 50, and100 μmol/L) were dissolved in aCSF and incubated with the slices subjected to 10 min OGD throughout the periods of preincubation, injured incubation, and reincubation, respectively. Slices were also incubated with these 4 iv anesthetics in oxygenated normal aCSF for 3 h to evaluate their effects on the TTC staining in rat cerebral cortical slices.

Assessment of TTC staining and LDH release Slices were immersed in 2 % TTC solution in a covered waterbath shaker at 37 ºC for 30 min, the wet weight was measured after rinsed twice by saline. An extracted solution (50:50, mixture of ethanol/ dimethylsulfoxide) was added with the proportion of 20 mL per 1 g slices. After 24-h extraction in a dark box, the extracted liquid was added to 96-well plates (200 μL per well), the absorbance (A) at 490 nm of each well was measured by the ELISA reader (Elx800, Bio-Tek). Percentage of tissue injury was calculated from the following equation: % tissue injury = 100 %× (1-$A_{injury}/A_{control}$). LDH releases in the incubated supernate were measured by spectrophotometry according to the kit specification.

Statistical analysis All data were expressed as mean±SD. SPSS statistical software 10.0 for windows was used and statistical analysis was evaluated by correlation analysis, analysis of variance (one-way ANOVA) followed by Student-Newman-Keuls test, the statistical significance was assumed if P values were less than 0.05.

RESULTS

Effects of OGD and the relations between TTC staining and LDH releases The A value represented by TTC staining was decreased gradually as the period of OGD increased. There was a statistical significance

between OGD groups and control group (P<0.01, Tab 1). The percentage of tissue injury induced by different periods of OGD had a positive correction with the LDH release in the supernate (r=0.961, P<0.01, Fig 1).

Tab 1. Comparison of TTC staining (*A* value) in rat cerebral cortical slices of different periods of oxygen-glucose deprivation (OGD) and the LDH releases in the incubated supernate. *n*=8 slices. Mean±SD. ^cP<0.01 *vs* control group, analyzed by one-way ANOVA.

Groups	TTC staining		LDH releases/ IU·g^{-1} (protein)
	A	Percentage of tissue injury/%	
Control	1.12±0.03	0	300±66
5 min	0.912±0.015^c	18.5±1.3	331±68
10 min	0.72±0.07^c	42.0±1.5	568±107
15 min	0.430±0.023^c	60.6±0.8	692±95
30 min	0.28±0.03^c	76.6±1.7	772±187
60 min	0.19±0.04^c	81±3	1001±191

Tab 2. Comparison of TTC staining in the rat cerebral cortical slices between the staining after 2 h of reincubation and staining immediately after OGD of the same period. *n*=8 slices in groups of TTC staining after 2 h of reincubation. *n*=10 slices in groups of TTC staining immediately after OGD. Mean±SD. ^cP<0.01 *vs* control group. ^fP<0.01 *vs* groups of TTC staining after 2 h of reincubation of the same period of OGD, analyzed by one-way ANOVA.

Groups	TTC staining after 2 h of reincubation		TTC staining immediately after OGD	
	A	Percentage of tissue injury/%	*A*	Percentage of tissue injury/%
Control	1.12±0.03	0	1.15±0.11	0
5 min	0.912±0.015^c	18.5±1.3	1.06±0.09^f	8±5^f
10 min	0.72±0.07^c	42.0±1.5	0.97±0.08^{cf}	15±8^f
15 min	0.430±0.023^c	60.6±0.8	0.94±0.11^{cf}	18±9^f
30 min	0.28±0.03^c	76.6±1.7	0.88±0.10^{cf}	22±9^f

Effects of 2 h reincubation on TTC staining The percentage of tissue injury in slices reincubated for 2 h was significantly increased compared with that stained immediately after the same period of OGD (P<0.01, Tab 2). Reincubation for 2 h in the oxygenated normal aCSF worsen the OGD injury in rat cerebral cortical slices.

Effects of ketamine, midazolam, thiopental, and propofol on 10 min of OGD injury There was no significant change in *A* value when slices were incubated with ketamine for 3 h. Different concentrations of ketamine completely prevented the decreases of *A* value induced by 10 min of OGD injury (P<0.01 *vs* OGD group, Tab 3).

There was no significant change in *A* value when slices were incubated with midazolam for 3 h (Tab 3). Midazolam 0.5 and 1 μmol/L lightly inhibited the decrease of *A* value induced by 10 min of OGD injury, while, midazolam 10 μmol/L significantly inhibited this

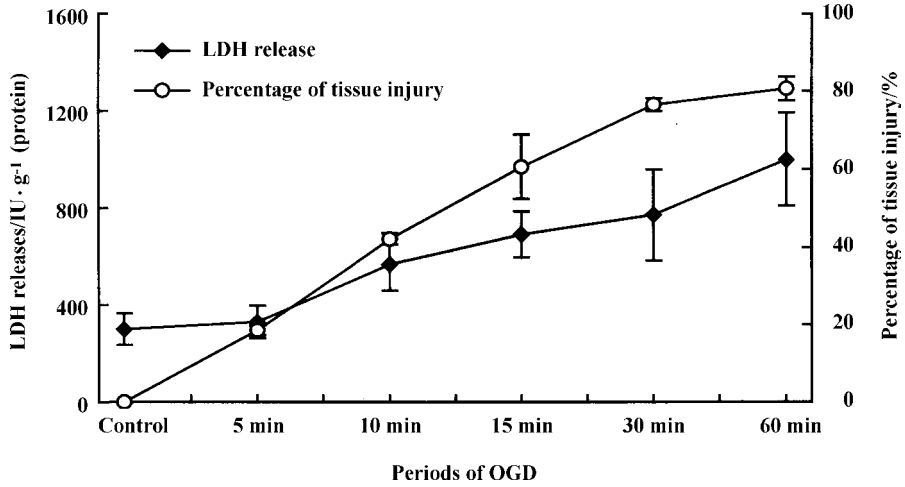

Fig 1. Comparison of the increases of percentage of tissue injury of different periods of OGD in rat cerebral cortical slices and the increase of the LDH releases in the incubated supernate, a positive correlation was seen in the two indices, *r*=0.961. *P*<0.01, analyzed by correlation analysis.

Tab 3. Effects of different concentrations of ketamine, midazolam, thiopental, and propofol on the TTC staining in rat cerebral cortical slices incubated with anesthetics for 3 h and in the slices subjected to 10 min of OGD. *n* indicates number of slices. Mean±SD. $^{c}P<0.01$ *vs* control group in the experiment of OGD injury. $^{c}P<0.05$, $^{f}P<0.01$ *vs* groups of OGD, analyzed by one-way ANOVA and SNK test.

Concentration/ μmol·L^{-1}	Slices incubated with anesthetics for 3 h		Slices subjected to 10 min of OGD injury	
	A	*n*	*A*	*n*
Ketamine				
Control	1.06±0.10	7	0.93±0.16	17
OGD			0.62±0.14^{c}	17
5	1.13±0.07	7	0.80±0.16	11
10	1.12±0.10	7	0.76±0.05	7
50	1.05±0.11	7	0.79±0.17	8
Midazolam				
Control	0.91±0.06	7	0.75±0.04	10
OGD			0.37±0.09^{c}	10
0.5	0.91±0.09	6	0.40±0.12^{c}	10
1	0.92±0.07	7	0.38±0.15^{c}	9
10	0.89±0.07	7	0.50±0.10^{ce}	8
Thiopental				
Control	0.90±0.04	8	0.93±0.21	8
OGD			0.23±0.05^{c}	9
25	0.89±0.06		0.36±0.11^{c}	9
100	0.92±0.06	7	0.29±0.05^{c}	9
400	0.91±0.06	7	0.52±0.06^{cf}	8
Propofol				
Control	0.59±0.04	7	1.00±0.19	14
OGD			0.52±0.15^{c}	14
5	0.60±0.03	7	0.44±0.08^{c}	7
50	0.601±0.018	7	0.37±0.05^{c}	8
100	0.58±0.04	7	0.12±0.04^{ci}	7

decrease ($P<0.05$ *vs* OGD group, Tab 3 and Fig 3), but its *A* values were still lower than that of control group ($P<0.01$ *vs* control group, Tab 3).

There was no significant change in *A* value when slices were incubated with thiopental for 3 h (Tab 3). Thiopental 25 and 100 μmol/L lightly inhibited the decrease of A value induced by 10 min of OGD injury, while, thiopental 400 μmol/L significantly inhibited this decrease ($P<0.01$ *vs* OGD group, Tab 3 and Fig 3), but its *A* values were still lower than that of control group ($P<0.01$ *vs* control group, Tab 3).

There was no significant change in *A* value when slices were incubated with propofol for 3 h (Tab 3). Different concentrations of propofol could not reduce the decrease of *A* value induced by 10 min of OGD injury, but propofol at high concentration of 100 μmol/L significantly enhanced this decrease ($P<0.01$ *vs* OGD group, Tab 3).

DISCUSSION

We found that 4 iv anesthetics had different effects. Ketamine completely prevented the OGD injury at low and high concentrations, midazolam and thiopental attenuated the damages at high concentrations, propofol had no protective effect, but augmented the OGD injury at high concentration. The free plasma concentrations (midian effective dose, EC_{50}) of ketamine, midazolam, thiopental, and propofol for general anesthesia are approximately 5, 0.1, 25, and 1 μmol/L, respectively. And these 4 lipophilic anesthetics are known to concentrate into brain, thus the lowest concentrations of these 4 iv anesthetics in our study are similar to those that occur in brain during anesthesia[7,8].

TTC staining has been widely used in the study of brain ischemia. In health tissue, TTC is converted to the red, insoluble formazan by succinate dehydrogenase in mitochondria, thus cells which are dead or which have impaired do not stain or stain less well than health cells[4]. Classical method of measuring the figure for infarct volume or percentage tissue loss is by photography and image analysis, but it may be difficult to accomplish accurately if there are areas where damage is patchy and not clearly indicated by a visible difference in TTC staining. Here we used the new method introduced by Preston *et al*[3] as incubating fresh slices with TTC solution, solvent extraction of red formazan and colorimetric measurement. The experimental results demonstrated that the increase of percentage of tissue injury calculated from *A* value had a positive correction with the increase of LDH releases, which is considered as a reliable biochemical indictor in the neuronal injury assessment[9], this indicated that the solvent extraction of red formazan and colorimetric measurement of *A* value using ELISA reader can objectively reflect the damage induced by OGD in rat cerebral cortical slices. Reincubation of slices with oxygenated normal aCSF worsen the deterioration inducing by OGD injury indicated that the OGD injury could imitate the experiment of brain I/R injury *in vivo*.

As an early consequence of OGD, neuronal aerobic metabolism and ATP production severely occurred,

the decrease of energy production and function of Na^+/K^+-ATPase leads to loss of active ion transport, destruction of transmembrane electrochemical ionic gradients, and membrane depolarization, which promotes presynptic glutamate release and impaired uptake. Excess extracellular glutamate produces neuronal excito-toxicity as activities of iontropic and metabotropic post-synaptic receptors, induces a massive increase of cytosolic calcium (Ca^{2+}) concentration, then triggers a cascade of intracellular events that leads to immediate or delayed neuronal death[10,11].

The neuroprotective effect of ketamine is contributed to its property of NMDA receptor blocking. Our results are identical to the conclusions of previous studies [12,13]. Many researchers found that ketamine damaged the posterior cingulated and retrosplenial cortices (PC/RS) with vacuolization in the neurons[14] and induced c-fos protein expression[15], those phenomena were postulated to be the origin of psychotomimetic side effects of ketamine. We could not find this neurotoxicity because the TTC staining only indicated the function of survival tissue, it needs further study by histopathological method.

The neuroprotection of midazolam of high concentration during ischemia may relate to its property of maintaining the normal function of glutamate transporter. The chief source of extracellular glutamate accumulation during ischemia is failure or reversal of glutamate transporters rather than synaptic vesicular release[16]. Sakai *et al*[17] found that midazolam above 3 μmol/L inhibited the reversal of glutamate uptake.

The result of thiopental's neuroprotection against OGD injury is consistent with the data from Zhan *et al*[6] as high concentration of thiopental mitigated increases in $[Ca^{2+}]_i$ caused by ischemia in brain slices *in vitro*. Several studies using the methods of electrophysiology and morphology reported that high concentration of thiopental attenuated neuronal damage induced by OGD in rat hippocampal slices[12,18]. A combination of direct and indirect attenuation of NMDA receptor-dependent processes may account for its neuroprotective efficacy[6,19].

Regarding the effect of propofol on brain ischemia, results are conflicting. Some studies demonstrated its neuroprotection and suggested that several mechanisms as activation of $GABA_A$ receptors, reduction of intracellular Ca^{2+} concentration, or scavenging of free radical species may participate in this effect[20-22]. However, other studies failed to show any neuroprotections[23]. Propofol did not attenuate ischemia/hypoxia induced glutamate accumulation *in vivo* and *in vitro* [24,25], not have a protective effect in recovery of population spikes of CA1 pyramidal layer following ischemia[12], or inhibit rat hippocampal CA_1 pyramidal cell swelling induced by ischemia *in vitro*[18]. It slight augmented the NMDA-mediated $[Ca^{2+}]_i$ response at 100 μmol/L[12]. Propofol enhanced NMDA-mediated damage in hippocampal slices[26]. Lipid emulsion (Intralipid) could activate NMDA receptors in cortical neurons[27]. Propofol used in our study was incorporated in a lipid carrier, so the neurotoxicity of propofol at high concentration of 100 μmol/L may be the result of over-activating of NMDA receptors by propofol or its lipid carrier. Further study with the pure propofol will be required to ascertain this supposition.

Although we can simply, objectively, and sensitively evaluate the neuroprotective effects of iv anesthetics against brain ischemia by the method of incubate fresh rat cerebral cortical slices with TTC solution, solvent extraction of red formazen and quantitative assessed by ELISA reader. We can not know exactly the role of a definite area or cell in the mechanism of these iv anesthetics neurprotection, Further studies associated with the methods of electrophysiology and histomorphometry are needed.

In summary, using the new quantitative method of TTC staining, solvent extraction and colorimetry, we demonstrate that ketamine, high concentration of midazolam, and high concentration of thiopental have neuroprotective effects against OGD injury in rat cerebral cortical slices, while high concentration of propofol augments OGD injury.

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