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P53 expression in ischemic rat models after the administration of ketamine and ketamine-xylazine

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ABSTRACT

Ketamine and ketamine-xylazine are often used as anesthetic drugs in animal models of ischemia. However, their neuroprotective and neurotoxic effects in ischemic animal models that have undergone tBCCAO are still under debate. The protein p53 is a pro-apoptotic factor involved in the cellular mechanism of ischemia. The interaction between death-associated protein kinase 1 (DAPK 1) and p53 is fundamental in determining whether cells experience necrosis or apoptosis in an ischemic stroke. This study was purposed to identify the presence or absence of differences between the p53 expressions in the brains of tBCCAO-induced ischemic rat models after the administration of ketamine and ketamine-xylazine. It employed a post-test control group design with four groups of adult male Wistar rats as the subject: (1) sham group operated with ketamine, (2) sham group operated with ketamine-xylazine, (3) models of tBCCAO-induced ischemia with ketamine, and (4) models of tBCCAO-induced ischemia with ketamine-xylazine. Ketamine was administered at the dose of 75mg/kg BW, while xylazine was at 8 mg/kg BW. The expression of p53 in rat brains was assessed by semi-quantification, specifically IHC staining with anti-p53 antibodies. P53 expression appeared as brownish stains in the cytoplasm of forebrain pyramidal neurons, and in this study, it was measured using the Allred score. The ANOVA test yielded a *p*-value of >0.05, implying the absence of difference between the p53 expressions in the brains of tBCCAO-induced ischemic rat models receiving ketamine and ketamine-xylazine.

Keywords: p53, Ketamine Xylazine, tBCCAO

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INTRODUCTION

The use of animal models in ischemic brain studies has been developing for several decades. One of the models for global ischemic brains is transient bilateral common carotid artery occlusion (tBCCAO). The tBCCAO has a higher significance than other ischemic models, mainly because its induced mortality rate is lower than that of focal ischemic brain models (MCAO) (Kaya *et al*., 2016). Also, it can be used to explain some ischemic condition in humans, such as ischemia caused by a heart attack, diabetes, or vascular dementia (Sanderson and Wider, 2013; Barbhuiya *et al*., 2015; Traystman, 2003; Kim *et al*., 2008; van der Spuy *et al*., 2015; Wang *et al*., 2016). The mechanism of ischemia can include necrotic and apoptotic cells and, more importantly, lead to imbalanced ions that can produce depolarization of neurons and glia, causing the hyperexcitability of NMDA receptor to occur (Fann *et al*., 2013; Mehta *et al*., 2007; Majid, 2014).

In the past decade, many studies of ischemic animal models have been using ketamine and ketamine-xylazine as anesthetics. Ketamine is a selective NMDA receptor inhibitor that works by decreasing calcium influx and brain injury (Zhang *et al*., 2007). Ketamine is sometimes combined with alpha-2 agonists, specifically xylazine (Leffa *et al*., 2015). Xylazine hydrochloride can act as an analgesic, sedative, and muscle relaxant (Gonca, 2015). Based on previous research, the neuroprotective and neurotoxic functions of ketamine and ketamine-xylazine remain debatable.

At the cellular level, apoptosis after cerebral ischemia involves a pro-apoptotic factor, p53. Injecting pifithrin-alpha (PFTa), a p53 inhibitor, weakens p53 nuclear transport and DNA binding, which have been proven to strengthen neurons and increase recovery after stroke. The interaction between death-associated protein kinase 1 (DAPK1) and p53 is substantial in determining whether the cells experience necrosis or apoptosis in ischemic stroke events. DAPK1 is related to P53DM and catalyzes p53 into pS^{23} that later moves into its core and activates the expression of pro-apoptotic gens. On the other hand, if pS^{23} moves into mitochondria and interacts with cyclophilin D, the cells will become necrotic. For these reasons, DAPK1-p53 interaction is the target of intervention in stroke therapy (Pei *et al*., 2014).

Until now, the effects of ketamine and the combination of ketamine-xylazine on the expression of the protein p53 in the brain of tBCCAO-induced ischemic rat models remain unknown. The purpose of this study was to identify differences between the p53 expressions of rat brains in tBCCAO-induced ischemic models after the injection of ketamine and ketamine-xylazine.

MATERIALS AND METHODS

Materials

The research subjects were adult male Wistar rats (*Rattus norvegicus*) selected with predefined inclusive and exclusive criteria. The inclusive criteria were healthy male rats aged three months old and weighed 200-250 grams. Healthy in this context was decided from physical conditions: the rats had clean, not-wet, and not-adhesive fur, moved actively, and ate, drank, and slept according to their cycle. Meanwhile, the exclusive criteria were sickness and death during the conducted study. The number of the subjects, determined based on the 3R principles, was precisely 18 rats. They were then divided into four groups: (1) sham group with ketamine but without tBCCAO, (2) sham group with ketamine–xylazine but without tBCCAO, (3) treatment group receiving ketamine and tBCCAO, and (4) treatment group receiving ketamine-xylazine and tBCCAO. Ketamine was administered at the dose

of 75 mg/kg (10% Alfamine, Alfasan Int., Woerden, Netherlands), while xylazine was at 8 mg/kg (2% Alfazyne, Alfasan Int., Woerden, Netherlands) IM.

Methods

Adaptation

The test rats were placed in a cage and allowed seven days to adapt to the laboratory conditions. Each cage, consisting of two rats, was set to room temperature with a light-dark cycle for 12 hours. The bright phase of the cycle started from 06.00 a.m. until 06.00 p.m., at which time the dark phase began. All test rats were given food every 06.00 a.m. and ad libitum access to water.

tBCCAO Induction

The procedures used to execute tBCCAO on Day 8 were based on Handayani *et al*. (2018; 2016). It started with administering ketamine or ketamine-xylazine, followed by placing the test rests on sterile platforms, which were Digital Jumbo Hotplate (LHT-2030D). The rectal temperature of each rat was maintained at 37 ± 1°C. Then, the anterior neck was disinfected using betadine solution, and a vertical incision was made along the median line of this neck. The common carotid artery underlying the submandibular gland was separated from the vagal nerves by obtuse dissection without harming these structures. For the treatment groups, the visible common carotid artery was ligated with a microvascular clamp (Serrefine small, curved, Q1Y:01No) for 20 minutes. This procedure was not applied to the sham groups. Afterward, a local analgesic therapy with 0.1 mL of 0.25% bupivacaine was carried out once a day. The incision sites were sewed with silk thread, and the areas around them were disinfected by betadine.

Euthanasia and brain tissue collection

After 24-hour reperfusion, the test rats were euthanized by transcardial perfusion. Firstly, they were anesthetized using IM injection of ketamine at the dose of 80-100 mg/kg BW. After the rats achieved a deep anesthetic state, an incision was made along the median line of the abdomen wall. Then, an incision along the axillary line was made until the thorax wall was open and the heart was visible. The left ventricle of the heart was incised, and a cannula was inserted until it reached the ascending aorta. An incision on the right atrium was made to eject blood. Afterward, NaCl perfusion liquid was channeled through the cannula. The descending aorta was nipped, allowing complete perfusion of the brain. The perfusion was maintained until the blood channeling out through the right atrium looked clear and the internal mammary artery around sternum turned white because it was filled with a clear liquid. Decapitation was carried out until the transcardial perfusion was complete, then the brain tissues were removed. Through the TTC staining, ischemic areas of the brain would appear faintly stained, whereas non-ischemic areas would turn red (Handayani *et al*., 2018).

Histological preparation

Paraffin blocks containing anterior brain tissues were sliced using a rotary microtome with a cut thickness of 5 um. IHC staining was performed on one slice using anti-p53 antibodies (Catalog No.: FNab06083). It started with deparaffinization using low concentrations of xylol and alcohol. The next slice of tissues was incubated in 3% H_2O_2 in 10% methanol for 20 minutes and then washed with distilled water and PBS three times. Then, the antigen was obtained using a buffer of citrate with pH 6 in a microwave. The slice of the tissues was heated at a high temperature (100^0C) for 10 minutes,

followed by medium to low temperature for 20 minutes. After that, the slice was allowed to cool down and washed again using PBS 3 times. The slice was then blocked with a protein of background sniper for 10 minutes, dropped using antibody (Ab) primer, and incubated for one night at a temperature of 4^{o} C before rewashed with PBS 3 times. Afterward, this slice was incubated in horseradish peroxidase-conjugated Streptavidin (SA-HRP Complex) for 10 minutes and washed again with PBS 3 times. Cells labeled p53 was identified using 3,3'-diaminobenzidine (1:100) for 5 minutes. Later, the slice was washed five times with distilled water and counterstained by Mayer's hematoxylin protocol for 1 minute. Then, it was washed with running water for 2 minutes, and dehydrated using different levels of ethanol, i.e., from 70% 80%, 90%, 95%, to 100%; this procedure lasted for 1 minute for each level. Finally, it was cleaned using xylene, added with Canada balsam, and coverslipped.

Histological observation

This observation used an Olympus CX21 light microscope with 1000x magnification and an OptiLab camera connected to a computer equipped with the OptiLab viewer program to record some pictures. The p53 expression was determined using the semi-quantification of p53 expressed in forebrain pyramidal neurons, specifically by IHC staining with an anti-p53 antibody. In this staining, the cytoplasm of the neuron turned brownish, and this expression was then further examined based on its Allred score, which gave the proportion of positive cells with a scale of 0-5 and color intensity with a scale of 0-3. If the sum of both parameters was 0-2, then the p53 expression was considered negative. Meanwhile, if the sum was 3-8, then it was considered positive. This study applied a blinding system in which only the first researcher was allowed to mark each subject with a particular code, while the first to fifth researchers carried out ischemia induction, decapitation process, blocking process, and IHC staining. Then, the sixth to eight researchers who had knowledge of the codes of the test rats were responsible for visual documentation and Allred score analysis. The statistical analyses involved all of the researchers after the group of each subject was revealed. Blinding was principally applied to minimize research biases.

Data Analysis

The data obtained were tested for their normality by the Shapiro-Wilk test. Differences between the p53 expressions of the groups were tested statistically by the Analysis of Variance (ANOVA). A post-hoc test was performed only if the ANOVA test yielded a *p*-value of less than 0.05, indicating a significant difference.

RESULTS AND DISCUSSION

This study has been approved by the Ethical Committee of the Faculty of Medicine, Universitas Islam Indonesia, with certificate number 0808/Ka.Kom.Et/70/KE/XI/2017. It was designed to reveal whether the brains of tBCCAO-induced ischemic rat models receiving different types of anesthetics, i.e., ketamine and ketamine-xylazine, showed different p53 expressions. It employed a post-test control group design with four groups adult male Wistar rats as the subject: (1) sham group operated with ketamine, (2) sham group operated with ketamine-xylazine, (3) model of tBCCAO-induced ischemia with ketamine, and (4) model of tBCCAO-induced ischemia with ketamine-xylazine. Ketamine and xylazine were administered at the doses of 75 mg/kg BW and 8 mg/kg BW, respectively. The expression of p53 in the rat brain was assessed by semi-quantification, i.e., IHC

staining with anti-p53 antibodies. P53 expression appeared in brownish stain in the cytoplasm of forebrain pyramidal neurons, and then it was measured using the ALLRED score.

The TTC staining results of the groups are shown in Figure. 1. The ischemic areas of the brain are faintly stained, whereas the non-ischemic area appeared in red.

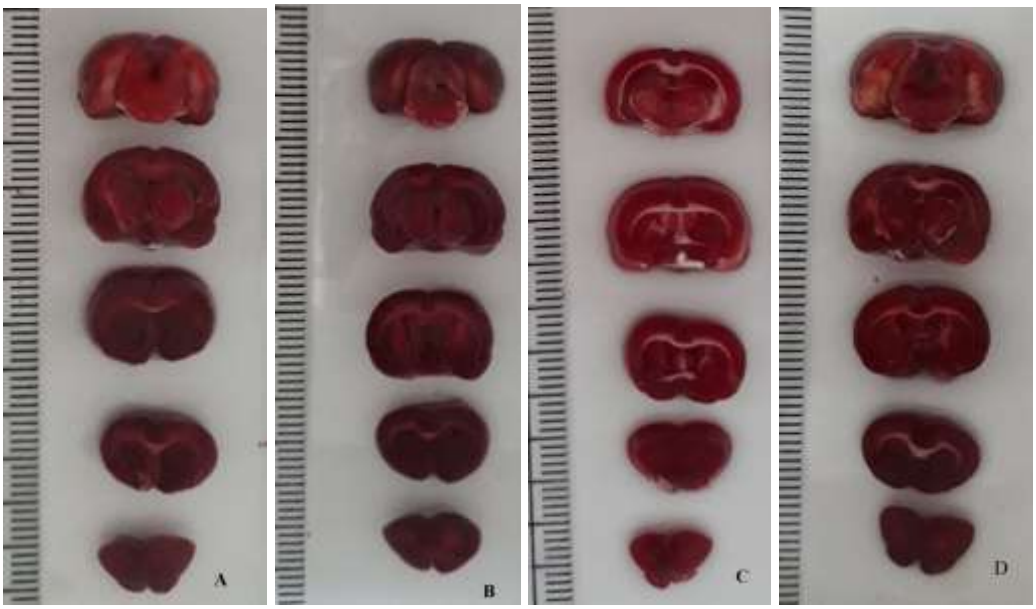

Figure 1. The macroscopic visualization of the test rat brains after TTC staining. The ischemic areas of the brain are faintly stained, whereas the red color shows the non-ischemic areas. (A) Group 1 (sham group operated with ketamine), (B) Group 2 (sham group operated with ketamine-xylazine), (C) Group 3 (ischemic group with ketamine), (D) Group 4 (ischemic group with ketamine-xylazine)

Based on the observation of the p53 expression, the brown color indicates a positive reaction (+). The microscopic visualization of the number of p53 expressions with 1000x magnification is presented in Figure 2.

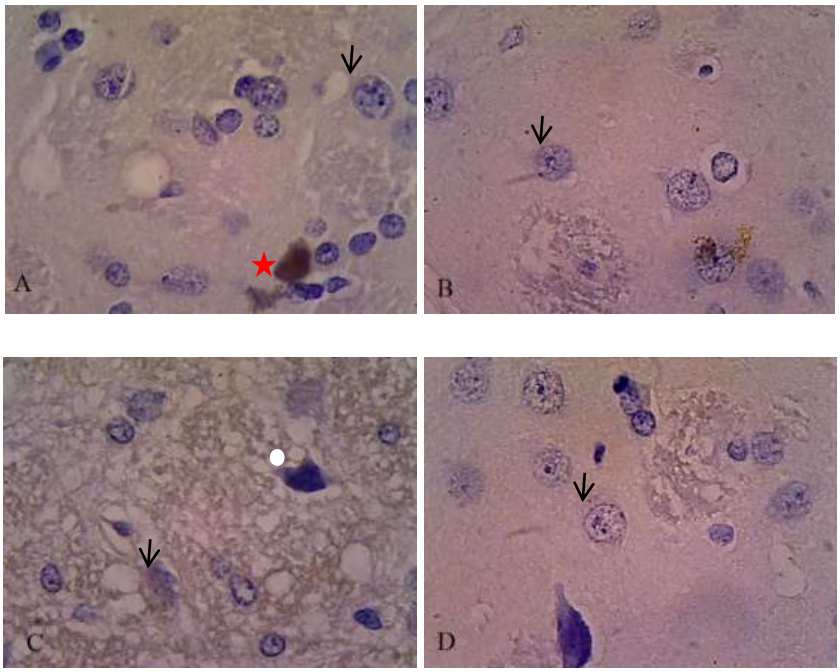

Figure 2. p53 expressions in the test rat brains with 1000x magnification. The brown color indicates a positive reaction (+). (A) Group 1 (sham group operated with ketamine), (B) Group 2 (sham group operated with ketamine-xylazine), (C) Group 3 (ischemic group with ketamine), (D) Group 4 (ischemic group with ketamine-xylazine) (black arrows point to neurons, red stars indicate positive, and white circles indicate negative)

The Allred scores of the p53 expressions were averagely 4.735 (group 1), 4.750 (group 2), 4.922 (group 3), and 4.945 (group 4). With mean values higher than 4, the expressions were then concluded as positive. P53 expression was the highest in group 4. The statistical t-test analysis yielded a *p*-value of 0.269 (>0.05), indicating no significant differences between the test groups (Table I).

Table I. The mean allred scores

Groups	Mean Scores	SD	*p*-value
1	4.735	0.13	
2	4.750	0.20	0.269
3	4.922	0.08	
4	4.945	0.33	

Note: Score 0-2 was negative, score 3-8, p53 expression was considered positive. (A) Group 1 (sham operated group with ketamine), (B) Group 2 (sham operated group with ketamine xylazine), (C) Group 3 (ischemic group with ketamine), (D) Group 4 (ischemic group with ketamine xylazine).

These findings prove that ketamine and ketamine-xylazine do not cause a difference in the p53 expression in the brain of tBCCAO-induced ischemic rat models ($p>0.05$). They also confirm that the administration of ketamine at the dose of 75 mg/kg BW and its combination with xylazine at the dose of 8 mg/kg BW cannot induce p53 expression in normal rat brains.

There was also no significant difference between the p53 expressions of tBCCAO-induced ischemic rats receiving ketamine and ketamine-xylazine. This result is consistent with Linou *et al*. (2015), which compare isoflurane with ketamine-xylazine (ketamine= 100 mg/kg BW IP; xylazine= 10 mg/kg BW IP) administered to pMCAO ischemic mice model. The study has found that the apoptotic neurons in mice with brain ischemia that receive isoflurane and ketamine-xylazine are not significantly different in number. Isoflurane and ketamine have similar characteristics, that is, as an NMDA receptor antagonist (Linou *et al*., 2015).

Some studies have pointed out that ketamine has neuroprotective properties. At certain doses, it can protect the nervous system in an ischemic animal model. Zhang *et al*. (2007) assert that at the dose of 50 mg/kg BW, ketamine protects the cerebral cortex of ischemic animal models (Zhang *et al*., 2007). Meanwhile, at the dose of 3 mg/kg BW, it can protect the frontal cortex of a hypoxic fetus (Rabaglino *et al*., 2016). The results of these previous studies are in line with this research in which no difference in p53 expression found between the tBCCAO group and the sham group (no tBCCAO, only ketamine).

On the other hand, ketamine is known to produce not only neuroprotective effects but also neuronal apoptosis. According to Yan *et al*. (2014), it can influence the neuronal apoptosis of the hippocampus in neonatal rats when its administration is repeated for three consecutive days. Also, ketamine increases the production of ROS, HIF-1α (*Hypoxia-inducible factor-1* subunit α), Bax levels, and the expression of p53 in neonatal hippocampal neurons. Liu *et al*. (2013) and Kristin *et al*. (2003) add that a high dose of ketamine can influence genotoxicity in the blood cells and brain cortex of mice. DNA damage continually occurs 24 hours after the administration of anesthetic ketamine (Leffa *et al*., 2015).

The p53 expression of the sham group receiving ketamine-xylazine revealed that this drug combination could induce apoptosis. There might be a decrease in blood flows to the brain when ketamine-xylazine was given until ischemia occurred. Ketamine-xylazine can decrease the metabolism of glucose in the brain (Prando *et al*., 2019).

On the other hand, the anesthetic ketamine-xylazine injected to the tBCCAO-induced ischemic rat models can produce protective responses because there is no significant difference between the p53 expressions of the sham group with xylazine and the tBCCAO-induced ischemic group with ketamine-xylazine. In a previous study, when ketamine-xylazine is given at the dose of 70 mg/kg BW (ketamine) and 6 mg/kg BW (xylazine), they can inhibit the inflammatory compounds of iNOS (inducible nitric oxide synthase); hence, protective responses to cells that experience hypoxia (Helmer *et al*., 2003). Another study shows that ketamine-xylazine can cause a decrease in blood flows to the cerebral cortex of rats. On the contrary, at the dose of 50 mg/kg BW, the use of ketamine alone cannot significantly change the blood supply to the brain (Lei *et al*., 2001).

For these reasons, the extent of the effects of ketamine and ketamine-xylazine depend on certain conditions, including the given dose, the frequency of administration, and the condition of the rat model (e.g., fetus and hypoxia).

CONCLUSION

There are no significant differences between the p53 expressions in the brains of tBCCAO-induced ischemic rat models that receive ketamine and ketamine-xylazine.

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