British Journal of Medicine & Medical Research
10(12): 1-12, 2015, Article no.BJMMR.19288
ISSN: 2231-0614

SCIENCEDOMAIN *international*
www.sciencedomain.org

Isoflurane and Ketamine/Xylazine Anesthesia do not Influence the Neuroprotective Effects of Simvastatin in a Model of Permanent Cerebral Ischemic Injury in C57BL/6J Mice

Maria Linou[1], Era Taoufik[2*], Georgios Kazakos[3], Efstathios Boviatsis[4], Lila Papadimitriou[5] and Ismene A. Dontas[6]

[1]Diagnostic Department, Hellenic Pasteur Institute, Athens, Greece.
[2]Laboratory of Cellular and Molecular Neurobiology, Hellenic Pasteur Institute, Athens, Greece.
[3]School of Veterinary Medicine, Aristotle University of Thessaloniki, Thessaloniki, Greece.
[4]2nd Department of Neurosurgery, ATTIKON University Hospital, Athens, Greece.
[5]Anesthesiology Department, Henri Dunant Medical Center, Athens, Greece.
[6]Laboratory for Research of the Musculoskeletal System, School of Medicine, University of Athens, Athens, Greece.

Authors' contributions

This work was carried out in collaboration between all authors. Authors ML and ET performed the experiments. Authors GK, EB, LP and IAD designed the study. Authors ML, ET and IAD wrote the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJMMR/2015/19288
Editor(s):
(1) Xin-an Liu, Neuroscience Department, The Scripps Research Institute, Scripps, Florida, USA.
Reviewers:
(1) Anonymous, Okayama University, Japan.
(2) Weiting Wang, Tianjin Institute of Pharmaceutical Research, Tianjin, China.
(3) Anonymous, King George's Medical College, Lucknow (UP), India.
Complete Peer review History: http://sciencedomain.org/review-history/11332

Original Research Article

Received 1st June 2015
Accepted 2nd August 2015
Published 9th September 2015

ABSTRACT

Laboratory mice with Middle Cerebral Artery Occlusion (MCAO) represent the main animal models for ischemic stroke research. Appropriate anesthetic protocols are essential, as anesthetic agents might affect the central nervous system (CNS) and therefore interfere with the outcome of pre-clinical ischemic stroke studies. In the present study we sought to investigate whether isoflurane, a

**Corresponding author: Email: etaoufik@pasteur.gr;*

widely used inhalational anesthetic, has any effect on MCAO mice pretreated with simvastatin, a well-known neuroprotective compound, compared with the administration of injectable ketamine/xylazine combination. Forty adult C57Bl/6J mice randomly allocated into four groups underwent ischemic injury by permanent coagulation of the Middle Cerebral Artery (MCA): Group A (n=11) animals were anesthetized with ketamine/xylazine, Group B (n=9) with isoflurane, Group C (n=9) with ketamine/xylazine after pretreatment with simvastatin 2h before permanent Middle Cerebral Artery Occlusion (pMCAO) and Group D (n=11) with isoflurane after similar pretreatment with simvastatin. The potential neuroprotective effect of the anesthetics was evaluated in terms of brain infarct volumes and neuron death. No significant differences, both quantitatively and qualitatively, were detected in brain lesions measured up to 7 days after pMCAO when comparing isoflurane inhalational anesthesia to ketamine/xylazine injectable anesthesia. Group C mice (simvastatin-treated ketamine/xylazine) had a significantly reduced brain infarct volume compared to Group A mice (non-simvastatin ketamine/xylazine) (P<.0005). Similarly Group D mice (simvastatin-treated isoflurane) had a significantly reduced brain infarct volume compared to Group B mice (non-simvastatin isoflurane) (P<.0005). No difference between morphology and number of apoptotic neurons was detected due to the two different anesthetic regimens. These results demonstrated the safe use of the established anesthetic agent isoflurane in mice where simvastatin is investigated as a neuroprotective compound.

Keywords: Maouse model; permanent middle cerebral artery occlusion; isoflurane; neuroprotection; simvastatin.

ABBREVIATIONS

Middle Cerebral Artery Occlusion (MCAO)
Central Nervous System (CNS)
Middle Cerebral Artery (MCA)
Permanent Middle Cerebral Artery Occlusion (pMCAO)

1. INTRODUCTION

Ischemic stroke represents one of the major causes of death and disability worldwide creating significant socio-economic burdens and leaving affected individuals with debilitating consequences including permanent disabilities. Ischemia results either from a permanent or transient interruption of the brain arterial blood supply, by an embolus or a thrombus [1,2]. It is well known that brain tissue has an absolute and continuous requirement for high oxygen and glucose as energy production depends almost exclusively on oxidative phosphorylation processes [3] and therefore one can imagine that halting this process in any way is deleterious for the CNS tissue. During the ischemic event the impaired blood flow does not allow the delivery of these substrates to the CNS, leading to energy depletion, over activation of glutamate receptors and release of excess glutamate, increase of intracellular calcium, loss of membrane potential and cell depolarization [4]. This deleterious cascade of events is initiated immediately after the ischemic insult and it results to cell death shortly after, whilst neuron death proceeds even at later phase resulting in an expanding affected area with increasing cell damage unless intervention comes to halt/prevent this progress [5].

Despite major advances in the pathophysiology, diagnosis, and follow-up [6] of ischemic stroke, the only approved treatment today offered remains the intravenous administration of recombinant tissue plasminogen activator (rt-PA) as long as it is given within the first 3 hours after stroke onset [7,8].

The reality is at least disappointing if we consider the number of thrombolytic, reperfusion and neuroprotecive compounds that have been developed and showed promising results in pre-clinical settings but failed in clinical trials [7,9]. Pre-clinical stroke research has a remarkably low translational success rate and the clinical need for novel neuroprotective therapeutics has gone largely unmet [10] whilst serious issues concerning the preclinical studies and available animal models were re-evaluated. However, this discrepancy led also to positive outcomes including improved study quality to include

considerable rigor, standardization and emphasis on minimization of experimental bias during animal experimentation [2].

Currently there is a large number of compounds showing promising effects. One of those is the family of statin proteins that have led to improved neurological outcome after acute cerebral ischemia [11] by having a positive impact on endothelial function, by modulating inflammatory responses and preventing the formation of thrombus [12]. More specifically, simvastatin, an approved drug for cholesterol reduction, has been shown to be a strong neuroprotectant in an embolic model of stroke [13] and in MCAO in rats [14] but also when combined with tissue plasminogen activator in experimental and human stroke [15,16] and with human umbilical cord blood cells [17] where it enhances vascular remodeling and functional outcome after MCAO in rats. In another study, simvastatin markedly decreased the OGD/reoxygenation-evoked death of cortical neurons by decreasing the intracellular level of 4-hydroxy-2E-nonenal (HNE) in neuronal cells, in rats [18].

In an effort to simulate stroke injury *in vivo*, a variety of animal models have been developed both in small laboratory animals and non human primates [19,20]. Even though all these models differ significantly in the way infarction is achieved, in whether this is reversible or not and in other multiple parameters, they all share one common feature, the absolute requirement for efficient anesthesia. For this microsurgery procedure anesthesia has to be long lasting, provide the required analgesic effect, allow recovery, minimize death incidence of experimental subjects and at the same time ensure that it does not interfere with parameters under investigation and the outcome of experimental procedures.

Pre-clinical studies for stroke are largely based on animal models that model specific aspects of ischemic injury. In humans the majority of strokes are atherothrombotic or embolic, accounting for 88% of cases. The closest surgical method to recapitulate such situations in rodents and other animal species (rabbits, dogs and non-human primates) is the occlusion of the middle cerebral artery that induces both subcortical and cortical damage by activating excitotoxic and apoptotic cellular processes [21,22]. Based on the level of pain and distress, this surgical procedure may be classified as severe (moderate in the hands of experts) [23] and is always conducted under general anesthesia. Injectable anesthetic agents are in ready to use solutions, can be used in combinations for better results, remain available at affordable cost and do not require the use of any specific equipment for their administration. However they have several disadvantages especially in small rodents as, they require high level of accuracy in dosing and as their action is long-lasting, rendering them unsafe, under conditions of poor monitoring of the animals. The most commonly used injectable anesthetics during middle cerebral artery occlusion in rodents are ketamine and xylazine which are co administered.

On the other hand, inhalational anesthetic agents are accurately administered at specific rate of flow, maintain an accurate anesthetic plane, and provide fast induction of anesthesia together with fast and at will recovery, ensuring the safest anesthetic conditions available in experimental settings. A major disadvantage is the pricing that is higher compared to injectable agents, the absolute requirement for specialized equipment and the strict monitor in order to maintain an accurate anesthetic plane. The most commonly used inhalational anesthetic is isoflurane [24] while there is a continuous development of related agents [25].

Despite the clear advantage of inhalational anesthetics, there is a rising concern for their use in middle cerebral artery occlusion experiments as there is data to support that they can alter the outcome of this experimental procedure. Specifically, in rodents that have been subjected to various models of brain injury, isoflurane has been shown to exert neuroprotective effects that can be either brief or prolonged [26,27]. This effect seems to be largely dependent on the concentration and duration of exposure to the anesthetic, the animal model used and other different parameters specific for each study. These results suggest that isoflurane anesthesia can undermine, hide or alter the effects of novel stroke treatments that are under investigation [28] and warrant caution when using anesthetics for pre-clinical testing of neuroprotective agents and when interpreting their data.

In this study, we aim to investigate the potential neuroprotective role of isoflurane in C57Bl/6J mice, in the ischemic model of pMCAO. Furthermore we sought to investigate whether isoflurane use could enhance or alter in any way the effect of simvastatin in mice pretreated with this well-known neuroprotective compound.

2. MATERIALS AND METHODS

2.1 Laboratory Animals

C57BL/6J mice (n=40) (Harlan, UK), 3 to 4 months old weighing at least 30 g were used for all ischemia procedures. Animals were bred under specific pathogen-free conditions (SPF) in the breeding unit of the Department of Animal Models for Biomedical Research at Hellenic Pasteur Institute. Mice were maintained at the experimental conventional unit of the same Institute, four to a polycarbonate cage (Tecniplast, Italy) with filter top and pre-defined dimensions of 332 x 150 x 130 mm and floor area of 335 cm2, according to the "Requirements for Establishments and for the Care and Accommodation of Animals, Annex III of the Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes" and bedding of wood shavings (Scobis Uno, Mucedola, Italy). External factors were maintained stable within approved limits and included a 12 h light/dark cycle, 50-60 % relative humidity, and temperature between 20º and 22ºC. Animals were administered filtered tap water by water bottles of clear-glass Polycarbonate (Tecniplast, Italy) and laboratory chow (4RF18 GLP certificate, Mucedola), *ad libitum*. The experimental protocol describing all animal procedures was approved by the National Veterinary Authority (license number 542/30-01-2013) according to the European Directive 2010/63/EU and the Greek Presidential Decree No. 56/2013 with which it is conformed.

2.2 Groups, Anesthetic and Neuro-protective Agent Treatments

All animals were randomly assigned to four different groups according to the exposure of selected anesthetics and simvastatin. In Group A (n=11) animals were anesthetized with ketamine and xylazine (at 100 and 10 mg/kg, IP, respectively), Group B (n=9) with isoflurane (4-5% for induction in a chamber and 1-2% for maintenance with the use of calibrated vaporizer), Group C (n=9) with ketamine and xylazine (at 100 and 10 mg/kg, IP, respectively) after pretreatment with simvastatin (LEPUR F.C.TAB 20 mg/tab, ELPEN, Greece, at 100 mg/kg, PO by oral gavage) 2h before pMCAO and Group D (n=11) with isoflurane (4-5% for induction in a chamber and 1-2% for maintenance with the use of calibrated vaporizer) after pretreatment with simvastatin (LEPUR F.C.TAB 20 mg/tab, ELPEN, Greece, at 100 mg/kg PO by oral gavage 2 h before pMCAO. All animals received the same pre-emptive multimodal analgesia: topical lidocaine and prilocaine (EMLA cream, ASTRA), and parenteral butorphanol and carprofen (Butador at 2 mg/Kg and Rimadyl at 5 mg/kg, SC) 1 h prior to pMCAO and post operatively, carprofen (Rimadyl, 5 mg/kg, SC) additionally every 24 hours for 7 days until the mice were sacrificed for brain analysis. It should be noted that during the pMCAO procedure one mouse of the ketamine/xylazine group died and was replaced, whilst all mice that received isoflurane survived.

Euthanasia was conducted by cervical dislocation by an experienced technician on animals anesthetized with isoflurane as above.

2.3 Permanent Middle Cerebral Artery Occlusion (pMCAO)

Surgical protocols were additionally approved by the Pasteur Institute ethics committee. Focal ischemia was induced by pMCAO as reported previously [29,30]. The mouse was placed in the supine position on a heating pad to ensure a body temperature of 37℃±0.5℃ (Harvard Apparatus, USA) and a rectal probe was used throughout the experimental procedure for monitoring temperature fluctuations. During ischemia, physiological parameters remained in the normal range (body temperature, 37±0.3℃; PaCO2, 40.9±4.2 mmHg; PaO2, 131.73±3.97 mmHg; pH, 7.08±0.08). Briefly, under a stereo dissecting microscope the left Middle Cerebral Artery (MCA) was exposed through a skin incision between the left eye and ear followed by a hole using a microdrill (Harvard Apparatus, USA) and was permanently occluded by bipolar electro coagulation (FST, Germany) distal to the bifurcation of the artery. To ensure complete occlusion the artery was dissected just above the point of coagulation using a micro-dissection scissors (FST, Germany). After coagulation, the skin lesion was stitched with 4-0 Vicryl absorbable sutures (Ethicon, USA) and the animals were placed in a recovery cage with a heating blanket underneath to avoid hypothermia until complete recovery from anesthesia for at least 4h, and then were allowed to return to their original cages and allowed to eat and drink *ad libitum*.

2.4 Histology and Immunocytochemistry

Brains were rapidly removed from the euthanized mice and placed in ice-cold isopentane solution (Merck, Germany) and were stored at -80℃ until further processing. Coronal brain sections (20 μm) were cut on a cryostat (Leica, Germany) and stained with thionin (Sigma-Aldrich, UK). Total infarct volume (mm^3) was calculated after integration of infarcted areas and correction for brain edema area on each section using the public domain ImageJ software with the distance (400 μm) between each section level analyzed (Valable et al., 2005). For immunocytochemistry, frozen sections were placed in blocking buffer containing 5% Normal Goat Serum and 0.1% Triton-X100 diluted in PBS and then incubated in primary antibody solutions overnight containing 1% Normal Goat Serum and 0.05% Triton-X100. Primary antibodies used were anti-caspase 3 (active) (Cell Signaling, UK, 1:600) and anti NeuN (Chemicon, UK, 1:400). Antibody binding was visualized using biotinylated secondary antibody followed by HRP-labeled avidin-biotin complex and DAB (VECTOR, UK).

2.5 Western Blot

Total protein extracts from non-occluded (contralateral) and ischemic cortex (ipsilateral) of representative mice from each experimental group (n=3 for ketamine/xylazine Group A and n=3 for isoflurane Group B, n=2 for ketamine/xylazine simvastatin pre-treated Group C and n=2 for isoflurane simvastin pre-treated Group D) 7 days after pMCAO were prepared by homogenization of tissues in ice-cold RIPA buffer containing a protease inhibitor cocktail (Roche, UK). Thirty micrograms of protein extract were boiled in sample buffer (Cell Signaling, UK) and resolved in 12.5% polyacrylamide gels under denaturating conditions and transferred to PVDF membranes (Pierce, UK). Blots were probed with an antibody against PARP (Santa Cruz, USA) at 1:500 dilution and detection was performed using a horseradish peroxidase-conjugated anti-rabbit antibody (Pierce, USA) at 1:2000 dilution and finally the ECL Plus detection system (GE Healthcare). Normalization was performed using an antibody against β-actin antibody (Cell Signaling) at 1:1000 dilution. Relative band intensities were quantitated using Image J Software.

2.6 Statistical Analysis

All data are presented as mean ± standard error of the mean (S.E.M.) for continuous variables. The Kolmogorov–Smirnov test was utilized for normality analysis of the parameters. Independent samples t-test was used for the comparison of anesthetic agents in the presence or absence of simvastatin and for the comparison of simvastatin use in relation to the two different anesthetic agents in relation to the average infarct volume variable. The same test was used for the comparison of the number of neuron nuclei and caspase 3 protein between groups. All tests are two-sided, statistical significance was set at $P < .05$. All analyses were carried out using the statistical package SPSS vr 17.00 (Statistical Package for the Social Sciences, SPSS Inc., Chicago, Ill., USA).

3. RESULTS AND DISCUSSION

3.1 Results

3.1.1 Ischemic lesion volume – groups A and B

Lesions were similar 7 days after occlusion between Groups A and B with an average lesion volume of 22.590±0.591 mm^3 in Group A and 22.071±0.565 mm^3 in Group B ($P > .05$) (Fig. 1A). Thionin staining of the coronal brain sections showed similar cortical lesions and did not show any differences between the two groups ($P > .05$) (Fig.1B).

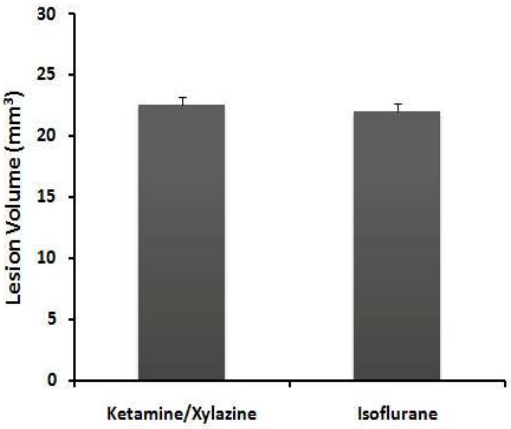


Fig. 1A. Ischemic lesion volume measurements 7 days post pMCAO. Quantitation of lesion volume (mm^3) in mice anesthetized with ketamine/xylazine (n=8, Group A) (1A left) and isoflurane (n=9, Group B) (1A right) using Image J Software revealed similar size of infarction between the two groups. Data are expressed as mean ± S.E.M. for each group analyzed

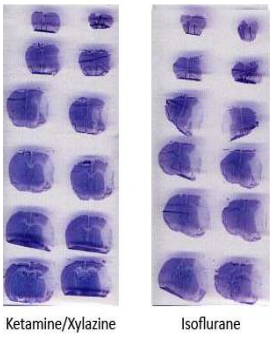


Fig. 1B. Thionin staining of Group A and Group B serial cryosections with 400 μm distance in between showing the similar extent of infarct between the two groups. Representative thionin staining from one animal per group is shown

3.1.2 Neuron death – groups A and B

Despite the absence of significant differences in lesion volume between the two different anesthetics, we performed further analysis of the lesions using immunocytochemical methods for the detection of the neuronal marker, NeuN, and of the apoptotic protein, caspase-3. No differences were observed both in the morphology (Fig. 2A) and number of neurons (Fig. 2B) within ischemic lesions between Groups A and B.

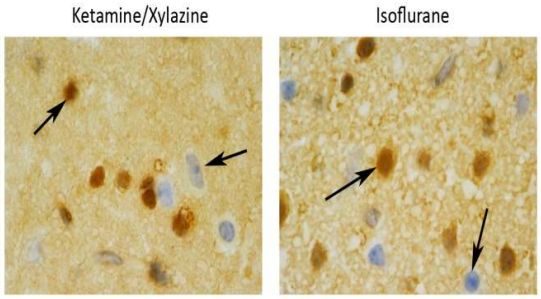


Fig. 2A. Analysis of neuronal death in ischemic lesions 7 days post pMCAO. Immunocytochemical analysis for the detection of the active form of caspase 3 confirmed that the neuronal cell death within lesions from ketamine/xylazine (n=3, Group A) and isoflurane (n=3, Group B) anesthetized mice was comparable, with condensed nuclei staining positive for active caspase-3 (brown) and cells that remain intact staining blue (60x magnification) (2A, representative sections shown)

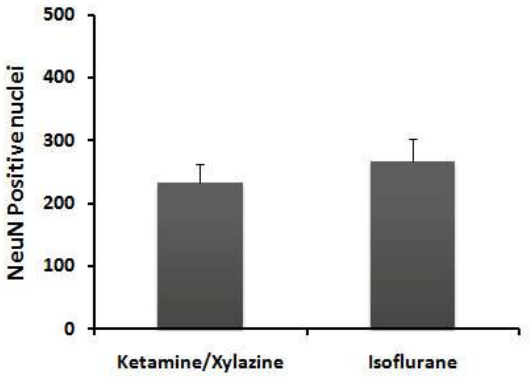


Fig. 2B. Quantitation of NeuN positive nuclei in both groups numbers of neurons in defined areas of the ischemic lesions were also similar in both groups (A and B) suggesting that neuron loss is not affected by the use of these anesthetics. Data are expressed as mean ± S.E.M. for each group analyzed

To further examine possible differences in cell death, we also assessed the levels of PARP cleavage as a marker of apoptosis, by Western blot in protein extracts after 7 days of pMCAO (n=3 for ketamine/xylazine Group A and n=3 for isoflurane Group B). In agreement with our immunocytochemical findings, PARP was efficiently cleaved after pMCAO (Fig. 2C) and its levels were similar between the two groups studied.

The above data confirm that in our pMCAO model in C57Bl/6J mice, injectable ketamine/ xylazine anesthesia and isoflurane inhalational anesthesia have similar effects in brain tissue, both quantitatively and qualitatively.

3.1.3 Ischemic lesion volume – groups C and D

Lesion volume analysis revealed that pre-treatment with simvastatin did not affect lesion volume regardless of the mode of anesthesia chosen (Group C 18.792±0.559 mm^3 vs D 18.353±0.468 mm^3, P=0.552 (Fig. 3).

3.1.4 Ischemic lesion volume – groups A vs C, B vs D regarding simvastatin treatment

It was observed that simvastatin exerted strong neuroprotection as lesion volume was significantly decreased in simvastatin-treated mice.

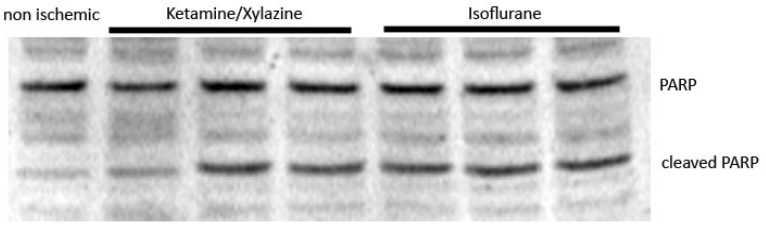


Fig. 2C. Western Blot analysis for PARP protein in non ischemic cortical protein extract (n=1), Group A (n=3) and Group B (n=3) ischemic protein extracts shows an increase in PARP cleavage after pMCAO that is similar between Group A and B

Specifically, simvastatin-treated ketamine/ xylazine mice (Group C) had a significantly reduced lesion volume (18.792±0.559 mm^3) compared to non-treated ketamine/xylazine mice (Group A: 22.590±0.591 mm^3) (P< .0005).

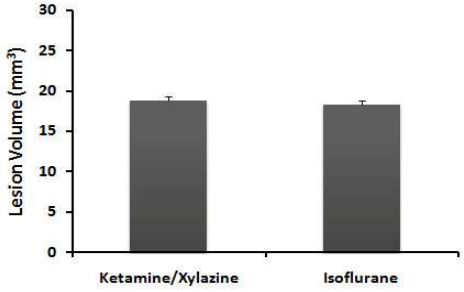


Fig. 3. Ischemic lesion volume 7 days post pMCAO in mice pre-treated with simvastatin. Quantitation of lesion volume (mm^3) in mice pretreated with simvastatin 1 h prior to pMCAO induction either using ketamine/xylazine (n=9, Group C) or isoflurane (n=11, Group D) using Image J Software revealed similar size of infarction between the two groups. Data are expressed as mean ± S.E.M. for each group analyzed

Similarly simvastatin-treated isoflurane mice (Group D) had a significantly reduced lesion volume (18.353±0.468 mm^3) compared to the non-treated isoflurane mice (Group B: 22.071±0.565 mm^3) (P< .0005) (Table 1, Figs. 4 A, B).

3.1.5 Neuron death – groups A vs C, B vs D regarding simvastatin treatment

Analysis of neuronal death in ischemic lesions 7 days post pMCAO in mice pretreated with simvastatin. Caspase 3 immunoreactivity was quantitated in defined areas of ischemic lesions and comparisons between Group C and A showed a significant reduction in the number of apoptotic cells (P< .001) (Fig. 5A) and a similar difference was shown when Groups D and B were compared (P< .001) (Fig. 5B). Data are expressed as mean ± S.E.M. for each group analyzed.

Caspase 3 immunoreactivities within lesions from simvastatin-treated mice (Group C: 28±3.4 and Group D: 42±2.6) were lower compared to untreated mice (Group A: 43±4 and Group B: 53±6) (Figs. 5C, D) but no difference between morphology or number of apoptotic neurons was detected due to the two different anesthetics (Figs. 5C, D).

3.2 Discussion

In the present study we compared isoflurane with one of the most commonly used injectable anesthetic combinations, ketamine/xylazine, for pMCAO by surgical coagulation in mice. Their potential neuroprotective effect was evaluated in terms of brain infarct volumes and neuron death.

No differences in brain lesion volume measured up to 7 days after pMCAO were detected when comparing isoflurane to ketamine/xylazine anesthesia, confirming available data where isoflurane use during ischemic procedures did not show any improved short or long term neurological outcome [27].

Our study was performed in adult C57Bl/6J mice using a model of permanent ischemic injury by coagulation of the MCA. In this model neuron death occurs independently of inflammatory infiltration, in contrast to ischemia/reperfusion models used, where isoflurane administration induces neuroprotection, by its effects on inflammation [31]. It was demonstrated that neuron death after pMCAO was not affected by isoflurane administration.

Table 1. Lesion volume mean values and standard error of mean (S.E.M.) in mm^3

Groups	Lesion Volume (mm^3)	S.E.M.
Group A (Ketamine/Xylazine)	22,590	0,591
Group B (Isoflurane)	22,071	0,65
Group C (Ketamine/Xylazine+ Simvastatin)	18,792	0,559
Group D (Isoflurane+Simvastatin)	18,353	0,468

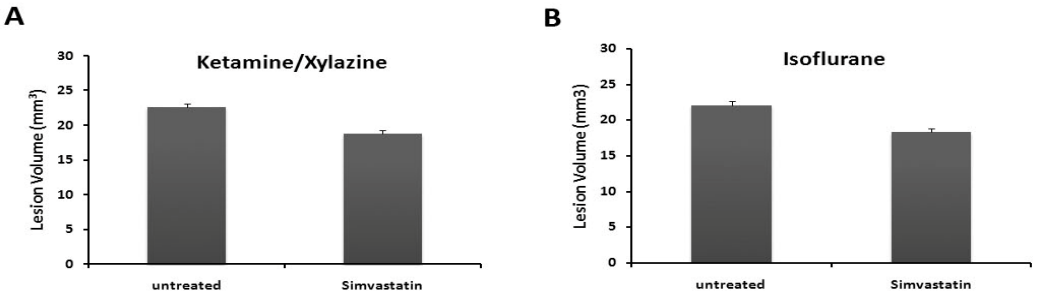


Fig. 4. Comparison of ischemic lesion volumes 7 days post pMCAO between Group C (n=9) and Group A (n=8) reveal significantly reduced lesion volume after simvastatin administration when ketamine/xylazine was used (*P*< .0005) (4A). Similar reduced ischemic volume was also observed when Group D (n=11) and Group B (n=9) were compared showing that simvastatin also conferred significant neuroprotection when isoflurane was used (*P*< .0005) (4B). Data are expressed as mean ± S.E.M. for each group analyzed

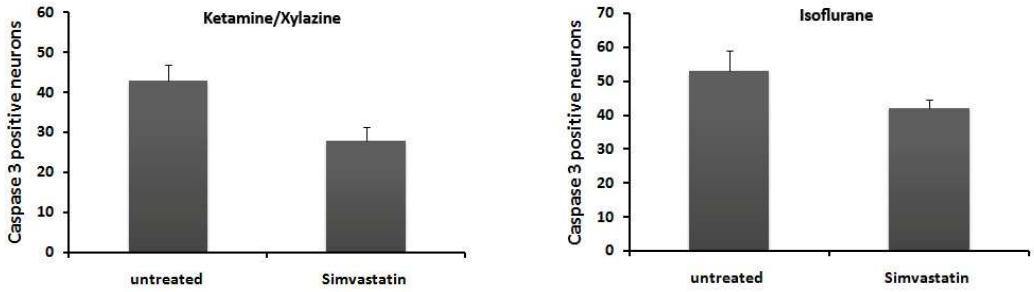
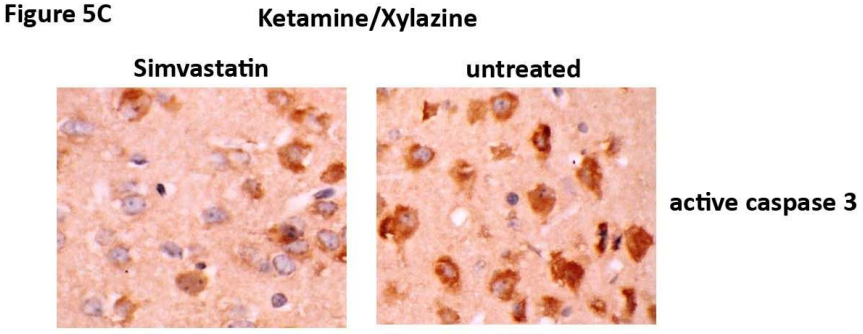


Fig. 5A, B. Analysis of neuronal death in ischemic lesions 7 days post pMCAO in mice pretreated with simvastatin. Caspase 3 immunoreactivity was quantitated in defined areas of ischemic lesions and comparisons between Group C and A and D and B showed a significant reduction in the number of apoptotic cells (*P*< .001). Data are expressed as mean ± S.E.M. for each group analyzed



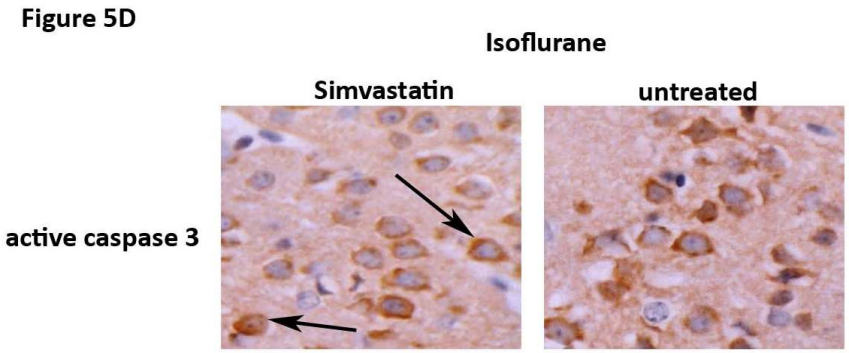


Fig. 5C, D Representative images of sections stained for active caspase 3 after pretreatment with simvastatin shows less immunoreactivity when compared to untreated mice both when ketamine/xylazine (5C) and isoflurane (5D) were used. Representative sections are shown for each group analyzed (60 x magnification)

In contrast to our findings, isoflurane use has been challenged by a number of studies that showed that it can induce neuroprotection [27,32,33] probably by antagonizing the NMDA glutamate receptor without excluding other mechanisms of action including potassium channels and NO mediated vasodilation [34,35]. In several studies the cerebroprotective effect of isoflurane is dependent on the duration and dose of the anesthetic, its pretreatment use, and the severity and extent of the study [36].

In another study preconditioning with a 30 min isoflurane administration in adult rats before MCAO resulted in an acute phase of neuroprotection with a time window of effect from a few minutes up to 3 hours [37].

The neuroprotective effects of isoflurane have been demonstrated using mainly histochemical and behavioral tests, in various models of ischemic injury mainly in rats and in reversible ischemic conditions with variable times of reperfusion [27]. This conferred a significant improvement in motor co-ordination and infarct volume and is in agreement with previous studies performed in neonatal rats [38,39] suggesting that studies that involve preconditioning with isoflurane are up to date the most consistent and in support of neuroprotecive effects.

Although ketamine has also been shown to have a neuroprotective effect as an NMDA glutamate receptor antagonist, it may be less neuroprotective than isoflurane in a rat model of experimental traumatic brain injury [40]. According to several studies there is no long-term outcome data demonstrating the persistent benefit of neuroprotection after ketamine anesthesia [41,42]. In the second part of our study, in order to ensure that in our experimental setting isoflurane does not modulate pharmacologically induced neuroprotection, we used simvastatin, a cholesterol lowering compound that belongs to the statin family of anti-inflammatory agents [43]. In contrast to groups not treated with simvastatin, where the infarct volumes did not have any significant difference between them, the infarct volume was significantly reduced when animals were pretreated with simvastatin, both in the isoflurane and the ketamine/xylazine group.

Simvastatin has been shown to be effective in an embolic model of stroke in rats, by establishing re-circulation in the injured brain and reducing perfusion deficits [13] and by modulation of NOS activity [4,12]. In our pMCAO model a pre-treatment two hours before occlusion in non-anesthetized mice was enough to confer long term (7 days) neuroprotection and reduced apoptotic cell death within lesions of the treated mice, regardless of the anesthetic used. The discrepancy between our results and those of Seto et al. [28] that also used C57Bl/6J mice and showed masking of neuroprotectants by isoflurane, could be explained by large differences in our experimental approaches, such as the use of prothrombotic occlusion instead of pMCAO. Moreover the compound tested involved the GluN2B or a4b2 nicotinic receptor antagonist and had direct neuroprotective activity lacking anti-inflammatory effects whilst the animal models employed involved freely awake moving mice.

In our study, isoflurane was also used as an anesthetic for the humane euthanasia of mice by cervical dislocation that was conducted by an experienced technician in order to kill the mice rapidly, humanely, with the minimum exposure to isoflurane. This brief period of isoflurane inhalation prior to dislocation may be considered a limitation as all groups inevitably were exposed to it. However, this exposure was brief, conducted 7 days post-operatively, and taking into account that the brains were removed immediately after dislocation, we believe that this inhalation did not have the time to interfere with the lesion findings.

4. CONCLUSION

Our study demonstrated that isoflurane administration did not affect ischemic lesion volume after pMCAO in mice, nor did it influence simvastatin-induced neuroprotection. Overall this data suggests that in the pMCAO C57Bl/6J mouse model, isoflurane use alone or in combination with a neuroprotective compound (simvastatin) did not affect the experimental outcome when compared with injectable anesthetics, providing further evidence to support the safe use of this inhalational anesthetic in pre-clinical studies of ischemic injury in mice.

While these findings in mice with pMCAO cannot be applied to all experimental brain ischemia settings, isoflurane use appears safe and efficient. Nevertheless future experimentation using various neuroprotective agents, especially compounds tested pre-clinically for stroke, is necessary before proceeding to clinical trials.

CONSENT

It is not applicable.

ETHICAL APPROVAL

All authors hereby declare that the European Directive 2010/63/EU “on the protection of animals used for scientific purposes” and the Greek Presidential Decree No. 56/2013 with which it is conformed were followed. All experiments have been examined and approved by the appropriate ethics committee of the National Veterinary Authority, license number 542/30-01-2013.

ACKNOWLEDGEMENT

The authors are grateful to Dr. Antonis Galanos for his valuable input regarding the statistical analysis of the study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Dirnagl U, Fisher M. International, multicenter randomized preclinical trials in translational stroke research: it's time to act. J Cereb Blood Flow Metab. 2012; 32(6):933-5.
2. Dirnagl U. Pathobiology of injury after stroke: the neurovascular unit and beyond. Ann N Y Acad Sci. 2012;1268:21-5.
3. Katsura K, Kristian T, Siesjo BK. Energy metabolism, ion homeostasis, and cell damage in the brain. Biochem Soc Trans. 1994;22(4):991-6.
4. Endres M, Dirnagl U, Moskowitz MA. The ischemic cascade and mediators of ischemic injury. Handb Clin Neurol. 2009; 92:31-41.
5. Moretti A, Ferrari F, Villa RF. Neuroprotection for ischaemic stroke: Current status and challenges. Pharmacol Ther. 2015;146C:23-34.
6. Silver B, Wulf Silver R. Stroke: diagnosis and management of acute ischemic stroke. FP Essent. 2014;420:16-22.
7. Tsivgoulis G, Katsanos AH, Alexandrov AV. Reperfusion therapies of acute ischemic stroke: Potentials and failures. Front Neurol. 2014;5:215.
8. Tissue plasminogen activator for acute ischemic stroke. The National Institute of Neurological Disorders and Stroke rt-PA Stroke Study Group. N Engl J Med. 1995; 333(24):1581-7.
9. Dirnagl U. Bench to bedside: the quest for quality in experimental stroke research. J Cereb Blood Flow Metab. 2006;26(12): 1465-78.
10. Grupke S, Hall J, Dobbs M, Bix GJ, Fraser JF. Understanding history, and not repeating it. Neuroprotection for acute ischemic stroke: From review to preview. Clin Neurol Neurosurg. 2015;129C:1-9.
11. Ni Chroinin D, Asplund K, Asberg S, Callaly E, Cuadrado-Godia E, Diez-Tejedor E, et al. Statin therapy and outcome after ischemic stroke: systematic review and meta-analysis of observational studies and randomized trials. Stroke. 2013;44(2):448-56.
12. Zacharia BE, Bruce SS, Carpenter AM, Hickman ZL, Vaughan KA, Richards C, et

al. Variability in outcome after elective cerebral aneurysm repair in high-volume academic medical centers. Stroke. 2014; 45(5):1447-52.
13. Shabanzadeh AP, Shuaib A, Wang CX. Simvastatin reduced ischemic brain injury and perfusion deficits in an embolic model of stroke. Brain Res. 2005;1042(1):1-5.
14. Zhu MX, Lu C, Xia CM, Qiao ZW, Zhu DN. Simvastatin pretreatment protects cerebrum from neuronal injury by decreasing the expressions of phosphor-CaMK II and AQP4 in ischemic stroke rats. J Mol Neurosci. 2014;54(4):591-601.
15. Campos M, Garcia-Bonilla L, Hernandez-Guillamon M, Barcelo V, Morancho A, Quintana M, et al. Combining statins with tissue plasminogen activator treatment after experimental and human stroke: a safety study on hemorrhagic transformation. CNS Neurosci Ther. 2013; 19(11):863-70.
16. Naci H, Brugts JJ, Fleurence R, Ades AE. Comparative effects of statins on major cerebrovascular events: A multiple-treatments meta-analysis of placebo-controlled and active-comparator trials. QJM : Monthly journal of the Association of Physicians. 2013;106(4):299-306.
17. Cui X, Chopp M, Zacharek A, Dai J, Zhang C, Yan T, et al. Combination treatment of stroke with sub-therapeutic doses of Simvastatin and human umbilical cord blood cells enhances vascular remodeling and improves functional outcome. Neuroscience. 2012;227:223-31.
18. Lim JH, Lee JC, Lee YH, Choi IY, Oh YK, Kim HS, et al. Simvastatin prevents oxygen and glucose deprivation/ reoxygenation-induced death of cortical neurons by reducing the production and toxicity of 4-hydroxy-2E-nonenal. J Neurochem. 2006;97(1):140-50.
19. Casals JB, Pieri NC, Feitosa ML, Ercolin AC, Roballo KC, Barreto RS, et al. The use of animal models for stroke research: a review. Comp Med. 2011;61(4):305-13.
20. Neuhaus AA, Rabie T, Sutherland BA, Papadakis M, Hadley G, Cai R, et al. Importance of preclinical research in the development of neuroprotective strategies for ischemic stroke. JAMA Neurol. 2014; 71(5):634-9.
21. Ginsberg MD, Busto R. Rodent models of cerebral ischemia. Stroke. 1989;20(12): 1627-42.
22. Onteniente B, Rasika S, Benchoua A, Guegan C. Molecular pathways in cerebral ischemia: Cues to novel therapeutic strategies. Mol Neurobiol 2003;27(1): 33-72.
23. Commission E. Expert working group on severity classification of scientific procedures performed on animals. Final Report, Brussels; 2009.
24. Constantinides C, Mean R, Janssen BJ. Effects of isoflurane anesthesia on the cardiovascular function of the C57BL/6 mouse. ILAR J 2011;52(3):e21-31.
25. Flecknell. Laboratory Animal Anaesthesia; 2009.
26. Zhao P, Peng L, Li L, Xu X, Zuo Z. Isoflurane preconditioning improves long-term neurologic outcome after hypoxic-ischemic brain injury in neonatal rats. Anesthesiology 2007;107(6):963-70.
27. Kitano H, Kirsch JR, Hurn PD, Murphy SJ. Inhalational anesthetics as neuro-protectants or chemical pre-conditioning agents in ischemic brain. J Cereb Blood Flow Metab. 2007;27(6): 1108-28.
28. Seto A, Taylor S, Trudeau D, Swan I, Leung J, Reeson P, et al. Induction of ischemic stroke in awake freely moving mice reveals that isoflurane anesthesia can mask the benefits of a neuroprotection therapy. Front Neuroenergetics. 2014;6:1.
29. Welsh FA, Sakamoto T, McKee AE, Sims RE. Effect of lactacidosis on pyridine nucleotide stability during ischemia in mouse brain. J Neurochem. 1987;49(3): 846-51.
30. Taoufik E, Valable S, Muller GJ, Roberts ML, Divoux D, Tinel A, et al. FLIP(L) protects neurons against in vivo ischemia and in vitro glucose deprivation-induced cell death. J Neurosci 2007;27(25): 6633-46.
31. Xu X, Kim JA, Zuo Z. Isoflurane preconditioning reduces mouse microglial activation and injury induced by lipopolysaccharide and interferon-gamma. Neuroscience. 2008;154(3):1002-8.
32. Khatibi NH, Ma Q, Rolland W, Ostrowski R, Fathali N, Martin R, et al. Isoflurane posttreatment reduces brain injury after an intracerebral hemorrhagic stroke in mice. Anesth Analg. 2011;113(2):343-8.
33. Chi OZ, Hunter C, Liu X, Weiss HR. The effects of isoflurane pretreatment on cerebral blood flow, capillary permeability, and oxygen consumption in focal cerebral

ischemia in rats. Anesth Analg. 2010; 110(5):1412-8.
34. Kawaguchi M, Drummond JC, Cole DJ, Kelly PJ, Spurlock MP, Patel PM. Effect of isoflurane on neuronal apoptosis in rats subjected to focal cerebral ischemia. Anesth Analg. 2004;98(3):798-805.
35. Heurteaux C, Guy N, Laigle C, Blondeau N, Duprat F, Mazzuca M, et al. TREK-1, a K+ channel involved in neuroprotection and general anesthesia. Embo J. 2004; 23(13):2684-95.
36. Wang C, Jin Lee J, Jung HH, Zuo Z. Pretreatment with volatile anesthetics, but not with the nonimmobilizer 1,2-dichlorohexafluorocyclobutane, reduced cell injury in rat cerebellar slices after an in vitro simulated ischemia. Brain Research 2007;1152:201-8.
37. Kapinya KJ, Lowl D, Futterer C, Maurer M, Waschke KF, Isaev NK, et al. Tolerance against ischemic neuronal injury can be induced by volatile anesthetics and is inducible no synthase dependent. Stroke 2002;33(7):1889-98.
38. McAuliffe JJ, Joseph B, Vorhees CV. Isoflurane-delayed preconditioning reduces immediate mortality and improves striatal function in adult mice after neonatal hypoxia-ischemia. Anesth Analg. 2007; 104(5):1066-77.
39. Zhao F, Jin T, Wang P, Kim SG. Improved spatial localization of post-stimulus BOLD undershoot relative to positive BOLD. Neuroimage. 2007;34(3):1084-92.
40. Statler KD, Alexander H, Vagni V, Dixon CE, Clark RS, Jenkins L, et al. Comparison of seven anesthetic agents on outcome after experimental traumatic brain injury in adult, male rats. J Neurotrauma. 2006;23(1):97-108.
41. Yan J, Jiang H. Dual effects of ketamine: neurotoxicity versus neuroprotection in anesthesia for the developing brain. J Neurosurg Anesthesiol. 2014;26(2): 155-60.
42. Himmelseher S, Durieux ME. Revising a dogma: ketamine for patients with neurological injury? Anesth Analg 2005;101(2):524-34. Table of contents.
43. Patel A. Simvastatin administration. Am J Nurs. 2012;112(8):20.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/11332