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The Effect of Remifentanil, $MgSO_4$, or Remifentanil-$MgSO_4$ as Neuroprotectors on BDNF, MAC, and Caspase-3 Levels in Wistar Rats with Traumatic Brain Injury

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Abstract

BACKGROUND: Traumatic brain injury (TBI) can lead to cell death and neurologic dysfunction. Meanwhile, Remifentanyl is an opioid with potent analgesia, while magnesium sulfate ($MgSO_4$) has antinociceptive properties that can prevent hemodynamic instability during laryngoscopy.

AIM: This study aims to examine the effect of remifentanil, $MgSO_4$ and their combination on BDNF, MAC, and Caspase-3 levels in Wistar rat models with TBI.

METHODOLOGY: An experimental study was conducted on 30 male Wistar rats which were randomly divided into five groups. The control group (G1) received normal saline, the induced group (G2) received normal saline after TBI induction using the modified Feeney method, and the treated group (G3, G4, and G5) received remifentanil, $MgSO_4$, and their combination after TBI induction. The rats' brain tissues were analyzed for BDNF, MAC, and Caspase-3 levels using ELISA. The data were analyzed statistically with ANOVA followed by *post hoc* Multiple Comparison Test ($p < 0.05$).

RESULTS: Treatment with remifentanil, $MgSO_4$ or the combination of both in TBI subjects reduced MAC and Caspase-3 but increased the BDNF level. The *post hoc* multiple comparisons showed significant differences in all groups except groups 3 and 5 in terms of MAC ($p = 0.190$) and Caspase-3 ($p = 0.999$). The combination of remifentanil-$MgSO_4$ increased BDNF levels significantly.

CONCLUSION: The administration of remifentanil, $MgSO_4$, or their combination can serve as a neuroprotector in Wistar rat models with TBI by lowering MAC and Caspase-3 as well as increasing BDNF levels.

Introduction

Traumatic brain injury (TBI) remains one of the health problems worldwide. Approximately 69 million people experience brain injury every year, and the total incidence per 100,000 people in North America, Europe, and Africa are 1299, 1012, and 301, respectively [1]. The mortality rate of TBI in people under 35 years old is 3.5 times that of cancer and heart disease combined [2].

Brain injury can lead to cell death and neurological impairment through direct physical damage of the tissue also known as primary injury as well as cellular and molecular pathophysiology mechanism or secondary injury. The majority of brain injuries lead to death from secondary injury. There is a serial process in secondary injury, such as inflammation, vasogenic edema, cytotoxic, increased influx of Ca^{2+}, and glutamate exytoxicity which can cause apoptosis [3]. There are two main pathways of apoptosis, namely, extrinsic and intrinsic which will meet at one point that is executioner caspase, such as caspase-3. Extrinsic apoptosis is triggered by external signals in the extracellular environment, for example, the release and recognition of Fas ligand induce cells to become apoptotic. This pathway commonly uses the activation of caspase-8 initiator which directly activates caspase-3 [4].

Furthermore, the complement system is divided into two major parts, namely, enzymatic cascade and lytic pathway. The enzymatic cascade produces molecules necessary to initiate the lytic pathway, while the soluble protein undergoes changes that allow its insertion into the lipid bilayer and eventually the formation of MAC [5]. MAC pores might cause cell death through osmotic flow, and it has been postulated that the pooled pores enable lysozyme to cross the outer membrane to degrade the peptidoglycan layer. Various protein translocations present in perforin, such as granzyme move from cytotoxic T-cell granules to target cell cytoplasm to induce apoptosis [6].

Concerning the treatment of TBI, several studies especially on animals showed promising results and there is evidence of a positive correlation between elevated BDNF expression and improved functional outcomes [2]. A previous study reported that injecting a

high level of collagen-binding domain BDNF in the lateral ventricle of rat models with infarction can regenerate neurons, reduce cell damage, decrease apoptotic process, and accelerate functional recovery [7].

Opioid-like remifentanil has beneficial effects in terms of analgesia and sedation; hence, it is commonly used for managing acute TBI. In addition, remifentanil is a potent short-acting μ-opioid receptor agonist [8], [9]. In cases of TBI where a craniotomy is performed, the primary goals of anesthesia management are maintaining the balance of cerebral blood flow and brain metabolism, avoiding the elevation of intracranial pressure, and maintaining stable hemodynamics to provide good surgical conditions, as well as promote rapid recovery. At present, there is no consensus on which anesthesia option is the most suitable for craniotomy [10], [11].

Magnesium sulfate ($MgSO_4$) is an alternative drug for preventing unstable hemodynamics after intubation. It was proven in a study that administering $MgSO_4$ before endotracheal intubation was effective in lowering blood pressure and heartbeat [12]. Magnesium ion is an intracellular cation which has significant roles in cellular metabolism. It affects certain secondary factors involved in the pathophysiology of TBI by blocking N-methyl-D-aspartate (NMDA) receptors and reducing glutamate release. Other neuroprotective mechanisms of magnesium include increasing brain blood flow, obstructing calcium channels, and inhibiting apoptosis [13].

Furthermore, $MgSO_4$ has been proven for having antinociception properties, mainly due to its antagonistic effect on NMDA receptors (NMDARs). It increases the duration of postoperative analgesia and can be used as an adjunct [14]. Magnesium has various favorable effects offering a variety of possibilities for its use in obstetric anesthesia and intensive care. Administered as a single intravenous bolus dose or a bolus followed by continuous infusion during surgery, magnesium lowers stress response to endotracheal intubation, and reduces intraoperative anesthetic and postoperative analgesic demands, while simultaneously preserving favorable hemodynamics. The use of magnesium as part of an intrathecal or epidural anesthetic mixture can lengthen the duration of anesthesia and reduce total post-operative analgesic consumption without adverse maternal or neonatal effects [15].

Meanwhile, μ-opioid receptor expression has been found in astrocytes. Its activation increases astrocyte cytoplasmic calcium and stimulates the release of the gliotransmitter glutamate, which generates slow inward currents through the activation of the neuronal NMDAR. Several clinical studies suggested that Mg^{2+} augments opioid activity, but its cellular mechanism remains unclear. NMDAR is a key ionotropic glutamatergic receptor, activated by excitatory amino acids-glutamate and aspartate and is widely dispersed in the nervous system. This receptor comprises two glycine-binding NR1 and two glutamate-binding NR2 subunits. It also contains a nonspecific cation channel allowing the influx of calcium (Ca^{2+}) and sodium (Na^{+}) ions. Furthermore, morphine including remifentanil induces the activation of μ-receptor leading to the PKC-induced phosphorylation of Ser890 residue of NMDARs C-terminus. This will culminate in the separation of both receptors, as well as the induction of NMDAR activity and calcium ions influx. Since Mg^{2+} are NMDAR antagonists, they might contribute to a decrease of the receptor induced by the activation of kinases, as well as calcium ion influx, leading to a reduction in opioid receptor phosphorylation and, consequently, opioid analgesia intensification [16], [17]. This trial was conducted to assess the effectiveness of magnesium sulfate as an alternative for intraoperative analgesia, compared to the use of opioids. Magnesium sulfate showed efficacy in controlling pain and autonomic responses during surgery. In addition, its use has shown efficiency in reducing opioid consumption [18].

The use of remifentanil aside from facilitating analgesia, sedation, and laryngoscopy can also protect neurons. There are only few studies about μ-receptor and the use of remifentanil in TBI. To date, there are no reports regarding the effect of remifentanil, $MgSO_4$, or their combination on BDNF, MAC, and Caspase-3 levels in subjects with TBI. Therefore, this study was conducted in rat models with TBI to investigate the effect of the aforementioned drugs on the levels of BDNF, MAC, and Caspase-3.

Methods

Animals

An *in vivo* true experimental study was performed using a randomized post-test-only controlled group design in 30 healthy white male Wistar rats, with a body weight of 200–300 g, age of 8–10 weeks, as well as normal blood pressure and heart rate. Meanwhile, the rats excluded include those that were ill and inactive during the 7 days of adaptation, died during the study period, and those which had been used in another study. The rats were randomly divided into five groups and then adapted for a week. These five groups comprised:

1. Group 1 (G1): did not get brain injury, only received NaCl intravenously
2. Group 2 (G2): had brain injury and received NaCl intravenously
3. Group 3 (G3): had brain injury and received remifentanil 0.01215 μg/150 g
4. Group 4 (G4): had brain injury and received $MgSO_4$ 729 μg/150 g
5. Group 5 (G5): had brain injury and received

the combination of $MgSO_4$ 729 μg/150 g with remifentanil 0.01215 μg/150 g.

TBI model

The modified Feeney technique was used to make rat models that suffered from brain injury. Furthermore, a double-blind technique was used considering that there were three different investigators, one for adaptation as well TBI induction processes; one for administering NaCl, $MgSO_4$, or remifentanil; and one for surgical procedure as well as sample collection.

Drugs and treatment

The following ELISA kits were used in this study: Rat brain-derived neurotrophic factor/BDNF ELISA Kit (BZ-08186740-EB), Rat B-cell lymphoma 2/Bcl-2 ELISA Kit (BZ-08187300-EB), Rat Bcl-2 associated X protein/Bax ELISA Kit (BZ-08184300-EB), Rat Caspase-3 ELISA Kit (BZ-08180820-EB), and Rat Soluble Terminal Complement Complex (SC5B9) ELISA Kit (MBS9355831). All ELISA Kits were purchased from FlexyLabs Instrument Indonesia.

$TMgSO_4$ administration was based on the optimal dose for humans, namely, 30mg/kgBW which then was adjusted with rat model conversion factor and its body weight. Hence, the dose obtained was 729 μg for 150 g rat model's weight by using the formula of optimal dose x rat's model's weight x conversion factor with 30 mg/kg × 0.15 kg × 0.162 = 0.729 mg = 0.729 μg. The remifentanil dose was calculated using a similar formula with the optimal doses for human of 0.5 μg/kgBW; hence, the dose obtained was 0.01215 μg.

Before TBI induction, all the rat models received Ketamine injection with a dose of 60 mg/kg BW. Sodium chloride injection for the placebo group and intravenous $MgSO_4$ for the treatment group were administered immediately after TBI induction, while remifentanil was given intravenously 4 h later.

The levels of BDNF, MAC, and Caspase-3 were measured with ELISA, while the statistical data analysis used One-Way ANOVA and *post hoc* multiple comparisons. The significance level was determined with $\alpha = 0.05$, while the Shapiro–Wilk test was used for data distribution analysis and the Lavene test for data variant analysis, with $p > 0.005$ considered significant.

Ethics statement

The animal study was reviewed and approved by The Research Ethical Committee, Regional General Hospital Dr. Moewardi, Surakarta, Indonesia with the Approval No. (1.123/XII/HREC/2021).

Results

During the study period, none of the rat models died or dropped out of the study. According to the Shapiro–Wilk test results, all obtained data were distributed normally ($p > 0.005$). Lavene test of MAC, BDNF, and Caspase-3 attained $p = 0.22$; 0.388; and 0.001, respectively. These indicated that BDNF had homogeneous variance, while MAC and Caspase-3 had heterogeneous variances. Moreover, ANOVA analysis obtained $p < 0.001$ in each group, showing there were significant differences between the five study groups (Figure 1). The results were then compared using *post hoc* multiple comparisons test which revealed significant differences between the groups (Table 1). This showed that administering $MgSO_4$ and remifentanil affected BDNF, MAC, and Caspase-3 levels of Wistar rat models with TBI.

Table 1: *Post hoc* test multiple comparison data results for each group

Variable	Paired Group	p-value
BDNF	G1 and G2	<0.001
	G1 and G3	<0.001
	G1 and G4	<0.001
	G1 and G5	<0.001
	G2 and G3	<0.001
	G2 and G4	<0.001
	G2 and G5	<0.001
	G3 and G4	<0.001
	G3 and G5	<0.001
	G4 and G5	<0.001
Caspase-3	G1 and G2	<0.001
	G1 and G3	<0.001
	G1 and G4	<0.001
	G1 and G5	<0.001
	G2 and G3	<0.001
	G2 and G4	<0.001
	G2 and G5	<0.001
	G3 and G4	<0.001
	G3 and G5	0.999*
	G4 and G5	<0.001
MAC	G1 and G2	<0.001
	G1 and G3	<0.001
	G1 and G4	<0.001
	G1 and G5	<0.001
	G2 and G3	<0.001
	G2 and G4	<0.001
	G2 and G5	<0.001
	G3 and G4	<0.001
	G3 and G5	0.190*
	G4 and G5	<0.001

Description: $p < 0.05$ is significant.

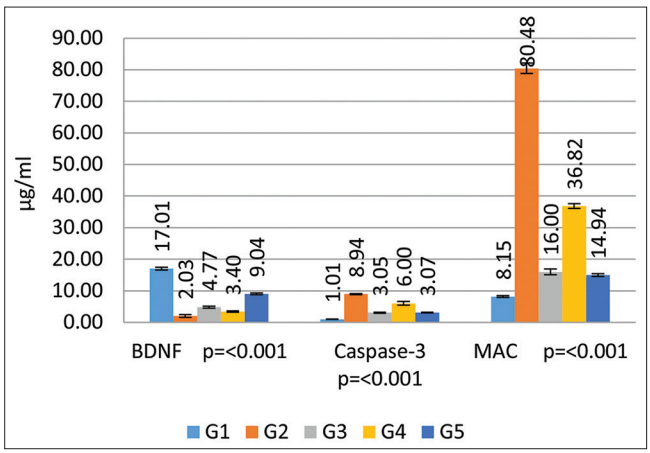

Figure 1. Mean Levels of BDNF, MAC, Caspase-3 and ANOVA Test Results

Discussion

Based on the statistical analysis, the mean level of BDNF, MAC, and Caspase-3 differed significantly. There was a great increase in MAC 80.48 μg/ml and Caspase-3 8.94 μg/ml in Group 2 compared to Group 1 with values of 8.15 μg/ml and 1.01 μg/ml, respectively. The mean level of BDNF reduced significantly in Group 2 with 2.03 μg/ml than that of Group 1 at 17.02 μg/ml. Furthermore, *post hoc* multiple comparisons of Groups 1 and 2 demonstrated significant differences meaning that in the TBI condition without any treatment, the levels of MAC and Caspase-3 increased, while BDNF decreased.

Ischemia occurs due to hypoxia in trauma injury which subsequently increases molecules that initiate the apoptotic process. Apoptosis is affected by MAC (calcium influx), Bcl-2, Bax, and Bid molecules. These molecules have an indirect important role in the domination process of both pro apoptosis (Bax and Bid molecules) and anti-apoptosis (Bcl-2 molecule), in which pro-apoptotic molecules influence reactive oxygen species (ROS) level. An elevation of ROS level will initiate cytochrome release and activate p53 protein culminating in DNA damage, caspase system activation including caspase-3, and apoptotic process [19].

A previous study in rats model of doxorubicin-induced acute cardiotoxicity showed an increase in apoptosis characterized by an elevation in caspase-3 [17]. It was also reported that patients with TBI had increased caspase-3 levels. High caspase-3 level is associated with a rise in mortality; hence, it can be used as a biomarker for predicting mortality in patients with TBI [20], [21].

Based on the results, the BDNF level was higher in Group 3 with 4.78 μg/ml, Group 4: 3.39 μg/ml, and Group 5: 9.04 μg/ml than that of Group 2: 2.03 μg/ml as the group control. This implies that there were significant differences between the groups ($p \leq 0.001$). In other words, the administration of remifentanil, $MgSO_4$, or the combination can increase the BDNF level. This result is consistent with a previous study which stated that the administration of morphine increased BDNF levels in embryo models of zebrafish due to elevated miR132 and decreased miR212 [22]. It was reported that reduced BDNF signaling through TrkB-FL causes memory impairment in transgenic mice [23]. The BDNF level can be used as an outcome predictor in TBI patients, especially those with severe conditions [24].

Regarding caspase-3 level, a significant decline was found in Group 3 with 3.05 μg/ml, group 4: 6.01 μg/ml, and Group 5: 3.07 μg/ml compared to Group 2 at 8.94 μg/ml. The *post hoc* test also obtained significant differences for the groups ($p \leq 0.001$) except between Groups 3 and 5 ($p = 0.999$). Therefore, the administration of remifentanil, $MgSO_4$, or the combination in subjects with TBI can lower caspase-3 levels. This is in line with a previous study which reported that remifentanil significantly reduced the activation of caspase-9 by increasing NMDAR activity through a cascade initiated by μ-receptor (MOR) in brain tissue of premature rat models [25].

Furthermore, there was a significant decrease in the MAC levels in group 3: 15.99 μg/ml, group 4: 36.82 μg/ml, and Group 5: 14.99 μg/ml compared to Group 2: 80.48 μg/ml. The MAC level differed significantly in all groups ($p \leq 0.001$) except between 3 and 5 ($p = 0.19$). According to a previous study, the inhibition of MAC formation by eradicating genetic C6 an important component, CD59a which is the main regulator and release of ornithodoros moubata complement inhibitor can reduce secondary neuron cell damage after TBI. This implies that MAC plays a key role in the pathophysiology of TBI [26].

Based on the results, the rat models that received the combination of $MgSO_4$ with remifentanil had significantly increased BDNF but reduced caspase-3 and MAC levels. This indicates that combined $MgSO_4$-remifentanil is more effective than the independent administration. The combined treatment had a synergistic effect in increasing BDNF. According to a previous report, remifentanil indirectly increases the expression as well as the release of IL-6 and has a moderate stimulation effect on the production of BDNF.(26) Meanwhile, $MgSO_4$ has been recognized for having its neuroprotective effect by increasing the production of BDNF in the placenta [27].

Overall, the results support a previous study on hypoxic-ischemic premature rat models given $MgSO_4$. This treatment reduced the apoptotic process by maintaining retinal ganglion cell and reducing the expression of vascular endothelial growth factor receptor-2 as well as glial fibrillary acidic protein (GFAP) [10], [28]. Another study showed that $MgSO_4$ has a protective effect on brain tissues of pre-eclampsia rat models by inhibiting signaling pathway of nuclear factor-κB (NF-κB)/intercellular adhesion molecule-1 [29] In rat models with cognitive disorders induced by aluminum chloride, the administration of $MgSO_4$ improved memory dysfunction [30]. Another study found that remifentanil or $MgSO_4$ when combined with sevoflurane produced adequate hypotensive anesthetic level. Nevertheless, $MgSO_4$ has a better analgetic effect and causes less shivering as well as nausea/vomiting after the surgery [31].

This study is limited because the doses of remifentanil and $MgSO_4$ were not varied.

Conclusion

The administration of remifentanil, $MgSO_4$, or their combination as a neuroprotector on Wistar rat

models with TBI significantly decreased MAC as well as caspase-3, but increased the BDNF levels. Based on the results, the combined administration had the most optimal outcome. Further investigations are suggested to use variations in the dose of remifentanil and $MgSO_4$ to obtain the optimal dose for TBI.

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