Original Article

Role of Remifentanil on MAC, Bcl-2, and Bax Levels in Traumatic Brain Injury Rat Models

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ABSTRACT

Introduction: Traumatic Brain Injury (TBI) conditions require immediate treatment such as the administration of analgesics and sedation, including remifentanil. TBI will affect the levels of several inflammatory and apoptotic proteins such as MAC, Bax, and Bcl-2. Remifentanil with its high affinity to μ-receptors is thought to affect the levels of these proteins and has neuroprotective properties to prevent secondary brain injury. **Objectives:** This research aims to determine the effect of giving remifentanil on MAC, Bcl-2, and Bax levels in rats with traumatic brain injury. **Materials and Methods:** Experimental research was conducted on 24 male Wistar rats, which were randomly divided into four groups. The Group-1 (G1) received an intravenous injection of NaCl, the Group-2 (G2) was given normal saline after TBI induction using the modified Feeney method. Furthermore, the Group-3 and Group-4 (G3 and G4) received fentanyl or remifentanil after TBI induction. The rat brain tissue was analyzed for MAC, Bcl-2, and Bax levels using the ELISA method. The data were analyzed statistically by ANOVA test, followed by Post-Hoc Multiple Comparison Test ($p<0.05$). **Results:** The MAC and Bax levels were reduced by the administration of fentanyl and remifentanil to TBI subjects, but increased Bcl-2 levels. The Post Hoc Multiple comparisons showed a significant difference between all group pairs ($p<0.05$). Meanwhile, the group given remifentanil showed more optimal results than those with fentanyl. **Conclusion:** The administration of remifentanil injection can act as a neuroprotector in TBI model Wistar rats by decreasing MAC and Bax levels and increasing Bcl-2 levels.

Keywords: Bax, Bcl-2, MAC, Remifentanil, Traumatic brain injury.

INTRODUCTION

Traumatic Brain Injury (TBI) is the leading cause of morbidity and mortality in children and adults, with an annual incidence of approximately 500–800 cases per 100,000 population in the United States and New Zealand.[1] Opioids such as fentanyl have beneficial effects in terms of analgesia and sedation. Therefore, they are indicated and often used during the acute management of traumatic brain injury.[2] Remifentanil is considered the analgesic and sedation of choice in TBI cases due to its numerous advantages over fentanyl.

The process of disease progression from primary to secondary TBI determines the patient's outcome. The primary TBI process begins with a breakdown of the blood-brain barrier, thereby triggering extravasation of peripheral circulating leukocytes, which can increase the inflammatory response. There are a series of processes in secondary injury, including inflammation, vasogenic edema, cytotoxicity, increased intracellular calcium (Ca^{2+}) influx, and glutamate excitotoxicity. Since increased Ca^{2+} activates several enzymes such as proteases, phospholipases, and endonucleases, they can damage cells and cause apoptosis.[3]

The main component of the inflammatory response that is often neglected is the complement system. It is also activated as part of the neuroinflammatory response in TBI, thereby causing MAC (Membrane Attack Complex) pooling of liquid-phase proteins to form pores in the lipid bilayer. MAC directly lyses pathogens and binds to the target cell's surface, which leads to cytolysis by forming transmembrane pores or channels in the cell membrane.[4,5]

Several proteins participate in the formation of MAC, first activated by C5b, which binds to C6 to form the C5b-6 complex. Subsequently, C7 forms the C5b-6-7 complex, which binds to C8 and consists of 3 chains, namely alpha, beta, and gamma. All

these will form the C5b-6-7-8 complex and C5b-6-7-8 will bind to C9 as a catalyst in C9 polymerization.[6] MAC pores can cause cell death by osmotic flux. Moreover, the translocation of various proteins, including granzymes, from cytotoxic T-cell granules into the cytoplasm of target cells induces apoptosis.[5]

The two main apoptosis pathways, namely extrinsic and intrinsic will meet at one point in the executable caspase activation pathway. In intrinsic apoptosis, the Bcl-2 protein regulates MAC formation as a cytochrome-c release channel. It also monitors the cellular status and acts synergistically to regulate the entrance to apoptosis.[6]

Bcl-2 Associated Protein X (BAX) is a member of the Bcl-2 family. Bax and Bak move between the cytosol and the mitochondrial outer membrane (MOM) at different rates in healthy cells. Under apoptosis conditions, Bax and Bak are activated and accumulate in MOM. They also oligomerize and mediate MOMP, leading to the release of proapoptosis factors such as cytochrome-c. Subsequently, the increased expression and activation of Bax and Bak will induce cell death via the intrinsic apoptosis pathway.[7]

Previous research has shown that remifentanil functions as an analgesic and has a protective effect on damaged cells. It was also discovered that the administration of remifentanil to rats induced by trauma with ibotenate inhibited the production of pro-inflammatory cytokines such as IL-1β, thereby inhibiting Bax and caspase-9 activity.[8] Moreover, remifentanil reduces myocardial cell injury by decreasing caspase-3 and increasing Bcl-2/Bax in a rat myocardial ischemia-reperfusion model.[9]

Remifentanil is a μ-Opioid Receptor (MOR) agonist with analgesic potency equivalent to fentanyl, which is 15-20 times stronger than fentanyl and has a similar blood-brain equilibration time to alfentanil. Therefore, it is often used in anesthetic practice and also has a fast onset of action in a relatively short duration. It can regulate the balance of intracranial pressure by maintaining the Cerebral Metabolic Rate of Oxygen ($CMRO_2$) and Cerebral Blood Flow (CBF) to maintain hemodynamic stability.[10,11]

With a faster onset of action, shorter half-life, and a more stable hemodynamic effect than its predecessor (fentanyl), remifentanil can be a new solution as balanced anesthesia in TBI cases. There is limited information on the administration of remifentanil in TBI cases. Therefore, this research aims to determine the effect of giving remifentanil as a neuroprotector in rats with TBI by observing MAC, Bax, and Bcl-2 levels.

MATERIALS AND METHODS

Animals

This research used a true experimental design in an *in vivo* laboratory with a Randomized Post-Test Only Controlled Group Design. The sample size for each group was determined based on the formula from Charan (2013),[12] namely:

E = Total number of animals – Total number of groups.

Note: E is the number of samples in each group; *E* value should be in the 10-20 range for meaningful results.

Based on calculations using the formula above, it was determined that the number of samples was five. Then added a loss of follow-up of 10-20% of the sample, so that in this study 6 rats were used for each group. The healthy white rats (*Rattus norvegicus*) used were male sex, age range 8-10 weeks, body weight 200-300 g, and normotensive. Meanwhile, the exclusion criteria were Wistar rats that were sick and inactive in the 7-day adaptation period, died during treatment, and had been used previously for research.[13] Subsequently, the 24 subjects were divided into four groups by simple random sampling and adapted for one week, with details:

Group 1 (G1): No treatment for brain injury and received intravenous injection of NaCl.

Group 2 (G2): Treated with brain injury and received an intravenous injection of NaCl.

Group 3 (G3): Treated with brain injury and fentanyl 0.926 μg/150g.

Group 4 (G4): Treated with brain injury and received remifentanil 0.463 μg/150g.

After being given treatment, the rats were observed for 4 hr. Then the rats were terminated and the rat brain tissue was taken to check MAC, Bcl-2, and Bax levels using the ELISA method.

Traumatic Brain Injury Model

The modified Feeney technique was used to make rat models that suffered from brain injury.[14] During the double-blind research, there was investigator 1 on the adaptation and treatment process of the traumatic brain injury model. Meanwhile, investigator 2 presents NaCl, fentanyl, or remifentanil, and investigator 3 is in charge of surgical procedures and sampling.

Drugs and Treatment

The dose of remifentanil used was based on the optimal dose in humans, namely 0.5 μg/kgBW. In rats weighing 150 g, the dose of remifentanil was given at 0.463 μg/150 g, after being calculated using the conversion factor for rats.[15] The dose of fentanyl used was based on the optimal dose in humans, namely 1 μg/kgBW. In rats weighing 150 g, the dose of fentanyl was given at 0.926 μg/150 g, after being calculated using the conversion factor for rats.[15]

Fentanyl and Remifentanil were administered intravenously in μg/mL units and on a nominal scale. MAC, Bax, and Bcl-2 levels were measured using ELISA with units of μg/mL on a ratio scale. The ELISA kits used include 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 Soluble Terminal Complement

Complex (SC5B9) ELISA Kit (MBS9355831). All ELISA Kits were purchased from FlexyLabs Instrument Indonesia.

Before the TBI treatment, all research groups were given Ketamine injections at 60 mg/KgBW. Fentanyl and remifentanil were given at 4 hr. Subsequently, statistical data analysis was carried out using the ANOVA and the *post hoc* Multiple Comparisons tests. Meanwhile, the degree of significance used is =0.05.

Ethics Statement

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

RESULTS

Based on the results, no rats died or dropped out during the research period. The distribution of the research data tested using Shapiro-Wilk showed significant results ($p>0.05$). Thus, all data in each group were normally distributed. Levene's test showed that MAC data had homogeneous variance ($p>0.05$), while Bax and Bcl-2 data had inhomogeneous data variances. Subsequently, MAC group data analysis used the One-Way ANOVA test, while the Bax and Bcl-2 groups used Welch ANOVA.

The ANOVA test analysis are shown in Figure 1, with a value of $p<0.001$ (significant) in each group. This shows that there are significant differences between the four treatment groups.

Furthermore, the *post hoc* Multiple Comparisons test was conducted to determine the difference between the treatment group pairs. The results of the *post hoc* test are presented in Figure 2, which were compared between G1, G2, G3, and G4, showing significant differences between all pairs of groups. This indicates that remifentanil administration affects MAC, Bax, and Bcl-2 levels in TBI model Wistar rats.

DISCUSSION

Based on the results and statistical tests, there were differences in the mean levels of MAC, Bax, and Bcl-2 in each group. There was an increase in the average MAC level in G2 (80.48 µg/mL) compared to G1 (8.15 µg/mL). The average Bax level also increased G2 (40.43 µg/mL) compared to G1 (7.77 µg/mL). The average levels of Bcl-2 showed a higher decrease in G2 (3.96 µg/mL) than G1 (19.77 µg/mL). Based on the Post Hoc test, MAC, Bax, and Bcl-2 data between G1 and G2 showed significant results ($p<0.001$). This shows that in TBI conditions and without any therapy, there will be an increase in MAC and Bax levels, as well as a decrease in Bcl-2 levels.

The ischemic injury induces complement activation, which leads to an increase in MAC. High MAC levels increase Ca influx and play a role in the apoptosis process. MAC pores can cause cell death by osmotic flux and passage of lysozyme across the outer membrane to degrade the peptidoglycan layer. Subsequently, there is a translocation of various proteins, including granzymes, from cytotoxic T-cell granules into the cytoplasm of target cells to induce apoptosis.[4] Under apoptosis conditions, Bax and Bak are activated and accumulated in MOM, affecting the release of proapoptosis factors, such as cytochrome-c.[7] Furthermore, the levels of Bcl-2 protein as an important regulator of intrinsic apoptosis and anti-apoptosis molecules will be suppressed.
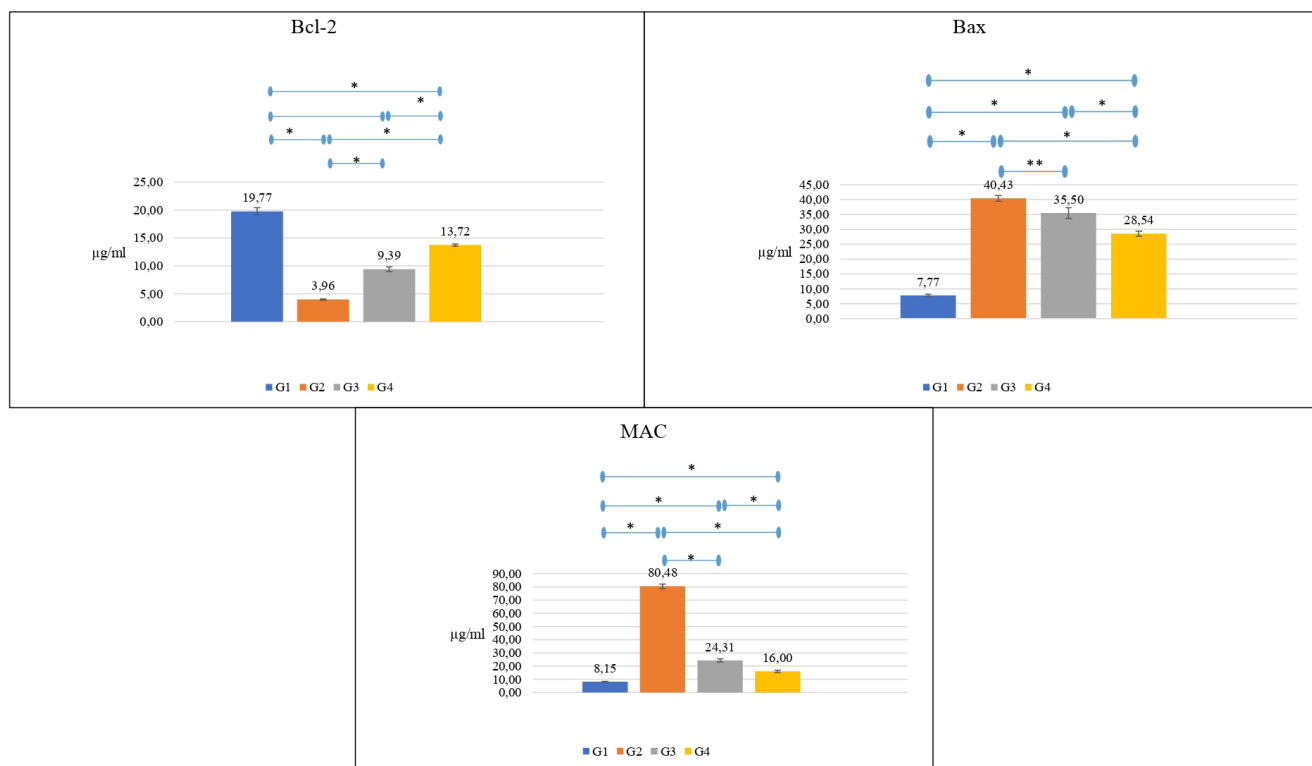
Effect of Remifentanil on MAC

Group 3 (G3) was given a fentanyl injection after the TBI procedure, while G4 received a remifentanil injection. Based on the average MAC level score, there was a decrease in scores in G3 (24.31 µg/mL) and G4 (15.99 µg/mL) compared to G2 (80.48 µg/mL). In the *post hoc* test, each group had a significant difference ($p<0.001$). This shows that the administration of fentanyl and remifentanil can reduce MAC levels. The reduction of MAC in the remifentanil group was more optimal than in the fentanyl group. Remifentanil differs from its predecessor (fentanyl) in the addition of an ester group which allows it to be rapidly metabolized by non-specific plasma and tissue esterases. It provides ultra-fast onset and offset action which allows it to be titrated quickly, have no cumulative effect, and recover quickly after administration is discontinued.[16]

MAC is implicated in the pathology of various acute and chronic neuroinflammatory diseases. MAC deposition is very evident at the edges of active plaques, indicating a close association with the ongoing pathology.[16] The previous research showed that the inhibition of MAC formation through genetic deletion of C6, inhibition of CD59a (a major regulator of MAC formation in rats), and providing *ornithodoros moubata* complement inhibitor (OmCI), which is a complement inhibitor that binds to C5, is neuroprotective by reducing secondary neuronal cell damage after brain trauma. This shows the key role of MAC in the pathophysiology of TBI.[17] Furthermore, the inhibition of the most downstream components of the terminal complement activation pathway and Membrane Attack Complex (MAC) formation
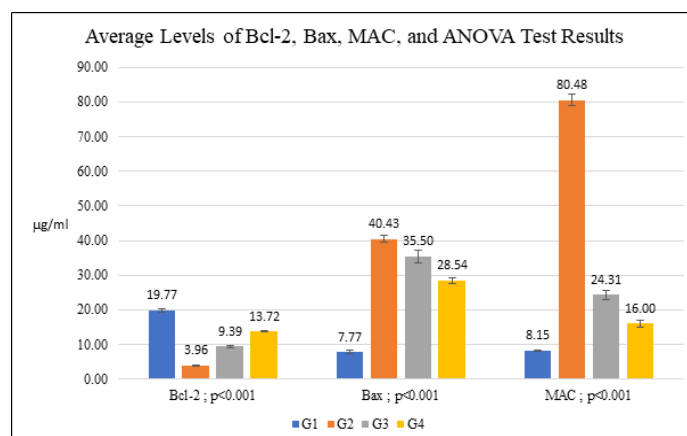
Figure 1: Average Levels of Bcl-2, Bax, and MAC and ANOVA Test Results in Each Group.

Figure 2: Post Hoc Test Results Multiple Comparison Data for Each Group. *$p<0.001$ (significant); **$p=0.02$ (significant).

will be sufficient to prevent secondary neurologic damage and neurologic deficits after TBI.[8]

Effect of Remifentanil on Bax

Based on the mean score of Bax levels, there was a decrease in scores in G3 (35.49 μg/mL) and G4 (28.54 μg/mL) compared to G2 (40.43 μg/mL). In the *post hoc* test, each group had a significant difference ($p<0.05$). This shows that the administration of fentanyl and remifentanil can reduce Bax levels. These results strengthen the previous research, where remifentanil administration to trauma-induced mice with ibotenate inhibited apoptosis death by restricting the production of pro-inflammatory cytokines such as IL-1β so that cortical Bax protein expression and caspase-9 activity can be suppressed.[9] Another study investigated the Bax/Bcl-2 ratio in rat models of focal mild TBI and pentylenetetrazole induced for convulsive effects showed an increase in the apoptosis process, as indicated by a high Bax/Bcl-2 ratio and caspase-3 compared to the control group.[18] Bax/Bcl2 ratio is considered a measure of cell susceptibility to apoptosis.[19]

Effect of Remifentanil on Bcl-2

The average score of Bcl-2 levels showed an increase in scores in G3 (9.39 μg/mL) and G4 (13.72 μg/mL) compared to G2 (3.96 μg/mL). In the Post Hoc test, each group had a significant difference ($p<0.001$). This shows that the administration of fentanyl and remifentanil can increase Bcl-2 levels. This is consistent with a previous report, where remifentanil administration reduced

myocardial cell injury by decreasing caspase-3 and increasing Bcl-2/Bax in a rat model of myocardial ischemia-reperfusion.[20] Subsequently, another investigation showed increased expression of Bcl-2 mRNA and Bcl-2 protein in the Cerebrospinal Fluid (CSF) of TBI patients, which was associated with neuroprotection and better outcomes.[21]

There was a greater decrease in average MAC levels in G4 (15.99 μg/mL) than in G3 (24.31 μg/mL). The decrease in average Bax levels in G4 (28.54 μg/mL) was greater compared to G3 (35.49 μg /mL), and the average increase in Bcl-2 levels in G4 (3.96 μg/mL) was greater than in G3 (13.72 μg/mL). In the Post Hoc test, there was a significant difference ($p<0.001$) between G3 and G4. These results indicate that remifentanil administration is more optimal in reducing MAC and Bax levels and increasing Bcl-2 levels compared to fentanyl.

Remifentanil is a μ-Opioid Receptor (MOR) agonist that has analgesic potency equivalent to fentanyl, which is 15-20 times stronger than alfentanyl and has a blood-brain equilibration time similar to alfentanil. Therefore, it is often used in anesthetic practice. The advantages of remifentanil include a fast onset of action, relatively short duration, and can regulate the balance of intracranial pressure by maintaining the Cerebral Metabolic Rate of Oxygen ($CMRO_2$) and Cerebral Blood Flow (CBF).[10]

In the case of injury, remifentanil will lead to activated -receptors and release CD59 protein, reducing MAC levels. It also indirectly suppresses the apoptosis and homeostatic process as well as

reduces apoptosis.[22] There is a need to inhibit C3 activation and MAC inhibition targeted at the injury site using the CD59-2a-CRIg pathway to prevent chronic inflammation and sustained neuronal loss by inhibiting microglial and astrocyte activation mechanisms. This reduces dendritic and synaptic density as well as inhibits neuroblast migration after TBI cases.[22,23]

Based on the results and supported by the theoretical explanation above, remifentanil administration is useful as a neuroprotector in traumatic brain injury, as shown by a decrease in MAC and Bax levels, as well as an increase in Bcl-2 levels. Researchers did not test the effects of varying doses of remifentanil in traumatic brain injury rat models and only conducted studies using male Wistar rats.

CONCLUSION

The administration of remifentanil acts as an analgesic and sedation. It also acts as a neuroprotector in experimental animal models of traumatic brain injury. This is shown by a decrease in MAC and Bax levels, as well as an increase in Bcl-2 levels.

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CONFLICT OF INTEREST

The authors declare that the research was conducted without any commercial or financial relationship, which can be construed as a potential conflict of interest.

ABBREVIATIONS

TBI: Traumatic brain injury; **MAC:** Membrane attack complex; **Bcl-2:** B-cell lymphoma 2; **Bax:** Bcl-2 associated protein X; **BAK:** Bcl-2 homologous antagonist/killer; **HCl:** Hydrochloride; **UV:** Ultraviolet; **RSD:** Relative standard deviation; **HPLC:** High-performance liquid chromatography; **HPTLC:** High-performance Thin layer liquid chromatography; **LC-MS:** liquid chromatography-mass spectroscopy; **AMPK:** AMP-activated protein kinase.

SUMMARY

Remifentanil and fentanyl have a high affinity for the μ-opioid receptor (MOR), one of their mechanisms of action is to inhibit Ca^{2+} channels. Blocking Ca^{2+} channels can reduce the inflammatory process that occurs. Remifentanil has a more rapid onset of action, a shorter half-life, and more stable hemodynamic effect than its predecessor (fentanyl). The results showed that the remifentanil group (G4) had higher Bcl-2 levels, and lower Bax and MAC levels than the fentanyl group (G3) which was statistically significant ($p<0.05$). This shows that remifentanil acts as a neuroprotector in experimental animal models of traumatic brain injury.

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