



Experimental Research

The role of diphenhydramine HCl on tumor necrosis factor- α levels in wistar rats with traumatic brain injury: An in vivo study

Venansius Ratno Kurniawan^a, Andi Asadul Islam^a, Willy Adhimarta^a, Andi Alfian Zainuddin^b, Djoko Widodo^b, Nasrullah^a, Andi Ihwan^a, Wahyudi^a, Muhammad Faruk^{c,*}

^a Department of Neurosurgery, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia

^b Department of Public Health and Community Medicine Science, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia

^c Department of Surgery, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia



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ABSTRACT

Background: Traumatic brain injury (TBI) is a major cause of death and disability worldwide that imposes a significant burden on both individuals and their families. Many of the symptoms experienced by patients with TBI are thought to be mediated by the neuroinflammatory process that occurs after the primary injury. Therefore, the present study aimed to determine the effect of diphenhydramine HCl (DPM) on serum levels of the inflammatory cytokine tumor necrosis factor- α (TNF- α) after TBI.

Materials and methods: This was an experimental study with a pre- and post-test control group design. A total of 10 adult Wistar rats were divided into 2 groups, the DPM group and the placebo group. The effect of DPM on serum levels of TNF- α was evaluated at 30 min, 2 h, and 24 h after the induction of experimental TBI in the rats using Marmarou's weight-drop model.

Results: TNF- α levels in the DPM group significantly decreased from 0 min to 24 h after TBI ($p = 0.004$). In the placebo group, TNF- α levels significantly increased from 0 min to 24 h after TBI ($p < 0.001$). Post hoc analysis found that TNF- α levels in the DPM group decreased significantly from 30 min to 2 h and from 2 h to 24 h after TBI ($p = 0.019$ and $p = 0.005$, respectively).

Conclusion: The results of this study suggest that administration of DPM causes a reduction in serum levels of TNF- α , indicating that DPM has a significant anti-inflammatory effect in experimental rats after TBI.

1. Introduction

The pathogenesis of traumatic brain injury (TBI) is a complex process caused by primary and secondary injuries that can lead to neurological deficits. Secondary brain injury occurs minutes to days after the primary brain injury and involves molecular, chemical, and inflammatory cascades that cause further brain damage [1]. Edema is a severe complication of numerous brain diseases, including TBI, and the pathogenesis of traumatic brain edema is complex [2], encompassing a wide range of cellular and molecular pathways [1–3].

Tumor necrosis factor- α (TNF- α) is an important mediator of tissue injury and inflammation. It is involved in the pathogenic processes of numerous central nervous system disorders, including cerebral

ischemia, Parkinson's disease, and TBI. A study on TBI induced using a weight-drop model found that acute inhibition of post-traumatic TNF- α production or administration of TNF-binding protein decreased edema, cortical tissue damage, and hippocampal neurodegeneration during the acute post-traumatic period [4].

Diphenhydramine HCl (DPM) is the first generation of H1 antihistamines used to manage allergic conditions by inhibiting histamine release. This drug also exhibits other pharmacological abilities, including sedative, anticancer, antiemetic, local anesthetic, and anticholinergic activities. DPM is also known to have significant antioxidant activity [4,5]. The purpose of this study was to investigate the role of DPM in inhibiting the post-TBI inflammatory reaction by assessing TNF- α levels as one of the important parameters involved in the

* Corresponding author. Department of Surgery, Faculty of Medicine, Hasanuddin University, Makassar, 90245, Indonesia. Jalan Perintis Kemerdekaan KM 11, Makassar, Indonesia.

E-mail addresses: ratnokurniawan10@gmail.com (V.R. Kurniawan), andiasadul@yahoo.com (A.A. Islam), willy.adhimarta@gmail.com (W. Adhimarta), a.alfian@med.unhas.ac.id (A.A. Zainuddin), djokwid@yahoo.com (D. Widodo), nasrullah.makassar@gmail.com (Nasrullah), aihwani77@yahoo.com (A. Ihwan), aamyudi@gmail.com (Wahyudi), faroe8283@gmail.com (M. Faruk).

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inflammatory process.

2. Methods

This study was a true experimental study on rats with a pre- and post-test control group design consisting of 1 treatment group and 1 control group, both sacrificed 24 h post-treatment. The study was conducted at the animal laboratory of the microbiology department at Hasanuddin University Medical Faculty, Makassar, Indonesia, in December 2021. Biomolecular examinations were performed at the microbiology laboratory of Hasanuddin University Medical Faculty, Makassar, Indonesia.

Approval for conducting the research was obtained from the Faculty of Medicine Ethics Committee, Hasanuddin University (number: 106/UN4.6.4.5.31/PP36/2022). This work was carried out under the ARRIVE (Animal Research: Reporting of In Vivo Experiments) Guidelines for Reporting Animal Research [6].

2.1. Animals

White male rats of the Wistar strain (*Rattus norvegicus*) [7] were used in this study. The study population was rats with TBI and the control group comprised rats without TBI. The sample was divided into 2 groups by simple randomization sampling. Group 1 consisted of rats with a TBI who were given DPM 5 min after sustaining a TBI. Group 2 consisted of rats with a TBI who were given a placebo (aquadest) 5 min after the head injury. Blood samples for both groups were taken at 0 h (before TBI), 30 min after TBI, 2 h after TBI, and 24 h after TBI, and craniectomy was performed 24 h after TBI.

The total number of experimental rats required was 10, based on the Federer formula [8]. The criteria for inclusion were (1) rats (*Rattus norvegicus*) of the Wistar male strain, (2) aged 2–3 months, (3) weighing 150–200 g, and (4) in good health. Exclusion criteria included rats that (1) died before the study was completed or (2) had damaged blood samples.

2.2. Diphenhydramine HCl

This study used 1% DPM (10 mg/mL) at a dose of 15 mg/kg body weight or 0.15 mL/100 g body weight.

2.3. Anesthetic drug preparation

A combination of 3 drugs was used to anesthetize the rats: ketamine HCl 5%, xylazine 2%, and acepromazine 2%. Based on the guidelines for the preparation of ketamine anesthesia cocktails for rats from the Institutional Animal Care and Use Program [9], the dose of each drug in the ketamine cocktails was 40–80 mg/kg ketamine, 8 mg/kg xylazine, and 4 mg/kg acepromazine maleate.

2.4. Anesthesia procedure

After randomization, 1 cc of venous blood was taken from the tails of all rats and was considered the 0-min measurement. The anesthetic procedure was then performed using the ketamine cocktail at a dose of 0.2 mL/50 g body weight given via intraperitoneal injection. The rats were held using the 2-hands technique by one researcher while a second researcher performed the intraperitoneal injection using a 1 cc syringe and a 25-gauge needle. The needle was inserted at the midpoint of the right lower quadrant of the abdomen at 45° to the craniomedial direction until the entire needle length was inserted (± 2 cm). Aspiration was performed to ensure negative pressure (needle tip into the peritoneal cavity) before the injection. The rats were observed for signs of peritonitis or other complications.

2.5. Traumatic brain injury procedure

TBI was induced using Marmarou's weight-drop model [10]. After the intraperitoneal anesthetic injection, the rats were fixed on the table. The craniectomy area of the scalp was shaved and cleaned with 10% povidone-iodine combined with lidocaine. The aseptic technique was performed according to the operative procedure. A midline incision was made on the rat's head and dissected until the skull was exposed (Fig. 1). A metal plate was then attached to the skull between the lambda and bregma using glue. The rats were placed on Marmarou's weight-drop device with the metal plate positioned just below the tube hole. The rat's body was fixed using an elastic bandage, and a TBI was induced by dropping a 30 g weight from a height of 80 cm onto the metal plate. After the TBI procedure, an interrupted suture was applied using 5/0 nylon thread to close the head wound [11].

Five minutes after the TBI, all rats in group 1 received an intravenous injection of 15 mg/kg body weight of DPM while all rats in group 2 received an intravenous injection of 0.9% sodium chloride as a placebo.

Two hours after the TBI, venous blood samples were taken from the tails of all rats.

2.6. Sacrifice procedure

At 24 h after TBI, sacrifice and craniectomy were performed in both groups. All rats were administered an intraperitoneal anesthetic ketamine cocktail as in the previous anesthetic procedure. A craniectomy was then performed to remove all brain tissue, and a thoracotomy procedure was performed to collect blood samples from the apex cords using a 25-gauge needle and 3 cc syringe.

2.7. Enzyme-linked immunosorbent assay procedure

Serum TNF- α levels were examined using the Rat-TNF- α ELISA Kit from AssayGenie, Dublin, Ireland (Catalog code: RTFI01177) according to the standard protocol. TNF- α levels are measured in units of pg/mL.

2.7.1. Statistical analysis

Data were analyzed using the Statistical Package for Social Sciences version 25 (Armonk, NY: IBM Corp). The normality test (Shapiro–Wilk test) for parametric data and the homogeneity test (Levene's test) for non-parametric data were performed with $p > 0.05$, indicating significant. If the data were normally distributed, the mean differences of the variables were compared. The independent t -test and repeated measures analysis of variance (ANOVA) were performed to compare TNF- α levels by type of treatment and within-group changes in TNF- α levels over time. A p -value of <0.05 was considered statistically significant. Post hoc analysis was performed to evaluate changes in serum TNF- α levels between consecutive examination times in each group, and a p -value of <0.05 was considered statistically significant.

3. Results

The animals' mean body weight is shown in Table 1. The mean body weight in the experimental group was 162.25 g. Using the Shapiro–Wilk normality test, the body weight data were found to be normally distributed ($p > 0.05$), so an independent parametric t -test was performed. Based on the parametric test and Levene's test for homogeneity, a p -value of 0.473 ($p > 0.05$) was calculated, as seen in Table 1. There was thus no statistically significant difference between groups in body weight, indicating that the subjects of this study were homogeneous. Therefore, it can be assumed that differences in the dependent variables were due to the effect of the induced TBI and exposure to DPM.

The effect of DPM on TNF- α levels in experimental rats with TBI at 0 min, 30 min, 2 h, and 24 h after TBI was analyzed using the independent t -test (Table 2). There was no significant difference in mean TNF- α levels at minutes 0 and 30 between the DPM and placebo groups ($p = 0.861$

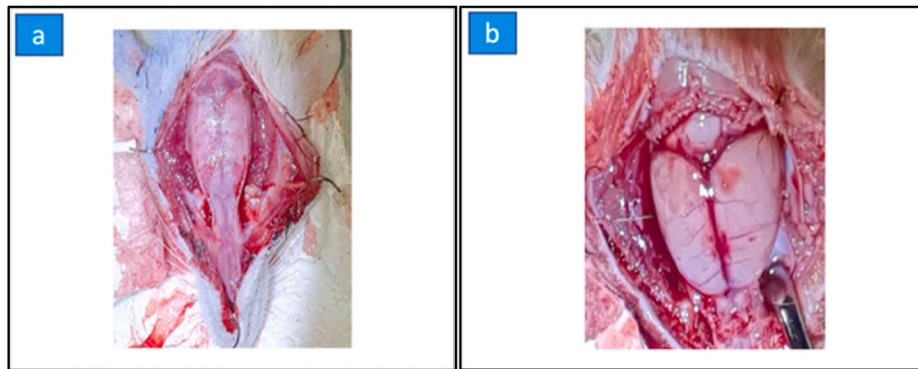


Fig. 1. Craniectomy procedure. (a) A pericranial and temporalis muscle dissection was performed to expose the skull, and (b) a circumferential craniectomy was performed using a high-speed drill and elevation of the skull to expose the dura mater tissue.

Table 1

Rattus norvegicus sample mean body weight.

Body weight (g; mean ± SD)		F	P
DPM	Placebo		
164.00 ± 9.97	160.50 ± 11.33	0.837 [#]	0.473*

Abbreviation: [#]Levene's test; *Independent *t*-test; DPM, Diphenhydramine HCl; SD, standard deviation.

Table 2

Independent *t*-test results comparing average TNF-α levels by type of treatment.

Time	TNF-α (pg/mL; mean ± SD)		p-value
	DPM	Placebo	
0 min	29.22 ± 4.96	28.74 ± 3.31	0.861*
30 min	32.23 ± 4.88	34.24 ± 2.10	0.423*
2 h	24.04 ± 0.80	41.15 ± 4.34	0.001*
24 h	21.67 ± 1.09	39.22 ± 2.37	<0.001*

Abbreviation: *Independent *t*-test; TNF-α, Tumor necrosis factor-α; DPM, Diphenhydramine HCl; SD, standard deviation.

and $p = 0.423$, respectively). Thus, the pretreatment conditions of the DPM rat group and the placebo rat group were similar or comparable. The DPM rat group demonstrated significantly lower mean TNF-α levels than the placebo rat group at 2 and 24 h after TBI ($p = 0.001$ and $p < 0.001$, respectively). TNF-α levels increased 30 min after TBI and continued to increase until 2 h after TBI in the placebo group before decreasing at 24 h. In contrast, in the DPM group, TNF-α levels

decreased 2 h after TBI and continued to decrease until 24 h after TBI (Fig. 2).

Repeated measures ANOVA was carried out to assess changes in TNF-α levels within each group from 0 min to 24 h after TBI. As shown in Table 3, TNF-α levels in the DPM group significantly decreased from 0 min to 24 h after TBI ($p = 0.004$). In the placebo group, on the other hand, TNF-α levels significantly increased from 0 min to 24 h after TBI ($p < 0.001$).

Post hoc analysis was performed to evaluate changes in serum TNF-α levels between consecutive examination times in each group (Fig. 3). In the placebo group, there was a significant increase in TNF-α levels at 30 min after TBI compared 0 min ($p = 0.014$). TNF-α levels also increased significantly from 30 min to 2 h after TBI ($p = 0.020$). From 2 h to 24 h after TBI, TNF-α levels in the placebo group decreased, though not significantly ($p = 0.409$). TNF-α levels thus increased from 30 min, peaked at 2 h, and decreased at 24 h post-TBI, though the decrease was not significant. Meanwhile, in the DPM group, TNF-α levels increased at 30 min after TBI compared to 0 min, though the increase was not statistically significant ($p = 0.363$). TNF-α levels in the DPM group decreased significantly from 30 min to 2 h and from 2 h to 24 h after TBI ($p = 0.019$ and $p = 0.005$, respectively).

4. Discussion

This study showed that DPM administration after TBI reduces TNF-α levels from 30 min after TBI, and the optimal or most significant decrease occurs between 2 and 24 h post-TBI. Macrophages, including microglia and infiltrating macrophages (peripheral macrophages), increase in number after TBI [12–14]. Macrophages appear to be abundant

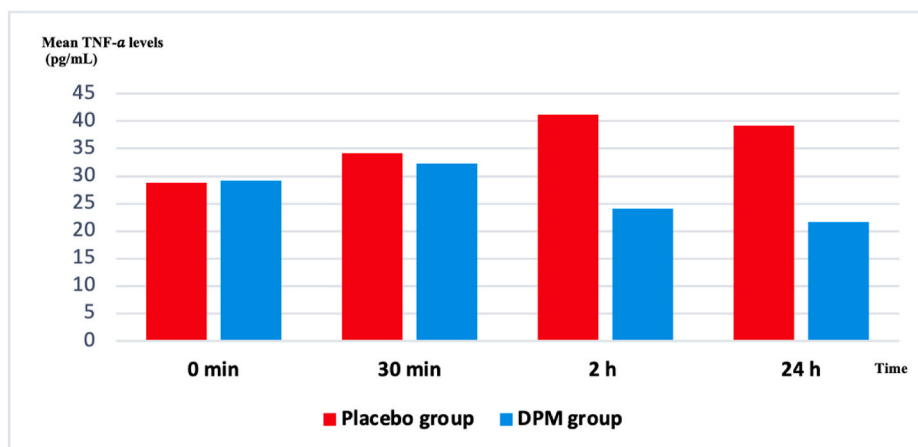
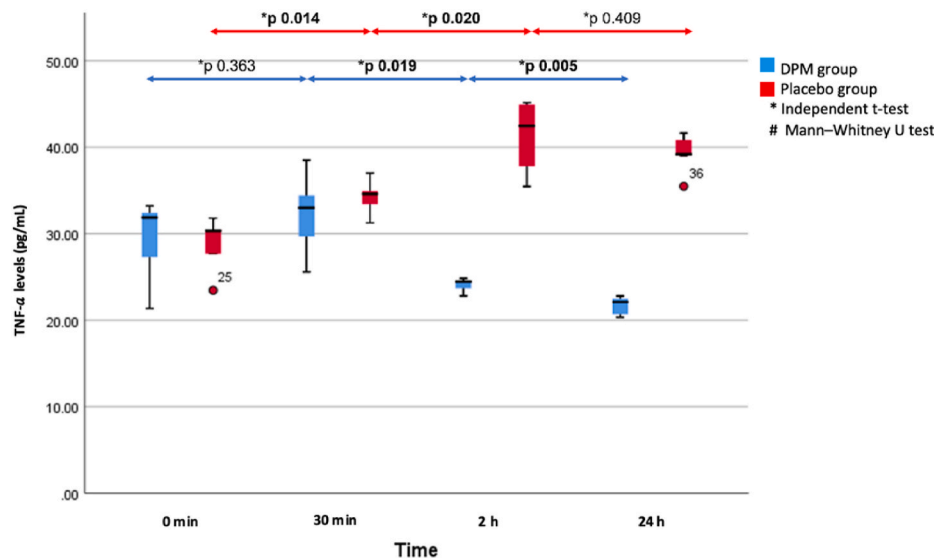


Fig. 2. Comparison of mean serum TNF-α levels by the length of time post-injury.

Table 3

Repeated measures ANOVA results comparing within-group changes in TNF- α levels over time.

	Group	Time				p-value
		0 min	30 min	2 h	24 h	
TNF- α (pg/mL; mean \pm SD)	DPM	31.85 (21.35–33.22)	32.99 (25.58–38.50)	24.45 (22.81–24.83)	22.10 (20.35–22.78)	0.004 [#]
	Placebo	28.74 \pm 3.31	34.24 \pm 2.10	41.15 \pm 4.34	39.22 \pm 2.37	<0.001*

Abbreviation: *Repeated measures ANOVA test; [#]Friedman test; TNF- α , Tumor necrosis factor- α ; DPM, Diphenhydramine HCl; SD, standard deviation.Fig. 3. Post hoc analysis of the mean change in TNF- α levels based on the length of time post-injury.

between 12 and 72 h post-trauma and are predominant in the damaged cortical region [15–17]. These cells accumulate in the injured area [15] as a result of at least 2 mechanisms. First, the release of chemokines and cytokines locally at the site of injury can attract macrophages. The second mechanism is through the activation of T cells. Upon arrival at the injury site, T cells are activated by direct contact with antigens presented by dendritic cells, macrophages, and microglia. This activation is a hallmark of the transition from a nonspecific innate immune response to a specific adaptive immune response involving, for example, the recognition of antigens by T cells [13,18,19]. Activation of microglia heightens the inflammatory response by not only causing the release of chemokines that weaken the blood–brain barrier, such as TNF- α , interleukin (IL)-1 β , IL-6, and IL-12, but also producing free reactive oxygen species and neurotoxic molecules, initiating other secondary cell death mechanisms [20,21]. The presence of interferon γ causes a dominant pro-inflammatory macrophage phenotype (M1), while IL-4 and IL-13 direct the differentiation of microglia into an anti-inflammatory macrophage phenotype (M2) [21].

This study showed that the administration of 15 mg/kg of diphenhydramine significantly reduced TNF- α levels up to 24 h post-TBI in the experimental group compared to the control group (placebo). The control group's TNF- α levels, meanwhile, increased from 30 min and reached a peak 2 h post-TBI. This indicates that TBI elicits an increase in TNF- α levels, which is caused not only by the degranulation of mast cells containing TNF- α but also by the activation of microglia and accumulation of peripheral macrophages that infiltrate the brain [13,15,22–25].

A previous study found that increased expression of neuronal TNF- α mRNA occurred at an early stage prior to leukocyte infiltration in rats with cortical ischemia following focal stroke [26–28]. Indeed, early TNF- α mRNA and protein expression were identified in activated microglia and macrophages following ischemia in the rat brain [29–31]. Furthermore, direct administration of TNF- α into the brain led to a dramatic increase in leukocyte adhesion to the vascular wall and

infiltration of these inflammatory cells into the tissue, though there was no neurotoxicity effect on the neurons at the injection site [32–35].

TNF- α levels in brain tissue undergo time-dependent changes. In a study on mice with mild TBI, Baratz et al. reported an increase in the protein levels of TNF- α in the brain; TNF- α levels were increased 6 h post-injury, saw a 2.5x increase (peak level) 12 h post-injury, and returned to baseline 18 h post-injury ($p < 0.0001$). At 12 h post-injury, TNF- α levels were elevated to 132.9 pg/mL compared to 53.4 pg/mL at baseline [36].

Immediately after TBI, astrocytes and microglia synthesize and release proinflammatory cytokines, particularly TNF- α , with acutely elevated mRNA and protein levels within 17 min post-injury found on postmortem examinations of brain tissue in patients who recently died after a TBI [37–40].

In experimental models of TBI, elevated TNF- α levels have been found in the early posttraumatic phase, with levels peaking within a few hours after the initial trauma. TNF- α also appears to be released more rapidly than other proinflammatory cytokines, initiating the activation of several cytokines and growth factors and the recruitment of immune cells [38,41,42].

In a previous study, the concentration of TNF- α was measured in the CSF and serum of patients with TBI at 24-h intervals, with the finding that TNF- α was significantly increased in the patients with TBI compared to the control group [43–46]. Hayakata et al. [47] examined the CSF of 23 patients with severe TBI, and peak TNF- α levels of 20–30 pg/mL were noted within 24 h. Stein et al. [48] analyzed CSF and serum samples from 24 patients at 12-h intervals for 7 days after sustaining a severe TBI. Intracranial pressure (ICP) and cerebral perfusion pressure (CPP) were continuously monitored so that the relationship between cytokine levels and subsequent changes in ICP or CPP could be investigated. They reported that an increase in serum (and not CSF) TNF- α concentrations were moderately correlated with an increase in ICP and a decrease in CPP.

TNF- α levels progressively increase in regions of ischemic brain damage after TBI [42,49–51], and high TNF- α levels are strongly associated with blood–brain barrier damage [52,53] and cell death after TBI and ischemia. In experimental models of induced neuronal damage caused by oxygen–glucose deprivation performed both in vitro and in animal models of brain ischemic damage induced by cerebral artery blockage or ligation, TNF- α has been identified as a mediator in the mechanism of neuronal death [54,55]. In several apoptotic pathways to neuronal death, peripheral aggregation has been associated with increased levels of TNF- α [32,56,57]. Administering DPM can reduce TNF- α levels by inhibiting the activation of microglial cells, peripheral macrophages, and neuronal cells known to be activated by histamine and substance P, which are released by degranulated mast cells.

A study conducted by Wang et al. [5] on an animal model of rats with periodontitis found that administration of DPM significantly reduced oxidative stress by reducing levels of superoxide anions, hydroxyl radicals, lipid hydroperoxides, and reactive non-radical components such as singlet oxygen and hydrogen peroxide. DPM is suspected to significantly increase antioxidant defense mechanisms by increasing free radical scavenging activity [58]. By inhibiting oxidative stress, DPM can in turn inhibit neutrophil recruitment, which is induced by oxidative stress [59]. In addition, administration of DPM reduces lysosomal enzymes, such as cathepsin B, cathepsin D, β -glucuronidase, and acid phosphatase, which can cause tissue damage [60].

Acute-phase proteins play an important role in the inflammatory process through the acute-phase reaction. The activity of these proteins is known to increase in response to cytokines such as IL-1, IL-6, and TNF- α [61,62]. Previous studies have shown that DPM can reduce levels of C-reactive protein and fibrinogen protein, revealing DPM's anti-inflammatory effect [61,63].

Other researchers investigating the effects of DPM treatment might consider our findings interesting. The lack of DPM efficacy data for TBI emphasizes the necessity of phase I–II clinical trials evaluating this treatment. As a new development in the treatment of TBI, we believe that our research can act as a foundational resource and source of information during decision-making regarding the administration of DPM. The limitations of this study included first that DPM was only administered once; it is thus necessary for future research to include serial drug administration. Second, the follow-up period was short at 24 h, which should be lengthened in future studies. Third, only one inflammatory cytokine was evaluated in this study. In the future, studies should examine other inflammatory cytokines that play a role in TBI (IL-1, IL-2, IL-6, IL-10, IL-12, interferon (IFN)- α , IFN- β , TNF- β , and histamine) by both ELISA and mRNA. Finally, a histopathological assessment was not performed.

5. Conclusion

TBI increases TNF- α levels and administration of DPM significantly reduces TNF- α levels in experimental rats after TBI, which suggests that DPM has a significant anti-inflammatory effect in experimental rats after TBI.

Ethical approval

All procedure for Animal experiment has been approved by Ethics Commission Faculty of Medicine, Hasanuddin University Number: 106/UN4.6.4.5.31/PP36/2022.

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Author contribution

Venansius Ratno Kurniawan, Andi Asadul Islam, Willy Adhimarta,

Andi Alfian Zainuddin, Djoko Widodo, and Nasrullah wrote the manuscript and participated in the study design. Venansius Ratno Kurniawan, Willy Adhimarta, Andi Ihwan, Wahyudi, and Muhammad Faruk drafted and revised the manuscript. Venansius Ratno Kurniawan, Wahyudi, and Muhammad Faruk performed head trauma treatment and surgery. Venansius Ratno Kurniawan, Andi Asadul Islam, Willy Adhimarta and Andi Alfian Zainuddin performed bioinformatics analyses and revised the manuscript. All authors read and approved the final manuscript.

Trail registry number

None.

Guarantor

Andi Asadul Islam.

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Declaration of competing interest

The authors declare that they have no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.amsu.2022.104399>.

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