Journal of Research in Pharmacy

Research Article

www.jrespharm.com

Effects of fermented calabash fruit (*Crescentia cujete* L.) on the Nissl's Body, C-RP and COX-2 in rat models with artificial-induced ischemic stroke

Yos Adi PRAKOSO[1] *, Achmadi SUSILO[2], Sitarina WIDYARINI[3], Puput Ade WAHYUNINGTYAS [1], Jasir Hakim HIDAYAH[4]

1 Department of Pharmacology, Faculty of Veterinary Medicine, University of Wijaya Kusuma Surabaya, Surabaya, Indonesia
2 Agrotechnology Study Program, Faculty of Agriculture, University of Wijaya Kusuma Surabaya, Surabaya, Indonesia
3 Department of Pathology, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia
4 Animal Research Facility, University of YARSI, Jakarta, Indonesia

* Corresponding Author. E-mail: yos.vet.docter@gmail.com (Y.A.P.); Tel. +62-812-5249-5940

Received: 29 August 2024 / Revised: 30 September 2024 / Accepted: 4 October 2024

ABSTRACT: Ischemic stroke is an essential disease in human life that causes disability and death. Ischemic stroke is commonly treated using clopidogrel, which potentially causes various side effects. Hence, alternative therapy against ischemic stroke must be elucidated, and this study used fermented calabash fruit (FC) due to its choline compound. This study aimed to analyse the effects of FC in rat models with artificial-induced ischemic stroke. This study conducted an experiment involving 40 male Sprague Dawley rats that were divided as follows: K1 (sham-operated) and K2-K4 (artificial-induced ischemic stroke groups). While, K2 (untreated); K3 (FC); and K4 (clopidogrel). The serum and brain were tested using enzyme-linked immunosorbent assays (ELISA) and immunohistochemistry against C-RP and COX-2, respectively. The data was then tabulated and analysed using SPSS. The results indicated that utilisation of FC improves the presentation of Nissl's bodies, increases the brain immune expression of COX-2, and decreases the level of circulatory COX-2 and C-RP compared to the other treatments ($P<0.05$). Surprisingly, the utilization of clopidogrel in group K4 promotes the decrease of level and immune expression of COX-2 compared to group K3 ($P>0.05$), but lower than group K2 ($P<0.05$). This study proved that FC benefits the Nissl's body presentation, increasing the brain's COX-2 immune expression and decreasing circulatory levels of C-RP and COX-2. An advance study is needed to evaluate the potential for toxicity and side effects after acute, subchronic, and chronic utilization of FCC.

KEYWORDS: C-RP; Clopidogrel; COX-2; Fermented calabash fruit-derived choline; Ischemic stroke

1. INTRODUCTION

Ischemic stroke is an essential disease for human life. The thrombus is a primary aetiology in ischemic stroke, with significant impacts on disability and death [1]. The pathogenesis of ischemic stroke has an intricate mechanism, such as changing immune expression and the level of cyclooxygenase-2 (COX-2) and C-reactive protein (C-RP). A previous study by Das [2] mentioned that the decrease in COX-2 is correlated with the occurrence of brain infarction. In contrast, Zhao et al. [3] found that high immunoreactivity of COX-1 and COX-2, elevates ischemic stroke susceptibility in Chinese populations. Moreover, C-RP is an acute-phase protein. C-RP significantly increase on day one after stroke and is involved in aggravating neuronal injuries [4]. Therefore, ischemic stroke management is essential to improve the prognosis of this disease.

The management of ischemic stroke involves rehabilitation and post-stroke treatment. Post stroke treatment is commonly using drugs such as clopidogrel. Clopidogrel is an antiplatelet medication that irreversibly affects platelets' P2Y12 adenosine diphosphate receptor [5]. This medication can improve vascular health, decrease platelet aggregation, and increase blood vessel viability [6]. Yang et al. [7] found that clopidogrel potentially increases the risk of haemorrhage in patients, which contributes to increasing

How to cite this article: Prakos YA, Susilo A, Widyarini S, Wahyuningtyas PA, Hidayah JH. Effects of fermented calabash fruit (Crescentia cujete L.) on the Nissl's Body, C-RP and COX-2 in rat models with artificial-induced ischemic stroke . J Res Pharm. 2025; 29(5): 2017-2022.

https://doi.org/10.12991/jrespharm.1765113
ISSN: 2630-6344

ischemic stroke pathogenesis. The haemorrhage caused by clopidogrel can be found intracranially and extracranially.

Hence, complementary therapy against ischemic stroke must be considered to reduce the potential side effects of clopidogrel. One alternative therapy is calabash fruit (*Crescentia cujete* L.), which contains several biochemical compounds. Wilujeng et al. [8] showed that fermented calabash fruit contains choline. Choline is essential in neurotransmitters and can function as a supplement in supporting metabolism and neuronal repair after injury [9]. In addition, Hidayah et al. [10] proved that choline derived from fermented calabash fruit (FC) is helpful for brain histopathological repair after induction of ischemic stroke in rat models. Hence, it is crucial to investigate the role of FC in analyzing the expression and levels of COX-2 and C-RP to evaluate the effectiveness of ischemic stroke treatment. This study examines the impact of FC on Nissl's bodies, C-RP, and COX-2 in rat models with artificially induced ischemic stroke.

2. RESULTS

Results indicated a significant increase in serum C-RP and COX-2 levels on day one after induction ($P<0.05$). The serum level of C-RP and COX-2 in K2 and K4 showed stagnation in all observation days, which is not different compared to each other ($P>0.05$), but it is different compared to K1 and K3 ($P<0.05$). In contrast, group K3 showed a dramatic decrease in serum levels of C-RP and COX-2 compared to K2 and K4 on days 7 and 14 ($P<0.05$) (Table 1). Moreover, the level of COX-2 within the rat brain showed a different pattern than the other groups ($P<0.05$). This study showed that K3 has the highest level of COX-2 in the brain tissue, while K2 is decreasing compared to K1. In addition, groups K1 and K4 are not different ($P>0.05$) (Table 1).

Table 1. Level of C-RP and COX-2 in the serum of rat models with artificial-induced ischemic stroke after treatment

Parameter	Group	Mean ± SD		
		Day 1	Day 7	Day 14
Serum C-RP (mg/dL)	K1	33.89±1.70	33.53±1.62	33.52±2.01
	K2	54.19±2.49*	55.18±1.99**	55.73±2.61***
	K3	55.59±1.92*	42.67±4.80*	37.66±3.88*
	K4	52.62±3.92*	52.49±2.60**	51.74±6.73**
Serum COX-2 (ng/dL)	K1	12.40±2.11	12.20±2.34	12.30±2.90
	K2	69.40±12.52*	73.70±6.39**	78.50±12.37**
	K3	72.10±10.08*	58.00±8.37*	32.60±12.72*
	K4	71.20±9.61*	72.10±8.68**	77.70±11.99**

*different superscript on the same column showed a significant differences ($P<0.05$).

The tissue analysis revealed that group K2 has the highest mean score of Nissl's body clumping compared to the others ($P<0.05$), and it is not different compared to K4 ($P>0.05$). Interestingly, group K3 was not different regarding Nissl's body clumping compared to K1 ($P>0.05$). In this study, the immune expression of COX-2 within brain tissue indicated a similar pattern to the level of COX-2 (Table 2). The qualitative images of Nissl's body and immune-expression of COX-2 were concomitantly embedded in Figure 1-2.

Table 2. Level of COX-2, clump score of Nissl's body and immunohistochemistry of COX-2 in the brain of rat models with artificial-induced ischemic stroke after treatment

Group	Level of COX-2 in brain (mean ± SD×10^3 Pg/mL)	Score (mean ± SD)	
		Nissl's body	Immune-expression of COX-2
K1	1.31±0.07	1.10±0.31	1.90±0.31
K2	0.97±0.37*	2.60±0.51*	1.20±0.42*
K3	2.38±0.57**	1.40±0.51	2.90±0.31**
K4	1.30±0.14	2.20±0.78*	1.60±0.51

*different superscript on the same column showed a significant differences ($P<0.05$).

Journal of Research in Pharmacy
Research Article

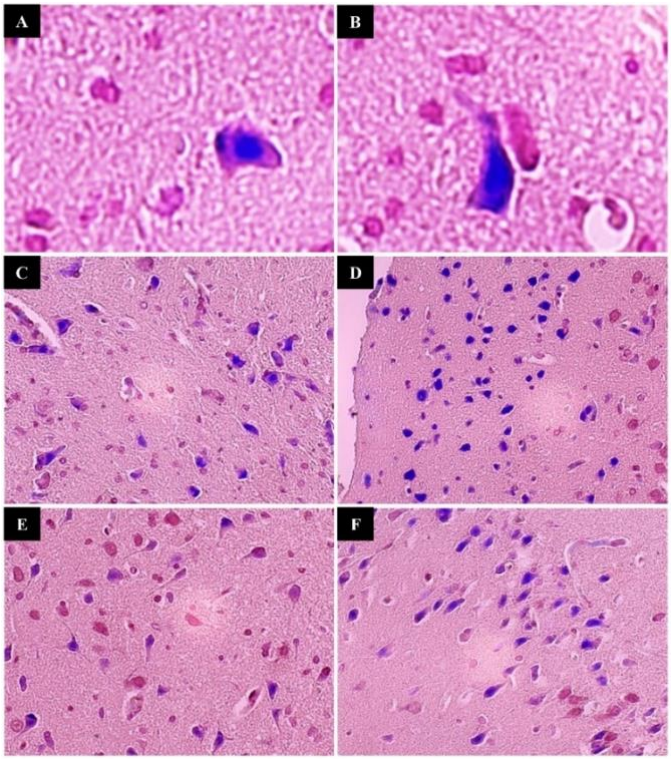

Figure 1. Presentation of Nissl's body after treatment. Typical neuron showed the Nissl's body with sandy presentation within cytoplasm (A); deep blue stained of necrosis neuron and the Nissl's body is not clearly presented (B); neuron of healthy rat group (C); artificial-induced ischemic stroke without treatment (D); FC group (E); and clopidogrel group (F). Thionine staining, 1000× (A-B), 400× (C-F).

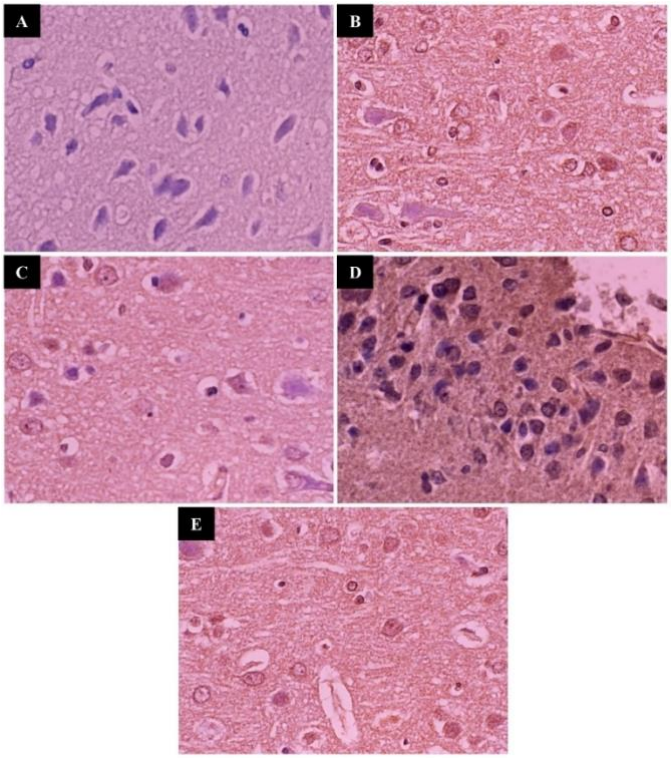

Figure 2. Immune-expression of COX-2 in the brain after treatment. No immune-expression of COX-2 in brain tissue without labelling (A); moderate immune-expression of COX-2 in neuron and brain parenchyma from healthy rat group (B); mild expression in artificial-induced ischemic stroke without treatment (C); strong expression from FC group (D); and mild expression from clopidogrel group (E). IHC antibody-anti-COX-2, 400× (A-E).

3. DISCUSSION

Stroke is a severe disease, and one cause is the formation of a blood clot called a thrombus. When a thrombus forms, it can create emboli [1]. The emboli can block blood flow to the tissues downstream, reducing the flow of nutrients and oxygen. In advances, thrombus triggers brain injuries, including ischemia and infarction. Brain infarction can be evaluated by the appearance of the clumping of Nissl's body, indicating neuronal necrosis [10]. In this study, ischemic stroke induction impacts on the immune-expression of COX-2. The decrease in immune expression of COX-2 after artificially inducing ischemic stroke resulted in a large number of parenchymal cell deaths.

Moreover, COX-2 is a pro-inflammatory cytokine that is essential in ischemic stroke. The increase of COX-2 is related to the oxidative pathway. Xu et al. [11] in their previous study found that COX-2 inhibition can be essential in promoting decreased brain infarct area in ischemic stroke. However, it is different from this study. This study shows a decrease in COX-2 immune expression and brain level in the untreated group. The mechanism involves because there is such interfering formation of endothelial nitric oxide synthase (eNO), lipoxin (LXs), prostacyclin (PGI2), and resolving, which promotes negative feedback on the immune expression of COX-2 [2].

Oliveira-Filho et al. [12] found that low expression of COX-2 in chronic conditions worsens the pathogenesis and risk of stroke in humans. In advance, the different methods used in this study (using 4 hours of ligation of the common carotid artery) might cause more severe damage to the carotid artery, and which is proved by the swollen common carotid artery and its branches on days 1, 7, and 14 after induction (unpublished data). The swollen common carotid artery increases eNO on the endothelial rather than brain tissue, decreasing the brain's COX-2 expression. A previous study described COX-2 as the most common mediator of inflammation during atherosclerosis [13]. Increased COX-2 levels within the serum samples support this hypothesis. The changes inside a brain influence the systemic circulation profile, especially the C-RP level. This was proved by the result of the C-RP level in the untreated group, which showed an increase in all observation times. The increase in C-RP is caused by its role as an acute phase protein (APP) attracted to various tissue injuries [7].

In this study, the fermented calabash has a choline. Choline is a primary source of acetylcholine that can be utilised as a neurotransmitter. Choline will be converted into acetylcholine during repeated cycling within the post and pre-synaptic terminals [14]. Hence, choline can be deposited inside a neuron and regulate cytokine production in brain tissue, especially COX-2. Zhang et al.'s findings [15] support this study by demonstrating the role of choline in reducing thermal and mechanical sensitivity, as opposed to diminishing pro-inflammatory cytokines, in the context of chronic constriction nerve injury (CCI) development and treatment. However, the potency of choline is different in the various tissues. In this study, choline derived from fermented calabash increases COX-2 immune expression in the brain. However, it also decreases circulatory COX-2 during an ischemic stroke, indicating anti-inflammatory activities. Tudor et al. [16] described that the decrease of COX-2 indicates an inhibition of the pathogenesis of inflammation.

This study proved that clopidogrel does not influence immune expression and the level of COX-2 in the brain and serum. This is indicated by the unchanged level of COX-2 in both the brain and serum after treatment. Those seriously impact unavoidable neuronal damage and limited neuronal repair. Paul et al. [17] described that clopidogrel induces massive proliferation of leucocytes within circulation that express COX-2 but decreases cytokine expression, especially CD3+. Kral et al. [18] explained that the decrease in platelet aggregation inhibits the monocyte, lymphocyte, and leucocyte differentiation and activation and decreases IL-1 and IL-6 that belong to pro-inflammatory cytokines. Using clopidogrel did not significantly affect neuronal and brain parenchymal repair. Clopidogrel showed a minimum repair of the presentation of Nissl's body clumping due to the drug's ability to only act as an antiplatelet aggregation rather than a neuro supplement [19]. Moreover, severe brain infarction causes high circulatory APP, including C-RP from the systemic inflammation pathway [20]. Moreover, a previous study also elucidated that CRP increase after ischemic stress in neuronal tissue [21].

4. CONCLUSION

FC has beneficial effects on the Nissl's body presentation, increases brain COX-2 immune expression, and decreases circulatory levels of C-RP and COX-2. An advance study is needed to evaluate the potential for toxicity and side effects after acute, subchronic, and chronic utilization of FCC.

5. MATERIALS AND METHODS

5.1 Ethics Committee Approval

Research Ethical Clearance Commission from FODM, Airlangga University approved and monitored the study. The authentication number from the ethical commission was 218/HRECC.FODM/III/2023.

5.2 Herbal Preparation

This study was conducted in Dept. Pharmacology, FVM, UWKS, Indonesia. The study was performed from March until December 2023. The calabash was collected from the private garden of UWKS, Surabaya. The calabash species was identified at Balai Besar Litbang Tanaman Obat dan Obat Tradisional, Indonesia, with voucher number KM.04.02/2/1248/2022. The fruit was peeled and the pulp was fermented. Further, the fermented product was detemnied regarding its choline level using LC-MS/MS referring to Wilujeng et al. [6]. The fermentation showed that the fermented product contained choline as much as 112.40±3.13 mg/kg.

5.3 Animal Experimentation and Design

This study conducted an experiment involving 40 male rats from Sprague Dawley strain, each 6-months old, weighing 250.68±2.57 grams. The metabolic cages (40 cm × 30 cm × 40 cm) were used to maintain the rat individually. The rats were given bedding made of oven husk. The light/dark interval was set at 12/12 hours, at 25^{o}C with 60% humidity. The rats were fed using a standard diet (RatBio, Indonesia) and water were provided ad libitum.

Prior to the induction, the rats were maintained for 7-days. The rats were separated into four groups, as follow: K1 group (sham-operated group); K2-K4 groups were induced by ischemic stroke using the ligation of common, internal and external carotid artery. The procedure of ischemic stroke induction was conducted following Prakoso et al.[4]. Moreover, the group K2 (untreated); K3 (treated with 2.94 mg/kg BW FC) [7]; K4 group (treated with 7.75 mg/kg BW clopidogrel) (Clopidogrel Bisulphate®, Hexpharm Jaya, Indonesia) [22]. The treatment was performed for 14 days, once daily.

5.4 Laboratory Test

The blood was collected several times, as follow: days 1, 7, and 14. The phlebotomy was performed via plexus retro-orbital under general anaesthesia. The general anaesthesia was given using ketamine in combination with xylazine. The blood was stored in an Eppendorf tube and the serum was collected. The serum was tested against C-RP and enzyme-linked immunosorbent assay (ELISA) against COX-2.

After 14-days, the rat was euthanised using a lethal doses of ketamine (150 mg/kg BW). The rat was necropsied and the brain tissue was collected. The brain was cut for histopathology and ELISA. For histopathology, the brain was fixed for 24 hours using 10% NBF. The brain was procesed for thionine staining (CAS25137-58-0, Santa Cruz Biotechnology Inc, USA), and immunohistochemistry (IHC) against antibody-anti COX-2 (sc-376861, Santa Cruz Biotechnology Inc, USA). The histopathology and IHC were analysed semi-quantitatively using scoring systems, including 1 = typical, 2 = mild changes/expressed, 3 = moderate changes/expressed, and 4 = strong expression.

The brains and serum were tested by ELISA using COX-2 (E-EL-R0792) from Elabscience, USA. The ELISA procedure was conducted using a manufacturer standard protocol.

5.5 Statistical Analysis

The data was analysed using SPSS version 26. The data of serum C-RP, COX-2 and brain level of COX-2 were analysed using ANOVA and was confirmed using Bonferroni test. However, the data of score of Nissl's body and immunohistochemistry of COX-2 were analysed using Kruskal-Wallis and Mann Whitney-U test as confirmation. The level of significance used in this study was $P<0.05$.

Acknowledgements: All the veterinary technicians from Dept. Pharmacology, FVM, UWKS were acknowledged for their assistance.
Author contributions: Concept – Y.A.P.; Design – Y.A.P.; Supervision – Y.A.P.; Resources – Y.A.P., A.S., S.W., P.A.W., J.H.H.; Materials – Y.A.P., A.S., S.W., P.A.W., J.H.H.; Data Collection and/or Processing – Y.A.P., S.W., P.A.W.; Analysis and/or Interpretation – Y.A.P., A.S.; Literature Search – Y.A.P., A.S., S.W., P.A.W., J.H.H.; Writing – Y.A.P., A.S., S.W., P.A.W., J.H.H.; Critical Reviews – Y.A.P., S.W.
Conflict of interest statement: The authors have no conflict of interests.

REFERENCES

[1] Ramos-Lima MJM, Brasileiro IC, Lima TL, Braga-Neto P. Quality of life after stroke: impact of clinical and sociodemographic factors. Clinics. 2018; 73: e418. https://doi.org/10.6061/clinics/2017/e418.

[2] Das UN. Can COX-2 inhibitor-induced increase in cardiovascular disease risk be modified by essential fatty acids?. J Assoc Physicians India. 2005; 53: 623–627.

[3] Zhao L, Fang J, Zhou M, Zhou J, Yu L, Chen N, He L. Interaction between COX-1 and COX-2 increases susceptibility to ischemic stroke in a Chinese population. BMC Neurol. 2019; 19 (1): 291. https://doi.org/10.1186/s12883-019-1505-1.

[4] Prakoso YA, Sigit M, Aliviameita A. Standardization of the simple methodology for experimentally induced ischemic stroke in rat models. World Vet J. 2023; 13 (4): 510-519. https://doi.org/10.54203/scil.2023.wvj54.

[5] Comin J and Kallmes D. Clopidogrel (plavix). AJNR Am J Neuroradiol. 2011; 32(11): 2002–2004. https://doi.org/10.3174/ajnr.A2913.

[6] Kuszynski DS and Lauver DA. Pleiotropic effects of clopidogrel. Purinergic Signal. 2022; 18(3): 253–265. https://doi.org/10.1007/s11302-022-09876-0.

[7] Yang Y, Huang Z, Zhang X. Efficacy and safety of clopidogrel and/or aspirin for ischemic stroke/transient ischemic attack: An overview of systematic reviews and meta-analysis. Medicine. 2021; 100 (50): e27804. https://doi.org/10.1097/MD.0000000000027804.

[8] Wilujeng S, Wirjaatmadja R, Prakoso YA. Effects of extraction, fermentation, and storage processes on the levels of choline derived from calabash fruit (*Crescentia cujete* L.). J Res Pharm. 2023; 27 (2): 620-626. https://doi.org/10.29228/jrp.344.

[9] Javaid S, Farooq T, Rehman Z, Afzal A, Ashraf W, Rasool MF, Alqahtani F, Alsanea S, Alasmari F, Alanazi MM, Alharbi M, Imran I. Dynamics of Choline-Containing Phospholipids in Traumatic Brain Injury and Associated Comorbidities. Int J Mol Sci. 2021;22(21):11313. https://doi.org/10.3390/ijms222111313.

[10] Hidayah JH, Prakoso YA, Widyarini S. Brain histopathological changes after treatment using calabash fruit (*Crescentia cujete* L.) in rat model with artificially induced ischemic stroke. Adv Anim Vet Sci. 2023; 11 (12): 2003-2009. https://doi.org/10.17582/journal.aavs/2023/11.12.2003.2009.

[11] Xu Y, Liu Y, Li K, Yuan D, Yang S, Zhou L, Zhao Y, Miao S, Lv C, Zhao J. COX-2/PGE2 Pathway Inhibits the Ferroptosis Induced by Cerebral Ischemia Reperfusion. Mol Neurobiol. 2022;59(3):1619-1631. doi: 10.1007/s12035-021-02706-1. Erratum in: Mol Neurobiol. 2022;59(12):7542-7543. https://doi.org/10.1007/s12035-021-02706-1.

[12] Oliveira-Filho J, Ornellas AC, Zhang CR, Oliveira LM, Araújo-Santos T, Borges VM, Ventura LM, Reis FJ, Aras R, Fernandes AM, Rosand J, Greenberg SM, Furie KL, Rost NS. COX-2 rs20417 Polymorphism Is Associated with Stroke and White Matter Disease. J Stroke Cerebrovasc Dis. 2015;24(8):1817-1822. https://doi.org/10.1016/j.jstrokecerebrovasdis.2015.04.018.

[13] Merhi M, Demirdjian S, Hariri E, Sabbah N, Youhanna S, Ghassibe-Sabbagh M, Naoum J, Haber M, Othman R, Kibbani S, Chammas E, Kanbar R, Bayeh HE, Chami Y, Abchee A, Platt DE, Zalloua P, Khazen G. Impact of inflammation, gene variants, and cigarette smoking on coronary artery disease risk. Inflamm Res. 2015;64(6):415-422. https://doi.org/10.1007/s00011-015-0821-1.

[14] Kansakar U, Trimarco V, Mone P, Varzideh F, Lombardi A, Santulli G: Choline supplements: An update. Front Endocrinol. 2023; 14: 1148166. https://doi.org/10.3389/fendo.2023.1148166.

[15] Zhang N, Li Y, Feng Z. Inhibition effect of choline and parecoxib sodium on chronic constriction nerve injury-induced neuropathic pain in rats. BMC Anesthesiol. 2023;23(1):22. https://doi.org/10.1186/s12871-022-01913-0

[16] Tudor DV, Bâldea I, Lupu M, Kacso T, Kutasi E, Hopârtean A, Stretea R, Gabriela Filip A. COX-2 as a potential biomarker and therapeutic target in melanoma. Cancer Biol Med. 2020;17(1):20-31. https://doi.org/10.20892/j.issn.2095-3941.2019.0339.

[17] Paul M, Paul JW, Hinwood M, Hood RJ, Martin K, Abdolhoseini M, Johnson SJ, Pollack M, Nilsson M, Walker FR. Clopidogrel Administration Impairs Post-Stroke Learning and Memory Recovery in Mice. Int J Mol Sci. 2023;24(14):11706. https://doi.org/10.3390/ijms241411706.

[18] Kral JB, Schrottmaier WC, Salzmann M, Assinger A. Platelet Interaction with Innate Immune Cells. Transfus Med Hemother. 2016;43(2):78-88. https://doi.org/10.1159/000444807.

[19] Kamarova M, Baig S, Patel H, Monks K, Wasay M, Ali A, Redgrave J, Majid A, Bell SM. Antiplatelet Use in Ischemic Stroke. Ann Pharmacother. 2022;56(10):1159-1173. https://doi.org/10.1177/10600280211073009.

[20] Xu LB, Yue JK, Korley F, Puccio AM, Yuh EL, Sun X, Rabinowitz M, Vassar MJ, Taylor SR, Winkler EA, Puffer RC, Deng H, McCrea M, Stein MB, Robertson CS, Levin HS, Dikmen S, Temkin NR, Giacino JT, Mukherjee P, Wang KKW, Okonkwo DO, Markowitz AJ, Jain S, Manley GT, Diaz-Arrastia R; TRACK-TBI Investigators. High-Sensitivity C-Reactive Protein is a Prognostic Biomarker of Six-Month Disability after Traumatic Brain Injury: Results from the TRACK-TBI Study. J Neurotrauma. 2021;38(7):918-927. https://doi.org/10.1089/neu.2020.7177.

[21] Finck T, Sperl P, Hernandez-Petzsche M, Boeckh-Behrens T, Maegerlein C, Wunderlich S, Zimmer C, Kirschke J, Berndt M. Inflammation in stroke: initial CRP levels can predict poor outcomes in endovascularly treated stroke patients. Front Neurol. 2023;14:1167549. https://doi.org/10.3389/fneur.2023.1167549.

[22] Sun Y, Venugopal J, Guo C, Fan Y, Li J, Gong Y, Chen YE, Zhang H, Eitzman DT. Clopidogrel Resistance in a Murine Model of Diet-Induced Obesity Is Mediated by the Interleukin-1 Receptor and Overcome With DT-678. Arterioscler Thromb Vasc Biol. 2020;40(6):1533-1542. https://doi.org/10.1161/atvbaha.120.314146.