Scientific Foundation SPIROSKI, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2021 Nov 02; 9(A):982-987.
https://doi.org/10.3889/oamjms.2021.7490
eISSN: 1857-9655
Category: *A - Basic Sciences*
Section: *Pathology*

The Effects of Glucagon and Insulin Combination toward on Neurodegeneration Following Traumatic Brain Injury in Rat Model

Akhmad Imron[1]*, Bethy Hernowo[2], Dany Hilmanto[3], Kahdar Wiriadisastra[1], Yulius Hermanto[1]

[1]Department of Neurosurgery, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia; [2]Department of Pathology, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia; [3]Department of Pediatric, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia

Edited by: Sinisa Stojanoski
Citation: Imron A, Hernowo B, Hilmanto D, Wiriadisastra K, Hermanto Y. The Effects of Glucagon and Insulin Combination toward on Neurodegeneration Following Traumatic Brain Injury in Rat Model. Open Access Maced J Med Sci. 2021 Nov 02; 9(A):982-987. https://doi.org/10.3889/oamjms.2021.7490
Keywords: Traumatic brain injury; Insulin; Glucagon; Glutamate; Glucose; Necrosis
***Correspondence:** Akhmad Imron, Department of Neurosurgery, Faculty of Medicine, Universitas Padjadjaran – Dr. Hasan Sadikin Hospital, Jl. Pasteur No.38, Sukajadi, Bandung 40161, Indonesia. E-mail: aimbd@yahoo.com
Received: 01-Oct-2021
Revised: 20-Oct-2021
Accepted: 22-Oct-2021

Funding: This research did not receive any financial support
Competing Interest: The authors have declared that no competing interest exists

Abstract

BACKGROUND: Traumatic brain injury (TBI) is a major cause of death and disability in the productive age. Glutamate excitotoxicity and hyperglycemia those occur following TBI are among the factors those influence secondary brain injury.

AIM: This study aimed to determine the effect of glucagon and insulin combination on neuronal necrosis following TBI.

METHODS: A total of 28 male wistar rats were randomized into four experimental groups: placebo, insulin, glucagon, and combination of glucagon and insulin. Each animal underwent controlled cortical impact model of TBI. The blood glucose and glutamate levels were measured before and 4 h following TBI. The brain tissues were collected to evaluate neuronal necrosis.

RESULTS: Glucagon or glucagon and insulin combination were able to prevent the increased of blood glutamate levels following TBI ($p < 0.05$). Glucagon administration was associated high blood glucose level (198.10 ± 32.58 mg/dL); a combination with insulin was able to minimize the increased of blood glucose level (166.53 ± 18.48 mg/dL). Combination of glucagon and insulin had a lower number of neuronal necrosis compare to the other groups ($p < 0.005$).

CONCLUSION: The combination of glucagon and insulin potentially exhibit neuroprotection effect on rats following TBI as being demonstrated by lower number of neuronal necrosis. This finding further indicates the role of glucose homeostasis in neuroprotection.

Introduction

Traumatic brain injury (TBI) remains the leading cause of death and disability in the young adults, making it a major problem in the field of public health and socioeconomics [1], [2], [3]. The incidence of TBI varies between 67 and 317/100,000 population, with a mortality rate about 4-8% in moderate TBI and about 50% in severe TBI [1], [2], [3].

Brain damaged in TBI is caused by primary injury as the result of direct insult that occurs immediately after trauma and secondary injury that occurs due to subsequent degeneration processes [4], [5]. Secondary brain injury occurs through several mechanisms, such as: disruption of cerebral blood flow (CBF), excitotoxicity, reactive oxygen species, inflammatory reactions, necrosis and apoptosis. TBI promotes accumulation of calcium ions in cells, hence activates protease enzymes such as calpain, protein kinase, and phosphatase those contribute to necrosis and apoptosis [4], [5].

Hyperglycemia in patients with TBI is associated with a higher risk of death and poorer outcome, this effect mainly due to aggravating of secondary brain injury. Therefore, controlling blood glucose level with insulin can improve the clinical outcome of patients with TBI [4], [5], [6], [7].

Glucagon acts as a neuroprotective agent in experimental TBI models. The neuroprotective effect of glucagon through decreasing glutamate concentration. Although glucagon causes an increase in glucose, the effect of lowering glutamate levels is higher as a neuroprotective. This indicates that the adverse effects of increasing glutamate concentrations outweigh the dangers of hyperglycemic effects. A decreased of circulating glutamate concentrations in rats could reduce brain damage after TBI [6], [7], [8]. Insulin lowers blood glucose levels, and circulating levels of amino acids, including glutamate. The combination of glucagon and insulin potentially enhances the neuroprotective effects. In this study, we sought to investigate the effect of glucagon and insulin combination as neuroprotective substances in TBI model.

Materials and Methods

Animals

Male wistar rats (250–300 g, 13–16 weeks) were obtained from the Animal Facility, Faculty of Medicine, Padjadjaran University. Animals were housed in a facility with constant humidity (60 ± 10%), temperature (25 ± 1°C), and a 12-h light/dark cycle (lights on at 7 am). Food and water were available *ad libitum* throughout the study. Animals were acclimatized to laboratory conditions for at least 1 week before undergoind surgery. All experimental procedures were performed according to the principles of laboratory animal care (National Institutes of Health publication, #85-23, revised in 1985). The experimental protocols were approved by the Ethical Committee of Faculty of Medicine, Padjadjaran University.

TBI model

TBI was induced in male wistar rats by controlled cortical impact using the modified method of Dixon *et al*. [9] Briefly, rats were anesthesized with intraperitoneal injection of thiophental at 40 mg/kgBW, then the rats were fixed on the operating table. Pulse oximetry was installed on the rat's tail to monitor oxygen saturation, to avoid hypoxia and hypercarbia. The scalp was shaved and cleaned with 10% povidone iodine at the craniectomy area. Skin incision was made to exposed the skull between bregma and lambda. A high speed drill was used to make a craniectomy with a diameter of 5–7 mm performed on the right side at coordinate of AP = + 3 mm and L= + 2 mm. A cylindrical tube 25 cm long, 5 mm in diameter directs an iron ball 4 mm in diameter, weighing 24 g dropped from a height of 21.8 cm [9]. Dropped once over the craniectomy area. The cylindrical tube was kept at an angle of 90° and was spaced 1 cm away from the brain surface to maintain compressed air. The force of the trauma is equal to the strength of the load multiplied by the height, equivalent to the force that causes a severe TBI. After being traumatized, the rats were awakened to prevent hypoxia and hypercarbia.

Experimental design and treatment

All animals were received intraperitoneal injection 25 min after the induction of TBI, and were treated similarly except subtances being tested. In this study, the animals were treated with vehicle (normal saline), glucagon (GlucaGen, Novo Nordisk, 175 μg i.p.), insulin (Humulin R, Eli Lily, 2 U/kgBW i.p.), and glucagon + insulin combination. Each group consist of seven animals. The examination of brain tissue was carried out 4 h after administration of intraperitoneal injection.

Blood examination

In this study, serial measurements of blood glucose level and blood glutamate were carried out 15 min and 4 h after the induction of TBI, by aspiration of blood from tail blood vein. The measurement of blood glucose level was carried out with rapid glucometer (Accu-Chek, Roche).

The measurement of blood glutamate level was carried out with spectrophotometry method. Briefly, the serum was diluted with assay buffer and added with reaction mix (Glutamate assay kit, Abcam, ab83389) subsequently incubated for 30 min at 37°c. The concentration was quantified at a wave length of 450 nm using Stat Fax 3300 (Inter Bio-Lab Inc.).

Histological examination

Four hours after the induction of TBI, the rats were perfused with 4% paraformaldehyde, subsequently brain tissues were post-fixed with buffered formalin overnight. The brain tissues were embedded in paraffin-block and processed for the histological examination by the blinded pathologist. Briefly, the tissues were sliced at 4 μm thickness and mounted on slide. The slides were deparaffinized, before routine staining with hematoxylin-eosin, and mounted with entelan. The microscopy images were observed with BX41 microscope (Olympus, Tokyo).

The histological examination was the degree of necrosis. The degree of necrosis was evaluated quantitatively through examination at 5 high power fields around the epicenter of the injured brain. The finding was reported as mean percentage of neuronal necrosis.

Immunohistochemistry

Briefly, the sections were deparaffinized and rehydrated. The antigenic retrieval was performed with microwave for 20 min. Membrane penetration was performed with Triton X-100 3%. Non-specific binding was blocked with 3% normal goat serum for 1 h. After, rinse with PBS, the sections were incubated with rabbit anti-calpain 1 (Abcam, ab39171) for 1 h. The sections were rinsed with PBS prior to incubation with biotinylated goat anti rabbit antibody (abcam, ab64256) for 20 min at room temperature, followed by incubation with streptavidin-peroxidase conjugate (Vectastain, PK-6100) for 10 min at room temperature. After rinsing with PBS, the staining was developed for 5 min in substate medium containing 0.05% 3,30-diaminobenzidine and 0,02% hydrogen peroxide (Vectastain, SK-4100) for 5 min. The sections were counter-stained with hematoxylin and mounted with entelan. The microscopy images were observed with BX41 microscope (Olympus, Tokyo). The

immunoexpression of calpain-1 was measured with histoscore method [10].

Statistical analysis

The data of blood examination were presented as mean ± SD, immunohistochemistry results were presented as median, and histological examination were presented as proportion. All statistical calculations were performed Graphpad Prism v 8.0, and $p < 0.05$ was considered statistically significant.

Results

Comparison of blood glucose levels

In this study, there was no increased of blood glucose level following the induction of TBI, as being observed in the placebo group that there was no significance difference in the mean of blood glucose level between pre (126.58 ± 11.71 mg/dL) and post TBI (146.87 ± 24.32 mg/dL). There was a trend of lower blood glucose level in insulin but statistically was not significant ($p > 0.05$). Rats those received glucagon (198.10 ± 32.58 mg/dL) and combination of glucagon and insulin (166.53 ± 18.48 mg/dL) had higher blood glucose post-induction of TBI (interaction $F(3, 48) = 10.33$, row $F(1, 48) = 16.26$, column $F(3,48) = 30.44$, $p < 0.001$, two-way ANOVA, post-hoc Sidak's multiple comparison test, $p < 0.01$, Figure 1).

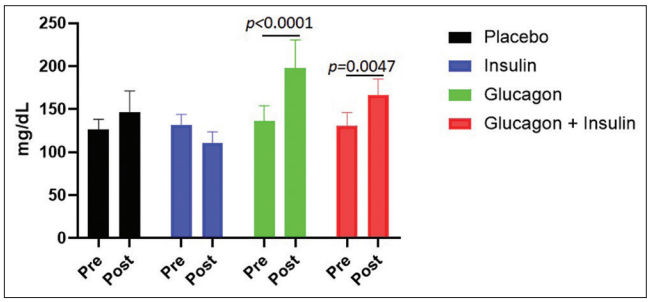

Figure 1: The mean of blood glucose level. In this study, there was a tendency for reactive hyperglycemia following TBI (pre = 126.58 ± 11.71 mg/dL and post = 146.87 ± 24.32 mg/dL, $p > 0.05$). There was a trend of lower blood glucose level in insulin but statistically was not significant ($p > 0.05$). Rats those received glucagon (198.10 ± 32.58 mg/dL) and combination of glucagon and insulin (166.53 ± 18.48 mg/dL) had higher blood glucose post-induction of traumatic brain injury (interaction $F(3, 48) = 10.33$, row $F(1, 48) = 16.26$, column $F(3,48) = 30.44$, $p < 0.001$, two-way ANOVA, post-hoc Sidak's multiple comparison test, $p < 0.01$)

Comparison of blood glutamate levels

In this study, there was an increased of blood glutamate level following the induction of TBI, as being observed in the placebo group that there was a significance difference in the mean of blood glutamate level between pre (11.32 ± 0.91 nmol/L) and post induction (13.92 ± 1.23 nmol/L). Rats those received insulin failed to prevent an increased of blood glutamate level (pre = 10.79 ± 1.23 nmol/L and post 13.51 ± 1.80 nmol/L), while glucagon or combination of glucagon and insulin administration were able to prevent an increase of blood glutamate following the induction of TBI (interaction $F(3, 48) = 8.67$, row $F(1, 48) = 7.02$, column $F(3,48) = 4.42$, $p < 0.05$, two-way ANOVA, post-hoc Sidak's multiple comparison test, $p < 0.01$, Figure 2).

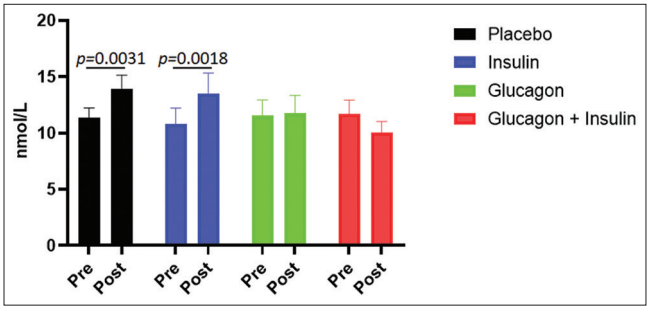

Figure 2: The mean of blood glutamate level. Following traumatic brain injury, there was an increased of blood glutamate level, as being observed in the placebo group. Rats those received insulin failed to prevent an increased of blood glutamate level, while glucagon or combination of glucagon and insulin administration were able to prevent an increase of blood glutamate following the induction of traumatic brain injury (interaction $F(3, 48) = 8.67$, row $F(1, 48) = 7.02$, column $F(3,48) = 4.42$, $p < 0.05$, two-way ANOVA, post-hoc Sidak's multiple comparison test, $p < 0.01$)

Comparison of neuronal necrosis

Through histological examination, we found that following the induction of TBI there was neuronal necrosis around the epicentrum of the impact (Figure 3a and b). In addition to neuronal necrosis, the hemorrhage was commonly observed in the specimens suggesting successful establishment of TBI model.

After the quantification, we found that the number of neuronal necrosis was lower in rats with combination of glucagon and insulin (51.00 ± 7.72%) compare with other groups (placebo = 66.14 ± 12.13%; insulin = 62.71 ± 12.72%; and glucagon = 59.43 ± 10.97%, $F(3,24) = 2.41$, $p = 0.0401$, one-way ANOVA, post-hoc Dunnett's multiple comparison test $p = 0.0141$, Figure 3c).

Immunoexpression of calpain-1

We further confirmed the occurrence of neuronal necrosis following the induction of TBI by performing immunostaining of calpain-1. The calpain-1 immunoexpression was observed in the neuronal cytoplasmic following the induction of TBI, suggesting the process of neuronal necrosis, particular at the area surrounding the epicentrum of the impact (Figure 4a and b). We quantified the immunoexpression of calpain-1 to evaluate the

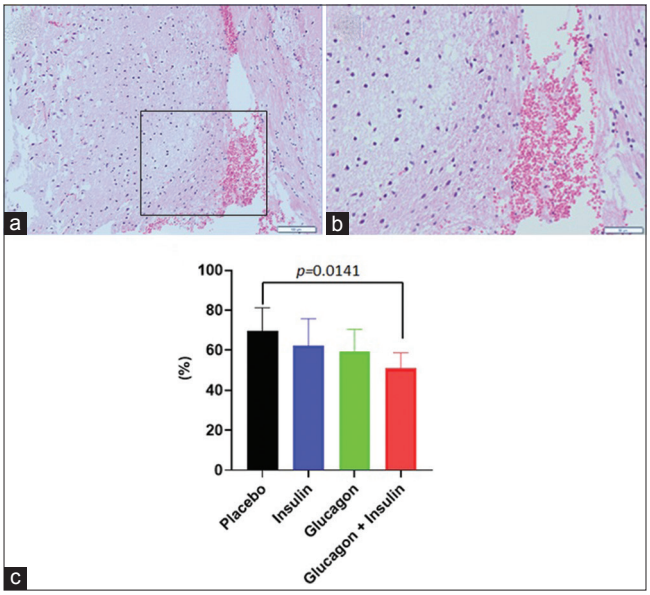

Figure 3: Examination of neuronal necrosis. (a and b) The neuronal necrosis was commonly observed around the epicentrum of the impact. (c) The number of neuronal necrosis was lower in rats with combination of glucagon and insulin (51.00 ± 7.72%) compare with other groups (placebo = 66.14 ± 12.13%; insulin = 62.71 ± 12.72%; and glucagon = 59.43 ± 10.97%, F(3,24) = 2.41, p = 0.0401, one-way ANOVA, post-hoc Dunnett's multiple comparison test p = 0.0141)). Scale bar a = 100 μm, b = 40 μm

efficacy of tested substances. In this study, rats those received a combination of glucagon and insulin has a lower histoscore value (7.71 ± 2.06) than other groups (placebo = 10.57 ± 1.72; insulin = 9.86 ± 1.95; glucagon = 9.29 ± 1.38, Krukal-Wallis statistic = 8.483, p = 0.0370, post-hoc Dunn's multiple comparison test p = 0.0154, Figure 4c).

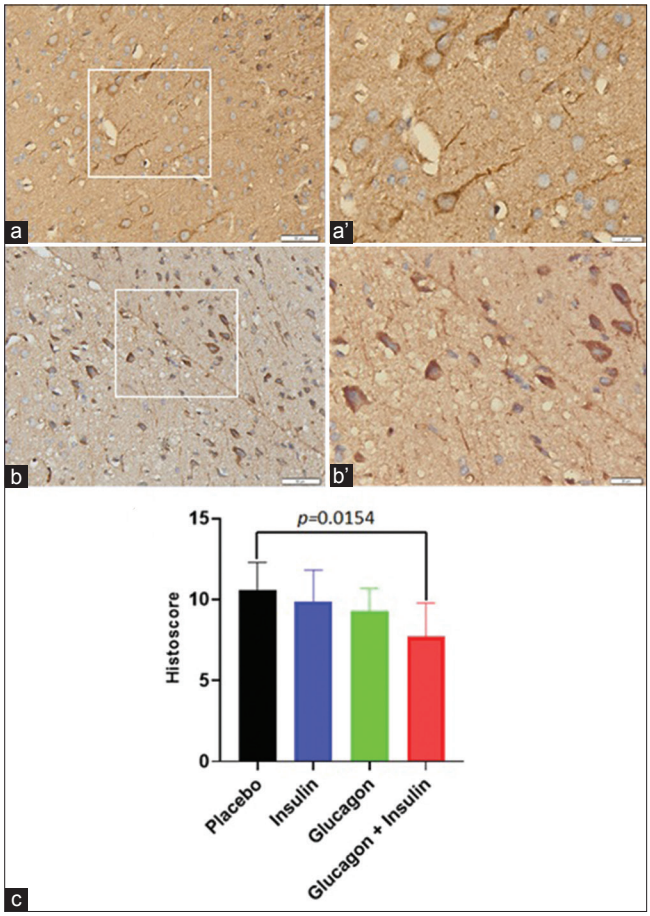

Figure 4: Immunohistochemistry of calpain-1. (a and b) Representative images of calpain-1 staining from rats brain. (a' and b') The magnification images from the white boxes. (c) Rats those received a combination of glucagon and insulin has a lower histoscore value (7.71 ± 2.06) than other groups (placebo = 10.57 ± 1.72; insulin = 9.86 ± 1.95; glucagon = 9.29 ± 1.38, Krukal-Wallis statistic = 8.483, p = 0.0370, post-hoc Dunn's multiple comparison test p = 0.0154). Scale bar a and b = 40 μm, a' and b' = 20 μm

Discussion

The efforts to improve neurological outcome for patients with TBI mainly act through minimizing secondary brain insults. Secondary brain insult is a complex process characterized by progressive neurodegeneration as consequences to initial insult, as results of changes in CBF, inflammation, metabolic disturbances, massive calcium ion influx, excitotoxicity, and activation of various cell death pathways.

In this study, we confirmed that TBI causes neuronal necrosis, particularly at the area surrounding the impact. The neuronal necrosis was confirmed through immunostaining of calpain-1 (a marker of necrosis), suggesting the successful establishment of animal model.

Glutamate excitotoxicity commonly occurs following TBI and known as a mediator for secondary brain injury [11]. Acutely following TBI, there is an unregulated release of glutamate that cannot be buffered or cleared effectively, resulting in damaging levels of glutamate in the extracellular space. The diffusion of glutamate outside the synapse due to impaired uptake may lead to increased extrasynaptic glutamate signaling and secondary brain injury through activation of cell death pathways [11]. Several neuroprotective agents are developed to target glutamate excitotoxicity [11], [12], [13], [14].

A previous study reported that glucagon administration could promote reduction of blood glutamate level and might exhibit neuroprotection effect [4], [5], [6]. In this study, we found that a combination of glucagon and insulin potentially exhibit a neuroprotection effect on rats following TBI as being demonstrated by lower number of neuronal necrosis. This finding further indicates the role of glucose homeostasis in neuroprotection. Hyperglycemic condition following TBI is associated with higher mortality [15], [16], this effect might attribute to accumulation of inflammatory cells and promotion of inflammation [17]. In line with previous studies, we observed that both glucagon and combination of glucagon and insulin were able

to minimize the increased of blood glutamate level, but only combination glucagon and insulin was able to minimize neuronal decrease. This might be due to hyperglycemic condition in rats with glucagon which attenuates the beneficial effect of neuroprotection of lowering glutamate level.

Elevated glutamate concentration is found in the cerebrospinal fluid (CSF) of the patients with TBI and predicts mortality following severe TBI [18], [19]. In TBI, there is a leakage of blood-brain barrier; therefore, a decrease in circulating glutamate might reduce glutamate in the CSF and the central nervous system. Our results and previous studies suggest that glucagon has overlapping neuroprotective effects on post-TBI glutamate elevation and combination with might provide additional benefit through preventing hyperglycemic condition [4], [5].

Limitation

In this study, we did not perform dose-response analysis to determine optimal dosage and therapeutic time window for the substances being tested. Rodents have a high endogenous recovery [20], [21]; hence, it was difficult to perform functional analysis to evaluate motoric function following TBI. Nevertheless, this study aimed to investigate about neuroprotection effect rather than the functional recovery.

Conclusion

The combination of glucagon and insulin potentially exhibit a neuroprotection effect on rats following TBI as being demonstrated by lower number of neuronal necrosis. This finding further indicates the role of glucose homeostasis in neuroprotection.

References

1. Ghajar J. Traumatic brain injury. Lancet. 2000;356(9233):923-9. http://doi.org/10.1016/S0140-6736(00)02689-1
   PMid:11036909
2. Langlois J, Rutland-Brown W, Wald M. The epidemiology and impact of traumatic brain injury. J Head Trauma Rehab. 2006;21(5):375-8. http://doi.org/10.1097/00001199-200609000-00001
   PMid:16983222
3. Dewan MC, Rattani A, Gupta S, Baticulon RE, Hung YC, Punchak M, *et al*. Estimating the global incidence of traumatic brain injury. J Neurosurg. 2019;130(4):1304-14.
4. Ng SY, Lee AY. Traumatic brain injuries: Pathophysiology and potential therapeutic targets. Front Cell Neurosci. 2019;13:528. http://doi.org/10.3389/fncel.2019.00528
   PMid:31827423
5. Werner C, Engelhard K. Pathophysiology of traumatic brain injury. Br J Anesth 2007;99(1):4-9. http://doi.org/10.1093/bja/aem131
   PMid:17573392
6. Fanne RA, Nassar T, Mazuz A, Waked O, Heyman SN, Hijazi N, *et al*. Neuroprotection by glucagon: Role of gluconeogenesis. J Neurosurg. 2011;114(1):85-91. http://doi.org/10.3171/2010.4.JNS10263
   PMid:20509730
7. Fanne RA, Nassar T, Heyman SN, Hijazi N, Higazi AR. Insulin and glucagon share the same mechanism of neuroprotection in diabetic rats: Role of glutamate. Am J Physiol Regul Integr Comp Physiol. 2011;301(3):668-73. http://doi.org/10.1152/ajpregu.00058.2011
   PMid:21677268
8. Zlotnik A, Gruenbaum BF, Klin Y, Gruenbaum SE, Ohayon S, Sheiner E, *et al*. The effects of insulin, glucagon, glutamate, and glucose infusion on blood glutamate and plasma glucose levels in naïve rats. J Neurosurg Anesthesiol. 2011;23(4):323-8. http://doi.org/10.1097/ANA.0b013e3182299b15
   PMid:21836527
9. Osier ND, Dixon CE. The controlled cortical impact model: Applications, considerations for researchers, and future directions. Front Neurol. 2016;7:134. http://doi.org/10.3389/fneur.2016.00134
   PMid:27582726
10. Jensen K, Krusenstjerna-Hafstrom R, Lohse J, Petersen KH, Derand H. A novel quantitative immunohistochemistry method for precise protein measurements directly in formalin-fixed, paraffin-embedded specimens: Analytical performance measuring HER2. Modern Pathol. 2017;30(2):180-93. http://doi.org/10.1038/modpathol.2016.176
    PMid:27767098
11. Bang SA, Song YS, Moon BS, Lee BC, Lee H, Kim JM, *et al*. Neuropsychological, metabolic, and GABA a receptor studies in subjects with repetitive traumatic brain injury. J Neurotrauma. 2016;33(11):1005-14. http://doi.org/10.1089/neu.2015.4051
    PMid:26414498
12. Dorsett CR, McGuire JL, DePasquale EA, Gardner AE, Floyd CL, McCullumsmith RE. Glutamate neurotransmission in rodent models of traumatic brain injury. J Neurotrauma. 2017;34(2):263-72. http://doi.org/10.1089/neu.2015.4373
    PMid:27256113
13. Mancini A, Chielini A, Di Capua A, Castelli L, Brogi S, Paolino M, *et al*. Synthesis and biological evaluation of a new class of benzothiazines as neuroprotective agents. Eur J Med Chem. 2017;126:614-30. http://doi.org/10.1016/j.ejmech.2016.11.053
    PMid:27923201
14. Ji SL, Cao KK, Zhao XX, Kang NX, Zhang Y, Xu QM, *et al*. Antioxidant activity of phenylethanoid glycosides on glutamate-induced neurotoxicity. Biosci Biotechnol Biochem. 2019;83(11):2016-26. http://doi.org/10.1080/09168451.2019.1637243
    PMid:31272310
15. Rau CS, Wu SC, Chen YC, Chen PC, Hsieh HY, Kuo PJ, *et al*. Stress-induced hyperglycemia, but not diabetic hyperglycemia, is associated with higher mortality in patients with isolated moderate and severe traumatic brain injury: Analysis of a propensity score-matched population. Int J Environ Res Public Health. 2017;14(11):1340. http://doi.org/10.3390/ijerph14111340
    PMid:29099813
16. Smith RL, Lin JC, Adelson PD, Kochanek PM, Fink EL, Wisniewski S, *et al*. Relationship between hyperglycemia and

outcome in children with severe traumatic brain injury. Pediatr Crit Care Med. 2012;13(1):85-91. http://doi.org/10.1097/PCC.0b013e3182192c30

PMid:21499170

17. Kinoshita K, Kraydieh S, Alonso O, Hayashi N, Dietrich WD. Contusion volume and neutrophil accumulation after moderate fluid-percussion brain injury in rats. J Neurotrauma. 2002;19(6):681-92. http://doi.org/10.1089/neu.1994.11.499

PMid:7861443

18. Stefani MA, Modkovski R, Hansel G, Zimmer ER, Kopczynski A, Muller AP, *et al*. Elevated glutamate and lactate predict brain death after severe head trauma. Ann Clin Transl Neurol. 2017;4(6):392-402. http://doi.org/10.1002/acn3.416

PMid:28589166

19. Ruppel RA, Kochanek PM, Adelson PD, Rose ME, Wisniewski SR, Bell MJ, *et al*. Excitatory amino acid concentrations in ventricular cerebrospinal fluid after severe traumatic brain injury in infants and children: The role of childe abuse. J Pediatr. 2001;138(1):18-25. http://doi.org/10.1067/mpd.2001.110979

PMid:11148507

20. Yang Li, Han B, Zhang Z, Wang S, Bai Y, Zhang Y, *et al*. Extracellular vesicle-mediated delivery of circular RNA SCMH1 promotes functional recovery in rodent and nonhuman primate ischemic stroke models. Circulation. 2020;142:556-74. http://doi.org/10.1161/CIRCULATIONAHA.120.045765

PMid:32441115

21. Dadwal P, Mahmud N, Sinai L, Azimi A, Fatt M, Wondisford FE, *et al*. Activating endogenous neural precursor cells using metformin leads to neural repair and functional recovery in a model of childhood brain injury. Stem Cell Rep. 2015;5(2):166-73. http://doi.org/10.1016/j.stemcr.2015.06.011

PMid:26235894