



BJP

Bangladesh Journal of Pharmacology

Research Article

**Protective effect of Kombucha tea
on brain damage induced by
transient cerebral ischemia and
reperfusion in rat**

Protective effect of Kombucha tea on brain damage induced by transient cerebral ischemia and reperfusion in rat

Najmeh Kabiri¹ and Mahbubeh Setorki²

¹Shahrekord University of Medical Sciences, Shahrekord, Iran; ²Department of Biology, Izeh Branch, Islamic Azad University, Izeh, Iran.

Article Info

Received: 11 March 2016

Accepted: 1 May 2016

Available Online: 6 July 2016

DOI: 10.3329/bjp.v11i3.27014

Cite this article:

Kabiri N, Setorki M. Protective effect of Kombucha tea on brain damage induced by transient cerebral ischemia and reperfusion in rat. Bangladesh J Pharmacol. 2016; 11: 675-83.

Abstract

The aim of study was to investigate the potential neuroprotective effects of Kombucha on cerebral damage induced by ischemia in rats (n=99). Cerebral infarct volume in the ischemic rats received Kombucha solution showed no significance alteration. However, the permeability of blood-brain barrier significantly decreased in both ischemic rats received 15 mg/kg Kombucha tea and Sham group. In addition, brain water content in the ischemic groups treated with Kombucha solution was significantly higher than the Sham group, although right hemispheres in all of the treated groups illustrated higher brain water content than the left ones. Brain anti-oxidant capacity elevated in the ischemic rats treated with Kombucha and in the Sham group. Brain and plasma malondialdehyde concentrations significantly decreased in both of the ischemic groups injected with Kombucha. The findings suggest that Kombucha tea could be useful for the prevention of cerebral damage.

Introduction

Stroke is the third leading cause of death and long-term disability in the industrial countries and even if they survive, the patients may be permanently disabled in memorizing, talking and walking. Strokes are induced by hemorrhage and ischemia, about 15% and 85%, respectively, and also ischemia itself is occurred by different factors such as thrombosis, embolism, and systemic perfusion (White et al., 2000). Ischemia causes neurological injury and subsequently the recirculation of blood to the injured tissues leads to reperfusion. During reperfusion, oxygen free radicals and other oxidative agents lead to higher cell damages than ischemia does. In addition, upon blood recirculation to the injured tissue, lack of oxygen and nutrients induce inflammation and oxidative damages, instead of sustaining normal activity. After establishment of cerebral blood flow, the returned blood flow and occlusion contribute to diffusion of oxygen into the cells, resulting necrosis and apoptosis (Lo et al., 2003).

Ischemia decreases oxygen and nutrients levels transported to the nervous system and consequently corrupts their function. In addition, oxygen deficiency contributes to decrease of ATP production in cells and consequently the cells switch to anerobic respiration pathway to achieve their needed energy, leading accumulation of lactate and cell death (Lo et al., 2003).

Now-a-days, medical plants possess a high state in the therapeutic protocols of many countries. Accordingly, many studies have been focused on anti-oxidants, as neuroprotective agents, to treat strokes. Early diagnosis and medical intervention could ameliorate the effects of ischemia as well as reduce cell death through inhibiting of apoptosis.

Anti-oxidants are substances preventing cell death through scavenging free radicals and eliminating the hazardous compounds generated during cell damage, thereby protecting healthy cells, especially their membrane and nucleus. Therefore, it is necessary to investigate the potential therapeutic effects of anti-

oxidants, more especially those originated from plants, on brain damages after stroke.

Kombucha tea drink is a functional fermented beverage produced through fermenting the tea using a symbiotic colony of bacteria and yeast. Due to its anti-oxidant properties, Kombucha tea is one of the plants likely possess protective effects against ischemia (Greenwalt et al., 1998; Malbaša et al., 2011; Ibrahim et al., 2013). Hence, the present study was aimed to evaluate the potential neuroprotective effects of Kombucha tea on the water content and hemostasis of Wistar rat brain.

Materials and Methods

Preparation of Kombucha solution

The tea (10 g) and sugar (80 g) were added into a one-liter sterile conical flask containing boiling water. Then, the solution was poured into some dishes and the fungus was cultured and incubated at $28 \pm 1^\circ\text{C}$ for 8-10 days. The surface of the dishes was covered with a piece of fabric and kept in a clean and dark room (Kastner and Freund, 1991).

Animal and treatments

Ninety-eight male Wistar rats (250-300 g) were kept under a controlled condition ($22 \pm 2^\circ\text{C}$ 12, L: 12D light cycle, and sufficient food).

The animals were separated into seven experimental groups ($n = 14$ for each group): Control group: No surgery and Kombucha administration, but received distilled water; Sham group: No Kombucha administration, but underwent surgery without blocking the carotid arteries; Ischemic group 1: No Kombucha administration; Ischemic group 2: Intraperitoneally injected with 5 mg/kg Kombucha solution; Ischemic group 3: Intraperitoneally injected with 15 mg/kg Kombucha solution; Kombucha treated group 1: Only intraperitoneally received 5 mg/kg Kombucha solution; Kombucha treated group 2: Only intraperitoneally received 15 mg/kg Kombucha solution.

Ischemia induction (Video Clip)

The rats were anesthetized by intraperitoneally injection of ketamin and xylazine (50 and 5 mg per kg, respectively). Middle cerebral artery occlusion was performed as described elsewhere (Long et al., 1989). Briefly, using microscopic surgery, a 3-0 nylon suture was introduced through the external carotid artery stump and advanced into the internal carotid until mild resistance where the tip was lodged in the anterior cerebral artery and blocked the blood flow to the middle cerebral artery. Finally, after an hour-ischemic period, reperfusion was started by withdrawing the suture (Rabiei et al., 2012).

Measurement of brain cerebral edema

The brains of the ischemic groups and the other ones used for evaluation of cerebral edema were dissected and the pons, cerebellums and olfactory bulbs were isolated, and then the sound and ischemic hemispheres were separated and weighted. The weighted samples were dried in an Oven (110°C for 24 hours). Brain water content was calculated using the following equation (1), and the difference of brain water contents between the two hemispheres used as an indicator of edema severity (Rabiei et al., 2012).

$$\text{BWC (\%)} = \frac{W_w - W_d}{W_w} \times 100 \quad \Delta\text{BWC} = \text{IBWC} - \text{SBWC}$$

Ischemic hemisphere water content (IBWC), sound hemisphere water content (SBWC)

Assessment of brain infarct volume

After scarifying the animals with chloroform, they were decapitated and the brain rapidly excised and cooled within a saline solution at 4°C for 5 min. The samples were placed into a brain matrix and then coronally sectioned (2 mm). To stain the sections, they were immersed in 2% 2,3,5-triphenyl tetrazolium chloride solution at 37°C for 15 min. The stained sections were digitally photographed using a digital camera (Lumix; Panasonic, Japan) and their unstained areas were measured using image analysis software (UTHSCSA Image Tool) and defined as infarct area.

Evaluation of blood-brain barrier permeability

Integrity of blood-brain barrier was evaluated through measuring Evans blue dye removed from brain. Evans blue (2%; 4 mg/kg body weight) was injected 30 min after induction of ischemia. Then, after blood reperfusion, the rats were deeply anesthetized and 250 mL of a warm normal saline (37°C was infused into their left ventricular catheters, thereby washing out the remnants of Evans blue from the circulation system. After decapitating, the brains were immediately removed to measure the weight of hemispheres. The hemispheres were separately homogenized in 2.5 mL phosphate buffered saline, and 2.5 mL of 60% trichloroacetic acid was added to precipitate their proteins. The mixture was stirred and centrifuged at 10,000 rpm for 30 min. Finally, Evans blue absorbance of the extracted supernatant was measured at 610 nm by spectrometer, and the concentration of the tissue Evans blue content was calculated according to its standard curve.

Determination of plasma anti-oxidant capacity

To determine serum anti-oxidant capacity of the experimental rats, their blood was collected to evaluate the biochemical parameter according to a common method. Briefly, a series of iron ion standard solutions was prepared and mixed with ferric reducing anti-oxidant power agent (FRAP), containing acetate buffer, 25 mL;

2,4,6-tripyridyl-s-triazine (TPTZ), 2-5 mL; and FeCl₃, 2.5 mL. For each of the experimental groups, the collected serum and FRAP were mixed and the total anti-oxidant capacity was determined based on ferrous form ($\mu\text{mol/L}$) using spectrometer at 593 nm (Rabiei et al., 2012).

Determination of brain anti-oxidant capacity

Brain anti-oxidant capacity of the experimental rats was measured according to Schmidt-Kaster method (Rabiei et al., 2012). Briefly, the brains were carefully removed, homogenized and centrifuged at 10,000 rpm, and then 50 μL of the supernatant was added to a freshly prepared TPTZ at 37°C. The mixture absorbance was measured by spectrometer at 593 nm.

Determination of plasma malondialdehyde level

P-MDA was determined in H₃PO₄-treated plasma after derivatization by thiobarbituric acid (TBA) and separation on HPLC. Briefly, 50 μL of the collected plasma was mixed in 50 μL of 0.05% BHT. Then, 400 μL of 0.44 H₃PO₄ and 100 μL TBA (42 mM) were consecutively added to the mixture and heated (100°C for 60 min) and cooled (0°C for 5 min). The mixture was homogenized and centrifuged (14,000 rpm for 5 min) after adding 250 μL of normal butanol, and finally, 20 μL of the supernatant was injected into HPLC (Block, 1999; Kamalakannan and Balakrishnan, 2015).

Determination of brain malondialdehyde level

To measure brain malondialdehyde level in brain, 1 mL of the collected tissues for each of the experimental groups was homogenized in 2.5% KCl (10% w/v) and incubated in a metabolic shaker (at $37 \pm 1^\circ\text{C}$ for 60 min). Thereafter, 1 mL of 5% trichloroacetic acid and 1 mL of

67% TBA reagent were added into the metabolic shaker. The final mixture was thoroughly homogenized again and centrifuged at 2,000 xg for 15 min; finally, after heating in a boiling water bath for 10 min, the obtained supernatant was cooled and its malondialdehyde level was measured at 535 nm by spectrometer (Rabiei et al., 2012).

Statistical analysis

All data were analyzed using SPSS 16 and expressed as the mean \pm standard deviation (SD). Multiple group comparisons of the means were carried out using a one way analysis of variance (ANOVA), and Dennett's and Tukey tests were used to compare the differences between the experimental groups and the control group ($p < 0.05$).

Results

Effects on cerebral edema

Cerebral edema in the ischemic rats intraperitoneally received 5 and 15 mg/kg Kombucha solution showed no significance difference, despite a dose-dependent reduction, when compared to the ischemic group with no Kombucha injection ($p > 0.05$; Figure 1).

Blood-brain barrier permeability

Permeability of the blood-brain barrier significantly decreased ($p < 0.05$) in the ischemic group treated with higher concentration of Kombucha solution (15 mg/kg) and also in the Sham group when compared to the ischemic group with no Kombucha injection. Moreover, a comparison between the left and right hemispheres

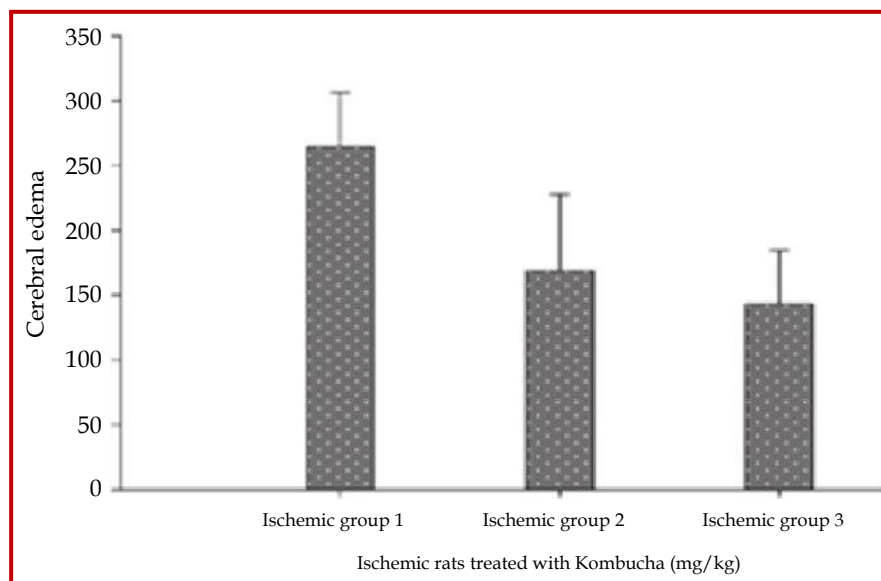


Figure 1: Cerebral edema in the ischemic groups, intraperitoneally injected with of Kombucha solution: And the ischemic group with no Kombucha treatment. Ischemia group 1, no Kombucha administration; ischemia group 2, intraperitoneally injected with 5 mg/kg Kombucha solution; and ischemia group 3, intraperitoneally injected with 15 mg/kg Kombucha solution

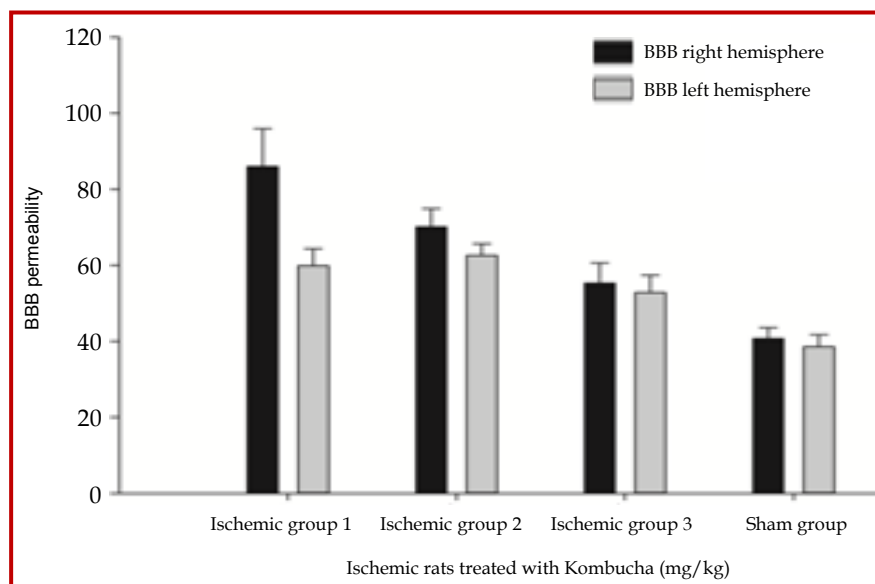


Figure 2: BBB permeability of the left and right hemispheres in the ischemic group1, received no Kombucha solution; ischemia group 2, intraperitoneally injected with 5 mg/kg Kombucha solution; ischemia group 3, intraperitoneally injected with 15 mg/kg Kombucha solution; and the Sham group, with no drug administration. Significance difference is denoted by asterisk (a). Data are expressed as mean \pm SD

showed that permeability of the left ones unchanged in the ischemic groups treated with Kombucha solution as well as Sham group when compared to the ischemic group received no Kombucha solution (Figure 2).

Effects on body water content

Body water content of the ischemic group received higher concentration of Kombucha solution (15 mg/kg) increased significantly in compare to the Sham group ($p < 0.05$). However, body water content in all of the ischemic groups was significantly higher than the Sham

group, although right hemispheres in all of the groups illustrated higher body water content than the left ones (Figure 3).

Plasma and brain anti-oxidant capacity

Anti-oxidant capacity of plasma showed no significance difference between the rats intraperitoneally received Kombucha solution and the control group (Figure 4). By contrast, brain anti-oxidant capacity significantly increased in the ischemic group treated with 15 mg/kg Kombucha solution and also in the Sham group ($p <$

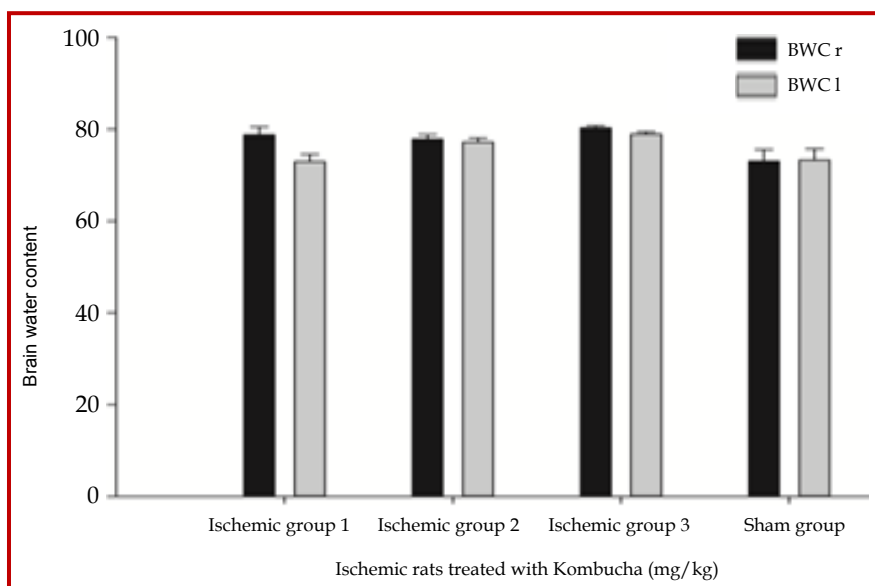


Figure 3: BWC of the right and left hemispheres of the ischemic group1, received no Kombucha solution; ischemia group 2, intraperitoneally injected with 5 mg/kg Kombucha solution; ischemia group 3, intraperitoneally injected with 15 mg/kg Kombucha solution; and the Sham group, with no drug administration. Data are expressed as mean \pm SD

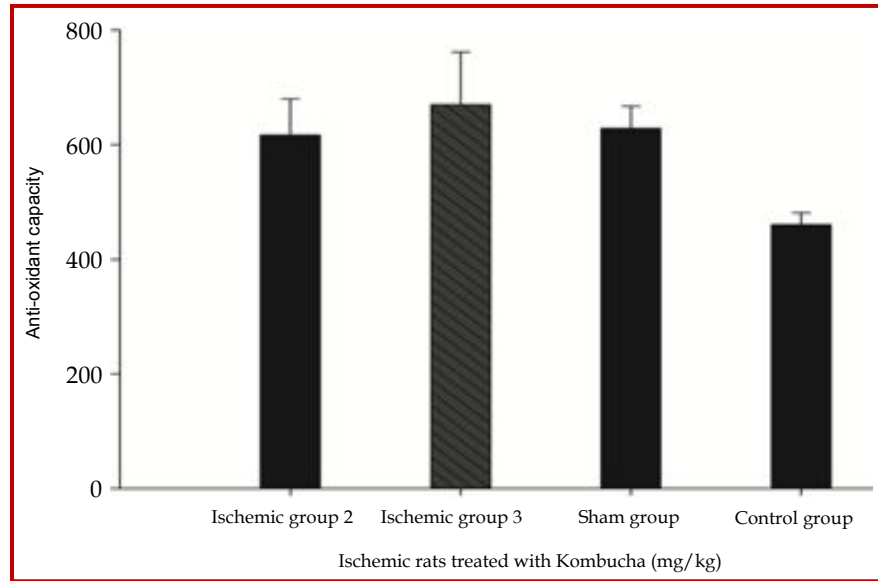


Figure 4: Plasma anti-oxidant capacity in the ischemic rat groups, intraperitoneally injected with 5 and 15 mg/kg of Kombucha solution; the control group, received no Kombucha solution; and the Sham group, with no drug administration. Data are expressed as mean ± SD

0.05; Figure 5).

Brain and plasma malondialdehyde level

Mean concentration of malondialdehyde in brain and plasma of the ischemic groups (Figure 6 and 7, respectively) treated with Kombucha tea significantly decreased when compared to the control group ($p < 0.05$).

Brain histopathological changes in the Ischemic groups

Coronal histopathological evaluation of cerebral infarct volume (unstained area) in the ischemic groups showed

approximately a same level of unstained area, as an indicator of infarct volume, 24 hours after ischemia induction (Figure 8).

Discussion

The results showed that Kombucha tea injection reduces cerebral edema, infarct volume and permeability of brain barriers through increasing blood-brain barrier integrity. Moreover, the concentrations of brain and plasma malondialdehyde significantly

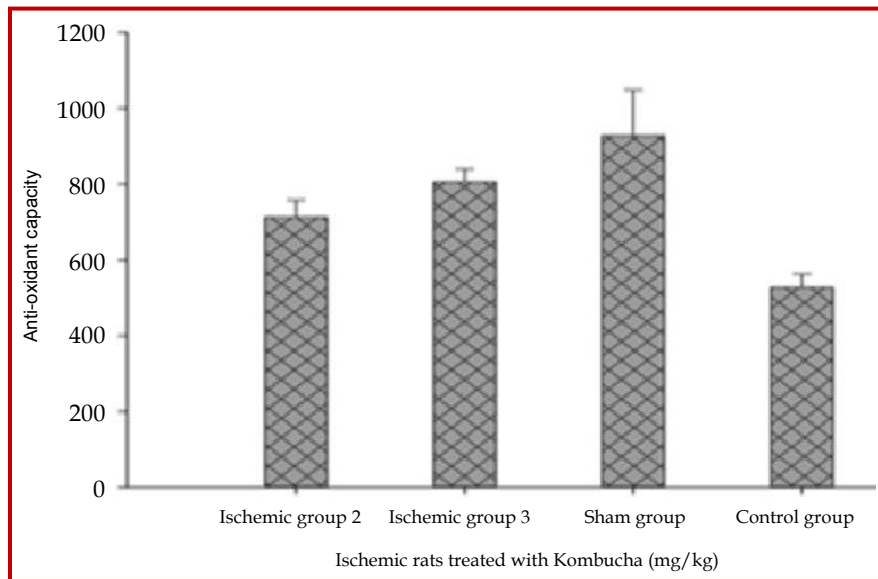


Figure 5: Brain anti-oxidant capacity in the ischemic rat groups, intraperitoneally injected with 5 and 15 mg/kg of Kombucha solution; Control group, received no Kombucha solution; and the Sham group, with no drug administration. Significance difference is denoted by asterisk (a). Data are expressed as mean ± SD

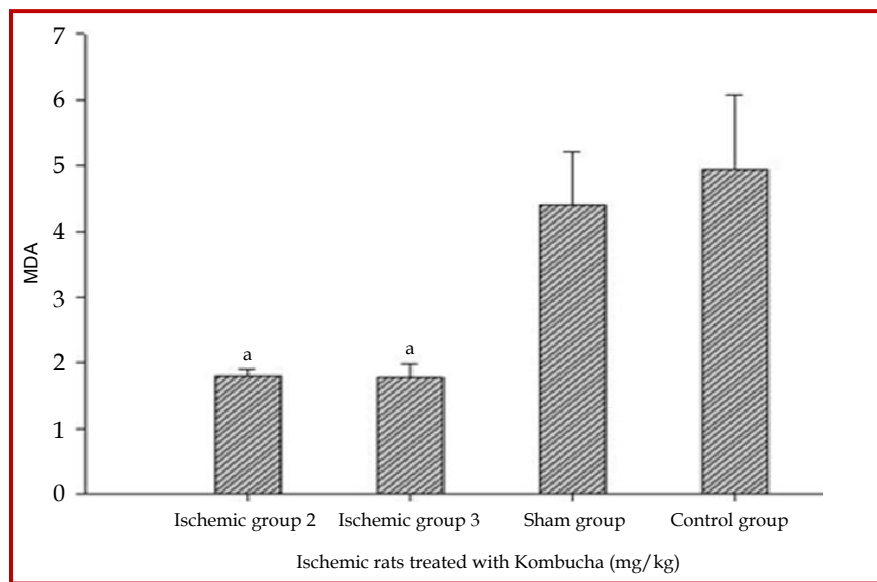


Figure 6: B-MDA in the ischemic rat groups, intraperitoneally injected with 5 and 15 mg/kg of Kombucha solution; Control group, received no Kombucha solution; and the Sham group, with no drug administration. Significance difference is denoted by asterisk (a). Data are expressed as mean \pm SD

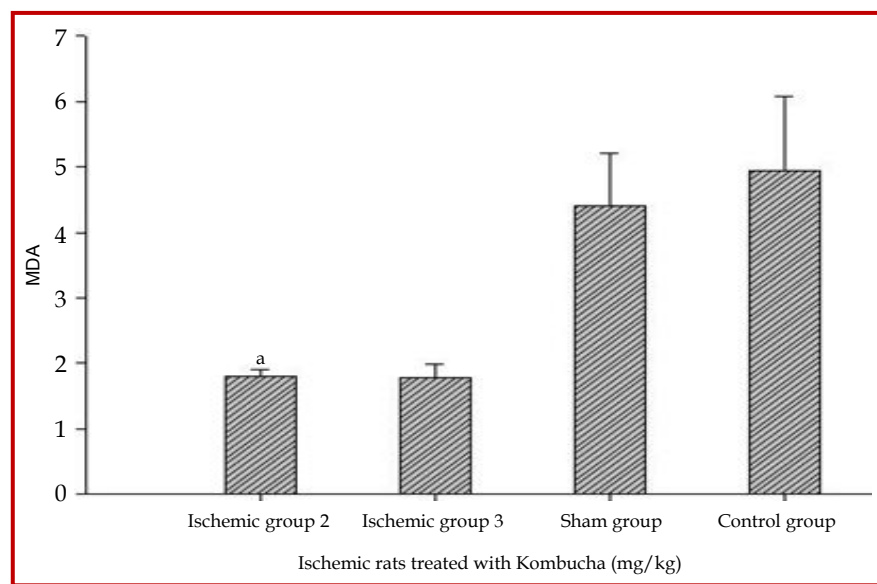


Figure 7: P-MDA in the ischemic rat groups, intraperitoneally injected with 5 and 15 mg/kg of Kombucha solution; Control group, received no Kombucha solution; and the Sham group, with no drug administration. Significance difference is denoted by asterisk (a). Data are expressed as mean \pm SD

increased in the group received no Kombucha solution.

During ischemia and reperfusion a series of cellular and molecular processes can result in damage to the nervous/non-nervous cells and brain capillaries. Of these events are cell cytotoxicity, oxidative stress, lipid peroxidation, inflammation and subsequently the breakdown of blood-brain barrier and generated cerebral edema. Brain ischemia and reperfusion engage multiple fatal terminal pathways involving loss of membrane integrity in partitioning ions, progressive proteolysis, and inability to check these processes

because of loss of general translation competence and reduced survival signal-transduction. Brain tissue is not well equipped with anti-oxidant defenses, so reactive oxygen species and other free radicals/oxidants, released by inflammatory cells, threaten tissue viability in the vicinity of the ischemic core (Block, 1999).

Blood-brain barrier integrity protects the neuronal micro-environment. When this barrier integrity is lost, inflammatory cells and fluid penetrate the brain, causing edema and cell death. Impermeability of the blood-brain barrier is maintained by micro-vascular

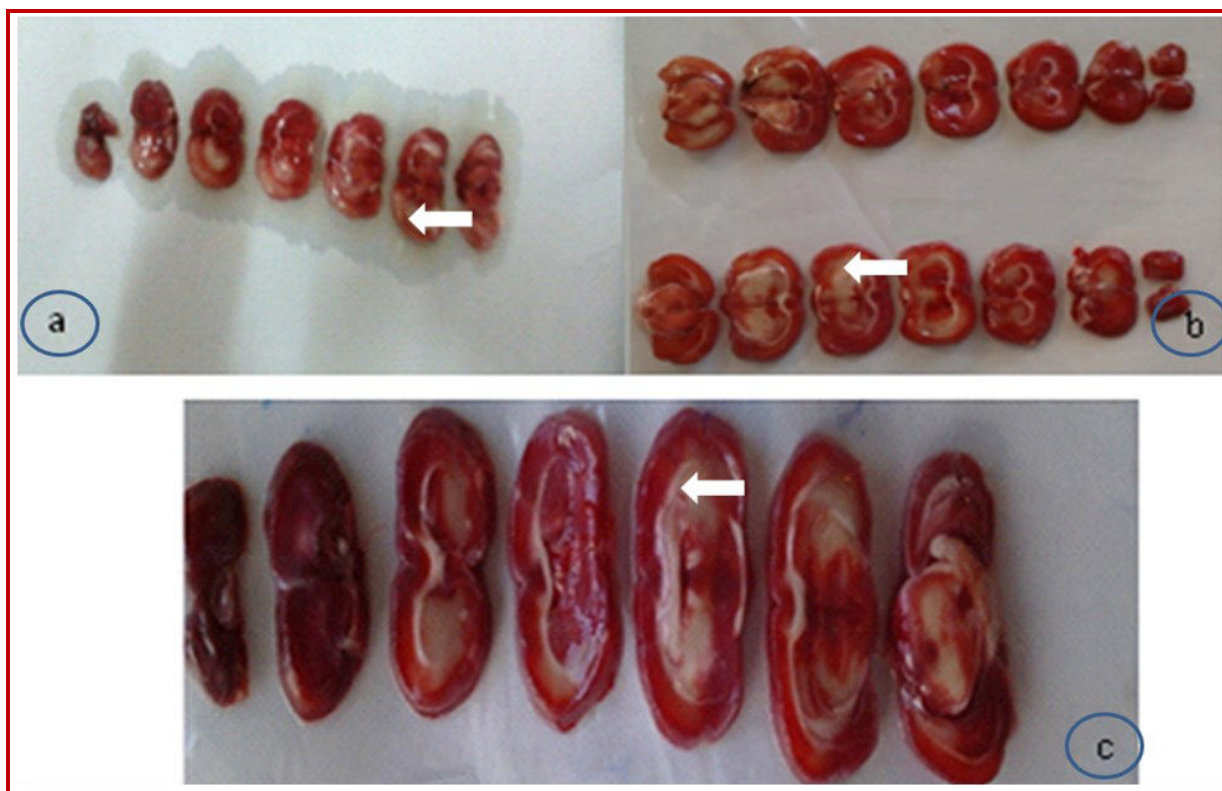


Figure 8: Brain histopathological changes in rats after a 60 min ischemia period. Arrows show infarct volume: (a) control group; (b) ischemia group 2, intraperitoneally injected with 5 mg/kg Kombucha solution; (c) and ischemia group 3, intraperitoneally injected with 15 mg/kg Kombucha solution

endothelial cells through their tight junctions and basal lamina. During cerebral ischemia, endothelial basal lamina dissolution starts as soon as 2 hours after the onset of ischemia and continues during reperfusion and is rapidly followed by an increase in blood-brain barrier permeability (Gasche et al., 2001). Ischemia and reperfusion stresses disrupt BBBs and increase cerebral vascular permeability, leading to the formation of brain edema.

The main source of free radicals generated during ischemia is probably resulted from the mitochondria damage and generated inflammatory responses (Kondo et al., 1997). The brain is protected against these radicals by free radical scavengers such as ascorbate, alpha-tocopherol as well as anti-oxidant enzymes including superoxide dismutase, glutathione peroxidase and catalase (Warner and Batinić Haberle, 2004). Although the expression of these enzymes is increased during ischemia, their capacity is limited to the levels of free radicals (Margaill and Lerouet, 2005). Oxidative stress can directly damage the membrane phospholipids and nucleotides and indirectly does through the mediation of cellular cascades. Thus, several studies have focused on anti-oxidants for the development of effective agents in treatment of stroke and cerebral protection. Anti-oxidants have been developed in many *in vitro* and *in vivo* experiments and some of them have been

investigated in clinical trials (McCarty, 2000).

Due to oxidative stress and inflammation, the main reasons for neuronal damage during ischemia and ineffectiveness of chemical drugs in ischemia treatment, concrete efforts have been focused on the anti-oxidant drugs.

The natural extracts are able to protect neurons from ischemic damage (Massaro et al., 2002). The efficiency of different natural extracts is depending on their bioactive metabolites with anti-oxidant activity, such as polyphenols (Carluccio et al., 2003). Polyphenolic compounds are able to inhibit endothelial dysfunction by reducing the expression of cell adhesion molecules (Carluccio et al., 2003), increasing of nitric oxide production and scavenging the free radicals in vascular endothelium (Moreno, 2003).

The beneficial effects of Kombucha tea stem from its polyphenols, gluconic acid, lactic acid, vitamins, amino acids, antibiotics and other nutrients produced during the fermentation process (Vijayaraghavan et al., 2000).

Bhattacharya et al. (2011) showed that Kombucha tea possesses more potential effectiveness than black tea to ameliorate TBHP-induced oxidative damages and cell death in murine hepatocytes. Ibrahim et al. (2013) showed the protective effect of Kombucha tea on liver

and kidney against oxidative stress caused by cadmium and gamma radiation. In addition, Gharib (2010) showed that Kombucha could improve the damages caused by environmental pollutants such as trichloroethylene.

Conclusion

Kombucha tea has protective effects in ischemia- and reperfusion-induced brain damages which may involve inhibition of oxidative damage and reduction in neuronal cell death. It may be a useful agent for the prevention of cerebral damage.

Ethical Issue

Animal handling manipulation procedures were based on the guidelines of the Animal Welfare Act (Rollin and Kessel, 1998).

Acknowledgement

The authors thank of Islamic University Izeh.

Conflict of Interest

The authors declare that there is no conflict of interest with regard to this work.

References

- Bhattacharya S, Manna P, Gachhui R, Sil PC. Protective effect of kombucha tea against tertiary butyl hydroperoxide induced cytotoxicity and cell death in murine hepatocytes. *Indian J Exp Biol*. 2011; 49: 511-24.
- Block F. Global ischemia and behavioural deficits. *Prog Neurobiol*. 1999; 58: 279-95.
- Carluccio MA, Siculella L, Ancora MA, Massaro M, Scoditti E, Storelli C, Visioli F, Distanto A, De Caterina R. Olive oil and red wine antioxidant polyphenols inhibit endothelial activation: Antiatherogenic properties of Mediterranean diet phytochemicals. *Arterioscler Thromb Vasc Biol*. 2003; 23: 622-29.
- Gasche Y, Copin JC, Sugawara T, Fujimura M, Chan PH. Matrix metalloproteinase inhibition prevents oxidative stress-associated blood-brain barrier disruption after transient focal cerebral ischemia. *J Cereb Blood Flow Metab*. 2001; 21: 1393-400.
- Gharib O. Review article: Does kombucha tea attenuate the hepato-nephrotoxicity induced by a certain environmental pollutant. *Egypt Acad J Biol Sci*. 2010; 2: 11-18.
- Greenwalt C, Ledford R, Steinkraus K. Determination and characterization of the anti-microbial activity of the fermented tea Kombucha. *LWT-Food Sci Technol*. 1998; 31: 291-96.

- Hadjinikolaou L, Kotidis K, Galiñanes M. Relationship between reduced elasticity of extracardiac vessels and left main stem coronary artery disease. *Eur Heart J*. 2004; 25: 508-13.
- Ibrahim NK. Possible protective effect of kombucha tea ferment on cadmium chloride induced liver and kidney damage in irradiated rats. *Int J Biol Life Sci*. 2013; 9: 8-11.
- Kamalakannan K, Balakrishnan V. Antioxidant and hepatoprotective activity of ethanolic and aqueous extracts of *Limonia elephantum* leaves, Bangladesh *J Pharmacol*. 2014; 9: 383-88.
- Kastner RS, Freund T. Selective vulnerability of the hippocampus in brain ischemia. *Neuroscience* 1991; 40: 599-636.
- Kondo T, Reaume AG, Huang TT, Carlson E, Murakami K, Chen SF, Hoffman EK, Scott RW, Epstein CJ, Chan PH. Reduction of CuZn-superoxide dismutase activity exacerbates neuronal cell injury and edema formation after transient focal cerebral ischemia. *J Neurosci*. 1997; 17: 4180-89.
- Lo EH, Dalkara T, Moskowitz MA. Mechanisms, challenges and opportunities in stroke. *Nat Rev Neur*. 2003; 4: 399-414.
- Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke* 1989; 20: 84-91.
- Malbaša RV, Lončar ES, Vitas JS, Čanadanović-Brunet JM. Influence of starter cultures on the anti-oxidant activity of kombucha beverage. *Food Chem*. 2011; 127: 1727-31.
- Margaill I, Plotkine M, Lerouet D. Anti-oxidant strategies in the treatment of stroke. *Free Radic Biol Med*. 2005; 39: 429-43.
- Massaro M, Basta G, Lazzarini G, Carluccio MA, Bosetti F, Solaini G, Visioli F, Paolicchi A, De Caterina R. Quenching of intracellular ROS generation as a mechanism for oleate-induced reduction of endothelial activation and early atherogenesis. *Thromb Haemost*. 2002; 88: 335-44.
- McCarty M. Up-regulation of endothelial nitric oxide activity as a central strategy for prevention of ischemic stroke—Just say NO to stroke! *Med hypotheses*, 2000; 55: 386-403.
- Moreno JJ. Effect of olive oil minor components on oxidative stress and arachidonic acid mobilization and metabolism by macrophages RAW 264.7. *Free Radic Biol Med*. 2003; 35: 1073-81.
- Nussmeier NA. A review of risk factors for adverse neurologic outcome after cardiac surgery. *J Extra Corpor Technol*. 2002; 34: 4-10.
- Pham-Huy LA, He H, Pham-Huy C. Free radicals, antioxidants in disease and health. *Int J Biomedical Sci*. 2008; 4: 89-96.
- Rabiei Z, Bigdeli MR, Rasouljan B, Ghassempour A, Mirzajani F. The neuroprotection effect of pretreatment with olive leaf extract on brain lipidomics in rat stroke model. *Phytomedicine* 2012; 19: 940-46.
- Rollin B, Kessel M. Guidelines for the treatment of animals in behavioural research and teaching. *Anim Behav*. 1998; 55: 251-57.
- Vijayaraghavan R, Singh M, Rao PV, Bhattacharya R, Kumar P, Sugendran K, Kumar O, Pant SC, Singh R. Subacute (90

- days) oral toxicity studies of Kombucha tea. *Biomed Environ Sci.* 2000; 13: 293-99.
- Warner DS, Sheng H, Haberle IB. Oxidants, anti-oxidants and the ischemic brain. *J Exp Biol.* 2004; 207: 3221-31.
- Wells PG, McCallum GP, Lam KC, Henderson JT, Ondovcik SL. Oxidative DNA damage and repair in teratogenesis and neurodevelopmental deficits. *Birth Defects Res Embryo Today.* 2010; 90: 103-09.
- White BC, Sullivan JM, DeGracia DJ, O'Neil BJ, Neumar RW, Grossman LI, Rafols JA, Krause GS. Brain ischemia and reperfusion: Molecular mechanisms of neuronal injury. *J Neur Sci.* 2000; 179: 1-33.
-

Author Info

Mahbubeh Setorki (Principal contact)

e-mail: doctor.setorgi@gmail.com

Your feedback about this paper

1. Number of times you have read this paper
2. Number of times you have seen the video clip
3. Quality of paper
4. Your comments