Beneficial Effects of Piperine in global cerebral ischemia/reperfusion model in irradiated rats

Samar Saad Azab*, Walaa Adel El-Sabbaghb, Mohamed M. Abdel-Daimc, Engy Refaat Rasheda*

*a: Department of Pharmacology and Toxicology, Faculty of Pharmacy, Ain Shams University, Cairo, Egypt
b: Department of Drug Radiation Research, National Center for Radiation Research and Technology, Egyptian Atomic Energy Authority, Cairo, Egypt c: Department of Pharmacology, Faculty of Veterinary Medicine, Suez Canal University, Ismailia Governorate, Egypt

Introduction

Stroke has been reported to affect over 15 million people around the world leading to cerebral damage and chronic disability (Liang et al., 2011). Reperfusion of ischemic tissue may cause systemic injury, leading to multiple organ dysfunctions or even death, re-entrance of blood into tissues causes the release of oxygen free radicals (FRs) and inflammatory mediators, contributing to both local and systemic injuries. These injuries might become obvious, particularly in myocardial, renal, pulmonary and brain tissues (Blaisdell 2002; Harman et al., 2012).

Ischemia/reperfusion (I/R)-induced cerebral injury has been reported as a leading cause of death and long-term disabilities with consequent impact on the quality of life. Among the factors involved in the pathogenesis of I/R injury are glutamate receptor-mediated excitotoxicity, energy failure, lactic acidosis, enhanced formation of free radicals, and cellular calcium homeostasis disturbance (Harukuni and Bhardwaj, 2006; Hicks and Jolkkonen, 2009; Abd El-Fattah et al., 2010). Cerebral ischemia in animal models leads to destruction of the blood–brain barrier (BBB) and edema (Keshavarz and Dehghani, 2017). This, consequently, results in the secretion of pro-inflammatory cytokines, TNF-α, IL-1β, and IL-6 in the ischemic region by activated immune cells, which drive and accelerate additional inflammatory processes via the activation of pro-inflammatory genes expression; such as, cyclooxygenase-2 (COX-2), nitric oxide synthase (NOS-2) and nuclear factor-kappa B (NF-kB) (Tu et al., 2010; Mohammadi et al., 2011). Induction have been described by various studies (Kojima et al., 1997; Leibowitz et al., 2011; Najafi et al., 2016). Radiation-induced
central nervous tissue (CNS) injury is thought to induce specific cytokines recovery/repair in response to radiation-induced oxidative stress (Tofilon and Fike, 2000).

Piper longum L. has been used as a crude drug in folk medicine to improve intestinal disorders, asthma, and poor peripheral blood circulation in Asia. In addition, piperine (1-piperoylpiperidine), the primary lipophilic principle in black pepper (Piper nigrum) and long pepper (Piper longum), has been reported to be effective against metabolic syndrome (Diwan et al., 2001) and displayed an anti-inflammatory activity (Pradeep and Kuttan, 2004). Piperine also possesses potent antidepressant-like properties (Li et al., 2007) and can activate whole-cell currents in rat sensory neurons; this suggests that piperine might be a good pharmacological candidate for acute neurodegenerative diseases (Szallasi 2005).

However, the role of piperine against I/R-induced cellular damage is still not well defined. Therefore, this study was designed to investigate the possible role of piperine in ameliorating the biochemical changes in the brain and serum induced by cerebral I/R in whole body γ-irradiated rats. The study is carried out in order to highlight the probable beneficial effects of the usage of a dietary antioxidant as adjuvant therapy in cancer patients receiving radiotherapy, especially those who have risk factor(s) for stroke; and in order to ameliorate one or more of the side-effects of radiotherapy and/or lower the morbidity induced by stroke.

**Experimental Animals:**
Male albino rats of Wistar strain, weighing 150–200 g were used in this study. They were purchased from the Egyptian organization for biological products and vaccines (Cairo, Egypt) and the animals were maintained at the animal house of the National Center for Radiation Research and Technology, Atomic Energy Authority. Animals were maintained at 25-28 °C with a natural light/dark cycle. Rats were provided with a standard rat pellets (El Nasr co. Egypt) and water ad libitum. All the experimental procedures conducted herein followed the ethics prepared by INSA, Animal Welfare Division of the Ministry of Environment & Forest, Council of International Organization of Medical Sciences (WHO/UNESCO), NIH and PHS. The experimental protocol of the present study has been approved by the ethics committee at Faculty of Pharmacy, Ain Shams University [Committee approval no 8].

**Irradiation process**
Rats were exposed to 6 Gy single dose whole body gamma irradiation. Irradiation was performed using Cesium-137 irradiation unit (Gamma cell-40) produced by the Atomic Energy of Canada Limited, at a dose rate of 0.5 Gy/min.

**Induction of global cerebral ischemia/reperfusion**
Rats were subjected to bilateral carotid artery occlusions for 60 min. using blunt mini clamps under urethane anesthesia (1g/kg; i.p.) (Guedes and de Vasconcelos, 2008) followed by declamping of both arteries to allow reperfusion for another 60 min. Animals temperature was estimated by rectal thermometer and maintained between 37°C and 38°C using heating lamp. Sham operated rats were treated identically without occluding carotid arteries (Vanella et al., 1990).

**Experimental design**
Animals were randomly divided into six experimental groups, eight rats each. Group I; normal sham operated group, the anaesthetized rats were subjected to all the surgical manipulations performed in the ischemic/reperfused groups, except that the occlusion of the common carotid arteries was not carried out. Group II; ischemic/reperfused group; cerebral I/R was induced in this group of rats as detailed above. Group III; irradiated sham operated group; rats were exposed to whole body γ-radiation at a dose level of 6 Gy and sham operated 24 hours later. Group IV; irradiated ischemic/reperfused group, I/R was induced in rats 24 hr following whole body γ-irradiation; Group V; piperine treated irradiated ischemic/reperfused group, rats were treated with piperine (10 mg/kg, orally for 15 days, single dose daily) suspended in corn oil (2 ml/Kg) (Vaibhav et al., 2012), on day 16, rats were irradiated and I/R was induced on day 17. Group VI; vehicle treated control group, rats were treated with corn oil (2 ml/Kg, orally for 15 days, single dose daily), then irradiated and rendered ischemic as detailed for Group V.

**Samples collection**
At the end of the reperfusion period, the animals were sacrificed by decapitation and their skulls were split on ice. The bodies of sacrificed rats were refrigerated till incineration. Serum samples were stored in aliquots at -20°C till use. The separated sera were used for the determination
of lactate dehydrogenase (LDH) activity (Howell et al., 1979) and total antioxidant capacity (TAC) via colorimetric reagent kit (Biodiagnostic, Egypt) (Koracevic et al., 2001).

The whole brain of each animal was separated, the left hemisphere was weighed and homogenized in ice cold normal saline (20% w/v homogenate) and was used for determination of brain contents of malondialdehyde (Yoshioka et al., 1979), reduced glutathione (Beutler et al., 1963) and total protein by colorimetric reagent kits and the supernatant was used for determination of NF-κB using ELISA kit specific for rats (MyBiosource Inc., USA), myeloperoxidase enzyme (MPO) activity using colorimetric activity assay kit (Leinco technologies Co.).

One ml of brain homogenate was mixed with an equal volume of cold tris-ethylenediamine tetracetic acid (EDTA) buffer pH 7.6, centrifuged at 105,000 xg for 15 min at 4°C using SORVALL1 ULTRA 80 ultracentrifuge (DuPont Co., Delaware, USA). The separated cytosolic fraction was used for the determination of cytosolic calcium concentration using the atomic absorption technique (Subramania 1995).

Autopsy samples (5 µm) were taken from the brain of rat in different groups and fixed in 10% formol saline (v/v) for twenty four hours. Washing was carried out using tap water then serial dilutions of alcohols (methyl, 70% ethyl, and absolute ethyl alcohol) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56 °C in hot air oven for twenty four hours. Paraffin/beeswax tissue blocks were prepared for sectioning at 4 microns thickness by sledge microtome. The obtained tissue sections were collected on glass slides, deparaffinized, and stained by hematoxylin & eosin stain for routine examination using a light electric microscope (Bancroft et al., 1996).

Data analysis

Results are expressed as means ±standard error (S.E.). Data were analyzed using one way ANOVA followed by Tukey-Kramer multiple comparison test. The p value was considered significant at p < 0.05. Graphpad prism 6 software was used to carry out these statistical tests.

Results

Results of the current work showed that each of whole body γ-irradiation, cerebral I/R induced a significant increase in serum LDH activity and brain cytosolic Ca²⁺ which amounted to 2- and 18-fold in I/R group and to about 2- and 17- fold in Rad group, as compared to the sham operated group, respectively. Moreover, combination of irradiation and cerebral I/R showed a further significant increase in both serum LDH activity and brain cytosolic Ca²⁺ as compared to the sham operated group amounting to 236% and 2065%, respectively (Figure 1).

On the other hand, an inflammatory response was observed in brain tissues of ischemic, irradiated and ischemic irradiated rats as compared to sham operated ones, which was indicated by a significant increase in MPO activity. NF-κB was observed in each of these groups. MPO activity amounted to 350%, 300% and 420% of the sham operated value, respectively. As for NF-κB expression, it reached 340%, 250% and 360% as compared to the sham operated group, respectively (Figure 2).
Fig. 2. Effect of Piperine on brain myeloperoxidase activity (MPO) (nmol/min/mg prt) (A) and brain nuclear factor-kappa B (NF-kB) (pg/mg prt) (B) in irradiated cerebral ischemic/reperfused male rats. Values are expressed as mean ± S.E.M and significance was ascertained as $P<0.05$; a: significant difference from Sh.O. group, b: significant difference from I/R group, c: significant difference from Rad + I/R group. [Sh.O.: Sham operated group; I/R: ischemic reperfused group; Rad: irradiated group; Rad+ I/R: irradiated ischemic reperfused group].

In line with this, a marked state of oxidative stress has been observed in rat brain as a result of I/R, irradiation and their combination. This state was indicated by the rise in brain MDA content with a concomitant depletion of GSH and TAC. Brain MDA amounted to 260%, 450% and 600% of the sham operated value in I/R, Rad and Rad + I/R groups, respectively. On the other hand, brain GSH content decreased to 51%, 57% and 44% of the sham operated value in I/R, Rad and Rad + I/R groups, respectively. Meanwhile, brain TAC decreased to 70%, 57% and 29% of the sham operated value in I/R, Rad and Rad + I/R groups, respectively (Table 1).

However, pretreatment of rats with piperine for 15 successive days prior to irradiation and induction of I/R resulted in a significant amelioration of brain damage. As for the inflammatory response, piperine induced a significant inhibition of MPO activity and NF-kB expression, their values amounted to 40% and 33% of the Rad + I/R group (Figure 2). In line with this, serum LDH activity and brain cytosolic Ca$^{2+}$ showed a significant decrement in piperine treated group amounting to 51% and 55% of the ischemic irradiated group (Figure 1). Regarding the oxidative stress, piperine induced a decrement in MDA and an increment in each of GSH and TAC, these parameters amounted to about 55%, 180% and 300% of the Rad+ I/R group value, respectively (Table 1).

Histopathological examination of brain tissues revealed that the brain of sham operated rats showed a normal cerebral cortex with normal neuronal cells that appeared rounded with large vesicular nuclei (Fig. 3 a). Meanwhile, brain of Rad group revealed neuronal degeneration and neuronophagia associated with satellitosis (Fig. 3 b) and proliferation of glial cells in addition to congestion of cerebral blood vessels. Brain of I/R rats revealed a wide spread neuronal cell necrosis in which the affected neurons appeared shrunken with intensely eosinophilic cytoplasm and small dark basophilic nuclei associated with neuronophagia of the necrotic neurons (Fig. 3 c) and vacuolation of the neurophils. Neuronophagia and satellitosis as well as focal cerebral hemorrhages were frequently demonstrated in this group. Severe histopathological alterations were demonstrated in Rad+I/R group characterized by eosinophilic neuronal cell necrosis associated with neuronophagia (Fig. 3 d), satellitosis and proliferation of glial cells. A significant improvement of histopathological alterations was observed in Rad+I/R + piperine group characterized by sparse necrotized neuronal cells (Fig. 3 e) with no evidence of cerebral hemorrhage. Brain of vehicle pretreated group revealed an individual neuronal degeneration (Fig. 3 f) associated with proliferation of glial cells.
TABLE 1. Effect of piperine on plasma total antioxidant capacity (TAC) and brain contents of malondialdehyde (MDA) and reduced glutathione (GSH) in cerebral ischemic/reperfused irradiated male rats

<table>
<thead>
<tr>
<th>Parameter Groups</th>
<th>Brain GSH (mg/g wet tissue)</th>
<th>Brain MDA (nmol/mg prt)</th>
<th>Plasma TAC (mmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sh. O.</td>
<td>1.41 ± 0.16</td>
<td>2.41 ± 0.36</td>
<td>47.26 ± 5.37</td>
</tr>
<tr>
<td>I/R</td>
<td>0.72 ± 0.09</td>
<td>6.26 ± 1.48</td>
<td>32.71 ± 2.98</td>
</tr>
<tr>
<td>Rad</td>
<td>0.81 ± 0.06</td>
<td>10.85 ± 1.63</td>
<td>27.29 ± 1.94</td>
</tr>
<tr>
<td>Rad + I/R</td>
<td>0.62 ± 0.07</td>
<td>14.69 ± 2.55</td>
<td>13.73 ± 1.64</td>
</tr>
<tr>
<td>Rad + I/R + Piperine</td>
<td>1.12 ± 0.21</td>
<td>8.10 ± 1.01</td>
<td>40.92 ± 3.78</td>
</tr>
<tr>
<td>Rad + I/R + Corn Oil</td>
<td>0.97 ± 0.14</td>
<td>11.29 ± 2.12</td>
<td>15.92 ± 3.12</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± standard error of mean (SEM), p <0.05; a: significant difference from Sh.O. group, b: significant difference from I/R group, c: significant difference from Rad + I/R group. [Sh.O.: Sham operated group; I/R: ischemic/reperfused group; Rad: Irradiated group; Rad+ I/R: irradiated ischemic/reperfused group].

Fig. 3. Brain of (a) Sham operated group showing normal rounded neuronal cells , (b) Rad group showing a neuronal degeneration and neuronophagia (arrow) associated with satellitosis (arrow head), (c) I/R group showing a neuronal cell necrosis with neuronophagia of the necrotic neurons (arrow), (d) Rad + I/R treated rats showing an eosinophilic neuronal cell necrosis (arrow) associated with neuronophagia (arrow head), (e) Rad + I/R + Piperine group showing sparse necrosed neuronal cells, (f) Rad + I/R + Corn oil group showing an individual neuronal degeneration. (H&E, X400)
Discussion

Ischemic reperfusion model in this study showed an increase in serum LDH activity, brain cytosolic Ca\(^{2+}\), MPO level and NF-κB this was in accordance with the results reported in previous studies (Matsuo et al., 1994; Stephenson et al., 2000; Xu et al., 2014). The ischemia-induced interruption of blood and oxygen supply leads to inhibition of aerobic glucose metabolism with a subsequent accumulation of lactate resulting in increased LDH activity to metabolize the formed lactate (Shaheen et al., 1996). Moreover, cells become dependent on anaerobic glycolysis for their ATP supply leading to an accumulation of lactate, protons, and NAD\(^+\) and, therefore, cause a drop in cytosolic pH. In an attempt to reestablish normal pH, the cell extrudes H\(^+\) ions in exchange for Na\(^+\) via Na\(^+\)/H\(^+\) exchanger (Murphy and Steenbergen, 2008). The Na\(^+\) ions are, in turn, exchanged for Ca\(^{2+}\) by the Na\(^+\)/Ca\(^{2+}\) exchanger. The endoplasmic/sarcoplasmic reticulum (ER/SR) Ca\(^{2+}\) store is also affected during I/R. In particular, Ca\(^{2+}\) reuptake into the ER/SR by the SERCA ATPase is impaired by I/R, whereas Ca\(^{2+}\) release through the ryanodine receptor is enhanced, both of which further exacerbate the lethal elevations in cytosolic Ca\(^{2+}\). These massive alterations in Ca\(^{2+}\) activate a variety of systems, all of which can contribute to cell death following I/R (Szydlowska and Tymianski, 2010; Sanada et al., 2011).

Increased intracellular Ca\(^{2+}\) also leads to the generation of calcium pyrophosphate complexes and the formation of uric acid, both of which belong to a group of “danger signals” that bind to the intracellular protein complexes called inflammasomes. Inflammasomes mediate, an increased production of cytokines, such as IL-1β and TNFα, which, in turn, activates transcription factors; e.g., Nuclear Factor-kappa B (NF-κB) to increase the expression of additional cytokines and chemokines, thereby precipitating a cytokine storm that exacerbates I/R injury (Kalogeris et al., 2012).

In the current work, I/R resulted in a decrease in rat brain total antioxidant capacity and brain GSH level as well as an increase in brain MDA level, reflecting an increase in oxidative stress state. In line with this, I/R-induced hypoxia and diminished energy supply have been reported to result in blood brain barrier rupture, reactive oxygen species (ROS) generation, recruitment of inflammatory molecules, and cell death by necrosis and apoptosis (Kataoka et al., 2004; Yasuda et al., 2011). Also, I/R has been reported to induce a marked enhancement of oxidative stress in a variety of organs (Muñoz-Casares et al., 2006, Abogresha et al., 2016). Mechanistically, reperfusion and reoxygogenation of tissues in the presence of hypoxanthine and xanthine oxidase lead to production of aggressive oxygen radicals (Marian et al., 2007; Yildirim et al., 2009).

Irradiated rats showed an exacerbated cerebral death after I/R as compared to the non-irradiated ones, this was in accordance with the findings of Abd El-Fattah et al. (2010) and Suresh et al. (2015). Irradiation has been proved to induce a neuro-inflammatory response, which spread from the site of injury to relatively remote regions of the central nervous system. Moreover, irradiation induces neural-precursor cell dysfunction by altering the microenvironment that regulates their fate and proliferative capacity. In addition, the remaining of non-irradiated neural precursor cells fail to differentiate into neurons in the irradiated hippocampus (Suresh et al., 2015).

In the present study, the inflammatory response observed in brain tissues following irradiation and ischemia is in accordance with earlier studies' findings which reported elevated levels of IL-1β, IL-6, and TNF-α in ischemic brain and serum. Further, these pro-inflammatory cytokines contents were much higher in ischemic brain tissues than those in serum. This indicates that I/R injury resulted in an elevated level of cytokines in brain and due to breakdown of blood–brain barrier (BBB), cytokines are also secreted into blood. In agreement with this, several studies have also reported that pro-inflammatory cytokines, including TNF-α and IL-1β are elevated in serum in the early phase of acute ischemic stroke (Yasuda et al., 2011; Oto et al., 2008). These molecules recruit more circulating leukocytes and promote accumulation of neutrophils, macrophages, and activated microglia, which infiltrate the ischemic region and lead to a further loss of neuronal cells and brain tissue, thereby possibly enlarging the cerebral infarct area (Chapman et al., 2009; Denes et al., 2010; Emsley et al., 2005; Gong et al., 2014).

In the current study, irradiation of animals affected the brain levels of GSH and MDA due to the oxidative stress which results from exposure to radiation. In addition, γ-irradiation induced more severe biochemical derangements in ischemic reperfused rats as evidenced by the
pronounced increase in both brain LDH activity and cytosolic Ca\(^{2+}\) level and exacerbation of brain oxidative stress status. The rise in enzyme activity in this study could be a result of radiation-induced inhibition of cellular respiration (Nosov et al., 1999) with a subsequent increase in the rate of glycolysis and LDH activity.

Piperine administration to I/R or Rad + I/R irradiated rats in this study showed a significant increase in total antioxidant capacity and reduced GSH level as well as a decrease in brain MDA level. Vaibhav et al. (2012) reported that piperine administration to rats followed by (I/R) injury showed a significantly less infarct area observed in rat brain after I/R injury. Piperine has been demonstrated in both in vivo and in vitro experiments to protect against oxidative damage by quenching free radicals and ROS and inhibiting lipid peroxidation (Yang et al., 2015; Mittal and Gupta, 2000). Selvendiran et al. (2004) investigated the impact of piperine on alterations of the mitochondrial antioxidant system and lipid peroxidation. Oral supplementation of piperine revealed a decrease in the extent of mitochondrial lipid peroxidation and concomitant increase in the activities of enzymatic antioxidants and nonenzymatic antioxidants. They reported that piperine modulates lipid peroxidation and increases the antioxidant defense system. Moreover, Vijayakumar et al. (2004) examined the effect of supplementation of black pepper or piperine on tissue lipid peroxidation and enzymic and non-enzymic antioxidants in rats fed a high-fat diet, and they observed that these spices can reduce high-fat diet-induced oxidative stress. They also observed that simultaneous supplementation with black pepper or piperine lowered thiobarbituric acid reactive substances and conjugated dienes levels and maintained SOD, catalase, GSH, glutathione-S-transferase, and reduced glutathione levels near to those of control rats.

Regarding the anti-inflammatory effect of piperine, observed herein, it is in accordance with the results reported in earlier studies (Kim et al., 2012; El-Ghazaly et al., 2016; Gupta et al., 2015). As for its supposed mechanism of action, piperine has been shown to inhibit the expression of cyclooxygenase-2 (COX-2) a key enzyme in the arachidonic acid (AA) metabolic pathway, resulting in a decreased production of prostaglandins E-2 (PGE-2) in the inflammatory responses (Kim et al., 2012). In line with this, a recent study investigated a possible anti-platelet mechanism of piperine through the modulation of the AA metabolic pathway. They compared the effects of piperine on the liberation of AA and the activities of AA-metabolizing enzymes in collagen-stimulated platelets and LPS-stimulated cells. In addition, piperine has been reported to inhibit collagen- and AA-induced platelet aggregation through the inhibition of their platelet activation cascades (Son et al., 2014).

**Conclusion**

Findings of the present study indicated that piperine supplementation provided promising beneficial effects against most of the biochemical changes induced by I/R in irradiated rat brain and serum. These effects are probably mediated through both its anti-inflammatory and antioxidant actions.

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