



Pomegranate extract protects against cerebral ischemia/reperfusion injury and preserves brain DNA integrity in rats



Maha A.E. Ahmed ^{a,*}, Engy M. El Morsy ^b, Amany A.E. Ahmed ^b

^a Department of Pharmacology and Toxicology, Faculty of Pharmacy, Misr University for Science and Technology (MUST), 6th of October City, Giza, Egypt

^b Department of Pharmacology and Toxicology, Faculty of Pharmacy, Helwan University, Ein Helwan, Helwan, Egypt

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ABSTRACT

Aim: Interruption to blood flow causes ischemia and infarction of brain tissues with consequent neuronal damage and brain dysfunction. Pomegranate extract is well tolerated, and safely consumed all over the world. Interestingly, pomegranate extract has shown remarkable antioxidant and anti-inflammatory effects in experimental models. Many investigators consider natural extracts as novel therapies for neurodegenerative disorders. Therefore, this study was carried out to investigate the protective effects of standardized pomegranate extract against cerebral ischemia/reperfusion-induced brain injury in rats.

Main methods: Adult male albino rats were randomly divided into sham-operated control group, ischemia/reperfusion (I/R) group, and two other groups that received standardized pomegranate extract at two dose levels (250, 500 mg/kg) for 15 days prior to ischemia/reperfusion (PMG250 + I/R, and PMG500 + I/R groups). After I/R or sham operation, all rats were sacrificed and brains were harvested for subsequent biochemical analysis.

Key findings: Results showed reduction in brain contents of MDA (malondialdehyde), and NO (nitric oxide), in addition to enhancement of SOD (superoxide dismutase), GPX (glutathione peroxidase), and GRD (glutathione reductase) activities in rats treated with pomegranate extract prior to cerebral I/R. Moreover, pomegranate extract decreased brain levels of NF- κ B p65 (nuclear factor kappa B p65), TNF- α (tumor necrosis factor-alpha), caspase-3 and increased brain levels of IL-10 (interleukin-10), and cerebral ATP (adenosine triphosphate) production. Comet assay showed less brain DNA (deoxyribonucleic acid) damage in rats protected with pomegranate extract. **Significance:** The present study showed, for the first time, that pre-administration of pomegranate extract to rats, can offer a significant dose-dependent neuroprotective activity against cerebral I/R brain injury and DNA damage via antioxidant, anti-inflammatory, anti-apoptotic and ATP-replenishing effects.

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Introduction

Stroke is a major cause of human disability and mortality (Moskowitz et al., 2010). The brain requires around 25% of constant cardiac output for its metabolic needs. Therefore, any deficiency in cerebral blood flow may cause cerebral ischemia and neurological dysfunctions (Deb et al., 2010). Previous studies showed that redox imbalance, inflammation and apoptosis are the major mechanisms of ischemia/reperfusion (I/R) injury (Ozbal et al., 2008; Yousuf et al., 2009). The high rate of oxidative metabolic activity and low concentrations of endogenous antioxidants cause exceptional vulnerability of the brain to oxidative insults (Chen et al., 2000).

The use of various natural products and herbal extracts to attenuate cerebral ischemia/reperfusion injury has been previously investigated. Experimentally, *Nigella sativa*, *Ginkgo biloba*, and garlic are some

examples of plants that showed protective effects against ischemic brain damage (Hosseinzadeh et al., 2007; Aguilera et al., 2010; Tulsulkar and Shah, 2013).

Punica granatum L. (Punicaceae), commonly known as pomegranate, is a shrub native to the Mediterranean region. The whole pomegranate fruit, its peel, or leaf extracts have powerful antioxidant and anti-liperoxidative activities (Kaur et al., 2006; Iqbal et al., 2008; Zhang et al., 2008). The plant shows several pharmacological effects, such as anti-bacterial (Prashanth et al., 2001), anti-diarrheal (Das et al., 1999), and anti-ulcer activities (Gharzouli et al., 1999). The beneficial activities of pomegranate are attributed to its active constituents mainly polyphenols, flavonoids and tannins (Faria and Calhau, 2011). The whole fruit extract shows superior actions to that of single constituents probably due to a synergistic effect (Jurenka, 2008). The antioxidant activity of pomegranate juice is threefold higher than that of red wine or green tea (Gil et al., 2000). Clinical studies showed that pomegranate juice possesses a higher antioxidant capacity than apple juice (Guo et al., 2008). Maternal consumption of pomegranate juice in the drinking water protected against experimental neonatal hypoxic brain injury

* Corresponding author. Tel.: +20 2 3835486; fax: +20 2 3835499.
E-mail address: mahapharm@yahoo.com (M.A.E. Ahmed).

(Loren et al., 2005). The mechanism of anti-inflammatory activities of pomegranate extract may include suppression of cyclooxygenase, lipoxigenase and matrix metalloproteinase (Schubert et al., 1999; Ahmed et al., 2005). Interestingly, natural compounds are well tolerated and can be consumed in high amounts (Jurenka, 2008). The safety of pomegranate and its constituents have been studied both experimentally and clinically and no evidence was observed for toxic effects on body organs including heart, liver, and kidney (Cerda et al., 2003; Aviram et al., 2004).

Accordingly, the aim of the present study was to examine whether pomegranate extract could protect against cerebral ischemia/reperfusion-induced brain injury in rats, and to investigate the possible underlying mechanisms.

Materials and methods

Chemicals and drugs

Pomegranate extract standardized to 40% ellagic acid was purchased from Holland & Barrett (UK). All other chemical reagents were purchased from Sigma-Aldrich chemical Co. (St. Louis, MO., USA).

Animals

Adult male Wistar rats weighing 230–250 g were housed in plastic cages at a controlled temperature (27 ± 3 °C) with alternating 12 h light and dark cycles. They were allowed standard chow pellets and drinking water ad libitum. The animals were left to accommodate for one week before the experiment. Experiments were performed between 8 and 11 a.m. This work was carried out according to the international guidelines for care and use of laboratory animals, and the experimental protocol was ethically approved by the Animal Care Committee, Faculty of Pharmacy, Helwan University.

Experimental design

Rats were randomly divided into four groups, ten animals each. Rats of the sham-operated control and ischemia/reperfusion (I/R) groups were orally administered 10% tween 80 saline solution by gavage for 15 days. Rats of the PMG250 + I/R or PMG500 + I/R groups received standardized pomegranate extract (250 mg/kg or 500 mg/kg) respectively, dissolved in 10% tween 80 saline solution by oral gavage for 15 days (Rosillo et al., 2012). One hour after the last dose, all rats except those of the sham control group were exposed to 60 min of cerebral ischemia followed by 60 min of reperfusion (Seif-el-Nasr and Fahim, 2001).

Induction of cerebral ischemia/reperfusion (I/R)

Overnight-fasted rats were anesthetized with chloral hydrate (360 mg/kg, i.p.) at the time of the operation. Rats of I/R, PMG250 + I/R, and PMG500 + I/R groups were all subjected to ischemia, where the right and left common carotid arteries were exposed by a midline ventral incision in the neck. The bilateral carotid artery was separated from the adjacent tissues and vagus nerve. Ischemia was induced by bilateral clamping of the common carotid arteries for 60 min. Following cerebral ischemia, the arteries were declamped to restore circulation. The skin was sutured with waxed silk stitches. Reperfusion was allowed for 60 min. Rats of the sham control group were exposed to the same procedure except for carotid occlusion. Rectal temperature was maintained at 37 °C throughout the experiment to prevent cerebral hypothermia (Seif-el-Nasr and Fahim, 2001).

Tissue collection

After I/R or sham operation, all rats were sacrificed by decapitation. The brains were carefully dissected out on ice, washed and homogenized in either ice-cold saline solution for MDA and NO determination, or phosphate buffered saline for all other parameters.

Determination of brain lipid peroxides content measured as malondialdehyde (MDA)

As a major indicator of oxidative stress, lipid peroxides were measured as MDA level in the brain homogenates (Mihara and Uchiyama, 1978). The principle of the assay depends on the reaction of MDA with thiobarbituric acid in acidic medium when incubated for 45 min at 95 °C. The resultant pink color was extracted by n-butanol and the absorbance was measured at 535 and 520 nm by a spectrophotometer. The difference in absorbance was calculated and MDA concentrations were determined from a standard curve.

Determination of brain nitric oxide (NO) content measured as total nitrates/nitrites

According to the method of Miranda et al. (2001), this assay determines total nitric oxide based on the chemical reduction of nitrate to nitrite by vanadium trichloride, followed by the colorimetric detection of nitrite as an azo dye product of Griess reaction at 540 nm. The concentration of NO in samples was determined from a standard curve.

Determination of brain superoxide dismutase (SOD) activity

SOD activity was determined by kit (R&D Systems, Inc., USA), according to the method of Beauchamp and Fridovich (1971). The assay is based on the conversion of NBT to NBT-diformazan by superoxide ions generated from the reaction of xanthine and oxygen catalyzed by xanthine oxidase. SOD reduces superoxide ion concentration, and hinders the appearance of NBT-diformazan. The absorbance was measured at 550 nm. SOD activity in samples was determined from a standard curve.

Determination of brain glutathione peroxidase (GPX) activity

Glutathione peroxidase (GPX) activity was measured by an EnzyChrom assay kit (BioAssay Systems, USA), according to the method of Paglia and Valentine (1967). GPX catalyzes the conversion of reduced glutathione (GSH) and hydrogen peroxide into GSSG and water. Glutathione reductase then converts GSSG into GSH by the aid of NADPH. The assay directly measures NADPH consumption. The decrease in optical density at 340 nm is directly proportional to the enzyme activity in the sample.

Determination of brain glutathione reductase (GRD) activity

Glutathione reductase reduces oxidized glutathione (GSSG) to its reduced form (GSH) which is an important cellular antioxidant. Therefore, measurement of glutathione reductase activity is used as an indicator for oxidative stress. EnzyChrom kit, BioAssay Systems, USA, was used for the assay of GRD activity. Based on Ellman's reaction, GSH generated from the reduction of GSSG by GRD in a sample is used to form a yellow colored product (Delides et al., 1976). The rate of change in the optical density, measured at 412 nm, is directly proportional to GRD activity in the sample.

Determination of brain cytokines (tumor necrosis factor- α and interleukin-10) level

TNF- α , and IL-10 cytokine contents were determined in brain homogenates by rat specific RayBio TNF- α (Bonavida, 1991), and RayBio IL-10 (Braun et al., 1999) ELISA kits (RayBiotech, Inc., USA). The assay procedure was followed as mentioned in the manuals and the absorbance of the yellow color was measured at 450 nm. Concentrations of TNF- α or IL-10 in samples were determined from the corresponding standard curve.

Determination of brain nuclear factor- κ B p65 (NF- κ B p65) level

CUSABIO ELISA kit (PRC) was used to carry out this assay (Bell et al., 1999). Briefly, brain tissues were homogenized in phosphate buffered saline. Cell membranes were broken by two freeze–thaw cycles, followed by centrifugation of the homogenates for 5 min at 5000 \times g at 2–8 °C. The supernatants were removed and assayed immediately. Samples and serial dilutions of the provided standard were added into wells of a microplate pre-coated with NF- κ B p65-specific antibodies. After incubation for 2 h at 37 °C to allow binding of NF- κ B p65 with the immobilized antibody, excess solution was discarded and a biotin-conjugated antibody specific for NF- κ B p65 was added into wells. After aspiration and washing, avidin conjugated horseradish peroxidase was added to wells, followed by another wash to remove any unbound avidin-enzyme reagent. Finally, TMB substrate solution was added into each well and incubated for 30 min at 37 °C, followed by the addition of the provided stop solution. The optical density of the developed color was measured within 5 min using a microplate reader at 450 nm. Samples concentrations of NF- κ B p65 were determined from a plotted standard curve.

Determination of brain caspase-3 level

The level of brain caspase-3 was measured by a USCEN ELISA kit (Life Science Inc., PRC). The finally developed yellow color was measured at 450 nm using a microplate reader (Fernandes-Alnemri et al., 1994). The concentration of caspase-3 in the samples was then determined from a standard curve.

Determination of brain ATP level

Brain ATP level was determined by an ELISA kit (Kamiya Biomedical Company, USA). The intensity of the yellow color was measured at 450 nm (Cailla et al., 1982). The ATP concentration of each sample was determined from a standard curve.

Determination of brain DNA damage by Comet assay (alkaline single-cell microgel electrophoresis)

Comet assay was performed according to the method of Singh et al. (1988). Briefly, 1 g of crushed brain samples was suspended in 1 ml ice-cold PBS, stirred for 5 min, then filtered. 600 μ l of low-melting agarose (0.8% in PBS) was thoroughly mixed with 100 μ l of cell suspension, followed by spreading 100 μ l of the mixture on agarose pre-coated slides. The slides were left to solidify at 4 °C, then they were immersed in chilled lysing solution (1% sodium sarcosinate, 2.5 M NaCl, 100 mM sodium edetate, 1% freshly prepared Triton X-100, and 10 mM Tris–HCl, at pH 10) for 1 h at 4 °C to lyse the cells and to permit DNA unfolding. The slides were then removed and placed in a horizontal electrophoresis chamber, filled with freshly prepared electrophoretic alkaline buffer (1 mM sodium edetate and 300 mM NaOH, pH \geq 13) for 20 min to allow unwinding of DNA. Electrophoresis was conducted for the next 20 min at 25 V and 180 mA. After electrophoresis was completed, the slides were washed gently to get rid of alkali and detergents, by flooding them slowly with 0.4 M Tris–HCl buffer (pH 7.5).

After 5 min, staining the slides was performed by adding 25 μ l of 20 μ g/ml ethidium bromide in distilled water solution at 4 °C on each slide. The DNA fragment migration patterns of 100 cells for each sample were observed with a fluorescence microscope, and images were captured by a Nikon CCD camera. The quantitative and qualitative extent of DNA damage in the cells were estimated using the Comet 5 image analysis software developed by Kinetic Imaging Ltd. (Liverpool, UK) by measuring the percentage of tailed cells (% tailed cells), the percentage of untailed cells (% untailed cells), the percentage of DNA in comet tail (% DNA), and tail length (μ m). Finally, tail moment was calculated by multiplying the tail length and percentage of migrated DNA.

Statistical analysis

Data is expressed as mean \pm S.E.M. One way analysis of variance (ANOVA) and Tukey–Kramer multiple comparisons test were used for statistical evaluation of data. Values of P < 0.05 were considered significant. Statistical analysis was carried out by GraphPad Prism software, Version 3.00 for Windows (San Diego, CA, USA).

Results

Effect of pomegranate extract on total brain lipid peroxides measured as malondialdehyde (MDA) in rats exposed to cerebral ischemia/reperfusion

As shown in Table 1, cerebral ischemia/reperfusion induced a significant increase in brain MDA content by 159% as compared to the sham control group. Pre-administration of pomegranate extract at both doses (250, 500 mg/kg) induced a significant decrease in brain MDA content by 23% and 27% respectively as compared to the I/R group.

Effect of pomegranate extract on brain nitric oxide (NO) content measured as total nitrate/nitrites in rats exposed to cerebral ischemia/reperfusion

As shown in Table 1, brain nitric oxide was significantly elevated by 87% following I/R as compared to the sham control group. Administration of pomegranate extract (250, 500 mg/kg) for 15 days prior to cerebral I/R caused a significant reduction in brain nitric oxide level by 23% and 27% respectively as compared to I/R group.

Effect of pomegranate extract on brain superoxide dismutase (SOD) activity in rats exposed to cerebral ischemia/reperfusion

As shown in Table 1, a significant reduction in brain SOD activity by 62% was observed in cerebral I/R group as compared to control. Pre-administration of pomegranate extract (250, 500 mg/kg) to cerebral I/R-exposed rats caused a significant enhancement of brain SOD activity by 65% and 100% respectively as compared to I/R group.

Effect of pomegranate extract on brain glutathione peroxidase (GPX) activity in rats exposed to cerebral ischemia/reperfusion

As shown in Table 1, brain GPX activity was significantly reduced by 48% following I/R as compared to the sham control group. Administration of pomegranate extract (500 mg/kg) for 15 days prior to cerebral I/R caused a significant elevation in brain GPX activity by 65% as compared to the I/R group.

Effect of pomegranate extract on brain glutathione reductase (GRD) activity in rats exposed to cerebral ischemia/reperfusion

As shown in Table 1, cerebral I/R induced a significant decrease in brain GRD activity by 37% as compared to the sham control group. Pre-administration of pomegranate extract (500 mg/kg) induced a significant increase in brain GRD activity by 41% as compared to the I/R group.

Table 1
Effect of pomegranate extract on brain contents of malondialdehyde (MDA), and nitric oxide (NO), and activities of superoxide dismutase (SOD), glutathione peroxidase (GPX), and glutathione reductase (GRD) in rats exposed to cerebral ischemia/reperfusion.

	Sham control	I/R	PMG250 + I/R	PMG500 + I/R
MDA ($\mu\text{M}/100$ mg wet tissue)	2.57 \pm 0.21	6.65 \pm 0.54 ^{***}	4.96 \pm 0.46 ^{**#}	4.01 \pm 0.32 ^{###}
NO (mM/100 mg wet tissue)	10.62 \pm 0.96	19.89 \pm 1.26 ^{***}	15.36 \pm 1.36 ^{*#}	14.45 \pm 1.07 [#]
SOD (U/100 mg wet tissue)	2.56 \pm 0.22	0.98 \pm 0.09 ^{***}	1.61 \pm 0.13 ^{**#}	1.96 \pm 0.17 ^{###}
GPX (U/100 mg wet tissue)	187.80 \pm 14.64	97.26 \pm 8.00 ^{***}	135.70 \pm 12.80 ^{*#}	160.50 \pm 14.42 ^{##}
GRD (U/100 mg wet tissue)	19.04 \pm 1.39	12.05 \pm 1.11 ^{**}	14.06 \pm 1.24 [*]	16.93 \pm 1.35 [#]

I/R (cerebral ischemia/reperfusion), PMG (oral pomegranate extract 250, 500 mg/kg). Results were represented as mean \pm S.E.M. (n = 10). ANOVA and Tukey's post hoc tests were used for statistical analysis of data.

* Significantly different from sham control group at $P < 0.05$.

** Significantly different from sham control group at $P < 0.01$.

*** Significantly different from sham control group at $P < 0.001$.

Significantly different from I/R group at $P < 0.05$.

Significantly different from I/R group at $P < 0.01$.

Significantly different from I/R group at $P < 0.001$.

Effect of pomegranate extract on brain tumor necrosis factor alpha (TNF- α) level in rats exposed to cerebral ischemia/reperfusion

As illustrated in Fig. 1, brain TNF- α level was significantly elevated by 193% following I/R as compared to the sham control group. Administration of pomegranate extract (250, 500 mg/kg) for 15 days prior to cerebral I/R caused a significant reduction in brain TNF- α level by 26% and 44% respectively as compared to the I/R group.

Effect of pomegranate extract on brain nuclear factor kappa B p65 (NF- κ B p65) level in rats exposed to cerebral ischemia/reperfusion

As demonstrated in Fig. 2, a significant increase in brain NF- κ B p65 content by 96% was observed in the cerebral I/R group as compared to control. Pre-administration of pomegranate extract (250, 500 mg/kg) to cerebral I/R elicited a significant decrease in the brain NF- κ B p65 content by 23% and 30% respectively as compared to the I/R group.

Effect of pomegranate extract on brain interleukin-10 (IL-10) level in rats exposed to cerebral ischemia/reperfusion

As illustrated in Fig. 3, cerebral I/R caused a significant decrease in brain IL-10 level by 41% as compared to the sham control group. Pre-

administration of pomegranate extract (500 mg/kg) induced a significant increase in the brain IL-10 level by 42% as compared to the I/R group.

Effect of pomegranate extract on brain caspase-3 level in rats exposed to cerebral ischemia/reperfusion

As shown in Fig. 4, cerebral ischemia/reperfusion caused a significant enhancement in brain caspase-3 level as compared to the sham control group. Pre-administration of pomegranate extract (250, 500 mg/kg) to cerebral I/R showed a significant decrease in the brain caspase-3 level by 27% and 45% respectively as compared to the I/R group.

Effect of pomegranate extract on brain adenosine triphosphate (ATP) level in rats exposed to cerebral ischemia/reperfusion

As illustrated in Fig. 5, cerebral I/R caused a significant decrease in the brain ATP level by 62% as compared to the sham control group. Pre-administration of pomegranate extract (250, 500 mg/kg) induced a significant increase in the brain ATP level by 71% and 110% as compared to the I/R group.

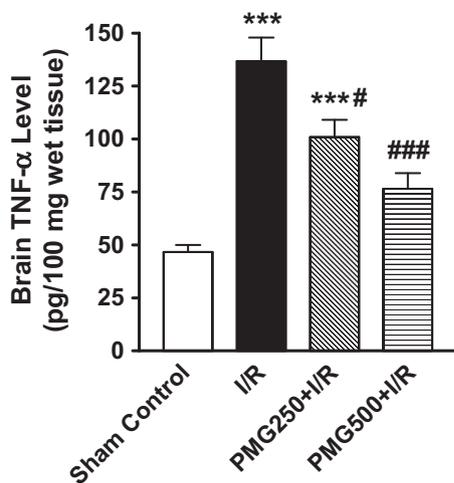


Fig. 1. Effect of pomegranate extract on brain tumor necrosis factor alpha (TNF- α) level in rats exposed to cerebral ischemia/reperfusion. I/R (cerebral ischemia/reperfusion), PMG (oral pomegranate extract 250, 500 mg/kg). Results were represented as mean \pm S.E.M. (n = 10). ANOVA and Tukey's post hoc tests were used for statistical analysis of data. *** Significantly different from sham control group at $P < 0.001$. #, ### Significantly different from I/R group at $P < 0.05$, and $P < 0.001$ respectively.

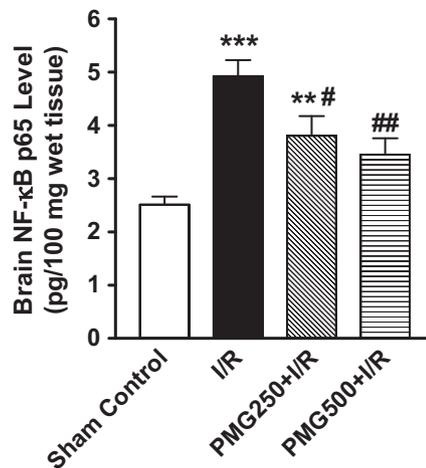


Fig. 2. Effect of pomegranate extract on brain nuclear factor kappa B p65 (NF- κ B p65) level in rats exposed to cerebral ischemia/reperfusion. I/R (cerebral ischemia/reperfusion), PMG (oral pomegranate extract 250, 500 mg/kg). Results were represented as mean \pm S.E.M. (n = 10). ANOVA and Tukey's post hoc tests were used for statistical analysis of data. **, *** significantly different from sham control group at $P < 0.01$, $P < 0.001$ respectively. #, ## significantly different from I/R group at $P < 0.05$, $P < 0.01$ respectively.

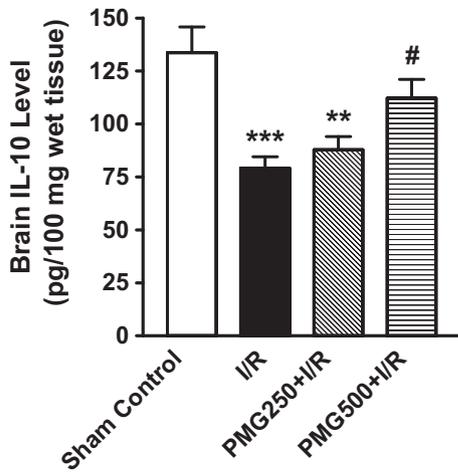


Fig. 3. Effect of pomegranate extract on brain interleukin-10 (IL-10) level in rats exposed to cerebral ischemia/reperfusion.

I/R (cerebral ischemia/reperfusion), PMG (oral pomegranate extract 250, 500 mg/kg). Results were represented as mean \pm S.E.M. ($n = 10$). ANOVA and Tukey's post hoc tests were used for statistical analysis of data. **,*** significantly different from sham control group at $P < 0.01$, $P < 0.001$ respectively. # significantly different from I/R group at $P < 0.05$.

Effect of pomegranate extract on DNA damage parameters as measured by comet assay in rats exposed to cerebral ischemia/reperfusion

As demonstrated in Fig. 6, the cerebral I/R group showed a significant increase in the percentage of tailed cells, tail length, percentage of fragmented DNA in the tail and tail moment by 175%, 140%, 94.7%, and 371% as compared to control. Pre-administration of pomegranate extract (250 mg/kg) to cerebral I/R-exposed rats elicited a significant decrease in the percentage of tailed brain cells, tail length, percentage of fragmented DNA in the tail and tail moment by 31%, 24%, 22%, and 39% respectively as compared to the I/R group. Whereas administration of pomegranate extract (500 mg/kg) to rats prior to cerebral I/R induction significantly reduced the aforementioned parameters by 46%, 38%, 31%, and 57% respectively as compared to the I/R group. On the other

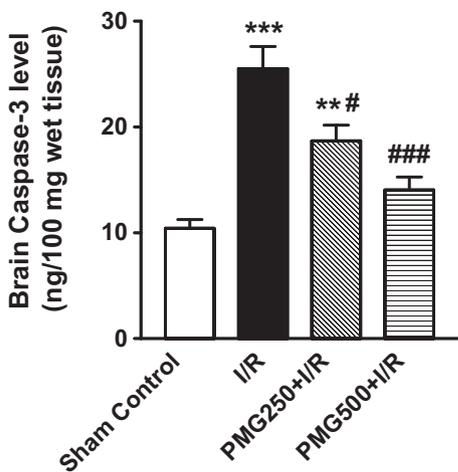


Fig. 4. Effect of pomegranate extract on brain caspase-3 level in rats exposed to cerebral ischemia/reperfusion.

I/R (cerebral ischemia/reperfusion), PMG (oral pomegranate extract 250, 500 mg/kg). Results were represented as mean \pm S.E.M. ($n = 10$). ANOVA and Tukey's post hoc tests were used for statistical analysis of data. **,*** significantly different from sham control group at $P < 0.01$, $P < 0.001$ respectively. #, ### significantly different from I/R group at $P < 0.05$, $P < 0.001$ respectively.

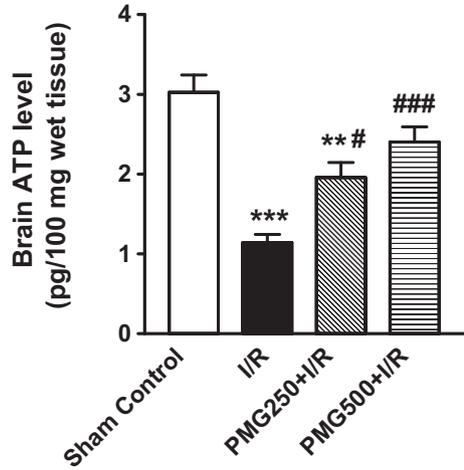


Fig. 5. Effect of pomegranate extract on brain adenosine triphosphate (ATP) level in rats exposed to cerebral ischemia/reperfusion.

I/R (cerebral ischemia/reperfusion), PMG (oral pomegranate extract 250, 500 mg/kg). Results were represented as mean \pm S.E.M. ($n = 10$). ANOVA and Tukey's post hoc tests were used for statistical analysis of data. **,*** significantly different from sham control group at $P < 0.01$, $P < 0.001$ respectively. #, ### significantly different from I/R group at $P < 0.05$, $P < 0.001$ respectively.

hand, a significant decrease in the percentage of untailed brain cells by 11% was shown in the cerebral I/R group as compared to control. Pre-administration of pomegranate extract (250, 500 mg/kg) induced a significant increase in the percentage of untailed cells by 6% and 9% respectively as compared to the I/R group.

Discussion

Cerebral Ischemia/reperfusion (I/R) is characterized by overproduction of reactive oxygen species and exhaustion of brain natural defense systems with consequent neuronal deterioration (Tian et al., 2008). In non-pathologic conditions, a normal balance exists between the generation and detoxification of free radicals by endogenous antioxidants (Schmidley, 1990). By virtue of SOD activity, reactive superoxide anions are converted to hydrogen peroxide, while reactive peroxides can be converted into water and molecular oxygen by catalase and glutathione peroxidase activities. On the other hand, glutathione reductase restores back reduced glutathione from its inactive oxidized form (Li et al., 2008). Sudden bursts of free radicals and reactive oxygen species observed during I/R overcome the capacity of endogenous antioxidants and thereby oxidative species accumulate and attack proteins, lipids and nucleic acids. This leads to lipid peroxidation, cellular membrane damage, impairment of cellular functions and DNA fragmentation (Schmidley, 1990; Thiyagarajan and Sharma, 2004).

MDA is considered the most sensitive marker of lipid peroxidation (Gutteridge, 1995). Kaundal and Sharma (2011) found that global cerebral ischemia/reperfusion is accompanied by enhancement of iNOS. Increased levels of NO can induce metabolic disturbance of the ischemic brain and enhance neuronal damage (Matsui et al., 1999). Nitric oxide can mediate early stages of ischemic cerebral damage (Tang et al., 2010). Moreover, nitric oxide can react with superoxide anions to generate the free radical peroxynitrite resulting in depletion of glutathione and neuronal death (Radi et al., 1991). The high lipid content of the brain and its rather low concentration of endogenous antioxidants render it highly susceptible to oxidative damage (Vatassery, 1998; Manzanero et al., 2013).

In the present study, the protective effects of pomegranate extract against I/R-induced brain damage can be attributed, at least in part, to its ability to reduce the brain levels of malondialdehyde and nitric oxide. The antioxidant properties of pomegranate extract may be mediated by enhancement of antioxidant enzyme activities and by

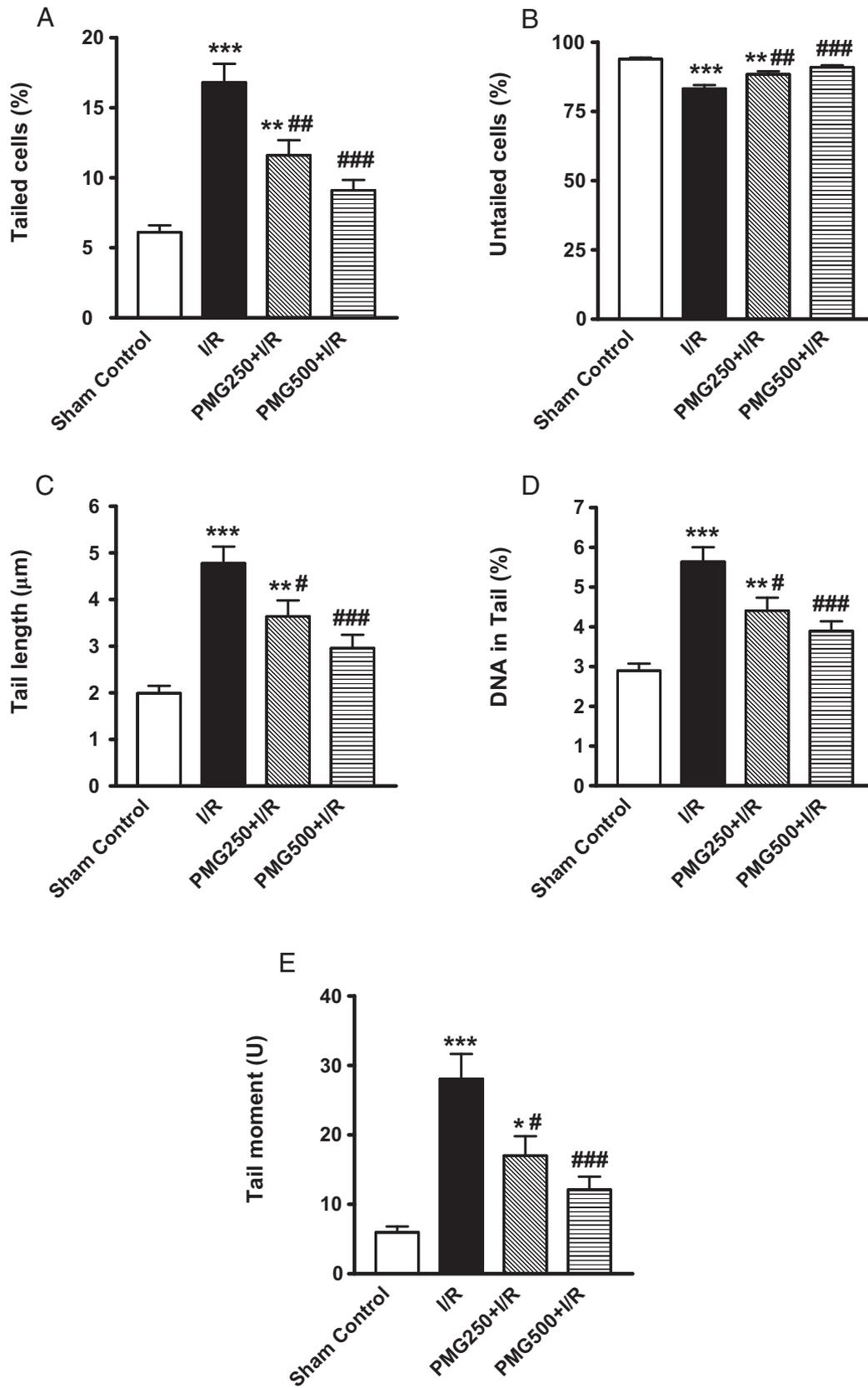


Fig. 6. Effect of pomegranate extract on DNA damage parameters as measured by comet assay in rats exposed to cerebral ischemia/reperfusion. I/R (cerebral ischemia/reperfusion), PMG (oral pomegranate extract 250, 500 mg/kg). Results were represented as mean \pm S.E.M. (n = 10). ANOVA and Tukey's post hoc tests were used for statistical analysis of data. *, **, *** significantly different from sham control group at $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively. #, ##, ### significantly different from I/R group at $P < 0.05$, $P < 0.01$, $P < 0.001$ respectively.

intrinsic free radical scavenging properties. Pomegranate extract contains many antioxidant compounds such as ascorbic acid, vitamin E, polyphenols, tannins, pro-anthocyanidins and flavonoids (Li et al., 2006; Rahimi et al., 2012). Pomegranate ellagitannins include punicalin and punicalagin. The latter can be hydrolyzed to ellagic acid, a natural phenol with powerful antioxidant activity, thus prolonging the release of this acid into the blood (Viladomiu et al., 2013). Interestingly, polyphenols, ellagitannins, flavonoids, and pro-anthocyanidins are absorbed from the intestinal tract into the circulation and are detectable in brain tissues (El Mohsen et al., 2006; Del Rio et al., 2010). Previous studies reported that pomegranate extract showed neuroprotective effects against Alzheimer's disease and depression (Abdel Moneim, 2012; Naveen et al., 2013).

Previous studies have revealed the effectiveness of pomegranate extract in reversing increased levels of MDA and NO (Kaur et al., 2006; Ashoush et al., 2013). These anti-lipoperoxidative and anti-nitrosative properties may be attributed to the remarkable enhancement of glutathione peroxidase, glutathione reductase, superoxide dismutase, and catalase activities in the rat plasma, and tissues (Kaur et al., 2006; Türk et al., 2008; Shaban et al., 2013). The antioxidant activity of pomegranate juice was found three folds higher than that of red wine or green tea (Gil et al., 2000). Rosillo et al. (2012) reported that rats treated with ellagic acid-enriched pomegranate extract showed attenuated colon inflammation due to suppressed iNOS expression.

Similarly, the expression of NF- κ B and inflammatory cytokines in brain tissues were detected after brain ischemia/reperfusion (Chen et al., 2009; Wang et al., 2014). Oxidative stress may activate the redox sensitive transcription factor NF- κ B, with the consequent transcription of pro-inflammatory genes and expression of pro-inflammatory cytokines (Reuter et al., 2010; El Assar et al., 2013). One of the most important cytokines released during ischemia/reperfusion is TNF- α (Yin et al., 2013). This pro-inflammatory cytokine may contribute to the pathogenesis of I/R injury by induction of cell adhesion molecules thereby facilitating leukocyte infiltration, enhancing reactive oxygen species production, and exaggerating the inflammatory response and cerebral damage (Hallenbeck, 2002; Wang et al., 2007; Yin et al., 2013). Lentsch et al. (1999) suggested that TNF- α level does not increase during ischemia but rather just after the start of reperfusion. In a sort of positive feedback, TNF- α enhances NF- κ B activation and thereby amplifies its deleterious effect. Binding of TNF- α to its receptors promotes I κ B serine phosphorylation and releases NF- κ B allowing its translocation to the nucleus, and transcription of various genes including TNF- α gene (Xu et al., 2003). Blockade of TNF- α receptors reduces brain infarct volume and cerebral edema after transient focal ischemia in rats (Hosomi et al., 2005).

Based on the current findings, pomegranate extract appears to exert inhibitory effect on NF- κ B activation and the downstream pro-inflammatory signaling pathway, possibly through its antioxidant effects. Thereby, offering protection against the deleterious effects of I/R and related inflammation. Suppression of TNF- α production may play a role in pomegranate-induced anti-inflammatory activity (Jung et al., 2006; Lee et al., 2008; Ouachrif et al., 2012). In addition, pomegranate extract was found to suppress NF- κ B translocation by inhibition of I κ B kinase, prevention of TNF- α -mediated activation, and induction of NF- κ B inhibitory factor (Bishayee et al., 2013; Adams et al., 2006).

On the other hand, the results of the present study showed that pre-treatment with pomegranate extract increased the brain levels of IL-10. The anti-inflammatory cytokine IL-10 is a crucial mediator in cerebral I/R recovery (Zhang et al., 1994). IL-10 knockout mice suffered from more profound infarctions after focal ischemia (Grilli et al., 2000), while the administration of IL-10 protected against brain ischemic injury and inhibited TNF- α production (Di Santo et al., 1995; Ooboshi et al., 2005). IL-10 decreases tissue inflammation either by attenuating the potency of mononuclear phagocytes to mediate inflammatory responses, by inhibiting secretion of many inflammatory cytokines and chemokines, or by opposing the effects of TNF- α (Schroeter and Jander, 2005; Ziebell and Morganti-Kossmann, 2010).

Similar to the present results, activation of apoptotic mechanisms and controlled neuronal death were previously observed following cerebral I/R (Sugawara et al., 2004). Apoptosis is achieved by a cascade of intracellular cysteine proteases called caspases (Faubel and Edelstein, 2005). Among caspases, caspase-3 is an executional apoptotic effector and has been found to increase soon after forebrain ischemia with a persistent increase after reperfusion (Li et al., 2010). Hippocampal caspase-3 gene expression has been reported after global ischemia (Ni et al., 1998). Activation of caspase-3 leads to cleavage of actin and loss of its ability to inhibit deoxyribonuclease (DNase) activity, with consequent DNA fragmentation (Mashima et al., 1995). Additionally, caspase-3 mediates the cleavage of proteins that are vital for DNA repair and cell stability, causing cell death primarily by apoptosis (Liu et al., 1997; Loetscher et al., 2001). Reactive oxygen species take part in the induction of apoptosis by promoting the activation of caspases leading to nuclear damage (Bratton and Cohen, 2001). Moreover, binding of TNF- α to the death receptor tumor necrosis factor- α receptor 1 (TNFR1) initiates caspase pathway activation (Love, 2003).

In the current study, the dose-dependent reduction in caspase-3 by pomegranate extract may be attributed to the antioxidant and anti-inflammatory activities of the active constituents of the extract. Pacheco-Palencia et al. (2008) reported that pomegranate extract may induce down-regulation of caspase-3 expression. Interestingly, the suppressive effect of maternal pomegranate extract supplementation on caspase-3 activation was observed in neonatal brain hypoxia in mice (Loren et al., 2005; West et al., 2007).

In this piece of work, pomegranate extract opposed cerebral I/R-induced inhibition of brain ATP. Mitochondria are mainly responsible for the production of cellular energy in ATP form. Disrupted blood flow causes oxygen depletion in ischemic tissues, which leads to impairment of mitochondrial oxidative phosphorylation (Sims, 1995). Following reperfusion, a transient mitochondrial recovery is observed followed by a secondary decrease in mitochondrial respiration and cellular ATP production in most brain regions (Blumberg et al., 1997). Excessive reactive oxygen species produced by mitochondria during reperfusion may induce mitochondrial derangement, damage electron transport complexes, impair cellular respiration and inhibit mitochondrial energy metabolism (Blomgren et al., 2003; Niatsetskeya et al., 2012; Li et al., 2014). Mitochondrial damage inhibits metabolic recovery and enhances apoptosis (Krajewski et al., 1999; Ye et al., 2011). During reperfusion rather than ischemia, mitochondrial permeability transition occurs in brain mitochondria, accompanied with mitochondrial swelling, and outer membrane rupture (Takayasu et al., 2007).

The neuroprotective effects of pomegranate polyphenols and ellagic acid may be mediated through the protection of tricarboxylic acid cycle enzymes from free radicals attack, and maintenance of mitochondrial energy production levels (Lee et al., 2010; Choi et al., 2012). Moreover, Ou et al. (2010) reported that ellagic acid can stabilize mitochondrial membranes, and protect against mitochondrial damage by acting as an electron donor.

Comet assay or alkaline single-cell gel electrophoresis assay is used to assess DNA damage (Singh et al., 1988). DNA fragmentation is one of the hallmarks of apoptotic cell death that occurs in response to various stimuli including oxidative stress (Nagata, 2000). Mechanisms leading to DNA fragmentation following ischemia may involve cleavage of chromosomal DNA by caspase-activated DNase (Cao et al., 2001).

In the present study, cerebral I/R caused significant elevation in the percentage of tailed cells, tail length, percentage of DNA in tail and tail moment, as markers of DNA damage. Lipid peroxidation produces reactive genotoxic products such as malondialdehyde, which can bind to and damage DNA (Blair, 2001; Crean et al., 2009). Interactions between DNA and reactive oxygen species produce DNA strand breaks and base modifications (Won et al., 1999). It was reported that free radical scavengers can effectively suppress the increased production of oxidative species and neuronal death caused by cerebral ischemia (Clark et al., 2001; Sharma et al., 2014). Previously, it was reported that

pomegranate juice administered to aged rats prevented blood mononuclear cell DNA damage (Guo et al., 2008). Moreover, in an in-vitro study, Hseu et al. (2012) showed that ellagic acid could protect against UVA-induced DNA damage in human keratinocytes, as assessed by the comet assay, via its antioxidant and anti-apoptotic effects. Noteworthy, the wide therapeutic window of natural compounds makes them highly attractive as novel therapies for the treatment of stroke and other neurodegenerative disorders (Zhao, 2005; Patel et al., 2008; Gupta et al., 2010).

Conclusion

In conclusion, pomegranate extract provides a natural protective tool against ischemia/reperfusion-induced brain injury in rats via antioxidant, anti-inflammatory, anti-apoptotic and ATP replenishing effects. Clinical studies are strongly recommended to evaluate the effectiveness of pomegranate extract in patients prone to stroke.

Conflict of interest

The authors declare that there is no conflict of interest, and that they have contributed equally to each part of this work.

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