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Neuroprotective Effect of Resveratrol Against Brain Ischemia Reperfusion Injury In Rats Entails Reduction of DJ-1 protein expression and Activation of PI3K/Akt/GSK3b survival Pathway

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Abstract:

In the current study, we aimed to investigate the mechanistic role of DJ-1/PI3K/Akt survival pathway in ischemia/reperfusion (I/R) induced cerebral damage and to investigate if the Resveratrol (RES) mediates its ischemic neuroprotection through this pathway. RES administration to sham rats, boosted GSH level and SOD activity and downregulated iNOS expression without affecting redox levels of DJ-1 forms nor components of PI3K/Akt pathway including PTEN, p-Akt or p/ p-GSK3β. However, RES pre-administration to I/R rats, reduced infarction area, oxidative stress, inflammation and apoptosis. Concomitantly, RES ameliorated the decreased levels of oxidized forms of DJ-1 and enhancing its reduction, increased the nuclear protein expression of Nfr-2 and led to activation of PI3K/Akt survival pathway. In conclusion, overoxidation of DJ-1 is a major factor that contributes to post I/R cerebral damage and its reduction by RES could explain the neuroprotection offered by RSE.

Key words: Resveratrol, brain, Ischemia reperfusion, Nrf-2, DJ-1, PI3K/Akt, oxidative stress
INTRODUCTION

Stroke is a disease of serious consequences to human health with high prevalence rate worldwide (Tang et al., 2012). It accounts the third common cause of disability among other diseases (Urban et al., 2010). Thus, searching for the exact mechanism(s) underlying cerebral ischemia injury, and finding accurate effective therapeutic strategies have become an urgent need to prevent neural damage before or after the onset of ischemic brain injury (Urban et al., 2010).

Although rapid reperfusion is a critical practice for the treatment of unexpected cerebral ischemic incidents, the occurrence of post-perfusion lesions is usually associated with exacerbation of brain injury in what is called ischemia/Reperfusion (I/R) injury (Eltzschig and Eckle, 2011). The well reported key players in the pathophysiology of I/R injury are oxidative stress, inflammation and apoptosis (Doyle et al., 2008; Broughton et al., 2009; de Vries et al., 2013). Hence, free radical ablation and reduction of inflammation as well as neural cell apoptosis for the prevention of post-ischemic tissue injury were confirmed to be useful in preventing I/R injury induced neural death (Broughton et al., 2009).

Within the cells, the major compartments are well equipped with various antioxidant molecules and enzymes to detoxify free radicals and maintain a balance between generation and removal of oxidative species (Ryter et al., 2007). These include glutathione system, superoxide dismutase (SOD) and catalase (CAT) (Fernandes and Holmgren, 2004; Lu and Holmgren, 2014).

However, during the past years, parkinson protein 7 (PARK7)/DJ-1 (DJ-1) has also been emerged in brain oxidative science as an important antioxidant regulator and cell survival molecule which is activated under various oxidative stress insults (Taira et al., 2004; Clements et al., 2006). Interestingly, in the brain ischemic models, DJ-1
acts intracellularly by means of paracrine and/or autocrine cues to coordinate extracellular signaling between neighboring neuronal cells to afford the neuroprotection (Kaneko et al., 2014a,b).

Indeed, DJ-1 acts as a stabilizer and regulator of Nuclear (erythroid-derived-2) like 2 factor (Nrf-2), a redox-sensitive transcription factor, which acts as a master regulator of antioxidant and detoxifying genes (Clements et al., 2006). Also, DJ-1 enhances cell survival through the binding of Cezanne, a negative regulator of NF-kappaB (McNally et al., 2011).

Moreover, DJ-1 controls various signaling pathways and can interacts with different signaling molecules. In this regards, DJ-1 has been reported to be a negative regulator of tumor suppressor proteins including P53 (Fan et al., 2008). Also, DJ-1 negatively regulates phosphatase and tensin homolog (PTEN) (McNally et al., 2011) which acts as a negative regulator of phophoinositide3-kinase (PI3K)/Akt signaling survival pathway (Kim et al., 2005).

DJ-1 has three cysteines located at amino acid numbers 46, 53, and 106. Although mild oxidation of C106 is necessary to activate DJ-1 (Canet-Aviles et al., 2004; Zhou et al., 2006), further over-oxidation of C106 is thought to render DJ-1 inactive and preventing from exerting its antioxidant effect (Ishikawa et al., 2009). Such effect is well described in patients with the sporadic form of Parkinson's and Alzheimer's diseases (Bandopadhyay et al., 2004; Choi et al., 2006).

Recently, several dietary supplements are widely used in the prevention of life-style related diseases including cerebral ischemia. Resveratrol (RES), is a naturally occurring plant phenol known as phytoalexins (Santos et al., 2013). RES is found in various plants, nuts, fruits, grapes and red wine, A growing body of evidences confirmed RES to act as a potent neuroprotective agent against cerebral ischemia.
induced injury (Ren et al., 2011; Zhou et al., 2014). Reports suggested that the cerebroprotective action of RES could be mediated by both its antioxidant and antiinflammatory effects (Orsu et al., 2013). The beneficial effects of against cerebral I/R injury are mediated by several mechanisms including, but not limited, to decreased oxidative stress and inflammatory markers like isoprostane, tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6), myeloperoxidase (MPO), and intercellular adhesion molecule-1 (ICAM-1) as well as increased antioxidative and anti-inflammatory markers like superoxide dismutase (SOD), catalase (CAT), and interleukin 10 (IL-10) (Santos et al., 2013; Orsu et al., 2013; Toth et al., 2014).

However, the expression pattern of DJ-1 and the changes of its oxidized/reduced forms during or after focal cerebral I/R injury are lacking in literature. Also, to best of our knowledge, the regulatory effect of RES on DJ-1/PI3K/Akt survival pathway after cerebral I/R injury is completely unknown. Hence, it was worthy to investigate these issues which are the core objectives in our study.
EXPERIMENTAL PROCEDURES

Drugs

Resveratrol (RES) powder (C14H12O3, Cat No. R5010) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was prepared by dissolving in a saline solution (0.9% NaCl) of 20% hydroxypropyl cyclodextrin (American Maize-Products Co., Hammond, IN, USA) to the desired final volume used in the experimental procedure (20mg/5ml).

Animals

Adult male Wistar rats (weighing 230 ± 10 g) were supplied by the animal house facility at King Khalid University, Abha, Saudi Arabia. The Animal Care and Use Committee of the King Khalid University approved all the experimental protocols which were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1978). All animals were housed in groups of 4 rats per cage in a temperature controlled environment (23 ± 1°C; 45%-50% relative humidity; fixed 12/12 h light/dark cycle, lights on at 08:00 h). Food and water were supplied ad libitum.

Experimental design

Rats were divided into four groups (n=18/group) as follows: 1) sham group, 2) sham+RES treated group, 3) I/R non-treated group and 4) I/R+RES treated group. RES was administered orally to desired treated groups (2&4) at a final neuroprotective dose of 20mg/kg as previously reported by Orsu et al. (2013). However, the other groups (1&3) received equivalent volume of normal saline. All treatments were administered to all rats for 30 consecutive days, on daily basis, one day before induction of focal cerebral I/R injury.

Induction of focal I/R injury
Brain focal ischemia was induced by middle cerebral artery occlusion (MCAO) in rats according to the method published by Belayev et al. (1996). In brief, rats were anesthetized with sodium pentobarbital (25 mg/kg, i.p.), ketamine (44 mg/kg, i.m.), xylazine (6.77 mg/kg, i.m.) and atropine (0.02633 mg/kg, i.m.). Body temperature was maintained by means of a heating pad at 37°C and monitored continuously with a rectal probe. Under an operating dissecting microscope, the external and internal right carotid arteries were exposed through a neck incision (1 cm). After tying the external carotid artery, a silicone-coated nylon filament (diameter: 0.37 mm, Doccal Corporation, Redlands, CA, USA) was then inserted into the external carotid artery and gently advanced into the internal carotid artery, approximately 18 mm from the carotid bifurcation, until mild resistance was felt, thereby indicating occlusion of the origin of the middle cerebral artery in the Willis circle. Then, the suture was tightened around the intra-luminal filament to prevent bleeding. To allow reperfusion, the nylon filament was removed, 90 minutes after MCAO. The sham group went under a similar procedure without inserting the filament into the internal carotid artery. The animals were allowed to awaken and were kept in their cages with free access to food and water for 24 hours.

**Brain water contents**

Twenty four hours after MCAO, rats from all groups (n=6) were killed by cervical dislocation after being anesthetized with sodium pentobarbital (75 mg/kg, i.p.). Their brains were dissected out. The pons and olfactory bulb were removed from them, quickly. The brains were weighted to obtain their wet weight (ww) and then dried at 110°C for 24 h for determining their dry weight (dw). Brain water content was calculated using the following formula: \([(ww-dw)/ww] \times 100\), as previously used as an index for brain edema (Vakili et al., 2005).
Evaluation of Infarction with TTC

Six rats from each group injected i.p. with sodium pentobarbital (40 mg/kg). Then, their brains were removed rapidly and frozen in -20°C for 30 min. Brains were dissected, and slices (2 mm thick) were acquired from frozen forebrains using a rodent brain matrix slicer. Brain slices were then stained with 2% 2,3,5-Triphenyltetrazolium chloride (TTC, Cat. No. T8877, Sigma-Aldrich, St. Louis, MO, USA) at 37°C for 20 min in dark. Normal brain tissues were stained red while the unstained or white area was considered to be the infarct area. Areas of red and white staining were measured in photographs obtained using a computer color multimedia image analysis system (Image J 1.46R, NIH, USA). The percent of infarction (infarction volume/brain) was calculated by summing all individual infarct area/slice which were calculated using the following equation: % infarct area/slice = infarct area/total area of slice × 100 (Cheng et al., 2012). Data were presented as mean ± SD of the sum of all infarcted areas of all slices.

TUNEL staining:

TUNEL staining was done to small parts of ischemic cortices using an apoptosis detection kit according to the manufacturer’s instructions (Cat. No. 11 684 817 910, Roche Diagnostics GmbH, Mannheim, Germany). In this test, the strong labeled TUNEL-positive cells were considered apoptotic, whereas lightly stained cells suggest necrosis and were not evaluated.
**Tissue collection:**

Part of the cerebral cortices of the remaining 6 rats in each group were homogenized in an appropriate buffer and used for the biochemical analysis, RT-PCR and immunoblotting. Before cortex collection, animals were perfused with 0.9% (w/v) saline containing heparin (1 U/ml). In brief, individual anesthetized rat (sodium pentobarbital, 75 mg/kg; i.p.) was placed and secure in a supine position on a heated cage for 5-10 minutes. A skin incision along the thoracic midline beneath the xiphoid process to the clavicle and 2 additional skin incisions from the xiphoid process along the base of the ventral ribcage laterally were made to expose the thoracic field completely. A cut through the thoracic musculature and ribcage between the breastbone and medial rib insertion points was made and extended rostrally to the level of the clavicles. The diaphragm was separated from the chest wall on both sides with scissor cuts. The reflected ribcage was pinned with 18G needles laterally to expose the heart. The pericardial sac was grasped gently with blunt forceps and the beating heart was secured with blunt forceps and a 1-2 mm incision was made in the left ventricle. Then, a 24G X 25.4 mm animal feeding needle (Harvard apparatus Cat. #52-4009) was inserted and threaded into the base of the aortic arch using a dissecting microscope. The needle base was clamped to the left ventricle above the incision site using a hemostat and the right atrium was immediately cut and at the first sign of blood flow, the infusion of heparinized saline started until the animal was completely free of blood. Then, the skull was opened and sections of the maximal infarction areas were quickly removed, washed with ice cold phosphate buffer saline (PBS) and stored at -80°C to be used later for biochemical determination, RT-PCR and immunoblotting as discussed below.

**Oxidative stress and lipid peroxidation determination:**
For oxidative stress and lipid peroxidation studies, frozen samples were thawed and homogenized in ice cold PBS with 5 mM betahydroxytoluene (BHT) at a dilution of 10:1 and then centrifuged at 800 × g for 5 min at 4°C to obtain supernatant which was stored and used later for the biochemical analysis. Lipid peroxidation in the cerebral homogenates were measured by the Thiobarbituric Acid (TBA) reaction using commercial kits (Cat No. NWK-MDA01, NWLSS, USA). Superoxide dismutase (SOD) activities in the homogenates were measured using a commercial kit (Cat. No. 706002, Cayman Chemical, Ann Arbor, MI, USA). All measurements and analysis were done as per kit manufacturer’s instructions.

**Measurement of GSH and GSSG**

Reduced glutathione (GSH) and Glutathione disulfide (GSSG) concentrations were measured in the frozen cerebral cortices of all groups using a commercial kit supplied by Cayman Chemical (Cat. No. 703002). This kit utilizes an optimized enzymatic glutathione reductase (GR) recycling method for quantification of GSH. For an individual reaction, 100 μl of tissue homogenate was added to an equal volume of the metaphosphoric acid and then centrifuged at 2000 × g for 2 min to remove protein. Then, 50 μl of 4 M triethanolamine was added for each milliliter of homogenate to neutralize the pH (7.4). For total GSH assay, 50 μl of sample was added to 150 μl of a reaction mixture containing 0.4 M 2-(N-morpholino) ethane-sulfonic acid, 0.1 M phosphate (pH 6.0), 2 mM EDTA, 0.24 mM NADPH, 0.1 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), and 0.1 unit GR. The reaction was carried out at 37°C for 25 min, and then total glutathione was determined by absorbance at 412 nm. To determine levels of GSSG, GSH was removed from the reaction by adding 10 μl of 1 M 2-vinylpyridine solution for each milliliter of homogenate. Then, the remaining GSSG in the reaction was quantified using GSSG as standard as. Each sample was
assessed in duplicates and the amount of reduced GSH was calculated according to this equation: GSH (μmol/g tissue)=total GSH-GSSG. The ratio of GSH/GSSG was used to indicate redox status that inferences the detoxification capacity.

**Estimation of Cytokine and intercellular adhesive molecules:**

Special ELISA kits to measure levels of cerebral macrophage inflammatory protein-2 (MIP-2, Cat. No. KRC1021, life technology, USA), murine homologue keratinocyte-derived chemokine (KC, Cat. No. KMC1061, invitrogen, USA), IL-1β (Cat. No. ab100768, Abcam) and intercellular cell adhesion molecule-1 (ICAM-1, Cat. No. ab100763, USA ) were used. All tests were done in accordance to the manufacturer’s instructions.

**Semi quantitative RT-PCR**

The procedure was optimized for semi quantitative detection using the primer pairs and conditions described in Table 1. Sequences of PCR primers used for the detection of DJ-1, Nrf-2, caspase 3, iNOS and Bcl-2 associated X protein (BAX) and were designed to cover all transcripts of the gene (if any) using BLAST, ensemble and clustal omega online software. β-actin was used as loading control. Total RNA was extracted from the frozen parts of cerebral cortices (30 mg) using an RNeasy Mini Kit (Qiagen Pty. Ltd., Victoria, Australia) according to manufacturer’s instructions. RNA purity was estimated by the 260/280 nm absorbance ratio. Single-strand cDNA synthesis was performed as follows: 30 μl of reverse transcription mixture contained 1 μg of DNase I pre-treated total RNA, 0.75 μg of oligo d(T) primer, 6 μl of 5x RT buffer, 10 mM dithiothreitol, 0.5 mM deoxynucleotides, 50 U of RNase inhibitor, and 240 U of reverse transcriptase (Invitrogen, USA). The reverse transcription (RT) reaction was carried out at 40°C for 70 min, followed by heat inactivation at 95°C for
3 min. The tested genes and the internal control (β-actin) were amplified by PCR using 2 μl RT reaction products from each sample in a 20 μl reaction containing Taq polymerase (0.01 U/ml), dNTPs (100 mM), MgCl₂ (1.5 mM), and buffer (50 mM Tris-HCl). PCR reactions consisted of a first denaturing cycle at 97°C for 5 min, followed by amplification cycles, consisting of denaturation at 96°C for 30 sec, annealing for 30 sec, and extension at 72°C for 1 min. A final extension cycle of 72°C for 15 min was included. Annealing temperature was adjusted as: 60°C for DJ-1, Nrf-2 and caspase 3, 62°C for iNOS and 55°C for both BAX, and β-actin. A control reaction without reverse transcriptase was included for every sample of RNA isolated to verify the absence of contamination and similar internal control was run on each gel. PCR products (10 μl) were electrophoresed on 2% agarose gels containing 100 ng/ml ethidium bromide and photographed with a Polaroid camera under ultraviolet illumination and intensities of resulted bands were evaluated by Image J software (1.46R, NIH, USA).

**Nuclear protein extraction:**

Nuclear protein extraction was done according to the established method of Zaho et al. (2006). In brief, parts the cerebral cortices of the maximum infarct area were homogenized in ice-cold hypotonic lysis buffer (10 mmol/L HEPES, pH 7.9; 10 mmol/L KCl, 1.5 mmol/L MgCl₂, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, and 1 mmol/L dithiothreitol) to which protease and phosphatase inhibitor were added. The mixtures were centrifuged at 4°C and the pellets were washed twice with lysis buffer and resuspended again in an ice-cold, hypertonic, nuclear extract buffer (20 mmol/L HEPES, pH 7.9; 420 mmol/L NaCl, 1.5 mmol/L MgCl₂, 1 mmol/L EDTA, 1 mmol/L EGTA, and 1 mmol/L dithiothreitol containing proteinase inhibitors). After centrifugation at 4°C, protein concentration in supernatant (nuclear extracts) was
determined by the Bradford method and used later for Nrf-2 protein immunoblotting in a procedure similar to the non reducing western blot mentioned below.

**Reducing and non reducing Western blot:**

For non reducing western blot analysis, total proteins of the frozen ischemic brain tissues were extracted with 1 ml RIPA buffer (150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS and 50 mM Tris, pH 8.0) to which protease and phosphatase inhibitor were added. The protein concentrations in this whole cell lysates were measured by Bradford assay (Bradford, M.M. (1976)). all samples were diluted in the loading buffer and heated at 95°C for 5 min and were then subjected to electrophoresis on 10% SDS-PAGE gel at 100 V for 2 h (60µg protein/well). After electrophoresis, and proteins transferring to nitrocellulose membranes, the membranes were rinsed briefly in Tris-buffered saline containing 0.05% Tween (TBST) buffer, blocked in blocking buffer (5% milk and 0.5% BSA) for 1 h, and washed three times with TBST buffer. Then, the membranes were incubated with different primary monoclonal and polyclonal antibodies against PTEN, phospho-PTEN (Ser380/Thr382/Thr383,1:1000, Cat. No. 9569), phospho-Akt (Ser473, 1:1000, Cat. No. 9271) and phospho-GSK3b (Ser9,1 :1000, Cat. No. 9336) (Cell Signaling Technology) for 2 h and then washed and reacted with corresponding secondary horseradish peroxidase–conjugated antibodies (Cell Signaling Technology) for other 2 h. Antigen-antibody complexes were then visualized using an Pierce ECL kit (Thermofisher, USA, Piscataway, NJ) photographed and bands intensities were quantified using Image J software (1.46R, NIH, USA). All experiments performed in triplicate to represent the mean ± SD of all data. A similar procedure was followed for blotting Nrf-2 in nuclear protein fraction.
(30µg of loading protein) using polyclonal anti Nrf-2 Antibody (H-300, Cat. No. sc-13032) diluted at a final concentration of 1:1000.

For reduced western blot, after homogenization, proteins from maximum infarcted area of all groups were precipitated with ice-cold 10%, (w/v) trichloroacetic acid (TCA) and were kept in ice for 1 h. samples were then centrifuged at 21,000 × g for 15 min to collect pellets which were washed then with acetone (−20 °C) and then centrifuged at 21,000 × g for 10 min, again. The pellets were solubilized in labeling buffer (670 mM Tris-HCl (pH 7.5), 2% SDS, 1 mM EDTA, and 10 mM AMS (Invitrogen) and incubated at 37°C for 1 h. The alkylation agent AMS reacts with –SH groups and adds ~500 Da in the molecular weight. DJ-1 redox forms were separated by 17 % polyacrylamide gel electrophoresis with non-reducing loading buffer (Go and Johns, 2009) as mentioned above in the non-reducing part and were blotted using antibodies against DJ-1 (Cell Signaling Technology, Danvers, MA, USA).

**Statistical Analysis**

Statistical analysis was performed using Graphpad Prism statistical software package (version 6). Data was presented as means with standard deviation (mean ±SD). Normality and homogeneity of the data were confirmed before ANOVA. Differences among the experimental groups were assessed by one-way ANOVA, followed by Tukey’s test.

**RESULTS:**

**Mortality rate:**

The mortality rate was very similar between both sham and sham+RES groups and 1 rats died in each group on the next day after induction of I/R injury and was mainly
due to bacterial infection. However, 25% (6/24) were died in the I/R group. RES administration to I/R group decreased the mortality rate to about 8.3 % (1/24) after I/R induction. Hence, based on the number of animals remained in I/R group, 18 animals from each group were selected randomly to be included in the current study and were further subdivided into 6 rats/various analysis as described before.

**Brain Infarction volume and water content:**

Twelve rats animals per group were sacrificed and the brains were removed for infarction analysis by TTC staining and for determination of water content, 24 h after induction of I/R injury (6/each analysis, Figure 1). The size of infarction was calculated as percentages of infarction volume. Brain water content was determined as difference between fresh and dry weights. While no signs of infarction and no significant difference with normal percentages of water content (about 69%) were seen in the sham groups received vehicle or RES, all animals underwent I/R injury developed prominent infarction in the ischemic cortex (27.6% of total area) and showed 43% increase in their brain water content as compared to sham operated group. On the other hand, infarction volume and brain water content were significantly reduced (8.6% and 20%, respectively) in the I/R rats pre-treated with RES for 30 days, as compared to I/R induced group.

**Oxidative Stress Markers**

As shown in Figure 2, significant decrease in the levels of Malondialdehyde (MDA, 33.3%) with concomitant increases in the activity of superoxide dismutase (SOD, 26.2%) and in total levels of intracellular glutathione (tGSH, 19.2%) were seen in the cerebral homogenates of sham group pre-treated with RES as compared to sham group received the vehicle. Associated with the increasesGSH levels, significant increases in both GSH (24.7%) and GSSG (20.8%) were seen in sham
group treated with RES as compared to sham operated received the vehicle. However, ratio of GSSG/GSH was significantly decreased in this group of rats. A massive increase in the levels of MDA (200%) with a concomitant massive decreases in the activities of SOD (43.6%) and levels of tGSH (37.6%), GSH (42.9%) and GSSG (18.2%) were seen in cerebral cortices homogenates obtained from I/R induced group as compared to sham operated group. This was associated with a significant reduction in the ratio of GSSG/GSH. On the other hand, RES pre-administration to I/R rats antagonized all changes in these parameters and reversed them toward their normal values as compared to I/R induced group. In this group of rats, even that ANOVA analysis revealed normal ratio of GSSG/GSH, all the other parameters were significantly higher than their corresponding levels seen I/R group but significantly lower than their levels obtained in the sham operated group.

**Levels of Inflammatory cytokines and ICAM-1.**

As shown in Table 2, the levels of macrophage inflammatory protein-2 (MIP-2), murine homologue keratinocyte-derived chemokine (KC), IL-1β and intercellular cell adhesion molecule-1 (ICAM-1) in Cerebral cortices homogenates were measured. No significant changes in the levels of these markers were seen between the sham operated group pre-treated with either vehicle or RES. However, levels of MIP-2, KC, IL-1β, and ICAM-1 were significantly elevated in the cerebral homogenates obtained from I/R rats by 86.2%, 198%, and 146.3% and 267.6%, respectively. On the other hand, significant decreases in the levels of these inflammatory markers were seen in the cerebral homogenates obtained from I/R rats pre-treated with RES compared with the I/R induced group. Interesting, RES pre-administration prior the induction of cerebral I/R injury completely ameliorated the levels of all these parameters and their
levels were not significantly different from their corresponding values measured in the sham operated group.

**Immunohistochemical detection of apoptosis**

Apoptosis in the infarct area was evaluated using immunohistochemical TUNEL staining test as shown in Figure 3. In sham operated group received vehicle or RES, apoptotic TUNEL test positive cell were almost absent. TUNEL-positive cells were abundant throughout the infarct areas of rats cerebral cortices obtained from I/R group. On the other hand, fewer TUNEL-positive cells were seen in the infract cortices obtained from I/R induced rats pre-treated with RES as compared to I/R induced group of rats.

**Levels of mRNAs expression**

Figure 4 shows the mRNAs levels of DJ-1, caspase 3, inducible nitric oxide synthase (iNOS) and Bcl-2 associated X protein (BAX) and Figure 8 (A) shows mRNA levels of Nrf-2 in the cerebral cortices obtained from all groups of rats as measured by RT-PCR. All tested transcripts were detected and resulted in fragments similar in size to those expected. The levels of the β-actin transcript remained relatively constant in all groups. In the sham operated groups or RES pre-treated sham group, levels of expressions of DJ-1, Nrf-2, caspase-3 and BAX mRNA were not significantly different. However, iNOS mRNA levels were significantly lower (48.2%) after administration of RES suggesting a regulatory inhibitory role of RES on iNOS synthesis. On the other hand, it is noteworthy that the significant increases in mRNA levels of DJ-1 (5 folds), (Nrf-2 (3.3 folds), iNOS (7 folds), BAX (6.4 folds) and caspase 3 (3.4 folds) were detected in the cerebral cortices obtained from I/R induced group of rats. Except for Nrf-2, which remained non significantly different from its
level detected in I/R group, all mRNA of these genes returned to their normal expression levels detected in sham operated group in the cerebral I/R induced group pre-administered RES.

**Protein expression:**

Non reduced western blot was used to measure the expression levels of PTEN, Phospho-PTEN (p-TEN), Akt, phospho-Akt (p-Akt), GSK3b and phospho-GSK3b (p-GSK3b) (**Figure 5-7, respectively**) and to measure nuclear expression of Nrf-2 (**Figure 8, B**). Non reducing western blot was used to differentiate and measure the levels of oxidized and reduced forms of DJ-1 in the infarcted cerebral area of all groups of rats (**Figure 9**). Internal control was used to normalized the readings among different blots. In this study, we couldn’t detect levels of p-PTEN in all tested samples, so data were not presented. However, RES pre administration to sham operated rats didn’t affect the expression of the above mentioned proteins and their levels were not significantly different from those blotted from the control group. However, significant higher levels of nuclear Nrf-2 were seen in sham groups pre-treated with RES. On the other hand, Higher significant level of PTEN with concomitant significant decreases in the levels of p-Akt and p-GSK3b as well as higher significant levels of oxidized DJ-1 and a significant decrease in the nuclear levels of Nrf-2 were seen in the infarct areas of I/R induced group of rats. Ratio of reduced to oxidized DJ-1 form was significantly reduced after I/R injury. On the other hand, RES pre-administration for 30 days to rats prior I/R induction resulted in normal levels of these proteins and reversed the ratio of reduced to oxidized DJ-1 to its normal level. Also, the maximum increases in the nuclear Nrf-2 levels were seen in this group of rats. ANOVA analysis showed normal levels of these proteins with their corresponding blotted in the sham operated rats.
DISCUSSION

The key novel finding of the current study is that post I/R cerebral infarction is associated with over-oxidation of DJ-1, a master upstream antioxidant transcription factor and cell signalling regulator, resulting in exaggerated oxidative damage and down-regulation of PI3K/Akt survival pathway. RES by direct its antioxidant potential antagonized ROS generation and enhanced expression of reduced form of DJ-1, thus activating survival pathway and reduced the damaging effects of cerebral post I/R injury.

For the first time, to our knowledge, RES at a dose of 20mg/kg suppressed post-ischemic brain -edema, 24 h after focal I/R injury. This is a very interesting evidence that shows, an anti--edematous and a protective effects of RES against blood-brain barrier damage after I/R injury in rats. In the same line, similar anti--edematous plant derived substances have been reported in literature to protect against I/R injury by compromising of blood-brain barrier. For example, in a very recent study, Vakili et al. (2014;2015) have shown an anti--edematous effect of Crocin against global cerebral ischemia induced blood-brain barrier damage in rats which could somewhat support the results of our study.

On the other hand, I/R injury is associated with exacerbation of redox imbalance, inflammation and apoptosis of major cells found in the infarcted areas (Ozbal et al., 2008; Yousuf et al., 2009). Similar to our findings reported in this study, reduced antioxidants capacity can lead to oxidative stress mediated brain injury, after I/R in small rodents (Alkan et al., 2008; Oztürke et al., 2008; Zheng et al., 2010). Most importantly, in the presence of superoxides radicals (O$_2^-$), enhanced expression of iNOS can exaggerate the production of peroxynitrite (ONOO$^-$) which has been found to be a main factor behind the depletion in glutathione levels after I/R injury. This
may explain the decreased GSH levels seen in our study which is mainly due to rapid consumption (Radi et al., 1991; Samdani et al., 1997; Tang et al., 2012). However, the effect of I/R injury on GSH synthesis pathway can't be expected based in this study as we couldn’t measure the expression levels of the enzymes involves in GSH synthesis.

Also, significant elevations of KC, MIP-2, ICAM-1 and IL-1β in the cerebral homogenates of I/R induced rats seen in this study account for the aggravated neutrophilia and cortical neutrophil accumulation and such effect has been recognized as an important factor in the development and progression of I/R injury (Mattson et al., 2001; Doyle et al., 2008). Indeed, neutrophils infiltration may promote the conversion of vulnerable yet viable cortical tissue to infarction through microvessel obstruction/thrombosis that compromises reperfusion (Ritter et al., 2000), production of ROS (Aizawa et al., 2006), and release of matrix metalloproteinases that degrade components of the vascular basement membrane and extracellular matrix (Gidday et al., 2006).

In the presence of sustained inflammation and oxidative stress, apoptosis is the predominant mechanism of neuronal cell death at later time points after I/R injury which promotes the gradual expansion of the ischemic lesion (Doyle et al., 2008; Broughton et al., 2009). To assess apoptotic processes after focal cerebral ischemia, we used biochemical analysis, TUNEL-assay and expression levels of BAX and caspase-3, all of which were reported previously as reliable enough to identify ROS generation and apoptotic cells (Huppertz et al., 1999). Interestingly, mRNA expression levels of caspase-3 and BAX were significantly higher in I/R rats and this was coincided with TUNEL positive apoptotic test. Such findings were previously
reported in rats after I/R injury (Choi et al., 2007; Xing et al., 2008; Li et al., 2010; Li et al., 2012; Yao et al., 2012).

Taken together, these data suggest that oxidative stress, inflammation and apoptosis are the key players in brain I/R induced brain damage and any agent that can ablate free radical generation and reduce inflammation is proposed to reduced the infarction area and protect against apoptosis. In the current study, RES antagonized oxidative stress, inflammation and a apoptosis seen in I/R rats and significantly reduced the infarcted cerebral area. Given the results of the control group treated with RES, RES enhanced SOD activity and GSH levels and lower the mRNA expression of iNOS without affecting levels of cytokines neither expression of BAX nor caspase 3 in the infract hemisphere. Such effects of RES have been also reported by many other authors who have shown an upregulatory effect of RES on cellular enzymatic and non enzymatic antioxidant systems (Wang et al., 2002; Robb et al., 2008; Simao et al., 2012). From these data, it is clearly obvious that antioxidant potential of RES as well as inhibition of iNOS synthesis, rather than its chemical antioxidant activity per se, underlies its antioxidant, anti-inflammatory and anti-apoptotic capacity.

On the other hand, DJ-1 acts to induce the expression of several antioxidant enzymes through translocation activation of the master transcription factor (Zhou and Freed, 2005; Clements et al., 2006). However, reduced/oxidized ratio of DJ-1 form is essential for its activity. While mild oxidation of Cystein 106 is necessary for DJ-1 to activate it (Canet-Aviles et al., 2004), further oxidation of C106 is thought to render DJ-1 inactive (Ishikawa et al., 2009; Zhou et al., 2006).

In the current study, concomitant with the decreased levels of endogenous antioxidants in rats cerebral homogenates after I/R induction, mRNA levels of DJ-1 and Nrf-2 were significantly increased suggesting activation of the defense
mechanism, However, coincided with this, significant decreases in protein levels of nuclear Nrf-2 fraction and total reduced form of DJ-1 were seen in cerebral cortex homogenates of I/R rats. Since reduction of DJ-1 has been found essential for the translocation of Nrf-2 to nucleus, we could suggest a novel role of ROS induced oxidized DJ-1 in the pathological process of cerebral I/R injury and reduced cellular antioxidant capacity through mediation of Nrf-2 translocation. However, such conclusion can’t be confirmed from this current western blot findings and mobility shift assay of this transcriptional factor should be evaluated to support our findings and suggestion. Supporting to these findings, RES administration to I/R rats didn’t affect mRNA levels of Nrf-2 and similar levels were seen as those seen in I/R group. Interestingly, reduced DJ-1 oxidation and enhanced reduced forms with a concomitant higher increase in nuclear levels of Nrf-2 and significant elevations of SOD and GSH were seen in the cerebral cortices of I/R pre-administered RES. Again as we said, further studies to evaluate nuclear translocation of Nrf-2 are required.

On the other hand, PI3K/Akt regulates the survival response against oxidative stress associated neuronal apoptosis (Hong et al. 2001; Manning and Cantley 2007). In the CNS, phosphorylation of Akt (Ser473) is required for its activation to act as a neuroprotector by promoting cell survival and suppresses apoptosis (Zhao et al. 2005; Zhang et al. 2007). An effect that is achieved by inhibition of several downstream substrates, including GSK3b (Cross et al. 1995; Hetman et al. 2000). The biological effects of Akt are determined by the balance between the activity of PI3K and PTEN (Torres and Pulido 2001). Dephosphorylation of PTEN increases PTEN activity and reduces PIP3 availability leading to dephosphorylation of Akt (Torres and Pulido 2001; Zhu et al. 2006). In this regards, in the CNS, PTEN has been demonstrated to
act as an important mediator of ROS production and of mitochondria dependent apoptosis (Zhu et al. 2006).

Interestingly, several studies have reported that DJ-1 protects against ROS damage by activating the PI3K/Akt pathway through binding inhibition with PTEN by (Kim et al., 2005; Kim et al., 2009). Hence, it was worthy to investigate the role of DJ-1/PI3K/Akt pathway in I/R injury and to investigate the regulatory role of RES on such pathway. In the current investigation, with the parallel increased in oxidized forms of DJ-1, there was significant increase in the levels of PTEN protein expression, ultimately resulted in the decrease expression levels of both p-Akt and p-GSK3b. However, with increased protein levels of DJ-1 reduced form, RES treatment reverses this situation and resulted in decreased levels of PTEN and enhanced levels of both p-Akt and p-GSK3b. Unfortunately, even if we ran the test, we couldn’t detect the expression levels of p-PTEN. However, based on the available data, overexpression of PTEN increased the sensitivity of hippocampal neurons to excitotoxicity (Gary and Mattson 2002), whereas knockdown PTEN or pharmacological down-regulation of its phosphorylation protected brain tissue from ischemic damage (Ning et al. 2004; Hong et al. 2006).

Together, these data indicate that inhibition of DJ-1 could be one of the main factor exaggerating tissue damage after I/R injury. On the other hand, RES pre-treatment, through reduction of ROS generation via enhancing antioxidants levels and downregulation of iNOS, ameliorated reduced to oxidized levels of DJ-1, resulting in its activation again. Such effect was associated with inhibition of PTEN activity and activation of PI3K/Akt survival pathway. However, the limitations in the current study are that cerebral homogenates was used to evaluate antioxidants levels and cerebral sections were used for immunoblotting evaluation of protein levels, both of
which contain various cell types normally fund in brain tissue. Hence, further studies including neural cell lines and knockdown experiments should be carried out to validate our findings.

**In conclusion**, Our data are the first to describe that oxidative neurodegeneration after focal cerebral I/R injury is associated with over-oxidation of DJ-1 and inhibition of PI3K survival pathway. RES seems to act as allosteric modulator of the reduced forms of DJ-1 due to its highly antioxidant properties.
Conflict of Interests
The authors declare that they have no competing interests.

Role of The Funding Source
The authors declare that this is self funded project

Authors’ Contribution
Ghada A. Abdel-Aleem, PhD, is the principle investigator for the study providing and contributed by writing the proposal, measurements of biochemical parameters, running all RT-PCR and western blot procedures preparation, assisted in immunohistochemical analysis, data collection and assisted in writing the paper.
Eman F. Khaleel, MD, assisted in writing the proposal, biochemical measurements and RT-PCR, data collection and analysis, writing the paper. Dalia G. Mostafa, MD, assisted in immunohistochemical analysis, measurement of some biochemical parameters, data collection and analysis and writing the paper. Lydia K. Elberier, MSc degree assisted in evaluation of the infarction area, writing the manuscript.

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REFERENCES


Table 1. Primers and conditions used in PCR reactions.

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer sequence (5′ to 3′)</th>
<th>AT (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DJ-1</td>
<td>Sense 5′CTGGCTAAAGGAGCAGAGGA3′, Antisense 5′ATCTTCAAGGCTGGCATCAG3′</td>
<td>60</td>
</tr>
<tr>
<td>Nrf-2</td>
<td>Sense 5′CCATGCCTTCTTCCACGAA3′, Antisense 5′AGGGCCCATGGATTTTCAGTT3′</td>
<td>60</td>
</tr>
<tr>
<td>Bax</td>
<td>Sense 5′GGTTGCCCTCTTTCTACTTT3′, Antisense 5′AGCCACCCCTGGTCTTG3′</td>
<td>55</td>
</tr>
<tr>
<td>iNOS</td>
<td>Sense 5′ACAACGTGGAGAAAACCCCAGGTG3′, Antisense 5′ACAGCTCCGGGCATCGAAGACC3′</td>
<td>62</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>Sense 5′GGTATTGAGACAGACAGTGG3′, Antisense 5′CATGGGATCTGTTTCTTTGC3′</td>
<td>60</td>
</tr>
<tr>
<td>β-actin</td>
<td>Sense 5′CGTTGACATCCGTAAAGAC-3, Antisense 5′TAGGAGCCAGGCAGTA-3</td>
<td>55</td>
</tr>
</tbody>
</table>
### Table 2: Levels of some inflammatory mediators in the control and experimental groups of rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>ICAM-1 (pg/ml)</th>
<th>IL-1β (pg/mg protein)</th>
<th>MIP-2 (pg/mg protein)</th>
<th>KC (pg/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>1358.3±221.7</td>
<td>8.56±1.87</td>
<td>12.12±2.12</td>
<td>100.12±12.42</td>
</tr>
<tr>
<td>Sham+RES</td>
<td>1543±267.7</td>
<td>8.45±1.82</td>
<td>11.54±1.66</td>
<td>93.4±13.45</td>
</tr>
<tr>
<td>I/R</td>
<td>5476±436.3</td>
<td>21.23±2.34</td>
<td>22.34±3.87</td>
<td>298±22.45</td>
</tr>
<tr>
<td>RES+I/R</td>
<td>1887±275.5</td>
<td>10.01±2.14</td>
<td>15.12±3.12</td>
<td>132±19.53</td>
</tr>
</tbody>
</table>

Values (mean ± SD) were obtained for each group of 6 animals.  

- **a** significantly different when compared to control group.  
- **b** significantly different when compared to Sham+RES group.  
- **c** significantly different when compared to I/R group ICAM-1: intracellular cell adhesive molecule, KC: keratinocyte-derived chemokine, MIP-2: macrophage inflammatory protein-2 and RES:Resveratrol.
Figure legends:

Fig. 1. Evaluation of cerebral infarction and total brain water content in all groups of rats 24 hours after induction of I/R injury. A: Photomicrographs obtained from slices of the area of maximum infarction. B: Volume of infarction calculated as = infarct area/total area of slice × 100 infarction per slice. Data were presented as mean + SD of the sum of all infarcted areas of all slices for 6 animals. C: total water content calculated as (wet Weight - dry weight). Data were presented as mean + SD for 6 animals. All analysis were considered significant at P<0.05. a significantly different when compared to Sham operated group. b significantly different when compared to Sham+RES group. c significantly different when compared to I/R.
Fig. 2. Levels of oxidative stress in maximum infarcted area obtained from the cerebral cortices homogenates of all groups of rats. Values (mean ± SD) were obtained for each group of 6 animals. a significantly different when compared to Sham operated group. b significantly different when compared to Sham+RES group. c significantly different when compared to I/R group. MDA: Malondialdehyde SOD: superoxide dismutase. tGSH: total glutathione. GSH reduced form of glutathione and GSSG: oxidized form of glutathione.
Fig. 3. Immunohistochemical staining for TUNEL-positive cells in the cerebral cortices in all groups of rats at the expected site of infarction. Strongly labeled deep brown cells were considered apoptotic TUNEL-positive cells. A: Sham operated group. B: Sham +RES treated group. C: I/R induced group and D: I/R+RES treated group. 400x.
Fig. 4. RT-PCR amplification products of DJ-1 mRNAs expression, (A), inducible nitric oxide synthase (B), caspase 3 (C) and BAX (D) in the maximum infarcted area or its corresponding area in cerebral cortices of all groups of rats. Analysis was performed using Image J software (1.46R, NIH, USA). All samples were run in duplicate and all gels were calibrated by an internal control sample. Values (mean ± SD) were obtained for each group of 6 animals. \(^a\)significantly different when compared to Sham operated group. \(^b\)significantly different when compared to Sham+RES group. \(^c\)significantly different when compared to I/R group.
Figure 5. Non reducing western blot protein levels of phosphatase and tensin homolog (PTEN) in the maximum infarcted areas or its corresponding area in the cerebral cortex of all groups of rats. All samples were applied at a final concentration of 60 µg/well. All samples were run in duplicate and all gels were calibrated by an internal control sample. Values (mean ± SD) were obtained for each group of 6 animals.  

- significantly different when compared to Sham operated group.  
- significantly different when compared to Sham+RES group.  
- significantly different when compared to I/R group.
Figure 6. Non reducing western blot protein levels of total Akt and P-Akt in the maximum infarcted areas of the cerebral cortex or its corresponding area of all groups of rats. All samples were applied at a final concentration of 60 µg/well. All samples were run in duplicate and all gels were calibrated by an internal control sample. Values (mean ± SD) were obtained for each group of 6 animals. a signifi-
cantly different when compared to Sham operated group. b significantly different when compared to Sham+RES group. c significantly different when compared to I/R group.
Figure 7. Non reducing western blot protein levels of GSK3b and p-GSK3b in the maximum infracted areas of the cerebral cortex of in all groups of rats. All samples were applied at a final concentration of 60 µg/well. All samples were run in duplicate and all gels were calibrated by an internal control sample. Values (mean ± SD) were obtained for each group of 6 animals. a significantly different when compared to Sham operated group. b significantly different when compared to Sham+RES group. c significantly different when compared to I/R group.
Figure 8. mRNAs (A) and nuclear protein (B) levels of Nrf-2 as measured by RT-PCR amplification and non reducing western blot, respectively. For western blot, all samples were applied at a final concentration of 30 µg/well. All products were within expected levels. Values (mean ± SD) were obtained for each group of 6 animals. *significantly different when compared to Sham operated group. †significantly different when compared to Sham+RES group. ‡significantly different when compared to I/R group.
Figure 9. Reduced western blot protein levels of reduced and oxidized forms of DJ-1 in the infarcted area of the cerebral cortex of in all groups of rats. All samples were applied at a final concentration of 60 µg/well. All samples were run in duplicate and all gels were calibrated by an internal control sample. Values (mean ± SD) were obtained for each group of 6 animals: a significantly different when compared to Sham operated group. b significantly different when compared to Sham+RES group. c significantly different when compared to I/R group.