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RESEARCH ARTICLE

Hot Pepper Extract Protects against Hypoglycemia-Induced Brain and Liver Injury in Mice

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ABSTRACT | This study aimed to investigate the potential neuroprotective effect of red hot pepper (*Capsicum annuum*) extract in hypoglycemic injury in mice. Hypoglycemia was induced by intraperitoneal (ip) injection of insulin (3 IU/kg) followed 30 min later by ip administration of the pepper extract at 0.25 and 0.5 g/kg. Mice were euthanized 3 h after insulin injection and their brains were dissected out for biochemical studies including determination of malondialdehyde (MDA), reduced glutathione (GSH), nitric oxide, paraoxonase-1 (PON-1), 5-lipoxygenase, and cholinesterase. Histological investigations for the brain and liver, and brain immunohistochemistry (glial fibrillary acidic protein; GFAP) were carried out. Results indicated that hypoglycemia significantly increased MDA and nitric acid levels and decreased GSH content in the brain. There was also significant inhibition of PON-1 and cholinesterase activities, but increased brain 5-lipoxygenase. Spongiform degeneration, vacuolations, and necrotic and apoptotic neurons were seen in the cerebral cortex. Glial cells were markedly decreased both in number and size with decreased GFAP staining. Additionally, necrosis of hepatocytes, and cytoplasmic vacuoles were observed. The biochemical alterations induced by hypoglycemia in the brain tissue were alleviated by the pepper extract resulting in decreased MDA and nitric oxide levels, restoration of GSH content, increased PON-1 and cholinesterase activities, and inhibition of 5-lipoxygenase. The pepper extract reduced the extent of damage to cortical neurons and increased the number, size, and length of astrocytes processes and GFAP expression. In addition, there was marked improvement in pathological changes induced by hypoglycemia in the liver by administering the pepper extract. The study indicates a protective effect for the hot pepper extract against brain and liver damage induced by hypoglycemia likely via inhibition of oxidative stress and 5-lipoxygenase.

KEYWORDS | *Capsicum annuum*; Glial fibrillary acidic protein; Hepatic injury; Hot pepper; Hypoglycemia; Insulin; 5-Lipoxygenase; Neuronal injury; Oxidative stress

ABBREVIATIONS | BChE, butyrylcholinesterase; GFAP, glial fibrillary acidic protein; GSH, reduced glutathione; H&E, hematoxylin and eosin; HPLC, high performance liquid chromatography; ip, intraperitoneal; MDA, malondialdehyde; PON-1, paraoxonase-1

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CONTENTS

1. INTRODUCTION

Capsicum fruits, peppers or paprika (family *Solanaceae*), are one of the most popular vegetables and spices in different parts of the world [1]. Out of the five known *Capsicum* species, *Capsicum annuum* is the most widely cultivated today [2]. Both sweet and hot varieties of peppers are common ingredients in human diet, though hot green or red peppers are far more widely used in some cuisines in spaghetti, soups, and Mexican-style dishes because of their flavoring and pungent properties [3, 4]. The highest intakes of hot peppers were reported to be 3 g/person/day in India [5] and 9 g/person/day in Korea [6]. Hot peppers owe their pungency to the presence of capsaicin, and dihydrocapsaicin [7] and peppers contain up to 1.5% of these pungent principles [8]. The main pungent principle is capsaicin, a derivative of nonenamide (8-methyl-*N*-vanillyl-6-nonenamide) [9]. Capsaicin levels in most peppers vary between 0.1 and 1% [10, 11].

Peppers, whether sweet or hot, are also rich in vitamin C and phytochemicals such as lutein, β-carotene, and capsanthin, and the flavonoid anthocyanins, which account for the green, yellow, orange, or red colors of ripe peppers [12, 13]. The carotenoids and flavonoids in peppers are endued with antioxidant effects [14–17] and thus are considered beneficial nutraceuticals for the prevention of aging-related neurodegenerative diseases [18, 19]. In preclinical studies, treatment with red hot pepper extract has been shown to exert neuroprotective effects in experimental Parkinson's disease by alleviating brain oxidative stress and neuroinflammation (e.g., 5-lipoxygenase activation) [20]. Moreover, in an experimental Alzheimer's disease induced by amyloid β infusion into the rat hippocampus, feeding with moderate and high potency red peppers inhibited

brain accumulation of amyloid β and improved the deficits in memory [21].

Insulin-induced hypoglycemia is a complication arising in patients with type I diabetics and poses serious neurological consequences including seizures and coma [22]. Depriving the brain of its energy substrate (i.e., glucose) can damage neurons, and rats subjected to severe hypoglycemia showed neuronal death and impaired memory performance [23]. One important mechanism that underlies the development of neuronal damage during insulin-induced hypoglycemia is oxidative stress [24, 25]. The brain is particularly vulnerable to oxidative burden. This is because of the high oxygen utilization by the brain and the consequent increase in the generation of reactive oxygen metabolites by the mitochondrial respiratory chain. Other sources of free radicals in the brain include the autoxidation of monoamine neurotransmitters. The high content of polyunsaturated fatty acids coupled with the paucity of brain antioxidant makes the brain tissue a preferred target for free radicals [26, 27]. It follows that supplementation of antioxidants would be important to keep the redox balance in the brain and to protect against reactive oxygen and nitrogen metabolites and other free radicals. In view of the popularity of consuming hot peppers in human diet and their antioxidant properties, the present study was to investigate the potential neuroprotective effect of red hot pepper extract in mice subjected to insulin-induced hypoglycemia.

2. MATERIALS AND METHODS

2.1. Animals

Swiss male albino mice (25–30 g in body weight) from the breeding colony at the National Research Center (Cairo, Egypt) were used in the study. Standard laboratory food and water were provided ad libitum. The study was performed in accordance with the recommendations of the Ethics Committee of the Institution and that of the U.S. National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.2. Drugs and Chemicals

Mixtard® 30 insulin (Novo Nordisk, France), consisting of human insulin soluble and human insulin isophane insulin (70/30), was used and diluted in 0.9% saline to obtain the necessary dose. The *Capsicum* fruits (hot red pepper) were purchased from local market in Giza province, Egypt. *Capsicum* fruits (100 g) were cut into ~2 cm lengths, dried, and grounded. Extraction was carried out with 70% methanol at room temperature. The extract was evaporated under reduced pressure and lyophilized.

2.3. HPLC Analysis of Capsaicin

Capsaicin was quantified with HPLC (high performance liquid chromatography). After extraction of the material, the extract was dissolved in deionized water. Both the mobile phase and the dissolved materials were filtered using a Millex-HX Nylon syringe filter (0.45 µm, 25 mm; Millipore, Bedford, MA, USA).The materials are subjected to chromatographic analysis with HPLC reverse phase with the following specifications: Shimadzu SCL-10Avp System controller, dual pump Shimadzu liquid chromatography (LC-10Avp), Shimadzu degasser (DGU-14A), Rheodyne manual injector, Shimadzu UV-Vis detector (SPD-10Avp), and a phenomenex RP-18 column (250 × 4.00 mm, 5 micron; UK). Elution was with acetonitrile/water/acetic acid at a ratio of 50:50:1 at a flow rate of 0.7 ml/min, run time 20 min, and temperature 25°C. The mobile phase solvents are HPLC grade and deionized H_2O. The compounds were detected with a UV detector and the chromatograms were recorded at 236 nm. Capsaicin was identified and quantified by comparing it with a standard (98% capsaicin purity, Fluka).

2.4. Study Design

Mice were randomly divided into four equal groups (6/group). Group 1 received saline and served as control. Groups 2–4 received intraperitoneal (ip) injection of regular insulin at the dose of 3 IU/kg followed 30 min later by ip administration of either saline (group 2) or the pepper extract at 0.25 and 0.5 g/kg (groups 3 and 4), respectively. Mice were euthanized 3 h after insulin injection and their brains and livers were quickly dissected out on an ice-cold plate, washed with ice-cold phosphate-buffered saline at pH 7.4, weighed, and stored at −80ºC until biochemical assays. Tissue homogenization was done with 0.1 M phosphate-buffered saline (pH 7.4) to give a final concentration of 10% weight/volume.

2.5. Biochemical Analyses

2.5.1. Determination of Lipid Peroxidation

Malondialdehyde (MDA), an end product of lipid peroxidation, was measured according to the method described by Nair and Turne [28]. In brief, thiobarbituric acid reactive substances (TBAS) react with thiobarbituric acid forming TBA-MDA adduct and the absorbance is read at 532 nm spectrophotometrically.

2.5.2. Determination of Nitric Oxide

Nitric oxide was measured using the Griess reagent according to Moshage et al. [29]. Nitrate is converted to nitrite by nitrate reductase. The Griess reagent then converts nitrite to a deep purple azo compound. The absorbance is read at 540 nm spectrophotometrically. Nitrite, a stable end-product of nitric oxide radical, is mostly used as an indicator for the production of nitric oxide.

2.5.3. Determination of Reduced Glutathione (GSH)

GSH was measured using a commercially available kit (Biodiagnostic, Egypt). In brief, following tissue homogenization, proteins were precipitated with trichloroacetic acid and the Ellman's reagent is used for the determination of the free sulfhydryl groups. The Ellman's reagent 5,5′-dithiobis(2-nitrobenzoic acid) is reduced by sulfhydryl groups to form 2-nitro-5-mercaptobenzoic acid. The latter has an intense yellow color and is determined spectrophotometrically at 412 nm. The method measures total non-protein thiols, with GSH being the most abundant form of non-protein thiols in cells.

2.5.4. Determination of Paraoxonase-1 (PON-1) Activity

PON-1 arylesterase activity was determined using phenylacetate as a substrate and the formation of phenol was measured spectrophotometrically at 270 nm and 25°C. One unit of arylesterase activity is defined as 1 μmol of phenol produced per min. Enzyme activity is calculated based on the extinction coefficient of phenol (1,310 M^{-1} cm^{-1} at 270 nm, pH 8.0 and 25°C) and expressed as kilo International Units/liter (kU/L) [30].

2.5.5. Determination of Cholinesterase Activity

Butyrylcholinesterase (BChE) activity was measured with a commercially available kit (Ben Biochemical Enterprise, Milan, Italy). BChE catalyzes the hydrolysis of butyrylthiocholine as a substrate into butyrate and thiocholine. The latter reacts with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) to produce a yellow chromophore which then could be quantified using a spectrophotometer [31].

2.5.6. Determination of 5-Lipoxygenase

5-Lipoxygenase was determined using a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) kit from Shanghai Sunred Biological Technology (Shanghai, China).

2.6. Histology

Brains of all animals were dissected immediately after euthanasia, and the specimens were then fixed in 10% neutral-buffered formalin saline for at least 72 h. Specimens were then washed in tap water for 30 min, dehydrated in ascending grades of alcohol, cleared in xylene, and finally embedded in paraffin. Serial sections of 6-μm thick were cut and stained with hematoxylin and eosin (H&E) for histopathological investigations.

2.7. Glial Fibrillary Acidic Protein Immunostaining

Immunohistochemistry for glial fibrillary acidic protein (GFAP) was performed on paraffin-embedded brain sections that were deparaffinized and rehydrated. Mouse monoclonal antibodies against GFAP were used for the detection of the GFAP expression. In brief, the paraffin sections were heated in a microwave oven (25 min at 720 W) for antigen retrieval and incubated with anti-GFAP antibodies (1:50 dilution) overnight at 4°C. After washing with phosphate-buffered saline (PBS), followed by incubation with biotinylated goat-anti-rabbit immunoglobulin G secondary antibodies (1:200 dilution; Dako, Carpinteria, CA, USA) and streptavidin/alkaline phosphatase complex (1:200 dilution; Dako) for 30 min at room temperature, the binding sites of antibody were visualized with 3,3′-diaminobenzidine (DAB, Sigma-Aldrich, ST. Louis, MO, USA). After

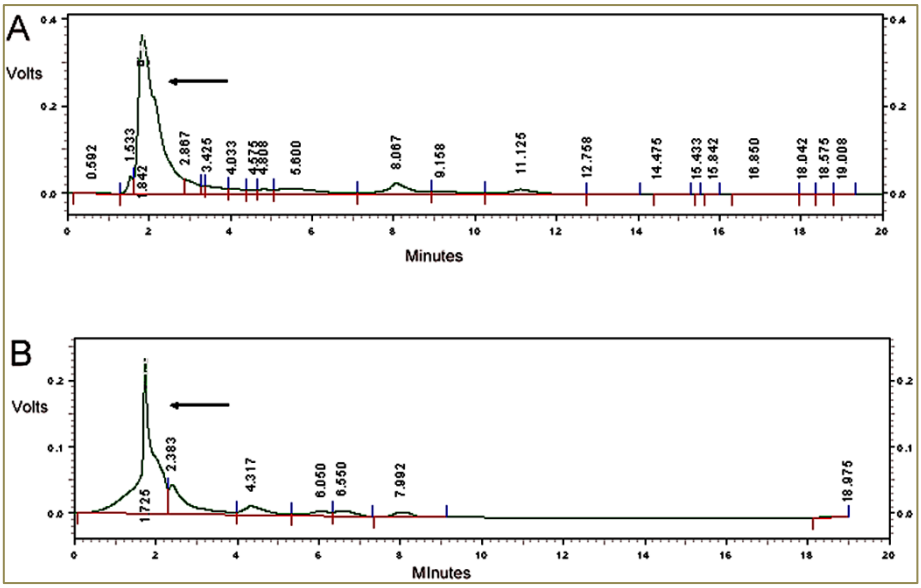

FIGURE 1. **HPLC identification of capsaicin in the red hot pepper extract.** (A) The chromatogram of a standard capsaicin solution. (B) The chromatogram of the red hot pepper extract. Arrows indicate capsaicin.

washing with PBS, the samples were counterstained with H&E for 2–3 min and dehydrated by transferring them through increasing ethanol solutions (30%, 50%, 70%, 80%, 95%, and 100% ethanol). Following dehydration, the slices were soaked twice in xylene at room temperature for 5 min, mounted, examined, and evaluated using a high-power light microscope.

2.8. Quantification of GFAP Immunostaining

An image processing and analysis system (A Leica QWin, Cambridge, UK) was used for interactive automatic measurement of the area percentage carried out on slides stained with GFAP by analyzing ten random fields per slide.

2.9. Statistics

Results are expressed as mean ± SEM. Statistical significance was determined using one-way ANOVA, followed by Duncan's multiple range test using the SPSS software (SAS Institute Inc., Cary, NC, USA). A probability value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1. Capsaicin Content of the Hot Pepper Extract

Capsaicin in the pepper extract was identified and quantified by comparing the peak retention time and area with those of the capsaicin standard under the same HPLC operating conditions. Standard curves were prepared using serial dilutions of capsaicin. HPLC chromatograms confirmed the presence of capsaicin in the extract. The chromatograms obtained for pepper showed one major peak, identified as capsaicin, and is indicated by arrow. The capsaicin content of the extract was found to be 1.2%. HPLC chromatograms for a standard capsaicin solution and the capsaicin content of the red hot pepper extract used in the study are presented in **Figure 1A** and **1B**.

3.2. Biochemical Results

3.2.1. Effect of the Pepper Extract on Oxidative Stress in Hypoglycemic Mice

Compared with the normoglycemic, saline-treated group, the hypoglycemic mice exhibited a 58.6% in-

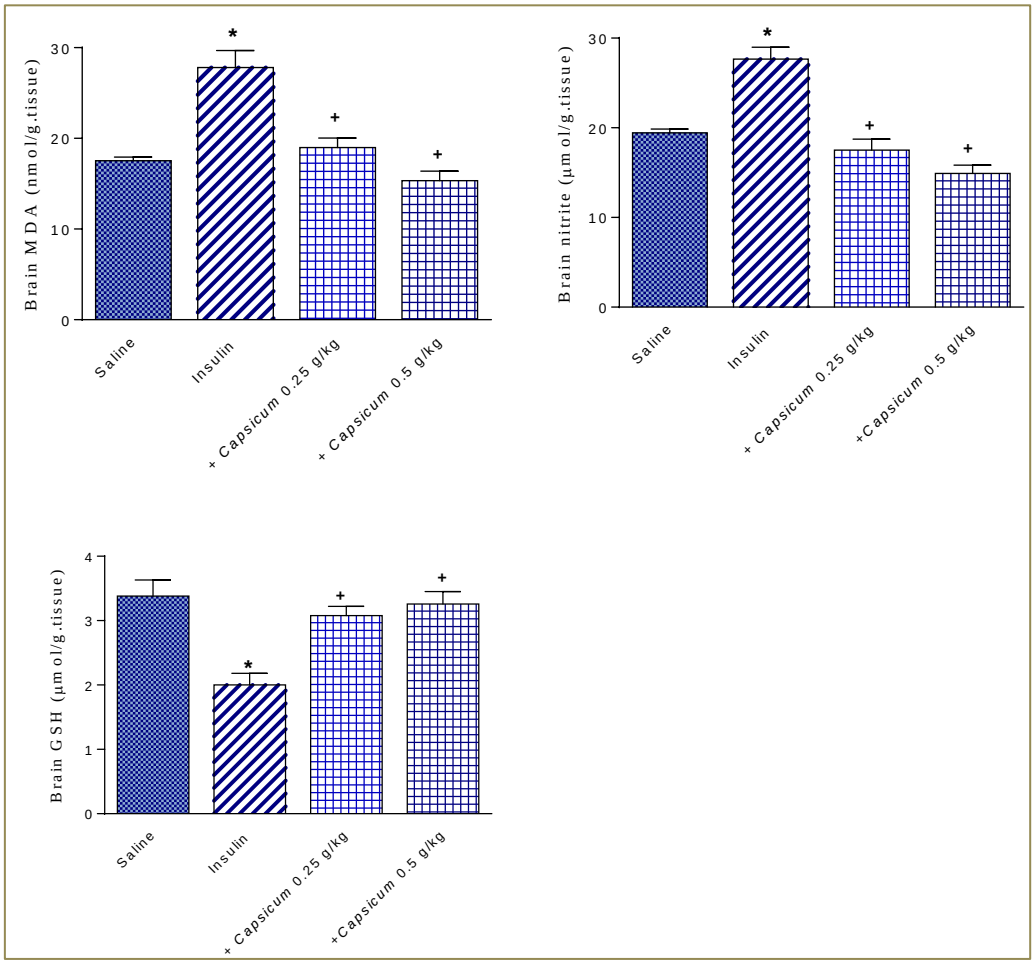

FIGURE 2. MDA, nitric oxide and GSH levels in the brain of control, hypoglycemic, and pepper extract-treated hypoglycemic mice. Data represent mean ± SEM (n = 6). *, $p < 0.05$ vs. saline control group; +, $p < 0.05$ vs. insulin control group.

crease in brain MDA content (27.82 ± 1.8 vs. 17.54 nmol/g tissue); a 42.3% increase in nitric oxide level (27.66 ± 1.33 vs. 19.44 ± 0.42 µmol/g tissue), and a 40.8% decrease in GSH level (2.0 ± 0.18 vs. 3.38 ± 0.25 µmol/g tissue). The pepper extract produced significant attenuation of the hypoglycemia-induced lipid peroxidation (–31.8% for 0.25 g/kg and –45.0% for 0.5 g/kg, as compared to insulin alone). Values were 27.82 ± 1.8, 18.98 ± 1.06, and 15.33 ± 1.05 nmol/g tissue for the insulin control and pepper-treated groups, respectively. The pepper extract also attenuated the increase in nitric oxide by –36.6% and –46.1%, respectively (17.52 ± 1.22, 14.91 ± 0.93 vs. insulin control value of 27.66 ± 1.33 µmol/g tissue). Moreover, treatment with the pepper extract resulted in 54.0% and 63.0% increments in the level of GSH compared with the insulin only treatment group (3.08 ± 0.14, 3.26 ± 0.19 vs. 2.0 ± 0.18 µmol/g tissue) (**Figure 2**).

3.2.2. Effect of the Pepper Extract on PON-1, BChE, and 5-Lipoxygenase in Hypoglycemic Mice

PON-1 activity in the brain decreased significantly by 48.4% following induction of hypoglycemia (6.7 ± 0.32 vs. 12.9 ± 0.61 kU/L). There was also a sig-

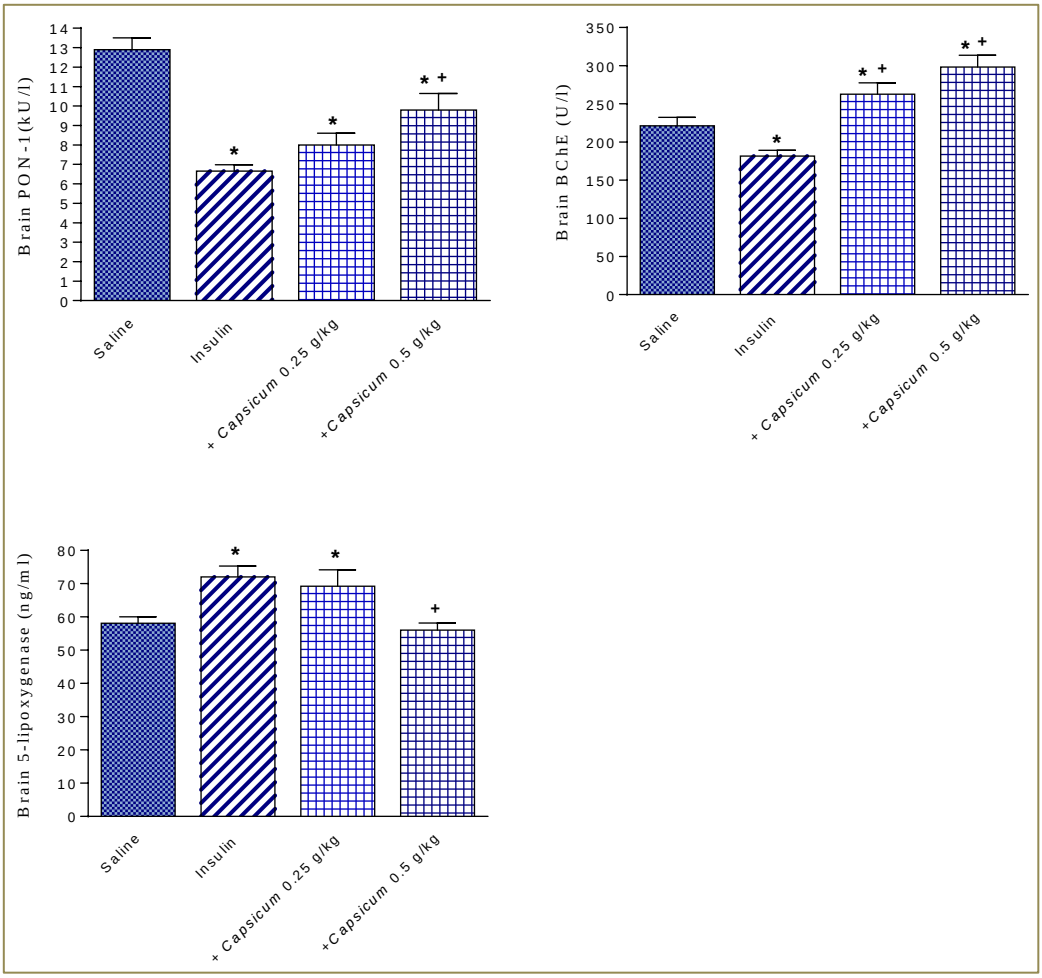

FIGURE 3. **PON-1, BChE, and 5-lipoxygenase activities in the brain of control, hypoglycemic, and the pepper extract-treated hypoglycemic mice.** Data represent mean ± SEM (n = 6). *, p< 0.05 vs. saline control group; +, p < 0.05 vs. insulin control group.

nificant decrease in brain BChE by 17.9% in the hypoglycemic mice compared with the saline control value (181.53 ± 7.71 vs. 221.14 ± 11.0 U/L). In contrast, brain 5-lipoxygenase increased by 24.0% in the insulin only treated group compared with the saline control (72.04 ± 3.20 vs. 58.1 ± 1.93 ng/ml). In insulin-treated mice, the administration of pepper extract caused significant increments in both PON-1 (19.4% for 0.25 g/kg and 46.1% for 0.5 g/kg, as compared to insulin alone; 8.0 ± 0.61, 9.79 ± 0.36 vs. 6.7 ± 0.32 kU/L) and BChE (44.7% for 0.25 g/kg and 61.5% for 0.5 g/kg, as compared to insulin alone; 262.78 ± 14.73, 293.2 ± 15.4 vs.181.53 ± 7.71 U/L). Meanwhile, the pepper extract given at 0.5 g/kg produced significant attenuation of the hypoglycemia-induced increase in brain 5-lipooxygenase by 22.3% (56.0 ± 2.17 vs. 72.04 ± 3.29 ng/ml) (**Figure 3**).

3.3. Brain Histology

Microscopic examination of brain tissue from a control rat showed the normal cortical morphology and neuronal structure (**Figure 4A** and **4B**). Histological examination of cerebral cortex of brain tissue treated with only insulin showed spongiform degeneration in the form of diffuse or focally clustered small round

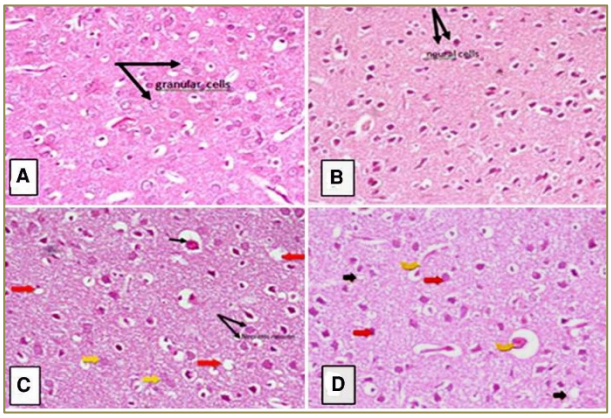

FIGURE 4. Microphotographs of representative sections of the cerebral cortex in different groups. (A and B) Saline control group shows normal histological features with well-formed neurons. (C) Insulin only treated group exhibits spongiform degeneration in the form of diffuse or focally clustered small round or oval vacuoles (red arrow) with gliosis and necrotic neurons (black arrow). Signs of degeneration in the form of karyolysis (orange arrow) are seen. (D) Insulin only treated group shows that some neurons appear apoptotic (red arrow) and congestion of cerebral blood vessel (yellow arrow) (H&E staining, ×400).

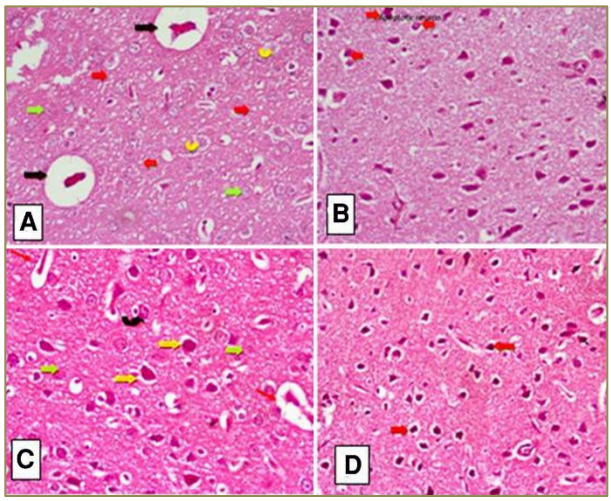

FIGURE 5. Microphotographs of representative sections of the cerebral cortex in different groups. (A) Insulin + the pepper extract at 0.25 g/kg group shows spongiform changes consisting of relatively small delicate vacuoles in the cortex (red arrow), and a vessel with membrane bound vacuoles (black arrow). Signs of degeneration in neurons in the form of karyorrhexis (yellow arrow) and karyolysis (green arrow) are seen. (B) Another field shows some apoptotic neuron and pyknotic nuclei (red arrow). (C) Insulin + the pepper extract at 0.5 g/kg group shows some improvement in pathological changes but signs of degeneration in neurons in the form of karyolysis (green arrow), and some eosinophilic neurons (yellow arrow) are seen. Dilation and congestion of cerebral blood vessel are also noticed (red arrow). (D) Another field shows pyknotic neurons (red arrow). See Figure 4 for microphotographs of saline control and insulin only groups (H&E staining, ×400).

or oval vacuoles with gliosis. Most of the neurons appeared necrotic, others apoptotic, and congested cerebral blood vessel was seen (**Figure 4C** and **4D**). Sections of the brain cerebral cortex of mice treated with insulin and the pepper extract at 0.25 g/kg showed that the cortex still suffered from pathological changes in the form spongiform changes consisting of relatively small delicate vacuoles in the cortex and thrombotic vessels. Signs of degeneration in some neurons in the form of karyolysis and karyorrhexis were observed. Some apoptotic and pyknotic neurons were also seen (**Figure 5A** and **5B**). Sections of the brain of the cerebral cortex of mice treated with insulin and the pepper extract at 0.5 g/kg exhibited some improvement in pathological changes in the form of absence of spongiform changes but signs of degeneration in neuron in the form of karyolysis and some eosinophilic neurons were still present. Dilation and congestion of cerebral blood vessels were seen (**Figure 5C** and **5D**).

3.4. GFAP Staining

Investigation of the cerebral cortex tissue shows that insulin alone caused the astrocytic cells to decrease markedly, both in number and size (**Figure 6B**) if compared with those in sections obtained from saline control mice (**Figure 6A**). This decrease in astrocytes was ameliorated by treating animals with the pepper extract at 0.5 g/kg as glial cells restored their normal number, size of their bodies, and length of their processes (**Figure 6C**).

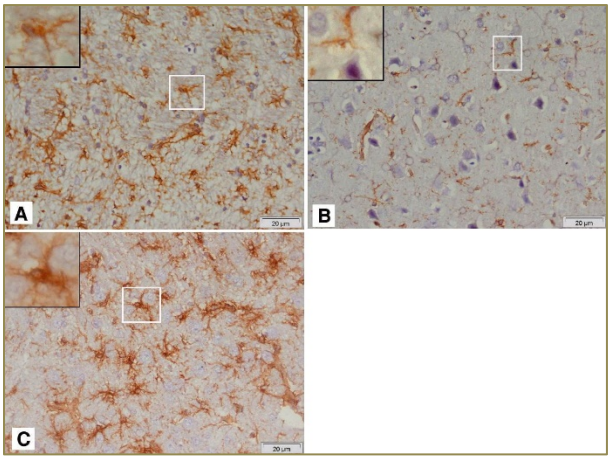

FIGURE 6. Microphotographs of representative sections of the cerebral cortex stained immunohistochemically with GFAP antibody in different groups. (A) Saline control group shows normal shape and number of astrocytes. (B) Insulin only group shows a marked decrease in size and number of glial cells. Notice the small size and the shortness of processes of astrocytes cells in the highly magnified upper left part of this section of the figure. (C) Insulin + the pepper extract at 0.5 g/kg group shows marked increase in number, size, and length of processes of astrocytes (GFAP staining, ×400).

3.5. Immunomorphometric Analysis

Quantitative morphometric analysis of the GFAP-stained sections shows GFAP in cytoplasm of viable astrocytes which is marked by the blue color in image analyzer system to be measured as an area%. The maximum expression was in the normal control group 4.459 ± 1.042 which received saline only. On the other hand, the lowest GFAP expression was 1.303 ± 0.161 in the group treated by insulin only denoting the destructive effect of hypoglycemia on glial cells. The area% of GFAP was increased, in the group that received insulin and the pepper extract at 0.5 g/kg, to 3.684 ± 0.258, a 182.7% increase compared with the insulin only group (**Table 1**).

3.6. Liver Histology

The liver of saline control mice revealed the normal characteristic architecture of hepatic lobules and central vein (**Figure 7A**). The microscopic examination of liver tissue of mice treated with insulin only revealed hepatocytes with cytoplasmic vacuoles, sings of degeneration in the form of necrosis and karyorrhehxis, and congestion of central vein and in blood sinusoids (**Figure 7B**). Mice treated with insulin and the pepper extract at 0.25 g/kg still suffered from pathological changes in the form of hepatocytes with cytoplasmic vacuoles and congestion of the central vein. Signs of degeneration in the form of karyolysis were also noted (**Figure 7C**). Examination of the liver tissue of mice treated with insulin and the pepper extract at 0.5 g/kg showed improvement in pathological changes, evidenced by no cytoplasmic vacuoles in hepatocytes and no signs of degeneration although minute vacuoles were seen (**Figure 7D**).

4. DISCUSSION

Oxidative stress has been implicated in the development of insulin-hypoglycemic neuronal injury [24, 25]. The present findings are consistent with this notion. Our results provide evidence for increased oxidative stress in the brain of hypoglycemic mice as indicated by the markedly raised lipid peroxidation end product MDA, an indicator of free radical attack on membrane lipids [32]. There was also decreased brain GSH content. GSH is the most abundant intracellular non-protein thiol and the ratio between its reduced and oxidized forms is important in maintaining the redox balance in the cell. GSH scavenges free radicals and other reactive oxygen metabolites, such as hydroxyl radical, lipid peroxyl radical, hypochlorous acid, and peroxynitrite both directly, and through reactions involving the enzymes glutathione reductase and glutathione peroxidase [33, 34]. The observed decrease in brain GSH in hypoglycemia would therefore indicate increased generation of free radicals and the consequent consumption of the scavenger molecule. The decrease in the level of GSH could also be due to energy failure and would render the cell more susceptible to an oxidative milieu. Other researchers also reported increased lipid peroxidation in the mice brain following insulin-induced hypoglycemia [24, 35]. In their study, Patockova et al. [24] reported significant increments in brain MDA 2–3 h after ip injection of regular insulin in mice. Here we show that the increase in lipid peroxidation and the decrease in GSH induced by

TABLE 1. The results of GFAP staining showing area% of the viable glial cells

Group	GFAP staining (area%)
Saline	4.459 ± 1.042
Insulin	1.303 ± 0.101*
Insulin + the pepper extract 0.5 g/kg	3.684 ± 0.258+

Data represent mean ± SEM (n = 10 fields/slide; 5 slides per group). *, p < 0.05 vs. saline group; +, p < 0.05 vs. insulin only group.

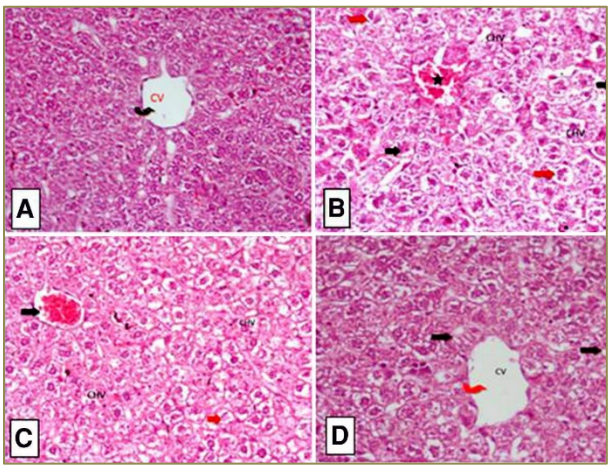

FIGURE 7. Microphotographs of representative sections of the liver in different groups. (A) Saline control group shows the classical hepatic architecture. Hepatocytes are arranged in cords radiating from the central vein (CV) and separated by blood sinusoids. (B) Insulin only group shows hepatocytes with cytoplasmic vacuoles (CHV), sings of degeneration in the form of necrosis (black arrow) and karyorrhexis (red arrow), and congestion of central vein (star) and in blood sinusoids. (C) Insulin + the pepper extract at 0.25 g/kg group shows hepatocytes with cytoplasmic vacuoles (CHV), karyolysis of some cells (red arrow), and congestion of central vein (black arrow). (D) Insulin + the pepper extract at 0.5 g/kg group shows improvement in pathological changes although minute vacuoles are still present (black arrow) (H&E staining, ×400).

insulin-hypoglycemia were restored to their control values by the pepper extract which are in agreement with an antioxidant activity for the red pepper constituents. In rat brain homogenates in vitro, aqueous extract of peppers (*Capsicum annum*, and *Capsicum chinense)* caused a significant decrease in the lipid peroxidation induced by the prooxidants sodium nitroprusside and quinolinic acid. The protective effect of hot pepper against sodium nitroprusside-induced lipid peroxidation was ascribed to its total phenol content [36].

Our results also indicate markedly increased brain nitric oxide in the hypoglycemic brain. The gaseous molecule nitric oxide is an important signaling messenger besides its role in maintaining blood flow. Nitric oxide is synthesized from L-arginine in a reaction that involves the enzyme nitric oxide synthase. The latter exists in two constitutive isoforms—endothelial and neuronal, while a third isoform is inducible in neurons and phagocytic cells by inflammatory and toxic stimuli and is held responsible for the generation of large amounts of nitric oxide for longer duration with its consequent deleterious action in impairing cellular respiration, resulting in energy failure and neuronal death [37, 38]. Suh et al. [39] suggested that in severe hypoglycemia, nitric oxide production causes the release of vesicular zinc that results in activation of NADPH oxidase and poly(ADP-ribose) polymerase-1 and neuronal death. In this study, the administration of the red hot pepper extract to insulin-hypoglycemic mice was associated with a significant decrease of brain nitric oxide, suggesting an inhibitory action for the extract on nitric oxide formation.

Our data also showed for the first time that PON-1 activity was markedly decreased in the brain of insulin-hypoglycemic mice. PON-1 is a calcium-dependent esterase and lactonase which is important in the detoxification of organophosphorus insecticides and other xenobiotics [40, 41]. Additionally, PON-1 is endued with antioxidant and anti-inflammatory actions. It is synthesized by the liver and bound mainly to low-density lipoprotein in the circulation [40, 42]. PON-1 is susceptible to oxidative inactivation and its activity could be increased

by antioxidants [43, 44] which provides a likely explanation for the observations in the present study.

The cholinesterase family of enzymes acts to hydrolyze the neurotransmitter acetylcholine and hence terminate its action in the neuronal synapse. BChE is widely distributed in the nervous system [45] and recent evidence suggests a role in Alzheimer's disease [46]. Our results indicate a modest, though a significant, decrease in BChE in the brain of insulin-treated mice. Meanwhile, the administration of the red hot pepper extract was associated with a marked increase in the enzyme activity. A similar finding has been reported where treatment with an extract of red hot pepper was able to revert the inhibition of brain BChE induced by rotenone [20]. Other researchers reported inhibitory activity for different peppers on both acetylcholinesterase and BChE activities in vitro but at high (mg/ml) concentrations [47, 48] which are unlikely to occur in the brain in vivo circumstances.

We also observed an increase in 5-lipoxygenase in the brain of insulin-hypoglycemic mice. The finding suggests an inflammatory component possibly involved in the development of neuronal injury during hypoglycemia. The 5-lipoxygenase catalyzes the oxidation of arachidonic acid to yield 5-hydroxyeicosatetraenoic and leukotrienes, including leukotriene B4 and cysteinyl leukotrienes which are potent mediators of inflammation and promote neurodegeneration. In this context, 5-lipoxygenase is upregulated in the aging brain and Alzheimer's disease [49, 50]. The 5-lipoxygenase enzyme is widely expressed in the central nervous system being localized mainly in neurons [51]. In this study, treatment with the pepper extract was able to alleviate the increase in brain 5-lipoxygenase. A similar 5-lipoxygenase inhibitory activity for hot pepper was also seen in the brain of rats treated with the nigrostriatal toxin rotenone [20]. This inflammatory inhibitory action of the red hot pepper extract is likely to contribute to the neuroprotection observed in these studies.

In the present study, the histopathological examination of the brain and liver tissues confirmed a deleterious effect for insulin hypoglycemia on tissue integrity and provided support for a protective effect for the pepper extract. The microscopic examination of the cerebral cortex tissue 3 h after inducing hypoglycemia revealed marked changes in the form of spongiform degeneration, neuronal necrosis, apoptosis, and gliosis. In the hypoglycemic brain, astrocytes, the predominant glial cells in the brain, also showed decreased number and size. These cells are important for the metabolic support of neuronal cells and their functional impairment is likely to cause profound consequences on neuronal functions and integrity. Other researchers observed neuronal death in the hippocampus, microglia activation, and increased myeloperoxidase immunoreactivity upon examination of the brain tissue 1 week after insulin hypoglycemia (10 U/kg, ip) [52]. The brain relies on a continued supply of glucose in order to carry on its metabolic functions. In severe hypoglycemia, energy deprivation initiates pathophysiological events such as increased release of glutamate and reactive oxygen metabolites, activation of poly(ADP-ribose) polymerase-1, and mitochondrial permeability transition, resulting ultimately in neuronal cell loss [53, 54]. Our results also implicate increased release of nitric oxide and 5-lipoxygenase activation in neuronal death. We also showed the development vacuolar degeneration and hepatocyte necrosis in the liver tissue of the hypoglycemic mice. These neuropathological alterations and liver tissue injury were markedly improved, though not totally prevented, by the pepper extract.

In this study, the capsaicin content of the extract was found to be 1.2%, suggesting that doses of capsaicin given were 3 and 6 mg/kg, respectively. The doses of the hot pepper extract (0.25 and 0.5 g/kg) and the inferred amounts of capsaicin used in this study are comparable to what humans consume in countries with highest intake (3 and 9 g pepper/person/day) [5, 6]. These human dietary doses of capsicum are equivalent to 0.27 and 0.81 g/kg, after conversion to mice doses according to Paget and Barnes [55].

Studies reported significant absorption of capsaicinoids from the rat stomach [56] and small intestine [57]. Capsaicinoids are absorbed partly in their intact form and are metabolized in the liver before reaching the systemic circulation [56]. Capsaicin administered by intragastric, intraduodenal, or intravenous route decreased bile flow and biliary protein secretion. HPLC analysis of the bile showed that capsaicin was excreted into bile at concentrations known to excite sensory nerve fibers [58]. Orally given capsaicin was shown to protect the rat liver from carbon tetrachloride toxicity [59]. Capsaicin given via intragastric or intraperitoneal route protected against the toxic effect of lipopolysaccharide on the liver tissue [60].

Meanwhile, intravenous administration of capsaicin led to very rapid distribution and elimination [61, 62]. Capsaicin could be detected in the rat brain, liver, and blood at ng/g concentrations 3 and 10 min after intravenous injection of 2 mg/kg [62]. In the present study, however, capsaicin was not measured in tissues after administering the hot pepper extract and thus it is not possible to conclude whether the effects observed are due to capsaicin or other components in red peppers that we did not analyze.

Red hot peppers are rich in phytochemicals other than capsaicin such as vitamin C, vitamin E, carotenoids, and phenolics, and red peppers have been shown to possess strong antioxidant properties [63]. These constituents of red peppers, although not measured in the present study, could thus have accounted for the protective effects observed in the current study.

In summary, the present study provides evidence that the administration of a red hot pepper extract shortly after inducing hypoglycemia with insulin was able to protect against the brain biochemical changes, neuropathological alterations, and liver damage induced by hypoglycemia. These protective effects of the pepper extract involve inhibition of oxidative stress and 5-lipoxygenase.

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