

Original article

Neuroprotection of Gueichih-Fuling-Wan on cerebral ischemia/ reperfusion injury in streptozotocin-induced hyperglycemic rats *via* the inhibition of the cellular apoptosis pathway and neuroinflammation

Yuh-Fung Chen^{a,b,*}, Kuo-Jen Wu^{a,#}, Wei-Shih Huang^{c,d,#}, Yow-Wen Hsieh^{b,e}, Yu-Wen Wang^a,Huei-Yann Tsai^b, Ming-Ming Lee^{f,†}^aDepartment of Pharmacology, China Medical University, Taichung 404, Taiwan^bDepartment of Pharmacy, China Medical University Hospital, Taichung 404, Taiwan^cDepartment of Neurology, China Medical University, Taichung 404, Taiwan^dDepartment of Neurology, China Medical University Hospital, Taichung 404, Taiwan^eDepartment of Pharmacy, China Medical University, Taichung 404, Taiwan^fDepartment of Health and Nutrition Biotechnology, Asia University, Taichung 413, TaiwanReceived 11th of September 2016 Accepted 30th of September 2016

© Author(s) 2016. This article is published with open access by China Medical University

*Keywords:*Gueichih-Fuling-Wan;
Cerebral Ischemia/
Reperfusion Injury;
Hyperglycemia;
Cellular Apoptosis;
Neuroinflammation.**ABSTRACT**

Background: Risks of stroke link with complications of hyperglycemia. Gueichih-Fuling-Wan (GFW), according to Chinese Medical Code literature, has the promotion of blood circulation and attenuates the swollen plot. Recent pharmacological studies have pointed out its efficacy in patients with cerebral ischemia or diabetes. Therefore, this study determined whether GFW has the protection against cerebral ischemia/reperfusion (I/R) injury in streptozotocin (STZ)-induced hyperglycemic rats and LPS-induced inflammation in BV-2 microglial cells.

Methods: Extracts of GFW were filtered and frozen to dry for use. Hyperglycemia was induced by intraperitoneal injection of 70 mg/kg STZ. Fourteen days after STZ injection, GFW (1, 2 and 4 g/kg) was orally administered once daily for seven days. Rats were subjected to cerebral ischemia/reperfusion and sacrificed for infarction analysis and neuronal apoptosis detection twenty-one days after STZ injection. MTT assay was used for cell viability; nitrite quantification and western blot analysis of iNOS and COX-2 were used to explore the effects of GFW on LPS-induced inflammation in BV-2 microglial cells.

Results: GFW significantly ameliorated cerebral infarction while dosage was more than 1 g/kg (by 38.03% at 2 g/kg and 52.44% at 4 g/kg), and attenuated neurological deficits by 23.48% (at 2 g/kg) and 47.25% (at 4 g/kg). Furthermore, GFW (2, 4 g/kg) notably decreased TUNEL- and cleaved caspase-3-positive cells in the immunohistochemical stain ($P < 0.01$ and $P < 0.001$, respectively). GFW remarkably increased in Bcl-2 and decreased in caspase-3 and Bax/Bcl-2 ratio protein expressions by Western blot. GFW (0.25, 0.5, 1 mg/ml) significantly reduced LPS-induced NO production in BV-2 microglial cells. And GFW attenuated iNOS and COX-2 expression in LPS-treated BV-2 cells. **Conclusions:** In summary, GFW has good bioactivities to protect cerebral I/R injury in hyperglycemic rats, which might be due to inhibition of cellular apoptosis and neuroinflammation.

1. Introduction

Atherosclerosis in the carotid and vertebralbasilar artery causing cerebral infarction is the chief cause of stroke worldwide [1].

Stroke, the leading cause of and disability and death, is a multi-factor disease that forms a possible end state for diabetes, atherosclerosis, and hypertension [2]. Statistically, ischemic stroke is significant major stroke damage, which induces the reduction of

*Corresponding author. Department of Pharmacology, College of Medicine, China Medical University, No. 91 Hsueh-Shih Road, Taichung 404, Taiwan.

Equal contribution to the first author

† Equal contribution to the corresponding author

E-mail address: yfchen@mail.cmu.edu.tw (Y.-F. Chen).

oxygen and glucose leading to neuronal excitotoxicity [3, 4]. The previous study reported that diabetic rats might be more susceptible to the cerebral vascular accident [5]. Increased likelihood of mortality and reduced functional recovery in acute hyperglycemia was higher than non-diabetic patients after ischemic stroke [6]. Epidemiological studies found diabetics 2-3 times as often as non-diabetics, a stroke rate twice that of the general population [7-9]. Patients with diabetes in the environment of high blood sugar are likely to suffer atherosclerosis and reduced blood flow, which lead to ischemia and cell death in the ischemic area. Diabetes alters central nervous system dysfunction associated with cognitive change and function abnormality, which increases the performance of reactive oxygen species and inflammatory mediators, and spawns neuronal apoptosis and neurodegeneration after reperfusion injury. It would exacerbate the likelihood of stroke damage [10-12]. Treatment options for stroke nowadays are limited and affect mainly only symptoms. Therapeutic applications are relevant for preventing or inhibiting neurological cell death for a variety of neurodegenerative conditions including ischemia, and stroke.

Inflammation contributes to stroke-related brain injury in both the core and the ischemic penumbra. It is believed to be especially dangerous after reperfusion. Numerous evidences show that post-ischemic inflammation induces the deleterious damage of neuronal cells after stroke, and the activation of microglia, in particular, has been thought as the main contributor by releasing proinflammatory and neurotoxic factors. LPS (lipopolysaccharide), the polysaccharide component of the cell wall of gram-negative bacteria, is the most frequently used model to investigate the inflammatory responses of microglia [13-15]. Stimulation of microglia with LPS induces the release of nitric oxide (NO)-mediated neuron death *in vitro* [16]. Inducible NO synthase (iNOS) is quickly transcribed and expressed in microglia after stimulation with bacterial LPS and cytokines [17]. Cyclooxygenase-2 (COX-2) also plays a predominant role in inflammation [18]. Excess oxygen free radicals could directly generate and damage cerebral tissue through the activation of apoptotic and necrotic cell signaling pathways. The molecular mechanisms of inflammation induced by LPS in microglial cells have been well reported [19-23]. In most neurodegenerative diseases, the neuronal loss is due to apoptosis. It could particularly be a possible mechanism for hyperglycemia-induced neuronal cell death, which is a successive occurrence of processing including condensation of chromatin, shrinkage of cell and nucleus, membrane bleb, and DNA fragmentation. Therefore, to interrupt the signaling networks that link to neuronal damage to apoptotic degradation is a possible treatment option in neurodegenerative disease [24-26]. Caspase enzymes and Bcl-2 family, two major families of proteins, are the key elements in apoptosis. Caspase-3 plays a pivotal role in apoptosis [27], whereas, there are two classes in Bcl-2 family to regulate apoptosis: the antiapoptotic proteins, including Bcl-2, Bcl-xL and proapoptotic proteins, including Bax and Bak [28].

Gueichih-Fuling-Wan (abbreviated as GFW), a traditional Chinese herbal medicine, has mainly treated gynecological diseases for thousands of years. GFW has the neuroprotective effect in diabetic rats through reducing advanced glycation end products (AGEs) accumulation and oxidative stress [29]. GFW also could improve vascular function and hemorrhological factors in spontaneously diabetic rats [30]. Recent studies show that GFW had the neuroprotective effect against cerebral ischemia in rats [31], and ameliorated memory deficits and neuronal apoptosis in streptozotocin-induced hyperglycemic rodents [32]. No paper has

been reported the neuroprotective effect of GFW on cerebral ischemia injury in hyperglycemic rats. Therefore, this study explored the neuroprotective mechanism of GFW in STZ-induced hyperglycemic rats with an ischemia/reperfusion brain injury *in vivo* and LPS-induced inflammation in BV-2 microglial cells *in vitro*.

2. Materials and methods

2.1. Reagents

Streptozotocin (STZ) was purchased from Sigma-Aldrich (St. Louis, MO), Zoletil[®] from Virbac Laboratories (Carros, France). BCA protein assay kit was from Thermo Fisher Scientific (Lafayette, CO). NovoLink Polymer Detection System Kit was from Leica Microsystems Inc. (Newcastle Upon Tyne, UK). Apo-BrdU-IHC[™] *In situ* DNA Fragmentation Assay Kit was from BioVision (Milpitas, CA); anti-Caspase-3, anti-Bcl-2, anti-Bax and anti- β -actin antibodies from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Extraction of GFW

Gueichih-Fuling-Wan (abbreviated as GFW), a traditional Chinese remedy, consists of five medicinal herbs in equal proportions: Cinnamomum cassia Blume; Paeonia lactiflora Pall; Paeonia suffruticosa Andr, Poria cocos (Schw.) Wolf; and Prunus persica (L) Batsch. Each medicinal herb (200 g) was extracted twice with 2 L boiling distilled water for two hours. Extracts were filtered and frozen to dry. The yield of extracts was 11.54 %, and GFW freshly prepared in distilled water before experiments.

2.3. Ethics statement

The experimental protocol was approved by the Institutional Animal Care and Use Committee of China Medical University (permit number 99-12).

2.4. Animals and drug administration

Male Sprague-Dawley (SD) rats with body weight 225-275 g were purchased from BioLASCO Co., Ltd. (Taipei, Taiwan). Animals, fed with regular chow, were housed in standard cages. The room temperature is at constant $22 \pm 1^\circ\text{C}$; relative humidity is $55 \pm 5\%$ and with 12 h inverted light-dark cycle. A minimum number of animals and duration of observations required for consistent data were used, rats randomly allocated into four groups ($n = 6/\text{group}$): hyperglycemia, hyperglycemia treated with GFW (1 g/kg, 2 g/kg, 4 g/kg, p.o.). Hyperglycemia was induced by intraperitoneal injection of streptozotocin (STZ) (Sigma; St. Louis, MO) at 70 mg/kg [33]. Three days after STZ injection, overnight fasting and plasma glucose was sampled from animal tail venous blood, and determined by using an automatic glucometer (ACCUCHEK Active, Roche Diagnostics Ltd.; Mannheim, Germany). Those animals, with plasma glucose level higher than 300 mg/dl, were considered for diabetes [32]. The day of STZ injection was designated Day 0. Fourteen days after STZ injection, GFW was orally administered once daily for seven days. Rats were subjected to cerebral ischemia/reperfusion and sacrificed for infarction analysis and neuronal apoptosis detection twenty-one days after STZ injection. GFW (1, 2 and 4 g/kg) was given orally, as shown in Fig. 1.

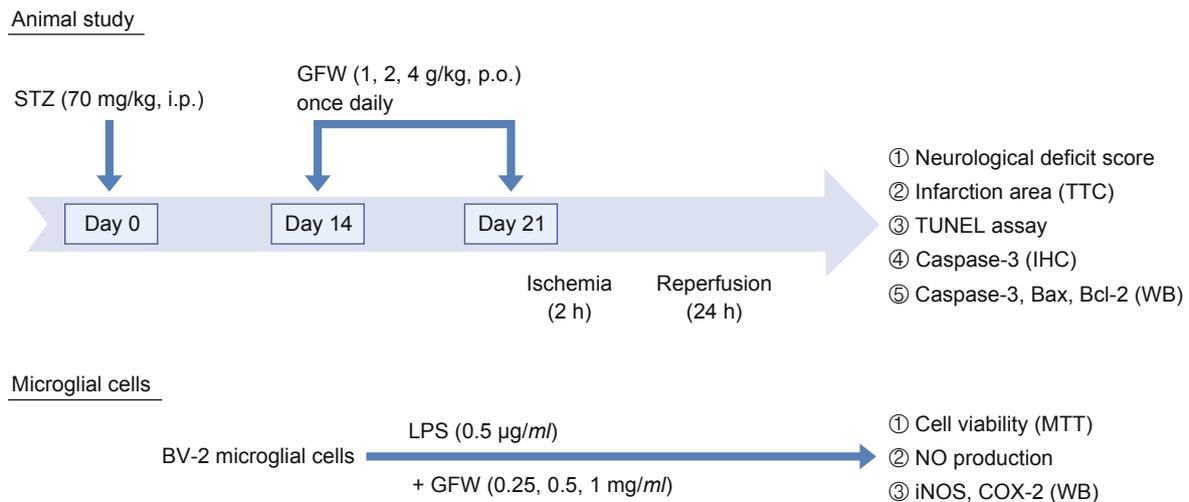


Fig. 1 - Schedule of drug treatment and determination of central neurological function *in vivo* and LPS-treated BV-2 microglia cells *in vitro*. Upper panel: GFW (1, 2, 4 g/kg) was orally administered once daily for seven days, and surgical cerebral ischemia was completed for 2 h on day 21 before reperfusion. The animal was sacrificed, and the central neurological functions were evaluated 24 h after reperfusion, infarction area (TTC), TUNEL assay, caspase-3 (IHC), caspase-3, Bax and Bcl-2 (western blot analysis) were performed in animal study. Lower panel: GFW (0.25, 0.5, 1 mg/ml) and LPS (0.5 µg/ml) were added to BV-2 microglia cells to evaluate cell viability (MTT), NO production, iNOS and COX-2 expression level (western blot) in microglia cells.

2.5. Surgical procedures of cerebral ischemia/reperfusion

Male SD rats were deeply anesthetized by an injection of 50 mg/kg of zoletil® (i.p.). Surgical cerebral infarction and ischemia/reperfusion were as previously described [34]. The rat was placed supine and both common carotid arteries were exposed and were tied off with a plastic line (0.1 mm in diameter). The different concentrations of distilled water, GFW (1, 2, 4 g/kg) are orally administered once daily for seven days before 3-vessels occlusion in rats, respectively. After 90 min of occlusion following by reperfusion for 24 h, the brain of each rat was removed after transcardial perfusion of 0.9% NaCl and 4% paraformaldehyde. Each brain was then placed into a plastic rat brain matrix and was coronal sectioned into 2 mm slices. The samples were stained with 2% 2,3,5-triphenyltetrazolium (TTC) solution in a 37°C for 20 min and were fixed with 10% formalin solution. The cerebral infarction areas of the first six sections from the frontal lobe were measured using an image analysis system (Image-Pro Plus 6.0 Media Cybernetics, USA). The ratio of infarct area to total brain area in each section of the rat brain was calculated, and the data were expressed as a percentage (%).

2.6. Assessment of neurological functions

The effects of GFW on neurological deficit score after ischemia for 2 h and following by reperfusion for 24 h in hyperglycemic rats with I/R brain injury were assessed as described according to a previous report [35]. The degree of neurological deficit is rated 0-5: 0 represents no neurological dysfunction and 1 represents contralateral forelimb consistently flexed. 2 represents decreasing grip ability on the contralateral forelimb with tail pulled and 3 represents spontaneous movement in all directions but circling toward contralateral side when pulled by the tail. 4 represents nonspontaneous walking with drowsiness, unconsciousness and 5

represents death.

2.7. TUNEL assay and immunohistochemical stain

Rats were deeply anesthetized by injection of zoletil® (50 mg/kg, i.p.) on day 22 after STZ injection. Intracardial perfusion with 200 ml of 0.9% saline followed by 4% paraformaldehyde in 0.1M PBS was performed before animals were decapitated. Brains were removed, immersed in 10% paraformaldehyde, sectioned at 2-mm intervals using a rodent brain matrix slicer (RBM-4000C; ASI Instruments; Warren, MI), processed and embedded in paraffin, cut 2.5 µm thick on a microtome and processed for TUNEL and immunohistochemical staining. TUNEL assay of brain sections used Apo-BrdU-IHC™ *In Situ* DNA Fragmentation Assay Kit (BioVision, Milpitas, CA). Incubated the brain slices with proteinase K for 20 min followed by 3 % H₂O₂ in methanol for 5 min to inactivation of endogenous peroxidase. TdT was added at room temperature and incubated overnight. Dark brown color indicated DNA breaks after incubation with DAB (3, 3'-diaminobenzidine tetrachloride) and hydrogen peroxide, followed by counterstaining with methyl green. Percentages of positive TUNEL staining cells within brain areas were estimated.

Brain slices were incubated with the anti-caspase-3 antibody (sc-7148, dilution 1:200, Biotechnology, Inc.; Santa Cruz, CA) overnight and immunohistochemical labeled by NovoLink Polymer Detection System Kit (Leica Microsystems Inc., Newcastle Upon Tyne, UK). Ratios of caspase-3-positive cells within brain areas were estimated.

2.8. Determination of expression of caspase-3, Bax and Bcl-2 by Western blot

Injection of zoletil® (50 mg/kg, i.p.) sacrificed rats for biochemical studies. Brain tissues were quickly removed, and the cere-

bral cortex and hippocampus were separated on the ice. A 10% homogenate was prepared in lysis buffer, centrifuged at 12,000 (rpm) for 30 min at 4°C. Use the BCA protein assay kit to determine the samples protein concentration. Seventy mg protein was separated on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were incubated for one hr with 5% dry skim milk in TBST buffer at room temperature to block non-specific binding, then with the anti-Caspase-3, anti-Bax, anti-Bcl-2, anti-β-actin antibodies. Later, membranes were incubated with alkaline-phosphatase-conjugated secondary antibody for one hour at room temperature; bands visualized with chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate in the presence of nitroblue tetrazolium.

2.9. Cell culture

Murine BV-2 microglial cells were kindly provided by Professor Jau-Shyong Hong (Neurobiology Laboratory/Neuropharmacology group, NIEHS/NIH, NC, USA). BV-2 microglial cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 100 units/ml penicillin, and 100 mg/ml streptomycin, and kept at 37°C in a humidified incubator with 5% CO₂ and 95% air.

2.10. MTT assay

The BV-2 microglial cells were used to explore the effects of GFW on LPS-induced inflammation. The cells were cultured in a 96-well plate at the density of 5×10^4 cells/well. BV-2 microglia cell cultures were administered with GFW and LPS for 24 h. MTT was added to each well, and the cells were incubated for 1 h. After culture media were discarded, DMSO was added to dissolve the formazan dye and the optical density was measured at 570 nm.

2.11. Nitrite quantification

NO generation was measured by the accumulation of nitrite in the culture medium. The colorimetric assay was used to determine nitrite with Griess reagent. BV-2 cells (5×10^4 cells per well) in 96-well plates in 200 ml culture medium were treated with LPS (0.5 μg/ml) for 24 hours. 100 μl of the isolated supernatant was added with an equal volume of Griess reagent in the 96-well plates for 10 min at room temperature and light avoidance. Standard solution of sodium nitrite prepared in the cell-culture medium was used to determine nitrite concentrations. The absorbance at 570 nm was read using an Elisa reader (Triad LT, DYNEX Technologies Inc, VA). Each experiment was duplicated three times.

2.12. Western Blot analysis of iNOS and COX-2

Cell lysates were prepared in lysis buffer, and the protein concentrations were determined by Bio-Rad protein assay kit (Richmond, CA, U.S.A.). Samples of protein (50 μg) were electrophoresed using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membrane. The iNOS, COX-2 were assayed by specific antibodies (Santa Cruz Biotech, Santa Cruz, CA, U.S.A. Cell Signaling Technology). An enhanced chemiluminescence detection kit was used in immunodetection (Amersham, Piscataway, NJ).

2.13. Statistical analysis

Data were expressed as the mean ± standard error. For single variable comparisons, Student's *t*-test was used. For multiple variable comparisons, data were analyzed by one-way ANOVA followed by Dunnett's test. *P* < 0.05 was considered significant.

3. Results

3.1. Effects of GFW on cerebral infarct volume and neurological function in STZ-induced hyperglycemic rats with I/R brain injury

After blocking blood flow of both common carotid arteries and right cerebral artery for 90 min, all rats developed cerebral infarction after 24 h reperfusion: visibly white and non-infarction areas red-purple by TTC staining (Fig. 2A). GFW decreased infarct volume in a dose-dependent manner (Fig. 2B). The average percentage of infarct area in STZ-induced hyperglycemic rats was 15.96 ± 0.76 %. The average percentages of infarct area treated with different dosages of GFW (1, 2, 4 g/kg) in hyperglycemic rats with I/R brain injury were inhibited by 15.06 ± 0.88 %, 9.89 ± 1.21 % (*P* < 0.01) and 7.59 ± 0.69 % (*P* < 0.001), respectively. The effects of GFW (1, 2, and 4 g/kg, p.o.) on neurological deficit score were assessed. Results showed that GFW significantly reduced neurological deficit score in hyperglycemic rats with I/R injury (Fig. 2C). GFW (1, 2, and 4 g/kg, p.o.) improved neurological deficit in the STZ-induced hyperglycemic rats with I/R brain injury by 3.18 ± 0.23 (1. g/kg), 2.64 ± 0.31 (2 g/kg) and 1.82 ± 0.23 (4 g/kg), respectively.

3.2. Effects of GFW on brain apoptosis in hyperglycemic rats with I/R brain injury by TUNEL stain and on caspase-3 expression in immunohistochemical staining

We used TUNEL to assay nucleosomal DNA fragmentation and on caspase-3 expression in immunohistochemical staining in the ischemic penumbra. Fig. 3 plots representative histological view of TUNEL stains and immunohistochemical staining in hyperglycemic rats. Treating with GFW (2 and 4 g/kg) observably decreased TUNEL-positive cells (Fig. 3B) and caspase-3 positive cells (Fig. 3C) in the hyperglycemic rats with I/R brain injury.

3.3. Effects of GFW on the expression of caspase-3, Bax and Bcl-2 in hyperglycemic rats with I/R brain injury

Fig. 4A showed the levels of protein expression as measured by Western blot. GFW could suppress caspase-3 expression in hyperglycemic rats with I/R brain injury in a dose-dependent manner (Fig. 4B, *P* < 0.001). GFW did not change Bax protein expression, but significantly enhanced Bcl-2 protein level. The Bax/Bcl-2 ratio was showed in Fig. 4C.

3.4. Effects of GFW on microglial cell viability, and NO production, iNOS, and COX-2 expression

Fig. 5 showed the effect of GFW (0.25, 0.5, 1 mg/ml) on microglial cell viability, and NO production, iNOS and COX-2 expression by Western Blot analysis. GFW (0.5, 1 mg/ml) significantly reduced NO production (Fig. 5B, *P* < 0.01, *P* < 0.001, respectively) without affecting the BV-2 cell viability (Fig. 5A). How-

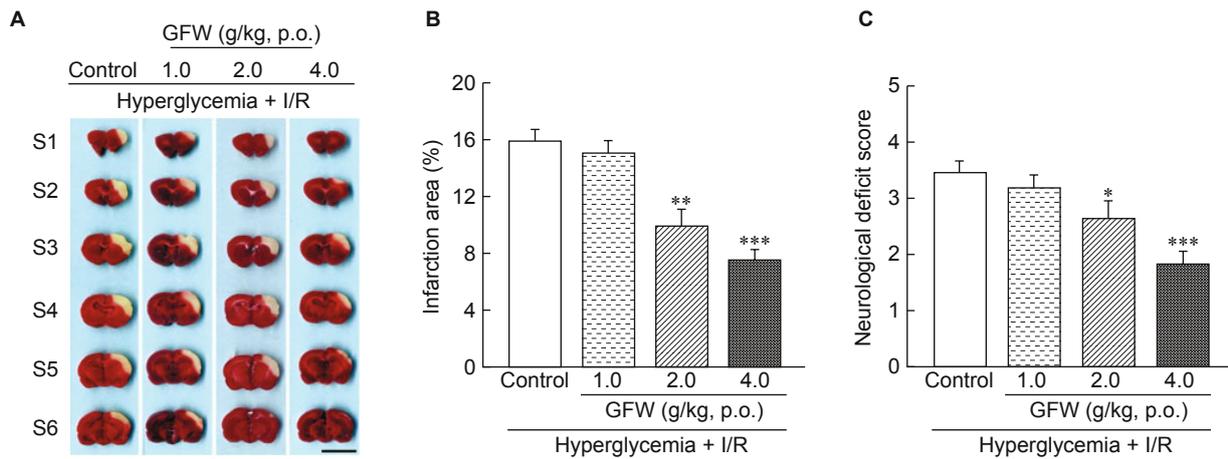


Fig. 2 - Effects of GFW on cerebral infarction area in hyperglycemic rats with cerebral I/R injury. [A] Coronal section of the brain after ischemia for 90 min and following by reperfusion for 24 h by TTC staining. Infarction areas appeared white and non-infarction areas appeared red-purple in color. S1àS6: Slices from the frontal lobe. GFW (1, 2, and 4 g/kg) was orally administered. Scale bar = 1 cm. [B] GFW quantitative data (1, 2, and 4 g/kg, p.o.) based on the infarction area. [C] Neurological deficit score in hyperglycemic rats with cerebral I/R injury. Each bar represents mean ± S.E. ** $P < 0.01$ compared to the normoglycemic group with I/R injury. *** $P < 0.01$ and **** $P < 0.001$ compared to hyperglycemic group with I/R injury. (n = 6 per group).

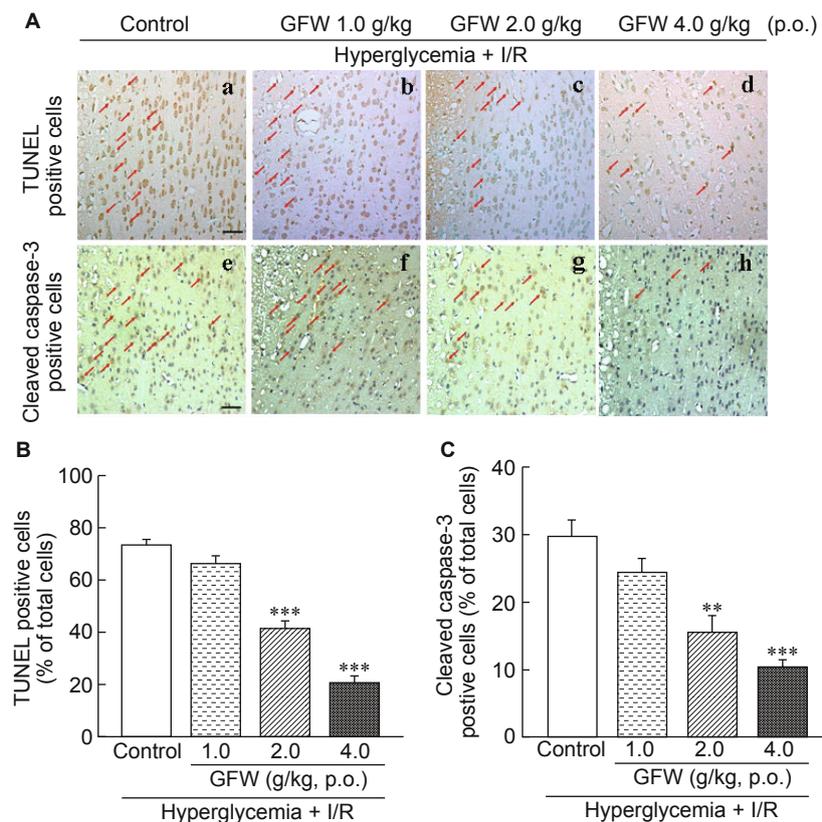


Fig. 3 - Effects of GFW on TUNEL positive cells and cleaved caspase-3 positive cells in hyperglycemic rats with I/R brain injury. GFW was orally administered and significantly inhibited TUNEL positive cells (3A: a, b, c, d) and cleaved caspase 3 positive cells (3A: e, f, g, h) in a dose-dependent manner, respectively. A : [a] and [e] Control, [b] and [f] treated with GFW 1 g/kg, [c] and [g] treated with GFW 2 g/kg, and [d] and [h] treated with GFW 4 g/kg. Bar charts of TUNEL positive cells [B] and cleaved caspase positive cells [C] were examined after ischemia for 90 min and followed by reperfusion for 24 h. in hyperglycemic rats. ** $P < 0.01$, *** $p < 0.001$ compared to hyperglycemic rats with I/R brain injury. (n = 6 per group). An arrow shows TUNEL positive cells. Scale bar = 50 μ m. Magnificance 200 \times .

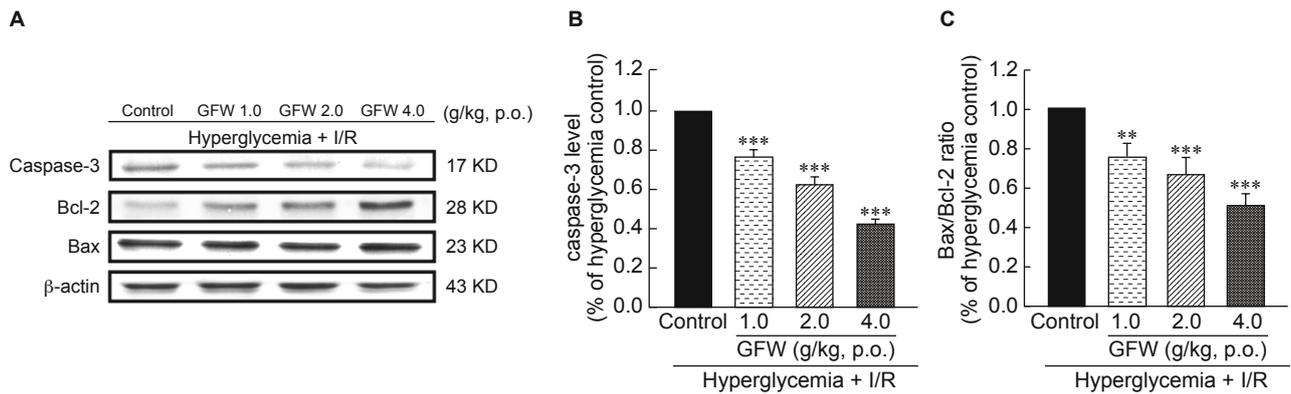


Fig. 4 - Effects of GFW on apoptosis-related protein expression [A], caspase-3 [B], and Bax/Bcl-2 ratio [C] in hyperglycemic rats with I/R injury. Bars represent mean \pm SE from six independent experiments. ** P < 0.01, * P < 0.001 compared to ischemia/reperfusion group.**

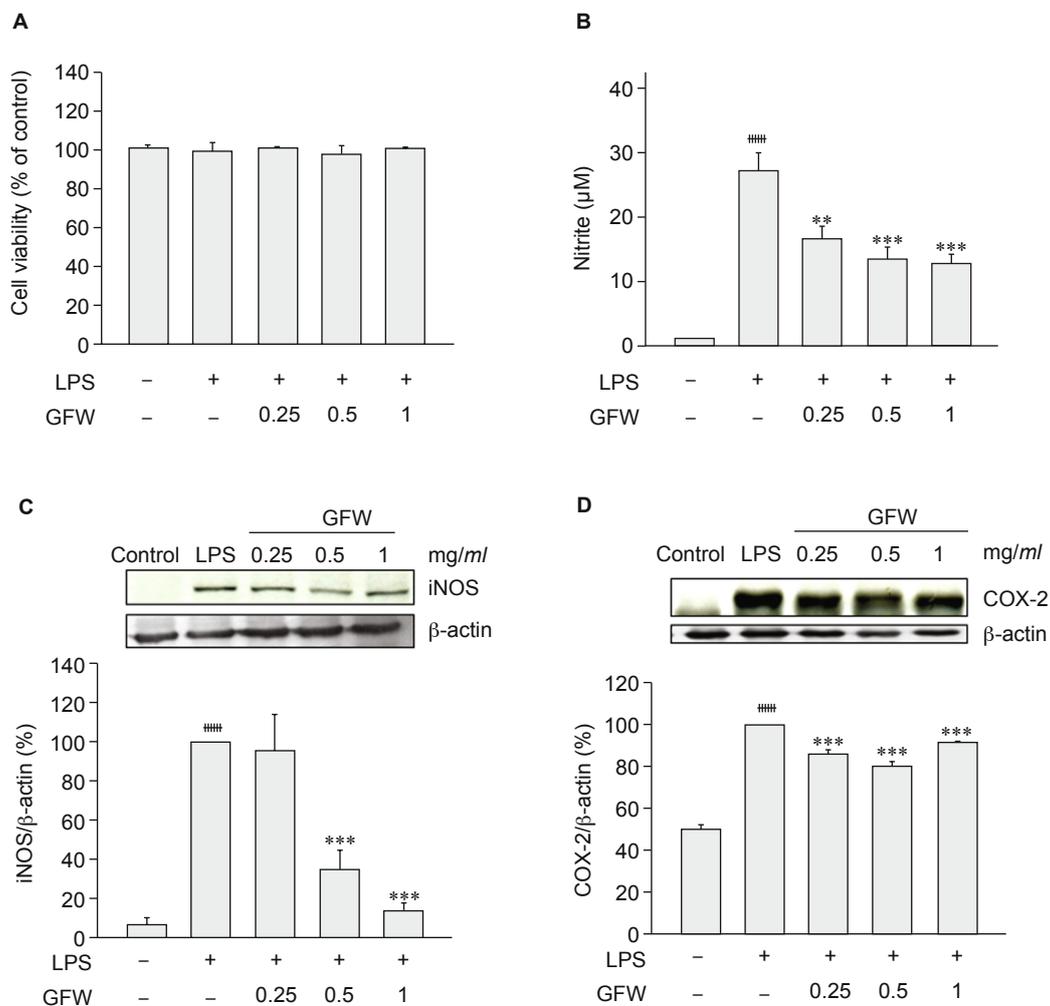


Fig. 5 - Effect of GFW on microglial cell viability, NO production, iNOS and COX-2 expression. GFW (0.25, 0.5, 1 mg/ml) significantly reduced NO production [B] without affecting the BV-2 cell viability [A] induced by LPS 0.5 μ g/ml. GFW attenuated expression of iNOS [C] and COX-2 [D] in LPS-challenged BV-2 microglial cells. BV2 cells were simultaneously treated different concentrations of GFW (0.25, 0.5, 1 mg/ml) and LPS 0.5 μ g/ml for 24 h. β -Actin was used as a control for protein loading. Data are mean \pm S.E. from more than three independent experiments. #### P < 0.001 compared with untreated group. ** P < 0.01, * P < 0.001, compared with LPS alone.**

ever, GFW could attenuate the expression of iNOS (Fig. 5C) and COX-2 (Fig. 5D) in 0.5 µg/ml LPS-challenged BV-2 microglial cells.

4. Discussion and conclusion

Cerebral ischemia and infarction are commonly associated with atherosclerotic disease and stroke in carotid and vertebrobasilar circulations [1]. Ischemic lesions divide core zone (ischemic core) and penumbra (ischemic penumbra); the latter includes the area as a mild-to-moderate reduction of cerebral blood flow. The core area is very low cerebral blood flow and produces irreversible neuronal damage [36]. Necrosis occurs mainly in the core area, apoptosis mainly distributed in the penumbra [37-39]. Cases of ischemic stroke changed penumbra gradually. The presence of high blood sugar is a significant increase in the degree of damage to the ischemic brain penumbra [40, 41]. This study focused on penumbra and assessed GFW efficacy on apoptosis in STZ-induced hyperglycemic rat brains after I/R injury, and determining apoptosis-related proteins in the penumbral area. In previous studies, stress-induced hyperglycemia is a modifiable risk factor for brain damage and associated with high mortality after stroke [6, 42]. Through diverse biochemical mechanisms, increased blood glucose concomitant with cerebral vascular injury may facilitate ischemia/reperfusion damage [43]. From the experimental results, infarcted brain tissue and noninfarcted brain tissue could be clearly observed on boundaries. Infarct area in STZ-induced hyperglycemia markedly increased.

As mentioned earlier, neurological deficit caused by I/R brain injury was associated with cerebral infarction size [44]. This study assessed neurological function: brain infarct was significantly reduced in hyperglycemic rats with I/R brain injury while treated with GFW. Greater vulnerability of the central nervous system in carotid artery ligation-induced cerebral ischemia means volume reduction of perceptual information, with disordered adaptation to environmental conditions and reproduction of conditioned reflexes [45].

Hyperglycemia-induced myocardial apoptosis also led to diabetic cardiomyopathy *via* mitochondrial cytochrome c-mediated caspase-3 activation pathway [25]. Many studies have suggested apoptosis playing an important role in cerebral ischemic pathogenesis. Diabetes following middle cerebral artery occlusion may increase the development of cerebral infarct injury and enhance apoptotic activity [46]. TUNEL stain is a standard method of DNA fragmentation associated marker with caspase-3 as another marker of apoptosis. In the Bcl-2 family, determination of anti-apoptotic proteins like Bcl-2 and pro-apoptotic ones like Bax can expedite understanding of apoptotic mechanism. Caspase-3 activation and alteration in the expression of Bax/Bcl-2 were evident in the hyperglycemic rats [47-49]. Induction of diabetes causes hippocampal neuronal cell death in which apoptosis is associated with an increasing Bax/Bcl-2 ratios as well as caspase-3 level [50]. In our previous study, hyperglycemia-induced apoptosis correlated with an increase in Bax/Bcl-2 ratio, and increased caspase-3 activity [32]. In this study, TUNEL and immunohistochemical stains averred GFW was significantly reducing TUNEL positive cells and caspase-3 protein expression in hyperglycemic rats with cerebral ischemia/perfusion injury. Western blot also showed GFW substantially attenuating caspase-3 expression and elevating Bcl-2 expression, with Bax expression unchanged. A reduced Bax/Bcl-2 ratio in STZ-induced hyperglycemia with cerebral I/

R brain injury which portends GFW reducing cerebral ischemia, infarction, and neurological deficit, possibly *via* a decrease in apoptosis. This result concurs with prior studies [47].

Traditional herbal remedies in use for thousands of years help to prevent and treat several diseases: *e.g.*, diabetes, stroke. For human health, they are still valuable and widely accepted due to various biological activity and low toxicity. GFW significantly attenuated infarct volume, neurological deficit and numbers of TUNEL as well as cleaved caspase-3 positive cells. This study is the first report to address the neuroprotective effects of GFW on apoptosis in the STZ-induced hyperglycemic rats with I/R injury.

Microglia and ischemic stroke is the double-edged sword [51]. An inflammatory response initiates within a few minutes to hours after ischemic stroke. Followed by microglia, astrocytes activation, and the production of chemoattractants, cytokines, chemokines [52-54], and subsequent infiltration of leukocytes [55, 56]. Microglial activation is the initial step of the inflammatory process in minutes [53, 57]. Two to three days following ischemia, the activation, and amplification of microglia reach the peak and continue for several weeks [58, 59]. Activated microglia, changes shape, and phenotype have the potential to phagocytose the presenting antigens, produce cytokines and matrix metalloproteinases that disrupt the blood-brain barrier [56]. Peripheral leukocytes infiltrate into the brain, further exacerbate inflammation and brain damage [51]. This study revealed GFW attenuated the infarct area of ischemic brain damage in STZ-induced hyperglycemic rats and inhibited microglial activation in LPS-induced BV-2 cells.

Based on our research data, we suggest that the neuroprotection of GFW in cerebral ischemia of STZ-induced hyperglycemic rats may be partly due to inhibition of Bax/Bcl2, caspase-3 signaling pathway, and neuroinflammation.

Acknowledgments

The authors would like to thank Ministry of Science and Technology (Taipei, Taiwan, R.O.C.) and China Medical University (Tai-chung, Taiwan, R.O.C.) for supporting this work (NSC96-2320-B-039-014-MY2, NSC-98-2320-B039-025-MY3, CMU101-ASIA-06 and CMU103-ASIA-10).

Conflicts of interest

The authors declare no conflicts of interest for this work.

Open Access This article is distributed under terms of the Creative Commons Attribution License which permits any use, distribution, and reproduction in any medium, provided original author(s) and source are credited.

REFERENCES

- [1] Hasso AN, Stringer WA, Brown, KD. Cerebral ischemia and infarction. *Neuroimaging Clin N Am.* 1994; 4: 733-52.
- [2] Tan JR, Koo YX, Kaur P, Liu F, Armugam A, Wong PT, *et al.* Mi-

- croRNAs in stroke pathogenesis. *Curr Mol Med*. 2011; 11: 76-92.
- [3] Lakhan SE, Kirchgessner A, Hofer M. Inflammatory mechanisms in ischemic stroke: therapeutic approaches. *J Transl Med*. 2009; 7: 97.
- [4] Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, *et al*. Heart disease and stroke statistics-2015 update: A report from American Heart Association. *Circulation* 2015; 131: e29-e322.
- [5] Wexle BC. Chronic diabetes followed by chronic cerebral ischemia induced by bilateral carotid artery ligation in arterosclerotic versus nonarterosclerotic rats. *Stroke* 1975; 6: 432-44.
- [6] Capes SE, Hunt D, Malmberg K, Pathak P, Gerstein HC. Stress hyperglycemia and prognosis of stroke in nondiabetic and diabetic patients: A systematic overview. *Stroke* 2001; 32: 2426-32.
- [7] Barrett-Connor E, Khaw KT. Diabetes mellitus: An independent risk factor for stroke? *Am J Epidemiol*. 1988; 128: 116-23.
- [8] Biller J, Love BB. Diabetes and stroke. *Med Clin N Amer*. 1993; 77: 95-110.
- [9] Jeerakathil T, Johnson JA, Simpson SH, Majumdar SR. Short-term risk for stroke is doubled in persons with newly treated type 2 diabetes compared with persons without diabetes. A population-based cohort study. *Stroke* 2007; 38: 1739-43.
- [10] Berry K, Wisniewski HM, Svarzbein L, Baez S. On the relationship of brain vasculature to production of neurological deficit and morphological changes following unilateral common carotid artery ligation in gerbils. *J Neurol Sci*. 1975; 25: 75-92.
- [11] Mooradian AD. Pathophysiology of central nervous system complications in diabetes mellitus. *Clin Neurosci Res*. 1997; 4: 322-6.
- [12] Ahmad A, Khan MM, Hoda MN, Raza SS, Khan MB, Javed H, *et al*. Quercetin protects against oxidative stress associated damages in a rat model of transient focal cerebral ischemia and reperfusion. *Neurochem Res*. 2011; 36: 1360-71.
- [13] Gao HM, Jiang J, Wilson B, Zhang W, Hong JS, Liu B. Microglial activation-mediated delayed and progressive degeneration of rat nigral dopaminergic neurons: relevance to Parkinson's disease. *J Neurochem*. 2002; 81: 1285-97.
- [14] Zheng LT, Ock J, Kwon BM, Suk K. Suppressive effects of flavonoid frestin on lipopolysaccharide-induced microglial activation and neurotoxicity. *Int J Immunopharmacol*. 2001; 8: 4.
- [15] Le W, Rowe D, Xie W, Ortiz I, He Y, Apple S.H. Microglial activation and dopaminergic cell injury: an *in vitro* model relevant to Parkinson's disease. *J Neurosci* 2001; 21: 8447-55.
- [16] Hemmer K, Fransen L, Vanderstichele H, Vanmechelen E, Heuschling P. An *in vitro* model for the study of microglia-induced neurodegeneration: involvement of nitric oxide and tumor necrosis factor- α . *Neurochem Int*. 2001; 38: 557-63.
- [17] Kim WG, Mohney RP, Wilson B, Jeohn GH, Liu B, Hong JB. Regional difference in susceptibility to lipopolysaccharide-induced neurotoxicity in the rat brain. Role of microglia. *J Neurosci*. 2000; 20: 6309-16.
- [18] Minghetti L. Cyclooxygenase-2 (COX-2) in inflammatory and degenerative brain diseases. *J Neuropathol Exp Neurol*. 2004; 63: 901-10.
- [19] Wang MJ, Lin WW, Chen HL, Chang YH, Ou HC, Kuo JS, *et al*. Silymarin protects dopaminergic neurons against lipopolysaccharide-induced neurotoxicity by inhibiting microglia activation. *Eur J Neurosci*. 2002; 16: 2103-112.
- [20] Bhat NR, Zhang P, Lee JC, Hogan EL. Extracellular signal-regulated kinase and p38 subgroups of mitogen-activated protein kinase regulate inducible nitric oxide synthase and tumor necrosis factor- α gene expression endotoxin-stimulated primary glial cultures. *J Neurosci*. 1998; 18: 1633-41.
- [21] Woo MS, Jang PG, Park JS, Kim WK, Joh TH, Lim HS. Selective modulation of lipopolysaccharide-stimulated cytokine expression and mitogen-activated protein kinase pathways by dibutyryl-cAMP in BV2 microglial cells. *Brain Res Mol Brain Res*. 2003; 11: 86-96.
- [22] Pawate S, Shen Q, Fan F, Bhat NR. Redox regulation of glial inflammatory response to lipopolysaccharide and interferon- γ . *J Neurosci Res*. 2004; 77: 540-51.
- [23] Xie Z, Smith CV, Van Eldik LJ. Activated glia induce neuron death *via* MAP kinase signaling pathways involving JNK and p38. *Glia*. 2004; 45: 170-9.
- [24] Waldmeier PC, Tatton WG. Interrupting apoptosis in neurodegenerative disease: potential for effective therapy? *Drug Discovery Today* 2004; 9: 210-8.
- [25] Cai L, Li W, Wang G, Guo L, Jiang Y, Kang YJ. Hyperglycemia-induced apoptosis in mouse myocardium: mitochondrial cytochrome C-mediated caspase-3 activation pathway. *Diabetes* 2002; 51: 1938-48.
- [26] Wyllie AH. Apoptosis: an overview. *Br Med Bull* 1997; 53: 451-65.
- [27] Green DR, Reed JC. Mitochondria and apoptosis. *Science* 1998; 281: 1309-12.
- [28] Yao Z, Shulan Z. Inhibition effect of Guizhi-Fuling-decoction on the invasion of human cervical cancer. *J Ethnopharmacol*. 2008; 120: 25-35.
- [29] Nakagawa T, Yokozawa T, Terasawa K, Nakanishi K. Therapeutic usefulness of Keishi-bukuryo-gan for diabetic nephropathy. *J Pharm Pharmacol*. 2003; 55: 219-27.
- [30] Goto H, Shimada Y, Sekiya N, Yang Q, Kogure T, Mantani N, *et al*. Effects of Keishi-bukuryo-gan on vascular function and hemorheological factors in spontaneously diabetic (WBN/kob) rats. *Phyto-medicine*. 2004; 11: 188-95.
- [31] Li TJ, Qiu Y, Mao JQ, Yang PY, Rui YC., Chen WS. Protective effects of Guizhi-Fuling-Capsules on rat brain ischemia/reperfusion injury. *J Pharmacol Sci*. 2007;105: 34-40.
- [32] Wu KJ, Chen YF, Tsai HY, Wu CR, Wood WG. Gueichih-Fuling-Wan, a traditional Chinese herbal prescription, ameliorates memory deficits and neuronal apoptosis in the streptozotocin-induced hyperglycemic rodents *via* the inhibition of mitochondrial apoptosis pathway. *J Evid Based Complementary Altern Med*. 2012, Article ID 656150, 11, doi 10.1155/2012/656150.
- [33] Wu KK, Huan Y. Streptozotocin-induced diabetic models in mice and rats. *Curr Protoc Pharmacol* 2008; 5.47.01-45.47.14, DOI: 10.1002/0471141755.ph0547s40
- [34] Chen YF, Wu KJ, Wood WG. Paeonia lactiflora extract attenuating cerebral ischemia and arterial intimal hyperplasia is mediated by paeoniflorin *via* modulation of VSMC migration and Ras/MEK/ERK signaling pathway. *J Evid Based Complementary Altern Med*. 2013, article ID482428, 12.
- [35] Candelario-Jalil E, Gonzalez-Falcon A, Garcia-Cabrera M, Leon OS, Fiebich BL. Wide therapeutic time window for nimesulide neuroprotection in a model of transient focal cerebral ischemia in the rat. *Brain Res* 2004;1007: 98-108.
- [36] Astrup J, Siesjo BK, Symon L. Thresholds in cerebral ischemia-the ischemic penumbra. *Stroke* 1981; 12: 723-5.

- [37] Li Y, Chopp M, Powers C, Jiang N. Apoptosis and protein expression after focal cerebral ischemia in rat. *Brain Res.* 1997; 765: 301-12.
- [38] Back T. Pathophysiology of the ischemic penumbra-revision of a concept. *Cell Mol Neurobiol.* 1998; 18: 621-38.
- [39] Li Y, Powers C, Jiang N, Chopp M. Intact, injured, necrotic and apoptotic cells after focal cerebral ischemia in the rat. *J Neurol Sci.* 1998; 156: 119-32.
- [40] Wang GX, Li G.R, Wang YD, Yang TS, Ouyang YB. Characterization of neuronal cell death in normal and diabetic rats following experimental focal cerebral ischemia. *Life Sci.* 2001; 69: 2801-10.
- [41] Li PA, Rasquinha I, He QP, Siesjo BK, Csiszar K, Boyd CD, *et al.* Hyperglycemia enhances DNA fragmentation after transient cerebral ischemia. *J Cereb Blood Flow Metab.* 2001; 21: 568-76.
- [42] Hamilton MG, Tranmer BI, Auer RN. Insulin reduction of cerebral infarction due to transient focal ischemia. *J Neurosurg.* 1995; 82: 262-8.
- [43] Lindsberg PJ, Roine RO. Hyperglycemia in Acute Stroke. *Stroke* 2004; 35: 363-4.
- [44] Garcia JH, Wagner S, Liu KF, Hu XJ. Neurological deficit and extent of neuronal necrosis attributable to middle cerebral artery occlusion in rats. Statistical validation. *Stroke* 1995; 26: 627-35.
- [45] Fateev IV, Bykov VN, Chepur SV, Pokrovskaya LA, Shemeleva NI, Vladimirova OO, *et al.* A model of cerebral circulation disorders created by staged ligation of the common carotid arteries. *Bull Ex Bio Med.* 2012; 152: 378-81.
- [46] Rizk NN, Rafols J, Dunbar JC. Cerebral ischemia-induced apoptosis and necrosis in normal and diabetic rats: Effects of insulin and c-peptide. *Brain Res.* 2006; 1096: 204-12.
- [47] Li ZG, Britton M, Sima AA, Dunbar JC. Diabetes enhances apoptosis induced by cerebral ischemia. *Life Sci.* 2004; 76: 249-62.
- [48] Brecht S, Gelderblom M, Srinivasan A, Mielke K, Dityateva G, Herdegen T. Caspase-3 activation and DNA fragmentation in primary hippocampal neurons following glutamate excitotoxicity. *Brain Res Mol Brain Res.* 2001; 94: 25-34.
- [49] Schulze-Osthoff K, Ferrari D, Los M, Wesselborg S, Peter ME. Apoptosis signaling by death receptors. *Eur J Biochem.* 1998; 254: 439-59.
- [50] Anarkooli IJ, Sankian M, Ahmadpour S, Varasteh ARZ, Haghiri H. Evaluation of Bcl-2 Family Gene Expression and Caspase-3 Activity in Hippocampus STZ-Induced Diabetic Rats. *Exp Diabetes Res.* 2008, Article ID 638467, 6; doi:10.1155/2008/638467
- [51] Patel AR, Ritzel R, McCullough LD, Liu F. Microglia and ischemic stroke: a double-edged sword. *Int J Physiol Pathophysiol Pharmacol.* 2013; 5: 73-90.
- [52] Barreto G, White RE, Ouyang Y, Xu L, Giffard RG. Astrocyte: targets for neuroprotection in stroke. *Cent Nerv Syst Agents Med Chem.* 2011; 11: 164-73.
- [53] Denes A, Vidyasagar R, Feng J, Narvainen J, McColl BW, Kauppinen RA, *et al.* Proliferating resident microglia after focal cerebral ischemia in mice. *J Cereb Blood Flow Metab.* 2007; 27: 1941-53.
- [54] Wang Q, Tang XN, Yenari MA. The inflammatory response in stroke. *J Neuroimmunol.* 2007; 184: 53-68.
- [55] Jin R, Yang G, Li G. Inflammatory mechanism in ischemic stroke: role of inflammatory cells. *J Leukoc Biol.* 2010; 87: 779-89.
- [56] Iadecola C, Anrather J. The immunology of stroke: from mechanisms to translation. *Nature Med.* 2011; 17: 796-808.
- [57] Lai AY, Todd KG. Microglia in cerebral ischemia: Molecular actions and interactions. *Canadian J Physiol Pharmacol.* 2006; 84: 49-59.
- [58] Thomas WE. Brain macrophages: evaluation of microglia and their functions. *Brain Res Rev.* 1992; 17: 61-74.
- [59] Lalancette-Hebert M, Gowing G, Simard A, Yuan CW, Kriz J. Selective ablation of proliferating microglial cells exacerbates ischemic injury in the brain. *J Neurosci.* 2007; 27: 2596-605.