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International Journal of Current Research
Vol. 6, Issue, 04, pp.6277-6287, April, 2014

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

MODULATION OF INFLAMMATORY AND OXIDATIVE MECHANISMS BY GINGKO BILOBA EXTRACT IN AN IN VITRO MURINE MODEL OF ISCHEMIA TO AUGMENT NEUROPROTECTION AND CELLULAR SURVIVAL

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ARTICLE INFO

Article History:
Received 15th January, 2013
Received in revised form
13th February, 2014
Accepted 03rd March, 2014
Published online 23rd April, 2014

Key words:

Ischemia,
Inflammation,
Tumor Necrosis Factor (TNF-a),
Oxygen glucose deprivation (OGD),
Stroke,
Caspase-3,
Gingko biloba,
iNOS,
Therapeutic.

ABSTRACT

Cerebral ischemia initiates a cascade of detrimental events including glutamate associated excitotoxicity, membrane lipid degradation, DNA damage, formation of reactive oxygen species and acute inflammation, which lead to the disruption of cellular homeostasis and structural damage of ischemic brain tissue. Inflammation is increasingly recognized to be the key element in pathological progression of ischemic stroke. Therefore, reducing oxidative stress and down regulating the inflammatory response are options that merit consideration as potential therapeutic targets for ischemic stroke. Consequently, agents capable of modulating both elements will constitute promising therapeutic solutions but as of now no such therapies have been translated to clinical application. Hence we hypothesized that contribution of oxidative and inflammatory response through TNF receptors in the course of ischemic cell death and its modulation by natural herbs may be of prime significance. Natural agent Gingko biloba extract (GBE) has been previously reported to be useful in the treatment of various cardiovascular diseases and neuronal degeneration. We used an in vitro murine model of ischemia (PC-12 cells exposed to oxygen–glucose deprivation[OGD]/reperfusion) and investigated the effects of this natural agent in neuroprotection and cell survival after ischemic injury.We observed that the treatment of cultures with GBE significantly increased cell viability and decreased NO generation in a dose-dependent manner. GBE (50 and 100 ug/ml) treatment of OGD exposed cellsmarkedly reduced the injury associated with increases in inducible nitric oxide synthase (iNOS), and active caspase-3 protein expressions as well as the mRNA expression of TNF-a. This effect may be partially mediated by the inhibition of TNF-a activation, followed by the inhibition of inflammatory responses in PC12 cells. Therefore, GBE treatment may represent a novel approach for lowering the risk of or improving function in ischemia-reperfusion brain injury-related disorders.

INTRODUCTION

Stroke is the third cause of death and leading cause of chronic disability throughout the world, affecting 15 million people each year [1]. Stroke is associated with disruption of the blood flow to the brain with rapid depletion of cellular energy and glucose, resulting in ionic disturbances which inturn initiate a cascade of processes. Stroke occurs due to reduced perfusion to a brain region, resulting in death or permanent neurological deficits including hemiplegia, numbness, loss of sensory and vibratory sensation, balance problems, ptosis, decreased reflexes, visual field defects, apraxia, and aphasia due to neuronal damage of pathways of the central nervous system (CNS), including the brain stem or cerebellum[2].

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Stroke induces production and release of cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1ß [IL-1ß], interleukin-6 (IL-6), [3,4]and inducible nitric oxide synthase (iNOS), by a variety of activated cell types; endothelial cells, microglia, neurons, leukocytes platelets, monocytes, macro-phages and fibro-blasts [5]. Cerebral ischaemia results in the loss of blood supply followed by a cascade of events including glutamate excitotoxicity, calcium overload, oxidative stress and inflammation, leading eventually to cell death by both necrosis and apoptosis. Many of the molecules involved in this complex series of biochemical events are potential therapeutic targets for the development of effective treatment for stroke [6-8]. Studies have shown that mechanisms including apoptosis, necrosis, inflammation, immune modulation and oxidative stress may lead to the development of the ischemic cascade. Recent advances in the stroke medicine have highlighted the role of acute transitory inflammation in the cellular pathology

following ischemic stroke [9]. Inflammation plays a key role in cerebral ischaemic injury [10-11]. Elevated levels of reactive oxygen species (ROS), generated by the cessation of cerebral blood flow, stimulate cells to secrete cytokines and chemokines which subsequently cause the secondary ischaemic damage [12]. It is imperative that acute inflammation might potentiate or perturb the already initiated exitotocxicity.

TNF-a has been predicted to be increased in the early as well as the late phases of ischemic stroke and preclinical studies have suggested a favourable outcome [13]. The advent of inflammation with the proven oxidative conditions can exacerbate the extent of neuronal death. Increasing evidence, from both clinical as well as experimental studies, suggests a major role for inflammation in the secondary injury events like activation of noxious cycles of inflammation, oxidant stress, and apoptosis that ultimately result in delayed death of neurons. Focal ischemia evokes a robust inflammatory response that begins within a few hours of onset and typifies the secondary or delayed response to ischemia. TNF-α is also upregulated in the brain after ischemia and plays a vital role in the execution of inflammatory cascade after ischemia. TNF-α is increased within a few hours of middle cerebral occlusion in rats. Protein expression is initially observed in neurons and later in glia [14-15].

TNF-α is a pleiotropic cytokine produced by many cell types, and is involved in blood-brain barrier, inflamm-atory, thrombogenic, and vascular changes associated with brain injury. TNF-α over-expressing rats had larger infarcts than wild-type at 24 h and seven days after cerebral ischemia [16]. Inhibition of TNF-α using antimurine-TNF-α antibody also reduced ischemic brain injury in mice [17] and TNF-a factor binding protein was protective against stroke in mice [18]. TNF-α may also protect the brain from ischemic injury by invoking ischemic tolerance probably via TNF receptor upregulation [19-20] as mice deficient in TNF receptors have larger infarcts [21]. SoInflammation plays a key role in cerebral ischaemic injury [3-4, 10-11]. Elevated levels of reactive oxygen species (ROS), generated by the cessation of cerebral blood flow, stimulate cells to secrete cytokines and chemokines which subsequently cause the secondary ischaemic damage [22]. This is characterized by the infiltration of leucocytes and microglial activation. Recent advances in the stroke medicine have highlighted the role of acute transitory inflammation in the cellular pathology following ischemic stroke [23]. It is imperative that acute inflam- mation might potentiate or perturb the already initiated exitotocxicity. Therefore, natural agents/drugs that have a wide spectrum of inhibitory actions on inflammation may be useful in rescuing neuronal cells, exposed to ischaemia. Ginkgo bilobaextract (GBE or EGb761), a defined complex mixture containing 24% ginkgo flavone glycoside and 6% terpenlactones (ginkgolides, bilobalide) that is extracted from Ginkgo biloba leaves, has commonly been used as a therapeutic agent for cardiovascular and neurological disorders [24]. It is then suggested that the pharmacological effects of GBE are closely related to its antioxidant ability to scavenge free radicals [25-26]. Ginkgo biloba contain compounds like kaempferol, quercetin and isoharmnetin which posses an antioxidant character[27]. The antioxidant activity of a ginkgo extract is determined mainly by flavonoids, which scavenge and destroy free radicals and the reactive forms of oxygen [28]. The activated oxygen forms such as peroxide, hydrogen peroxide, hydroxyl radical and singlet oxygen have been shown to cause inflammation [29]. Clinical studies showed that Ginkgo extracts exhibit therapeutic effects in a variety of diseases including Alzheimer's disease, memory loss, cerebral and ocular blood flow occlusion, premenstrual problems and altitude sickness [30]. Moreover, it has been previously shown that GBE could reduce cytokine-stimulated endothelial adhesiveness by downregulating NF-kB and AP-1 activation [31]. This supportsthe notion that the natural compound Ginkgo biloba may have potential implications in clinical stroke. In the present study, we examined the detailed mechanisms underlying the inhibitory effects of GBE on inflammatory, oxidative and apoptotic responses induced by OGD in PC12 cells. We also studied the contribution of inflammation through TNF receptors in the course of ischemic cell death and its modulation by GBE.

MATERIALS AND METHODS

Cell culture

PC-12 cells, a rat pheochromocytoma cell line were cultivated in Dulbecco's Modified Eagle's Medium [DMEM], supplemented with 0.2% sodium bicarbonate, 100 units/ml penicillin G sodium, 100 µg/ml streptomycin sulfate, 0.25 µg/ml amphote-ricin B, 2.5% fetal bovine serum and 15% horse serum. Cultures were maintained at 37^0C in 5% CO2–95% atmosphere under high humid conditions. Medium was changed twice a week and cultures were split at a ratio of 1:6 once a week. Cells were assessed for cell viability by assessing the loss of membrane integrity by Trypan blue dye exclusion test. Only the batches showing more than 95% viability were used in experiments during the study.

Ischemic stroke induction in PC-12 cells

Oxygen glucose deprivation as an invitro model of stroke was developed as per Singh *et al*. 2009 [32]. Briefly, cells were collected by centrifugation at 600 rpm for 5 min followed by two washings with sterile 1X PBS with physiological PH (pH 7.4), cells were re-suspended in culture medium having all the standard components except glucose, and allowed to grow at 37 °C in hypoxic condition (5%-CO_2, 94%-N_2, 1%-O_2) with high humid atmospheric conditions for 6 h. Immediately following the OGD injury for 6 hours, fresh medium containing glucose (final concentration 4.5 mg/ml) was added to the cultures and were incubated under normoxic conditions (reoxygenation) for an additional time period up to 24 h of total experiment duration. Control cultures were maintained under normoxia (regular oxygen atmospheric conditions).

Preparation of ethanol extract from Ginkgo biloba leaves

The standardized *Ginkgo biloba* leaf extract EGb761 used in experiments was from Schwabe Pharmaceuticals (Karlsruhe, Germany). And the Ginkgo bilobaextract (GBE) was prepared by cutting the leaves into 2–5 mm pieces followed bydrying. After which, leaves (100 g) were mixed with a solvent (900 ml;

96% v/v aqueous ethanol), refluxed gradually for 30 min in Soxhlet extractor and left aside to cool overnight at an ambient temperature. The suspension was filtered over Büchner funnel and the residue was washed twice, each time with 100 ml of the same solvent. The filtrates were combined and the solvent was evaporated under reduced pressure. The extract of a dark green greasy consistency was transferred in a conical flask filled with argon and stored in the dark in a refrigerator at –20 °C. During the extraction and sample preparation, the laboratory glass was covered with aluminium foil to protect the extract against the light.

MTT reduction assay for viability

In brief, cells were seeded in poly-L-lysine pre-coated 96-well culture plates and allowed to adhere properly for 24 h at 37◦C in a CO2 incubator (5% CO2–95% atmosphere at high humidity). Then the medium was aspirated and replaced with glucose-free medium and exposed to OGD injury for 6h as described earlier. Cells after OGD were exposed to 20 µl of MTT stock solution (5mg/ml). This was followed by incubation for 1 h and when purple-colored precipitates were visible under the microscope, medium was carefully discarded. For solubilization of formazan crystals (MTT formazon) 200 µl of DMSO was added to each well and cells were incubated in dark at room temperature for 2 h. The purple colour of formazan crystals developed was measured at 570 nm by a microplate reader.

Flow cytometry using Annexin V/PI staining

For the quantitative assessment of apoptosis, Annexin V-FITC and PI double staining, followed by flow cytometry was used. Cells after harvesting from the cultures were stained using Annexin V-FITC and a PI double-staining kit (BD USA) in accordance with the manufacturer's protocol. Cells were then analyzed immediately using flow cytometry (FACS BD USA). The signals from apoptotic cells were localized in the lower right quadrant of the resulting dot-plot graph.

Measurement Nitrite

As an indicator of nitric oxide production, the amount of nitrite accumulated in culture supernatant was determined with a colorimetric assay using Griess reagent [1% sulfanilamide, 2.5% H3PO4, 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride]. Equal volumes of culture supernatant (50µl) and Griess reagent were mixed and incubated for 10 min at room temperature in the dark. The absorbance at 540 nm was determined with the SpectraMax Plus microplate spectro-photometer. The concentration of nitrite in samples was determined from a sodium nitrite standard curve.

Measurement of ROS production

The production of intracellular ROS was measured by using ROS dependent fluorescence of 2′,7′-dichlorofluorescin diacetate (DCFDA). To monitor intracellular accumulation of ROS, the fluorescent probe DCF-DA was used according to the method described by Le Bel *et al*. 1992 [33]. At the indicated time points, the treated cells wereincubated with 5µM DCF-DA for 30 min at 37°C. Cells were subsequently washed twice with HBSS and collected. DCF fluorescence intensity of 100µl cell suspension was quantified with a fluorometer (GENios, USA) using 485 nm excitation and 530 nm emission on a fluorescence plate reader. The results are given as percents relative to the oxidative stress of the control cells set to 100%. All experiments were performed in triplicate.

Measurement of lipid peroxidation

Malonyl dialdehyde [MDA], as the most abundant lipid peroxidation product from PC12 cells, was measured by the thiobarbituric acid colorimetric assay as developed by Mihara and Uchiama 1978 [34]. Briefly, after treatment, the cells were washed 3X with ice-cold HBSS and then scraped in PBS containing 0.5mMEDTA and 1.13mM butyl hydroxytoluene. The cell lysis was performed by means of six cycles of freezing and thawing. One milliliter of 10% [w/v] trichloroacetic acid was added to 450 µl of cellular lysate. After centrifugation at 3,000 rpm for 10 min, 1.3 ml of 0.5% [w/v] thiobarbituric acid was added, and the mixture was heated at 100°C for 20 min. After cooling, MDA formation was recorded (optical density=530 and 550 nm) in a PerkinElmer spectrofluorimeter, and the results were calculated and represented as percentage of control cells. All experiments were performed in triplicate.

Flow Cytometric Detection for apoptosis

Apoptotic cells were quantified by determining DNA content of cells by propidium iodide staining by flow cytometry developed by Telford *et al*. 1991 [35]. Briefly, after experimental treatment, the cell pellets were resuspended in ice cold 70% ethanol and fixed at 4°C for 24 h. The cells were then centrifuged, and ethanol was removed by washing thoroughly with PBS. The cell pellets were resuspended in 1 ml DNA staining reagent containing 50 µg/ml RNase, 0.1% triton X-100, 0.1 mM EDTA (pH 7.4), and 50µg/ml PI. The staining was stable at 4°C for 30 min. Red fluorescence (DNA) was detected through a 563–607 nm bandpass filter by using a FACS 440 flow cytometer (Becton Dickinson). In flow cytometric histograms, apoptotic cells gave DNA fluorescence in the sub-diploid regions, which were well separated from the normal G1 peak. Ten thousand cells in each sample were analyzed, and the percentage of apoptotic cell accumulation in the sub-G1 peak was calculated. Each measurement was carried out in triplicate.

Nuclear Staining for Apoptosis

Hoechst 33258 was used to assess DNA fragmentation as a marker for apoptosis. PC12 cells were grown in 24-well plates on poly-L-lysinecoated coverslips. After differ-rent treatment for 24 h, cells were fixed with 4% paraformaldehyde in PBS (120 mM NaCl, 19 mM Na2HPO4, 6 mM KH2PO4), pH 7.4, for 30 min. Cells were washed with PBS and permeabilized with 0.1% Triton X-100 in PBS for 10 min and washed again. Coverslips were incubated with Hoechst 33258 (60 ng/ml) for 10 min. Slides were rinsed briefly with PBS, air-dried, then mounted in antifluorescein fading medium (Perma Flour, Immunon, PA, USA). Fluorescence microscope (BX50-FLA, Olympus) was used for analysis. Cells with condensed nuclei

were scored as apoptotic. The percentage of apoptotic cells in relation to the total number of cells was determined from 10 random fields per slide, from three independent experiments.

Bcl-2 and Bax Protein Expression by flowcytometry

The level of Bcl-2 and Bax protein was measured by flow cytometry as described in (Liu and Zhu 1999). Briefly, cells were collected after treatment for the indicated time points and washed with cold PBS. After fixation with 2% paraformaldehyde for 30 min and permeabilization with 0.5% Triton X-100, cells were incubated with primary antibodies for Bcl-2 or Bax for 20 min, respectively, followed by incubation with corresponding fluorescein isothio- cyanate conjugated secondary antibodies for 20 min at room temperature in the dark. After the cells were washed with PBS, the antigen density was measured by flow cytometry. Cell Quest software was used for analysis of flowcytometric data.

iNOS and Caspase-3 protein expression

Briefly PC12 cells were exposed to OGD injury for 3h and then after reperfusion were incubated in fresh medium and treated with [100 ug/ml] GBE for 24hrs. Then cells treated with GBE were collected, homogenized and lysates were centrifuged. The supernatant (50 µg protein) of each sample was then subjected to SDS-PAGE, and transferred onto PVDF membranes for analysis of iNOS expression. After incubation in blocking buffer and being washed three times with TBST buffer [10 mM Tris-base, 100 mM NaCl, and 0.1% Tween 20; pH 7.5], the blots were treated with an anti-iNOS monoclonal antibody (mAb;1: 3000, BD Biosciences, CA US), and an anti-active caspase-3 pAb (1: 250; Biovision, Mountain View, CA US), or an anti-α-tubulin mAb (1: 2000; Santa Cruz Biotech, CA US) in TBST buffer overnight. Blots were subsequently washed with TBST and incubated with secondary HRP-conjugated goat anti-mouse mAb (Amers-ham) for 1 h. The blots were then washed, and immunoreactive protein was detected using film exposed to enhanced chemilumi-nescence detection reagents [ECLsystem; Amersham]. α-tubulin was used to demonstrate equal loading in each lane.

Isolation of total RNA and RT-PCT for TNF-a mRNA expression

Briefly PC12 cells were exposed to OGD injury for 3h and then after reperfusion were incubated in fresh medium and treated with [100 ug/ml] GBE for 24hrs. Then cells treated with GBE were collected, homogenized and lysate were centrifuged followed by analysis of TNF-a mRNA expression by RT-PCR. Total RNA was isolated from cells by a commercially available kit according to the manufacturer's instructions (TRIzol, Gibco). For each RT-PCR, 0.5 mg of the RNA sample and 0.2 uM of primers were reverse-transcribed and amplified in a 50-ul reaction mixture of commercially available reagents (SUPERSCRIPT One-Step RT-PCR with PLATINUM Taq Kit, Invitrogen) and the amplification was done in a thermal cycler (GeneAmp PCR system 2400, PerkinElmer). For visualization and quantification by densitometry of each RT-PCR, a 10-ul aliquot was subjected to electrophoresis on a 1.5% agarose gel using a mini horizontal submarine unit [HE 33] containing 0.5 mg/ml ethidium bromide to allow UV-induced fluorescence. To normalize the amount of the cDNA template used in each PCR reaction GAPDH level was used.

Statistical analysis

In vitro experiments were all carried out in triplicate and conducted at least three times in order to ensure accuracy of experimental results and conclusions. Parametric data packages from SPSS were used for analysis (Student t-test). The main effects of treatment and time, as well as interactions among the two experimental factors was evaluated using a mixed-model analysis of variance [MANOVA] for each measure.A p value of < 0.05 was considered to be statistically significant.

RESULTS

Gingko biloba Extract (GBE) isneuro-protective against OGD induced toxicity in PC-12 cells

Some earlier studies have shown that the auto-oxidation of flavonoids produces semiquinone radicals and superoxide anion and higher concentrations of Gingko biloba extract

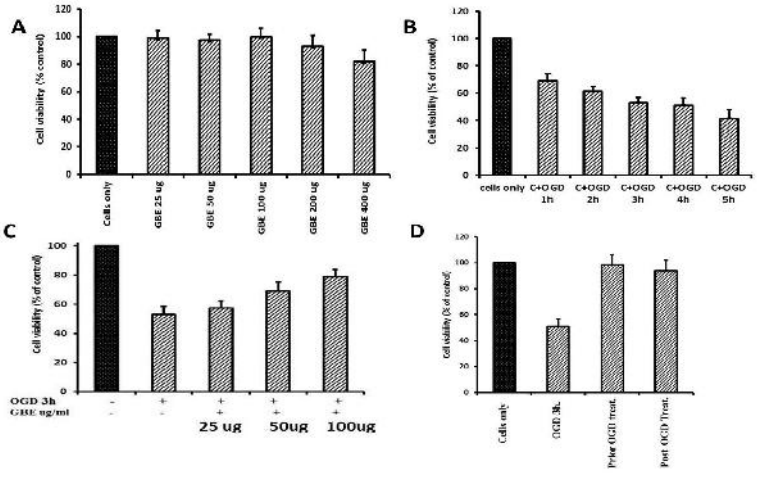

Figure 1[A-D] Treatment of PC12 with Gingko biloba [GBE] inhibits OGD-induced cell injury. A PC12 cells were pretreated with different concentrations of GBE alone for 24 h, and then cell viability was examined by MTT assay. B PC12 cells were exposed to different time periods for OGD injury , and then cell viability was examined by MTT assay. C PC12 cells after OGD injury for 3h were incubated with fresh medium and treated with different concentrations of GBE for 24h and cell viability was checked by MTT assay. D PC12 cells were either [Prior OGD injury] pretreated with 100ug/ml GBE for 3h and then OGD injury was given for three hours and cells were then incubated for 24 h in fresh medium and cell viability was checked by MTT assay or [post OGD injury] cells were given OGD injury for 3h and then incubated in fresh medium with GBE for 24h and cell viability was checked by MTT assay. The data are shown as means ± SEM of three independent experiments. #$p<0.05$ compared to the non-treated cells and *$p<0.05$ relative to OGD-treated cells

[GBE] could be cytotoxic to the PC12 cells due to the more generation of ROS during auto-oxidation [Hodnick *et al*.1986, 1998; Miura *et al.*, 1998]. Hence we first determined the safe/optimal dose of GBE on PC12 cell viability. There was no notable change in the cell viability of PC12 cells with 25–100µM of GBE for 24 h and high dosages of GBE [200, 400 µg] decreased cell viability in nontreated PC12 cells [Fig.1A]. Therefore 100µM of GBE dose was employed for the subsequent experimental paradigms. Next we optimised the time period for the OGD injury to PC12 cells by exposing the PC12 cells to different periods of OGD injury and found that 3h time of OGD is optimum to induce neurotoxicity [Fig. 1B]. Next we checked the effect of GBE extract on cell viability after OGD injury and found that GBE was rescuing the OGD injuryed PC12 cells in a dose dependant manner [Fig 1C]. Further we compared the effect of GBE on PC12 cells prior and post OGD injury and found that GBE is neuroprotective against OGD induced toxicity in both the cases [Fig 1D]. GBE with a concentration of 100 µM provided maximal protection against OGD injury producing a 50% increase in cell survival. Therefore, in all further experiments, we used the optimum time of 3h of OGD injury to PC-12 cells with GBE 100µM]. So we observed that GBE is neuroprotective against OGD induced toxicity.

MTT assay. C PC12 cells after OGD injury for 3h were incubated with fresh medium and treated with different concentrations of GBE for 24h and cell viability was checked by MTT assay. D PC12 cells were either [Prior OGD injury] pretreated with 100ug/ml GBE for 3h and then OGD injury was given for three hours and cells were then incubated for 24 h in fresh medium and cell viability was checked by MTT assay or [post OGD injury] cells were given OGD injury for 3h and then incubated in fresh medium with GBE for 24h and cell viability was checked by MTT assay. The data are shown as means ± SEM of three independent experiments. #p<0.05 compared to the non-treated cells and *p<0.05 relative to OGD-treated cells.

GBE Attenuates OGD-Induced Increase in Intracellular

ROS Level: OGD-induced oxidative stress was measured both as ROS formation and as quantification of lipid peroxidation of cell membrane. Although ROS formation and Malonyl dialdehyde [MDA], as the most abundant lipid peroxidation product from PC12 cells, was measured by the thiobarbituric acid colorimetric assay [34]. MDA were detectable at 2h and 4h after treatment, respectively, but maximal effect was observed at 12 h. Therefore, we evaluated the effect of GBE at 12h. As shown in Fig.2a, when PC12 cells were exposed to OGD injury, the intracellular ROS level significantly increased [about three fold] compared with untreated cells as indicated by the increase in RFU, revealing that OGD enhanced ROS production in PC12 cells. Treatment of [50–100µg] GBE obviously attenuated an increase in ROS caused by OGD in a concentration-dependent manner. Meanwhile, as shown in Fig.2b, the exposure of cells to OGD increased LPO by approximately four fold relative to non-OGD -exposed control cells.

Treatment of cells with [50–100µg] GBE decreased OGD-induced LPO in PC12 cells significantly in a concentration-dependent manner. And the antioxidative effects of GBE with high concerntration [200µg] were not significant compared with 100µg GBE. When given alone, 100µg GBE did not alone induce the intracellular ROS accumulation and LPO in OGD-exposed cells. Moreover, as shown in Figure 2[C], the pretreatment of PC12 cells with GBE to show maximum inhibition of ROS generation after OGD injury was dependent on the duration of GBE pretreatment. The data shown in Fig.1a and Fig 1b, shows the antioxidant abilities of GBE to reduce OGD induced intracellular ROS and LPO in PC12 cells.

GBE Prevents OGD-Induced Apoptosis in PC12 Cells

As observed in the previous experiment that GBE could inhibit ROS generation and LPO we next investigated the ability of GBE on cellular apoptosis by FACS analysis to estimate DNA

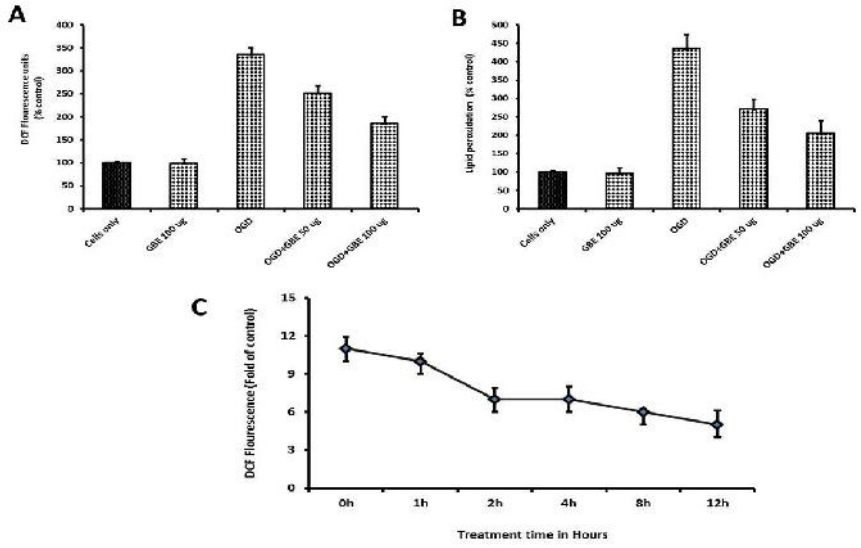

Figure 2. Effect of GBE on intracellular ROS level and LPO in PC12 cells following OGD challenge. PC12 cells were exposed to OGD injury for 3h and then incubated for 12h with 50 and 100ug of GBE. [A] ROS levels were measured by FACS analysis of DCF-DA stained PC12 cells. [B] Lipid peroxidation [LPO] levels were measured by analysis of MDA. [C]PC12 cells were incubated [pretreatment] with 100ug/ml of GBE for the indicated time period before OGD injury and after the indicated time points cells were exposed to OGD injury for 3h and then incubated in fresh medium for 24h. Intracellular ROS production was measured at excitation wavelength of 485nm and emmision wavelength of 530nm using Flourescence micro plate reader. The data were expressed as means ± SEM of the percentage of untreated control cells from three independent experiments. #p<0.05 compared to the nontreated cells and *p<0.05 compared to the OGD-alone-treated cells by Student'st test.

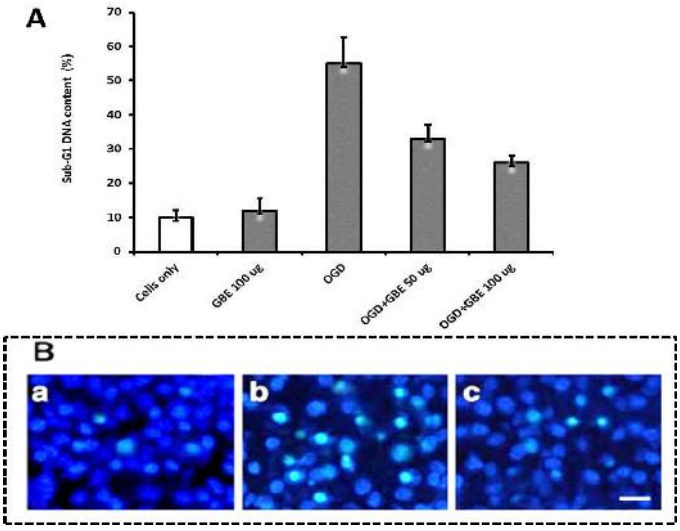

Fig. 3. Effect of GBE treatment on OGD-induced apoptosis in PC12 cells. [A] PC12 cells were pretreated with different concentrations of GBE [50-100ug/ml] and after OGD injury were incubated in fresh medium for 24 h, DNA fragment was assessed by FACS analysis. The percentage of apoptotic cell accumulation in the sub-G1 peak was calculated by analyzing ten thousand cells in each sample. #p<0.05 compared to the non-treated cells and *p<0.05 compared with the OGD alone-alone-treated cells [Student's t test]. B Morphological analysis of nuclear chromatin in by Hoechst 33258. Fig 3B a Control conditions. b After exposure to OGD, cells displayed condensed chromatin and apoptotic nuclei. c Cells treated with 100µg GBE and OGD did not obviously exhibit such nuclear condensation. Scale bars=10µm.

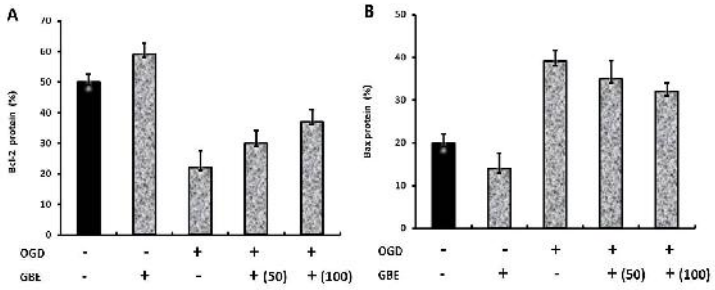

Fig 4. Effects of GBE treatment on the expression of Bcl-2 and Bax in OGD-exposed PC12 cells. PC12 cells after OGD injury were treated with GBE [50 and 100ug/ml] and then incubated for 12h and the protein expression of [A] Bcl-2 and [B] Bax were analyzed by flow cytometry. Data were expressed as mean values ± SEM of triplicate experiments. #p<0.05 compared to the nontreated cells and *p<0.05 compared with the OGD-alone-treated cells [Student's t test]

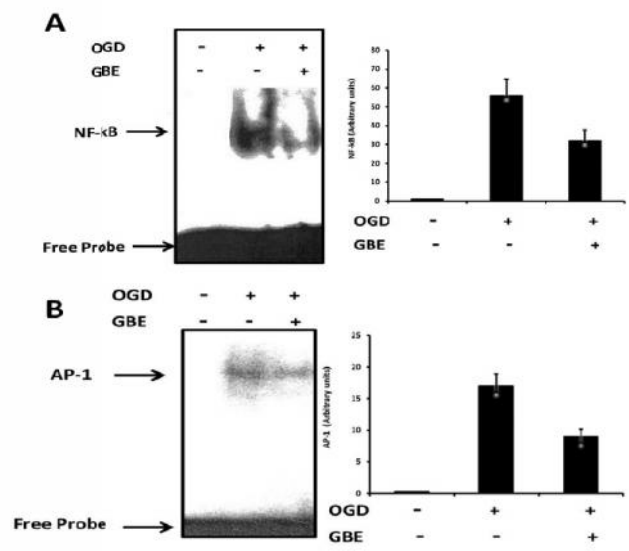

Figure 5. Inhibitory effects of GBE on OGD–induced NF-kB [A] or AP-1 [B] activation in PC12. Cellswere preincubated for 12 hours with 50ug/mL GBE followed by 3h OGD injury. Nuclear protein extracts were prepared, and a gel shift assay was performed using radiolabeled oligonucleotides containing consensus NF-kB or AP-1 binding sequences. Unlabelled oligonucleotide control experiments were also done separately to confirm that the presence of bands is specific to NF-kB or AP-1. The results are from 3 separate experiments. *P<0.05 compared with OGD effect.

fragmentation, which are also called sub-G1 cells as an index of apoptosis. As shown in Fig. 3a, OGD injury dramatically increased the degraded sub-G1 DNA content characteristic of apoptosis cells compared with untreated cells. The increase of DNA fragment by OGD exposure was reduced by treatment with [50–100μg] GBE in a concentration dependent manner. This was further confirmed by the nuclear staining assay using chromatin dye Hoechst 33258. Figure 3b shows a typical image of vehicle-treated cells with round intact nuclei. In contrast, OGD exposed cells showed bright nuclear fragmentation typical of apoptosis and GBE pretreatment inhibited the morphological changes significantly. Since the DNA fragment in an individual cell indicates the occurrence of apoptotic cell death, these observation suggested that GBE was capable of preventing OGD-induced cell apoptosis.

Ginkgo Biloba Extract Inhibits OGD induced Activation of NF-kB and AP-1

The results of EMSA showed that OGD exposure of PC12 cells resulted in the appe-arance of both NF-kB [Figure 3A] and AP-1 [Figure 3B] shifted bands. These bands were specific for NF-kB or AP-1 binding, because they were undetectable when a 100-fold excess of unlabeled NF-kB or AP-1 oligonucleotide was included [Figure 3C]. Pretreatment with GBE [100 ug/mL] significantly reduced the densities of NF-kB shifted bands induced by OGD injury;P<0.05][Figure 3A]. GBE could also significantly reduce the AP-1 shifted bands induced by OGD.

Effects of GBE on iNOS protein expre-ssion in PC12 cells after OGD injury

When PC-12 cells after OGD injury were assessed for iNOS protein expression showed that OGD injury in PC12 induced the iNOS production but upon treatment with GBE [50ug/ml] significantly reduced the iNOS protein expression as compared to controls. Results of Western blotting of OGD injured PC12 cells are shown in Fig.3 GBE [50 mg/kg] treatment Significantly [P < 0.05] suppressed the expression of iNOS in OGD injured cells [Fig. 3, lane 3]. In Figure 3, the iNOS band, was detected as a major band of approximately 135 kDa, showed significant increases in OGD injured PC12 as compared to controls. With the treatment of GBE [50 mg/kg], iNOS expre-ssion was markedly reduced.

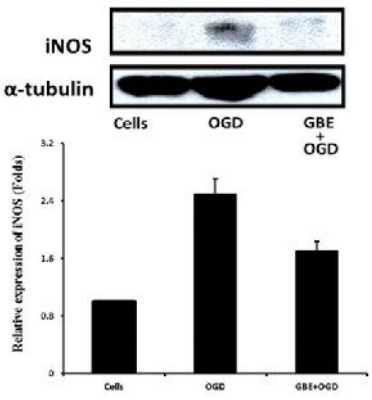

Fig. 6 Effects of GBE on the expressions of inducible nitric oxide synthase [iNOS] in PC12 cell after OGD injury. PC-12 cells after OGD injury for 3h were incubated with GBE [50ug/ml] in fresh medium for 24hrs. Cells were seperately collected, homogenized and centrifuged. The supernatant [50 ug protein] was then subjected to SDS-PAGE, and transferred onto membranes for analysis of iNOS expressions. The results are representative examples of three similar experiments. Data are presented as the means ± S.E.M. **P < 0.01 compared to the cells only [lane 1]; # P < 0.05 and ## P < 0.01 compared to the OGD injuryed cells [lane 2]. Equal loading in each lane is demonstrated by similar intensities of α-tubulin.

Effect of GBE on active Caspase-3 protein expression and TNF-a mRNA expression in OGD PC12 cells

In addition, negative immunostaining was obtained for active caspase-3 in the sham-operated group [Fig. 7A, lane 1]. At 24 h after OGD-reperfusion, strong staining of active caspase-3 [17 kDa] was observed in ischemic cerebral tissues [lane 2] compared to levels obtained in the corresponding area of the control cells [lane 1]. Again, GBE [50 ug/ml] abolished the elevation of active caspase-3 [Fig. 7A, lane 3].Transient OGD resulted in a significant and more-sustained increase in the expression of TNF-a mRNA in the injured OGD cells compared to controls [Fig. 7B].

DISCUSSION

Cerebral ischemia initiates a cascade of detrimental events including glutamate-associated excitotoxicity, membrane lipid degradation, DNA damage, intracellular calcium accumulation, formation of reactive oxygen species (ROS), and acute inflammation, which lead to the disruption of cellular homeostasis and structural damage of ischemic brain tissue. Therefore, reducing oxidative stress (OS) and downregulating the inflammatory responseare options that merit consideration as potential therapeutic targets for ischemic stroke. Consequently, agents capable of modulating both elements will constitute promising therapeutic solutions because clinically effective neuroprotectants have not yet been discovered and no specific therapy for stroke is available to date. This inflammatory reaction involves accumulation of neutrophils, monocytes and leukocytes in the ischemic brain in animal models and in human focal stroke. TNF-α a pleiotropic pro-inflamma-tory cytokine is rapidly upregulated in the brain after injury and acts by binding to its receptors, TNF-R1 (p55) and TNF-R2 (p75), on the cell surface. One of the first indications that TNFα is an important mediator of stroke is the correlation of its expression with stroke damage. But the development of a variety of approaches to prevent this post-ischemic inflammation shows great promise to augment therapies aimed at reperfusion. As of now, no such therapies have been translated to clinical application, but the treatment of inflammation in stroke remains a viable target for drug development.

Many recent studies have shown that oxidative stress is a major cause of cellular injuries in a variety of human diseases including neurodegenerative disorders [36]. Reactive oxygen species such as hydrogen peroxide, superoxide anion, and hydroxyl radical could damage biological molecules, which can ultimately lead to apoptotic or necrotic cell death [37-38]. Thus, removal of excess reactive oxygen species or suppression of their generation by antioxidants may be effective in preventing oxidative cell death.

Recently, researchers have made considerable efforts to search for natural antioxidants with neuroprotective potential [39-40]. Gingko biloba extract is known to have antioxidant, anticancer,

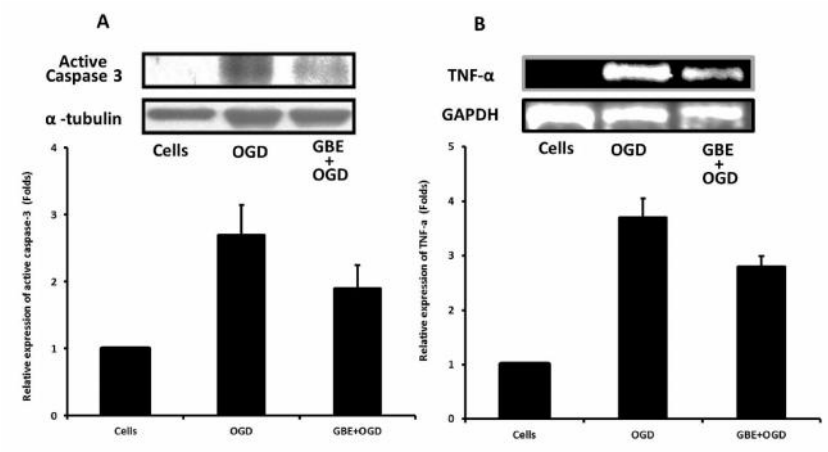

Fig 7. Effects of GBEon the expressions of [A] active caspase-3 protein and [B] tumor necrosis factor [TNF]-α mRNA in PC12 cells after OGD injury:[A] PC12 cells were exposed to OGD injury and then treated with [100 ug/ml] GBE and incubated in fresh medium for 24hrs. Then cells treated with GBE collected, homogenized, and centrifuged. The supernatant [50 µg protein] was then subjected to SDS-PAGE, and transferred onto membranes for analysis of active caspase-3 expression. The results are representative examples of three similar experiments. Data are presented as the means ± S.E.M. *P < 0.05 compared to the cells only [lane 1]; # P < 0.05 compared to the OGD exposed cells [lane 2]. Equal loading in each lane is demonstrated by similar intensities of α-tubulin. **[B]** PC12 cells were exposed to OGD injury and then treated with [100 ug/ml] GBE and incubated in fresh medium for 24hrs. Then cells treated with GBE collected, homogenized, and centrifuged. The supernatant [50 µg protein] was then subjected to SDS-PAGE, and transferred onto membranes for analysis of active caspase-3 expression. The results are representative examples of three similar experiments followed by analysis of TNF-α mRNA expression by RT-PCR. The GAPDH level was used to normalize the amountof the cDNA template used in each PCR reaction. The results are representative examples of three similar experiments.

and anti-inflammatory effects, and it has been reported that GBE can protect neuronal cells from oxidative stress. In this study, we first provided evidence that pretreated cells with GBEresulted in resistence to OGD toxicity in PC12 cells. The results indicate that GBE significantly attenuated OGD-induced neurotoxicity through the inhibition of oxidative stresses and regulation of the expression of Bcl-2 protein family. Our present studies confirmed that exposing PC12 cells to OGD injuryresulted in a dose dependent viability loss and pretreated with different concentrations of GBE greatly decreased the cell viability loss. These results indicated that GBE pretreatment did significantly protect PC12 cells from OGD-induced cytotoxicity. Many studies have shown that elevated ROS and LPO levels are linked to injurious effects such as loss of fluidity, inactivation of membrane enzymes, increases in permeability to ions, and eventually disruption of cell membrane leading to release of cell organelles [41-42]. Our results confirmed that OGD exposure could cause increase of ROS production and lipid peroxidation. Meanwhile, pretreatment with GBE could reduce the ROS formation and LPO in cells following OGD exposure, as compared with that in the control. Taken together, these results suggest that GBE may exert neuroprotective effect through an antioxidant pathway, which is in agreement with the result of other reports that have indicated that antioxidant strategies may protect cells from OGD-induced toxicity. Excessive reactive oxygen species also cause DNA damage by directly attacking DNA or by activation of endonucleases that degrade DNA, which ultimately contribute to apoptotic or necrotic cell death [43]. So, we next explored whether GBE has a protective effect against neuronal cell apoptosis. Exposure of PC12 cells to OGD triggered DNA damage as assessed by a DNA-sensitive dye with a flow cytometer, showed a significant sub-G1 peak in the histograms, indicating the presence of an apoptotic component in OGD-induced cell injury. GBE exhibited more potent protection against OGD-induced PC12 apoptosis, which indicates that the antioxidant defense of the PC12 cells was reinforced, at least in part, by.

Bcl-2 family is important proteins which are involved in both the positive and negative regulation of cell apoptosis [44]. Among them, Bcl-2 and Bcl-XL are antiapoptotic, while Bax, Bcl-Xs, Bad, Bak, and Bik are proapoptotic. The balance of pro- and antiapoptotic proteins may be critical to the survival of individual neurons. For example, increased Bax and/or lowered Bcl-2 expression has been shown to reduce mitochondrial membrane potential and increase reactive oxygen species production in neurons, which have been defined as early events in theprocess of apoptosis [45]. Bcl-2 was also found to inhibit lipid peroxidation and oxidative DNAand/or protein damage elicited by various proapoptotic stimuli capable of producing reactive oxygen intermediates [46-47]. Other studies demonstrated that Bcl-2 could act as channel proteins in the mitochondrial membrane. It is conceivable that the channel property of Bcl-2 or Bax may control the mitochondrial permeability transition or other early mitochondrial perturbation [48-49]. And Bax may facilitate the passage of some important proteins, such as cytochrome c or other apoptosis-inducing factors that trigger the activation of caspase cascade and result in apoptosis [50-51]. In agreement with the previous studies, our current study demonstrated a remarkable increase of the Bax and decrease of Bcl-2 protein levels in PC12 cells after OGD exposure and treatment with GBE, suggesting that the downregulation of Bcl-2 or upregulation of Bax altered mitochondrial membrane permeability triggered mitochondrial cytochrome c release to cytosol and promoted apoptosis. Pretreatment with GBE significantly attenuated OGD-induced upregulation of Bax/Bcl-2 ratios, suggesting that GBE could protect PC12 cells against OGD-induced apoptosis and the modulation of apoptosis-related gene expression might at least partly contribute to the antiapoptotic effect of GBE. In summary,

GBE could ameliorate OGD-induced oxidative stress and apoptosis in PC12 cells. The protective effects of GBE were related to inhibition of ROS production and LPO and regulation of apoptosis related gene expression of Bcl-2 and Bax first, and these regulations might adjust the mitochondrial membrane permeability, attenuate cytochrome c release to cytosol.

Conclusion

Gingko biloba extract [GBE] has been previously has been reported to be useful in the treatment of various cardiovascular diseases. In conclusion this study demonstrates that OGD injury induces iNOS, and active caspase-3 protein expressions, and TNF-a mRNA expression, which may represent the response of neurons suffering from ischemic injury. In this study, we observed that elevations of active caspase-3 and iNOS expressions could be significantly suppressed by GBE. In addition, TNF-a which is one of the key immunomodulatory and proinflammatory cytokines upregulated during ischemia [52]. Administration of TNF-a during ischemic brain injury has been shown to augment the injury, as evidenced by increased tissue damage and neurological deficits. In addition to inflammation, TNF-a has also been shown to be involved in apoptosis [53]. Here we demonstrated that GBE can downregulate the transcription of TNF-a during ischemia. Therefore, inhibition of active caspase-3 expression by GBE may occur, at least partially, through the inhibition of TNF-a expression in ischemic injury. This effect may be mediated, at least in part, by the inhibition of TNF-alpha activation, followed by the inhibition of inflammatory responses (i.e., iNOS expression) and apoptosis formation (active caspase-3) resulting in a reduction in OGD induced injury in PC12 cells. Therefore, GBE treatment may represent a novel approach for lowering the risk of or improving function in ischemia-reperfusion brain injury-related disorders.

The most important findings of this study suggest that the neuroprotective effect of GBE on PC12 cells in OGD-induced injured cells is probably mediated by the inhibition of TNF-a, followed by the inhibition of inflammatory responses (i.e., iNOS) and apoptosis (active caspase-3). The rationale for the use of GBE is based on the fact that multiple deleterious processes in different cell types of organelles are initiated during ischemia-reperfusion injury which ultimately synergistically moves toward irreversible injury. Therefore, treatment using GBE is not limited to one factor but involves many mechanisms, most of which may be interrelated. For example, GBE-induced neuroprotection is related to inflammation, NO, and apoptosis. We speculate that the correction of these molecules and morphological changes may lead to neurobehavioral improvement in patients; thus, treatment using GBE may represent an ideal approach for improving function after ischemia-reperfusion injury.

Disclosure/conflict of interest

The authors declare no conflict of intrest.

Acknowledgement

This research was entirely supported by the Sheikh Abdullah Bin Tuwaijiri Chair for Applied Research in Stroke (SATSC), under Majmaah University.We would like to thank Dr. Naser Bin Ali Al-Jarallah for his kind support through out the study.

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