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Dose-dependent neuroprotective effect of oriental phyto-derived glycyrrhizin on experimental neuroterminal norepinephrine depletion in a rat brain model

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ABSTRACT

The dose-dependent neuroprotective role of licorice-derived glycyrrhizin during subacute neuroterminal norepinephrine (NE) depletion was studied in rat brain. Experimental design included thirty 5-week-old male rats randomly divided into five groups. Compared to the saline-injected control group, the group receiving daily *intraperitoneal* injection of fusaric acid (FA; 5 mg/kg/b.w.) for 30 days showed pharmacological depletion of NE. The neuroprotective effects of three successively increasing oral doses of glycyrrhizin were examined in FA-treated rats. Neurochemical parameters and histo-/immunohistopathological changes in the hippocampus were examined. FA generated global hippocampal stress with altered neurobiochemical parameters, accompanied by immune-confirmed inflammatory tissue damage, and noticeable behavioral changes. Although glycyrrhizin after FA-induced intoxication did not correct the recorded drop in the NE level, it decreased the dopamine levels to control levels. Similarly, glycyrrhizin at a high dose restored the serotonin level to its normal value and blocked the FA-induced increase in the level of its metabolite, 5-hydroxyindoleacetic acid. The FA-induced rise in γ-aminobutyric acid (GABA) and histamine was alleviated after administration of a high dose of glycyrrhizin. This was accompanied by improvements in the bioenergetic status and neuronal regenerative capacity through recovery of ATP and brain-derived neurotrophic factor levels to the pre-intoxicated values. High doses of glycyrrhizin also ameliorated the FA-generated behavioral changes and oxidative damage, manifested by the reduction in the expression of cortical pro-apoptotic caspase 3 in the same group. This study suggests that glycyrrhizin can potentially mend most of the previously evoked neuronal damage induced by FA intoxication in the brain of an experimental rat model.

1. Introduction

Licorice root (*Glycyrrhiza glabra*) is mostly known as a popular flavoring agent in the food industry. Licorice extracts have been used in herbalism and traditional medicine with a long history of medicinal uses ("Licorice root". National Center for Complementary and Integrative Health, US National Institutes of Health. September 2016. Retrieved 20 December 2017). For centuries, licorice, the root of *Glycyrrhiza glabra*, has been applied towards several medical purposes, including wound healing, anti-ulcerative, anti-inflammatory and anti-bacterial effects [1]. Currently, its supplements are mainly used for digestive disorders and ulcers [2]. Recently, more than 100 compounds were detected in *Glycyrrhiza glabra* by comprehensive two-dimensional liquid chromatography-mass spectrometry [3], these included two

Abbreviations: NE, norepinephrine; FA, fusaric acid; GABA, γ-aminobutyric acid; b.w., body weight; GJCs, gap junction channels; CNS, central nervous system; MAPK, mitogen activated protein kinase; DA, dopamine; 18β-GA, 18β-glycyrrhetinic acid; 5HT, 5-hydroxytryptamine; NAc5HT, N-acetylserotonin; 5HIAA, 5-hydroxyindoleacetic acid; 5HTOL, 5-hydroxytryptophol; NODCAR, National Organization for Drug Control and Research; IACUC, Institutional Animal Care and Use Committee; HPLC, high-performance liquid chromatography; PBS, phosphate buffered saline; DAB, 3,3′-diaminobenzidine tetrahydrochloride; *i.p.*, *intraperitoneally*; OFT, open field test; MDA, malondialdehyde; AChE, acetylcholinesterase; 8-OHDG, 8-hydroxydeoxyguanosine; BDNF, brain-derived neurotrophic factor; H & E, haematoxylin and eosin; GLM, general linear model; Cx43, connexin 43; SOD, Superoxide dismutase

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Table 1
The effect of glycyrrhizin dose levels on hippocampal monoamines in fusaric acid-induced neurotoxicity in brain rat model.

Groups	Parameters			
	NE (μg/g tissue)	DA (μg/g tissue)	5HT (μg/g tissue)	5HIAA (μg/g tissue)
Control	0.448 ± 0.012a	1.65 ± 0.045b	0.354 ± 0.01a	0.098 ± 0.003b
FA	0.184 ± 0.005c	2.94 ± 0.095a	0.205 ± 0.006c	0.201 ± 0.005a
FA + LL	0.167 ± 0.004c	1.88 ± 0.051b	0.255 ± 0.007bc	0.175 ± 0.005 ab
FA + LMD	0.198 ± 0.005c	1.79 ± 0.048b	0.301 ± 0.008 ab	0.166 ± 0.005b
FA + LHD	0.284 ± 0.008b	1.68 ± 0.048b	0.344 ± 0.01a	0.109 ± 0.003b

Data are expressed as Mean ± S.E. for 6 rats/group.
a, b, c. Having different superscript letters in the same column indicates significant difference ($P < 0.05$).

Table 2
The effect of glycyrrhizin dose levels on hippocampal amino acids in fusaric acid-induced neurotoxicity in brain rat model. Data are expressed as Mean ± S.E. for 6 rats/group.

Groups	Parameters		
	GABA (μg/g tissue)	GLU (μg/g tissue)	His (μg/g tissue)
Control	6.84 ± 0.187c	3.61 ± 0.103a	2.088 ± 0.057c
FA	9.21 ± 0.251a	3.98 ± 0.108a	3.937 ± 0.107a
FA + LL	8.41 ± 0.237a	3.629 ± 0.096a	2.72 ± 0.072bc
FA + LMD	7.3 ± 0.196b	3.713 ± 0.104a	2.022 ± 0.056c
FA + LHD	6.54 ± 0.175c	3.119 ± 0.089a	1.929 ± 0.054c

a, b, c. Having different superscript letters in the same column indicates significant difference ($P < 0.05$).

major alkaloid families of glycosylated flavanones and triterpene saponins. In addition to its enrichment with isoliquiritigenin, which blocks the production of dopamine with possible antidote activity against cocaine abuse, licorice is enriched in glycyrrhizin and its aglycone glycyrrhetinic acid, which are associated with its major biological activities [4,5]. The considerable amount of 18β-glycyrrhetinic acid (18β-GA) in licorice has multiple beneficial and therapeutic effects. Glycyrrhetinic acid can block 11β-hydroxysteroid dehydrogenase with a concomitant reduction in cortisol levels [6].

In the gut, glycyrrhizin is hydrolysed to 18β-GA prior to further conjugation to form 3β-monoglucuronyl-18β-glycyrrhetinic acid in the liver [7]. The consumption of mycotoxin-contaminated cereals, *e.g.,* corn or rice, as staples increases the risk of different types of illnesses, including cancer and neurodegenerative diseases. Mycotoxins are highly toxic secondary products of the metabolism of some fungi mainly belonging to *Aspergillus*, *Penicillium*, and *Fusarium* spp. [8]. *Fusarium* toxins, beauvericin, fusaproliferin, moniliformin, and fusaric acid (FA) are among the 300 fungal metabolites potentially toxic to humans and animals. FA is a toxic metabolite produced by numerous *Fusarium* species in maize and other cereal grains including barley, wheat millet, and sorghum, and a phytotoxin that likely inhibits dopamine (DA) β-hydroxylase (the enzyme that converts DA to norepinephrine (NE)) in addition to inhibiting cell proliferation and DNA synthesis [9,10]. FA and its analogues have also been reported to be quorum-sensing inhibitors [11]. Early reports suggested that FA, the neglected mycotoxin, induces a stimulatory dopaminergic effect on the enzyme responsible for the conversion of serotonin, or 5-hydroxytryptamine (5HT), to pineal N-acetylserotonin (NAc5HT), with a concomitant decrease in pineal 5HT and increase in pineal DA [12]. The neurochemical effects of FA were previously reported to alter brain and pineal neurotransmitters in a rat model with increases in brain 5HT, 5-hydroxyindoleacetic acid, tyrosine, and DA levels and concomitant decreases in NE levels. In the pineal gland, however, FA induces an increase in DA with a parallel decrease in NE. NAc5HT is also increased, whereas, levels of pineal 5HT and its two major metabolites, 5-hydroxyindoleacetic acid (5HIAA) and 5-hydroxytryptophol (5HTOL), decreased. The reported elevation in brain and pineal DA levels and the decrease in NE might explain the inhibitory effect of FA on both tyrosine hydroxylase and DA-β-hydroxylase, respectively [13]. On a molecular basis, FA dose-dependently induces oxidative stress in the moderately vulnerable hepatocellular carcinoma (HepG2) cell line with accelerated lipid peroxidation and concomitant intracellular ATP depletion, which disrupts mitochondrial biogenesis and activates apoptosis [14].

Thus, the possible neuroprotective effects of licorice-derived glycyrrhizin need to be resolved. In the present study, we investigate the possible protective effects of glycyrrhizin against FA-induced neuronal injury in rat brain hippocampus, the most vulnerable regulatory organ of emotions, memory and spatial navigation.

2. Material and methods

2.1. Animals

Experiments were performed on weaning rats (Sprague Dawley) aged 5 weeks weighing 100–120 g housed in cages (6/cage) under controlled conditions. Animals were supplied from the animal house of the National Organization for Drug Control and Research (NODCAR), Giza, Egypt. The animals were fed *ad libitum* and allowed to acclimate to the environment for two weeks before the experiment. The animals were housed at 22 ± 2 °C with light/dark cycles. Animal use and care for the experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of NODCAR as well as the Cairo University Animal Care and Use Committee (approval number CU/II/F/60/18).

2.2. Materials

Glycyrrhizic acid (glycyrrhizin) was purchased from Xian Plant Bio-Engineering Co. (100% natural disodium glycyrrhizinate 99%). All other chemicals, solvents and reagents were purchased from Sigma and were of HPLC grade.

2.3. Identification and characterization of glycyrrhizic acid by HPLC

Chromatographic conditions Column: Hypersil, ODS, C18 mobile; mobile phase A: 0.05 mol/L ammonium acetate, mobile phase B: acetonitrile; gradient elution: 15 min: 32% B; 30–60 min: 75% B. Wavelength: 250 nm. Flow rate: 2 mL/min; Temperature: 30 °C, injection volume: 20 μL [15].

2.4. Experimental design

Thirty male rats were used in the study and were randomly divided into five groups of equal number with the same average weight. The study lasted for 30 days. Group I was the control group. These rats received saline solution (0.5 mL/100 g of body weight (b.w.)) at the same volume as the drug solutions given the other groups. Group II served as the positive group and received FA injected *intraperitoneally*

Table 3
The effect of glycyrrhizin dose levels on hippocampal nitric oxide radical (NO), antioxidant defense enzymes (SOD and TAC), lipid and DNA peroxidation in fusaric acid-induced neurotoxicity in brain rat model.

Groups	Parameters				
	NO (μmole/g tissue)	SOD (U/g tissue)	TAC (nmole/g tissue)	MDA (nmol/g tissue)	8OHdG (pg/g tissue)
Control	0.363 ± 0.014d	58.36 ± 2.108a	19.674 ± 0.679a	38.32 ± 1.045c	284 ± 7.61c
FA	0.665 ± 0.023a	41.95 ± 1.389d	14.384 ± 0.553c	49.62 ± 1.355a	331 ± 9.058a
FA + LL	0.478 ± 0.015b	39.54 ± 1.314d	12.129 ± 0.454c	44.26 ± 1.255b	342 ± 9.725a
FA + LMD	0.424 ± 0.012bc	44.36 ± 1.535c	14.51 ± 0.49c	34.62 ± 0.916d	306 ± 8.122bc
FA + LHD	0.391 ± 0.013cd	51.6 ± 1.603b	16.946 ± 0.522b	36.87 ± 1.045cd	311 ± 8.313b

Data are expressed as Mean ± S.E. for 6 rats/group.
a, b, c.: Having different superscript letters in the same column indicates significant difference ($P < 0.05$).

(*i.p.*) at a dose of 5 mg/kg b.w. daily for 30 days according to Chaouloff [12]. Group III was given low doses of glycyrrhizin dissolved in distilled water (100 mg/kg b. w.) + FA for 30 days. Group IV was given glycyrrhizin at a medium dose (200 mg/kg b. w.) + FA for 30 days. Group IV was treated with a high dose of glycyrrhizin (300 mg/kg b. w.) + FA for 30 days. Rats were anaesthetized with thiobarbital (30 mg/kg b. w.) *i. p.* prior to sacrifice by cervical dislocation, and brain cortex and hippocampus samples were recovered.

2.5. Behavioral testing

The open field test (OFT) has been long used as an appropriate test for measuring situational neurodegeneration in rodents. The rats were taken to a test room in their home cages, and tested for 3 min once at a time. The open field apparatus was constructed and handling was done according to previous studies [16]. Parameters extracted from OFT were identified as including: grooming-the duration time which animal spent licking or scratching it while stationary; rearing-the frequency of standing on their hind legs in the maze; center square duration-the duration time of rats spent in the central square; peripheral square duration-the duration time of rats spent in the peripheral square; urination-the number of puddles or streaks of urine; and defecation-the number of fecal boli produced.

2.6. Biochemical and neurochemical parameters

The monoamine and amino acid levels in the brain hippocampus were determined by chromatography [17]. Cellular lipid peroxidation was monitored by a chromatographic method for the detection of malondialdehyde (MDA), and acetylcholinesterase (AChE) activity in tissue homogenate of the collected hippocampus and was monitored using a colorimetric method [18]. The concentration of nitric oxide radicals was estimated as a sum of nitrite and nitrate via HPLC to evaluate possible FA-induced oxidative stress [19]. The endogenous antioxidant, SOD, was spectrophotometrically measured as a percent of inhibition against pyrogalol-induced yellow colour development [20].

Table 4
The effect of glycyrrhizin dose levels on hippocampal cell energy in fusaric acid-induced neurotoxicity in brain rat model. Data are expressed as Mean ± S.E. for 6 rats/group.

Groups	Parameters		
	ATP (μg/g tissue)	ADP (μg/g tissue)	AMP (μg/g tissue)
Control	56.98 ± 1.60a	18.67 ± 0.522a	6.3 ± 0.168bc
FA	23.5 ± 0.651d	15.36 ± 0.414b	8.76 ± 0.24a
FA + LL	20.9 ± 0.567d	13.95 ± 0.388b	7.26 ± 0.197 ab
FA + LMD	29.92 ± 0.834c	18.14 ± 0.479a	5.58 ± 0.158c
FA + LHD	37.18 ± 1.06b	14.15 ± 0.395b	6.72 ± 0.192bc

a, b, c. Having different superscript letters in the same column indicates significant difference ($P < 0.05$).

The total enzymatic and nonenzymatic antioxidant defense system [total antioxidant capacity (TAC)] was determined by ferric reducing antioxidant power (FRAP) assay using spectrophotometry [21]. Global DNA damage was monitored as a function of the 8-hydroxydeoxyguanosine (8-OHDG) levels using a previously described chromatographic method [22]. The bioenergetic status of the brain hippocampus was screened by a chromatographic method for the detection of energy currency of different adenosine phosphate levels (levels of ATP, ADP and AMP) by the methods described in our early study [23]. The fluctuation in the level of brain-derived neurotrophic factor (BDNF) was estimated using a rat-specific immunoassay kit.

2.7. Histopathological and immunohistochemical analysis

Light microscopic examination was used to examine two brain areas (the cortex and hippocampus). The brain tissues were collected from the different groups, fixed in 10% neutral buffered formalin and then processed to obtain 4-μm-thick paraffin-embedded sections. The sections were stained with haematoxylin and eosin (HE) and then examined under a microscope [24]. A compiled immunohistochemistry analysis of the level of expression of caspase-3 was performed on the hippocampus according to the methods described in a previous report [25]. Brain tissue sections were de-paraffinized in xylene and rehydrated in graded alcohol. Hydrogen peroxide (Thermo Scientific, USA) was added to block endogenous peroxidase activity. Antigen retrieval was performed by pre-treatment of the tissue sections with 10 mM citrate in a microwave oven for 10 min. Sections were then incubated for 2 h with the primary antibody, rabbit *anti*-caspase-3 polyclonal antibody (Cat. No. PA1-29157, Thermo Fisher Scientific Co., USA), at a 1:2000 dilution in Tris-HCl, pH 7.6, followed by a rinse in phosphate buffered saline (PBS) and further incubation with the goat anti-rabbit IgG heavy and light chain horseradish peroxidase conjugate (ab205718; Abcam, Cambridge, UK) for 10 min. After another rinse in PBS, the sections were finally incubated with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma). The slides were counterstained with haematoxylin and then mounted. Primary antibodies were replaced

Table 5
The effect of glycyrrhizin dose levels on hippocampal BDNF and AChE in fusaric acid-induced neurotoxicity in brain rat model.

Groups	Parameters	
	BDNF (ng/g tissue)	AChE (U/g tissue)
Control	43.95 ± 1.196a	5.45 ± 0.147a
FA	17.69 ± 0.483d	1.78 ± 0.047e
FA + LL	22.95 ± 0.619d	2.32 ± 0.066d
FA + LMD	29.36 ± 0.776c	3.16 ± 0.084c
FA + LHD	38.51 ± 1.031b	4.75 ± 0.128b

Data are expressed as Mean ± S.E. for 6 rats/group.
a, b, c. Having different superscript letters in the same column indicates significant difference ($P < 0.05$).

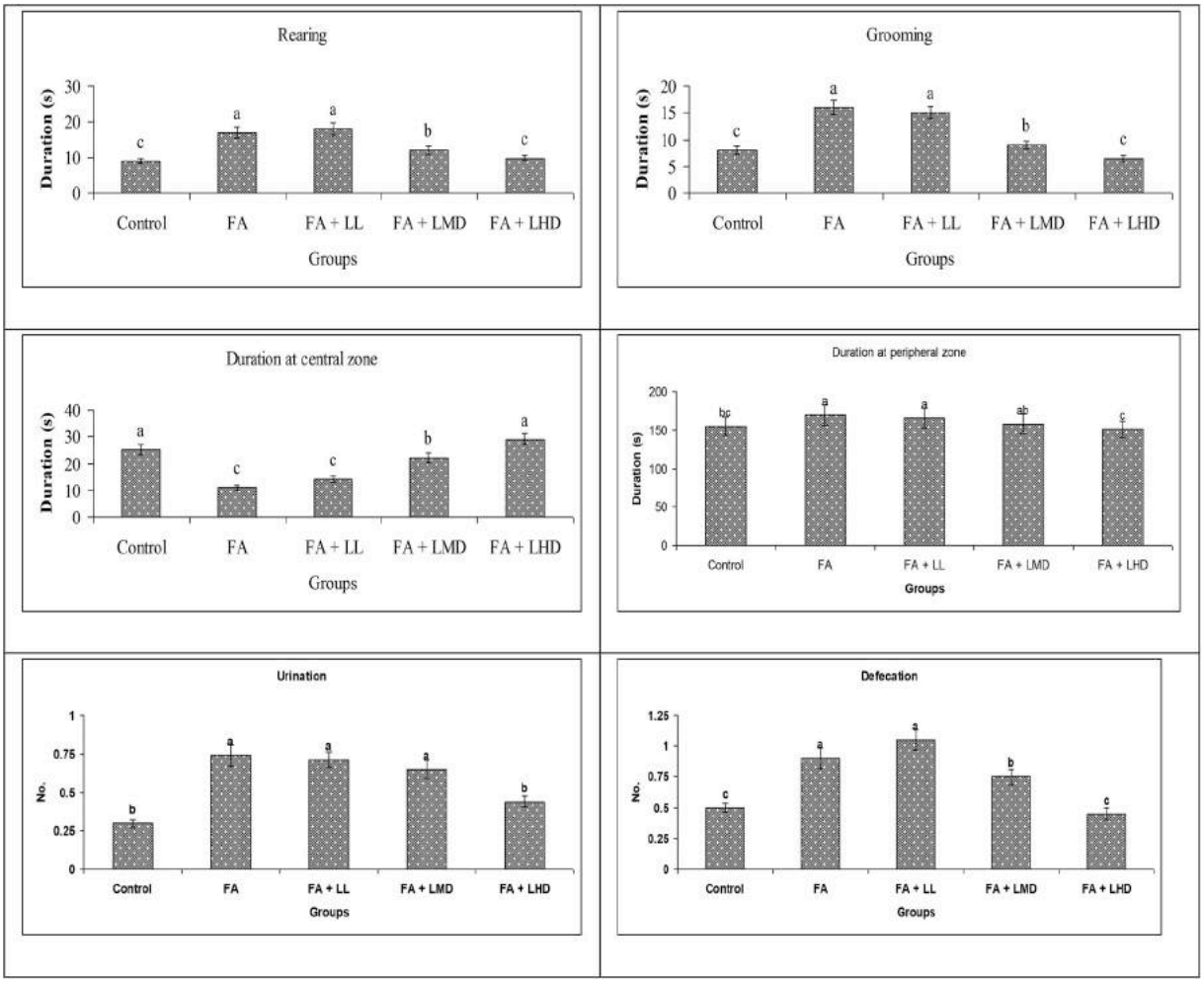

Fig. 1. The effect of glycyrrhizin dose levels on open field test in fusaric acid-induced neurotoxicity in the rat brain model.
FA induced rat hyperactivity confirmed by an open field behavioral test with a significant increase in rearing, grooming, defecation, and urination, and duration time in a peripheral zone. In contrast FA showed markedly decrease in duration in a center zone. Low dose of glycyrrhizin plus FA showed the same trend of FA and did not show any behavioral amelioration. High dose showed a marked amelioration and medium doses showed a marked amelioration but still near positive induction for defecation and duration at a central zone.

with PBS for negative controls. Quantitative evaluation of our immunohistochemistry data was performed using software for image analysis (ImageJ IJ 1.46r) to convert the developed intensity of blue-colour (as negative function of caspase 3 expression) into numerical data.

2.8. Statistical analysis

Statistical analysis of the obtained behavioral data was performed using nonparametric statistics Kruskal Wallis test followed by Mann Whitney *U* test. Other parameters were determined using the general linear model (GLM) produced by Statistical Analysis Systems Institute (SAS, 1989). Significant differences among means were evaluated using Duncan's Multiple Range Test. The following linear model was applied:

$Yij = \mu + \alpha i + \xi ij$

Yij = Observation measured

μ = Overall mean

ξIj = Experimental error assumed to be randomly distributed (σ2 = 0)

3. Results

As expected, FA-induced inhibition of dopamine β-hydroxylase resulted in a marked depletion of NE and an increase in brain hippocampus DA levels in the FA-intoxicated group (Table 1). Administration of increasing doses of glycyrrhizin in groups III, IV, and V did not alleviate the half-reduction in NE level in the brain that was observed in group II. In contrast, DA levels returned to normal control values ($P < 0.05$) in brain hippocampus after treatment with licorice extract at increasing doses in groups III, IV, and V.

In addition, FA intoxication showed a global disruption in the serotoninergic system in the rat hippocampus, with a significant decrease in 5HT levels parallel with a significant doubling in the levels of its main metabolite 5HIAA. These alterations, however, were corrected in a dose-dependent manner (groups III to V) after administration of glycyrrhizin.

As shown in Table 2, the profound inhibitory effect of FA intoxication on the production of the stimulatory neurotransmitter NE, observed in Table 1, was accompanied by a significant surge in the level of γ-aminobutyric acid (GABA), the chief inhibitory neurotransmitter in the rat hippocampus, as well as a rise in the level of histamine. These alterations, however, returned to normal values by administration of licorice extract.

The results in Table 3 confirm the neuro-intoxication-induced global

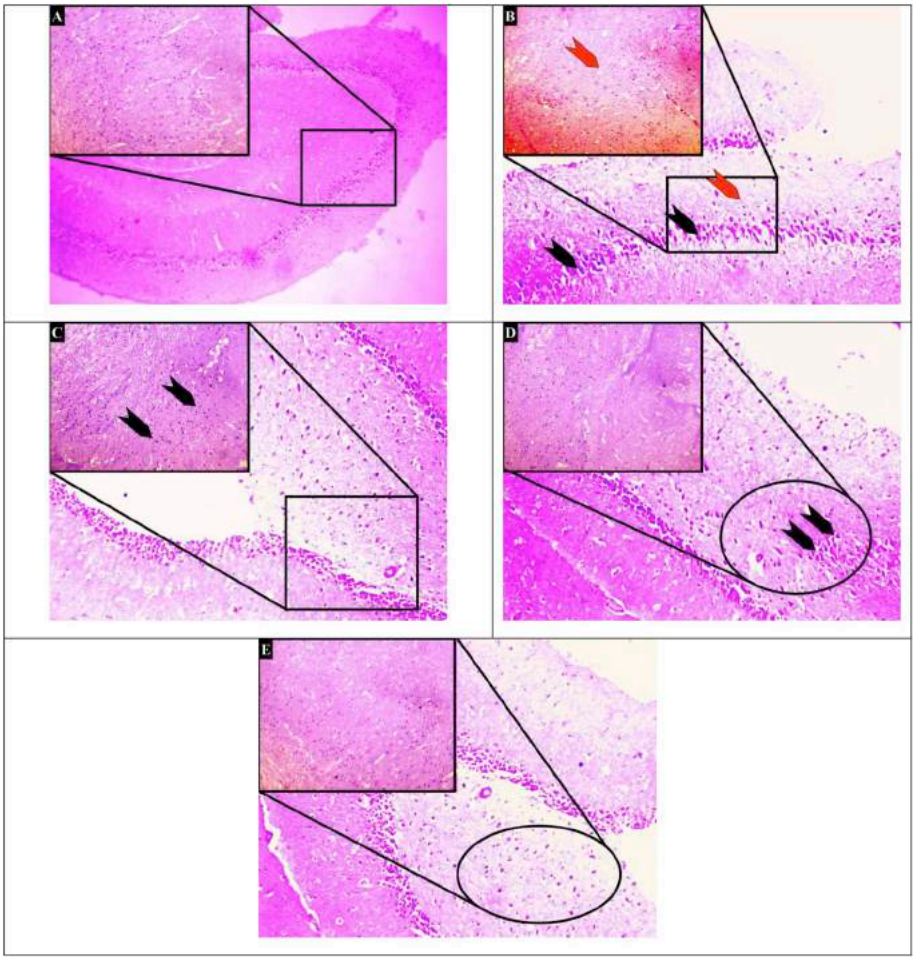

Fig. 2. The effects of different doses of glycyrrhizin on hippocampal histopathology in a FA-induced neurotoxicity rat model.
Figs. (A to E): Photomicrographs of the hippocampus stained by H & E in groups under study show normal structure in control group (A). A large number of damaged neurons, distortion of the pyramidal cells, pykanotic pyramidal cell (black head arrow) and neuropil vacuolation (red head arrow) were reported in FA treated group (B). The groups treated with FA plus glycyrrhizin in low and medium doses (C and D) have mild tissue injury with moderate neuronal damage and few recorded pykanotic pyramidal cells, and neuropil vacuolation. The last group treated with FA plus glycyrrhizin in high dose (E) revealed marked tissue recovery with normal neurons and pyramidal cell similar to control. . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

deterioration in hippocampal activity as manifested by an elevation in tissue NO free radical with subsequent increase in MDA, a specific marker of accelerated membrane lipid peroxidation. This resulted in accelerated cellular apoptosis, indicated by a local rise in the 8OHdG level in group II treated with FA for 30 days. Moreover, FA markedly decreased the endogenous antioxidant system containing SOD and TAC. The medium and high doses of glycyrrhizin, but not the low dose, normalized the negative effects of FA on the NO, SOD, TAC, MDA and 8OHdG levels.

The alterations observed in the neuro-intoxicated group (group II) were complicated by distorted cerebral bioenergetics indicated by a half-reduction in the level of ATP (the main energy currency in the brain) after intoxication, which persisted even after administration of the various doses of glycyrrhizin (Table 4).

The data shown in Table 5 suggest deterioration in cognitive function in the neuro-intoxicated group indicated by a significant drop in the BDNF level. However, glycyrrhizin treatment resulted in a dose-dependent increase in BDNF to levels not significantly different from that in the control group. These changes, indicative of changes in cognitive function, were accompanied by an expected over accumulation of acetylcholine as evidenced by the down regulation of its terminator enzyme, acetylcholine esterase. These neuro-biochemical findings were met with observed behavioral changes in the FA-intoxicated group (Fig. 1) manifested by rat hyperactivity. These behavioral changes were markedly ameliorated after a high dose of glycyrrhizin. The data obtained were further confirmed by histological findings observed in hippocampus and cerebral cortex (Figs. 2 and 3) as well as hippocampal immunostaining used to monitor the different degrees of apoptosis (Fig. 4 and Table 6).

4. Discussion

Licorice root (*Glycyrrhiza glabra*) possesses multiple biomedical ingredients implicated in antioxidative, anti-inflammatory, antiviral, and neuroprotective effects. Licorice root is enriched with glycyrrhizin and its aglycone glycyrrhetinic acid, which result in its major biological activities [4,5]. Previous results indicated a neuroprotective role of isoliquiritigenin isolated from *Glycyrrhiza* species on 6-hydroxydopamine-induced neurotoxicity in a dopaminergic cell line [26]. However, the potential neuroprotective role of glycyrrhizin against FA-induced neurochemical disruptions in the rat brain model has not been fully resolved. Importantly, the data presented demonstrates a possible neuroprotective role of glycyrrhizin against FA-induced NE depletion in the rat brain. FA is a known *Fusarium* species-derived picolinic acid with an inhibitory effect on DA β-hydroxylase, which converts DA to NE [27]. The identification of a novel neuroprotective drug needs to consider issues of both blood-brain barrier permeability and toxicity. The use of licorice-derived glycyrrhizin as a potential neuroprotective agent in our FA-induced NE-depletion model has dual merits. First, the hydrophilic nature of this metabolite promises protection against any intoxication with oral administration at high doses and affords considerable bioavailability to cross the blood-brain barrier. Second, glycyrrhizin has been previously shown to act as a neuroprotective agent [28,29]. Previous work has indicated that following oral administration, glycyrrhizin is first hydrolysed into 18β-GA by intestinal bacteria. After complete absorption from the gut, β-glycyrrhetinic acid is then conjugated to glucuronic acid in the liver, forming 3β-monoglucuronyl-18β-glycyrrhetinic acid [7]. A recent study demonstrated that after glycyrrhetinic is absorbed into the blood it can cross the

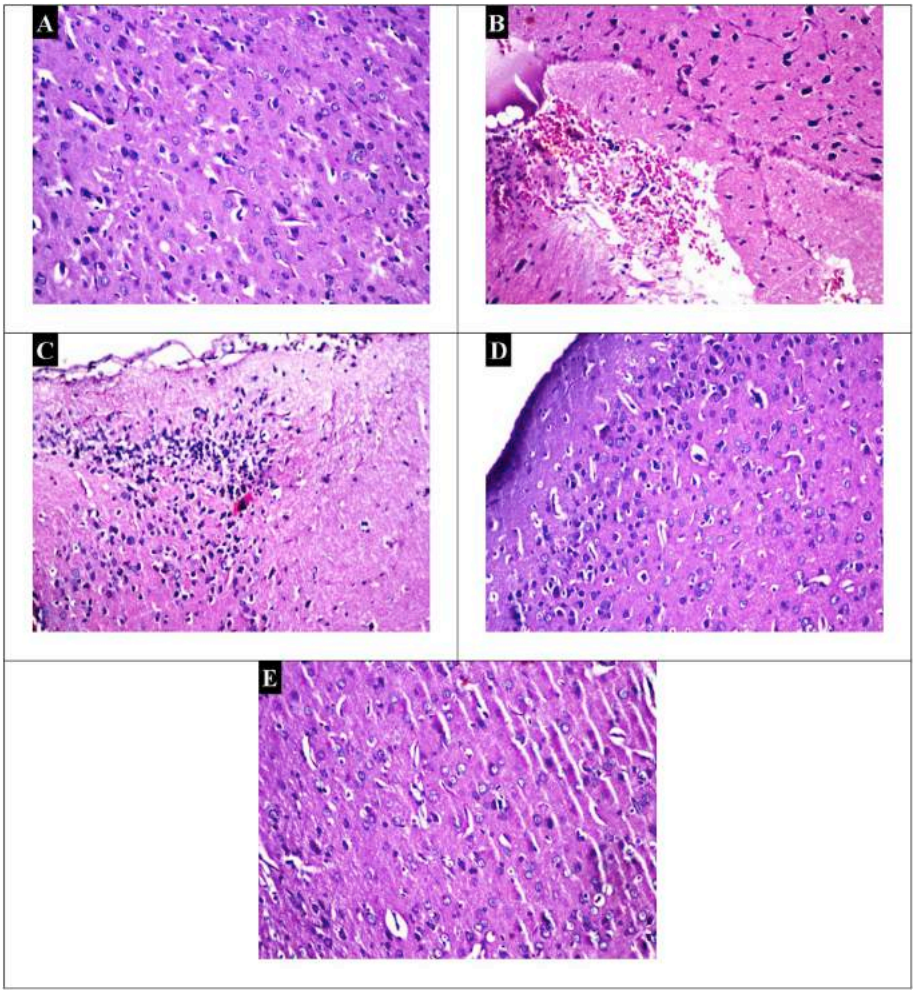

Fig. 3. The effects of different doses of glycyrrhizin on cerebral cortex histopathology in a FA-induced neurotoxicity rat model.
A. Cerebral cortex: control group shows no alteration with a normal histological structure of the neurons and intact pyramidal and glial cells and well-defined neurofibrillar network (H & E 400X). B. FA-intoxicated group showed a focal hemorrhage associated with nuclear pyknosis and neuro-degenerative changes (H & E 400X). C. FA plus low dose of glycyrrhizin treated group showed nuclear pyknosis and degeneration in the neurons with glial cells proliferation (H & E 400X). D. FA plus medium dose of glycyrrhizin treated group showed mild histopathological alterations (H & E 400X). E. FA plus high dose of glycyrrhizin treated group showed marked neuronal recovery with minimal histopathological alterations (H & E 400X).

mammalian blood-brain barrier [30]. The modulatory effect of glycyrrhizin on gap junction channels (GJCs) has been well established in previous studies [31]. GJCs are clusters of intercellular channels that serve as neuronal wiring and are referred to as electrical synapses when found between nerve cells [32]. Because the electrical activity in the brain emerges from neuronal communication, GJCs not only serve as neural wiring that pass ions but, in different cell types, also distribute energy currency between cells in the form of ATP [33]. Stressed central nervous system (CNS) cells retrieve their derailed functions by increasing local expression of neuronal and glial gap junction proteins in the injured brain [34]. Alleviating gap junction dysfunction has recently been shown to mitigate depression [35]. Physiologically, local GJC closures and uncoupling in conditions of injury in the brain suggest the idea of a common mechanism, available to limit the extent of a lesion and increase chances of cell survival [36]. Therefore, GJC modulation has a neuroprotective role through easing the propagation of toxic products during CNS ischaemic insults. Glycyrrhetinic acid exerts its inhibitory effect on GJCs by blocking hyperpolarization mediated by calcium-activated potassium channels [31]. This blocking ability of GJCs has been shown to attenuate the progression of toxic and metastatic elements and retard tumor growth in breast cancer [37] and, therefore, might minimize the global neurochemical disruption after FA load, as observed in our study. Furthermore, a recent study demonstrated that treatment with glycyrrhizin improves recovery of neurological function, reduces lesion volume, and inhibits the release and expression of high-mobility group box 1 after traumatic brain injury by modulating microglia/macrophage polarization [28]. Other findings demonstrate possible protection against ischaemia/reperfusion injury in excitable cells after the partial blocking of GJC-related Cx43 hemichannel opening [38]. Further assessment of the neuroprotective effects of GJC blockers [39] shows that the GJC blocker carbenoxolone (the glycyrrhetinic acid derivative) reduced latencies in the rat brain during traumatic injury. Moreover, treatment with carbenoxolone showed down regulation for expression of autophagy-related gene beclin-1 and upregulation of the expression of glutamate transporter 1 in the rat hippocampus.

The data described above demonstrates the importance of restraining gap junction-related neuronal communication to minimize neuronal autophagy and enhance cognitive deficit repair during brain injury. Concomitant with these data, our *in vivo* findings show that administration of the licorice-derived glycyrrhizin generally corrects FA-induced neuronal damage. 18β-Glycyrrhetinic acid significantly promotes the neuroprotective effect of microglia by decreasing the pro-inflammatory profile in the CNS through the suppression of the MAPK signalling pathway and promotes CNS remyelination in experimental autoimmune encephalomyelitis mice [37]. Therefore, the observed dose-dependent tissue recovery and restoration of neurochemical parameters after glycyrrhizin administration observed in groups III and IV are unsurprising. Licorice-derived glycyrrhizin is one of the most potent compounds that exhibit high-energy binding to DA receptors [40]. This high affinity for DA receptors may induce competitive feedback inhibition of DA production, resulting in the observed gradual decrease in the DA levels after licorice extract administration that reaches the pre-FA-intoxicated value in group IV. Glycyrrhizin exerts an anti-inflammatory effect by reversing the effect of histamine on mucin expression as well as inflammatory cytokine production, while

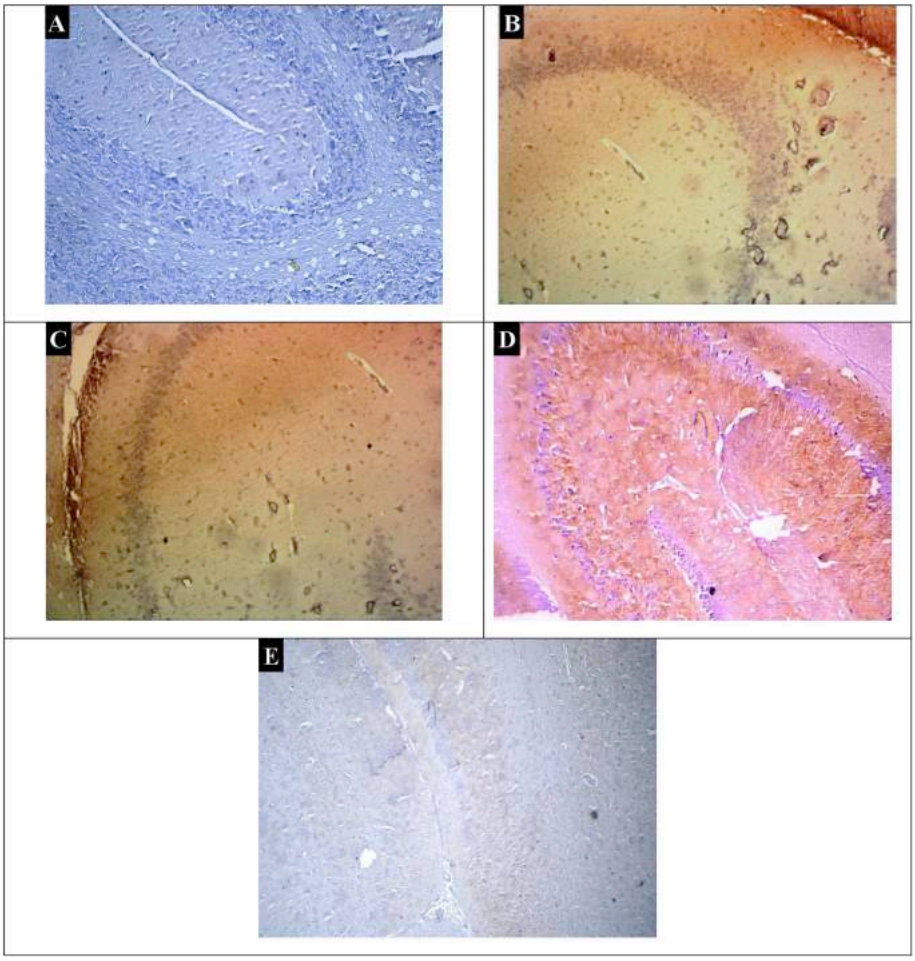

Fig. 4. The effects of different doses of glycyrrhizin on hippocampal apoptotic marker in a FA-induced neurotoxicity rat model.
Figs. (A to E): Immunohistochemical staining of caspase 3 in the brain hippocampus (400X). Immunohistochemical analysis of control rats showed no expression of caspase 3 (negative immunohistochemical reaction) (A). Immunohistochemical analysis of the brain area in the FA group (B) showed strong expression of caspase 3 (positive immunohistochemical reaction). Immunohistochemical analysis of the brown area in the group treated with FA and a low dose of glycyrrhizin (C) showed strong expression of caspase 3 (positive immunohistochemical reaction). Immunohistochemical analysis of the brown area in the group treated with FA and a medium dose of glycyrrhizin (D) showed mild expression of caspase 3 (mild positive immunohistochemical reaction). Immunohistochemical analysis of the brown area in the group treated with FA and a high dose of glycyrrhizin (E) showed faint expression of caspase 3 (weak immunohistochemical reaction). ImageJ IJ 1.46r software digitalized the blue-background intensity as a negative function of the degree of caspase 3 expression (F) to provide quantitative analysis of the numerical data after different treatments. The ameliorating effects of different doses of glycyrrhizin on hippocampal immunohistochemical stain of caspace-3 intensity in fusaric acid-induced neurotoxicity in brain rat model (Mean, Std. Dev., Int. Den.) are: Control (202, 28, $1.4x10^8$); FA (164, 24, $1.1x10^8$); FA + LL (139, 27, $9.6x10^8$); FA + LMD (182, 29, $1.3x10^8$); FA + LHD (195, 28, $1.4x10^8$). The high blue intensity in control group (A; control) after being abolished in FA group (B; FA) shows dose-dependent restoration after glycyrrhizin administration in low (C; FA + LL), medium (D; FA + LMD) and high (E; FA + LHD). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

The ameliorating effects of different doses of glycyrrhizin on hippocampal immunohistochemical staining of Caspace-3 intensity in fusaric acid-induced neurotoxicity in brain rat model.

	Mean	Std. Dev.	Int. Den.
Control	202	28	140,000,000
FA	164	24	113,000,000
FA + LL	139	27	961,000,000
FA + LMD	182	29	126,000,000
FA + LHD	195	28	135,000,000

improving cellular hydration and regeneration by overexpression of aquaporin 5 proteins and phosphorylated cyclic adenosine monophosphate-responsive element binding protein, respectively [41]. The reduction in the histamine level after glycyrrhizin administration in group IV to a level below that found in group 1 indicates that glycyrrhizin rapidly decreases histamine production in mast cells [42].

Brain bioenergetics were greatly disrupted after FA injection in group II, as indicated by a marked depletion in cellular ATP. In addition to being a cellular energy currency, ATP acts as an important brain neurotransmitter by inducing fast excitatory postsynaptic currents. Glycyrrhizin partially restored the level of this reserve in group V. Connexin 43 (Cx43) is the main constituent of both gap junctions and hemichannels in mammalian cells. A previous study [43] found that inhibition of the hemichannel induced by 18β-GA evoked an increase in intracellular ATP and calcium ions in a mammalian cell line. This observation may explain the partial restoration of ATP levels after licorice extract administration in FA-intoxicated rats. Previous work demonstrated that FA contributes to accelerated apoptosis with lipid peroxidation in parallel with disrupted bioenergetics in mammalian cell lines [14]. The observed *in vivo* FA-induced neurotoxicity, resulting in increased lipid peroxidation and accelerated DNA damage in our findings, is similar to a previous report showing FA-induced accelerated cell death with overexpression of the pre-apoptotic Bax protein and different caspase proteins, decreased expression of the anti-apoptotic Bcl-2 protein, and a parallel reduction in ATP levels in a mammalian-derived cell line [44]. The elevated levels of MDA and 8OHG, as biomarkers of accelerated lipid peroxidation and DNA oxidative damage, observed in our study after FA intoxication, would only be expected to return to normal levels after a long latency.

Licorice-derived glycyrrhizin is structurally similar to membrane cholesterol, with a shared capability to change membrane fluidity and potentiate adrenergic action through a reduction in β2-adrenergic receptor internalization [45]. These effects may explain the observed licorice extract-induced neuroprotection after marked depletion of NE caused by FA intoxication. Glycyrrhizin also blocks acetylcholinergic and histaminergic receptors in certain mammalian tissues [46], further augmenting the ability of licorice extract-derived glycyrrhizin to activate AChE [47]. AChE catalyses the breakdown of ACh and is mainly located in neuromuscular junctions and cholinergic synapses due to its main function to terminate synaptic transmission. In contrast, BDNF

contributes to the sustenance of long-term memory [48] while promoting neurogenesis via the growth of new neurons and synapses and differentiation of neural stem cells into new neurons. An increase in BDNF occurs in parallel with motor performance [49], which results in an upregulation in BDNF in the brain during certain types of physical exercise, leading to exercise-induced neurogenesis and improvements in cognitive function [50]. BDNF upregulation and improved motor and cognitive functions were found with regular exercise in a genetically simulated animal model of Parkinson's disease [51]. Furthermore, a GJC blocker positively impacted the expression of BDNF in an animal model of neurodegeneration [52]. This protective effect is consistent with our finding of a gradual rise in BDNF levels and the correction of behavioral changes with increasing doses of glycyrrhizin (groups III to V) when compared to the BDNF levels after FA intoxication observed in group II.

Our examination of the efficiency of glycyrrhizin to modulate glutamate-derived neurotransmitters after neuro-intoxication is beneficial because glycyrrhizin derivatives have been confirmed to effectively protect hippocampal neuronal cells against glutamate-induced oxidative stress in mouse-derived cultured cells [53]. GABA is the primary inhibitory neurotransmitter in the mature brain and reduces neuronal activity of target cells through its binding to GABA receptors present on the target cell subsurface. Nearly half of all synapses of the human brain express some type of GABA receptors. The enzyme glutamate decarboxylase converts one of the main neurotransmitters, glutamate, to its principal neuro-inhibitory counterpart, GABA. There is no direct connection between FA toxicity and GABA metabolism [54], but glutamate is believed to more efficiently cross the blood-brain barrier than GABA [55,56]. This difference in blood-brain barrier permeability may explain the unchanged levels of glutamate and the significant changes in GABA levels after the different treatments throughout the study. Importantly, GABA enhances the catabolism of 5HT into N-acetylserotonin (the precursor of melatonin) in rats [57].

5HT is a neurotransmitter produced by the Raphe nuclei located along the midline of the brainstem, with axons extensively reaching every part of the CNS involved in many brain and body functions, and is commonly known as the substance of well-being and happiness. In addition to mood regulation, 5HT is implicated in many other brain functions, including satiety control, circadian rhythm regulation, and cognitive and learning activities. 5HT is synthesized from tryptophan amino acids and stored in small nerve terminal vesicles. Upon stimulation, 5HT is released from serotonergic neurons into the synaptic cleft to bind its 5HT receptors on the postsynaptic neuron. 5HT is then deactivated by removal from the synaptic space by binding to the 5HT transporter protein. Low 5HT levels are associated with several depressive disorders that can be alleviated by selective 5HT reuptake inhibitors. Non-pharmacological methods of raising brain 5HT, including tryptophan-rich diets and herbal medicine, have shown promising results in recent studies [58]. The transmission of 5HT between different cells is more likely through gap junctions [59]. Our finding of lowering the level of 5HT and increasing the level of its metabolite 5HIAA is concomitant with a previous pharmacological finding that FA has an antagonizing effect against 5HT [60] with a marked ability to decrease pineal 5HT and tyrosine [61]. The preservation of the 5HT level after glycyrrhizin treatment, however, supports the novel use of 18β-GA-based herbal medicine to provide neuroprotection against hippocampus-related neurodegenerative disorders [62]. Moreover, the observed glycyrrhizin–induced decrease in GABA level and the consequent return of 5HT level to its normal control value might explain its amelioration effect on the behavioral changes associated with FA-intoxication.

5. Conclusions

In conclusion, our *in vivo* results using medium and high doses of licorice-derived glycyrrhizin as a potential remedy against FA-induced neurotoxicity showed an overall global neuroprotection manifested by enhanced adrenergic and serotonergic neurotransmission with anti-inflammatory effects and neuroplasticity in the hippocampal region of the brain in a rat animal model. Further studies are required to validate our findings in a higher mammalian model.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbi.2019.05.045.

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