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Preventive and curative effects of grape seed powder on stroke using *in vitro* and *in vivo* models of cerebral ischemia/reperfusion

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ABSTRACT

Stroke is a worldwide concern. Many studies pointed out relevant preventive effect of grape seed powder (GSP) against deleterious brain ischemia/reperfusion (I/R) injury, but curative effect has been scarcely approached.

The present work aimed at studying the preventive and curative effect of GSP against stroke using *in-vitro* and *in-vivo* models. Primary neuron-astrocyte cocultures were used to evaluate *in-vitro* GSP protective and curative effect on oxygen-glucose-deprivation (OGD). A murine I/R model, in which GSP was administered as delayed post stroke drug, to evaluate its potential clinically translatable therapy was used and behavioral tests were conducted after 15 days. Ultra-structure of hippocampus dentate gyrus using Transmission Electron Microscopy (TEM) was also undertaken.

GSP prevented OGD-induced toxicity and cell death in a dose dependent manner and was neuroprotective as assessed by sustained cell viability (70 % ± 1 for OGD + GSP and 37 % ± 2 for OGD) and modulated cytokines and brain derived neurotrophic factor (BDNF) expression. GSP also promoted behavioral outcomes by increasing step-down inhibitory time from 17s ± 4 to 50s ± 11 and rat overall activities by improving scores in open field test to near control level. Furthermore, GSP protected hippocampus dentate gyrus area from I/R-induced drastic alterations as assessed by reduced autophagic vacuoles.

1. Introduction

Stroke is a major public health disorder and a leading cause of long lasting disability and mortality in humans. Despite numerous efforts worldwide to hold back this pathology, the number of stroke patients is still increasing and prevention of high risk population is considered as the best way to overcome such a health concern and socio-economic burden of our healthcare system. Ischemic stroke, which is the most common form of stroke, is characterized by a wide array of early detrimental events including oxidative stress, excitotoxicity, inflammation, free radical production and ionic dyshomeostasis that promote edema and ultimately programmed cell death pathways including apoptosis, necroptosis, and autophagy [1,2].

Excitotoxicity, initiated by altered glutamate release and reuptake leads to overactivation of ionotropic glutamate receptors, allowing the intracellular lethal burst of Ca^{2+} along with energy failure and shut-down of Na^{+}/K^{+}ATPases leading to membrane depolarization, glutamate accumulation into the extracellular space and opening of NMDA associated Ca^{2+}channels. The burst in calcium also triggers the activation of proteases such as calpains and lipases, nucleases and enzymes that are implicated in the early generation of ROS, a well established hallmark of stroke pathogenesis. Increased ROS production induces the release of pro-inflammatory cytokines and cellular mediators such as NO whose interaction with free radicals generates the highly toxic peroxynitrite [3], a powerful free radical capable of inducing severe lipid and protein oxidation into the brain, highly prone to oxidative stress due to its low anti-oxidative defence and high polyunsaturated fatty acids content [4].

Grape Seed Powder (GSP) is a natural and complex mixture containing bioactive polyphenolic compounds such as flavonoids, non flavonoids, stilbenes and anthocyanins [5] exhibiting a large spectrum of pharmacological and multi-organ protective effects such as anti-inflammatory [6], and anti-oxidative [7] as well as liver [8],brain [9],heart [10] and lung protection [11] among others. Many studies pointed out the beneficial health effects against stroke of nutraceuticals in particular those containing high polyphenol content [12] as they are implicated in ROS lessening and scavenging activity [13–15]. For instance epicatechin [16], resveratrol [17]and quercetin [18] were protective against the deleterious effects of ischemic insult in various in vivo and *in vitro* experimental models [19].

In a previous work we demonstrated that high and repeated dosing GSP (2.5 g/kg) prevented efficiently rat brain from I/R insult mainly

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Table 1
Primers.

	Forward	Reverse
TNF-α	TCTCTTCAAGGGACGAGGCT	CCCGGACTCCGTGGTTTTT
IL_6	CCGTCCTGTTGGGGTACAG	GCTCCTTAGGAACGGCAACA
BDNF	CGGATTTGTCCGAGGTGGTA	TCGTCAGACCTCTCGAACCT
$TGF_{\beta1}$	CTCCCGCAAAGACTTCACCC	GGTTGAGGGAGAAAGCAGCA
IL_{10}	TCCCTGGGAGAGAAGCTGAA	GTAGATGCCGGGTGGTTCAA

through its anti-oxidative properties [9]. The present investigation approached both the preventive and curative effects of GSP using an in-vitro model of primary brain cell cultures subjected to OGD and an in vivo model of I/R injured rats with a special emphasis on behavioral tests.

2. Material and methods

2.1. GSP processing and analysis

GSP was processed from a local grape cultivar (carignan) of *Vitis vinifera* from northern Tunisia. Briefly seeds were separated from skins, air dried and grounded with an electric grinder (FP3121 Moulinex) until a fine powder was obtained. GSP safety was checked following certification that it does not contain harmful level of microorganisms (bacteria and fungi) and pesticides. GSP was dissolved into 10 % ethanol (v/v), vigorously shaked and centrifuged (10.000 *g*, 15 min, 4 °C) and the resulting supernatant containing soluble polyphenols was used throughout the study. Total polyphenol content was determined according to the Folin-Ciocalteu (FC) colorimetric method and expressed as mg gallic acid (GA) equivalent/g dry weight (mg GAE/g DW). Total flavonoids were established according to [20] and expressed as mg catechin equivalent (CE)/g dry weight (mg CE/g DW). Condensed tannins were determined using the vanillin method. Briefly, 50 µL of diluted extract was mixed with 3 ml of vanillin (4 %) and 1.5 ml pure HCl and absorbance measured at 500 nm after 15 min and total content expressed as mg catechin equivalent (CE)/g dry weight (mg CE/g DW) using a calibration curve with catechin.

2.2. In vitro antioxidant properties

Total antioxidant capacity (TAC) was determined using phosphomolybdate and expressed as mg ascorbic acid equivalent (AAE)/g dry weight (mg AAE/g DW). Chelating capacity was determined following the ability of the extract to inhibit Fe^{2+}-ferrozine complex formation as described previously [21]. Ferric ion reducing antioxidant potential was determined using the FRAP method according to [22].

2.3. Polyphenol composition of GSP: GC–MS analysis

Polyphenol composition of GSP was determined using the trimethylsilyl (TMS) derivatization method [23]. Briefly, 0.45 mg of lyophilized fraction was hydrolyzed with 1 ml of 2 M TCA at 70 °C for 2 h in a sealed tube in nitrogen atmosphere. Released compounds were converted to their trimethylsilyl (TMS) derivatives by adding 100 µL pyridine (sigma-aldrich) and 100 µL of N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) to the dried sample.1 µL of silylated sample was injected via a split injector (250 °C, split ratio of 20:1, split flow of 18 mL/min), into a HP-5 [(5 % Phenyl)-methylpolysiloxane] column (30 m, 0.25 mm, 0.25 µm). The initial column temperature of 150 °C (1 min) was increased to 180 °C at the rate of 1 °C/min, from 180 to 250 °C at 2 °C/min and from 250 to 300 °C at 15 °C/min (held for 10 min). The ion source of the mass spectrometer (electronic impact) was set at 250 °C, the carrier gas was Helium N60 (1 mL/min). The detector condition was 70 eV and temperatures were 150 °C for the MS Quad, 230 °C for the MS Source, and 250 °C for the transfer line. Quantification was made using an internal standard of D-myo-inositol and expressed as ratio of assay versus standard area.

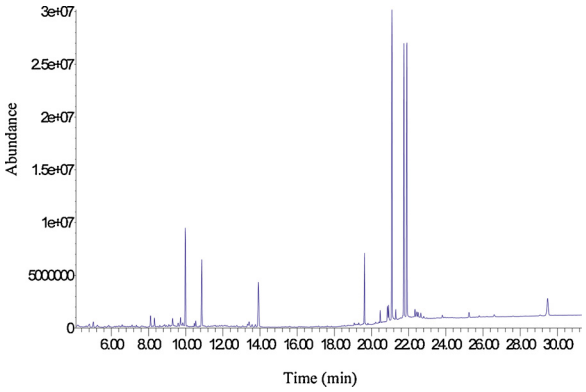

Fig. 1. Typical GC–MS chromatogram pattern of polyphenolics in 10 % ethanol GSP extract.

Table 3
Qualitative composition of GSP: polyphenolics containing 10 % ethanol extract.

Compound	Relative abundance (%)
Tyrosol	2.56
Syringaldehyde	1.76
Vanillic acid	1.68
Protocatechuic acid	3.74
7,9-di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione	2.75
p-Coumaric acid	2.08
Gallic acid ethyl ester	3.27
Gallic acid	14.38
Ferulic acid	1.81
Squalene	2.74
Epicatechin	25.21
Catechin	24.41
flavan-3-ol	3.79
Quercetin	9.82

2.4. Neuron-astrocyte co-culture

Primary brain cell cultures were established from cortical rat foetuses (E18) as described previously [24]. Briefly, dissected cortexes were trypsinized for 10 min at 37 °C and mechanically triturated for dispersion. After centrifugation, cells were suspended in neurobasal medium supplemented with B-27, L-Glutamine, transferrin and PSN antibiotic mixture (GIBCO). To follow brain cell proliferation, cells were daily fixed on coated coverslips using 4 % paraformaldehyde for 20 min at room temperature and identified using immunofluorescence staining with anti-GFAP for astrocytes, anti-Neun for neurons and DAPI for both cell types.

Table 2
Quantitative composition of GSP : total polyphenol, flavonoids and condensed tannins in 10 % ethanol extract.

	Yield (g/100 g GSP)	Tannins (mg CE/g GSP)	Flavonoids (mg CE/g GSP)	Polyphenols (mg GAE/g GSP)
GSP (10 % ethanol)	2.57 ± 0.06	1.00 ± 0.01	2.40 ± 0.02	4.71 ± 0.05

Table 4
In vitro antioxidant properties of GSP: total antioxidant capacity (TAC), DPPH radical scavenging activity, chelating power and FRAP test of 10 % ethanol extract (Standards: EDTA, BHT and ascorbic acid).

	TAC (mg AAE/g DW)	DPPH IC50 (μg/mL)	chelating power IC50 (mg/mL)	FRAP EC50 (mg/mL)
GSP (10 % ethanol)	18.75 ± 0.01	229.00 ± 0.02	1.38 ± 0.01	0.5 ± 0.04
ascorbic acid		0.06 ± 0.08		
BHT			105.69 ± 0.01	
EDTA				0.09 ± 0.01

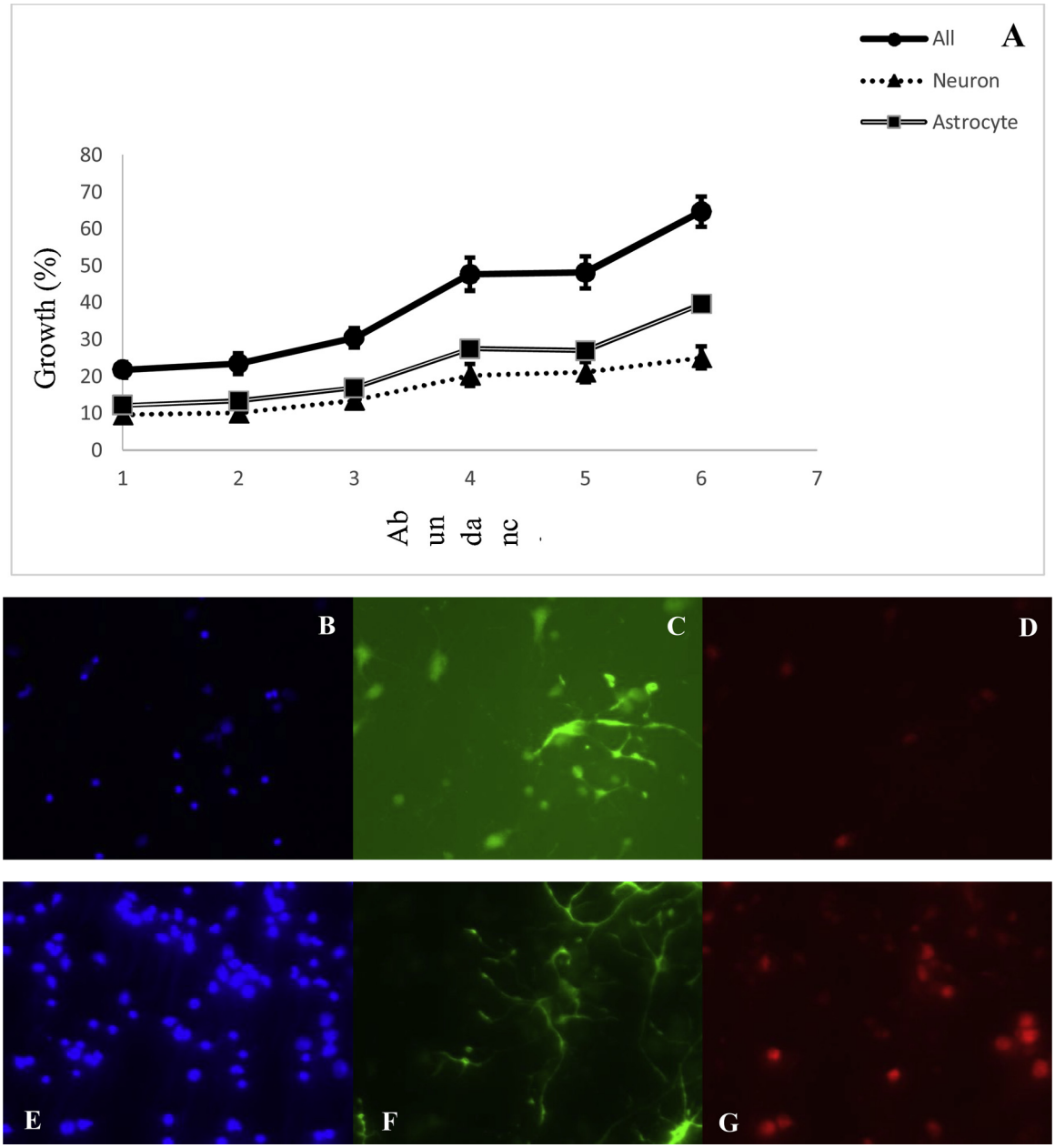

Fig. 2. Proliferation of (astrocyte + neuron) co-cultures. (A) growth curve, (B) DAPI labeling day one (neurons/astrocytes), (C) GFAP labeling day one (astrocytes), (D) NeuN labeling day one (neurons), (E) DAPI labeling day six, (F) GFAP labeling day six, (G) NeuN labeling day six.

2.5. Cell viability and cytotoxicity assays

Cell viability was evaluated using Cell Proliferation Reagent WST-1 (Roche) following the manufacturer's instructions and cytotoxicity using the ToxiLight Non-destructive Cytotoxicity BioAssay Kit (Lonza) as described by the manufacturer.

2.6. OGD assay

For OGD experiments cells were transferred to Sterofundin medium (Braun, Melsungen, Germany), incubated at 37 °C in a hypoxic chamber containing 1 % O_2, 5 % CO_2 and 94 % N_2O and then re-incubated under standard cell culture conditions for 24 h.

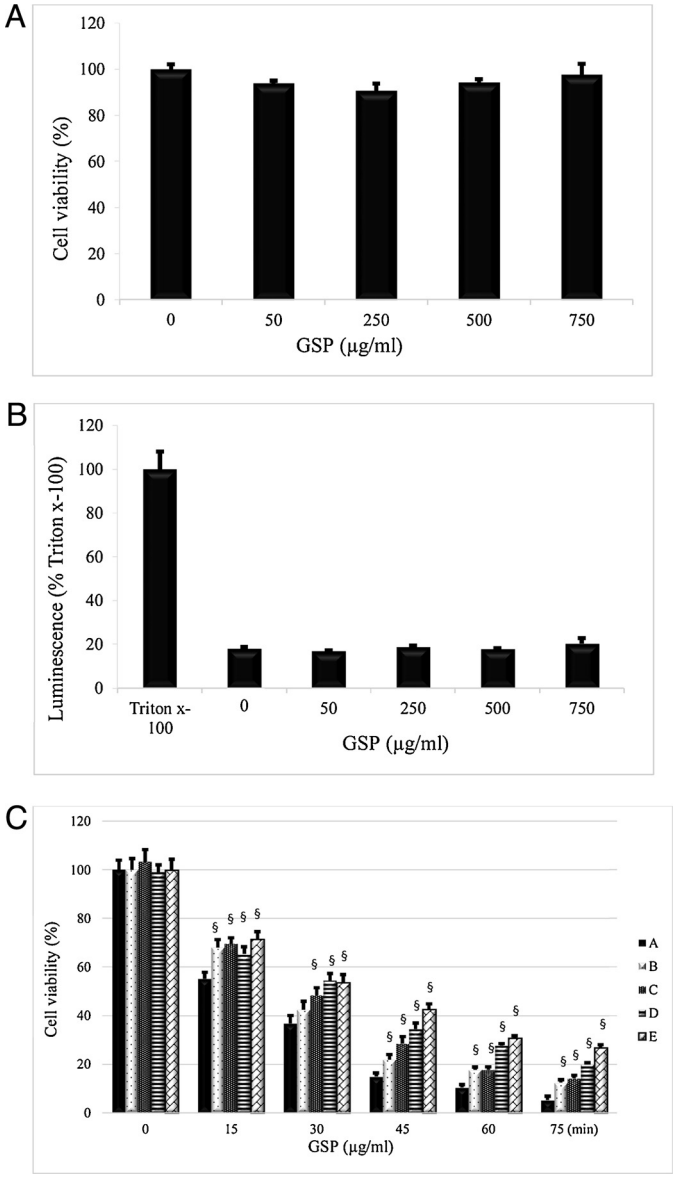

Fig. 3. A: Effect of increasing doses of GSP on primary brain cells viability in control condition. B: Effect of increasing doses of GSP on primary brain cells toxicity in control condition. C: Time and dose-related *in vitro* preventive effect of GSP on cell viability in OGD condition. (A) control (0 µg/ml GSP), (B) 50 µg/ml GSP, (C) 250 µg/ml GSP, (D) 500 µg/ml GSP, (E) 750 µg/ml GSP. (§) indicates significance versus control without GSP.

2.7. Quantitative PCR

RNA was extracted from cells using RNeasy Mini Kit (Qiagen) following manufacturer's instructions. Briefly, contaminant DNA was removed using DNase Set (Qiagen) and RNA was retro-transcripted to cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems). Reaction mixture (25 µl) contained 5 µl of cDNA (100 ng), 12.5 µl of Maxima SYBR Green/ROX qPCR Master Mix (2X) (Biomatik), and 0.3 µM of each gene specific primer (Table 1). The program consisted in 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s and each RT-qPCR was conducted in triplicate. The gene coding for β-actin was used as a reference gene for data normalization and the relative expression of the studied genes was calculated according to the 2-ΔCT method [25].

2.8. Rat brain I/R injury

Thirty two male Wistar rats (206–226 g, 10 weeks old) from Pasteur Institute Tunis, were used in accordance with the NIH recommendations. Rats were acclimated for14 days, provided with food and water ad libitum and maintained in animal facility at fixed temperature of 22 ± 2 °C with a 12-hlight–dark cycle. Briefly rats were randomly divided into four groups of eight animals each, anesthetized with urethane (200 mg/kg) and both common carotid arteries were dissected, clamped for 30 min followed by reperfusion (I/R) for half of the animals constituting (I/R) and (GSP + I/R) groups followed by (C) and (GSP) sham control groups without I/R. Animals were then kept in individual micro filter top cages, treated with betadine and amoxicillin (50 mg/kg) to avoid infection and daily intraperitoneally administered with either high dosage GSP (2.5 g/kg bw) or vehicle (10 % ethanol) till day 15 post-injury where animals were subjected to behavioral study.

2.9. Cerebral infarct size

TTC staining was used to evaluate cerebral infarction size as described by [26]. Two animals from each group were sacrificed after I/R injury, their brains rapidly collected and frozen at −80 °C for 30 min, then cut into coronal sections of 2-mm thick that were incubated in 2 % TTC solution for 30 min at room temperature and fixed in 4 % paraformaldehyde overnight. Microcaptures were acquired using a Canon EOS 1100D.

2.10. Behavioral testing

The step down inhibitory assay was performed using a locally manufactured device able to deliver stimuli discharge of 0.5 mA and 125 Hz according to [27]. On day 14 rats had training sessions and step-down inhibitory test was evaluated at day 15. Exploration activity was measured using an open field and behavioral system of 100 cm diameter, 50 cm height containing a painted grid. Each animal was disposed at the center of the open field and crossing or rearing measured for 10 min.

2.11. Hippocampal ultrastructure

Three animals from each group were euthanized using ketamine and xylazine high dosage injection, brain hippocampi carefully dissected, immediately fixed in 3 % glutaraldehyde (buffered at pH 7.4) and post-fixed in 2 % osmium tetroxide for two hours. Following dehydration, hippocampi were embedded in Epon 812 and ultrathin sections of dentate gyrus area realized, stained with 1 % uranyl acetate and lead citrate and observed using a JEM-1010 transmission electron microscope (TEM).

2.12. Statistics

Results are expressed by mean ± SEM for each group. Statistical analysis was performed using two-way ANOVA followed by post hoc Sidak test and $p < 0.05$ was considered significant. (*) indicates significance versus C and (§) versus I/R or OGD.

3. Results

3.1. Polyphenolics composition of GSP

As all our prior investigations were conducted using 10 % ethanol extract of GSP, we focused on its specific characterization. Total yield was 2.57 ± 0.06 gDW/100 g GSP, whereas polyphenol, flavonoid and condensed tannin content were 4.71 ± 0.05 mg GAE/g DW, 2.40 ± 0.02 mg CE/g DW and 1 ± 0.01 mg CE/g DW respectively (Table 2). The major phenolic compounds of GSP were epicatechin and

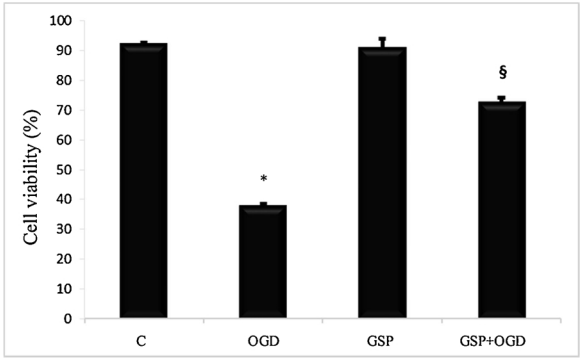

Fig. 4. *In vitro* curative effect of GSP. Brain cells were subjected (OGD, GSP + OGD) or not (C, GSP) to 45 min OGD and treated (GSP, GSP + OGD) or not (C, OGD) with high dosage GSP (750 µg/ml) for 3 days. (*) indicated significance versus control and (§) versus OGD.

catechin representing 25.21 and 24.41 % respectively whereas gallic acid represented 14.37 % followed by quercetin at 9.81 % (Fig. 1, Table 3).

3.2. In vitro antioxidant properties of GSP

We investigated the i*n-vitro* antioxidant activities of GSP and found that TAC reached 18.75 ± 0,01 mg AAE/gDW. DPPH scavenging activity exhibited an $IC_{50}$of 229.00 ± 0.02 µg/mL and a half maximal chelating power concentration of 1.38 ± 0.01 mg/ml whereas the FRAP test indicated that GSP exhibited a half maximal effective concentration of 0.5 ± 0.04 mg/ml (Table 4).

3.3. Neuron-astrocyte co-culture

According to microscopic observations, co-cultured neuron-astrocyte from E18 were able to grow under these specific cell culture conditions. Immunofluorescence staining of neurons and astrocytes indicated that both cells were able to proliferate albeit at differential growth rates (Fig. 2). Indeed, astrocytes growth rate was higher than neurons especially at day 3 and 5 and at day 4 total cell number in the co-culture reached 2.5 times the initial number.

3.4. Cell viability assay

GSP did not affect cell viability (Fig. 3A) nor did it exert any toxicity (Fig. 3B) over a wide-ranging dosage from 50 to 750 µg/ml.

3.5. GSP prophylaxis on OGD altered cell viability

OGD experiments were assessed at day 4 co-culturing and GSP treatment started 3days before the onset of OGD. OGD reduced cell viability in a time dependent fashion to 50 % after 15 min and to 8 % after 75 min. GSP improved cell viability in a dose dependent fashion and the best protection was obtained at the highest dose of 750 µg/ml reaching 23 % survival after 75 min OGD (Fig. 3C).

3.6. Curative effect of GSP on OGD induced cell death

We further asked whether GSP was able to improve cell viability when administered post OGD injury. Following 45 min OGD, cells were treated with 750 µg/ml GSP for three days and their viability evaluated. OGD decreased cell survival to 41 % and GSP clearly improved it up to 78 % (Fig. 4).

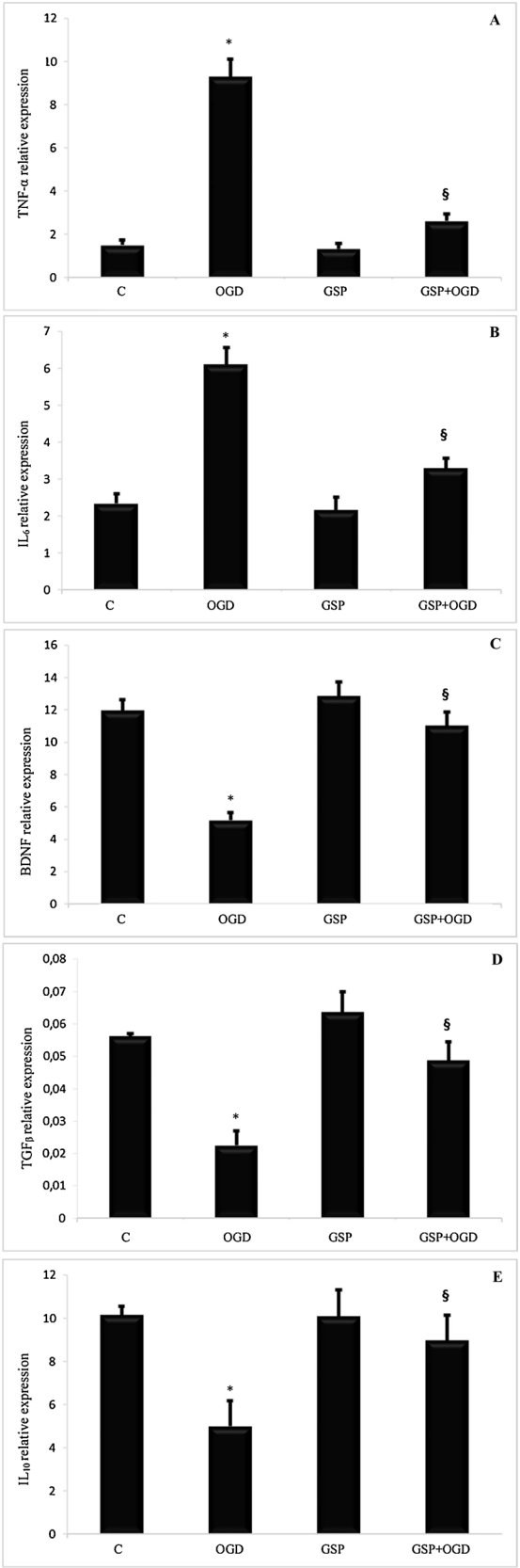

Fig. 5. Effect of OGD and GSP on brain inflammation. Brain cells were subjected (OGD, GSP + OGD) or not (C, GSP) to 45 min OGD, pretreated (GSP, GSP + OGD) or not (C, OGD) with high dosage GSP (750 µg/ml) for 3 days and expression of pro-inflammatory TNF-α (A) and IL-6 (B), BDNF (C), or anti-inflammatory TGF-β (D) and IL-10 (E) determined by RT-qPCR.. (*) indicated significance versus control and (§) versus OGD.

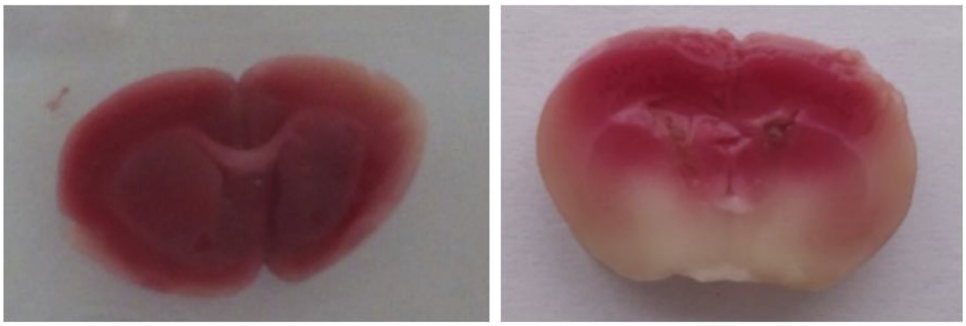

Fig. 6. Brain infarct size evaluation after TTC staining of non-I/R (C) or I/R-injured (I/R) animals for 30 min followed by one hour reperfusion.

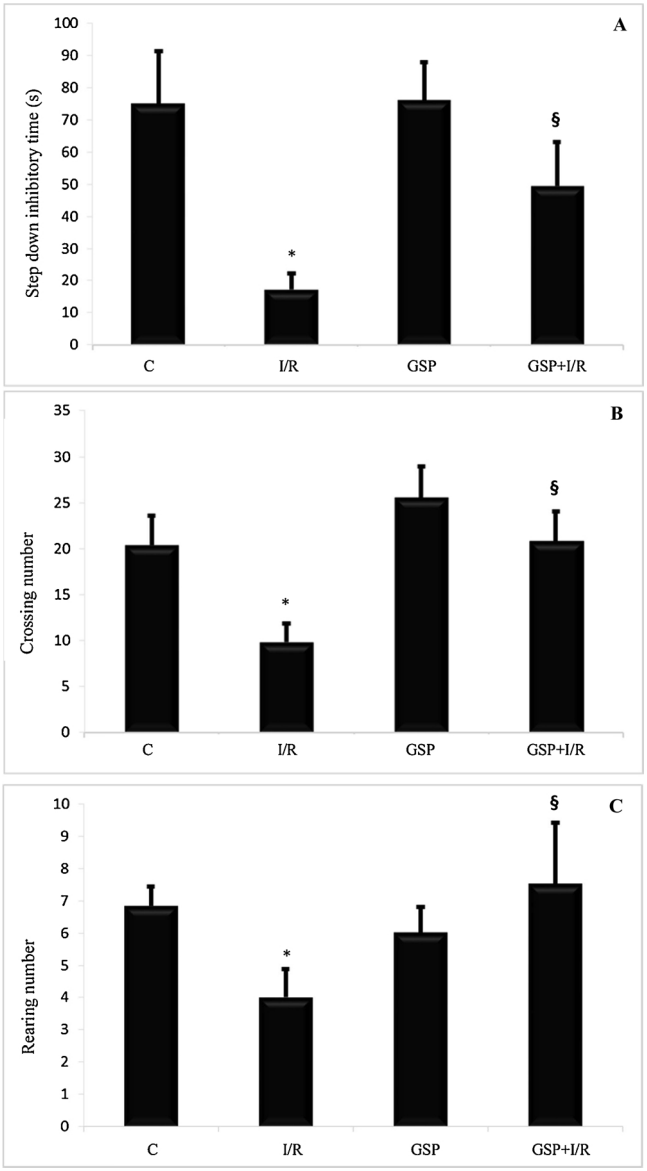

Fig. 7. Effect of GSP on I/R-induced behavioral testing. Animals were subjected (I/R, GSP + I/R) or not (C, GSP) to 30 min I/R injury and then treated (GSP, GSP + I/R) or not (C, I/R) with high dosage GSP (2.5 g/kg) for 15 days and tested for step down inhibitory (A), crossing (B) and rearing (C) behavioral tests. (*) indicated significance versus control and (§) versus I/R.

3.7. Anti-inflammatory effect of GSP

Pro-inflammatory and anti-inflammatory genes expression were assessed after 45 min OGD (Fig. 5). Expression of pro-inflammatory TNF-α (Fig. 5A) and IL-6 (Fig. 5B) increased 6 and 2.5 times respectively, and pretreatment with GSP prevented efficiently from almost all disturbances to near control level. Expression of brain derived neurotrophic factor (BDNF) also decreased upon OGD and was restored to near control level following GSP treatment (Fig. 5C). Expression of anti-inflammatory TGF-β (Fig. 5D) and IL-10 (Fig. 5E) decreased upon OGD and were restored to near control level upon GSP.

3.8. Cerebral infarct size

Brain sections from I/R injured animals (I/R) revealed clear damages identified by a pale color corresponding to an infarct volume of 29 % when compared to brain sections of non injured animals (C) which appeared uniformly red colored (Fig. 6).

3.9. Behavioral testing

We further asked whether GSP post-treatment was able to cure from stroke outcome in an in vivo murine model of I/R injury. Animals were subjected to global brain I/R for 30 min, then treated or not with high dosage GSP (2.5 g/kg bw) for 15 days and behavioral tests evaluated at the end of the treatment (day 15, Fig. 7). Step down inhibitory assay showed that animals subjected to I/R spend a very short time before crossing to another compartment of the cage. GSP improved stroke outcome, in such a way that treated animals spent much more time than control to reach the other compartment (Fig. 7A). Rats were also subjected to the open field test in order to evaluate their exploratory behavior and overall activity. Ischemia decreased both crossing (Fig. 7B) and rearing tests (Fig. 7C) to roughly the half of control group level whereas GSP clearly improved both behavioral and overall activities of rats as it allowed to restore rearing and crossing scores to near control level (Fig. 7).

3.10. Hippocampal ultrastructure

Micrographies from hippocampal dentate gyrus area of sham (Fig. 8A) and GSP control (Fig. 8C) revealed well defined cytoplasm, organelles and nuclear architecture, whereas I/R brains exhibited smaller picnotic nucleus containing condensed chromatin, altered mitochondria and numerous autophagic vacuoles as autophagosomes and autolysosomes reflecting highly suffering even dying cells (Fig. 8B). Interestingly GSP protected efficiently from almost all I/R-occuring disturbances in this brain area (Fig. 8D).

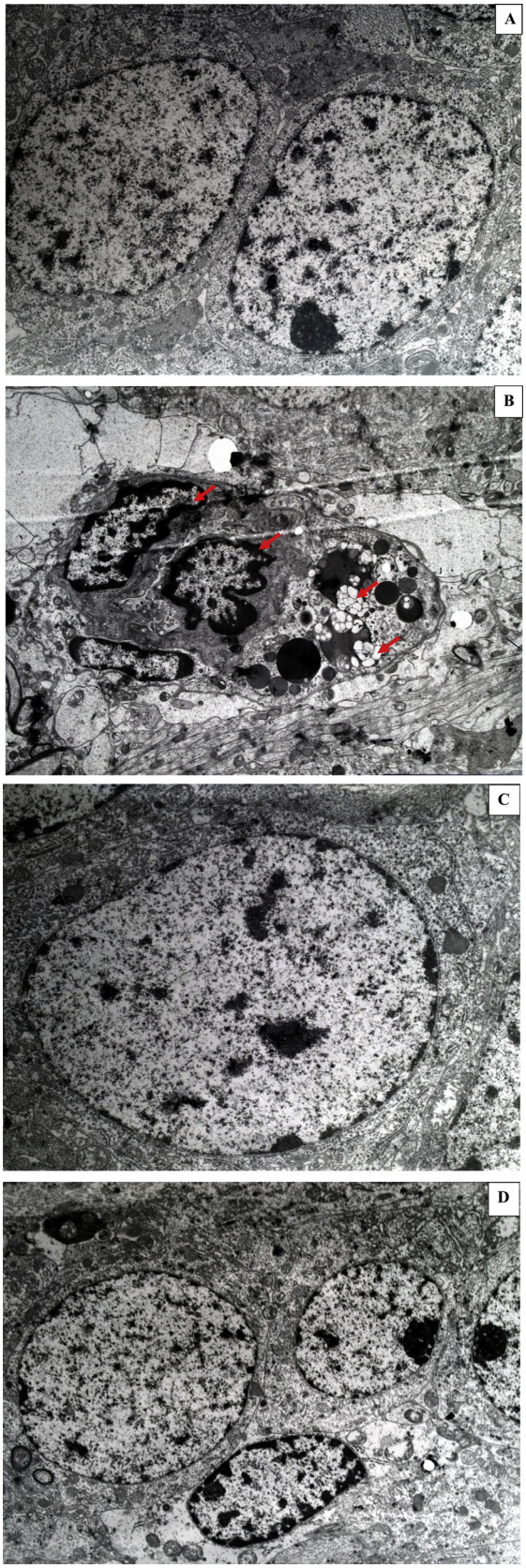

Fig. 8. Micrographies illustrating the protective effect of GSP on I/R- induced structural disturbances in rat hippocampal dentate gyrus area. Sham (A) and GSP (C) controls exhibited intact nuclear membranes and organelles and I/R (B) harbored disrupted nuclear membranes and collapsed organelles whereas GSP + I/R (D) zone appeared protected and close to sham control.

4. Discussion

In a previous in vivo study, we investigated the prophylactic effect of high and repeated dosing GSP (2.5 g/kg) against brain I/R injury and demonstrated its powerful protection against the deleterious effects of I/R-induced oxidative stress [9].The current work investigated the *in vitro* preventive effect of high dosage GSP in an OGD model of primary brain cell cultures as well as its curative effect in both in-vitro and in-vivo model of stroke with a special emphasis on its behavioral aspect.

Preliminary experiments using the OGD model indicated the ability of neurons and astrocytes to proliferate in a co-culture system for 6 days, even though neurons proliferation occurred at a lower rate. As at day 4 co-cultured cells harbored numerous cell projections this time was chosen to perform OGD injury. We first checked the safety of GSP towards (neuron + astrocyte) co-cultures in control condition and found that high dosage GSP (50–750 μg/ml) was not cytotoxic nor did it affect cell viability. Moreover GSP prevented efficiently brain cell co-cultures subjected to drastic OGD in a time and dose dependent way, which fully supported its well recognized protective effect on brain ischemia disorders [28–30]. Moreover as GSP was also effective when administered post OGD treatment, this result emphasized its putative use as a curative agent or as an adjuvant to pre-existing therapies as tPA-induced thrombolysis [31] or as protease inhibitor like ulinastatin [32].

A putative mechanism of GSP neuroprotection could rely on its anti-inflammatory properties. GSP exerted a powerful anti-inflammatory effect as it counteracted OGD-induced pro-inflammatory cytokines expression as TNF-α and IL-6 or anti-inflammatory cytokines expression as IL-10 and TGF-β. In this regard, it has been observed that IL-6 [33] and TNF-α [34] appear to intensify cerebral injury whereas TGF-β [35] and IL-10 [36] may be neuroprotective. TNF-α is involved in cell apoptosis and necroptosis through the activation of TNF R1 inducing signaling via the NF-κB pathway that involves the poly-ubiquitinylation of receptor interacting protein kinase 1 (RIPK1) and NEMO [37]. GSP attenuated OGD-increased pro-inflammatory cytokine IL-6 implicated in endothelial barrier dysfunction and capillary leakage [38].

OGD also decreased the expression of TGF-β after 24 h. The anti-inflammatory or pro-inflammatory role of TGF-β is still controversial as TGF-β was shown to exacerbate cerebral I/R oxidative stress injury [39] and to protect hippocampal neurons from cerebral ischemia [40]. In addition, TGF-β prevents from blood brain barrier disruption [41] and such a discrepancy on TGF-β roles in stroke may be linked to the distinct spatial and temporal induction of TGF-β receptors suggesting their involvement in different functions [42]. We also found that OGD depressed IL10 and that GSP restored the cytokine level till sham control. IL10 has been implicated in autophagy [43], and in neuroprotection afforded by flavonoids to rat subjected to I/R injury [44]. Although it is difficult to straighfully compare *in vitro* and in vivo data [45], our results strengthened the positive effect of GSP on I/R injury via the induction of anti-inflammatory and repression of pro-inflammatory cytokines and overally emphasized the complexity of inflammatory processes network upon stroke.

Furthermore, GSP prevented OGD-induced decrease in BDNF expression. Such a positive effect has been previously described for quercetin [46], rosmarinic acid [47] or resveratrol [48]. BDNF plays a key role in brain ischemia as it inhibits caspase-3 activation and cell apoptosis that occurred upon hypoxia-ischemic injury in vivo [49]. BDNF also increases the expression of anti-apoptotic Bcl-2 [50], and protects the brain from hypoxia-induced neurotoxicity through the activation of ERK- and AKT-signaling pathway [51].

To our opinion the most relevant finding of the current study is the curative effect of high dosage GSP observed both *in vitro* and *in vivo*. GSP protected cerebral cells from post ischemic death after 45 min OGD and 24 h recovery. Since *in vivo* model mimics more suitably the pathophysiological events that occur during stroke, we investigated the curative effect of GSP in a rat model of I/R injury and found that fifteen

days after ischemia, GSP significantly improved behavioral scores and memory of rats as evidenced by the step down inhibitory test which suggested a positive effect of GSP on brain areas implicated in memory functioning as the hippocampal dentate gyrus area. Using TEM approach we analyzed the ultrastructure of hippocampus which plays a key role in memory and neurodegenerative diseases. GSP protected the dentate gyrus area from major alterations in cellular architecture and nuclear damages characterizing excitotoxic cell death as necroptosis figures or autophagic phagosomes, which could be linked at least partly to GSP-induced inhibition of calpain activity [9] or to enhancement of neurotrophic factors such as BDNF as shown in the current work and in agreement with previous data [52].

Since several years, our laboratory recurrently reported the beneficial health effects of high dosage GSP in several stressful biotic experimental settings as high fat diet-induced obesity and lipotoxicity in heart [10], lung [53], pancreas [54], liver [55], brain [9], kidney [56], spleen [57], or abiotic stressful conditions as lithium [58] or arsenic [59] toxicity. The angular stone of all these studies being constituted by the tremendous safety of high and repeated dosing GSP which allowed a good bioavailability to occur especially when targeting highly protected organs as the brain [60]. As a support of these in vivo data [60], the present work emphasized the lack of any toxicity of tremendous GSP dosing (750ug/ml for *in vitro* and 2.5 g/kg for *in vivo* experiments). The great efficiency of GSP is likely linked to the presence of numerous low molecular weight polyphenols as catechin, epicatechin, gallic acid, quercetin or resveratrol that act synergistically to ensure multifaceted and multitargeted effects of GSP leading to modulation of cytokine-dependent signaling pathways and lessening neuroinflammation.

In conclusion our findings emphasize the utmost relevance in the use of high and repeated dosing GSP for the prevention and treatment of stroke. Furthermore, polyphenols encapsulation using solid lipid nanotechnologies is currently developed to optimize their brain bioavailability and pave the way to realistic clinical trials to be performed.

Authors contribution

Safwen Kadri and Mohamed El Ayed performed the experiments; Ferid Limam and Ezzedine Aouani analyzed the data, wrote the paaper; Meherzia Mokni supervised the experiments and corrected the manuscript.

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Declaration of Competing Interest

None.

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