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ORIGINAL ARTICLE

Comparison of the neuroprotective effects of gossypin on cisplatin-induced neurotoxicity in vitro and in vivo

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Abstract

Background Cisplatin (CIS), a widely used chemotherapeutic agent, is known for its severe neurotoxic side effects. This study investigates the neuroprotective effects of gossypin (GOS), a bioflavonoid with antioxidant and anti-inflammatory properties, against CIS-induced neurotoxicity.

Methods and results In-vitro and in-vivo experiments were conducted.50 male Mus Musculus mice were divided into five groups (n:10). Gossypin was administered at varying doses to (in-vitro: 50, 75, 100 µM; in vivo: 5, 10, 20 mg/kg/day) evaluate its protective effects. Results showed gossypin significantly improved cell viability dose-dependently, normalized oxidative stress markers Superoxide dismutase (SOD) activity and glutathione (GSH) and malondialdehyde (MDA) levels, reduced pro-apoptotic and inflammatory genes (CASP-3, CASP-9, TNF-α, NF-kB, iNOS), and increased anti-apoptotic markers (BCL2/BAX ratio, nNOS). Histopathological analysis revealed gossypin mitigated CIS-induced brain tissue damage.

Conclusions Gossypin exhibited significant neuroprotective effects against CIS-induced neurotoxicity via antioxidant (TNF-α), anti-inflammatory (NF-kB), and anti-apoptotic (BCL2/BAX ratio, nNOS) mechanisms by regulating various key regulatory genes, suggesting that it may be a promising adjuvant therapy to protect against the neurotoxic side effects of cisplatin in cancer treatment.

Keywords Cisplatin · Neurodegenerative · Natural products · Apoptosis · Inflammation · Oxidative stress

Introduction

Cancer is a significant medical challenge, affecting individual well-being and healthcare economics globally. Treatment varies by type and progression, including surgery, chemotherapy, radiation, immunotherapy, targeted therapy, hormone therapy, gene therapy, and photodynamic therapy. Chemotherapy remains a predominant and effective strategy, using cytotoxic or alkylating agents to combat cancer cells [1].

CIS, a pioneering metal-based chemotherapeutic, is noteworthy for its widespread application, with reports suggesting that it is utilized in treating half of cancer patients worldwide [1]. CIS is versatile, treating a broad spectrum of solid tumors, including those affecting the testicles, ovaries, bladder, lungs, cervix, head and neck, stomach, and more [2, 3]. Its antineoplastic efficacy is attributed to its ability to bind with both genomic DNA (gDNA) and mitochondrial DNA (mtDNA), resulting in DNA lesions that hinder DNA, mRNA, and protein synthesis, disrupt DNA replication, and trigger signal transduction pathways leading to cell death via necrosis or apoptosis [4].

However, CIS's full therapeutic potential is often not realized due to adverse effects and the development of drug resistance. Resistance mechanisms involve reduced drug uptake, drug neutralization by protein binding, enhanced DNA repair capabilities, and alterations in apoptosis-related proteins [5]. The toxic side effects of CIS are significant

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concerns, with nephrotoxicity, ototoxicity, hepatotoxicity, gastrointestinal issues, and neurotoxicity being the most severe [2, 6–8]. Notably, CIS-induced neurotoxicity has been substantiated through various animal studies, highlighting the need for cautious application and monitoring during treatment [9, 10]. Despite CIS toxicity, the testing of various natural protective agents with low side effect profiles and mostly strong anti-inflammatory and antioxidant properties has increased considerably [11, 12].It would be beneficial to understand the mechanism of these side effects of CIS as a dose-limiting factor that negatively affects patients' quality of life and to eliminate and reduce these side effects if possible.

Gossypin (3,5,8,3,4-pentahydroxy-7-O-glucosylflavone 8-glucoside) isolated from *Hibiscus vitifolius* is a bioflavnoid. It has antioxidant, anti-inflammatory [13] and analgesic [14] properties. In addition, it has been reported to protect pancreatic β-cells from glucotoxicity in diabetes [15]. It was also said to have neuroprotective activity in a model of cerebral ischemia in rats [16]. In a neurotoxicity study with rat cortex primary cells, it was reported that gossypin is neuroprotective and does this by suppressing oxidative stress [17]. In addition, gossypin has not only antioxidant but also anticancer effects. Its antiproliferative and apoptotic effects have been demonstrated in gastric cancer [18], melanoma [19], and prostate cancer (PC-3 cells) [20]. Since it shows both antioxidant and anticancer effects, it suggests that its combination with CIS may reduce its side effects with a neuroprotective impact while supporting the chemotherapeutic effect.

An effective treatment for CIS toxicity is still needed. Gossypin, due to its chemical structure and protective mechanisms, might be an ideal neuroprotective agent, though its CIS toxicity hasn't been studied.While the number of studies on gossypin in the literature is low, the antioxidant properties of gossypin are prominent in these studies. Our study is the first to investigate its effects against neurotoxicity due to CIS in vivo and in-vitro.

Materials and methods

Ten newborn (5-hour-old) animals were used for primary neuron culture formation (Kafkas University local animal care unit). 50 Mus Musculus mice (25–30 g) were used in the in-vivo toxicity model. The animals were treated with 12 h of light and dark in plastic cages with sawdust floors, and one-week adaptation care was provided with regular feed and water at average room temperature (24 ± 2 ^{0}C). All study and animal care procedures were performed by the decisions of the local animal ethics committee of Kafkas University, numbered KAU/HADYEK/2019-066 and KAU/HADYEK/2019-070. All methods were performed in accordance with the above-mentioned guidelines and regulations. This study is reported in accordance with the ARRIVE guidelines (https://arriveguidelines.org).

Primary neuron culture

The newborn Sprague Dawley rat puppies were quickly decapitated, their cortex neurons were removed, and they were kept in the ready neurobasal medium (CAS number: SCM003 (Sigma-Aldrich In. (St. Louis, MO)). After adding 1:1 trypsin and keeping it in the incubator for 30 min, it was centrifuged 3 times, the supernatant was discarded each time, and a new medium was added. The medium was prepared by adding a neurobasal medium of 1000:1 penicillin 50:1 B27 and 10:1 FBS (fetal bovine serum) in a separate tube. Cells were added to the conditioned medium. 150 µl of medium were added to each chamber of the 96-well plates. Cells taken from neonatal cortex neuron culture will be seeded into 96 well plate. 5 wells will be allocated to each group. In total, MTT will be formed from 10 groups. For PCR analysis, cells were added to 6-well plates in 2000 ml of medium using the same medium and method. Cells were kept in the incubator for ten days to adhere to the bottom of the wells, to coat the bottom, and to grow. With the same method, a 6-well plate was planted for biochemical analysis. CIS doses were kept similar to our previous study [21]. The culture medium was changed every 3 days. The cell cultures were examined at an inverted microscope (Leica) for approximately 24 h. Then, IC_{50} was determined in this cell line.

Determination of cell proliferation

Briefly, approximately 5000 cells/well were seeded in 96-well plates and incubated ten days for proper attachment. The experimental procedure was started when the culture reached approximately 75–80% density in the culture flask. Doses of gossypin (10–100 µM) (Sigma Aldrich (CAS Number 652-78-8)) were administered 2 h before CIS toxicity. Cell proliferation was determined by applying the MTT method 24 h after CIS (Sigma Aldrich (CAS Number 15663-27-1)). Gossypin dosage was determined as a wide range based on the results we obtained from our previous cell lines [13, 20, 22].The cells incubated with the MTT kit (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) (11465007001, Sigma-Aldrich Chemie Gmbh Munich, Germany) method by the manufacturer's protocols, as in my previous studies, were measured with a microplate reader spectrophotometer (Epoch Microplate Spectrophotometer, BioTek, USA) at 620 nm absorbance value in 3 replicates. The cell viability was then calculated

using the following equation: Inhibitory rate (%)= (1 – [ODtreatment-ODcontrol])* 100%. By taking the average of these measurements, the IC_{50} value of primary neuron cells for gossypin was determined from the results. While creating the biochemical and PCR groups, three different doses were used, considering the IC_{50} value.

Quantitative determination of mRNA expression by real-time PCR

For PCR analysis, 2000 ml of medium was added to 6-well plates (approximately 200,000 cells/well). Cells were kept in the incubator for ten days to adhere to the bottom of the wells, cover the bottom, and grow.

PCR groups (6th and 12th-hour groups are indicated below with the same). Three groups were formed based on IC50 values for group determination.

(1) Control, (2) Control+ % 0.1 lik DMSO, (3) CIS 50 µM, (4) CIS 100 µM, (5) CIS 50 µM+Goss 50 µM, (6) CIS 50 µM+Goss 75 µM, (7) CIS 50 µM+Goss 100 µM, (8) CIS 100 µM+Goss 50 µM, (9) CIS 100 MM+Goss 75 MM, 10. CIS 100 MM+Goss 100 MM.

CIS was administered two hours after Gossypin administration. After 6 and 12 h, the cells were removed by scraping with a plate scraper and centrifuged at 1000 Rpm for 2 min. Total RNA extraction and cDNA synthesis were performed according to the methods described in our previous studies [13, 23], according to the manufacturer's instructions, and TNF-α (Mm00562055_m1), NF-KB (Mm01399572_m1), iNOS (Mm00561646_m1), nNOS (Mm00583793_m1), Bcl (Mm00821025_g1), Bax (Mm01480161_g1), Caspase-3 (Mm00563902_m1), Caspase-9 (Mm00581212_m1) and β-actin (Mm00667869_m1) mRNA expression as a housekeeping gene were analyzed. Compared with the control group, All data were expressed as fold change in expression compared with the cell groups using the $2^{-\Delta\Delta}$Ct method. The differences between the groups' oxidative stress, inflammation, and apoptosis were evaluated.

Biochemical parameters

For oxidative stress analysis, 2000 ml of medium was added to 6-well plates (approximately 200,000 cells/well) using the same medium and method. Cells were kept in the incubator for ten days to adhere to the bottom of the wells, cover the bottom, and grow. CIS was administered 2 h after Gossypin administration. After 6 and 12 h, the cells were scraped with a plate scraper, and the supernatant was removed by centrifugation at 1000 Rpm for 2 min. The cell lysate was subjected to the same steps as the brain tissues by the manufacturer's instructions, and SOD activity, GSH, and MDA level parameters were measured. Briefly, the Mouse cortex, cerebellum tissues, and primary cell lysates were stored at -80˚C. 100 mg of tissue from each sample was homogenized with Tissue Lyser in a specific homogenate buffer on ice. It was then centrifuged according to the instructions in the kit. For biochemical studies, SOD activity, MDA levels, and GSH levels were measured from each supernatant by ELISA kit measurement methods. Superoxide dismutase (SOD) activity and glutathione (GSH) and malondialdehyde (MDA) levels were measured manually from the supernatants, as described in our previous studies [13].

Hoechst 33,258 staining assay

The nuclear morphology of cells treated with CIS and GOS was observed with a Hoechst 33,258 staining assay. Primary neuron cells were seeded in 6-well plates and cultured for 10 days. CIS was administered 2 h after Gossypin administration. The method was performed as in our previous study [23]. Changes in nuclear morphology were monitored with a LEICA inverted microscope (Leica, DMIL LED, Germany).

Animals

Ethical statements and animals

Ten newborn Balb/C Mice were used for the in-vitro part of the study. For the in-vivo phase of our research, 50 male Mus Musculus mice (25–30 g) were obtained from the Atatürk University Experimental Animals Research and Application Center. We studied at Atatürk University's Experimental Animals Research and Application Center. In our study, 50 male mice were randomly divided into five equal groups and taken into a separate study compartment. The animals were treated with 12 h of light and dark in plastic cages with sawdust floors, and one-week adaptation care was provided with regular feed and water at average room temperature (24±2 ^{0}C). All study and animal care procedures were performed by the decisions of the local animal ethics committee of Kafkas University, numbered KAU/HADYEK/2019-066 and KAU/HADYEK/2019-070.

Experimental protocols of neurotoxicity

After a week of acclimatization, the experimental animals were divided into five groups (comprising six rats each) as follows:

1. Healthy Control (n=10) i.p saline+oral % 0.1 lik DMSO.
2. CIS 10 mg/kg/ day (n=10) (i.p).
3. CIS 10 mg/kg/ day+Gossypin 5 mg/kg/ day (n=10) (i.p+oral).

4. CIS 10 mg/kg/ day + Gossypin 10 mg/kg/ day ($n = 10$) (i.p + oral).
5. CIS 10 mg/kg/day + Gossypin 20 mg/kg/ day ($n = 10$) (i.p + oral).

In our study, CIS and gossypin were applied to 4 groups except the healthy group.

CIS (CIS-DICHLORODIAMINEPLATINUM(II), 99.99%,(TRACE METAL BASIS), Sigma Aldrich, MO, Germany) 10 mg/kg/day was administered intraperitoneally for 14 days.

Gossypin (Cat. no: 652-78-8, Sigma Aldrich, Germany) dissolved in 0.1% DMSO and administered with oral gavage of 5, 10, 20 mg/kg/day for 14 days [16].

On the 15th day, all mice were anesthetized with 30 mg/kg of thiopental sodium, the brain tissues were removed, some of them were treated with liquid nitrogen for biochemistry and molecular analysis, then kept at -80 ^{0}C for examination, and the other part was preserved in formaldehyde for histopathological examinations. Brain tissues were divided into two hemispheres. Ten brain tissues were allocated for histopathology and ten for biochemistry and molecular analysis randomized.

Histopathological examinations

Hematoxylin-eosin staining procedure At necropsy, tissue samples from the cerebral cortex and cerebellum were taken and fixed in 10% formaldehyde solution. The method was performed as in our previous study [13].

Immunohistochemical staining For immunohistochemical analysis, the cerebral cortex and cerebellum region tissues were stained with apoptosis-initiating BAX and inflammation-initiating NFkB (Bax and NFkB, Santa Cruz Biotechnology). The method was performed as in our previous study [24].

Statistical analysis

Values were expressed as mean ± S.D. Numerical data obtained from biochemical analyses and Real-Time PCR results were analyzed with the One Way ANOVA Post Hoc Duncan test. SPSS-20 for Windows was used for all statistical analyses. P values less than 0.05 were considered significant.

Results

Primary neuron cells viability results

In the primary neuron culture, as shown in Fig. 1, gossypin conserves the viability of primary neuron cells in a time- and dose-dependent manner. Gossypin, IC50 values in primary

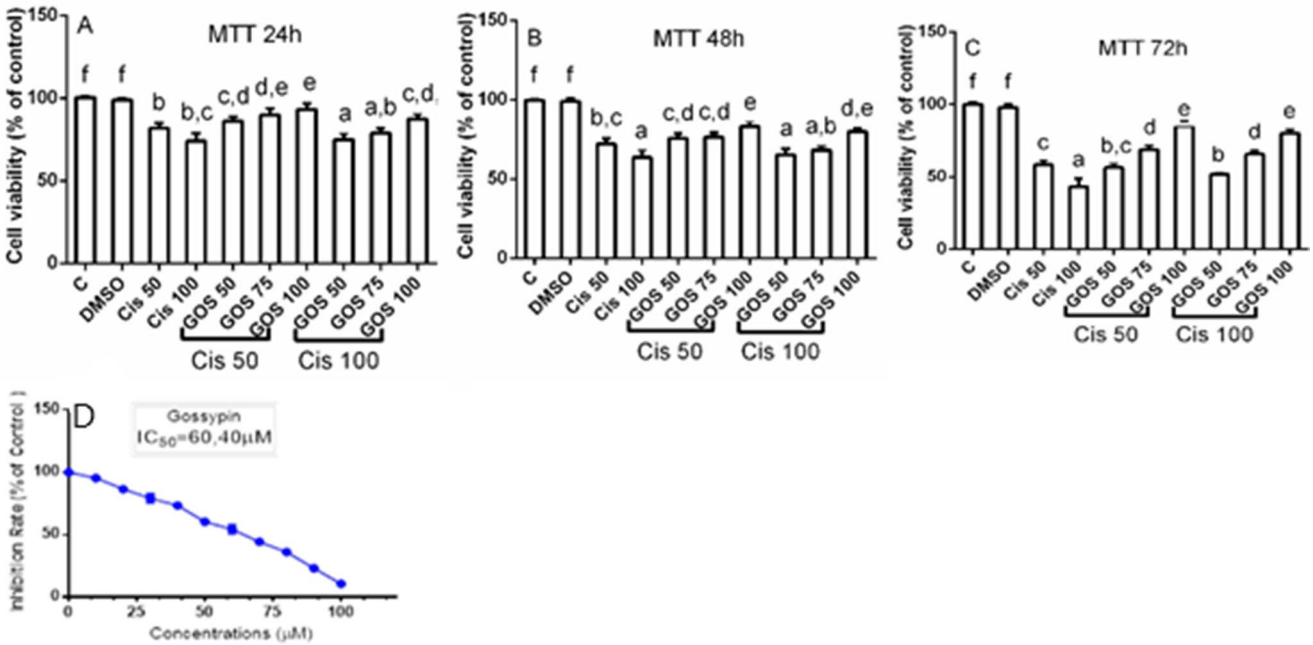

Fig. 1 Primary Neuron Culture Time-Dependent Cell Viability Results. Primary Neuron culture IC_{50} Results in 24 h. (**A**) 24 h, (**B**) 48 h, (**C**) 72 h, (**D**) GOS IC50. $^{a-f}$ Values not sharing a common superscript differ significantly at $P < 0.05$ Duncan's Multiple Range Test (DMRT). ($p < 0.05$). CIS 50: CIS 50 µM, CIS 100: CIS 100 µM, GOS 50: Gossypin 50 µM, GOS 75: Gossypin 75 µM, GOS 100: Gossypin 100 µM

neuron culture were 60,40 µM (Fig. 1) at 24 h. Especially the application of 100 µM dose of Gossypin, the viability level, which decreased to 50% with the application of 100 µM cisplatin, increased up to 80% (Fig. 1).

Oxidative stress markers in primary neuron cells and brain tissues

Compared to the control group, SOD activity (34.60 ± 1.18[a]) and GSH levels (2,14 ± 0,07[a]) were decreased, while MDA levels (5,11 ± 0,11[e]) were increased with CIS application in the primary neuron cells. With the application of Gossypin, these parameters approached their normal levels. For the 12th hour, respectively, It was found to be 70,88 ± 3,75[e], 4,59 ± 0,25[e], 3,17 ± 0,22[b] (Supplementary Fig. 1).

Similar findings were found in brain tissue analyses of mice. Decreased SOD activity and GSH levels and increased MDA levels with CIS application were dose-dependently regulated by GOS application. In addition, the TNF-α level, measured by the ELISA method regarding inflammation, also supported our findings (Fig. 6).

Primary neuron cells and brain tissues mRNA expression results

In comparison to the control group, we found significant increases ($p < 0.05$) in pro-apoptotic markers, CAS3 (5,85 ± 0,28[f]), inflammatory markers TNF-α (6,99 ± 0,49[a]), NF-kB (2,68 ± 0,09[g]) and iNOS (7,85 ± 0,09[h]) after cisplatin challenge. In addition, significant reductions ($p < 0,05$) in anti-apoptotic markers, CAS9 (1,79 ± 0,12[g]) gene expression, Bcl/Bax ratio (0,28 ± 0,03[a]), and nNOS (0,36 ± 0,02[a]) after cisplatin injection were observed. Moreover, our results revealed that gossypin treatment significantly decreased (2,54 ± 0,22[b]) CAS3 (2,54 ± 0,22[b]) gene expressions, CAS9 (1,31 ± 0,02[c, d]), TNF-α (2,97 ± 0,19[a]), NF-kB (1,73 ± 0,14[d, e]) and iNOS (4,32 ± 0,25[e]). And increased Bcl/Bax ratio (0.80 ± 0.05d) and nNOS (0,75 ± 0,03[c]) gene expression (Figs. 2 and 3).

TNF-α, NF-kB, iNOS, CASP-3, and CASP-9 values increased significantly with CIS application to mice, while nNOS and Bcl-2/BAX values were significantly decreased compared to control. These values were normalized depending on the dose (especially GOS 20) with the administration of Gossypin (Figs. 4 and 5).

Results of in-vivo hematoxylin eosin and immunohistochemistry findings

The most striking change was observed in Purkinje cells when the cerebellum tissues were examined in staining with hematoxylin-eosin. Purkinje cell bodies consisted of well-circumscribed nuclei and nucleoli in the control group. In the CIS group, unlike the control group, Purkinje cell nuclei were pycnotic, and there was an increase in eosinophilia in the cytoplasm. It was observed that the nucleus and cell

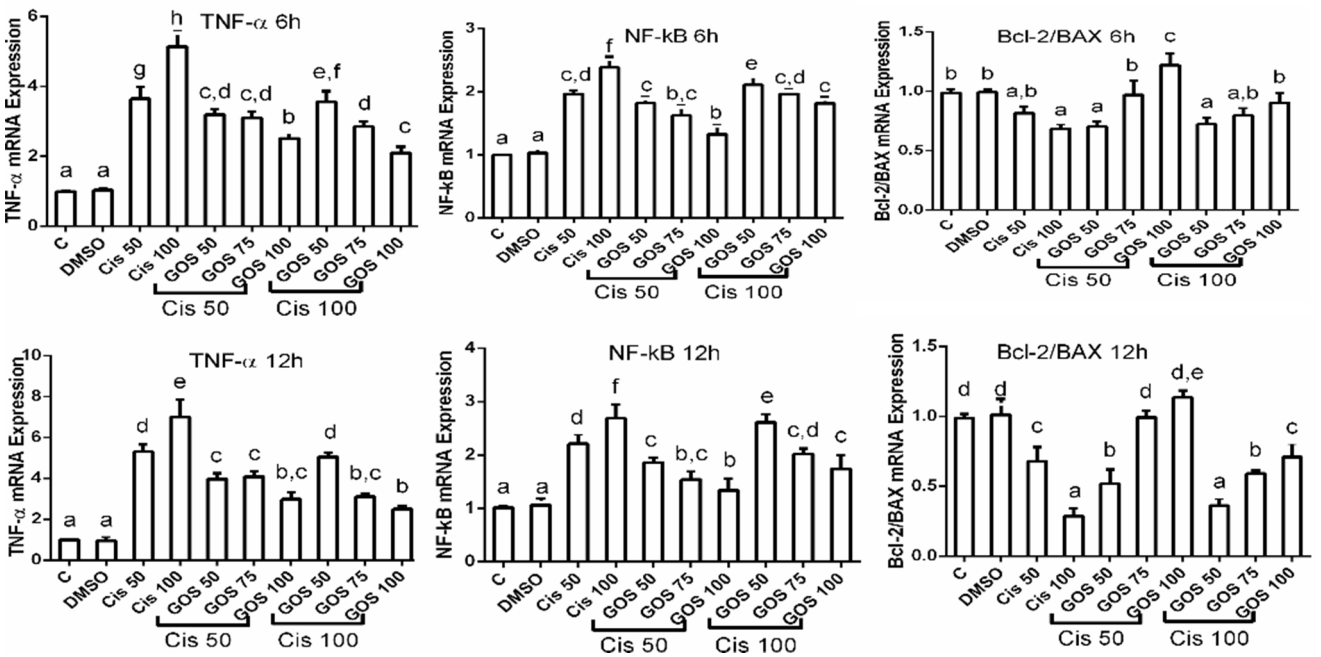

Fig. 2 Primary Neuron Culture TNF-α, NF-kB and Bcl-2/Bax mRNA Expression Results at 6 and 12 h.(Mean ± SE). [a–f] Values not sharing a common superscript differ significantly at $P < 0.05$ Duncan's Multiple Range Test (DMRT). ($p < 0.05$). CIS 50: CIS 50 µM, CIS 100: CIS 100 µM, GOS 50: Gossypin 50 µM, GOS 75: Gossypin 75 µM, GOS 100: Gossypin 100 µM

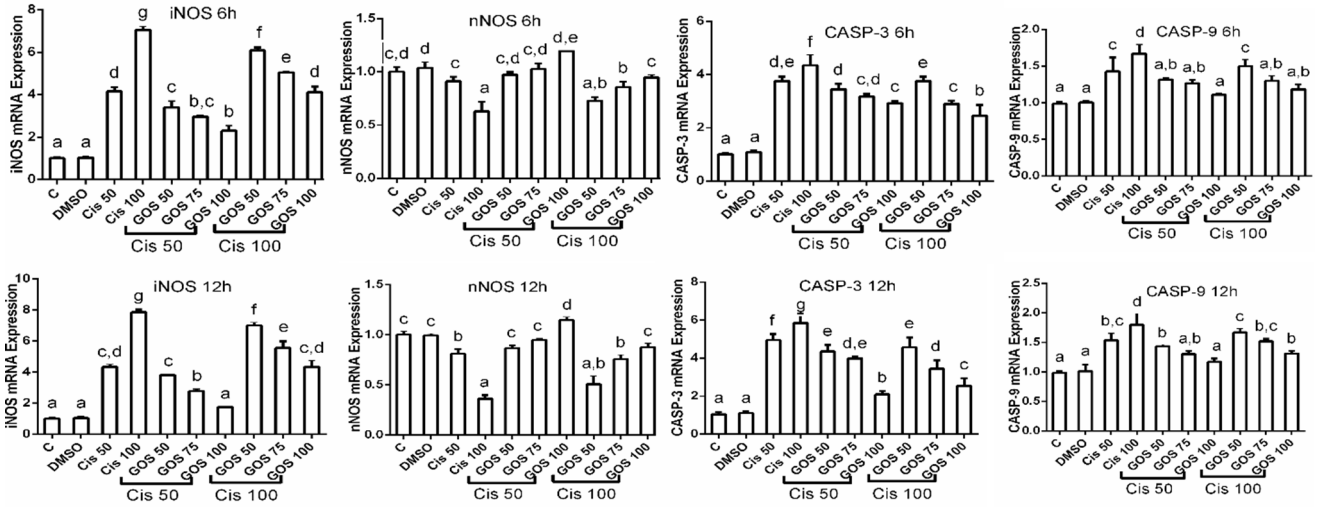

Fig. 3 Primary Neuron Culture nNOS, iNOS, CAS-3 and CAS-9 mRNA Expression Results at 6 and 12 h.(Mean±SE). $^{a-f}$ Values not sharing a common superscript differ significantly at $P<0.05$ Duncan's Multiple Range Test (DMRT). ($p<0.05$). CIS 50: CIS 50 µM, CIS 100: CIS 100 µM, GOS 50: Gossypin 50 µM, GOS 75: Gossypin 75 µM, GOS 100: Gossypin 100 µM

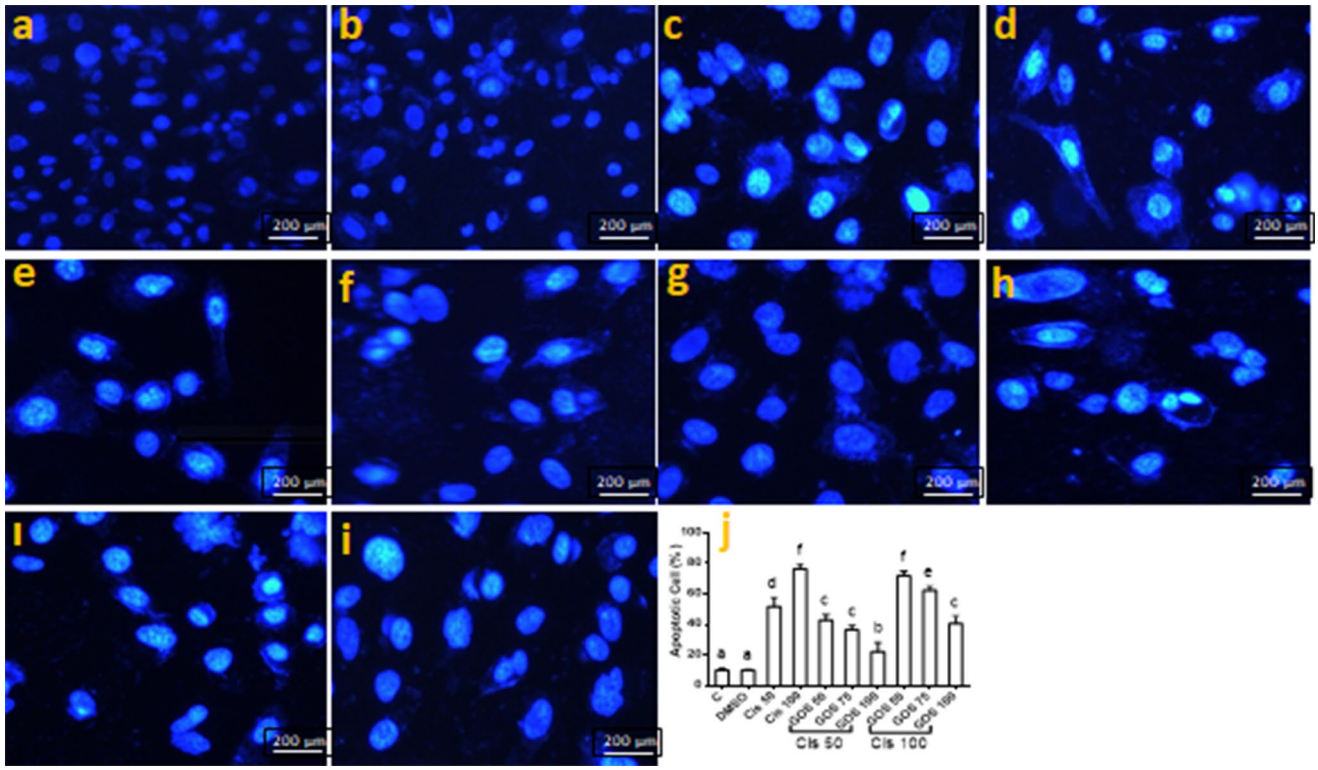

Fig. 4 Fluorescent microscope findings and apoptotic cell rates after Hoechst 33,342 staining in primary neuron culture cells. **a** Control, **b** DMSO (%0.01), **c** CIS 50: CIS 50 µM, **d** CIS 100: CIS 100 µM, **e** CIS 50+GOS 50: Gossypin 50 µM, **f** CIS 50+GOS 75: Gossypin 75 µM, **g** CIS 50+GOS 100: Gossypin 100 µM. **h** CIS 100+GOS 50: Gossypin 50 µM, ı. CIS 100+GOS 75: Gossypin 75 µM, **i** CIS 100+GOS 100: Gossypin 100 µM. **j** Apoptotic cell (%)

border were improved, and the cytoplasm became close to control in Purkinje cells, which was dose-dependent in the GOS-treated groups (Fig. 7).

The CIS group was compared with the control group, and the most prominent degeneration was seen in the capillaries. In the CIS group, it was observed that the capillary wall integrity was impaired, and the endothelial cells clustered into the lumen. The capillary degenerations were smoothed in the GOS-applied groups depending on the dose. Purkinje and capillary base changes in hematoxylin-eosin stained

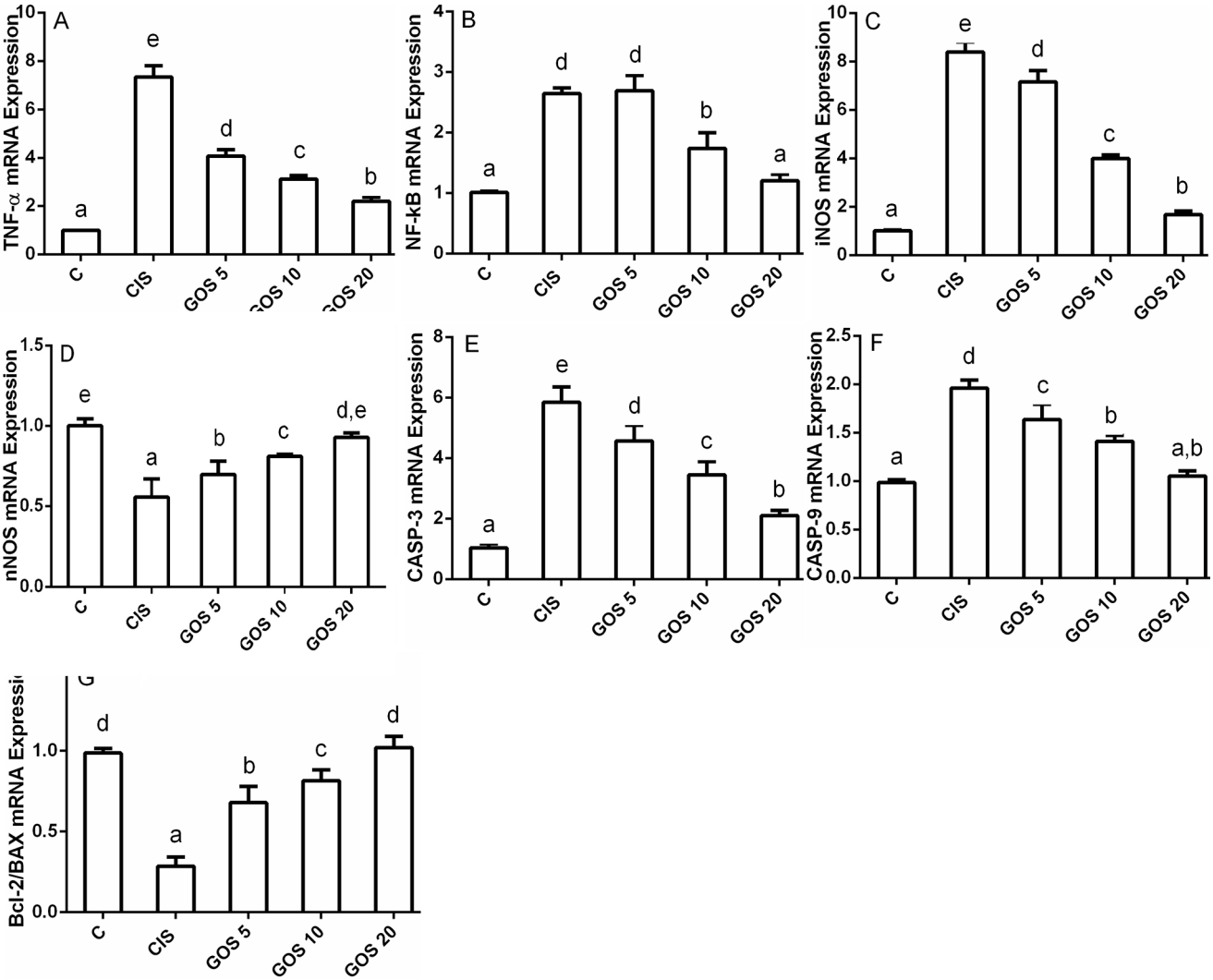

Fig. 5 In-vivo parameters (**A**) TNF-α, (**B**) NF-kB, nNOS, (**C**) iNOS, (**D**) nNOS, (**E**) CAS-3, (**F**) CAS-9 and, (**G**) Bcl-2/Bax mRNA levels.Values not sharing a common superscript differ significantly at $P<0.05$ Duncan's Multiple Range Test (DMRT). C: Control, CIS: CIS 10 mg/kg, GOS 5: Gossypin 5 mg/kg, GOS 10: Gossypin 10 mg/kg, GOS 20: Gossypin 20 mg/kg

sections were scored for Purkinje cell degeneration and capillary degeneration. (Supplementary Table 1)

Immunohistochemical findings

Immunopositive Purkinje cells in the ganglion cell layer of the cerebellum tissues were counted by marking with BAX primary antibody in the brain tissues (Fig. 7). A statistically significant difference was found between the groups ($P<0,0001$). The number of positive cells was increased in the CIS group compared to the control group ($P<0,0001$). It was observed that the number of immunopositive Purkinje cells decreased statistically depending on the increasing dose of gossiping ($p=0,0004$, $p=0,0055$, $p=0,0053$, respectively) (Fig. 7).

Positive cells were counted in the dentate gyrus of the hippocampus, especially in the subgranular zone (Fig. 6). When the groups were compared for the number of positive cells, the difference was statistically significant ($P<0,0001$). The number of positive cells was increased in the CIS group compared to the control group ($P<0,0001$). Compared to the control group, it was observed that the number of positive cells increased due to the decreased dose of gossypin ($p=0,0020$, $p=p<0,0001$, $p=0,0020$, respectively) (Fig. 6).

When the H-score analysis of the BAX primary antibody marking of the images taken from the cerebral cortex was examined, it was observed that there was a statistically significant difference between the groups ($p<0,0001$). The H-score ratio increased in the control group compared to the CIS group, which was statistically significant ($p<0,0001$). When the control group and GOS groups were compared,

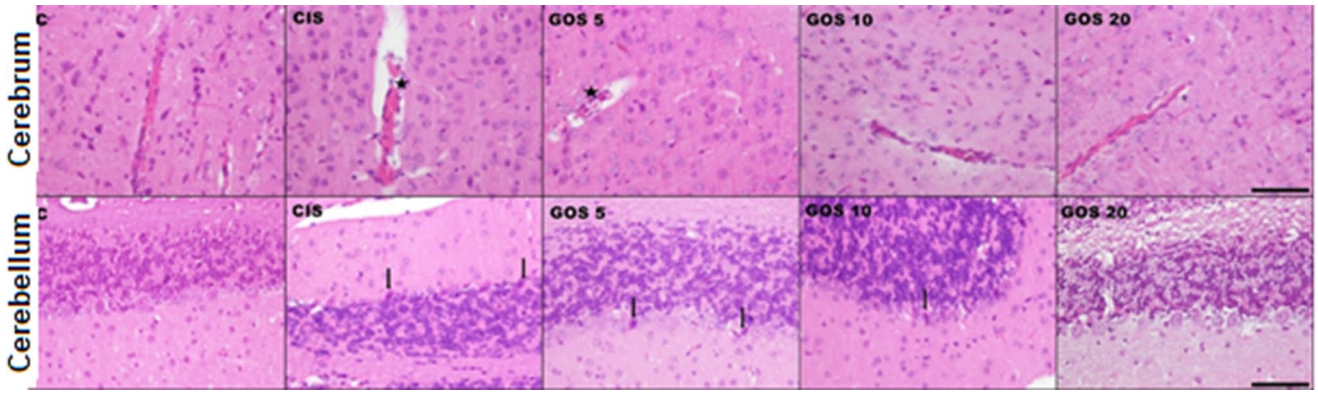

Fig. 6 Brain Micrographs Stained With Hematoxylin Eosin. C: Control, CIS: CIS 10 mg/kg, GOS 5: Gossypin 5 mg/kg, GOS 10: Gossypin 10 mg/kg, GOS 20: Gossypin 20 mg/kg. OK: Picnotic cells, star; cells clustered into the lumen in capillaries. Bar: 200 µm

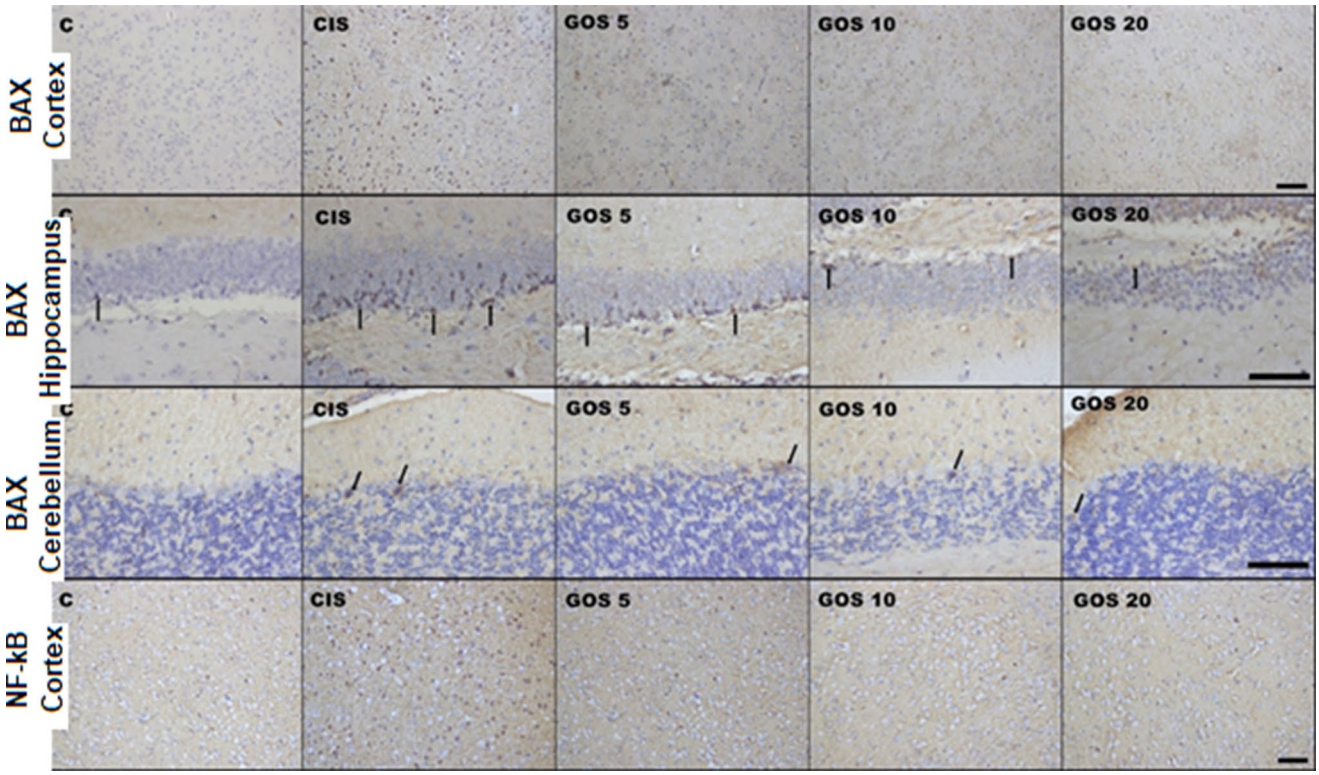

Fig. 7 Labeling with BAX and NF-kB Primary Antibody. Brown areas indicate immunopositivity. Arrow: positive cells (hippocampus and cerebellum). C: Control, CIS: CIS 10 mg/kg, GOS 5: Gossypin 5 mg/kg, GOS 10: Gossypin 10 mg/kg, GOS 20: Gossypin 20 mg/kg. OK: Picnotic cells, star; cells clustered into the lumen in capillaries. Bar: 200 µm

it was observed that the positivity decreased with increasing dose ($p<0{,}0001$, $p<0{,}0001$, $p=0{,}0003$), respectively (Fig. 7).

The H-score ratios obtained from labeling with NFKB primary antibody were statistically significant between the groups ($p<0{,}0001$). When the control group was compared with the CIS group and GOS groups, it was observed that the H-score changed statistically Supplementary Fig. 3) (CIS; $p<0{,}0001$, GOS 5; $p<0{,}0001$, GOS 10; $p<0{,}0001$, GOS 20; $p<0{,}0001$) (Fig. 7).

Discussion

The research thoroughly investigated gossypin for its antioxidant, anti-inflammatory, and anticancer properties using a comprehensive array of in-vitro and in-vivo methodologies, followed by a detailed analysis of the findings. The efficacy of CIS is dose-dependent, with higher doses yielding more pronounced therapeutic outcomes. However, dosage escalation is limited by severe toxicities, primarily nephrotoxicity, ototoxicity, and neurotoxicity [25]. Nonetheless, no

prophylactic measures have been established to counteract CIS's ototoxic or neurotoxic effects [26]. Clinically, saline hydration and osmotic diuresis are preventative strategies that have shown partial effectiveness against nephrotoxicity. Nonetheless, no prophylactic measures have been established to counteract CIS's ototoxic or neurotoxic effects. The most common approach to mitigate CIS-induced neurotoxicity involves either dose modification or cessation of treatment [26]. Among platinum-based chemotherapeutic agents, CIS is recognized as the most neurotoxic.

CIS-induced neurotoxicity is cumulative and dose-dependent [27]. In our study, CIS 50 and CIS 100 increased cell death, ROS, inflammatory and apoptotic genes, and damage in primary neuron cultures, especially in CIS 100 groups. Neurotoxicity limits CIS chemotherapy, highlighting the need for protective agents. We determined gossypin's IC50 value as 60.40 µM and selected doses of 50, 75, and 100 µM. Applying CIS and gossypin to neuron cultures, we assessed cell viability over 24, 48, and 72 h. CIS showed a time-dependent cytotoxic effect, increasing with CIS 100. Gossypin protected neurons dose-dependently, with CIS 100+GOS 100 group showing 80% viability at 72 h, compared to 43% for CIS 100 alone.

The pathophysiological processes associated with CIS-induced toxicity are not yet fully elucidated. Oxidative stress is a pivotal mechanism, with studies observing reduced levels of endogenous antioxidants like SOD and GSH, and elevated oxidative biomarkers such as MDA in CIS-treated tissues. This oxidative damage can cause structural alterations in neural tissue, leading to demyelination and myelin degeneration. Other mechanisms contributing to CIS toxicity include inflammatory responses, lipid peroxidation, and apoptotic cell death. CIS increases pro-inflammatory cytokines like TNF-α and IL-1β in astroglial cells of the spinal cord [28]. Normally, cellular homeostasis balances ROS production and neutralization by antioxidants like GSH, SOD, and CAT. However, excessive oxidative stress can damage proteins, lipids, and DNA, potentially leading to cellular transformation and carcinogenesis.

Furthermore, CIS exhibits a particular affinity for neuronal structures within the peripheral and central nervous systems. Neuronal cells are especially vulnerable to oxidative stress due to their limited antioxidant capabilities and high lipid concentration, predisposing them to oxidative damage [29, 30].

Cancer cells experience heightened oxidative stress from ROS compared to normal cells due to oncogene activation, which drives uncontrolled cell proliferation, enhanced metabolic demands, and mitochondrial dysfunctions. Gossypin has been shown to have anti-inflammatory activity, potentially related to neuroprotection in the brain by inhibiting inflammation mediators. Its free radical scavenging activity is directly responsible for its antioxidant and neuroprotective effects [16].

In our study, SOD activity, GSH, and MDA levels were measured using ELISA kits for in-vitro biochemical studies at 6 and 12 h, and on homogenates from primary neuron culture cells and brain tissue. Results showed significant decreases in SOD activity and GSH levels, and significant increases in MDA levels in the CIS groups compared to the control group, especially in the in-vitro CIS100 group. Gossypin dose-dependently normalized CIS-induced damage in both in-vitro and in-vivo groups. Our in-vitro findings align with hippocampal tissue concentrations observed in in-vivo studies, confirming that CIS treatment increases lipid peroxidation and reduces glutathione levels [31]. Consequently, interventions that inhibit lipid peroxidation and elevate intracellular antioxidants could help maintain neuronal integrity, crucial for cognitive functions and memory retention.

The impact of CIS on the balance between neurotoxic and neuroprotective mechanisms, specifically iNOS/nNOS, was examined. CIS-treated cohorts showed elevated neurotoxic iNOS and reduced neuroprotective nNOS levels. NO acts as both a neurotoxin and a neuroprotector [32]. CIS-induced ROS upregulate iNOS and downregulate nNOS [33]. iNOS's harmful effects may involve caspase-dependent and independent pathways, while nNOS's benefits are mediated by hypoxia-inducible factor-1, a regulator of neuroprotective genes [34].

TNF-α is a important cytokine in brain injury pathophysiology, synthesized by astrocytes, neurons, and microglia during neuroinflammatory processes [35]. Inflammation is critical in cisplatin-induced toxicity, with elevated NF-κB and TNF-α levels observed in CIS-treated subjects. TNF-α is upregulated early after nerve injury, triggering local inflammation [36]. CIS-treated primary neuron culture cells showed increased TNF-α, iNOS, and decreased nNOS mRNA levels. Gossypin normalized these changes dose-dependently. In vivo studies confirmed elevated TNF-α levels in brain tissue supernatant, supporting our findings. NF-κB activation induces proinflammatory mediators, including TNF-α and iNOS [37].

CIS showed NF-κB phosphorylation compared to control, significantly increasing TNF-α, iNOS, and nNOS mRNA expression levels. Gossypin inhibited NF-κB activation, suppressing CIS-induced inflammation in primary neuron culture cells. Our in-vivo study supports this. Mitochondrial dysfunction and apoptosis are pivotal in CIS-induced cytotoxicity [38, 39]. Overproduction of ROS triggers apoptosis via extrinsic and intrinsic pathways. Bax, a pro-apoptotic protein, plays a vital role in this process [40]. Mitochondrial impairment causes redox disequilibrium and apoptosis, particularly in peripheral neurons [41]. CIS-induced oxidative

stress depletes mitochondrial protein thiol groups, impairing calcium sequestration and reducing mitochondrial membrane potential [42]. The mitochondrial apoptosis pathway, modulated by Bcl-2 family proteins, involves an increase in Bax and a decrease in Bcl-2. The Bcl-2/Bax ratio is crucial for assessing cellular apoptosis and viability [43].

Previous studies have shown CIS affects several molecular factors in cytotoxicity, including activation of p53 and modulation of Bcl-2 family proteins like BAX. CIS releases cytochrome c from BAX-displaced mitochondria [44, 45]. CIS-induced apoptosis involves p53 activation, Bax translocation, cytochrome c release, and caspase-3 and caspase-9 activation [46]. In our in-vitro study, the BCL2/BAX ratio in CIS-treated groups was almost 3/4 lower compared to the control group. Gossypin improved this ratio dose-dependently, especially at the 12th hour, with more significant improvement in CIS50 groups than CIS100 groups. In our in-vivo study, CIS significantly reduced the BCL-2/Bax ratio and activated CASP-3 cleavage. Previous studies also show gossypin has antiapoptotic activity [20].

Our study confirms the anti-apoptotic effects of gossypin in mouse cerebral neurons. Gossypin reduced cell death by increasing Bcl-2 expression and decreasing Bax expression, while inhibiting CASP-3 and CASP-9 mRNA levels. Apoptotic cells were visualized using Hoechst 33,342 staining, showing fragmented nuclei in CIS-treated cells. Gossypin treatment significantly decreased apoptotic nuclei, indicating a dose-dependent protective effect against neuronal apoptosis. Control cells showed no apoptotic nuclei.

The beneficial effects of gossypin against CIS-induced apoptosis were supported by NF-κB and BAX IHC staining in brain tissue, providing evidence of its neuroprotective effect through antiapoptotic mechanisms.

Conclusions

Our results support gossypin as a neuroprotective anti-apoptotic agent during cisplatin-induced neurotoxicity. Gossypin regulates the apoptotic pathway by reducing nitro-oxidative stress and inflammatory markers caused by cisplatin. These findings help understand the anti-apoptotic and antioxidant properties of gossypin. The limitations of our study include the inability to perform in-vivo behavioral tests and the inability to specifically stain primary neuronal cells. In future studies, the time intervals can be extended and the results can be supported with behavioral tests to bring the study closer to the clinical study stage.

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Data availability No datasets were generated or analysed during the current study.

Declarations

Ethical approval The animal study was reviewed and approved by the Animal Experimentation Committee of the Kafkas University (KAU/HADYEK/2019-066 and KAU/HADYEK/2019-070.)

Consent for publication All authors agreed to the publication of this paper.

Competing interests The authors declare no competing interests.

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