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ORIGINAL PAPER

Non-cytotoxic Concentration of Cisplatin Decreases Neuroplasticity-Related Proteins and Neurite Outgrowth Without Affecting the Expression of NGF in PC12 Cells

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Abstract Cisplatin is the most effective and neurotoxic platinum chemotherapeutic agent. It induces a peripheral neuropathy characterized by distal axonal degeneration that might progress to degeneration of cell bodies and apoptosis. Most symptoms occur nearby distal axonal branches and axonal degeneration might induce peripheral neuropathy regardless neuronal apoptosis. The toxic mechanism of cisplatin has been mainly associated with DNA damage, but cisplatin might also affect neurite outgrowth. Nevertheless, the neurotoxic mechanism of cisplatin remains unclear. We investigated the early effects of cisplatin on axonal plasticity by using non-cytotoxic concentrations of cisplatin and PC12 cells as a model of neurite outgrowth and differentiation. PC12 cells express NGF-receptors (trkA) and respond to NGF by forming neurites, branches and synaptic vesicles. For comparison, we used a neuronal model (SH-SY5Y cells) that does not express trkA nor responds to NGF. Cisplatin did not change NGF expression in PC12 cells and decreased neurite outgrowth in both models, suggesting a NGF/trkA independent mechanism. It also reduced axonal growth (GAP-43) and synaptic (synapsin I and synaptophysin) proteins in PC12 cells, without inducing mitochondrial damage or apoptosis. Therefore, cisplatin might affect axonal plasticity before DNA damage, NGF/trkA down-regulation, mitochondrial damage or neuronal apoptosis. This is the first study to show that neuroplasticity-related proteins might be early targets of the neurotoxic action of cisplatin and their role on cisplatin-induced peripheral neuropathy should be investigated in vivo.

Keywords Cisplatin · GAP-43 · Synapsin I · Synaptophysin · Neurite outgrowth · NGF · PC12 cells · SH-SY5Y cells

Introduction

Cisplatin (*cis*-diamminedichloroplatinum II) is a highly-effective chemotherapeutic agent that is widely used, alone or combined with other agents, in the treatment of several types of cancer; however it induces severe dose-limiting toxicities including peripheral neurotoxicity [13, 30, 39]. Other platinum compounds also induce peripheral neurotoxicity, but cisplatin is the most neurotoxic among them [2]. The neurotoxicity of cisplatin is both cumulative and dose-dependent [27].

The symptoms of cisplatin-induced neurotoxicity are sensory-related and occur symmetrically in hands and feet, in a so-called "gloves-and-stocking" distribution [5, 30]. Symptoms include numbness, tingling, pins and needles, burning, decreased or increased sensitivity and pain [21]. There are no effective measures to prevent or treat the neurosensory damage induced by cisplatin and symptoms may persist even after treatment discontinuation; most patients improve over time, but the recovery might be incomplete [6, 17].

Cisplatin-induced neurotoxicity has been associated with damage of mitochondrial and genomic DNA, but the precise mechanism is not clear [7, 23, 32]. First, cisplatin causes a distal dying-back axonal degeneration that might progress to degeneration of cell bodies and apoptosis [3, 25]. It is possible that axonal degeneration induce peripheral neuropathy

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regardless loss of cell bodies. In fact, despite neuronal apoptosis, symptoms occur nearby distal axonal branches [34]. In this scenario, early mechanisms of axonal damage might be involved. In fact, a study showed that NGF inhibits cisplatin-induced neuronal apoptosis in vitro, without reducing DNA adduct formation [29]. Another study showed decreased levels of circulating NGF in patients treated with cisplatin and with neuropathic symptoms [31]. Additionally, it has been demonstrated that cisplatin inhibits neurite outgrowth in vitro [24], but the mechanism is unclear. Altogether, these findings suggest that cisplatin might affect NGF and axonal plasticity. The present study focus on these mechanisms of axonal damage that might involve NGF and neuroplasticity, but not necessarily DNA damage. The effects of cisplatin on neuroplasticity are unknown. We used a PC12-cell-neuronal model to address this issue.

PC12 are neuron-like cells that, when exposed to NGF, differentiate acquiring biochemical and morphological characteristics of sympathetic neurons [20]. They express NGF-high-affinity receptors (trkA) and respond to NGF by forming neurite extensions, branches and synaptic-like vesicles. Therefore, PC12 cells are a useful system to study the action of neurotoxic agents on neuronal differentiation and the involvement of NGF/trkA pathway [16, 20, 38].

Based on these premises, the present study investigates the effects of non-cytotoxic concentrations of cisplatin on axonal growth (GAP-43) and synaptic (synapsin I and synaptophysin) proteins, as well as in NGF expression in PC12 cells. We also evaluated neurite outgrowth in SH-SY5Y neuronal model, which has a different profile for neurotrophin receptors (trkB) and do not respond to NGF stimulation [22]. This is the first study to address neuroplasticity-related proteins as axonal targets of cisplatin.

Materials and Methods

Chemicals

Reagents were obtained from Sigma-Aldrich® (St. Louis, MO, USA), unless differently stated. Cell culture media were purchased from GIBCO®. Reagents for Western blot analysis were obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Working concentrations of cisplatin were prepared in 0.9% saline solution prior to assays. All solutions were prepared with ultra-pure water purified by a Milli-Q Gradient system (Millipore, Bedford, USA). In this study, high purity reagents were used (analytical grade minimum).

Cellular Cultures

PC12 and SH-SY5Y cells were obtained from the American Type Culture Collection, ATCC (Rockville, MD).

PC12 cells were maintained at 37 °C in humidified atmosphere containing 5% CO_2 and 95% air. The medium used was Dulbecco's Modified Eagle Medium (DMEM; GIBCO®) supplemented with 10% heat-inactivated horse serum (GIBCO®), 5% heat-inactivated fetal bovine serum (FBS; GIBCO®), and 1% antibiotic mixture (5 mg/mL penicillin, 5 mg/mL streptomycin and 10 mg/mL neomycin, PNS GIBCO®). Medium was changed every 3 days. To harvest PC12 cells, medium was removed and cells were detached with trypsin/EDTA solution (GIBCO®). Trypsin was inactivated by the addition of supplemented medium and after centrifugation, cells were suspended in the growth medium and plated at the density required by each assay.

SH-SY5Y cells were grown in F12 nutrient mixture (F12 HAM; Sigma Cell Culture, St. Louis, MO) supplemented with 15% fetal bovine serum (GIBCO) and 1% PNS. Cells were cultured in 75 cm^2 tissue-culture flasks at 37 °C under a humidified atmospheric condition of 5% CO_2 and 95% air. Medium was replaced every day. Confluent cultures were detached with trypsin/EDTA solution (Gibco®), inactivated with growth medium, centrifuged, and subcultured (1:2; every 2–3 days). Third-passage cells with 80% confluence were used in the experiments.

Determination of the Working Concentration of Cisplatin

PC12 cells (2.0×10^5 cells/well) were plated in 24-well plates coated with poly-L-lysine (Sigma-Aldrich®, St. Louis, MO, USA) and incubated for 24 h for better adhesion. After this period, the medium was replaced by F-12K Nutrient Mixture Kaighn's Modification (GIBCO®) supplemented with 1% horse serum, 1% antibiotic mixture and NGF (100 ng/mL). Cells were treated with serial dilutions of cisplatin (1, 5, 10, 20, 50 and 100 μM), incubated for 24 or 72 h and tested for cell viability and neurite outgrowth, respectively. The tested range of concentrations were based on a previous related study [24]. The lowest concentration of cisplatin able to decrease the number of differentiated cells, without causing significant cytotoxicity was chosen as the working concentration.

Neurite Outgrowth Assays

PC12 cells (2.0×10^5 cells/well) were plated in 24-well, poly-L-lysine-coated microplates (Sigma-Aldrich®, St. Louis, MO, USA) and incubated for 24 h for adhesion. Then, medium was replaced by F-12K Nutrient Mixture Kaighn's Modification (GIBCO®) supplemented with 1% horse serum, 1% antibiotic mixture and NGF (100 ng/mL). Cells were treated with cisplatin 5 μM and incubated for 72 h.

SH-SY5Y neuroblastoma cells were incubated in 24-well plates (3×10^4 cells/well) in F12 HAM supplemented with

15 % FBS and 1 % antibiotic mixture (penicillin/Streptomycin/Neomycin, PSN GIBCO®) for 24 h for adhesion. After this period, the medium was replaced (F12 HAM supplemented with 1 % FBS, 1 % PSN) and cells were incubated for 5 days, during which the medium was renewed every 2 days. Two different experimental conditions were analyzed: (i) simultaneous treatment with cisplatin and retinoic acid (RA), and (ii) 24 h-pretreatment with cisplatin before addition of RA. In the first condition, cells were simultaneously treated with RA 10 µM and cisplatin 5 µM and incubated for 5 days. In the second condition, cells were treated with cisplatin 5 µM for 24 h, then the medium was replaced (to remove cisplatin) prior to the addition of RA 10 µM and further incubation for 5 days. The removal of cisplatin allows to investigate if the possible effect of cisplatin would derive or not of the simple inactivation of RA by a direct reaction with cisplatin.

For both cell lines, the morphometric analysis was performed on the images obtained under inverted-phase-contrast microscopy (Carl Zeiss Axio Observer A1 inverted microscope, ×400 magnification), after incubation for 24, 48 and 72 h. Quantitation was performed by using the Image J Software [35]. Cells with at least one neurite with a length equal to (or higher than) the cell body diameter were considered as differentiated [11].

MTT Assay—Dose-Response Curve

The MTT assay is based on the conversion of MTT to formazan (purple) by mitochondrial dehydrogenases of viable cells [19]. An aliquot of 200 µL of cell suspension (2.0×10^4 cells/well) was incubated with different concentrations of cisplatin (1, 5, 10, 20, 50, 100, 200 µM) in 96-well microplates at 37 °C, for 24 h, in a humid atmosphere with 5 % of CO_2. Then, plates were incubated with 20 µL of MTT solution (5 mg/mL) for 3 h at 37 °C. After that, plates were centrifuged (1000 rpm, 5 min), the supernatant was discarded and 200 µL of DMSO was added to solubilize formazan. The absorbance was measured at 570 nm on a microplate reader (Multiskan FC, Thermo Scientific).

LDH Release

PC12 cells (1.0×10^6 cell/well) were seeded in 12-well plates and incubated (at 37 °C, 5 % CO_2) for 24 h before treatment. Then, cells were exposed to different concentrations of cisplatin (1, 5, 10, 20, 41.66, 83.33, 166.33, 333.33 µM) for 24 h and the medium was collected in order to analyze the level of cytoplasmic lactate dehydrogenase (LDH) released into the medium, a marker of membrane integrity loss. Determination of released LDH activity was performed according to the instructions of the manufacturer (Colorimetric Lactate Dehydrogenase Activity Assay Kit TOX7, Sigma-Aldrich®, St. Louis, MO, USA).

Caspase-3 Activity

An aliquot of 1 mL of cell suspension (1.0×10^7 cell/well) was incubated with cisplatin 5 µM in 6-well plates (at 37 °C, for 24 h). After that, medium was removed and cells were assayed for caspase-3 activity by using the Colorimetric Caspase 3 Assay Kit (Sigma-Aldrich®, St. Louis, MO, USA) according to the manufacturer's instructions. The activity of caspase-3 was assessed by monitoring the absorbance at 405 nm.

Western Blot Analysis

Preparation of Cell Lysate

PC12 cells (2.0×10^5 cells/well) were plated in 24-well plates coated with poly-L-lysine (Sigma-Aldrich®, St. Louis, MO, USA) and treated as describe above "Neurite Outgrowth Assays". After 72 h of incubation, cells were detached with trypsin, transferred to the microcentrifuge tubes and centrifuged (1000 rpm, 5 min, 4 °C). The supernatant was discarded and the cell pellet was suspended in 40 µL Tris-Triton lysis buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, Triton X-100 1 %, 10 % glycerol, 0.1 % SDS, 0.5 % deoxycholate, 1:200 Protease Inhibitor Cocktail and 1 % Phosphatase Inhibitor Cocktail). After 10 min, the cell lysate was centrifuged at 12,000 rpm for 10 min at 4 °C and the supernatant was stored in freezer (−80 °C) until the assay. A 10 µL aliquot of cell lysate was assayed for protein determination by the Bradford method.

Determination of Protein in Cell Lysate (Bradford)

The Protein Assay Dye Reagent (Bio-Rad) was used according to the manufacturer's instructions. Lysates and color reagent were diluted with water (1:5) and a calibration curve of BSA (40, 100, 200 and 400 mg/mL) was used. The absorbance (595 nm) was determined in a microplate reader (Multiskan FC, Thermo Scientific) and the concentrations of protein were calculated based on the calibration curve response and multiplied by the dilution factor (5).

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were added to an equal volume of Laemmli sample buffer (65.8 mM Tris, pH 6.8, 26.3 % glycerol, 2.1 % SDS, 0.01 % bromophenol blue, 5 % β-mercaptoethanol) and heated to 98 °C for 5 min. Aliquots of 35 µL containing

10 µg total protein were applied to 10% polyacrylamide gel (10 wells) and separated by SDS-PAGE (1 h, 160 V, Tris/glycine/SDS buffer).

Transfer

Proteins were transferred to nitrocellulose membranes (1 h, 0.37 A, Tris/glycine buffer).

Immune Reaction

Membranes were blocked (30 min, RT, 300 rpm) with 5% milk or BSA solution in Tween 20/TBS buffer (TTBS). The membranes were incubated with the primary antibodies: anti-GAP-43 (1:1250), anti-Synaptophysin (1:400) or anti-Synapsin I (1:1000) overnight at 4 °C, 300 rpm. Then, the membranes were washed with TTBS and incubated (1h, RT, 300 rpm) with the secondary antibody conjugated with horseradish peroxidase (anti-mouse or anti-rabbit IgG—HRP. 1:20,000). The membranes were washed with TTBS and TBS and treated with 3 mL of chemiluminescence enhancer detection reagent (1:1) and images were captured by using ChemiDoc system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Quantitation was performed on the images based on the optical densitometry (OD) of the bands by using the open source software Image J software [35]. Finally, the membranes were stripped (2% SDS, 62.5 mM Tris pH 6.8 and 100 mM mercaptoethanol) and reprobed for loading control with anti-β-actin (1:3000). OD values of GAP-43, synapsin I and synaptophysin bands were divided by the OD values of β-actin for normalization of the results [26].

Determination of NGF Expression

PC12 cells (2.0×10^5 cell/well) were plated in 24-well plates and incubated at 37 °C for 24 h prior to the following additions: cisplatin 5 µM, NGF 100 ng/mL (positive control). Untreated cells were used as negative control. Then cells were incubated at 37 °C for 72 h. After treatment, an aliquot of 100 µL of culture supernatant was analyzed by using Enzyme-Linked Immunosorbent Assay kit RAB0381 (Sigma-Aldrich®, St. Louis, MO, USA) as recommended by

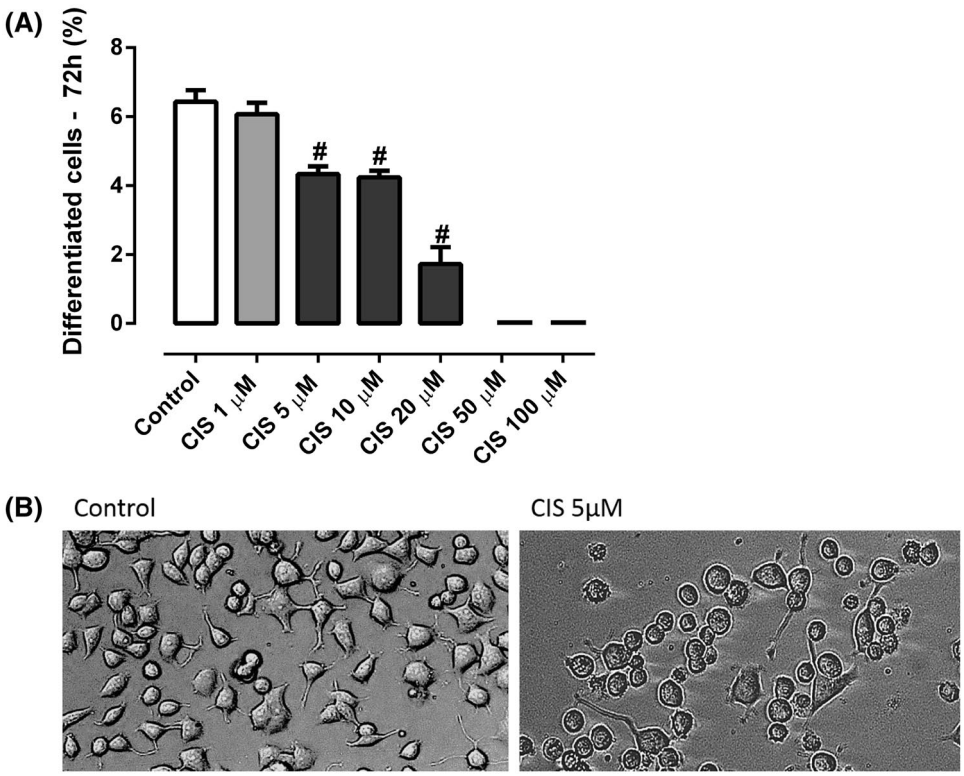

Fig. 1 Effect of different concentrations of cisplatin on the differentiation of PC12 cells after 72 h-incubation. Additions: NGF 100 ng/mL; cisplatin (100-5 serial dilution except 1 µM). **a** Bar graph. *Each bar* represents the mean ± SEM obtained from three independent experiments; each experiment was performed in triplicates. #Significantly different from the control group ($p < 0.05$). Cisplatin 1 µM had no effect, cisplatin (5–20 µM) significantly decreased differentiation and cisplatin (50 and 100 µM) completely blocked cell differentiation in relation to the control. **b** Photomicrographs showing the morphological changes of PC12 cells in control and in the group treated with 5 µM cisplatin, after 72 h of incubation. Cells in the control are square-shaped, larger and have more neurite extensions than cells exposed to 5 µM cisplatin (spherical-shaped)

the manufacturer. The absorbance at 450 nm was determined in a microplate reader (Multiskan FC, Thermo Scientific).

Statistical Analysis

Data were expressed as means ± SEM. Statistical analysis was carried out by using One-way ANOVA (Analysis of Variance) for multiple comparison, followed by the Bonferroni post-test (GraphPad Prism Software, version 5.0 for Windows, San Diego, California, USA). Differences between pairs were analyzed by using the non-parametric Mann–Whitney test. Values of $p < 0.05$ were considered significant. Experiments were repeated three times using cell cultures from different days. The experiment of each day was performed in triplicate.

Results

Effect of Cisplatin on the Differentiation of PC12 Cells

No significant difference was observed on the differentiation of cells treated with 1 μM cisplatin (6.07 %) in relation to control (6.43 %). Decreased percentage of differentiated cells was observed in cells treated with 5 μM cisplatin (4.33 ± 0.22 %), 10 μM cisplatin (4.24 ± 0.18 %) and 20 μM (1.72 ± 0.49 %) as compared to control; higher concentrations of cisplatin (50 and 100 μM) completely blocked cell differentiation (Fig. 1a). Figure 1b shows the morphological changes between the control and cells treated with 5 μM cisplatin, after 72 h of incubation. Cells in the control have large, square-shaped bodies and form more

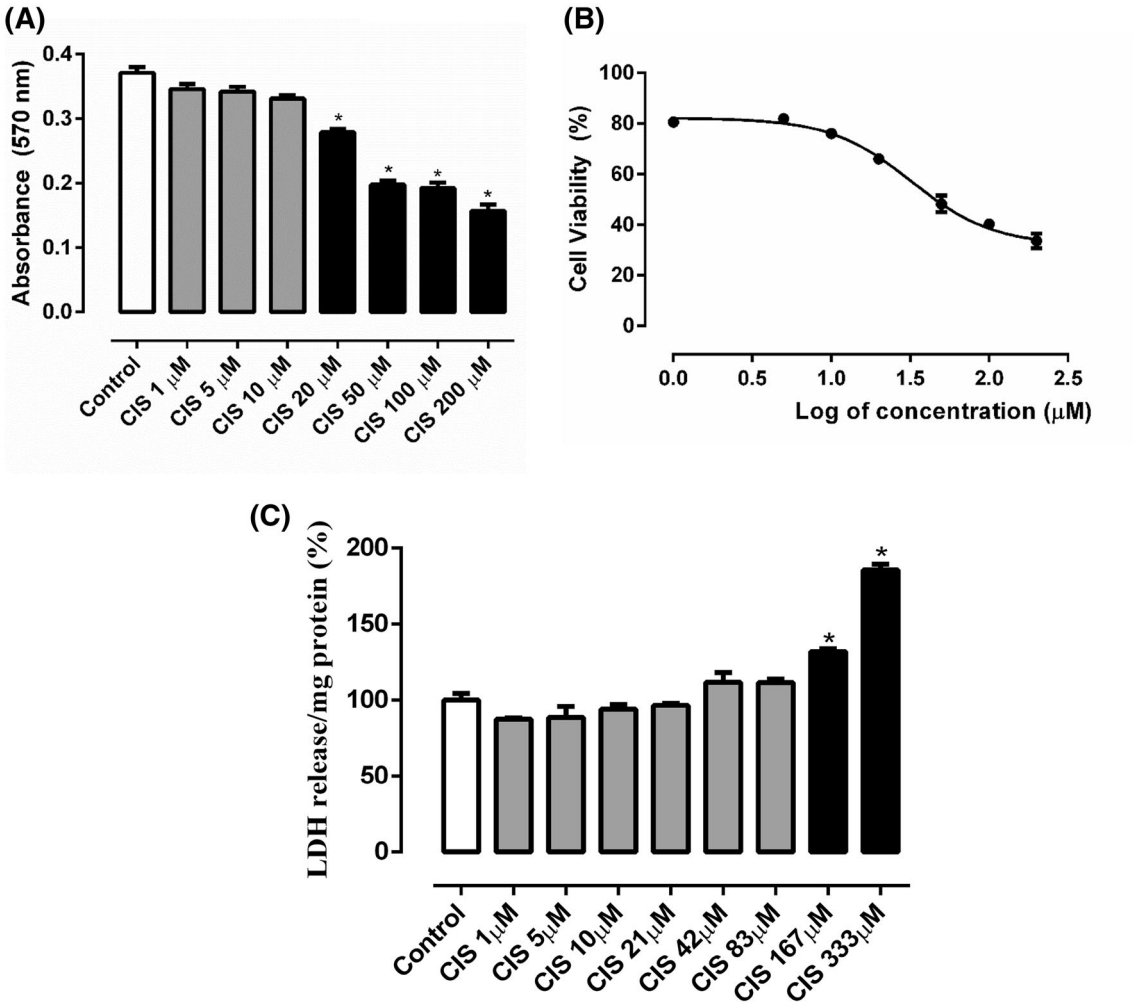

Fig. 2 Cytotoxicity of different concentrations of cisplatin after 24 h-incubation. **a** MTT reduction assay: bar graph. **b** Dose-response curve: non-linear curve of the cellular viability (%) versus log of cisplatin concentrations. Equation: [Y = 100/(1 + 10^(LogIC50-X) × hillslope], in which hillslope is −1.682 and LogIC50 is 1.509. The calculated IC50 is 32.27 μM. **c** LDH assay: results are presented as means ± SEM obtained from three independent experiments; each experiment was performed in triplicate. Controls were normalized to 100 %. *Significantly different from the control group ($p < 0.05$). The mitochondrial toxicity of cisplatin started at 20 μM as shown by the MTT assay, whereas membrane integrity loss started at 167 μM as shown by the LDH release assay

neurite extensions, while cells exposed to 5 µM cisplatin are mostly spherical and have fewer neurites as compared to the control.

Cytotoxicity of Cisplatin

As shown in Fig. 2a (MTT assay), no significant reduction in the mitochondrial activity was observed in cells treated with 1 µM (94.86±2.12%), 5 µM (93.84±2.10%) or 10 µM (90.86±1.42%) of cisplatin. When cells were exposed to 20 µM of cisplatin (76.53±1.50%) or higher concentrations such as 50 µM (54.24±1.83%), 100 µM (52.78±2.39%) and 200 µM (43.13±2.66%), the viability of PC12 cells decreased significantly as compared to control (101.8±2.55%).

The non-linear curve of the cellular viability (%) versus log of cisplatin concentrations is presented in Fig. 2b. The absorbance from MTT assay was normalized (0–100% range) and the concentrations of cisplatin were transformed into the corresponding logarithm. IC50 (32.27 µM) was calculated by using the following equation: Y = 100/(1 + 10^(LogIC50-X) × hillslope).

The viability of PC12 cells exposed to serial dilutions of cisplatin was also assessed by monitoring of the release of the cytosolic enzyme LDH, which indicates loss of the integrity of the cytoplasmic membrane. Only the two highest concentrations of cisplatin (166 and 333 µM) significantly increased the release of LDH as compared to control.

Based on the neurite outgrowth (Fig. 1) and cytotoxicity (Fig. 2) assays, the working concentration of cisplatin (5 µM) was chosen. This was the lowest concentration of cisplatin that significantly decreased cell differentiation without causing significant cytotoxicity.

Effect of Cisplatin on Caspase-3 Activity

Cisplatin 5 µM (0.16±0.014) did not alter the activity of caspase-3 as compared to control (0.14±0.014) (Fig. 3).

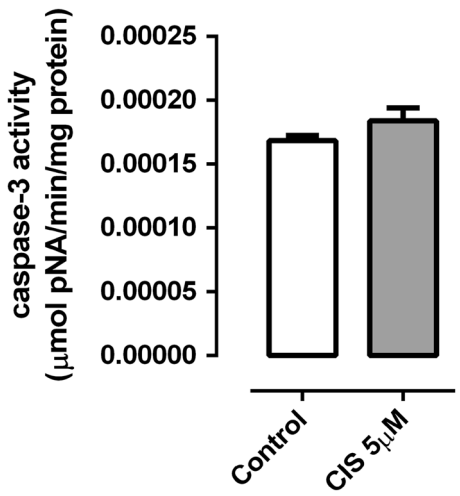

Fig. 3 Effect of cisplatin 5 µM on caspase-3 activity. Results are presented as means ± SEM obtained from three independent experiments; each experiment was performed in triplicate. Cisplatin 5 µM did not change caspase-3 activity as compared to the control

Effect of Cisplatin 5 µM on the Differentiation of Retinoic-Acid-Stimulated SH-SY5Y Neuroblastoma Cells

Retinoic acid increased the percentage of differentiated cells as compared to controls in all periods of incubation. When cells were incubated simultaneously with RA 10 µM and CIS 5 µM, the percentage of differentiated cells were reduced (Fig. 4). Figure 4f shows the increased neurite outgrowth and the morphological changes in cells treated with RA and the partial inhibition of these effects in cells treated simultaneously with cisplatin and RA.

Pretreatment with RA and cisplatin 5 µM reduced the percentage of differentiated cells in all periods of incubation (Fig. 5). Figure 5f shows the increased neurite outgrowth and the morphological changes induced by RA and the partial inhibition of this effect in cells treated with cisplatin prior to the addition of RA.

Effect of Cisplatin on the Expression of GAP-43, Synapsin I and Synaptophysin

Cisplatin 5 µM reduced the expression of GAP-43 (1.06±0.16), synapsin I (1.14±0.06) and synaptophysin (1.11±0.18) as compared to control (2.17±0.33; 2.02±0.21; 2.39±0.33, respectively). Results are presented in Figs. 6, 7a, b and 8a, b, respectively.

Expression of NGF

Cisplatin 5 µM reduced the expression of NGF (0.018±0.0019), but it was not significantly different from control (0.021±0.0032) as depicted in Fig. 9.

Discussion

Cisplatin-induced neurotoxicity has been associated with damage of mitochondrial and genomic DNA, but the precise mechanism is not clear [7, 23, 32]. Cisplatin causes a *Wallerian-type* axonal degeneration that precedes the neuronal apoptosis [21, 33]. Axons are very susceptible to the mitochondrial DNA damage induced by cisplatin [32], but cisplatin also seems to interfere with NGF and neurite outgrowth [24, 29, 31]. These findings suggest that cisplatin might impair axonal plasticity. The progression of cisplatin-induced neurotoxicity might have a similar time course

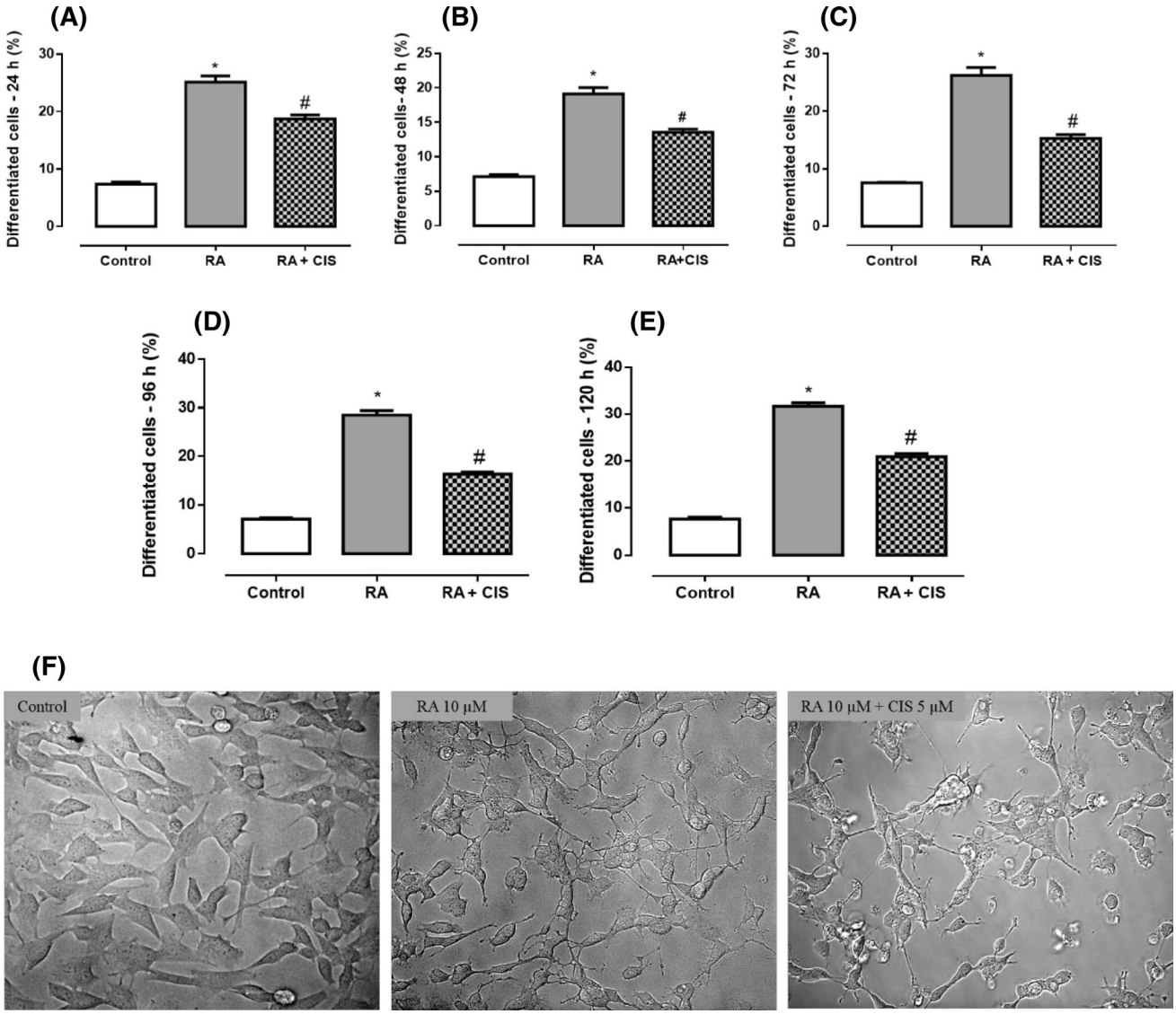

Fig. 4 Effect of cisplatin on the differentiation of SH-SY5Y cells after simultaneous addition of cisplatin 5 µM and retinoic acid (RA) 10 µM, and incubation for different periods: **a** 24, **b** 48, **c** 72, **d** 96 and **e** 120 h of incubation. Simultaneous treatment with 10 µM RA and 5 µM cisplatin reduced the percentage of differentiated cells in all periods of incubation. *Bars* represent means ± SEM of three independent experiments; each experiment was performed in triplicate. *Significantly different from control ($p < 0.05$); #significantly different from RA 10 µM ($p < 0.05$). **f** Phase-contrast photomicrographs after 120 h of incubation

observed in other neurotoxic processes, such as that induced by MPP^+ (neurotoxin relevant to Parkinson's disease), i.e., inhibition of differentiation occurs at early stages before death of neurons [14].

In order to address the early axonal damage induced by cisplatin, we evaluated the effects of a non-cytotoxic concentration (5 µM) of cisplatin on neurite outgrowth and on neurite-associated proteins (GAP-43; synapsin I and synaptophysin) in PC12 cells, widely used neuronal model to assess the outgrowth of neurites. Neurites are processes extended by cultured neurons or neuron-like cells upon trophic stimulation and they are precursors of axons and dendrites.

First, we selected the appropriate concentration of cisplatin by evaluating the effect of several concentrations on differentiation and viability of PC12 cells. Cisplatin (1 µM) was not cytotoxic nor affected neurite outgrowth. The lower non-cytotoxic concentration of cisplatin that significantly decreased neurite formation was 5 µM. Another study in PC12 reported the same effects at similar concentrations [24]. To evaluate cytotoxicity, we performed two well-known assays, MTT reduction and LDH release. MTT assay monitors mitochondrial function, while LDH assay monitors the integrity of cell membrane [28]. According to our results, cisplatin induced mitochondrial damage from 20 µM concentration (IC50 = 33 µM) and induced LDH

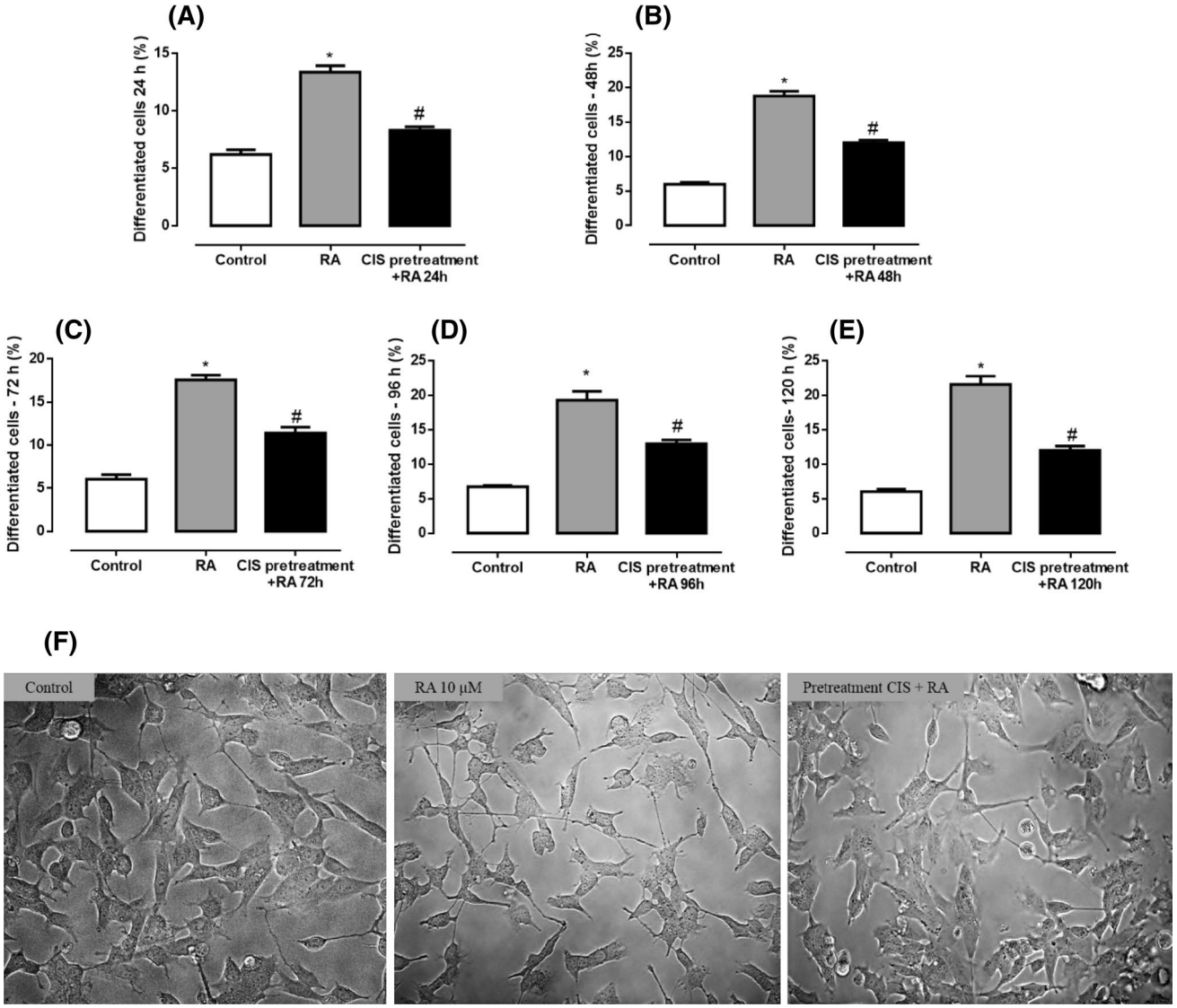

Fig. 5 Effect of pretreatment with cisplatin 5 µM on the differentiation of SH-SY5Y cells stimulated with retinoic acid (RA) 10 µM. Periods of incubation: **a** CIS +RA 24, **b** 48, **c** 72, **d** 96 and **e** 120 h. Treatment with 5 µM cisplatin prior to treatment with RA reduced the percentage of differentiated cells in all periods of incubation. *Bars* represent means ± SEM of three independent experiments; each experiment was performed in triplicate. *Significantly different from control ($p < 0.05$); #significantly different from RA 10 µM ($p < 0.05$). **f** Phase-contrast photomicrographs after 120 h of incubation

release from 167 µM concentration. No mitochondrial damage was observed in cells treated with 5 µM cisplatin. Additionally, we addressed apoptosis by evaluating the effect of cisplatin 5 µM on the activity of caspase-3, the final executioner in the apoptotic cascade. No significant difference was observed in relation to control. Altogether, LDH, MTT, caspase-3 and neurite outgrowth assays clearly showed that the selected concentration (5 µM) of cisplatin does not cause mitochondrial damage, cell body loss or apoptosis. On the other hand, it significantly impaired the differentiation induced by NGF in PC12 cells. Therefore, this concentration was selected to assess the early neurotoxic events induced by cisplatin.

PC12 cells express NGF-high-affinity receptors (trkA) and differentiate in the presence of NGF [37]. In fact, PC12 cells constitutes a useful system for studying trkA function [38]. In order to evaluate the involvement of NGF/trkA pathway, we investigated changes in the expression of NGF in PC12 cells and additionally, we evaluated neurite outgrowth in a second neuronal model (SH-SY5Y cells) with a distinct phenotype for neurotrophin receptors. SH-SY5Y cells do not express trkA; therefore, they do not respond to NGF stimulation. On the other hand, they express functional trkB in the presence of RA, and differentiate in the presence of RA alone or combined with another neurotrophin, BDNF [22]. In SH-SY5Y cells, cisplatin decreased

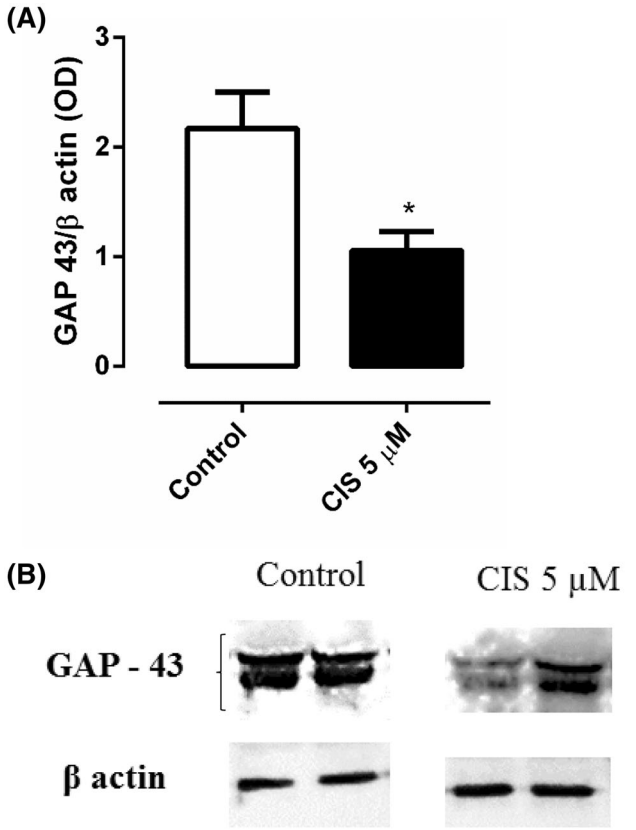

Fig. 6 Effect of cisplatin 5 μM on the expression of GAP-43. **a** Bar graph of the optical densitometry of the bands of GAP-43 normalized with β-actin (loading control). **b** Western blot bands. Cisplatin 5 μM reduced the expression of GAP-43 as compared to the control. *Bars* indicate means ± SEM of three independent experiments; each experiment was performed in triplicate. *Significantly different from control ($p < 0.05$)

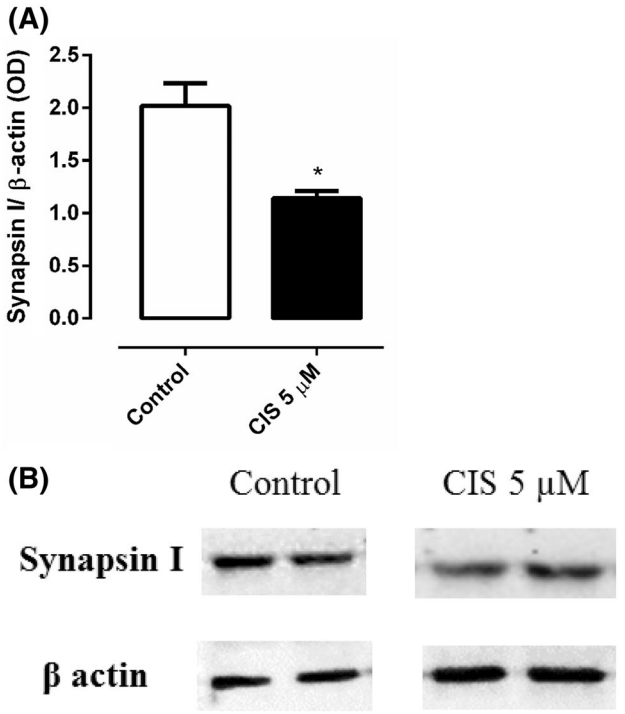

Fig. 7 Effect of cisplatin 5 μM on the expression of Synapsin I. **a** Bar graph of the optical densitometry of the bands of synapsin I normalized with β-actin (loading control). Cisplatin 5 μM reduced the expression of synapsin I as compared to the control. **b** Western blot bands. *Bars* indicate means ± SEM of three independent experiments; each experiment was performed in triplicate. *Significantly different from control ($p < 0.05$)

neuritogenesis in relation to control in both treatment protocols, i.e., (i) simultaneous treatment with cisplatin and RA, and (ii) 24 h-pretreatment and removal of cisplatin, which excludes the possibility of the inactivation of RA by cisplatin. These results also suggest that trkA receptors are not early targets of cisplatin, since cisplatin decreased neuritogenesis in both cell lines, PC12 and SH-SY5Y, regardless the expression of trkA receptors. It might occur later, but data on this issue are controversial. A study suggested that cisplatin decreases the expression of trkA receptors in sensory neurons [1]; whereas another study reported that no significant change was observed in the expression of mRNA for trkA in a rat model of peripheral neurotoxicity [8].

Additionally, cisplatin did not significantly change the expression of NGF in PC12 cells, which corroborates the results in SH-SY5Y cells, i.e., that the early neurotoxic effects of cisplatin are not mediated by the NGF/trkA pathway. On the other hand, studies have shown reduced levels of circulating NGF in rat models of peripheral neuropathy and in chemotherapy patients with neurological deficits [8, 12]. A study in mice showed that cisplatin reduced the levels of NGF in several peripheral NGF-producing tissues, and that administration of exogenous NGF restored the neurological deficits induced by cisplatin in these animals [1]. Altogether, the previous and present findings suggest that cisplatin might affect the synthesis and availability of NGF at later stages of the neurotoxic process, when the neurological deficits are already in course, but not at early stages. It is possible that down-regulation of NGF results from the action of cisplatin on DNA [1], an event that might occur after axonal damage. Another explanation is that peripheral NGF-producing tissues might be more susceptible to cisplatin-DNA binding than neurons [8] or neuronal models such as PC12 cells.

Although cisplatin seems not to decrease NGF at early stages, it is possible that neuroplasticity-related proteins are affected by cisplatin in a process not mediated by NGF. To investigate this hypothesis, we evaluated the effect of 5 μM cisplatin on the expression of three neuronal proteins related to axonal growth (GAP-43) and synaptic communication (Synapsin I and Synaptophysin) [11]. Cisplatin reduced the expression of these three proteins. The growth-associated protein GAP-43 is a protein that is preferentially distributed in the growth cone and elongating axon during development

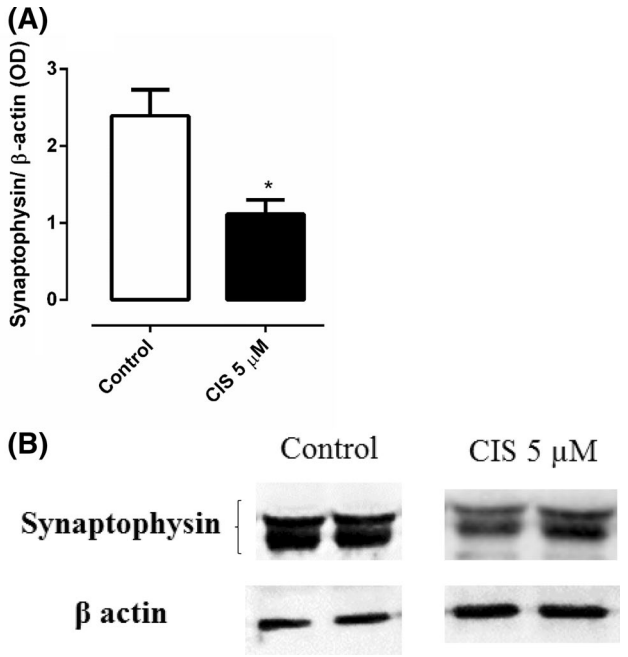

Fig. 8 Effect of cisplatin 5 μM on the expression of Synaptophysin. **a** Bar graph of the optical densitometry of the bands of Synaptophysin normalized with β-actin (loading control). **b** Western blot bands. Cisplatin 5 μM reduced the expression of synaptophysin as compared to the control. *Bars* indicate means ± SEM of three independent experiments; each experiment was performed in triplicate. *Significantly different from control ($p < 0.05$)

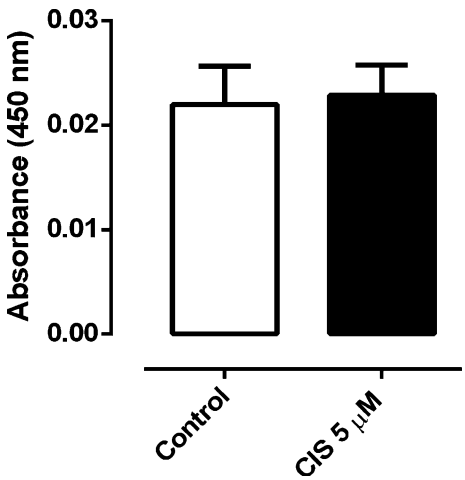

Fig. 9 Effects of cisplatin 5 μM on the expression of NGF in PC12 cells after 72 h of incubation. Cisplatin 5 μM decreased the expression of NGF but the difference was not significant. *Bars* indicate means ± SEM of three independent experiments; each experiment was performed in triplicate. No significant difference was observed between the groups

and regeneration of neurite network, and formation of new synapses [9]. Synapsin I and synaptophysin, are localized in the presynaptic membrane and are respectively used as molecular markers for nerve terminal maturation and synaptogenesis [11, 40]. Down-regulation of these proteins suggest that cisplatin is able to impair peripheral neuroplasticity before inducing neuronal apoptosis. There is no data about the effects of cisplatin on these proteins, except for one study [18] that showed up-regulation of mRNA for GAP-43 in peripheral neurons of rats treated with cisplatin; however, the effect occurred only after a cumulative dose of 14 mg/kg. One hypothesis for these opposing findings is that, at first, cisplatin would decrease neuroplasticity and later, after severe damage, neurons would respond by stimulating regenerative processes to increase the expression of the same proteins.

Little is known about the neuroplasticity of the peripheral system [15, 36] and about the effects of cisplatin on axonal proteins. The results of the present study suggest that the impairment of axonal plasticity by cisplatin might have implications on the development/progression of neuropathies. In vitro systems are rapid and reliable tools to investigate the potential neurotoxicity of chemicals as well as the molecular mechanisms responsible for it [4, 10]. However, it is important that the mechanisms and potential targets shown under the homogeneous and controlled environment of cell culture be complemented in vivo, under the variety of nerve cells, integrated functions and compensatory mechanisms. Further studies should investigate the effect of cisplatin on peripheral neuroplasticity in animal models.

Conclusion

Cisplatin has the potential to impair axonal growth and synaptic communication and it might occur before DNA damage, NGF/trkA down-regulation, mitochondrial damage, cell bodies loss and apoptosis. This is the first study to show that neuroplasticity-related proteins might be early targets of the neurotoxic action of cisplatin and their role on cisplatin-induced peripheral neuropathy should be investigated in vivo.

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