Does Sinapic Acid Provide Neuroprotection Against Cisplatin-Induced Toxicity in HT-22 Cells?

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Abstract: Objective: The aim of this study was to explain the benefits and possible protective mechanisms of sinapic acid (SA) against cisplatin-induced oxide-inflammatory damage in HT-22 rat hippocampal cells by biochemical and molecular methods. Materials and methods: Sinapic acid (SA) was applied at different concentrations (100, 400, and 800 μM) before cisplatin treatment on HT-22 cells under in vitro conditions to elicit neuroprotective activity. Half an hour after SA treatment, 5.5 μM cisplatin was added to all wells except the control group and incubated for 24 hours. Cell viability was determined by 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and cytotoxicity was determined by lactate dehydrogenase (LDH) assays. Oxidative stress was evaluated by total antioxidant capacity (TAC), catalase (CAT), glutathione reductase (GSH), malondialdehyde (MDA), and superoxide dismutase (SOD) assays. In addition, the effect of SA on Caspase-3 gene regulation in HT-22 cells was investigated by real-time PCR. Results: Cisplatin decreased cell viability by approximately 40% and increased LDH level in HT-22 cells. In SA-treated groups, cell viability increased and LDH level decreased dose-independently. SA showed neuroprotective activity by inhibiting the cytotoxic activity of cisplatin and increasing the antioxidant activity in cells. Similarly, Caspase-3, which was up-regulated by cisplatin, approached the control value upon SA administration. SA eliminated the neurotoxicity of cisplatin and significantly reduced cell death and oxidative stress. Conclusion: The results of this study indicate that SA protects HT-22 cells against cisplatin by inhibiting both the formation of oxidative stress and induction of cell apoptosis.

Keywords: Catalase; Caspase-3; Oxidative stress

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1. Introduction

Cisplatin (CP) is a platinum-based chemotherapeutic agent. It is widely used in the treatment of many solid tumors such as lung, stomach, ovarian, testicular, colorectal, and bladder cancers [1]. Despite its widespread use, CP does not act in a targeted manner in the body. Instead, it acts on healthy cells as well as cancerous cells and causes significant neurotoxic side effects such as peripheral neuropathy, impaired recognition, tremor, and ataxia, which limit its clinical use [2]. Oxidative stress, DNA damage, mitochondrial dysfunction, and increased production of proinflammatory cytokines play an important role in the neurotoxicity caused by CP [3]. Although some clinical and preclinical studies have reported that CP causes cognitive dysfunction, learning difficulties, and memory loss [2,4], information on the effects of CP on the hippocampal structure, biochemical changes, and cognitive behaviors is still limited. Therefore, in this study, we investigated the effects of CP on proinflammatory cytokines and oxidative stress markers in hippocampal cells and rats.
Minimizing the neurotoxic damage caused by frequent use of CP has recently become an important research topic [5]. Due to their antioxidant properties, polyphenolic compounds have recently been suggested to be useful in reducing the toxic effect of CP on brain tissue [6]. Sinapic acid (SA) is a polyphenol derived from hydroxycinnamic acid, which is cinnamic acid with 3,5-dimethoxyl and 4-hydroxyl structures in the phenyl group. It has been reported that SA has pharmacological activities such as anti-inflammatory, antioxidant, antibacterial, and antitumor [7]. At the same time, it has been reported to stabilize the increase in lipid peroxidation in neurodegenerative diseases such as Alzheimer’s and Parkinson, suppress oxidative stress, and show neuroprotective effect by preventing cell apoptosis [7,8]. However, there has been no study on the effect of SA on CP-induced hippocampal neurotoxicity.

CP-induced neurotoxicity is mediated by various mechanisms. In this study, the benefits and possible protective mechanisms of SSA against oxido-inflammatory damage induced by cisplatin in HT-22 rat hippocampal cells by biochemical and molecular methods are discussed.

2. Materials and methods

2.1. Chemicals and reagents
CP (CAS 15663-27-1), Phosphate buffer solution (PBS), Dulbecco’s Modified Eagles Medium F12 (DMEM-F12), Fetal Bovine Serum (FBS), dimethylsulfoxide (DMSO), 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Trypsin (with EDTA) and antibiotics were obtained from Sigma-Aldrich (St. Louis, MO, USA), and Elisa kits were obtained from Elabscience (Houston, USA). Primers and reagents required for gene expression by real time-PCR were obtained from Roche (Darmstadt, Germany).

2.2. Cell culture experiments

2.2.1. HT-22 cell culture
HT-22 (CVCL_0321) was purchased from ATCC for our study and stored at -196°C until use. For the experiment, the thawed cells were first centrifuged for 5 min. Cells were suspended in fresh medium, 10% fetal bovine serum (FBS) and 1% antibiotic (penicillin, amphotericin B and vestreptomycin) and seeded in different well plates for each assay. Cells seeded in 96-well plates (Corning, USA) for MTT and LDH activity assays and 6-well plates (Corning, USA) for PCR assays were stored in an incubator (5% CO₂; 37 °C) under appropriate ambient conditions (9).

2.2.2. In vitro drug administration
Firstly, different concentrations of SA (100, 400 and 800 μM) were administered to ensure neuroprotective activity (10). Half an hour later, 5.5 μM CP was applied to all groups except the control group (11). 24 hours were incubated under optimum conditions. After 24 hours of incubation, the study was terminated for analyses (12).

2.2.3. MTT assay
After 24 h of treatment, MTT (10 μL) was added to each well of a 96-well plate and the plate was incubated in a CO₂ incubator for 4 h. After 4 h, DMSO (100 μL) solution was added (to liquefy formazan crystals). The optical density was read at 570 nm by a spectrophotometer.

2.2.4. Lactate dehydrogenase (LDH)
LDH level was examined in the LDH detection kit. HT-22 cells at a density of 103-106 cells/well were seeded in 200 μL medium in a 96-well plate and and incubated for 24 h. The cells were then removed from the incubator to measure the LDH level. 6 wells were prepared for each concentration. TritonX-100 (10%)
and assay buffer were added, and the wells were incubated at room temperature for one hour. After centrifugation, the cell supernatant was transferred to a new 96-well assay plate. LLDH reaction solution was added to each well and the plate was incubated at 37 °C for 30 min with gentle shaking on an orbital shaker. A microplate reader was used to measure the absorbance optical density (OD) at 490 nm [13].

### Table 1. Gene sequences of specific primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>β-actin</td>
<td>5’-CCAACCGCGAGAAGATGA-3’</td>
<td>5’-CCAGAGGGCTACAGGGGATAG-3’</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>5’-CAGTGGAGGCGACTTCTTG-3’</td>
<td>5’-ATCCAGTCGCTTTGTGCCA-3’</td>
</tr>
</tbody>
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### 2.3. Biochemical analyses

#### 2.3.1. Total antioxidant capacity (TAC)

TAC levels were measured spectrophotometrically (Multiskan™ GO Microplate Spectrophotometer). The number of oxidants and antioxidants in the sample was determined based on the intensity of the color.

#### 2.3.2. Analysis of catalase (CAT), glutathione (GSH), malondialdehyde (MDA), superoxide dismutase (SOD), TNF-α, and IL-1β assay

CAT, GSH, MDA and SOD, TNF-α, and IL-1β were determined by ELISA kits (Elabscience, USA). ELISA analyses were performed according to the manufacturer’s instructions. Absorbance was measured with a spectrophotometer at 450 nm.

#### 2.3.3. Real-time PCR

Total RNA was extracted with the High Pure RNA Isolation Kit (Roche Mannheim Germany). Cells were extracted with absolute methanol. We performed a real-time Polymerase Chain Reaction (PCR) using a High Capacity Single-strand cDNA Synthesis Kit and specific primers for RT-PCR (AMW) from Roche (Darmstadt, Germany). The sequences of the genes used are listed in **Table 1**. Results were expressed as fold changes in relative expression changes between SA (100, 400 and 800 μg/mL), CP (positive control), and control group (healthy cells). We normalized the expression of target genes to beta-actin (reference control gene) using the ΔΔCt method [14].

### 2.4. Statistical analysis: Data obtained from experiments were analyzed with SPSS 26.0. All data are expressed as mean ± SD. Differences between experimental groups were compared by One-Way ANOVA. P < 0.05 were considered significant.

### 3. Results

#### 3.1. MTT test results

After 24 hours of application, the cytotoxicity results of all groups were determined by MTT assay and the cell viability graph is shown in **Figure 1**. Cell viability in the control group (negative control) was considered as 100%. In 5.5 μM CP, the cell viability was 62.47% after 24 hours, which meant that the cell viability was significantly decreased in 5.5 μM CP (P < 0.001). This approximately 40% decrease in viability proves the cytotoxic effect of CP. In 100 μM SA, the cell viability was 85.31% (P < 0.05). After 24 hours, the highest cell viability was found to be 97.01% in 400 μM SA (P < 0.001). In our study, SA offered an effective treatment option at all doses. However, the most prominent neuroprotective activity was detected at a concentration of 400 μM. 400 μM SA eliminated the cytotoxic effect of CP and had a near 100% cell viability.
3.2. LDH results
Since LDH is released by necrotic cells, it is an excellent metabolic marker of cell viability. The effect of SA treatment on the LDH activity of HT-22 cells was determined using an LDH kit (Figure 1). The LDH activity of treated cells were expressed as % of the standard (shown as 100%). The LDH level in 5.5 μM CP was 26.96%, which was significant compared to the control group ($P < 0.01$). For neuroprotective activity, the LDH level decreased gradually in a dose-dependent manner in the SA-treated groups before 5.5 μM CP administration. The best result was found in the concentration of of 400 μM SA. In this group, LDH level was found to be 10.29, which was close to the control (8.73), and protected the cells against CP cytotoxic effect. However, LDH level also decreased significantly in the other two concentrations ($P < 0.05$ and $P < 0.01$). These data show that SA significantly decreases the cytotoxic effect of cisplatin.

![MTT results (a) and LDH activity results (b).](image)

Figure 1. MTT results (left) and LDH activity results (right). Data are expressed as mean ± SD. **$P < 0.01$ indicates significant differences compared to the control; # $P < 0.05$, ##$P < 0.001$ indicate significant differences compared to CP.

3.3. Redox status in HT-22 cells treated with SA

3.3.1. TAC results
The spectrophotometrically determined TAC value of HT-22 cells in the control group was 12.66 mmol Trolox/L, while the TAC value in 5.5 μM CP was 2.99 mmol Trolox equivalent/L (Figure 2). Compared to the control group, cisplatin decreased cellular antioxidant activity and induced cytotoxicity ($P < 0.01$). The antioxidant activity of cells was significantly increased by SA. The TAC value was 8.99 mmol Trolox/L in 100 μM SA, 11.815 in 400 μM SA and 10.97 mmol Trolox/L in 800 μM SA (Figure 2). TAC value in 400 μM group approached the control group at the highest level.
3.3.2. CAT, GSH, MDA, and SOD levels

As shown in Figure 3, the CAT, GSH and SOD levels decreased significantly in 5.5 μM CP compared to the control, while MDA levels increased significantly ($P < 0.001$). The cells treated with showed a significant increase in SA, CAT, GSH, and SOD activities, and decrease in MDA levels compared to the 5.5 μM CP ($P < 0.001$). Antioxidant activity, which decreased with cisplatin administration, reached normal levels in SA groups. These findings were consistent with TAC, MTT, and LDH results (Figure 3).
3.4. Inflammation status in HT-22 cells treated with SA

IL-1β and TNF-α mediate the development of numerous inflammatory diseases and IL-1β is typically activated when TNF-α is produced. According to our results, CP treatment significantly increased IL-1β and TNF-α levels in cells (24.49 pg/ml) \((P < 0.01)\). SA treatment decreased cellular inflammation. This decrease was dose-independent, and the most significant result was obtained in 400 μM SA. These findings were consistent with the results other tests in this study (Figure 4).
Figure 4. TNF-α and IL-1β results. Data are expressed as mean ± SD. Data are expressed as mean ± SD. **P < 0.01 indicates significant differences compared to the control; #P < 0.05, ##P < 0.001 indicate significant differences compared to CP.

3.5. Real-time PCR results
Caspase-3 gene expression was measured by real-time PCR analysis after 24 h of treatment. The results of this study showed that CP 5.5 μM significantly up-regulated Caspase-3 expression (P < 0.01). Specifically, a 1.69-fold increase is one of the most important indicators of cisplatin-induced apoptotic factors in HT-22 cells. SA reversed the apoptotic stimulus occurring in the cells regardless of the dose change and down-regulated Caspase-3 activity, thus protecting the cells from the apoptotic effect of cisplatin (Figure 5).
4. Discussion

In this study, the effect of SA against CP-induced hippocampal neuronal damage in HT-22 cells was investigated by biochemical and molecular methods. When we evaluated the neurotoxicity induced by CP using MTT and LDH assays, we found that 5.5 μM CP caused significant toxicity compared to control cells at the end of 24 h. Our results were consistent with other studies on the toxic effects of cisplatin on neuronal cell viability [2]. We found that SA significantly reduced CP-induced hippocampal toxicity. So far, there has been no information on the protective effect of SA against CP-induced neurotoxic damage in hippocampal cells was found. In vitro and in vivo studies have reported that CP causes oxidative damage and lipid peroxidation by increasing free radical in brain tissue [3,15]. MDA is the end product of lipid peroxidation. MDA exacerbates the damage caused by lipid peroxidation by causing cross-linking and polymerization of membrane components [16,17]. The fact that high MDA levels were found in cisplatin-treated cells in our study shows that our findings are compatible with other studies. It is also known that impaired redox balance is closely related with CP-related neuronal toxicity [18,19]. Therefore, the effect of cisplatin on SOD, GSH, CAT, and TAC levels, which are important indicators of antioxidant capacity, was investigated in our study. As seen from our findings, a significant decrease in SOD, GSH, CAT, and TAC levels was detected in parallel with the increase in MDA in hippocampal cells as a result of cisplatin administration. In in vivo and in vitro studies, it was reported that cisplatin significantly decreased antioxidant enzyme levels and total antioxidant capacity in hippocampus tissue [2,20]. SA significantly suppressed CP-induced increase in MDA and decrease in SOD, GSH, CAT and TAC levels in HT-22 cells. Verma et al. showed that SA protected hippocampus tissue from oxidative stress by increasing antioxidant enzyme levels and preventing MDA increase in Alzheimer’s disease rat models [7]. The results of this study support the previous studies and suggest that SA protects the hippocampus tissue against oxidative damage thanks to its antioxidant properties. It has been reported that the increase in free radical production in hippocampus damage accelerates neuroinflammation by triggering excessive production of proinflammatory cytokines such as TNF-α and IL-1β in neuronal cells [2,3]. In our study, TNF-α and IL-1β levels in hippocampal cells were found to increase with CP. In the study conducted by Cankara et al. [2], it

Figure 5. Caspase-3 gene regulation results, expressed as mean ± SD. **P < 0.01 indicates significant differences compared to the control; #P < 0.05, ##P < 0.001 indicate significant differences compared to CP.
was reported that there was a significant increase in TNF-α and IL-6 proinflammatory levels following oxidative stress as a result of CP application in HT-22 cells. SA treatment significantly decreased TNF-α and IL-6 levels compared to the cisplatin group, indicating that it antagonized CP-induced hippocampal inflammation. In the literature, it has been reported that SA reduces neurodegeneration by suppressing proinflammatory cytokine release [21]. Shahmohamady et al. (22) showed that SA improves inflammation in hippocampal tissue by inhibiting the increase of TNF-α inflammatory cytokines. It has been shown that oxidative stress induces apoptosis in cisplatin-induced hippocampal toxicity [14]. At the same time, it is known that high TNF-α and IL-1β levels caused by cisplatin in the hippocampus are related to apoptosis [23]. In our study, cisplatin caused a significant increase in the expression level of Caspase-3, an important component of the mitochondrial apoptotic pathway, in hippocampal cells. In in vivo studies, it was reported that Caspase-3 mRNA level increased significantly in hippocampus tissue [2]. In addition, Binnetoglu et al. [24] found an increase in Caspase-3, 9, and TNF alpha expression levels due to cisplatin toxicity in primary neuron culture. However, in our study, SA administration significantly suppressed the increase in caspase-3 caused by cisplatin. Our findings support the findings of other studies by showing that increased Caspase-3 level in neurodegenerative diseases is decreased by SA [25].

5. Conclusion

In conclusion, the results of this study suggest that SA is an effective molecule in the prevention of CP-induced neurotoxicity in HT-22 hippocampal cells. SA preserves the viability of HT-22 cells against cisplatin toxicity and reduces apoptosis through the mitochondrial intrinsic pathway by reducing oxidinflammatory damage. However, further in vivo and clinical studies are needed in addition to in vitro studies to clarify the molecular basis of the neuroprotective properties of SA.

Disclosure statement

The authors declare no conflict of interest.

References


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