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Oxytocin administration improves DNA damage and total oxidative stress parameters in vincristine and cisplatin-induced cortical neuron toxicity

Oksitosin uygulaması vinkristin ve sisplatin kaynaklı kortikal nöron toksisitesinde DNA hasarını ve toplam oksidatif stres parametrelerini iyileştirir

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ABSTRACT

Objective: Oxytocin (OXT) has been reported to have promising therapeutic potential due to its antioxidant properties in vincristine (VCR) and cisplatin (CP) induced peripheral neuropathy in both *in vivo* and *in vitro* studies. The cerebral cortex is responsible for sense, perception, and memory. Damage to these parts of the brain can lead to impairment of central nervous system functions. However, the effectiveness of OXT in toxicity caused by vincristine and cisplatin in cortical neurons has not been reported. In this study,we aimed to investigate the effects of OXT in VCR and CP-induced cortical neuron cell culture toxicity via biochemically measuring TAS-TOS levels and immunohistochemically determining 8-OHDG expression.

Methods: Cortical neuronal cells were exposed to different concentrations of VCR and CP, and also after the neuronal cells were exposed to OXT (1 µM) for 5 minutes, VCR and CP concentrations were

ÖZET

Amaç: *İn vivo* ve *in vitro* deneysel çalışmalarda oksitosin (OXT)'in antioksidan özelliği sayesinde vinkristin (VCR) ve sisplatinin (CP) neden olduğu periferik nöropatide umut verici terapötik potansiyele sahip olduğu bildirilmiştir. Serebral korteks duyu, algı ve hafıza gibi işlevlerden sorumlu olan beyin bölgesidir. Beynin bu bölgesinin hasar görmesi merkezi sinir sistemi işlevlerinin bozulmasına neden olabilir. Buna rağmen, kortikal nöronlarda VCR ve CP'nin neden olduğu toksisitede OXT'nin etkinliğini bildiren herhangi bir çalışmaya rastlamadık. Bu çalışmada in vitro olarak VCR ve CP'nin neden olduğu kortikal nöron toksisitesinde OXT'nin etkilerini biyokimyasal olarak TAS-TOS düzeylerini ölçerek ve immünohistokimyasal olarak 8-OHDG ekspresyonunu belirleyerek araştırmayı amaçladık.

Yöntem: Kortikal nöron hücrelerine VCR ve CP'ye ayrı ayrı ve farklı konsantrasyonlarda uygulanırken aynı zamanda 5 dakika boyunca OXT'ye (1 µM) maruz bırakıldıktan sonra, hücrelere ayrı ayrı ve farklı

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applied to the cells. Cell viability was determined using the MTT method. TAC and TOS were measured for antioxidant/oxidant activity. The expression of 8-OHdG was investigated as an indicator of oxidative DNA damage.

Results: Administration of OXT before CP and VCR exposure was able to protect against neuronal cytotoxicity. TAS levels increased positively correlated with in cells exposed to OXT+ VCR and OXT+ CP, while TOS levels and DNA damage (8-OHdG levels) negatively correlated.

Conclusion: OXT alleviated the toxic effects of VCR and CP-induced cortical neuron toxicity by increasing the TAS levels, while decreasing TOS levels and DNA damages. According to the results of this study, OXT has the potential to be used protective agent for anticancer agents induced toxicity. However, further studies are needed to clarify mechanisms of action of OXT.

Key Words: Oxytocin, vincristine, cisplatin, cortical neuron, DNA damage, oxidative stress

konsantrasyonlarda VCR ve CP uygulandı. Hücre canlılık testi, MTT yöntemi kullanılarak yapıldı. Antioksidan/oksidan aktiviteyi belirlemek için TAC ve TOS düzeyleri ölçüldü. 8-OHdG ekspresyonu, oksidatif DNA hasarının bir göstergesi olarak incelendi.

Bulgular: Kortikal nöron hücrelerine VCR ve CP uygulaması doz bağımlı olarak sitotoksisiteye neden olurken nöron hücrelerine VCR ve CP maruziyetinden önce OXT uygulaması nöronal sitotoksisiteye karşı koruma sağladı. TAS seviyeleri, OXT+VCR ve OXT+CP'ye maruz kalan hücrelerde pozitif korelasyon gösterirken, TOS seviyeleri ve DNA hasarı (8-OHdG seviyeleri) negatif korelasyon gösterdi.

Sonuç: OXT, VCR ve CP'nin kortikal nöronlarda neden olduğu toksisiteyi TAS düzeyini artırarak, TOS düzeyi ve DNA hasarını azaltarak gösterdiği saptanmıştır. Bu çalışma sonuçlarına göre OXT antikanser ajanların neden olduğu toksisitede protektif ajan olarak kullanılabileceği düşünülmüştür. Ancak, OXT'nin etki mekanizmalarını açıklığa kavuşturmak için daha ileri çalışmalar yapılmalıdır.

Anahtar Kelimeler: Oksitosin, vinkristin, sisplatin, kortikal nöron, DNA hasarı, oksidatif stres

INTRODUCTION

Oxytocin (OXT), a nine-amino-acid nano peptide hormone, is released from the hypothalamus (supraoptic and paraventricular nucleus) (1). While the best-known functions of OXT are uterine smooth muscle contraction and lactation, OXT has been demonstrated major roles in the growth and viability of endothelial, nerve, and glial cells in recent years. Also, OXT has a major role in endocrine and paracrine activities including neuromodulation (2,3). Abnormalities in OXT signaling pathways are relevant to many disease etiologies. OXT neuronal activities have been found significantly increased at the early stage of sepsis and cancer patients to initiate immune defense, thereby playing the role of immune surveillance (4,5). At the same time, exogenous OXT administration is very effective in improving oxidative stress by its antioxidant capacity. The antioxidant properties mechanisms of OXT work by preventing lipid peroxidation and oxidative apoptosis thanks to tyrosine and tryptophan residues in its structure (6). This may explain the capacity of OXT to act as a "natural

medicine" protecting against stress conditions and many illnesses (1). The unique properties of OXT we mentioned above, suggest that OXT maybe improve adverse conditions that could cause neuronal toxicity.

Anticancer drugs have been widely used for years, but the most important feature that distinguishes anticancer drugs from other drugs is the frequency and severity of side effects at therapeutic doses (7). Vincristine (VCR) and cisplatin (CP) are the most common chemotherapy methods in cancer treatment by targeting the proliferation of tumor cells and their ability to metastasize (8,9). VCR interferes with tumor cell proliferation through binding the ß-subunit of tubulin and inhibiting microtubule formation (8), while CP forms deoxyribonucleic acid (DNA) -platin adducts to impede cancer cells proliferation (9). VCR and CP are not specific to cancer cells and VCR and CP also cause oxidative stress in normal healthy cells lead to multiple organ toxicity (10,11). The pathophysiology of the neurotoxicity caused by VCR and CP are not fully known. However, VCR and CP accumulate irreversibly in neurons and increase free radicals causing lipid, protein, and DNA damage, lead to chronic pain in the peripheral nerves, irreversible nerve damage, memory retrieval, and mood disorders. Many studies are carried out to reduce the side effects caused by VCR and CP by using antioxidant substances together with VCR and CP (12,13). Zhu et al. demonstrated OXT is beneficial for VCR-induced chronic pain and decreases nerve damages (14), while Bilmez et al. showed that OXT is a protective agent with antioxidant properties in cisplatin-induced ototoxicity (15).

With this background, we hope to contribute to the attempts for improving related to VCR and CP-relevant cancer treatment protocols by using OXT. Therefore, oxidative damage was determined biochemically with TAS-TOS analysis, immunohistochemically with 8-OHdG expression.

MATERIAL and METHOD

Cell culture and application of the drugs

Frozen cortical neuron cells were obtained from the Pharmacology Department of Medical Faculty of Atatürk University (Erzurum, Turkey). Firstly, after thawing cryotubes were centrifuged (5 min, 1200 rpm). The cells were cultured in a neurobasal medium supplemented with 10% FBS, 2% B27, and 0.1% antibiotics (penicillin, streptomycin, and amphotericin B) at 37 °C in 5% CO_2 (16). The cells were seeded at a density of (2×10^5 cells/well) and then exposure to OT (1 µM) (17) for 5min, various concentrations of VCR (0.5, 1 and 2 µg/ml) (18) and CP (5, 10, 15 µg/ml) (19) were applied to primary cortical neurons for 24 hours.

MTT assay

The cortex neuronal cells viability assay was performed by using 3-[4,5-dimethylthylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) cell proliferation kit (Sigma, USA) (1). At the end of the exposure, MTT solution (5 mg/ml, 10 µl = 10% of total good volume) was added to each well and incubated at 37 °C for 4 h. The medium was then carefully removed and the formazan crystals dissolved with 100 µL of DMSO for 5 min. Thereafter, the absorbance was measured at 570 nm by a microplate reader (20).

TAC and TOS analysis

The total antioxidant capacity (TAC) and total oxidant status (TOS) were evaluated spectrophotometrically utilization of Erel methods by modifying with TAS and TOS commercial kit (Rell Assay Diagnostics, Gaziantep, Turkey) (21,22). The results of TAC and TOS were indicated as µmol Trolox Eq/L and µmol H_2O_2 Eq/L respectively. TAC method based on the measurement of the antioxidant impact of the sample against the free radical reactions, which is begun by the generated produced hydroxyl radical. The alteration of absorbance at 660 nm is dependent on the total antioxidant level of the sample (21). At the same time, the TOS method based on the

measurement of oxidant molecules in the sample oxidizes the ferrous iron chelator complex into ferric ion. The color density is associated with the total amount of oxidant molecules available in the sample and can be assessed at 530 nm (22).

TAC and TOS analysis

Cortical neuron cells were cultured in a chamber tissue culture glass slides and were administrated with agents at 75% confluence for 24 h. Once the chamber was removed, slides were rinsed with phosphate buffer, then the cells were fixed by 2% paraformaldehyde and permeabilized in methanol. Then slides were rinsed with phosphate buffer again % serum was used as a blocking agent. The primary antibody of 8-OHdG was incubated overnight. Then incubation with an appropriate biotinylated secondary antibody. 3,3'-diaminobenzidine (DAB) was used as a chromogen for staining and hematoxylin for counterstaining. Slides were examined with confocal microscopy and evaluated as having none (-), very light (+), mild (++), moderate (+++), severe (++++), very severe (+++++) necropsy according to the histopathological findings (16).

RESULTS

MTT assay results

Cells viability assay was used to analyze the metabolic activity. It evaluates the degradation of tetrazolium salts facilitated by mitochondrial dehydrogenases. The quantity of surviving cells is proportional to the formed formazan product. To investigate the effect of OXT on cell viability in cortex neuron culture, cortical neuron cells were treated with OXT (1 μM) for 5 min and also with common anticancer drugs, VCR (0.5, 1 and 2 μg/ml) and CP (5, 10, 15 μg/ml) (Figure 1). The highest survival rate was at a concentration of OXT (1 μM) which was determined as 95% whereas the viability rate of the highest concentration of CP (10 μg/ml) and VCR (2 μg/ml) were 80% and 81% respectively (p<0.05) (Figure 1). The combination of CP and VCR with OXT shows better results compared with the individual CP and VCR results. The viability rate of OXT combination with high dose VCR (2 μg/ml) and CP (10 μg/ml) were 86% (p>0.05). The highest survival rate among OXT combination was %92 (OXT 1 μM + VCR 0.5 μg/ml).

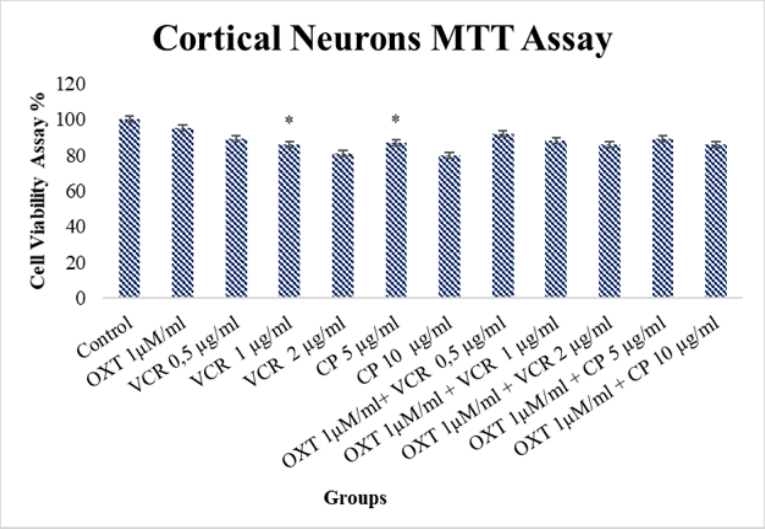

Figure 1. In vitro viability percentages of OXT (1 μM), VCR (0.5, 1 and 2 μg/ml) and CP (5, 10, 15 μg/ml) on primary cortex neurons (n=6/group).
* Significant differences at p<0.05 compared with the control group; ** Significant differences at the p<0.001compared with the control group. (OXT: Oxytocin, VCR: Vincristine, CP: Cisplatin)

Total antioxidant capacity (TAC) assay results

TAC assay was performed after 24 h exposure to CP, VCR, and OXT (Figure 2). TAC was decreased in a concentration-dependent manner following exposure to pure CP and VCR. The highest antioxidant capacity among pure drugs were measured at the lowest concentration of both VCR (0.5 µg/ml) and CP (5µg/ml) groups (5.8 and 5.6 Trolox equiv./mmol/L respectively) (p>0.05) and the lowest antioxidant capacity was observed in the two concentrations of VCR 2 µg/ml and CP 10 µg/ml respectively (p<0.05).

Total oxidant status (TOS) assay results

TOS assay was performed following 24 h exposure to CP, VCR, and OXT (Figure 3). TOS has increased following exposure to both CP and VCR this elevation in oxidant status was concentration-dependent (p<0.05). The highest oxidant rate was seen at the CP concentration of 10 µg/ml and was determined by changes in the 4.2 H_2O_2 equiv./mmol/L. Oxidant status in all concentration groups did not show a significant difference compared to control group values (p>0.05).

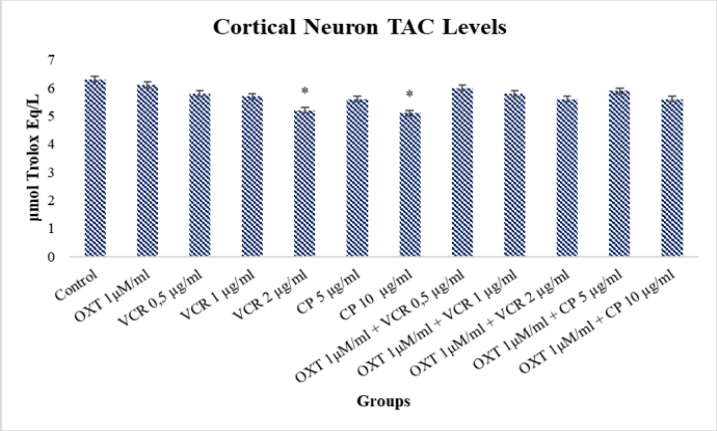

Figure 2. In vitro TAC capacity effects of OXT (1 µM), VCR (0.5, 1 and 2 µg/ml) and CP (5, 10, 15 µg/ml) on primary cortex neurons (n=6/group).
* Significant differences at p<0.05 compared with the control group; ** Significant differences at the p<0.001compared with the control group. (OXT: Oxytocin, VCR: Vincristine, CP: Cisplatin)

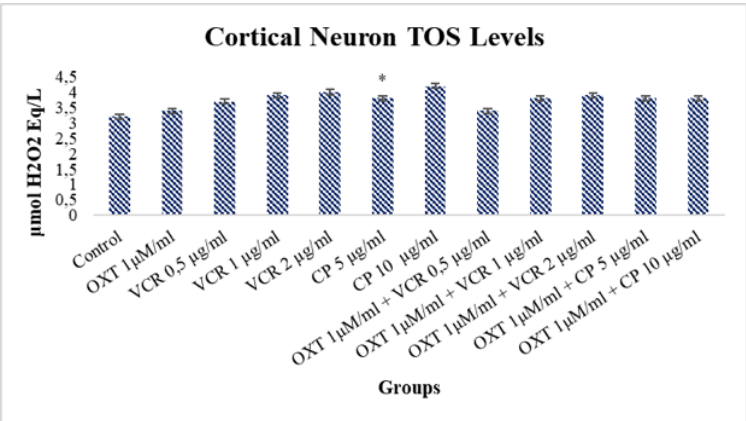

Figure 3. In vitro capacity effects of OXT (1 µM), VCR (0.5, 1 and 2 µg/ml) and CP (5, 10, 15 µg/ml) on primary cortex neurons (n=6/group).
* Significant differences at p<0.05 compared with the control group; ** Significant differences at the p<0.001compared with the control group. (OXT: Oxytocin, VCR: Vincristine, CP: Cisplatin)

Results of immunohistochemical investigation of 8-OHdG for cortex neuron culture

Immunohistochemically, 8-OHdG staining was used to determine the DNA damage in the primary cortex neuron culture. When cortex neuron culture was examined, 8-OHdG expression was not determined in neurons in the control and OXT 1 µM groups (Figure 4a-b). In the VCR 0.5 µg/ml group, while necrosis was determined in a small number of neurons, a very mild level of 8-OHdG expressions was detected in neuron cytoplasm (Figure 4c). In the VCR µg/ml group, intermediate levels of necrosis and 8-OHdG expressions in neuron cytoplasm were determined (Figure 4d). In the VCR 2 µg/ml group, necrosis and severe levels of 8-OHdG expressions were found in a large number of cells (Figure 4e). In the CP 5 µg/ml group was observed necrosis in some neurons and moderate 8-OHdG expressions in neurons cytoplasm (Figure 4f). In the CP 10 µg/ml group showed necrosis and severe 8- OHdG expressions in numerous neurons cytoplasm (Figure 4g). In the CP 15 µg/ml group, severe levels of necrosis and 8-OHdG expressions were determined in neurons (Figure 4h). In the VCR 0.5 µg/ml + OXT 1 µM groups were shown that necrosis in a few neurons and a very mild level of 8-OHdG expressions in neurons (Figure 4i). In the VCR 1 µg/ml + OXT 1 µM groups, necrosis was observed in one or two neurons and a mild level of 8-OHdG expressions was showed neuron cytoplasm (Figure 4j). In the VCR 2 µg/ml + OXT 1 µM groups, necrosis and moderate levels of 8-OHdG expressions were detected in neurons (Figure 4k). In the CP 5 µg/ml + OXT 1 µM groups, multiple necroses and a mild level of 8- OHdG expressions were determined in neurons (Figure 4l). In the CP 10 µg/ml + OT 1 µM groups, was determined necrosis in some neurons and moderate level 8-OHdG expression in neuron cytoplasm (Figure 4m). In the CP 15 µg/ml + OXT 1 µM groups, necrosis in some neurons and severe level 8- OHdG expression in neuron cytoplasm were detected.

Table 1. 8-OHdG –positive stained cells in cortical neuron cells in culture

	Necrosis in cortical neuron culture	8-OHdG in cortical neuron culture
Control group	-	-
OXT 1 µM group	-	-
VCR 0.5 µg/ml group	+	++
VCR 1 µg/ml group	+++	+++
VCR 2 µg/ml group	++++	++++
CP 5 µg/ml group	++	++
CP 10 µg/ml group	+++	++++
CP 15 µg/ml group	+++++	+++++
VCR 0.5 µg/ml + OXT 1 µM group	+	+
VCR 1 µg/ml + OXT 1 µM group	++	++
VCR 2 µg/ml + OXT 1 µM group	+++	+++
CP 5 µg/ml + OXT 1 µM group	+	++
CP 10 µg/ml + OXT 1 µM group	++	+++
CP 15 µg/ml + OXT 1 µM group	+++	++++

None (-), very light (+), mild (++), moderate (+++), severe (++++), very severe (+++++)

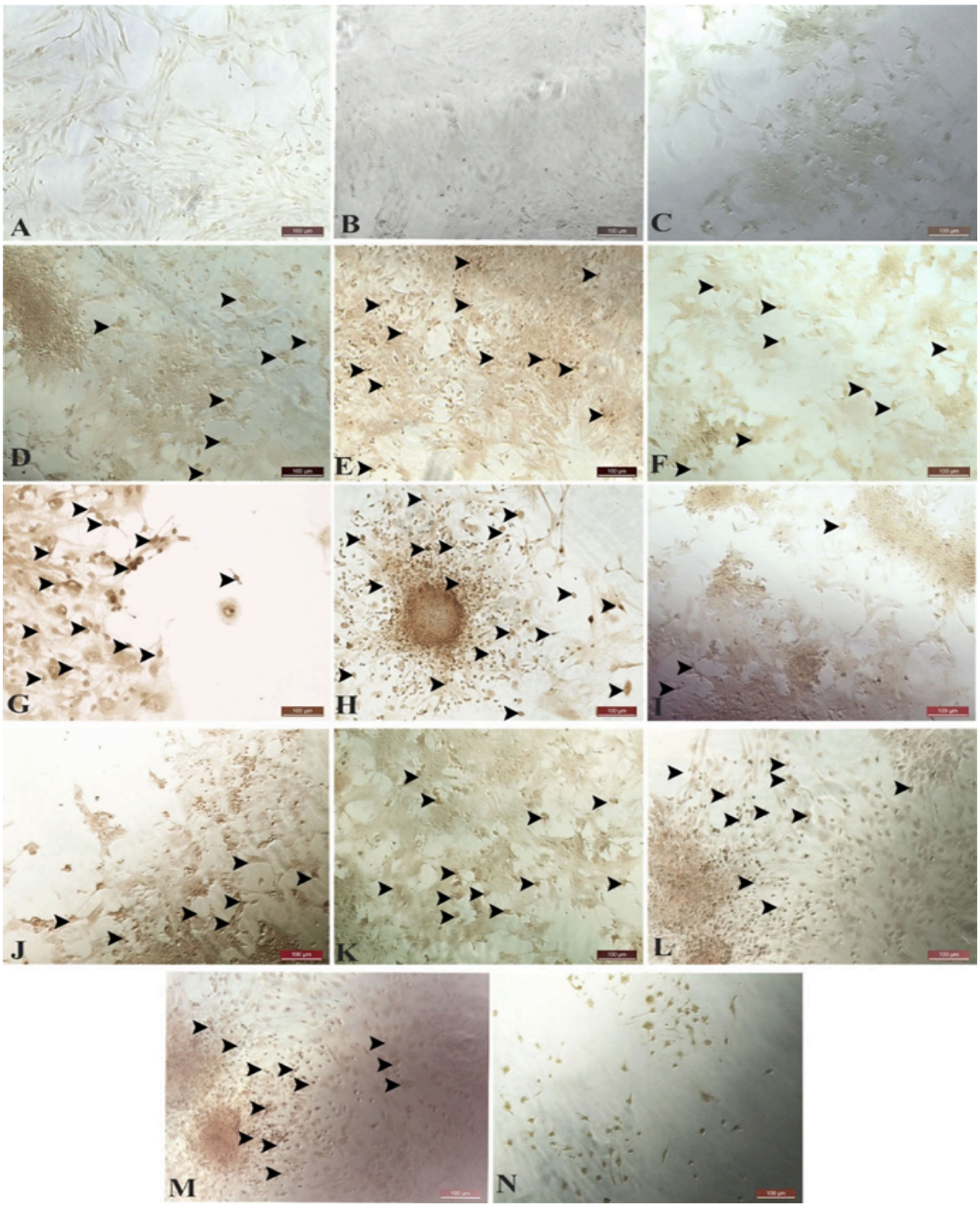

Figure 4. 8-OHdG expression in primary cortex neuron cells. a,b) Control and OXT 1 µM groups: 8-OHdG expression is negative; c) VCR 0.5 µg/ml group: 8- OHdG expression at mild levels in neurons; d) VCR 1 µg/ml group: Moderate 8-OHdG expression in neurons (arrow heads); e) VCR 2 µg/ml group: Severe 8- OHdG expression in neurons (arrow heads); f) CP 5 µg/ml group: Moderate 8-OHdG expression in neurons (arrow heads); g) CP 10 µg/ml group: 8- OHdG expression at severe levels in neurons (arrow heads); h) CP 15 µg/ml group : Very severe 8-OHdG expression in neurons (arrow heads); i) VCR 0.5 µg/ml + OXT 1 µM groups: very mild 8-OHdG expression in neurons (arrow heads); j) VCR 1 µg/ml + OXT 1 µM groups: 8- OHdG expression at mild levels in in neurons (arrow heads); k) VCR 2 µg/ml + OXT 1 µM groups: moderate 8-OHdG expression in neurons (arrow heads); l) CP 5 µg/ml + OXT 1 µM groups: mild 8-OHdG expression in neurons (arrow heads); m) 8-OHdG expression at moderate levels in neurons; n) Severe 8-OHdG expression in neurons (arrow heads). IHC-P, Bar: 100 µm

DISCUSSION

VCR and CP have different side effects on the brain (12-15). As the cerebral cortex is responsible for sensation, perception, memory, association, though, toxicity caused by VCR and CP in these parts of the brain may explain the impairments of these central nervous system functions (23, 24) . OXT has been highlighted as a powerful antioxidant property in many previous neurological scientific studies (14,15).

The present study was to identify the protective effect of OXT against VCR and CP-induced cortical neuron culture. The histological technique was used for confirming the protective effect of OXT on in vitro cortical neuron cells. Some preclinical studies have reported that OXT has a protective effect on peripheral neuropathy caused by VCR and CP. Zhu et al. showed that VCR caused degeneration of neurites of primary DRG neurons in vitro and after administration of OXT, neurites' length enhanced (14). Akman et al. declare that CP induces an increase in lipid peroxidation and decreases glutathione level in rats' plasma. Also, CP reduced the amplitude in the electromyographic recording taken from the sciatic nerves of rats. However, the treatment of OXT significantly improved the electromyographic alteration and improved antioxidative capacity (25). The researcher did not give any information about the effects of OXT in VCR and CP-induced toxicity in cortical neurons. We found that the highest doses of VCR and CP (2 µg/ml and 10 µg/ml respectively) induce the highest toxicity to neurons. However, administration of OXT five minutes before the VCR and CP treatment dramatically and significantly prevented neurons from toxicity (Figure 1). Consistent with our study, Wing et al showed that induced pluripotent stem cell-derived cortical were treated with VCR and CP resulted in a decrease in cell viability as a mechanism for neurotoxicity (26). The literature proposes that different mechanisms are responsible for the neurotoxicity caused by VCR and CP, but oxidative damage stands out among them (27). Vashistha et al. findings showed that reduced glutathione and increased thiobarbituric acid reactive species levels are associated with VCR- induced neuropathic pain (28). Abdel-Wahab et al. demonstrated that the antioxidant status of the brain is importantly inhibited evidenced by the decrease in the TAC, the level of glutathione, as well as the activities of catalase and superoxide dismutase in the brain upon applying with CP (13). In our study, the amounts of TOS levels in cortical neuron culture of the CP at 10 µg/ml group were found the highest while TAS levels were found lowest. We also determined significantly elevated TOS and decreased TAS level at 2 µg/ml VCR concentration in cortical neuron culture (Figure 2 and 3). These results support the hypothesis that the mechanism of VCR and CP-induced neurotoxicity is associated with oxidative stress. Suppression of the antioxidant status may be a result of the enhanced production of oxidants as mentioned earlier. OXT reduces ROS in the brain membranes, inhibits low-density lipoprotein oxidation, and prevents lipid peroxidation (14,15). Lefter et al. reported the neuroprotective effects of OXT following the valproic-acid autistic rat model most probably by increasing the antioxidant enzyme and decreasing lipid peroxidation (6). Recently, Erdoğan et al. reported that OXT significantly improved lipid peroxide levels along with motor performance, electrophysiological and histopathological changes in a rat model of vincristine-induced neuropathy (29). In our study, (Figure 2 and 3) pure 1 µM OXT did not show any significant increase in TAC and also TOS in cortical neuron culture comparison to the control group. On the contrary, OXT combination with the cancer drugs showed a significant increase in TAC in cortical neuron culture comparison to the control group ($p<0.05$), however, attenuated the increase in TOS significantly at 10 µg/ml concentration of CP presenting the antioxidant effects of OXT. Also elevated free oxygen radicals interact with DNA and result in the production of 8–OHdG, which is responsible for DNA damage (30). In vivo model of peripheral neurotoxicity caused by VCR, an increase in the levels of 8–OHdG was observed to cause oxidative stress, oxidative DNA damage, and also VCR prevents neuronal survival (31). Moreover, Sakat et al. showed that CP treatment-induced immunopositivity of 8-OHdG in spinal ganglion cells (32). In the present study, VCR and CP applied group demonstrated a remarkable increase in 8-OHdG levels were compared to the control groups. According

to these results, it is thought that oxidative stress induced by VCR and CP elevates the 8-OHdG mediated DNA methylation possibly because of decreasing the activities of antioxidant enzymes. Although several studies are linking VCR and CP tissue toxicity to 8-OHDG, limited studies were associating the increase of 8- OHDG with neuronal cell culture. Besides, no information was found that OXT inhibited the increase of 8- OHDG in cortical neuron culture. However, it has been reported that OXT diminishes DNA damage in the comet assay of peripheral blood lymphocytes by in vitro exposure to H_2O_2 (33). These data support our study that OXT alleviates the expression of 8-OHDG, which increases as a result of toxicity caused by VCR and CP, in cortical neuron culture through its antioxidant effects. Our microscopic investigation of cortical neuron culture reveals immunohistochemistry findings consistent with our TAS-TOS analysis results.

In conclusion, VCR and CP lead to oxidative damage in cortical neuron culture, and these changes induce significant immunopathological damages. OXT has been demonstrated to prevent the toxic effects of VCR and CP-induced neuronal toxicity. Further studies are needed to fully determine the effects of OXT against VCR and CP-induced toxicity on the neuron.

ETHICS COMITTEE APPROVAL

* This study does not require Ethics Comittee Approval.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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