



RESEARCH ARTICLE

Oxytocin Promotes C6 Glial Cell Death and Aggravates Hydrogen Peroxide-induced Oxidative Stress

Ahmet Sevki Taskiran^{1*}, Merve Ergul²

¹Departments of Physiology, School of Medicine, Sivas Cumhuriyet University, Sivas, Turkey

²Departments of Pharmacology, School of Pharmacy, Sivas Cumhuriyet University, Sivas, Turkey

*Corresponding Author: Ahmet Sevki Taskiran, **Email:** ahmettaskiran@cumhuriyet.edu.tr, **Tel:** +90 346 219 1010, **Fax:** +90 346 219 1602

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Abstract:

Background. Recent studies have shown that oxytocin plays a vital role in neurons and glial cells. However, its effect on hydrogen peroxide (H₂O₂)-induced oxidative stress as well as cyclooxygenase-1 (COX-1) and COX-2 in glial cells are still unclear.

Objective. This study aims to examine the effect of oxytocin on glial cell viability, oxidative stress, COX-1, and COX-2 in C6 glial cells after exposure to H₂O₂.

Methods. In this study, C6 glioma cell line was used to study the effect of oxytocin on the glial cell in four cell groups. The control group was untreated. Cells in the H₂O₂ group were treated with 0.5 mM H₂O₂ for 24 h. Cells in the oxytocin group were treated with various concentrations (0.25, 0.5, 1, and 2 µg/mL) of oxytocin for 24 h. Cells in the oxytocin+H₂O₂ group were pre-treated with various concentrations (0.25, 0.5, 1, and 2 µg/mL) of oxytocin for 1 h before 24-h exposure to 0.5 mM H₂O₂. Cell viability was evaluated using XTT assay. Total antioxidant status and total oxidant status (TOS), COX-1, and COX-2 levels in the cells were measured by commercial kits.

Results. Oxytocin with various concentrations significantly decreased the viability of C6 cells after H₂O₂-induced oxidative stress ($P < 0.01$). It also significantly increased the levels of TOS, COX-1, and COX-2 in C6 cells after H₂O₂-induced oxidative stress ($P < 0.001$).

Conclusion. Oxytocin increases glial cell death after H₂O₂-induced oxidative damage in C6 cells, along with upregulation of COX-1 and COX-2 levels.

Keywords: Oxytocin, Oxidative stress, Cell death, Cyclooxygenase-1, Cyclooxygenase-2, C6 glioma

1 Introduction

Reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂), are produced from normal cellular metabolism. They play vital roles in the process of signal transmission [1]. However, excessive H₂O₂ production damages the cellular component and also exerts genotoxic effects [2]. Besides, a surplus of ROS results in oxidative damage, leading to cellular dysfunction and cell death [3].

Due to its high metabolism and lipid composition, the brain is the most susceptible tissue to oxidative damage [4]. Therefore, oxidative stress is a significant factor in neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease [5,6]. Glial cells are non-neuronal cells that maintain homeostasis as well as support and protect neurons in the central nervous system (CNS) [7]. Moreover, activation of the glial cells causes neuroinflammation and oxidative stress that destroy the neuronal cells.

Therefore, the health of glial cells plays an important role in the development of neurodegenerative disorders, especially Alzheimer's disease and Parkinson's disease [8].

Oxytocin is a neuropeptide hormone consisting of nine amino acids. It is known for its physiological functions, for example, uterus constriction during parturition and milk ejection reflex during lactation [9]. Moreover, it has been found that oxytocin is related to mammalian social behaviors such as effective parental and aggressive behaviors [10]. Of note, oxytocin is distributed by neuronal transport system to distant brain regions, where oxytocin serves as a paracrine or autocrine hormone. Oxytocin receptors are present in the CNS [11], and they were also discovered in cultured neuroblastoma and glial cell lines [12]. More recently, *in vitro* and *in vivo*, studies have demonstrated that oxytocin could affect cell survival and death [13]. Moreover, oxytocin promotes the survival and maturation of newborn neurons in the hippocampus [14] and the oxytocin receptors play a role in modulating cellular growth of glial cell [15]. Furthermore, it has also been suggested that oxytocin receptors are closely associated with oxidant-antioxidant systems [16].

Despite that, the effect of oxytocin on oxidative stress in C6 glial cells and underlying mechanisms are still unclear. In the present study, we examined the effect of oxytocin on H₂O₂-induced oxidative stress in C6 glial cells and levels of cyclooxygenase-1 (COX-1) and COX-2.

2 Materials and methods

2.1 Cell culture and chemicals

C6 glioma (CRL107) cell lines were obtained from American Type Culture Collection and cultured in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, Altrincham, UK) containing 10% Fetal Bovine Serum (Sigma-Aldrich Co., St Louis, MO, USA), 1% L-glutamine (Sigma-Aldrich Co., St Louis, MO, USA), and 1% penicillin/streptomycin (Sigma-Aldrich Co., St Louis, MO, USA). The cells were maintained at 37°C in the 5% CO₂ humidified atmosphere. Oxytocin and H₂O₂ (Sigma-Aldrich Co., St Louis, MO, USA) were dissolved in DMEM, and stock solutions were prepared before treatment.

2.2 Cell viability assay

XTT assay (Roche Diagnostic, MA, USA) was used to assess cell viability. C6 glioma cells were seeded in 96-well plates at a density of 1×10^4 cells per well in 100- μ L DMEM and grown overnight before the addition of oxytocin. The effect of oxytocin on C6 glioma cell viability was investigated in four groups. The control group was not treatment with either H₂O₂ or oxytocin. Cells in the H₂O₂ group were treated with 0.5 mM H₂O₂ for 24 h. Cells in the oxytocin group were treated with various concentrations (0.25, 0.5, 1, and 2 μ g/mL) of oxytocin for 24 h. Cells in the oxytocin+H₂O₂ group were pre-treated with various concentrations (0.25, 0.5, 1, and 2 μ g/mL) of oxytocin for 1 h before exposure to 0.5 mM H₂O₂ for 24 h. After incubation, the medium was removed, and each well was washed two times with phosphate-buffered saline (PBS). In the last step, 100 μ L DMEM without phenol red and a mixture of 50 μ L XTT labeling solution were added to each well, and then the plates were maintained at 37°C for 4 h. The plates were shaken, and the absorbance was detected using an ELISA microplate reader (Thermo Fisher Scientific, Altrincham, UK) at 450 nm. All experiments were performed three times, and the cell viability was measured as a viable cell amount percent compared to control, that is, the untreated cells.

2.3 Preparation of cell homogenates

The cells for each group were collected in sterile tubes. They were centrifuged at 2000 rpm for approximately 10 min. Following the removal of supernatants, the cell pellets were suspended in PBS (pH 7.4) to produce cell suspension with a concentration of approximately 1 million/ml. The cells were disintegrated through repeated freeze-thaw cycles to let out the inside components. They were centrifuged at 4000 rpm for 10 min at a temperature of 4°C. Then, the supernatants were collected for biochemical analysis. Bradford protein assay kit (Merck Millipore, Darmstadt, Germany) was used to determination of total protein levels in samples.

2.4 Measurement of total antioxidant status (TAS) and total oxidant status (TOS)

The levels of TAS from the supernatants of the cells for each group were measured using commercial

kits (Rel Assay, Antep, Turkey). The experiments were conducted according to the manufacturer's guidelines that were previously developed by Erel [17]. Standards of kit and cell supernatants premixed with reaction reagent (reagent I) were added into the wells. Then, staining reagent (reagent II) was added and incubated for 5 min at 37°C. After incubation, the absorbance was read at 660 nm. The method measures the reaction rate of free radicals by quantitating the absorbance of colored dianisidyl radicals formed during free radical reactions, which happen simultaneously with the production of hydroxyl radicals in Fenton reaction. The results were expressed in micromolar Trolox equivalents per milligram tissue protein ($\mu\text{mol Trolox Eq/mg protein}$) on the basis that antioxidants in the samples should suppress coloring in proportion to their concentrations [17].

The levels of TOS from the supernatants of the cells for each group were measured using commercial kits (Rel Assay, Antep, Turkey). The

experiments were conducted according to the manufacturer's guidelines that were previously developed by Erel [18]. Standards of kit and cell supernatants premixed with reaction reagent (reagent I) were added into the wells. Then, staining reagent (reagent II) was added and incubated for 5 min at 37°C. After incubation, the absorbance was read at 530 nm. Since ferrous ion is oxidized to ferric ion when adequate quantities of oxidants are available in the medium, the method quantifies TOS levels by measuring the ferric ions in the samples with the use of xylenol orange. H_2O_2 was used for the calibration of the assay [18]. Therefore, the results of the assay were expressed in micromolar H_2O_2 equivalents per milligram tissue protein ($\mu\text{mol H}_2\text{O}_2 \text{ Eq/mg protein}$).

2.5 Measurement of COX-1 and COX-2

The levels of COX-1 and COX-2 from the supernatants of the cells for each group were

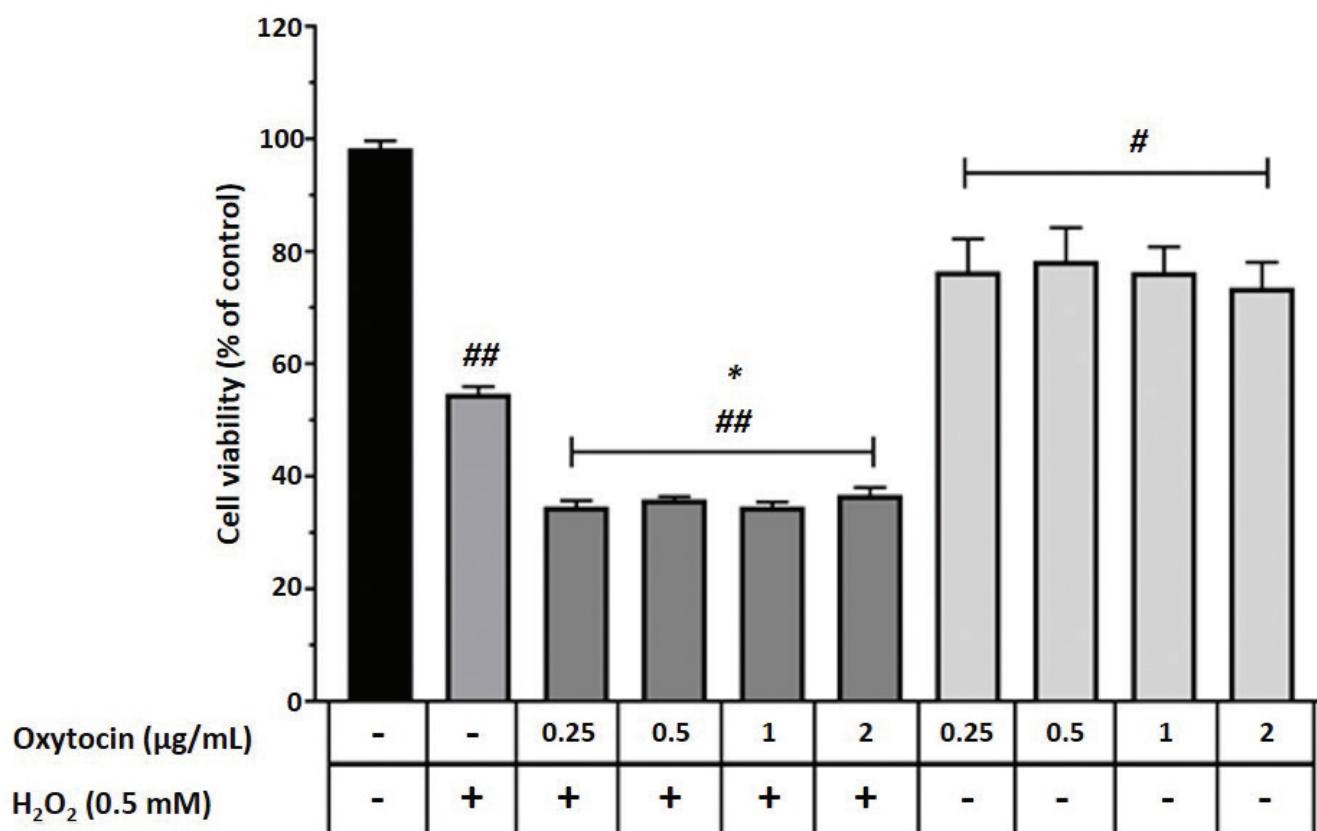


Figure 1. Effect of oxytocin on C6 cell survival after H_2O_2 -induced oxidative damage. The C6 cell was pre-treated with various concentrations of oxytocin (0.25 – 2 $\mu\text{g/mL}$) before H_2O_2 -induced (0.5 mM) oxidative damage. The data are expressed as mean \pm standard error mean. # $P < 0.01$ and ## $P < 0.001$ as compared to the untreated cells of control group; * $P < 0.05$ compared to the cells treated with H_2O_2 only.

measured using rat ELISA commercial kits (YL Biont, Shanghai, China). The experiments were conducted according to the manufacturer's instructions. In brief, standard and tissue samples were added into the wells and incubated for 60 min at 37°C. After the washing step, staining solutions were added and incubated for 15 min at 37°C. The stop solution was added and absorbance was read at 450 nm. Standard curves were plotted to determine the value of samples. The coefficients of variation within and between plates were <10%.

2.6 Statistical analysis

The results were expressed as a mean \pm standard error of the mean. The data analyses were performed with SPSS Version 25.0 for Windows. The data were evaluated using a one-way analysis of variance (ANOVA). A post-hoc Tukey test was utilized to identify the differences between the experimental groups.

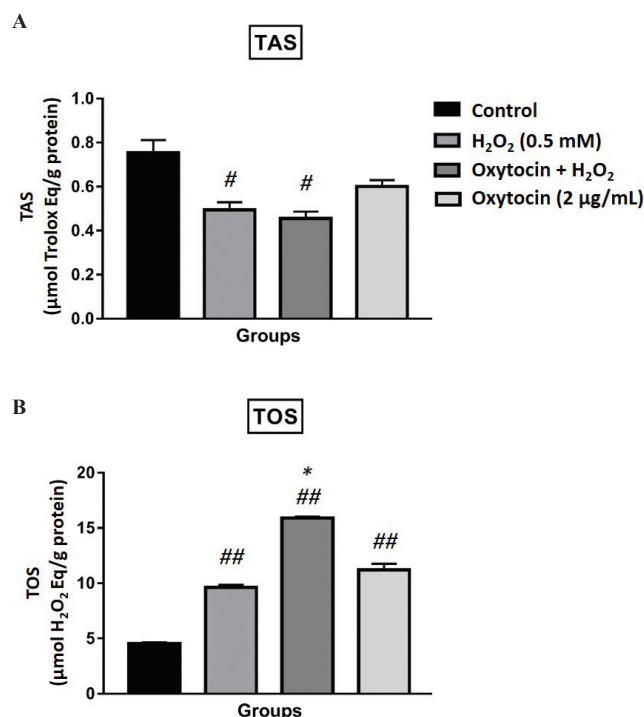


Figure 2. Effect of oxytocin on TAS and TOS levels in C6 cells after H₂O₂-induced oxidative damage. The data are expressed as mean \pm standard error mean. [#] $P < 0.01$ and ^{##} $P < 0.001$ compared to the untreated cells of control group; ^{*} $P < 0.001$ compared to the cells treated with H₂O₂ only.

Differences with $P < 0.05$ were considered statistically significant.

3 Results

3.1 Effect of oxytocin on cell survival after H₂O₂-induced oxidative damage

In this study, it was tested that the increasing doses of oxytocin (0.25 – 2 μg/mL) compromises the survival of C6 cells, including those treated with H₂O₂-treated C6 cells. The cells were initially treated with increasing doses (0.25, 0.5, 1, and 2 μg/mL) of oxytocin for 1 h and then incubated or not incubated with 0.5 mM H₂O₂ for the next 24 h. As shown in **Figure 1**, the tested doses of oxytocin decreased cell survival in C6 cells as compared with untreated cells of the control group ($P < 0.01$). On the other hand, preincubating the C6 cells with H₂O₂ for 24 h significantly reduced cell survival as compared with the untreated cells ($P < 0.001$; **Figure 1**). Moreover, oxytocin, in all doses, significantly decreased C6 survival compared with H₂O₂-treated C6 cells ($P < 0.05$; **Figure 1**).

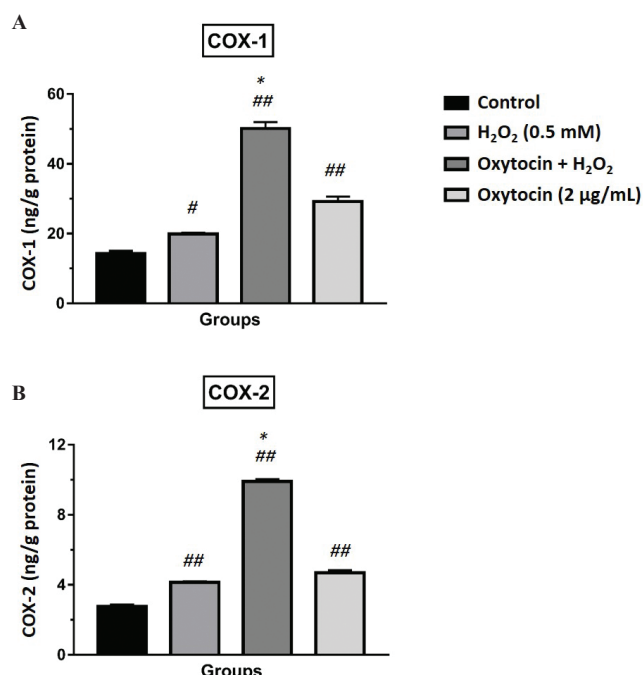


Figure 3. Effect of oxytocin on COX-1 and COX-2 levels in C6 cells after H₂O₂-induced oxidative damage. The data are expressed as mean \pm standard error mean. [#] $P < 0.05$ and ^{##} $P < 0.001$ compared to the untreated cells of control group; ^{*} $P < 0.001$ compared to the cells treated with H₂O₂ only.

3.2 Effect of oxytocin on TAS and TOS levels after H₂O₂-induced oxidative damage

The cells were initially treated with the single dose (2 µg/mL) of oxytocin for 1 h and then incubated or not incubated with 0.5 mM H₂O₂ for the next 24 h. As shown in **Figure 2A**, the treatment of H₂O₂ decreased TAS level in C6 cells as compared with untreated cells of the control group ($P < 0.01$). However, the oxytocin did not change TAS in C6 cells as compared with H₂O₂-treated C6 cells ($P > 0.05$). Moreover, preincubating the C6 cells with H₂O₂ for 24 h significantly increased TOS level as compared with untreated cells ($P < 0.001$; **Figure 2B**). Furthermore, the oxytocin significantly raised TOS level in C6 cells compared with untreated cells ($P < 0.001$; **Figure 2B**). On the other hand, the TOS level of oxytocin+H₂O₂ group was significantly higher than that of the only H₂O₂ group ($P < 0.001$; **Figure 2B**).

3.3 Effect of oxytocin on COX-1 and COX-2 levels after H₂O₂-induced oxidative damage

The cells were initially treated with the single doses (2 µg/mL) of oxytocin for 1 h and then incubated or not incubated with 0.5 mM H₂O₂ for the next 24 h. Preincubating the C6 cells with H₂O₂ for 24 h significantly increased COX-1 and COX-2 levels as compared with untreated cells of the control group ($P < 0.05$ to $P < 0.001$; **Figure 3**). On the other hand, oxytocin significantly raised COX-1 and COX-2 levels in C6 cell compared with untreated C6 cells ($P < 0.001$; **Figure 3**). Moreover, COX-1 and COX-2 levels were significantly higher in the oxytocin+H₂O₂ group than the H₂O₂ group ($P < 0.001$; **Figure 3**).

4 Discussion

The present study provides the first evidence of the possible effects of oxytocin on H₂O₂-induced oxidative damage in C6 glial cells. Here, we showed that the pretreatment with oxytocin increased H₂O₂-induced oxidative damage in C6 cells. Moreover, oxytocin pretreatment also raised TOS levels and led to increased COX-1 and COX-2 levels after H₂O₂-induced oxidative damage in C6 cells.

Oxytocin is one of the essential peptide-based hormones in the CNS which can commonly be found in peripheral and central neurons [11].

Several studies have demonstrated that oxytocin exerts protective effects on CNS disorders such as autism and epilepsy [19,20]. On the other hand, it has been reported that oxytocin is essential for pain modulation in the CNS [21]. Moreover, as demonstrated in the clinical and experimental studies, oxytocin levels are related to neurodegenerative diseases such as Alzheimer's and Parkinson's [22,23]. These studies highlight the importance of oxytocin in the CNS. Another beneficial effect of oxytocin is the protection of lymphocytes from H₂O₂-induced toxicity [24]. On the contrary, the present study showed the negative effects of oxytocin on glial cells; we found that oxytocin decreased C6 cell viability. A possibility that oxytocin behaves differently in its biological effects is probably the characteristics or type of the cells we experimented with in this study. Oxytocin is known for its inhibition of proliferation of neoplastic cells of either epithelial (such as mammary and endometrial), nervous or bone origin, which are all expressing oxytocin receptors [13]. Since glioma is a type of tumor, it is inadvertent to assume that the reduced viability of C6 glioma cells is due to the cancer cell specificity of oxytocin. Nevertheless, the discordance of these findings with others deserves further investigation.

Oxidative stress is defined as an imbalance between oxidants and antioxidant defense systems. This imbalance is characterized by excessive ROS production, which harms tissues and disturbs the physiological function of the organism [25]. Besides, the brain is one of the vulnerable organs to oxidative damage [4]. Much evidence shows that oxidative stress plays a vital role in the occurrence of neurodegenerative diseases and CNS disorders [26]. Previous studies have claimed that oxytocin has antioxidant properties and protects different tissues from oxidative damage [27,28]. In contrast to these studies, oxytocin did not show antioxidant properties in this study; instead, it aggravates the levels of oxidative stress after H₂O₂-induced oxidative damage in C6 cells. This effect could be related to the different characters of tissues and cell lines.

COX-1 and COX-2 are isoenzymes that catalyze the conversion of arachidonic acid into prostaglandins. COX-1 and COX-2 are involved in the pathogenesis of CNS disorders by rising

neuroinflammation in the brain [29]. Previous studies have shown that oxytocin upregulates the COX-1 and COX-2 levels in different tissues [30,31]. In this study, exposure to oxytocin resulted in upregulated levels of COX-1 and COX-2 after H₂O₂-induced oxidative damage in C6 cells line which is in consistence with previous studies. Following the oxytocin-induced aggravation of oxidative stress in glial cells, the function of neurons could be indirectly affected in a negative manner.

According to our findings, oxytocin plays an important role in glial cell survival. Since glial cells are critical for neurodegenerative diseases, its receptor could be a therapeutic target for the treatment of neurodegeneration-related diseases. However, more studies are needed.

The current study has several limitations. This study was performed using C6 rat glioma cells, rather than primary glial cells, which could confound the findings owing to the tumor characteristics of C6 cells. Oxytocin receptor blockers were not used in this study to validate whether the effects of oxytocin are indeed related to the receptor. The experiments of this study do not lead to a clearer illumination of the mechanisms underlying the effects of oxytocin on C6 glial cells. More methods, including immunohistochemistry and calcium imaging studies, are needed to clarify the underlying mechanisms of oxytocin in glial cells.

5 Conclusion

The present study shows that oxytocin aggravates H₂O₂-induced oxidative damage in C6 glial cells. These effects may occur possibly through the deterioration of oxidative stress or the actions of COX-1 and COX-2. Therefore, oxytocin could exert either a protective effect or harmful effect to the cells of CNS. However, further investigation is required to explore the two-faced characteristics of oxytocin and the mechanisms behind its effects.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

A.S.T. and M.E. conceived, designed, performed, and analyzed the experiments. A.S.T. wrote the paper. A.S.T. and M.E. reviewed drafts of the paper. All authors read and approved the final manuscript.

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