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Trolox Reduces Neuroblastoma-Induced Oxidative Stress and Inflammation

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ABSTRACT

AIM: To determine the effects of different concentrations of Trolox on the cytotoxic, oxidant, antioxidant, and cytokine levels in the SH-SY5Y cell line.

MATERIAL and METHODS: SH-SY5Y cells were grown in an appropriate medium and under appropriate conditions. Trolox was added to the cell line at concentrations of 50, 100, 200, 400 and 800 μM and incubated for 24 and 48 hours. Subsequently, methyl thiazolyl tetrazolium (MTT) and proinflammatory cytokine level assays were performed, and the cytotoxicity of oxidative stress was assessed.

RESULTS: Administration of 200 μM of Trolox reduced the cancer cell viability to 77.76%. Furthermore, Trolox exhibited a concentration-dependent effect on the SH-SY5Y cell line. Administration of 200 μM of Trolox also reduced the oxidant activity, increased the antioxidant capacity, and decreased the proinflammatory cytokine levels in neuroblastoma (NB) cells, which were consistent with the cytotoxicity test results.

CONCLUSION: The results of this experimental study demonstrated a Trolox concentration of 200 μM produces an anticancer effect on NB cell-line.

KEYWORDS: Inflammatory Neuroblastoma, Oxidative stress, Trolox

ABBREVIATIONS: NB: Neuroblastoma, **TAC:** Total antioxidant capacity, **TOS:** Total oxidant status

INTRODUCTION

Neuroblastoma (NB) is a tumor of the sympathetic nervous system that accounts for approximately 15% of all pediatric cancer-related deaths and 7%–8% of childhood malignancies (16,24). NB is the most common childhood cancer that is preferred as a model. It originates from neural crest cells and exhibits a variety of clinical behaviors that range from spontaneous remission to death with rapid tumor progression (19). These properties are also characteristic of stem or progenitor-like cells that are found in cancer. Disturbances can occur during the differentiation of progenitor cells into mature cells (19). Antioxidant drugs have a therapeutic effect on various types of cancer, such as breast and brain cancer (2,23). Trolox, an analog of vitamin E, forms a bond with peroxyl and alkoxyl radicals (9). A single concentration (<5%) of Trolox has been used as an antioxidant supplement for cardiac differentiation (2). In this study, we aimed to increase the therapeutic effect of Trolox via its antioxidant properties on NB cell lines.

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MATERIAL and METHODS

Cell Cultures

The SH-SY5Y cell line used in our study was obtained from the American Type Culture Collection (ATCC, USA). In cell culture, SH-SY5Y cells were grown in an appropriate nutrient medium and under appropriate conditions. The cell suspension was centrifuged for five minutes at a speed of 1200 revolutions per minute. Following the resuspension of the cells in new media, Dulbeco's modified eagle's medium (DMEM F-12), 10% fetal bovine serum (FBS), and 1% antibiotics (penicillin, streptomycin, and amfoterisin B), the cells were collected in a flask with a capacity of 25 cm^2 (Corning, USA). At 37 degrees Celsius and 5% carbon dioxide, the flask that had been constructed was incubated. Following the removal of trypsin-ethylenediamine-tetraacetic acid (EDTA) (0.25% trypsin-0.02% EDTA) and centrifugation, the flask was filled with cells until it covered eighty percent of the flask. The supernatant was discarded, and the cell solution was distributed onto tissue culture plates with 96 wells at a volume of 100 microliters per well, which corresponds to 10 000 cells per well (9). Negative control group is cell only and positive control group is 1% DMSO solvent.

Drug Administration

When the cells in the plates reached a density of 80%, Trolox concentrations were determined, and an experimental design was created. Trolox concentrations of 50, 100, 200, 400, and 800 µM were added to the culture plates. The cells were incubated at 37°C with 5% CO_2. Ten replicates were used for each concentration. The methyl thiazolyl tetrazolium (MTT) assay protocol in a previous study was modified and used in this study (3,12).

MTT Assay

The experiment was concluded by introducing 10 µL of the MTT solution (SigmaAldrich) after 24 and 48 hours of exposure. MTT measurements were obtained according to the protocols of a previous study (3,6).

Oxidative Stress Analysis

The TAC and TOS were measurements according to the protocols of a previous study (3,5,6).

Estimation of Interleukin-6 and Tumor Necrosis Factor-Alpha Levels

The levels of the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) were estimated according to the instructions provided by the kit's manufacturer (BT Lab, China). The IL-6 and TNF-α levels in the treated cells were measured using spectrophotometry at a wavelength of 450 nm (BioTek Instruments, USA).

Statistical Analysis

Data are expressed as the means and standard deviation. All data were analyzed using one-way ANOVA. All statistical analyses were performed using SPSS (version X;). A $p < 0.05$ and p 0.001 indicated statistical significance when compared with the positive control group.

RESULTS

MTT Assay Results

The MTT method was used to evaluate the cytotoxic effects of Trolox on the NB cell line (Figure 1). Cell death in the experimental groups were compared with those in the positive control group at concentrations of 50, 100, 200, 400, and 800 µM after 24 and 48 hours of exposure (Figures 1A and 1B). The highest anticancer activity was observed at a concentration of 200 µM, in which the cell viability was 77.76% at 48 hours. Trolox concentrations of 100, 400, and 800 µM were also effective. However, these concentrations did not significantly affect the TAC and TOS results. The experiment was repeated

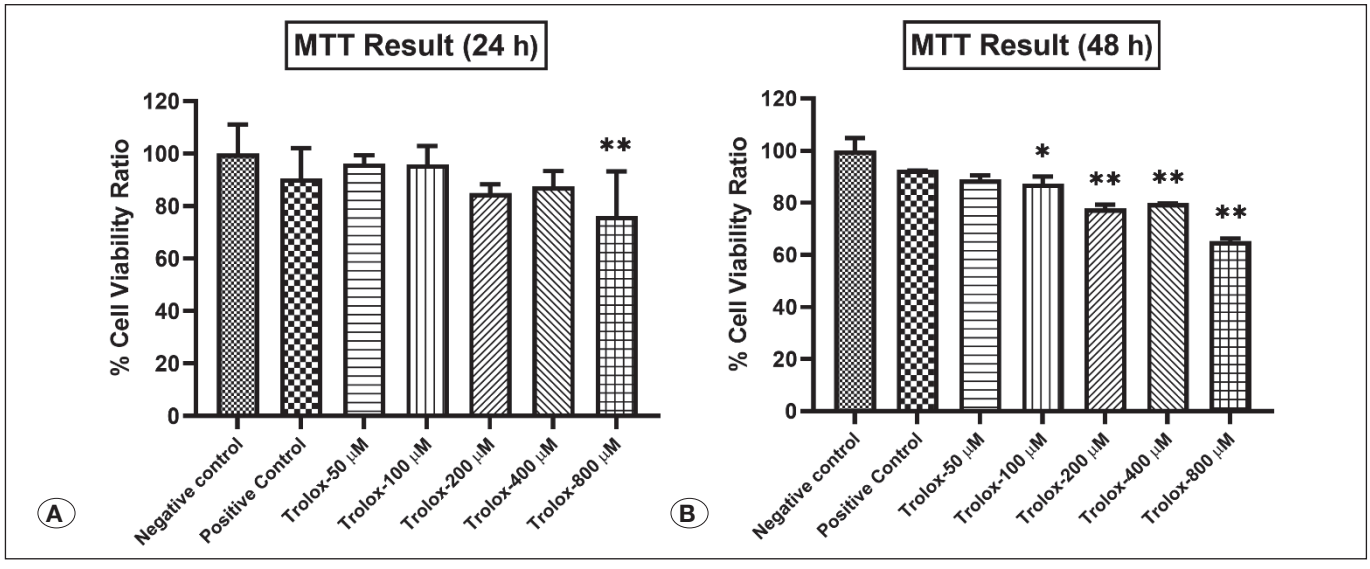

Figure 1: Effects of Trolox on the cell viability (MTT) of neuroblastoma cells. **A)** 24 h MTT result. **B)** 48 h MTT results. *$p < 0.05$, **$p < 0.001$ indicates a significant difference between the test and positive control groups. Data are expressed as mean ± standard deviation.

with cells treated with Trolox for 48 hours because the 48-hour MTT findings were more significant.

TOS and TAC Results

At high Trolox concentrations of 400 and 800 μM the TAC activity increased. At Trolox concentrations of 50, 100, and 200 μM, the TOS activity decreased (Figure 2A). At a Trolox concentration of 200 μM, the TAC increased the most (Figure 2B). However, at concentrations of 50, 100, 400, and 800 μM, there was not much of an increase in TAC.

IL-6 and TNF-α Levels

There was statistically significant decrease in the IL-6 levels at Trolox concentrations of 200 and 400 μM when compared with the levels in the positive control group (Figure 3). At Trolox concentrations of 100 and 800 μM, a non-significant decrease in IL-6 level was observed. The TNF-α level significantly decreased at a Trolox concentration of 200 μM ($p < 0.05$), and it increased at Trolox concentrations of 100, 400, and 800 μM.

DISCUSSION

Trolox, a vitamin E analog, is a direct scavenger of peroxyl and alkoxyl radicals. Thus, Trolox exhibits an antioxidant effect. Antioxidants have demonstrated therapeutic effects in various types of cancer, such as breast, prostate, colon,

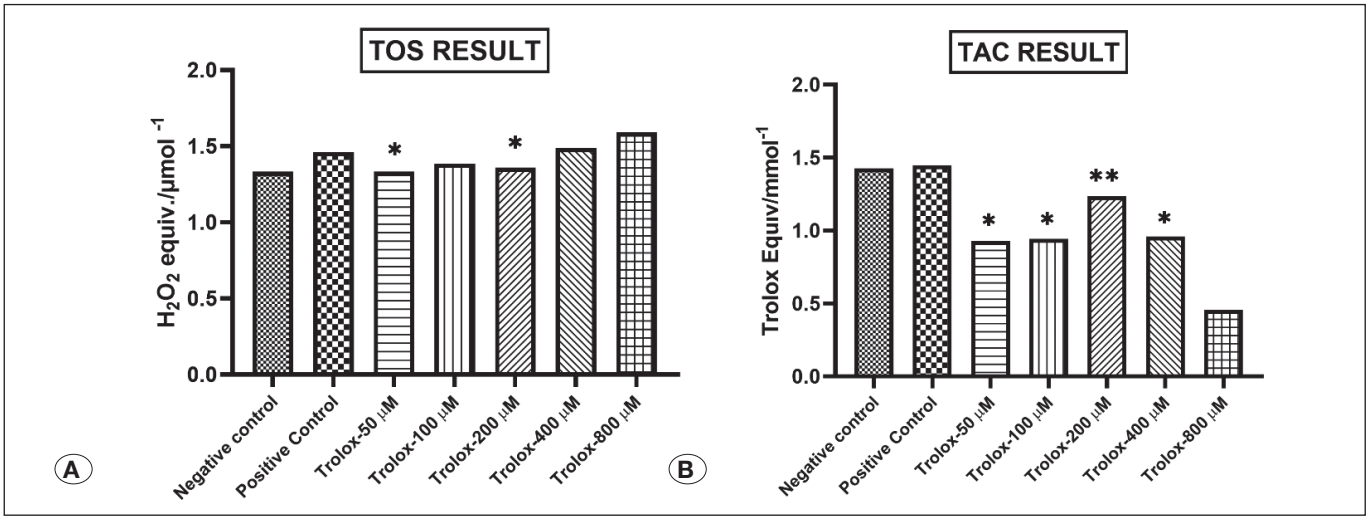

Figure 2: Total oxidant status and total antioxidant capacity results. **A)** Total oxidant status in the cell culture fluid were read spectrophotometrically at 530 nm. **B)** Total antioxidant capacity in the cell culture fluid that were read spectrophotometrically at 660 nm. *p<0.05, **p<0.001 indicates a significant difference between the test and positive control groups. Data are expressed as mean ± standard deviation.

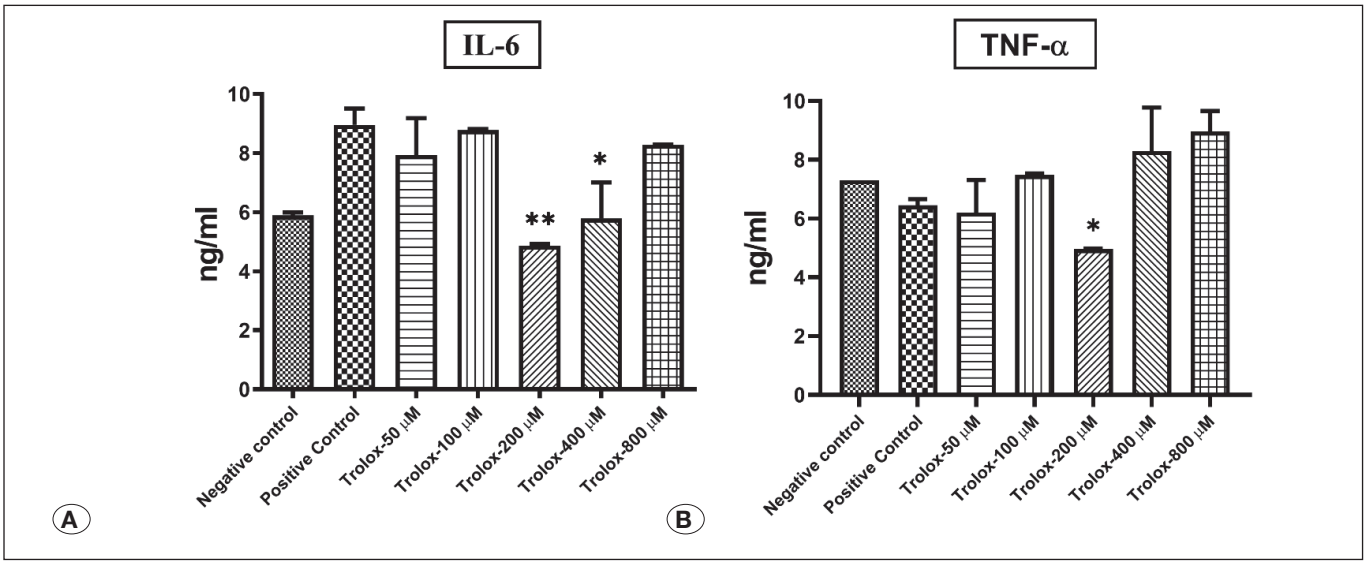

Figure 3: Effects of bromelain on the pro-inflammatory parameters **A)** IL-6 and **B)** TNF-α in the neuroblastoma cells.*p < 0.05, **p < 0.001 indicates a significant difference between the test and positive control groups. Data are expressed as mean ± standard deviation.

and brain cancers. NB is a pediatric tumor that arises from neural crest cells (7). Oxidative stress and increased levels of inflammatory cytokines are involved in the pathogenesis of several diseases. Because the antioxidant property of Trolox is well-established in other cancers, its anticancer activity in an NB cell line was evaluated (12). NB is a common cancer in infants. Chromosomal abnormalities in the tumor make treating NB a challenge. Therefore, a comprehensive treatment for neuroblastomas is still required (12). The low success rate of NB treatments and the high recurrence rate have prompted the search for other treatment options. Therefore, the NB cell line was chosen in our study. Oxidative stress is related to several cancers (7). In a study conducted in children with solid and liquid cancers, an imbalance was identified between oxidative stress and antioxidant mechanisms (4). One of these cancers was NB. Studies have demonstrated that ROS formed in the 2nd week, when oxidative stress is the highest in NB, support tumor growth and progression (15,18). However, some malignant cells exhibit a reduced capacity to detoxify ROS (6, 21). Oxidative stress at 2, 4 and 6 weeks is reportedly significantly higher in NB than in controls (13). Furthermore, oxidative damage increased by 166%, 110% and 87% at 2, 4 and 6 weeks, respectively, when compared with the controls (13). Okamoto et al. determined that the cell death induced by caspase activation of lidocaine is suppressed by the administration of Trolox (250 μM) and n-acetyl cysteine (NAC; 10 mM) (14). Kim et al. pretreated the NB cell line SH-SY5Y with Trolox (1 μM) or GSH-EE (1 mM) 1 hour before staurosporine treatment. They found that Trolox or GSH-EE significantly inhibited LDH release, ROS production, and caspase-3 activation (10). In another study, Trolox (at concentrations of 0.1, 0.3 and 1 μM), NAC (at concentrations of 0.1, 0.5 and 1 mM), and sylibin (at concentrations of 10, 20 and 50 μM) did not produce a significant difference between the NB SH-SY5Y cells and control cells. However, when the doses of Trolox (1 μM), NAC (1 mM), and Sylibin (50 μM) were increased, they provided protection against glyphosate (herbicides) and aminomethylphosphonic acid (AMPA) cytotoxicity, reduced ROS production, and inhibited caspase-3/7 activity (11). In another study, alpha-cypermethrin (α-CYPER) increased the lipid peroxidation at the nitric oxide level in the neuroblastoma cell line SH-SY5Y. However, this decreased after the administration of melatonin, Trolox and NAC (17). In our study, we evaluated the therapeutic effect of Trolox on oxidative stress and pro-inflammatory cytokine levels in the NB cell line. The MTT assay was used to evaluate the cell metabolic activity (8). The MTT revealed that a Trolox concentration of 200 μM was cytotoxic and reduced the viability of NB cells by 77.76%. However, its effectiveness at concentrations of 50, 100, 400, and 800 μM is low.

The TOS activity was highest at Trolox concentrations of 50 and 200 μM. This indicates the anti-cancer activity of Trolox was the most effective at these concentrations. Application of Trolox at 50, 100, and 200 μM concentrations reduced the TOS level in the cancer-induced ROS. However, no therapeutic effect was detected in the MTT assay and inflammatory cell levels at Trolox concentrations of 50 and 100 μM. Thus, a Trolox concentration of 200 μM produced an anticancer effect via its antioxidant property. Furthermore, the highest antioxidant effect was observed at a Trolox concentration of 200 μM. However, the TAC at Trolox concentrations of 50 and 100 μM was low, indicating no antioxidant effect at these concentrations. Choe et al. reported that a single concentration of Trolox (<5%) exhibits antioxidant properties in cardiac differentiation (2). In this study, at a Trolox concentration of 200 μM, the oxidative capacity decreased and the antioxidant capacity increased, and which regulated the oxidative stress in the NB cell line.

Monocytic and macrophagic infiltration may be observed in NB tumors. CD14, CD16, IL-6, and IL-6R are released from these cells (1,22). In one study, activation of the NF-κB pathway led to the production of pro-tumorigenic cytokines such as IL-6, G-CSF, IL-1 and TNF-α (20). These cytokines stimulate NB cell proliferation, leading to increased tumor growth and angiogenesis.

The antioxidant Trolox produces a therapeutic decrease in the inflammatory cytokine levels in addition to regulating oxidative stress. In our study, the IL-6 level was significantly lower at Trolox concentrations of 200 and 400 μM than in the control group. Furthermore, the TNF-α level was significantly lower at a Trolox concentration of 200 μM than in the control group. However, at Trolox concentrations of 100 and 800 μM the proinflammatory cytokine levels were higher, producing a toxic effect. Zhen et al. reported that IL-10 and TGF-β levels were effectively reduced by Trolox in NB cancer cells (24). The estimation of the pro-inflammatory cytokine levels revealed that Trolox reduced the cancer-induced increase in cytokine levels in addition to having an anticancer effect.

Our study findings demonstrated that application of 200 μM of Trolox to NB cells effectively reduced oxidative stress by reducing the oxidant capacity and increasing the antioxidant levels. Thus, it produces an anticancer effect by suppressing proinflammatory cytokine levels. Therefore, it can be used in the treatment of cancer.

CONCLUSION

In conclusion, we found that 200 μM of Trolox can inhibit cell cytolysis in in vivo NB cells. Furthermore, 200 μM of Trolox regulates oxidative stress levels by decreasing TOS levels and increasing TAC levels. Trolox administration also increases the expression of IL-6 and TNF-α in SH-SY5Y cells. Thus, a Trolox concentration of 200 μM produces an anti-tumor as well as anti-inflammatory effect. Because the downregulation of Trolox may inhibit tumor growth, further studies should be conducted to determine if Trolox can be used in the treatment of NB.

Declarations

Funding: This study was not supported by any organisation.

Availability of data and materials: All supporting data are included within the main article. For the original data, please contact the corresponding author.

Disclosure: Authors declare no conflict of interest.

Ethics approval and consent to participate: Ethics committee approval is not necessary.

AUTHORSHIP CONTRIBUTION

Study conception and design: AH, GA, FY, HC

Data collection: AH, GA, FY, FD, DA, ABS

Analysis and interpretation of results: AH, GA, FY, HC

Draft manuscript preparation: AH, GA, FY, HC

All authors (AH, GA, FY,HC, FD, DA, ABS) reviewed the results and approved the final version of the manuscript.

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