

ANTI-CANCER EFFECT OF NEOCUPROINE ON
OXIDATIVE STRESS AND INFLAMMATORY LEVELS
INDUCED BY NEUROBLASTOMA CANCER CELLS

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Abstract

Neocuproine is a free radical scavenger. Neuroblastoma cancer is seen mostly in children. It accounts for approximately 15% of all pediatric cancer deaths. Treatments against SH-SY5Y may be ineffective due to the development of drug resistance, resulting in poor survival in high-risk patients. The goal of this study was to find out how the anti-cancer concentration of Neocuproine affected the levels of oxidants, antioxidants, and cytokines in the SH-SY5Y cell line. Five different concentrations of Neocuproine (12.5, 25, 50, 100, and 200 μ M) were applied to the cell line for 24 h. MTT cytotoxicity tests, total oxidant status and total antioxidant capacity tests, and anti-inflammatory cytokine tests were used to look at the uses of Neocuproine. In our results, 100 μ M Neocuproine application reduced cancer cell viability up to 69.76%. In addition, it was determined that 100 μ M Neocuproine reduced oxidant activity, increased antioxidant capacity, and regulated anti-inflammatory cytokine levels in neuroblastoma cells, consistent with the cytotoxicity test. As a result, it was concluded that Neocuproine has an anti-cancer effect on the SH-SY5Y cell line.

Key words: inflammatory, neocuproine, neuroblastoma, oxidative stress, SH-SY5Y

Introduction. Neuroblastoma (NB), is the most common tumour in children. It is also a high-grade malignancy and originates from the primitive neural

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crest [1]. More than 70% of neuroblastoma patients have metastatic disease. Although multidisciplinary treatment is available, the 5-year progression-free survival of high-risk NB patients is only 36–56%. Therefore, new therapies are needed to improve treatment outcomes for NB patients [1]. Early metastasis suggests that neuroblastoma cells are highly competent. A thorough understanding of the regulatory mechanism underlying neuroblastoma cell migration and invasion will help identify new therapeutic targets.

Antioxidant drugs may have therapeutic effect on cancer [2]. In breast cancer and lung adenocarcinoma, interleukin (IL)-10 and transforming growth factor (TGF)- β 1 are known to trigger tumour growth through pathways including tumour migration and invasion [3,4]. Neocuproine is a widely used copper chelator that increases cellular uptake of copper and exhibits cytotoxicity [5]. In the presence of Neocuproine, Cu(II) shows increased cytotoxicity, and Cu-Neocuproine complexes have been shown to bind to isolated DNA [6]. Furthermore, the addition of Neocuproine may increase the lipophilicity of the complexes, thus improving cellular uptake. Neocuproine Cu(I) complex stabilizes copper ions and renders them unable to allow redox cycling between the Cu(I) and Cu(II) states [7]. Thus, the formation of free radicals is significantly reduced, and important biomolecules are protected from pro-oxidative damage [8]. In our study, we aimed to investigate the therapeutic effects of Neocuproine against oxidative and inflammatory damage caused by SH-SY5Y cancer cells.

Materials and methods. Cell cultures. The SH-SY5Y cell line was acquired from the Department of Medical Pharmacology at Ataturk University, which is located in Erzurum, Turkey. 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, Dulbecco'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 cm² (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 on the plates achieved 80% density, the Neocuproin concentrations were determined, and the experimental design was created. Concentrations of 12.5, 25, 50, 100, and 200 μ M Neocuproin were administered to the culture plates. Cells were incubated for 24 h at 37 °C in 5% CO₂. Each concentration was tested with ten duplicates.

MTT assay. The MTT test is a colourimetric test that evaluates cell metabolic activity [10]. After 24 h of exposure time, the experiment was finished by adding 10 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution. Then the plates were incubated for 4 h at 37 °C in a CO₂ incubator. One hundred μ L of dimethyl sulfoxide (DMSO) solution was incorporated into all wells to dissolve formazan crystals. The density of the formazan crystals was read at a wavelength of 570 nm by the BioTek Instruments Microplate Spectrophotometer reader [9].

Total Oxidant Status (TOS) and Total Antioxidant Capacity (TAC) analysis. Because oxidant and antioxidant effects can be added together, the effects of these molecules are measured as a whole because it is hard to measure the effects of each oxidant and antioxidant substance separately [9]. Total Antioxidant Capacity (TAC) and Total Oxidant Status (TOS) analyses were performed with cell culture fluids obtained after application. In this respect, utilizing commercially available kits may provide data that was obtained (Rel Assay Diagnostics, Gaziantep, Turkey).

Total Oxidant Status (TOS). The evaluation is made by calculating spectrophotometrically (BioTek Instruments, USA). The intensity of the colour is linked to the quantity of oxidants status. The ingredients in the TOS kits were Reactive 1, Reactive 2, Standard 1, and Standard 2. In order to detect the TOS standard, 500 μ L Reactive 1 was incorporated into the wells in which 75 μ L plasma specimen was present, and later reading the original absorbance value at 530 nm, 25 μ L Reactive 2 was incorporated into the equal well, and secondary absorbance was read at 530 nm at the end of the waiting duration of 10 min at room temperature. Standard 2 in the kit was used for Standard 2. By using the absorbance values acquired and the following formula, TOS standards were detected in μ mol H₂O₂ equiv/L [11].

$$\begin{aligned} \text{TOS} &= \Delta_{\text{example}} / \Delta_{\text{ST2}} \times 20, \\ \Delta_{\text{ST2}} &(\Delta_{\text{standard 2}} = \text{ST2 second reading} - \text{ST2 first reading}), \\ \Delta_{\text{Sample}} &(\Delta_{\text{Sample}} = \text{Sample second reading} - \text{Sample first reading}). \end{aligned}$$

Total Antioxidant Capacity (TAC). The ingredients of the kit were Reactive 1, Reactive 2, Standard 1, and Standard 2. In order to detect the TAC standard; 500 μ L of Reactive 1 was incorporated into the wells, including a 30 μ L specimen, and the initial absorbance was read at 660 nm. After, 75 μ L of Reactive 2 was incorporated into the equal wells and released to wait at room temperature for 10 min. At the end of the waiting duration, the secondary absorbance value was read at 660 nm. While distilled water was used for Standard 1, Standard 2 in the kit was used for Standard 2. The absorbance values acquired were established according to the following formula

$$\text{TAC} = (\Delta_{\text{ST1}} - \Delta_{\text{example}}) / (\Delta_{\text{ST1}} - \Delta_{\text{ST2}}) [11],$$

ΔST1 ($\Delta\text{standard 1} = \text{ST1 second reading} - \text{ST1 first reading}$),
 ΔST2 ($\Delta\text{standard 2} = \text{ST2 second reading} - \text{ST2 first reading}$),
 ΔSample ($\Delta\text{Sample} = \text{Sample second reading} - \text{Sample first reading}$),

and TAC standards were detected in mmol Trolox Equiv/L.

IL-10 and TGF- β pro-inflammatory cytokine markers. The determination of interleukin 10 (IL-10) and tumour growth factor- β (TGF- β) was conducted in accordance with the instructions provided by the manufacturer. A commercial kit from BT Lab, China, was utilized for this purpose. The levels of IL-10 and TGF- β in the treated cells were measured using spectrophotometry at a wavelength of 450 nm (BioTek Instruments, USA).

Statistical analysis. All results were analyzed by one-way ANOVA using SPSS and IBM 21.00 software. $*p < 0.05$, $**p < 0.001$ shows the difference compared to the negative control group. Data are expressed as the means $\pm$ SD.

Results. MTT assay results. The cytotoxic effects of Neocuproine on SH-SY5Y cell lines were investigated by the methyl thiazolyl tetrazolium (MTT) method (Fig. 1). Accordingly, cell viability at 12.5, 25, 50, 100, 100, and 200 μM

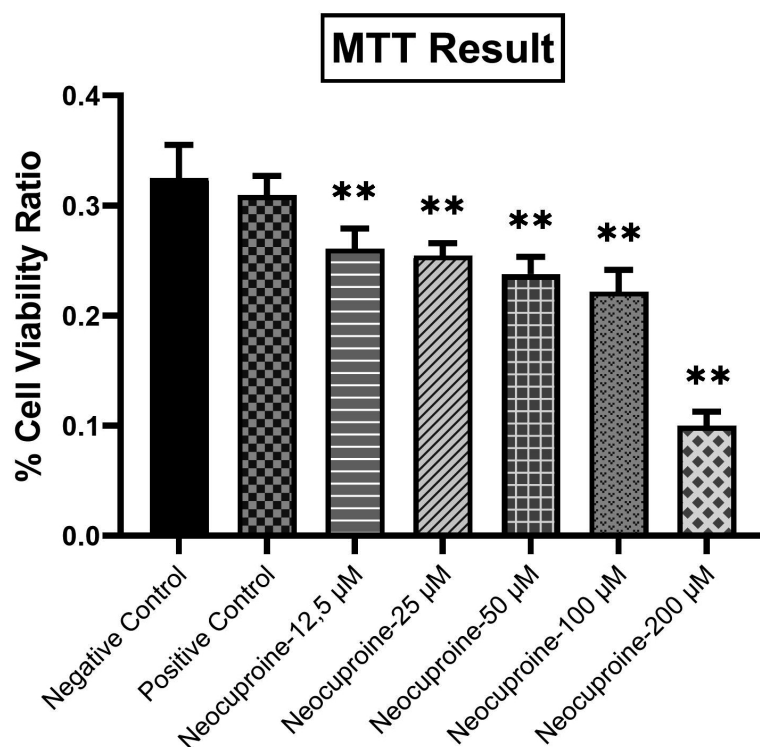


Fig. 1. Effects of Neocuproine on the cell viability (MTT) of neuroblastoma cell lines. $**p < 0.001$ shows the difference compared to the negative control group. Data are expressed as the means $\pm$ SD

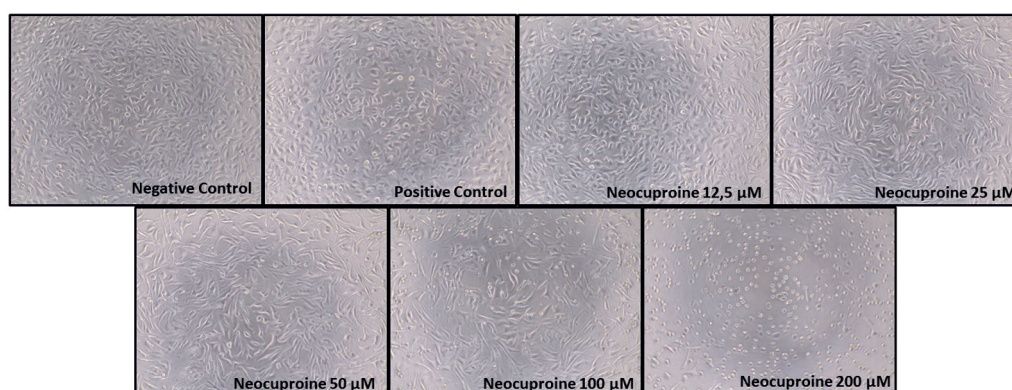


Fig. 2. After a treatment with Neocuproine over a period of 24 h, SH-SY5Y cells

concentrations after 24 h of exposure was compared with the positive control group (Fig. 1). The 100 μM concentration was observed to have 69.76% viability. Although 12.5, 25 and 50 μM Neocuproine concentrations were also significant, the highest anti-cancer activity concentration was determined as 100 μM . According to the experimental results, the 200 μM Neocuproine concentration was also found to be effective, but this concentration value was at a toxic level, and its activity was not statistically significant in TOS result. Therefore, according to the results of the MTT analysis, the most effective concentration was determined to be 100 μM when compared with other concentrations and controls (Fig. 2).

TOS and TAC results. Oxidative stress was evaluated by total oxidant status and total antioxidant capacity tests. Compared to the positive control group, 12.5, 25, 50, 100, and 200 μM concentration values were found to increase total oxidant status, while the 100 μM concentration increased it less (Fig. 3A). According to the total antioxidant capacity results, especially 50, 100 and 200 μM concentrations increased the total antioxidant capacity (Fig. 3B). In the treatment that was used, it was seen that concentrations of 12.5, 25, 50, 100, and 200 μM raised the level of TOS in the cancer cells' reactive oxygen species. Although MTT and inflammatory results of 12.5, 25 and 50 μM concentrations showed therapeutic effect, 100 μM concentration was found to be more effective in treatment.

IL-10 and TGF- β anti-inflammatory cytokine results. It was found that IL-10 levels went up statistically more at concentrations of 50 and 100 μM compared to the positive control group in the anti-inflammatory cytokine results (Fig. 4A). At 25 and 200 μM concentrations, a non-significant decrease was observed. According to TGF- β results, the 100 μM concentration value was significantly increased ($p < 0.05$), while non-significant changes were observed at other concentrations (Fig. 4B).

Discussion. Neocuproine prevents the formation of free radicals and has a therapeutic effect against oxidative damage [8]. Antioxidants are preferred as

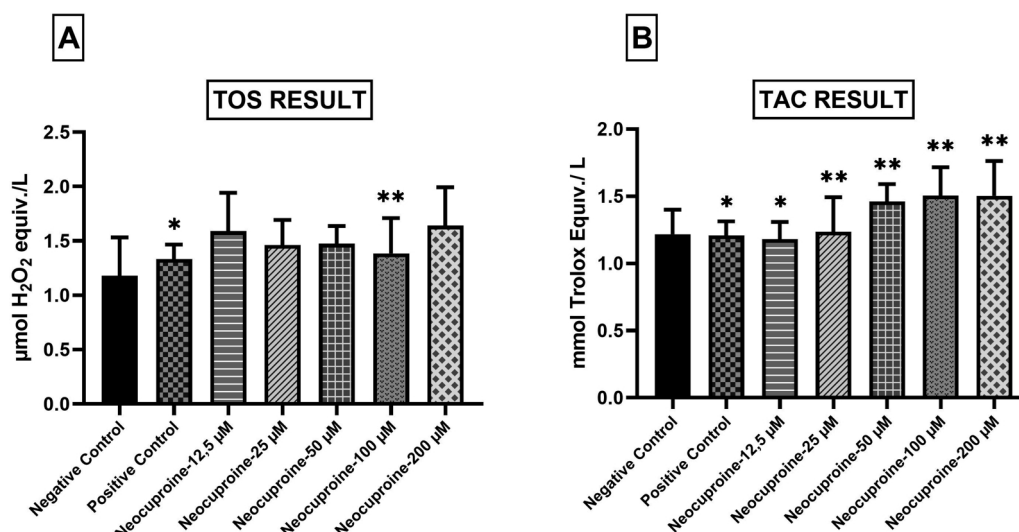


Fig. 3. TOS and TAC assay results. A) Total oxidant status test values read spectrophotometrically at 530 nm in cell culture fluid; B) Total antioxidant capacity test values read spectrophotometrically at 660 nm in cell culture fluid. * $p < 0.05$, ** $p < 0.001$ shows the difference compared to the negative control group. Data are expressed as the means $\pm$ SD

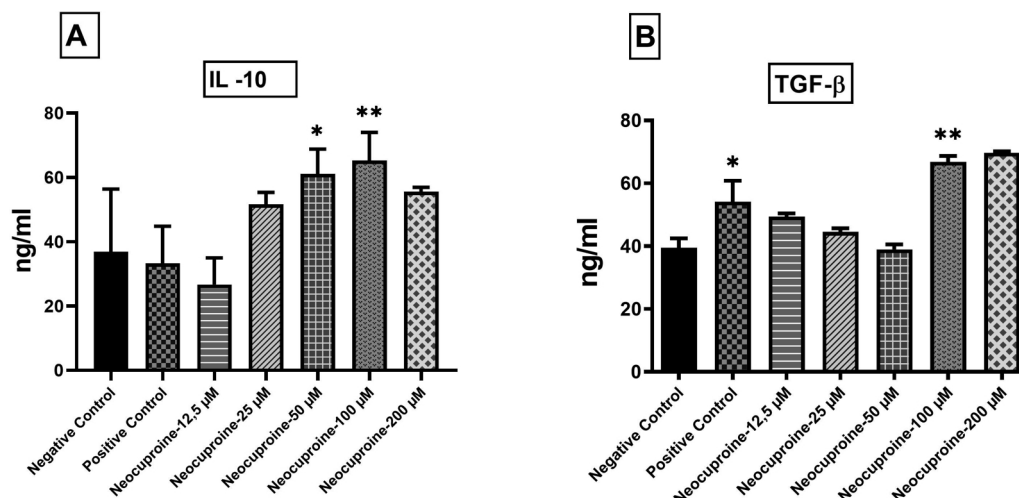


Fig. 4. Effects of neocuproine on the anti-inflammatory parameters (IL-10 and TGF- β) of neuroblastoma cells. A) IL-10 results B) TGF- β results. * $p < 0.05$, ** $p < 0.001$ shows the difference compared to the negative control group. Data are expressed as the means $\pm$ SD

therapeutic agents in cancer treatment. Oxidative stress and inflammatory cytokine regulation play an important role in the treatment of many diseases, especially cancer [12]. Neuroblastoma is a pediatric tumour arising from neural crest cells [12]. Since individual chromosome abnormalities occurring in the tumour make the treatment of neuroblastoma difficult, there is a need for a comprehen-

sive treatment method that will affect neuroblastoma cancers. Therefore, the SH-SY5Y cell line was selected for our study. In this study, the therapeutic effect of Neocuproine, which has free radical scavenging properties, on oxidative stress and anti-inflammatory cytokine levels in neuroblastoma cell lines was investigated. After looking at the MTT results, 100 μ M Neocuproine was found to have a cytotoxic effect, which means that 69.76% of the SH-SY5Y cells tested were viable. Concentrations of 12.5, 25 and 50 μ M were found to have low activity and a concentration of 200 μ M was found to be a toxic concentration.

Although other concentrations were also effective, the highest oxidant activity was found at 100 μ M concentrations, which are the two most effective concentrations of Neocuproine in terms of anti-cancer activity. In the treatment used, 12.5, 25, 50, 100 and 200 μ M concentrations were found to increase the TOS level in the reactive oxygen species of cancer cells, but it was concluded that the most effective treatment concentration was 100 μ M. Although MTT and inflammatory results of 12.5, 25 and 50 μ M concentrations showed therapeutic effect, 100 μ M concentration was found to be more effective in treatment. In addition, according to our total antioxidant results, it was determined that the 100 μ M concentration value had the highest antioxidant effect. In our study, it was found that 100 μ M Neocuproine reduced oxidative capacity, increased antioxidant capacity in neuroblastoma cancer cells, and regulated oxidative stress.

The free radical scavenger Neocuproine regulates oxidative stress in SH-SY5Y cells. However, it also showed a therapeutic effect on inflammatory cytokine levels. In our anti-inflammatory results, it was determined that 50 and 100 μ M Neocuproine were effective on the IL-10 level, and 100 and 200 μ M Neocuproine were effective on the TGF- α level, compared to the control group. It was observed that Neocuproine at concentrations of 12.5 and 50 μ M further reduced anti-inflammatory cytokine levels and showed toxic effects. ZHEN et al. [1] reported that the applied treatment was effective on IL-10 and TGF- β levels. According to our anti-inflammatory IL-10 and TGF- β cytokine results, Neocuproine was seen to have an anti-cancer effect and regulate cytokine levels.

Our study showed that Neocuproine, a free radical scavenger, lowers the ability of oxidants to do damage and controls oxidative stress by raising the levels of antioxidants in SH-SY5Y cells. In addition, it was found to have a therapeutic effect on anti-inflammatory cytokine levels. Therefore, Neocuproine will be preferred as an anti-cancer agent in SH-SY5Y, and further studies are needed.

Conclusions. In conclusion, our study showed that 100 μ M Neocuproine inhibited cell cytolysis in the SH-SY5Y cell line in vitro. One hundred μ M Neocuproine regulated the level of oxidative stress by decreasing TOS levels and increasing TAC levels. The treatment with Neocuproine also changed the amounts of cytokines IL-10 and TGF- β in SH-SY5Y cells, which help fight inflammation. Neocuproine down-regulation suppresses tumour growth and may be a novel anti-cancer agent in SH-SY5Y cancer cells.

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