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Original research article

Cytotoxic effect of tempol on human osteosarcoma

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Abbreviations
ATCC – American type culture collection
CC3 – cleaved caspase-3
DET – deoxyelephantopin
ELISA – enzyme linked immunosorbent assay
OS – osteosarcoma
PBS – phosphate buffer saline
ROS – reactive oxygen species
SCC-25 cell line – human squamous carcinoma cell line
SEM – standard error of the mean
TPL – tempol

Keywords: Bax; bcl-2; caspase-3; cytotoxic; osteosarkoma; tempol

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Abstract

Background and purpose: *In addition to the antioxidant activity of tempol, its high concentrations also act as a pro-oxidant that prevents the growth of cancer cells. The objective of our study is to determine if tempol exhibits cytotoxic properties that can be utilized in the cure of osteosarcoma.*

Materials and methods: *The human SCC-25 cell-line was provided from the American Type Culture Collection (ATCC) via cell culture. The cells were subjected to a 4 mM concentration of tempol for a duration of 48 hours. The cells were subjected to centrifugation, and the resulting liquid portion, known as the supernatant, was collected. The assessment of cell viability was conducted using the MTT test. The Bradford technique was employed to quantify the total protein content. The ELISA method was used to evaluate the expression and functionality of cleaved bax, caspase-3, and bcl-2. An unpaired Student's t-test was used to confront the parameters of the experimental and control groups.*

Results: *Bax, bcl-2 and cleaved caspase-3 levels in the control groups, respectively; It was determined as 1.035±0.1121, 7.083±0.8002 and 0.8017±0.2342. Bax, bcl-2 and cleaved caspase-3 levels in tempol applied groups were determined as 4.667±0.7601, 1.833±0.4766 and 8.018±0.8563, respectively.*

Conclusion: *As a result, tempol can be evaluated as an antitumor agent in the treatment of osteosarcoma with its cytotoxic effect.*

INTRODUCTION

Osteosarcoma (OS) is a primary malignancy that primarily impacts the skeletal system and tends to occur in individuals within the age range of children, teenagers, and young adults *(1)*. OS is abnormal bone growth arising from mesenchymal tissue (consisting of spindle-shaped stromal cells) *(2, 3)*. It is more common in the metaphysis of long tubular bones. The majority of patients are present with only a single lesion *(3)*. The formation of osteosarcoma is influenced by a combination of genetic, epigenetic, and environmental variables that facilitate the differentiation of mesenchymal stem cells into bone precursor cells. These molecular pathways could serve as the foundation for the creation of novel therapeutic alternatives for this tumor *(2)*.

New drugs that can end the malignant behavior of cells are also needed in the treatment of osteosarcoma *(4)*. Tempol (4-hydroxy-2,2,6,6 tetramethylpiperidine-N-oxyline) is a fixed nitroxide radical, water-soluble superoxide dismutase mimetic agent that is cell membrane permeable *(5-7)*. Tempol (TPL) counteracts the impact of superoxide radicals and directly interacts with carbon-centered and peroxy radicals *(5)*, therefore hindering the conversion of hydrogen peroxide into hydroxyl

radical *(5, 8)*. Therefore, Tempol reduces oxidative stress by reducing the release of oxidant free radicals *(8)*. Tempol protects cells from damaging effects such as oxidative stress and liver toxicity resulting from inflammation *(8, 9)*. Tempol is used to treat cancers that depend on the generation of reactive oxygen species (ROS) in the surrounding microenvironment *(10)*.

Cytotoxicity is a phenomenon that causes varying degrees of damage to cells depending on the dose of the drug and the duration of exposure. End of research, determining the amount of live/dead cells is the most important part of the experimental cytotoxicity study *(11)*.

Apoptosis is a programmed cell death process that is essential for maintaining the balance and stability of an organism *(12)*. Caspase-3, a crucial component in the process of programmed cell death, is a key focus of cancer treatment. Once activated, caspase-3 initiates the activation of death protease and subsequent degradation of proteins, and the cycle results in apoptosis *(13, 14)*. The bax/bcl-2 ratio the expression of caspase-3 is linked to the ratios of RNA and protein *(14)*. The bcl-2 and bax proteins are key members of the bcl-2 protein family, and they have a crucial function in controlling apoptosis. Bax is a protein that enhances apoptosis, while bcl-2 is a protein that prevents apoptosis from occurring *(12)*.

This survey aims to find the potential cytotoxic effects of tempol as a therapeutic approach for human osteosarcoma.

MATERIALS AND METHODS

Cell culture

The human SCC-25 cell-line was acquired from the ATCC. The squamous cancer cells were cultivated using Ham's F12 medium and Dulbecco's Modified Eagle's Medium (DMEM) (Gibco). The medium contained 15 mM HEPES (2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid), 1.2 g/L sodium bicarbonate, 2.5 mM L-glutamine and 0.5 mM sodium pyruvate (HyClone). Additionally, the medium was enhanced with 10% fetal bovine serum (FBS) and 400 ng/ml hydrocortisone (HyClone). The cells were cultured in a controlled environment with high humidity at a temperature of 37°C, with a CO_2 concentration of 5% *(15)*.

Cell homogenization

A population of cells, with a density of 5 × 104 cells per square centimeter, was subjected to a concentration of 4 mM tempol (Sigma-Aldrich) for a duration of 48 h. The cells were bathed with phosphate buffer saline (PBS) and then broken down in radio-immunoprecipitation assay (RIPA) buffer (containing 0.5% TritonX-100, 1mmol/L dithiothreitol, 20 mmol/L EGTA, 1 mmol/L Na3VO4, 50 mmol/L Tris–HCl [pH 7.4], 25mmol/L NaF and 150 mmol/L NaCl) for 15 minutes on ice. The combination was then centrifuged at 15000 rpm for 20 minutes, resulting in the separation of the liquid fraction (supernatants) from the solid fraction (pellets), which was subsequently discarded *(15)*.

Cell viability assay (MTT assay)

The viability of SCC-25 cells was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) assay. Cells were seeded in 96-well plates at a density of 1 × 104 cells per well. After 48 h of exposure to a combination of varying concentrations of tempol (2, 4, and 8 mM), the cell culture medium was removed. Then, cells were treated with 100 µL of MTT solution (dissolved in 0.5 mg/mL DMEM) and incubated at 37 °C with 5% CO for 2 h. The MTT solution was then discarded and 100 µL of DMSO was added to each well. Optical density (OD) was measured at 550 nm using a microplate reader (EL800, Bio-Tek Instruments, Inc.). Viability was calculated by determining the ratio of the mean OD from each measurement result to that of the control group *(15)*.

Enzyme linked immunosorbent assay (ELISA)

The expressions of bcl-2 and bax proteins (Abcam) and activity of cleaved caspase-3 were analyzed using enzyme-linked immunosorbent assay (ELISA) kits in line with the manufacturer's protocols *(15)*.

Statistical analysis

The significance of differences in MTT levels and protein expressions between the groups was evaluated using Student's t-test. The data is shown as the mean value accompanied by the standard error of the mean (SEM), denoted by ±. Statistically $P < 0.05$ is significant.

RESULTS

Figure 1 depicts the outcomes of cell viability (%) when SCC-25 cancer cells were subjected to different levels of tempol (2, 4, and 8 mM) for a 48h period. It is evident that higher doses of tempol resulted in a notable decrease in cell proliferation compared to the control group after the 48h time frame ($^*P < 0.05$).

The experiment involved subjecting SCC-25 cancer cells to a concentration of 4 mM of tempol for a duration of 48 hours. The control group comprised untreated SCC-25 cancer cells. The 48-hour IC_{50} dose of tempol in SCC-25 cancer cells was found to be 114.3733 µg/mL. Cleaved caspase-3 levels were determined as 0.8017±0.2342 (control) and 8.018±0.8563 (tempol). Bax levels in the control and tempol applied groups were found to be 1.035±0.1121 and 4.667±0.7601, respectively. The administration of

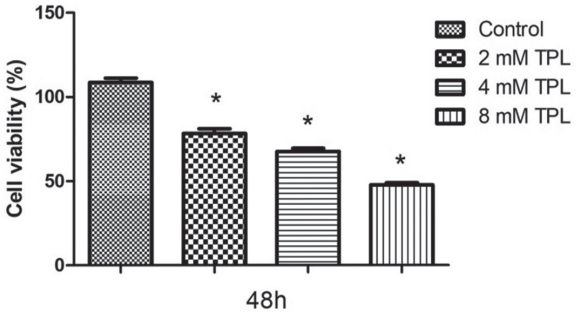

Figure 1. *Viability levels in SCC-25 cancer cells following the administration of increasing doses of Tempol. (n = 7, mean ± SEM) (*: for control P < 0.05).*

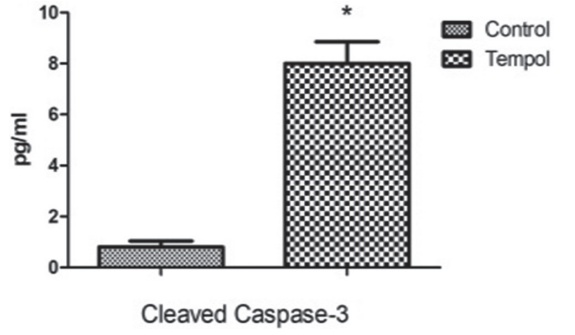

Figure 2. *Cleaved caspase-3 levels (P_{cc3} < 0.0001) in SCC-25 cancer cells. (n = 7, mean ± SEM) (*: for control P < 0.05).*

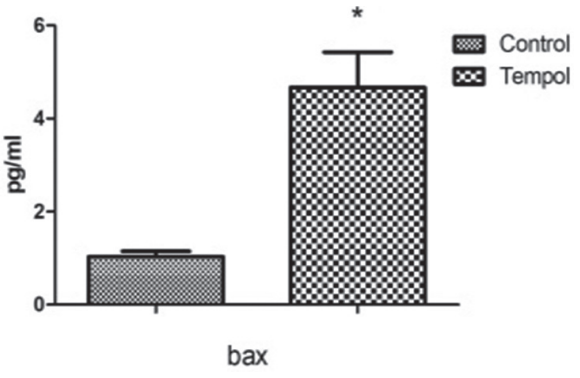

Figure 3. *Bax levels (P_{bax}=0.0002) in SCC-25 cancer cells. (n = 7, mean ± SEM) (*: for control P < 0.05).*

tempol resulted in a significant rising in the levels of cleaved caspase-3 (Figure 2) and bax (Figure 3) in SCC-25 cancer cells, as confronted to the control group (P < 0.05).

Conversely, the application of tempol led to a notable falling in the levels of the bcl-2 (Figure 4) in SCC-25 cancer cells, confronted to the control group (P < 0.05). Bcl-2 levels were determined as 7.083±0.8002 and 1.833±0.4766 in the control and tempol groups.

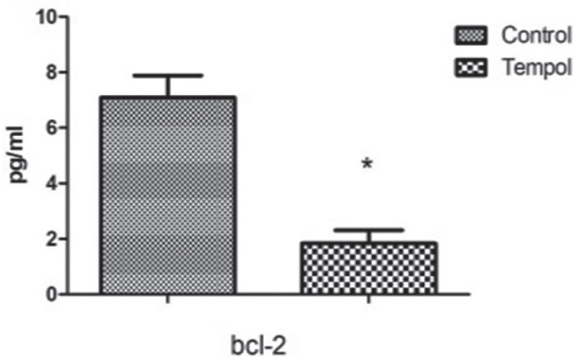

Figure 4. *Bcl-2 levels (P_{bcl-2} = 0.0002) in SCC-25 cancer cells. (n = 7, mean ± SEM) (*: for control P < 0.05).*

DISCUSSION

Osteosarcoma is the prevailing kind of bone malignancy, and the available therapy choices are restricted. Neoadjuvant chemotherapy with surgical resection has become the standard treatment *(16)*. Apoptosis is an important physiological process that maintains cellular homeostasis by eliminating cells with bad characteristics *(17)*. Several anti-tumor medications demonstrate cytotoxic properties by inducing apoptosis in cancer cells *(4)*. Tempol initially affects the mitochondrial membranes of both lung cancer and normal cells before inducing apoptosis. Tempol is a compound that undergoes a redox cycle and helps in the metabolism of ROS by quickly switching between different forms: nitroxide, hydroxylamine, and oxoammonium cation. As a result, depending on its quantity within cells, tempol might potentially engage in redox reactions *(7)*. Cell death caused by Tempol is related to elevated amounts of ROS within the cells *(18)*. Tempol toxicity causes disruption of mitochondrial function, and with the resulting increase in ROS production, oxidative stress is induced and a process that results in apoptosis occurs in the cell *(7)*. In cancer cells, decreased bax expression and/or bcl-2 overexpression leads to cells escaping apoptosis and continuing to proliferate *(12)*. Comprehending the correlation between bax and bcl-2 is crucial for the advancement of therapies that can reinstate regular apoptic control and provoke the demise of cancerous cells *(12, 19)*. An imbalance resulting from the dysregulation of bax and bcl-2 can decrease caspase-3 activity and contribute to resistance to apoptosis *(20)*.

Our study demonstrates that tempol induces cytotoxicity in bone tissue, leading to an increase of bax expression and cleaved caspase-3 activity, likewise a decrease of bcl-2 expression. This showed that tempol induced apoptosis in cancer cells and had a cytotoxic effect on osteosarcoma cells. Different studies have stated that the use of tempol alone and/or in combination with other cancer drugs suppresses proliferation in cancer cells and may be effective in cancer treatment by increasing apoptosis *(7,*

21-23). The study conducted by Zou *et al.* determined that deoxyelephantopin (DET) triggers apoptosis in osteosarcoma cells by generating ROS, impairing mitochondrial activity, and activating caspases *(4)*. Our and Zou *et al.* studies also showed that the active substances used had cytotoxic effects on cancer cells with similar mechanisms of action *(4)*.

CONCLUSION

This work demonstrates that administering tempol as a premedication substantially enhances the cleaved caspase-3 expression and bax/bcl-2 ratio, leading to apoptosis and exerting a cytotoxic impact on tumor cells. The results of our study support the potential use of tempol as an anticancer drug for treating osteosarcoma.

REFERENCES

1. ISAKOFF M S, BIELACK S S, MELTZER P, GORLICK R 2015 Osteosarcoma: Current treatment and a collaborative pathway to success. J Clin Oncol 33: 3029-3035. https://doi.org/10.1200/JCO.2014.59.4895
2. RATHORE R, VAN TINE B A 2021 Pathogenesis and current treatment of osteosarcoma: Perspectives for future therapies. J Clin Med 10: 1182. https://doi.org/10.3390/jcm10061182
3. ZHAO X, WU Q, GONG X, LIU J, MA Y 2021 Osteosarcoma: a review of current and future therapeutic approaches. BioMed Eng OnLine 20: 24. https://doi.org/10.1186/s12938-021-00860-0
4. ZOU J, ZHANG Y, SUN J, WANG X, TU H, GENG S, LIU R, CHEN Y, BI Z 2017 Deoxyelephantopin induces reactive oxygen species-mediated apoptosis and autophagy in human osteosarcoma cells. Cell Physiol Biochem 42: 1812-1821. https://doi.org/10.1159/000479537
5. CHONPATHOMPIKUNLERT P, HAN J, TOH K, ISODA H, NAGASAKI Y 2011 Tempol protects human neuroblastoma SH-SY5Y cells against ß-amyloid-induced cell toxicity. European Journal of Pharmacology 650: 544-549. https://doi.org/10.1016/j.ejphar.2010.10.028
6. NEIL S, HUH J, BARONAS V, LI X, MCFARLAND HF, CHERUKURI M, MITCHELL J B, QUANNDT J A 2017 Oral administration of the nitroxide radical tempol exhibits immunomodulatory and therapeutic properties in multiple sclerosis models. Brain, Behavior and Immunity 62: 332-343. https://doi.org/10.1016/j.bbi.2017.02.018
7. PARK W H 2022 Tempol inhibits the growth of lung cancer and normal cells through apoptosis accompanied by increased O2 •– levels and glutathione depletion. Molecules 27: 7341. https://doi.org/10.3390/molecules27217341
8. PINAR N, KAPLAN M, OZGUR T, OZCAN O 2018 Ameliorating effects of tempol on methotrexate-induced liver injury in rats., Biomedicine & Pharmacotherapy 102: 758-764. https://doi.org/10.1016/j.biopha.2018.03.147
9. ABOUZIED M M, ELTAHIR H M, TAYE A, ABDELRAHMAN M S 2016 Experimental evidence for the therapeutic potential of tempol in the treatment of the acute liver injury. Mol Cell Biochem 411: 107-115. https://doi.org/10.1007/s11010-015-2572-2
10. PROCTOR P H 2015 Tempol and preventing breast cancer. ResearchGate, https://doi.org/10.13140/RG.2.1.1787.2084
11. TOKUR O, AKSOY A 2017 In vitro cytotoxicity assays. Harran Univ Vet Fak Derg 6 (1): 112-118. https://doi.org/10.31196/huvfd.325794
12. HUANG Y K, CHANG K C, LI C Y, LIEU A S, LIN C L 2023 AKR1B1 Represses glioma cell proliferation through p38 MAPK mediated bcl-2/bax/caspase-3 apoptotic signaling pathways. Curr Issues Mol Biol 45: 3391-3405. https://doi.org/10.3390/cimb45040222
13. MCCOMB S, CHAN K, GUINOT A, HARTMANNSDOTTIR H, JENNI S, DOBAY M P, BOURQUIN J P, BORNHAUSER B C 2019 Apoptosis requires feedback amplification of upstream apoptotic signals by effector caspase-3 or -7. Sci Adv 5: eaau9433. https://doi.org/10.1126/sciadv.aau9433
14. MOGHADAM N S, KAZEMINEZHAD B, DARGAHI L, AHMADIANI A 2010 Maternal oral consumption of morphine increases bax/bcl-2 ratio and caspase 3 activity during early neural system development in rat embryos. J Mol Neurosci 41: 156-164. https://doi.org/10.1007/s12031-009-9312-6
15. CELIK E, KAPLAN H M, SINGIRIK E 2020 The impact of propranolol on apoptosis in cutaneous squamous cell carcinomas. Bratisl Med J 121(11): 801-804. https://doi.org/10.4149/BLL_2020_131
16. DURFEE R A, MOHAMMED M, LUU H H 2016 Review of osteosarcoma and current management. Rheumatol Ther 3: 221-243. https://doi.org/10.1007/s40744-016-0046-y
17. FLUSBERG D A, SORGER P K 2015 Surviving apoptosis: Life-death signaling in single cells. Trends Cell Biol 25: 446-458. https://doi.org/10.1016/j.tcb.2015.03.003
18. HAN Y H, PARK W H 2012 Tempol inhibits growth of As4.1 juxtaglomerular cells via cell cycle arrest and apoptosis. Oncol Rep 27: 842-848. https://doi.org/10.3892/or.2011.1518
19. KUNAC N, FILIPOVIC N, KOSTIC S, VUKOJEVIC K 2022 The expression pattern of bcl-2 and bax in the tumor and stromal cells in colorectal carcinoma. Medicina 58: 1135. https://doi.org/10.3390/medicina58081135
20. SHI L, CHEN J, YANG J, PAN T, ZHANG S, WANG Z 2010 MiR-21 Protected human glioblastoma U87MG cells from chemotherapeutic drug temozolomide induced apoptosis by decreasing bax/bcl-2 ratio and caspase-3 activity. Brain Res 1352: 255-264. https://doi.org/10.1016/j.brainres.2010.07.009
21. MONTI E, SUPINO R, COLLEONI M, COSTA B, RAVIZZA R, GARIBOLDI M B 2001 Nitroxide tempol impairs mitochondrial function and induces apoptosis in HL60 cells. J Cell Biochem 82 (2): 271- 276. https://doi.org/10.1002/jcb.1160
22. WANG M, LI K, ZOU Z, LI L, ZHU L, WANG Q, GAO W, WANG Y, HUANG W, LIU R 2018 Piperidine nitroxide tempol enhances cisplatin-induced apoptosis in ovarian cancer cells. Oncol Lett 16 (4): 4847- 4854. https://doi.org/10.3892/ol.2018.9289
23. KAPLAN H M, PAZARCI P 2024 Antiproliferative and apoptotic effects of tempol, methotrexate,and their combinations on the MCF7 breast cancer cell line. ACS Omega 9(6): 6658-6662. https://doi.org/10.1021/acsomega.3c07624