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Research Article

COMBINED EFFECTS OF ZOLEDRONIC ACID AND SODIUM PENTABORATE PENTAHYDRATE ON PROLIFERATION, MIGRATION AND APOPTOSIS OF HUMAN NEUROBLASTOMA CELL LINE

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Abstract: *Neuroblastoma occurs in childhood with high aggressiveness and is one of the most common solid tumors. Although there are many different strategies to fight neuroblastoma including surgical treatment, chemotherapy, radiotherapy, and immunotherapy, ultimately successful treatment has not been evaluated yet. Effective, safe, and less toxic options must be investigated. Zoledronic acid (ZOL) is a type of amino-bisphosphonates and has been used in bone-related diseases for more than 20 years and the anti-tumor ability of the ZOL is known. Boron is a natural product and many regenerative properties of boron compounds such as myogenic, osteogenic, and odontogenic induction potential have been discovered. Besides, the boron compound also displayed anti-cancer characteristics in different studies. In the current study, we evaluated the possible synergistic effects of the ZOL and Sodium pentaborat tetrahydrate (SPT) on the neuroblastoma cells, SH-SY5Y. As a result, ZOL and SPT combination exhibited the most favorable anti-proliferative, pro-apoptotic and anti-migratory effects compared to the ZOL and SPT alone and control groups. Moreover, molecular evidence has indicated that while expression of the proliferative gene, NFκB was significantly decreased in the combination group compared to all other groups, pro-apoptotic genes, were overexpressed. To sum up, obtained results from the recent study lead it necessary to carry out more detailed studies.*

Keywords: *Neuroblastoma, Zoledronic acid, Sodium pentaborat tetrahydrate, Apoptosis*

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1.Introduction

Neuroblastoma is a childhood cancer with very aggressive potential and can migrate many parts of the body, such as bone, bone marrow, liver, lymph node, and skin [1,2]. This aggressiveness has caused almost 15% of cancer-related deaths in early childhood [3]. High-dose chemotherapy, stem cell treatment, radiotherapy, and immunotherapy cannot provide an ultimately successful treatment for neuroblastoma [4]. Effective, safe, and less toxic options are needed.

Bisphosphonates (BPs) are the analogs of the inorganic pyrophosphate and have a high affinity for bone. Because of this unique property, Clinicians use the BPs to inhibit bone resorption in osteoporosis treatment [5]. There are many types of BPs; however, especially Zoledronic acid (ZOL) displays a positive effect when used to treat metastatic bone disease in children. Besides, ZOL was successfully used in chemotherapy-related osteonecrosis patients [6]. It also caused beneficial outcomes for the reduction of skeletal-related events in breast cancer patients [7]. It has also been proven that ZOL application with endocrine therapy reduced breast cancer progression and metastasis to bone. Moreover, this combination increased the survival rate compared to only endocrine therapy. Due to the anti-tumor, anti-metastatic and anti-angiogenic properties of the ZOL, it triggers apoptosis and blocks tumor-cell invasion [8].

Boron (B) is a natural product and found in various human tissues and plays essential roles in hard tissue, especially bone development and maintenance [9]. The importance of boron is increasing by the day because of its biological properties. B is included in many cancer treatment processes such as prostate, breast, lung, and cervical cancers [10]. Both consumption as a food and usage as a chemotherapeutic agent of the B display positive effects against the cancer cells [10–13]. Inhibition of critical pathways, blocking cell division, and inducing apoptosis are the compelling characteristic features of the Boron compounds in cancer treatment [10].

In this study, to discover new, safe and efficient therapy for the treatment of neuroblastoma, we combined a boron compound sodium pentaborat tetrahydrate (SPT) and zoledronic acid (ZOL) due to having some similar characteristics such as bone affinity and anti-cancer. We evaluated the anti-proliferative, anti-migrative, and pro-apoptotic potential of the combination on the neuroblastoma cells, SH-SY5Y.

2. Materials and Methods

2.1. Reagents and Cell Lines

Dulbecco's Modified Eagle Medium with 4.5 g/L D-glucose (DMEM, Invitrogen, UK) was used. The medium was completed with 10% fetal bovine serum (FBS, Invitrogen, Gibco, UK) and 1% of penicillin/streptomycin/amphotericin (PSA, Invitrogen, Gibco, UK). SH-SY5Y (CRL-2266^{TM}, ATCC) cells were selected as model cells for neuroblastoma. For passaging of the cells, 0.25% trypsin/EDTA (#25200-056, Invitrogen, Gibco, UK) was applied to the cells. Zoledronic acid monohydrate (ZOL) was obtained from Sigma–Aldrich (#SML0650, St. Louis, MO, USA). 6mM ZOL was prepared as the main stock in dH_2O and diluted in DMEM for each experiment. The main stock of the ZOL was stored at -20 ºC for a long period. Sodium pentaborat tetrahydrate (SPT) was purchased from Ziegler & Co. GmbH (Wunsiedel, Germany) and prepared in DMEM at a 1 mg/mL concentration and diluted before use. SPT was freshly prepared before each experiment.

2.2. Cell Viability (MTS) Assay

MTS assay (3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (#G3582, CellTiter96 AqueousOne Solution; Promega, Southampton, UK)) was performed for determining the effects of ZOL and SPT alone and in combination on the cell viability of the SH-SY5Y cells. Firstly, different doses of ZOL (10, 25, 50, 75, and 100 μM) were applied to the cells, which were seeded onto 96-well plate ($5x10^3$ cells/well). The cell viability was calculated at 24, 48, and 72 h

after administration by ELISA microplate reader (Biotek, Winooski, VT) at 495 nm. According to the results, 10 µM ZOL were selected and applied on the cells alone and combined with different doses of SPT, including 67.5, 125, and 250 µg/mL. The cell viability was also calculated at 24, 48, and 72 hours after administration. According to MTS results, 10 µM ZOL and 67.5 µg/mL SPT were selected for the subsequent experiments.

2.3. Annexin V & Dead Cell Assay

To confirm the apoptotic situation of the SH-SY5Y cells under ZOL and SPT alone and in combination, Muse® Annexin V & Dead Cell Kit (Merck Millipore, USA and Canada) was carried out. The cells which were seeded onto the 6-well plate ($0.1x10^6$ cells/well), were exposed to 10 µM ZOL and 67.5 µg/mL SPT alone and in combination. 24h later, the cells were collected and exposed with the kit and analyzed with Muse® Cell Analyzer (Merck Millipore, USA and Canada).

2.4. Hoechst 33342 Staining

DNA-binding fluorescent dye Hoechst 33342 was used for evaluating nuclear morphological changes of the SH-SY5Y cells under ZOL and SPT alone and in combination. The cells which were seeded onto the 6-well plate ($0.1x10^6$ cells/well), were exposed to 10 µM ZOL and 67.5 µg/mL SPT alone and in combination for 24h. For fixation, 4% Paraformaldehyde (PFA) was used at +4ºC for 30 minutes. Finally, the wells were stained with the 1.6mM fluorescent dye at 37 ºC for 10 minutes in the dark. After staining, the wells were visualized through a fluorescence microscope (AXIO Vert. A1, Zeiss, Germany).

2.5. Cell Cycle Assay

To detect the cell cycle position of the cells after administration of ZOL and SPT alone and in combination, Muse® Cell Cycle Kit (Merck Millipore USA and Canada) was performed. The cells were seeded onto the 6-well plate ($0.1x10^6$ cells/well). The next day, 10 µM ZOL and 67.5 µg/mL SPT were administrated alone and in combination. 24h later, the cells were collected and exposed with the kit and analyzed with Muse® Cell Analyzer (Merck Millipore, USA and Canada).

2.6. Scratch Assay

The migration capacities of cells under ZOL and SPT alone and in combination were evaluated with the scratch assay that is a model experiment. For this aim, the cells were seeded onto the 6-well plate ($0.1x10^6$ cells/well). After reaching ~100% confluency, the wound model was formed by using a sterile 200 µL pipette. Then, 10 µM ZOL and 67.5 µg/mL SPT were administrated alone and in combination on SHSY5Y cells, and the wells were visualized by the Zeiss PrimoVert light microscope with an AxioCam ERc5s camera (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA) at 0, 24 and 48 h. Total closure rates of each treatment group were calculated.

2.7. Quantitative Polymerase Chain Reaction (qPCR)

The effects of ZOL and SPT on the expression levels of the apoptosis and cell survival-related genes, including AKT, BAX, Caspase 3, Caspase 7, and NFκB were evaluated. The cells were seeded onto the 6-well plate ($0.1x10^6$ cells/well). After 24h, 10 µM ZOL and 67.5 µg/mL SPT were applied alone and in combination on the cells. The next day, the total RNA isolation was done by using the High Pure RNA Isolation Kit (Roche). The isolated total RNA was used to synthesize cDNA by Transcriptor

High Fidelity cDNA Synthesis Kit (Roche). SYBR Green, the specific primers for related genes, and synthesized cDNA were mixed and qPCR was performed by running the iCycler RT-PCR system (CFX Real-Time System; Bio-Rad, Singapore).

2.8. Statistical Analysis

For statistical analysis, a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test was selected and GraphPad Prism (version 7.00; GraphPad Software, Inc., San Diego, CA, USA) is used. The values of *p<0.05 were decided statistically significant results.

3. Results

3.1. Cell Viability (MTS) Assay

To evaluate the effects of different concentrations of ZOL on the cell viability of SH-SY5Y cells, MTS assay was performed for 72h. 24h after exposure, all treated ZOL concentrations significantly decreased the cell viability of the cells (Fig. 1A). The lowest dose (10 µM) was selected for combination treatment. Then, three different doses of SPT (67.5, 125, and 250 µg/mL) were used alone and in combination with 10 µM ZOL in MTS assay. 24h after exposure, while 67.5 µg/mL SPT increased cell viability of the cells, 10 µM ZOL decreased to %77.5±15.8. Moreover, the combination of the 10 µM ZOL and 67.5 µg/mL SPT decreased to 48.11±6.6. In other treatment groups, synergistic effects were observed in a dose-dependent manner (Fig. 1B). 10 µM ZOL and 67.5 µg/mL SPT alone and in combination were decided for the subsequent experiments.

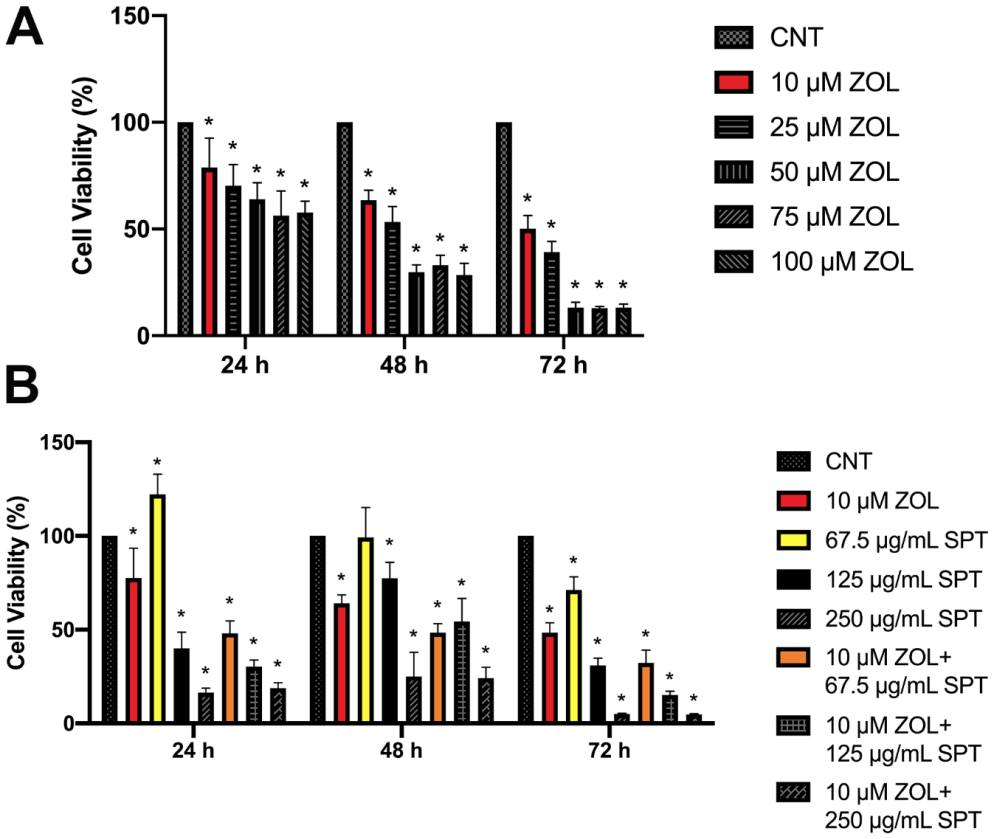

Figure 1 A. The effects of ZOL on the cell viability of SH-SY5Y cells. **B.** The effects of ZOL and SPT alone and in combination on the cell viability of the SH-SY5Y cells. CNT: Control, ZOL: Zoledronic acid, SPT: Sodium pentaborat tetrahydrate, *: $p < 0.05$.

3.2. Annexin V & Dead Cell Assay

Annexin V & Dead Cell Assay was performed to detect the apoptotic situation of the cells when they were treated to 10 µM ZOL and 67.5 µg/mL SPT alone and in combination during 24h. There were no significant differences in the aspect of live cell and apoptotic cell ratios among the CNT (%86.3-%12.7), ZOL (%84.3-%14.7), and SPT (%78.3-%20.3) groups, while the combination caused a significant decrease in the live-cell ratio (%68.3) and increased apoptotic cell ratio (%30.7) compared to the CNT group (Fig. 2A, B).

3.3. Hoechst 33342 Staining

Administration of the 10 µM ZOL and 67.5 µg/mL SPT in combination for 24 h caused apoptotic nuclei formation and white arrows showed the fragmented nucleus. CNT and 67.5 µg/mL SPT-treated groups exhibited similar morphological characteristics with a well-rounded, spherical and integrated nucleus. In addition, apoptotic nuclear characteristics were slightly raised in the 10 µM ZOL-treated group compared to the CNT group (Fig. 2C).

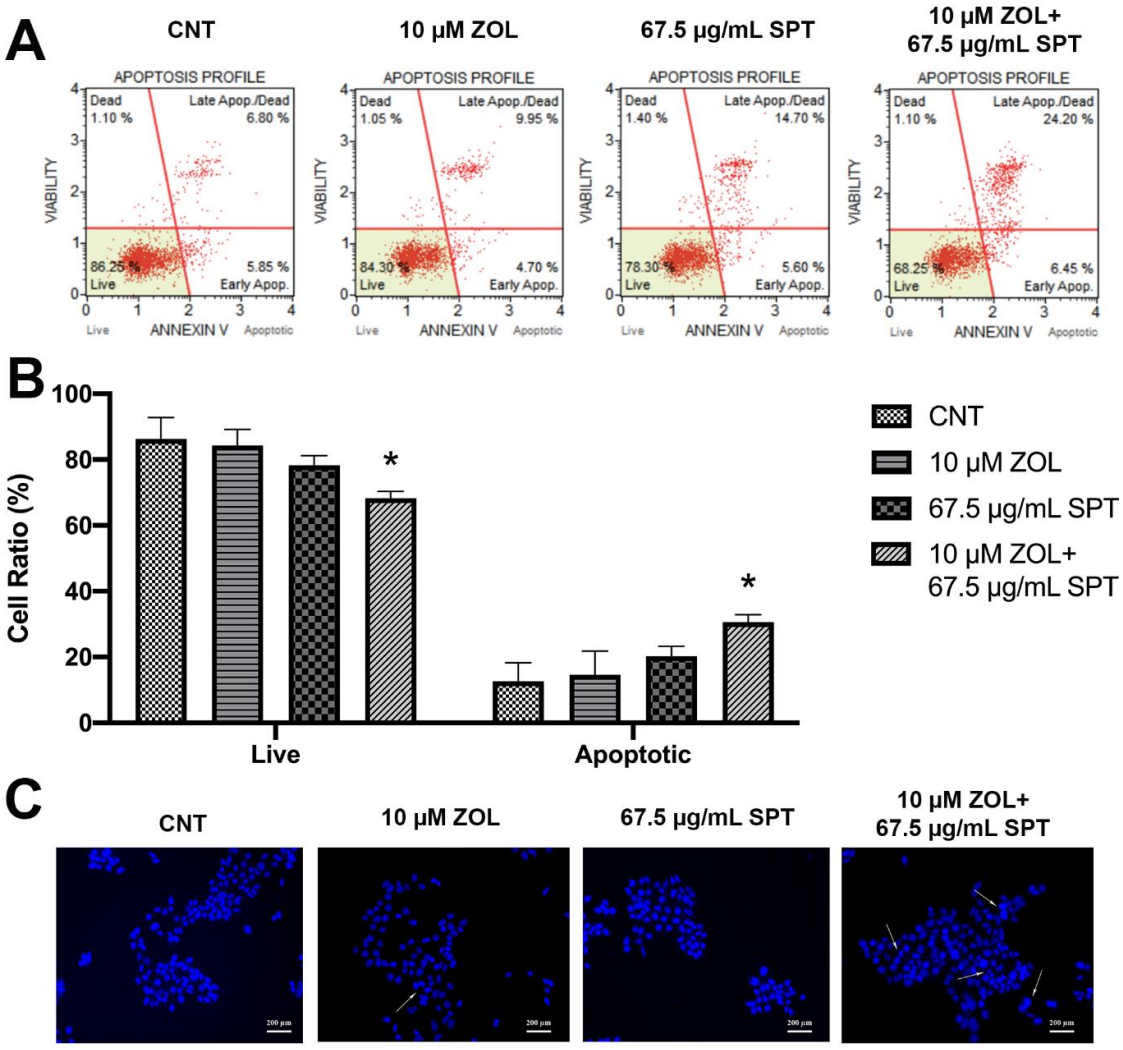

Figure 2 A. Apoptotic situation of the SH-SY5Y cells under ZOL and SPT alone and in combination. **B.** Bar graph indicates the percentage of viable and apoptotic cells. **C.** apoptotic nuclei formation of the SH-SY5Y cells under ZOL and SPT alone and in combination, white arrows showed the fragmented nucleus. CNT: Control, ZOL: Zoledronic acid, SPT: Sodium pentaborat tetrahydrate, *: $p < 0.05$.

3.4. Cell Cycle Assay

To investigate the cell cycle distribution of the SH-SY5Y cells when they were treated with 10 µM ZOL and 67.5 µg/mL SPT alone and in combination during 24h, a cell cycle assay was performed. All treated groups accumulated the cells in the G2/M phase while reduced the G0/G1 and S phase cell ratio compared to the CNT group. However, there was no significant difference among the treated groups in cell-cycle phase distribution (Fig. 3A, B).

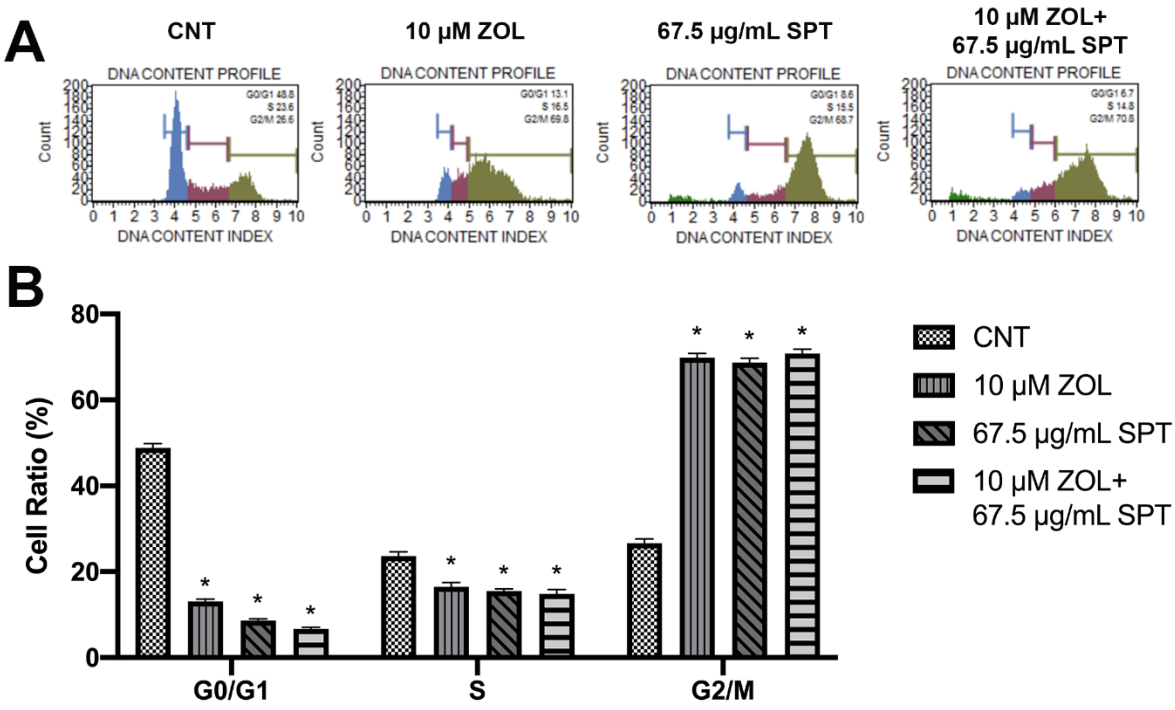

Figure 3 A. Cell cycle distribution of the SH-SY5Y cells under ZOL and SPT alone and in combination. **B.** Bar graph indicates the percentage of the cell cycle distribution. CNT: Control, ZOL: Zoledronic acid, SPT: Sodium pentaborat tetrahydrate, *: $p < 0.05$.

3.5. Scratch Assay

The migration capacity of the SH-SY5Y cells exposed to 10 µM ZOL and 67.5 µg/mL SPT alone and in combination for 24h, was evaluated in scratch assay which is a model experiment. The wound closure rate of the cells significantly decreased when the cells were treated with ZOL alone and in combination with SPT while SPT alone did not display any significant effect compared to the CNT group. At 24h, closure rates of the CNT and SPT groups were 60.8% and 56.5%, respectively, 40.6% and 22.4% closure rates were observed in ZOL and combination groups. Almost complete closure was observed in CNT and SPT group, while ZOL (48.7%) and combination (29.4%) caused lower closure rates at the end of the 48h treatment (Fig. 4A, B).

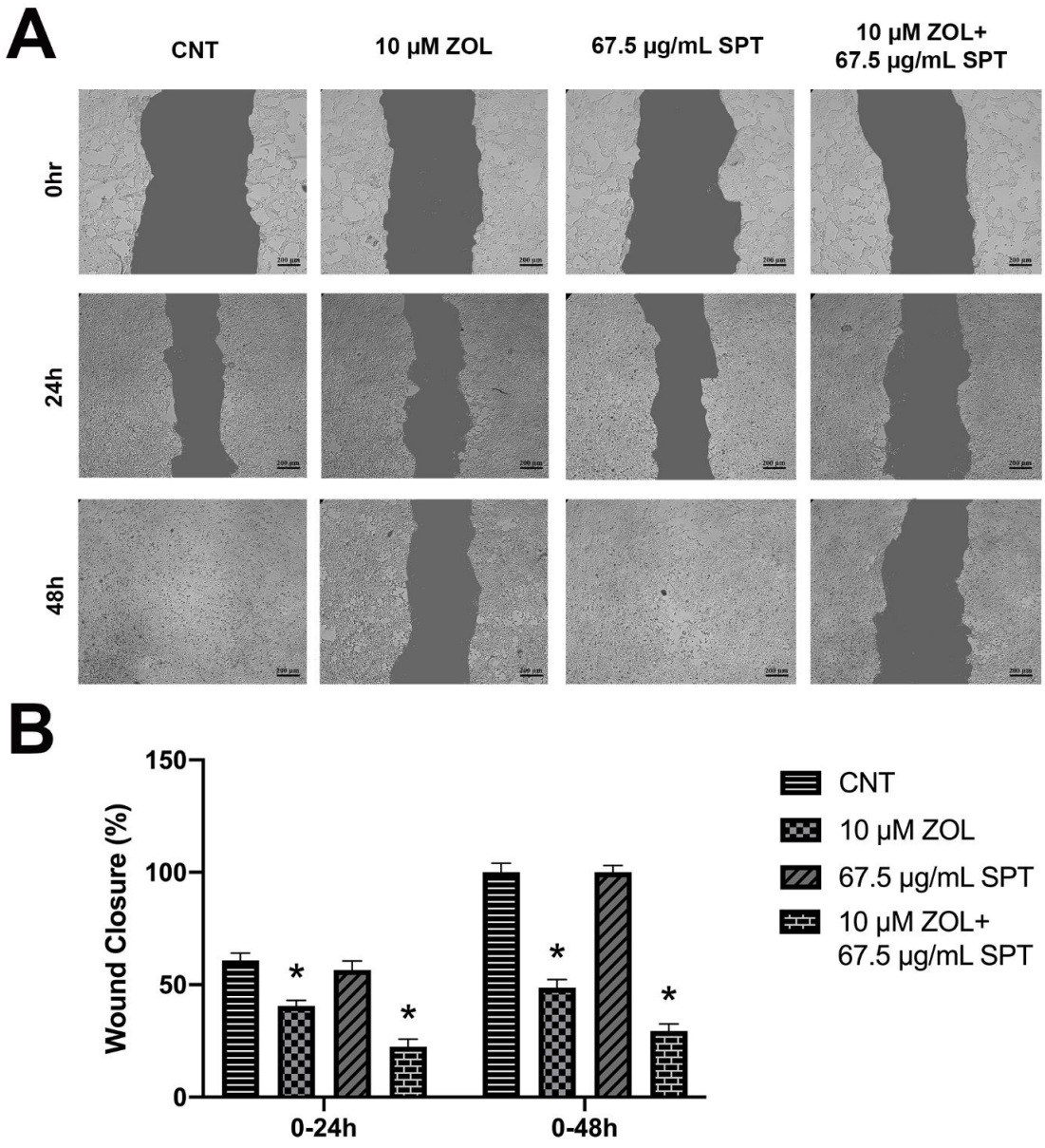

Figure 4 A. Scratch assay photographs of the SH-SY5Y cells under ZOL and SPT alone and in combination at 0, 24, and 48. **B.** Bar graph indicates the percentage of wound closure. CNT: Control, ZOL: Zoledronic acid, SPT: Sodium pentaborat tetrahydrate, *: $p < 0.05$.

3.6. Quantative Polimerase Chain Reaction (qPCR)

qPCR was performed to observe the expression levels of the proliferation and apoptosis-related genes under 10 µM ZOL and 67.5 µg/mL SPT alone and in combination treatment. While AKT expression did not change in all treatment groups, only combination treatment significantly downregulated NFκB expression compared to the CNT group. Expression levels of BAX, CAS3, and CAS7 which are pro-apoptotic genes, were significantly upregulated in combination groups compared to the CNT group. Besides, ZOL treatment alone significantly increased the expression level of BAX compared to the CNT group (Fig. 5).

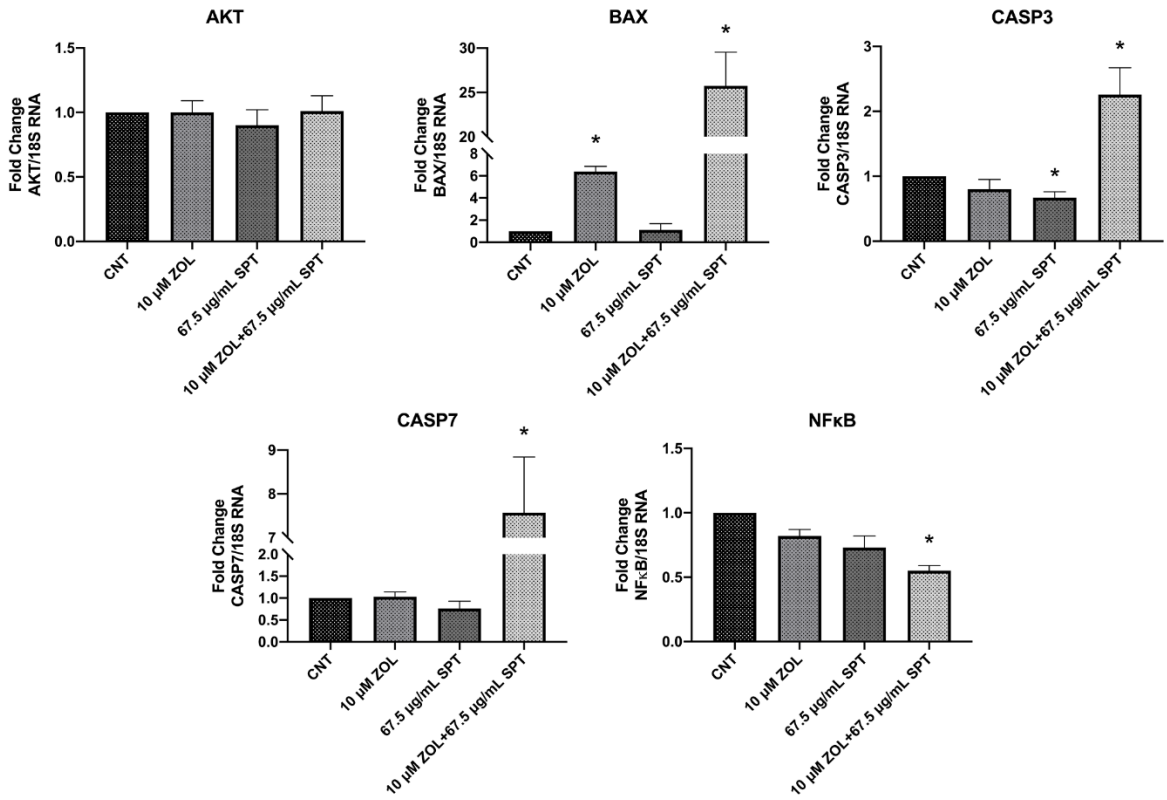

Figure 5. Expression levels of the proliferation and apoptosis-related genes of SH-SY5Y cells under ZOL and SPT alone and in combination. CNT: Control, ZOL: Zoledronic acid, SPT: Sodium pentaborat tetrahydrate, *: $p < 0.05$.

4. Discussion

Neuroblastoma is frequently observed extracranial tumor type in children. The tumor derives from a forming precursor cell in the peripheral (sympathetic) nervous system and causes different clinical courses [14]. There are many different therapeutic approaches for neuroblastoma including cytotoxic chemotherapy, external beam radiation, and therapy surgery. Besides, induction of differentiation or apoptosis of the neuroblastoma cells and inhibition of the angiogenesis for blocking the tumor growth may also be promising approaches for the treatment [3].

Amino-bisphosphonates (N-BPs) have been used FDA-approved for more than 20 years in bone-related diseases such as osteoporosis and bone metastases [15]. The anti-tumoral activity of zoledronic acid (ZOL) which is a type of N-BPs is very well known [16]. Besides, it has displayed immune-modulatory and anti-angiogenic activities in animal models [17]. In a recent study, a cell viability assay was performed with different concentrations of the ZOL for 72h and all tested doses caused the cytotoxic effect on the SH-SY5Y cells. Usage of the ZOL as an adjuvant against breast cancer increased survival rate and decreased recurrence in post-menopausal patients [18]. Boron is a natural product and found in many human tissues. Up to now, developmental benefits of the B have been shown in various studies [19,20]. B compounds have been used in prostate, lung, cervical, and breast cancer and caused positive effects against cancer [11,13,21–23]. The supporting effect of B was demonstrated in patients who received chemotherapy [11]. To evaluate the possible synergistic effects of ZOL and Sodium pentaborat tetrahydrate (SPT) on SH-SY5Y cells, 10uM ZOL that is selected according to cell viability assay and different concentrations of the SPT were applied on the cells for 72h. SPT did not display a negative

effect on the cell viability in the first 2 days alone, while in combinations with ZOL significantly decreased cell viability of the SH-SY5Y compared to alone ZOL and control group.

Apoptotic profile and nuclear morphological changes of the cells exposed to the agents are important points to decide cytotoxic activity of the agents against the cancer cells [24]. ZOL and SPT combination significantly increased apoptotic cell death and apoptotic nuclei formation compared to other groups. Besides, all treated groups blocked the cell cycle of SHSY5Y by causing accumulation at the G2/M phase. G2/M phase accumulation has been observed in another pro-apoptotic characteristic of the DNA damaging agent [25].

Neuroblastoma has a high metastatic rate of 70% at the time of diagnosis [26]. Therefore, the anti-migratory effect of the agent to be used in the treatment of neuroblastoma is crucial. To evaluate the migration capacity of the cells under certain conditions, the scratch assay is performed as a model experiment [27]. While SPT exhibits similar migratory activity to the control group, ZOL alone and in combination administrations caused anti-migratory effects on the SH-SY5Y cells. Besides, the most favorable effect was observed in the combination group.

Proliferation and apoptosis are very complex mechanisms controlled by many genes. Interaction between AKT and NFκB exhibits proliferative effect however is also responsible for apoptosis [28,29]. ZOL and SPT did not change AKT expression, while NFκB was significantly down expressed in the combination group compared to the other groups. There are many studies about pro-apoptotic activities of the AKT and Caspases [30,31]. Over expressions of the pro-apoptotic genes can provide the induction of apoptosis which must be one of the main goals to fight against cancer. ZOL treatment alone significantly increased BAX level compared to the control group, while other ZOL and SPT alone administrations did not increase pro-apoptotic gene levels. However, AKT, CASP3, and CASP7 were significantly over-expressed in the combination group compared to the CNT group.

5. Conclusion

Overall, the effects of administration of ZOL and SPT alone and in combination on the SH-SY5Y cells were evaluated by various experiments and promising results were obtained. Combination therapy displayed the most favorable anti-proliferative, pro-apoptotic and anti-migratory effects on the neuroblastoma cells. The possible synergistic effects of the ZOL and BOR have been already observed and seem to be promising for the planned subsequent experiments for the future.

The compliance to Research and Publication Ethics

This study was carried out by obeying research and ethics rules.

Acknowledgment

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The Declaration of Conflict of Interest/ Common Interest

No conflict of interest or common interest has been declared by the author.

The Declaration of Ethics Committee Approval

The author declares that this document does not require an ethics committee approval or any special permission. Our study does not cause any harm to the environment.

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