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Anti-proliferative effects of salmon calcitonin on SH-SY5Y neuroblastoma *in vitro*

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ABSTRACT

Aim: We aimed to examine the potential cytotoxic effect of salmon calcitonin, which is one of the components that regulates mineral metabolism and prevents the increase in the amount of calcium, on SH-SY5Y cells, a neuroblastoma cell line.

Methods: SH-SY5Y cells were cultured in DMEM medium in the presence of 37°C and 5% CO_2 in conventional culture flasks. MTT assay was applied to investigate the effect of calcitonin individually on SH-SY5Y cells by treatment different concentrations for 24 h and performed.

Results: In cells cultured with salmon calcitonin applied at different concentrations (0.1, 1, 3.125, 6,25, 12.5, 25, 50 and 100 nM/ml), anti-proliferation was statistically significant at concentrations of 50 and 100 nM/ml compared to the control group. It showed that 50 nM/ml and 100 nM/ml had the highest cytotoxic effect on SH-SY5Y for 24 h

Conclusions: Considering the proliferation curve of SH-SY5Y, the results show that salmon calcitonin treatment potentiated the proliferative activities by inhibiting cell viability in SH-SY5Y cells at concentrations of 50 and 100 nM/ml. Further studies exploring salmon calcitonin's protective effects may prove successful and maybe it is a promising agent for cancer treatment.

Key words: Salmon calcitonin, SH-SY5Y, anti-proliferative effect, neuroblastoma.

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Introduction

It is known that cancer is the most important cause of death worldwide. There are extensive studies on the development of less toxic anti-cancer drugs that continue intensively to find potential targets and promising anti-cancer approaches [1, 2]. Neuroblastoma is one of the extra cranial solid tumors seen in one in 7000 live births among childhood cancers [3]. It is a biologically diverse tumor with varying clinical course and prognosis depending on age at diagnosis, histology, and molecular pathway characteristics [4]. In its treatment, alkylating agents with serious side effects such as cisplatin are used [5]. In addition, despite aggressive treatments, treatment success is very low due to high drug resistance [6].

Since SH-SY5Y can be transformed into various types of functional neurons by the addition of specific compounds, it is preferred as a suitable model for studies on

neurodegenerative disorders and modeling neurodegenerative diseases.

Salmon calcitonin (sCT) is a single-chain peptide hormone containing 32 amino acids with a molecular weight of approximately 3500 Da. There are four types of sCT used in the clinic: human, pig, eel, and sCT derived [7]. The main role of calcitonin is to regulate mineral metabolism and help to eliminate the increase in calcium level called 'calcium stress' [8]. Evidence has been suggested that sCT is closely related to the glutamatergic system such as NMDA and AMPA receptors and causes glutamate release [9, 10, 11].

In the literature, antiproliferative effects on the different one of sCT were not found. However, there are studies on calcitonin (CT). One of these demonstrated expression (calcitonin-induced) of transforming growth factor (TGF-21) as an antiproliferative on Lactotrophs in rats [12]. In another study, it was stated that CT was associated with the breasts [13]. They determined that calcitonin inhibited it at a rate of 1/4 in primary tumors (expression of uPA mRNA).

In a different study, it was shown that CT and its receptor (CTR) significantly reduced tumor growth with its autocrine supplement, and they also stated that it is a potential option in invasive cancers. In the details of the study, they emphasized that the urokinase type plasminogen activator and survivin were at a low rate, thus activating the uPA-uPAR axis and the PI-3-kinase-Akt-survivin pathway [14]. However, the effects of sCT on the CNS still remain unclear. There are many studies on the neuroblastoma cell line in the literature [15, 16]. No specific data were found on the effect of sCT on SH-SY5Y. In this study, we aimed to investigate the anti-proliferative effect of sCT in SH-SY5Y neuroblastoma cells, which are frequently preferred in neurotoxicity and neurodegenerative processes.

Materials and metods

We confirm that no permission from an ethical committee is necessary for the cell cultures utilized in the study. The study was carried out *in vitro* and was carried out using Bolu Abant İzzet Baysal University, Faculty of Medicine, and Physiology Department research laboratory and cell culture laboratory facilities.

Drugs and reagents

sCT used in the study was obtained from CT (natural, 98%, Santa Cruz Biotechnology, Inc. CAS 47931-85-1 Dallas, TX, USA). Fetal bovine for cell culture experiment (Sigma-Aldrich, Schnelldorf, Germany), penicillin-streptomycin as antibiotic (Capricorn Scientific Ebsdorfergrund, Germany), trypsin, EDTA solution (Hyclone, Logan, UT, USA), dimethyl sulfoxide (DMSO), phosphate buffered water (PBS) and Dulbecco's Modified Eagle Medium/F-12 (DMEM/F-12) medium (Pan-Biotech, Aidenbach, Germany). In the *in vitro* assay step, sCT was diluted to different concentrations by dissolving in high-grade water to obtain final concentrations.

Cell culture

The SH-SY5Y human neuroblastoma cell line purchased from ATCC (ATCC CRL-2266, Manassas, VA, USA) was used in this study. The cell culture protocol was performed as described in previously published studies [17]. Briefly, cells were rapidly thawed in a 37°C water bath and then centrifuged at 3000 rpm for 4 minutes. After centrifugation, all cells were cultured in 75 cm flasks containing supplemented DMEM/F12 mixture/full medium supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin at 37°C in a humidified incubator with 5% CO_2. The next day, the growth medium

was changed to remove any DMSO that may be present in the freezing medium. Cells were maintained at log phase and medium was replaced with fresh medium every 3-4 days. Cell morphologies and growth rates of the cell line were monitored daily under an inverted microscope (Olympus CKX41, Tokyo, Japan) and passaged by separation with 0.25% Trypsin-EDTA when cells reached 70-80% confluence.

Drug administration - Salmon calcitonin treatment:

After reaching the appropriate confluence, the cells were passaged and 100 µl equal volumes of sCT concentrations prepared to be 0.1, 1, 3.125, 6,25, 12.5, 25, 50 and 100 nM/ml were given to the cells and used defined times incubated. For each dose, 2 wells were inoculated and cells were incubated in a humid environment at 37ºC in a 5% CO_2 air mixture.

Cell viability assay

Trypan blue was used to determine cell viability and cell numbers. BioRad TC20 Automated Cell Counter (California, USA) was used to measure cell viability and cell numbers. Cytotoxicity experiments were performed in duplicate and GraphPad Prism 5.0 software (GraphPad Software, San Diego, USA) was used for data analysis. All cells were counted and approximately $1.5x10^4$ cells were seeded into wells in a total volume of 200 µL in 96-well plates. Plates were then incubated at 37 °C for 24 hours for cell attachment.

First, the dose was determined, for this the cells in the wells were harvested with 0.5% trypsin and centrifuged. After discarding the supernatant portions, they were suspended with 1 ml of medium and counted with an Automated Cell Counter. The ID50 (Inhibition dose 50%) dose for sCT was determined by recording the total cell counts. Eight different doses were administered to see the effects of sCT on SH-SY5Y.

When the cell line was ready for 70-80% confluence in flasks, they were seeded into 96-well plates and after 24 h of holding, different dilutions of sCT (0.1, 1, 3.125, 6,25, 12.5, 25, 50 and 100 nM/ml) were added) was incubated for 48 hours. Cells incubated in 10% FBS were used as positive control and the viability of the cells was evaluated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl- tetrazolium bromide) (Serva, Germany) method. Briefly, 10 µl of MTT reagent was added to each well and the 96-well plate was incubated at 37°C for 4 hours, then DMSO was added to the cells. Absorbance values were read with a colorimetric reader (spectrophotometer) (Epoch BioTek Instru-ments, Inc., Highland Park, USA) at 570 nm. The most appropriate proliferative and inhibitory doses of sCT for cells were determined. Thus, the effect of sCT on the viability of cultured cells and the duration of the effective dose were determined. Negative control (Medium control) = salmon calcitonin concentration + DMEM/F12 medium and Positive control (DMSO control) = %10 DMSO was applied. Data from each sCT dose were compared with the mean of the negative control. Control group means and data taken at each dose are given as the mean ± SD of each in two parallels. We used our control group for the test of MTT, in which we evaluated the viability of cells. Untreated cells exposed to maximum solvent concentrations with DMSO were used to determine the metabolic activities of this control group.

Statistical analysis

Statistical analyzes were done with SPSS 26.0 package statistics program (New York, USA). Multi-dimensional statistical evaluations were made on the obtained data. Since the measurement values did not show homogeneous distribution, non-parametric tests were used. The Kruskal-Wallis analysis of variance test was used to evaluate the significance of the difference

between the groups. The difference between two independent groups consisting of continuous variable values was determined with the Mann-Whitney U Test. A probability value of < 0.05 was accepted significant.

Results

In this study, we investigated the anti-proliferative effect of human neuroblastoma cell line SH-SY5Y using sCT as the active ingredient. Neuroblastoma is an extra cranial solid tumor. SH-SY5Y is also widely used in experimental neurological studies, neurodegenerative processes, neurotoxicity and neuroprotection [18]. Eight different doses (0.1, 1, 3.125, 6.25, 12.5, 25, 50 and 100 nM/ml) of sCT were used in the study to demonstrate anti-proliferative effects on SH-SY5Y cell. In culture medium (5% CO_2, 37 °C), SH-SY5Y cells showed normal polygonal morphology (Figure 1).

The anti-proliferative effect of especially 50 and 100 (nM/ml) sCT from eight different doses is clearly noticeable (p<0.05). When these two doses are compared with the control group, 100 nM/ml seems to be 1.16 times more effective than 50 nM/ml. Negative control value 0.27; 0.289; 0.208 (mean=0.25) and Positive control value 0.124; 0.148; 0.168 (mean=0.14). Applications of sCT below these two doses did not show an anti-proliferative or proliferative effect (Figure 2).

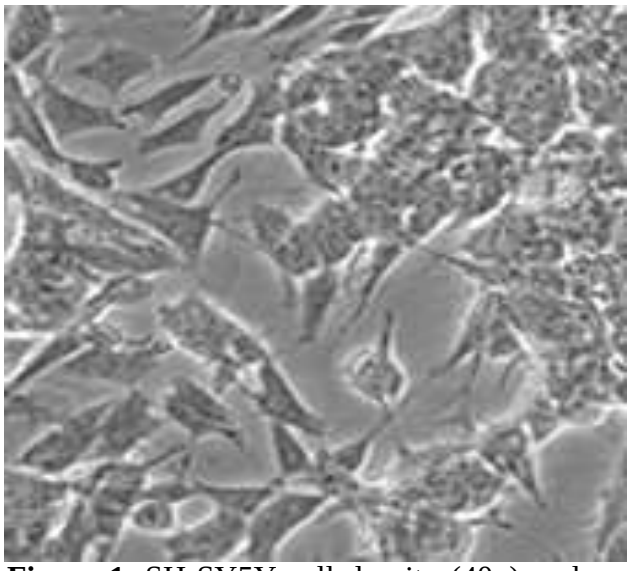

Figure 1. SH-SY5Y cell density (40x) under a light-inverted microscope.

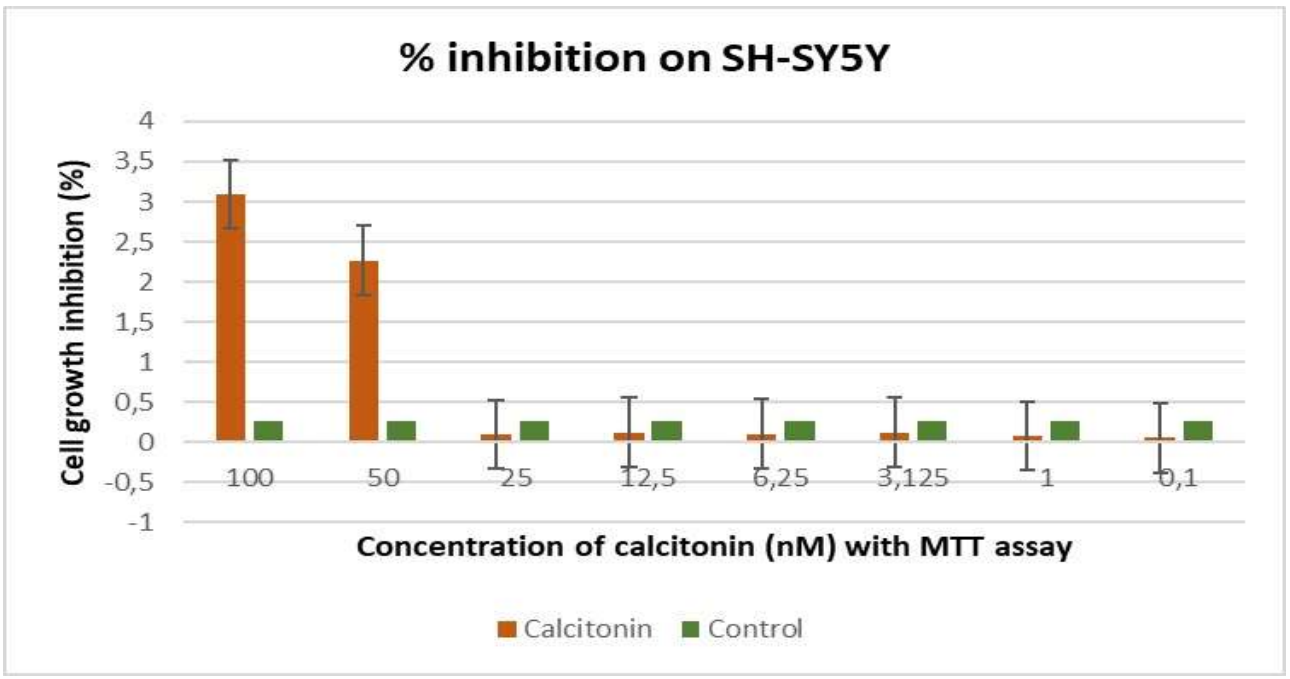

Figure 2. The anti-growth effect using MTT assay after treatment with the salmon calcitonin for 24 h.

sCT administrations below these two doses did not show an anti-proliferative or proliferative effect (Figure 2). According to the results, it is clearly noticed that sCT has an anti-proliferative effect on SH-SY5Y cells at doses of 50 and 100 nM/ml. At other doses and control, the effect is below zero. As shown in Figure 2, treatment of the SH-SY5Y cell line with the sCT displays concentration dependence decline in cell viability. Our results showed that the particularly suppressive effect begins in the first 24 h (p<0.01 vs. the control group). The high dose of sCT has a significantly suppressive effect (p=0.003 versus control group). The lower dose of sCT, there appears some effect on the SH-SY5Y proliferation but it is not that significantly effect (p=0.874). These findings showed that a dose-dependent inhibition of SH-SY5Y proliferation by sCT.

Discussion

Despite the advances in cancer research, one of the problems encountered is that chemotherapy methods can cause serious side effects by damaging cancer cells as well as healthy ones [19]. For this reason, anti-cancer drug researches with low toxicity continue to maintain their importance. Neuroblastoma is becoming more widely known, but there are still no reliable therapies for it. As a result, researchers are looking for new, efficient medications to treat neuroblastoma [20]. The drug sCT is a therapeutic option for many years, which is used in the treatment of postmenopausal (5 years) osteoporosis in women, bone diseases and Paget's disease. Although it has been found to have some antiproliferative, apoptotic effects on some cancers are known, it has not been clarified yet sCT has a friendly or foe effect on cancer [8].

In the current study we investigated the anti-proliferative effects of sCT in SH-SY5Y cell lines. Although the benefits of sCT on some cancer treatments are known [8, 13, 14], there are limited data on neuroblastoma cell line (SH-SY5Y) at different doses is available in the literature. According to the findings of Sabbisetti et al., who report that the effects of sCT are accelerated at concentrations higher than 50 nM, the results presented here showed that sCT could inhibit the growth of SH-SY5Y at higher doses (50-100 nM/ml) for 24 h [21]. Furthermore, according to our research, SH-SY5Y cells treated with sCT at different concentrations for 24 h had a cell number inhibition. It is believed that the reason of the high dose depence is due to the sCT's some properties such as genetic, physiological, and pharmacokinetic variation among different cancer and/or different cell lines.

These findings suggested that sCT might be a medication with some promise for the treatment of neuroblastoma *in vitro*. Additionally, contrasting studies with some calcitonin subtypes have suggested that advantageous effects on nervous system. Researchers showed that sCT is closely related to the glutamatergic system and glutamate releasing [10, 11]. Also one year later, Taşkıran et al. investigated the effect of sCT on glutamate-induced cytotoxicity in C6 glial cells involved in the inflammatory and nitric oxide pathways, due to the main mechanisms and impact on glutamate-induced cytotoxicity are still unknown. As a results of their research, sCT inhibits nitric oxide and inflammatory pathways to protect against glutamate-induced cytotoxicity in C6 glial cells. For those with neurodegenerative symptoms, sCT may be a helpful supportive medication [22]. Therefore, our findings confirm the evidence of inhibition effect of sCT on SH-SY5Y cells. Studies conducted both *in vivo* and *in vitro* have shown that sCT has beneficial effects on the different system especially nervous system and some cancer types such as breast, prostate [23].

One study highlights that CT is a stimulant for angiogenesis [24].

Although it has been mentioned that he possibility of sCT causing cancer has been studied and CT can provide invasion of some tumor by metastases *in vivo*. However, these results contradict other published studies on CT biology [25, 26, 27], so we advise conducting more research on this topic to verify us believe further. Wells et al., Ng et al. and Thomas et al. report significant effect of sCT on different cancer types in their studies [27, 28, 29]. Likewise, it is clear from the information we provided in the current study, sCT shows antiproliferative effects on SH-SY5Y cell line *in vitro* and this research paralles the previous studies. Therefore, sCT may be a tropic agent in cancers especially neuroblastoma. The treatment of the SH-SY5Y cell line with sCT reduced cell viability depending on the dosage for 24 h. The results shown here make it abundantly evident that the presence of sCT had a favorable impact on SH-SY5Y proliferation.

Conclusion

To our knowledge, this is the first study which sCT has been evaluated for its antiproliferative effect on SH-SY5Y for different doses *in vitro*. Thus, the findings provided here sCT is a possible therapeutic candidate for *in vitro* treatment of human neuroblastoma cells. Finally, we advise further future research is needed to understand the mechanism of action of sCT in comparison to SH-SY5Y and other neuroblastoma cell lines.

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Ethical statement*: Ethics committee decision was not taken as it was a cell culture study.*

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