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EXPERIMENTAL STUDY

Investigation of the mechanisms involved in anticancer effect of glucosamine sulfate on SH-SY5Y cell line

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ABSTRACT

AIM: Glucosamine derivatives have been found to have anticancer effects in many cancer cell lines in previous investigations. The effect of glucosamine sulfate on neuroblastoma, however, is uncertain. The potential cytotoxic effects of glucosamine sulfate on the SH-SY5Y cell line were investigated in this study. The underlying mechanisms of this cytotoxicity have also been studied.

MATERIAL AND METHODS: In this study, the SH-SY5Y cell lines were used. The cells were treated with various concentrations of glucosamine sulfate (0.3125, 0.625, 1.25 and 2.5 μg/mL) and the viability of the cells was determined using the XTT assay after 24 hours. The quantities of cleaved PARP, BCL-2, 8-Hydroxy-desoxyguanosine (8-oxo-dG), cleaved caspase 3, Bax, total oxidant, and total antioxidant in the cells were determined by ELISA kits.

RESULTS: At doses of 0.3125, 0.625, 1.25 and 2.5 μg/mL, glucosamine sulfate dramatically reduced cell viability in SH-SY5Y cells ($p < 0.001$). ELISA tests demonstrated that 1.25 μg/mL glucosamine sulfate considerably increased the amounts of 8-oxo-dG, cleaved caspase 3, Bax, cleaved PARP and total oxidant. However, 1.25 μg/mL glucosamine sulfate treatment did not change the quantity of BCL-2 protein.

CONCLUSIONS: Altogether, glucosamine sulfate produced considerable cytotoxicity in SH-SY5Y cells by triggering oxidative stress, inducing DNA damage, and finally causing apoptosis. In addition, more research is needed to determine the efficacy of glucosamine sulfate as an anticancer drug in the treatment of neuroblastoma *(Fig. 5, Ref. 39)*. Text in PDF *www.elis.sk*

KEY WORDS: glucosamine sulfate, cell viability, SH-SY5Y cell, oxidative stress, dna damage, apoptosis, ELISA.

Introduction

The most common extracranial solid tumor in the young children is neuroblastoma, which accounts for around 15 % of all cancer mortality in children. Neuroblastoma is an autonomic nervous system tumor in children that forms from precursor cells generated from neural crest tissues (1). Despite comprehensive neuroblastoma treatment, high-grade neuroblastoma tumours have a bad prognosis, with overall survival rates ranging from 20 % to 40 % (2). Because neuroblastoma is a biologically and clinically heterogeneous disease, targeted therapies to this tumor are dependent on the biological prognostic markers and stage of the disease (3). In light of these situations, new treatment techniques for neuroblastoma and more investigation for effective and appropriate class compounds are needed.

SH-SY5Y cells are human neuroblastoma cells isolated from a bone marrow biopsy obtained from a 4-year-old girl suffering from neuroblastoma. SH-SY5Y cells are frequently used in scientific studies as a model for neuronal activity and differentiation *in vitro* (4).

Glucosamine is an amino sugar that is increasingly used as a nutritional supplement by osteoarthritis patients. Because of its ability to prevent tumor growth both *in vitro* and *in vivo*, glucosamine is an attractive natural chemical (5, 6). With the exception of mild intestinal problems, glucosamine has been shown to have no significant side effects or toxicity in humans (6, 7). This amino sugar is also predicted to play a significant role in cancer medication. Quastel and Cantero reported the first evidence of glucosamine's anti-cancer action in 1953. In mice with Sarcoma 37 tumors, daily injections of glucosamine resulted in a reduction of cell mass and hemorrhagic regions (8). For the anti-cancer properties of this amino sugar, different mechanisms have been suggested (6). However, the precise biochemical pathways by which glucosamine fights cancer are still unknown.

Anticancer properties of glucosamine have recently gained increasing interest. It is a powerful lytic agent for a variety of cancers, with minimal toxicity to healthy cells (9). Glucosamine has been shown to inhibit the growth of human prostate tumor DU145 cell lines via the STAT3 signaling pathway (10). Through the suppression of N-linked glycosylation, glucosamine can also cause anti-cancer effects (11). Glucosamine exerts an antitumor effect in retinal pigment epithelial cells by suppressing epidermal

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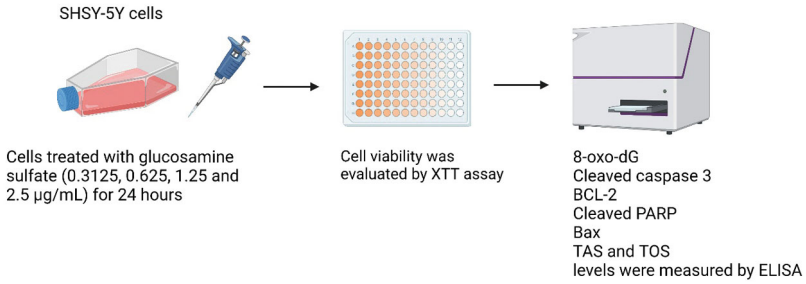

Fig. 1. Experimental design of the study (Created with BioRender.com).

growth factor-induced proliferation (12) and causing cell cycle arrest (13). D-glucosamine ($GlcNH_2$) at specific concentrations has been shown to eradicate tumor cells while having no effect on normal cells (9). It's thought that combining $GlcNH_2$ with membrane-active medicines could enhance its anticancer activity especially in the case of neuro-oncology (14). It was demonstrated that glucosamine sulphate can suppress leukemia HL60 cell proliferation and stimulate differentiation of HL60 cells into granulocytic or monocytic lineages (14). However, the effects of glucosamine sulphate on neuroblastoma cells, let alone the mechanisms, remain unknown. The goal of this research was to examine the antiproliferative effect of glucosamine sulphate on the SH-SY5Y cell line, as well as the mechanisms involved in this activity.

Materials and methods

Cell culture and cell lines

SH-SY5Y (neuroblastoma) and L929 (mouse fibroblast) cell lines were obtained from the American Type Culture Collection. (ATCC, USA). These two cell lines were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) and placed in humidified atmosphere of 5 % CO_2 incubator at 37 °C. 10 % fetal bovine serum (FBS) (Sigma-Aldrich) and 1 % antibiotic mixtures of penicillin and streptomycin were added to this medium. Before the procedure, glucosamine sulfate (Sigma-Aldrich) was dissolved in DMSO (Dimethyl sulfoxide) and diluted in the culture medium to a final DMSO concentration of less than 0.1 percent.

Cell viability test

Using XTT assay (Roche Diagnostic, MA, USA), the effect of glucosamine sulfate on the viability of SH-SY5Y and L929 cell lines was examined (Fig. 1). Before the addition of glucosamine sulfate, the two cell lines were cultured at a concentration of 1×10^4 cells per well and incubated overnight. After that the different concentrations (0.3125, 0.625, 1.25 and 2.5 µg/mL) of glucosamine sulfate were added to cells for 24 h. Cells which were not treated were used as a control. After incubation, 50 µL of XTT mixture was supplemented to each well. Following 4-hour incubation, the cells were shaken and the absorbance was measured using a microplate reader (Thermo Fisher Scientific, Altrincham, United Kingdom) at 450 nm. After three repetitions of each experiment, cell viability was calculated as a percentage of living cells against untreated cells (15).

The measurement of 8-oxo-dG, cleaved caspase 3, BCL-2, cleaved PARP and Bax quantities

The human ELISA kits of 8-Hydroxy-Desoxyguanosine (8-oxo-dG) (BT Lab, catalog #E1436HU), cleaved caspase 3 (BT Lab, catalog # E6970HU), BCL-2 (BT Lab, catalog #E1832HU), cleaved PARP (BT Lab, catalog #E6971HU) and Bax (BT Lab, catalog #E1825HU) were used to examine the quantities of 8-oxo-dG, cleaved caspase 3, BCL-2, cleaved PARP and Bax in glucosamine sulfate-treated and untreated SH-SY5Y cells. In short, SH-SY5Y cells were cultivated into a 6-well plate and treated with 1.25 µg/mL glucosamine sulfate for 24 hours. SH-SY5Y cells that had been treated with glucosamine sulfate and those that had not were gathered and diluted in PBS. Then they were frozen and thawed three times. Following that, the quanti-

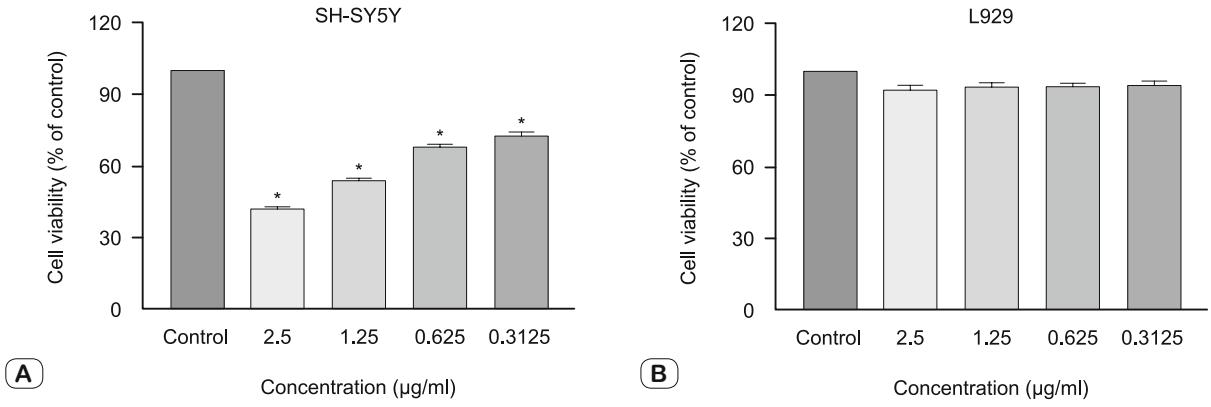

Fig. 2. The anti-proliferative effects of glucosamine sulfate on SH-SY5Y cells (A) and L929 cells (B). The findings are calculated as a percentage of viable cells versus control. The results are presented as the mean ± SEM. *p < 0.01 as compared to the control group.

ties of 8-oxo-dG, cleaved caspase 3, BCL-2, cleaved PARP and Bax in cell lysates were examined following the manufacturer's directions. Bradford protein assay kit (Merck Millipore, Darmstadt, Germany) was used to calculate the total protein quantities in glucosamine sulfate-treated and untreated SH-SY5Y cells (16).

Total antioxidant status (TAS) and Total oxidant status (TOS) assessment in glucosamine sulfate-treated and untreated SH-SY5Y cells

The total antioxidant status assay kit (Rel Assay Diagnostics, Turkey) and total oxidant status assay kit (Rel Assay Diagnostics, Turkey) were used to examine TAS and TOS quantities in glucosamine sulfate-treated and untreated SH-SY5Y cells, respectively. SH-SY5Y cells were treated with 1.25 µg/mL glucosamine sulfate for 24 hours and the manufacturer's directions were implemented. For TAS and TOS, the data were expressed as mmol Trolox Equiv./L and mol H_2O_2 Equiv./L, respectively (17).

Statistical analysis

The laboratory findings were stated as mean±standard error. The results obtained from the cell viability tests were tested using the one-way ANOVA test with post hoc test. The results obtained from 8-oxo-dG, cleaved caspase 3, BCL-2, cleaved PARP and Bax, TAS and TOS levels tests were tested using Independent Samples t Test. The level of significance was set at $p<0.05$. GraphPad Prism 8.0 software (USA) was used for data analysis and graphical presentations.

Results

Glucosamine sulfate treatment inhibited the proliferation of SH-SY5Y cells

At first the anti-proliferative effect of glucosamine sulfate was explored in SH-SY5Y cells. Glucosamine sulfate significantly suppressed the proliferation of SH-SY5Y cells at 0.3125 µg/mL and higher doses as compared with control ($p<0.01$). The IC_{50} value of glucosamine sulfate in SH-SY5Y cells was found to be 0.37 µg/mL for 24 hours (Fig. 2a). Moreover, the cytotoxic activity of glucosamine sulfate was examined in L929 cells, which are non-cancerous, and the results showed that glucosamine sulfate had no effect on L929 cells (Fig. 2b).

The effect of glucosamine sulfate on Bax, cleaved caspase 3, BCL-2 and cleaved PARP quantities in SH-SY5Y cells

ELISA was used to evaluate the expression of proteins associated with apoptosis in SH-SY5Y cells, such as Bax, cleaved caspase 3, cleaved PARP, and BCL-2. The treatment with glucosamine sulfate (1.25 µg/mL) for 24 hours significantly elevated Bax ($p<0.05$), cleaved PARP ($p<0.05$), and cleaved caspase 3 ($p<0.05$) quantities. In contrast, glucosamine sulfate had no effect on BCL-2 quantity ($p>0.05$) (Fig. 3).

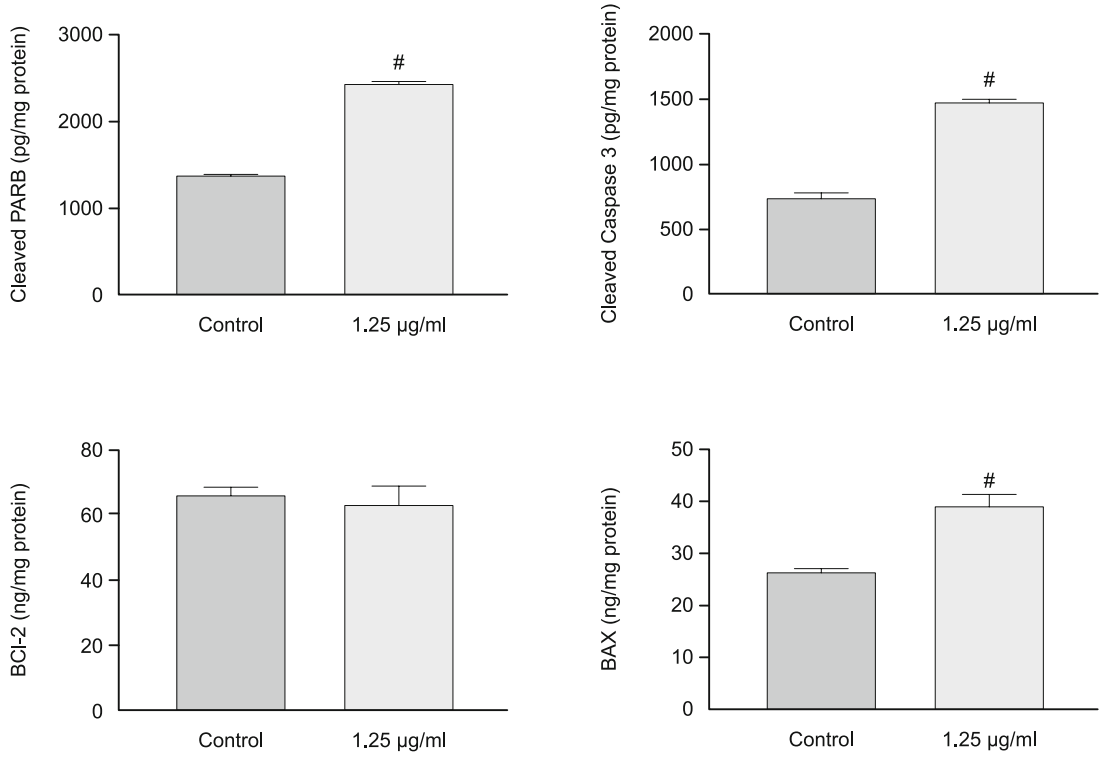

Fig. 3. Glucosamine sulfate at dose of 1.25 µg/mL enhanced apoptosis of SH-SY5Y cells. The cleaved caspase 3, BCL-2, cleaved PARP and Bax quantities were calculated using the ELISA kits. Results are represented as mean±SEM. $^{\#}p<0.05$ as compared to the control groups.

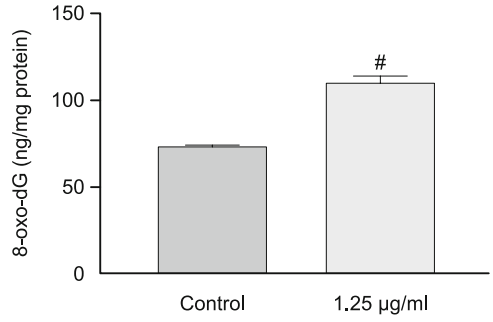

Fig. 4. Glucosamine sulfate at dose of 1.25 µg/mL enhanced DNA damage of SH-SY5Y cells. The 8-oxo-dG level was calculated using the ELISA kit. Results are represented as mean±SEM. #p<0.05 as compared to the control groups.

The effect of glucosamine sulfate on 8-oxo-dG quantity in SH-SY5Y cells

ELISA was also used to assess 8-oxo-dG expressions in SH-SY5Y cells in order to determine the DNA-damaging effects of glucosamine sulfate. Treatment with glucosamine sulfate (1.25 µg/mL) for 24 hours significantly increased the quantity of 8-oxo-dG ($p<0.05$) (Fig. 4).

The effect of glucosamine sulfate on TAS and TOS quantities in SH-SY5Y cells

The effect of glucosamine sulfate on TAS and TOS quantities in SH-SY5Y cells was determined using a TAS and TOS assay kit. Glucosamine sulfate had no effect on TAS quantity ($p>0.05$). On the other hand, the drug caused increase in TOS quantity in SH-SY5Y cells ($p<0.05$). TAS was determined as 0.469±0.018 in the control group and 0.475±0.002 in 1.25 µg/mL glucosamine sulfate-treated cells. Additionally, TOS was determined as 26.890 ±0.194 in the control group and 54.160±3.035 in 1.25 µg/mL glucosamine sulfate-treated cells (Fig. 5).

Discussion

Despite the use of advanced chemotherapy and radiotherapy in cancer treatment, drug resistance, recurrence, and metastasis have impeded neuroblastoma treatment in many developing and developed countries throughout the world (4). Glucosamine sulfate is a synthetic compound produced from glucosamine ($C_6H_{13}NO_5$), an amino derivative of glucose that is found in numerous polysaccharides and is the fundamental structural unit of chitin. In osteoarthritic patients, glucosamine sulfate is used to induce cartilage formation (18, 19).

Studies showed that chitin and chitosan oligomers caused growth inhibition of tumor cells (20). Since chitosan is just a polymer of N-Acetyl-D glucosamine, it was theorized that glucosamine sulfate, a glucosamine derivate, could also limit cancer cell proliferation. Up to now, the anticancer effect of glucosamine sulfate on neuroblastoma is still unknown. As a result, we evaluated the suppressive effects of glucosamine sulfate on SH-SY5Y cell line as well as possible biochemical mechanisms.

First, the XTT assay was applied to examine whether glucosamine sulfate has a dose-dependent cytotoxic effect on the SH-SY5Y cell line. Our findings revealed that glucosamine sulfate caused a significant inhibition of SH-SY5Y cell growth in a concentration-dependent manner and the IC_{50} value was 0.37 µg/mL The toxicity of antitumoral drugs on normal cells remains a serious problem, limiting therapeutic possibilities. The cytotoxicity of glucosamine sulfate on non-cancerous L929 cell line was examined and the findings confirmed that glucosamine sulfate had no substantial cytotoxicity on L929 cells. Apoptosis is widely known for playing a role in the cellular pathogenesis of cancers and for influencing the outcome of pharmacological therapies (21). To confirm the apoptotic effect of glucosamine sulfate on SH-SY5Y cells, the quantities of cleaved PARP, cleaved caspase 3, Bax, and BCL-2 were determined by ELISA. The induction of cell apoptosis, which is a primary goal of cancer treatment, is one of the defense mechanisms against tumor development and progression (22).

The mitochondrial route of apoptosis involves permeabilization of the outer mitochondrial membrane (MOMP), which results in the release of cytochrome c and other proteins from the mitochondrial intermembrane gap. The Bcl-2 family regulates the mitochondrial outer membrane, which is important for apoptosis. Pro-apoptotic Bcl-2 family members, such as Bax and Bak, induce MOMP and promote the release of pro-apoptotic factors from mitochondria, whereas apoptogenic Bcl-2 family members, such

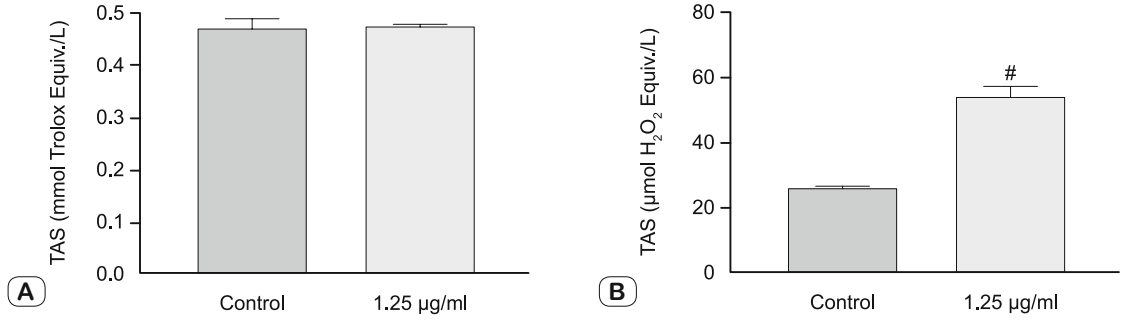

Fig. 5. Glucosamine sulfate at dose of 1.25 µg/mL didn't cause a significant change in the TAS level (A) but increased the TOS level (B) of SH-SY5Y cells significantly. Results are represented as mean ± SEM. #p <0.05 as compared to the control group.

as Bcl-2 and Bcl-xL, block the release of apoptotic proteins from mitochondria and thereby prevent the MOMP (23–25).

Caspases are expressed in a precursor form (inactive) with a certain molecular weight in resting cells. Caspases, on the other hand, are partly proteolytically cleaved and activated in cells when they are exposed to apoptotic stimuli (26). Once activated, they split large number of cellular proteins, such as PARP and other essential proteins (27). PARP is also an important component of DNA repair pathways, mainly in the reparation of base excisions, and its cleavage or repression stimulates cell death by exploiting a DNA repair deficiency (28). In this study, 1.25 µg/mL glucosamine sulfate treatment markedly augmented pro-apoptotic Bax, cleaved caspase 3, and cleaved PARP expression while not alerting anti-apoptotic BCL-2 expression. The 8-oxo-dG ELISA technique was utilized to investigate the DNA fragmentation in SH-SY5Y cells after 24 hours of glucosamine sulfate administration to see if the cytotoxic effect of glucosamine sulfate is associated with DNA damage. 8-oxo-dG is a well-known biomarker for DNA oxidative damage (29). Treatment with glucosamine sulfate at 1.25 µg/mL considerably increased the amount of 8-oxo-dG in SH-SY5Y cells, suggesting that glucosamine sulfate has cytotoxic and apoptotic properties.

Most anticancer medications act by creating oxidative stress in cancerous cells, which is assumed to be the cause of the majority of macromolecular changes in the cell. Reactive oxygen species (ROS) can attack proteins, membrane lipids, and DNA, among other macromolecules (30). Because the TOS is one of numerous measures used to estimate oxidative stress, it is frequently employed to assess the total oxidative status of cells. The TAS is also used to evaluate the overall antioxidant status of cells (31). In this context, we studied whether glucosamine sulfate could cause cytotoxicity by elevating TOS levels. In comparison to untreated cells, exposure to glucosamine sulfate for 24 hours augmented TOS levels; yet, there was no considerable alteration in TAS levels. An increase in TOS and no alteration in TAS levels suggested that treatment with glucosamine sulfate obviously stimulated oxidative stress in the glucosamine sulfate-treated SH-SY5Y cells. In addition, the damage of mitochondrial membrane and excessive increase of ROS can trigger the intrinsic apoptosis route (32). In consideration of our mitochondrial membrane potential data, it can be said that glucosamine sulfate triggered apoptosis via the mitochondrial route.

In agreement with our results, Zhe Wang and colleagues reported that glucosamine sulfate (GS) suppressed proliferation and caused apoptosis in the K562 cells (human chronic myelogenous leukemia). GS-induced apoptosis was accompanied by caspase-3 activation, poly (ADP-ribose)-polymerase cleavage, and cyt c release. The levels of Bcl-2 protein expression, on the other hand, remained unchanged (33). It was shown that D-glucosamine caused cytotoxic effect in rat C6 glioma cells (9, 34). It was shown by Jung and colleagues' study that glucosamine-hydrochloride caused substantial anti-proliferative, growth suppressive, and apoptosis-inducing effects in the YD-8 cells, and these effects seem to be achieved through activation of mitochondrial-dependent caspase and induction of endoplasmic reticulum stress (35). According to Zhanwu Yu's study, glucosamine was found to inhibit lung cancer cell growth, probably by altering FOXO1 and p-FOXO3 transcriptional activity (36). Hiraku et al. discovered that aminosugars generated H202, which caused DNA damage and mediated apoptosis, inhibiting tumor growth (37). Sumoto et al. indicated that ROS are implicated in DNA damage caused by aminosugars (38). Karagozlu et al. proposed that COS, a soluble chitooligomers prepared by chemical depolymerization or enzymatic hydrolysis of chitosan, triggered apoptosis in AGS tumor cells through mitochondrial pathways by up-regulation of Bax expression and caspases activation (39).

Conclusions

Glucosamine sulfate significantly suppressed SH-SY5Y cell reproduction in a concentration-dependent way without causing significant cytotoxic effects on L929 cells. Glucosamine sulfate significantly elevated pro-apoptotic Bax, cleaved caspase 3, and cleaved PARP protein expressions. Glucosamine sulfate also significantly elevated 8-oxo-dG quantities in SH-SY5Y cells and thus the cytotoxic effect of glucosamine sulfate may be linked to DNA damage. Treatment with glucosamine sulfate caused a rise in TOS supporting its cytotoxic effects. Overall, glucosamine sulfate has the potential to be a new and effective anticancer option due to its promising results in cancer cell suppression and lack of toxicity in normal cells. However, the lack of meaningful clinical research is the most significant impediment to its commercial development.

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