

Genistein: A Preliminary *In Vitro* Evaluation on IMR-32 Neuroblastoma Cells

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ABSTRACT: Neuroblastoma (NB) is a frequent pathology among children with a serious prognosis. Although there is currently a chemotherapeutic treatment, over the years resistance to existing therapy has developed, necessitating new therapeutic approaches. The current study aimed to evaluate *in vitro* genistein (GEN) on human neuroblastoma cells-IMR-32, a possible candidate for treating NB. The results indicated that GEN does not affect healthy cells (HaCaT), but has a cytotoxic effect on tumor cells, at concentrations of 50 and 75µM, significantly reducing viability. Moreover, depending on the dose, GEN degraded the cell membrane by releasing LDH and caused changes in the cell shape as well as at the nuclear level similar to apoptosis. The data provide an important perspective on the therapeutic effect of GEN at the NB level, opening the way to new directions in treating this pathology with natural compounds.

KEYWORDS: Neuroblastoma, genistein, cytotoxicity, IMR-32 cells.

Introduction

Neuroblastoma (NB) is a tumor that derives from the neural crest of the peripheral nervous system and is the main cause of death among children.

NB is different from other solid tumors, presents a high risk of metastases, does not respond to standard classical anti-cancer treatment and most of the time even possesses drug resistance [1,2].

Thus, NB remains a clinical challenge with poor survival despite multimodal treatment.

Therefore, there is a growing need for new therapeutic options and new agents with fewer disadvantages [3,4].

Many of these agents belong to the class of natural compounds, which were shown to possess promising antitumor effects in previous studies [5-7].

Genistein (GEN) is an isoflavone mostly found in soy and contained in soy-based food preparations that are consumed especially by Asian countries [8].

Following epidemiological studies, a lower incidence of certain types of cancer, such as prostate and breast cancer, has been observed in the Asian population, compared to the Western

population, due to the high intake of isoflavones (25-50mg) [9,10].

Genistein has attracted the attention of scientific researchers due to its therapeutic properties on human pathologies, such as cancer.

The basis of genistein's antitumor action mechanism is its potential to induce apoptosis, to stop the cell cycle, as well as its antimetastatic, anti-inflammatory, and antioxidant actions [11].

Over the past decades, several studies have been published on the potential anticancerous of genistein both *in vitro*, in cell cultures, and *in vivo*, in animal models [12,13].

NB is a rare tumor with a high degree of heterogeneity and the availability of preclinical models derived from patients represents a promising method in evaluating potential new forms of treatment.

In vitro models are important in cancer research because they are tools for evaluating the effects of compounds on tumor development and growth, in investigating the mechanisms of pathology and the biochemical pathways underlying cancer [14].

In vitro studies include detailed experiments, carried out safely using various components of an organism, most often cells [15].

Thus, the aim of the current study was the *in vitro* evaluation of GEN on human keratinocytes-HaCaT and especially on human neuroblastoma cells-IMR-32.

In the first phase, the safety profile on the healthy line was investigated, and then the cytotoxic effect on tumor cells, by determining the action on cell viability, on the integrity of the cell membrane as well as on the morphology of the cell, but also in detail on the morphology of the nucleus.

Materials and Methods

Reagents and instruments

The active substance evaluated genistein (GEN), together with PBS (phosphate buffer saline), trypsin-EDTA (ethylenediaminetetraacetic acid) solution, DMSO (dimethyl sulfoxide), the antibiotic mixture (Penicillin-Streptomycin), were procured from Sigma Aldrich, Merck KGaA (Darmstadt, Germany).

The medium utilized for the growth of the cell culture, DMEM (Dulbecco's Modified Eagle's medium), EMEM (Dulbecco's Minimal Essential Medium), and FBS (fetal bovine serum) were acquired from ATCC (American Type Cell Collection, Manassas, VA, USA).

The lactate dehydrogenase (LDH) cytotoxicity kit and Hoechst 33342 were obtained from ThermoFisher Scientific (Waltham, MA, USA), and the MTT kit was bought from Roche Holding AG (Basel, Switzerland).

All reagents in the present study comply with the standards for use in cell culture experiments and were used according to the manufacturers' recommendations.

The instruments used were the Olympus IX73 inverted microscope (Olympus, Tokyo, Japan), Cytation 5 (plate reader), and Lionheart FX (automated microscope), acquired from BioTek Instruments Inc. (Winooski, VT, USA).

Cell culture conditions

The analyses used two cell lines: HaCaT-healthy human immortalized keratinocytes from CLS Cell Lines Service and IMR-32 human neuroblastoma cells acquired from ATCC.

The cells were grown in the specific medium, DMEM for the HaCaT cell line and EMEM for the IMR-32 cell line, supplemented with 10% FBS and 1% antibiotic combination.

The cell lines were cultured in an incubator dedicated to cell cultures, under standard conditions (37°C and 5% CO₂).

Cellular viability test

The impact on cell viability (HaCaT and IMR-32) after treatment with GEN (5, 10, 25, 50, and 75µM) was evaluated using the MTT assay after an interval of 24h.

The cells were cultivated in 96-well plates (10⁴ cells/well), and after stimulation of 24h, the medium was replaced with a fresh one, and 10µL of MTT reagent was added for 3 hours.

Finally, the solubilizing solution (100µL) was added for half an hour at room temperature and then the absorbances were measured at 570nm using Cytation 5 (BioTek Instruments Inc., Winooski, VT, USA).

Cell morphology assay

To assess the influence of the GEN on cellular morphology, the microscopic evaluation of IMR-32 cells was conducted after 24h of treatment with 5, 10, 25, 50, and 75µM.

Potential alterations were observed using the Olympus IX73 inverted microscope (Olympus, Tokyo, Japan).

The captured photos were examined using the cellSens Dimensions v.1.8. Software (Olympus, Tokyo, Japan).

Cytotoxic assay-Lactate dehydrogenase (LDH) test

The possible cytotoxic effect of GEN (5, 10, 25, 50, and 75µM) in IMR-32 cells was studied using the LDH method.

For this analysis, the cells were cultured in a 96-well plate (10⁴ cells/well) and stimulated with the QUE when the needed confluence was attained (80-90%).

After 24 hours, a volume of 50µL from each well was moved into a new 96 well-plate, over which 50µL of the reaction mixture was added and incubated for 30 minutes at room temperature.

In the last step, the stop solution (50µL) was added and the absorbances were read at 490 and 680 nm using Cytation 5.

Nuclear Staining

To determine the action induced by the GEN at the nuclear level in IMR-32 cells, Hoechst 33342 staining (1:2000 solution in PBS) was realized.

The cells were grown in 12 well-plates and treated with GEN (75µM) for 24 hours.

The staining solution was added to each well for 7 minutes and then washed with PBS (3x).

An automated microscope Lionheart FX was used to take representative images of nuclei, to detect the changes that occurred after the application of the GEN.

Statistical Analysis

The one-way ANOVA assay followed by Dunnett’s multiple comparisons post-test described the statistical differences between multiple treatment groups.

The results are marked as mean±standard deviation (SD) using GraphPad Prism version 9.4.0 (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

The statistically significant differences between the data were noted with*, as follows: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

Results

The first step in evaluating the effect of GEN on HaCaT and IMR-32 cells was performed by

MTT colorimetric analysis after 24 hours of treatment.

Initially, the action on the healthy human keratinocyte line was explored to observe a potential toxic effect.

Following the analysis, there was no negative impact on the viability of HaCaT cells, with the viability decreasing dose-dependently up to 95%, and at the first three concentrations tested producing a slight cell proliferation of up to≈105%.

While on the IMR-32 tumor line, a significant decrease in viability was observed, at the concentration of 75μM reaching a percentage of≈65% (Figure 1).

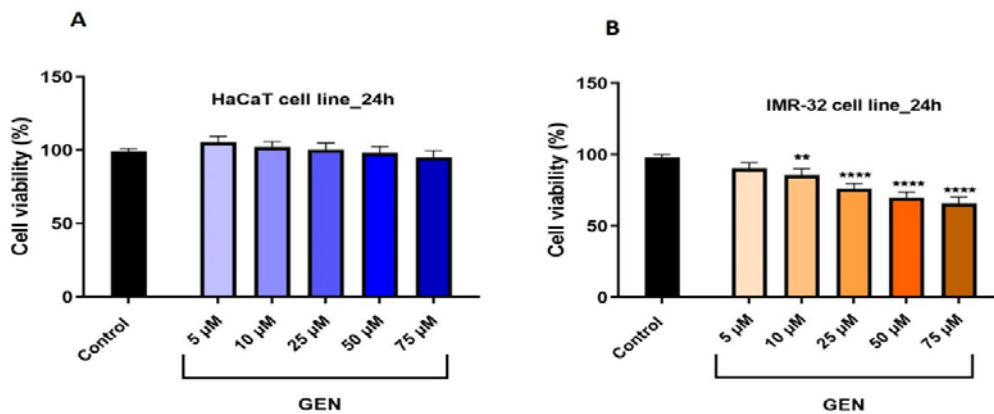


Figure 1. The cell viability percentages after 24 hours of treatment with GEN (5, 10, 25, 50, and 75μM) on HaCaT (A) and IMR-32 (B) cells. The statistical differences between the untreated and the treated group (**p<0.01; ****p<0.0001) were determined through one-way ANOVA analysis followed by Dunnett’s multiple comparisons post-test.

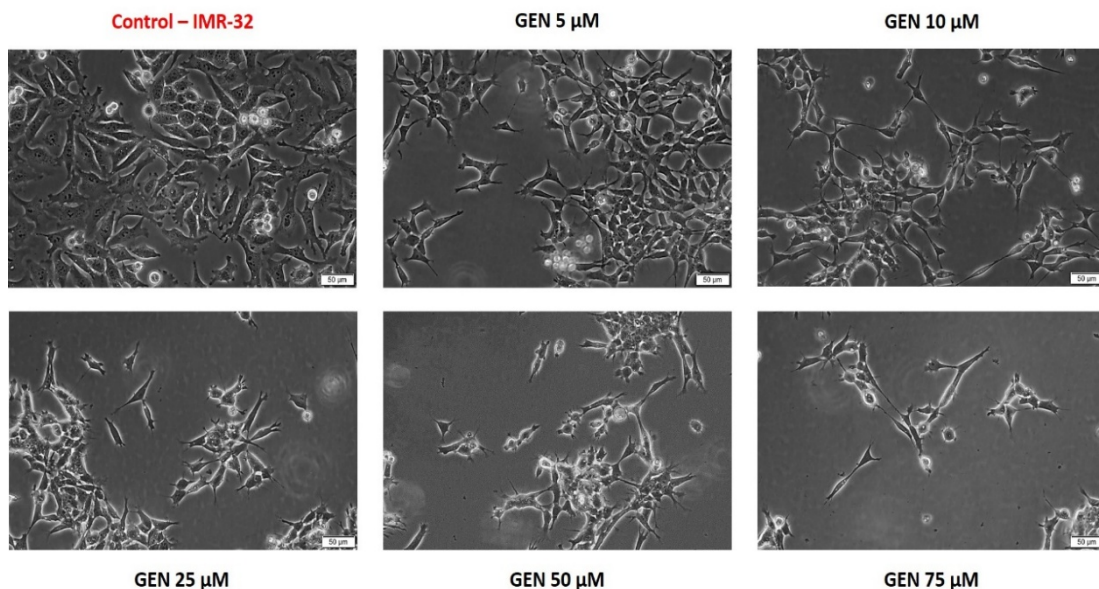


Figure 2. Representative images of the IMR-32 cells’ morphology after 24 hours of the treatment with GEN (5, 10, 25, 50, and 75μM). The scale bar indicates 50μm.

Because no effect on the viability of HaCaT cells was observed, the activity of GEN on human neuroblastoma cells was further investigated.

Thus, 5 increasing concentrations of GEN were tested to identify the action on the morphology and confluence of IMR-32 cells.

At the concentration of 5 and 10 μM , a decrease in the confluence was evident, while with the increase in the dose, changes in the morphology were also observed, the cancer cells lost their elongated shape and became round, even detaching from the plate (Figure 2).

To outline in detail the effect of GEN on IMR-32 cells, the cytotoxic effect was evaluated, i.e. the impact on the integrity of the membrane through the LDH test, and the amount of cytoplasmic LDH released was quantified after 24 hours.

The results showed that from the concentration of 25 μM , increasing percentages of cytotoxicity are recorded, reaching the highest dose tested at a percentage of 13.13% compared to untreated cells, as can be seen in Figure 3.

Finally, the impact on nuclear morphology was evaluated by the Hoechst method at a concentration of 75 μM after 24 hours of treatment.

At the highest dose, signs of cell apoptosis were evident after the application of GEN.

Thus, deformations of the nuclei were visible, with the formation of apoptotic bodies (Figure 4).

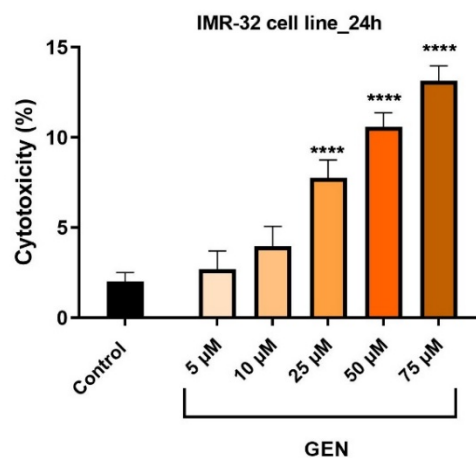


Figure 3. Lactate dehydrogenase leakage percentages after 24 hours of treatment of GEN (5, 10, 25, 50, and 75 μM) on IMR-32 cells. The statistical differences between the untreated and the treated group (**** $p < 0.0001$) were determined through one-way ANOVA analysis followed by Dunnett's multiple comparisons post-test.

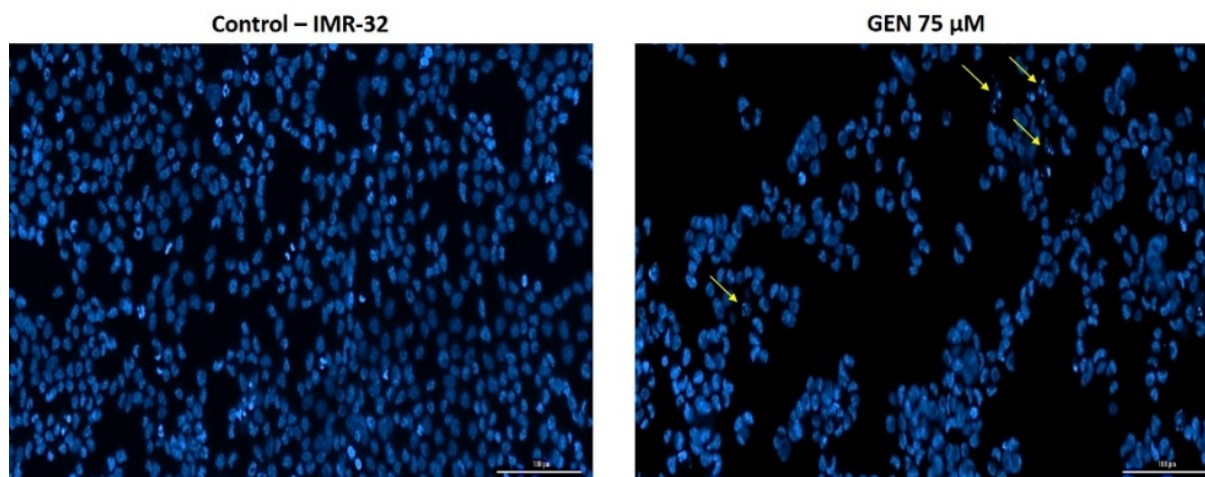


Figure 4. The changes in nuclear morphology after 24h of the treatment with GEN (75 μM) on IMR-32 cells. The yellow arrows show changes in cell nuclei. The scale bar indicates 100 μm .

Discussions

Despite numerous studies, cancer remains one of the most serious diseases with the most challenges worldwide, being one of the most important causes of death in industrialized countries.

In recent years, various anticancer drugs have been developed to treat cancer; however, despite progress, cure rates for cancerous pathologies remain low [16].

Currently, there is a need for more effective and less harmful treatments [17].

Therefore, natural compounds can be considered potential agents for creating more beneficial anticancer drugs [18-20].

Remarkably, more than 50% of the drugs used in cancer come from natural sources derived from plants [21].

Previous studies have shown that these natural compounds may have the ability to reduce the side effects of chemotherapy medications and

could even increase host immunity, decreasing the rate of cancer recurrence [17,22].

Genistein can be part of this category; this isoflavone can act on various molecular targets and modulate numerous signaling pathways, changing the final response of tumor cells [12,23,24].

It is important to take a multidimensional approach to the toxicological evaluation of GEN, referring to cytotoxicity, antitumor properties, and potential benefits, to outline the safety profile and toxicological implications of the active compound.

The first step of the study was to evaluate the toxicity of GEN on the HaCaT cell line, to eliminate a potential cytotoxic effect on the healthy human keratinocyte line. The data obtained showed that GEN does not affect HaCaT cells, rather at the lowest dose, it stimulates cell viability, followed by a slight decrease in viable cells up to 95% with increasing dose.

In a previous study, the effect of GEN (up to 100 μ M concentration) on the HaCaT cell line was evaluated after application for 24 h, 48h, and 7 days, respectively.

Following the MTT assay, cell viability was reduced dose-dependently, reaching a percentage of \approx 80% after 24h, a value of 55% after 48h, and 20% after 7 days of treatment, respectively.

Furthermore, pretreatment with GEN (100 μ M) for 120 minutes inhibited TNF- α -induced NF- κ B nuclear translocation of p65 in HaCaT cells [25].

In the research conducted by Wang et al., GEN (50, 100 μ M) inhibited the TNF- α -induced rise in NF- κ B level in human keratinocytes (HaCaT) compared to the TNF- α -only group and inhibited TNF- α -induced phosphorylation of I κ B- α [26].

Genistein has been investigated in diverse studies regarding its effect in combating neuroblastoma. The results of our study demonstrated that when GEN is applied, cell viability is reduced in a dose-dependent manner.

At the highest concentration tested (75 μ M), cell membrane damage occurred; also at the highest dose applied, GEN induced changes in cell morphology and confluence, with IMR-32 cells detaching from the plate and changing shape.

Moreover, at 75 μ M, signs of apoptosis were recorded on the neuroblastoma tumor cell line.

Zheng et al examined the action of genistein on human neuroblastoma (SK-N-SH) cell proliferation induced by endocrine disruptors,

such as bisphenol A (BPA) and di-2-ethylhexyl phthalate (DEHP). SK-N-SH tumor cells were treated with 17 β -estradiol (1ng/ml), DEHP (100 μ M), and BPA (2 μ g/ml) in combination or not with GEN (12.5 μ M).

The BPA and DEHP groups had 30% higher viability compared to the untreated group at 48 hours.

Moreover, the number of cells did not increase significantly in the groups with additional treatment with GEN, the same trend being observed at 72 hours.

The expression of the phospho-Akt protein was increased in cells stimulated with BPA or DEHP compared to the untreated group after 72 hours, while no significant increase in the expression of phospho-Akt was identified in the cells where GEN was applied.

Furthermore, cells were arrested in the G2/M phase by GEN. Thus, GEN has been shown to inhibit proliferation induced by estradiol and endocrine disruptors in an Akt pathway- and cell-cycle-dependent manner [27]

In another study, the *in vitro* effect of GEN and rotlerin on two neuroblastoma lines (SH-SY5Y and Kelly) was tested.

The results of the study demonstrated that the application of GEN and rotlerin led to an important decrease in cell proliferation, the ability to heal wounds, as well as the formation of colonies on tumor cells at concentrations of 5 μ M and 30 μ M.

The two compounds also increased apoptosis and produced G1 cell cycle arrest in SH-SY5Y and Kelly cell lines [28].

The group led by Li et al demonstrated that GEN is an epigenetic modifier that can enhance the expression of CHD5 and p53, and reduce the hypermethylation levels of CHD5, having a possible role in inhibiting the growth of neuroblastoma.

Thus, it was stated that GEN can inhibit the growth of NB *in vivo*, preventing the risk of tumorigenesis by inhibiting the formation of tumor microvessels [29].

The study led by Mohan et al induced autophagy in neuroblastoma cells (SK-N-BE2 and IMR-32) by administering rapamycin because autophagy is considered to be a possible survival mechanism in malignant NB.

The results showed that the combination of GEN and LC3 shRNA plasmid transfection can inhibit rapamycin-induced autophagy and thus control the growth of human malignant NB cells in cultures and animal models [30].

In addition to the effects on NB, GEN has been studied on other cancer cell lines, where it demonstrated its therapeutic effect through different mechanisms, such as cell cycle arrest, anti-apoptotic, anti-proliferative, and anti-metastatic effects.

Therefore, there are numerous studies in which GEN shows its anticancer properties on tumor lines of breast, colon, cervical, lung, and pancreatic cancer, but also on glioblastoma [31-38].

Therefore, natural anticancer compounds are an important current topic in the medical field and have led to a multitude of valuable scientific discoveries, even in the case of neuroblastoma [39].

Conclusion

In conclusion, the results of the study support that GEN possessed a dose-dependent cytotoxic effect on human neuroblastoma cells (IMR-32) compared to the healthy cell line (HaCaT).

The obtained effect is defined by the reduction of cell viability, LDH leaks, changes in cell morphology, as well as by nuclear changes characteristic of apoptosis.

Therefore, these data expose a new perspective on the antitumor effects of GEN and provide a step toward future studies to elucidate the mechanism of action underlying these beneficial effects in NB.

Author contributions

Conceptualization, A.H., A-D.S. and S.A.; Methodology, A-D.S., G.M. and I-A.P.; Software, A-D.S., G.M. and C.T.; Investigation, A-D.S., G.M. and S.A.; Resources, A.H., D.S.A., D.V. and S.A.; Writing-original draft preparation, A.H., A-D.S., I-A.P., and G.M.; Writing-review and editing, CT, D.A., D.V. and S.A.; Visualization, A.H. and S.A.; Supervision, C.T., D.S.A. and S.A.; Project administration, A-D.S. and S.A.; Funding acquisition, G.M. and S.A.

Alina Heghes and Alexandra-Denisa Semenescu share the first co-authorship.

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Conflicts of interest

The authors declare no competing interests.

Institutional Review Board

The study was conducted according to the guidelines of the Declaration of Helsinki.

Consent Statement

Not applicable.

Data availability

All data presented in the manuscript are available from the authors upon request.

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