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Effect of Vitamin C antioxidant, Ag and TiO2 Nano-Particles on Homo sapiens Circular RNA 0001518 Expression in BALB/c Mice with Brain Tumor

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Abstract

Background and Aim: Brain cancer is a leading cause of mortality, posing significant treatment challenges. CircRNAs are emerging as innovative biomarkers and therapeutic targets for various diseases, including cancer. This study evaluates the half-maximal inhibitory concentration (IC50) of Vitamin C, Ag, and TiO2 nanoparticles (NPs) against the GL-261 tumor cell line and their impact on expression of hsa_circ_0001518 in cancerous and healthy mice.

This investigation aims to determine the impact of Vitamin C antioxidants and Ag and TiO2 NPs, individually and in combination, on the gene expression patterns of CircRNAs in brain cancer.

Methods: The GL-261 cell line was cultured and treated with Vitamin C (25, 50, 75, and 100 µg/ml), Ag (11, 24, 42.24, and 65 µg/ml), and TiO2 NPs (5, 7, 10.5, and 17.5 µg/ml). The MTT test was performed to determine cell viability. The expression of investigated circular RNA after treatment with the IC50 of Vitamin C in healthy and brain tumor-bearing mice was assessed by qRT-PCR.

Results: The IC50 values after 48h were 81.25 µg/ml for Vitamin C, 45.47 µg/ml for Ag NPs, and 8.73 µg/ml for TiO2 NPs. qRT-PCR results indicated that the combination of the antioxidant and NP significantly decreased circ0001518 expression in brain tumor-bearing animals more than any single treatment.

Conclusion: The combination of TiO2 and Vitamin C, as well as Ag and Vitamin C, significantly alters the expression of the investigated circ and shows potential as a novel therapeutic approach for brain cancer.

Keywords: Brain Cancer, Vitamin C antioxidant, TiO2 Nanoparticles, Ag Nanoparticles, hsa-circ-0001518

1. Introduction

Cancer is one of the main causes of death worldwide, putting a significant burden on the public health system(1). Among the various types of cancer, brain cancers are the main cause of cancer-related mortality in children as well as the second leading cause of cancer-related death overall (2). There are different types of brain tumors, such as meningioma, pituitary, and glioma (3). A glioma originates from glial cells, which help support the function of the other brain cell type, namely the neuron(4). Over the past two decades, research on brain tumors has advanced significantly, enhancing our understanding of their fundamental biology and pathogenesis. This extensive research has provided deeper insights into the molecular and genetic mechanisms underlying tumor initiation, progression, and resistance to treatment. Studies have revealed crucial information about the heterogeneity of brain tumors, the significance of the tumor microenvironment, and the impact of genetic

mutations on cellular processes such as apoptosis, proliferation, and invasion. These findings have paved the way for development of novel therapeutic strategies and improvement of existing treatment modalities (5). Despite significant advances in neurosurgery, radiotherapy, and chemotherapy, cell heterogeneity and various genetic mutations affecting cell cycle control, apoptosis, cell proliferation, and cell invasion lead to resistance to treatment approaches. The development of new drugs and the use of combination therapies may present effective techniques to combat brain cancer (6).

Non-coding RNAs (ncRNAs) are RNA molecules transcribed from the genome that do not encode proteins. Despite their lack of protein-coding ability, ncRNAs perform critical regulatory functions in a variety of biological processes, including gene expression, chromatin remodeling, and the modulation of cellular signaling pathways. These molecules include microRNAs, long non-coding RNAs, and circular RNAs, each contributing uniquely to the regulation of gene expression and the maintenance of cellular homeostasis. Long ncRNAs and circular RNAs are two essential groups of large ncRNAs that play a crucial role in numerous pathophysiological processes, including cancer (7) . In this context, circular RNAs can influence cancer-promoting processes such as cell proliferation, invasion, apoptosis, and chemoresistance. As a result, several compounds have been proposed as potential therapeutic targets in the fight against Glioblastoma [6]. One of the circRNAs is homo sapiens circular RNA 0001518 (hsa-circRNA-0001518), which is encoded by the F-Box and Leucine-Rich Repeat Protein 17 gene (*FBXL17*) and is found on chromosome5(8). According to predictions made by the circ-Base bioinformatics database (http://www.circbase.org), this cirRNA is linked to brain cancer by influencing the B-cell lymphoma 2 (*Bcl-2*) gene and changing the activity of several miRNAs. Several preclinical and clinical studies have identified the B-cell lymphoma 2 (*Bcl-2*) protein family as essential regulators of therapy-induced cell death(9). Many treatment options have been proposed to regulate apoptosis in tumor cells; one possible strategy involves the use of various antioxidants and vitamins.

Antioxidants are potent drugs that can attenuate ROS-mediated cellular injury in healthy tissues and mitigate the negative effects of chemotherapy. However, it has been indicated that 20–85% of individuals diagnosed with cancer take antioxidant supplements, with the majority of users being post-treatment breast cancer patients (10, 11). Antioxidants can remarkably regulate various signaling pathways and different signaling biomolecules. Curcumin is an effective chemical when used with several other drugs, such as 5-cisplatin, paclitaxel, doxorubicin, FU, and celecoxib in various malignancies. Vitamins E and C are dietary antioxidants that have a variety of functions, from cancer prevention to anti-inflammatory properties (12, 13).

These antioxidants can reduce cell growth, induce apoptosis in tumor cells, and inhibit cell proliferation. Vitamin C (L-ascorbic acid) can strongly exhibit greater cytotoxic sensitization in cancerous cells compared to normal cells. Vitamin C serves as a prodrug by producing ascorbate radicals and H2O2, inducing oxidative stress and subsequently destroying cancer cells when administered intravenously (14). High doses of ascorbic acid, either alone or in combination with traditional anticancer medications, have been shown to dramatically limit cancer development (15). Vitamin C has the ability to selectively destroy cancer cells, and its activity is determined by parameters such as cancer type and the signaling system involved in tumor formation (16). Vitamin C acts as a powerful reducing agent, a free radical scavenger, and a protector of cell membrane against primary oxidative damage in tissues and extracellular fluids. L-ascorbic acid is essential for collagen biosynthesis and the production of numerous other biologically important molecules, modulation of enzyme activity, detoxification of xenobiotics, and prevention of carcinogenic nitrosamine formation. It also plays a critical role in the immune system. Accumulating evidence indicate that cancer patients commonly exhibit vitamin C deficiency (13).

In the present research, we investigated the expression level of hsa_circ_0001518 in brain tumor-bearing BALB/c mice compared to healthy mice in order to examine the impact of the vitamin C antioxidant.

Recently, nanoparticles (NPs) have become the major trend in nanomedicine for cancer treatment (17, 18). Nanotechnology refers to the ability to control, modify, examine, and manufacture objects and devices with nanoscale precision. These nano-sized objects, known as "nanoparticles", which are made of the same material, have different properties and functions. The small size, unique surface, increased conditionality, and adaptability of nanoparticles continue to expand their new medicinal applications. The identification of nanoparticles is essential for understanding and controlling their synthesis and application (17, 18) The structural properties of NPs make them excellent choices for targeting and influencing the growth of abnormal cancer cells. They can potentially enter abnormal cells that cause DNA damage and gene defects (19).

2. Methods

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Mice

In this investigation 120 male BALB/c mice, aged six to seven weeks, were sourced from the Animal Breeding Stock Facility of the Pasteur Institute in Karaj, Iran. Before the experiment, the mice were acclimated to the laboratory environment under a 12-hour day/night cycle with a regulated temperature of 23 ± 2°C and humidity of 50-60%. The mice had unlimited access to clean water and conventional rodent food. All animal-related procedures were approved by the Ethical Committee of Islamic Azad University, Tehran Medical Sciences Branch (IR.IAU.TNB.REC.1398.154) and were conducted in accordance with all relevant standards and legislation.

Experimental Planning

To determine the IC50 of antioxidant Vitamin C and create brain cancer models, the GL261 tumour cell line used in this study was obtained from National Cell Bank of Iran, Pasteur Institute. Antioxidant Vitamin C was provided by Sigma-Aldrich (product number: A0278, USA). The nanoparticles used in this study were prepared and characterized according to the specifications shown in Figures 1 and 2. Cells were maintained under standard culture conditions (37°C in a humidified atmosphere containing 5% CO2) in RPMI-1640 medium with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin.

An orthotopic glioblastoma model was established at Shahid Beheshti Research Center by stereotactic injection of 1 × 10^5 GL261 cells suspended in 0.1 ml sterile PBS into the frontal lobe of mice. Following anesthesia, a midline scalp incision was made and a burr hole was drilled in the skull. The cells were then injected using a stereotaxic apparatus at predetermined coordinates relative to bregma. The mice were divided into two main groups (Healthy and Cancerous) and each main group was further divided into six subgroups (n=10 per subgroup). They received 81.25 µg/ml of Vitamin C, 45.47 µg/ml of silver (Ag), and 8.73 µg/ml of titanium dioxide (TiO2) nanoparticles (IC50 dosages) administered either individually or in combination in the cancerous groups. Subgroup 1 served as the control and received saline solution. Subgroup 2 received vitamin C, subgroup 3 received AgNps, and subgroup 4 received TiO2 Nps, while subgroups 5 and 6 received combinations of these treatments. All treatments were administered at a total injection volume of 1 ml per animal. The same treatment grouping was performed for both healthy and cancerous mice. After two weeks of treatment, the animals were anesthetized and tissue samples were harvested for examination.

MTT Assay

The cytotoxicity effects of Vitamin C antioxidants on GL261 cells were evaluated using the tetrazolium -based (MTT) test. Cells (5x10^4) were seeded into 96-well plates and allowed to attach for 24 h. The culture medium was then replaced with fresh medium containing different doses of Vitamin C (25, 50, 75, and 100 µg/ml), and the cells were incubated for an additional 48 h. Subsequently, 20 µl of MTT solution (5 mg/ml) was added to each well and incubated for 4 hours. The formed Formazan crystals were dissolved in 100 µl of DMSO, and the optical density was measured at 570 nm using a plate reader (BioTek-ELx800, USA). Cell viability was determined by comparing treated cells with untreated control cells. The IC50 values were calculated using GraphPad Prism 6 software. All experiments were conducted in triplicate. The calculated IC50 doses were non-lethal to mice during the 14-day observation period; hence, a lethal dose was not established due to ethical considerations.

RNA Extracting and Synthesis of cDNA

Two weeks post-treatment, all animals were sacrificed using ketamine-xylazine (KX). Tumors and brain tissues from both the control and treated groups were collected, homogenized in ice-cold phosphate-buffered saline, and centrifuged at 12,000g for 15 minutes at 4°C. RNA was extracted from the pellet using the Ribo EX kit (GeneAll Biotechnology, South Korea) and quantified applying a Thermo Scientific Nanodrop 2000 spectrophotometer. Complementary DNA (cDNA) was synthesized from the extracted RNA using the BioFACT cDNA Synthesis kit (South Korea) under the following temperature conditions: 25°C for 10 minutes, 42°C for 60 minutes, and 70°C for 10 minutes.

Quantitative Real-Time Polymerase Chain Reaction (PCR)

Levels of the investigated circ were determined using Q-RT PCR in normal and brain cancer groups treated with IC50 doses of Vitamin C, Ag, and TiO2 nanoparticles. SYBR Green Master Mix (TAKARA, Japan) was used in triplicate on a Roche Light-Cycler TM 96. The expression levels were normalized to ACTB (β-actin) using the 2^–ΔΔCt technique. Primer3plus software was employed to design the primer sequences (table 1).

Table 1. The RT-qPCR primer sequences

Target transcript	Primer type	Sequence (5'→3')
ACTB	Forward	GATCAAGATCATTGCTCCTCCTG
	Reverse	CTAGAAGCATTTGCGGTGGAC

Circ-0001518	Forward	GGCAGAACAGGAAGTTGGTC
	Reverse	GACAGAGAATGGGGCAGAAA

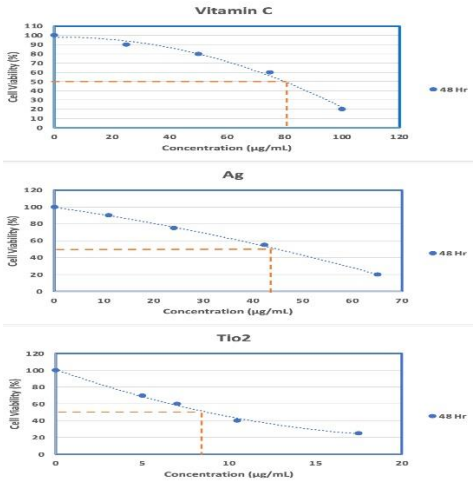
Statistical Evaluation

Statistical analyses were conducted using IBM SPSS Statistics (version 21) and GraphPad Prism (version 6.0). The One-Sample Kolmogorov-Smirnov test was employed to evaluate the normality of the expression data. Differences in circ expression levels were assessed using one-way ANOVA, and p-values <0.05 were considered statistically significant.

3. Results

MTT Assay for Measuring Cellular Toxicity

The in vitro cytotoxicity effects of Vitamin C, Ag, and TiO2 nanoparticles (NPs) on GL-261 tumor cell lines were evaluated using the MTT test to determine the optimal concentrations (IC50) for subsequent animal studies (Figure 3). As displayed in Figure 3, the IC50 values for Vitamin C, Ag, and TiO2 NPs were 81.25 µg/ml, 45.47 µg/ml, and 8.73 µg/ml, respectively.



Levels of circ Expression

qRT-PCR analysis was performed to determine the expression levels of circRNA (0001518) in healthy and cancerous mice treated with IC50 doses of Vitamin C, Ag, and TiO2 NPs, either alone or in combination. As illustrated in Figure 4, treatment with the IC50 concentration of Vitamin C resulted in up-regulation of expression in healthy mice and down-regulation in cancerous animals. The expression levels of circ-0001518 were also up-regulated with the use of Ag and TiO2 NPs alone in the healthy group. However, the expression level in healthy mice was up-regulated to a greater extent following treatment with Vitamin C in combination with either Ag or TiO2. In contrast, in cancerous mice treated with the same combinations, the expression level was down-regulated.

4. Discussion

Vitamin C is widely recognized as an antioxidant and has been investigated for its potential role in cancer therapy. Previous studies have shown that Vitamin C can enhance the cytotoxic effects of conventional anticancer treatments and reduce therapy-associated side effects. Epidemiological evidence suggests that dietary antioxidants, such as Vitamin C, β-carotene, folate, and α-tocopherol, may reduce the incidence of brain tumors (Zhang et al.). Moreover, Vitamin C has been explored as an adjuvant in glioblastoma treatment to mitigate treatment-related toxicity while promoting cytotoxic effects at specific doses.

Metal oxide nanoparticles, including AgNPs and TiO_2 NPs, have also demonstrated notable anticancer activity in brain cancer models. Rezaei et al. have reported dose- and time-dependent cytotoxic effects of various nanoparticles, including dextran-coated iron oxide NPs and NiO NPs, in cancer cell lines. These findings are consistent with our IC50 results, which showed a dose-dependent reduction in GL-261 cell viability following treatment with Vitamin C, AgNPs, and TiO_2 NPs.

The treatment doses used in the in vivo experiments were selected based on previously reported safe and effective ranges in murine tumor studies. These concentrations were well tolerated and were designed to correspond as closely as possible to the IC_{50} values determined in our in-vitro experiments, thereby enabling a biologically relevant comparison between the cellular and animal findings.

Circular RNAs play important roles in the regulation of apoptosis, migration, and metastasis by modulating key signaling pathways and gene networks. (Tables 2 and 3) Several circRNAs have been proposed as diagnostic or prognostic biomarkers in glioma. For instance, hsa_circ_0088732 has been reported to promote epithelial–mesenchymal transition, migration, and invasion via the hsa_circ_0088732/miR-661/RAB3D axis. A summary of circRNAs and their associated pathways in glioma is presented in Tables 2 and 3 (20).

Notably, our results revealed opposite regulatory effects of Vitamin C, AgNPs, and TiO_2 NPs on circ0001518 expression in normal and tumor-bearing mice. This differential response may be attributed to fundamental differences in redox homeostasis, stress-response signaling, and circRNA biogenesis between healthy and cancerous cells. Cancer cells typically exhibit elevated oxidative stress and altered RNA-binding protein activity, including dysregulation of QKI, ADAR1, and FUS, which are known regulators of circRNA back-splicing (21,22).

Furthermore, nanoparticles such as AgNPs and TiO_2 NPs can induce stronger oxidative and inflammatory responses in cancer cells due to their impaired antioxidant defenses, leading to activation of p53- and JNK-mediated apoptotic pathways. These pathways have been associated with suppression of circRNAs involved in cell survival and proliferation (23-26). In contrast, normal cells may activate adaptive stress-response mechanisms rather than cytotoxic signaling cascades, resulting in divergent circRNA expression patterns.

Table 2. Representative circRNAs and related signaling pathways in brain cancer (glioma) (23)

circRNA	Dysregulation	Sponge target/ mechanism	Downstream genes and signaling pathway	Phenotype
circPINTexon2	Down	Encode PINT 87aa	Work as an anchor of PAF1 complex and inhibit downstream genes CPEB1, SOX2, c-myc	Tumorigenicity
circHIPK3	up	miR-654	IGF2BP3	Proliferation, invasion
hsa_circ_0076248	up	miR-181a	SIRT1	Tumorigenesis Apoptosis, invasion
circ_0034642	up	miR-1205	BATF3	Proliferation, migration, invasion, apoptosis
circSMARCA5	Down	SRSF1	VEGFA	Migration, angiogenesis
circSHPRH	Down	Encode SHPRH-146aa	Protect SHPRH which ubiquitinates PCNA	Proliferation, tumorigenicity
circFBXW7	Down	Endode FBXW7-185aa	reduced the half-life of c-Myc by antagonizing USP28-induced c-Myc stabilization	Proliferation, cell cycle
circNFIX	Up	miR-34a-5p	Notch1 Notch signaling	Proliferation, migration, Invasion, apoptosis
circHIPK3	Up	miR-124-3p	STAT3	Proliferation, invasion,
circMMP9	Up	miR-124	CDK4, AUPKA	Proliferation, migration, Invasion
CircNT5E	Up	miR-422a	NT5E, SOX4, PI3K, P-AKT, p-smad2 PI3K/AKT signaling Smad2 signaling	Proliferation, migration, invasion
hsa_circ_0046701	Up	miR-142-3P	ITGB8	Proliferation, invasion
has_circ_001946	Down	miR-671-5p	CDR1	Proliferation, migration, Invasion, apoptosis
hsa_circ_0012129	Up	miR-661	/	Proliferation, migration, invasion
cZNF292	Up	/	Wnt/β-catenin signaling and related genes including cyclinA, p-CDK2, VEGFR, EGFR	Proliferation, cell cycle angiogenesis
hsa_circ_0000177	Up	miR-638	FZD7, Wnt/β-catenin signaling	Proliferation, migration, invasion
circTTBK2	Up	miR-217	HNF1β/Derlin1 PI3K/AKT and ERK signaling	Proliferation, migration, Invasion, apoptosis
hsa_circ_14359	Up	miR-153	p-AKT PI3K/AKT signaling	Proliferation, migration, Invasion, apoptosis
circU2AF1	Up	miR-7-5P	NOVA2 PI3K/AKT and ERK signaling	Proliferation, migration, Invasion, apoptosis
circCFH	Up	miR-149	AKT1 PI3K/AKT signaling	Proliferation
circSHKBP1	Up	miR-544a/miR379	FOXP1/FOXP2/AGG1 PI3K/AKT and ERK signaling	Proliferation, migration, angiogenesis
has_circ_002136	Up	miR-138-5p	SOX13/SPON2	Migration, invasion angiogenesis
circDICER1	Up	miR-103a-3p. miR-382-5p	ZIC4/HSP90 PI3k/AKT signaling	Proliferation, migration, angiogenesis
has_circ_0074362	Up	miR-1236-3P	HOXB7	Proliferation, migration, invasion

cir-ITCH	Down	miR-214	ITCH/wnt/β-catenin signaling	Proliferation, migration, invasion
hsa_circ_0001649	Down	/	BCL2/caspase3 signaling	Apoptosis

Table 3. The migration and invasion-related and proliferation-related circular RNAs in brain cancer (glioblastoma) (23)

circRNAs	Expression	Mechanism	Biological function
hsa_circ_0008344	Up	—	Promote cell proliferation, colony formation and inhibit cell apoptotic rate, Promote cell migration and invasion
circ-MMP9	Up	miR-124/CDK4	Promote cell proliferation, Promote cell migration and invasion
hsa_circ_0029426	Up	miR-197	Promote cell proliferation, inhibit cell apoptosis, Promote cell migration and invasion
hsa_circ_0074027	Up	miR-518a-5p/IL17RD	Promote cell proliferation, colony formation and inhibit cell apoptotic rate, Promote cell migration and invasion
hsa_circ_0067934	Up	PI3K-AKT	Promote cell proliferation, Promote cell migration and invasion
circ-PITX1	Up	miR-379–5p/MAP3K2	Promote cell proliferation, inhibit cell apoptosis,
circ-FOXO3	Up	miR-138-5p/miR-432-5p/NFAT5	Promote cell proliferation, Promote cell migration and invasion
hsa_circ_0001801	Up	miR-628-5p/HMGB3	Promote cell proliferation, Promote cell migration and invasion
circ-EPB41L5	Up	miR-19a/EPB41L5	Promote cell proliferation, colony formation, Promote cell migration and invasion
circ-ENTPD7	Up	miR-101-3p/ROS1	Promote cell proliferation, Promote cell migration and invasion
hsa_circ_0043278	Up	miR-638/HOXA9	Promote cell proliferation, Promote cell migration and invasion
circ-SMO	Up	SMO-193aa	Promote cell proliferation
circ-SKA3	Up	miR-1	Promote cell proliferation
circ-PARP4	Up	miR-125a-5p	Promote cell proliferation, Promote cell migration and invasion
circ-PITX1	Up	miR-584-5p/KPNB1	Promote cell proliferation, Promote cell migration and invasion
circ-ABCC3	Up	miR-770-5p/SOX2	Promote cell proliferation, inhibit cell apoptosis, Promote cell migration and invasion
hsa_circ_0006168	Up	miR-628-5p/IGF1R	Promote cell proliferation, colony formation and inhibit cell apoptotic rate, Promote cell migration and invasion
hsa_circ_0001588	Up	miR-211-5p/YY1	Promote cell proliferation, Promote cell migration and invasion
circ-FLN1	Up	miR-199-3p	Promote cell proliferation,
circ-SERPINE2	Up	miR-361-3p/miR-324-5p/BCL2	Promote cell proliferation, colony formation and inhibit cell apoptotic rate,
circ-LGMN	Up	miR-127-3p/LGMN	Promote cell proliferation, Promote cell migration and invasion
circ-NF1	Up	miR-340	Promote cell proliferation
circ-NT5E	Down	miR-422a	Inhibit cell proliferation, Inhibit cell migration and invasion
hsa_circ_0001946	Down	miR-671-5p/CDR1	Inhibit cell proliferation and promote cell apoptosis, Inhibit cell migration and invasion
circ-MTO1	Down	miR-92/WWOX	Inhibit cell proliferation, Inhibit cell migration and invasion
circ-AKT3	Down	AKT3-174aa/PI3K/AKT	Inhibit cell proliferation
hsa_circ_01844	Down	—	Inhibit cell proliferation and promote cell apoptosis
circ-CDR1as	Down	p53/MDM2	Inhibit cell proliferation

5. Conclusion

Our findings suggest that Vitamin C, Ag, and TiO2 NPs, particularly when used in combination, can alter the expression of our investigated circRNA in brain tumor-bearing mice, indicating their potential as therapeutic candidates for brain cancer. Further research is required to elucidate the mechanisms underlying the cytotoxic effects of these agents and to

explore the therapeutic potential of combining Vitamin C with other nanoparticles to enhance anticancer efficacy.

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Ethical Considerations and Compliance with Ethical Guidelines

All cell lines used in this study were obtained from authenticated and reputable cell repositories and were handled in accordance with institutional bio safety and ethical guidelines. Standard laboratory practices were followed to prevent contamination and misidentification of cell lines, including routine authentication and mycoplasma testing. All experiments were conducted following established bio safety protocols to ensure responsible and ethical research practices.

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Conflict of interest

The authors declare no conflict of interest.

AI Using Declaration

Chat GPT was used for language and grammar corrections. The final output was read and modified by all authors.

Author's contributions

All authors equally contributed to preparing this article.

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