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RESEARCH PAPER

Investigation of the antiproliferative effect of ceramide and sphingomyelin on various cell lines

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Abstract

Sphingolipids participate in the structure of the cell membrane and have bioactive roles in many cellular events, including cell proliferation, differentiation, and programmed cell death. Changes in sphingolipid metabolism contribute to the formation and progression of the cancer phenotype in different cancer types and multi-drug resistance. In this study, we aimed to examine the antiproliferative effects of ceramide and sphingomyelin on different cancer cell lines. The results showed that ceramide and sphingomyelin act similarly and have antiproliferative effects on three different cancer cell lines and a normal cell line in a dose-dependent manner. In contrast, this effect decreases when low concentrations are applied. In DMSO solvent, ceramide was most effective in C6 cells (IC50 = 32.7 µM) and least in CCD-18Co (IC50 = 56.91 µM). In ethanol, it showed highest sensitivity in CCD-18Co (IC50 = 0.33 µM). Sphingomyelin in DMSO had IC50 values of 0.25 µM in C6 and HT29 and 0.45 µM in CCD-18Co. In ethanol, it was more effective in cancer cells (IC50 = 0.25–0.28 µM) but less cytotoxic in CCD-18Co. In conclusion, these two molecules have antiproliferative activities in a dose-dependent manner, however, further molecular investigations are needed to understand the mechanism of actions.

Introduction

Cancer is among the most critical health problems, with widespread morbidity and mortality. According to the global cancer statistics, it is estimated that 19.3 million cancer cases were seen in 2020, and approximately 10 million people died due to cancer (Sung et al., 2021). Although significant progress has been made in cancer treatments in recent years, more effective and new treatment strategies are still needed. Therefore, identifying and characterizing new molecular targets or improving the therapeutic effects of existing therapies are the focus of most research.

Lipids, beyond being structural components of cell membranes, play critical roles in energy storage, acting as signaling molecules, and regulating processes such as cell growth, differentiation, and death (Martin-Perez et al., 2022).

Among the primary types of lipids are sphingolipids, sphingolipids, triglycerides, fatty acids, and sterols, with particular attention drawn to the sphingolipid class (Gencer, 2019). While these lipids are known as structural components of cellular membranes, they also act as bioeffector molecules mediating physiological and pathological processes such as cell division, differentiation, proliferation, angiogenesis, metastasis, inflammation, and the initiation of apoptotic processes (Ogretmen, 2018). Within this class, ceramide and sphingomyelin emerge as central components in modulating the biological behavior of cancer cells (Futerman and Hannun, 2004; Li & Zhang, 2015; Zhou et al., 2024).

Ceramide is generated through the hydrolysis of sphingomyelin by sphingomyelinase enzymes involved in cellular signaling pathways (Kolesnick, 2002; Merz et al., 2024). Specifically, ceramide plays a significant role in regulating intracellular stress pathways, apoptosis, and growth regulation, thereby influencing cellular fate

(Ding et al., 2024; Yan et al., 2024). Increased ceramide levels trigger cell death and limit cancer cell proliferation, while decreased ceramide levels contribute to the upregulation of pro-apoptotic proteins, promoting increased cell proliferation (Bienias et al., 2016). These molecules, with their pro-apoptotic and anti-apoptotic properties, play a pivotal role in determining cellular fate between survival and death (Ding et al., 2024; Yan et al., 2024).

The primary mechanism behind ceramide's ability to mediate these effects lies in its role in triggering intrinsic and extrinsic apoptotic signaling pathways, thereby initiating the caspase cascade (Bansode et al., 2011; Mesicek et al., 2010; Patwardhan & Liu, 2011; Song et al., 2022). On the other hand, sphingomyelin, another vital lipid compound, resides in the cell membrane and can be converted into ceramide. Like ceramide, sphingomyelin metabolism not only contributes to the structural integrity of cell membranes but also modulates numerous signaling pathways (Airola & Hannun, 2013; Bienias et al., 2016; Falluel-Morel et al., 2008; Huang et al., 2011; Merz et al., 2024; Morad & Cabot, 2013; Wang et al., 2024).

Disruption of the balance in ceramide and sphingomyelin metabolism leads to loss of cell cycle control, affecting the growth and survival capacity of cancer cells (Morad & Cabot, 2013).

Therefore, since changes in the mechanism of action of ceramide and sphingomyelin contribute to the formation and progression of different cancer phenotypes in various tumors, disruptions or disorders in these processes they undertake, support oncogenic functions and help the development and maintenance of the microenvironment of cancer cells (Butler et al., 2020). In this study, we used ceramide and sphingomyelin and dissolved in two different solvents (DMSO and ethanol) to determine their potential antiproliferative effects by performing Sulphorodamine B assays (SRB) in three different cancer cell lines (C6, HT-29, OV2008) and a normal cell line (CCD-18Co). The therapeutic potential roles of ceramide and sphingomyelin lipids have been demonstrated by their ability to exhibit both proliferative and antiproliferative effects on cell viability, depending on the concentration of the compounds and the solvent used. This highlights the critical importance of solvent selection and concentration optimization in the application of ceramide and sphingomyelin compounds to cancer and normal cells.

Materials and Methods

Cell lines and reagents

In this study, we used C6 (rat glioma), OV2008 (ovarian), HT-29 (colorectal) cancer cell lines, and CCD-18Co (normal colon fibroblast) cell lines. OV2008 ovarian cancer cells were cultured in RPMI 1640 medium (Sigma-Aldrich), HT-29 and C6 cell lines were cultured in Dulbecco's Modified Eagle Media (DMEM, Sigma-Aldrich) and CCD-18Co normal colon cells were grown in Eagle's Minimum Essential Medium (EMEM, Sigma-Aldrich). All mediums were supplemented with 10% Fetal Bovine Serum (FBS), 2 mM L glutamine, 100U/ml penicillin and 100 µg/ml streptomycin and cells were incubated at 37 °C in a humidified 5% CO_2 incubator (Berkel et al., 2020). Cells were regularly checked for contamination and viability was determined with 0.4% (w/v) trypan blue solution (Biological Industries). Ceramide and Sphingomyelin were commercially obtained from Sigma-Aldrich.

Preparation ceramide and sphingomyelin concentrations

Ceramide and sphingomyelin were dissolved in either DMSO or ethanol. In this study, a 12,500 µM stock concentration of ceramide was prepared in DMSO, while a 50 µM stock concentration was prepared in ethanol, followed by dilution steps. For the solubilization of sphingomyelin compound in DMSO, a 25 µM stock concentration was prepared, and dilution steps were carried out, whereas for solubilization in ethanol, an 80 µM stock concentration was prepared, followed by dilution. After the solubilization of ceramide and sphingomyelin compounds in DMSO and ethanol, 2 µL of each was added to each well containing 200 µL of media and cells. The application of 2 µL of solvents to the wells minimized the potential toxicity that the solvents could induce.

These two molecules were further diluted in cell culture mediums before applied to the cells. Ceramide was dissolved in DMSO at concentrations of 10, 40, 60, 100, 120, and 125 µM or in ethanol at concentrations of 50, 100, 150, 200, 250, 300, 400, and 500 nM. Sphingomyelin was dissolved in DMSO at concentrations of 12.5, 25, 50, 75, 100, 150, 200, and 250 nM or in ethanol at concentrations of 12.5, 25, 50, 400, 600, and 800 nM.

Sulforhodamine B (SRB) assay

Sulforhodamine B (SRB, Chiton Red 620) assay was first developed by Skehan et al. (1990) for the determination of drug-induced cytotoxicity and cell proliferation. Initially, 7500 cells were seeded in 96 well plates and incubated for 24 h. Different concentrations of ceramide and sphingomyelin were applied to the cell lines and incubated for an additional 48 h. SRB assay was performed as described in Al Janabi and Hadjira (Al-Janabi et al., 2020; Hadjira et al., 2021). Briefly, cells were fixed by adding 20% of Trichloroacetic acid (TCA) and incubated for 1.5 h at 4°C. Cells were then washed three times with 300 µL of ddH_2O and left to dry for 30 min at 50 °C. After the wells were completely dried, each well was treated with 0.4 % SRB dye (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at room temperature, then washed three times with 300 µL of 1% acetic acid (CH_3COOH) and kept at 50°C for 30 min. At the final step, cells were incubated with 10 mM Tris Base solution for 30 min room temperature, and absorbance values at

492 nm wavelength were recorded in a microplate reader. The analysis of control experiments was calculated using the formula:

$$\% \text{ Cell viability} = \frac{\text{Absorbance of drug} - \text{treated cells in each well}}{\text{Absorbance of control cells}} x100$$

OD values corresponding to the control cells were taken as 100 for each cell line and the response of treated cells was calculated accordingly. SRB dye was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Statistical analysis

Data in graphs are expressed as mean ± SEM. Statistical comparisons and graphs were made using Prism (GraphPad Software) (Cacan & Ozmen, 2020). Statistical significance was determined using Student's t-test, and IC50 values were calculated by regression analysis (*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$).

Results

Effect of ceramide on cell viability

The effect of ceramide in which dissolved in DMSO on cell viability was determined at concentrations of 5, 10, 20, 40, 60, 100, 120, and 125 µM in four cell lines. Cell viability start to decrease significantly at the concentration of 40 µM compared to the control samples in all cell lines (Figure 1). Ceramide showed a significant cytotoxic effect when applied at 40 µM and higher concentrations, and the decrease in cell viability was found to be statistically significant in all three tumor cell lines. Similar effects were observed in CCD-18Co normal cell line as well; however, the number of viable cells were slightly higher in this normal cell line as compared to tumor cells. (Figure 1).

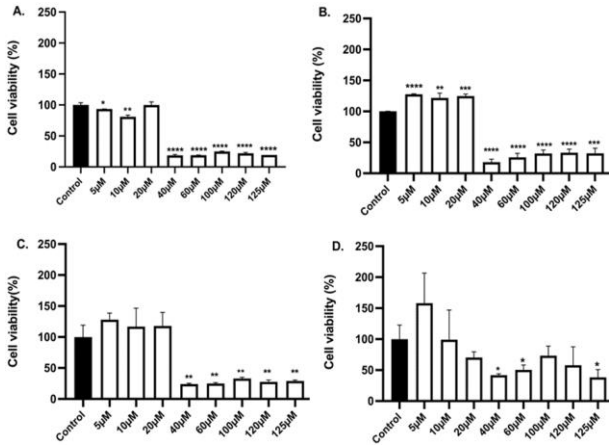

Figure 1. Effects of different ceramide (dissolved in DMSO) concentrations on cell viability. A. C6 (rat glioma), B. OV2008 (ovarian cancer), C. HT-29 (human colorectal adenocarcinoma), D. CCD-18Co (normal colon fibroblast cell). The data in the graphs were expressed as mean ± SEM. *: $p \leq 0.05$; **: $p \leq 0.01$; ****: $p \leq 0.0001$). Statistical comparisons were performed using GraphPad Prism 7 software.

We also calculated IC50 values and found C6 (32.7 µM) cell line the most sensitive cell line and CCD-18Co (56.91 µM) was the most resistant cell line to ceramide treatment. The effect of ceramide on OV2008 and HT-29 cell lines was determined similarly and IC50 values were calculated as approximately 42 µM. (Table 1). The ceramide compound dissolved in DMSO exhibited lower IC50 values in cancer cell lines compared to the healthy cell line (CCD-18Co); however, the difference between them was quite limited. This observation suggests that, when dissolved in DMSO, ceramide does not demonstrate selective cytotoxicity, inducing similar levels of toxicity in both healthy and cancer cell lines.

Table 1. IC50 values for Ceramide and Sphingomyelin (All values are given in µM)

Cell Line/Solvent	Ceramide		Sphingomyelin	
	DMSO	Ethanol	DMSO	Ethanol
C6	32.70	0.36	0.11	0.25
OV2008	41.69	0.45	0.08	0.28
HT-29	42.16	0.45	0.08	0.25
CCD-18Co	56.91	0.33	0.07	0.48

We next determined the effect of ceramide which dissolved in ethanol on cell viability. Here, we used concentrations of 50, 100, 150, 200, 250, 300, and 400 nM in 4 different cell lines and an additional concentration of 500 nM in HT-29 and CCD-18Co cell lines. While ceramide started to show a significant decrease in cell viability at 250 nM concentration in C6 (*: $p \leq 0.05$) cell line, a significant decrease was observed in OV2008 (**: $p \leq 0.01$), and HT-29 (*: $p \leq 0.05$) cell lines following 400 nM concentration (Figure 2). IC50 concentrations calculated by regression analysis of ceramide dissolved in ethanol were calculated (Table 1). According to IC50 concentrations, the most sensitive cell line to ceramide dissolved in ethanol was CCD-18Co (0.33 µM), while the least resistant cell lines were OV2008 (0.45 µM) and HT29 (0.45 µM) cell lines (Table 1). Here, we interestingly observed more toxic effects in the CCD-18Co cell line as compared to DMSO solvent.

In our study, it was observed that ceramide exhibited remarkably low IC50 values in both cancer (C6, OV2008, and HT-29) and healthy cell line (CCD-18Co) when dissolved in ethanol. This finding indicates that ceramide, when dissolved in ethanol, demonstrates a high cytotoxic effect with low selectivity between healthy and cancer cells.

Effect of sphingomyelin on cell viability

The effect of sphingomyelin which is dissolved in DMSO on cell viability was examined in the same cell lines at concentrations of 12.5, 25, 50, 75, 100, 150, 200, and 250 nM, respectively. A significant decrease in cell viability was observed at 100 nM concentration in C6 (****: $p \leq 0.0001$) and HT-29 (**: $p \leq 0.01$) cell lines, while OV2008 (****: $p \leq 0.0001$) cells showed a significant decrease following 150 nM sphingomyelin treatment (Figure 3). CCD18-Co cell line started to show a significant decrease at 50 nM (*: $p \leq 0.05$)

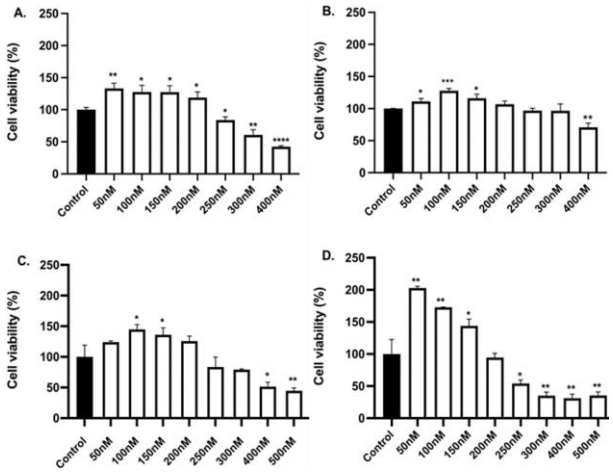

Figure 2. Effects of ceramide dissolved in ethanol on cell viability. A. C6 cell line, B. OV2008 cell line, C. HT-29 cell line, D. CCD-18Co cell line. The data in the graphs were expressed as mean ± SEM. *: p ≤ 0.05; **: p ≤ 0.01; ***: p ≤ 0.001; ****: p ≤ 0.0001). Statistical comparisons were performed using GraphPad Prism 7 software.

concentration and about 50% of these cells start to die at this concentration. The IC50 values of sphingomyelin dissolved in DMSO were showed in the table (Table 1). According to IC50 concentrations, the most sensitive cell line to sphingomyelin dissolved in DMSO was CCD-18Co (0.07 µM), while the most resistant cell line was C6 (0.11 µM). The effect of sphingomyelin was determined similarly in OV2008, and HT-29 cell lines, and IC50 values were calculated as approximately 0.08 µM. Sphingomyelin dissolved in DMSO exhibited remarkably low IC50 values in both healthy and cancer cell lines. However, due to the minimal differences between these values, the dissolution of sphingomyelin in DMSO does not demonstrate cellular selectivity.

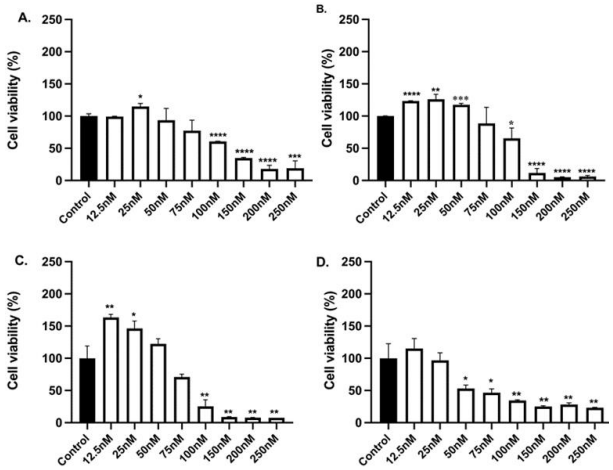

Figure 3. The effects of sphingomyelin dissolved in DMSO at different concentrations on cell viability. A. C6, B. OV2008, C. HT-29 and D. CCD-18Co cell lines. The data in the graphs were expressed as mean ± SEM. *: p ≤ 0.05; **: p ≤ 0.01; ***: p ≤ 0.001; ****: p ≤ 0.0001).

The effect of sphingomyelin dissolved in ethanol on cell viability was also determined. Cells were treated with 12.5, 25, 50, 400, 600, and 800 nM concentrations. The results revealed that all used cell lines showed significant decrease in cell viability following 400 nM sphingomyelin treatment which dissolved in ethanol (Figure 4). The decrease was statistically significant in C6 (****: p ≤ 0.0001), OV2008 (**: p ≤ 0.01), HT-29 (**: p ≤ 0.01), and CCD-18Co (**: p ≤ 0.01), cell lines.

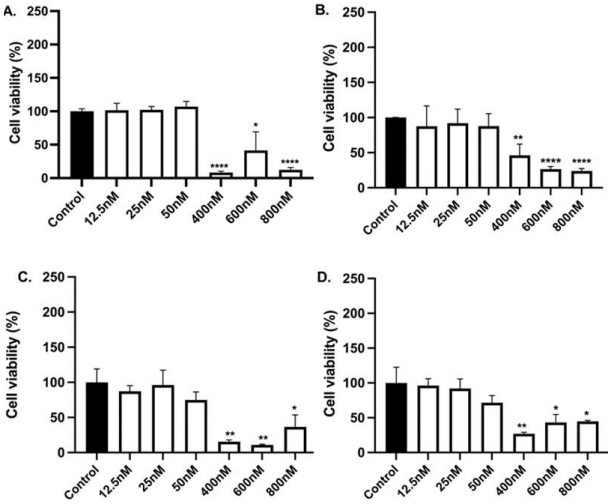

Figure 4. Effects of sphingomyelin dissolved in ethanol at different concentrations on cell viability. A. C6, B. OV2008, C. HT-29 and D. CCD-18Co cell lines. The data in the graphs were expressed as mean ± SEM. *: p ≤ 0.05; **: p ≤ 0.01; ****: p ≤ 0.0001).

Interestingly, CCD-18Co normal cells didn't show a significant decrease in cell viability at higher concentrations. The IC50 concentrations calculated by regression analysis of sphingomyelin dissolved in ethanol are given in the table. According to IC50 concentrations, the most sensitive cell lines to sphingomyelin dissolved in ethanol were C6 and HT-29 cell lines (0.25 µM), while the least affected cell line was CCD-18Co (0.45 µM). The IC50 value of the OV2008 cell line was 0.28 µM.

The results obtained from the application of sphingomyelin dissolved in ethanol and DMSO indicate that when sphingomyelin is dissolved in DMSO, no significant differences in IC50 values were observed between the healthy cell line (CCD-18Co) and the cancer cell lines (OV2008, HT-29, and C6). However, when sphingomyelin was dissolved in ethanol, it effectively reduced the viability of cancer cell lines by 50% at concentrations ranging from 0.25 to 0.28 µM, without reducing the viability of the CCD-18Co healthy cell line by 50%. Furthermore, the IC50 value for sphingomyelin dissolved in ethanol was higher in the CCD-18Co cell line compared to its application in DMSO, indicating increased selectivity when applied in ethanol.

Our findings suggest that sphingomyelin in an ethanol environment and at an appropriate concentration could serve as a more effective and selective anti-cancer agent. These results highlight the importance of formulating sphingomyelin with suitable solvents such as ethanol in cancer treatment. Additionally, they provide valuable insights into

enhancing the efficacy of cancer therapies with lower IC50 values while minimizing toxicity to healthy tissues.

Discussion

One of the major challenges in cancer therapy is the disruption of the delicate balance between cell proliferation and programmed cell death. Ceramide and sphingomyelin, the two principal members of the sphingolipid family, are bioactive lipids involved in diverse cellular processes, including signal transduction, apoptosis, cell cycle regulation, and stress responses (Zhakupova et al., 2025). These molecules are known to exert antiproliferative and proapoptotic effects in tumor cells while promoting survival and proliferation in normal cells, thus representing promising therapeutic targets (Alizadeh et al., 2023; Morad and Cabot, 2013; Kolesnick, 2002; Shen et al., 2025).

The biological activities of these lipids are not only concentration-dependent but are also significantly influenced by the solvent systems in which they are dissolved. Lipophilic environments such as DMSO or ethanol may affect membrane permeability and the intracellular localization of ceramide and sphingomyelin, thereby modulating their cytotoxic or proliferative effects (Nguyen et al., 2025; Xie et al., 2025). In this context, the present study investigated the concentration- and solvent-dependent effects of ceramide and sphingomyelin on cell viability in four different cell lines, including one healthy cell line (CCD-18Co) and three cancer cell lines (C6, OV2008, and HT-29).

Ceramide showed a bimodal effect on cell viability depending on concentration and solvent. When applied in DMSO, low concentrations slightly increased cell viability, whereas higher concentrations (particularly 100 µM) significantly reduced viability in cancer cell lines (66–75%) while causing relatively lower toxicity (26%) in healthy cells. In contrast, ceramide dissolved in ethanol demonstrated reduced selectivity, with lower cytotoxicity in cancer cells (29–58%) but higher toxicity in healthy cells (65%). These findings suggest that ceramide, when delivered in DMSO, can selectively target cancer cells with minimal effects on normal cells, and may therefore serve as a more favorable formulation for therapeutic purposes.

Sphingomyelin exhibited similar solvent- and concentration-dependent effects. High concentrations of sphingomyelin led to significant reductions in cell viability across all cell lines, particularly when dissolved in DMSO (81–96% in cancer cells), though this also resulted in substantial toxicity in the healthy cell line. Conversely, ethanol-dissolved sphingomyelin caused slightly lower cytotoxicity in cancer cells (76–92%) but was less damaging to healthy cells. This suggests that ethanol may provide a more selective solvent environment for sphingomyelin, potentially enhancing its therapeutic specificity.

Our findings are supported by prior studies. For example, Fillet et al. (2003) and Ji et al. (2010) reported dose-dependent cytotoxicity of ceramide in HCT116, OVCAR, L3.6, and MCF-7 cell lines. Other studies further demonstrated that higher concentrations of ceramide induce significant decreases in cell viability, whereas lower concentrations had negligible effects (Chang et al., 2018; Zhu et al., 2003; Selzner et al., 2001). In a study by Toman et al. (2002), ceramide exhibited a bimodal response in neuronal models, where low doses enhanced cell survival and differentiation, while higher doses induced cell death. This biphasic response aligns with our in vitro results, highlighting the importance of dose optimization in therapeutic applications.

Collectively, these findings emphasize the dual role of ceramide and sphingomyelin as both cytotoxic and pro-survival agents, depending on dosage and formulation. Their selective effects on cancer cells, when optimized, point to their promising utility as modulators of cell fate in targeted cancer therapy.

Conclusion

This study demonstrates that ceramide and sphingomyelin possess both proliferative and antiproliferative properties under in vitro conditions, and that these effects are strongly influenced by concentration and solvent type. The observed selective cytotoxicity in cancer cell lines especially when ceramide is administered in DMSO at an optimal concentration underscores the critical importance of solvent and dosage selection in therapeutic design. Furthermore, the capacity of these sphingolipid molecules to promote proliferation in healthy cells suggests their potential application in tissue regeneration. To advance their clinical relevance, future studies should focus on delineating the molecular signaling pathways involved and validating the in vivo anticancer efficacy of ceramide and sphingomyelin. These bioactive lipids hold considerable promise as regulators of cell proliferation, apoptosis, stress responses, and differentiation, offering substantial opportunities for the development of targeted therapies and biomarker-driven treatment strategies in oncology.

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Author Contributions

Conceptualization: N.Y., E.C., Data Curation: S.B.S, A.K., Formal Analysis: S.B.S, A.K., E.C., Funding Acquisition: N.Y., S.B.S., E.C., Investigation: S.B.S, A.K., Project Administration: E.C., Resources: S.B.S, E.C., Supervision: N.Y., E.C., Visualization: A.K., E.C., Writing - original draft: S.B.S, A.K., E.C., Writing -review and

editing: E.C. All authors discussed the results and commented on the manuscript.

Conflict of Interest

The authors declare there is no conflict of interest.

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