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ANTIPROLIFERATIVE EFFECTS OF VITAMIN K2 IN OSTEOSARCOMA CELLS: COMPARISON OF DIFFERENT CYTOTOXICITY ANALYZES

K2 VİTAMİNİNİN OSTEOSARKOMA HÜCRELERİNDE ANTİPROLİFERATİF ETKİLERİ: FARKLI SİTOTOKSİSİTE ANALİZLERİNİN KARŞILAŞTIRILMASI

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Öz

Amaç
Bu çalışmanın amacı, K2 vitamininin antiproliferatif etkilerini saptamak için yaygın olarak kullanılan dört sitotoksisite analizinin (XTT, nötral kırmızı alım, kristal viyole ve propidyum iyodür boyama) doğruluk potansiyelini karşılaştırmaktır.

Gereç ve Yöntem
Osteosarkoma hücre hattı olan Saos-2 hücreleri, 48 ve 72 saat boyunca K2 vitaminine (10 µM, 20 µM, 30 µM, 40 µM, 50 µM, 100 µM ve 200 µM) maruz bırakıldı. Tetrazolyum tuzu testi (XTT), nötral kırmızı alım testi (NR), kristal viyole testi ve propidyum iyodür (PI) boyama yapılarak hücrelerin canlılık oranlarına göre K2 vitamini sitotoksisitesi belirlendi ve farklı dozların IC_{50} değerleri hesaplandı. Analiz sonuçları tek yönlü varyans analizi (ANOVA) ve Tukey testiyle değerlendirildi.

Bulgular
K2 vitamininin osteosarkoma hücreleri üzerinde sitotoksik etkileri olduğu sırasıyla XTT, NR, kristal viyole testi ve PI yöntemleri kullanılarak belirlendi ve IC_{50} değerleri 48 saatlik maruziyet için sırasıyla 61,93; 40,21; 62,11; 70,57 µM ve 72 saatlik maruziyet için sırasıyla 75,44; 68,22; 41,66; 88,01 µM olarak saptandı.

Sonuç
İstatistiksel analizler çalışmada uygulanan testlerden elde edilen sonuçların farklılık gösterdiğini ortaya koymuştur. Ayrıca propidyum iyodür boyama sonucundaki canlılık oranlarının diğer testlerle elde edilen sonuçlara kıyasla çarpıcı bir şekilde daha yüksek olduğu belirlenmiştir. Sitotoksisite analizlerinde sonuçların yanlış yorumlanmasını önlemek için farklı zamanlarda ve farklı konsantrasyonlarda inkübasyonlar gerektiği ve ayrıca tetrazolyum tuzu temelli testlerin metabolik olmayan diğer testlerle desteklenmesi gerektiği sonucuna varılmıştır.

Anahtar Kelimeler: Hücre canlılık testleri, İn vitro sitotoksisite, K vitamini, Osteosarkoma

Abstract

Objective
The aim of this study was to compare four commonly used cytotoxicity assays (XTT, neutral red uptake, crys-

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tal violet assay, and propidium iodide staining) which analyzed the antiproliferative effects of vitamin K2.

Material and Method
Saos-2 cells, an osteosarcoma cell line, were exposed to vitamin K2 at different concentrations (10 µM, 20 µM, 30 µM, 40 µM, 50 µM, 100 µM and 200 µM) for 48 and 72 hours. Tetrazolium salt test (XTT), neutral red uptake (NR) assay, crystal violet assay, and propidium iodide (PI) staining were performed to determine cytotoxic potential of vitamin K2 in terms of the cell viability and IC_{50} values. The results were evaluated with one-way analysis of variance (ANOVA) and the Tukey test.

Results
Cytotoxic effects of vitamin K2 on osteosarcoma cells were analyzed with XTT, neutral red, crystal violet assay, and propidium iodide, respectively. IC_{50} values were determined exposure to 61.93; 40.21; 62.11; 70.57 µM vitamin K2 for 48 and 75.44; 68.22; 41.66; 88.01 µM vitamin K2 for 72 hours.

Conclusion
Statistical analysis revealed that there is a significant statistical difference between four tests used in this study. In addition, it was determined that the viability rates in propidium iodide staining were higher than other tests for cytotoxicity analyses. It has been concluded that incubations at different concentrations were required to prevent misinterpretation of results in cytotoxicity analyses, and tetrazolium salt-based tests should be supplemented with other non-metabolic tests.

Keywords: Cell viability tests, In vitro cytotoxicity, Osteosarcoma, Vitamin K.

Introduction

Osteosarcoma (OS) is the most malignant and common type of primary bone tumor. It is generally seen in children, adolescents, and young adults (1). With the advancement of chemotherapy, the long-term treatment rate after surgery for nonmetastatic osteosarcoma increased from 25% to 60%, and the average 5-years survival is around 70% (2).

Many studies in recent years have investigated the anti-tumor effects of vitamin K (VK). These studies have shown that vitamin K inhibits growth and induces apoptosis in cancer cells, including leukemia, hepatocellular carcinoma, lung, breast, bladder, and prostate cancers (3-8). Vitamin K is an essential fat-soluble vitamin that is effective in the synthesis of active forms of coagulation proteins and is of vital importance in ensuring normal coagulation (9). The vitamin K family consists of three groups; phylloquinone (K1), menaquinone (K2) and menadione (K3) (10). Menaquinones have both pro-osteoblastic and anti-osteoclastogenic properties. It also has the effect of improving bone quality rather than preserving bone mineral density (11). In this study, we aimed to investigate the cytotoxic effects of VK2 on osteosarcoma (Saos-2) cells by different cytotoxicity assays.

Cytotoxicity studies are conducted to assess cell death caused by a substance by measuring cell number if the substance has a cytotoxic potential (12). Cell-based cytotoxicity studies have emerged as an alternative to animal experiments and have become frequently preferred in toxicology laboratories due to their ease of application and compatibility with data obtained from in vivo studies. Cytotoxicity varies depending on the dose and exposure time of the substance under investigation (13). Dye exclusion, colorimetric assays, fluorometric assays, and luminometric assays are commonly used methods for the determination of cytotoxicity (14).

XTT (2,3-Bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide) one of the enzymatic test methods widely used in cytotoxicity evaluations, was first described by Scudiero et al. (15). This assay is based on the ability of the tetrazolium salt XTT to be reduced to orange-colored formazan compounds by metabolically active cells. The orange-colored formazan is soluble in water, and its density can be measured with a spectrophotometer. There is a linear relationship between the density of formazan and the number of viable cells (14).

The neutral red uptake assay is a method frequently used in cytotoxicity tests that allow quantitative estimation of the number of viable cells in cultures (16). Neutral red, a weakly cationic dye, penetrates cell membranes by non-ionic passive diffusion (active transport) and accumulates in lysosomes (17), where it binds to the anionic and/or phosphate groups of the lysosomal matrix by electrostatic hydrophobic bonds (18,19). The dye is then extracted from living

cells using an acidified ethanol solution, and the absorbance of the solubilized dye is measured using a spectrophotometer (20).

Another method of detecting the continued adhesion of cells is staining living cells with a crystal/cresyl violet dye that binds to proteins and DNA. Cells that undergo cell death lose their adhesion and subsequently disappear from the cell population, reducing the amount of crystal violet staining in a culture. This protocol describes a rapid and reliable screening method suitable for examining the effect of chemotherapeutics or other compounds on cell survival and growth inhibition (21).

One of the most common methods of assessing cell viability is the measurement of cell membrane integrity. Dyes such as trypan blue and propidium iodide cannot freely pass through the membrane of living cells (13). PI is a nucleic acid-specific dye that emits red fluorescence that can only stain dead cells, that is, cells with impaired membrane integrity (22). For this reason, it is widely used in the evaluation of cell death and apoptosis or the determination of DNA content in cell cycle analysis.

Based on these findings, our aim in this study is to compare the effects of four different cytotoxicity methods on the same cell line.

Material and Method

Preparation of Cells

The Saos-2 cells received from the nitrogen tank were thawed in a 37°C water bath and centrifuged at 1800 rpm for 10 minutes. The supernatant was removed and the pelleted cells were grown in Dulbecco's Modified Eagle Medium High glucose (Lonza, USA) supplemented with 10% fetal bovine serum (Biochrom, Germany), 1% penicillin-streptomycin (Biochrom, Germany), and 1% antifungal at 37°C in a humidified atmosphere supplemented with 5% CO_2. Cells that became 80-90% confluent were passaged at the desired density ($5x10^3$) into 96-well plates with fresh medium for analysis. The densities, viability, and proliferation rate was monitored daily under an inverted microscope.

Preparation of Vitamin K2

Vitamin K2 (V9378, Sigma-Aldrich, USA) used in the study was supplied in powder form. VK2 was dissolved in dimethyl sulfoxide (DMSO) (OriGen, USA). Stock solutions prepared at a concentration of 10 mM were sterilized by 0.2 µM filters, divided into disposable aliquots and stored at -20ºC. 10 µM, 20 µM, 30 µM, 40 µM, 50 µM, 100 µM and 200 µM vitamin K2 concentrations were prepared by diluting 500 µM intermediate stock solution with the cell culture medium. Control groups (cultured in medium) and DMSO control groups (cultured in 0.1-2% DMSO containing medium) were also included in the study. However, since no statistically significant change in cell viability was observed in the DMSO control groups, the data are not shown.

Preparation of Osteosarcoma Cells for the Tests

Osteosarcoma cells seeded in 96-well cell culture plates ($5x10^3$) were incubated for 24 hours in 5% CO_2 by adding 100 µl of medium per well. After the cells adhered, the medium was removed. Fresh mediums containing vitamin K2 at concentrations of 10 µM, 20 µM, 30 µM, 40 µM, 50 µM, 100 µM and 200 µM were added and incubated at 37°C for 48 and 72 hours. Cell viability percentages were calculated based on the control group. The tests were repeated three times at different times.

XTT

After osteosarcoma cells were prepared 50 µl of XTT reaction solution (Biotium, 30007) was added to each well. After being wrapped in aluminum foil to avoid light and incubated for 2 hours at 37°C in an environment containing 5% CO_2 and 95% humidity, measurements were made on the Elisa reader (Thermo Scientific, Varioskan Flash Multiplate Reader) at 475 nm and 630 nm (background) wavelengths. The values obtained by subtracting the background absorbance from the signal absorbance to obtain normalized absorbance values were used in cell viability calculations.

Neutral Red Uptake Assay

The osteosarcoma cells were prepared as described above. According to the protocol of the test, at the end of the period, an amount of 0.33% neutral red solution (Sigma-Aldrich, N-2889) was added to 10% of the volume of the culture medium and allowed to incubate for 2-4 hours. At the end of the incubation period, the culture medium was carefully aspirated and the cells were quickly washed with PBS. 100 µl of neutral red dissolving solution (50% ethanol, 1% acetic acid, 49% dH_2O) was added to each well to dissolve the red precipitates. The plates were mixed on a microplate shaker at medium speed for 15 minutes to completely dissolve the red precipitates. The absorbance of the wells was measured at 540 nm and 690 nm (background) wavelengths and the values obtained by subtracting the absorbance value measured at 690 nm from the absorbance value measured at 540 nm were used in cell viability calculations.

Crystal Violet Assay

Osteosarcoma cells were prepared in 96 well plate and at the end of the incubation period, the medium was removed and washed with PBS. After adding 50 µl of 0.5% crystal violet solution (Sigma-Aldrich, USA) per well, it was kept at room temperature for 20 minutes and then washed 4 times with distilled water. The plates were then left to dry at room temperature for at least two hours with the lid open. 200 µl of methanol was added to the per well and the lid was closed. After waiting at room temperature for 20 minutes, the measurement was made on the Elisa reader at 590 nm (21).

Propidium Iodide Staining

Unlike other methods, cells were seeded in 6-well ($3x10^5$) cell culture plates. Cells were trypsinized after culture period. Then cells were harvested from the culture plates and centrifuged at 1800 rpm for 10 minutes. The medium was added, and cells were washed to eliminate the trypsin effect. The supernatant was discarded, 100 µl medium was added to the tube, and cells were resuspended and analyzed by flow cytometry after adding 1 µl of PI (Biolegend, CA) (23).

Calculation of IC_{50} Value

Viability absorbance values obtained as a result of cytotoxicity tests of osteosarcoma cells in which vitamin K2 was applied were normalized with the control value (without VK2). Absorbance values were graphed using Graphpad Prism 8.0 program and IC_{50} (*Half-maximal inhibitory concentration*) values were calculated.

Statistical Analysis

Statistical analysis was performed using the IBM SPSS 25.0 package program. It was observed that the data were normally distributed after the conformity of the groups to the normal distribution was made according to the Shapiro-Wilk test. Comparisons between groups were evaluated with One-Way analysis of variance (ANOVA). The differences between the means and the control group were evaluated with the Tukey test. Comparison data were given as mean ± standard deviation. The statistical significance level was accepted as $p<0.05$.

Results

Saos-2 cells were exposed to different concentrations of vitamin K2 for 48 and 72 hours and cytotoxicity tests were made by XTT, NR assay, crystal violet assay and PI staining. The IC_{50} values calculated for each incubation time (48 and 72 hours) are shown separately in Table 1.

In order to calculate the viability ratios of cells in each method; the absorbance value in each concentration for each sample divided to the absorbance value of the untreated control and then multiplied by 100.

The cytotoxic effects of vitamin K2 on Saos-2 cells were determined by the XTT method for 48 and 72-hours, and the results are shown in Figure 1. According to the pairwise comparisons between the groups, cell viability decreased significantly between the control group and the 100 µM; 200 µM groups ($p<0.05$).

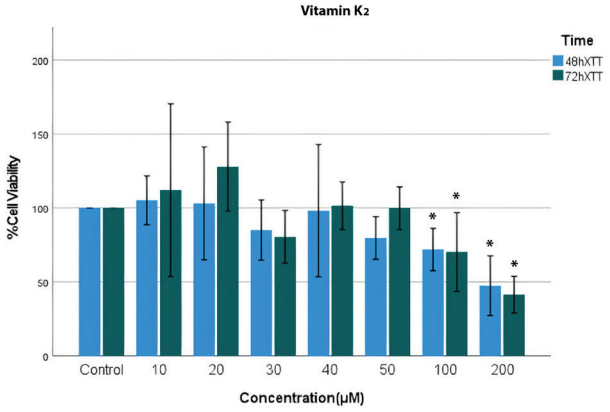

Figure 1:
Cytotoxic effects of vitamin K2 on Saos-2 cells.
Cell viability was evaluated compare to control using XTT method. $p<0.05$ was accepted as statistically significant.

Table 1 IC_{50} values of the applied methods.

IC_{50} values (µM)	48 h	72 h
XTT	61.93	75.44
NR assay	40.21	68.22
Cresyl violet	62.11	41.66
PI	70.57	88.01

The cytotoxicity results after exposure of VK2 for 48 and 72 hours for NR assay were shown in Figure 2. According to the pairwise comparisons between the groups, cell viability decreased significantly between the control group and the 200 µM groups ($p<0.05$).

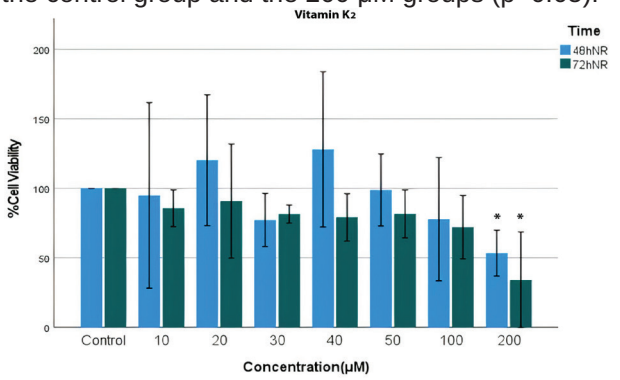

Figure 2:
Cytotoxic effects of vitamin K2 on Saos-2 cells.
Cell viability was evaluated compare to control using NR method. $p<0.05$ was accepted as statistically significant.

The cytotoxicity results, after exposure of VK2 for 48 and 72 hours for crystal violet test method, were shown in Figure 3. According to the pairwise comparisons between the groups, cell viability decreased significantly between the control group and the 50 µM; 100 µM and 200 µM groups ($p<0.05$).

The cytotoxicity results, after exposure of VK2 for 48 and 72 hours PI method, are shown in Figure 4. According to the pairwise comparisons between the groups, cell viability decreased between the control group and the 200 µM groups for 72 h ($p<0.05$).

When we analyzed four cytotoxicity tests to determine the antiproliferative effects of vitamin K2; XTT, NR, and crystal violet results show a dose-dependent decrease in cell viability compared to control for both 48 and 72 hours, but PI results show high cell viability (Figure 5).

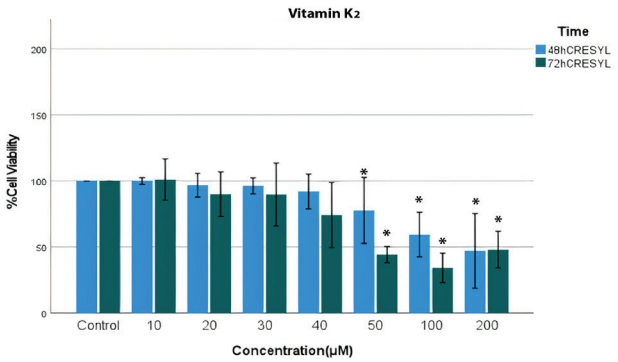

Figure 3:
Cytotoxic effects of vitamin K2 on Saos-2 cells.
Cell viability was evaluated compare to control using crystal violet assay method. $p<0.05$ was accepted as statistically significant.

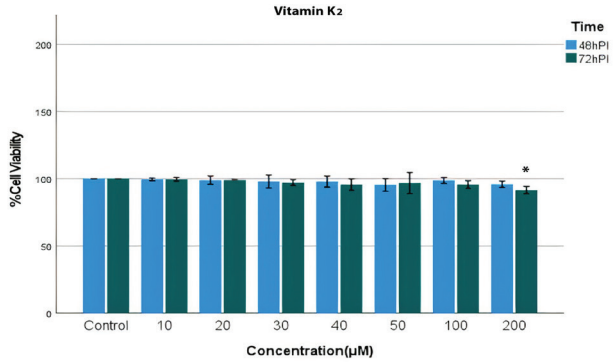

Figure 4:
Cytotoxic effects of vitamin K2 on Saos-2 cells.
Cell viability was evaluated compare to control using PI method. $p<0.05$ was accepted as statistically significant.

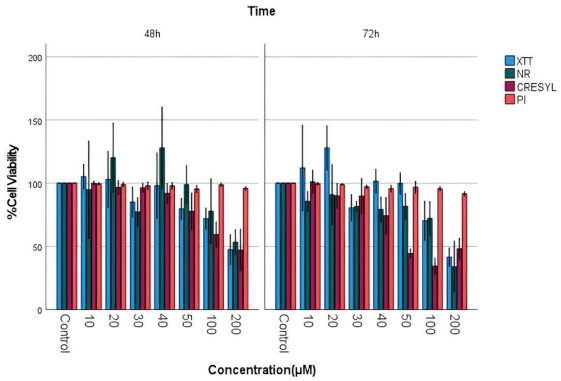

Figure 5:
Effect of vitamin K2 at 10-200 µM concentrations on Saos-2 cells for 48 and 72 hours using four different cytotoxicity assays.

Discussion

Cytotoxicity tests provide basic information about the behavior of a substance that is likely to be a drug or whose toxic effect is unknown and form a source for future animal or clinical trials. For this reason, the accuracy and reliability of the data obtained from the studies are very important. The accuracy of the data depends not only on the method but also on many variables such as the knowledge and experience of the researcher, the type of cell used, the pH of the medium, ion concentration (calcium, potassium, sodium), environmental factors (temperature, humidity), and the duration of the cell cycles (12). Numerous methods have been developed to measure parameters related to cell death, and each method has its advantages and disadvantages.

However, researchers think that none of these methods is sufficient alone and it is appropriate to prefer combinations of different methods, taking into account the variability of the methods (24). Cell death is a highly heterogeneous process; since the signaling pathways leading to cell death can differ even in relatively similar experimental environments, it is at the discretion of each researcher to decide which biochemical parameters are the most appropriate to monitor the mechanical characterization of cell death in the experimental setup (24, 25).

Although many studies have been conducted in recent years on the anticancer effects of vitamin K, which is known to have important roles in blood coagulation and bone metabolism, studies on its effects on osteosarcoma cells are very rare (26). In our study, the effect of vitamin K2 on the survival of osteosarcoma cells was investigated with different in vitro cytotoxicity analyses to fill this gap, and IC_{50} values were found at different doses.

According to our results, we determined the lowest IC_{50} value in osteosarcoma cells with the NR test at 40.21 for 48 hours, and the highest IC_{50} value in the PI method at 88.01 for 72 hours. In a similar study by Zenmyo et al., it was shown that MG-63 osteosarcoma cells were applied 5 µM vitamin K2 for 48 hours to inhibit cell proliferation, but the IC_{50} value was not specified (27). In another study by Otsuka et al. on hepatocellular carcinoma cells, cells were exposed to VK2 for 5 days, and decreased growth of cells was reported, with an IC_{50} value (45 µM) close to our result (28). When the apoptosis induction of VK2 in lung cancer cell lines was examined, it was reported that it suppressed growth in a dose-dependent manner and the IC_{50} value was calculated as 7.5-75 µM (29). Yokoyama et al. reported that VK2 suppressed growth in small cell lung cancer, and the IC_{50} value was found to be 16-64 µM (30). It has been reported that VK2 inhibits the proliferation of hepatocellular carcinoma cells by caspase 8 activation and induction of apoptosis involving p53, and the IC_{50} value is 9.73 µM in 24 hours (31). On the other hand, it has been reported that cells are minimally affected after VK2 (100 µM) is administered at high concentrations to non-cancer cells. All these results show that vitamin K2 has a low cytotoxic effect in non-cancer cells, while it has anticancer activity in different cancer cells (5). Since studies examining the effects of vitamin K2 on osteosarcoma cells are rare, it is thought that the difference between the IC_{50} values found when we look for its cytotoxic effects in different cancers is due to the different fetal bovine serum and proliferation conditions, which are effective on cell development. In addition, since the sensitivity, variability, and contents of the tests used in cytotoxicity analyzes are different from each other, they may affect the data. Therefore, the same cell lines may give different responses in different environments.

Fotakis and Timbrell compared four different cytotoxicity assays (LDH, a protein, NR, and MTT assays) by exposing hepatocellular carcinoma cells to cadmium chloride, observing different sensitivity for each assay. They stated that NR and MTT assays are most sensitive in detecting cytotoxic events (32). In another study, three different cytotoxicity assays (NR, resazurin test and TB staining) were performed to determine $VOSO_4$ cytotoxicity in the CHO-K1 cell line. While NR and resazurin tests performed after 24 hours of exposure showed similar sensitivity; the NR test after 48 hours showed higher sensitivity than the resazurin test in detecting vanadyl-induced toxicity. The TB test, performed after 24 and 48 hours of exposure was found to be less sensitive than other tests (20). When the analyzes we used in our study were evaluated, it was determined that PI staining was less sensitive than other tests. Therefore, the present results highlight the role of lysosomes, mitochondria, DNA and proteins in the mechanism of VK2-induced toxicity in Saos-2 cells. When the studies are evaluated in terms of the sensitivity of the tests taken into account, it is observed that different results are obtained. It has been reported by studies that the accuracy of the data does not depend only on the method but on many variables such as the knowledge and experience of the researcher, the type of cell used, the pH of the medium, the ion concentration (calcium, potassium, sodium), environmental factors (temperature, humidity), and the duration of the cell cycles (12).

In conclusion, it should be noted that incubations of various concentrations at different times are necessary to avoid over-or under-estimate the toxicity of a substance and to distinguish between effects on particular organelles or overall cytotoxicity. It is recommended to use more than one test to increase the reliability of the results obtained and to determine cell viability in vitro studies.

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Conflict of Interest Statement
The authors have no conflicts of interest to declare.

Ethical Approval
This article does not contain any studies with human or animal subjects.

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Availability of Data and Materials
Data available on request from the authors.

Authors Contributions
BÖ: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Funding acquisition; Project administration; Writing-original draft.

TÖS: Conceptualization; Funding acquisition; Resources; Validation; Writing-review & editing.

HMÖ: Formal analysis; Validation; Writing-review & editing.

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