Journal of Research in Pharmacy

Research Article

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Caffeine Increased Antitumor Effects of Paclitaxel (PTX) in MCF-7 and MDA-MB-231 Breast Cancer Cells

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Received: 03 August 2023 / Revised: 20 September 2023 / Accepted: 20 September 2023

ABSTRACT: Although progress has been made in the diagnosis and treatment of breast cancer, which is one of the most important causes of women's health, it is still one of the leading causes of death in women. One of the most prominent causes for this is illness resistance to the medications employed in treatment. For this reason, the trend towards combination therapy research has increased in addition to conventional therapy. In this study, we aimed to investigate the effect of co-administration of caffeine (CAF) and paclitaxel (PTX), which is frequently used in breast cancer, on MDA-MB-231 and MCF-7 cells. For this purpose, 4 groups were determined as control, CAF, PTX and CAF+ PTX. MTT assay was used to assess cell viability and the appropriate dose for CAF was determined. The apoptotic effect of the drug combination on cell lines was evaluated with the TUNEL method, and it was determined at what stage it paused cell division by cell cycle analysis. According to the study's findings, the results indicated that CAF induced apoptosis in breast cancer cells and the best effect was in the group administered with PTX. Furthermore, it was discovered that CAF and PTX in the MCF-7 cell lines, both together and separately, blocked cell division in the S phase in MCF-7 cell lines. These results are promising for future studies that will prove the usefulness of CAF as an adjuvant in the treatment of breast cancer.

KEYWORDS: Caffeine; paclitaxel; breast cancer; apoptosis; cell cycle.

1. INTRODUCTION

Breast cancer (BC) has become the most commonly diagnosed cancer, with a significant increase globally, and represents a major challenge to public health [1]. It is the leading cause of cancer mortality among females, with an estimated 685,000 deaths from breast cancer in 2020 [2]. BC is a heterogeneous disease, and the prognosis differs between cell subtypes [3, 4]. MCF-7 cells are commonly preferred in hormone-sensitive BC in-vitro studies [5]. Triple-negative breast cancer (TNBC) is an aggressive subtype of BC that lacks amplification of the estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2), accounting for 12-20% of all breast cancers [6, 7]. The absence of these receptors renders endocrine and HER2-targeted therapies ineffective, and the prognosis remains poor [8]. MB-231 is a well-known TNBC representative with aggressive behaviors such as metastasis, cell aggregation, and treatment resistance [9].

PTX is a chemotherapeutic drug that is frequently used to treat cancers such as uterine and breast cancer. PTX inhibits the cell cycle and induces apoptosis in BC cells by altering microtubule dynamics during cell division. [10]. However, there have been reports of severe side effects of PTX during treatment, including neurotoxicity. Furthermore, BC resistance to PTX treatment and other chemotherapeutics is responsible for poor clinical outcomes in BC patients [11, 12]. Decreasing chemotherapeutic drug adverse effects and boosting drug efficacy are critical components of treatment. Taking medications in conjunction with natural ingredients may support therapeutic efficacy synergistically [13].

Caffeine (CAF) is a natural component of our daily diet that acts as a central nervous system stimulant as well as a protein kinase inhibitor. [4, 14, 15]. Recently, studies have highlighted the association between CAF and cancer. CAF has been shown to increase the lethal impact of chemotherapy, impairing the ability of cancer cells to repair damage to themselves [16]. In addition, it has been reported that CAF induces apoptosis

How to cite this article: Aydın F, Önder GÖ, Göktepe Ö, Bitgen N. Caffeine Increased Antitumor Effects of Paclitaxel (PTX) in MCF-7 and MDA-MB-231 Breast Cancer Cells. J Res Pharm. 2023; 27(6): 2242-2449.

ISSN: 2630-6344

http://dx.doi.org/10.29228/jrp.513

[17] and enhances the toxicity of antitumor agents [18]. Moreover, cell cycle delays cause increased chemotherapeutic resistance in cancer cells. CAF overcomes chemotherapy-induced delays in cell cycle progression and thus supports the treatment against the formation of drug resistance [19]. All these described actions suggest that CAF may be useful as an adjuvant treatment in the prevention or treatment of cancer. For this reason, the MCF-7 and MDA-MB-231 cell lines, which are widely utilized in vitro, were chosen for our study. To determine how CAF influences the efficiency of PTX in these cell lines, cell proliferation, apoptosis, and cell cycle analyses were performed.

2. RESULTS

2.1. Cell Viability

CAF's effects on cell line viability were observed in BC cell lines (MCF-7 and MDA-MB-231) using MTT assay. The obtained results showed that the exposure of CAF at concentrations of 60 to 160 µM for 24, 48, and 72 h significantly inhibited the cell viability of MCF-7 and MDA-MB-231 cells. As a result of the investigations, the IC50 value of CAF for both MCF-7 and MDA-MB-231 cell lines at 24 h was founded as 100 µM (Figure 1).

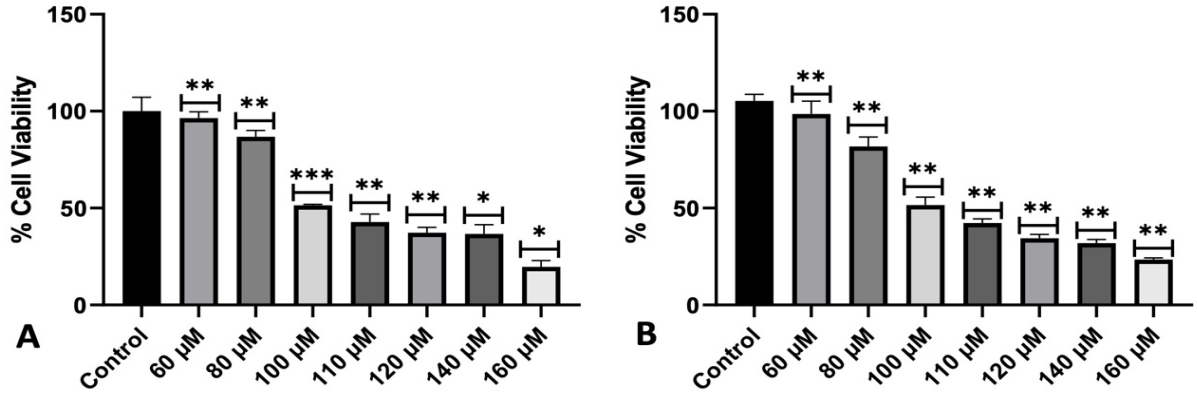

Figure 1. Cell viabilities of the MCF-7 (A) and MDA-MB-231 (B) lines after 24 hours of CAF administration.

2.2. TUNEL Staining Assay

The TUNEL technique was utilized to investigate if the anticancer activities of CAF and PTX related to apoptosis. TUNEL-positive cells in MCF-7 and MDA-MB-231 cell lines, which are key indicators of apoptotic conditions, are shown in Figures 2 and 3, respectively. The green reflections in the pictures are TUNEL+ apoptotic bodies. The results of the TUNEL experiment showed that CAF and PTX exhibited antitumor activity against breast cancer cells both together and individually.

The statistical analysis of TUNEL data for CAF and PTX is shown in Table 1. In MCF-7 cells, after the application of CAF to breast cancer cells alone, there was a significant increase in TUNEL+ apoptotic cell immunoreactivity in the CAF group compared to the control group ($p=0.0225$). In the PTX group, a statistically significant increase in the presence of TUNEL-positive apoptotic cells was noted compared to the control group ($p=0.0099$). A statistically significant increase was observed in apoptotic cell density in the CAF and PTX groups compared to both the CAF ($p=0.0106$) and PTX groups ($p=0.0243$) (Table 1). When comparing the control group in MDA-MB-231 cells, it was discovered that there was a significantly higher level of TUNEL apoptotic cell immunoreactivity in the CAF group ($p<0.0001$). The TUNEL apoptotic cell immunoreactivity was found to be considerably higher in the PTX group compared to the control group ($p=0.0034$). The CAF + PTX group's TUNEL + apoptotic cell immunoreactivity was revealed to be statistically substantially higher than the CAF group ($p<0.0001$). When the CAF + PTX group was compared to the PTX group, there was an increase in TUNEL + apoptotic cell immunoreactivity, although this increase was not statistically significant ($p>0.9999$).

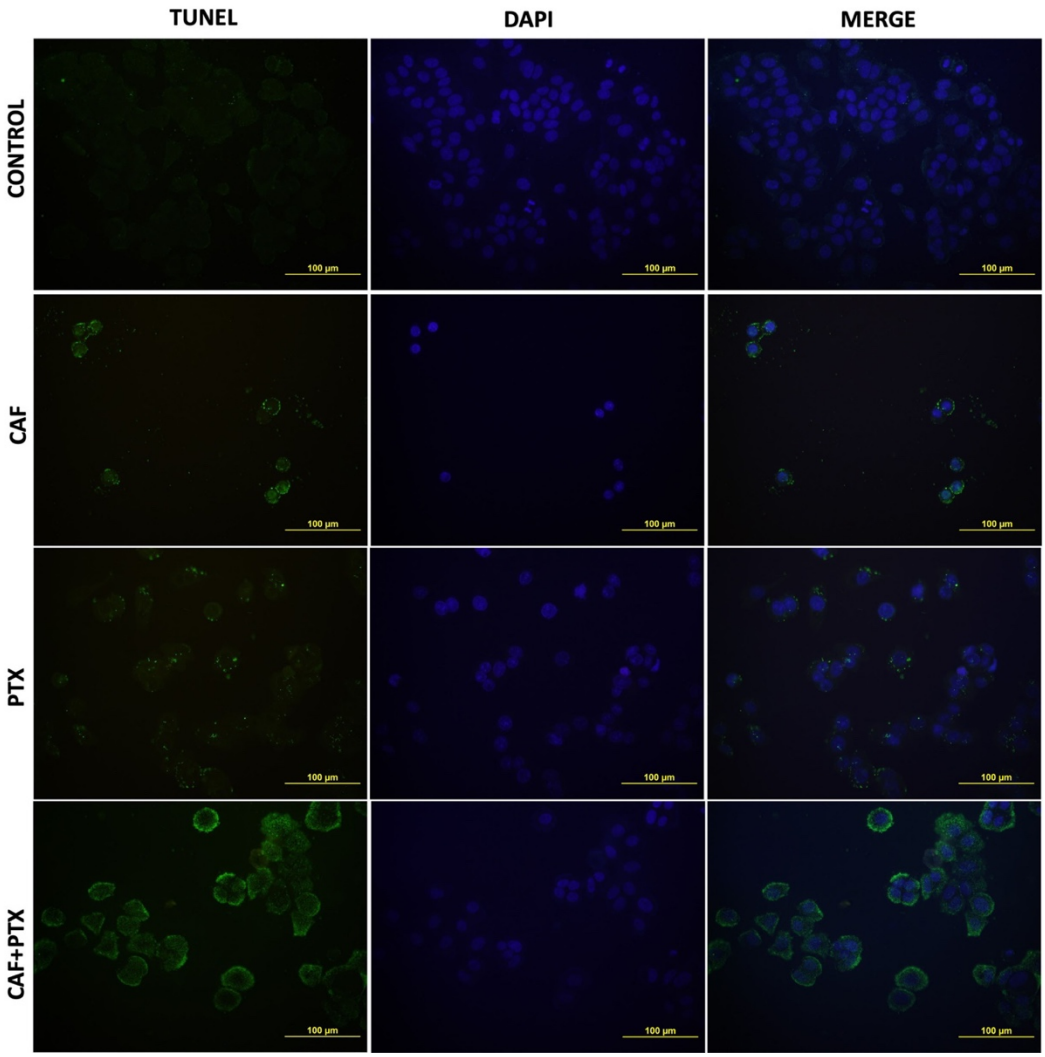

Figure 2. TUNEL images of the MCF-7 cell line treated with CAF and PTX. In the first and third columns, the apoptotic bodies released in the cells were detectable under the fluorescent microscope as green reflections.

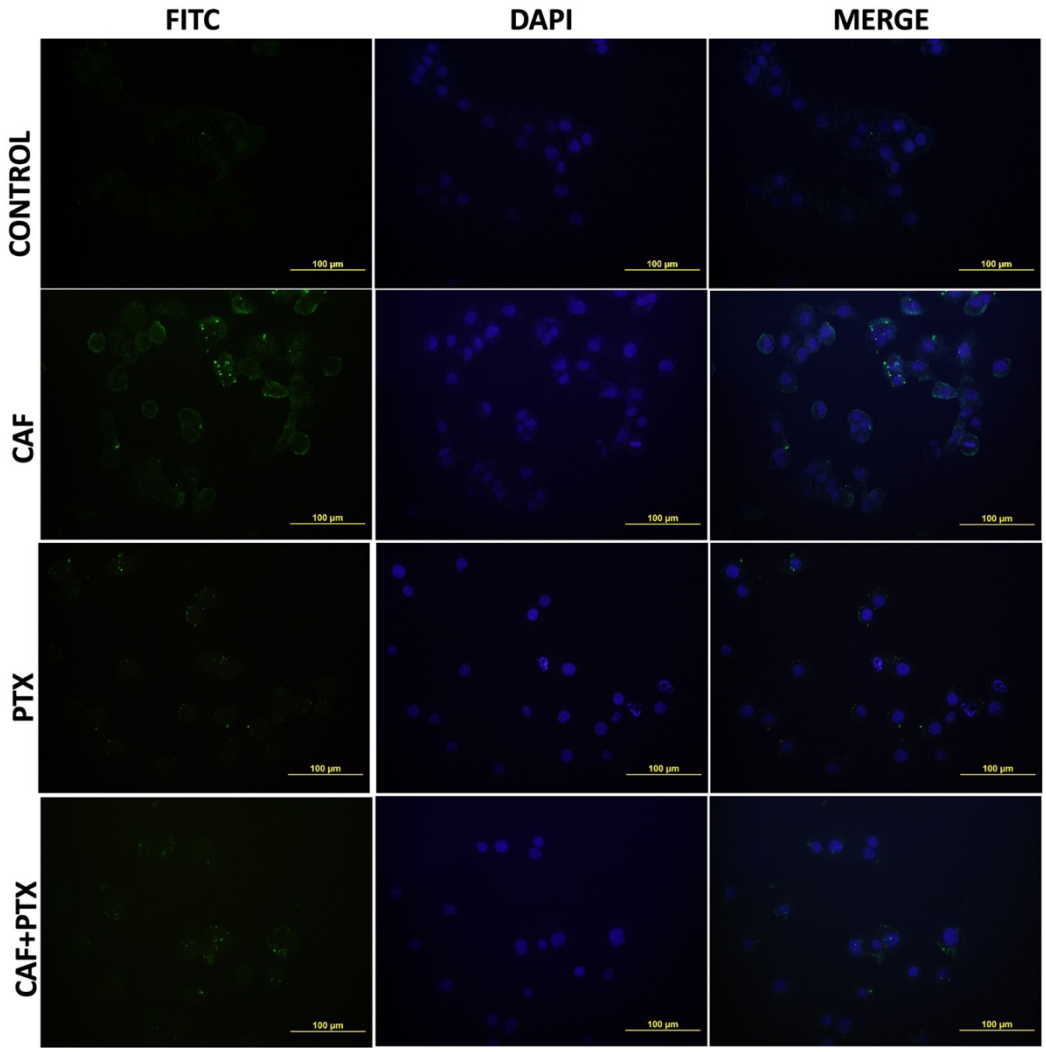

Figure 3. TUNEL images of the MDA-MB-231 cell line treated with CAF and PTX. In the first and third columns, the apoptotic bodies released in the cells were detectable under the fluorescent microscope as green reflections.

Table 1. TUNEL statistical analysis results of breast cancer cell lines treated with CAF and PTX.

	Groups				
	CONTROL	**CAF**	**PTX**	**CAF+PTX**	**p**
MCF-7	(4.68 ± 0.96)[a]	(6,47 ± 0.87)[b]	(6,65 ± 0.77)[b]	(8,43 ± 2.10)[c]	<0.0001
MDA-MB-231	(2.46 ± 0.49)[a]	(7.13 ± 1.09)[b]	(4.06 ± 0.83)[c]	(4.17 ± 1.00)[c]	<0.0001

Data are expressed as the mean ± standard deviation. p refers to the significance of the difference between the groups. The same lowercase letters in the same row show similarity between groups, and different lowercase letters indicate differences between groups.

[a] p value of <0.05 was used for significance.

2.3. Cell Cycle Analysis

When the groups were compared, it was shown that there was a statistically significant increase in S-phase in MCF-7 cells in CAF and PTX groups compared to the control group (**p<0.01). Also, it was seen that in the CAF + PTX group cell line blocked cell division in S-phase according to the control group (***p<0.001) (Figure 4A). In MDA-MB-231 cells, the results showed that there was a statistically significant increase in G0/G1 phase in CAF + PTX group compared to the control group (***p<0.001). It was determined that CAF and PTX in the MDA-MB-231 cell line blocked cell division in G0/G1 phase both together and individually (Figure 4B).

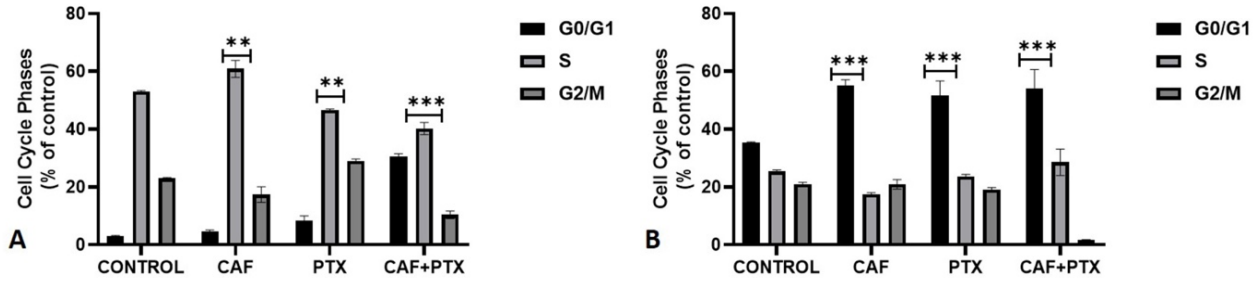

Figure 4. Effect of CAF and PTX on cell cycle arrest in (A) MCF-7 and (B) MDA-MB-231 cells *p<0.05, **p<0.01, ***p<0.001 vs the control group.

3. DISCUSSION

Cancer cells have the ability to multiply very quickly. Chemotherapy drugs target rapidly dividing cancer cells. However, the cells quickly begin to show resistance to them. Because they can change their genetic structures and activate mechanisms to protect themselves under stress [20, 21]. In recent years, because of the recent developments in molecular biology and carcinogenesis in cancer treatment, many anticancer agents have been used in combination with surgical treatment, chemotherapy, and radiotherapy [22]. PTX is an anticancer drug with an impact on the stabilization of microtubules that has been used to treat many cancer types, including breast cancer [23]. The mechanism of action of PTX as an antimitotic drug was documented by many studies [24-26]. However, cells treated with PTX often develop drug resistance. Chemotherapy resistance leads many cancer treatments to fail to have the desired impact on patients and causes the disease to progress [27]. Studies on the use of alternative products in conjunction with chemotherapy medications are of interest to provide a more efficient and enhanced form of cancer therapy [28].

In recent years, several studies have been published describing the synergistic effects between plant-derived bioactives and chemotherapeutics. These effects are generally explained by four different mechanisms: the synergistic multitarget effects, the pharmacological modulation, the intervention with resistance mechanisms, and the elimination or neutralization potential [29]. The biological effects of CAF are widely known, and recently there has been increasing evidence for the synergistic effects of this compound with different conventional drugs in various clinical situations [29, 30]. It has also recently been reported that the combination of anti-cancer drugs with CAF increases the effectiveness of the treatment. Pascua et al. investigated the synergistic effect of CAF with cisplatin and reported that CAF combined with cisplatin increased the cytotoxicity of cisplatin on cancer cells by changing the effect of MDA-MB-231 and MCF-7 on energy metabolism [4]. Motegi et al. [31] verified that CAF created synergistic antitumor effects with or without doxorubicin. They evaluated the antitumor effects of CAF by measuring caspase-3/7 or annexin V protein gene expression. The results indicated that CAF induced apoptosis in hemangiosarcoma cell lines.

Previous studies have shown that the CAF increases the effectiveness of anticancer agents by influencing drug-induced cell cycle arrest. Abe et al. reported that CAF induced G0/G1 cell-cycle arrest and suppressed cell proliferation in osteosarcoma and fibrosarcoma [32]. Hashimoto et al. also observed that CAF induced cell-cycle arrest appeared in the G1 phase and decreased the proportion of cells in the S phase [33].

In our study, we showed that PTX combined with CAF creates synergistic apoptotic effects on the most studied MDA-MB-231 and MCF-7 breast cancer cell lines. Our study confirmed that the number of apoptotic cells was higher in the CAF + PTX group compared to the PTX group by TUNEL staining. Cell-cycle arrest is

a survival mechanism in cells exposed to chemotherapeutic medicines, and cell cycle progression resensitizes cancer cells to medications [34].

4. CONCLUSION

The findings clearly demonstrated that cell division in S-phase was blocked in the MCF-7 cell line in the CAF + PTX group compared to the control group. It was also seen that CAF and PTX in the MDA-MB-231 cell line blocked cell division in the G0/G1 phase both together and individually. Finally, we discovered that CAF coupled with PTX is an effective treatment for breast cancer by triggering apoptosis in two well-known breast cancer lines, MDA-MB-231 and MCF-7. This study provides an idea of the increasing interest in this field of research and the promising role of CAF in cancer therapy.

5. MATERIALS AND METHODS

5.1. Cell Lines

The human BC cell lines MDA-MB-231 and MCF-7 were purchased from Betul Ziya Eren Genome and Stem Cell Centre (Kayseri, Turkey). The complete media was prepared in Dulbecco's modified Eagle medium (DMEM, Sigma Aldrich, USA) supplemented with 1% L-glutamine (Thermo Fisher Scientific, Rockford, USA), 10% fetal bovine serum (FBS, Gibco, South America) and protected with 1% penicillin/streptomycin (Capricorn Scientific, Germany) solution. The cells were seeded in flasks at 37 ^{o}C in a free gas exchange environment with atmospheric air containing 5% CO_2.

5.2. Preparation of CAF and PTX

CAF powder (CAF, Sigma, MO, USA) was diluted in dimethyl sulfoxide (DMSO, Sigma, MO, USA). 2 mM CAF solution was prepared as stock solution. The dose of PTX (300 mg/50 mL, Sandoz, Novartis) supplied in liquid form was determined as 20 nM after literature review [34-36].

5.3. Cell Viability Assessment

Cells were seeded in 96-well plates (5×10^4 per well in 100) and incubated with CAF at different concentration and time points. The cells were then incubated for 4 hours with a 1 mg/mL of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT, Sigma Aldrich, USA) solution. After removing the MTT solution, the formazan crystals produced by viable cells were dissolved in 100 mL of DMSO. To determine cell viability, absorbance measurements were taken at 595 nm with an ELISA reader.

5.4. Experimental Groups

After the IC50 dose was determined, four groups were identified as control, CAF, PTX and CAF + PTX, respectively. The cells were received from the IC50 dose of CAF for CAF group, 20 nM PTX for PTX group and the IC50 dose of CAF and 20 nM PTX for CAF+PTX group.

5.5. TUNEL Staining Assay

For the TUNEL staining assay, cells were fixed with 10% formaldehyde first and then stained using the ApopTag Fluorescein In Situ Apoptosis Detection Kit (Merck, Darmstadt, Germany) [37]. To observe cells, an Olympus fluorescence microscope (Olympus BX51, Tokyo, Japan) was used (magnification, x 400). A total of 10 fields of view were randomly selected for analysis.

5.6. Cell Cycle Analysis

The cell cycle status was analyzed with the Muse Cell Cycle Kit (Luminex, TX, USA). Cells were collected by trypsinization and centrifuged at 300 g for 5 min. For fixation, 70% cold ethanol was used at -20 ^{o}C. The cells were incubated with cell cycle reagents for 30 minutes in the dark. The data was analyzed with A Muse cell analyzer and Muse analysis software (Merck, Darmstadt, Germany).

Acknowledgements: We thank the Erciyes University Genome and Stem Cell Center where many stages of the study took place.

Author contributions: Concept – F.A., G.Ö.Ö., Ö.G., N.B.; Design – F.A., G.Ö.Ö., Ö.G., N.B.; Supervision – F.A., G.Ö.Ö., Ö.G., N.B.; Materials – F.A., G.Ö.Ö., Ö.G., N.B.; Data Collection &/or Processing – F.A., G.Ö.Ö., Ö.G., N.B.; Analysis &/or Interpretation – G.Ö.Ö., Ö.G., N.B., F.A.; Literature Search – F.A., G.Ö.Ö., Ö.G., N.B.; Writing – F.A., G.Ö.Ö.; Critical Reviews – F.A., G.Ö.Ö., Ö.G., N.B.

Conflict of interest statement: The authors declared no conflict of interest in the manuscript.

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