

Berberine Synergizes with Cisplatin via Inducing Apoptosis on A549 non-Small Cell Lung Cancer Cells

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ABSTRACT

Objective: Lung cancer is the most common cause of morbidity and mortality. Platinum-based chemotherapy, which is the primary line of treatment, offers limited benefit due to drug resistance and side effects. Berberine (BBR), which is characterised by its potent and safe anticancer activity, represents a promising combination option in chemotherapy. To overcome the limitations in lung cancer chemotherapy, we investigated whether BBR and cisplatin (CIS) exert synergistic effects on non-small cell lung cancer cell line (A549) based on cytotoxicity and apoptotic response markers.

Methods: The potential cytotoxic effects of the combination treatment were evaluated using the MTT and Chou-Talalay methods. Elisa assays were also performed to measure the levels of the proapoptotic protein Bax and the effector protein caspase (Cas)-3.

Results: The results showed that BBR alone reduced A549 cell viability in a dose-dependent manner and synergized with CIS (CI =0.34±0.05 at IC50 concentrations). Elisa results showed that the combined treatment (both at IC50 concentrations) modulated apoptotic signalling pathways in A549 cells. Bax and Cas3 protein levels were dramatically enhanced in A549 cells treated with CIS +BBR compared to control (0.5% DMSO) (p < 0.001).

Conclusion: Our results suggest that BBR can synergistically enhance the therapeutic effect of CIS in A549 cells. The potential therapeutic efficacy of BBR as part of a combination in current chemotherapy should be supported by in-depth research and clinical studies on the molecular mechanisms associated with cancer.

Keywords: Berberine (BBR), Cisplatin (CIS), synergism, A549, apoptosis



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INTRODUCTION

Cancer is one of the diseases with the highest mortality rate in the world [1]. It is predicted that cancer incidence will increase dramatically in the coming years due to demographic changes such as population growth and aging. According to the GLOBOCAN update, an estimated 10 million people will die from cancer in 2020. Lung cancer ranks first among cancer-

related causes of death, with an estimated mortality rate of 18%. The global cancer burden is expected to increase by an estimated 47% (64-95% for developing countries and 32-56% for developed countries) in 2040 compared with 2020. However, this rate may worsen due to socioeconomic and demographic changes [2].

Histologically, there are two subtypes of lung cancer: small cell lung cancer (about 15% of cases) and non-small cell lung cancer (NSCLC, about 85% of cases) [3]. NSCLC is often diagnosed at advanced stages when metastases have already formed, contributing to a poor prognosis and a low overall survival rate [4]. The main lung cancer treatment modalities include chemotherapy, radiation, and/or surgery [5]. Platinum-based drugs are commonly used in first-line therapy to eradicate NSCLC [6]. However, this chemotherapy alone results in only modest improvement in patient survival. Similarly, a review of lung cancer treatment reported that platinum-based combination therapy with gemcitabine, paclitaxel, tyrosine kinase inhibitors, or angiogenesis inhibitors showed no limited or significant difference in overall survival [7]. In this way, the need for new agents and/or combined treatment strategies to improve efficacy, safety, and resistance issues in the treatment of lung cancer is emphasized. Nowadays, the beneficial effects of various bioactive phytochemicals in chemotherapy are the focus of interest, and studies on this topic are increasingly being conducted [8-11].

Berberine (BBR), long known and used in traditional medicine, could be a promising therapeutic option for lung cancer because it is an effective and safe anticancer phytochemical. BBR is a quaternary benzisoquinoline alkaloid that has been isolated from many medicinally important plants such as *Berberis vulgaris*, *Hydrastis canadensis*, and *Coptis chinensis* [12]. Over the years, research has shown that BBR has a wide range of therapeutic applications against a variety of diseases, including cancer. It has been suggested that BBR may exert therapeutic effects by regulating various molecular targets in different cancer cells, including gastric, colon, prostate, breast, and lung cancers [13-

15]. Several studies have shown that BBR strongly suppress the proliferation, growth, and metastasis of NSCLC through various mechanisms, including cell cycle arrest, apoptosis, and triggering autophagy and cell death [7,16]. In addition, BBR is thought to interact directly with DNA, telomerase, topoisomerase I, p53, NF- κ B, nucleic acids including MMPs and estrogen receptors, as well as a variety of genes and proteins [13]. It has also been shown to prevent the development of resistance to chemotherapy, tumor metastasis, and recurrence through various molecular mechanisms, including immunotherapy [17-19]. In the literature, in addition to the anticancer effect of BBR alone, its synergistic effect with chemotherapeutic agents has also been reported in various types of cancer [20-24]. Taken together, this strengthens the hypothesis that BBR can sensitize lung cancer cells to standard chemotherapeutic agents through different mechanisms.

There appears to be a need for newer therapies to improve treatment outcomes in lung cancer. There is strong evidence that BBR, both alone and in combination with chemotherapy, can play an important role. However, few studies have been conducted on the effect of BBR in combination with CIS in lung cancer [25]. The current study examined the effects of BBR on the cytotoxicity of standard chemotherapeutic agents CIS and the effects of co-treatment on apoptosis markers in A549 lung cancer cells to contribute to studies aimed at increasing the efficacy of lung cancer treatment.

MATERIALS AND METHODS

Chemicals

CIS (10 mg/20 ml, 1.665 mM concentrated solution for intravenous infusion) was purchased commercially from Kocak Pharma (Tekirdag, Turkey). BBR was purchased from Cayman Chemical Co (Michigan, USA) and stored at a concentration of 40 mM in dimethyl sulfoxide (DMSO; from Merck Co, Darmstadt, Germany) as stock solution at -20°C. Working concentrations were freshly prepared by dilution with cell culture medium to a final concentration of DMSO \leq 0.5%. Dulbecco's phosphate-buffered saline (PBS), glutamine, Dulbecco's Modified Eagle Medium (DMEM) with high glucose content, methylthiazolyldiphenyltetrazolium bromide (MTT), penicillin-streptomycin, and trypan blue were purchased from Sigma Aldrich Co (St. Louis, USA). Heat-inactivated fetal bovine serum (FBS) was purchased from Capricorn Scientific (Ebsdorfergrund, Germany) and trypsin-EDTA from Thermo Fisher Scientific Inc (Waltham, MA, USA). Human Bax and Cas3

Main Points;

- Non-small cell lung cancer (NSCLC) is a type of cancer that is usually diagnosed at advanced stages and has a poor prognosis and low survival rate.
- Platinum-based chemotherapy, which is the mainstay of treatment, offers limited benefit due to drug resistance and side effects.
- Berberine, which attracts attention for its potent anticancer activity, showed synergistic cytotoxic effect with cisplatin and induced apoptosis in A549 cells.
- Berberine could enhance treatment efficacy as part of chemotherapy in the treatment of NSCLC.

Elisa kits from Bioassay Technology Laboratory (Birmingham, UK) were used for the experiments, and all analyzes were performed according to the manufacturer's protocols.

Cell Culture

A549 cells (human lung adenocarcinoma cell line) were provided by the American Type Culture Collection (ATCC; Rockville, MD, USA). Cells were maintained in DMEM cell culture medium. All media were supplemented with 10% FBS and 1% penicillin/streptomycin solution in a 95% humidified incubator with 5% CO₂ at 37 °C.

Cytotoxicity Test

The cytotoxic effect of the applied treatments on A549 cancer cells was determined using the MTT assay described by Mossmann [26]. Briefly, cells were plated at a density of 1×10^4 cells per well (counted with trypan blue) in 96-well plates. After 75% attachment, cells were first treated for 24 hours with various concentrations of CIS (1.95, 3.91, 7.81, 15.62, 31.25, 62.5, 125, or 250 μ M) or BBR (3.91, 7.81, 15.62, 31.25, 62.5, 125, 250, 500, 1000, or 2000 μ M) to determine their half-maximal inhibitory concentration (IC₅₀). Control cells were incubated with 0.5% DMSO. After 24 hours, the cells were incubated with MTT solution (5 mg/mL in PBS) for 4 hours at 37°C in the dark. The medium was then discarded and 100 μ L of dissolution solution (DMSO) for formazan crystals was added. The color changes were measured at 570 nm using a microplate reader (Epoch, Biotek, USA). IC₅₀ values of CIS and BBR were calculated by nonlinear regression (curve fitting) of cytotoxicity data using the dose-response inhibition equation (log inhibitor vs normalized response variable slope) with GraphPad Prism 9.5.0 (GraphPad Software, San Diego, CA, USA).

Determination of Synergy

To determine the effects of the combined treatment of CIS and BBR, cells (1×10^4 /well) were seeded in 96-well plates and incubated for 24 hours. The cells were then incubated again for 24 hours with different concentrations of CIS or BBR or combinations thereof (with different concentrations in non-constant ratios). Based on the MTT data, the combination indices (CIs) were defined according to the isobologram and median effect equality method developed by Chou and Talalay [27] using CompuSyn V1.0 software (ComboSyn, Inc.). According to this method, CI values below 1.0 indicate synergy and values above 1.0 indicate antagonism.

Enzyme-linked immunosorbent assay (Elisa) for the detection of apoptotic proteins

Commercially available Elisa kits were used for the analysis of Bax and Cas3 levels in the supernatants. All steps were performed according to the manufacturer's instructions. OD values were analyzed using a microplate reader (at 450 nm). Using the standard concentrations, a graph was generated and used to calculate the protein concentrations of the samples. The protein concentrations of each treatment group were calculated as pg/mg protein.

Statistical Analysis

Quantitative data were expressed as mean \pm standard deviation (n=3). Significant comparisons between groups were performed with ANOVA followed by post hoc comparisons with Tukey's HSD test. All statistical analyses were performed with GraphPad Prism 9.5.0. The statistical significance threshold was set at $p < 0.05$.

RESULTS

BBR inhibited proliferation of A549 lung cancer cells

The cytotoxic effect of BBR was investigated using the MTT assay on A549 lung cancer cells. Cells were treated with different concentrations of BBR (3.91-2000 μ M) for 24 hours. The results showed that BBR inhibited cell viability and proliferation in a dose-dependent manner. The IC₅₀ values of BBR were determined to be 131.90 μ M for 24 hours (Table 1). In addition, the IC₅₀ value of CIS (1.95-250 μ M) was 7.21 μ M for 24 hours (Table 1). The IC₅₀ values were used in the next steps of our study.

Table 1. IC₅₀ of BBR and CIS in A549 for 24 h

Groups	IC ₅₀ (μ M)	95% Confidence Interval (Min-Max)	r ²
BBR	131.90	79.41-212.00	0.843
CIS	7.21	6.12-8.51	0.956

Abbreviation: CIS, Cisplatin; BBR, Berberin.

The combination of BBR and CIS has a strong synergistic effect on A549 cells

To investigate whether BBR has synergism with CIS on lung cancer cell viability, combination groups were formed based on IC₅₀ values, which included lower and higher concentrations of IC₅₀. Cells were grown with concentrations of BBR IC₅₀x2, IC₅₀, and IC₅₀/2 (approximately 264, 132, and 66 μ M) in combination with IC₅₀ and IC₅₀/2 concentrations of CIS

(approximately 7 and 3.5 μM , respectively). After 24 hours of incubation, absorbance values obtained by the MTT assay were entered into the CompuSyn program. CI values were calculated according to the Chou-Talalay method used for preliminary evaluation of combinations (Table 2). We found that the combination of BBR and CIS suppressed A549 cell growth more than monotherapy, and the CI value was less than 1.0 in all groups studied. On the basis of these data, we concluded that the combination of BBR and CIS had a strong cytotoxic effect at all concentrations studied. We found the lowest CI value (0.34 ± 0.05), ie, the strongest synergism, in the group treated with both CIS and BBR at IC₅₀ concentrations.

Table 2. The combination index (CI) values of combinations of BBR with CIS in A549 lung cancer cells.

Combinations	CI values
CIS (7 μM) + BBR (264 μM)	0.40 ± 0.07
CIS (7 μM) + BBR (132 μM)	0.34 ± 0.05
CIS (7 μM) + BBR (66 μM)	0.47 ± 0.05
CIS (3.5 μM) + BBR (264 μM)	0.85 ± 0.10
CIS (3.5 μM) + BBR (132 μM)	0.85 ± 0.07
CIS (3.5 μM) + BBR (66 μM)	0.79 ± 0.07

Abbreviation: CIS, Cisplatin; BBR, Berberine; CI, Combination index.

Co-treatment with BBR promoted CIS-induced apoptosis in A549 lung cancer cells

Commonly used markers for in vitro detection of apoptosis include the level of Bax, a pro-apoptotic protein, and the activity of Cas3, an effector caspase. To determine whether CIS +BBR co-treatment can induce apoptosis, A549 lung cancer cells were treated with IC₅₀ concentrations of CIS or CIS +BBR for 24 hours, and Bax and Cas3 protein levels were measured by Elisa (Figure 1).

The amount of Bax protein, which plays an important role in regulating apoptosis, was calculated with means and standard deviations of 23.07 ± 0.79 , 27.69 ± 1.06 , 64.65 ± 4.28 pg/mg for the control, CIS, and CIS +BBR groups, respectively. Concurrent treatment of CIS with BBR significantly increased the amount of Bax protein compared to control and CIS ($p < 0.001$). The means and standard deviations of Cas3 protein levels were calculated as 61.70 ± 2.35 , 100.54 ± 1.53 , 180.52 ± 10.50 pg/mg for C, CIS and CIS +BBR groups, respectively. CIS co-treatment with BBR significantly increased Cas3 protein levels compared to control and CIS groups ($p < 0.001$). Taken together,

these findings indicate that co-treatment shows synergism for apoptosis-inducing activity in A549 cells based on Bax and cas3 protein levels.

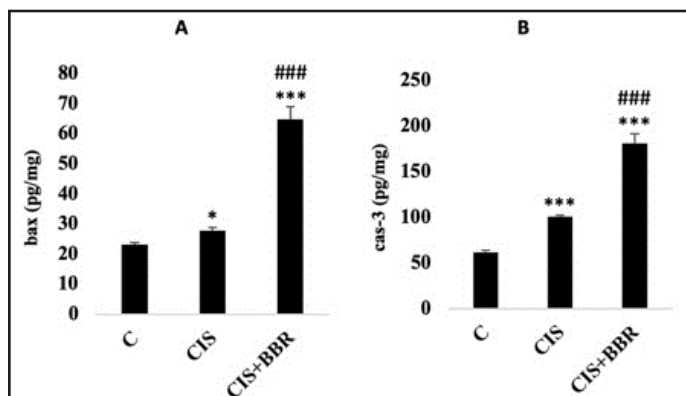


Figure 1. The effect of CIS alone or in combination with BBR on various proteins bax (A) and cas-3 (B) amounts associated with apoptosis in A549 lung cancer cells for 24 h. The results are represented mean \pm standard deviation of three independent experiments. C: DMSO-treated (0.5%) control, CIS: Cisplatin (at IC₅₀ doses), BBR: Berberine (at IC₅₀ doses). (*compared to C $p < 0.05$; *** compared to C $p < 0.001$; # compared to CIS $p < 0.05$; ### compared to CIS $p < 0.001$).

DISCUSSION

Lung cancer is a major cause of morbidity and mortality [2]. Platinum-based chemotherapy, which is now a mainstay of treatment, offers limited benefit due to drug resistance and side effects [28]. Studies on combination therapies with various bioactive phytochemicals are increasingly being conducted to improve treatment efficacy [9,11]. BBR is a promising therapeutic option for lung cancer due to its potent and safe anticancer activity. BBR is known to exert antiproliferative effects on many human cancer cells through various molecular mechanisms [7,16]. However, the therapeutic efficacy of BBR in combination with platinum-based chemotherapy in lung cancer has not been adequately studied. Determining the molecular mechanism underlying the potential synergistic effect is critical for cancer treatment. In the present study, we researched the cytotoxic effects of CIS + BBR combination treatment in A549 cells and its potential effects on apoptotic signalling pathways.

In our study, the cytotoxic effect of BBR and CIS on A549 cells was first evaluated by a 24-hour MTT assay, and the IC₅₀ values were 131.9 μM and 7.2 μM , respectively. The results demonstrated that both BBR and CIS suppressed cell proliferation in cells in a concentration-dependent manner.

Consistent with our results, several studies have revealed that BBR can have an antiproliferative effect on lung cancer cells [24,29,30]. Kumar et al. reported that BBR decreased the viability of A549 cells (66.38%) even at the lowest concentration tested (3.125 μM), and the viability of cells treated with 50 μM BBR was 49% during 48 h incubation by MTT assay [29]. IC50 values of 24.5 and 21.0 $\mu\text{g/ml}$ for 72 hours of incubation were obtained for NSCLC cells H460 and H1975, respectively [24]. A current study reported that cell death was significantly induced in A549 and PC9 lung cancer cells treated with BBR (0-160 μM) in a concentration- and time-dependent manner. IC50 values of 80-100 μM were also reported using the CCK8 assay for 48 hours [31]. It was noted that the calculated IC50 values vary in the literature. It is clear that the cytotoxic profiles may vary depending on the different assays applied and the cell lines used.

Furthermore, we investigated the synergistic cytotoxic effects in A549 cells at different concentrations of the combination groups based on the IC50 values. According to the MTT data, the CI value was less than 1 in all studied groups, indicating synergism. These results indicate that BBR can sensitize CIS cytotoxicity in A549 cells. The lowest CI value, i.e., the strongest synergism, was obtained in the combination group at the IC50 concentrations of both CIS and BBR. Therefore, we used IC50 values in the molecular mechanism experiments. It has been reported in the literature that BBR as part of the combination can show synergistic effects in various cancer chemotherapies in humans. In a study supporting our findings, a potential synergistic effect of BBR (at non-cytotoxic doses, 0-10 μM) with CIS was demonstrated by significantly reducing the colony-forming potential of A549 lung cancer cells [23]. In addition, BBR was reported to increase doxorubicin (DOX)-mediated resistance and sensitize lung cancer cells to DOX. The molecular data from this study showed that BBR can suppress the activation of *Signal Transducer and Activator of Transcription 3* (STAT3, a protein that plays a critical role in malignant transformation and progression), preventing cell proliferation and inducing apoptosis in DOX-resistant lung cancer cells (H460 and H1975) [24]. A recent study has shown that the combined treatment of BBR with osimertinib (inhibitor of *epidermal growth factor receptor* (EGFR) tyrosine kinase) has a synergistic anticancer effect on lung cancer cells and may act as a gene (*MET*) inhibitor related to the resistance mechanism [11]. Some studies have shown that BBR in combination with melatonin [31], cinnamaldehyde [32], and icotinib [33] has the potential to exert anticancer effects by targeting several genes

and/or signaling pathways that play important roles in lung cancer cell growth, invasion, and metastasis. Besides the chemocytotoxic potential of BBR, the potential for radiosensitization has also been reported in NSCLC [34].

Besides lung cancer, a very recent study reported that BBR induced synergistic cell growth inhibition in human epidermoid carcinoma cells (A431) with Erlotinib -inhibitor of EGFR tyrosine kinase- using tumor xenograft models *in vitro* and *in vivo*. This anticancer effect was associated with greater inhibition of pAKT and pEGFR, as well as inhibition of Bcl-2 and cyclin D expression. This study supports the combination of BBR with erlotinib as a novel strategy for the treatment of patients with EGFR-positive tumors [20]. BBR induces ionizing radiation mediated cytotoxicity associated with cell cycle arrest in G2/M phase and autophagic cell death. In the *Lewis* lung carcinoma mouse model, synergistic treatment of BBR (1 and 2 mg/kg) with ionizing radiation resulted in a reduction in tumor volume (approximately 48% and 22%, respectively) [34]. In addition, randomized trials in patients with NSCLC treated with radiotherapy showed that BBR therapy (20 mg/kg once daily for 6 weeks) improved baseline lung function and dramatically decreased the incidence of radiation-induced lung injury. The expression of soluble intercellular adhesion molecule-1 and transforming growth factor-beta-1, which are included in the pathogenesis of radiation-induced inflammation, was also reduced by this combination therapy [35]. Taken together, these studies support the investigation of the potential of BBR as an adjuvant in lung cancer. The findings propose that BBR may have a synergistic effect with platinum-based therapy, but the molecular mechanism underlying this synergistic effect has not been fully clarified.

In various *in vitro* and *in vivo* lung cancer models, BBR has been shown to contribute to both apoptosis and autophagy in lung cancer cells by upregulating the expression of apoptotic and/or pro-apoptotic signaling pathways/proteins and targeting AMPK/mTOR/ULK1 signaling pathways [30,36,37]. Apoptosis induction is thought to be an important pathway for the synergistic anticancer effects that occur with bioactive phytochemicals in chemotherapy [7,16]. In our study, we demonstrated that CIS +BBR co-treatment effectively inhibited A549 cell proliferation through *in vitro* induced apoptosis. Analysis of known apoptotic proteins revealed that they can induce cellular apoptosis. We found that this co-treatment increased the level of Bax protein, which promotes apoptosis, compared to control and CIS ($p < 0.001$).

Increased Bax levels generally lead to enhanced mitochondrial membrane permeability and the release of proapoptotic factors such as cytochrome c from mitochondria into the cytosol, which initiates the activation of the caspase cascade and promotes the progression of apoptosis [38]. In support of our findings, our study also analyzed higher Cas-3 protein levels compared to the control and CIS groups ($p < 0.001$). In a study consistent with our results, BBR was reported to inhibit proliferation of NSCLC cells (A549 and PC9), promote apoptosis, and suppress metastasis via the MMP-2, Bcl-2/Bax, and Jak2/VEGF/NF-Kb/ AP -1 signaling pathways [36]. Another study recently reported that it strongly suppressed ROS -mediated ASK1/JNK activation, Cas3 cleavage, cytochrome c release, mitochondrial membrane depolarization, and dose- and time-dependent cell growth in NSCLC cell lines (A549 and PC9) treated with BBR (40, 80, 120 $\mu\text{g}/\text{mL}$). The study data correlated with nude mouse xenograft tumor experiments [30]. Ni et al [39] reported that BBR decreased the proliferation of NSCLC cells and suppressed colony formation in vitro, and inhibited the growth of NSCLC tumors in lung tumor models, resulting in prolonged survival. They also reported that BBR suppressed the growth of NSCLC cells by suppressing DNA repair and replication [39]. Overall, the therapeutic effects of BBR on cancer cells and the induction of apoptosis seem to be a promising combination candidate for chemotherapy of NSCLC patients.

Despite its potent anticancer potential, BBR is subject to several distinct limitations that restrict its clinical use. An example of these limitations is a study that showed that BBR alone or in combination with 5 FU reduced the growth of lung cancer stem cells (H460) but increased the survival of cells in the secondary population [40]. This study requires further investigation before BBR can be incorporated into clinical therapy. One of the main disadvantages of BBR is its low bioavailability. Therefore, high doses are required to achieve therapeutic goals. Many studies have attempted to develop approaches such as the use of absorption enhancers or co-solvents, salt formation, modification of the structure of BBR, and/or nano-based delivery systems to overcome this problem and increase therapeutic efficacy [12,22,41]. For example, a study on breast cancer cells (4T1) reported that the nanodrug design consisting of DOX and BBR effectively inhibited tumor growth and also significantly suppressed lung metastasis by blocking the HMGB1-TLR4 axis. This nanodrug design study indicates that it will shed light on the development of biomimetic nanodrugs for effective and safe chemotherapy [22]. Optimization of these approaches

and development of an effective strategy for combined use in chemotherapy may be critical for the treatment of lung cancer.

Our results show that BBR can sensitize CIS cytotoxicity in A549 cells and has a synergistic anti-cancer effect by inducing apoptosis together with CIS. Considering the low bioavailability of BBR, which limits its therapeutic use, technological approaches with higher bioavailability of BBR should be investigated in research studies to address this issue. We are aware of the limitations of our study. In addition to studying cytotoxicity and the apoptotic process, other techniques can be used to investigate various possible cellular pathways such as cell cycle checkpoints, antioxidant defense system, genotoxicity, and inflammation. Our results showing the complementary role of BBR in enhancing the therapeutic efficacy of CIS in the treatment of lung cancer provide promising new data for the relevant literature. This study may pave the way for in vivo and clinical research to develop therapeutic strategies for lung cancer.

CONCLUSION

It has been shown that BBR at an IC50 concentration can enhance the anticancer effect of CIS by inducing caspase-dependent apoptosis in lung cancer cells. BBR may play an important role in increasing treatment efficacy, reducing side effects, and lowering treatment costs, alone or as part of chemotherapy in various human cancers. The existing data still need to be supported by *in vivo* and clinical studies. Considering the low bioavailability of BBR, various treatment strategies can be developed by formulating nanotechnology-based systems to address this issue.

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Author's Contributions: MBK; conception, design, data collection and/or processing, analysis and/or interpretation,

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