Journal of Research in Pharmacy
Research Article
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Investigation of the antibacterial, antibiofilm and cytotoxic effects of boron compounds in a *Streptococcus mitis* infection model on HepG2 liver cell

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Received: 17 January 2023 / Revised: 10 April 2023 / Accepted: 11 April 2023

ABSTRACT: Liver abscesses are most commonly attributed to *Escherichia coli* and *Klebsiella pneumoniae*, but because *Streptococcus mitis* is a commensal bacterium with low virulence activity, it is overlooked by its antimicrobial resistance and virulence factors. In this study, we tried to evaluate the synergistic effect of boron compounds and the effect on biofilm in the infection model we created with *Streptococcus mitis* NCTC 12261 on the HepG2 liver cell line. Minimal inhibitory concentration (MIC) values were determined on *Streptococcus mitis* at doses of 31.25 µg/ml of Sodium Perborate Metahydrate (SPM), Zinc Borate (ZB) 62.5 µg/ml and Etidote 125 µg/ml. Etidote+SPM, Etidote+ZB, and ZB+SPM combinations showed the most effective fractional inhibitor concentration value synergistic effect at minimum doses of 32 µg/ml+32 µg/ml in all combinations, while it showed an effect on biofilm at doses of 1028 µg/ml+ 1028 µg/ml HepG2 Cell culture.

KEYWORDS: Biofilm, Boron Compounds, Liver Cell, Synergistic Effect, Cytotoxic effect

1. INTRODUCTION

Streptococcus mitis and commensal viridans normally found in the human oropharynx is a streptococcus. *Streptococcus mitis* can cause a number of invasive diseases in humans and emerges as a cause of bloodstream infections in neutropenic and immunocompromised patients and patients undergoing cytotoxic anti-cancer chemotherapy [1-6]. They can colonize the human oropharynx by a mechanism that includes the expression of adhesins, immunoglobulin A proteases and toxins, and modulation of the host immune system. These diverse colonization factors allow *Streptococcus mitis* to compete for space and nutrients against its more pathogenic oropharyngeal microbial neighbors. However, it is likely to use the same colonization and immune modulation factors as virulence factors that support the opportunistic pathogenesis of *Streptococcus mitis* in vulnerable, immunocompromised patients [7]. In recent studies, *Streptococcus mitis* strains isolated from bacteremic patients were found to be resistant to commonly used antibiotics [8]. Patients tend to be infected with their own common strains, and the mortality rate from *Streptococcus mitis* bacteremia varies between 6-30% when looking at the United States data [9]. In light of the studies and the information obtained, it is not known whether *Streptococcus mitis* is commensal or pathogenic and the mechanism of resistance it develops against antibiotics. In studies with boron compounds, which have been studied in alternative treatment methods instead of antibiotics, it has been determined that they show antibacterial activity against microorganisms. The unique electron deficiency and coordination property of boron compounds has led to a wide variety of applications in chemistry, energy research, materials science, and life sciences. The use of boron-containing compounds as pharmaceutical agents has a long history, and recent developments have taken encouraging steps. Boron agents have been used for both radiotherapy and chemotherapy. In radiotherapy, boron neutron capture therapy (BNCT) has been investigated to treat various tumor types such as glioblastoma multiforme (GBM) of the brain, head, and neck tumors. Following the clinical introduction of bortezomib as an anti-cancer agent, the benzoxaborole drugs, Tavborole, and

How to cite this article: Celebi D, Celebi O, Baser S, Taghizadehghalehjoughı A. Investigation of the Antibacterial, Antibiofilm and Cytotoxic Effect of Boron Compounds in a *Streptococcus mitis* Infection Model on HepG2 Liver Cell. J Res Pharm. 2023; 27(6): 2277-2284.

ISSN: 2630-6344
http://dx.doi.org/10.29228/jrp.516

crisaborole, have been approved for clinical use in the treatment of onychomycosis and atopic dermatitis [10]. In this study, we aimed to evaluate the synergistic effect of boron compounds and their effect on biofilm in the infection model we created on the HepG2 Liver Cell Line with the *Streptococcus mitis* NCTC 12261 strain, which shows pathogenicity in immunocompromised patients.

2. RESULTS

2.1. Microbiological analysis

The minimal inhibition concentration (MIC) values of boron compounds on *Streptococcus mitis* are shown in Table 1.

Table 1. Minimal inhibition concentration (MIC) value at which boron compounds inhibit the growth of *Streptococcus mitis*

Boron Compounds	MIC (µg/ml)
Sodyum Perborate Metahidrate (SPM)	31,25
Zinc Borate (ZB)	62,5
Etidote	125

The fractional inhibition concentration (FIC) at which these compounds act on *Streptococcus mitis* with the combination of Etidote+SPM, Etidote+ZB, and ZB+SPM, and the optical density (570/OD) results of the combinations made with the microdilution plate method are given in Table 2.

Table 2. Fractional inhibition concentration (FIC) and biofilm optical density values of the combination of boron compounds on *Streptococcus mitis.*

Bacteria Strains ATCC No	Boron Compounds	Biofilm Highest OD Value	Positive Control	Negative Control	∑FIC	Dose max.-min.(µg/ml)	Interpretation
Streptococcus mitis ATCC 12261	Etidote	1,351	1,367	0,19	0,42-0,18 0,98-0,77	(128-32)+32 (256-128) +64	Synergy Additive
	SPM						
	Etidote	0,848	0,725	0,094	0,45-0,28 0,51-0,99	(256-32) +(64-32) (512-256)+(256-64)	Synergy Additive
	ZB						
	ZB	0,195	0,178	0,078	0,38 0,56-0,98	64 +32 (64-512)+(1028-32)	Synergy Additive
	SPM						

*∑FIC **:** Fractional inhibition concentration
* SPM: Sodyum Perborate Metahidrate
*ZB: Zinc Borate

2.2. Cell culture analysis

The cytotoxic effects of SPM 62,5 µg/ml + Etidote 125 µg/ml, SPM 62,5 µg/ml + Zinc Borat 31,25 µg/ml, and Zinc Borat 31,25 µg/ml + Etidote 125 µg/ml were determined after 24 hours using the MTT method (Figure 1). All data were compared with the control group. The cell viability rate of the control group was 100%. The highest viability of the SPM 62,5 µg/ml + Etidote 125 µg/ml group was measured at 85% ($P>0.05$). The lowest viability was observed SPM 62,5 µg/ml + Zinc Borat 31,25 µg/ml, and Zinc Borat 31,25 µg/ml + Etidote 125 µg/ml (viability rate was 75% ($P<0.001$) and 71% ($P<0.001$) respectively) (Figure 1).

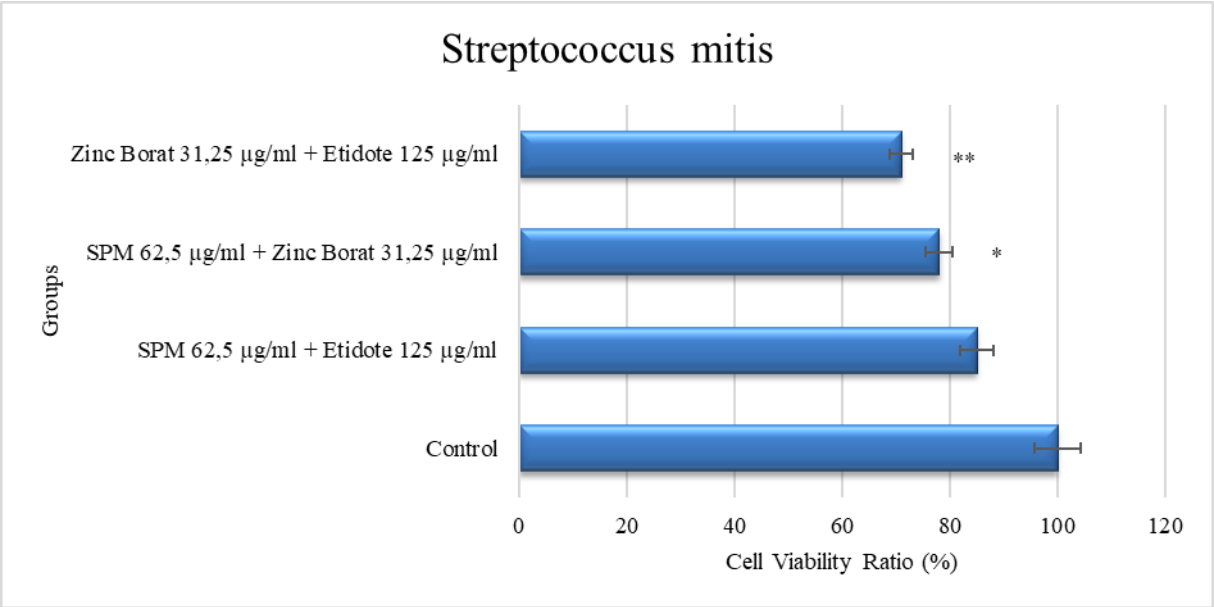

Figure 1: Cell viability ratio of HepG2 cells after 24 h. The viability ratios of the SPM 62,5 µg/ml + Etidote 125 µg/ml, SPM 62,5 µg/ml + Zinc Borat 31,25 µg/ml and Zinc Borat 31,25 µg/ml + Etidote 125 µg/ml groups were compared with that of the control group (** P<0.001).

2.3. Immunohistochemical analysis

Control group, When cell lines were examined by immunofluorescence staining method; Expressions of 8-OHdG and H2A.X were evaluated as negative. SPM+Etidote group, When cell lines were examined by immunofluorescence staining method; moderate intracytoplasmic 8-OHdG and H2A.X expressions were observed. SPM+ZB group, When cell lines were examined by immunofluorescence staining method; severe cytoplasmic 8-OHdG and H2A.X expressions were detected. ZB+Etidote group: When cell lines were examined by immunofluorescence staining method; Severe intracytoplasmic 8-OHdG and H2A.X expressions were observed (Figure 2) (Table 3).

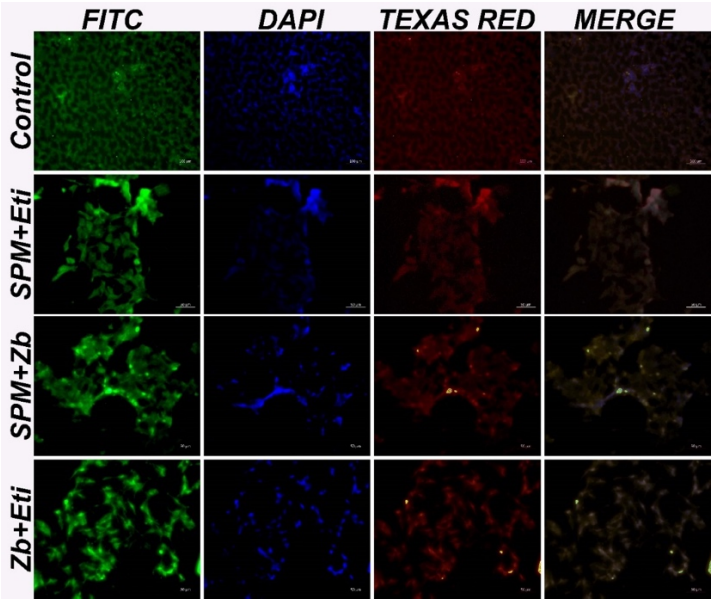

Figure 2. Cell lines, 8-OHdG expressions (FITC) and H2A.X expressions (Texas Red), IF, Bar:50µm. SPM 62,5 µg/ml + Etidote 125 µg/ml, SPM 62,5 µg/ml + Zinc Borat 31,25 µg/ml and Zinc Borat 31,25 µg/ml + Etidote 125 µg/ml

Table 3. Statistical analysis of immunofluorescent staining results

	8-OHdG	H2A.X
Control	21.38±3.74[a]	20.12±5.42[a]
SPM+Etidote	39.86±5.39[b]	33.18±4.98[b]
SPM+ZB	55.12±5.74[c]	52.15±6.43[c]
ZB+Etidote	58.45±5.53[c]	54.38±4.23[c]

*a, b, c: different letters in the same column were considered as statistically significant difference ($p<0.05$)

3. DISCUSSION

Streptococcus mitis, a member of the *Streptococcus mitis* viridans group of streptococci (VGS), is most commonly found in the throat, nasopharynx, and oral cavity. It is generally considered to have low virulence and pathogenicity. However, *Streptococcus mitis* endocarditis and meningitis have been reported [11,12]. In the United States, liver abscesses are most commonly polymicrobial and attributed to *Escherichia coli* and *Klebsiella pneumoniae* [13]. In a study examining the antibacterial and antibacterial activity of copper ions on bacteria; It was determined that *Streptococcus mitis, Streptococcus mutans, Streptococcus saliva, Streptococcus sanguis, Actinomyces viscosus,* and *Actinomyces naeslundii* inhibited the growth rate in broth culture. It has been determined that while copper ions inhibit the growth of bacteria at 10^{-3} M concentrations, 10^{-4} M and lower concentrations have little or no effect. It has been determined that it inhibits the growth of bacteria in the mouthwash used as an oral mouthwash in vitro[14]. The antibiotic resistance patterns of viridans group streptococci (VGS) vary greatly depending on the species and patient population. Although penicillin is used as the first choice in the treatment of all VGS and infections caused by *Streptococcus mitis*, resistance to beta-lactam group antibiotics and other antimicrobial agents has increased in recent years. In general, *S. mitis* is more resistant to antimicrobials than other VGS strains. On the other hand, *Streptococcus mitis* isolated from the CSF culture of our case was found to be sensitive to all tested antibiotics [15]. In a case report on meningitis and white matter lesions due to *Streptococcus mitis* in a child, it was thought that meningitis that occurred in the patient might be related to a sinus infection, since there was a recent history of sinusitis and the MR examination revealed continued inflammation, especially in the maxillary sinuses. It has been stated that patients with meningitis due to *Streptococcus mitis* should be evaluated in detail cardiological, since VGSs may be a cause of meningitis in patients with infective endocarditis. Although *Streptococcus mitis* is a rarely isolated agent in acquired community-acquired meningitis, it should be considered in the differential diagnosis of immunocompetent children with meningitis who have a previous history of predisposing infection and white matter changes in brain MRI [16]. Although *Streptococcus mitis* is a bacterium that can cause infection in the mouth, throat, nasopharynx, and even in the brain, there are also case reports that have been reported as an infectious agent in liver abscesses. Since it is stated that this bacterium may be sensitive to the antibiotics used, alternative treatment options should also be considered [15]. In this study, we tried to evaluate the synergistic effect of boron compounds and their effect on biofilm in the infection model we created with *Streptococcus mitis* NCTC 12261 on the HepG2 liver cell line. According to our result, the SPM + Etidote group did not change the cell viability ratio significantly. There is a limited study are done on SPM and Etidote. Human & Environmental Risk Assessment on ingredients of European household cleaning products determined toxicity will acquire up to 140 µg/kg body weight the dose is too high in compare our cell culture, MIC and FIC result [17]. The resistance developed by *Streptococcus mitis* against antibiotics, virulence factors, and the presence of biofilm has led to the search for alternative treatment methods. Minimal inhibitory concentration (MIC) values were determined on *Streptococcus mitis* at doses of 31.25 µg/ml of Sodium Perborate Metahydrate (SPM), Zinc Borate (ZB) 62.5 µg/ml and Etidote 125 µg/ml. Etidote+SPM, Etidote+ZB, and ZB+SPM combinations showed the most effective fractional inhibitor concentration value synergistic effect at minimum doses of 32 µg/ml+32 µg/ml in all combinations, while it showed an effect on biofilm at doses of 1028 µg/ml+ 1028 µg/ml.

4. CONCLUSION

As a result of our results, it is seen that boron compounds can be preferred as an alternative treatment option in non-cytotoxic dose ranges against the resistance developed by *Streptococcus mitis* against antibiotics, virulence factors, and the presence of biofilm.

5. MATERIALS AND METHODS

5.1. Bacteria Strain

In our study, *Streptococcus mitis* NCTC 12261(Colindale, London, U.K.) strain was used. Suspensions equivalent to strain 0.5 McFarland turbidity were prepared.

5.2. Chemicals and Reagents

Tryptic Soy Broth Sigma (St. Louis, MO, USA), glucose, Sigma (St. Louis, MO, USA), Etidote Sigma (St. Louis, MO, USA), Sodyum Perborate Metahidrate (SPM) Sigma (St. Louis, MO, USA), Zinc Borate Sigma (St. Louis, MO, USA), 9% Isotonic Sodium Chloride Solution Sigma (St. Louis, MO, USA), Blood Agar Sigma (St. Louis, MO, USA), 96 well cell culture plate flat bottom (Orange Scientific), NaCl Sigma (St. Louis, MO, USA),, methanol solution (for HPLC, ≥99.9%, Sigma-Aldrich), glacial acetic acid (≥99.85%, Sigma Aldrich), 2% crystal violet solution (from the Gram color kit Liofilchem), Dulbecco Modified Eagles Medium (DMEM) Sigma (St. Louis, MO, USA), Fetal calf serum (FBS) Sigma (St. Louis, MO, USA), phosphate buffer solution (PBS) Sigma (St. Louis, MO, USA), antibiotic antimitotic solution (100 x) Sigma (St. Louis, MO, USA), L glutamine Sigma (St. Louis, MO, USA), and trypsin-EDTA Sigma. (St. Louis, MO, USA).

5.3. Bacteria Production

Streptococcus mitis NCTC 12261 bacterial stock was added to 100 µl of Tryptic Soy broth (TSB) medium and after 24 hours of incubation at 37°C, 150 rpm, its production was carried out. Then, 200 µl of the growth medium was taken and inoculated into fresh TSB, and the stock medium was made ready for the study.

5.4. Minimum Inhibition Concentration Values

MIC values of Sodium Perborate Metahydrate (SPM), Zinc Borate (ZB), and Etidote compounds against *Streptococcus mitis* NCTC 12261 were determined using the microdilution method. Dose ranges were determined as (1000-31,25 µg/ml). Mueller Hinton Broth (MHB) medium were inoculated into 96-well plates with 180 µl of each dilution. Then, 20 µl of *Streptococcus mitis* NCTC 12261(10^6 CFU/ml) was added to each well and incubated at 37°C. After 24 hours, TTC water-soluble salt solution, a biological indicator, was added to each well (5 mg $\cdot ml^{-1}$) and the plates were incubated for 2-3 hours [18].

5.5. Biofilm Analysis

180 µl of the compounds prepared with TSB medium, whose value (1028-32 µg/ml) was determined, were inoculated into a flat-bottomed 96-well plate. Glucose-enriched TSB medium was used as the negative control and *Streptococcus mitis* NCTC 12261 strain was used as the positive control. Then, 20 µl (10^6 CFU/ml) of *Streptococcus mitis* NCTC 12261 strain was inoculated into each well except the negative well. It was incubated at 37°C for 48 hours. Biofilm analysis was performed in 3 repetitions [18].

5.6. Combination Application of Streptococcus mitis NCTC 12261 with SPM, ZB, Etidote Compounds

The most effective MIC concentrations of SPM, ZB, and Etidote compounds were prepared in combination with each other. In the analysis performed similar to the biofilm evaluation test principle, *Streptococcus mitis* NCTC 12261 strain was inoculated into MHB medium enriched with glucose and incubated at 37°C for 48 hours. Bacteria were expected to grow. In addition, the medium was made fresh by adding TSB medium to the plates at 24-36 hour intervals. After 48 hours, the liquid in the plates was evacuated. And 200 µl of glucose-enriched culture medium containing TTC (5 mg $\cdot ml^{-1}$) was added to each well and incubated at 37°C for 3-4 hours. The intensity of the red color at the end of the resulting test was considered an indicator of viable cell number and was measured at 570 nm. Results were compared with controls. And the test was applied as 3 repetitions.

5.7. Microdilution panels

The solutions were prepared by calculating the final concentrations of SPM, ZB, and Etidote compounds on the prepared panels. Dose ranges were determined as (1028-32 µg/ml). Intermediate dilutions were prepared at a concentration of four times the final concentration desired in the well. 100 µl of TSB medium was dispensed into all wells. First, 100 µl of SPM was diluted in half and dispersed, then 100 µl was added to the wells which were diluted sequentially with ZB, Etidote 1028 µg $\cdot ml^{-1}$. The medium was prepared as a negative control and bacterial wells were prepared as a positive control. Except for the negative control well, 5 µl of antimicrobial agents were dispensed into the plates. This process was repeated for the other ZB and Etidote and applied as 3 repetitions.

5.8. Fractional Inhibitor Concentration Index-Combination (FIC)

It was applied according to the FIC index formula used to determine the effectiveness of the combinations. And the results were determined according to the formula.
A: Antimicrobial 1 used in combination
B: Antimicrobial 2 used in combination

Calculation of the FIC index:

$$FIC\ A = \frac{\text{MIC numerical value of A in the presence of B}}{\text{MIC numerical value of A alone}}$$

$$FIC\ B = \frac{\text{MIC numerical value of B in the presence of A}}{\text{MIC numerical value of B alone}}$$

Σ FIC index = FIC A + FIC B
Σ FIC index ≤ 0.5: synergy
Σ FIC index >0.5 and <1: additive
Σ FIC index ≥1 and 4 ≤ : ineffective (indifference)
Σ FIC index >4: antagonism

5.9. Cell Culture

HepG2 cell (HB-8065, ATCC) cultures were obtained from Bilecik Seyh Edebali University Medical Pharmacology Department (Bilecik, Turkey) for this study. Briefly, cells were resuspended in fresh medium (DMEM), 10% fetal bovine serum (FBS), and 1% antibiotic (penicillin, streptomycin, and amphotericin B) (Sigma Aldrich, St. Louis, MO, USA). Cells were then seeded in 24-well plates (Corning, Inc.) as previously described and stored in an incubator (5% CO_2; 37°C). After reaching 85% confluence on a 0.5 McFarland scale, the bacterial suspension was added to the cell culture. After 30 minutes, treatments were administered for 24 hours. At the end of the experiment (following treatment with boric acid and potassium metaborate for 24 hours), 10 µL of MTT solution (Sigma Aldrich, St. Louis, MO, USA) was added to each well plate and the samples were incubated for 4 hours. h; 100 µL of DMSO (Millipore Sigma) was added to all wells to dissolve the Formazan crystals. The optical density of the solutions was read at 570 nm using a Multiskan™ GO microplate spectrophotometer (Thermo Fisher, Porto Salvo, Portugal) [18].

5.10. Immunohistochemical analysis

Cells cultivated in cell culture were incubated for 30 minutes in a paraformaldehyde solution for 30 minutes. The cells were then incubated in 3% H_2O_2 for 5 minutes. 0.1% Triton-X solution was dripped onto the cells washed with Pbs and left for 15 minutes. After the incubation period, protein blocks were dripped onto the cells and kept in the dark for 5 minutes. Then, the primary antibody (8-OHdG cat no: sc-66036, Dilution Ratio: 1/100 US) was dropped and incubated in accordance with the instructions for use. An immunofluorescence secondary antibody was used as a secondary marker (FITC Cat No: ab6785 Diluent Ratio: 1/500, UK) and kept in the dark for 45 minutes. Then, the second primary antibody (H2A.X Cat No: I 0856-1, Dilution Ratio: 1/100, US) was dripped onto the tissues and incubated in accordance with the instructions for use. An immunofluorescence secondary antibody was used as a secondary marker (Texas Red Cat No: ab6787 Diluent Ratio: 1/1000 UK) and kept in the dark for 45 minutes. Then, DAPI with mounting medium (Cat no: D1306 Dilution Ratio: 1/200 UK) was dripped onto the sections and kept in the dark for 5 minutes, then the sections were closed with a coverslip. The stained sections were examined under a fluorescent microscope (Zeiss Axıo Germany) [18].

5.11. Statistical analysis

In order to determine the intensity of positive staining from the pictures obtained as a result of the dyeing; 5 random areas were selected from each image and evaluated in the ZEISS Zen Imaging Software program. Data were statistically defined as mean and standard deviation (mean±SD) for % area. Mann-Whitney U test was performed to compare positive immunoreactive cells and immunopositive stained areas

with healthy controls. As a result of the test, an AP value of <0.05 was considered significant and the data were presented as mean ± SD.

Acknowledgements: This study received no financial support from anywhere. Thanks to the entire research team for their scientific contributions and insights in the preparation of the study with the principles of high ethics, honesty and openness.

Author contributions: Concept – D.C., O.C., S.B., A.T.; Design – D.C., O.C., S.B., A.T.; Supervision – D.C., O.C., S.B., A.T.; Resources – D.C., O.C., S.B., A.T.; Materials – D.C., O.C., S.B., A.T.; Data Collection and/or Processing – D.C., O.C., S.B., A.T.; Analysis and/or Interpretation – D.C., O.C., S.B., A.T.; Literature Search – D.C., O.C., S.B., A.T.; Writing – D.C., O.C., S.B., A.T.; Critical Reviews – D.C., O.C., S.B., A.T.;

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

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