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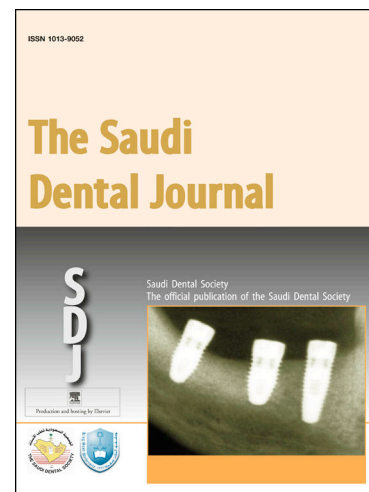
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## Effect of dental antiseptic agents on the viability of human periodontal ligament cells

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- Cell viability is key for tissue regeneration
- Dental antiseptics were cytotoxic in periodontal ligament cells
- Real-time impedimetry proved to be a non-invasive method to monitor cell viability

## Abstract

### Objectives

We aimed to study whether or not various dental antiseptic agents affect the viability and proliferation of human periodontal ligament cells (PDLCs).

### Materials and methods

Human PDLCs were isolated from a total of 10 surgically extracted impacted third molars and were cultured *in-vitro*. The cells were exposed to commonly used dental antiseptics, including chlorhexidine, cetylpyridinium chloride, triclosan, povidone-iodine and sodium bicarbonate for ultra-short-term (10, 20, 30 sec), short-term (10, 20, 30 min) and long-term (24, 48 h) at various concentrations. Cell morphology was observed with light microscopy. Cell viability was studied with impedimetric real-time xCELLigence and resazurin-based alamarBlue® assays. We used one-way ANOVA with Tukey's and Bonferroni test ( $p < 0.05$ ) for statistical analysis.

### Results

Both alamarBlue® and xCELLigence analysis results were in agreement that ultra-short-term contact with cetylpyridinium chloride  $\geq 0.01$  mg/ml, chlorhexidine  $\geq 1$  mg/ml, triclosan  $\geq 1$  mg/ml and povidone-iodine  $\geq 1$  mg/ml as well as long-term exposure to cetylpyridinium chloride  $\geq 0.001$  mg/ml, chlorhexidine  $\geq 0.01$  mg/ml, triclosan  $\geq 1$  mg/ml, povidone-iodine  $\geq 1$  mg/ml and sodium bicarbonate  $\geq 10$  mg/ml was able to reduce the viability of human PDLCs significantly. According to the half-maximal inhibitory concentration (IC<sub>50</sub>) the rank of cytotoxicity was cetylpyridinium chloride > chlorhexidine > triclosan > povidone-iodine > sodium bicarbonate.

### Conclusions

Our findings suggest that the tested antiseptic agents were cytotoxic to human PDLCs at lower than practically applied concentrations in dental interventions.

### Keywords

Periodontal ligament cells, dental antiseptic compounds, cell viability, impedimetry

## 1. Introduction

Chemical reduction of plaque and infection control is essential in routine dental care and pre- or post-operative regime. Antimicrobial agents prevent or arrest the growth or action of microorganisms after topical administration. Preferably, they should have a broad spectrum with rapid onset and long-lasting effect and should not be toxic to host cells as far as possible (Schulman, 1993). However, the broad spectrum of antimicrobial agents causes potential toxic effects not only to the infectious microbes but also to the host cells.

Chlorhexidine is the most widely used synthetic cationic bis-biguanide antiseptic against gram-negative and gram-positive bacteria and fungi (Solderer et al., 2018). It was reported to be cytotoxic to various kinds of cells, including fibroblast (Wyganowska-Swiatkowska et al., 2016), (Goldschmidt et al., 1977), (Peacock et al., 1991), myoblast (Liu et al., 2018), osteoblast (Lee et al., 2010), mouse osteoblast precursor cells (Song et al., 2019), odontoblast-like cells (Lessa et al., 2010) and stem cells exfoliated deciduous teeth (SHED) (Tu et al., 2015). Cetylpyridinium chloride is a cationic quaternary compound commonly added to mouthwashes (Teng et al., 2016). It was reported to be cytotoxic to keratinocytes (Hagi-Pavli et al., 2014) and osteoblast precursor cells (Song et al., 2019). Povidone-iodine is an iodophor against bacteria, mycobacteria, fungi, viruses and protozoa (Greenstein, 1999), (Zamora, 1986). The cytotoxicity of povidone-iodine to osteoblasts (Schmidlin et al., 2009) and fibroblasts (Thomas et al., 2009) was testified before. Triclosan is an antimicrobial and antifungal phenolic compound, available in some dental products such as mouthwash and toothpaste. Triclosan was cytotoxic to neural stem cells (Park et al., 2016). Sodium bicarbonate does not have a direct antimicrobial effect but it raises oral pH and prevents aciduric bacteria's overgrowth (Dodd, 2000). Some experts suggested baking soda solution for acute leukemia patients undergoing chemotherapy because it relieves oral dryness and reduces oral bacterial colonization (Choi and Kim, 2012).

Periodontal ligament (PDL) contains heterogeneous stem cell populations (Seo et al., 2004) (Gay et al., 2007) and participates in the periodontal tissue healing and regeneration process (Hu et al., 2017), (Grimm et al., 2011). They are highly possible to contact antiseptic compounds during or post-dental procedures directly. Therefore, periodontal ligament cells (PDLs) are suitable cell models for in vitro cytotoxic studies for dental materials (López-García et al., 2019). We examined the viability of PDLs when exposed to the abovementioned antiseptic for ultra-short-, short- and long-term. The present study's null hypothesis was that dental antiseptics do not affect the viability of PDLs.

## 2. Materials and methods

### 2.1 Cell isolation

Extracted third molars were collected from the Department of Oral Diagnostics, Faculty of Dentistry, Semmelweis University. The procedure was performed with the approval of the Ethical Committee of the Hungarian Medical Research Council. The ethical approval number is EFOP-3.6.2-16-2017-00006. PDLs were isolated based on the previous study's protocol (Kadar et al., 2009) with minor modifications. Tissue samples were digested in 1mg/ml collagenase type-I solution (Gibco, USA) and expanded in MEM alpha (Lonza, Belgium) medium supplemented with 10% FBS (Lonza, Belgium), 1% penicillin/streptomycin (Invitrogen, USA) and 1% glutamine (Invitrogen, USA). Cells were passaged with 0.25% trypsin EDTA solution when reached 70% confluence, and passages between 2-5 were used for the experiments. In this study, we used samples from a total of 10 different individuals.

### 2.2 Preparation of antiseptic solutions

Stock solutions of cetylpyridinium chloride, chlorhexidine, povidone-iodine and sodium bicarbonate were solubilized in d.i water and triclosan was solubilized in dimethyl sulfoxide (DMSO); and appropriate

dilutions of 22  $\mu$ l were added per well in 96-well plate cultures. Final concentrations were: cetylpyridinium chloride (Sigma-Aldrich, USA) 0.0001, 0.001, 0.01 and 0.1 mg/ml; chlorhexidine (Sigma-Aldrich, USA) 0.001, 0.01, 0.1 and 1 mg/ml; triclosan (Sigma-Aldrich, USA) 0.01, 0.1, 1 and 2 mg/ml; povidone-iodine (ABCAM, UK) 0.1, 1, 2 and 4 mg/ml; sodium bicarbonate (Sigma-Aldrich, USA) 0.01, 0.1, 1 and 10 mg/ml. Controls received d.i water or x100 times dilution of DMSO regarding solvents.

### 2.3 Fluorescence-based alamarBlue® assay

Cells were plated at 96-wells at a density of  $10^4$  cells/well with 200  $\mu$ l media and incubated at 37 °C in 5% CO<sub>2</sub> for 24 h allowing cell adhesion. Then, 22  $\mu$ l/well antiseptic agents were added with a range of concentrations and incubated for ultra-short-term (10, 20, 30 sec), short-term (10, 20, 30 min) and long-term (24, 48 h) at 37 °C in a 5% CO<sub>2</sub>. Subsequently, the fluids were aspirated and washed with PBS, then added 200  $\mu$ l fresh medium per well. Resazurin sodium salt (Sigma-Aldrich, USA) 0.15 mg/ml was solubilized in phosphate-buffered saline (PBS), and 25  $\mu$ l/well solutions were added and incubated at 37 °C, protected from light for 6 h. Fluorescence intensity was measured with Spectrophotometer at 565 and 590 nm wavelengths. Cell morphology was examined by an inverted microscope Axio Observer A1 (Zeiss, Germany) after 24 h exposure to the compounds. Each experiment was conducted three times with samples from different individuals with a minimum of 3 wells.

### 2.4 Real-time impedimetric cell analysis (RTCA)

We utilized the xCELLigence SP (Roche, USA) system to monitor cell behavior under stimuli of antiseptics. A baseline measurement was done with 100  $\mu$ l/well medium at the E-96 plate for 1 h. Subsequently,  $10^4$  cells/well added with 100  $\mu$ l medium and incubated at 37 °C in 5% CO<sub>2</sub> overnight, allowing cell adhesion. Next, 22  $\mu$ l antiseptic solutions in different concentrations were added to each well. Ultra-short-term monitoring was recorded every second for a 1-minute duration. Long-term tracking was performed continuously for 48 h. The xCELLigence system is based on electronic impedance reading from the gold plated sensor electrodes that fused to the plates' bottom and represented by cell index (CI). When there is an absence of living cells or suspension of dead cells, the CI value is close to zero (AECA Biosciences, 2013). Each experiment was repeated three times with samples from different individuals with a minimum of 3 wells. Data were plotted as the normalized cell index (NCI), which is calculated as the CI at a given time point divided by the CI time at the normalization time point. Data were evaluated with RTCA 2.0 software.

### 2.5 Statistical analysis

Each experiment was conducted three times with samples from different individuals (biological replicates) with a minimum of 3 wells (technical replicates). The CI, NCI, CI slope and half-maximal inhibitory concentration (IC<sub>50</sub>) values for xCELLigence measurement were calculated automatically by the RTCA 2.0 software. The IC<sub>50</sub> value from alamarBlue® assay was calculated using nonlinear regression by OriginPro 8.5 (OriginLab, USA) software. Numerical data were expressed as a mean  $\pm$  standard deviation. Statistical differences between groups were carried out using OriginPro 8.5 software using one-way ANOVA with Tukey's and Bonferroni test.  $p < 0.05$  (x),  $p < 0.01$  (y) and  $p < 0.001$  (z) were considered to indicate statistically significant differences.

## 3. Results

### 3.1 Cell morphology

We observed cell morphology after 24 h of direct contact with the antiseptic solutions. Unexposed control cells showed a long spindle shape and were well-attached to the culture plate (Supplementary Fig. 2). Cetylpyridinium chloride 0.0001 mg/ml did not alter cell morphology or confluence; 0.001 mg/ml

treated cells appeared relatively smaller than control, some cells were round-shaped; 0.01 - 0.1 mg/ml caused detachment (Supplementary Fig. 2A). Chlorhexidine 0.001 mg/ml did not affect cell morphology; a majority of cells were detached due to 0.01 and 0.1 mg/ml chlorhexidine, and 1 mg/ml produced dense precipitation (Supplementary Fig. 2B). Triclosan 0.01 mg/ml did not alter cell morphology and confluency; 0.1 mg/ml reduced cell density; 1 and 2 mg/ml caused cell detachment (Supplementary Fig. 2C). Povidone-iodine 1, 2, and 4 mg/ml lowered cell density, and cells appeared thinner than control (Supplementary fig. 2D). Sodium bicarbonate 10 mg/ml exposed cells were in low numbers and altered shape (Supplementary fig. 2E).

### 3.2 alamarBlue® assay

Cells were exposed to antiseptic agents for ultra-short-term (10, 20, 30 sec), short-term (10, 20, 30 min) and long-term (24 h and 48 h). Cell viability was quantified as fluorescence intensity % relative to control-unexposed. Cetylpyridinium chloride 0.0001 mg/ml did not affect cell viability; ultra-short-term contact with 0.001-0.1 mg/ml caused up to 15% reduction in cell viability and long-term contact with 0.01-0.1 mg/ml led to less than 20% viability (Fig. 1A). Chlorhexidine 0.01 mg/ml long-term, 0.1 mg/ml short-term exposure affected lower than 50% viable cells, and 1 mg/ml ultra-short-term caused lower than 20% viability. In contrast, the cell viability was unchanged with ultra-short or short contact with 0.001 mg/ml chlorhexidine but was somewhat increased by long-term contact (Fig. 1B). Triclosan 1 and 2 mg/ml lowered cell viability down to 80% in ultra-short-term, down to 40-60% in the short-term, and after 48 h, the cell viability was less than 20%. Lower concentrations of triclosan 0.01 and 0.1 mg/ml did not decrease cell viability, and long-term exposure led to a slight but significant increase in it (Fig. 1C). Povidone-iodine 0.1 mg/ml did not disturb viability in ultra-short- or short-term. More than 20-sec contact with povidone-iodine 1 and 2 mg/ml povidone-iodine caused significantly reduced cell viability. With povidone-iodine 4 mg/ml, cell viability dropped instantly down to 40% and lower. Cells in contact with low concentrations of povidone-iodine 0.1 and 1 mg/ml also showed significantly stimulated cell growth after 24 h (Fig. 1E). Sodium bicarbonate 0.1-1 mg/ml did not change cell viability compared with control in ultra-short and short-term. They showed increased cell growth after long-term culturing. The alamarBlue® assay indicated that more than 30 min direct contact with 10mg/ml baking soda was cytotoxic to human PDLs (Fig. 1E).

The half maximal inhibitory concentrations (IC<sub>50</sub> value) for antiseptic compounds on PDLs described in Table 1.

### 3.3 Impedimetric analysis

The real-time ultra-short-term impedimetric signal was recorded every second for a 1-minute duration. It illustrated that cetylpyridinium chloride 0.01 and 0.1 mg/ml ( $p < 0.01$ ), chlorhexidine 1 mg/ml ( $p < 0.01$ ), triclosan 1 and 2 mg/ml ( $p < 0.01$ ), and povidone-iodine 1, 2 and mg/ml ( $p < 0.001$ ;  $p < 0.01$ ;  $p < 0.05$ ) were able to significantly decrease cellular impedance within 1 minute (Fig.2).

In long-term monitoring, cetylpyridinium chloride 0.0001 mg/ml did not affect cell index; 0.001 mg/ml caused gradual decrease ( $p < 0.001$ ); and due to 0.01 and 0.1 mg/ml cell index reached close to zero (Fig. 3A, 3B). Chlorhexidine 0.001 mg/ml reduced cell index ( $p < 0.05$ ) after 24 h and gradually decreased NCI due to 0.01 mg/ml reached close to zero after around 24 h. The cell index remained stagnant for 0.1 mg/ml and 1 mg/ml chlorhexidine added wells (Fig. 3C, 3D). Triclosan 0.01 and 0.1 mg/ml treated cells produced significantly lower than control ( $p < 0.05$ ) but the similarly ascending curve of NCI throughout 48 h, while higher concentrations 1 and 2 mg/ml caused a descending curve of NCI to reach close to zero after about 12 h (Fig. 3E, 3F). Povidone-iodine 0.1 did not alter cell growth, whereas 1, 2 and 4 mg/ml caused an instant drop in NCI. After around 24 h, the NCI curve for povidone-iodine 1 mg/ml treated groups showed a slowly ascending curve, while NCI for 2 and 4 mg/ml exposed groups remained close to

zero. Sodium bicarbonate 0.01-1 mg/ml did not affect cell viability. At a high concentration of 10 mg/ml, it dropped NCI to zero after 12 h, and the NCI remained close to zero during the rest of the measurement (Fig. 3I, 3J).

The half maximal inhibitory concentrations (IC<sub>50</sub> value) for antiseptic compounds on PDLCs described in Table 1.

	IC <sub>50</sub> (mg/ml) at 48 h	
	AlamarBlue® assay	RTCA assay
Cetylpyridinium chloride	0.0022 ± 0.001	0.001 ± 0.00008
Chlorhexidine	0.0037 ± 0.0002	0.0014 ± 0.00018
Triclosan	1.25 ± 0.007	0.57 ± 0.3
Povidone-iodine	1.4 ± 0.17	0.7 ± 0.4
Sodium bicarbonate	2.4 ± 0.1	7.4 ± 1.9

**Table 1** Half-maximal inhibitory concentration (IC<sub>50</sub>) of various dental antiseptic agents on human PDLCs in 48 h. Data are represented as mean ±SD obtained from alamarBlue® assay and xCELLigence analysis, each repeated three times (biological replicates) with three technical replicates.

#### 4. Discussion

Among examined antiseptic compounds, cetylpyridinium chloride was most cytotoxic to PDLCs in a dose- and time-dependent manner. Shorter than 1 min contact with 0.01 and 0.1 mg/ml significantly reduced cell viability, and prolonged exposure resulted in cell death. At a lower concentration, 0.001 mg/ml, it was able to reduce cell proliferation. The IC<sub>50</sub> value of cetylpyridinium chloride on PDLCs (0.001-0.002 mg/ml in 48 h) was much lower than the practically recommended concentration of 0.5-1 mg/ml (Haps et al., 2008).

Chlorhexidine 1 -50 mg/ml are recommended in dental practice depending on the type of products and treatment (Shyamacharan et al., 2017). In our study, chlorhexidine 1 mg/ml reduced PDLCs viability instantly, and 0.01 mg/ml interrupted cell proliferation. Its IC<sub>50</sub> value on human PDLCs (0.001-0.003 mg/ml) was much lower than practically recommended concentrations. Impedimetry showed 0.1-1 mg/ml chlorhexidine caused a stagnant CI, and the dense precipitation was observed with microscopy. It can be explained that chlorhexidine forced immediate precipitation in the animal serum-supplemented culture (Hidalgo and Dominguez, 2001); it acted as an insulator on the electrode surface.

Some countries have banned triclosan from hygienic products because of its potential health risks such as antimicrobial resistance and endocrine disruption (Wolf, 2017). Nevertheless, it is available in dental products such as mouthwash and toothpaste, mostly with 3 mg/ml concentration (Fernández et al., 2017), (Riley and Lamont, 2013). Our results presented that triclosan higher than 1 mg/ml decreased cell viability within a minute and suppressed proliferation in the long-term.

Povidone-iodine is applied in the oral cavity in a wide range of concentrations between 10-100 mg/ml. In our study, the IC<sub>50</sub> of povidone-iodine on PDLCs was 0.7-1.4 mg/ml.

More than 30-min exposure to sodium bicarbonate at 10 mg/ml caused significantly decreased cell viability, and long-term continuous exposure led to cell death.

The advantages of xCELLigence system versus the traditional end-point assay are a continuous readout that gives a possibility to analyze any time point of the measurement and labor efficiency. To our knowledge, the current study was the first to perform ultra-short-term impedimetric analysis for cytotoxicity analysis of antiseptic agents. The current study's limitation is that *in-vitro* monolayer cell



culture cannot accurately represent a periodontal wound condition. More *in vivo* studies are needed to examine and enhance dental antiseptic compounds' safety and efficacy when applied near open incisions or intra-wound application.

## 5. Conclusions

Antiseptic agents exerted a cytotoxic effect on PDLCs in a dose-dependent manner and reduced cell viability and proliferation at lower than practically used concentrations. Among tested compounds, cetylpyridinium chloride and chlorhexidine were highly cytotoxic to PDLCs.

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## CRediT authorship statement

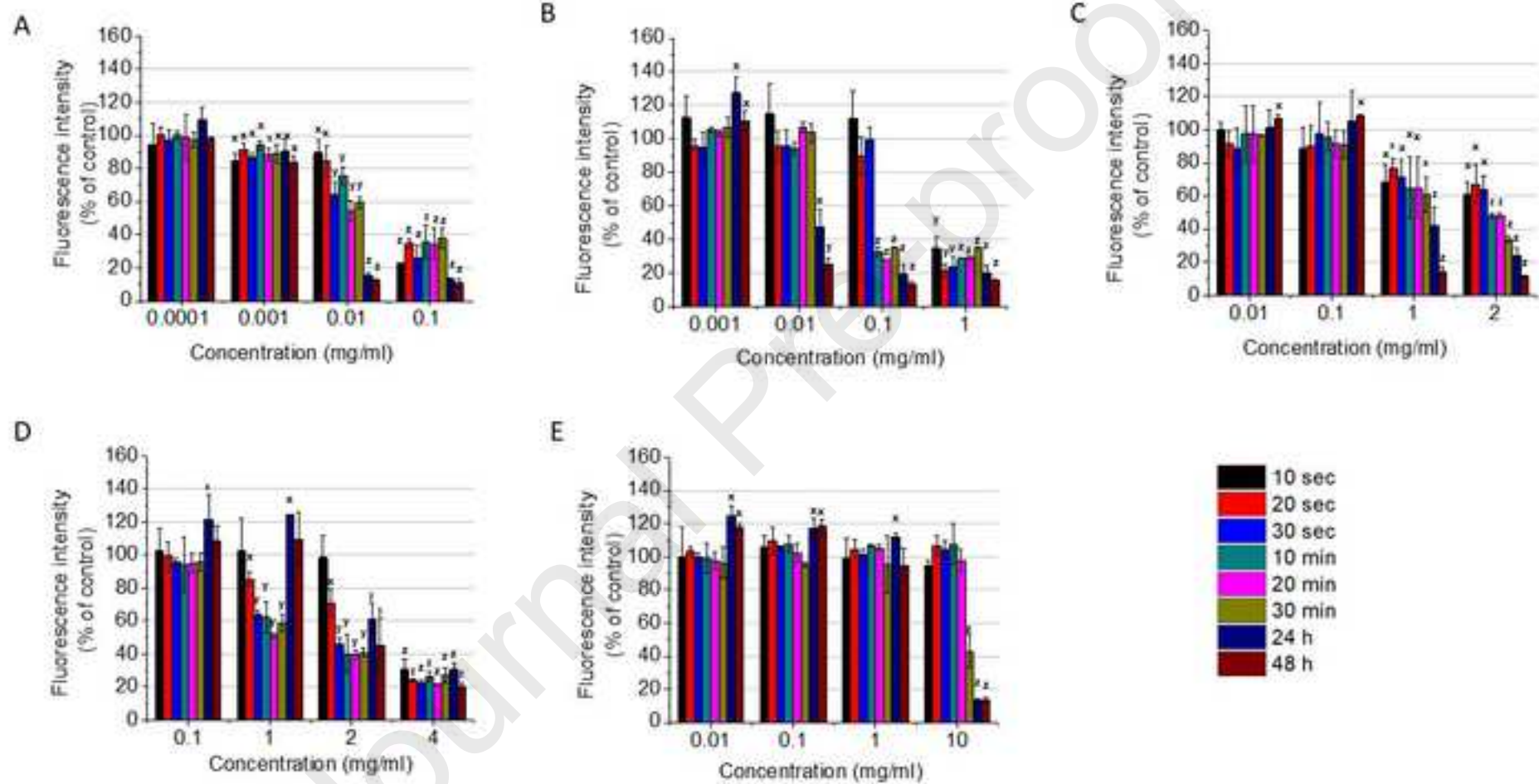
**Zambaga Khorolsuren:** Data curation; Formal analysis; Investigation; Methodology; Roles/Writing - original draft; Writing - review & editing. **Orsolya Lang:** Formal analysis; Investigation; Methodology; Software. **Janos Vag:** Conceptualization; Validation; Writing - review & editing. **Laszlo Kohidai:** Conceptualization; Funding acquisition; Project administration; Resources; Supervision; Roles/Writing - original draft; Writing - review & editing.

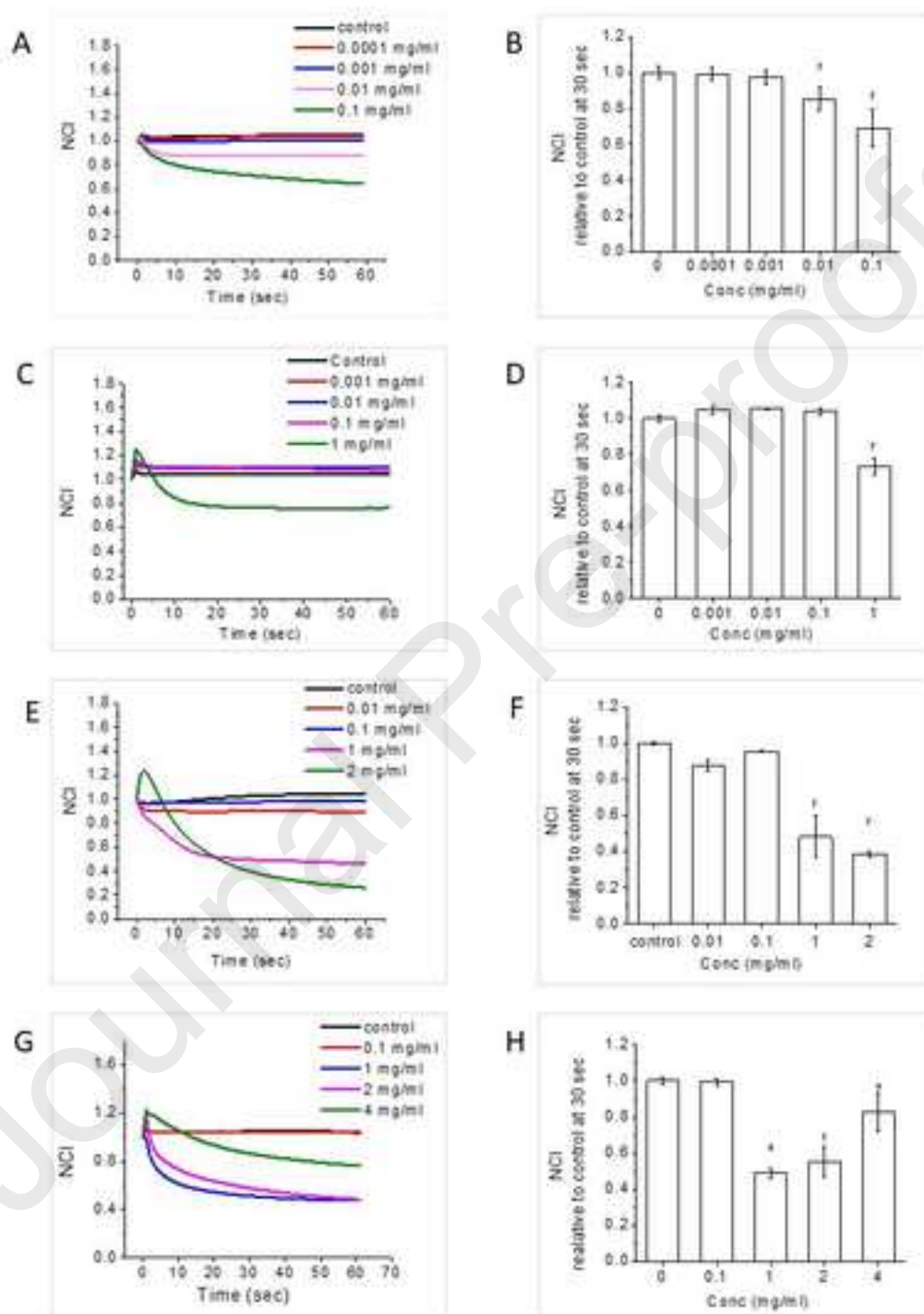
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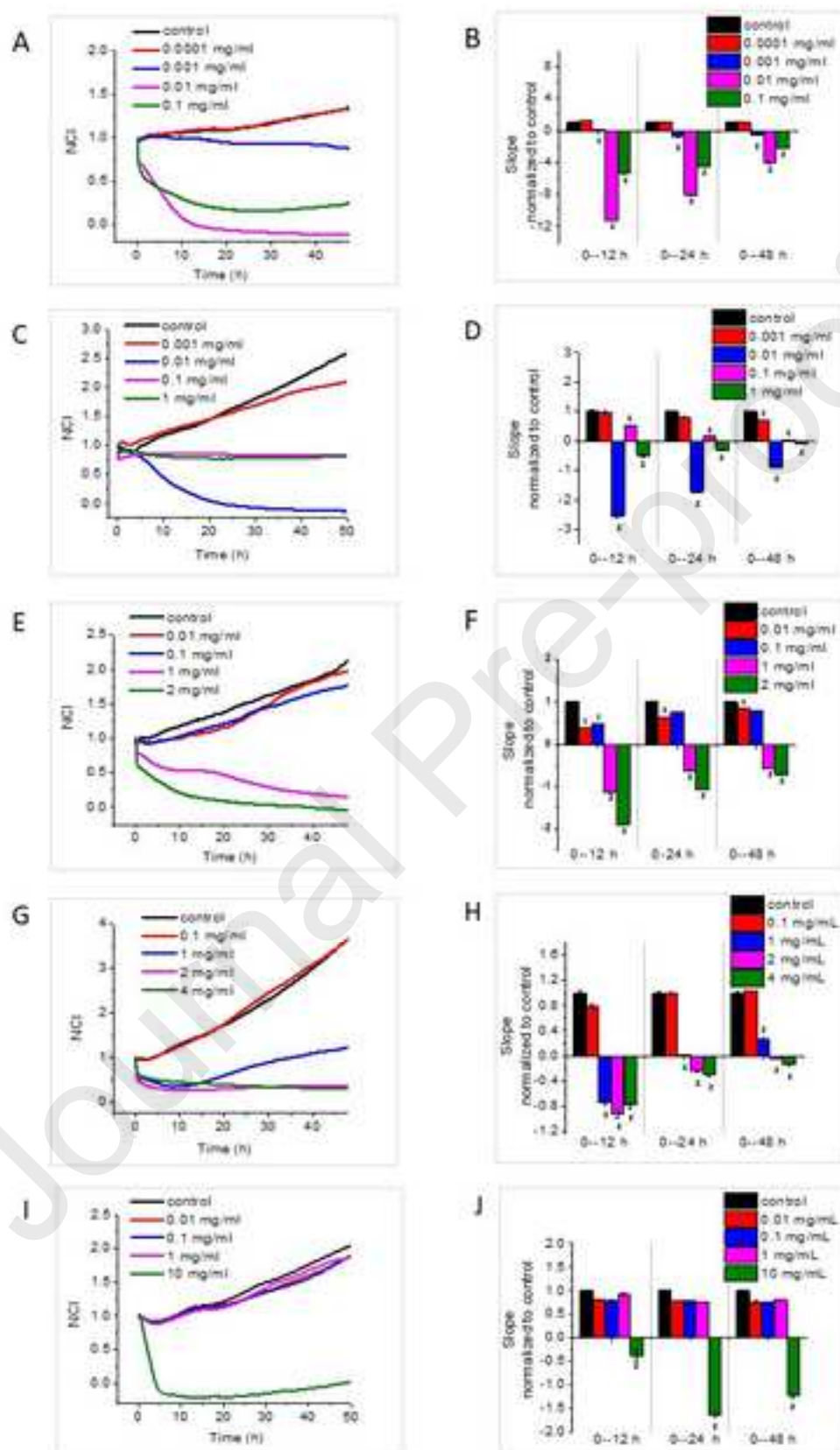
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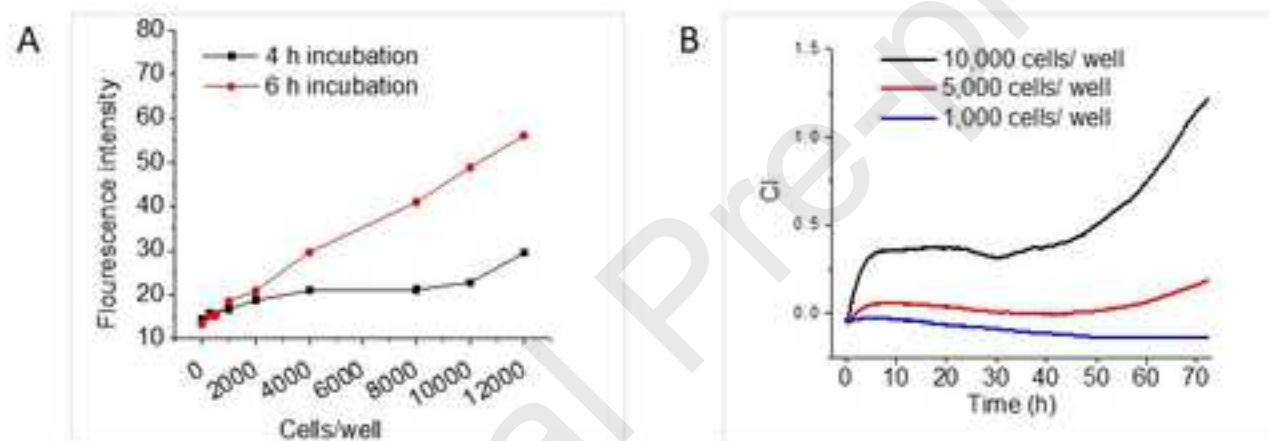
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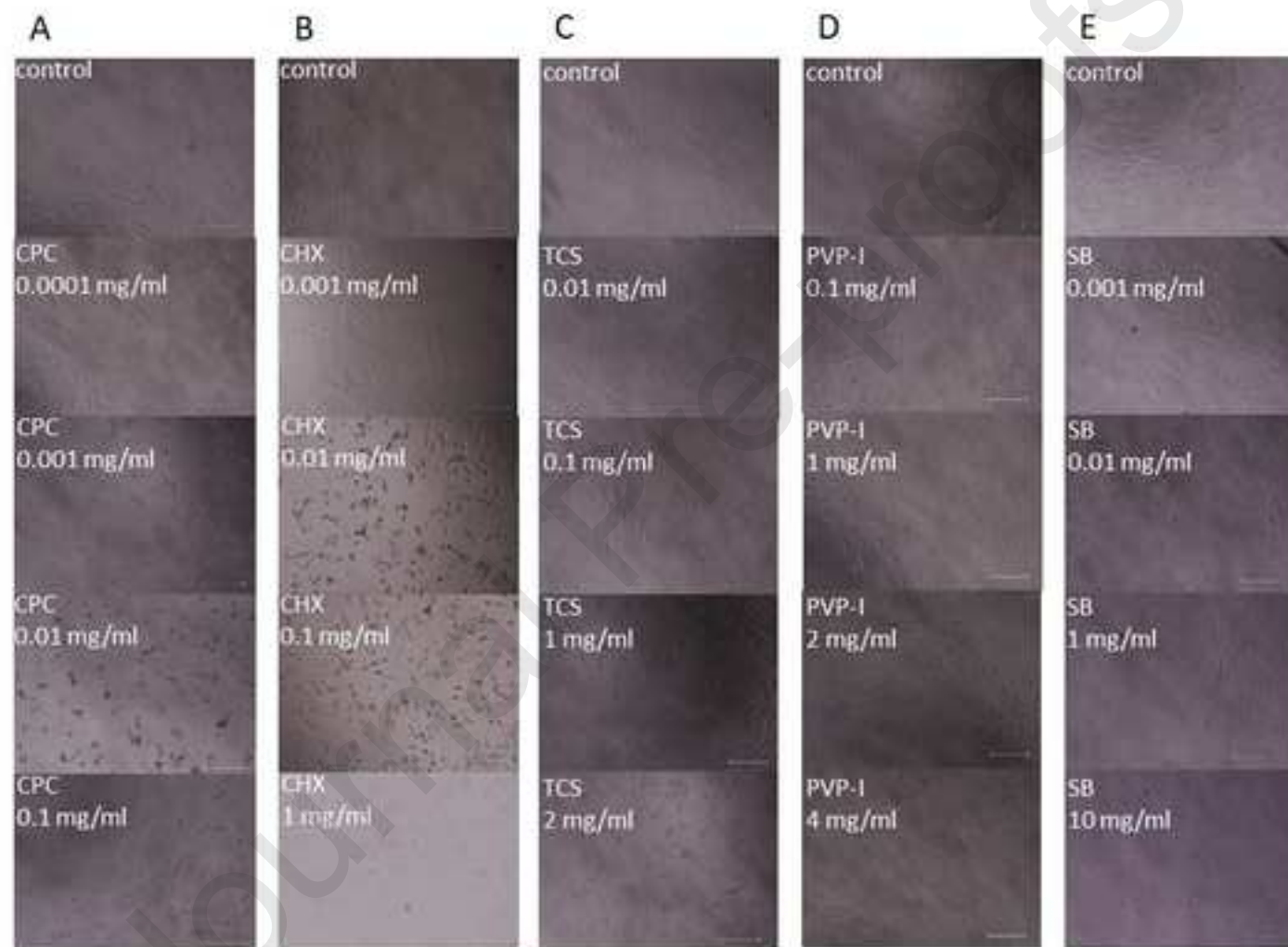














	IC50 (mg/ml) at 48 h	
	alamarBlue® assay	RTCA assay
Cetylpyridinium chloride	0.0022 ± 0.001	0.001 ± 0.00008
Chlorhexidine	0.0037 ± 0.0002	0.0014 ± 0.00018
Triclosan	1.25 ± 0.007	0.57 ± 0.3
Povidone-iodine	1.4 ± 0.17	0.7 ± 0.4
Sodium bicarbonate	2.4 ± 0.1	7.4 ± 1.9

Table 1. The half-maximal inhibitory concentration (IC50) of various dental antiseptic agents on human PDLCs in 48 h. Data are represented as mean ±SD obtained from alamarBlue® assay and xCELLigence analysis, each repeated three times (biological replicates) with three technical replicates.

## Effect of various mouthwash antiseptic agents on viability and proliferation of human periodontal ligament cells

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### Author declaration

*[Instructions: Please check all applicable boxes and provide additional information as requested.]*

#### 1. Conflict of Interest

Potential conflict of interest exists:

We wish to draw the attention of the Editor to the following facts, which may be considered as potential conflicts of interest, and to significant financial contributions to this work:

The nature of potential conflict of interest is described below:

**X No conflict of interest exists.**

**Authors wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.**

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Budapest, 20/11/2020

**Prof. Hamdan S. Alghamdi**  
**King Saud University Department of Periodontics**  
**and Community Dentistry, Riyadh, Saudi Arabia**

**Editor-in-Chief**  
**The Saudi Dental Journal**

Dear Professor Algamdi,

With reference to the title of our manuscript:

*„Effect of various mouthwash antiseptic agents on viability and proliferation of human periodontal ligament cells”*

we are sending it for considering to be published as a full research paper in the

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The materials (text (+highlights, +abstract, +legends) with 1 table +5 figures +3 supplementary files) of our manuscript were uploaded to the online Editorial Manager system of the Journal.

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Best regards

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Assoc. Professor, Med. Habil.

Budapest, 03/03/2020

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**King Saud University Department of Periodontics**  
**and Community Dentistry, Riyadh, Saudi Arabia**

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### **Author Statement**

Dear Professor Algamdi,

With reference to our manuscript:

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which revised version was uploaded for considering to be published as a full research paper in the

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Authors hope that the Editorial Board and the Reviewers will agree on the interest and novelty of this study and will accept manuscript material for publication .

Best regards

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