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ORIGINAL ARTICLE

Azithromycin with scaling and root planing versus scaling and root planing alone in the treatment of periodontitis: A randomized controlled trial

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KEYWORDS

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CD68 antigen;
M1 M2 macrophages;
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Scaling and root planning

Abstract *Background:* The growing interest in the possibilities of macrophages modulation with therapeutic purposes promotes new approaches for periodontitis treatment.

Aim: The aim of this randomized controlled open clinical study was to evaluate the early clinical and immunological effects of the long-course azithromycin as an adjunct to scaling and root planing in periodontitis.

Methods: 50 patients (with stage I-III, grade A/B periodontitis) and 22 periodontally healthy volunteers as the reference group were recruited. Following scaling and root planing (SRP), the patients were randomly assigned to one of two treatment modalities: SRP only (n = 25) and adjunct azithromycin (Az) treatment (n = 25). The patients were monitored at baseline, and 30 ± 5 days after therapy. Clinical attachment loss (CAL), periodontal probing depth (PPD) and bleeding on probing (BoP) were evaluated. Secondary outcome measures included mean changes in single-positive CD68 + and CD163 + macrophages (Mφs) density and ratio, evaluated by immunohistochemistry, and IL1-β, IL-6, IL-10, TGF-β levels, detected by ELISA.

Results: At 1 month both groups showed significant improvements of CAL, PPD and BoP, without significant added benefit in terms of CAL, PPD and BoP of Az. But Az increased the density of CD68 + and CD163 + Mφs ($P < 0.0001$), decreased the CD68+/CD163 + ratio ($P = 0.043$), decreased IL-1β ($P < 0.01$), IL-6 ($P < 0.001$) levels, and increased IL-10 ($P < 0.0001$) and

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TGF-β ($P < 0.001$) levels compared to SRP and periodontitis at baseline.

Conclusion: The long course of Az demonstrated modulation of CD68 + and CD163 + Mφs towards M2 polarization, which may play a significant role in achieving favorable long-term treatment outcomes. ClinicalTrials.gov

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1. Introduction

Periodontitis is an immune-mediated disease that causes progressive destruction of the tooth-supporting structures. Susceptible patients present with aberrant immune responses, leading to temporary disease activity in certain sites, along with the progression of periodontal destruction (Silva et al., 2015).

The benefits of the azithromycin (Az) in periodontitis treatment have been confirmed by a number of clinical investigations (Fujise et al., 2014; Jagannathan et al., 2019; Nepokupnaia-Slobodianuk and Skripnikov, 2014; Oliveira et al., 2019). New treatment dynamics includes high dosages and long treatment periods, as recommended for biofilm-related diseases (Del Pozo, 2018). Additionally, the effectiveness of Az depends not only on its antibacterial activity but also on its potential immunomodulatory action.

Az is a well-known macrolide antibiotic that inhibits bacterial protein synthesis, quorum-sensing, and reduces biofilm formation. It accumulates effectively in cells, particularly phagocytes, and is delivered in high concentrations to sites of inflammation (Parnham et al., 2014). Furthermore, the immunomodulating and regenerative impact of azithromycin has been demonstrated in periodontitis (Fujise et al., 2014).

Macrophages (Mφs) should be responsible for different levels of inflammation and tissue remodeling topically in periodontitis (Silva et al., 2015, Zhou et al., 2019). Importantly, Mφs orchestrate all immune responses and have the ability to exhibit the polar-opposite M2/healing and M1/killing functions (Mills et al., 2015). Classically activated Mφs, or M1, are pro-inflammatory, involved in bacterial killing and promote inflammation through increased production of cytokines such as interleukin-1 beta (IL-1β), IL-6, IL-12, IL-23, and tumor necrosis factor alpha (TNF-α). In contrast, alternatively activated, or M2, play a role in the resolution of inflammation and tissue repair, characterized by the production of IL-10 and transforming growth factor beta (TGF-β) (Shapouri-Moghaddam et al., 2018). M1- or M2-predominant Mφs can induce Th1/Th2 or other types of responses (Mills et al, 2000). The M1/M2 ratio and the expression profile of these cells can change in different diseases, leading to their transformation into pathogenic ones (Satoh, 2018).

Despite the confirmed disturbance of the M1/M2 ratio in periodontitis (Zhou et al., 2019), no attempts have been made to modify it. We hypothesized that Az could inhibit periodontal inflammation by shifting the local gingival Mφs subpopulations. The aim of this randomized controlled add-on open clinical study was to evaluate the early clinical and immunological effects of the long-course Az as an adjunct to scaling and root planing (SRP) in periodontitis.

2. Material and methods

2.1. Ethical guidelines

All eligible patients (ClinicalTrials.gov ID: NCT05506371, 08/17/2022) were informed of the nature, potential risks and benefits of their participation in the study, and signed an informed consent form. The study protocol was reviewed and approved by the Ethical Committee of Poltava State Medical University (August 23rd, 2021, No. 207). The research was conducted in full accordance with the Helsinki Declaration of 1975, as revised in 2013.

2.2. Patient population

Fifty patients with periodontitis (23 women and 27 men; mean age: 46.76 years, range: 19–82 years old) and 22 healthy volunteers (11 women, 11 men; mean age: 26.1 years, range: 14–67 years old) were recruited from the population referred to the Department of Postgraduate Education for Dentists of Poltava State Medical University (Poltava, Ukraine). Analysis of the effects of smoking, age, or gender was not assessed in this study.

2.3. Inclusion and exclusion criteria

Diagnosis of periodontitis in stages I-III grade A/B and periodontal health were identified using the criteria of the World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions, 2017.

Inclusion Criteria for periodontitis patients.

- Presence of at least 15 natural teeth, at least 10 sites with a periodontal pocket depth (PPD) $\geq$ 4 mm and/or clinical attachment loss (CAL) of $\geq$ 4 mm at the same site, and satisfactory general health.
- Grade A or B as patterns of the periodontitis progression (radiographic bone loss expressed as percentage of root length divided by the age of the subject $<$ 0.25 to 1.0).

Inclusion criteria for healthy volunteers.

- Presence of more than 20 teeth (for each tooth, the probing pocket depth $\leq$ 3 mm).
- Absence of clinical gingival inflammation at intact, or reduced periodontium.
- Bleeding on probing $<$ 10%.

Exclusion Criteria for periodontitis patients.

- Taking antibiotics or anti-inflammatory medications within the previous 3 months.
- Periodontal therapy within the previous 6 months.
- Pregnancy or breastfeeding.
- Severe, uncontrolled (decompensated) diseases of the internal organs, or neuropsychiatric disorders.
- Other conditions that determined the patients' inability to understand the nature and possible consequences of the study.

Periodontal first and second step therapy (Sanz et al., 2020) were performed using ultrasonic scaler, Gracey curettes and

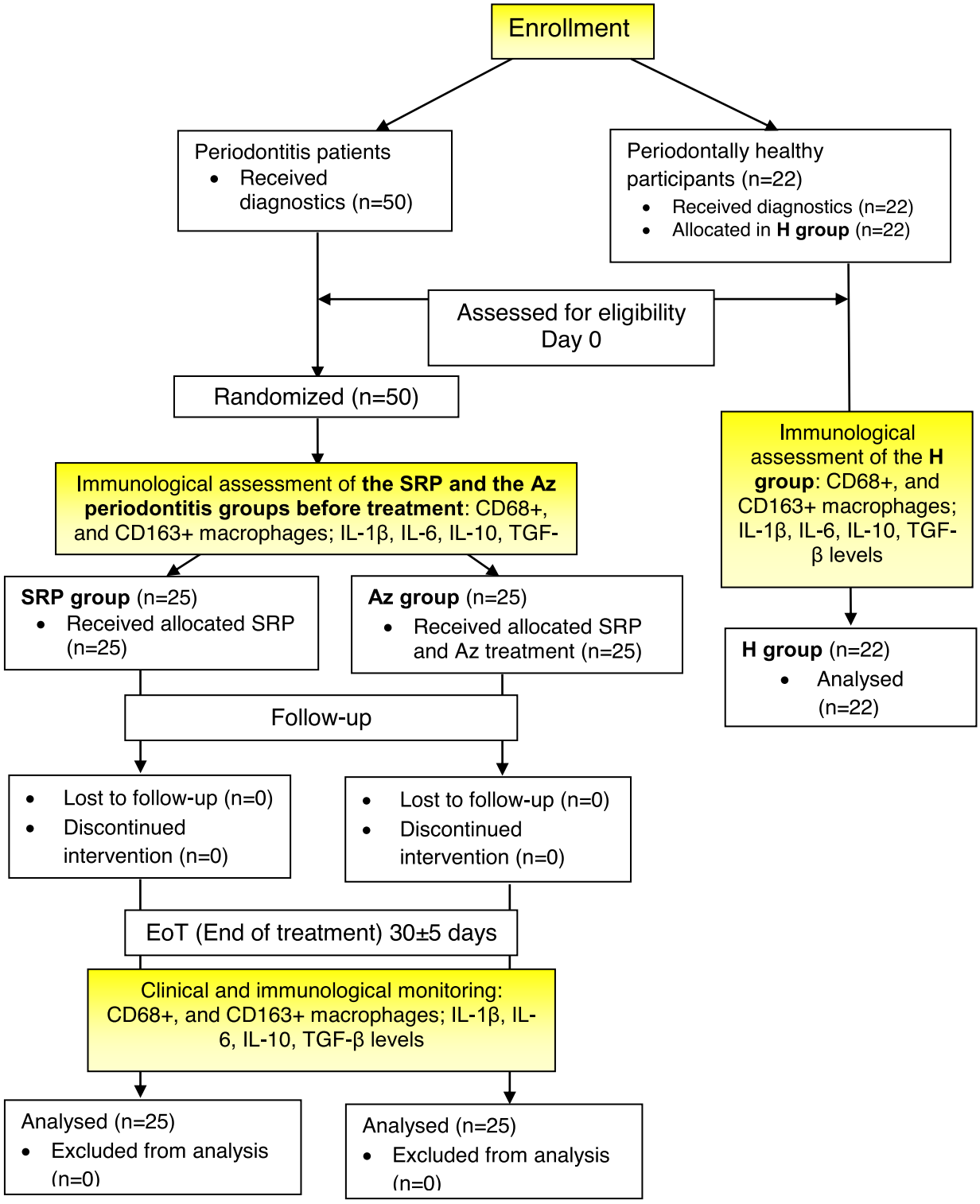

Fig. 1 Flowchart of the study design.

air-abrasive polishing. A compliance control measure included pill counting.

2.4. Experimental design and treatment protocol

Eligible patients with periodontitis were randomly (simple randomization) allocated to groups according to gender and age after the basal examination using a computer-generated list (Fig. 1). The comparison group underwent scaling and root planing (SRP group, n = 25) as a one-stage full-mouth procedure. The Az group (n = 25) additionally received oral azithromycin ("Health" Pharmaceutical Company, Kharkiv, Ukraine) by the long-term course in a dose of 500 mg q.d. for 7 days, followed by 500 mg q.w. for the next 3 weeks, as described earlier (Nepokupnaia-Slobodianuk and Skripnikov, 2014, Del Pozo, 2018). The patients of the Az group started taking azithromycin the day before the SRP session.

All patients were clinically examined at baseline, before treatment, and after 1 month ± 5 days by the same examiner.

Examiner calibration was determined using the Kappa coefficient, with a threshold of ≥ 0.85 indicating agreement. Ten patients with at least five teeth with PPD and CAL ≥ 5 mm on proximal sites were selected. Each patient was examined twice by a manual periodontal probe (0106. DT06.CP10, DenTag, Maniago, Italy), with a 48 h interval between the first and second assessments.

For all periodontitis patients, sex and age were recorded, and periodontal parameters CAL, PPD, and bleeding on probing (BoP) were measured at six periodontal sites around each tooth including the third molars to the nearest 1 mm. All measurements and periodontal treatment were performed by the same experienced clinician.

Periodontally healthy participants in the H group (n = 22) served as the reference to data about Mφs and cytokine levels. The signs of gingival inflammation, probing depth exceeding 3 mm and BoP greater than 10% were excluded to insure periodontal health.

2.5. Immunological monitoring

2.5.1. Collection of gingival tissue samples

For precise immunohistochemical analysis, gingival biopsies (~3 mm^2) were excised from the most severe clinically appropriate site at baseline and 1 month ± 5 days after SRP. Local anesthesia ensured patient comfort during the harm-free biopsy procedure. Biopsy sites were selected from areas commonly associated with dental and periodontal procedures. Biopsies from periodontally healthy patients were obtained during tooth extraction for reasons like caries complications, wisdom teeth, and orthodontic indications. Half of the biopsies were fixed in 4% formalin for 24 h, dehydrated, and embedded in paraffin, while the other half were prepared for cytokine assays.

2.5.2. ***Immunohistochemistry and antibodies***

Paraffin sections, 2–3 μm in thickness, were deparaffinized and dehydrated. Heat-induced epitope retrieval was performed in citrate buffer, pH 6, then processed with blocked reagent, incubated with mouse monoclonal CD68 (1:30, clone PG-M1) or CD163 antibodies (1:100, clone 10D6), stained with the 2-steps plus Mouse/Rabbit PolyVueTM HRP/DAB Detection System (all from Diagnostic BioSystems, The Hague, The Netherlands), and counterstained with Mayer's hemalum. Phosphate-buffered saline was used as a negative control, and lymph node tissue was used as a positive control.

2.5.3. Evaluation of immunohistochemical staining

CD68 + and CD163 + Mφs were counted under light microscope Axio Lab.A1 (Carl Zeiss, Göttingen, Germany) (×400) in infiltrated areas of 5 selected regions per slide. The number of Mφs per 10 000 μm^2 was calculated as density. The evaluation of immunohistochemical staining was performed by the same experienced examiner.

2.5.4. Tissue culturing

Part of gingival tissue samples were cultured in a humidified incubator at 37 °C in the presence of 5 % CO_2. Biopsies were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % (vol/vol) fetal bovine serum (Thermo Fisher Scientific, Gibco™, US), 1 % (vol/vol) penicillin–streptomycin (Gibco™, US), and 0.2 % (vol/vol) fungizone amphotericin B (Gibco™, US) during 48 h (Hai et al., 2006). Each biopsy sample was placed in a separate well of Costar® 24-well Plates (Corning Life Sciences, Kennebunk, US), containing 0,4 ml of supplemented DMEM. After incubation, the tissues were removed and culture media were centrifuged at 800 g and 4 °C.

2.5.5. Cytokine assay

Cytokine assay in the tissue culture medium were determined using ELISA. IL1-β, IL-6, IL-10, and TGF-β concentrations were measured using kits from MyBioSource (San Diego, US). Cytokine concentrations were expressed as recommended by the manufacturer.

2.6. Outcome variables

Mean changes in CAL, PPD, and BoP were defined as the primary outcome variables.

Mean changes in CD68 + and CD163 + Mφs density, CD68+/CD163 + ratios, levels of IL1-β, IL-6, IL-10, and TGF-β were defined as the secondary outcome measures.

2.7. Statistical analysis

Sample size calculation was performed using CAL as the primary outcome variable. A significance level of α = 5% α and a power level of 80% was defined. Considering a difference ≥ 1 mm between groups in CAL changes and a standard deviation of 0.8 mm, 14 participants per group were necessary to detect potential differences (Haffajee and Socransky, 1986). For PPD, CAL and BoP statistical evaluations, the patient was the unit of measurement. Statistical analysis was performed using Prism 5 software (GraphPad Software, San Diego, US) with ANOVA, Chi-square test, and correlation. P values < 0.05 were considered significant in all analyses.

The null hypothesis tested was that Az has no influence on clinical measurements, density, the CD68+/CD163 + ratio, and cytokines levels.

3. Results

3.1 Clinical monitoring

This trial started in September 2017 and finished in June 2019, but clinical monitoring of patient continues to obtain long-term results. The diagnosis of chronic periodontitis of I-III stages Grade A/B was confirmed in all patients. All patients successfully completed the study. Fig. 1 shows the study design.

Before periodontitis treatment, the SRP and Az groups were homogeneous in terms of clinical parameters, except for mean CAL occasionally (Table 1).

SRP led to non-significant means of CAL and PPD reduction, but a significant BoP reduction compared to the baseline.

Az was well tolerated by all participants without any observed or self-reported adverse effects. It led to non-significant mean CAL reduction, significant PPD reduction from 3.7 to 3.2 (95%CI [0,084—0,93]), and significant BoP reduction compared to the baseline.

The between-groups difference was observed only in mean CAL, which persists from the baseline and was doubtfully related to the Az treatment. The between-group PPD difference did not reach statistical significance (Table 1).

3.2. Immunological monitoring

The histology of biopsy samples was represented by two epithelial surfaces of marginal gingiva: oral and sulcular/junctional. Oral epithelium covered non-infiltrated dense connective tissue. The sulcular epithelium covered the infiltrated connective tissue area.

General characteristics of CD68 + and CD163 + Mφs include granular staining, as well as differences in size, shape and reactivity. Mφs were localized subepithelial close to the basal lamina in cellular infiltrates or scattered.

The densities of CD68 + and CD163 + Mφs were lower, and the CD68+/CD163 + ratio was higher before periodontitis treatment compared to the H group (Fig. 2, a,c,e,f).

Az intake led to a significant increase in the density of both Mφs subpopulations (Fig. 2, a-e).

In the SRP group, the density of CD68 + and CD163 + Mφs increased non-significantly (Fig. 2, e).

In the Az group the CD68+/CD163 + ratio was significantly decreased versus periodontitis at baseline and tiny higher versus SRP treatment, and did not differ from the H group (Fig. 2, f). Az intake led to the loss of the direct correlation between CD68 + and CD163 + Mφs density, which was observed at baseline, in healthy gingiva, and after SRP treatment (Fig. 2, g-j).

The feature of the H group was weak immunoreactivity in 40% of samples (Fig. 3, a). Strong immunoreactivity was observed in Mφs localized under sulcular epithelium (Fig. 3, b).

In periodontitis-affected gingiva levels of IL-1β, IL-6 were increased, and IL-10, TGF-β levels were decreased ($P < 0.001$), compared to the H group (Fig. 4).

The Az intake significantly decreased IL-1β and IL-6 levels, and significantly increased IL-10 and TGF-β levels compared to SRP and periodontitis before treatment (Fig. 4).

The SRP significantly increased IL-10 and TGF-β levels compared to the baseline (Fig. 4).

Table 1 Comparative analysis of clinical periodontal parameters over time and between treatment modalities.

Group and patients' distribution	**Time-point**	**Clinical attachment loss** mean ± SD (standard deviation), mm	**Periodontal probing depth** mean ± SD (standard deviation), mm	**Bleeding on probing** mean, %	**Comparisons between groups**
Azythromicin group: 14 women and 11 men; mean age: 47 years, (range: 19–82 years old)	0	4.5 ± 0.89	3.7 ± 0.68	62	P_1 = **0.001** 95%CI [0.26–1.28] P_2 > 0.05 P_3 < **0.001** 95% CI [0.07–0.12]
	End of treatment (30 ± 5 days)	4.1 ± 0.98	3.2 ± 0.56	13	P_1 = **0.001** 95% CI [0.06–1.08] P_2 > 0.05 P_3 > 0.05
Scaling&root planning group: 9 women and 16 men; mean age: 47 years (range: 20–74 years old)	0	3.7 ± 0.79	3.3 ± 0.63	52	–
	End of treatment (30 ± 5 days)	3.5 ± 0.61	3.0 ± 0.41	14	–
Comparisons among examination times		$P_{1\ Az}$ > 0.05 $P_{1\ SRP}$ > 0.05	$P_{2\ Az}$ < **0.001** 95%CI [0,08—0,93] $P_{2\ SRP}$ > 0.05	P_3 < **0.001**	–

P value calculated by one-way ANOVA and post-hoc Bonferroni tests and Chi-square test (for bleeding on probing). Statistically significant differences ($P < 0.05$): P_1 – for clinical attachment loss, P_2 – for periodontal probing depth, and P_3 – for bleeding on probing comparisons.

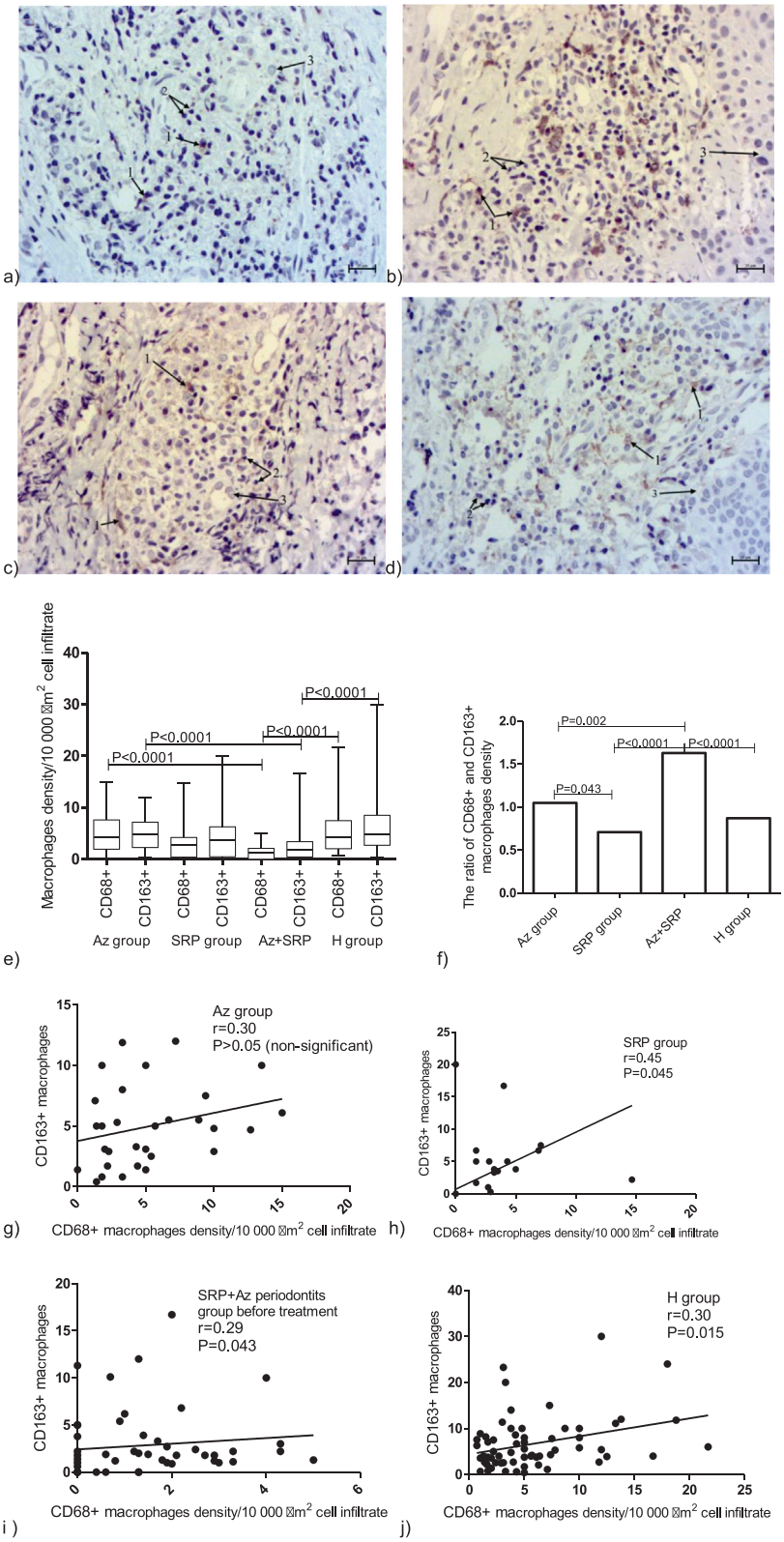

Fig. 2 Macrophages in periodontitis-affected and clinically healthy gingiva. Counterstaining: Mayer's hemalum, scale bars 20 μm: 1-immunopositive macrophages; 2- infiltrating cells; 3- epithelial cells; Az + SRP - the Az and the SRP groups before periodontitis treatment. a) CD68 + Mφs among infiltrating cells in the Az and the SRP groups before periodontitis treatment; b) CD68 + Mφs in the Az group after treatment; c) CD163 + Mφs among infiltrating cells in the Az and the SRP groups before periodontitis treatment; d) CD163 + Mφs in the Az group after treatment; e) Intra- and between-group comparisons of immunopositive Mφs density. P value calculated by one-way non-parametric ANOVA and posthoc Bonferroni tests, $P < 0.001$; f) Intra- and between-group comparisons of CD68/CD163 ratios. P value calculated by Chi-square test; Correlations between CD68 + and CD163 + Mφs density: g) in the Az group after treatment; h) in the SRP group after treatment; i) in the Az and the SRP groups before periodontitis treatment; j) in the H group.

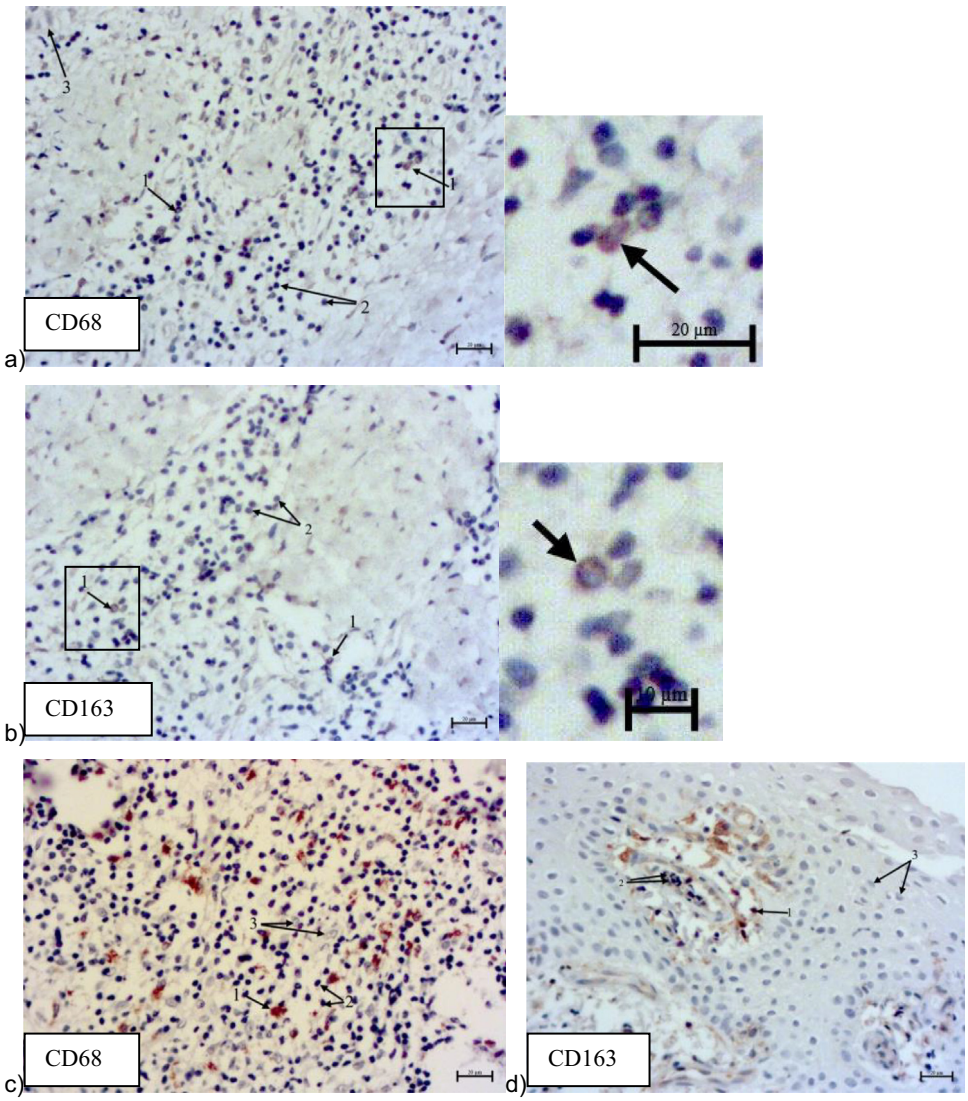

Fig. 3 Macrophages in clinically healthy gingiva. Counterstaining: Mayer's hemalum, scale bar 20 µm. a) Contour-like and weak CD68 + and b) CD163 + immunoreactivity: 1- immunopositive Mφs; 2- infiltrating cells; 3- epithelial cells. c) Strongly immunopositive CD68 + Mφs and; d) CD163 + Mφs with dendritic morphology.

4. Discussion

The primary objective of this study was to assess the clinical efficacy of long-course Az when used in conjunction with SRP in periodontitis treatment. Az was administered using a specific dosage regimen that has been previously supported for the treatment of biofilm-related infections. This study is the first to investigate the impact of Az on periodontitis in combination with Mφs and cytokine levels.

The study predominantly revealed:

- a nonsignificant improvement in CAL between treatment modalities;
- significant PPD improvement during Az administration without clinically meaningful difference between the groups;
- significant improvement of BoP regardless of the treatment;
- significant increase in CD68 + and CD163 + Mφs density after Az administration with a decrease in the CD68 + /CD163 + ratio, and significant changes in IL-1β, IL-6, IL-10, and TGF-β levels, providing evidence for the additional immunological efficacy of Az. Therefore, the null hypothesis can be rejected.

Our findings showed that both treatment modalities resulted in a reduction of mean CAL, PPD, and BoP, but without significant differences, indicating no additional clinical benefit of Az. These results are consistent with a recent report by Morales et al. (2021), but review by Martande et al. (2016) reported a higher overall mean effect of Az. Furthermore, the interest in Az remains high (Andrada et al., 2020).

The immunomodulatory action of Az on Mφs has been supported by previous studies, demonstrating functional impairment of Mφs and a shift toward an anti-inflammatory

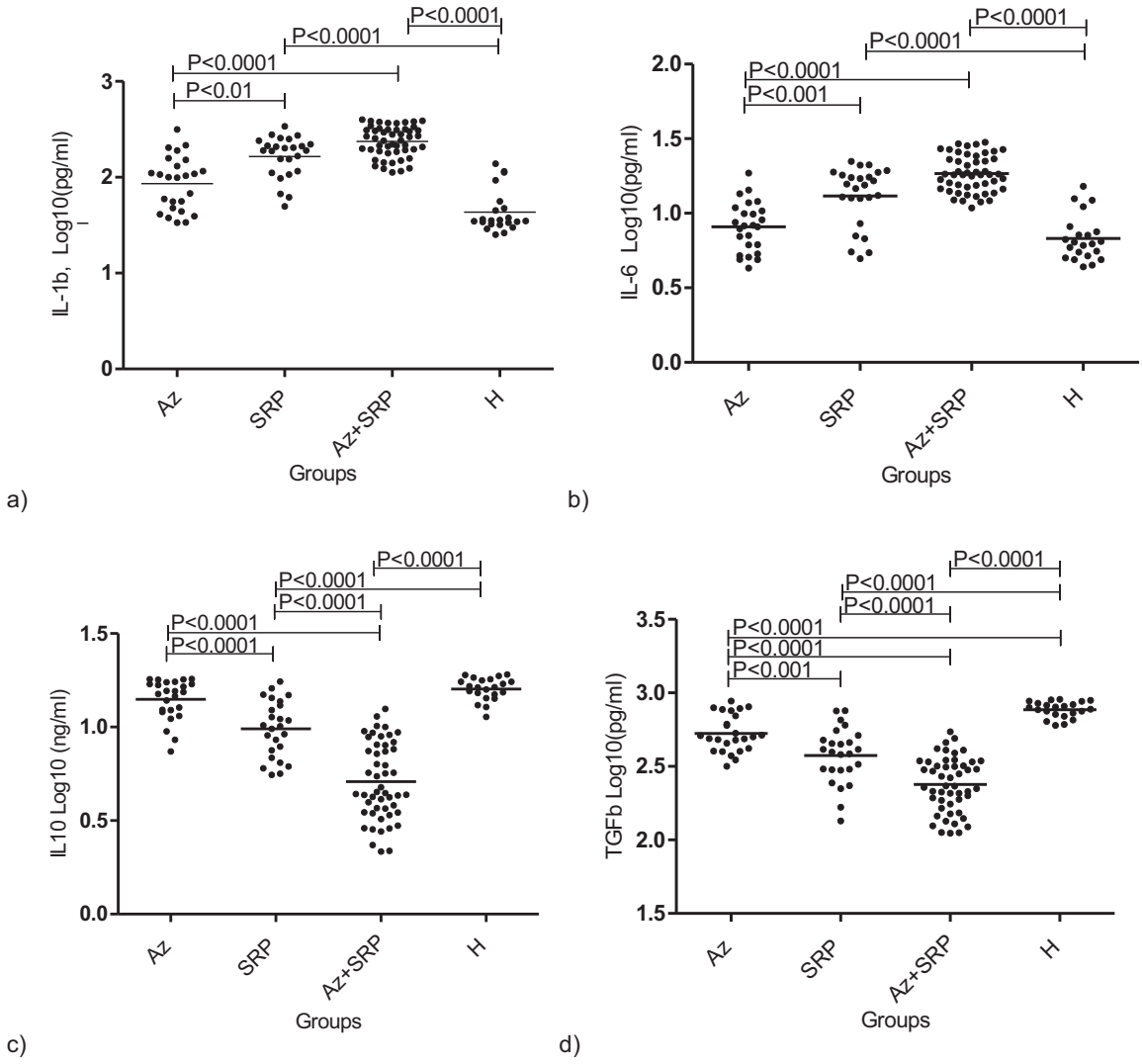

Fig. 4 Chemokine levels before treatment in periodontitis, after two regiment of treatment and in healthy gingiva. P value calculated by one-way non-parametric (IL-1β) or parametric ANOVA (other cytokines) & post hoc test; statistically significant differences ($P < 0.05$); *y* axis log10-scaled; Az + SRP - the Az and the SRP groups before periodontitis treatment. a) IL-1β levels comparison; b) IL-6; c) IL-10; d) TGF-β.

phenotype (Parnham et al., 2014; Haydar et al., 2019). Additionally, Vrančić et al. (2012) demonstrated that Az attenuated metabolism, production, and expression of pro-inflammatory cytokines in human Mφs stimulated with lipopolysaccharide.

To assess the expecting modulation of Mφs, the single positive CD68 + and CD163 + Mφs subpopulations were estimated (Allam et al., 2011, Barros et al., 2013, Fabriek et al., 2005, Rakic et al., 2022), further complemented with M1 (IL1-β, IL-6), M2 (IL-10, TGF-β) cytokines levels. Garaicoa-Pazmino et al. (2019) used CD68 as a marker for total Mφs in periodontitis-affected gingiva. Allam et al. (2011) demonstrated expression of the CD163 on CD68 + cells isolated from peripheral blood from the bottom region of periodontitis lesions, suggesting the presence of inflammatory M1 within the CD68 + Mφs subpopulation. And CD163 remains a reliable marker for detecting M2 in gingival connective tissue (Yang et al., 2018). M1-related cytokines include IL-1β, IL-6, while M2-related cytokines include IL-10 and TGF-β (Zhu et al., 2015). Therefore, CD68 + Mφs with IL-1β and IL-6, and CD163 + Mφs with IL-10 and TGF-β can serve as morphological equivalents of the M1 and M2, respectively.

This study confirmed that periodontitis is associated with increased levels of IL-1β and IL-6, and decreased levels of IL-10 and TGF-β compared to healthy gingiva, consistent with earlier findings (Zhou et al., 2019; Zhu et al., 2019). The study also revealed an increased CD68 + /CD163 + ratio. Interestingly, lower densities of CD68 + and CD163 + Mφs were defined in periodontitis, which could be explained by the reduced polarization of Mφs in this condition, as suggested by Garaicoa-Pazmino et al. (2019).

The additional immunological efficacy of Az observed in this study is consistent with findings from other studies that have reported changes in cytokine levels (Skrypnykov et al., 2014, Pan et al., 2019, Dutzan et al., 2009, Choi et al., 2004, Gur et al., 2022). However, the increase in CD68 + Mφs density could be attributed to the use of overlapping antibodies

and attenuation of M1 functions under Az influence (Jiang et al., 1998, Ramprasad et al., 1995, Parnham et al., 2014). For comparison, the highest density of CD68 + and CD163 + Mφs with weak immunoreactivity was found in healthy gingiva, suggesting different activity levels.

Altogether, these findings support the observed clinical benefits and suggest a limited additional immunological benefit of Az.

The study had limitations, including a short follow-up and a narrow range of Mφs markers. The benefit of Az may be associated with its immunotropic action in addition to its antibacterial properties. Correlation analysis between clinical parameters and Mφs and cytokine levels was not possible due to ethical reasons, leading to representative morphological studies.

Further investigations should involve long-term clinical monitoring.

5. Conclusions

Periodontitis-affected gingiva showed a dominance of M2 macrophage function, and while Az did not provide additional clinical benefits, it demonstrated modulation of Mφs, potentially influencing long-term outcomes.

Ethics approval and consent to participate

The study was approved by the Ethical Committee of Poltava State Medical University (August 23rd, 2021, No. 207).

Availability of data and materials

All data generated or analyzed during this study are included in this manuscript.

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CRediT authorship contribution statement

Viktoriya I. Shynkevych: Investigation, Conceptualization, Data curation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Svitlana V. Kolomiiets:** Investigation, Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing. **Igor P. Kaidashev:** Investigation, Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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