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Research Article

Experimental biochemical and histological activity of sodium hypochlorite gel on induced periodontitis in rats

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Abstract. Objective: the purpose of the present study was to investigate the clinical, biological and histological outcomes of two treatment methods for periodontal disease induced in Wistar rats using a sodium hypochlorite buffered gel in comparison to scalling and root planning alone and no treatment at all. Material and method: the study was performed on forty male Wistar rats. The animals were divided into four groups: TEST1 (n=10), scalling and root planning (SRP), TEST2 (n=10), SRP + sodium hypochlorite gel, TEST3 sodium hypochlorite gel, and CONTROL (n=10) no treatment after disease induction. The animals were clinically evaluated at the beginning (T0), after periodontitis induction (T1) and seven days after treatment (T2). The hematological parameters Interleukin-1 alpha (IL-1α), high sensitive C Reactive Protein (hsCRP), and Tumor Necrosis Factor-alpha (TNF-α) were measured. Seven days after treatment (T2), the animals were sacrificed, and samples were prepared for histological evaluation. Results: Although, the clinical and biochemical outcome of the evaluated procedures for the test groups were similar, histologic evaluation demonstrated a better treatment outcome in terms of healing for TEST 2 group with the association of SRP and local application of the antiseptic sodium hypochlorite gel. Conclusion: the findings of this research show that the association of hypochlorite and the initial periodontal therapy leads to better healing in terms of tissue and cellular repair, this being the first study evaluating the effects of hypochlorite on periodontal tissue in an experimental model.

Key Words: hypochlorite gel; periodontitis; nonsurgical periodontal treatment; inflammation; cytokines

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Introduction

Periodontitis is a chronic immunoinflammatory disease of the periodontium, with a high prevalence in the general population, which has as a final result, in the absence of treatment, the progressive loss of the supporting tissues of the tooth, namely the periodontal ligament, the gingival soft tissues and the underlying alveolar bone (Pihlstrom et al 2005; Ferreira et al 2017; Sanz et al 2020). In addition to the major impact that periodontal disease has on the health and quality of life, the annual costs for the treatment of periodontal disease are estimated to exceed $14 billion per year in the U.S. Chronic inflammation of the periodontium is initiated by periodontitis bacteria organized in complex subgingival biofilms. The biofilm contains a proportion of commensal gram-negative anaerobic bacteria, but opportunistic pathogens of the oral cavity are also found, including *Porphyromonas gingivalis* (*P. gingivalis*) (Fernandes et al 2010). As a response reaction to periodontal pathogens, polymorphonuclear neutrophils (PMNs) release reactive oxygen species through the respiratory cycle (Chapple 1997; Oz and Puleo 2011), proteinases and other factors that can alter the host's tissues (Enwonwu 1994). These molecules further promote an oxidative destruction of gingival tissues, periodontal ligament and activate osteoclastic bone resorption (Waddington et al 2000; Oz and Puleo 2011). These secreted agents also cause the production and release of numerous proinflammatory cytokines, including (IL)-1ß, IL-6, and tumor necrosis factor (TNF-α) along with other biomolecules whose concentrations is increased in the crevicular fluid and periodontal tissues in periodontal patients. These components contribute, directly or indirectly, to the progression of the disease (Ebersole and Taubman 1994; Garlet et al 2004; Bretz et al 2005). The levels of pro-inflammatory markers are reduced after periodontal therapy, differently, depending on the applied treatment and the host organism, the reactivity of each, being different from the initiated destruction and implicitly from the expression of the periodontal disease (Sharma and Pradeep 2006; Oz and Puleo 2011). Although, periodontitis is caused by bacterial infection, there are a lot of susceptibility factors that can influence the progression of the disease (Salvi and Lang 2005; Schenkein 2006; Oz and Puleo 2011). They can be favoring factors that act locally and modifying factors that act on a general

level throw an increased level of pro-inflammatory molecules (Chapple et al 2018).

Mechanical, non-surgical debridement of soft and hard deposits at the root surface, known as scaling and root planning (SRP) is the gold standard treatment for any periodontal pathology of infectious origin (Sanz et al 2012; Jurczyk et al 2016). This procedure can be performed with ultrasonic devices with or without the aid of manual curettes under anesthesia. This mechanical cleaning is often associated with the application of local antiseptics or antibiotics (Hancock and Newell 2001; Antonelli et al 2019). During maintenance, periodontitis can be controlled with optimal oral hygiene, associated with supra and subgingival professional oral cleaning by the periodontologist. In this stage the antiseptics can be used as an adjunctive treatment if needed. Over the past 10 years, numerous subgingival antiseptics and antibiotics have been tested as complementary measures to non-surgical therapy. These include: chlorhexidine, azithromycin, metronidazole, doxycycline, minocycline, tetracycline, povidone iodine, silver nanospheres or sulphonic acids (Matesanz-Pérez et al 2013; Lupșe et al 2021) but none of them proved their efficacy over time. The use of antiseptics as adjunctive treatment is not yet provided as gold standard, this being the reason why they are not implemented on clinical practice at a large scale. An alternative approach that is newer on the market for improving the results of scaling and root planning (SRP) could be the topical application of a sodium hypochlorite solution or gel. The sodium hypochlorite has a wide and fast spectrum of bactericidal action and is non-toxic if applied in low concentrations (Slots 2002). Information on its effect on the dissolution of necrotic tissue vs. healthy tissue in a low concentration has existed for a long time, this antiseptic having a wide range of use in dental medicine, especially in endodontics (Jurczyk et al 2016). Perisolv®, (Regedent AG, Zurich, Switzerland) is an active biochemical gel that is recommended for easing and optimizing the efforts of the periodontologist on the mechanical non-surgical treatment of periodontitis. The active components of Perisolv gel are represented by three amino acids (leucine, lysine and glutamic acid) along with sodium hypochlorite (NaOCl). By adding amino acids to the sodium hypochlorite solution, a mixture with oxidative effect that will react with the necrotic tissue will result, namely with the modified proteins that are found in the areas of necrosis associated with the epithelium of the periodontal pocket, but also in the bacterial plaque, respectively in the biofilm. During treatment, mechanical debridement is complemented by chemical reactions to dislodge bacterial biofilm and remove granulation tissue. Due to the action of Perisolv only on denatured proteins, this method affects neither healthy dentin nor intact root cementum (Cederlund et al 1999; Wennerberg et al 1999). The increased pH has a reducing effect on the hardness of bacterial deposits, which makes the debridement process easier. The gel is applied directly to the periodontal pocket, where it dissolves the altered tissues, also having a bacteriostatic effect. All the studies conducted on this product are human clinical trials, which have shown the effect of this product used as an adjuvant therapy to root surfacing (Bizzarro et al 2016; Megally et al 2020; Ramanauskaite et al 2020; Iorio-Siciliano et al 2021). None of the studies initiated so far are aimed at evaluating histological changes in periodontium after applying the gel. Moreover, the clinical results are promising, but there is no information related to the resolution time of the inflammatory process and the histological modifications.

The present study aims to investigate the biochemical and histological parameters variation of two treatment methods of induced periodontitis using the sodium hypochlorite buffered gel in comparison to SRP alone and no treatment at all. For the purpose of this study, the following null hypothesis was tested: the outcomes of the treatment methods do not differ in terms of clinical, biological and histological changes.

Materials and Methods

The experiment was conducted at the Centre for Experimental Medicine – The laboratory Animal Facility - Iuliu Hațieganu University of Medicine and Pharmacy. All protocols described below were approved by the Institutional Ethics Committee of the University of Medicine and Pharmacy Iuliu Hațieganu Cluj Napoca (Protocol no. 87/03.03.2017) and The National Sanitary Veterinary and Food Safety Authority (authorization no. 39/10.03.2017). They were performed and conducted in accordance with present laws regarding animal welfare and ethics in animal experiments (Directive 86/609 EEC/1986; Romanian Law 205/2004; Romanian Law 206/2004; Romanian Law 471/2002; Romanian Law 9/2008; Romanian Order 143/400). This study was conducted on 40 adults male Wistar rats with an initial mean weight of 385.15 grams and a standard deviation of 46.10. The animals were selected according to the inclusion criteria (male sex, Wistar race, weight between 300-500 grams, 18-20 weeks of age). The exclusion criteria for this study were visible signs of disease or animals that were primarily used in other experimental activities. The animals were kept in plastic type II-L open-top cages (Tecniplast Buguggiate, Italcages, Varese, Italy). They had an acclimatization housing period of 2 weeks before beginning the experiment. Five animals were housed in each cage and maintained under a 12-hour light/dark cycle in a temperature- and humidity-controlled room (23 ± 1 °C and 50±5% relative humidity) with access to standard rat chow pellets and water ad libitum. Animals were numbered and randomly assigned to four groups: one control group and three test groups. All groups received the same housing and feeding regimen. The induction of periodontitis was done using the molar ligature protocol (Tomina et al 2022) for the Control group, test 1, 2 and 3 group Fig.1, Fig. 2. For the Control group (CONTROL, n=10) after the induction of periodontitis the ligatures were removed without further treatment. In all test groups, before applying the treatment, the sutures used for periodontitis induction were removed. For Test group 1 (TEST1, n=10) subjects were treated with scalling and root planning after experimental periodontitis induction; for Test group 2 (TEST2, n=10) subjects were treated with scalling and root planning associated with the application of Perisolv in the periodontal pockets after experimental periodontitis induction; as for the test group 3 (TEST3, n=10) the subjects were treated only with the application of a local sodium hypochlorite gel in the periodontal pockets after experimental periodontitis induction. Each subject was assigned a code according to the group they belonged to. The code was noted on the subject, on the subject s chart and used for further references. Three blood samples of 1.5–2 mL were obtained from each animal by retro-orbital sinus puncture after

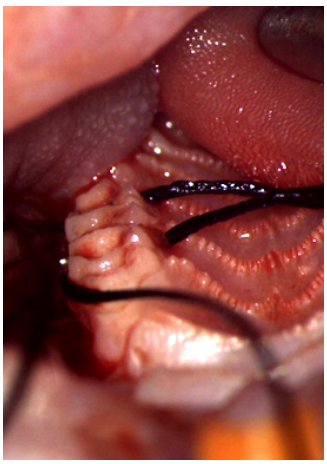

Fig. 1 Ligature application

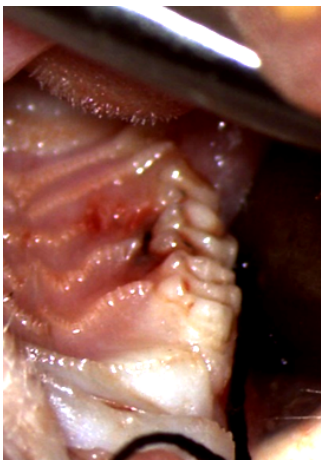

Fig. 2 Suture in position

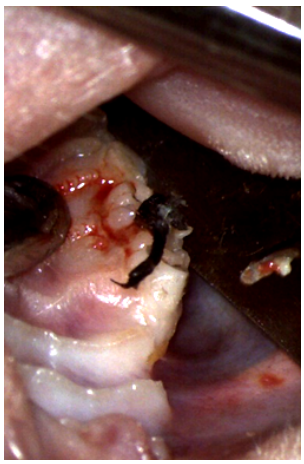

Fig. 3 Ligature removal

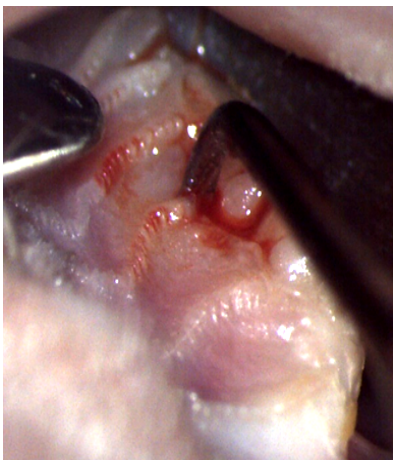

Fig. 4 Scalling and root planning

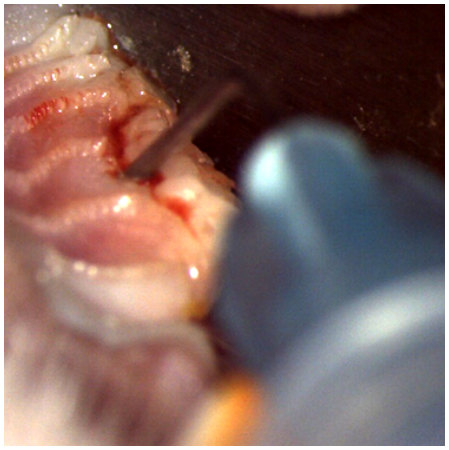

Fig. 5 Application of antiseptic sodium hypochlorite-based gel

general sedation with isoflurane (Aerrane, Baxter, Berkshire, UK) using a standard vaporizer (EZ-Anesthesia®; E-Z Systems, Bethlehem, PA, USA). The first blood sample was obtained on the first day of the experiment (T0), the second sample was obtained on the 7th day of the experiment (T1) and the third sample was obtained at the end of the experiment (T2). The blood was stored in tubes with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. The total blood count was completed within 2 h of sampling, and the rest of the blood (for T0 and T1) was centrifuged for 10 min at 3000 rpm. Plasma was separated and kept in Eppendorf tubes at -200 C until further use in the immunoassays. The following parameters were recorded after the hematological analyses: total leukocytes, neutrophils, lymphocytes, monocytes, eosinophils, and platelets. To quantify the host inflammatory response, interleukin-1 alpha (IL-1 alpha), tumor necrosis factor-alpha (TNF-alpha) and high-sensitivity C-reactive protein (hsCRP) plasma concentration measurements were performed. Blood was analyzed using a Sysmex XT-1800i automated hematology analyzer (Sysmex Corporation, Tokyo, Japan). The IL-1 alpha, TNF-alpha, and hsCRP analyses were performed using kits supplies for research: Rat Interleukin 1-alpha ELISA, Rat TNF-alpha ELISA, and Rat hsCRP ELISA (Bio Vendor® Research and Diagnostic products, Brno, Czech Republic). General anesthesia was performed by an intraperitoneal injection with a mixture of 10% ketamine and 2% xylazine (2:1), 0.12 ml/100 gr body weight, using a 1 ml syringe with a 26 G – 0.45x12 mm detachable needle (Omnifix®-F Solo, BBraun, Melsungen, Hessen, Germany). The dose was calculated according to the weight measurments. The application of the suture was performed around the second upper molar with the help of microsurgical tools and under magnification. Seven days after placing the ligatures, after blood sampling, the silk ligature was removed under anesthesia Fig. 3. Control group didn't receive any further treatment while the three test groups received the following treatment: Test 1 – scalling and root planning of the molar using a proper micro-mini-five Gracey currete (Hu-Friedy, Chicago, IL, USA) by 10 traction movements from distal to mesial surface, both buccal and palatal Fig. 4. The interproximal areas were instrumented with the same currete by 10 traction movements in an apical-coronal direction. Test 2 – Application of a local sodium hypochlorite gel (Perisolv®, Regedent AG, Zurich, Switzerland) in the periodontal pockets. The gel was obtained by mixing the contents of the two syringes in the kit: syringe 1, with opaque appearance: gel containing amino acids (glutamic acid, leucine, lysine), sodium chloride, carboxymethylcellulose, titanium dioxide, milli-Q ultrapure water, as well as sodium hydroxide; syringe 2, with transparent appearance: sodium hypochlorite solution 0.95%. The two components were mixed until a homogeneous aspect was obtained (10-15 back and forth movements), as recommended by the producer and the mixture obtained was placed in the periodontal pocket using a plastic cannula Fig. 5. The product was allowed 30 seconds to act without being washed; after, scalling and root planning was performed as described for Test 1 group. Test 3 Application of a local sodium hypochlorite gel (Perisolv®, Regedent AG, Zurich, Switzerland) in the periodontal pockets as described above for test group 2 without further instrumentation. The excess product was wiped with a sterile gauze after 30 seconds. All treatment procedures were performed by the same experienced operator. At the end of the experiment, seven days after treatment, the subjects were sacrificed by an overdose of anesthetic 10% ketamine and 2% xylazine, 0,5ml/100 gr body weight after general sedation with isoflurane (Aerrane, Baxter, Berkshire, UK), and the samples were prepared for histological handling.

Clinically, the evaluation was made at the beginning of the study, after periodontitis induction and at the end of the experiment. The parameters assessed were body weight, probing depth, gingival bleeding score, aspect, contour and color of the soft tissues surrounding the teeth, tooth mobility.

Table 1. Clinical parameters, before/after periodontal disease induction and after treatment

Group	Variables	Values			*p*-value*		
		T0	T1	T2	T1/T0	T2/T0	T2/T1
TEST 1	Mobility	0	3	2.6±0.48	0.002	0.04	0.046
	Inflammation	0	2.9±0.7	2±0.66	0.04	0.007	0.023
	Weight (grams)	396.40±58.68	398.50±50.11	416.50±40.003	0.799	0.074	0.018
TEST 2	Mobility	0	3	2.4±0.48	0.002	0.004	0.014
	Inflammation	0	3.1±0.7	1.9±0.53	0.004	0.004	0.016
	Weight (grams)	380.20±55.61	400.10±38.92	435.00±34.72	0.052	0.005	0.005
TEST 3	Mobility	0	3	0.8±0.6	0.002	0.011	0.004
	Inflammation	0	3.2±0.6	1.6±0.66	0.004	0.004	0.004
	Weight (grams)	398.60±30.18	417.60±36.93	457.00±42.24	0.139	0.008	0.005
CONTROL	Mobility	0	3	2.6±0.48	0.002	0.04	0.046
	Inflammation	0	2.9±0.83	2.4±0.66	0.005	0.004	0.025
	Weight (grams)	365.40±32.90	414.00±28.84	421.00±27.76	0.005	0.005	0.121

Data are presented as mean ± standard deviations; * Wilcoxon Signed Ranks Test Asymp. Sig. (2-tailed)

In the total blood count, the following parameters were recorded: total leukocytes, neutrophils, lymphocytes, monocytes, eosinophils, and platelets. To quantify the host inflammatory response, interleukin-1 alpha (IL-1 alpha), tumor necrosis factor-alpha (TNF-alpha) and high-sensitivity C-reactive protein (hsCRP) plasma concentration measurements were performed.

The molars to which the ligature and treatment was applied were dissected together with the part of the jaw containing them at a distance of 3 mm mesial and distal and 10 mm apical to the free gingival margin by sectioning with a milling cutter and histological examined. The harvested pieces were fixed in 10% formalin for 7 days, decalcified with trichloroacetic acid for 4 weeks, dehydrated in ethyl alcohol, clarified with 1-butanol and embedded in paraffin. Care was taken to obtain histological sections in which the 2nd molar, the alveolar bone crest, and the coronal and root pulp chambers were clearly identified. When these criteria were met, serial sections with a thickness of 6 μm were made, and the most representative sections were colored by Goldner's trichrome stain method. Histological preparations were examined using a confocal microscope (Olympus BX 41, lens 12.5/0.25, Tokyo, Japan) equipped with an Olympus E-330 (Tokyo, Japan) digital imaging camera.

The statistical handling was performed using IBM® SPSS® Statistics 24.0 (IBM Inc., New York, USA) and the Microsoft Excel application. The data are presented as the mean ± standard deviation of the mean (SD). The hypothesis of absence of statistically significant difference in the data obtained for all the examined variables in the four different groups, subgroups/treatments and periods in the teeth with induced periodontitis was tested. Normal distribution was assessed using the Kolmogorov Smirnof and Shapiro–Wilk test, where p values <0.05 suggested that the data came from a normally distributed population. Although, the majority of data came from normally distributed population, due to the small number of subjects per group, non-parametric statistical tests were used. The comparison between the evaluated parameters of the four groups on the 3 different evaluation intervals was performed with Wilcoxon signed-rank test. A Kruskal-Wallis test was used to determine whether or not there is a statistically significant difference between the medians of the four groups. For the comparison of each test group with the control group the Mann-Whitney test was used.

Results

Clinical parameters

The registered parameters were: a) gingival contour; b) gingival aspect; c) gingival color; d) gingival inflammation; e) tooth mobility and f) weight. This parameters were also evaluated in a previous published paper (Tomina et al 2022) were the differences between the evaluated variables at the beginning (T0) and after periodontal disease induction (T1) were described. For quantification of the clinical inflammation and tooth mobility, the scoring system described in the above mention paper was used. After periodontal disease induction (T1), the aspect of the soft tissue was the same for all the studied groups. Its normal aspect changed to cyanotic, with the presence of edema, food debris and bleeding. On the 14th day of the experiment (T2), after periodontal treatment, a series of differences between the control group and the test groups were observed. The control group, maintained its irregular aspect of the free gingival margin with the presence of bleeding on probing and edema which was less pronounced compared to T1. For the test groups, the gingival appearance returned back to normal with discrete bleeding on probing and a slight displacement of the free gingival margin of about 0.5 mm apically from its original position. The parameters (mobility, inflammation, weight) were compared on the three major timeframes as seen in Table 1.

Mobility

Regarding the variable mobility there were statistically significant differences in all four groups with the same results at the examination time T1 (Fig. 6) with respect to the tooth mobility at the beginning of the experiment T0 (p=0.02) due to the fact that this parameter is represented by a scale and not a specific value for each subject within one group. Regarding the

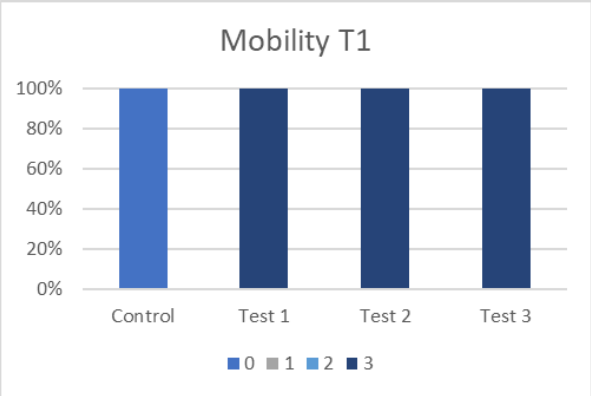

Fig. 6. Mobility values at T1

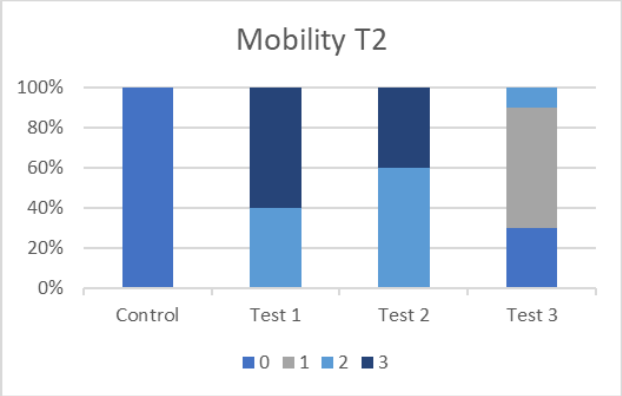

Fig. 7. Mobility values at T2

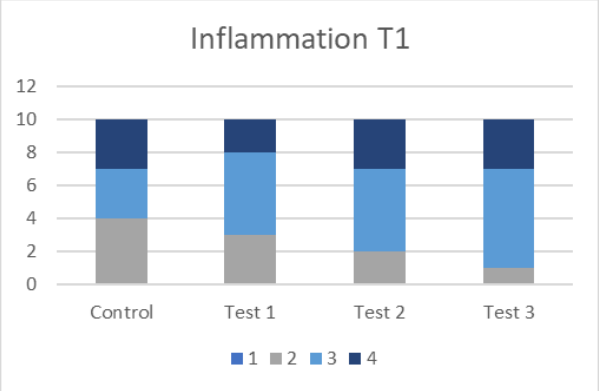

Fig. 8. Inflammation values at T1

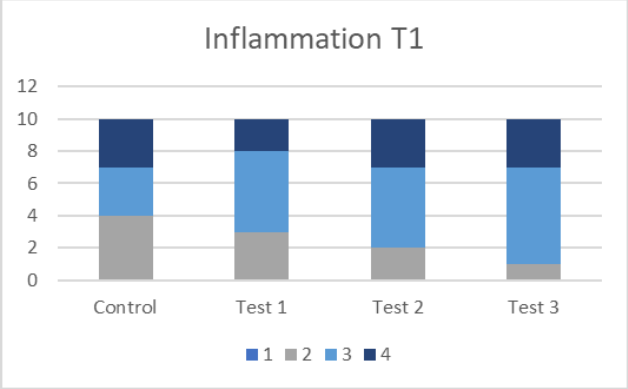

Fig. 9. Inflammation values at T2

intra group comparison of the same variable at the end of the experiment, after periodontal treatment T2 (Fig. 7), all values decreased with a statistically significant difference relative to T1. The highest score for tooth mobility was found for TEST 1 and control group (2.6± 0.48, p=0.046), while the lowest score at the end of the experiment was observed in TEST 3 group (0.8± 0.6, p=0.004).

Inflammation

Regarding inflammation, the pattern is similar for all four groups. The quantification of inflammation was made using the Gingival Bleeding index described by Liu et al (Xu and Wei 2006) and used by the authors in a previous published paper for model validation (Tomina et al 2022). The value of this parameter almost tripled for all the groups at T1 (Fig. 8) with respect to the initial situation at T0, while, after periodontal treatment (T2) all the values dropped but not at the level they were at the beginning (T0) (Fig. 9). All the values are statistically significant when compared by pairs (T1 vs T0, T2 vs T0 and T2 vs T1). The biggest difference of values within this pattern is observed for TEST 3 group (from 0 in T0 to 3.2±0.6 in T1 and 1.6±0.66 in T2, p=0.004) while the other groups have similar values.

Weight

The weight (Table 1) had an increase in all four groups without respecting a clear pattern. For TEST 1 group the only statistically significant increase was observed between T2 and T1 (p=0.018). For TEST 2 and TEST 3 groups the increase was statistically significant between T2 and T0 and between T2 and T1 while for the control group the difference was significant between T1 and T0 and between T2 and T0 (p=0.005) while the results of the difference between T2 and T1 weren't statistically relevant.

Blood testing

The values of the evaluated inflammatory markers (hsCRP, TNF-alpha, and IL1-alpha) were compared at T0, after periodontal disease induction (T1) and at the end of the experiment (T2) for the four groups included in the study (Table 2).

In all test groups, all parameters increased statistically significant with a pick in T1, after periodontal disease induction, and afterwards, all the values decreased with a statistical significance while maintaining a higher value than the one they had at the beginning of the experiment (T0). Regarding the CONTROL group, the values of the TNF-α and IL1-α continued to increase after the silk thread was removed, without a statistical significance while hsCRP followed the test groups pattern and significantly decreased after the peak reached in T1. The values of the parameters in the blood count (leukocytes, neutrophils, eosinophils, lymphocytes, monocytes, platelets) were also compared at the beginning of the experiment (T0), after periodontal disease induction (T1) and at the end of the experiment (T2) for all groups included in the study. For the control group, the mean values of the leukocytes were observed to have statistically increased after the induction of the periodontal disease and after treatment compared to baseline. Also, the monocytes and platelets values have a statistically increased value at the end of the experiment compared to T1. All the other values of the parameters evaluated in the control group were observed to be steady. For the test groups, the same pattern as for proinflammatory markers was observed, with a peak in T1 and a decrease

Table 2. Pro-inflammatory markers at T0, T1 and T2

group	Variables	Values			*p*-value*		
		T0	T1	T2	T1/T0	T2/T0	T2/T1
TEST 1	hsCRP(pg/mL)	53.38±2.7	108.49±8.10	77.65±5.94	0.005	0.005	0.005
	TNF-α (pg/mL)	43.68±3.47	100.19±8.01	81.18±9.82	0.005	0.005	0.005
	IL1-α (pg/mL)	38.29±2.00	66.94±9.89	47.81±4.18	0.005	0.005	0.005
TEST 2	hsCRP(pg/mL)	55.05±5.82	112.32±12.45	83.2±12.87	0.005	0.005	0.007
	TNF-α (pg/mL)	43.63±3.56	97.67±14.4	67.73±5.54	0.005	0.005	0.005
	IL1-α (pg/mL)	39.94±2.39	67.00±9.80	49.36±6.21	0.005	0.005	0.005
TEST 3	hsCRP(pg/mL)	57.41±2.93	113.62±13.25	77.15±8.40	0.005	0.005	0.005
	TNF-α (pg/mL)	46.63±3.18	98.22±12.55	64.04±10.8	0.005	0.005	0.005
	IL1-α (pg/mL)	37.39±2.32	69.89±7.31	55.25±7.76	0.005	0.005	0.005
CONTROL	hsCRP(pg/mL)	52.07±3	103.86±13.67	78.48±6.06	0.005	0.005	0.005
	TNF-α (pg/mL)	40.89±2.57	91.1±10.38	94.1±9.83	0.005	0.005	0.508
	IL1-α (pg/mL)	45.17±5.62	68.02±8.19	70.09±9.7	0.005	0.005	0.683

Data are presented as mean ± standard deviations; * Wilcoxon Signed Ranks Test Asymp. Sig. (2-tailed)

Table 2. Pro-inflammatory markers at T0, T1 and T2

group	Variables	Values			*p*-value*		
		T0	T1	T2	T1/T0	T2/T0	T2/T1
TEST 1	Leukocytes [10^3/μL]	9.97±2.56	15.41±3.3	11.16±2.85	0.005	0.357	0.005
	Neutrophils (%)	21.85±2.89	25.44±2.96	22.55±2.73	0.005	0.168	0.005
	Eosinophils (%)	2.54±0.79	1.78±0.5	2.95±0.72	0.011	0.85	0.005
	Lymphocytes (%)	70.67±7.96	75.87±8.15	72.53±7.17	0.007	0.169	0.022
	Monocytes (%)	8.34±2.27	8.7±1.76	8.32±1.75	0.284	0.759	0.168
	Platelets [10^3/μL]	1037.6±110.83	1223.7±112.81	1149.5±76.80	0.005	0.059	0.169
TEST 2	Leukocytes [10^3/μL]	10.98±3.26	14.76±2.47	12.09±2.56	0.005	0.169	0.005
	Neutrophils (%)	22.34±2.42	26.86±1.41	24.87±2.72	0.005	0.005	0.012
	Eosinophils (%)	2.88±1.05	2.17±0.69	2.86±0.63	0.103	0.978	0.028
	Lymphocytes (%)	70.25±4.9	74.02±6.40	71.15±2.69	0.059	0.445	0.074
	Monocytes (%)	8.19±1.42	8.58±1.27	8.26±1.68	0.126	0.54	0.342
	Platelets [10^3/μL]	1064.1±91.59	1283.3±147.99	1153.9±159.34	0.005	0.013	0.005
TEST 3	Leukocytes [10^3/μL]	10.48±2.36	14.65±2.84	13.00±2.78	0.005	0.005	0.005
	Neutrophils (%)	22.52±3.49	26.15±2.6	23.32±5.07	0.013	0.575	0.022
	Eosinophils (%)	2.71±0.98	2.32±0.79	2.7±0.75	0.153	0.959	0.05
	Lymphocytes (%)	71.41±5.37	75.6±5.00	71.37±7.88	0.005	0.386	0.114
	Monocytes (%)	7.39±1.59	7.83±0.98	7.4±1.17	0.153	0.959	0.202
	Platelets [10^3/μL]	1098.2±177.94	1315.5±156.1	1177.10±159.06	0.005	0.093	0.013
CONTROL	Leukocytes [10^3/μL]	9.92±1.07	14.8±1.84	13.81±1.95	0.005	0.005	0.059
	Neutrophils (%)	20.23±6.08	23.11±4.14	24.97±8.67	0.074	0.093	0.919
	Eosinophils (%)	2.83±1.38	2.36±0.97	2.66±1.26	0.112	0.919	0.284
	Lymphocytes (%)	70.70±10.93	68.63±5.44	76.5±8.76	0.386	0.153	0.013
	Monocytes (%)	6.88±1.49	6.52±1.36	7.32±2.16	0.414	0.678	0.057
	Platelets [10^3/μL]	1077.10±164.44	1245.00±137.19	1220.10±216.54	0.047	0.169	0.575

Data are presented as mean ± standard deviations; * Wilcoxon Signed Ranks Test Asymp. Sig. (2-tailed)

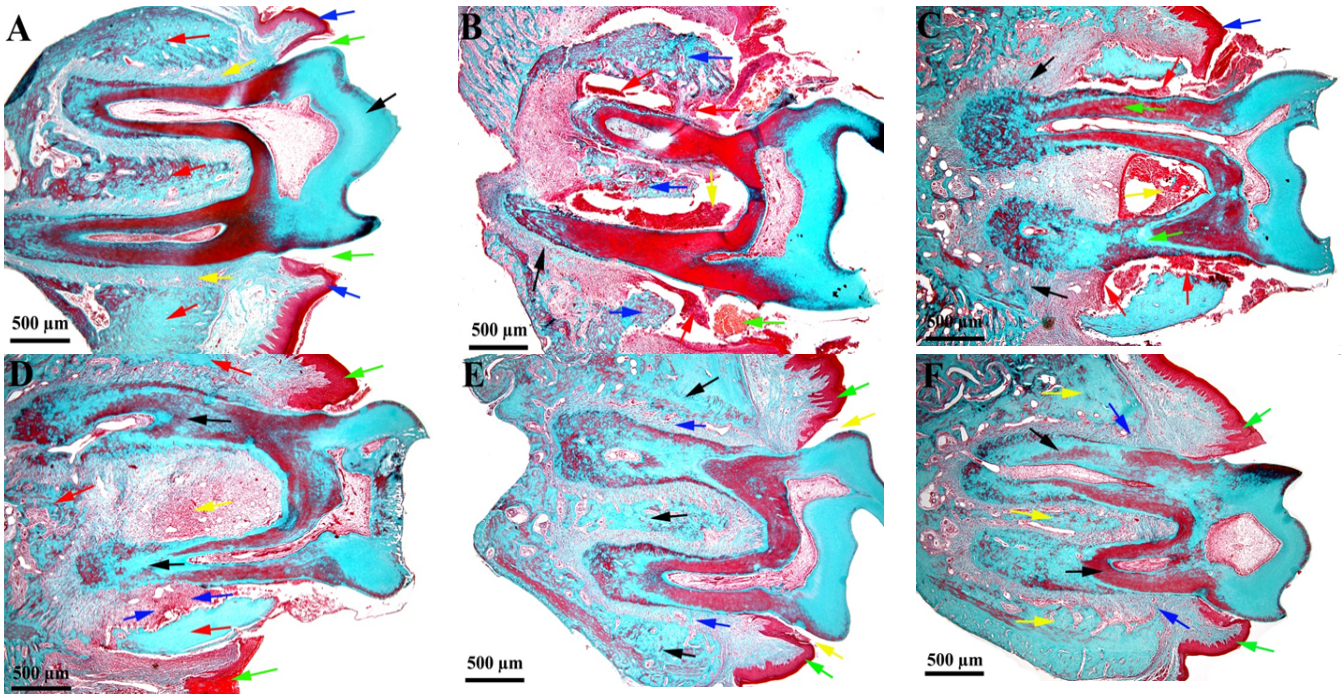

Fig. 10. Histological aspects; A – rat molar physiological aspect; B – rat molar induced periodontitis ; C – CONTROL group; D-TEST 1; E – TEST 2; F – TEST 3 ; Goldner's trichrome stain; (A) – black arrow – molar's crown; blue arrow – gingiva; green arrow– gingival sulcus; red arrow – alveolar bone; yellow arrow – alveolar ligament; (B) – green arrow – silk thread; blue arrow – alveolar bone; yellow arrow – interradicular necrosis; red arrow – alveolar ligament necrosis; black arrow– alveolar ligament; (C) – green arrow – molar's root; blue arrow – gingival epithelium; yellow arrow – interradicular necrosis; black arrow– alveolar ligament; red arrow – alveolar ligament necrosis; (D) – black arrow – molar's root; blue arrow – alveolar ligament necrosis; yellow arrow – interradicular inflammatory infiltrate; red arrow – alveolar bone; green arrow – gingiva; (E) – black arrow – alveolar bone; blue arrow – alveolar ligament; yellow arrow – gingival sulcus; green arrow – gingival epithelium; (F) – black arrow – molar's root; blue arrow – alveolar ligament; yellow arrow – alveolar bone; green arrow – gingiva.

in value afterwards as shown in Table 3, without many statistically significant differences between T2 and T0.

Histologic evaluation

The histologic evaluation was performed at the end of the experiment for all groups included in the study. (Fig. 10 D-F). For a better understanding of the results, an imagine with the physiological histology of the rat molar periodontal structures (Fig. 10 A) and one representing the destruction caused by periodontal disease induction were used (Fig. 10 B). For each group the most representative imagine for the group characteristics was chose to be presented.

Control group (Fig. 10 C)

The applied ligature caused large-scale lesions that extended in depth over about 2/3 of the length of the roots, on both surfaces (buccal and oral), having a greater magnitude on the buccal side. The lesions comprised all neighboring structures, starting with the junctional epithelium, the periodontal ligament, connective tissue and even the alveolar bone. The gingival sulcus on the oral side presented necrotic detritus but from the level of the free gingival margin in depth, its quantity decreases significantly. On the buccal surface the lesions are of greater magnitude, the gingival sulcus being replaced with necrotic tissue. The periodontal ligament is destroyed on the first 2/3 of the root being present only in the apical third. The alveolar bone appears affected to a very large extent, the alveolar ridges are extinct, in the area there are only dismantled bone fragments surrounded by necrotic detritus. The remaining bone fragments are no longer viable due to the total absence of osteocytes, blood vessels and the emptiness of osteoplasts.

TEST 1 group (Fig. 10 D)

For Test1 group, morphological lesions are also present, but their severity and extent are smaller. The lesions are present on both the oral and buccal sides of the tooth, but with a relatively large difference between the two areas. On the buccal side, the gingival epithelium is almost completely restored, except for the one at the bottom of the sulcus where isolated lesions still persist. The connective tissue beneath the epithelium is easily reacted and presents numerous active fibroblasts. There is a moderate amount of detritus in the gingival sulcus. The crest of the oral alveolar bone and the alveolar ligament are basically restored with a poor organization on the collagen fibers of the alveolar ligament. On the buccal side, the lesions are much more pronounced and extensive. The gingival epithelium is extinct on a certain surface, after which it is present, it has a certain thickness but on a relatively large area it shows obvious degenerative changes. The lesions here are extensive, going up to the deep third of the root. Bone fragments are present, surrounded by debris and granulation tissue. The alveolar ligament is present only in the deep third of the root. The alveolar bone crest is destroyed so that the viable bone is found only in depth but with a more trabecular structure. In the extent of the injured area there is a newly proliferated trabecular bone.

TEST 2 group (Fig. 10 E)
For Test 2 Group, the recovery/healing processes are advanced in both soft and hard components, but they are not fully completed. The gingival epithelium is restored in a very large proportion, it covers the gingival sulcus, both on the oral and buccal sides. At the level of the oral surface, the restored soft structures are attached to the root cementum with minor apical displacement of about 0.5 mm from cemental enamel junction (CEJ). On the buccal side (where the lesions were larger and more extensive) the attachment is positioned approximately 1.5-2 mm apically with respect to the CEJ. Under the epithelium lining the gingival sulcus, there is still a slight reaction of the underlying connective tissue (chorion), but on a small area towards the depth of the sulcus. The periodontal ligament is largely restored starting from the bottom of the gingival sulcus and around the roots on both the oral and buccal sides. However, it does not have the typical structure; the collagen fibers are not oriented quite as thoroughly as in the tooth that has not suffered any injuries and has slightly more blood vessels. The bone lesions rising from experimental periodontitis have been repaired to a very large extent, but are not yet completed. There are also areas where there are polymorphic cavities surrounded by injured bone. The fact that there are active repair and reshuffle processes is also confirmed by the presence of osteoclasts that remove the affected bone to be replaced by newly formed one. At the level of the alveolar ridges, both at the internal and external sides, active processes of proliferation and bone remodeling are present.

TEST 3 group (Fig. 10 F)
Test 3 group had a good evolution, with the largely repair of the affected tissues following experimental periodontitis. The gingival epithelium is largely restored and it continues at the level of the gingival sulcus, to its depth, both on the oral and buccal side. Keratin layer on the gingival ridge, and especially in the upper half of the gingival sulcus, is not yet restored, and the epithelium has extensions that tend to penetrate in depth to the bottom of the gingival sulcus. The reaction in the subepithelial chorion is moderate. The attachment of the tooth to the neighboring structures is apically displaced with respect to the CEJ, both on the oral and buccal side. The periodontal ligament is largely restored on both sides of the tooth, but it does not yet have the structure it had before the experimental periodontitis. The connective fibers of the ligament are fewer, thinner with less firm orientation. In addition, there are, especially towards the alveolar bone, relatively large areas in which the attachment to the bone is not yet completed. These spaces are occupied by either blood vessels or young connective tissue that actively participates in bone repair processes. At the level of the alveolar bones, polymorphic cavities bordered by non-viable bone are present. These spaces are numerous and larger comparing to Test 2 group. Such spaces are also present at the level of the bone in the interradicular space and in addition here there is a reaction in some specimens given by the persistence of small bone fragments and even epithelial proliferations in the uppermost part of the interradicular space. Active processes of bone proliferation and remodeling are present in the alveolar bone ridges.

Discussion

The present study examined the influence of using a commercial antimicrobial sodium hypochlorite gel-based application on the outcome of induced periodontal disease treatment in rats following the clinical, biochemical and histologic evaluation. From our knowledge, this is the first study that assesses the treatment outcomes taking into consideration the pro-inflammatory markers (TNF-α, IL-1α and hsCRP) and histological findings regarding sodium hypochlorite gel usage in periodontitis treatment.
Periodontal disease is a chronic inflammatory process that leads to the destruction of periodontal supporting tissues, through the release of lipopolysaccharide and numerous proteases (Swerts et al 2017) from bacteria present in the dental biofilm. The tissue inflammation is associated with the increased number of neutrophils, high values of hsCRP, and the activation of several pro-inflammatory mediator as IL-1, IL-6, IL-8 and tumor necrosis factor (TNF-α) (Kara et al 2013) among others. They are responsible for modifying the bone homeostasis that leads to the triggering of the alveolar bone resorption processes with an increased activity of matrix metalloproteinases (MMP). The MMP are involved in the connective tissue degradation (Meisel et al 2014), due to exacerbated immune response (Karim et al 2012). The inflammatory phase should lead to the destruction of the bacteria. When that is accomplished the resolution phase should follow to regain the physiological state. Due to the characteristic of the dental biofilm and the characteristic of the periodontal tissue the bacteria cannot be destroyed, leading to a chronic inflammatory phase.
Periodontal treatment is used to dismantle the microbial biofilms and consequently stop the destruction of the periodontal tissues in order to avoid their loss (Cappuyns et al 2012). There are situations, where isolated periodontal mechanical therapy is not effective or doesn't reach the sole purpose of stopping the infection by reducing the periodontal pockets and maintain a proper level of the gingival bleeding index. This suggests that systemic factors, not discovered or referred to during treatment, actively interfere with the development and progression of the disease and treatment results (Saglam et al 2014). Thus, the use of locally delivered antiseptics could be an adjuvant therapy to conventional treatment (Meisel et al 2014; Swerts et al 2017) in order to obtain a better clinical result.

Clinical oral evaluation
The signs of gingivitis in the rat model resemble the signs of gingivitis in humans. In humans, if not treated, it will progress deeper in the tissues and will cause attachment loss and bone resorption. Periodontitis appears whenever the attachment is lost due to inflammation (Lindhe et al 2009). In rat-induced periodontitis, this processes are initiated by mechanical trauma produced by the presence of the ligature (Graves et al 2008; Tomina et al 2022). Regarding the objective oral examination, the tooth mobility, periodontal inflammation and weight were considered to be statistically evaluated in this study. Tooth mobility had a statistically significant relevance at T1, after periodontal disease induction which means that the experiment of inducing the pathology was correct and in accordance with other publications in the literature (Ionel et al 2015; Swerts et al 2017; Tomina et al 2022). After periodontal treatment application, regardless of the procedure, all the values dropped significantly except the one for the CONTROL group, which didn't receive any treatment. This aspect suggested that without treatment, tooth mobility will decrease at a slower rate, due to the fact that inflammatory mechanisms are still active,

leading to a higher destruction of the periodontal supporting tissues. Inflammation, quantified by the gingival bleeding score, increased after periodontal disease induction while after treatment had a significant decrease for all the tested groups. The highest value of inflammation at the end of the experiment was observed for the control group which reinforces the statement that although the source was removed, the pathological processes progress, but at a slower rate. The lowest value of GBI was observed in TEST 3 group, while TEST 1 and TEST 2 groups had similar values. This can be explained by the fact that both TEST 1 and TEST 2 groups received mechanical debridement which adds the traumatic stress on the fragile periodontal tissues. The parameter weight had some fluctuations without any clinical relevance for the purpose of the present study. Overall the clinical outcomes of the test groups are similar to the ones found in humans after periodontal disease treatment in terms of inflammation reduction and clinical aspect (Bizzarro et al 2016; Megally et al 2020; Ramanauskaite et al 2020; Iorio-Siciliano et al 2021); the majority of the human studies focus their research on probing depth and clinical attachment levels as outcomes of non-surgical periodontal therapy associated with the application of sodium hypochlorite, parameters that cannot be standardized in rat models induced periodontitis.

Biochemical evaluation

All the examined values of the pro-inflammatory markers significantly increased after periodontal disease induction and decreased significantly after treatment, regardless of the applied procedure. For the control group though, the hsCRP value significantly dropped after the silk thread removal, while the TNF-α and IL-1α continued to increase without a statistical significance though suggesting that the macrophages are no longer stimulated by the presence of the silk thread but the deep inflammatory process is progressing. Regarding the intergroup comparison, beside the hsCRP, which had comparable results in all groups on the three examination times, the values of TNF-α and IL-1α were statistically significant on T2 when comparing each test group with the control group and steady between test groups at the same examination times T1 and T2. The clinical relevance is that regardless of the treatment applied, SRP alone, SRP associated with local application of Perisolv or Perisolv alone, the inflammation and destructive processes will regress.

The influence of local inflammation on homeostasis was evaluated by the total count of leukocytes. For all the test groups, the total count of leukocytes increased within groups from T0 to T1 ($p<0.05$) and decreased after treatment from T1 to T2. The only exception was the total count of the eosinophils which had an opposite pattern, decreasing in T1 compared with T0 and increasing in T2 compared to T1 with a statistic significance ($p<0.05$). This can suggest a massive proliferation of granulocytes inside the affected tissue with the initiation of the phagocytic processes (Freire and Van Dyke 2013) that will be reversed in T2 after periodontal treatment application. Regarding the control group, the difference in count between eosinophils in T 2 vs T1 is not statistically relevant ($p=0.284$) while the neutrophils and lymphocytes follow an increasing pattern from T0 to T1 and T2 which suggests that infection is still out of control and progressing. The data regarding the total blood count are similar to the ones found in literature (Dascalu Rusu et al 2022), without assessing the pro-inflammatory markers as well, especially the ones used in this study which are key to diagnose, asses and complete a holistic diagnostic of periodontitis (El-Shinnawi and Soory 2013).

Histologic evaluation

The present study demonstrated with the use of clinical and biochemical parameters that the simple removal of the irritative thread does not stop the pathological processes which continues to evolve at a slower pace. The lesions are gradually amplified by covering practically all the neighboring structures. After the onset of periodontitis, the chance to self-healing is extremely small. SRP ensures the removal of detritus and biofilm from the affected area, which is a significant help for the reparatory mechanisms in their effort to restore the affected structures. After the SRP is performed, phisiological processes are triggered, but they evolve simultaneously with the destructive ones that still act for a certain period of time. In advanced pathological processes, healing cannot be achieved only by thorough mechanical instrumentation of the affected area. To validate the action of the sodium hypochlorite buffered gel, the histological samples of TEST2 and TEST3 groups were analyzed. We resorted to these two therapeutic alternatives, SRP + gel (TEST 2 Group) and Perisolv (TEST 3 Group) alone in order to quantify what is the real contribution of the two components on the development of reparative processes after experimental periodontitis. In both test groups where the gel was applied (TEST 2 and TEST 3, group) the healing processes were, at the end of the experimental period, in an superior stage of healing, even if there were certain differences between the two groups. The tested gel, managed to stop pathological processes and create optimal conditions for the reparative processes. Stopping the evolution of pathological processes is of particular importance, especially for bone structures in the composition of the alveolar ridges that appear really thinned at the time of gel application. If the cortical bone doesn't fracture, no matter how thin they are, they retain the height of the previous intact alveolar crest. By stopping the pathological processes and having the proper height, the reparative processes will gradually restore the alveolar ridges that eventually reach the parameters close to those had before the experimental induction of periodontal disease. With all the particularly good results obtained by applying the gel, between the two groups to which it was applied, there were some differences between them. For TEST 3 group, where only the gel was applied as treatment, the reparative processes were delayed. Even if the repair process it is later induced with no chance of being improved, due to the epithelial seal that will attach more apically in this situation not permitting a proper connection of the connective tissue afterwards. We refer here to the fact that in TEST 3, the repair is ended in such a way that the insertion of the gingival components to the tooth is made significantly apical than in TEST 2 group. The fact that this aspect is present only in TEST 3 group, highlights the element that the experimental treatment option that ensured the most correct and complete healing of the lesions occurring after the experimental periodontitis, was the one used for TEST2. The antiseptic effect is provided by the sodium hypochlorite-based gel, mechanical debridement proved very beneficial preparing the area for the action of the antiseptic product. The vast

majority of the studies found in the literature use the hematoxylin eosin (HE) staining (Ionel et al 2015, 2017; Mester et al 2019; Dascalu Rusu et al 2022) for the histological assessment of disease and treatment of periodontitis induced in rats. This method is cheap and fast but it has some limitations when it comes to periodontal structures. The periodontal ligament is mostly composed of collagen fibers. Whenever and aggressor penetrates the natural barrier of protection in this area, an inflammation cascade develops which, untreated ends up in different stages of necrosis. Masson-Goldner trichrome staining is a method of staining muscle fibers and collagen in tissues in which the Fast-Green dye binds to collagen and causes it to turn green. Masson-Goldner's trichrome is also used to visualize the increase in collagen accumulation associated with functional tissues taken for scar tissue, but also for the differentiation of smooth muscle and collagen fibers (Szunyogova and Parson 2016). For this reason, the descriptive histology for this research was made with Golden trichrome staining.

Conclusions

The group treated with sodium hypochlorite buffered gel associated with conventional non-surgical initial therapy showed the highest rate of healing in terms of inflammation reduction, quantified by the clinical signs, gingival bleeding index, weight fluctuation, tooth mobility and the overall aspect of the gingival structures. Moreover, the pro-inflammatory markers like hsCRP, IL 1- α and TNF- α were statistically lower for TEST 2 group. The histologic evaluation showed the highest rate of tissue repair for the same group which suggests that the association of sodium hypochlorite gel to the conventional non-surgical initial therapy could add a clinical benefit for patients if we extrapolate the data to humans.

Despite the limits of this study (low number of animals per group, relatively short follow-up period, the lack in uniformity of data distribution, the absence of immunohistochemical staining and examination of the cellular composition of the periodontal pockets), the null hypothesis was rejected and the overall conclusion is that induced periodontitis in rats will not heal by intrinsic means if left untreated. The results of this study can be interpreted as preliminary in order to advance hypothesis and complex study designs for further investigations.

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