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Preclinical and histological study of boron-containing compounds hydrogels on experimental model of periodontal disease

IOANA MITRUŢ[1)], MELANIA OLIMPIA COJOCARU[2)], ION ROMULUS SCOREI[3)], ANDREI BIŢĂ[3,4)], GEORGE DAN MOGOŞANU[4)], MIHAI POPESCU[5)], DANIEL-ALIN OLIMID[6)], HORIA OCTAVIAN MANOLEA[1)]

[1)]*Department of Dental Materials, Faculty of Dental Medicine, University of Medicine and Pharmacy of Craiova, Romania*

[2)]*Department of Oral Rehabilitation, Faculty of Dental Medicine, University of Medicine and Pharmacy of Craiova, Romania*

[3)]*Department of Biochemistry, BioBoron Research Institute, S.C. Natural Research S.R.L., Podari, Dolj County, Romania*

[4)]*Department of Pharmacognosy & Phytotherapy, Faculty of Pharmacy, University of Medicine and Pharmacy of Craiova, Romania*

[5)]*Department of Pediatric Dentistry, Faculty of Dental Medicine, University of Medicine and Pharmacy of Craiova, Romania*

[6)]*Department of Biology, University of Craiova, Romania*

Abstract

Periodontitis is a disease that affects a wide group of people, and there has been an increased interest in the research of finding useful materials that help reduce inflammation and the further loss of tissue. In this study, we have tested a boron-containing compound (BCC) Calcium Fructoborate (CaFB) and Boric Acid (BA) hydrogels on the gingival level on Wistar rats. First, we have induced the periodontal disease at the lower incisors, we have applied the hydrogels and after a week, we have euthanized the rats. Next, the oral soft tissue reaction was clinically and then histologically investigated. Our study has shown good clinical response of the oral tissue, and we have noticed lower levels of inflammation on the experimental groups treated with the BCCs hydrogels. Despite the generally good response of the biological structures to the presence of BA and CaFB on periodontal level, more scientifically proved information is needed to obtain the desired biological responses in all clinical situations.

Keywords: experimental model, inflammation, periodontal disease, ligature, Calcium Fructoborate, Boric Acid.

Introduction

Periodontitis is a highly prevalent, chronic immuno-inflammatory disease of the periodontium that results in the progressive loss of gingival tissue, the periodontal ligament, and adjacent supporting alveolar bone. Periodontitis was associated with systemic diseases, such as diabetes, auto-immune diseases, and cardiovascular complications [1, 2].

There has always been an interest in developing a scientific basis for understanding the pathological processes behind this disease and some animal models, like rodents and rats have proved to be relevant for experimental periodontal research [1].

Antibacterial activity of boron, which is a bioactive trace element frequently found in diets plentiful in foods such as fruits, vegetables, and nuts, has been reported by many advanced research teams [3–10]. A regulatory effect of boron-containing compounds (BCCs) in the inflammatory and immune response has also been demonstrated. It has been shown to reduce the formation of an inflammatory infiltrate and bone loss in rats, measured histologically and by micro-computerized tomography [11–14].

It was found that Calcium Fructoborate (CaFB) decreased the amount of intracellular reactive oxygen species induced by exposure to oxidative stress (exogenous hydrogen peroxide) [5, 15, 16]. CaFB has also been reported to stimulate the differentiation of osteoblasts from bone narrowing, as well as to restrict superoxide formation within cells [5, 17].

Aim

The aim of this paper was the study of the clinical effectiveness of BCCs, mainly CaFB and Boric Acid (BA). Thus, we followed two aspects in this study: on the one hand, the clinical evaluation of the response of the oral soft tissues to the presence of BCCs hydrogels, and on the other, the histological assessment of the tissues. Another objective was to compare the effects of the local application of an experimental hydrogel formulation based on CaFB on the evolution of the healing of a periodontal defect compared with other classical treatment solutions and the identification of the optimal method of local application of the therapeutic products.

Materials and Methods

Reagents and chemicals

CaFB standard (synthesized according to Hunter's US Patent) was provided by VDF FutureCeuticals (Momence, IL, USA). The purity of CaFB (2.7%, expressed in boron) was provided by multinuclear magnetic resonance (mNMR) using liquid and solid state ^{11}B- and ^{13}C-NMR. BA standard was obtained commercially from Merck Millipore. The

chromatographic grade solvents and reagents, such as 2-Propanol, Ethanol (99%) and Water (LiChrosolv®) were purchased from Merck (Darmstadt, Germany). Synthalen K and Chlorogenic Acid were purchased from Sigma-Aldrich (Munich, Germany).

BCCs hydrogels preparation

A hydrogel containing 10% CaFB and 1.5% Synthalen K as a thickening agent was formulated. As comparison, the same formulation with 10% BA was used.

Preparation of standard and sample solutions

An aqueous 1 mg/mL stock solution of BA was prepared, while the concentration for the stock solution of CaFB was 5 mg/mL. The standard working solutions were obtained by diluting the stock solutions with water to achieve the concentration range of 0.2–0.6 µg/band for BA and 1–3 µg/band for CFB, respectively [18]. The samples were obtained by dissolving 20 mg of BA and CaFB hydrogels in 10 mL of water. Standards and samples were applied on the high-performance thin layer chromatography (HPTLC) plate according to Table 1 and Figure 1.

Table 1 – ***Application order and volume for standard and sample solutions***

Track No.	Compound	Volume [µL]
1.	Blank (water)	2
2.	BA	2
3.		3
4.		4
5.		5
6.		6
7.	CaFB	2
8.		3
9.		4
10.		5
11.		6
12.	BA hydrogel	2
13.		2
14.	CaFB hydrogel	4
15.		4

BA: Boric acid; CaFB: Calcium Fructoborate.

Equipment

For the quantitative evaluation and sample application, a CAMAG TLC Scanner III and Linomat V (CAMAG, Muttenz, Switzerland) controlled by a computer system with the visionCATS software (visionCATS ver. 2.5) were used. For derivatization, the CAMAG Chromatogram Immersion Device 3 was employed for automated dipping of the plate. The images were acquired with a Canon EOS 700D DSLR camera (Canon). A Bandelin Sonorex Digiplus DL 102 H ultrasonic bath (Bandelin Electronic GmbH & Co. KG, Berlin, Germany) was also used for the sample preparation.

HPTLC analysis

The analysis was performed on HPTLC silica gel G 60 F_{254} glass plates (20×10 cm, 0.2 mm layer thickness, Merck DC). Application of standard and sample solutions was achieved using the semiautomatic TLC sample spray band applicator (CAMAG Linomat V) (Table 1). The developing distance was up to 50 mm from the bottom edge of the plate, with a mixture of 2-Propanol–Water (8:2, *v/v*, pH 7.53) as the mobile phase, in a vapor-equilibrated chromatographic tank (CAMAG 20×10 cm twin trough chamber). After developing, the plate was dried and dipped using the Immersion Device into a 0.01% Chlorogenic Acid ethanolic solution, dried again for about one minute, and then visualized at 365 nm. The quantification analysis of the samples was achieved using the CAMAG TLC Scanner III [18].

CaFB mass spectrometry (MS) confirmation

To confirm that the active compound from therapeutic hydrogel truly was CaFB, we eluted one of the bands from the sample directly into the mass spectrometer and we obtained the expected mass spectrum. The settings used for MS analysis were as follows: the mobile phase was Methanol–Ammonium Acetate 10 mM aqueous solution (9:1, *v/v*); negative mode, electrospray ionization (ESI); probe temperature, 450°C; capillary voltage, 0.8 kV; cone voltage, 25 V [19].

Animals

In order to perform the study, 16 Wistar adult rats were selected, weighing between 250 and 350 g, from the Animal Facility of the University of Medicine and Pharmacy of Craiova, Romania. The animals were kept in well ventilated cages and marked according to each group. The cages were maintained at a temperature of 25±1°C, with an alternation of 12 hours light/dark. The rats were fed standard food, and water *ad libitum*. For each animal was kept a record of the experiment to which it was subjected. All experiments were conducted in accordance with local guidelines on the welfare of experimental animals and with the approval of the Ethics Committee of the University of Medicine and Pharmacy of Craiova (No. 80/16.04.2019).

The experimental groups were divided in control, P (in which the periodontal defect was created without receiving no further treatment), P + SRP (SRP – scaling and root planning; the periodontal defect was created, followed after 14 days by mechanical treatment), P + SRP + BA (periodontal defect followed by mechanical treatment and application of an BA hydrogel), and P + SRP + CaFB (periodontal defect, mechanical treatment and application of a CaFB hydrogel).

All animals received general anesthesia, administered intraperitoneally, before any subsequent intervention. The administration of anesthetic was in the form of a solution of Ketamidor 100 mg/mL 20 IU (0.2 mL) and Xylazin Bio 2% 0.3 mL. The injection was made slightly to the right of the white abdominal line.

Induction of experimental periodontal disease

The periodontal defect was performed on the lower incisors because of the easy access and a larger gingival sulcus [1]. In the beginning, the depth of the gingival groove was measured with the help of a periodontal probe at the level of each incisor in six points (MV, V, DV, ML, L, DL) for each animal. With the help of modified dental instruments, a silk thread used for gingival retraction,

size 00, was inserted into the gingival groove, and held in position by a suture with a sterile non-absorbable 4/0 silk thread.

Experimental design

After 14 days from the application of the ligature, the animals in the study groups were again anesthetized and the suture and gingival retraction thread were removed. The periodontal defect obtained in each animal was quantified by noting the depth of the periodontal pouch measured with the help of a periodontal probe at the level of each tooth in six points (MV, V, DV, ML, L, DL), but also the presence and location of the areas of inflammation.

In the next step of the study, we selected only the animals where we have managed to induce a periodontal defect with a periodontal pocket of 3–5 mm depth and with signs of clinical inflammation (bleeding and moderate congestion of the gingiva).

For the P + SRP, P + SRP + BA, and P + SRP + CaFB experimental groups, the next step was to perform the mechanical cleaning of the created periodontal defect (SRP), using a 5/6 Mini Five® Gracey periodontal curette, using a standardized movement by the same operator for each animal, consisting of three vertical movements on each face of the affected teeth in a gingival-incisal direction. The group P was left untreated.

Next, for the P + SRP + BA and P + SRP + CaFB experimental groups, after performing the mechanical treatment, the therapeutic hydrogel corresponding to the respective study group was applied by introducing it directly into the periodontal defect.

From each study group, the animals were sacrificed one week after the removal of the sutures and the treatment performed, in order to evaluate the way in which the healing of the experimental periodontal defect occurred. Euthanasia of the animals was performed according to the standards in force by administering an anesthetic overdose.

Preclinical assessment procedure

The first method of evaluation was the direct preclinical examination, performed immediately after euthanasia of the laboratory animals in order to prepare the samples for the histological study. A minimal, but careful, direct clinical observation could allow the highlighting of the areas of inflammation. The periodontal defect obtained in each animal was quantified by noting the depth of the periodontal pouch measured using a periodontal probe at the level of each tooth in six points (MV, V, DV, ML, L, DL), but also the presence and location of inflammation areas.

Sampling procedure

We obtained samples consisting of the two lower incisors together with the marginal periodontium and the alveolar bone with a representative fragment of the adjacent tissue structures after a previous sectioning with the scalpel, at least 1 cm away from the inserted material. After harvesting, samples were immediately transferred to glass containers appropriate in volume and size, in a 10% neutral buffered formalin solution.

Histological assessment of the oral soft tissues

The samples were further processed using classical histological technique for paraffin inclusion, a technique that allowed us to perform 3–5 µm thick serial sections. The sections were deparaffinized, hydrated and stained with Hematoxylin–Eosin (HE), the most widely used method for highlighting tissues and with Masson's trichrome. The sections were examined using an Olympus CX 20 microscope attached to a camera and computer.

Results

HPTLC analysis of CaFB and BA hydrogels. CaFB MS spectrum

The densitometer rendered a calibration curve from the polynomial regression (BA calibration function: $y = -8.629\times10^{-15}x^2 + 2.199\times10^{-8}x - 5.739\times10^{-4}$; CaFB calibration function: $y = -2.603\times10^{-16}x^2 + 3.047\times10^{-9}x + 3.262\times10^{-5}$) of the weights and areas of the scanned standard bands, which was then used to automatically interpolate the amount found in the samples, from their scanned areas. The correlation coefficients (BA, R=99.922%: CaFB, R=99.766%) and the coefficients of variation (BA, CV 1.26%; CaFB, CV 1.96%) exhibited excellent values.

The amounts of BA and CaFB from therapeutic hydrogels was determined by the HPTLC method. On the HPTLC chromatogram, BA and CaFB exhibited single and distinctive bands (R_f 0.554±0.03 and R_f 0.460±0.03, respectively) (Figure 1). The amount found in the samples were 10.3 g for BA hydrogel and 10.25 g for CaFB in 100 g hydrogel, thus confirming that the formulations contained 10% active ingredient.

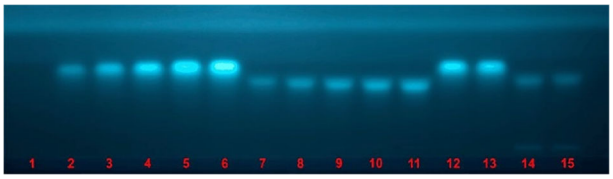

Figure 1 – ***HPTLC chromatogram of the BCCs hydrogels captured under UV light (365 nm). Lane 1: Blank (water); Lanes 2–6: BA standard; Lanes 7–11: CaFB standard; Lanes 12 & 13: BA hydrogel; Lanes 14 & 15: CaFB hydrogel. BA: Boric acid; BCCs: Boron-containing compounds; CaFB: Calcium Fructoborate; HPTLC: High-performance thin layer chromatography; UV: Ultraviolet.***

Using MS analysis, the m/z value obtained for the Fructoborate anion was $[M–H]^-$ 367.21 (main ion) (Figure 2).

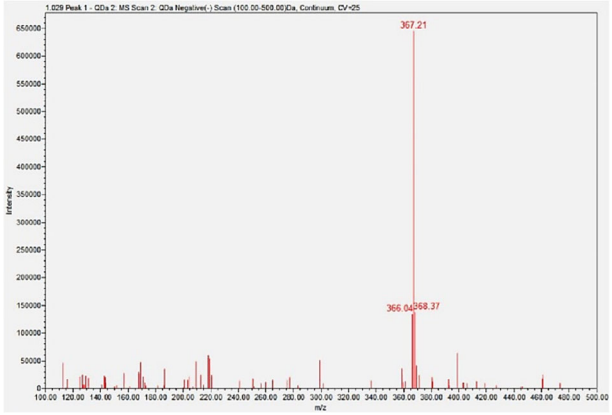

Figure 2 – ***Mass spectrometry profile of the Calcium Fructoborate (CaFB).***

Preclinical assessment

We have managed to induce periodontal disease to all study groups. The periodontal pockets have been only on the buccal sides and with different variations of the severity of the pockets.

The ligature model together with the gingival thread induced an intense inflammatory response in all groups, along with purulent discharge in some cases. The clinical observations after the treatment application highlighted the reduction of inflammation to different degrees, in all study groups but with significant differences depending on the group but especially on the initial severity of the periodontal defect.

Clinically, we have noticed a tendency of healing of the periodontal lesions after the removal of the irritation factor represented by the retraction chords maintained at that level. The simple removal of the irritation factor in the P group, without being followed by another step led after two weeks to the decrease of bleeding and the color of the mucosa, but with the persistence of a tumefaction on the buccal mucosa of the alveolar bone, well defined by a fibrous ring attached to the animal tooth (Figure 3, A and B). The application of the SRP treatment led to the decrease of the inflammatory effects, with the lack of swelling and minimal redness and tendency of bleeding (Figure 4, A and B).

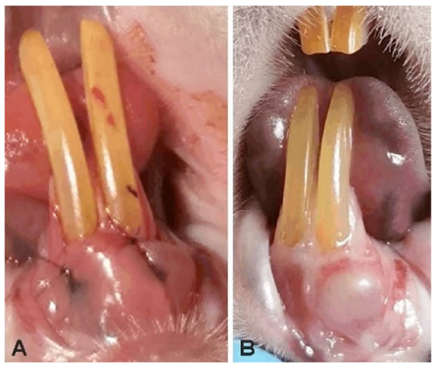

Figure 3 – ***Clinical aspect of the protocol for inducing periodontal disease in the P group (A) and clinical aspect seven days after the suture removal (B); a large tumefaction area on the left vestibular alveolar process, with some fibrous connective tissue at cervical level near the incisor. P: Periodontal defect created without receiving no further treatment.***

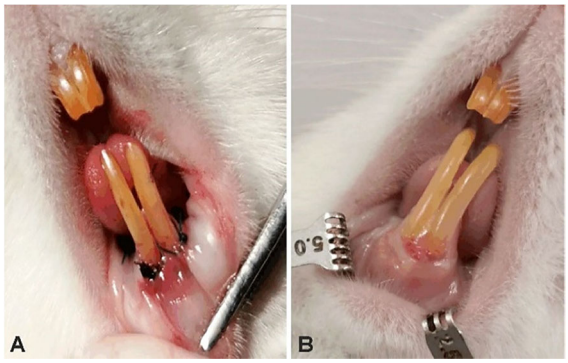

Figure 4 – ***Clinical aspect of the periodontal defect in the P + SRP group (A) and clinical aspect seven days after the suture removal and scaling and root planning (B); the reduction of the inflammatory response and a gingival retraction of both incisors can be seen. P: Periodontal defect created without receiving no further treatment; SRP: Scaling and root planning.***

The application of BA and CaFB therapeutic hydrogels let to the disappearance almost entirely of the inflammatory effects with a small buccal gingival retraction, but with the appearance of the fibrous ring attached to cervical level in the P + SRP + CaFB group (Figures 5 and 6 – A and B).

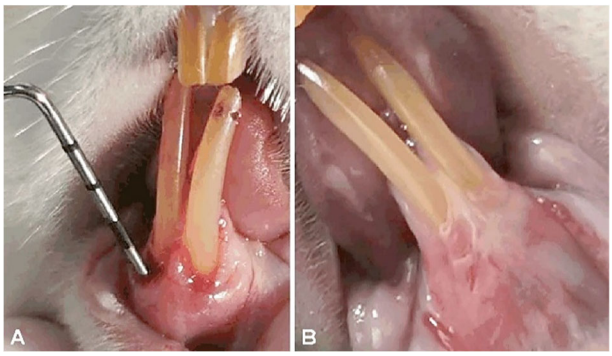

Figure 5 – ***Clinical aspects of the periodontal defect before (A) and after (B) the application of 10% Boric Acid (BA) therapeutic hydrogel: the evolution of the defect from a 3 mm periodontal pocket with moderate gingival inflammation to the reattachment of the gingiva, and the disappearance of both the pocket and the inflammatory response.***

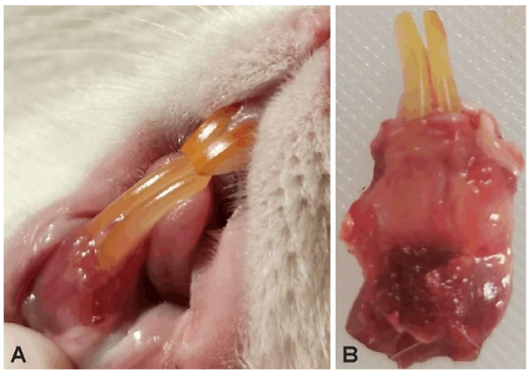

Figure 6 – ***Clinical aspects of the periodontal defect before (A) and after (B) the application of 10% Calcium Fructoborate (CaFB) therapeutic hydrogel: the evolution of the defect from a 2 mm periodontal pocket with moderate gingival inflammation to the reattachment of the gingiva, and the appearance of a fibrous tissue ring at cervical level.***

Histological assessment

Generally, the histological analysis showed the presence of the inflammatory infiltrate, with angiogenesis vessels, and a tendency of the surrounding tissues to isolate the abscess and the areas of necrosis.

The control groups and the group treated only with mechanical procedures of scaling and root planning presented moderate to severe inflammation, large lymphocyte density and vascular congestion. In addition, the presence of abscess with destructed cells and necrotic areas were noticed (Figures 7 and 8 – A and B).

The application of the therapeutic hydrogels containing BA and CaFB led to the reduction of the inflammatory reaction, the cellular density in the chorion and the formation of angiogenesis vessels (Figures 9 and 10 – A and B).

The histological analysis showed a better response to the BCCs hydrogels, than the mechanical treatment. Between the CaFB and BA hydrogels, we noticed a greater reduction of the inflammatory response in the CaFB group.

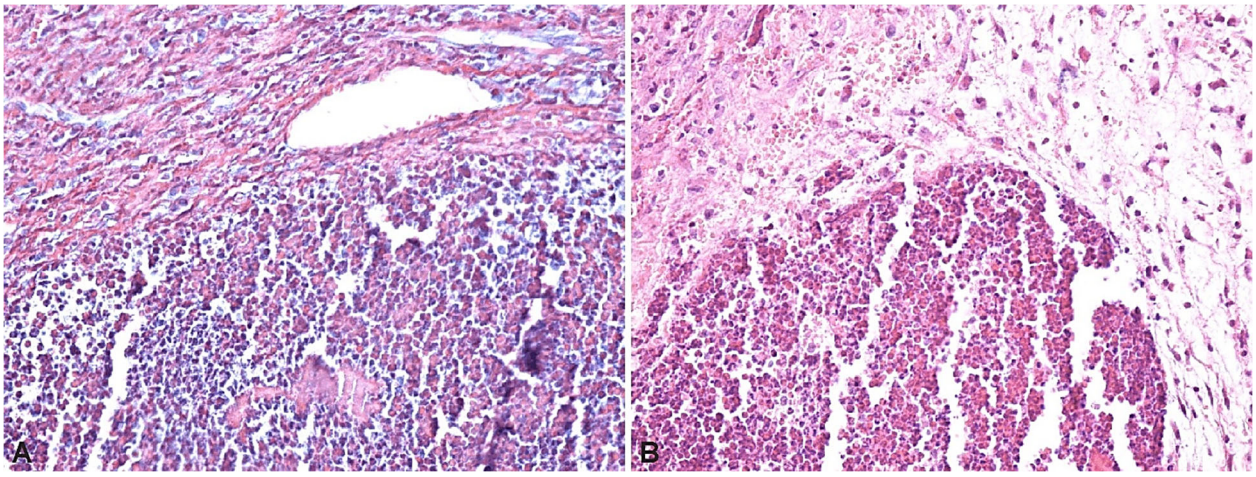

Figure 7 – *(A and B) Histological aspect of periodontal tissue of the control group, which did not receive any treatment: strong inflammatory reaction, large abscess with necrotic areas and vascular congestion. Massons' trichrome staining: (A) ×200. HE staining: (B) ×200.*

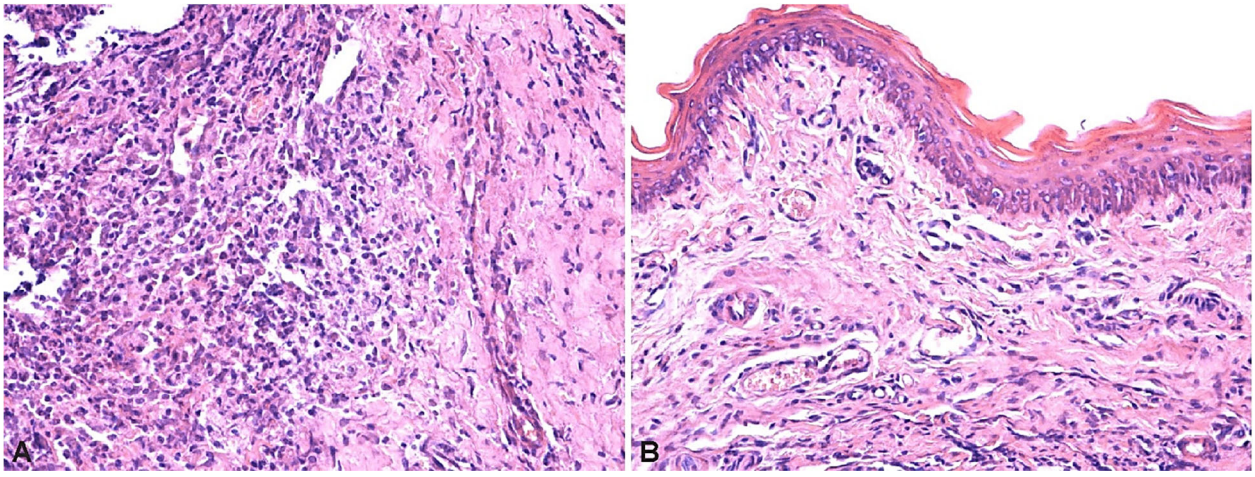

Figure 8 – *(A and B) Histological aspects of the periodontal tissue of the P + SRP group, which received only mechanical treatment of scaling and root planning: moderate inflammatory infiltrate in the chorion, with vascular congestion and angiogenesis vessels. HE staining: (A and B) ×200. P: Periodontal defect created without receiving no further treatment; SRP: Scaling and root planning.*

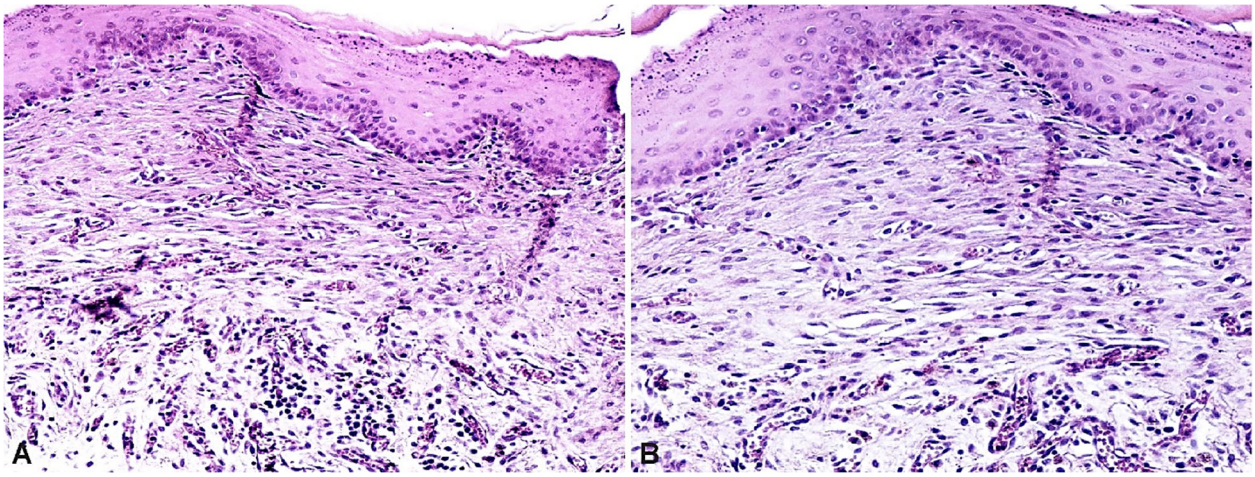

Figure 9 – *(A and B) Histological aspects of the soft tissue one week after treatment with 10% Calcium Fructoborate (CaFB) therapeutic hydrogel: reduced inflammatory reaction, with a large cellular density in the chorion and angiogenesis vessels. HE staining: (A and B) ×100.*

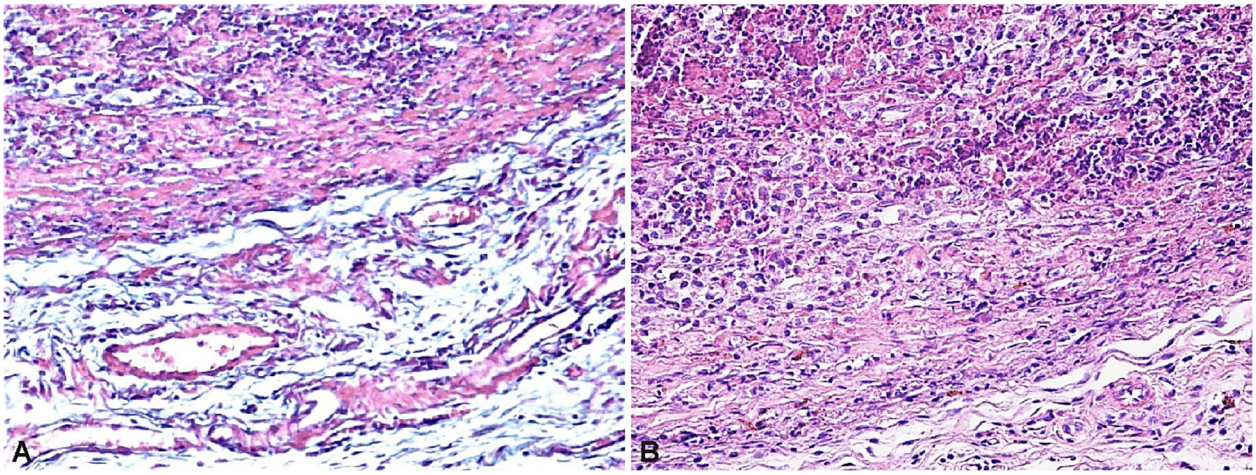

Figure 10 – ***(A and B) Histological aspect of the periodontal tissue one week after the application of 10% Boric Acid (BA) therapeutic hydrogel: moderate inflammatory infiltrate, with a limitation of the abscess by the surrounding tissues, angiogenesis vessels. Massons' trichrome staining: (A) ×100. HE staining: (B) ×100.***

Discussions

Animal models used to induce periodontal disease help to obtain a better understanding of the physiology, the pathogenesis of diseases and the action of new drugs. Elucidating the periodontal disease pathophysiology and developing targeted therapies is the main purpose of experimental models. To this end, we focused our efforts on characterizing and evaluating the host response to different hydrogels used on gingival inflammation at a histological, molecular level, aiding in the selection of the best possible therapy for helping to reduce gingival inflammatory response.

Periodontal disease is the result of bacterial aggression that triggers inflammation and mobilizes the immune defense system [20].

Although periodontal pathogens are the main responsible for the etiopathogenesis of periodontitis, there is a growing body of evidence suggesting the pivotal role played by oxidative stress [21]. In patients who received periodontal therapy combined with adjunctive antibiotic therapy, oxidative stress status decreased from a very high level to a medium one [22].

The histological studies suggest a hypothesis based on the rearrangement of collagen fibers in the extracellular matrix of the periodontium following periodontal disease. This process is characterized by the formation of thicker and spaced fibers together with a significant reduction in the area occupied by fibrillar collagen in the gingival connective tissue.

It is also stated that a new configuration of the fibers in the connective tissue would contribute to increase the progression of the periodontal destruction, leading to loss of gingival protection allowing pathogenic microorganisms to invade and reach deeper areas of periodontal tissue [23].

Mechanical therapy consisting of scaling and root planning is a standard procedure for the treatment of periodontal disease. However, mechanical therapy may fail to eliminate periodontal pathogenic species because of limited access to the root surface and tissue-invading properties of some periodontopathogenic bacteria [11].

Antimicrobials, including Povidone–Iodine and Chlorhexidine, have been used with limited success in the treatment of periodontal diseases because of potential toxicity and the unique anatomy of the periodontal pocket. Chlorhexidine has often been used as an adjunct to mechanical therapy because of its broad-spectrum antimicrobial activity. However, antimicrobial activity on the subgingival microflora and clinical benefits were shown to be limited [11].

BCCs administration has been thought to reduce oxidative stress by increasing the glutathione (GSH) reserves that neutralize the oxidants [24, 25]. Additionally, the administration of BCCs increases the levels of GSH, thereby maintaining the toxic effects of malathion. Additionally, BCCs regulates enzymatic activity associated with the immune system [26].

It has been proposed that BCCs help preserve the structures of numerous macromolecular compounds, as well as the membrane and protein complexes [27, 28]. Recent studies on mice have proved the interaction of BCCs with T- and B-cell receptors resulting in the proliferation of lymphocytes and the growing of the macrophages to release inflammatory mediators as a primary mechanism. Other clinical studies have showed the ability of CaFB to modulate molecular markers of inflammation, mainly C-reactive protein (CRP) [15, 19]. Rogoveanu *et al.* (2015) showed that CaFB at 112 mg/day compared to placebo for 30 days produced a significant decrease of interleukin (IL)-6 and IL-1β, CRP and monocyte chemoattractant protein-1 (MCP-1) [16, 29]. In our study, although the application of both BCCs hydrogels had benefic effects on the evolution of the induced periodontal defects, the CaFB hydrogel showed better results clinically and histologically.

Animal models have distinct advantages because they can mimic cellular complexities that occur in humans *in vivo* and are often more accurate than *in vitro* studies that take place on plastic surfaces with limited numbers of cell types present.

Various animal models have been used to investigate the host–bacteria interaction and to evaluate the pathogenesis of periodontitis. Animal models of periodontal disease have contributed new knowledge to the biological sciences. An important feature of the experimental models used to study human infectious diseases is the ability to simulate

an infectious process similar to that observed in humans, while mimicking the pathogenesis of the natural disease [30].

To initiate experimental periodontal disease in rodents, ligature is one of the most widely used models in periodontal research. In rats, alveolar bone loss occurs predictably after seven days. A limitation of this model is the mechanical injury caused during the placement of a ligature that could aggravate periodontal tissue destruction and physiological bone remodeling [30].

The ligature model developed here was effectively used to induce intense inflammation and gingival abscesses. Our results revealed in the histological sections inflammatory infiltrate, with necrosis areas in certain cases. The changes also included angiogenesis vessels and loss of connective tissue attachment. We concluded that these rats are susceptible to periodontal disease using the ligature induced method, and the insertion of the gingival thread in the periodontal groove helped by being a continuous irritation factor. The principle of this model is based on the adherence of microorganisms around the ligature and the thread, which serves as a niche for bacterial colonization, leading to the initiation of the periodontal injury.

Some authors consider this model to be the most representative of human periodontitis [31]. The disadvantages of the ligature model are related to the mechanical trauma caused during the ligature placement and with the decrease in disease severity over time. For maintenance of the disease intensity over time, some have used ligature incubated with *Porphyromonas gingivalis* and repositioned the thread daily in an apical position to maintain the ligature in intimate contact with the marginal tissues [32, 33].

Conclusions

BCCs continue to show their potential as a treatment option for gingivitis and periodontitis. The results of our study showed a good clinical response of the periodontal tissue to both the BA and CaFB hydrogels. The histological study revealed the reduction of inflammatory response when using the hydrogels, compared to the traditional methods. The CaFB hydrogel led to the appearance of a thicker fibrous tissue ring and a greater histological reduction of the inflammatory response compared to the BA hydrogel. Since BCCs have shown its efficacy, more scientifically proved information is needed to adapt the manufacturing technology of the compounds and to extend the research in other areas in the field of oral health.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgments

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BCCs hydrogels were prepared in the Laboratory of BioBoron Research Institute, S.C. Natural Research S.R.L., Podari, Dolj County, Romania. The HPTLC–MS analysis was performed within the Department of Pharmacognosy & Phytotherapy, Faculty of Pharmacy, University of Medicine and Pharmacy of Craiova, Romania. The interventions on the experimental animals were made within the Animal Facility and the histological study was made within the Research Center for Microscopic Morphology and Immunology, University of Medicine and Pharmacy of Craiova.

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Corresponding author
Ion Romulus Scorei, Professor, Biochem, PhD, Department of Biochemistry, BioBoron Research Institute, S.C. Natural Research S.R.L., 31B Dunării Street, 207465 Podari, Dolj County, Romania; Phone +40351–407 543, e-mail: romulus_ion@yahoo.com

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