Dental Follicle Stem Cells as a Biomaterial for Periodontal Regeneration

A *proof-of-concept* study

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The goal of periodontal therapy is to regenerate the periodontal structures: cement, periodontal ligament and alveolar bone. Stem cell-based tissue engineering raised novel therapeutic strategies for periodontal repair. In vivo dental follicle stem (DFSC) cells give rise to cementoblasts, osteoblasts and fibroblasts. Based on this idea, we have assessed DFSC potential to regenerate the periodontal structures, and by that to elaborate a new biomaterial. An experimental study was performed on male Wistar rats which were subjected to a procedure of periodontitis induction through placing silk thread ligatures around the lower incisors, under general anesthesia. Clinically, the changes of the periodontal tissue (bleeding on probing, dental mobility, dental plaque, presence of pus) induced by the periodontitis progression were daily assessed. The subjects were divided in two groups: a control and a study group. After 7 days from placing silk thread ligatures, subjects from the study group received an injection in the gingival sulcus. The injected biomaterial contained dental follicle stem cells seeded on fibrinogen. Bleeding on probing at the end of the treatment period was significantly reduced in the study group (study vs control group - 0% vs 100%, p=0.01.). In terms of dental mobility statistical significant results were obtained (p=0.04): for 20% from the subjects from the study group dental mobility was absent, while in the control group all subjects presented different degrees of mobility (33.3% degree II- v-o/m-d, 66.6% degree III-v-o/m-d/ax). In the control group pus was present in 66.7% from the subjects, and absent in the study group (p=0.10). Dental plaque was present in 40% of the subjects from the study group and in 100% in the control group. The clinical and histological results of our study demonstrate that dental follicle stem cells are a valuable cell source for tissue engineering the periodontal tissue.

Keywords: biomaterial for periodontal regeneration; dental follicle stem cells

The significant impact of periodontal disease on the general health and quality of life of patients requires an appropriate management of this disorder for the recovery of affected functions and the prevention of potential complications of this disease. The final aim of periodontal therapy is the predictable regeneration of the functional periodontal system, which involves at least three unique tissues: cementum, periodontal ligament and alveolar bone. Research over the past years has suggested new opportunities regarding regenerative periodontal therapy as a result of extending knowledge in the field of tissue engineering [1,2].

Cell migration is a dynamic and complex process which is essential for the adequate development of organs and tissues [3,4]. Cellular mechanisms for tissue regeneration are based on the capacity of precursor cells to differentiate into various cell lines, as well as on the multipotent differentiation capacity of stem cells in the desired target tissues [5].

The various aspects related to the tissue engineering applications as well as interface aspects was analyzed by different research groups with applications not just in dentistry [6-9], but also for other clinical specializations like orthopedics [10-14], cardiovascular surgery [15-17], neurosurgery [18-20], abdominal surgery [21-24], and other surgical specializations [25-27].

Recent findings in the field of cell biology support the presence of mesenchymal stem cells in dental tissues, including dental pulp, periodontal ligament and dental follicle. Dental stem cells are accessible and high-quality multipotent mesenchymal stem cells, the use of which does not involve the same ethical considerations and controversies as that of embryonic stem cells. Dental stem cells are represented by stem populations with a mesenchymal appearance, derived from the neural crest, having a different origin compared to bone marrow-derived mesenchymal cells, which originate in the mesoderm [28]. The dental follicle is an ectomesenchymal fibrous tissue

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that surrounds unerupted teeth and modulates osteoclastogenesis and osteogenesis, processes involved in tooth eruption [28, 29]. The origin of the periodontium, including cementum, periodontal ligament and alveolar bone, is found in follicle stem cells, this development cascade confirming the presence of stem cells in the dental follicle [30-32]. Dental follicle-derived stem cells have a remarkable proliferation rate and under specific culture conditions, they have the capacity to differentiate into multiple cell lines such as osteoblasts, cementoblasts, adipocytes and cells with a neuronal appearance [33, 34]. Due to the previously mentioned characteristics, follicle stem cells have been proposed as biological grafts in regenerative periodontal therapy [1, 35]. Dental inclusion is frequently found in third molars and canines, and their extraction is indicated for the prevention of local pathological processes or for orthodontic purposes. Included teeth usually contain dental follicle considered to be a candidate source for stem cell isolation [30].

Working hypothesis and objectives

Considering the previously presented premises, this study aimed to evaluate the periodontal regeneration potential of follicle stem cells in a Wistar rat experimental model of periodontitis. The preparation used for treatment consisted of a gel containing follicle stem cells in fibrinogen solution, cells isolated from the follicular sac of an included maxillary canine. The study monitored the effects of treatment by local injection of stem cells on the subjects' periodontal and systemic status, with relevance for the elaboration of a new therapeutic approach in periodontal disease.

Experimental part

Materials and Methods

Experimental protocol

The experimental study performed was a pilot study which included 10 male Wistar rats aged 6-8 weeks, with a weight of 150-200 g. The experiment was carried out according to ethical considerations and legislation regarding animal research, with the approval of the Ethics Committee of the Iuliu Haieganu UMPh, Cluj-Napoca. The animals were kept for acclimation for a week before the initiation of the experiment under vivarium conditions.

Induction of periodontal disease, experimental groups

Periodontal disease was induced by placing silk ligatures around the lower incisors under general anesthesia. The ligatures were maintained for 7 days, during which lesions with a characteristic periodontitis appearance developed. After removal of ligatures and local cleaning, the animals were randomly distributed into two groups:

Group PG – control (n=5): untreated subjects;

Group STM-F (n=5): subjects underwent a therapeutic protocol consisting of the injection of a follicle stem cell-based gel in the gingival sulcus of the lower incisors.

Blood sample collection

Blood samples of 1.5-2 mL were collected from each subject by retro-orbital sinus puncture, under general anesthesia. The samples were collected at two reference time points of the study, as follows: on the first day of treatment (after induction of periodontal disease – M1), and at the end of the experimental period (M2). The blood was collected and stored until processing in anticoagulant (EDTA) tubes.

Preparations used and treatment performed

The follicular sac was collected from an 18-year-old patient, from the upper left maxillary canine in total intraosseous inclusion. Cells were isolated from the follicular sac, and were subsequently characterized phenotypically by immunocytochemistry, flow cytometry for stem cell marker expression and RT-PCR analysis [7].

Seven days before administration, follicular stem cells were removed from nitrogen and thawed. Cells were cultured in glucose / F12 HAM medium, fetal bovine serum (FCS), penicillin + streptomycin, glutamine, non-essential amino acids (NEA), pyruvate and β-mercaptoethanol (Sigma-Aldrich, USA).

On the day of intervention, follicular stem cells were trypsinized and counted with a Thoma chamber, and 10^5 cells were resuspended in fibrinogen solution (Sigma-Aldrich, USA).

Fig. 1. The gel containing follicle stem cells in fibrinogen solution

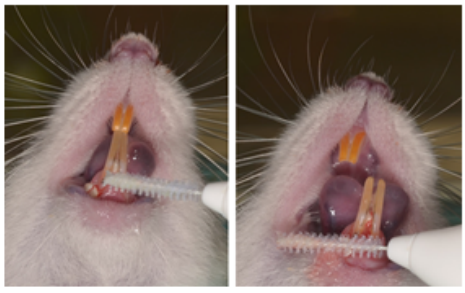

Fig. 2. Cleaning of subjects in group STM-F before injection

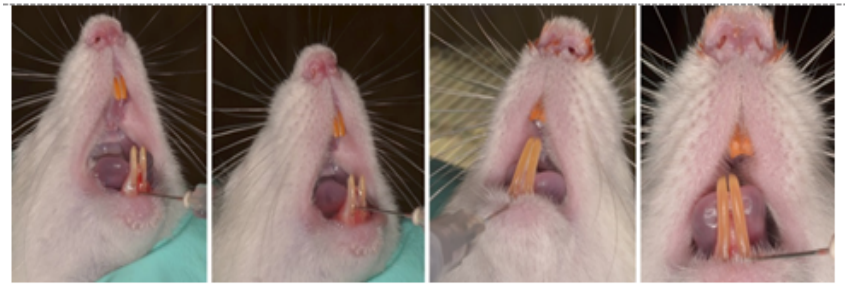

Fig. 3. Injection of the gel containing follicle stem cells in fibrinogen solution

Follicle stem cells were stored and transported on ice until their injection (Fig. 1).

After disinfection of the puncture site, the gel containing follicle stem cells in fibrinogen solution 3mg/ml was injected. The injected amount was 0.1 mL/subject. Injection was performed on the first day and on the third day of the experimental treatment period (Fig. 2, Fig. 3).

Analytical methods

Clinical examination

The clinical evaluation of the subjects involved the daily record of parameters relevant for the subjects' periodontal status: bleeding on probing, presence of bacterial plaque deposits, presence of purulent secretion in the sulcus, and tooth mobility. Each separate parameter was assigned scores allowing the statistical analysis of results. Gingival bleeding was assessed after a probe-free touch of the gingival margin: 0- the absence of gingival bleeding, 1- moderate gingival bleeding, 2-abnormal gingival bleeding. The presence of the bacterial plaque and purulent secretion in the sulcus was noted as 1, and their absence was denoted 0.

Dental mobility was assessed as follows: 0- absent, 1- buccolingual mobility, 2-buccolingual and mesiodistal mobility, 3- buccolingual, mesiodistal and apicocoronally mobility.

Hematological and immunological determinations

The blood parameters recorded before and after treatment (M1/M2) were the following: total number of leukocytes, neutrophils, monocytes, lymphocytes, eosinophils, basophils and platelets, as well as the inflammatory marker IL-6.Blood samples were analysed using a Sysmex XT-1800 automated hematology analyser (Sysmex Corporation, Japan). The IL-6 assay was performed using a commercial Rat IL-6 ELISA kit (Abbexa, Cambridge, UK).

Histopathological examination

After the fixation of the samples in neutral formalin 10% for 72 h, these were decalcified in a mixture of 8% formic acid and 8% chlorhydric acid (1/1) for 3 weeks. When decalcification was completed the samples were trimmed longitudinally and dehydrated through successive baths of Isopropyl alcohol, clarified in xylene and embedded in paraffin wax [36].

Pieces of 4 ìm thickness were cut using a rotary microtome (Leica RM2135). The examination under an Olympus BX41 microscope was performed after the sections were stained with hematoxylin-eosin (H&E). The bright field microscopic images were taken with an Olympus UC30 camera and processed using Olympus Stream Basic image analysis software [37].

Statistical analysis

The statistical processing was performed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA) and the Microsoft Excel application. The data are presented as mean±standard deviation of the mean (SD). Normal distribution was assessed using Shapiro-Wilk test and p values <0.05 were considered as statistically significant.

Results and discussions

Results of clinical examination

The degree of bleeding on probing registered at the end of the experimental period was significantly lower in the group treated with follicle stem cells (STM-F) compared to the control group (PG), the recorded values being the following: STM-F vs PG - 0% vs 100%, $p=0.01$. The comparison of the two groups regarding tooth mobility evidenced the following statistically significant results ($p=0.04$): in 20% of subjects in group STM-F, tooth mobility was absent, while all subjects in group PG had different degrees of tooth mobility (33.3% 2nd degree - v-o/m-d, 66.6% 3rd degree - v-o/m-d/ax). Subjects in group STM-F had 1st degree tooth mobility in a proportion of 80%. Purulent secretion from the sulcus was present in 66.7% of subjects in the control group, and in no subject in group STM-F (0%) ($p=0.10$). Bacterial plaque was present in 40% of subjects in group STM-F and in all subjects in group PG (100%), without statistically significant differences between the recorded values ($p=0.19$).

The dynamics of clinical parameters between the two evaluated time points (M1 and M2) for the group treated with follicle stem cells (STM-F) and the control group (PG) is presented in table 1.

Table 1

ANALYSIS OF CHANGES IN CLINICAL PARAMETERS FROM M1 TO M2 IN GROUPS STM-F/PG

Group	Parameter	Difference of the means ± SD (M1-M2)	p
STM-F	Gingival bleeding	1.40±0.55	**0.04**
	Bacterial plaque	0.60±0.55	0.08
	Purulent secretion	0.60±0.55	0.08
	Tooth mobility	0.40±0.55	**0.16**
PG	Gingival bleeding	1.00±0.00	0.08
	Bacterial plaque	0.00±0.00	1
	Purulent secretion	0.33±0.58	0.32
	Tooth mobility	-0.67±0.58	0.16

In group STM-F, a statistically significant decrease in bleeding on probing after treatment was observed. The value of the score quantifying bleeding on probing decreased on an average by 1.40±0.55 from the first record, at the beginning of treatment (M1), to the end of the experimental period (M2), with a statistically significant difference between the obtained values ($p=0.04$). The scores quantifying the presence of bacterial plaque and purulent secretion in dental and periodontal structures decreased from M1 to M2 by an average of 0.60±0.55, without statistically significant differences between the obtained values ($p=0.08$). The score for tooth mobility also decreased by an average of 0.40±0.55 ($p=0.16$).

For subjects in the control group (PG), clinical parameters did not statistically significantly change from

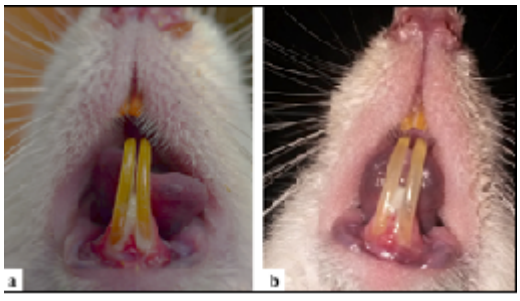

Fig. 4. Clinical appearance before (a) and after local treatment with the gel containing follicle stem cells (b)

M1 to M2. An increase by an average of 0.67±0.58 in the score assessing tooth mobility from M1 to M2 was recorded, without significant differences (p=0.16).

Figure 4 illustrates the macroscopic appearance of the periodontal region in subjects treated with the gel containing follicle stem cells.

Results of histopathological examination

The median histological score evaluated at the end of the experimental period for subjects in group STM-F was significantly lower compared to the median histological score for subjects in group PG (21[19-26]vs 26[26-26], p=0.02) (Figure 5).

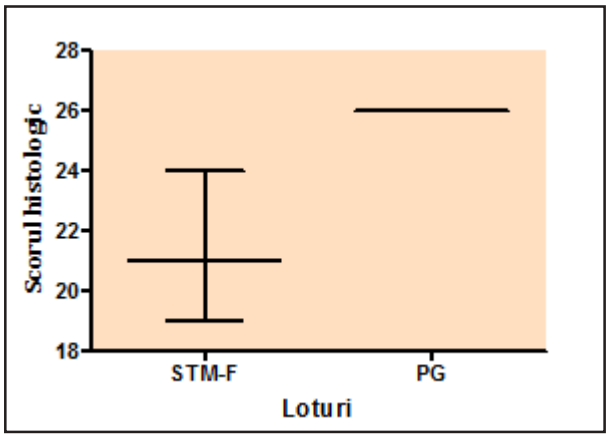

Fig. 5. Comparative analysis of the histological score for the group treated with follicle stem cells (STM-F) and the control group (PG)

Table 2

HISTOLOGICAL PARAMETERS IN GROUPS STM-F AND PG AT THE END OF THE EXPERIMENTAL PERIOD

Parameters	Group	Mean ± SD	p
Final histological score	**STM-F**	21.40±3.05	**0.02**
	PG	26.00±0.00	
Inflammation	**STM-F**	2.10±1.24	0.63
	PG	2.33±1.15	
Granulation tissue	**STM-F**	1.90±1.14	0.61
	PG	2.00±0.00	
Newly formed vessels	**STM-F**	1.50±1.00	0.16
	PG	2.33±0.29	
Fibroblasts	**STM-F**	1.60±0.89	0.69
	PG	1.33±1.15	
Bone formation	**STM-F**	2.10±1.60	**0.05**
	PG	0.00±0.00	
Osteoblasts	**STM-F**	1.60±1.14	0.22
	PG	0.67±0.58	
Bone resorption	**STM-F**	0.90±0.55	0.39
	PG	1.33±0.58	
Osteoclasts	**STM-F**	0.80±0.45	0.44
	PG	1.00±0.00	
Gingival hyperplasia	**STM-F**	1.30±0.97	0.17
	PG	2.67±1.44	

SD – standard deviation; STM-F – group treated with the gel containing follicle stem cells; PG – untreated periodontitis group

The evaluation of parameters included in the histological score shows that the score quantifying bone regeneration was higher in subjects treated with the gel containing follicle stem cells (STM-F) compared to subjects with untreated periodontal disease (PG), with a statistically significant difference between the groups (p=0.05). The comparison of the scores of the other parameters included in the healing score (inflammatory infiltrate, bone resorption, presence of osteoclasts and granulation tissue) evidenced no statistically significant differences between the studied groups. The results are shown in table 2.

Descriptive histology

The histological aspects of periodontal tissues of rats in group STM-F are illustrated in Figure 6. The image 6A shows a section comprising an alveolar bone and gingival tissue area. The black arrow indicates the injection site of the gel containing follicle stem cells. The indicated region is well delimited and vascularized; the presence of pleomorphic cells with a blastic appearance (fusiform, star-shaped, rhomboid), as well as a protein extracellular matrix with a loose appearance can be seen. A mixture of osteoblasts (mononuclear cells) and osteoclasts is illustrated in the detail image in figure 6B. The presence of osteo-cartilaginous tissue (black triangles) indicates bone remodeling processes (fig. 6C). The bracket in image 6D marks mixed inflammatory tissue and granulation tissue; inflammatory infiltrate is reduced in the area marked with asterisk, where cartilaginous nuclei can be observed.

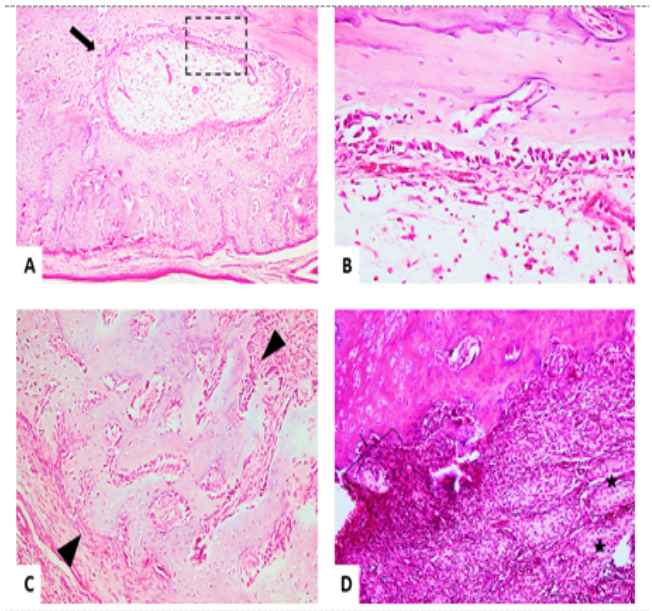

Fig. 6. Histopathological aspects of periodontal tissue of rats treated with the gel containing follicle stem cells – STM-F; H&E staining

Results of hematological and immunological determinations

The mean value of the inflammatory marker IL-6 decreased for subjects in group STM-F from M1 (11.35±16.04) to M2 (3.5±1.78), without statistically significant differences (p=0.46). Lymphocytes decreased on an average by 5.35±13.75 from M1 to M2 (p=0.47). Slight increases in the values of some parameters from M1 to M2 were recorded, as follows: total leukocytes increased on an average by 1.28±2.20 (p=0.27), neutrophils increased by an average of 0.27±14.38 (p=0.97), monocytes increased by a mean of 4.53±4.48

(p=0.07), and eosinophils by 0.50±1.58 (p=0.59). The mean value of platelets decreased from M1 to M2 by an average of 1.00±136.25 (p=0.99). In the control group PG, increases in the mean values of total leukocytes by an average of 2.3±0.79 (p=0.11) and of neutrophils by an average of 2.60±7.21 (p=0.60) were recorded.

The effects of treatment on systemic inflammatory markers were evaluated by a comparative analysis of the previously mentioned hematological and immunological parameters for groups STM-F and PG at the end of the experimental period. The results are presented in table 3.

Statistically significant differences were observed at the end of the treatment period (M2) between groups STM-F and PG regarding the mean platelet value, which was significantly lower in the treated subjects (p=0.03). The mean values of total leukocytes, lymphocytes and monocytes were also higher in subjects of the control group compared to treated subjects, but no statistically significant differences were identified between the obtained values (Table III). The mean value of the inflammatory marker IL-6 in group STM-F was higher at M2 compared to group PG (3.50±1.78 vs 2.33±0.46, p=0.34).

Table 3

COMPARATIVE ANALYSIS OF HEMATOLOGICAL PARAMETERS EVALUATED IN GROUPS STM-F AND PG AT THE END OF THE EXPERIMENTAL PERIOD (M2)

Parameter	Group	Mean ± SD	p
Leukocytes (x 10^3 µL)	**STM-F**	7.61±1.68	0.20
	PG	9.17±1.19	
Neutrophils (%)	**STM-F**	31.48±9.42	0.72
	PG	27.17±3.79	
Eosinophils (%)	**STM-F**	1.30±1.27	0.18
	PG	0.20±0.00	
Basophils (%)	**STM-F**	0.09±0.10	0.39
	PG	0.00±0.00	
Lymphocytes (%)	**STM-F**	56.70±8.65	0.92
	PG	57.43±10.61	
Monocytes (%)	**STM-F**	10.40±2.70	0.26
	PG	15.20±6.91	
Platelets (x 10^3 µL)	**STM-F**	1112.50±94.77	0.03
	PG	1422.67±38.00	
IL-6 (pg/mL)	**STM-F**	3.50±1.78	0.34
	PG	2.33±0.46	

SD – standard deviation; STM-F – periodontitis/local treatment with the gel containing follicle stem cells; PG – untreated periodontitis

The aim of this experiment was to demonstrate that dental follicle stem cells are an abundant source of cells involved in the regeneration of periodontal tissues. The identification of alternative mesenchymal stem cell sources such as the dental follicle has a considerable importance because bone marrow aspiration is an invasive procedure, and the frequency and differentiation potential of bone marrow-derived mesenchymal stem cells decrease significantly with age [4,29, 38]. Follicle tissue collection is an easy procedure that can be performed in surgical interventions for the extraction of included teeth or even less invasive procedures such as exposure of included teeth for orthodontic purposes. Compared to other stem cell sources in the oral cavity, such as dental pulp, the advantages of dental follicle are the larger size of the tissue, which can be easily handled, and a higher proliferation capacity than that of dental pulp stem cells [39].

In vivo, follicle stem cells generate cementoblasts, osteoblasts and fibroblasts. Considering this hypothesis, we evaluated the potential of follicle stem cells to regenerate periodontal tissue in a Wistar rat model of experimentally induced periodontitis. The follicular sac was collected from a human included maxillary canine. Cells were isolated and characterized by immunohistochemical staining, flow cytometry analysis and RT-PCR analysis for the identification of the stem cell gene expression [7]. The positivity for stem cell-specific markers, along with the high proliferation rate, suggests an intermediate stage between embryonic and adult stem cells of cells obtained from the human dental follicle. RT-PCR analysis indicated almost no risk of malignant proliferation of these cells after transplantation. Human leukocyte antigen (HLA-DR) expression was not identified in the evaluated follicle stem cells. The lack of HLA-DR expression confers immunosuppressive properties to these cells, with a major impact in clinical transplantation. The absence of rejection of follicle stem cells suggests a decrease in the incidence of the rejection reaction between the graft and the host, according to previous literature reports [40, 41].

The treatment applied in our study involved the injection of a preparation with follicle stem cells in fibrinogen solution in the lower incisor region of rats in which periodontal disease was experimentally induced by placement of ligatures. A significant improvement in the subjects' periodontal status was found after treatment, with a significant reduction of local inflammatory processes, indicated by a reduction of bleeding on probing and tooth mobility compared to the untreated control group.

The histological score used to assess the efficiency of treatment involved the recording and encoding of representative parameters for the certification of inflammatory and bone regeneration processes. The evaluated parameters were: inflammation, granulation tissue, newly formed vessels, fibroblasts, osteoblasts, osteoclasts, bone formation and resorption. The codes assigned to each parameter were added up, and a final histological score resulted. A higher value of this score suggests a more advanced stage of periodontal lesions, while a lower score indicates the initiation of tissue remodeling and regeneration processes. The formulated histological score is reproducible and is in accordance with other histological scores used in the literature [42]. The mean value of the histological score for subjects in group STM-F was significantly lower compared to the value recorded in the control group (p=0.02). The score quantifying bone formation was significantly higher for subjects treated with follicle stem cells compared to untreated subjects (p=0.05), thus suggesting the efficiency of treatment in the initiation of bone regeneration processes, in experimental periodontal disease.

Histopathological analysis performed in our study evidenced osteochondral proliferation, which is relevant for supporting the regenerative potential of follicle stem cells. The results are in agreement with those of other literature studies, which suggested the capacity of follicle stem cells to regenerate tissues affected by periodontal disease [1,38].

Immunological determinations showed that the mean value of the inflammatory marker IL-6 decreased by an average of 7.85±16.12 (p=0.46) following local treatment with follicle stem cells in group STM-F. Lymphocytes also decreased by an average of 5.35±13.75 from the first day of treatment to the end of the experimental period, in

treated subjects. The comparison of the values of hematological and immunological parameters for groups STM-F and PG, at the end of the experiment, evidenced statistically significant differences regarding the mean value of platelets. Thus, for subjects treated locally with follicle stem cells, the final mean value of platelets was significantly lower compared to untreated subjects ($p=0.03$). For the other hematological parameters assessed, no significant differences were found between groups STM-F and PG. We could not compare the data obtained in our study to the results of other literature studies, because we did not identify any results of studies evaluating systemic inflammatory response in local therapy by injection of follicle stem cells in periodontal lesions.

Experimental animal models provide important research data, but their applicability to the human species still raises controversy given the species-related characteristics, as well as disease susceptibility and individual response to treatment. The data of this study should be complemented by extending research on the regenerative potential of follicle stem cells both in experimental models and in comprehensive clinical studies, which might be premises for the development of new therapeutic modalities in periodontal disease.

Conclusions

The histopathological examination performed evidenced bone regeneration processes, confirmed by specific osteochondral proliferation aspects in subjects treated locally with follicle stem cells. Clinical examination showed an improvement in their periodontal status, with a significant reduction of local inflammatory processes. The results of our study suggest that follicle stem cells are an abundant source of cells for tissue engineering, with applicability in regenerative periodontal therapy.

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