ORIGINAL PAPER

RJME
Romanian Journal of Morphology & Embryology
http://www.rjme.ro/

Histological findings from rat calvaria defect augmented with platelet-rich fibrin by using two consecutive periosteal incisions

ALEXANDRU FLORIAN GRECU[1,2], DRAGOŞ GEORGE POPA[3], CRISTIAN VIRGIL LUNGULESCU[4], EDUARD MIHAI CIUCĂ[5], ADRIAN CAMEN[5], DANIELA MARINESCU[6], OLIVIU NICA[1,3], CRISTINA JANA BUSUIOC[7], FENG IFRIM CHEN[8], MARIUS EUGEN CIUREA[3]

[1] *PhD Student, Doctoral School, University of Medicine and Pharmacy of Craiova, Romania*
[2] *Department of Orthopedics and Traumatology, Emergency County Hospital, Craiova, Romania*
[3] *Department of Plastic Surgery and Reconstructive Microsurgery, University of Medicine and Pharmacy of Craiova, Romania*
[4] *Department of Oncology, University of Medicine and Pharmacy of Craiova, Romania*
[5] *Department of Oral and Maxillofacial Surgery, University of Medicine and Pharmacy of Craiova, Romania*
[6] *Department of Vascular Surgery, University of Medicine and Pharmacy of Craiova, Romania*
[7] *Department of Histology, University of Medicine and Pharmacy of Craiova, Romania*
[8] *Department of Traditional Medicine, "Carol Davila" University of Medicine and Pharmacy, Bucharest, Romania*

Abstract

Background and Objectives: Platelet-rich fibrin (PRF) is a new generation of biomaterial that proved to be an effective tool in numerous clinical uses. This study aims at expanding the range effectiveness of PRF in promoting bone healing by histological evaluation. *Materials and Methods*: We performed a pair of two calvaria defects on 35 Wistar rats. The left defect was left empty as a control and the right defect was augmented with PRF. After 45 days, the experiment was terminated and the calvaria were collected and underwent morphological and histological analysis. *Results*: New bone formations have been shown to be prevalent in the PRF augmented defect. *Conclusions*: PRF increases the body's natural ability to heal and regenerate bone.

Keywords: platelet-rich fibrin, tissue engineering, regeneration, decalcification, bony callus.

Introduction

Platelet-rich plasma (PRP) was first developed [1] in the 90s and proved to be a very popular and reliable tool for numerous aspects of the healing process [2, 3]. Despite early promising developments, there were studies showing that the presence of anticoagulants, especially bovine sourced thrombin, in the PRP negatively impact its wound healing capabilities. Because the anticoagulant prevents the formation of clots, the healing process is altered, as this is a crucial step in physiological healing [4]. This issue was addressed by the development of platelet-rich fibrin (PRF) [5].

PRF is a form of platelet concentrate [6], with plastic properties, given to it by the polymerization of fibrinogen into fibrin [7] that traps the platelets in the three-dimensional matrix, while being formed. It is obtained by centrifugation of blood in a glass/non-glass Vacutainer, without anticoagulants or other additives. Growth factors that are located on the surface of the platelets are released during platelet activation, thus locally stimulating healing and growth. Besides the advantages given by the intrinsic properties of the platelets, PRF proved to contain a high concentration of leukocytes as well, that prevent the occurrence of infection [8]. The way that PRF is delivered at the site of the injury and its mechanical proprieties of being easily manipulated, pressed and cut to size proved to be a noteworthy healing tool.

PRF related research has been ongoing since the its first production [9] with diverse applications. Due to the broad range of possible effects of the growth factors present on the surface of platelets, PRF has been used, for example, in tendon healing [10], rotator cuff injuries [11], nerve injuries [12], gingival recession [13], periodontal defect healing [14, 15], as an antimicrobial agent [16], wound healing [17] and wound healing set on a diabetic background [18], medication-related osteonecrosis of the jaw[19], implant osseointegration [20], regenerative dentistry [21, 22] or plastic and reconstructive surgery [23].

Aim

The aim of this study is to histologically evaluate the effect that PRF has on a site of bone lesion by using animal experiment on rats.

Materials and Methods

The aim of this study was obtained by using 35 male Wistar rats. For this experiment, approval of the Animal

Welfare and Bioethics Committee of the University of Medicine and Pharmacy of Craiova (Romania) was obtained.

All the rats were housed in larger than standard cages, with a cage density of one rat per cage [24], food and water provided *ad libitum*.

The rats used in the experiment presented at a weight of 320 g (range: 220–420 g) and selected to be of adult age, without being older than six months.

Process of PRF procurement

The speed at which rat blood coagulates had to be taken into consideration when performing the experiment. Thus, the centrifuge (PRF Duo Centrifuge® – Process) had to be primed with counterweights and set up according to the PRF obtaining protocol [25], in advance of the actual experiment.

The technique used to draw blood from the rats was terminal cardiac puncture [26], due to the imbalance between the necessary 10 mL of blood for satisfactory PRF clot formation and the low blood quantity of total blood from a rat. The advantage of using this technique was represented by the fact that we could produce sufficient PRF membrane from a single terminal cardiac puncture procedure for 4–5 experiments.

The PRF donor rats were anesthetized with an association of Ketamine 50 mg/kg and Xylazine 5 mg/kg injected intramuscularly. Complete anesthesia installation was tested by performing the corneal reflex test and the toe squeeze test. Corneal dehydration was prevented by generous application of artificial tears.

The rat was shaved on the anterior left torso. Then, it was placed on its back, on a heated mat. The point of needle insertion was chosen in relation to the apexian shock and the median sternum line.

After determining and marking the place of needle insertion, the area was sanitized using a surgical alcoholic solution. The needle was moved slightly above or below the determined point, in cases where the tip of the needle superimposed a rib. In order to achieve cardiac puncture, the needle was angled at 25–35°, with the tip oriented towards the head of the rat. After the percutaneous needle insertion, cardiac puncture was achieved after a travel of the needle of 1.5 cm. The blood was drawn directly into a PRF Choukroun Vacutainer®, by using a BD Vacutainer® Eclipse™ blood collection needle connected to a BD Vacutainer® one-use holder for easy manipulation. A 10 mL Vacutainer fill was achieved in 3–4 seconds. This was quickly transferred to the pre-prepared centrifuge (1300 rpm – 400 g/8 minutes spin time) and the centrifugal process was initiated in a median time of less than 10 seconds from blood draw initiation.

After blood collection, the blood donor rat was sacrificed by administering a threefold dose of anesthetic mixture. At the end of the centrifugal process, the Vacutainer was stored for 10 minutes in a tube stand, away from direct sunlight. The PRF clot was then removed, separated from the red blood clot (RBC) formed at the bottom and placed inside a PRF box, meant for transforming the PRF clot into a PRF membrane by pressing. If ideal moisture conditions are maintained by using the exudate of the PRF, this could be used for up to 2.5–3 hours.

Surgical protocol for inducing calvaria bone defects

Prior the surgical intervention, the test rats were anesthetized with the same protocol as the blood donor rats. Their skin covering the calvaria was shaven and antiseptic solution was applied.

The calvaria were exposed by operating a longitudinal incision of 3 cm in the middle of the calvaria. The periosteum was first incised on the left side. The bone defect was performed through this periosteum opening by using a trephine with a diameter of 3 mm attached to a motorized electric handpiece, under continuous irrigation with sterile saline solution so as to prevent bone necrosis. The bone inside the defect was removed by using a small Hohmann bone elevator, taking great care to assure that no dura was still attached to the removed bone. The defect was left as it was and the periosteum was sutured on top of it so as to have an evaluation of a 3 mm defect in rat calvaria defect, without any augmentation. This defect will be called "left-control defect".

A right side incision was subsequently performed in the periosteum, of the same length as the left one. The defect was performed in an identical manner to the left one and the residual bone removed as well. A PRF graft was cut to size to fit the defect and was placed inside. The periosteum was sutured on top, taking great care not to mobilize the graft from its in-defect position. This defect will be called "right-test defect". The skin was sutured and a local anesthetic was injected around the surgical wound.

Experiment endpoint and sample harvesting for histological assessment

The test rats were sacrificed 45 days after the bone defect inducing procedure by administering a 3× dosage of normal anesthetic mixture. An incision was performed along the line of the previous surgical scar. The periosteum was widely exposed. The joint tendinous aponeurosis of the superficial head of the masseter muscle and temporal muscle was removed at its insertion, such as to free the calvaria from any muscle insertions. The calvaria were removed as uninterrupted bone by using a dental drill tool connected to a motorized handpiece, thus the calvaria piece contained both left-control and right-test defects. This piece was lightly cleaned of any loose soft tissue that might still be attached. The periosteum was left *in situ*. An indentation was performed using the drill tool on the left side of the collected sample.

The samples were transferred in a sterile tube containing 10% formalin solution, where they were kept for two weeks. After this, the samples were transferred in a solution containing ethylenediaminetetraacetic acid (EDTA) and kept on a laboratory shaker. The solution was exchanged every three days. This was continued until the bone sample had achieved decalcification. This was evaluated as achieved when the sample turned into a rubbery consistence.

The process of paraffin embedding was initiated by dehydration of the samples by using increasing concentrations of alcohol and continued by xylene cleaning. They were then embedded in paraffin, and, by using a microtome device, sliced into 5 μm thick slices that were

then fixed to glass slides. Paraffin was removed; the samples underwent a hydration process and stained with Hematoxylin and Eosin (HE). Histological assessment and sample photography were performed with a Nikon 55i microscope device and a digital camera attachment.

Results

Surgical results

Our protocol separates itself from other existing protocols by the decision to perform two separate incisions in the periosteum, one at a time (Figure 1). This manner of manipulating the periosteum incisions greatly decreased the uncertainty of any PRF graft mispositioning inside the right-test defect. This was due to the consecutive nature of the periosteum incisions in relation to each other thus allowing for the left periosteum incision to be closed and sutured, essentially restoring the continuity of the periosteum before enacting the subsequent right-test defect (Figure 2). The periosteum between the two incisions was not raised from the bone, thus acting as a periosteum isthmus, isolating the two defects from each other.

The small incision, performed exactly at the site of the right-test defect, incurred small flaps that would allow for little to none supplementary movement of the periosteum upon the suturing procedure which in turn minimizes the risk of PRF graft mobilization.

The added risk of dealing with two separate incisions resulted in no complications in our study. We have successfully shown that the periosteum did not show any sign of periosteal stripping or periosteal shredding in relation to the underlying bone defects, further proving the value of this technique.

Our technique also augmented the beneficial proprieties of the PRF graft. This was proven by the consistent signs of healing that were visible in the right-test defect, when compared to the non-augmented left-control defect.

Our results proved that it is possible to enact a surgical protocol that allows for secure PRF membrane graft placement.

The PRF used in our experiment can be substituted with any bone healing adjuvant material undergoing research in any further studies thus improving the outcome and reliability of any study concerning material that aid bone healing.

Histological findings

The histological examination showed that bone defect recovery began at the periphery of the bone, from the normal bone area, to the center lesion. Major structural changes were observed at the periosteal level and we consider them to have occurred in the initial stages of repairing bone defects. Therefore, in the "right-test defect", the microscopic examination has revealed numerous ossification centers, which occurred as a result of osteo-forming activity of the periosteum. Periosteal fibroblasts have rapidly multiplied and some of them have trans-formed into osteoblasts (Figure 3). The osteoblasts disseminated and formed several ossification centers separated by a young connective tissue similar to granulation tissue. These centers of primary ossification were thus populated with young cells with large, hypochromic nuclei and abundant cytoplasm (Figure 4). Around them, the extracellular matrix was poorly mineralized, having a slightly eosinophilic color.

In other areas, osteoblasts matured, becoming osteo-cytes, oval-like cells with more intense colored (hyper-chromic) nuclei and less cytoplasm. The extracellular matrix became more abundant, more eosinophilic. As osteoblasts or transformed into osteocytes, it has been observed that ossification densities occurred in some ossification centers that led to the formation of the first bone blades (Figures 5 and 6). With the ossification process advancing, ossification centers have merged and generated larger bone structures where lamellar bones have become more apparent. These newly formed bony blades initially presented with an irregular pattern of bone blades that slowly metamorphose into regular bone blades.

In the "right-test defect", we noticed that the primary ossification process did not completely fill the lesion area, and bone remodeling elements appeared. Osteoclasts were observed in bone tissue (Figure 7). These osteoclasts are evidence that the bone healing area of the "right-test defect" was trapped in the final bone formation process.

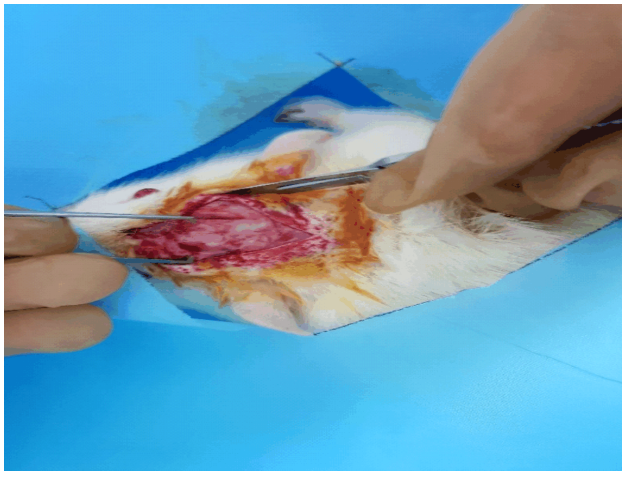

Figure 1 – ***Macroscopic image during surgery: periosteal incisions.***

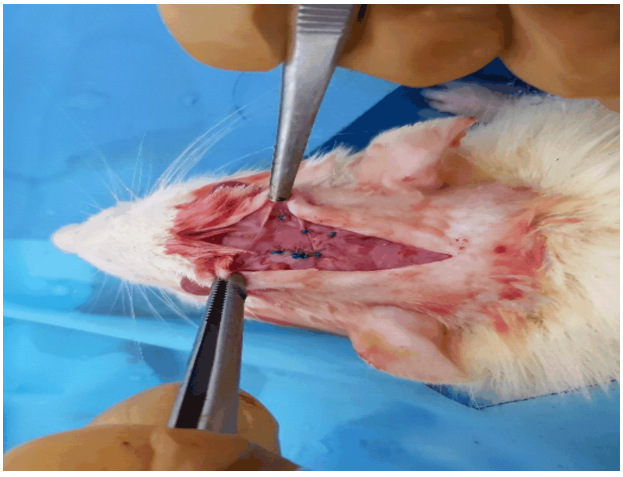

Figure 2 – ***Sutured periosteal incisions.***

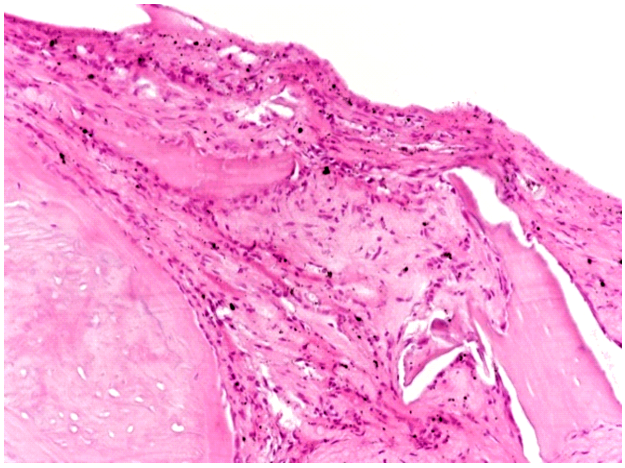

Figure 3 – ***Periosteal connective tissue with moderate inflammatory infiltration and incipient ossification areas in "right-test defect" (HE staining, ×100).***

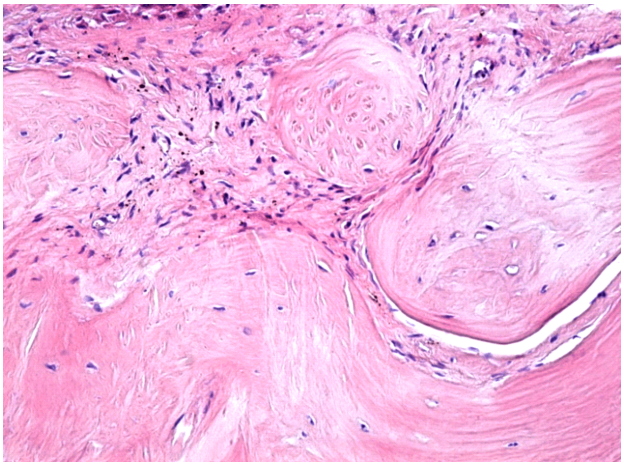

Figure 4 – ***Image of multiple formation of primary ossification centers by transformation of periosteal fibroblasts into osteoblasts (HE staining, ×100).***

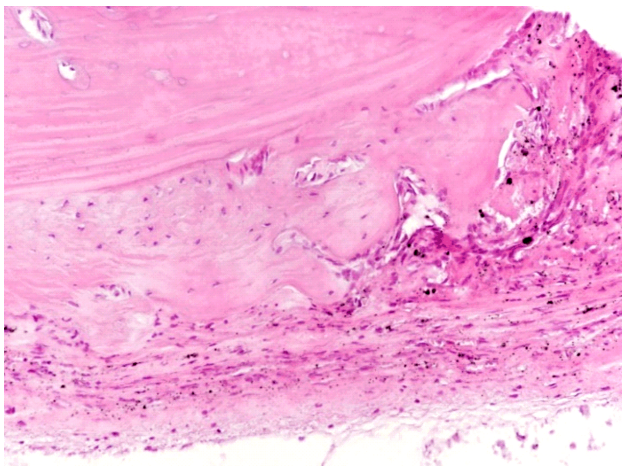

Figure 5 – ***Microscopic image of an ossification area with numerous osteoblasts and osteocytes appearing under, in "right-test defect" (HE staining, ×100).***

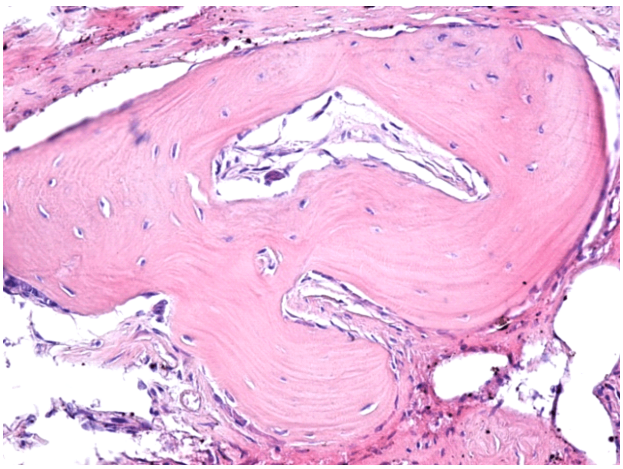

Figure 6 – ***Bone structure well structured, mainly formed from osteocytes and bone lashes in "right-test defect" " (HE staining, ×200).***

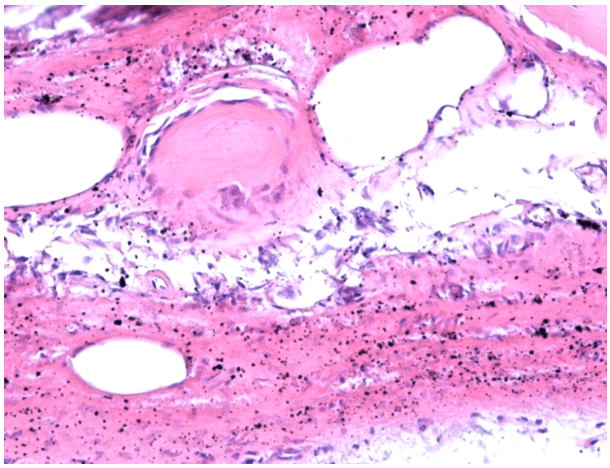

Figure 7 – ***Osteoclasts generating areas of bone remodeling, in "right-test defect" (HE staining, ×100).***

Histological assessment focused on both of the two defects revealed typical signs of bone healing in both types of defect.

However, the "right-test defect" was consistently found to have better represented marginal healing, with one case showing particularly fast bone regeneration, with the microscope sample showing a bone bridge that arched over the entire diameter of the defect (Figure 8).

The histological findings did not emphasize any reaction typical of a foreign body reaction.

In our study, we noticed that the primary ossification process was not homogeneous across its entire section and was not identical in all animals. Sometimes, there were observed defects of primary ossification characterized by lack of bone loss in some areas (Figure 9). We believe that these "defects" of primary ossification will disappear during the process of secondary ossification and bone remodeling.

Significant microscopic changes were also observed in the periosteum in "right-test defect". It had an increased thickness, with numerous fibroblasts, fibrocytes and collagen fibers in its structure. These changes show its importance in the ossification process (Figure 10).

When compared to the "right-test defect", the "left-control defect" showed signs of delayed bone formation with either no bone densification occurring in the conjunctive tissue filling the defect (Figure 11), or singular bone formation, with no outgrowths typical of an ongoing healing process (Figure 12). Thus, we can conclude that the left-control defect did not show any of the rapid healing processed occurring in the right-test defect.

Given the identical aspect of the two defects regarding size, inducing technique and local environment, we can conclude that the addition of PRF proved to be a valuable resource in promoting bone defect repair and fill.

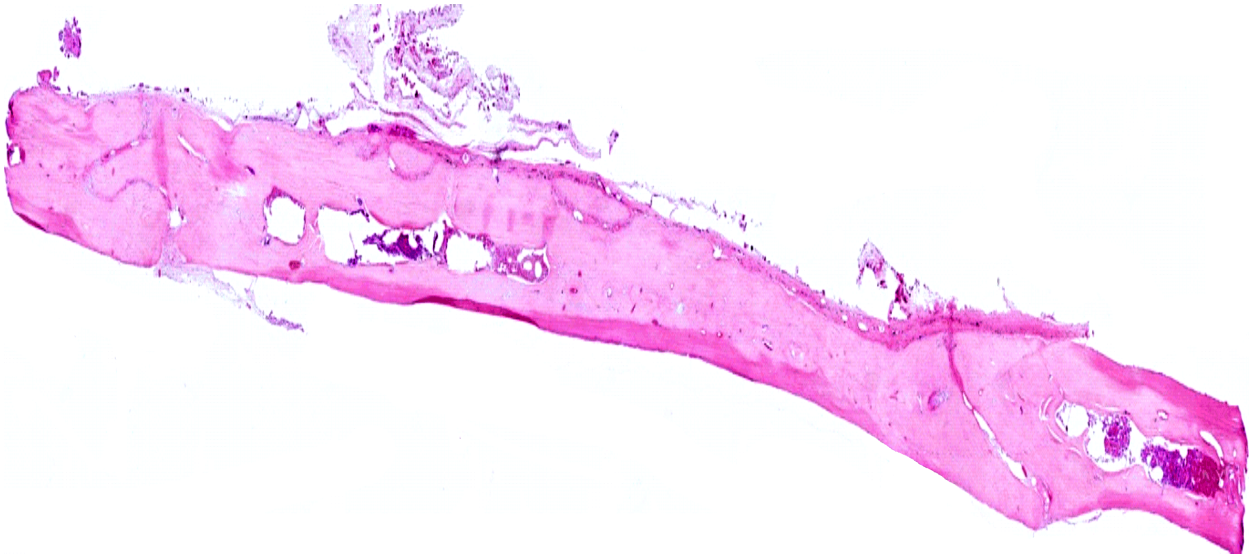

Figure 8 – ***Microscopic image where complete filling of the bone defect can be observed in "right-test defect" (HE staining, ×40).***

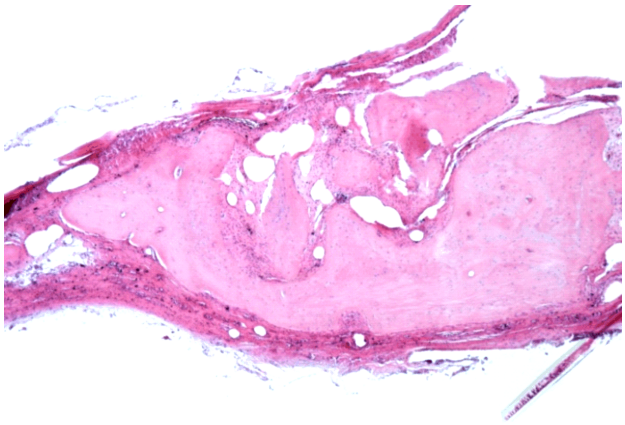

Figure 9 – ***Primary ossification process with "defects" (HE staining, ×100).***

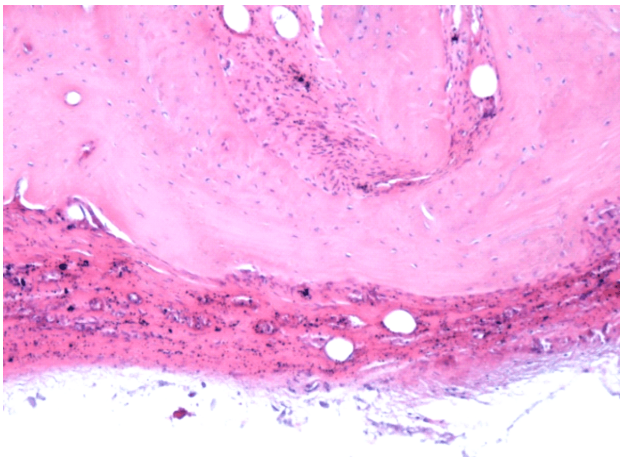

Figure 10 – ***Microscopic image of the periosteum in the adjacent area of the ossification process. Increasing the thickness of the peritoneum by increasing the amount of fibroblasts and collagen fibers in "right-test defect" (HE staining, ×200).***

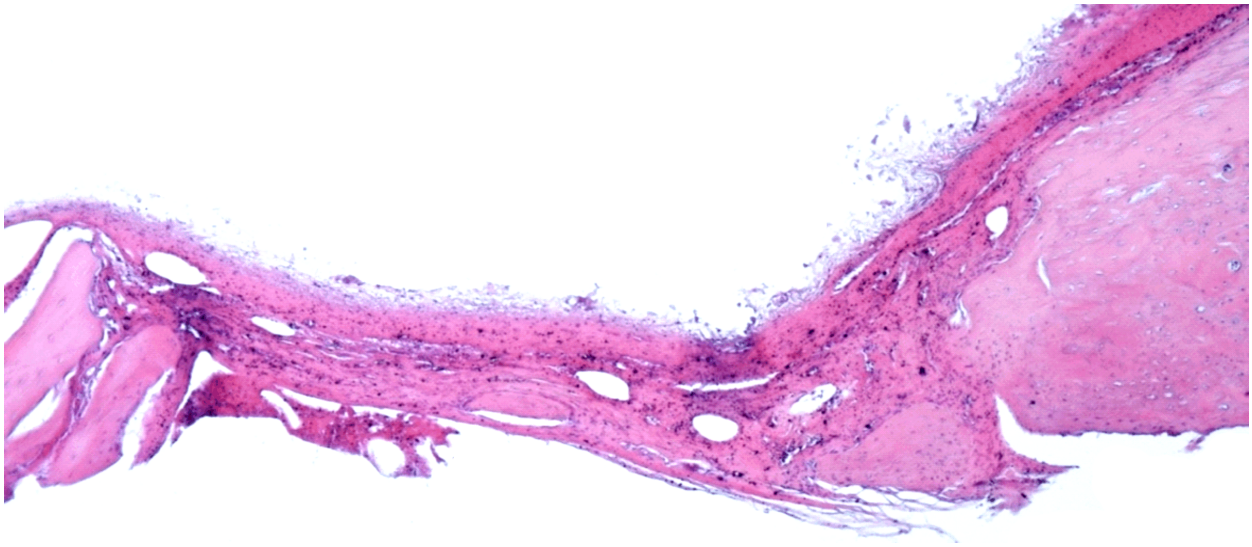

Figure 11 – ***Left-control defect presenting with little to none ossification centers (HE staining, ×100).***

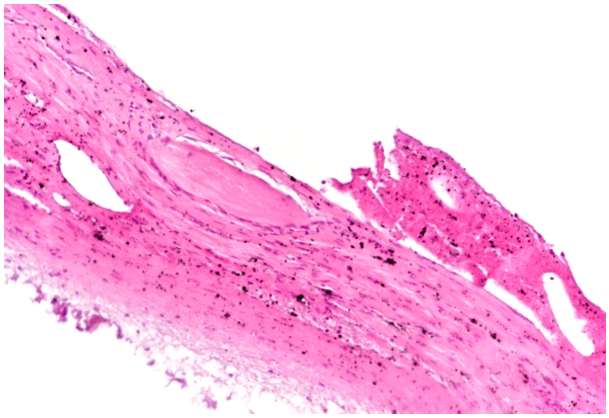

Figure 12 – ***Singular ossification center in the left-control defect (HE staining, ×100).***

Discussions

Bone defects caused by various pathologies (such as trauma, tumors or infections) significantly affect the quality of life of the patient and represent a major burden on society in general and on the healthcare system in particular. Repairing and regenerating these defects represents a considerable challenge for physicians [27].

The healing of bone lesions is an extremely complex process that depends on a variety of factors: the presence or absence of the periosteum, the differentiation of the osteogenic cells, the inflammatory reaction, the local vascularization, existent endogenous or exogenous osteo-forming factors, and others [28, 29].

PRF is a new form of biomaterial that has shown promise in the field of accelerating and improving tissue healing [30]. Bone healing is the only healing process that has the ability to generate complete healing of the affected organ, as opposed to other healing processes that usually heal with scar tissue as a side product [31]. Fracture healing and bone defect filling are subjects of great interest, with researchers worldwide searching for solutions to clinical scenarios of poor or insufficient healing [31–33]. Given the high social and economic impact of a bone fracture, different opportunities are being investigated in order to improve outcomes related to bone healing [34, 35]. Platelet concentrates represent a new promising field of bone related research [36, 37], with further studies being necessary to evaluate the possible benefits of PRF addition to a fracture site [38].

The first step in researching a material is to perform animal experimentation. Several aspects must be taken into consideration when pertaining to experimental bone research performed on animals [39]: evaluate the experimental technology from a biological and mechanical standpoint; simulate the target clinical conditions, subsequent possible variations of it; perform a quantitative determination from a capacity, volume and effectiveness perspective.

The experimental model of two defects induced side by side in the same individual rat allowed for a self-contained environment that gave both defects the same baseline healing conditions, such as to allow for any potential differences in healing of the PRF grafted defect to be attributed to the presence of the PRF alone.

In our experiment, we did not observe any complications that could have arisen from performing two distinct incisions in the periosteum, such as infection or periosteum dehiscence. Despite this, the relatively small sample size reduces the statistical significance of this finding. Further studies, with larger sample sizes are required in order to quantify any risk associated with this procedure.

It is important to note that we did not find any significant presence of granulomatous inflammatory response typical of a foreign body reaction [40]. This was probably caused by the as – autologous nature of the PRF graft that was used in the experiment and confirms the complete compatibility of PRF between the individual Wistar rats used in the experiment.

Despite the general observed tendency of both defect types to have bone healing occur predominantly from the outside of the defect inwards, we were able to observe a significantly higher number of ossification occurring in the mass of fibroconnective tissue in the right-test defect, thus proving the higher degree of healing caused by the presence of PRF in the bone defect.

The reason for choosing the Vacutainer blood draw kit as opposed to drawing blood with a syringe and then transferring it to a Vacutainer was that the more handling of the blood required, the greater the risk of infection and other associated risks [41], as well as the extra time required for syringe to Vacutainer transfer. Our chosen technique allowed for the least amount of blood handling while being fast and precise.

The calvaria were harvested in a single piece in order to prevent the occurrence of any sample fragmentation. If this had occurred, the most likely place for the fragment to crack was at the place of least resistance, represented in our case by any of the two defects. Our experiment was aimed at evaluating any bone healing and new bone formation that had occurred inside the two defects, thus any fracture line that overlapped the perimeter of any of the defects would have greatly influenced the results.

The harvested samples were marked by mechanical marking by using a dental handpiece because it allowed for proper defect identification in the Department of Histology, while preserving the structural integrity of the two defects. Accurate marking of the sample was of paramount importance because our study greatly relied of making correct comparisons.

This study use of laboratory rats for experimentation has proven particularly useful, due to the extensive histological assessment that we were able to perform by euthanizing the rats and harvesting the sample in one piece. We consider that this method would have been significantly more difficult to perform with any other animal species. If another animal species had been used and we would have used a needle biopsy of the defect instead of the full sample histological and microscopic assessment [42], it would have made the assessment far less accurate and thus greatly impede on the reliability of the results.

The limited time frame of this study did not allow for reliable allocation of our 3 mm defect as a critical sized defect or as a subcritical sized defect (SCSD) [43, 44]. This also confirms the literature regarding this subject,

which states that healing of a rat calvaria defect occur by marginal healing and by osseous processes occurring in the mass of fibroconnective tissue. During the healing process, these masses enlarge in size until they conflate and, with the area covered by marginal healing, cover the entire defect, if the defect was a SCSD [45]. This is because despite the fact that maximum healing occurs at the four weeks mark, it continues until 24 weeks [46, 47]. This implies that, in order to testify for the inclusion of any calvaria defect into one of the two categories, the study must be stopped and samples must be sent for assessment after the healing process had ceased.

Conclusions

Our study has successfully proven that the addition of PRF to a bone defect of the rat calvaria increases the healing rate when compared to an identical defect without any healing augments and that the use of the descried innovative surgical technique enhances the chance of calvaria bone defect healing.

Conflict of interests

The authors declare that they have no conflict of interests.

References

[1] Marx RE, Carlson ER, Eichstaedt RM, Schimmele SR, Strauss JE, Georgeff KR. Platelet-rich plasma: growth factor enhancement for bone grafts. Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 1998, 85(6):638–646.

[2] Kon E, Filardo G, Di Martino A, Marcacci M. Platelet-rich plasma (PRP) to treat sports injuries: evidence to support its use. Knee Surg Sports Traumatol Arthrosc, 2011, 19(4):516–527.

[3] Charneux L, Demoulin C, Vanderthomment M, Tomasella M, Ferrara MA, Grosdent S, Bethlen S, Fontaine R, Gillet P, Racaru T, Kaux JF. [Platelet-rich plasma (PRP) and disc lesions: a review of the literature]. Neurochirurgie, 2017, 63(6):473–477.

[4] Mourão CF, Valiense H, Melo ER, Mourão NB, Maia MD. Obtention of injectable platelets rich-fibrin (i-PRF) and its polymerization with bone graft: technical note. Rev Col Bras Cir, 2015, 42(6):421–423.

[5] Choukroun J, Adda F, Schoeffler C, Vervelle A. Une opportunité en paro-implantologie: le PRF. Implantodontie, 2001, 42:55–62.

[6] Badran Z, Abdallah MN, Torres J, Tamimi F. Platelet concentrates for bone regeneration: current evidence and future challenges. Platelets, 2018, 29(2):105–112.

[7] Miron RJ, Bishara M, Choukroun J. Basics of platelet-rich fibrin therapy. Dent Today, 2017, 36(4):74–76.

[8] Owen CA, Campbell EJ. The cell biology of leukocyte-mediated proteolysis. J Leukoc Biol, 1999, 65(2):137–150.

[9] Dohan DM, Choukroun J, Diss A, Dohan SL, Dohan AJ, Mouhyi J, Gogly B. Platelet-rich fibrin (PRF): a second-generation platelet concentrate. Part I: technological concepts and evolution. Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 2006, 101(3):e37–e44.

[10] Sánchez M, Anitua E, Azofra J, Andía I, Padilla S, Mujika I. Comparison of surgically repaired Achilles tendon tears using platelet-rich fibrin matrices. Am J Sports Med, 2007, 35(2): 245–251.

[11] Fu CJ, Sun JB, Bi ZG, Wang XM, Yang CL. Evaluation of platelet-rich plasma and fibrin matrix to assist in healing and repair of rotator cuff injuries: a systematic review and meta-analysis. Clin Rehabil, 2017, 31(2):158–172.

[12] Şenses F, Önder ME, Koçyiğit ID, Kul O, Aydin G, Inal E, Atil F, Tekin U. Effect of platelet-rich fibrin on peripheral nerve regeneration. J Craniofac Surg, 2016, 27(7):1759–1764.

[13] Kuka S, Ipci SD, Cakar G, Yılmaz S. Clinical evaluation of coronally advanced flap with or without platelet-rich fibrin for the treatment of multiple gingival recessions. Clin Oral Investig, 2018, 22(3):1551–1558.

[14] Du J, Mei S, Guo L, Su Y, Wang H, Liu Y, Zhao Z, Wang S, Liu Y. Platelet-rich fibrin/aspirin complex promotes alveolar bone regeneration in periodontal defect in rats. J Periodontal Res, 2018, 53(1):47–56.

[15] Castro AB, Meschi N, Temmerman A, Pinto N, Lambrechts P, Teughels W, Quirynen M. Regenerative potential of leucocyte- and platelet-rich fibrin. Part A: intra-bony defects, furcation defects and periodontal plastic surgery. A systematic review and meta-analysis. J Clin Periodontol, 2017, 44(1):67–82.

[16] Badade PS, Mahale SA, Panjwani AA, Vaidya PD, Warang AD. Antimicrobial effect of platelet-rich plasma and platelet-rich fibrin. Indian J Dent Res, 2016, 27(3):300–304.

[17] Miron RJ, Fujioka-Kobayashi M, Bishara M, Zhang Y, Hernandez M, Choukroun J. Platelet-rich fibrin and soft tissue wound healing: a systematic review. Tissue Eng Part B Rev, 2017, 23(1):83–99.

[18] Ding Y, Cui L, Zhao Q, Zhang W, Sun H, Zheng L. Platelet-rich fibrin accelerates skin wound healing in diabetic mice. Ann Plast Surg, 2017, 79(3):e15–e19.

[19] Inchingolo F, Cantore S, Dipalma G, Georgakopoulos I, Almasri M, Gheno E, Motta A, Marrelli M, Farronato D, Ballini A, Marzullo A. Platelet rich fibrin in the management of medication-related osteonecrosis of the jaw: a clinical and histopathological evaluation. J Biol Regul Homeost Agents, 2017, 31(3):811–816.

[20] Öncü E, Bayram B, Kantarci A, Gülsever S, Alaaddinoğlu EE. Positive effect of platelet rich fibrin on osseointegration. Med Oral Patol Oral Cir Bucal, 2016, 21(5):e601–e607.

[21] Miron RJ, Zucchelli G, Pikos MA, Salama M, Lee S, Guillemette V, Fujioka-Kobayashi M, Bishara M, Zhang Y, Wang HL, Chandad F, Nacopoulos C, Simonpieri A, Aalam AA, Felice P, Sammartino G, Ghanaati S, Hernandez MA, Choukroun J. Use of platelet-rich fibrin in regenerative dentistry: a systematic review. Clin Oral Investig, 2017, 21(6):1913–1927.

[22] Shah R, M G T, Thomas R, Mehta DS. An update on the protocols and biologic actions of platelet rich fibrin in dentistry. Eur J Prosthodont Restor Dent, 2017, 25(2):64–72.

[23] Yu P, Zhai Z, Jin X, Yang X, Qi Z. Clinical application of platelet-rich fibrin in plastic and reconstructive surgery: a systematic review. Aesthetic Plast Surg, 2018, 42(2):511–519.

[24] Kimura LF, Mattaraia VGM, Picolo G. Distinct environmental enrichment protocols reduce anxiety but differentially modulate pain sensitivity in rats. Behav Brain Res, 2019, 364:442–446.

[25] Giannini S, Cielo A, Bonanome L, Rastelli C, Derla C, Corpaci F, Falisi G. Comparison between PRP, PRGF and PRF: lights and shadows in three similar but different protocols. Eur Rev Med Pharmacol Sci, 2015, 19(6):927–930.

[26] Parasuraman S, Raveendran R, Kesavan R. Blood sample collection in small laboratory animals. J Pharmacol Pharmacother, 2010, 1(2):87–93.

[27] Grayson WL, Bunnell BA, Martin E, Frazier T, Hung BP, Gimble JM. Stromal cells and stem cells in clinical bone regeneration. Nat Rev Endocrinol, 2015, 11(3):140–150.

[28] Coman M, Hîncu M, Şurlin P, Mateescu G, Nechita A, Banu M. Comparative histomorphometric study of bone tissue synthesized after electric and ultrasound stimulation. Rom J Morphol Embryol, 2011, 52(1 Suppl):455–458.

[29] Coman M, Hîncu M. Study of bone cells by confocal microscopy in fractures stimulated by ultrasound. Rom J Morphol Embryol, 2013, 54(2):357–360.

[30] Barbon S, Stocco E, Macchi V, Contran M, Grandi F, Borean A, Parnigotto PP, Porzionato A, De Caro R. Platelet-rich fibrin scaffolds for cartilage and tendon regenerative medicine: from bench to bedside. Int J Mol Sci, 2019, 20(7):1701.

[31] Einhorn TA, Gerstenfeld LC. Fracture healing: mechanisms and interventions. Nat Rev Rheumatol, 2015, 11(1):45–54.

[32] Foulke BA, Kendal AR, Murray DW, Pandit H. Fracture healing in the elderly: a review. Maturitas, 2016, 92:49–55.

[33] Wang M, Yang N, Wang X. A review of computational models of bone fracture healing. Med Biol Eng Comput, 2017, 55(11): 1895–1914.

[34] Grecu AF, Grecu DC, Nica O, Ciuca EM, Camen A, Ciurea ME. A novel method of obtaining platelet rich fibrin from rats and quantifying platelet count. Curr Health Sci J, 2019, 45(1):104–110.

[35] Wang Y, Fang X, Wang C, Ding C, Lin H, Liu A, Wang L, Cao Y. Exogenous PTHrP repairs the damaged fracture healing of PTHrP+/- mice and accelerates fracture healing of wild mice. Int J Mol Sci, 2017, 18(2):337.

[36] Shi Z, Zhou H, Pan B, Lu L, Liu J, Kang Y, Yao X, Feng S. Effectiveness of teriparatide on fracture healing: a systematic review and meta-analysis. PLoS One, 2016, 11(12):e0168691.
[37] Dülgeroglu TC, Metineren H. Evaluation of the effect of platelet-rich fibrin on long bone healing: an experimental rat model. Orthopedics, 2017, 40(3):e479–e484.
[38] Akyildiz S, Soluk-Tekkesin M, Keskin-Yalcin B, Unsal G, Ozel Yildiz S, Ozcan I, Cakarer S. Acceleration of fracture healing in experimental model: platelet-rich fibrin or hyaluronic acid. J Craniofac Surg, 2018, 29(7):1794–1798.
[39] Marcazzan S, Weinstein RL, Del Fabbro M. Efficacy of platelets in bone healing: a systematic review on animal studies. Platelets, 2018, 29(4):326–337.
[40] Muschler GF, Raut VP, Patterson TE, Wenke JC, Hollinger JO. The design and use of animal models for translational research in bone tissue engineering and regenerative medicine. Tissue Eng Part B Rev, 2010, 16(1):123–145.
[41] Molina-Ruiz AM, Requena L. Foreign body granulomas. Dermatol Clin, 2015, 33(3):497–523.
[42] Reddy VK, Lavoie MC, Verbeek JH, Pahwa M. Devices for preventing percutaneous exposure injuries caused by needles in healthcare personnel. Cochrane Database Syst Rev, 2017, 11:CD009740.
[43] Balaure PC, Holban AM, Grumezescu AM, Mogoşanu GD, Bălşeanu TA, Stan MS, Dinischiotu A, Volceanov A, Mogoantă L. *In vitro* and *in vivo* studies of novel fabricated bioactive dressings based on collagen and zinc oxide 3D scaffolds. Int J Pharm, 2019, 557:199–207.
[44] Vajgel A, Mardas N, Farias BC, Petrie A, Cimões R, Donos N. A systematic review on the critical size defect model. Clin Oral Implants Res, 2014, 25(8):879–893.
[45] Manolea HO, Crăiţoiu MM, Mogoantă L, Dascălu IT, Moraru AI, Agop Forna D, Mercuţ R. An evaluation of a collagen-based material osseointegration. Rom J Morphol Embryol, 2017, 58(1):161–165.
[46] Lee DJ, Kwon J, Current L, Yoon K, Zalal R, Hu X, Xue P, Ko CC. Osteogenic potential of mesenchymal stem cells from rat mandible to regenerate critical sized calvarial defect. J Tissue Eng, 2019, 10:2041731419830427.
[47] Honma T, Itagaki T, Nakamura M, Kamakura S, Takahashi I, Echigo S, Sasano Y. Bone formation in rat calvaria ceases within a limited period regardless of completion of defect repair. Oral Dis, 2008, 14(5):457–464.

Corresponding authors

Oliviu Nica, PhD Student, Department of Plastic Surgery and Reconstructive Microsurgery, University of Medicine and Pharmacy of Craiova, 2 Petru Rareş Street, 200349 Craiova, Romania; Phone +40746–020 496, e-mail: oliviu.nica@gmail.com

Feng Ifrim Chen, Associate Professor, MD, PhD, Department of Traditional Medicine, "Carol Davila" University of Medicine and Pharmacy, 8 Eroilor Sanitari Avenue, 050474 Bucharest, Romania; Phone +40722–303 681, e-mail: adsm.adsm@yahoo.com

Received: December 15, 2018

Accepted: June 1, 2019