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

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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, 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 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. Concomitant dehydrating 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 dosage of normal anesthetic mixture. An incision was performed along the line of the previous surgical scar. The peristeme 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. The 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 peristeme 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 peristeme 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 administrating 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. The piece was lightly cleaned of any loose soft tissue that might still be attached. The peristeme 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”.

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 5Si 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 osteoforming activity of the periosteum. Periosteal fibroblasts have rapidly multiplied and some of them have transformed 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 osteocytes, oval-like cells with more intense colored (hyperchromic) 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.
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 processes 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 periosteum 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).
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-differenting 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 on making correct comparisons.

This study used 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 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 described 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**


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