Original Paper

A Novel Method of Obtaining Platelet Rich Fibrin from Rats and Quantifying Platelet Count

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ABSTRACT: Background and objectives: Rat experimentation is the first line of research by which a medical hypothesis is usually tested. Platelet Rich Fibrin (PRF) is a relatively new bio-material that has shown promise to enhance healing in the field of bone research and tissue engineering. In order to perform PRF based experiments on rats, a proper protocol of obtaining PRF from rats needs to be established. Materials and Methods: 35 Wistar rats were used to obtain PRF by using cardiac puncture blood draw and quick subsequent centrifugation. The PRF samples were analyzed and compared to standard literature PRF composition. Results: PRF samples analysis showed persistent results pertaining to known PRF composition. Conclusions: Our experiment has shown that our protocol of obtaining PRF is capable of providing high quality PRF from rats.

KEYWORDS: Platelet-rich fibrin, tissue engineering, proof of concept study, experimental animal models, platelet count

Introduction

Platelet Rich Fibrin (PRF) is a new biomaterial that is obtained by a process of centrifugation from the patient's own blood [1] that sets itself apart from the rest of platelet concentrates by the process of fibrin polymerization that encapsulates thrombocytes within it. The growth factors on the surface of the thrombocytes are slowly released and thus promote the healing processes that occur at the site of PRF application.

Research has been ongoing since its discovery with research areas including musculoskeletal injuries [2,3], oro-maxillo-facial surgery [4] or plastic surgery [5]. The common denominator in these field of research is that they make use of PRF's capacity to be manipulated in its membrane form. This propriety gives the attending physician the possibility to use PRF in a multitude of clinical scenarios. The full range of therapeutic uses of PRF have yet to be researched. In order to comply with scientific rigor any experimental use of PRF must first be performed in a controlled laboratory scenario, usually using rats as experimental subjects.

The rat is a good candidate for experimental bone research because it can fulfil the above-mentioned requirements besides having numerous other advantages such as: the relative ease with which it can be procured, manipulated, stored, fed and monitored. These advantages have propelled the rat at the forefront of bone healing research, with rat experimentation consisting of 38% of the total number of experiments[6] (as opposed to rabbits 19%, mice 13%, sheep 11%, dogs 9%, goats 4% and other 5%). This leads to a vast literature regarding numerous aspects of medical research with covered aspects including liver research [7], osseointegration of different materials [8,9] or stroke research [10,11,12,13].

The aim of this paper is to establish a protocol for obtaining PRF from rats that can be used by researchers in any field of laboratory or clinical scenarios.

Materials and Methods

For the purposes of this study we used 35 male Wistar rats. Approval of the Committee for Animal Welfare and Bioethics from the University of Pharmacy and Medicine from Craiova, Romania was obtained and the experiment was conducted at the Laboratory for Animal Research belonging to the University.

The weight of the rats was 320g in average (range: 220-420g). In order to comply with current good practices guidelines for the purpose at hand the rats were selected to be at least 6 months of age [14].

Due to the natural fast coagulation of rat blood all the materials had to be prepared for use and the centrifuge set up beforehand. The centrifuge (Process for PRF Duo Centrifuge®) was set according to the protocol for 1300rpm (400g) x 8min spin time [15].

The centrifuge was also primed with 3 sterile saline Vacutainers acting as counterweights for the PRF Vacutainer.

Given the 12-15ml total circulating blood in a rat [16] and the 10ml required for PRF preparation, the blood harvesting technique of choice was cardiac puncture [17]. This technique took advantage of the antigenicity compatibility [18] in between Wistar rats in order to produce sufficient PRF membrane from a single donor rat to suffice for 2-5 experiments with PRF, depending on the use.

Anesthesia was performed by subcutaneous injection of a mixture of Ketamine and Xylazine (dosage of 50mg/kg and 5mg/kg respectively). Ethical standards were complied, regarding the comfort of the test animals, by performing reflex tests such as the toe pinch test and corneal reflex [19] to ensure that full anesthesia is reached. Artificial tears were applied after the anesthesia set in order to prevent corneal dryness. This was reapplied when necessary.

We used a PRF Choukroun VacuTainer®, Vacutainer Eclipse Blood Collection Needles® and BD Vacutainer Single Use Needle holder® in order to obtain the necessary blood sample.

The anesthetized donor rat was placed in a dorsal recumbency position and we highlighted the apexian shock by using a marker. Starting from this point we drew a segment perpendicular to the median sternum line. We continued by finding the middle point of this segment and drew another perpendicular line starting from this middle point, in the caudal direction. We marked a point approximately 1cm distal on this line and we marked it as the place of needle insertion (Fig.1).

Fig.1. Drawn lines according to the instructions

The needle was placed at 25-35° from the horizontal plane and the tip of the needle was cranially orientated (Fig.2).

Fig.2. Needle positioning

If the insertion point was exactly over a rib, we adjusted the positioning in order to be in the intercostal space beneath the rib. We continued by carefully inserting the needle. After the needle was inside the heart we attached the vacutainer to the needle and waited for it to fill. This took a maximum of 3-4 seconds. The vacutainer was then transferred into the centrifuge and the PRF production protocol was initiated as quick as possible. In our experiments we have found that a time longer than 7-9 seconds from initial vacutainer connection to its placement in the centrifuge resulted in coagulated blood, that rendered the sample unsuitable for our purposes.

This was the reason for having the centrifuge prepared beforehand at the designated protocol of 1300rpm (400g)/8 minutes spin time.

After the centrifugal process had finished, we removed the vacutainer and placed it in a tube stand where it sat for another 5 minutes, at room temperature, away from any direct light. After this, the vacutainer presented with 3 macroscopic layers:

- the plasma layer (Platelet Pure Plasma-called PPP1 for future calculations)
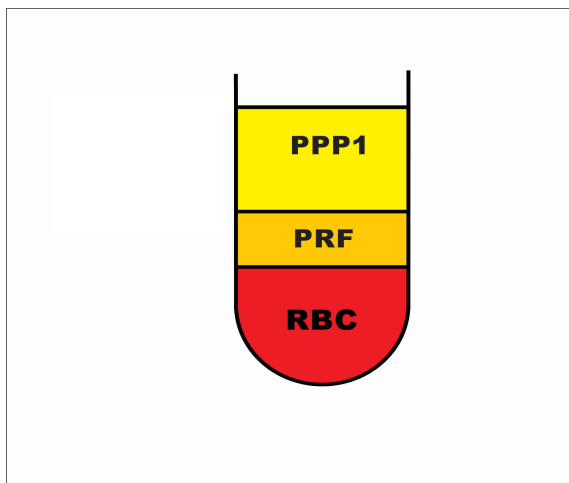- the PRF clot
- the red blood clot (RBC). (Fig.3)

Fig.3. PRF centrifugation protocol, blood separation

We removed the cap of the vacutainer and, by using an anatomic surgical clasp we grabbed the PRF clot from the middle 1/3 of the liquid and carefully removed it from the tube. This mobilized the PRF clot together with the RBC clot. The two clots had to be separated at the junction line. If the procedure is difficult to perform with absolute precision (caused by an angled or somewhat irregular junction line), it is always advisable to perform the cut with a little

tolerance by including a maximum of 1mm vertical height of RBC clot. This is performed because of the known spatial distribution of the platelets inside the clots (the spatial distribution of the platelets permeates the PRF-RBC clots junction by infiltrating in the upper most layer of the RBC) [20]. By performing the cut in this manner we were able to maximize the number of available platelets in the PRF clot.

After obtaining the PRF clot we placed it in a PRF box [21] in order to transform the clot into a membrane by pressing the exudate out. If kept wet, inside the sterile PRF box, with the adjacent exudate eliminated by the PRF clot, the resulted PRF membrane could be used for a period of up to 2.5-3 hours (Fig.4).

Fig.4. PRF Box

In order to measure the platelet concentration of the resulted PRF we used indirect deduction based on a separate production of Platelet Rich Plasma (PRP). We produced the PRP by centrifuging (12min at 450g) 10ml of blood collected in an identically shaped VacuTainer, primed with anticoagulant. After the centrifugal process the blood had separated into three macroscopic layers:

- plasma layer (Platelet Pure Plasma-noted PPP2 for future calculations);
- the thin buffy coat (platelet rich layer-PRP);
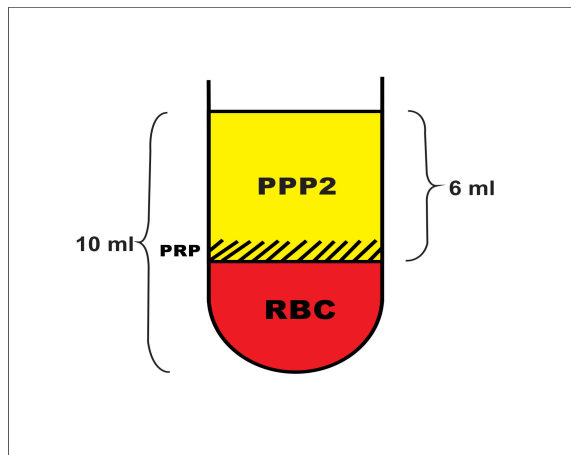- the RBC layer (Fig.5).

Fig.5. PRP centrifugation protocol, blood separation

We collected the PRP+PPP2 and measured the platelet concentration of the resulting mixture and analyzed the platelet count of this mixture.

Results

We based our results on the following laboratory results measuring platelet concentrations:

Mean platelet count of whole rat blood: $850,000/mm^3$, with a range of $702,000/mm^3$ to $998,000/mm^3$;

PPP1: trace platelets, for the purposes of calculation, $0/mm^3$, totaling a mean of 4.2ml;

PRP+PPP2: mean platelet count $1.246.666/mm3$, totaling 6ml;

In order to find out the quantity of platelets that were lost in the red clot, if any, we perform the following calculations:

Absolute platelet number in whole blood 10 ml:

850,000 platelets in 1 mm3 | x1000

850,000,000 platelets in 1000 mm3 (=1ml) | x10

8,500,000,000 platelets in 10 ml of whole blood

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Absolute platelet count in 6 ml PPP2 + PRP:

1,246,666 platelets in 1 mm3 | x1000

1,246,666,000 platelets in 1000 mm3(=1ml) | x6

7,479,996,000 platelets in 6 ml of PPP2 + PRP

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Lost platelets:

8,500,000,000-7,479,996,000 = 1,020,004,000 platelets lost

$$1,020,004,000 \approx \frac{12}{100} \times 8,500,000,000$$

➔   12% of platelets were lost in the red clot during the centrifugation process;

The red blood fractions (RBC) generated after PRF and PRP centrifugations were equal (Fig.6). We could thus extrapolate that:

PRF + PPP1 = PRP + PPP2 = 6 ml.

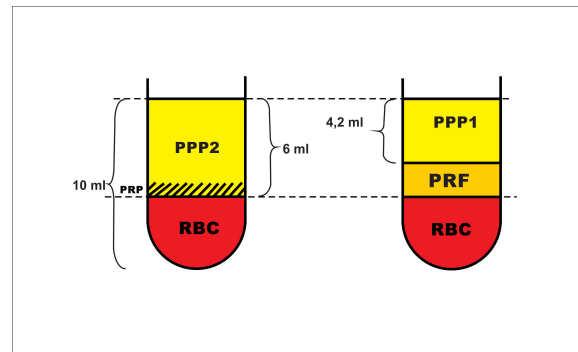PPP1 = 4.2 ml ➔ PRF = 6-4.8 = 1.8 ml

Fig.6. Schematic representation of PRP and PRF centrifugation of a 10 ml blood sample

PPP1 Platelet concentration was ~ 0 ➔ all of platelets that could be found in the PRP + PPP2 could now be found concentrated in the PRF. (**Fig.7**)

6 ml PRP + PPP → 1,246,666 /mm3

1.8 ml PRF → x/mm3

Given the inverse rule of 3 we have

$$x = \frac{6*1,246,666}{1.8} = 4,155,553/mm3$$

Platelet concentration increase in PRF compared to whole blood:

$$4,155,553 \approx \frac{489}{100} \times 850,000$$
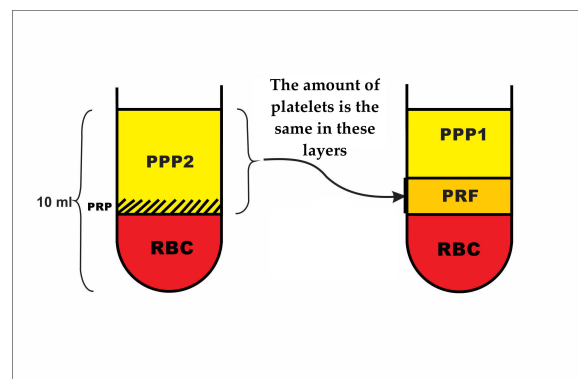
➔ 4.89x increase in platelet concentration

Fig.7. Platelet distribution in the PRP and PRF

Thus, we could conclude that the mathematical calculations outlined resulted in a Platelet concentration of 4,155,553/mm3. When compared to the platelet concentration of whole blood we observed an increase in platelet concentration of 489% in the PRF clot. This proved that the protocol described above

successfully produced PRF, with a relative platelet concentration of 4.89x compared to the baseline platelet concentration. The calculations delineated in Appendix A show that 12% of the available platelets in whole blood were lost in the RBC.

The advantages and disadvantages of our protocol can be seen in Table 1.

Table 1. Advantages and disadvantages

Advantages	Disadvantages
Standardized method	Terminal procedure for the donor rat
Easily reproducible using step by step instructions	Technique is sensible to user variation in method
Proven platelet concentration increase of ~ 4.9 x	
Possibility to produce a large PRF membrane with a single centrifuge spin	
Identical PRF graft for 4-5 experimental procedures	

Discussion

PRF preparation protocols are based on the end point use. This can consist of the use of PRF as a naturally degradable membrane that acts as a barrier for guiding tissue regeneration, a source of growth factors delivered in a concentrated form or as scaffolding for tissue engineering. This results in the need for PRF to deliver both mechanical and biological proprieties. We performed this study in order to determine the best production method quality PRF.

The novel character of our protocol consists of the high quantity and quality of PRF that we were able to produce with a single donor rat. Upon review of the literature we have found other examples of researchers that have obtained PRF from rats for various research purposes, such as bone healing [22,23,24,25], nerve injury [26,27], intestinal anastomoses [28,29,30,31], Achilles tendon injuries [32] or perforated tympanic membrane [33]. The usual way of procuring blood from rats was by sub-orbital dripping or venous blood collection from the lateral tail vein [34]. These blood collecting techniques are able to produce a limited amount of blood and consequently a small PRF graft. The same can be said regarding another minimally invasive blood collecting technique from the rat, percutaneous drawing from the cranial vena cava [35]. This can generate additional issues such as difficulties to identify the PRF clot in the Vacutainer, difficulties in grabbing and subsequent manipulation of it. This in turn generated a very small PRF membrane that was very difficult to manipulate, rendering the process unfeasible, especially for large defects.

Our protocol also potentially eliminates the variability intrinsic to each donor animal by generating up to identical 4-5 PRF membranes that in turn can be used for 4-5 experiments. This can be achieved by cutting the PRF membranes into pieces that can fit the size for the experiment at hand. Thus, the PRF grafts used in these experiments are identical to each other, as they are cut from the same graft. By eliminating one of the variables of any experiment we can greatly improve the reliability of the outcome [36].

The use of a PRF box is a relatively new procedure that replaces the old spoon compression technique of obtaining PRF membranes manually and further enhancing the use of the obtained PRF membranes.

Our study relies on indirect mathematical induction and represents a personal variation of the standard subtraction technique of counting platelets and determining platelet concentration from PRF [37].

Further studies on different centrifugation protocols are also required to gather research regarding PRF variants, such as Intelligent PRF (I PRF), Advanced PRF (A-PRF), Leukocyte Rich PRF (L-PRF) [38].

Approximatively 12% of platelets were lost in the red blood fraction of the centrifugated vacutainer and thus unavailable for the platelet concentration process that occurred in the plasma layer, thus further studies need to be made in order to refine the PRF obtaining protocol, in a way that maximizes the available platelets.

Conclusions

Our study has successfully proven this method of obtaining PRF as a viable option with considerable advantages, especially for, but not limited to, research that involves the procurement of large batches of up to 4-5 identical PRF membranes.

Acknowledgment

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