RESEARCH ARTICLE

Preclinical Evaluation of Efficacy of Processed PRP and Fresh PRP in Diabetic Wound Healing

Vyas Pooja U.[1*], Khobragade Deepak S.[1], Mundhada Dharmendra R.[2], Shrivastava Sandeep P.[1], Vyas Ujwal B.[1], Pethe Anil M.[1]

[1]*Department of Pharmaceutics, Datta Meghe College of Pharmacy, DMIHER Sawangi (M) Wardha, Maharashtra, India*
[2]*Department of Pharmaceutics, Agnihotari College of Pharmacy, Wardha, Maharashtra, India*

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ABSTRACT

Background: One in four people with diabetes will experience diabetic wounds at some point in their lifespan, which is among the diabetes complications that have the worst effects on quality of life. The study's objective was a comparative preclinical study of the efficacy of fresh platelet-rich plasma (PRP) Vs L-PRP in diabetic wound.

Methods: Twenty four rabbits were used to study the efficacy. Diabetes was generated in the rabbits, and the diabetic wound's perilesional region received PRP treatment. The comparative evaluation by done by counting the wound area and rate of healing.

Results: There was more than three folds rise in growth factors in lyophillised-PRP than compared to fresh PRP. The rate of wound healing was much fast in lyophilised PRP group. In the control group the wound was unhealed by 30th day and also showed pus cell formation and symptoms of infection. However, it was completely healed on 25th day when treated with L-PRP stored at 8°C.

Conclusion: The outcomes prompted clinical research to compare L-PRP to fresh PRP's efficacy.

Keywords: Diabetic wound, Growth Factors, L-PRP, F-PRP, Lyophilization, Wound healing

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INTRODUCTION

By 2045, 630 million individuals worldwide are expected to have been diagnosed with diabetes, according to the International Diabetes Federation (IDF).[1] One in four people with diabetes will experience diabetic wounds at some point in their lifespan, which is among the diabetes complications that have the worst effects on quality of life.[2-4] In the EU, treating chronic wounds costs 2% of the yearly health budget. Between 200 and 2000 pounds are spent on outpatient care for 4 months, and 40 million are spent on ulcer therapy each year.[5,6]

An effective barrier is intended to be restored through the complicated process of healing, which also involves hemostasis, inflammation, and remodeling. Different cell types and levels of cytokines and/or growth factors control this entire process. Every stage of the healing process is either directly or indirectly affected by the inflammatory phase as a result of the release of growth factors from platelets and macrophages following injury.[7-9] Platelets contain more than 30 cytokines, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), transforming growth factor-beta (TGF-beta), vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF) which are essential for wound repair.[10,11] Since platelet concentrates can speed up wound healing by inducing molecular and cellular reactions, their usage is growing in favor.[12,13] Platelet-rich plasma (PRP) is described as having a high concentration of platelets (four to nine times the basal amount) in a small volume of plasma.[14] PRP preparation methods have been detailed in a variety of ways up until this point, including commercial systems and in-house methods. The results of PRP research are varied since there is a dispute over the pace and duration of centrifugation and whether activators and leukocytes should be added to PRP preparations. Muraglia et al. attempted to standardize heterologous PRP products, focusing on enhancing cell proliferation, supplementing culture media and maintaining genetic integrity.[14,15]

PRP has not shown to be effective in treating diabetic wounds in some studies.[16,17] These investigations only

**Author for Correspondence: email*

measured platelet counts using various protocols based on internal techniques. Though, the concentration of growth factors also plays a significant role in PRP, making a protocol that can encourage both a high concentration of growth factors and platelets useful. In cell culture experiments, our team recently released a study[18] demonstrating the preservation of concentration and functional PRP growth factor parameters after freeze-drying.[19-21]

Because L-PRP is ready to use after reconstitution, its use in clinical practice can be intriguing. To treat chronic wounds in rats, Pietramaggiori and colleagues compared utilizing human fresh frozen or L-PRP and controls. In contrast to controls, PRP preparations enhanced granulation tissue, sparking novel concepts in the field of regenerative medicine.[22] As shown by the preservation of function by *in-vitro* testing, the possible utilization of growth factors formed from platelets and the ability to extend the shelf-life of platelet concentrates by freeze-drying become a particularly ideal strategies for boosting wound healing treatment.[14,15,18]

The objective of this research is to advance our understanding of healing diabetic wounds by presenting PRP as biological means of accelerating the healing process. In preliminary research, we compared fresh and L-PRP at two temperature points.

We anticipate that by offering a treatment that is based on proof and can be used in clinical practice, the therapeutic approach will be enhanced, nonhealing diabetic wounds will heal more quickly, patient quality of life will increase, and the cost of healthcare for the general public will decrease.

MATERIALS AND METHODS

Experimental Animal and Sample Size

The sample size for this study was 24 New Zealand rabbits divided into six groups (Table 1) as per the purposive sampling technique for the complete study[23] (12 males and 12 females) provided by the Central Preclinical (Animal) Research facility of Datta Meghe College of Pharmacy. DMIHER. The approval of the animal ethics committee was received wide letter no- DMIMS (DU)/IAEC/2020-21/07. The rabbit's weight was in between 1kg to 2.5kg.[24] The animals have caged two rabbits with the same sex in one cage with a partition to avoid contact between them. The animals were housed under typical conditions with unrestricted access to food and water, a temperature of 22°C, and a 12 hour cycle of light and darkness. The Datta Meghe Institute of Higher Education and Research's Institutional Ethics Committee authorized the procedures, and they were carried out in accordance with the ethical guidelines for using animals in research.

Table 1: Groups of rabbits for wound healing study

Study group	No of rabbit	Dosing
Control group	4	Wound will be allowed to heal naturally.
Test group 1	4	Wound will be treated with saline solution
Test group 2	4	Wounds will be treated with standard wound healing cream
Test group 3	4	Wounds will be treated with fresh prp
Test group 4	4	Wounds will be treated with lyophilized prp stored at room temperature
Test group 5	4	Wounds will be treated with lyophilized prp stored at 8ºc
Total	24	

Blood Withdrawal Method

Rabbits were confined in a wooden enclosure. Using a 22G to 25 G needle or a 22G butterfly connected to a syringe, up to 8 mL of blood was drawn from the auricular marginal veins of each ear.[25]

The epidermis was cleaned with alcohol and the hair on the ear were removed. A cream containing lidocaine was used to anesthetize the area gently. The entire thickness of the epidermis became numb after 45 minutes.

Blood was carefully drawn, and gathered in test tubes with CPDA anticoagulant solution. To avoid the development of hematomas and blood clots, cotton gauze was tightly applied to the site of venipuncture for at least one minute or until bleeding stops. The rabbit was watched for the next few hours to ensure homeostasis is perfect.

Preparation of Fresh PRP (F-PRP)

The F-PRP was prepared by the standard double centrifuge method. 8 mL of heparinized blood was centrifuged at 1200 rpm (soft spin) for 10 minutes. and the second centrifugation after removing the RBC layer was done for a further ten minutes at 2000 rpm (hard spin) and 21°C. The 1.3 mL of PRP was obtained after removing the Poor PRP layer.[26,27] Leukocyte, erythrocyte, and thrombocyte total counts were calculated following each sedimentation using a hematology analyzer (ABX Micros ESV60, Horiba).

Preparation of Lyophilized -PRP (L-PRP)

The processed PRP from the diabetes blood of rabbits was prepared using the lyophilization technique (MAC lyophiliser, Freeze Dryer. Cat no: MSW-137, Sr. No. 2511 Macro Scientific Works Pvt. Lmt). The temperature and vacuum cycle used was -35ºC at a vacuum of -897 mmhg gauge for 24 hours. Such 7 cycles were repeated for 7 days. After 7 days the dry, crystalline light-yellow powder was collected and stored in a dry air tight container. L-PRP was reconstituted for the tests using sterile water for injection.[18] The same was repeated by collecting the blood every 3rd day till ample amount of powered PRP was obtained for all the further analysis to be done by storing it at room temperature and 8ºC.

Reconstitution of Lyophilized PRP

Around 50 mg of lyophilized power of PRP was reconstituted in 1.5 mL (one time dose) of sterile water for injection by agitating it for 1-minute at 50 rpm. The solution was tested randomLy for platelet counts and concentration of growth factors.

Inducing Diabetes in Experimental Animals

Diabetes was induced by administrating diabetes inducing drug streptozotocin (STZ) based on body weight.[28] Before administering streptozotocin injections, animals were starved overnight. Diabetes mellitus was induced by single intraperitoneal injection of freshly prepared solution of STZ (60 mg/kg b.w.) in 0.1 M citrate buffer of pH 4.5, to the rabbits after overnight fasting for 12 hours induction of diabetes was confirmed by determining the blood glucose level. As per the literature, the range glucose level in a non-diabetic rabbit is below 300 mg/dL and the diabetic rabbit is above 300 mg/dL.[29]

Creating Wound in Experimental Animal

Coin method (Rs 10 coin) was used to create the desired wound.

Animal Preparation

The rabbits are weighed and typically fasted for several hours before surgery to reduce the risk of anesthesia-related complications. The rabbits are then anesthetized using a combination of ketamine and xylazine and placed on a surgical table in a supine position.

Wound Induction

The skin of the rabbit is cleaned with the help of povidone iodine solution. An Indian coin of Rs 10 is sterilized and placed on the rabbit's dorsal skin to mark the wound's size with the help of a permanent marker. A circular wound of a marked size is induced on the rabbit using a surgical scissors. The wound can be made by incision method. The wound's size is measured using a measuring tape/transparent sheet and graph paper. The area of wound is calculated using the software mentioned above. 2 wounds were created per rabbit.[30]

Postoperative Care

The rabbits are kept in a clean environment and the wound is monitored regularly to prevent infection.

Application of PRP on the Wound

The rabbit was kept in rabbit restrainer to avoid movement during PRP application. The fresh and Lyophilised PRP was prepared. Before reconstitution, the powder is removed and kept to attend room temperature. Our earlier study has shown that this helps in platelet activation due to the freeze-thawing process and helps in release of growth factors for it. Both the PRPs were taken in a 1-m syringe and applied on along the borders of the wound, injecting the needle superficially without penetration in the blood vessel. The process was repeated every 3rd day from the day of wound creation as per the literature.

Quantification of Growth Factors in PRP

Using the Enzyme Link Immunosorbent Assay kit, GF were measured in L-PRP and fresh PRP (Genosys Informatics Wuyan, China). EGF, PDGF-AA, TGF-β1, IGF-1, and VEGF were the growth factors that were examined because they are essential for the diabetic condition's wound healing process. The protocol was carried out in accordance with the manufacturer's instructions.

Measurement of Wound Healing

The PRP (fresh PRP and L-PRP), wound healing cream and saline water were applied to the defined groups every 4th day as per the STAR technique protocol.[30-32] In order to measure wound contraction, a transparent plastic sheet was applied to the lesion, and the wound margins were marked at predetermined intervals of time: days 0,3,7, 11, 15, 19, 23, 27 and 30th day as per literature. After digitization, Image J software was used to quantify the size of the wound. On these particular days, wound measurements and photos were taken without scab removal. To calculate wound sizes as percentage of initial wound area, value in cm^2 on day 0 was taken as 100% and other days were measured in relation to day 0, as shown in example below:

Day 0 (cm^2)- 100%
3rd day (cm^2) – X

Statistical Analysis

The statistical tests were run with a significance threshold of 5% (p 0.05). GraphPad version 5 was used for the analyses.

RESULTS AND DISCUSSION

PRP Analysis

According to platelet count and growth factor content, fresh and lyophilized-PRP were differentiated. Fresh PRP contained 5614 x 10^3 cells/l of platelets, and Table 2 displays the growth factor amounts and the platelet counts at various storage temperatures.

When compared to fresh PRP, the lyophilized-PRP had greater concentrations of all growth factors ranging from 1.59 to 3.7-fold. After lyophilization, there was a 95.2% loss in platelet number. This may be due to the rupture of the platelet

Table 2: Conc. of GFs in various types of PRPS

	Fresh PRP		*L-PRP at room temp*		*L-PRP at 8°c*		*Fold GF lyoph/fresh*
	Growth factor (pg/uL)	*Platelet counts*	*Growth factor (pg/uL) a*	*Platelet counts*	*Growth factor (pg/uL) b*	*Platelet counts*	
PDGF-AA	50988.2	2,81,217.2	188656.3	13498.5	188650.9	13450.7	3.7
VEGF	1499.7		2384.5		2386.9		1.59
EGF	1278.9		3325.14		3329.1		2.60
TGF-β1	1609.1		4135.38		4137.5		2.57
IGF-1	37689.4		109676.1		109678.2		2.91

The mean of reading of column mentioned as a, and b is taken and divided by the amount of concentration of GF recorded in fresh PRP to estimate the fold of concentration rose after lyophilization.

structure in the intense process of lyophilization which resulted in a bust of release of growth factors inside it.

We know that between 40 and 90 gp% of the platelets are damaged during the lyophilization procedure, but not all of them. Additionally, PRP's action is primarily drawn from growth factors rather than platelets. In this manner, a rise in the concentration of growth factors was seen, which was expected given that lyophilization had damaged the platelets, causing them to lyse and release growth factors, which raised the level of these proteins.

Wound healing evaluation

The above Figure 1 shows the % change in the size of wounds in various groups from day 0 to day 30. The diabetic wound was unhealed till day 30th for control group, group 1 and 2. Infact there were traces of puss formation resulting into infection for group 1. However, there was earlier healing of wounds in Group 3, 4 and group 5 on 26th and 24th day, respectively. Hence the wound was unhealing even on day 30th for control group, group 1 and 2. In contrast, the wound was completely healed in groups using PRP on day 26th for F-PRP and day 27th for L-PRPs. Also, it was observed that when the rate of wound healing is compared among the L-PRPs, the rate of wound healing in L-PRP at room temperature is less as compared to that stored at 8ºc.

In this research, the administration of PRP or L-PRP did substantially change the kinetics of wound healing as determined by contraction when compared to controls on all estimated days.[5,9,12] With the exception of D7, L-PRP displayed the tiniest area despite not being noticeably different. We hypothesize, this result is high concentration of VEGF supplied to the wound region by L-PRP. This study's measurement of growth factors in L-PRP was three times faster than that of regular PRP, which was a significant discovery.

PRP is frequently used to treat wounds, but it is still debatable because preclinical studies have produced conflicting findings, and there hasn't been any agreement yet. Mehrjerdi et al. examined topical benefits of autologous PRP without leukocytes versus saline in the healing of canine wounds (2008).[16] In a daily application of polyurethane film over chronic rabbit lesions, Ostvar *et al*. compared practice of PRP without leukocytes to saline. Through microscopic and histological analysis, it was revealed that the group treated with PRP had a faster healing time, which was correlated with an increase in mean vascular density, showing the treatment's beneficial effects on wound healing.[33]

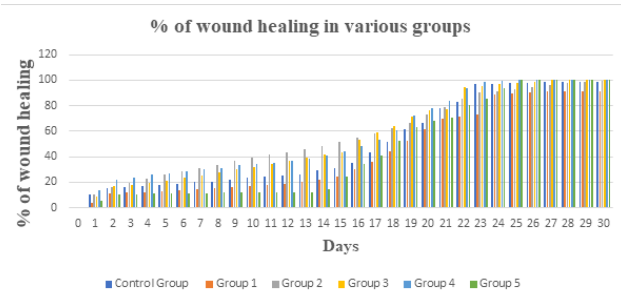

Figure 1: % of wound healing in various groups

Due to the brief half-life of platelet concentrate, use of frozen platelets or lyophilized has been researched. Focusing on cytokines and growth factors found in PRP granules, a variety of therapeutic applications for PRP are presently being researched. Given the capability to keep constant preparation and the fact that multiple PRP samples can be obtained from a single blood sample, L-PRP may be a useful strategy. In reality, we created an autologous powdered product with a significant growth factor concentration. Despite the limited amount of simple and preclinical research in this area, it is crucial for clinical practice.

The fields of freeze-dried and wound recovery have benefited from the work of Pietramaggiori and associates. An increase in region of granulation tissue in former showed that lyophilized PRP with and without stabilizing agents was more successful than control at treating wounds in diabetic mice. All of the formulations had the same amount of GF (TGF-1, PDGF-AB, VEGF and EGF), but in vitro testing revealed that lyophilized PRP increased cellular proliferation.[14,15,18] In contrast to untreated group, several doses of recombinant VEGF and use of L-PRP hasten wound closure in distinct experiment comparing single or multiple injection applications in wound region of diabetic mice with fresh PRP, trehalose-containing L-PRP, or no therapy.[34] The wound model used in this research was one of its limitations, and we were unable to rule out the possibility of a more beneficial effect on the management of chronic wounds.

CONCLUSION

In conclusion, lyophilization is suggested as a viable choice in clinical practice based on the outcomes of treating a diabetic wound in rabbit with PRP or L-PRP. Particularly for patients who require numerous applications, this is very intriguing. Since PRP has been used successfully to treat wounds, it is highly desirable to extend shelf life of PRP products through lyophilization. The capacity of the therapy to encourage more vascularization, which is essential for wound healing, may be related to an increase in VEGF concentration in L-PRP. Frozen-dried PRP produced positive results in animals by significantly increasing the number of blood vessels and myofibroblasts.

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