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RESEARCH ARTICLE

PLATELET CONCENTRATIONS AND GROWTH FACTOR YIELD FOLLOWING TWO DIFFERENT METHODS OF PLATELET RICH PLASMA PREPARATION IN NIGERIAN LOCAL DOGS

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Abstract

This study was carried out to evaluate the sequestration efficiency and growth factor yield of two different methods of platelet rich plasma (PRP) preparations in 20 matured Nigerian local dogs. The first method employed a common method of PRP preparation in most local laboratories in Nigeria while the second method involved the use of Plateltex^{R} kit/Act which is a standard PRP preparation kit. Ten milliliter blood was collected from the cephalic vein of each dog for each method and was subjected to 2-step centrifugations at varying revolutions per second. Activation of the PRP prepared using the first method was done with calcium chloride while the Plateltex kit-prepared PRP (PPRP) was activated with batroxobin/calcium gluconate. The platelet counts of the whole blood and the two differently prepared PRP for the dogs were recorded and the results compared. Platelet derived growth factor (PDGF) and transforming growth factor (TGF) concentrations of each of the prepared PRP were quantified using enzyme-linked immunosorbent assay (ELISA) methods. The result showed that the platelets and growth factor concentrations of PPRP were higher than those of calcium chloride activated PRP (CPRP) which were in turn higher than those of the whole blood. This variation could partly be attributed to the varying intensity and duration of centrifugation employed during the preparation of the PRPs. The significantly ($p < 0.05$) higher concentration of PDGF and TGF in PPRP when compared to CPRP could probably be as a result of premature degranulation of platelets prior to calcium chloride activation in that method. The study clearly elucidated the superiority of plateltex kit-preparation method over the commonly used calcium chloride preparation methods in most of Nigerian laboratories.

INTRODUCTION

Platelet rich plasma (PRP) is being used more and more in treatment of several clinical conditions and as an adjunctive material in tissue engineering with variable therapeutic outcomes probably due to complete lack of standardization in methods and devices used in PRP preparations (Mazzucco *et al.,* 2008). Equally, there are few randomized controlled trials and most of the reports were case reports involving few animal models and lacked controls. The PRP is made by embedding concentrated platelets within a semisolid network of polymerized fibrin. This can be achieved through blood sequestration and activation.

Although, the preparation of autologous PRP in man is well known, its preparation in some animal models is difficult due to the small volume of blood available. Several methods and devices are also available for the production of PRP but so intriguing is the concern about the equivalence and the differences among the methods or the devices available with regards to their efficiency in platelet concentrations, PRP collection and subsequently their growth factor contents (Kevy and Jacobson, 2004, Borzini and Mazzucco, 2005, Leitner *et al.*, 2006, Everts *et al.*, 2006). Prior to clinical use of any preparation method or device, its technical performance must be tested and if possible, the results compared with those of other methods or devices with the same intended use (Mazzucco *et al.*, 2008).

In many developing countries, the use of many uncertified devices and techniques for PRP preparation is common with a lot of unpredictable clinical outcomes. The use of different activation agents for PRP activation is documented and many researchers feel that this may have a strong effect on the quality of the PRP gel produced as well as the rate of release of their growth factor contents. One of such activation agents commonly used especially in rats is calcium chloride. Plateltex^{R} kit/Act^{R} is a standard PRP preparation kit which uses batroxobin/calcium gluconate as activation agent.

The aim of this study was to compare the sequestration efficiency and growth factor yield of Plateltex^{R}-PRP preparation method which uses batroxobin/calcium gluconate as activating agent with the local-PRP preparation method which uses calcium chloride as an activating agent.

Preparation of PRP using $CaCl_2$ as an activating agent

Animals

Twenty healthy dogs (8-12 months old) were used for this study after acclimatization for two weeks. They were kept in department of Veterinary Surgery small animal house. The dogs were fed jollof rice, gari with chicken soup and bambara beans. Clean drinking water was provided to them *ad-libitum*.

Methodology

Using a 20ml syringe preloaded with 1.3ml anticoagulant citrate dextrose (ACD), 10ml of blood was aseptically collected from the cephalic vein of each dog. One milliliter of each of the blood samples was set aside for cell counting while the remaining 9ml were transferred to labeled tubes. The tubes containing the samples were then centrifuged for 15minutes at 72g resulting in three layers: the inferior layer composed of red cells, the intermediate layer composed of white cells and the superior layer made up of plasma. The plasma with the buffy coat layer was collected using sterile pipette into graduated test tubes. These samples were then centrifuged again for another 5minutes at 1006g to obtain two part plasma: the upper platelet poor plasma (PPP), and the lower platelet rich plasma (PRP). The PPP was aspirated to avoid mixing up with PRP. The PRP was then aspirated with pipette and placed into sterile tubes for activation. Before activation, 0.5ml of the PRP was set aside for platelet and growth factor quantification. The platelet counts in the whole blood and the PRP samples were by direct manual method according to Brecher and Cronkite, (1950).

Activation of the PRP:

This was done by addition of 0.5ml of a 5% solution of $CaCl_2$ into each of the PRP tubes. The gelation was determined by the visualization of the clot and the gel remaining attached to the tubes after turning it upside down.

Preparation of PRP using Plateltex prep^{R}/Plateltex act.^{R}

Blood collection devices in the kits (one per dog) were used to collect blood from the cephalic vein of each of the dogs making sure that each tube was attached to the collecting system only after inserting the needle into the vein. From each of the dogs, 10 ml of blood was collected into the provided tubes. The collected blood was gently turned upside down several times for homoginity. Finally, the tubes were placed in a centrifuge in a way to have them properly balanced and the first centrifugation ran at 160g for 10 minutes. The tubes were removed from the centrifuge, gently opened and placed in a rack. The PRP in the tubes were gently aspirated starting from the top and ending with the buffy coat above the red cells. The PRP tubes were then placed in the centrifuge and centrifuged a second time at 1200 g for 10 minutes. The tubes were then removed from the centrifuge and placed in a rack. Pouch 3 in the kit was then opened and the plastic needle mounted on the 10 mL syringe. The graduated tubes were uncapped and with the 10 ml syringe the PRP was gently aspirated starting from top so that about 2ml of PRP will remain at the bottom. The plastic needle was then removed and discarded. The rack was gently shaken to reshuffling the PRP concentrate with the remaining PPP. The PRP concentrate obtained was then collected with another 10 ml syringe.

Activation of the PRP using Plateltex act^{R}:

With the aid of a 3ml syringe 1ml of calcium gluconate was aspirated from the vial and transferred into the vial containing freeze-dried batroxobin and shaked. The mixture of batroxobin/calcium gluconate was then aspirated with the 3ml syringe. With 10ml syringe the prepared PRP concentrate was aspirated and put in a dish for the gel preparation. The PRP was mixed with 0.5ml of the solution of baroxobin/calcium gluconate and gently shaked and allowed to form gel.

Evaluation of the platelet concentrations of the PRPs

The platelet counts in the whole blood and the PRP samples were done by direct manual method according to Brecher and Cronkite, (1950.) The platelet counts of the whole blood and the two differently prepared PRP were recorded and the results compared using ANOVA -SPSS. Significant differences were considered at 5% level.

Evaluation of the growth factor content of the PRPs:

Platelet derived growth factor (PDGF) and transforming growth factor (TGF) concentrations of each of the prepared PRP were quantified using enzyme-linked immunosorbent assay (ELISA) methods (Mazzucco *et al*., 2008) .This was performed according to the manufacturer's instructions.

Standards and the test samples were dispensed into the wells of growth factor antibody-coated plates. After incubation and removal of unbound substances, an enzyme-coupled secondary antibody was added. After washing, the substrate solution was then added. The colorimetric reaction was then stopped and the optical density measured.

Statistical analysis

The results of the optical densities,(concentrations of PDGF and TGF) in the whole blood, Plateltex prepared PRP (PPRP) and calcium chloride prepared PRP (CPRP) were recorded and presented as mean ± sem and analyzed using analysis of variance (ANOVA) (SPSS).

Significance was accepted at 5% probability level.

Results

The mean platelet counts, PDGF and TGF concentrations in the whole blood, CPRP and PPRP are shown in (Table 1).The mean platelet concentrations in the whole blood, CPRP and PPRP were 4.13±0.18($x10^5$/µl), 12.46±0.59($x10^5$/µl), and 20.47±0.92($x10^5$/µl) respectively. The platelet concentrations of CPRP and PPRP were approximately three and five times respectively above the baseline. The means of the platelet concentrations of the whole blood, CPRP and PPRP varied significantly ($p < 0.05$).

The mean PDGF concentrations in the whole blood, CPRP and PPRP were 3.10±0.8 (ng/ml), 8.30±0.35 (ng/ml) and 14.73±0.31 (ng/ml) respectively while those of the TGF were 34.95±0.84 (ng/ml), 43.60±1.05 (ng/ml) and 90.55±1.97 (ng/ml) respectively. There were also significant variations ($p < 0.05$) in the mean PDGF and TGF concentrations in the whole blood, CPRP and PPRP.

TABLE 1: Mean (±) Platelet, PDGF and TGF concentrations in whole blood, $CaCl_2$ PRP and Plateltex PRP

Substance/growth factor	Mean (± sem)concentration		
	Whole blood	CPRP	PPRP
Platelet $x10^5$/ µl	4.13±0.18[a]	12.46±0.59[b]	20.47±0.92[c]
PDGF(ng/ml)	3.10±0.8[a]	8.30±0.35[b]	14.73±0.31[c]
TGF(ng/ml)	34.95±0.84[a]	43.60±1.05[b]	90.55±1.97[c]

a, b, c = Means on the same row with different superscript varied significantly ($p < 0.05$)

CPRP = Calcium chloride prepared PRP

PPRP = Plateltex prepared PRP

PDGF = Platelet derived growth factor

TGF = Transforming growth factor

Discussion

In this experiment, efforts were made to prepare PRP using two standard methods and compare the performance of these methods in terms of their degree of sequestration of platelets and the growth factors (PDGF and TGF). Significant variation ($p < 0.05$) in the concentration of platelets and growth factors were observed in this study. The platelets and growth factor concentrations of PPRP were higher than those of CPRP which were in turn higher than those of the whole blood. This variation may partly be attributed to the intensity and duration of centrifugation employed during the preparation of the PRPs. The two methods enabled the preparation of PRP for the release of high concentration of platelet derived growth factors, but showed different harvesting capacities for the collection of concentrated platelets. Addition of batroxobin/calcium gluconate in the PPRP and calcium chloride in CPRP as activating agents of the PRPs resulted in the release of high concentrations of PDGF and TGF when compared to the

control (whole blood). The concentrations of growth factors (PDGF and TGF) were higher in the PPRP than the CPRP suggesting that the different platelet harvesting methods had different level of effect on the platelet concentrations, activation of the PRP and subsequent release of platelet growth factors. It is therefore critical to validate and understand any PRP preparation method before the PRP is administered to patients. A fundamental prerequisite of PRP efficacy is the intrinsic quality of the concentrate. Relevant factors are the platelet concentration and the growth factor content. This is because it has been stipulated that for PRP to be therapeutically effective it must contain at least four to eight folds of platelet above the base line (Anon, 2012). In this study PPRP had platelet concentration of 20.47 ± 0.92 almost 5 times that of the baseline thus suggesting a greater potential of contributing to wound healing at injured sites. Under normal circumstances, wound healing is initiated through platelet activation and aggregation in the presence of calcium and thrombin, generating a fibrin clot in which a matrix for migration of tissue forming cells would be formed (Lin *et al.*, 1997).

The significantly ($p<0.05$) higher concentration of PDGF and TGF in PPRP when compared to CPRP could probably be as a result of premature degranulation of platelets prior to calcium chloride activation in that method. It is highly probable that platelets from calcium chloride PRP released their α-granules much more quickly than platelets from batroxobin/calcium gluconate activated PRP. It could also be because the gelation time for PPRP was markedly shorter than that of the CPRP thereby allowing more time to elapse between the additions of calcium chloride and gelation in CPRP (Mazzucco *et al*., 2008). There were probably differences in the strength and biomechanical stress exerted by the two methods on the platelets hence influencing the growth factor recovery and availability at the end of the processes as suggested by Leitner *et al.*, (2006).This could be the reason why less PDGF and TGF were measured in the CPRP supernatants compared to the PPRP.

Results of this study indicated that PRP can be concentrated three and five times in CPRP and PPRP respectively but there was discrepancy in the quantity of growth factors released after the PRP activation. With the significantly ($p < 0.05$) higher concentration of platelets and greater release of growth factors in the PPRP compared to CPRP, the standard PRP preparation method was better and would likely provide greater availability of growth factors and other useful constituents of PRP to the microenvironments of any injured tissues where it may be applied and probably faster healing than the PRP prepared by the local method.

Competing interests

The authors declare that they have no competing interests.

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