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Evaluation of lyophilized platelet powder for angiogenesis in chorio allantoic membrane assay

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Abstract
Platelet-rich plasma (PRP) accelerates wound healing, as it is an excellent source of growth factors. An optimal scaffolding material should elicit minimal inflammatory response and promote cell binding, proliferation and activity of cells. The scaffold has to possess appropriate mechanical properties for physical support during tissue development despite their high porous structure. In this study, Canine PRP prepared were subjected for lyophilization and evaluated for its potential angiogenesis on developing chicken embryo model. The PRP has much angiogenic activity inducing Chorio allantoic membrane (CAM) tissue invasion. The PRP in lyophilized form shows promising application in chronic ulcers for wound healing with potential biocompatibility.

Keywords: Plasma rich plasma, bio-scaffold, CAM, angiogenesis

1. Introduction
Bio-polymeric materials commonly used in everyday life are widely applied especially in the biomedical field as well as in cosmetic formulations. Three dimensional scaffolds for tissue engineering can be prepared by freeze drying, gas forming foam, 3D printing, thermal-induced phase separation, electrospinning, laser treatment and the precipitation of crystals. Collagen-based gel is used for skin treatment and collagen-based gel stimulates bordering healthy cells to initiate the wound healing (Peng *et al*., 2004) [8]. According to (Yassin *et al*., 2019) [14], platelet-rich plasma (PRP) accelerates wound healing, as it is an excellent source of growth factors. The PRP powder prepared by lyophilization is reported to have antibacterial activity and showed wound size reduction on induced wounds in rats (Wasterlain *et al*., 2016) [12]. PRP acts as a Growth factor agonist and has both mitogenic and chemotactic properties on different cells found in wounds such as fibroblasts, keratinocytes and epithelial cells. It also stimulates the production of growth factors at the wound site, enhances wound closure and promotes angiogenesis and re-epithelialization (Gonchar *et al*., 2017) [4]. PRP / Platelet Concentrate (PC) acquires a solid viscoelastic fibrin network called platelet gel (PG), acting as a temporal scaffold for the proteins of the cells. PG has been explored for corneal healing and bone and soft tissue regeneration (Sionkowska, 2011) [10].

2. Materials and Methods
2.1 Preparation of Platelet Rich Plasma (PRP)
PRP was separated from apparently healthy dog blood samples at the Blood Bank of the Madras Veterinary College with due ethical concern. Blood samples were collected in tubes containing 3.2 percent sodium citrate as an anticoagulant. After blood collection, the tubes were turned upside down 6-8 times to allow complete mixing. Then the PRP tubes were centrifuged for 10 min at 1000g in a refrigerated centrifuge. The blood was separated into three different layers: the upper layer, which consisted of platelet poor plasma (PPP), the middle layer which contained the PRP and the lower layer, which comprised red blood cells (RBC). These three layers of RBC's, PRP and PPP were visible by naked eye. PRP was prepared by double centrifugation method. The PRP obtained in first centrifugation was subjected for second centrifugation without additive.

2.2 Preparation of lyophilized PRP powder
The separated PRP was pre-frozen at -80 ˚C for 24 hrs and then lyophilized using Christ Alpha 1-2 LD plus mini freeze dryer.

After lyophilization, the lyophilized PRP powder was stored in a freezer at -20 ˚C after sealing in a freezer vial to prevent transformation and contamination (Nakatani *et al*., 2017) [7].

2.3 Preparation of the chicken eggs for the CAM assay and placing of the PRP

Fertilized eggs obtained from PRS, Madhavaram were pre-incubated in an egg incubator for 9 days at 37 ˚C and 55percent relative humidity. On the embryonic day 11(ED11), the eggs were rested in a position for 3 hours to ensure that the embryo is on the top. The top of the egg was marked with a pencil and carefully wiped the egg shell with 70% ethanol without turning it. The protocols described earlier **(**Woloszyk *et al*., 2016) [13] were followed with few modification to suit our lab. Eggs were placed in a 60mm petri-dish on top of a piece of cellotape to stabilize the egg. A small hole was made on the shell with the help of an egg driller and removed 4ml of albumen using a syringe and a needle to lower the developing embryo. The scissor was carefully inserted to cut an oval hole while turning the egg with the other hand. Then opened the shell partially with forceps and placed the pinch of lyophilized PRP powder into the opening carefully and placed it on the CAM and removed the forceps. The partially opened egg shell was sealed with cellotape and ensured that there is no gap. Molten wax was applied with a cotton swab on the eggshell around the cellotape. The eggs were placed in the incubator and incubated at 37 ˚C and 55 per cent humidity. Candling of the eggs was done every 24 hours for observing liveability of the embryo. On ED 14 and 18 candling of the egg was done and observed vascularization of the CAM. Eggs were placed in the fridge overnight at 4 ˚C before sacrifice. On the next day removed the CAM with the scaffold sample using the scissors and washed with Phosphate Buffer Saline. The CAM was fixed in 10% formaldehyde for histochemical studies.

3. Results

The platelet rich plasma separation protocol standardized showed clear visible amber coloured plasma which on centrifugation was clarified with contaminating cells (Fig1). The PRP was pooled and subjected for lyophilization. The lyophilized powder ability to contribute in angiogenesis was confirmed in chorioallantoic membrane assay (Fig2). The double centrifugation method yield PRP without cellular contamination

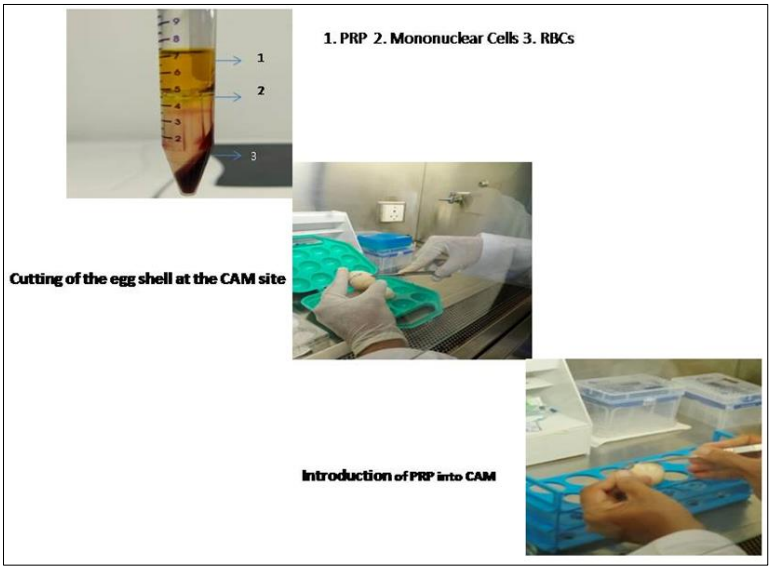

Fig 1: PRP preparation for CAM assay

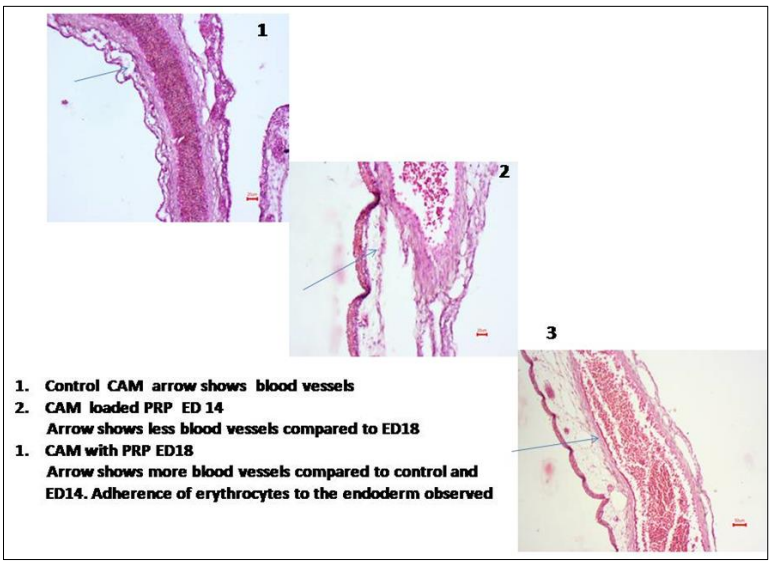

Fig 2: Histolochemical analysis of chorid alotoic membrane

4. Discussion

Platelet rich plasma is currently recommended for regenerative medical applications for the treatment of several musculoskeletal injuries and as a surgical co-adjuvant in both human (Pascale *et al*., 2015) [3] and veterinary (Corti, 2014) [2] medicine. In general, the rationale behind the use of this biomaterial lies in the fact that the GF and cytokines contained within are gradually released and diminish inflammation, promote neo vascularization, increase extracellular matrix protein deposition, and induce cell migration and proliferation (De Pascale *et al*., 2015 and Brossi *et al.*, 2015) [3, 1] and these substances could enhance tissue regeneration (Gonzalez *et al*., 2016) [5].

Dried powder forms a better drug delivery system as they could absorb water and humidity from the wound secretions (Kim *et al*., 2009) [6] and offer sustenance for tissue formation, but also can shield a wound by becoming an efficient fence against external contamination and adhere and travel through the tissue networks. The lyophilized PRP acts as a scaffold supports the delivery and retention of cells and different biochemical factors. It lets cells interact and connect by facilitating proper cell attachment and migration and permits the flow of vital cell nutrients and released products.

An optimal scaffolding material should elicit minimal inflammatory response and promote cell binding, proliferation and activity of cells. The scaffold has to possess appropriate mechanical properties for physical support during tissue development despite their high porous structure.

Visualization of angiogenesis aiming at tissue replacement and or regeneration for predicting and understanding the outcome of new regenerative approaches is one of the key challenges in regenerative medicine. The CAM assay is a simple and cost effective and highly reproducible method which does not require ethical approval when performing experiments in chicken embryos until embryonic day 14 (Ronaldo *et al*., 2019) [9]. This technique could be applied for the direct comparison of the activity of biomolecules on angiogenesis. The present study can constitute a valuable tool for studying in detail cell-mediated vascularization efficiency (Thamilarashi, *et al*., 2014) [11].

5. Conclusion

The PRP has much angiogenic activity inducing CAM tissue invasion and could be a promising wound healing candidate for chronic ulcerous wounds. CAM assay is found to be a simple, cost effective and highly reproducible technique for the study of angiogenesis.

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