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Formation and Assessment of Platelet Rich Plasma Treatment

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Abstract- The literature currently describes many various methods of creating Platelet-Rich- Plasma (PRP); however, according to Robert Marx's widely accepted definition a platelet rich solution is three times (3x) greater in platelet concentration compared to the same volume of whole blood with a double centrifugation process is PRP. In this experiment the creation of PRP was optimized using two previously published PRP protocols from Messora et. al. (2011). Furthermore, the literature minimally describes the possibility of activating cytokine and vesicular release from PRP using collagen scaffolds for full thickness wound repair. To date, there are publications that discuss the collagen scaffold and PRP combination therapy use in connective tissues repair, but this leaves out epithelial tissue repair where PRP could also assist in the healing response. Here the creation of an electrospun collagen scaffold is created and used to activate the optimized PRP on-demand. PRP levels were validated through manual cell counting methods and flow cytometry. Once quantitative assessment of the PRP was completed, activation of the PRP took place. This activation process was validated throughlight-microscopy (LM) and scanning-electron-microscopy(SEM).

Keywords:Platelet-Rich- Plasma, light-microscopy, scanning-electron-microscopy

Introduction

Platelet rich solutions also called platelet releaseate have been clinical available and utilized since the late 1970 and early 1980's (Ferrari, 1987). The literature and clinical utility of these solutions has endured much controversy regarding the creation of PRP. Currently there are single centrifugation techniques, double centrifugation techniques and various speeds at which PRP is being created none of which are standardized (Delong et al, 2012). Some of these methods can be detrimental to the creation process of the PRP. For instance, if a PRP solution is being created and spun at a speed above 1,000 times gravity it then sheers the platelets causing vesicular release into the centrifugation tube, which may begin the cytokine degradation process during the creation rather than the implementation of the PRP (Bausset, 2012). Here an optimal Platelet-Rich solution called Platelet-Rich-Plasma (PRP) was discussed, created and qualitatively and quantitatively assessed using the modified methods of two researchers Nagata et al. (2009) and Messora et. al. (2011). Assessment of the PRP came from manual counting methods and automated counting using flow cytometry to ensure proper platelet concentrations were achieved, thus adhering to the Marx definition of PRP. The optimized PRP was then activated using electrospun collagen scaffolds. Activation was determined through morphological and molecular characteristics using light microscopy and scanning electron microscopy (SEM) along with enzyme linked immunosorbant assay (ELISA) testing. The literature and the knowledge of the coagulation pathway allows one to appreciate how collagen scaffolds, at the molecular level, can facilitate activationofthePRP.Thisactivated PRP can then beappliedto in

vitro and in-vivoassays to evaluate its effect on wound healing, as described in the current and subsequent chapters.

Research Aim

To create an optimized PRP solution and activate the PRP with a collagen scaffold.

Research Hypothesis

If an optimized PRP solution is created and combined with an electrospun collagen scaffold then it will cause platelet activation and subsequent vesicular release.

Materials and Methods for Platelet-Rich-Plasma

The purpose of this study was to create PRP from whole blood; building and modifying on current procedures found in existing scientific literature. The PRP was prepared from acid citrate dextrose (ACD) anti-coagulated whole porcine whole blood with a ratio of 15:85 ACD to whole blood. ACD was determined to be the ideal anticoagulated based on literature findings (Lei, et. Al., 2009) and individual PRP creation experiments performed during this initial aim. The table below demonstrates the different types of blood evaluated and the number of trials and anticoagulants used during the initial creationtrials.

Table 1: The highlighted region denotes the ideal anticoagulant needed for PRP creation. This was based on literature support and experimental trials performed during this initial research

Blood type	Blood Received from	Number of trials	Anticoagulant
Murine	NAU tissue transfer murine takedown (~10 ml)	2 samples	EDTA
Porcine	APS in MN (~1000 mL)	3 samples	Heparin
Porcine	APS in MN (~1000 mL)	8 samples	ACD
Porcine	Lampire Biological in MN (~1000 mL)	15 samples	ACD

Upon the completion of the anticoagulated studies, porcine-pathogen-free, ACD- Blood was obtained from Lampire Biological Laboratories. The PRP was made using a double centrifugation technique altered from Nagata et al. (2009) and Messora et. al. (2011). Ice packed whole blood arrived in sterile intravenous bags (I.V.) (figure 1) and was immediately placed in a refrigerator set to 3°C. All blood samples were sent with microbial free content reports, demonstrated in figure 2.

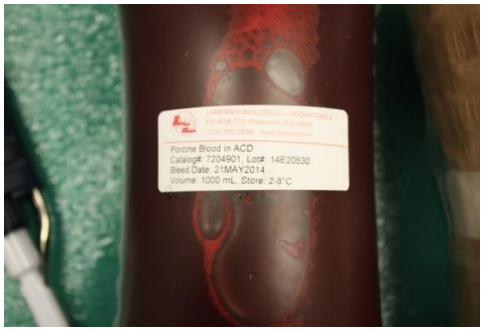

Figure 1: ACD blood arrival inIVbag

An initial 2µL whole blood smear was performed to observe the morphological quality of the whole blood. Once the whole blood smear was performed, the smeared glass slide was stained using a method derived from Rowley Biochemical Inc. "Wright Stain Method" and observed under a Leica light microscope. The slide was submerged in Wrights stain for 1 minute then transferred to a pH 7 phosphate buffer solution for 2 minutes then placed under a Leica light microscope for morphological-qualitative viewing. If the platelets were not viewed in an activated state or aggregating the blood was deemed acceptable to use for the PRP creation.Forty (40) mL of ACD-whole blood was transferred from the IV bag/collection tubes and placed into the sixteen tubes respectively. The tubes were placed in a precooled Thermoscientific ST16R centrifuge set to 4°C at 200x G and an acceleration anddeceleration of 5x G for 20 minutes to separate the plasma layer, white blood cells/platelet layer (buffy coat) and erythrocyte layer (figure 2).

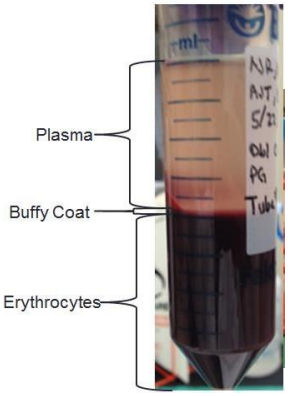

Figure 2: Results after first centrifuge

Once the first spin was complete, the buffy coat and a small portion of both erythrocytes and plasma were transferred to a

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15mL BD. The tubes were placed back into the same Thermoscientific ST16R precooled centrifuge set at 4°C for a second spin at 400x G and an acceleration and deceleration of 5x G for 15 minutes to separate once more (figure 3).

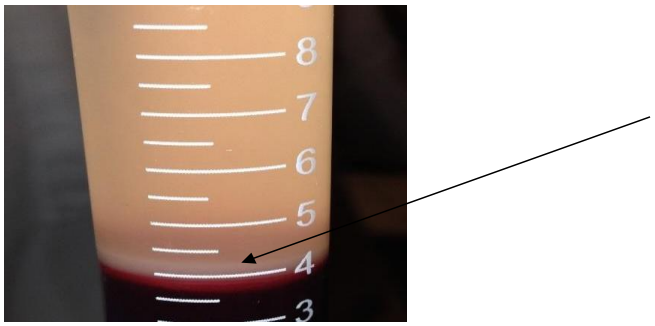

Figure 3: Separation after 400xG centrifugation, the arrow is identifying the PRP zone After the second centrifugation was performed and the layers were welldifferentiated, approximately 2µL of the platelet-rich plasma were extracted from thebuffy coat using a P10 Gilson pipetteman. The 2µL drop of PRP was placed on a glass slide, smeared and stained using Rowleys method then analyzed under a Leica light microscope (Figure 4). This creation process was standardized for all PRP samples throughout the various aims of the project. A complete standard-operating-procedure (SOP) of PRP can be found in the appendix of this dissertation.

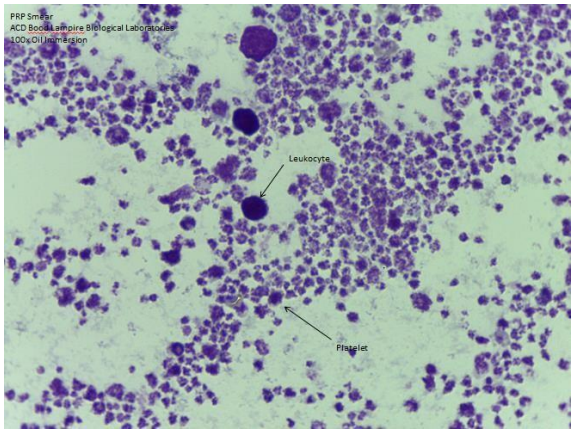

Figure 4: Slide image (100x, wrights stain) of porcine PRP under light microscope

Materials and Methods for Manual Counting Methods

Whole blood and PRP platelet quantification was performed via manual counting methods using a method altered from Tasker et al. (1999) & (2001). Blood smears were performed and stained, as previously described, for both whole blood and PRP. There were 20 slides of each group created, yielding a total of 40 slides (n= 20 whole blood and n= 20 PRP). To reduce investigator bias, one blinded researcher was selected and trained to view blood smears under light microscopy for approximately two weeks. Once trained, the same individual then collected ten randomized monolayer images using a Leica DM750 with a mounted Leica ICC50 HD camera for each slide (a total of 400 images) ata magnification of 100x oil immersion. The images were taken in the upper portion of the monolayer (figure 5).

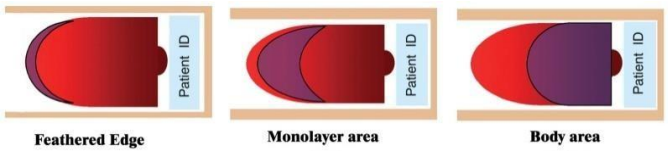

Figure 5: Various zones of a blood smear. Image adapted from Faheem (2014) All platelets within the 100x oil immersion field were manually counted and theplatelet count from each of the 10 images (for the selected slide) was averaged. Figure 6 and 7 below depict an example of counted images of whole blood and PRP,respectfully.

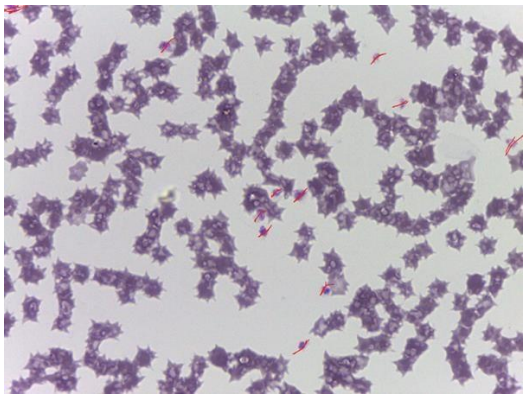

Figure 6: A counted ACD- porcine whole blood smear (100x wright stain).The red hash lines denote the counting of the platelets.

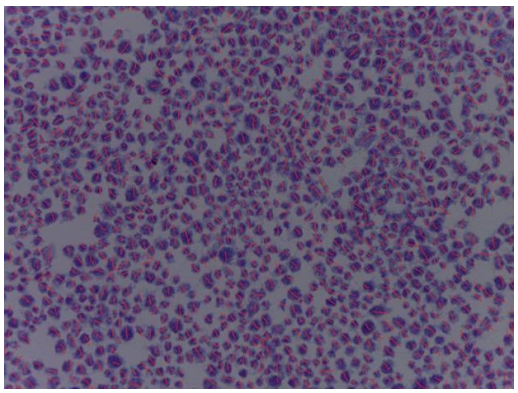

Figure 7: A counted ACD-porcine PRP smear (100x wright stain).

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A students T-test with an alpha level of 0.05 was performed between the whole blood and PRP manual counted samples.

Materials and Methods for Flow Cytometry

Following manual counting methods the PRP was further validated against whole blood using flow cytometry. A BD Accuri C6 flow cytometer was used to compare the PRP and whole blood sample. The samples of PRP were created using the methods previously described. Varying PRP and whole blood aliquot volumes were combined with 1x Phosphate Buffer Solution (PBS) until the volume of sample reached 1 ml, the volumes are outlined below in table 1. The samples were then analyzed via the flow cytometer with the threshold set at 10,000 (events given for one trial) by recommendations of BD Accuri C6 software users guide and the gates were manually created per BD Accuri guidelines and the publication by Masters and Harrison (2013). The image below demonstrates the actual set location of thegates.

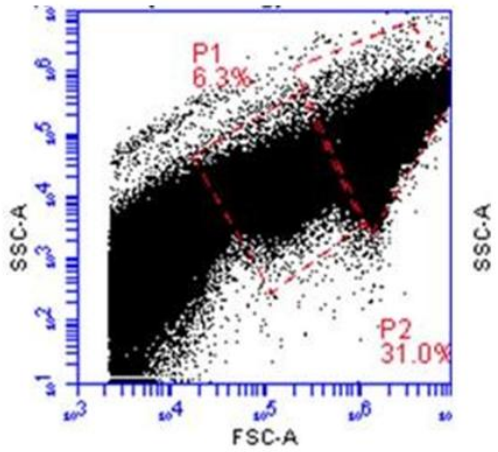

Figure 8: P1 and P2 denote the set gates for all flow samples ran. These were determined by the literature (Masters and Harrison, 2013). The gates (P1 and P2) are based on the size of the events (sample that passes through the laser). The FSC and SSC are referring to the forward and side scattering lasers.

A students T-test with an alpha level of 0.05 was performed between the whole blood and PRP flow cytometry samples.

Background on Flow Cytometry

Cellular counting is an important research and clinical tool that aids in prognosis, research findings and clinical diagnosis. Prior to automated diagnostic methods laboratories would routinely perform manual methods. This process employed a trained technician who would understand the cell(s) he or she would be identifying and subsequently counting. Although this is a method that is still used today, it is costly, time intensive and variable depending on the technician performing the assignment (Usaj et al, 2011). These reasons have led to the development of automated cell counting systems including flow cytometry.

Flow cytometry uses laser optics to determine the size and quantity of various cells. Once the sample is loaded into the flow cytometer the fluidics system transports the sample in a fluid stream to the laser to be analyzed. As the sample nears the laser, the nozzle tip forces the particles to individually pass through the laser in a process called hydrodynamic focusing (Rahman et al., 2010). The passing of the particle or cell through the laser causes the light to scatter in both a forward scatter and side scatter. The forward scattering light, typically light scattered at less than 20^{o}, is proportional to the cell size, thus allow for determination of different cell type, i.e. leukocytes, erythrocytes, and platelets (Rahman et al., 2010). The side scattering, light scattering at approximately 90^{o}, is proportional to the cell's internal complexity or granularity, allowing for determination of cells that may have the same size, such as the varying types of leukocytes (Tasker et al., 2001) (Rahman et al., 2010).

The light scattering is detected by photodetectors that generate a small current when contacted by a photon. The related voltage has an amplitude that is proportional to the number of photons sensed by the photodetectors. This voltage is then amplified andconverted into signals that are large enough to be plotted and counted (Rahman et al., 2010).

Results

The purpose of the quantification of the PRP against whole blood was to determine if PRP was actually being created according to the Marx definition. The grape demonstrate the manual count averages of each whole blood and PRP glass slide smears

The graph below shows the average whole blood platelet count compared to PRP count.

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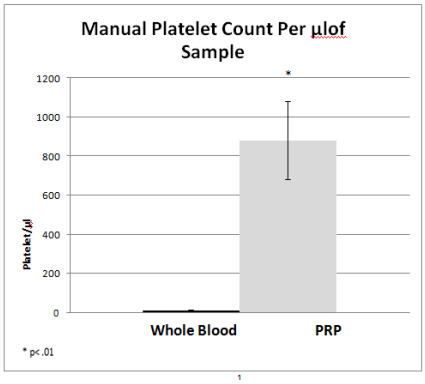

Figure 9: Manual platelet counts between whole blood smears and PRP smears The manual platelet count of the PRP was significantly ($p<0.01$) higher and morethan three times that of the whole blood, which adhered to the definition set forth by Marx (Marx, 2001). To verify the manual counting methods, an automated flow cytometry method was utilized. Below is a graphical representation of the flow data. Similar to the manual methods the PRP samples were significantly higher and muchmore than three times greater (12 times) than that of the whole blood counts. Thisfurthervalidates that PRP is being created according to Marx's definition and that the manual methods were validated.

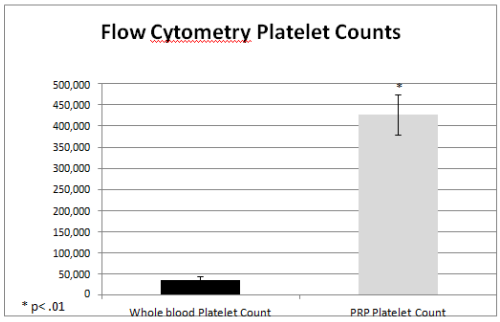

Figure 10: Platelet counts of whole blood and PRP based on dilutions.

a variety of *in-vitro* assays including platelet activation using collagen scaffolds. These scaffolds combined with activated PRP will allow for a novel treatment modality in full-thickness dermal wounds. The next section discusses the creation of collagen scaffolds and how they activate the optimally created PRP.

Background on Collagen and Electrospun Collagen Scaffolds

Collagen is the most abundant protein found in the human body. It is used for structural support and connection of tissues (Bailey et al., 1979) (Di Lullo et al. 2002). To date there are many isoforms of collagen. One location where collagen is found in high quantities is the integument. Studies have determined that the integument is primarily comprised of type I and type III collagen fibers making up approximately 80% of dry skin weight (Smith, 1986) (Brett, 2008). The synthesis of collagen begins with a multigene transcription process, specifically 34 genes are involved in collagen formation primarily belonging to the *COL* gene family (U.S. National Library of Medicine, 2015). Following the initial step of mRNA transcription a procollagen molecule is formed using vitamin C as a cofactor (Nusgens et al., 2001). Once the post-translational modification using vitamin C is complete, one final step is needed to create a fully functional strand, this includes the golgi apparatus adding oligosaccharides to the procollagen (Tasab, 2000). This process then allows the procollagen to be packaged and ready for vesicular transport out of the cell. Extracellular-membrane-collagen-peptidases cleave portions of the procollagen off thus creating a tropocollagen. Lysyl oxidases then crosslinks hydroxylysine and lysine residues forming the collagen fiber(s) (Siegal et al.,1970).

In addition to being an important structural protein, collagen plays a large rolein many other cellular functions seen during a traumatic wound event. Theseprocessesinclude differentiation, aiding in protein synthesis, fibroblast and keratinocyte cellular migration and chemotaxis/migration to the wound bed, aiding in the healing event (Montesano et al., 1983) (Madri & Marx, 1992) (Albini & Adelmann-Grill, 1985). Both researchers and clinicians have capitalized on this knowledge and begun to create and use collagen dressings on wound beds (Babu, 2000) (Brett, 2008). These collagen dressings have been demonstrated to expedite the wound healing event (Brett, 2008).

In the current study a Type I bovine collagen scaffold was electrospun and used as an activator of the PRP. The basis for this activation process is a result of the known and established biological interaction of collagen and the platelets during the coagulation process (Baumgartner, 1977). During this activation process, two platelet surface protein receptors interact with the collagen. Intergrin $\alpha_2\beta_1$ allows for the platelet to adhere to the collagen and glycoprotein VI is able to recognize the quaternary structure of collagen (Kehral et al., 1998). This adhesion and recognition allows for vesicular release from the activated

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platelets, which aids in the inflammatory event and the initial stages of wound healing. The combination of PRP and the collagen scaffolds allow for a decrease in wound healing time as a result of collagen presence and an increase in growth factors/cytokines from the PRP granule release. The presence of the collagen allows forthe extracellular matrix to be recreated and therefore helps to facilitate cellular adhesion. The increase in growth factor and cytokines recruits the needed cells including fibroblasts and phagocytic cells for the wound healing event. These cells aid in remodeling the ECM and assisting in clearing the wounded tissue of pathogenic agents.

Materials and Methods for Electrospun Collagen Scaffolds

In order to create electrospun collagen scaffolds, lyophilized collagen was solubilized in 1,1,1,3,3,3,-hexafluoro-2-propanol (HFIP) to create a 7.5% collagen solution. The solution was gradually heated to 40^{o}C in order to completely dissolve the collagen. The 7.5% collagen solution was collected in a syringe and the syringe was loaded into a pump. The nozzle of the electro-spinner was set 12cm from the target. The pump was set to a flow rate of 1ml/hr. The electro-spinner was subsequently charged to 25kV and then allowed to spin for 1 hour. Upon completion, forceps were utilized to separate the scaffold from the target. The collagen scaffold was crosslinked and sterilized for 1 hour on each side using UV light.

Materials and Methods for SEM

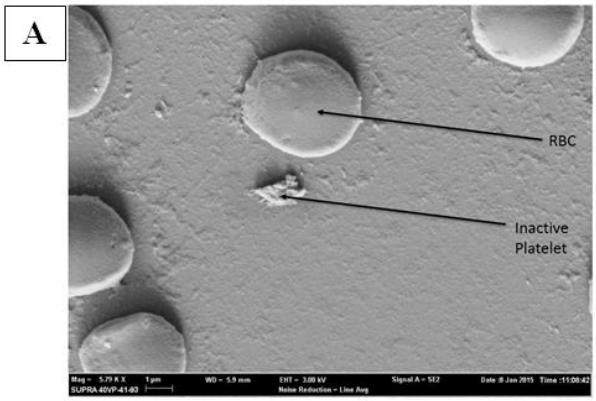
The following various blood products were imaged using scanning electron microscopy (SEM) to demonstrate inactive and active states; whole blood, platelet-rich- plasma (PRP) and PRP with an Advanced BioMatrix aqueous Type I PureCol bovine collagen (PRP-c) created scaffold.

Initially the three various blood products were created using the standard PRP research protocol. Following the creation or gathering of the products 1 µl of each product was pipetted onto a glass slide cover slip; this was performed 4 times to all multiples of each blood product (n= 4 whole blood smear, n=4 PRP smears, and n=4PRP-c smears). All glass slides were allowed to air-dry then each smear was placed in 2.5% gluteraldehyde solutions overnight at 4ºC. Once gluteraldehyde fixation was complete, each smear was washed 3 times with a 1% PBS solution for 10 minutes. A secondary fixation was then completed using a 1% (Osmium Oxide) OsO_4 solution for 1 hour. Following 1 hour, each slide was rinsed with Millipore water 3 times for 10 minutes each rinse. Following the rinsing, the various slides went through a dehydration procedure.

(Hexamethyldisilazane) HmDS and 100% ETOH solution for 15 minutes followed up by a 2 100% HmDS. They were then allowed to dry for 15 minutes. The various glass smears were then mounted to SEM studs using carbon mounting stickers. Once the smears were mounted they were ready for gold sputter coating preparation. The samples were initially placed on the pedestal. Then the Pyrex cylinder was placed in center of the base plate. Next, the sputterhead was placed down on Pyrex cylinder. The Denton Vacuum Desk II Cold Sputter Etch Unit was then turned on and pressure reached 100 millitorr. The argon gas was then turned on until the pressure reached 500 millitorr. Thegas flow was then decreased until the pressure reached 50-100 millitorr (this was completed twice to flush the system). Once the system had been flushed the gas flow was adjusted so the pressure was stabilized between 70 and 100 millitorr. The sputter coat time of 30 seconds was then entered in the Denton unit and the sputter coat process started. The current was held relatively consistent at 45 milliamps. After the 30 seconds, the machine stopped automatically and the machine was turned off. A two minute wait period applied so that inhalation of the argon did not occur.

Results

This supports the idea that the PRP can be activated on demand, through the use of electrospun scaffolds, and is thus appropriate for pre-clinical and potential future clinical use.



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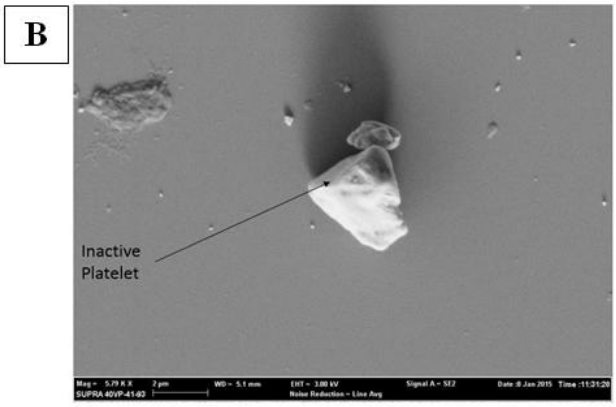

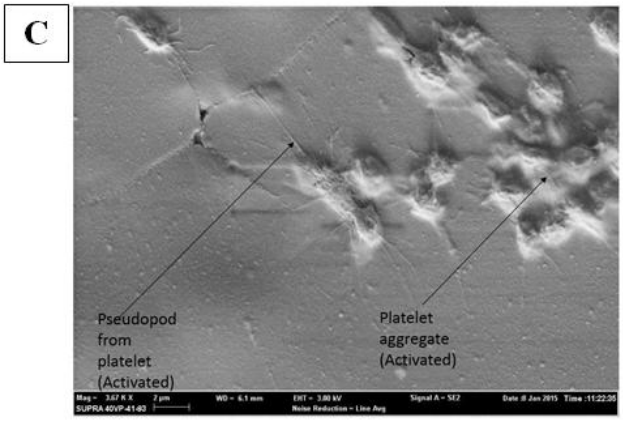

Figure 12: pseudopod formation.

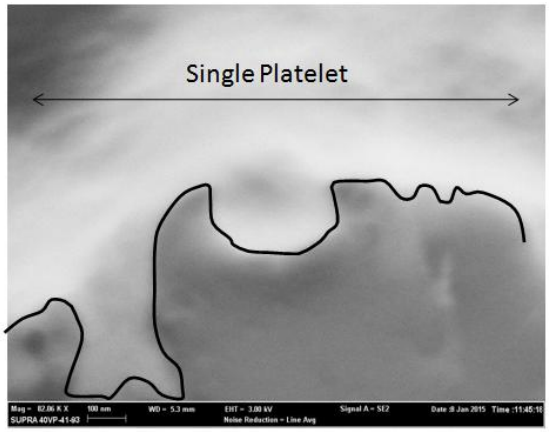

Figure 13: 86 K magnification

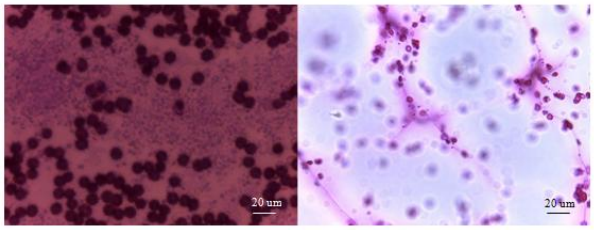

Figure 14: Histology slide (100x mag).

Conclusion

Based on the findings above, it was determined that the research hypothesis, If an optimized PRP solution is created and combined with an electrospun collagen scaffold then it will cause platelet activation and subsequent vesicular release was supported. This was demonstrated through a variety of *in-vitro* tests and assays including manual counting methods, automated counting methods (flow cytometry), ELISA activation assay, imaging techniques: SEM and light microscopy. The support of this hypothesis warranted the implementation of testing this optimal PRP in a mock wound healing model called the scratch assay.

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