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Preparing Activated Platelet-Rich Plasma for Culturing Human Adipose-Derived Stem Cells

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TITLE:

Preparing Activated Platelet-Rich Plasma for Culturing Human Adipose-Derived Stem Cells

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KEYWORDS:

Platelet-rich plasma, double spin method, adipose-derived stem cells, proliferation, human, platelet

SUMMARY:

In this report, we describe a double-spin method for preparing activated platelet-rich plasma (PRP). Using autologous thrombin, human adipose-derived stem cells (hASCs) were cultured. Activated PRP was shown to promote proliferation of hASCs.

ABSTRACT:

Activated platelet-rich plasma (PRP) prepared from whole blood via centrifugation demonstrated a proliferation-stimulating effect in several kinds of cultured cells, implying a possible use in regenerative medicine. Here, a double-spin method was used to prepare PRP from whole blood. PRP was further activated by autologous thrombin. The platelet count was measured in the activated PRP and the proliferation-stimulating effect in human adipose-derived stem cells (hASCs) was examined. The resulting platelet count was 11.5-times higher in PRP than in whole blood plasma. The proliferation of hASCs was markedly enhanced by incubation with 1% PRP. The described method can be used to reproducibly prepare PRP with a high concentration of platelets. PRP prepared by this method markedly promotes proliferation of hASCs.

INTRODUCTION:

Activated platelet-rich plasma (PRP) is prepared by centrifugation of whole blood and is shown to contain platelets well above baseline levels[1]. Autologous RPP has been widely used in surgical treatment, including wound healing[2], bone injury[3], and aesthetic surgeries[4,5]. After activation of platelets in PRP, the α-granules present in platelets release several growth factors, such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), insulin-like growth factors

(IGFs), transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), and others[1,6,7]. These growth factors play an important role in cell proliferation[8], migration[9], and differentiation[9].

To date, several studies have reported the proliferation-stimulating effect of PRP in different kinds of cells[10-16]. One of these cell types is human adipose-derived stem cells (hASCs); hASCs exist in human adipose tissue and can be easily collected in large numbers. The regenerative effect of hASCs further suggests a potential use in clinical applications[8]. Our previous studies reported that compared to non-activated PRP, activated PRP had a marked proliferative effect on hASCs and human dermal fibroblasts (hDFs)[8]. In addition, we reported that PRP promotes the proliferation of hDFs through an ERK1/2 signaling pathway[17]. Recently, we also reported that PRP promotes the proliferation of hASCs through the ERK1/2, JNK and Akt signaling pathways[18]. In hASCs, PRP plays an important role as a supplement that promotes proliferation. Knowledge about the effect of PRP on hASCs will help the development of large-scale culture methods and enable further studies on the mechanism of proliferation in hASCs.

In this report, we introduce and describe a method for preparing PRP from whole blood using centrifugation. This method uses the double spin method to easily prepare a stable sample of PRP. To assess the biological function of PRP, we measured concentrated platelet counts and the concentration of several proliferation factors. We also confirmed the growth stimulatory effect by using the prepared PRP for culturing hASCs.

PROTOCOL:

The study was approved by the Ethics Review Board of Kansai Medical University in accordance with the ethical guidelines of the Helsinki Declaration of 1975. All specimens were collected and used with informed consent from the donors.

1. Preparation

1.1. After obtaining informed consent, take blood from healthy adult donors. Here, blood was collected from four male blood donors between the ages of 28-38.

1.1.1. Recommend donors to drink 500 mL of water 6 h before blood collection.

NOTE: The reference ranges for hemoglobin (Hb), red blood cell (RBC) count, and platelet (PL) concentration in healthy adults are described by Vajpayee[19]. In order to be suitable for blood donation, the donor's blood analysis is required to be within these ranges.

2. Blood collection

2.1. Have the blood donors sit during the procedure.

2.1. Wear gloves. Fasten a tourniquet on the donor's upper arm while the donor makes a fist.

2.2. Once a suitable vein is found, sterilize the skin twice with 70% alcohol and insert the needle.

2.3. Use a 21 G syringe to collect blood. Do not loosen the tourniquet during the blood draw.

2.3.1. Use an 8.5 mL blood collection tube to collect the blood. Observe the state of the blood in the tube and change to a new tube as soon as the blood flow slows down.

2.3.2. Collect a sufficient amount of blood to make the PRP. Collect four tubes of anticoagulated blood with a total volume of 34 mL. Invert the tubes 10 times to mix the blood with the anticoagulant.

2.3.3. Use a 10 mL serum blood collection tube (see **Table of Materials**) without anticoagulant to collect blood for making the activator (see Step 4). Collect 10 mL of non-anticoagulant blood.

3. Double-spin method to make PRP

3.1. Take 40 µL of anticoagulated whole blood for the platelet count. Use filtered pipette tips to protect the pipette from blood contamination.

3.1. Centrifuge the anticoagulated whole blood for 7 min at 450 x *g* and room temperature. This is the first spin to obtain layered blood samples. The upper layer is the plasma fraction, the middle layer is the thin buffy boat, and the bottom-most layer consists of red blood cells.

3.2. Use a marker to make a line at 2 mm below the buffy coat.

3.3. Use a 20 mL syringe with a long cannula to collect the plasma just to the 2 mm mark below the buffy coat. The yellow plasma with buffy coat contains platelets, leukocytes, and some erythrocytes. Collect the plasma from two tubes into a serum blood collection tube. Combine the contents of 4 tubes into 2 tubes for a total volume of 16 mL of plasma collected.

3.4. Centrifuge the plasma with the buffy coat for 5 min at 1,600 x *g* and room temperature. This is the second spin. The supernatant of the layered blood samples is platelet-poor plasma (PPP).

3.5. Use a 20 mL syringe with a long cannula to transfer the PPP to a 50 mL serum blood collection tube containing 1 mL of liquid as a measure. Platelets that accumulated in the thrombocyte pellet in the remaining 1 mL plasma were used as the PRP. The total volume of PRP collected was 2 mL.

3.6. Vortex the PRP in each tube and then pool into one tube (total 2 mL).

3.7. Take 400 µL of the PRP for a platelet count.

3.8. Aliquot the PRP into several 1.5 mL tubes, containing approximately 400 µL in each tube. There is a total of four tubes. Approximately 500 µL PRP per tube is recommended.

4. Prepare the activator

4.1. Allow the 10 mL of blood without anticoagulant in a serum blood collection tube (step 2.3.3) to sit for 30 min at room temperature.

4.1. Centrifuge the blood sample without anticoagulant for 8 min at 2,015 x *g* and room temperature in a laboratory centrifuge.

4.2. Collect the supernatant as autologous thrombin.

4.3. Mix 0.5 M $CaCl_2$ and autologous thrombin in a 1:1 (v/v) ratio as an activator. For example, 500 µL of $CaCl_2$ was mixed with 500 µL of autologous thrombin. Make a total volume of 1 mL of the activator.

5. PRP activation

5.1. Mix PRP and the activator in a 10:1 (v/v) ratio. Mix 400 µL of PRP with 40 µL of the activator in each 1.5 mL tube, and then incubate for 10 min at room temperature. This is activated PRP in a coagulated form.

6. Storage of PRP

6.1. Centrifuge activated PRP at 9,000 x *g* for 10 min in a 4 °C laboratory centrifuge.

6.2. Collect the supernatant via pipette into a proper volume syringe.

6.3. Filter the supernatant through a 0.22 µm membrane.

6.4. Store the final PRP at −80 °C until use.

7. Measurement of platelet concentrations and growth factor levels

7.1. Count the platelet number in whole plasma and PRP using an automated hematology system (see **Table of Materials**).

7.2. Analyze concentrations of PDGF-BB, IGF, and EGF levels in whole plasma and activated PRP using commercially available ELISA kits (see **Table of Materials**).

8. Cell proliferation assay

8.1. Isolate hASCs using a previously described method[18].

8.1.1. Seed hASCs at a density of 1.0 x 10^4 cells/well in 24-well culture plates and incubate in

DMEM containing 10% FBS and antibiotics overnight at 37 °C. Use hASCs from passages 7-9 in experiments.

8.2. Replace cell media with serum-free DMEM. After 6 h, add PRP at the designated concentrations and incubate further for 48 h at 37 °C.

8.3. Incubate with WST-8 solution (see **Table of Materials**) for 1 h at 37 °C. Read absorbance at 450 nm with a multi-well plate reader.

8.4. Construct a standard curve: Seed hASCs at densities of 0, 6,250, 12,500, 25,000, 50,000, and 100,000 cells/well in 24-well plates in DMEM with 10% FBS for 3 h. Read the absorbance at 450 nm after incubation with WST-8 solution for 1 h at 37 °C

8.4.1. Draw the standard curve by plotting the number of cells versus the $A_{450\ nm}$.

8.5. Estimate the number of hASCs from the absorbance based on the standard curve.

REPRESENTATIVE RESULTS:

Enriched concentrations of platelet and PDGF-BB in PRP

Concentrations of platelets and PDGF-BB in PRP increased 11.5-fold and 25.9-fold, respectively, as high as those in whole plasma. However, the concentrations of EGF in PRP were not changed and IGF was only 70% of that of whole plasma (**Table 1**). The experiments were replicated four times by the double-spin method.

Enhanced proliferation of hASCs by PRP stimulation

Cell proliferation was increased by treatment with 0.2% PRP ($P < 0.05$ vs control), and to a greater extent with 1% PRP ($P < 0.05$ vs control and $P < 0.05$ vs 0.2% PRP). **Figure 1A** demonstrates that proliferation of hASCs was stimulated by PRP in a dose-dependent manner. The enhanced proliferation of hASCs by PRP stimulation was confirmed by phase-contrast microscopy (**Figure 1B**). Data were provided as the mean value ± standard deviation (SD). The Mann–Whitney U test was used to evaluate differences among groups. $P < 0.05$ was considered statistically significant.

FIGURE LEGENDS

Figure 1: Enhanced proliferation of hASCs by PRP stimulation. Cells were incubated with PRP in serum-free DMEM for 48 h. Cell proliferation was determined with WST-8 by reading the absorbance at 450 nm. (**A**) PRP stimulated hASC proliferation in a dose-dependent manner (n = 4). *$P < 0.05$. (**B**) Phase-contrast micrographs showing an increased growth of hASCs by stimulation with PRP.

Table 1: Platelet concentrations and growth factor levels in the serum and PRP. Concentrations of platelets and PDGF-BB in PRP were 205.3 ± 34.8 x 10^{10}/L and 38.8 ± 0.8 ng/mL, which increased 11.5-fold and 25.9-fold, respectively, as high as those in whole plasma. However, the concentrations of EGF in PRP were not changed and IGF was only 70% of that of whole plasma.

DISCUSSION:

After PRP activation, several growth factors, such as PDGF, EGF, IGF, TGF-β, and VEGF[1,6,7] "activate" cells and tissues of wounds promoting wound healing[20,21]. In the case of cosmetic reconstruction surgery, activated cells have been shown to induce healing and improve aesthetics[22,23]. Alternatively, human adipose-derived stem cells can be used without PRP stimulation in aesthetic and reconstructive fields[24-26]. PRP can also be combined with insulin in culture, favoring chondrogenic and osteogenic differentiation of human adipose-derived stem cells in three-dimensional collagen scaffolds[27].

PRP can be prepared using different methods, depending on the institution or physician. The issues observed in PRP preparation include use of PRP without activation and low platelet concentrations. The main goal of PRP preparation is collecting as many platelets as possible from blood samples of individual patients. In this way, cytokines can be extracted after activation in order to observe the effect on cells and wounded areas.

An important factor in PRP preparation is the concentration of platelets in the collected whole blood. Two centrifugation methods, the single spin and double spin methods, are used for PRP preparation. Centrifugation is specified by the number of rotations (centrifugal gravity) and centrifugal time (minutes). Generally, 900–3200 rotations/min as the number of rotations and 6–18 min as the centrifugal time have been reported. PRP preparation using the double spin yields a platelet concentration that is enhanced 4–7.9-fold[4,28,29] over the baseline concentration. Thus, the double spin method yields higher platelet concentrations than the single spin method. Marx recommended the double spin method due to the increased platelet yield and efficacy as the single spin method cannot effectively separate and concentrate platelets for clinical treatments[28]. We also recommend the double spin method and have used the method for in vivo studies and clinical applications[9,30-33].

In the double spin method, red blood cell and plasma layers are separated during the first centrifugation. Platelets exist between these two layers (in the buffy coat: containing white blood cells and platelets). We applied suction to collect the contents of this layer (until 2-3 mm below this layer) and transferred it to another test tube. This layer was subsequently separated by a second centrifugation into a yellow layer, mainly consisting of plasma, separating an upper white blood cell layer (PPP), and a lower red layer (PRP) containing concentrated platelets.

The single spin method has the advantage of easier, more rapid automation. However, the reported concentration rate at the present stage for the single spin method is 359%, which indicates that it can only concentrate platelets to approximately 3.38%[34]. Although the double spin method has disadvantages of requiring more time and effort due to two centrifugation steps, it can prepare platelets with a higher concentration rate than the single spin method.

Measurement of platelets and growth factors in PRP is important to assess the effects of their concentration from whole blood[8,35]. After preparing PRP by several methods from several blood samples from a single person, Castelioa et al.[35] concluded that comparisons of platelet and growth factor concentrations were necessary to assess each PRP preparation. Kushida et al.[31]

collected whole blood from a single donor to prepare PRP using seven different commercial PRP separation systems and then to compare platelets and growth factor concentrations. There are two types of commercial separation systems: one uses a fully automated centrifugal separator, while the other requires centrifugation of manually collected platelet fractions. The representative centrifugal separator used in the double spin method is described by Kushida et al.[31]. The advantage of this system is greater uniformity due to reduced potential for technical errors and ease of PRP preparation. The main disadvantages are the high costs for the kit and the centrifugal separator.

The PRP preparation method introduced in this report is a double spin method that yields a high platelet concentration. An additional advantage of this method is that less expensive equipment is required including readily available syringes, blood-collecting vessels, and a common centrifugal separator.

Upon adding autologous thrombin and calcium chloride to activate PRP, α granules in platelets release high concentrations of PDGF, TGF-β, EGF, and VEGF. Kakudo et al.[8,9] reported that they prepared PRP by the same double spin methods as described here, and then added autologous thrombin and calcium chloride to activate PRP. Kakudo et al.[8] also reported that the amount of PDGF and TGF-β1 released when PRP was activated in the same manner was markedly higher than those in whole blood or pre-activated PRP.

When adding PRP into cultured cells, including adipose stem cells, the following three methods were important: 1) Use the double spin method to prepare PRP with high platelet concentrations; 2) Activate PRP to obtain PRP with high growth factor concentrations; 3) Before adding PRP, thoroughly remove debris by strong centrifugation and/or filtration. This paper presents a useful PRP preparation method that adheres to the three conditions above and can quickly and economically obtain activated PRP that will amplify adipose stem cells.

ACKNOWLEDGMENTS:
Not applicable.

DISCLOSURES:
The authors declare that they have no competing financial interests.

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Figure 1

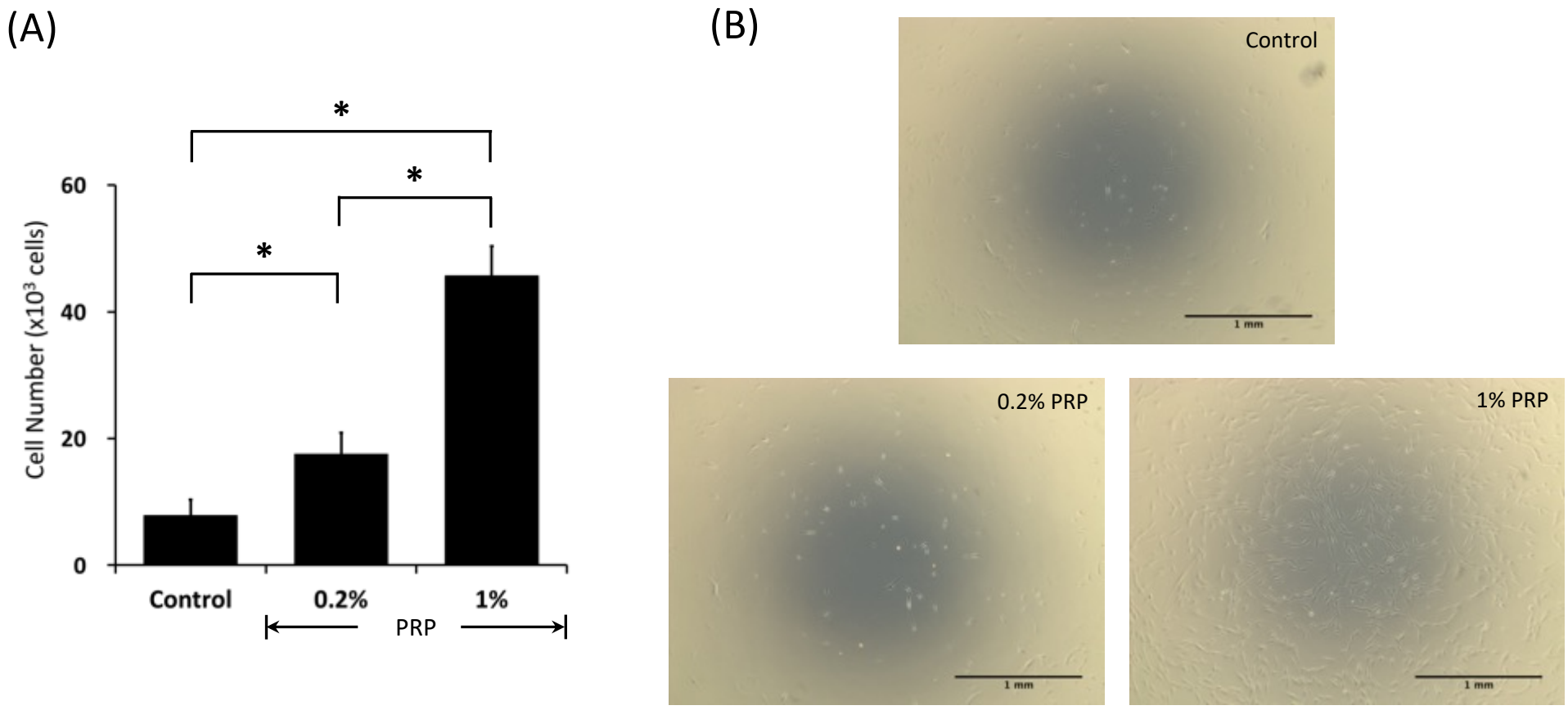

Table 1

	Platelets (x 10^{10}/L)	PDGF-BB (ng/mL)	EGF (pg/mL)	IGF-I (ng/mL)
Plasma	17.92 ± 1.4	1.5 ± 0.1	537 ± 7	224 ± 4
PRP	205.3 ± 34.8	38.8 ± 0.8	640 ± 21	147 ± 3
Ratio (PRP/Plasma)	11.5	25.9	1.2	0.7

Name of Material/ Equipment	Company
20 mL Syringe, Terumo syringe lock type	Terumo, Tokyo, Japan.
50 mL Tube, Polypropylene Conical Tube	Corning, NY, USA.
Automated Hematology System	Sysmex Corp., Tokyo, Japan
Blood Collection Needle, SafeTouch PSV set with luer adapter, 21 G x 3/4”	Nipro, Osaka, Japan.
Blood Collection Tube, ACD Solution A Blood Collection Tube, 8.5 mL	BD Vacutainer, NJ, USA.
Blood Collection Tube, Serum Blood Collection Tube/monovette, 10 mL	BD Vacutainer, NJ, USA.
Calcium Chloride, 1 mEq/ mL,	Otsuka Pharmaceutical Factory, Tokushima, Japar
Cannula, BS non-bevel needle, 18 G (1.2 mm) x 75 mm	BS Medical, Tokyo, Japan.
Cell Counting Kit-8	Dojindo Molecular Technologies, Kumamoto, Jap
Centrifuge	Kokusan, Tokyo, Japan.
Centrifuge	Eppendorf, Hamburg, Germany.
EnSpire 2300 Multilabel Reader	PerkinElmer, Inc., Waltham, MA, USA
Filter Unit	Merck Millipore, Co. Cork, Ireland.
Human EGF Quantikine ELISA Kit	R&D Systems, Minneapolis, MN, USA
Human IGF-I Quantikine ELISA Kit	R&D Systems, Minneapolis, MN, USA
Human PDGF-BB Quantikine ELISA Kit	R&D Systems, Minneapolis, MN, USA
Pipette Tip, ART 1000 Reach Barrier Tip	Thermo Scientific, MA, USA.
Pipette, Nichipet EXII 100-1000 µL	Nichiryo, Saitama, Japan.
Sterling Nitrile Power-Free Exam Gloves	Kimberly-Clark
Yamazen Alcohol for Disinfection	Yamazen Pharmaceutical, Osaka, Japan.

Catalog Number	Comments/Description
SS-20LZP	
352070	
XE-2100	
32-384	
364606	
366430	
3215400A1061	
BS-81007	Not for sale
CK04	
H-19F	
5415R	
SLGP033RS	
DEG00	
DG100	
DBB00	
2079-05-HR	
00-NPX2-1000	
50707	
A7L07	

Dear Journal of Visualized Experiments Editorial Office, review editor:

Thank you for your letter and comments concerning our manuscript entitled: “Method of Preparing Activated Platelet-Rich Plasma for Culturing of Human Adipose-Derived Stem Cells” by Lai, Kakudo, Morimoto, Ma and Kusumoto. We have considered the comments carefully and made revisions that we hope will meet with your approval. We look forward to the publication of our manuscript in the Journal of Visualized Experiments.

Our responses to the editorial comments are as follows:

• *Comment 1: I have tried to fix some of the grammatical errors, but from the protocol onwards these were too numerous to fix (almost every sentence needs fixing). Please employ a professional language editing service; several statements are impossible to understand. JoVE will not copyedit for you.*

→We consult International Science Editing (http://www.internationalscienceediting.com) for editing this manuscript.

• *Comment 2: Line 108. How is counting performed?*

→We described the platelet counting in Line 167: 7.1. Count platelet number in whole plasma and PRP using an automated hematology system (see table of materials).

• *Comment 3: Line 111. At what temperature?*

→If it is without special instructions, the centrifugation are all under room temperature.

• *Comment 4: Line 117. Long needle? Mention gauge*

→We described the Long Cannula in the *Table of Materials.* Cannula, BS non-bevel needle, 18G (1.2mm) x 75mm.

• *Comment 5: Line 117. Please clarify: some RBCs are also collected, correct?*

→Yes, during this step, some RBCs are also collected.

• *Comment 6: Line 122. At what temperature?*

→Same with Comment 3, it is under room temperature.

• *Comment 7: Line 125. Unclear what is meant and what is being done here.*

→As described in the manuscript, “*Platelets that accumulated in the thrombocyte pellet in 1 mL plasma remained were used as PRP*”. The “*Serum Blood Collection Tube containing 1 mL liquid*”

is used as a measure.

The schematic diagram is as follows.

• *Comment 8: Line 126. Gauge?*

→Same with Comment 4, the Long Cannula was described in the *Table of Materials.* Cannula, BS non-bevel needle, 18G (1.2mm) x 75mm.

• *Comment 9: Line 126. Is this tube empty?*

→Yes, the 50 mL tube is empty.

• *Comment 10: Line 127. Unclear why 1 mL*

→Because the platelets are accumulated in the lower part of the layered plasma. Depend on different protocols, the platelets collection methods are different. Thus, we decide 1 mL of plasma in the bottom need to be collected.

• *Comment 11: Line 132. How is counting performed?*

→As we described in Line 167~168, "*7.1. Count platelet number in whole plasma and PRP using an automated hematology system (see table of materials).*"

• *Comment 12: Line 134. Here you say 400, then a few words later you say 500. Which is it?*

→Blood collected for prepare PRP this time are not sufficient for 500 μL per tube, thus we describe 400 μL this time. Still, 500 μL PRP per tube is recommended.

• *Comment 13: Line 139. Cannot comprehend.*

→This sentence means, use a Serum Blood Collection Tube to collect 10 mL non-anticoagulant whole blood.

• *Comment 14: Line 142. At what temperature?*

→Same with Comment 3, it is under room temperature.

• *Comment 15: Line 157.?*

→It is a typing mistake, the sentence should be "6.1. Spin activated PRP at 9000 × g for 10 min, in a 4 °C laboratory centrifuge."

• *Comment 16: Line 159. Mention needle gauge.*

→This step, we use pipette to collect supernatant to a syringe chamber. We didn't use the needle.

• *Comment 17: Line 175. What is the source of the cells? Please add to the table of materials.*

→We isolate hASCs from human adipose tissue that obtained by plastic surgery. The isolation method was described previously[1].

• *Comment 18: Line 175. Mention culture temperature, humidity, CO2 etc*

→hASCs were cultured in 37℃, CO2 0.5%, humidity as normal.

• *Comment 19: Line 179. What are the concentrations?*

→The concentration of PRP are 0.2% and 1%, that was described in Line 206~207 of Result Section.

• *Comment 20: Line 182. Do you remove the DMEM+PRP before added WST-8?*

→We direct add WST-8 reagent without remove the DMEM+PRP.

• *Comment 21: Line 187. Replace the product name with a generic alternative.*

→As you suggested, we change "Cell Counting Kit-8" into "WST-8".

• *Comment 22: Line 202. Please provide this as an excel file. Table 1 appears to have 2 tables. These should be Table 1 and Table 2.*

→Actually, there is just one table. We correct this table and submit a excel file also.

• *Comment 23: Line 207. Provide fig 1 as an individual figure fie. All panels must be labeled and presented on a single page.*

→As suggested, we combined Fig 1A & 1B into a single file, and labeled them on a single page.

• *Comment 24: Line 215. Define the error bars.*

→/

• *Comment 25: Line 219. Add legends for the tables.*

→？？？

• *Comment 26: Line 241. Please supply machine-independent relative centrifugal force (g) values instead.*

→These speeds are used in previous studies. We don't have enough information to convert them into the centrifugal gravity.

• *Comment 27: Line 257. Remove commercial names*

→These contents are cited from previous studies about different commercial PRP separation system. Thus, we use the commercial names as it was.

• *Comment 28: Line 272. Remove commercial names. They can be listed in the table of materials instead.*

→Same with Comment 26, these contents are cited from previous studies about different commercial PRP separation system. Thus, we use the commercial names as it was.

• *Comment 29: Line 273. Add citation number*

→As suggest, we add the citation number.

we are looking forward to your reply.

Best regards,

Sincerely yours,
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Reference

1. Lai F, Kakudo N, Morimoto N, Taketani S, Hara T, Ogawa T, Kusumoto K: **Platelet-rich plasma enhances the proliferation of human adipose stem cells through multiple signaling pathways.** *Stem Cell Res Ther* 2018, **9:**107.