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Conflict of interest

The authors declare no conflict of interest.

healing.

Materials and Methods: We prepared L-PRPs using different centrifugation methods and assessed their regenerative potential in an *in vivo* rat model. Bilateral critical size tibial bone defects were created and filled with either: single spin L-PRP, double spin L-PRP, or filtrated L-PRP. Empty defects and defects treated with collagen scaffolds served as controls. Rats were euthanized after two weeks, and their tibias were collected and analysed using micro-CT and histology.

Results: Double-spin L-PRP contained higher concentrations of platelets than single-spin L-PRP and filtrated L-PRP. Filtration of single-spin L-PRP resulted in lower concentrations of minerals and metabolites. *In vivo*, double-spin L-PRP improved bone healing by significantly reducing the size of bone defects (1.08±0.2 mm ^{3}) compared to single-spin L-PRP (1.42 ±0.27 mm filtrated- L-PRP (1.38±0.28 mm ^{3}). There were fewer mast cells, lymphocytes, and macrophages in defects treated with double-spin L-PRP than in those treated with single-spin or filtrated- L-PRP.

Conclusion: The preparation method of L-PRP affects their composition and potential to regenerate bone.

Key words: platelet concentrates, platelet-rich plasma (PRP), critical size defect, bone formation, bone healing, rat surgery.

therapeutic effects.

Principal findings: We have found the extent of healing in bone defects to be directly related to the concentration of platelets in L-PRPs. Double spin L-PRP resulted in better bone healing compared to single spin or filtrated L-PRP.

Practical implications: The use of double spin L-PRP may enhance their regenerative potential in clinical practice.

1. Introduction

Bone fractures represent a major health concern worldwide. Every year, around 8 million US patients suffer from bone fractures and may develop further complications such as delayed healing and non-union (Victoria, Petrisor, Drew, & Dick, 2009; Yamamoto, D'Avila, & Luz, 2013). Furthermore, pathological bone defects can cause significant morbidity. Different types of bone grafts including autogenous bone grafts, allografts, xenografts, and alloplasts as well as biological materials such as platelet concentrates (PCs), have been used to restore bone defects and manage bone fractures (Albanese, Licata, Polizzi, & Campisi, 2013; Kumar, Vinitha, & Fathima, 2013; Lawson & Biller, 1982).

PCs are autologous materials obtained from centrifuged whole blood; they are classified into four main types based on their content of leukocytes and fibrin architecture: Pure Platelet-Rich

angiogenesis, and tissue regeneration(Faez Saleh Al-Hamed et al., 2019). These GFs, which are released from platelet alpha granules, include transforming growth factor beta (TGF-beta), fibroblast growth factor (FGF), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF) and insulin-like growth factor (IGF) (Masuki et al., 2016). Indeed, in addition to GF-rich alpha granules, platelets express dense granules, which release molecules that could have a negative effect on bone healing such as serotonin, adenosine triphosphate (ATP), and polyphosphate (Badran, Abdallah, Torres, & Tamimi, 2018). Thus, the balance between platelet pro-osteogenic and anti-osteogenic properties is important in defining the quality of PC preparations.

PCs have been extensively used in dentoalveolar and maxillofacial surgery (F. S. Al-Hamed et al., 2019; F. S. Al-Hamed, Tawfik, Abdelfadil, & Al-Saleh, 2017; Dragonas, Schiavo, Avila-Ortiz, Palaiologou, & Katsaros, 2019). PCs has been reported to enhance soft tissue healing, reduce pain and discomfort, but in regard to bone regeneration, there was a great heterogeneity of the available studies and limited number of RCTs, which did not lead to a robust conclusion (Donos, Dereka, & Calciolari, 2019; Sanz et al., 2019). This could be explained by differences in study designs and types of PCs (Badran et al., 2018; Del Corso et al., 2012; Harrison, 2018). The concentration of platelets in PCs could play a key role in bone healing. *In vitro* studies showed that PCs with a high platelet concentration improved osteoblast proliferation, although these

protocol, a blood sample is collected in a tube with anticoagulant and immediately centrifuged. This short step separates red blood cells from platelet-rich plasma, whereas in the double spin protocol, whole blood is centrifuged twice, resulting in higher concentrations of platelets in PRPs (El-Husseiny, Saleh, Moustafa, & Salem, 2020). Furthermore, the use of single spin and double spin PRP protocols may produce variable concentrations of metabolites and other molecules, which could affect their regenerative potential.

The clinical evidence shows high heterogeneity among different studies in regard to the efficacy of different types of PCs on bone regeneration. In addition, different PRP preparation protocols may produce variable concentrations of platelets, metabolites and other molecules, which could affect their regenerative potential. Therefore, we aimed to investigate how differences in PCs composition would influence their ability to regenerate bone. In this study, we chose to test the changes in the composition of platelet rich plasma (PRP), not the platelet rich fibrin (PRF) as its unfeasible to modify platelet concentration or to filter small molecules when using PRF due to the formation of the fibrin clot. We first prepared PRP using a single step centrifugation protocol (single spin L-PRP) or two step protocol (double spin L-PRP). To assess the effect of L-PRP-derived small molecules and metabolites on bone healing, we developed a new centrifugal filtration protocol using dialysis centrifugal concentrators to produce filtrated L-PRP. The regenerative performance of different PRPs was assessed *in vivo* using a rat model of critical size tibial bone defects.

this study, of them 11 rats were used for Leukocyte and Platelet-Rich Plasma (L-PRP) preparation and the remaining 24 rats were used for bone defect surgeries.

2.2.1 L-PRP Preparation and characterization

A total of 11healthy Sprague Dawley rats were used for Leukocyte and Platelet-Rich Plasma (L-PRP) preparation. Blood samples were collected via cardiac puncture in 10 ml acid citrate dextrose tubes (BD Vacutainer™). From each rat, two tubes were collected. The blood retrieved was immediately used to produce three different types of L-PRP as follows: single-spin L-PRP, double-spin L-PRP, and filtrated- L-PRP. In this study, a centrifuge with a fixed angle rotor (centrifugation radius = 12 cm, Eppendorf 5810R) was used. The centrifugation forces (g) ranged from 100 g to 4000g (1092rpm to 5460 rpm) (Miron, Pinto, Quirynen, & Ghanaati, 2019; Perez et al., 2014; SLICHTER & HARKER, 1976). The different plasma samples obtained from different rats were pooled together to eliminate the inter-individual differences (such as differences in complete blood count between rats), thus this makes it easier to compare the differences in the preparation protocols (Figure 1).

Single-spin L-PRP

Blood samples were centrifuged at 160g-400g for 15 minutes at room temperature. This centrifugation separated blood into two components: the red blood cells (RBCs) in the bottom and the single spin L-PRP in the top of the centrifugation tube. Then, the RBCs layer was

Then, the RBCs layer was discarded, and the platelet-rich plasma was collected in a separate tube. The platelet-rich plasma was centrifuged at 4000g for 30 minutes. Then, the platelet pellet was resuspended in 50% original volume of plasma to prepare double-spin **L-PRP**, while the supernatant layer was discarded. The platelet activator ($CaCl_2$, 23mM) was a surgery to produce **L-PRP** gel.

Filtrated L-PRP

This procedure was developed to obtain a similar concentration of platelets as that obtained with single spin **L-PRP**, while modifying its composition by reducing the concentration of **L-PRP** metabolites and small molecules. First, single spin **L-PRP** was obtained as described above. The platelet activator ($CaCl_2$) was added in order to release the small molecules and allow their removal with the dialysis centrifugal concentrator before the **L-PRP** clots**.** The small molecules and metabolites were removed using a dialysis centrifugal concentrator (Amicon Ultra-15, Millipore, Sigma) with a 3 kDa cut-off. Saline was added to maintain consistent volume and concentration of platelets.

A range of centrifuging forces (500g-4000g) and times (5-40 min) , as well as of volumes of saline (1:1, 1:0.5 ratio) and activator (0 mM, 3mM, 23mM) were tested and optimized in order to maintain a constant concentration of platelets while modifying the composition of **L-PRP** (Fig S1,S 2).

competitive ELISA-kit (Abcam 155035, Canada); (Almishri, Shaheen, Sharkey, & Swain, 2019).

The chemical elements compositions of single spin L-PRP, double spin L-PRP, filtrated L-PRP, and filtrate samples were measured using inductively coupled plasma optical emission spectrometry (ICP-OES, Thermo Scientific iCAP 6500, Cambridge, UK) as described previously (Faez Saleh Al-Hamed et al., 2021; Hudson et al., 2015). Metabolites of different samples were extracted and analysed based on targeted metabolite analysis using ion paring liquid chromatography–mass spectrometry (LC-MS) or gas chromatography-MS (GC-MS) as described previously (Faez Saleh Al-Hamed et al., 2021; Vincent et al., 2015).

2.2.2 Collagen scaffolds preparation

In this study, collagen scaffold was used as a carrier for the filtrate material and as a control. Collagen sponges were prepared as described previously (Varley et al., 2016).

***In vivo* experiments:**

2.3.1. Animals

A total of 24 healthy female Sprague Dawley rats (10-12 weeks-old, Charles River Laboratories, Montreal, QC), weighing 200–250 g were housed (2 animals per cage) in the Genome Animal Facility of McGill University. Water and a rodent diet were provided *ad libitum*, and rats were monitored daily by a veterinarian in the animal facility. Additional seven rats were used for **L-PRPs** preparation as described in the characterization section.

divided by using sealed envelope method into 6 subgroups where the following materials were tested for their efficacy to promote bone healing: single spin L-PRP, double spin L-PRP, filtrated L-PRP, collagen-filtrate scaffold, collagen scaffold, and empty defect (Figure 2, Figure S3).

Postoperatively, rats were monitored daily and caprofen (5mg/kg) was used to control pain for the first three days after the surgery. Out of the 24 rats that underwent bone surgeries, 4 rats were euthanized during the first three days due to open wounds and bone fractures and thus were excluded them from the analysis. Rats were euthanized after two weeks using an overdose of CO2, and their tibiae were collected and analysed using micro-CT and histology. The same bone samples used for the Micro-CT analysis, were also used for the histological analysis.

2.3.1 Micro-computed tomography

Micro-computerized tomography (Micro-CT) analysis was conducted as described previously by our team (Al-Subaie et al., 2016). Briefly, tibiae with bone defects were scanned using a micro-CT (Sky-Scan1172; Bruker, Kontich, Belgium) set at a 12.7 µm resolution, a 50 kV voltage, a 0.5 rotation step, a 10 random movement and a 0.5 mm aluminum filter. The region of interest (ROI) was defined as the original defect (2.5 mm Ø) and analysed by the CT-analyser software (SkyScan; Bruker, Kontich, Belgium). Three-dimensional (3-D) bone parameters were calculated based on the 3-D reconstructed images.

Leica). Five sagittal sections, which include the center of the defect, were obtained from each bone sample. Sections were stained with Tartrate-Resistant Acid Phosphatase (TRAP), Hematoxylin and Eosin (H&E), and Acidified Toluidine Blue (aTB) stains to assess the number of osteoclasts, mononuclear immune cells (macrophages and lymphocytes), and mast cells respectively. Von Kossa stain was used to measure the percentage of new bone formation.

2.4 Blinding: The retrieved L-PRPs samples and bone samples were anonymously labeled. Micro-CT, whole blood count, minerals, and metabolite analyses were performed by a researcher blinded to the group allocation.

2.5 Statistical analysis

Sample size was calculated based on 95% confidence interval and a statistical power of 80% to be able to reject the null hypothesis that the regenerative ability of different PCs is comparable. Considering a 20% mean change of the defect size to be clinically relevant and a 12 % potential standard deviation was assumed based on previous studies, a total of 6 defects were determined to be adequate for each treatment group (Ahmed Al Subaie et al., 2016; Malhotra, Pelletier, Yu, Christou, & Walsh, 2014).

The primary outcome was defect size measured in mm^3. The secondary outcomes were cortical thickness, trabecular thickness, trabecular number, and trabecular separation. Normality of data was tested using Shapiro-Wilk test. Analysis of Variance (ANOVA test) was used when

3. RESULTS

3.1. Compositional differences in PCs

The main characteristics of L-PRPs obtained by single-spin, double-spin and filtration are presented in Table 1 and Figure 3. As expected, double-spin L-PRP had higher platelet concentrations (3162±1179 x 10^9/L) in comparison with single-spin L-PRP (1077±134 x 10^9/L) and whole blood (632±146 x 10^9/L). Filtration of single-spin L-PRP did not alter significan their platelet counts and the levels of Fe, P, Zn, and Ni as compared with single-spin L-PRP. However, filtrated L-PRP had significantly decreased levels of Cu, K, Mg, Na, Se and serotonin, and increased levels of Ca than single-spin PC. Double-spin L-PRPs were comparable to single-spin L-PRPs in terms of their chemical element levels, with the exception of higher levels of P and Se, and serotonin.

Metabolite analysis showed a decrease in the relative concentration of metabolites in filtrated L-PRP and filtrate samples such as nucleosides, citric acid cycle intermediates, pentose phosphate pathway, and water-soluble oxidative stress indicators (except glutathione) compared to single or double spin L-PRPs.

3.2. The effect of different types of L-PRPs on bone healing

Micro-CT analysis showed that bone-defects treated with double-spin L-PRP presented with smaller defect sizes (1.08 mm^3 ±0.2 mm^3) compared to defects treated with filtrated L-PRP (1.3

Histological and histomorphology analyses (**Fig. 6**) showed that defects treated with single-spin L-PRP (4.1±1.8 x 10^3 cell/mm^2), filtrated L-PRP (4.2±1.1 x 10^3 cell/mm^2), coll combination (4.9±1.6 x 10^3 cell/mm^2) or collagen alone (3.7±1.9 x10^3 cell/mm^2) macrophage and lymphocyte infiltration compared to empty defects (6.3±1.1 x 10 p>0.05), whereas the double-spin L-PRP presented lower number of macrophages and lymphocytes (3.4±1.0·10^3 cell/mm^2, p=0.01).

The percentage of mineralized bone in filtrated L-PRP (43±10%), single spin PC (44±7%), double spin L-PRP (48±5%) was similar to empty defect (43±5%), whereas higher than collagen-filtrate combination (29±11%, p = 0.03, 0.04, 0.01, respectively) or collagen alone (27±16%, p= 0.049, 0.08, 0.03, respectively).

Mast cell infiltration was significantly higher in filtrated L-PRP (165±111 cell/mm^2) spin L-PRP (84±7 cell/mm^2) compared to double-spin L-PRP (20±2 cell/mm^2; 0.001 respectively), or empty defect (34±4 cell/mm^2; p= 0.040 and 0.001, respe Furthermore, collagen-filtrate composite presented higher mast cell infiltration compared to collagen alone (86±4 vs 29±5 cell/mm^2, p=0.003).

4. Discussion

This is the first study to assess the effect of different L-PRP compositions on bone healing while controlling for inter-individual variability by using head-to-head comparisons of various L-PRP obtained from the same animals. Taken together, our results suggest that increased platelet

et al., 2012; Nagata et al., 2010). Such increase in platelet concentration could explain the better bone regeneration observed in defects treated with double-spin L-PRP. In agreement of our findings, it has been reported that a medium concentration of platelets (2.65 10^9/mL), which was obtained in the current study by using double spin L-PRP, promoted osteoblast proliferation, high concentrations of platelets (8.21 10^9/mL ± 0.4 10^9/mL) osteogenic proliferation, whereas a low concentration (0.85 10^9/mL ± 0.16 10^9/mL) obtained by using single L-PRP and filtrated L-PRP, had no effect on osteogenesis (Chen et al., 2013; Kawasumi et al., 2008).

Bone regenerated with double spin L-PRP presented lower trabecular separation, and higher trabecular number compared to non-treated defects, which is in agreement with other studies showing that it also improves the trabecular pattern and reduces trabecular separation in humans and animals, respectively (Alissa, Esposito, Horner, & Oliver, 2010; Huang et al., 2019). Also, the size of defects treated with double spin L-PRP was significantly reduced compared to defects treated with single spin or filtrated L-PRP, but not compared to empty defects. The reduction in the defect size was around -0.4 mm^3, which was statistically significant compared to def treated with single spin L-PRP or filtrated-L-PRP. However, the small difference in the defect size seems to be less clinically relevant. These positive effects could be explained by the rich content of L-PRP-derived regenerative proteins such as GFs [e.g. platelet-derived growth factor (PDGF), transforming growth factor (TGF), Insulin-like growth factor (IGF), and bone

metabolites critical to the citric acid cycle, pentose phosphate pathways (PPP), and nucleotides compared to single or double spin L-PRPs. Such differences occurred due to the use of the filter and adding saline and $CaCl_2$ to the filtrate samples prior to second centrifugation. The citric acid cycle is a key essential metabolic pathway for body energy supply (Akram, 2014). The pentose phosphate (PP) pathway is an essential component of cellular metabolism, which maintains carbon homeostasis, anabolism, and provides precursors for nucleotide and amino acids (Stincone et al., 2015).

Our results showed that collagen scaffolds interfere with bone defect healing and adding the filtrate material did not modify their effect on bone healing. These results could be explained by the slow degradation of collagen scaffolds that could be attributed to a high degree of crosslinking. Furthermore, the reduced mechanical strength and osteoinductive potential of collagen scaffolds limit their applications for bone regeneration procedures (Zhang, Wu, Chen, & Lin, 2018). In agreement with our results, the implantation of collagen scaffold in rats did not induce bone formation, whereas the incorporation of bioactive glass into collagen scaffolds has been found to improve scaffold bioactivity and osteogenesis (Zhang et al., 2018).

Our histomorphology results showed comparable inflammatory cell infiltration between defects treated with collagen-filtrate composite and collagen alone except mast cell infiltration which was significantly increased in collagen-filtrate composite. Mast cells have been identified as regulatory elements of inflammation and bone turnover during bone healing and their numbers

as PCs (Ibrahim et al., 2017). Taken together, these findings suggest that mast cell quantity and activity is influenced by PRP composition.

In the present study, the regenerative potential of different type of PRPs were assessed using a tibial bone defect rat model. This bone defect model is considered as a minimally invasive surgery and reduces animal suffering. We have already used it in our previous studies with minimal complications (Faez Saleh Al-Hamed et al., 2020; Faez Saleh Al-Hamed et al., 2021). However, in the current study, 4 rats were euthanized because of bone fractures. This occurred due to misplacement of the drilled defect which weakened the bone and thus lead to bone fracture.

Overall, the application of different L-PRP showed non-significant differences in regard to defect volume and BV/TV compared to empty defects (control group). This could be due to the fact that this study was performed in a young group of rats that have high regenerative ability as well as the tested groups were assessed within the same animal. This may mask the real effect of L-PRP compared to controls. Also, due to the small sample size, we may not be able to see differences in the healing between L-PRP and controls. Therefor, further studies with larger sample size are required. Furthermore, the comparable healing observed in the empty defects raises a concern regarding the suitability of this bone defect model. However, this model is considered a reliable model to study the effect of medications on bone healing based on previous

This study was the first to show the effect of changes in L-PRP composition on bone healing by understanding the differences between single-spin L-PRP, double-spin L-PRP, and filtrated L-PRP as well as their bone regenerative potential. The main limitation of this study was the effects of **L-PRP**s on bone healing was assessed only at one-time point, which was two weeks after surgery. This two-week time point was selected as it allows accurate assessment of the early stages of bone healing in terms of osteoblastic activity induced by medications or biological materials as reported in our previous studies(Faez Saleh Al-Hamed et al., 2020; Faez Saleh Al-Hamed et al., 2021; A. Al Subaie et al., 2016). Furthermore, assessing bone healing at shorter timepoints (< one week) don't show anything on Micro CT because there is no mineralization yet and longer timepoints don't show differences because the defect is completely healed as we performed the experiment in a young group of rats which have rapid healing ability. However, further studies will be needed to study the long-term plasma on bone healing. Also, in this study, we assessed on the effect of composition changes of PRPs on bone healing, not the soft tissue healing. Therefore, further studies are required to study the effect of changes in PRPs composition on soft tissue healing.

Conclusion

Changes in compositional components of L-PRPs affects their potenial to regenerate bone. High platelet concentration enhanced the healing of bone defects. Adding the filtrate material to collagen scaffolds did not modify their effect on bone healing.

Von Kossa, TRAP, and ALP stained bone samples. LA assessed in Micro-CT analysis and literature review. RR helped in plasma preparation and serotonin analysis. SS, prepared collagen scaffolds and revised the manuscript. JR performed histological assessment of mast cell infiltration. OE performed histological assessment of immune cell infiltration. HM helped in bone surgery experiments. QG helped in bone surgery. TB helped in ELISA analysis. LB, JT, and LR performed the serotonin release experiment. ST revised the manuscript, ML, contributed to the study design, revised and edited the manuscript, MK contributed to the study design, revised and edited the manuscript, ZB and FT contributed to the study design, interpretation, edited and revised the manuscript. All authors approved the final version of the manuscript.

Data availability statement

The data that support the findings of this study are available as a supplementary file.

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metabolomics, and histopathology Centres at MUHC for their help in PRP preparation and analysing the data.

6. Figure Legends:

Figure 1: Photographs showing bone defect surgery. (a) a unicortical bone defect was created in the lateral surface of tibial metaphysis and left empty as a control. (b) Bone defect filled with single spin L-PRP. (c) Bone defect filled with double spin L-PRP. (d) Bone defect filled with filtrated L-PRP. (e) Bone defect filled with collagen scaffold. (f) Bone defect filled with collagen scaffold and the filtrate material.

Figure 2: A diagram showing the steps followed in the preparation of single spin L-PRP, double spin L-PRP, filtrated L-PRP, and filtrate samples.

Figure 3: PC composition analysis. (a) Hierarchical clustering heatmap showing the fold changes in the levels of selected metabolites measured in single spin L-PRP, double spin L-PRP, filtrated L-PRP, and filtrate. Red color indicates high concentration, while blue color indicates low concentration. (b) concentration of chemical elements. (c) serotonin level. The letter "a" indicates significant difference compared to filtrate, the letter "b" indicates significant difference compared to compared to filtrated L-PRP, and the letter "c" indicates significant difference compared to single spin L-PRP.

Figure 4: Sagittal, coronal, trans-axial, and 3-D μ-CT images of bone defects showing impaired bone healing in defects treated with collagen-filtrate composite and collagen alone compared to other treatment groups. Defects treated with double spin L-PRP showed reduced defect volume compared to other treatment groups.

Figure 5: Micro-CT data analysis of bone defects treated with different treatments for the following parameters: (a) defect volume, (b) trabecular number (Tb.N), (c) trabecular thickness (Tb.Th), (d) bone volume/tissue volume (BV/TV), (e) cortical thickness, and (f) trabecular separation (Tb.Sp). The letter "a" indicates significant difference compared to collagen groups. The letter "b" indicates significant difference compared to double spin L-PRP.

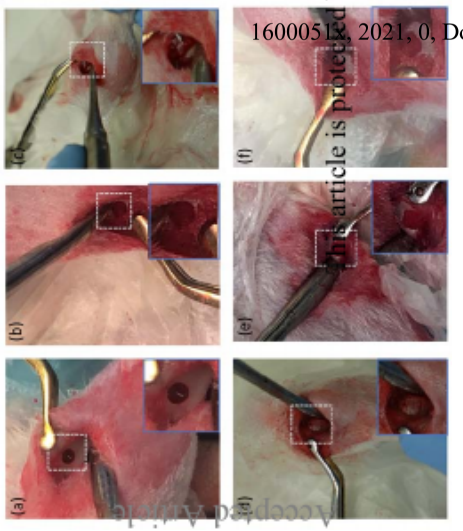
"d" indicates significant difference compared to filtrated L-PRP. The letter "e" indicates significant difference compared to collagen alone.

7. Table Legend:

Table 1: Complete blood count of whole blood and different L-PRPs.

RBCs x1 0^{12} /L	5.65±0.58	0.22±0.23	0.39±0.06	0.18

CBC, complete blood count, WBCs, white blood cells, RBCs, red blood cells

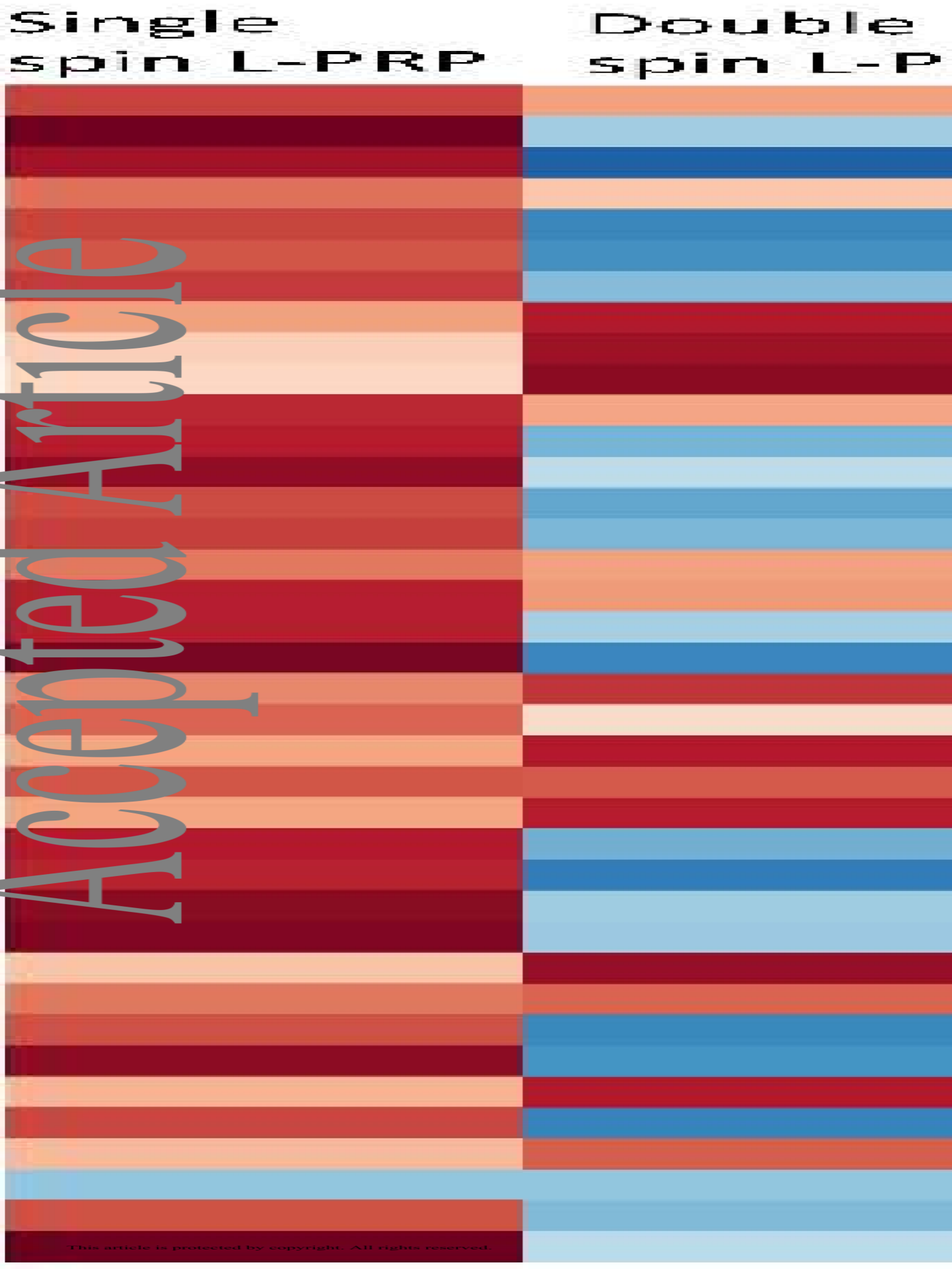


Whole blood

1st

(160

(a)



Empty

Sagittal

Coronal

Transaxil

3-D

(a)

Von- Kossa

(b)

T.B

(c)

H&E