IMPACT OF DIFFERENT PRP CENTRIFUGATION SPEEDS ON IMMUNOHISTOCHEMICAL EXPRESSION OF RANK, RANKL AND ALKALINE PHOSPHATASE IN EXPERIMENTALLY INDUCED PERIODONTITIS

Mahitabe Elgamily * and Mona Denewar *

ABSTRACT

Objectives: This study was conducted to evaluate the effect of two single centrifugation speeds used in platelet rich plasma (PRP) preparation on the expression of osteoclastogenic markers RANK, RANKL and the osteogenic marker Alkaline phosphatase (ALP) in experimentally induced periodontitis in a rat model.

Material and Methods: Thirty four adult male Wistar rats, weighing 150-200 g were selected. Four rats for PRP preparation. Ligatures were placed subgingivally around the lower incisors of thirty rats in figure 8 manner. Ligature control was performed daily then after 14 days ligature removal was done and the rats were subsequently divided into 3 equal groups (n=10); group I: control non treated, group II: injected with 0.2ml PRP prepared with 800 rpm, group III: injected with 0.2ml PRP prepared by 3000 rpm. Five rats of each group were sacrificed after 3 and 6 weeks, the specimens were processed, then evaluated using haematoxylin and eosin, RANK, RANKL and ALP. The results were subjected to digital morphometric assessment followed by statistical analysis.

Results: Both PRP treated groups showed better alveolar bone regeneration compared to control non treated group especially at the 6th week. Group II had better regenerative capacity compared to group I and III as it showed significant increase in the amount of ALP and significant decrease in the RANK and RANKL osteoclastogenic markers.

Conclusion: Local injection of PRP with both tested centrifugation speeds enhanced the regenerative capacity of bone in experimentally induced periodontitis. However, the lower centrifugation speed (800 rpm) have better regenerative effect.

KEYWORDS: Periodontitis, RANK, RANKL, Alkaline Phosphatase, PRP.

* Lecturer of Oral Biology, Faculty of Dentistry, Mansoura University
INTRODUCTION

Periodontal disease is a multifactorial complex disease of the periodontium which may be caused by periodontopathic bacteria accumulated on the tooth surface, it is characterized by loss of attachment of connective tissue with subsequent damage of the tooth supporting structures involving alveolar bone resorption\(^1\,^2\). Periodontitis is mediated by the inflammatory reaction in the host against the bacteria in the dental tissue \(^3\). The disruption of the balance between osteoblast and osteoclast activities that is formed by bacterial products and inflammatory cytokines is the main underlying cause of inflammatory induced bone loss. Complex inflammatory signals and cytokines regulate osteoclastogenesis\(^4\).

Subgingival gram-negative organisms producing lipopolysaccharides cause inflammation of the periodontal tissues involving a local response, which implies infiltration of polymorph nuclear leukocyte, liberation of reactive oxygen species and inflammatory mediators\(^2\,^5\).

The target of periodontal therapy is the elimination of the inflammatory process, prevention of regression and regeneration of the lost periodontal tissue by construction of new cementum, new periodontal ligament and new bone that became a challenge for clinicians \(^6\). The gold standard for the managing periodontitis is scaling and root planning. However, mechanical methods are not enough in the treatment of severe destructive periodontal cases. Limitation of this approach led to development of alternative methods as an adjunct to the prime one \(^7\).

Periodontal healing necessitates a sequence of interactions between the responsible cells such as gingival fibroblasts, epithelial cells, osteoblasts and periodontal ligament cells. Any disturbance of vascularization during this process results in platelets clustering, and the release of numerous growth factors into tissue from platelets. There is growing evidence that the growth factors and the cytokine content in platelets play a crucial role in wound healing. Platelets also secrete vitronectin, fibrin and fibronectin which act as a matrix for connective tissue. This led to the idea of utilizing platelets as a therapeutic tool for improvement of tissue repair, especially in the periodontium\(^8\).

Platelet rich plasma is identified as a high concentration of platelets suspended in a small volume of plasma. The positive effects of PRP are accredited to its mitogenic, angiogenic and proliferative abilities of growth factors, such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and transforming growth factor (TGF) \(^9\,^10\). The first generation of platelet concentrates is platelet rich plasma. It can be prepared using centrifugation which could be done in either one or two steps (i.e., single or double spin) using several forces and centrifugation times. Moreover the centrifugation time was ranging from 8 to 30min \(^11\). The mostly used centrifugation forces range from 100 to 1000 rpm \(^12\,^13\), however, other protocols used forces as high as 3,731 rpm \(^13\).

So the aim of this study is to evaluate the effect of two single centrifugation speeds used in PRP preparation on the expression of osteoclastogenic markers RANK, RANKL and the osteogenic marker ALP in experimentally induced periodontitis in a rat model.

MATERIAL AND METHODS

All experimental procedures were done according to the guides of Ethical Committee of Faculty of Dentistry, Mansoura University, Egypt.

Animals

Thirty four adult male, pathogen free, Wistar rats, weighing 150-200 g were selected for this study. They were acclimatized for at least 3 days before starting the study. Rats were housed in individual polypropylene cages in Medical Experimental Research Center in Faculty of Medicine, Mansoura
University. The rats were kept under controlled conditions (22±3ºC temperature, 55-60% relative humidity) with a 12:12-h light-dark cycle in a light-controlled room. They received standard soft high-carbohydrate diet and water for rodents.

**Study design:**

**Preparation of platelet rich plasma:**

Rat PRP was obtained by drawing the whole blood of 4 male Wistar rats by a cardiac puncture into tubes containing 3.8% sodium citrate. Then shaking of the tubes was done by figure eight “8” pattern 20 times gently in order to make proper mixing of blood with sodium citrate (anticoagulant) without breaking down of platelets. The PRP was obtained from this anticoagulated blood after centrifugation at 800 rpm and at 3000 rpm for 15 min at 25ºC. Three milliliters of blood resulted in 150 mL of PRP. The platelets present in the whole blood and in PRP were counted automatically using a hematology analyzer\(^{[14]}\). PRP preparation was performed at the time of beginning of treatment.

General anesthesia was performed to thirty rats via intraperitoneal injection of a mixture of Xylazine (5mg /kg) and Ketamine (75mg /kg) (Egyptian Inter Pharmaceutical Industries Co, 10th Ramadan City, Egypt)\(^{[15]}\).

**Induction of periodontitis:**

After anesthesia was performed the lower incisors were ligated with 4/0 non-resorbable sterile silk thread rounded needle in figure “8” pattern. This ligature acted as a gingival irritant for 14 days with subsequent development of periodontitis\(^{[16]}\). After placing the ligatures, the animals were kept in the same conditions and observed for 14 days. Ligature control was performed daily and the animals were checked in terms of proper nutrition. After 14 days the 30 rats were partially anesthetized and subjected to removal of ligatures, then they were divided randomly into 3 equal groups (N= 10)

- **Group I:** control group (periodontitis without treatment).
- **Group II:** treated with supra-periosteal injection of 0.2ml of PRP prepared by 800 rpm centrifugation force.
- **Group III:** treated with supra-periosteal injection of 0.2ml of PRP prepared by 3000 rpm centrifugation force.

Five rats from each group were sacrificed after 3\(^{rd}\) and 6\(^{th}\) week from the day of ligature removal and the beginning of the treatment for the treated groups. Specimens were properly fixed, decalcified in 10% neutral EDTA and then they were dehydrated and paraffin embedded, finally 4 μm sections were made for subsequent staining with haematoxylin and eosin (H&E) and immunohistochemistry.

Regarding immunohistochemical staining the sections were blocked in 10% normal goat serum then overnight incubation with primary antibodies against RANK, RANKL and ALP antibodies (1:300) (abcam) was done at 4°C; and then stained with HRP-conjugated secondary antibodies (ZSJQ-BIO, Beijing, China). The utilized substrate for color development was Diaminobenzidine and counterstaining with hematoxylin\(^{[17]}\). Finally, digital morphometric and statistical analysis were performed for the results.

**Statistical analysis:**

Analysis of the data was done using Statistical Package for Social Science software with computer program version 26 (SPSS, Inc., Chicago, IL, USA). The data were presented as means and standard deviations. One-way Analysis of variance (ANOVA) and tukey were used for comparing quantitative parametric data of more than two different groups while student’s t-test(unpaired) was used to compare between two different groups. P value less than 0.05 was considered statistically significant.
RESULTS

Haematoxylin and eosin stain results:

**Group I (Control group):**

After three weeks alveolar bone still revealed severe resorption evidenced by the presence of Howship’s lacunae on the bone surface filled with osteoclasts followed by reduction of bone thickness. After six weeks, repair areas began by the appearance of new osteoid tissue filling some resorption lacunae, reversal lines could also be seen (Fig.1).

**Group II (Treated with PRP 800 rpm):**

After three weeks few resorption lacunae could be seen on the surface of the alveolar bone, but after reaching six weeks no evidence of osteoclastic activity could be seen on the bone surface. All the lacunae were filled with osteoid tissue enclosing newly entrapped osteocytes and osteoblasts were arranged on the surface of the newly formed bone (Fig.1).

![Fig. (1): Histopathological overview of the 3 study groups at different time points.](image)

(A) Control group at 3 weeks; deep resorption lacunae in the alveolar bone filled with osteoclasts (black arrow). (B) Control group at 6 weeks; new osteoid bone is formed (white arrow), some resorption lacunae still present enclosing osteoclasts (black arrow); reversal line is evident (blue arrow). (C) PRP 800 rpm at 3 weeks showing few resorption lacunae (black arrow). (D) PRP 800 rpm at 6 weeks all resorption lacunae are filled with osteoid tissue, the bone surface is nearly normal with osteoblasts lining it. (E) PRP 3000 rpm at 3 weeks, resorption lacunae are evident (black arrow); there is new osteoid deposition and beginning of repair (white arrow). (F) PRP 3000 rpm at 6 weeks many resorption lacunae were partially filled with osteoid (white arrow), some lacunae still empty (black arrow), reversal line is evident (blue arrow). (H&E staining, X 400) (Scale bar= 25)
**Group III (Treated with PRP 3000 rpm):**

After three weeks osteoclastic activity was still evident on the bone surface but signs of repair began to be seen as deposition of new bone in some areas to fill the resorbed lacunae, after six weeks most of the resorption lacunae were filled with osteoid, the reparative capacity and the rate of new bone deposition was better than the control group but less than group II which indicates the reparative effect of PRP against periodontitis induced bone resorption (Fig. 1).

**Immunohistochemical stain results:**

Immunohistochemical analysis of RANK, RANKL and ALP stained bone sections indicated the protective effect exerted by PRP with both centrifugation speeds on ligature-induced experimental periodontitis models (Fig. 2). To elucidate the osteoblastic activity, ALP was stained as osteogenic marker, as well as RANK and RANKL as osteoclastogenesis molecular markers. The digital image analysis and the statistical evaluation revealed that ALP level was alleviated in both PRP treated groups at the two tested time points three and six weeks as compared to control group. Group II (PRP 800 rpm) showed the highest values regarding the ALP level it was significantly higher than both group I and group III, which indicated a marked increase in osteoblast activation (Fig. 2,3) (Table 1).

On the contrary RANK and RANKL staining were the complete opposite they were significantly higher in the control group at three and six weeks this indicates the greater amount of bone resorption in untreated periodontitis. Both PRP treated groups had lower expression level of RANK and RANKL than the control group, but group II (PRP 800 rpm) was significantly lower in their expression than group III (PRP 3000 rpm) in both tested time points which indicated less osteoclastic activity and more susceptibility to repair (Fig.2,3) (Table 1).

![Fig. (2): Immunohistochemical overview of the 3 study groups at different time points stained with ALP, Rank and Rankl. ALP expression was highest at 800 rpm group. RANK & RANKL expression was highest in the control group followed by the 3000 rpm group (Scale bar =25).](image-url)
Periodontitis is among the most prevalent inflammatory diseases\(^{18}\). Numerous studies investigating the treatment modalities are being conducted, they differ according to the severity of the disease. Previously, all efforts focused on elimination of pathogens, control of inflammation\(^{19}\), and modulation of the patient immunity\(^{20}\). However, the exciting treatments have been proven to be unsatisfactory, and the need for novel modalities became a must.

Ligature induced rat experimental periodontitis offers a reliable model with site-specific, time-dependent alveolar bone resorption. This model allows the accumulation of bacteria around the ligature with subsequent inflammation and plaque formation, leading to rapid induction of periodontitis and alveolar bone loss, it also mimicked conditions of enhanced oxidative stress\(^{17,21}\).

The ligature model was formed for years in rat molars, but it was difficult and require special equipment to provide good visibility and accessibility. For these reasons, Ionel et al. performed a ligature model periodontitis in the rat incisor. The results confirmed the appearance of gingivitis and the first symptoms of periodontitis from day three. After 14 days significant alveolar bone loss was developed\(^{16}\). This model provides simplicity, visibility, accessibility to the daily

### TABLE (1): Comparison of percent area of ALP, RANK and RANKL expression between different groups:

<table>
<thead>
<tr>
<th></th>
<th>Alkaline phosphatase</th>
<th>RANK</th>
<th>RANKL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 weeks</td>
<td>6 weeks</td>
<td>3 weeks</td>
</tr>
<tr>
<td>Control</td>
<td>1.80±0.36</td>
<td>3.17±0.63*</td>
<td>18.81±3.76</td>
</tr>
<tr>
<td>PRP 800</td>
<td>13.34±2.67*</td>
<td>20.53±4.11**</td>
<td>9.43±1.89*</td>
</tr>
<tr>
<td>PRP 3000</td>
<td>5.40±1.08bc</td>
<td>7.54±1.51bc</td>
<td>15.48±3.10c</td>
</tr>
</tbody>
</table>

Data expressed as mean±SD, significance≤0.05

- a: significance between Control & PRP 800 groups
- b: significance between Control & PRP 3000 groups
- c: significance between PRP 800 & PRP 3000 groups
- *: significance of 6 weeks vs 3 weeks within each group

Fig. (3): Bar chart for mean ± SD of ALP, RANK and RANKL positively stained area % for different groups at different periods of time.
checking of ligature and the most important is reproducibility so it was successfully repeated by Mohammed et al. and Mester et al. [22, 23]. The elimination of the pathogenic stimulus represented in this model by the ligatures removal may be considered similar to the basic periodontal disease treatment modality by the removal of dental biofilm and calculus, allowing the assessment of the repair process [24].

Application of new treatment modalities such as PRP began to be used in periodontal regeneration after the discovery of its biocompatibility and the high amount of growth factors that could promote the growth and differentiation of the periodontal ligament and alveolar bone cells, it also showed an interesting feature that it is sticky due to its high fibrin content which may work as a hemostatic agent and stabilize the graft material and the blood clot in cases of periodontal defects [25].

The utilization of one centrifugation step in the current study was to preserve the platelet count because Perez AG et al. revealed in their studied about double spinning techniques that some erythrocytes are unavoidably transported from the first spin and subsequently present in the PRP volume after the second spin. The occurrence of these remaining RBCs lead to pellet generation at the bottom of the tube, this pellet adsorb WBCs and platelets on its surface and that the manual mixing was insufficient to totally resuspend the platelets and large alteration in the platelet count resulted [12].

Using inactivated PRP in the present study was based on previous works indicating that inactivated PRP enhanced the proliferation of mesenchymal stem cells (MSCs) and that it significantly increased the osteoinductivity of bone matrix only if it is used without thrombin activation. Furthermore, it was discovered that PRP could be activated when exposed to collagen, which is prevalent in periodontal ligaments, thus leading to a sustained continuous release of growth factors. Additionally, it was discovered that bovine thrombin, used for activation, have significant immunologic side effects [26]. We also didn’t utilize collagen type I for activation as it stimulates a rapid release of growth factors that remains stable up to 24 h then degraded [27]. Utilizing sodium citrate in the present study was due to previous suggestions regarding it as an anticoagulant without negative impacts on preparation of PRP than the other types of anticoagulants that were stated to be harmful to the platelets [28].

Osteoblasts and osteoclasts are responsible for the balance between bone resorption and bone formation which is dependent on the RANK-RANKL-OPG axis. In the present study we used RANK and RANKL as markers of osteoclastogenesis and ALP as an indicator of bone synthesis activity [16]. RANK is the activation receptor of NF-kB and it is found on the surfaces of osteoclasts. RANKL, is considered as RANK ligand and is a transmembrane protein appeared in variable cell types, especially activated T cells and osteoblasts. RANKL influences the cell to physically interact with osteoclasts precursors and finally binds to its receptor RANK with subsequent induction of hard tissue resorption [29]. The results of the current study showed that RANK/RANKL activity significantly increased in periodontitis group (control) compared to both PRP treated groups. These changes in RANK/RANKL expressions that provoked resorption might be due to increased pro-inflammatory mediators’ levels (TNF-α, IL-1β, IL-6) and oxidative stress which are found during periodontitis as mentioned by Kose et al. [30].

The expression of both osteoclastogenic markers was less in the group treated with PRP prepared with 800 rpm than the group treated with 3000 rpm. This level of expression indicates less activation of the osteoclasts in the PRP 800 rpm treated group and subsequently better effect in alleviation of periodontitis associated bone resorption. We could explain that PRP may inhibit osteoclastogenesis via downregulation of RANKL expression in the periodontal ligament cells and it could also promote osteogenesis. Moreover, Kobayashi et al.
Mahitabe Elgamily and Mona Denewar

E.D.J. Vol. 67, No. 1

demonstrated that PRP was able to release supra-
physiological doses of platelet-derived growth
factors, TGF-β1, EGF, VEGF, and IGF at varying
concentrations for up to 10 days, also PRP was
found to be extremely biocompatible with all cell-
types [25]. All these factors have a stimulating effect
via chemotactic and mitogenic effects on pre-
osteoblastic and osteoblastic cells as mentioned by
Yu et al. [31]. Another recent study done by Wang
et al. revealed that the Wnt signaling pathway
involved in PRP-induced inhibition of osteoclast
differentiation [32].

Several studies have investigated the role of
PRP in inducing bone formation via stimulation of
osteoblastic differentiation [33]. ALP is an enzyme
produced by osteoblast cells and it is necessary for
mineralization, so it is considered as an important
indicator of osteoblastic activity and bone formation.
The results of the present study showed that ALP
expression significantly increased in PRP treated
groups compared to periodontitis group which
showed nearly negative expression. This result is
an indicator of the increased osteoblastic activity in
PRP treated groups. The enhancement of the ALP
levels in the PRP 800 rpm treated group was better
than that with the PRP 3000 rpm treated group. The
results regarding the enhancement of ALP level in
both PRP treated groups are consistent with previous
studies done by Kose et al. and Kanno et al. [30, 34].

On the other hand, Kobayashi et al. demonstrated different results, their study revealed
that PRP don’t affect the ALP activity in the PDL
cells and it significantly downregulated the in vitro
mineralization capacity of PDL cells. The study
also concluded that despite the little potential of the
PRP to induce osteoblast differentiation, it favors
gingival fibroblast regeneration [25].

Platelet-derived growth factors which are
abundant in PRP could activates bone regeneration
and initiates connective tissue healing by raising the
number of healing cells, activating angiogenesis,
and triggering macrophages [34]. On the other
hand, TGF-β which is generated by macrophages,
fibroblasts and specially by platelets have a great
role in connective tissue regeneration; it is also
a powerful inhibitor of osteoclastogenesis and
bone resorption, promoting bone formation [35, 36]
triggering adjacent cells, such as pre-osteoblasts,
to differentiate into mature osteoblasts favoring
the initiation of bone remodeling, tissue healing,
and bone mineralization [34]. PRP when locally
applied, increase tissue and bone repair acting on
osteoprogenitor cell recruitment, proliferation, and
differentiation [37].

The better results of the PRP 800 rpm treated
group versus the 3000 rpm treated group may be
explained by Nikolidakis and Jansen results who
revealed that the least mechanical forces could
separate the plasma with a great benefit of avoiding
damage of platelets which may be caused by higher
centrifugation rates with the consequence of losing
high amount of the growth factors [38]. Also, there
were reports indicating that spins greater than 800
rpm might cause decrease in the amount of some
growth factors [39].

All the previous results support our deduc-
tion that PRP may decrease alveolar bone loss in
the ligature-induced periodontitis rats via decreasing
osteoclastogenesis related molecules RANKL/
RANK expression and inducing osteoblastic
differentiation. PRP may be used in periodontitis as a
complementary approach to the conventional peri-
odontal therapy. However, further research must be
performed to identify the precise elements involved
in the influence of PRP in periodontitis.

CONCLUSION

Local injection of PRP with both tested centrifuga-
tion speeds enhanced the regenerative capacity of
bone in experimentally induced periodontitis. How-
ever, the lower centrifugation speed (800rpm) has
better regenerative effect.
IMPACT OF DIFFERENT PRP CENTRIFUGATION SPEEDS ON IMMUNOHISTOCHEMICAL

REFERENCES


