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Histopathological assessment of the preventive effect of leukocyte-platelet-rich fibrin on bisphosphonate-related osteonecrosis of the jaw following dental extraction: An animal study

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ABSTRACT

Background: Leukocyte- and platelet-rich fibrin (L-PRF) could be considered a preventive measure in Bisphosphonate-Related Osteonecrosis of the Jaw (BRONJ). The present experiment aimed to assess the preventive effects of L-PRF on osteonecrosis of the jaw in rats.

Methods: In this interventional animal study with a split-mouth design, 28 rats were randomly allocated to saline (negative control), bisphosphonate (positive control), and Bis + L-PRF (case) groups. Bilateral extraction of maxillary molar teeth was performed followed by random application of L-PRF to one of the extraction sockets treated with Zoledronic acid for four weeks. Clinical occurrence of BRONJ and histopathologic evaluations were done, and data were subjected to the Kruskal-Wallis test, Mann-Whitney *U* test and exact Fisher test performed using SPSS 25. The significance level was set at 0.05.

Results: The application of L-PRF resulted in a 41.67% reduction in osteonecrosis centers and the number of osteoclast cells. Also, Kruskal Wallis test results showed a significant difference among the three groups regarding the frequency distribution of inflammation severity. However, no significant difference was detected regarding the frequency distribution of the blood vessels (Kruskal Wallis test, *P*-value = 0.649).

Conclusion: It could be inferred that possible preventive effects on the clinical occurrence of osteonecrosis could be expected from the application of L-PRF.

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1. Introduction

Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is defined as exposure of bone in the maxillofacial region for at least eight weeks in patients with a history of treatment using anti-angiogenic and anti-resorptive medications with no history of accompanied radiotherapy [1]. Recent studies have shown that dental extraction is among the most common oral surgical procedures increasing the risk of this phenomenon up to ten times [2].

There is no gold-standard treatment for BRONJ, and in most cases, consequently, management of BRONJ mainly consists of conservative interventions, including local debridement, systemic antibiotic therapy, and application of antimicrobial mouth rinses with the ultimate aim of pain control and minimizing the progress or incidence of bone necrosis [1].

Leukocyte and platelet-rich fibrin (L-PRF) is the second generation of autologous platelet grafts and is composed of a fibrin matrix enriched with leukocyte cells, growth factors, proteins, and cytokines [3]. Available platelets produce a broad spectrum of proteins and growth factors playing a vital role in bone remodeling and soft tissue repair [3]. L-PRF is claimed to be superior to PRP in numerous ways. Firstly, L-PRF has no chemical materials, which results in a purely natural coagulation process. Secondly, L-PRF has a lower absorption rate, and as a result of its firm fibrin matrix, it provides slow and continuous release of essential growth factors during one to four weeks resulting in enhanced tissue repair [4–7].

Hartlev and associates studied the surgical method of MRONJ treatment accompanied by PRF applied in membrane layers and showed that the application of PRF membrane in the surgical treatment of grade-2 osteonecrosis might be successful [8].

Kim and colleagues evaluated L-PRF in the management of BRONJ. They applied L-PRF followed by removing necrotic and infected bone in 34 patients who were observed weekly for six weeks and then monthly for six months. They reported that L-PRF could be considered applicable for managing BRONJ; however, no definitive conclusion was drawn regarding the efficacy of this method [9].

In 2017, Maluf et al. assessed the efficacy of L-PRF in the treatment of MRONJ in two patients. They placed L-PRF in the extraction socket and followed the participants for two years. Clinical and topographic evaluation of the samples showed that L-PRF could be regarded as an effective means of treating MRONJ by acting as a physical barrier to bacterial invasion and secondary infection [10].

Finally, Asaka and associates examined the application of L-PRF in reducing the delayed healing process of the extraction socket in 102 patients receiving oral bisphosphonate medications for about 32 months. Radiographic and clinical evaluation after three months demonstrated a significant preventive effect of L-PRF on osteonecrosis of the jaw as a result of dental extraction in these patients [11].

Most of the available articles related to L-PRF have studied this method as a treatment for BRONJ, and few studies are available evaluating the preventive effect of L-PRF on BRONJ following dental extraction. Concerning the antimicrobial and angiogenic effects of L-PRF, the present study aimed to histopathologically assess the efficacy of L-PRF in the prevention of BRONJ following dental extraction in an animal model.

2. Material and methods

This interventional study was implemented on 28 healthy male Wistar rats in the *Animal Laboratory of Dentistry Faculty of Isfahan University of Medical Sciences, Isfahan, Iran*. The formula for the comparison of two proportions was used for calculating the sample size. A minimum difference of 0.476 in the proportion of necrotic areas between the two groups was considered statistically significant with

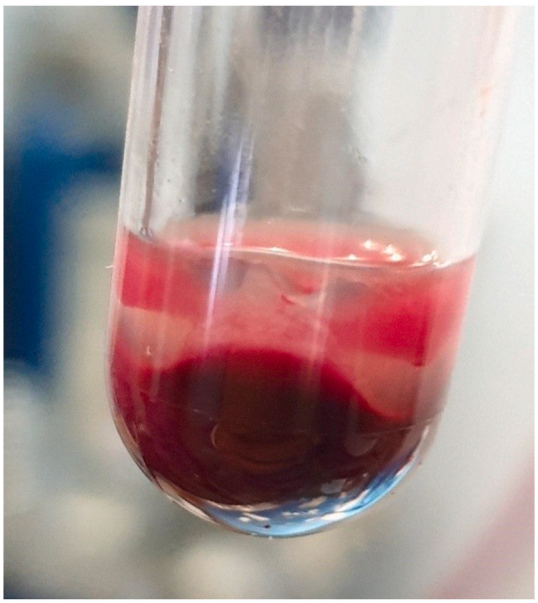

Fig. 1. L-PRF formed in the middle part of the test tube.

α-error of 0.05 and 1-β = 0.80. Simple randomization was done using online computer software (http://www.randomization.com) that provided a randomized order for the intended intervention for each rat.

Animal care was in accordance with the guidelines of the *Regional Ethical Review Board of Isfahan University of Medical Sciences,* including avoiding unnecessary injury to the sample animals (Ethical code number: IR.MUI.RESEARCH.REC.1400.419).

Before the intervention, all rats were kept in a laboratory environment for ten days and fed with a specific food regimen and prepared food. Rats received standard laboratory nutrition, making food and water available 24h of the day in a peaceful place under a controlled temperature of 22 ± 2° of centigrade and moisture of 40–60%, along with the light-dark cycle of 12:12 h.

Based on the randomized order, animals in the bisphosphonate intervention group (n = 28 in 14 rats) received weekly intraperitoneal injections of 0.06 mg/kg Zoledronic acid diluted with 0.9% sodium chloride solution (Zolena, Ronak, IRI) for four weeks, while in the saline group (n = 14 in 14 rats) 0.06 mg/kg normal saline with the approximate volume of 0.2 ml was injected using the same route. Afterward, all rats were subjected to general anesthesia using intraperitoneal injections of 75 mg/kg Ketamine and 2 mg/kg Acepromazine. Flunixin 5% was applied for analgesic purposes both during and after surgery; however, no antibiotics were prescribed regarding its confounding effects on the outcomes. Animals in the bisphosphonate intervention group were divided into two groups with a split-mouth design: The normal healing positive control group (n = 14) and the L-PRF intervention group (n = 14). Two ml of blood was taken from the tail vein of the intervention group and collected in the 9 ml plastic-glass test tubes with no anticoagulant agents and immediately placed into the centrifuge device (Intra- Spin system, Intra - Lock, Boca- Raton, FL, USA) with the program speed of 2800 PRM for 12 min. During this time, the maxillary first molar teeth of rats placed in the supine position were extracted with minimum trauma following proper flap elevation. Heart and respiratory rates were monitored during the procedure. Gel form L-PRF in the middle part of the test tube was isolated using a scissor and randomly placed in the extraction socket of the right or left maxillary first molar. Then a figure of eight suture was used for primary wound closure (Figs. 1 and 2). Rats were then observed, while food and water were provided 24 h a day; rats were examined routinely by a veterinarian colleague. If there was an uncontrollable injury and pain, rats were excluded from the study by being executed as a result of overdose inhalation of the Halothane 15 mg/kg.

Four weeks following the successful completion of the interventions, all animals were executed using Halothane. Next, the upper jaws of the rats were isolated and placed in Formalin 10%, followed by decalcification using EDTA. Afterward, samples were divided into two halves from the midline, and the bone in the extraction socket area was cut and placed into Paraffin. Axial cuts in the middle part of the socket were provided with an average width of 4 μm and then colored using Hematoxylin & Eosin method.

Histologic evaluation was done by a blind pathologist (Professor of Oral and Maxillofacial Pathology) using an optical microscope assessing the following parameters using Immunohistochemistry (IHC) and by utilizing monoclonal and polyclonal antibodies to determine the intensity and distribution of the target cells:.

- Osteonecrotic foci: Each focus was defined as 8 to 10 adjacent empty lacunas in the alveolar bone (Figs. 3 and 4). The mean number of foci was calculated in five separated microscopic fields without overlap with the 40× of magnification [12].
- The mean number of osteoclast cells was calculated in five separated microscopic fields without overlap from the alveolar bone surrounding the extraction socket with the 40× of magnification [13] (Fig. 5).
- Fibroblast density was defined as degree 0 (=less than 30 cells), degree 1 (=31–50 cells), degree 2 (=51–75 cells), and degree 4 (=more than 76 cells) [14].
- The intensity of the inflammation was assessed by calculating the number of lymphoplasmocytes in five separated microscopic fields without overlap from the alveolar bone surrounding the extraction socket with the 40× of magnification and classified as

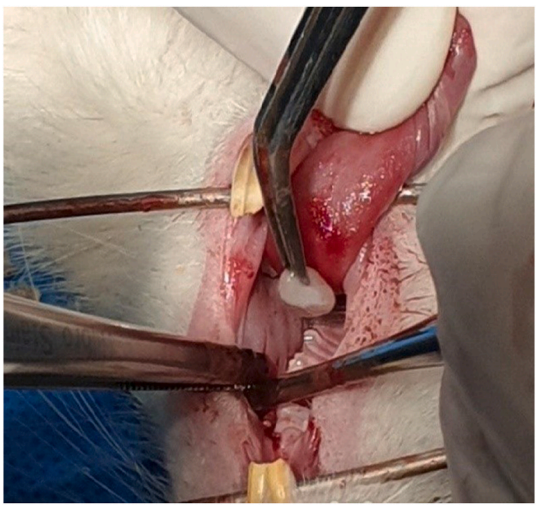

Fig. 2. Gel form L-PRF isolated by a scissor and placed randomly in the extraction socket of the right or left maxillary first molar in rats receiving Bisphosphonate.

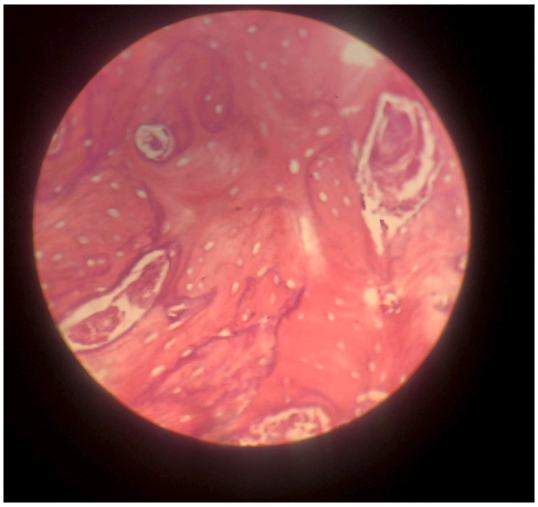

Fig. 3. Necrotic bone center empty of osteocyte cells (× 400 magnification).

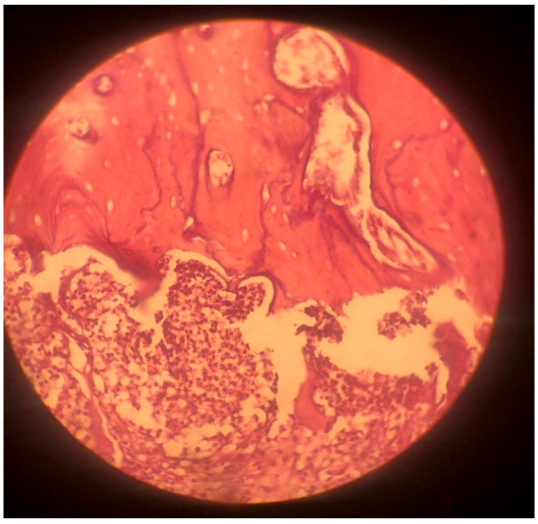

Fig. 4. Necrotic bone with a serrated margin and areas of neutrophil infiltration (× 400 magnification).

degree 0 (=no swelling), degree 1 (= less than 10 lymphoplasmocytes), degree 2 (=10 to 30 lymphoplasmocytes), and degree 3 (=more than 30 lymphoplasmocytes) [15].

- Blood vessels were evaluated on the alveolar socket surface and defined as the number of arterioles in five separated microscopic fields without overlap with the 40× of magnification and classified as degree 1 (=less than ten arterioles) and degree 2 (=more than ten arterioles) [15].

3. Statistical methods

Data was subjected to the descriptive analysis, *Fisher Exact test*, *Mann-Whitney test, and Kruskal-Wallis test* performed with SAS statistical software version 9.4 (SAS Institute, Cary, NC, USA)..

4. Results

From the total number of 42 samples, two in the saline control group and four samples (two rats) in the bisphosphonate groups were lost during the experiment and excluded from the study.

The frequency of clinical occurrence of BRONJ was highest in the bisphosphonate group (6 out of 12), followed by one-third of the 12 samples in the bisphosphonate + L-PRF group, and only one out of 12 in the saline group. There was a decrease in the presence of

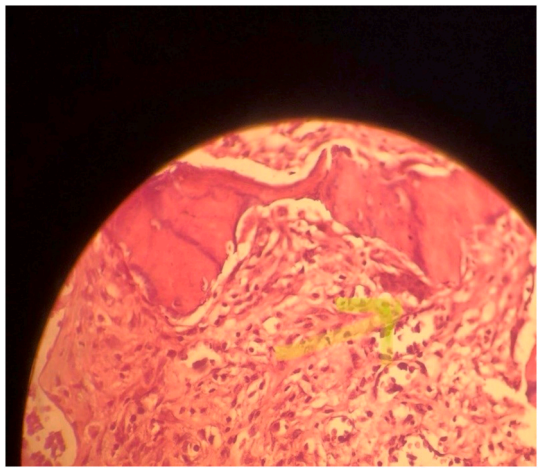

Fig. 5. Osteoclast cell (× 400 magnification).

osteonecrotic centers in 33.3% of cases and an increase in 16.7% of samples in the Bisphosphonate + L-PRF group compared to the Bisphosphonate group. Half of the samples showed no changes in this regard. Consequently, the Fisher Exact test showed that this difference was not considered statistically significant (*P*-value = 0.109).

The mean number of osteonecrotic foci in each group is reported in Table 1. There was a decrease in the number of osteonecrotic centers in 41.7% of cases in the Bisphosphonate + L-PRF group compared to the Bisphosphonate group. 58.3% of samples in this group also showed a similar or an increased number of osteonecrotic centers compared to the Bisphosphonate group. Wilcoxon Signed Rank test showed no significant difference between Bisphosphonate + L-PRF and Bisphosphonate groups regarding the number of the osteonecrotic foci in the histopathologic evaluation (Z = − 0.141, *P*-value = 0.888).

The mean number of osteoclast cells in each group is reported in Table 2. One-third of the samples in the Bisphosphonate + L-PRF group showed an equal number of osteoclast cells compared to the Bisphosphonate group. One-quarter showed an increase in number, and the rest showed a decrease in the number of osteoclast cells when compared to the Bisphosphonate group. The difference in this value between Bisphosphonate and Bisphosphonate + L-PRF groups was not considered statistically significant (Wilcoxon Signed Rank test, Z = −0.431, *P*-value = 0.667).

The density of fibroblast cells is reported in Fig. 6. It was shown that a higher grade of the density of fibroblast cells was detected in bisphosphonate and Bisphosphonate + L-PRF compared to the control saline group. Comparing the Bisphosphonate + L-PRF group to the Bisphosphonate group revealed a decrease in 16.6% of cases, while in 25% of the samples, there was an increase in the density of fibroblast cells. 58.3% of the samples in the Bisphosphonate + L-PRF groups had similar fibroblast density to the Bisphosphonate group. However, Kruskal-Wallis test results showed that There were no significant differences between Saline, Bisphosphonate, and Bisphosphonate + L-PRF groups regarding the frequency distribution of the density of fibroblast cells (*P*-value = 0.669).

The frequency distribution of inflammation severity is demonstrated in Fig. 7. Grade 0 of inflammation (No inflammation) was more commonly detected in the Bisphosphonate + L-PRF group compared to the Bisphosphonate group (33.3% vs. 25%). On the other hand, moderate inflammation was more commonly reported in the Bisphosphonate group compared to the Bisphosphonate + L-PRF group (41.7% vs. 33.3%). Generally, Kruskal Wallis test results showed a significant difference among the three groups regarding the frequency distribution of inflammation severity. Moreover, the Mann-Whitney *U* test reported significant differences between "Bisphosphonate and Saline" (*P*-value of 0.041) and "Bisphosphonate + L-PRF and Saline" (*P*-value of 0.015) while comparing Bisphosphonate + L-PRF to Bisphosphonate group showed 41.6% decrease, 16.6% increase, and 41.6% similar inflammation and the related analytic test showed that this difference was not considered statistically significant (Mann-Whitney *U* test, *P*-value = 0.704).

Also, only an 8.3% decrease in the mean number of blood vessels was reported in the Bisphosphonate + L-PRF group compared to the Bisphosphonate group. Since 91.6% of cases showed no change in this variable, analytic test results showed no significant difference among the three groups regarding the frequency distribution of the blood vessels (Kruskal Wallis test, *P*-value = 0.649).

Table 1
The mean number of osteonecrotic foci in each group.

Groups	Number of samples	Mean ± SD
Saline	12	0.25 ± 0.86
Bisphosphonate	12	1.33 ± 1.61
Bis + L-PRF	12	1.50 ± 2.27

Table 2
The mean number of osteoclast cells in each group.

Groups	Number of samples	Mean ± SD
Saline	12	2.83 ± 3.61
Bisphosphonate	12	1.42 ± 1.73
Bis + L-PRF	12	1.00 ± 1.53

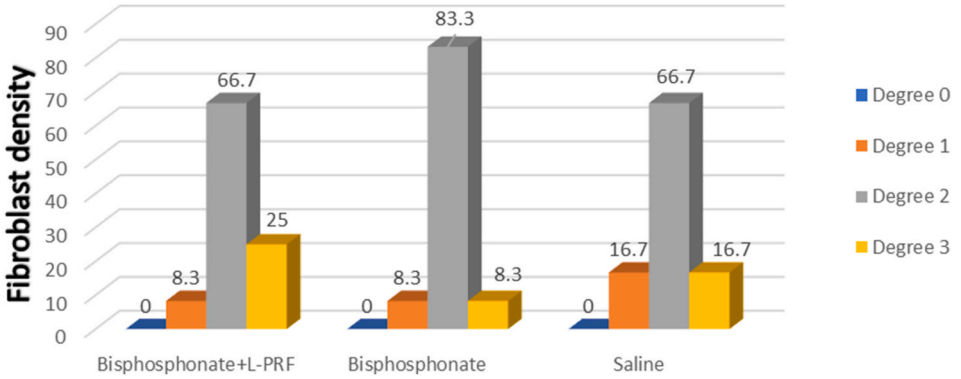

Fig. 6. The frequency distribution of fibroblast density in each group.

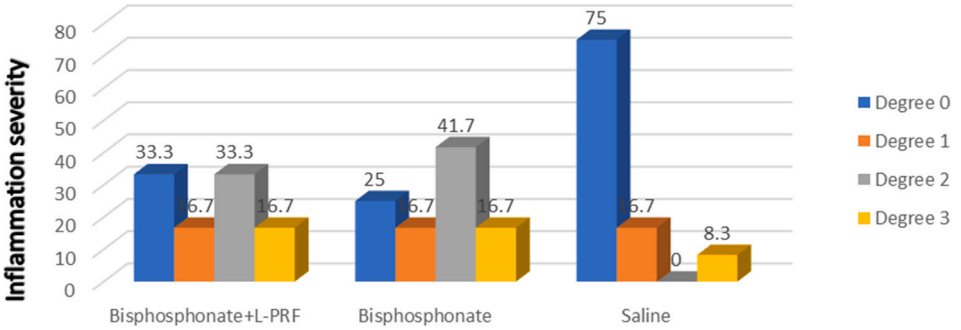

Fig. 7. The frequency distribution of inflammation intensity in each group.

5. Discussion

Recent studies have suggested different approaches regarding the prevention and treatment of BRONJ. Among these methods, the application of autologous platelet concentrates such as L-PRF has been vastly investigated. The outcomes of the present paper showed that the application of L-PRF might prevent the clinical occurrence of osteonecrosis and improve inflammatory responses following dental extraction in rats treated with bisphosphonates.

According to the results of some studies, the application of PRF following bone surgeries and dental extraction could have positive effects on the healing process reducing postoperative complications in patients with a history of taking bisphosphonates and BRONJ [11,16,17]. A study by Maluf shows that L-PRF as an adjunctive therapy along with bone debridement could be helpful in the treatment of MRONJ and alleviation of its symptoms [10]. Some other studies emphasize the preventive role of APCs in BRONJ after surgical interventions in bisphosphonate takers [18]. The Results of these studies are consistent with the outcomes of our study regarding the effectiveness of PRF in the prevention and treatment of BRONJ.

According to the study by Kargarpour et al., PRF can prevent the differentiation of osteoclasts from the bone marrow hematopoietic cells [19]. It was also stated in a study by Kumar that PRF combined with BCP26 could prevent the differentiation of osteoclasts and cause their apoptosis in chronic periodontitis [20]. Although the results of this study showed that L-PRF could reduce the number of osteoclasts, insignificant differences between the two groups could be related to the small sample size in our study.

According to a recent study by Steller et al., applying PRF reduces the adverse effect of zoledronic acid on the viability of fibroblasts and increases their proliferation and migration [21]. Similarly, in the present study, the outcomes showed that the distribution of fibroblastic intensity was more similar to the normal rats unaffected by bisphosphonate following the application of L-PRF in rats treated with bisphosphonate compared to those not receiving L-PRF.

According to the study of Kaneko and Santini, zoledronic acid is an amino bisphosphonate that increases inflammation by

activating M1 macrophages [22,23]. According to Wang's study, L-PRF reduces the secretion of inflammatory cytokines from Schwann cells, thereby exhibiting anti-inflammatory effects [24]. Also, Mudalal and colleagues showed that L-PRF reduces the release of inflammatory cytokines such as IL-6, IL-1β, and TNF-α; hence, it could be beneficial in the prevention and treatment of inflammatory diseases. They also found that L-PRF can reduce gingival inflammation [25]. Moreover, Nasirzadeh et al. showed in their study that PRF could change the polarization of macrophages from M1 to M2 with anti-inflammatory activity and the resultant increase in bone production during fracture repair [19]. The results of the present study also confirm these outcomes supported by the 41.6% reduction in inflammation intensity due to the application of L-PRF in rats affected by bisphosphonate.

In this study, there was no significant relationship between the number of blood vessels and the three sample groups (P = 0/649). According to previous studies, bisphosphonate reduces the serum level of vascular endothelial growth factors and angiogenesis in the bone marrow and periosteum [1,26]. The results of the present study are in contrast with these studies. Comparing the bisphosphonate + L-PRF samples to the bisphosphonate group, the number of blood vessels in 91.6% of them remained unchanged, and 8.3% of them showed a decrease, which indicates the ineffectiveness of L-PRF on the level of blood vessels. Previous studies show that PRF contains vascular endothelial growth factor (VEGF), which plays a role in angiogenesis and blood supply of damaged tissue. PRF also provides a condition for the expansion of small blood vessels and the migration of endothelial cells, which are necessary for the construction of new blood vessels [27]. The results of the present study are not consistent with these studies. PRF plays a role in reducing the number of osteoclasts, and osteoclasts are needed for the passage of blood vessels through the bone matrix [19,28], which can justify the contradictory results of the present study in this regard.

Our study had certain strengths and limitations. Regarding the split-mouth design, the effects of confounding variables were minimized. Other confounding factors, including the difficulty of surgery, operation time, and surgeon experience, control group by applying saline as a placebo was taken into consideration to ensure the effect of bisphosphonate were also controlled by having only one experienced surgeon performing all surgeries. Also, a negative medication in producing histologic alterations of osteonecrosis. A combination of L-PRF and medications (including antibiotics) is recommended for future studies. Besides, studying other animal species with larger body mass and genetically more similar to human species is recommended.

6. Conclusion

The outcomes of the present study showed possible positive effects of the application of L-PRF on the prevention of some bisphosphonates-related complications including the clinical occurrence of osteonecrosis and inflammation; however, no significant preventive effects including reducing the number of osteoclasts, anti-angiogenesis, and antifibrotic effects were reported in this regard. Given the relatively small number of samples, future studies on human samples with bigger sample sizes are recommended for drawing a more definitive conclusion in this regard.

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Ethics approval statement

The *Regional Ethical Review Board of Isfahan University of Medical Sciences* approved this study with the code number: "IR.MUI.RESEARCH.REC.1400.419".

Author contribution statement

Milad Etemadi Sh; Farnaz Shooshtarian; Golnaz Tajmiri: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

MohammadSoroush Sehat: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Data availability statement

Data will be made available on request.

Additional information

No additional information is available for this paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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