



## The expression of matrix metalloproteinase-13 (MMP-13) on xenograft and PRF in bone regeneration

Efeito da combinação de xenoenxerto e PRP na expressão de metaloproteínase de matriz -13 (MMP-13) durante regeneração óssea

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**How to cite:** Augustina EF, Rivai NR, Gifary HM. The expression of matrix metalloproteinase-13 (MMP-13) on xenograft and PRF in bone regeneration. *Braz Dent Sci.* 2023; 26(2): e3366. <https://doi.org/10.4322/bds.2023.e3366>

### ABSTRACT

**Objective:** inflammation may play a role in bone loss by altering the bone remodelling process, favouring bone resorption by osteoclasts over bone synthesis by osteoblasts. Matrix metalloproteinase 13 (MMP-13) has the ability to activate osteoclasts, leading to bone resorption. Regenerative treatments have been widely used in periodontology. When combined with Platelet-rich fibrin (PRF), xenografts will give better results in bone regeneration. The aim of this study was to evaluate the effect of xenograft combined with PRF on MMP-13 expression in a bone defect using an experimentally created bone defect. **Material and Methods:** eighteen New Zealand rabbits were assigned to three groups. Each group consisted of six New Zealand rabbits. A critical bone defect with a diameter size of 5 mm was created in the right tibia of each rabbit in group 1 (application: xenograft), group 2 (application: PRF), and group 3 (application: xenograft and PRF). The PRF was produced from 5 ml of blood taken from each rabbit's ears. After 30 days, the rabbits were euthanized. The tissue samples were evaluated by immunohistochemical staining. **Results:** group 3 showed the lowest mean expression of MMP-13 (4.50) compared to group 1 (20.50) and group 2 (11.70). Group 3 showed a significant difference in the MMP-13 expression compared to group 1 and group 2 ( $P = 0.000$ ) ( $P < 0.05$ ). **Conclusion:** this research showed that the combination of xenograft and PRF had the lowest expression of MMP-13. The application of a xenograft and PRF has better osteogenesis ability in bone regeneration.

### KEYWORDS

MMP-13; Xenograft; Platelet-rich fibrin; Bone regeneration; Inflammation.

### RESUMO

**Objetivo:** inflamação pode interferir na perda óssea através de alterações no processo de remodelação, favorecendo a reabsorção óssea pelos osteoclastos ao invés da síntese pelos osteoblastos. A metaloproteínase de matriz 13 (MMP-13) ativa osteoclastos causando reabsorção óssea. Tratamentos regenerativos têm sido amplamente usados na periodontia. Quando combinamos Plasma rico em plaquetas (PRP) e xenoenxerto levam a melhores resultados de regeneração óssea. O objetivo deste estudo foi avaliar os efeitos de xenoenxerto combinado com PRP na expressão de MMP-13 em defeitos ósseos experimentais. **Material e Métodos:** dezoito coelhos Nova Zelândia foram distribuídos em 3 grupos de 6 coelhos cada. Um defeito ósseo de 5 mm de diâmetro foi feito na tíbia direita dos animais do grupo 1 (xenoenxerto), grupo 2 (PRP) e grupo 3 (Xenoenxerto+PRP). O PRP foi obtido pela coleta de 5mL de sangue das orelhas dos coelhos. Após 30 dias, os coelhos foram eutanasiados. As amostras foram submetidas a coloração imunohistoquímica. **Resultados:** o grupo 3 apresentou a menor expressão de MMP-13 (4.50) quando comparado ao grupo 1 (20.50) e ao grupo 2 (11.70). O grupo 3 mostrou diferença estatística significativa em relação a expressão de MMP-13 quando comparado aos grupos 1 e 2 ( $p=0.000$ ) ( $p < 0.05$ ). **Conclusão:** esta pesquisa mostra que a combinação de xenoenxerto e PRP teve a menor expressão de MMP-13. A combinação de xenoenxerto e PRP têm maior habilidade de osteogênese na regeneração óssea.

### PALAVRAS-CHAVE

MMP-13; Xenoenxerto; Plasma rico em plaquetas; Regeneração óssea; Inflamação.

## INTRODUCTION

Bone defects due to infectious and other pathological processes can lead to inflammatory processes, such as the bone destruction caused by periodontal disease. Proinflammatory mediators, such as Interleukin-1 (IL-1), Interleukin-6 (IL-6) and tumour necrosis factor  $\alpha$  (TNF $\alpha$ ), trigger the expression of matrix metalloproteinases (MMPs). MMPs actually play major roles in normal tissue remodelling processes, such as in embryonic development, bone growth, resorption and wound healing [1]. MMPs also cause pathological tissue destruction [2].

MMP-13 is a coupling factor that periosteoclastic cells (osteoblast/bone lining cells/osteoblast precursors/early reversal cells) produce to aid the breakdown of the osteoid prior to the attachment and resorption of osteoclasts [3]. MMP-13 produced by osteoclasts and polymorphonuclear (PMN) causes collagen degradation in the connective tissue and alveolar bone and affects the remodelling and degradation of the periodontium [1]. MMP-13 can trigger the activation of osteoclasts and is also expressed in osteoblastic cells adjacent to osteoclasts at sites of active bone resorption [2], gingival crevicular epithelium, gingival fibroblasts, macrophages and plasma cells [4]. MMP-13 affects the activity of osteoclasts and bone resorption, thus contributing to the destruction of periodontal tissue [5].

Bone graft materials are used to support bone healing and are often used in guided tissue regeneration (GTR) [6]. Bone grafts must be biocompatible, bioresorbable and adequately porous to be suitable for vascular ingrowth [7], and they must also be suitable for osteoconduction, osteoinduction and osteogenesis for bone tissue regeneration. The addition of a bone graft is expected to accelerate bone regeneration [8]. Xenografts are bone graft materials that are derived from a genetically unrelated species to the host [9]. New bone formation is slower with xenografts than with allografts [10]. Xenografts on bone defects can cause the differentiation of mesenchymal cells and reduce cytokine levels and control inflammatory response [11].

Xenografts have been widely used in tissue regeneration, but in clinical use, many studies report that the use of xenografts alone leads to less than optimal healing and tissue repair [12]. Research shows that xenografts give better results when combined with the use of membrane

materials or other growth factors [13]. Xenografts are known to have a long absorption capacity [14]. As shown in many studies, a xenograft remains in the tissue nine months after application [3]. Therefore, xenografts are used in combination with materials that can improve the regenerative properties of the xenograft [15].

PRF is simple to produce, does not use chemicals and produces growth factors such as platelet-derived, vascular endothelial and insulin-like growth factors. The literature shows that PRF can continuously increase the proliferation of all types of cells, especially osteoblasts [16]. These growth factors can regenerate bone tissue and release growth factors continuously for up to ten days [17]. PRF is also an immune regulation node with inflammation retrocontrol ability [14].

Several studies have reported that PRF can support hard or soft tissue regeneration. However, combining xenograft and PRF has never been proven to decrease MMP-13 expression in bone defect inflammation. This study aimed to evaluate the effect of xenograft and PRF on MMP-13 expression in a bone defect using an experimentally created bone defect in rabbits.

## MATERIALS AND METHODS

### Animal selection and study design

This study was an experimental in vivo study using New Zealand rabbits. The rabbits selected were eight-to-ten-week-old males, each weighing 1.5-2.5 kg. The rabbits were kept in 60 x 60 x 60 cm cages in isolation rooms with good air ventilation. A normal chow diet and water were provided ad libitum [18]. This study received ethical approval from the Ethical Committee of Dental Research, Airlangga University, reference number 184/KKEPK.FKG/XII/2021.

The samples were chosen at random and were determined using the Hosmer-Lemeshow test sample formula. Eighteen rabbits were assigned to three groups. Each group consisted of six rabbits. A critical bone defect with a diameter size of 5 mm was created in the right tibia of each rabbit. In group 1, the defect was grafted by xenograft (bovine, Indonesia) 0.05 gr; in group 2, the defect was grafted by PRF. PRF was produced from 5 ml of blood taken from each rabbit's ears. In group 3, the defect was grafted by xenograft and PRF.

## PRF

The PRF was produced from 5 ml of blood taken from the ears of each rabbit. The blood was put in a glass tube and centrifuged for ten minutes at 2500 rpm to form the PRF. Whole blood was centrifuged to separate it into an upper liquid layer of platelet poor plasma, a middle gel-layer and a red blood cell (RBC) layer, with the PRF being isolated by cutting the upper section of the RBC. The PRF was placed in a filter, then cut into small pieces and mixed with the xenograft using a spatula [17,19].

## Experimental procedure

Group 1 was grafted by xenograft. First, intramuscular anaesthesia with ketamine 15–25 mg/kg (Anesject, Indonesia) was injected into the right tibia. A lengthwise incision was made in the right tibia. A separation of the bone was performed, and a defect with a diameter size of 5 mm and a depth of 3 mm was created using a micromotor bur. The weight of each xenograft (bovine bone, Indonesia) was 0.05 gr in every defect in the right tibia [20]. The wound was then stitched with inner and outer layer stitching using silk thread. The wound was closed using medical plaster (Hypafix, Indonesia). After two

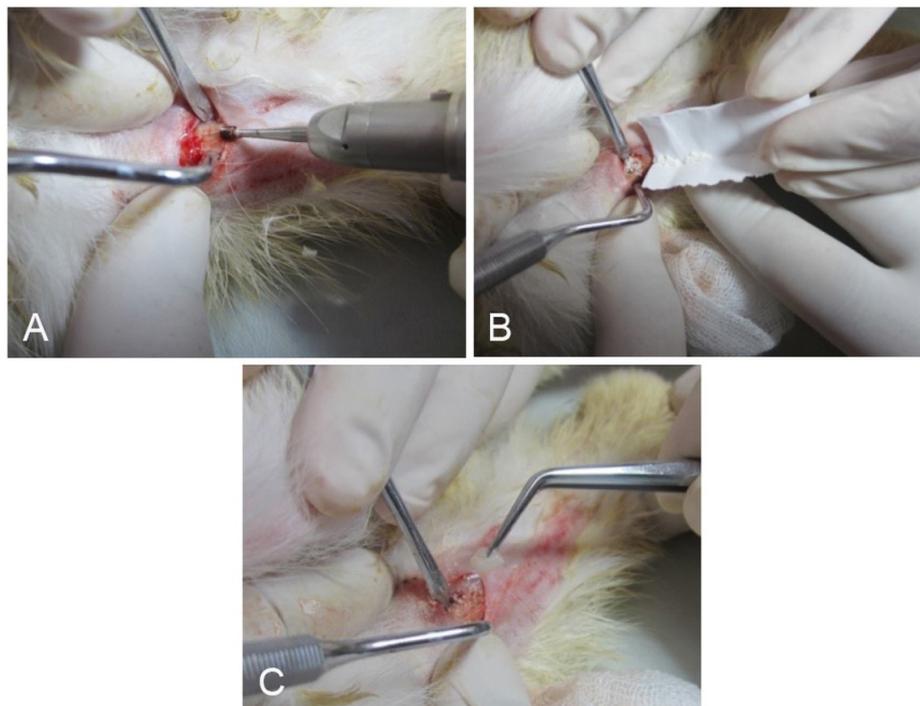
weeks, the outer layer was removed and left for 30 days [21].

In group 2, 5 ml of blood from each rabbit's ears was taken to make the PRF. The PRF weight was 0.05 gr in every defect of the right tibia. The same procedure was performed as for group 1. The wait was 30 days.

In group 3, 5 ml of blood was taken from each rabbit's ears to make the PRF. The PRF weight was 0.025 gr, and the xenograft (bovine bone, Indonesia) weight was 0.025 gr in each defect of the right tibia. The same procedure was performed as for group 1. The wait was 30 days (Figure 1).

## Tissue isolation

Intramuscular anaesthesia with ketamine 15–25 mg/kg (Anesject, Indonesia) was injected into the right tibia. Tissue was taken from the right tibia and put in a 10% neutral buffered formalin fixation solution. The fixation was done in two stages. After the first 48 hours, the fixation solution was replaced, and the tissue was cut smaller to aid penetration. At this stage, the tissue was left in the solution for another 48 hours. Observation of the expression of MMP-13 was completed using the immunohistochemical technique. First, the slides were washed with phosphate buffered saline (PBS) pH 7.4 once



**Figure 1** - The experimental procedure on the animal: bone defect creation in the right tibia (A), application of xenograft to the bone defect (B), application of PRF to the bone defect (C).

for five minutes. Then, blocking endogenous peroxide 3% H<sub>2</sub>O<sub>2</sub> was used for 20 minutes, and then the slides were washed with PBS pH 7.4 three times for five minutes.

After that, the unspecific protein was blocked using 5% fetal bovine serum containing 0.25% Triton X-100, and then the slides were washed with PBS pH 7.4 three times for five minutes. Then, the slides were incubated in a monoclonal antiMMP-13 (Santa Cruz) 1:1000 dilution for 60 minutes and washed with PBS pH 7.4 three times for five minutes. Then, the slides were incubated with antirabbit HRP (Biogear), conjugated for 40 minutes and washed with PBS pH 7.4 three times for five minutes. Then, the slides were dripped with diaminobenzidine, incubated for ten minutes and washed with PBS pH 7.4 three times for five minutes and H<sub>2</sub>O for five minutes.

Counterstaining was completed using Mayer's Hematoxylin Solution, which was incubated for ten minutes and then washed with tap water. The slides were rinsed with dH<sub>2</sub>O and dried. Mounting was completed using an Entellan cover with cover glass. The samples were observed under a light microscope.

### Measuring MMP-13 expression

A total of 18 slides were divided into three examination groups, with six slides per group. Each tissue sample was cut into slices with a thickness of 4  $\mu$ m. Then, a haematoxylin eosin (HE) examination was performed; the osteoblast structure was observed, and an immunohistochemical examination of MMP-13 expression was completed. The examination was performed using random numbering. The examination and measuring of MMP-13 expression were performed by observing the brown colour of the cytoplasm cell, which had been modified for osteoblastic cells [20,22]. Each slide used 1000  $\times$  magnification and 20 fields of view to measure the average.

The number of osteoblasts and osteoclasts were observed using HE. The results were calculated under a light microscope with a 1000  $\times$  magnification, each containing 1500 cells, then the HE was used for structural comparison.

### Statistical analysis

The expression of MMP-13 was analysed using the Kolmogorov–Smirnov normality test.

Then, the result was tested by a one-way analysis of variance to determine any differences between groups and by a post hoc test, Tukey's honestly significant difference test, with a significance value of  $p < 0.05$ .

## RESULT

### Quantity of osteoblasts and osteoclasts

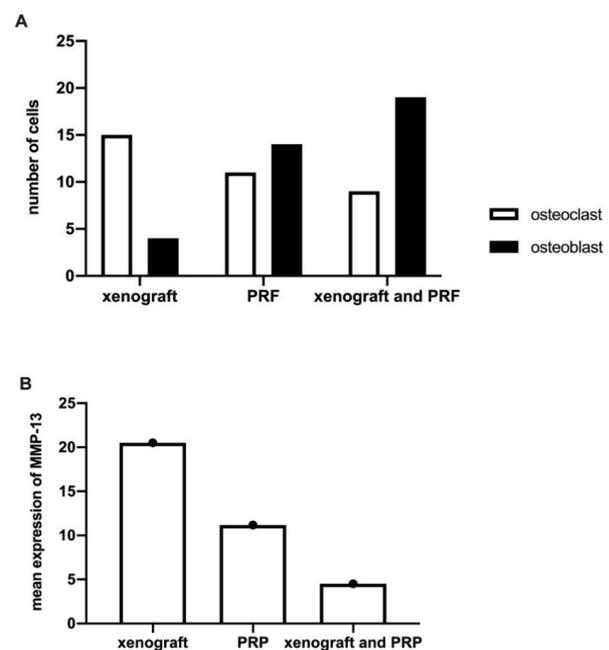
The osteoclasts were observed and compared with the osteoblasts. As shown in Figure 2, the highest number of osteoclasts was in group 1 (15), then group 2 (11) and group 3 (9). The highest number of osteoblastic cells was in group 3 (19), then group 1 (4) and group 2 (14).

### MMP-13 expression

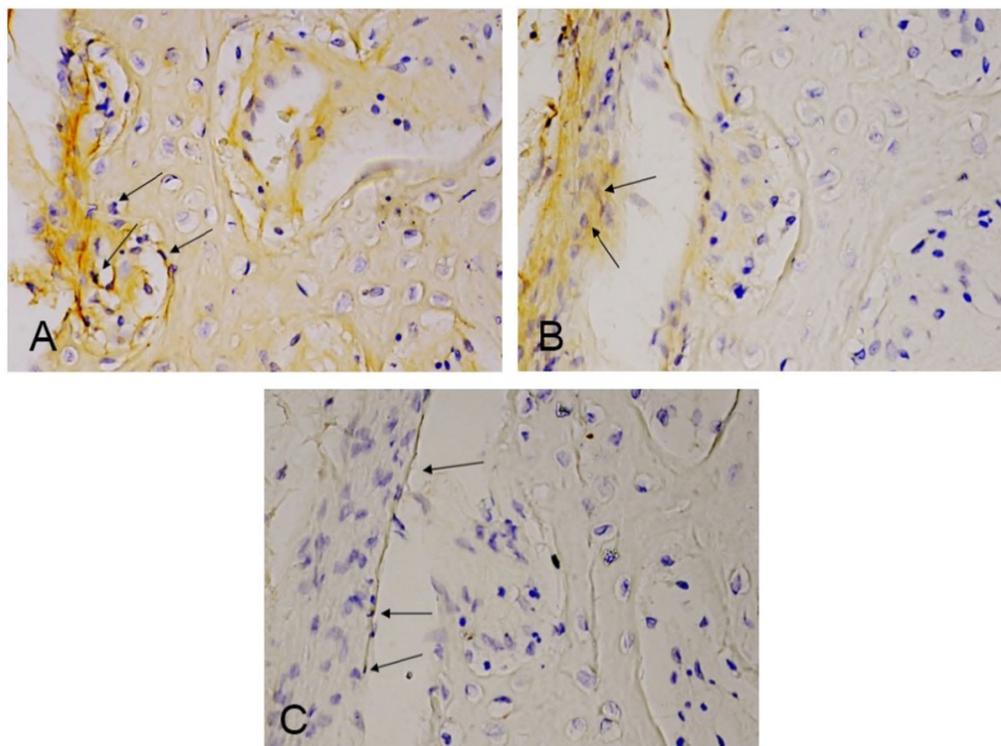
MMP-13 expression in the wound areas is presented in Figure 3. Group 3 showed the lowest mean expression of MMP-13 (4.50) compared with group 1 (20.50) and group 2 (11.70) (Figure 2). In group 3, there was a difference significant in MMP-13 expression compared with group 1 and group 2 ( $P = 0.000$ ) ( $P < 0.05$ ).

## DISCUSSION

Based on the immunohistochemical examination, the mean expression of MMP-13 in group 1 was 20.50 (Figure 2). Xenografts are



**Figure 2** - The number of osteoblasts and osteoclasts in the bone defect using HE examination (A), the mean of expression of MMP-13 in the bone defect (B).



**Figure 3** - The immunohistochemical analysis of expression of MMP-13. The application of xenograft (A), PRF (B), xenograft and PRF (C). The analysis of expression was completed in a light microscope with a magnification of 1000 × in 20 different fields.

known to have a long absorption capacity [23] and remain in the tissue nine months after having been applied [12]. Histologically, new bone formation was lower than by allograft. The high expression of MMP-13 in group 1, as demonstrated by research, showed that in xenograft-treated bone, bone remodelling is accompanied by chronic inflammation at a low level [24].

When chronic inflammation occurs, inflammatory mediators such as IL1 and TNF $\alpha$  increase [10]. The increased IL-1 and TNF $\alpha$  will stimulate increased MMP-13 expression [4], where MMP-13 or the collagenase-3 enzyme has a vital role in bone biology [25]. MMP-13 degrades not only type II collagen but also types I, III and X, which are essential components of bone [4]. IL-1 and TNF $\alpha$  receptors, through ligand binding, will recruit receptors that bind to proteins and continue the stimulus into the cell. To bind to the receptor, IL-1, and TNF $\alpha$  through a phosphorylation mechanism in cells, mediated by one of the proteins, namely mitogen-activated protein kinase (MAPK). MAPK facilitates the induction of MMP-13 due to the presence of these inflammatory mediators [7]. Inflammatory mediators present in these tissues cause increased MMP-13 expression. This is consistent with the theory that the high expression of MMP-13 in

gingival crevicular fluid in periodontitis patients suggests a role for increased MMP-13 in the bone resorption process [4].

In group 2 (addition of PRF), the mean number of MMP-13 expression was lower (11.17) than in group 1. It can be seen that PRF has anti-inflammatory properties by releasing inflammatory mediators slowly; the number of inflammatory mediators is controlled, so the MMP-13 expression also decreases. Physiologically, the fibrin matrix of PRF can retain several growth factors and inflammatory mediators and release them slowly to the defected area. Leukocytes and inflammatory mediators, such as IL1 $\beta$ , IL6, IL4 and TNF, are retained in the PRF, thus providing an anti-inflammatory effect [26].

The lowest MMP-13 expression, 4.50, was found in group 3 (xenograft and PRF treatment). PRF has properties that improve wound healing, increase bone regeneration, stabilise graft position and hemostasis [27]. With controlled inflammation, inflammatory mediators decrease, and the expression of MMP-13 also decreases, increasing bone formation and the number of osteoblasts. PRF is combined with a xenograft; although it does not have an osteoinductive ability, the inorganic material of this graft can make

an attachment and proliferate the osteoblastic cells [28]. According to research by Simon (2012), when combined with the use of membrane material, GTR or other growth factors, a xenograft gives a better result than when used alone [13].

HE staining showed a higher number of osteoblasts in group 2 and group 3 than in group 1. The formation of osteoblasts in the tibial bone defect of rabbits treated with xenograft, PRF and xenograft and PRF shows the process of bone regeneration. This is because PRF contains concentrations of growth factors and other mediators that can improve wound healing and increase bone regeneration. This can be seen from clinical indicators, which include reduced pocket depth, better attachment (an increased clinical attachment level) and a more filled infrabony defect area [29].

The tibia bone of the New Zealand rabbit was used as a sample because it has a similar bone anatomy and basic morphology to humans. It was hoped that the results of this study would demonstrate that the regenerative results of adding regenerative materials to the defect show a microscopic correlation between the formation of osteoblastic cells in the rabbit tibia bone and the human alveolar bone [30].

Some limitations need to be considered in the interpretation of the results. First, further research is needed to determine the cellular mechanism of PRF for the tissue healing process, especially in bone defects in dentistry. In addition, research is needed to determine the effect of using PRF and other regenerative materials as tissue regeneration therapy materials, especially in the field of dentistry.

## CONCLUSION

The results of this research showed that the combination of xenograft and PRF had the lowest expression of MMP-13. The application of xenograft and PRF has better osteogenesis ability in bone regeneration.

## Author's Contributions

EFA: Conduct research, data analysis, create the script. NRR: Conduct research, data analysis, create the script. HMG: Conduct research, data analysis, create the script.

## Conflict of Interest

The authors have no proprietary, financial or other personal interest of any nature or kind in any product, service and/or company presented in this article.

## Funding

This research did not receive any specific grant from funding agencies in the public, commercial or not-for-profit sectors.

## Regulatory Statement

This study received ethical approval from the Ethical Committee of Dental Research, Airlangga University, reference number 184/KKEPK.FKG/XII/2021.

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Date submitted: 2021 Dec 31  
Accept submission: 2022 Nov 11