




RESEARCH ARTICLE

REVISED Pre-Clinical Study: Immunohistochemical evaluation of matrix metalloproteinase-13 on rabbit (*Oryctolagus cuniculus*) socket healing after application of platelet-rich fibrin with and without hydroxyapatite [version 2; peer review: 2 approved]

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Abstract

Background: Tissue engineering technology has been used globally and proven to accelerate wound healing. This study aimed to analyse the effect of adding hydroxyapatite (HA) as a scaffold to platelet-rich fibrin (PRF) as a growth factor in accelerating the wound healing process as seen from the expression of matrix metalloproteinase-13 (MMP-13).











Methods: This research is an animal experiment conducted on 18 rabbits (*Oryctolagus cuniculus*). Rabbits were randomly divided into the following three groups of treatment: (G1) the application of PRF group, (G2) the application of PRF+HA group and (C) the control group without any application. Furthermore, each treatment group was split randomly into three groups of observation time. Periodontal tissue biopsy was performed to analyse the histopathological features that were examined on the basis of the level of MMP-13 immunoexpression.



Results: MMP-13 immunoexpression in the PRF+HA group showed better histoscore results, indicating a substantial reduction in MMP-13 values compared with other groups. The healing process was shown to increase with increasing observation time ($p < 0.05$), and the PRF+HA group outperformed the PRF and control groups. On day 3, MMP-13 exhibited a dark brown colour of Immunohistochemistry (IHC), which indicated an increase in the expression value of MMP-13 in the early stages of healing, namely, inflammation. On day 14, light brown IHC was seen, especially in group 2, as a reference that the remodeling process had begun.

Conclusions: This study indicates that the administration of PRF and HA was capable of reducing the MMP-13 expression that significantly

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Any reports and responses or comments on the article can be found at the end of the article.

accelerates the socket healing process. Hydroxyapatite is an alloplastic material that has inherent bioactive properties that support osteoconduction, can bind MMPs, and showed faster healing results based on the observation time as documented by immunohistochemistry.

Keywords

Socket Healing, Matrix Metalloproteinases-13, Platelet-Rich Fibrin, Hydroxyapatite



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REVISED Amendments from Version 1**Abstract:**

The conclusion in the abstract.

Methods:

The production company of the material used, animal criteria, formulation of each material, assessment of pathologist standards, statistically analysis.

Result:

The legend of the table.

Discussion:

More literature in the discussion section.
Limitation of the study.

Any further responses from the reviewers can be found at the end of the article

Introduction

Tooth extraction is one of the most common treatment procedures in dentistry. This procedure may cause resorption of the alveolar bone, gingival recession around the extraction area and periodontal abnormality and may also result in an aesthetic problem because of post-extraction anatomical and physiological changes.^{1,2} The wound healing process is influenced by various molecules, inflammation mediators, integrins, growth factors and matrix metalloproteinases (MMPs). MMPs has a function in every phase of wound healing by making modifications to the wound matrix, enabling cell migration essential in the remodeling process. Furthermore, MMPs are responsible for collagen degradation, and tissue engineering is one of the treatment options for regenerating periodontal tissues and alveolar bone. Progenitor cells, scaffolds, and growth factors are three essential components of tissue engineering.^{3,4}

MMP-13 has an important role in the early diagnosis of arthritis by analysing its pathological activity on the basis of histopathological features.⁵ Fibroblast cells are abundant in the stroma, and these cells play a role in making the extracellular matrix (ECM) function properly. During the wound healing, inflammatory, proliferative and remodeling stages, fibroblasts adapt to their environment and respond to and emit signals locally. At the time of injury, fibroblasts can replace the injured tissue. During pathological conditions, the ECM is produced in excess, and collagen is deposited irregularly, often leading to irreversible organ dysfunction or a disfiguring appearance.⁶

Bone grafts are used to replace and regenerate the lost bone. Scaffolding materials studied have been widely employed to stimulate bone growth. A promising scaffold must have biomaterials similar to the original bone structure.⁷ Platelet concentrates, such as platelet-rich fibrin (PRF), are utilised in different clinical fields, particularly in a medical procedure involving the mouth and jaws, the concentrates contain a high concentration of growth factors, which are essential in wound healing, particularly bone regeneration. Thus, they are considered as another treatment adjuvant.⁸⁻¹² Research that utilised platelets as growth factor therapy has affirmed that the use of PRF is better in wound healing compared with platelet-rich plasma (PRP), which is seen from various diagnostic tools.¹¹ In dentistry, PRF has proven to accelerate wound healing.¹³

Hydroxyapatite (HA) is a fundamental part of hard tissues, namely, bones and teeth. HA ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) is a material to assist with bone regeneration, it has great biocompatibility, does not cause unnecessary inflammation phenomena. It is non-poisonous, has great osteoconduction, it also has a high affinity for binding with other materials, and is a manufactured material generally utilised in medication and dentistry.^{11,14} In addition, HA can be employed as a scaffold in bone tissue regeneration. Growth factors and scaffold products induce the release of inflammatory molecules and mediators, such as tumour necrosis factor-alpha (TNF- α), interleukin (IL)-1, IL-6 and MMP-13, to render a better catabolic effect on chondrocyte metabolism and to speed up the inflammatory process.¹⁵ Scaffold functions as an early ECM essential for cell proliferation, migration and differentiation, while growth factor has a vital role in the wound healing process, an acute tissue response to trauma and several cell physiological processes. This study aimed to analyse the effect of adding HA as a scaffold to PRF as a growth factor in accelerating the wound healing process as observed from the expression of MMP-13.

Methods**Ethical clearance**

All experimental procedures involving animals were conducted in accordance with ARRIVE guidelines 2.0 on the care and use of laboratory animals to ameliorate any suffering for the animals.¹⁶ The treatment of the experimental animals

was according to the regulations regarding the convention on international trade in endangered species of wild fauna and flora. A research proposal containing research procedures was submitted to the ethics committee and passed the ethical committee issued by the University of Padjadjaran Bandung, Indonesia (Number: 132/UN6.C1.3.2/KEPK/PN/2017).

Study design

This analytic, quantitative research used an animal experimental laboratory design, and a post-test-only control group study design was conducted. The sample size was determined using the Mead equation formula calculation.¹⁷

$$E = N - B - T$$

$$N = 15-25$$

$$N = 18$$

Where E = Degrees of freedom of error components it should be in the range of 10–20

N = Total number of individuals or units in the research

B = Blocking component, representing environmental effects allowed in the design

T = Treatment component, corresponding to the number of experimental groups.

Subjects were 18 rabbits (*Oryctolagus cuniculus*) chosen according to the following: healthy, male, adult (5–6 months), good inferior anterior teeth and 300–400 g of weight. The study employed three treatment groups and three observation times. The treatment groups were as follows: the control group (C), the PRF group (G1) and the PRF+HA group (G2), and the observation times were the following Day 3, Day 7 and Day 14 based on the inflammation, proliferation and remodeling phases of the healing process. Each animal obtained different treatments with distinct observation times. The experimental animals were randomly divided into three treatment groups and then within the groups split into three groups on the basis of the time of observation. The allocation of each animal to an experimental group was performed on the basis of a simple random choice. Each group was marked on the back area using markers (C, G1 and G2) and placed in a different cage according to the time of observation. Cage A for Day 3, B for Day 7 and C for Day 14.

After subject selection, a rabbit adaptation was performed for 7 days, and all the animals fasted for 12 hours before tooth extraction. Thereafter, tooth extraction was performed, PRF was applied, and then HA (Gama-cha) was added. A commercial brand of HA, which is commonly used in dentistry treatment, was chosen for this study. During the experimental period, all the experimental animals were given standard feeds and cages met the ethical standards. All the research processes (research procedures and general conditions) were recorded by the research team in the research logbook every day which contained group allocations at various stages of the experiment starting from preparation, treatment, data collection, and data analysis.

Platelet preparation

Three milliliters of homologous blood were acquired from the ear cartilage of each rabbit collected using a vacuum blood collection plastic tube which were kept without an anticoagulant. From that point onward, the blood was quickly centrifuged (small centrifuges Hettich EBA 200S, rotor angulation 33°, radius at the clot 54mm) at 3200 rpm, relative centrifugal force (RCF) is 1600 g for 10 minutes. The blood was isolated into three layers, and the top layer was separated from the other layers by transferring to another centrifugation tube and then was centrifuged again at 3200 rpm, RCF 1600 g for 15 minutes.^{10,18} Fibrin clots like gel were put on dry gauze to absorb excess serum for around 10 seconds, 2 mm of PRF (PRF: HA=1:1) were applied to the tooth socket. Reducing RCF increased the release of growth factors as well as the concentration of leucocytes and platelets.

Tooth extraction process in animal models

Extraction was performed under anaesthesia (ketamine and xylazine) to relieve pain. During the extraction process, the experimental animals were conditioned as comfortably as possible and were put to sleep on a support board. The enclosed action area was not visible to other experimental animals. Further, the tooth extraction was executed with labial and lingual luxation movements using pedodontic forceps for the inferior anterior region with the principle of minimal injury. After the extraction, the socket was irrigated using a 0.09% saline solution and was drained using a sterile tampon. The socket was filled with PRF (G1) and PRF+HA (G2), and nothing was given to the control group. After the application was made, the wound was closed with simple stitches using a 3.0 silk stitches thread and a curved needle with a simple

interrupted suture method. The periodontal tissue biopsy specimens were taken on the basis of the observation time (Days 3, 7 and 14). Termination was conducted before taking the specimens using ketamine at a dose of 200 mg/kg rabbit body weight.

Immunohistochemical of MMP-13 processing and evaluation

Immunohistochemical tests were performed to analyse the immunoexpression of MMP-13. The current research used an MMP-13 primary antibody kit, a labelled streptavidin-biotin (LSAB) secondary antibody kit by Mybiosource, polyclonal antibody (MyBioSource, Cat# MBS837431, RRID: AB_2895530), and positive control of breast cancer. The immuno-expression of MMP-13 appeared to be positive when the connective tissue/stromal fibroblast around was brown in colour by looking at the distribution and intensity. Histocore/final score is the multiplication of distribution with intensity, and the values for the distribution are as follows: 4 = cells positive > 75%, 3 = cells positive 51%–75%, 2 = cells positive 25%–50% and 1 = cells positive < 25%. The value for intensity is 0 = no colour, 1 = current colour, weak (light brown), 2 = present colour, moderate (brown) and 3 = present colour, strong (dark brown).^{10,19,20} Intra-examiners calibration was conducted using Leica microscope and imaging system programme. This research was conducted in Laboratory Pathology Anatomy Hasan Sadikin General Hospital Bandung, Indonesia, and the immunoexpression scores of MMP-13 were evaluated by a qualified pathologist who is blinded to the clinical outcome examined, unaware of the time point, subject, clinical and imaging information.

Statistical analysis

Data analysis was conducted using the Statistical Package for Social Science (SPSS) software (IBM SPSS Statistics, RRID:SCR_019096). Normal distribution data analysis was performed using the Shapiro–Wilk test if not normally distributed, and non-parametric tests were performed. The evaluation of MMP-13 among the groups and observation times was performed statistically with the Kruskal–Wallis test. All the data were uploaded to the repository.^{21–23}

Results

The addition of HA to PRF displayed faster wound healing in the socket. The total histocore in group 2 was lower than that in group 1 and the control group. **Table 1** shows differences in MMP-13 immunoexpression between groups. Histocore Days 7 and 14 had a significant difference ($p < 0.05$). On the third day, the MMP-13 values were the same in all the groups. On Day 7, the MMP-13 value began to decrease, where group 2 showed a significant decrease. On Day 14, it was observed that MMP-13 had a lower histocore, especially in group 2.

Table 1. Histocore difference of MMP-13 between groups (n=18).

MMP-13	Treatment group			p-Value
	C*	G1*	G2*	
Distribution				
Day 3	4.00 (4.00-4.00)	4.00 (4.00-4.00)	3.75 (3.00-4.00)	0.368
Day 7	3.50 (3.00-4.00)	3.50 (3.00-4.00)	3.50 (3.00-4.00)	0.400
Day 14	3.00 (3.00-3.00)	4.00 (3.00-4.00)	3.00 (3.00-3.00)	0.111
Total	3.50 (3.00-4.00)	4.00 (3.00-4.00)	3.00 (2.00-4.00)	0.648
Intensity				
Day 3	3.00 (3.00-3.00)	3.00 (3.00-3.00)	3.00 (3.00-3.00)	1.000
Day 7	3.00 (3.00-3.00)	3.00 (3.00-3.00)	2.50 (2.00-3.00)	0.004
Day 14	3.00 (3.00-3.00)	2.00 (2.00-3.00)	2.00 (2.00-2.00)	0.026
Total	3.00 (3.00-3.00)	3.00 (2.00-3.00)	2.50 (2.00-3.00)	0.001
Histocore				
Day 3	12.00 (12.00-12.00)	12.00 (12.00-12.00)	11.25 (9.00-12.00)	0.368
Day 7	11.25 (9.00-12.00)	9.75 (9.00-12.00)	7.00 (6.00-8.00)	0.013
Day 14	9.00 (9.00-9.00)	8.50 (8.00-9.00)	6.00 (6.00-6.00)	0.009
Total	11.25 (9.00-12.00)	10.00 (8.00-12.00)	8.00 (6.00-12.00)	0.011

*Median value (range).

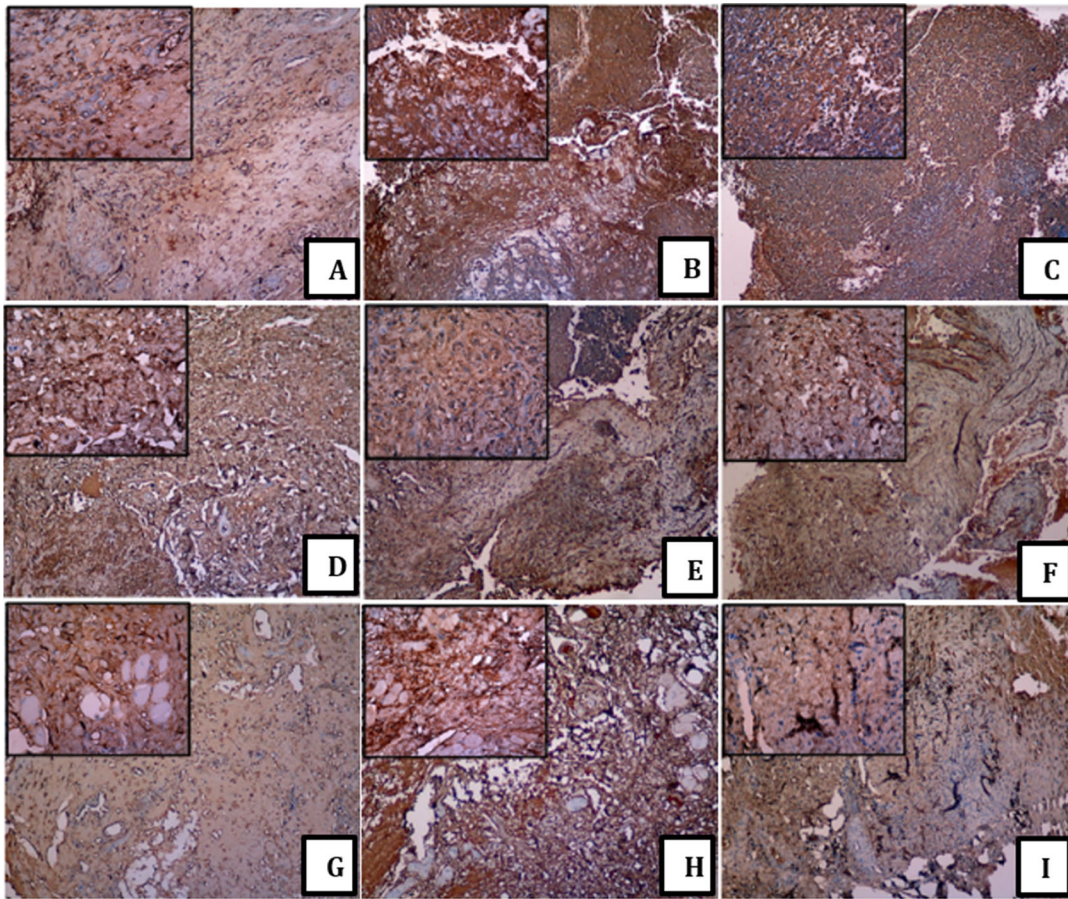


Figure 1. Immunohistochemistry of MMP-13 among a group. (A) control group day 3; (B) control group day 7; (C) control group day 14; (D) group 1 day 3; (E) group 1 day 7; (F) group 1 day 14; (G) group 2 day 3; (H) group 2 day 7; (I) group 2 day 14. With a 100× and 400× magnification using Leica Microscope.

The brown staining addresses the showed antigen, while the blue colour was the counterstain of the cores. Socket healing featured MMP-13 protein in the cytoplasm of the stromal fibroblast cells. In addition, the undeniable levels of MMP-13 protein were solely in the cytoplasm of the fibroblast cells in all the groups for day 3 [Figure 1 (A), (D) and (G)]. The light brown colour on fibroblast represented the low IHC staining of the MMP-13 expression (Days 7 and 14 and groups 1 and 2) [Figure 1 (F), (H) and (I)]. The brown colour on the fibroblast represented the moderate IHC staining of MMP-13 expression (B), (C) and (E). The large and small image inserts exhibited the portion of each treatment group and the observation time at magnifications 100× and 400×. Furthermore, administration platelets increased the MMP-13 expression in line with HA levels. The histopathological picture showed the expression of MMP-13 with a dark brown appearance in the stromal fibroblast area, which was evenly distributed in all areas with strong intensity (A, D and G). In contrast to the IHC picture, which looked light brown, especially in pictures (F) and (I), it displayed a reduced distribution and intensity (see Figure 1).²³

Table 2. Differentiation of each treatment groups by observation times on Day 3, Day 7, and Day 14.

Treatment group	P Value*
	MMP-13
C	0.017
G1	0.016
G2	0.010

*Based on Kruskal Wallis test.

Table 2 showed that administration of PRF and PRF+HA could reduce MMP-13 levels during the observation time. There was a significant difference in examination between observation times. Of all group, group 2 saw a significant difference based on the healing time.

Discussion

The present study validated that MMP-13 could determine healing activities. Hydroxyapatite is the primary mineral aspect of vertebrate bones and teeth, and it's far a good cloth for growing as a simple, efficient, and environmentally pleasant approach of forming biofunctional scaffolds and implant coatings with sizeable biocompatibility, bioactivity, mechanical strength, and the cap potential to feature as a drug delivery system.²⁴

This preclinical study confirmed the hypothesis that the application of HA in post-extraction socket healing can accelerate the healing process and decrease the level of MMP-13 immunoexpression based on the stages of wound healing, namely the stages of inflammation, proliferation, and progression. On Day 3, MMP-13 was highly expressed, proving that up to 4–5 days after an injury, there will be an inflammation phase. On the next day, the expression began to decrease, especially in group 2, meaning that HA could accelerate the work of growth factors in the wound healing process. On Day 14, there was a decrease in expression, indicating that the inflammatory process was finished and that tissue regeneration or the stages of proliferation and progression began. In this study, there was a significant difference based on the observation time from Day 3 to Day 14.^{25,26}

This result related to the theory that growth factors are an important part of the bone restoration response delivery system and delivering exogenous growth factors to the injured site improves healing outcomes significantly. Scaffold systems have the potential to provide safer, simpler bone regeneration therapies than the systems currently utilised within the clinic.²⁷ Platelets and growth factors comprise a scaffold component in the form of a fibrin matrix that could increase the bioactivity of inflammatory mediators to accelerate wound healing. Platelets and leukocyte cytokines are essential in biomaterial biology, and the fibrin matrix that supports it is the determining factor responsible for increasing the potential use of PRF. The primary source of angiogenesis is derived from fibrin gel. The fibrin matrix supports injured tissues, which affects the metabolism of epithelial cells and fibroblasts. Additionally, PRF acts as a physiological fibrin matrix, functioning as cell stems and allowing the remodeling of fibrin into being more dense connective tissue resistant. Therefore, the PRF membrane simultaneously increases the angiogenesis and the thickness or closure of the epithelium necessary for cutaneous healing. In this way, the use of platelet concentrate autologous is a promising application in the field of periodontal tissue regeneration and the alveolar bone that can be utilised in clinical circumstances that require quicker healing.²⁸

MMP-13 has been widely used as a marker and therapy in the medical field. MMP-13, an important member of the MMPs family, performs well-measured functions through degradation of type II collagen in articular cartilage and bone in osteoarthritis, concerns the molecular metastases of oral cancer, and should alter vasculogenic mimicry and endothelial-dependent vessel formation in large cell lung cancer.^{29–31} Similar studies using the expression of MMP-13 have proven that MMP-13 plays a role in every phase of wound healing by making modifications to the wound matrix, enabling cell migration essential in the remodeling process. MMPs are responsible for collagen degradation. Tissue engineering is one of the helpful endeavours in recovering periodontal tissues and alveolar bone. The three fundamental parts in tissue engineering are progenitor cells, scaffolds and growth factors. Proteinases play an essential role in wound healing by controlling cell-grid connections and the accessibility of bioactive particles. In the research conducted by Toriseva M. *et al.* (2012), the role of matrix MMP-13 on granulation tissue development was significantly reduced in mice. Granulation tissue in MMP-13/– mice exhibited delayed myofibroblast organisation, an increased microvascular density and an almost complete absence of large vessels. In addition, the gene expression profile identified genes that were differentially expressed in the granulation tissue of MMP-13/– mice involved in biological functions, including inflammatory response, angiogenesis, cell movement, cell growth, proliferation and proteolysis. This study exhibited the role of MMP-13 in wound healing by organising cell exercises that are significant in the growth and maturation of granulation tissues, including myofibroblast work, inflammation, angiogenesis and proteolysis.³²

HA applications are widely used for wound healing, even the modifications continue to be developed. Hassan HH, *et al.* (2021) states that following compositional modification, cellulose acetate nanofibers containing modified HA for wound healing utilization demonstrated a high degree of response with proliferation and growing behaviors.³³ Samadian HA, *et al.* (2018) used HA modification dressing that resulted in the highest collagen synthesis, re-epithelialization, neovascularization, and cosmetic appearance, according to histological and histomorphometric examinations of the wounds.³⁴ Wardhana AS, *et al.* use ellagic-hydroxyapatite acid to promotes osteogenesis in bone defects by increasing the amounts of osteoblasts and therefore the expression of osteoprotegerin and osteocalcin.³⁵ HA is a scaffold that can bond with MMPs and reduce its activities in vitro. Secretion and MMP activities are regulated well, and in normal tissues,

MMP is expressed on a fundamental level. If the tissue remodeling phase is necessary, such as in wound healing, MMP can be expressed and activated easily. A few diverse cell types communicated MMPs inside the skin (keratinocytes, fibroblasts, endothelial cells and inflammatory cells, such as monocytes, lymphocytes and macrophages). MMP expression can be prompted to see different signs, including cytokines, hormones and contact with other cell types or with ECM.³ MMP-13 cleaves Connective Tissue Growth Factor (CTGF) and releases deliveries a few parts, which are more strong than the parent particle to actuate fibrosis.³⁶

MMPs' roles in the pathogenesis of oral and systemic diseases, which are endopeptidases that are active against extracellular macromolecules, have recently been identified as potential utility biomarkers for the diagnosis and follow-up of oral and systemic diseases, allowing for the early assessment of malignancy risk as well as the monitoring of disease progression and treatment response. There are several inflammatory mediators involved in the wound healing process following a tooth extraction, with matrix metalloproteinases (MMPs) playing a significant role. Collagenase is the primary enzyme involved in collagen degradation. MMP-13, also known as collagenase-3, is capable of insulating fibrillar collagen. MMP-13 is more effective at cleaving type II collagen than type I or III collagen. Unlike other collagenases, MMP-13 is a strong gelatinase with telopeptides activity.^{37,38} However, there are several other, more specific markers that require further investigation. MMP-13 is a collagenase that has a relatively limited appearance in adult normal tissue. The study's limitation is that the evaluation was done solely by looking at the level of MMP-13 immunoexpression. This study has limitations in optimizing the work of the material inserted into the socket after tooth extraction. Simple sutures are performed to prevent the material from escaping from the socket, mixing with saliva, and being lost. However, the sutures can come off if the experimental animal is too active to move its teeth and tongue. Although the assessment was blinded, it is preferable to use consensus pathology rather than the judgment of a single pathologist. Some versions involving more than one pathologist are possible. During trials, slide rereading may also occur to measure shifts between and between readers. Allowing pathologists to communicate and apply their knowledge at all phases of the trial may result in more realistic expectations and more rewarding outcomes.

The study's limitation is that the evaluation was done solely by looking at the level of MMP-13 immunoexpression. Further research will be required to assess the changes in the alveolar bone using intra-oral radiographic examination. Further research can also be carried out with a longer observation period to estimate the expression of other molecular markers of the periodontal tissue and alveolar bone and are also needed to confirm the effective dose of the biomaterial used when ready for use in human clinical trials.

Conclusion

In conclusion, the administration of PRF and HA was capable of reducing the MMP-13 expression that significantly accelerate the socket healing process. This study that used platelet concentrates as growth factors and hydroxyapatite as scaffold is the best combination and can be utilised as an alternative therapy. Moreover, hydroxyapatite is an alloplastic material that has inherent bioactive properties that support osteoconduction, can bind MMPs, and showed faster healing results based on the observation time as documented by immunohistochemistry.

Author contributions

MMD and MR conceived the plan of this research. MMD wrote the manuscript. MMD analysed the data and made the figures. MMD edited the manuscript. MMD is responsible for research in the animal laboratory, MR did the analysis of MMP-13 evaluation, and MMD supervised the whole research. All authors revised and approved the manuscript for final submission.

Data availability

Underlying data

Figshare: Underlying data for 'Pre-Clinical Study: Immunohistochemical evaluation of matrix metalloproteinase-13 on Rabbit (*Oryctolagus cuniculus*) socket healing after application of platelet-rich fibrin with and without hydroxyapatite'

Histopathological of IHC MMP-13

<https://doi.org/10.6084/m9.figshare.16529847>.²³

This project contains the following underlying data:

= MMP-13 C 3 100×.tiff (immunoexpression of MMP-13 control group in Day 3 observation at 100× magnification).

- = MMP-13 C 3 400×.tiff (immunoexpression of MMP-13 control group in Day 3 observation at 400× magnification).
- = MMP-13 C 7 100×.tiff (immunoexpression of MMP-13 control group in Day 7 observation at 100× magnification).
- = MMP-13 C 7 400×.tiff (immunoexpression of MMP-13 control group in Day 7 observation at 400× magnification).
- = MMP-13 C 14 100×.tiff (immunoexpression of MMP-13 control group in Day 14 observation at 100× magnification).
- = MMP-13 C 14 400×.tiff (immunoexpression of MMP-13 control group in Day 14 observation at 400× magnification).
- = MMP-13 PRF 3 100×.tiff (immunoexpression of MMP-13 PRF group in Day 3 observation at 100× magnification).
- = MMP-13 PRF 3 400×.tiff (immunoexpression of MMP-13 PRF group in Day 3 observation at 400× magnification).
- = MMP-13 PRF 7 100×.tiff (immunoexpression of MMP-13 PRF group in Day 7 observation at 100× magnification).
- = MMP-13 PRF 7 400×.tiff (immunoexpression of MMP-13 PRF group in Day 7 observation at 400× magnification).
- = MMP-13 PRF 14 100×.tiff (immunoexpression of MMP-13 PRF group in Day 14 observation at 100× magnification).
- = MMP-13 PRF 14 400×.tiff (immunoexpression of MMP-13 PRF group in Day 14 observation at 400× magnification).
- = MMP-13 PRF+HA 3 100×.tiff (immunoexpression of MMP-13 PRF+HA group in Day 3 observation at 100× magnification).
- = MMP-13 PRF+HA 3 400×.tiff (immunoexpression of MMP-13 PRF+HA group in Day 3 observation at 400× magnification).
- = MMP-13 PRF+HA 7 100×.tiff (immunoexpression of MMP-13 PRF+HA group in Day 7 observation at 100× magnification).
- = MMP-13 PRF+HA 7 400×.tiff (immunoexpression of MMP-13 PRF+HA group in Day 7 observation at 400× magnification).
- = MMP-13 PRF+HA 14 100×.tiff (immunoexpression of MMP-13 PRF+HA group in Day 14 observation at 100× magnification).
- = MMP-13 PRF+HA 14 400×.tiff (immunoexpression of MMP-13 PRF+HA group in Day 14 observation at 400× magnification).

Figshare: Histoscore of IHC MMP-13

<https://doi.org/10.6084/m9.figshare.16531398>.²¹

This project contains the following underlying data:

- = Histoscore C PRF PRF+HA (Multiplication of distribution and intensity of MMP-13 among group: control, PRF, and PRF+HA)
- = Histoscore C 3, 7, 14 (Multiplication of distribution and intensity of MMP-13 in control group based on observation time)
- = Histoscore PRF 3, 7, 14 (Multiplication of distribution and intensity of MMP-13 in PRF group based on observation time)
- = Histoscore PRF+HA 3, 7, 14 (Multiplication of distribution and intensity of MMP-13 in PRF+HA group based on observation time)
- = Histoscore 3, 7, 14 (Multiplication of distribution and intensity of MMP-13 among group based on observation time)

Reporting guidelines

Figshare: ARRIVE checklist for 'Pre-Clinical Study: Immunohistochemical evaluation of matrix metalloproteinase-13 on Rabbit (*Oryctolagus cuniculus*) socket healing after application of platelet-rich fibrin with and without hydroxyapatite'

<https://doi.org/10.6084/m9.figshare.16640299>.¹⁶

This project contains the following underlying data:

- = ARRIVE guidelines checklist full

Figshare: Figure of Table for 'Pre-Clinical Study: Immunohistochemical evaluation of matrix metalloproteinase-13 on Rabbit (*Oryctolagus cuniculus*) socket healing after application of platelet-rich fibrin with and without hydroxyapatite'

<https://doi.org/10.6084/m9.figshare.16641181>.³⁹

This project contains the following underlying data:

- = Table 1 (Table of histoscore difference of MMP-13 between groups)
- = Table 2 (Table of differentiation of each treatment groups by observation times on Day 3, Day 7, and Day 14)

Data are available under the terms of the [Creative Commons Zero "No rights reserved" data waiver](#) (CC0 1.0 Public domain dedication).

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Current Peer Review Status:  

Version 2

Reviewer Report 02 September 2022

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Niswati Fatmah Rosyida 

Department of Orthodontics, Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta, Indonesia

The authors have revised the points that must be corrected in the first review. Therefore the quality of the article has improved.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: biomaterial; orthodontic tooth movement, orthodontics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 11 August 2022

<https://doi.org/10.5256/f1000research.137236.r147248>

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Joao Vitor dos Santos Canellas 

Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Rio de Janeiro State University, Rio de Janeiro, Brazil

The authors addressed each of the points made by the first revision, improving the quality of the paper considerably.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Evidence-based dentistry, Systematic reviews, meta-analysis, clinical trials, and Platelet-rich fibrin.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 04 July 2022

<https://doi.org/10.5256/f1000research.77809.r142579>

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Joao Vitor dos Santos Canellas 

Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Rio de Janeiro State University, Rio de Janeiro, Brazil

The authors make quite an effort trying to evaluate in a preclinical study the use of PRF with and without HA in socket healing. However, there are some points that need clarification.

Comments:

1. The title and the abstract of this study are appropriate, direct, and descriptive.
2. The authors reported that the sample size was determined using the Mead equation. However, they should describe how the sample size was determined exactly, including the values used to achieve the sample of 18 rabbits. Small sample sizes may make it difficult to determine if the outcome is a true finding or a type II error.
3. The authors reported that the animals were randomly divided into three groups, but they did not explain the method used to assign rabbits interventions. Bias arising from the randomization process can occur if the method of allocation is not adequately or not truly random.
4. Important differences among commercial HA result from chemical proprieties and the manufacturing process. Authors should detail what HA was used in this research. Many studies reported that two similar alloplastic biomaterials could produce different clinical results.
5. Authors should remove the sentence "...The limitation of this study is that, after tooth extraction, intervention was executed on the basis of the treatment group, and then simple stitches was performed. However, stitches can loosen up if the experimental animal is too active in moving its teeth and tongue, even making the stitches to come off." From the method section. This information should be included in the discussion section.

6. A detailed description must be reported in all studies related to PRF to increase the transparency of research. Please, provide the centrifugation device used, as well as the rotor angulation, radius at the clot, and tube characteristics. Each of mentioned parameters may influence regeneration success.
7. Bias in the measurement of the outcomes can occur when the researcher involved in outcome analysis is not blind to the assigned interventions. Please inform if the qualified pathologist was blinded. If not, include this information in the study limitation paragraph.
8. Authors use a surrogated outcome, "MMP-13 immunoexpression" to evaluate alveolar changes after tooth extraction. Discuss the limitations of this examination and other possible limitations of the present preclinical study.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Evidence-based dentistry, Systematic reviews, meta-analysis, clinical trials, and Platelet-rich fibrin.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 01 Aug 2022

META DAMAYANTI, Unisba, Bandung, Indonesia

The authors thank reviewer for the excellent review of the paper and for the in-depth suggestions made to improve the quality of the article. The followings are the responses to

the recommendations of the reviewer. The valuable suggestions and corrections are incorporated cautiously, and the paper is made more transparent while revising the manuscript. All changes have been made in response to the reviewer. I have just submitted version 2. Furthermore, to answer point-by-point:

The authors reported that the sample size was determined using the Mead equation. However, they should describe how the sample size was determined exactly, including the values used to achieve the sample of 18 rabbits. Small sample sizes may make it difficult to determine if the outcome is a true finding or a type II error.

The exact determined sample has been updated as suggested to avoid an error. This approach further allowed us to reduce the required number of animals according to the principle of the three R's (replacement, reduction, and refinement) and ARRIVE 2.0 guidelines for animal handling.

The authors reported that the animals were randomly divided into three groups, but they did not explain the method used to assign rabbits interventions. Bias arising from the randomization process can occur if the method of allocation is not adequately or not truly random.

We applied simple random sampling to divide into three groups of treatment and then three groups of observation time. Randomization processes for the animal study have been described in greater detail in the new version manuscript.

Important differences among commercial HA result from chemical properties and the manufacturing process. Authors should detail what HA was used in this research. Many studies reported that two similar alloplastic biomaterials could produce different clinical results.

We agree that there are differences between commercial HA. Because we already have experience with HA. We use HA material with the brand that is most often used by dentists today, namely a modification made from the Gama-Cha brand. We recognize the limitations of the ingredients, and we agree that further research is needed to see how commercial HA administration differs from homemade biocompatible products.

Authors should remove the sentence "...The limitation of this study is that, after tooth extraction, the intervention was executed on the basis of the treatment group, and then simple stitches was performed. However, stitches can loosen up if the experimental animal is too active in moving its teeth and tongue, even making the stitches to come off." From the method section. This information should be included in the discussion section.

We have deleted the sentences regarding the statement above. We agree with the comment that it should be removed. This aspect has consequently been removed from the text, and more explained in the discussion section.

A detailed description must be reported in all studies related to PRF to increase the transparency of research. Please, provide the centrifugation device used, as well as the rotor angulation, radius at the clot, and tube characteristics. Each of mentioned parameters may influence regeneration success.

Thank you for your helpful report. Your suggestions have allowed us to improve our manuscript and present our data more accurately. We will add to the new version paper,

and more detailed documentation will also be provided.

Bias in the measurement of the outcomes can occur when the researcher involved in outcome analysis is not blind to the assigned interventions. Please inform if the qualified pathologist was blinded. If not, include this information in the study limitation paragraph.

Thank you for your comment. Blind observers (pathologists) assigned random areas of IHC photographed using a Leica microscope without knowing the group identity on histopathology slides. We added this more detailed explanation to the materials and methods section. Revisions have been included in the revised text.

Authors use a surrogated outcome, "MMP-13 immunoexpression" to evaluate alveolar changes after tooth extraction. Discuss the limitations of this examination and other possible limitations of the present preclinical study.

The limitations of this examination and other possible limitations of the present preclinical study have been included in the revised text.

Competing Interests: No competing interests were disclosed.

Reviewer Report 07 February 2022

<https://doi.org/10.5256/f1000research.77809.r119475>

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Niswati Fatmah Rosyida 

Department of Orthodontics, Faculty of Dentistry, Universitas Gadjah Mada, Yogyakarta, Indonesia

1. Abstract:

- Kindly rewrite the conclusion in the abstract, because the conclusion is different from the result of this study.

2. Method:

- Kindly mention the production company of the material used.

- "The animals were not selected on the basis of sex and age because they did not affect the treatment". Kindly comment on this statement. Please add the literature which proves that statement.

- Please mention the reason not to control the age of animals?

- Kindly describe the preparation of PRF+HA. Mention the formulation of PRF and PRF+HA which

were used in this study, the concentration of each material, the dosage, and dosage form.

- "...the immunoexpression scores of MMP-13 were evaluated by a qualified pathologist." - please describe how intra-examiner calibrations were measured.

- "...if normally distributed, and parametric tests were performed. The evaluation of MMP-13 among the groups and observation times was performed statistically with the Kruskal-Wallis test". Please add the reason for using a nonparametric test (Kruskal Wallis).

3. Results:

- Kindly revise the legend of the table.

- Please add an asterisk or similar sign to highlight where there are significant differences between the groups.

- Kindly revise the legend of the table. Add the information of magnification.

4. Discussion:

- "...there was a significant difference based on the observation time from Day 3 to Day 14." - kindly add literature in paragraph two of the discussion which strengthens this result.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: biomaterial; orthodontic tooth movement

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 01 Aug 2022

META DAMAYANTI, Unisba, Bandung, Indonesia

We want to thank the invited reviewer for their valuable comments and suggestions. We feel that the scientific value of our manuscript has increased significantly. This revised version of the manuscript includes the following significant adjustment as suggested by the reviewer. We have proofread the manuscript a couple of times. Thank you for your review. I have just submitted version 2. To answer point-by-point:

Abstract:

- Kindly rewrite the conclusion in the abstract, because the conclusion is different from the result of this study.

A revised abstract about the conclusion has been added in the new versions.

Method:

- Kindly mention the production company of the material used.

References for the product of material used have been added in the revised version. Also, we generated and utilized the commonly used product.

- "The animals were not selected on the basis of sex and age because they did not affect the treatment". Kindly comment on this statement. Please add the literature which proves that statement.

Thank you for making us aware of this. We followed your suggestion, and we added this to the script. As in all studies here, we mentioned that gender had no effect because we assumed no hormonal influence on oral wound healing. However, I will add gender-specific criteria because our study used male and adult animal models. However, for age, we have revised it by adding an age range as an inclusion criterion.

- Please mention the reason not to control the age of animals?

Thanks for mentioning this. I missed a clear statement of inclusion criteria, and a more detailed age range will also be corrected.

- Kindly describe the preparation of PRF+HA. Mention the formulation of PRF and PRF+HA which were used in this study, the concentration of each material, the dosage, and dosage form.

Thank you for letting us know that this explanation has gotten lost somehow. The methodology has been clarified, such as formulations of PRF and PRF+HA, the concentration of each material, the dosage, and the dosage form used in the study. Materials and methods have been described more accurately as suggested in the new versions.

- "...the immunoexpression scores of MMP-13 were evaluated by a qualified pathologist." -

please describe how intra-examiner calibrations were measured.

Evaluation by a qualified pathologist has been described as well as how intra-examiner calibration is measured, including several points to address the importance of this research study evaluation method.

-"...if normally distributed, and parametric tests were performed. The evaluation of MMP-13 among the groups and observation times was performed statistically with the Kruskal-Wallis test". Please add the reason for using a nonparametric test (Kruskal Wallis).

In the revised text, we have corrected the error of the normal to abnormal distribution, so that a non-parametric test with the Kruskal-Wallis test was performed. Because in this study we want to examine differences in stratified groups. Additions suggested by reviewers have also been added detailed in the methods section.

Results:

- Kindly revise the legend of the table.

Thank you for your detailed comment. We replaced the revised table in the new version paper.

- Please add an asterisk or similar sign to highlight where there are significant differences between the groups.

Thank you for the suggestions. We have been adding a similar sign to highlight significant differences between the groups and are now uniform throughout the manuscript to increase readability.

- Kindly revise the legend of the table. Add the information of magnification.

The legend of the table and information on magnification has been fully addressed in the revised text.

Discussion:

- "...there was a significant difference based on the observation time from Day 3 to Day 14."

- kindly add literature in paragraph two of the discussion which strengthens this result.

The comment in the discussion section to strengthen the result has been addressed in the revised text by providing more literature.

Competing Interests: No competing interests were disclosed.

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