ORIGINAL ARTICLE

Effectiveness Of 1% Metformin Gel And Xenograft On Bone Regeneration In Post Extraction Socket Through Osteoblast And Osteoclast Dynamics

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ABSTRACT

Introduction: Basic components in engineering tissue is cells, scaffolding and signaling molecules. Bone graft is a bone reconstruction treatment that is a proponent in tissue engineering. Metformin is a diabetic medication also can be used as a signaling molecule in tissue engineering, regenerative agent in bone and soft tissue. Bone tissue is dynamic, with a decrease in osteoclast cell numbers and an increase in osteoblast cell numbers. The goal of this research is to determine the effect that metformin, with 1% formulation in bone regeneration in post-extraction sockets, which is determined through osteoblastic and osteoclastic dynamic. **Method:** Twenty-seven Cavia Cobaya had their incisivus extracted, then divided into negative control group with no treatment given, positive control group treated with xenograft, and Treatment group treated with xenograft plus 1% metformin gel. On day 7,14 and 28 rats were sacrificed, and histologically examined. **Result:** A decrease in osteoclast cells followed by an increase in osteoblast cell following 1% metformin gel and xenograft application **Conclusion:** Application of 1% Metformin Gel followed by Xenograft placement provide a major decrease in osteoclast cells and an increase in osteoblast cells.
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INTRODUCTION

Tooth extraction causes the tooth socket to undergo bone resorption and remodeling, thus potentially causing loss of bone structure and volume.[1] As cited from previous study, bone resorption occurred more rapidly in the buccal area with a reduction of alveolar ridge volume experiencing reduction by approximately 50% of its original volume.[2] Loss of this bone structure can affect the stability, retention, and support of dental prostheses, causing various complications later on.[3] Due to the negatively profound effect of extraction on the alveolar bone over- time, there has to be an active measure in order to preserve the ideal dimensions and the overall condition of the alveolar bone, this can be done by applying treatments, namely Socket Preservation.[4]

Socket preservation is an attempt to limit the degradation of alveolar bone along the socket post extraction by applying bone graft, membrane barrier, and/or biological agents in the extraction socket. Therefore, the success of this procedure would be closely tied with the severity of both the trauma and inflammation, the morphology of the alveolar socket, the closure of the extraction wound, the use of graft material in the socket, and the use of regenerative materials. Bak et al., 2010 found that metformin significantly reduced alveolar bone loss in mice with periodontitis by causing two times more mineralization of MC3T3-E1 cells, thus increasing osteoblasts.[5] Socket preservation can only partially prevent bone resorption, and delayed healing may occur at the graft site, with unpredictable bone quality.[6] For this reason, it is necessary to add materials to improve wound healing along with bone and soft tissue regeneration. There are various types of bone graft materials that can be inserted into the socket to prevent loss of bone structure and volume, including

autograft, allograft, xenograft, and alloplast materials.[7] However, each material has its own drawbacks .[8]

Bone is a complex structure that is constantly goes through a cycle of formation and deformation during an individual's lifetime.[9] The process by which osteoclasts remove older or damaged bone and replace it with newly created bone by osteoblasts is known as bone remodeling. The cells that resorb bone are called osteoclasts, and they are produced by hematopoietic stem cells (HSCs). Osteoclasts release proteolytic enzymes including cathepsin K (CTSK) and acids that solubilize collagen and other matrix proteins during bone resorption. Mesenchymal precursors are linked to the osteoprogenitor lineage by the sequential action of transcription factors. This results in the development of osteoblasts, which are the cells that produce bone, into osteocytes.[10] Alkaline phosphatase, osteocalcin, and type I collagen are examples of extracellular proteins that osteoblasts create. Type I collagen accounts for about 90% of the protein in bone matrix.[11] The extracellular matrix becomes mineralized when calcium phosphate accumulates as hydroxyapatite, having previously been discharged as unmineralized osteoid.[12]

Communication between osteoblasts and osteoclasts is crucial for regulating bone remodeling during bone homeostasis. Through interactions involving EFNB2-EPHB4, FAS-FASL, and NRP1-SEMA3A to control cell proliferation, differentiation, and survival, osteoblasts and osteoclasts come into direct contact with one another. TGF-β and IGF-1 are released from the bone matrix during osteoclast-mediated bone resorption, which triggers osteoblast-mediated bone formation. Osteoblasts release WNT5A, OPG, and WNT16, which suppress osteoclast activity, as well as M-CSF, RANKL, and WNT5A, which encourage osteoclast formation and development. On the other hand, osteoclasts release SEMA4D, which inhibits osteoblast differentiation, and S1P, CTHRC1, and C3, which promote osteoblast differentiation.[13]

Metformin is a diabetic drug that has been used for a long time, and is proven to have a positive effect on diabetics without significant side effects.[14] Several studies have also shown the potential of metformin as an anti-aging, anti-cancer, anti- inflammatory agent by changing the NF-κB and TNF-α signaling pathways, antibacterial in the gastrointestinal environment, and helping tissue regenerative, especially periodontal tissue. Metformin can increase osteogenic differentiation, facilitate proliferation, migration, and protect periodontal ligament stem cells from oxidative stress. Metformin can also reduce alveolar bone loss, increase osteoblast differentiation, and induce osteoblastic differentiation in pluripotent stem cells, derived from mesenchymal stem cells.[13]

Clinically, in dentistry metformin has been used as a local drug delivery, mixed with composite resin to induce pulpal stem cells, and intracanal medicament for the treatment of apical periodontitis. Metformin with a concentration of 1% is proven to have the best effect on bone and soft tissue regeneration.[15] The results from the research by Khalifehzadeh S et al., 2019 also support the clinical improvements resulting from the use of metformin in treating periodontal defects in humans and combined the use of metformin and PRGF and demonstrated significant radiographic improvement. [16]

However, so far there has been no study using metformin with a combination of graft materials in post-extraction socket cases. This study aims to determine the effect 1% metformin and xenograft on bone regeneration in post-extraction sockets through osteoblast and osteoclast dynamics.

EXPERIMENTAL MATERIALS AND METHOD

Gel Preparation

Metformin hydrochloride pure active ingredient 2 gr was purchased from a regular pharmacy in Makassar, South Sulawesi, Indonesia. Gellan Gum, Sucralose, Methyl paraben, and Propyl paraben strawberry flavor were obtained as samples. The additional chemicals that were acquired, such as mannitol, sodium citrate, and citric acid, were all of analytical quality. The formulation's necessary ingredients were all precisely weighed. 50 milliliters of distilled water kept at 95 degrees Celsius were used to spread dry gellan gum powder. To help hydrate Gellan gum, the dispersion was agitated using a magnetic stirrer for 20 minutes at 95°C. The Gellan Gum solution was continuously stirred as the necessary amount of mannitol was added, and the temperature was kept above 80°C. Next, while stirring, sucralose, citric acid, and preservatives (methyl and propyl paraben) were added. Finally, 10 mL of distilled water was used to dissolve the necessary amount of sodium citrate, which was then added to the mixture. During the manufacturing process, the weight of the gel is constantly measured and corrected to 100 g using purified water. Metformin, Gellan Gum, and additional ingredients were combined and enclosed in an airtight plastic bag. To create a gel, the mixture was cooled to room temperature. Gellan gum was used in four distinct concentrations (0.2, 0.3, 0.4, and 0.5%), and two different sodium citrate concentrations (0.3 and 0.5%) were used in each gel preparation.[16]

Animals

Twenty-seven two-month old male Cavia cobaya weighing at 250-300 gr underwent acclimization one week before the study. This research was conducted after obtaining an ethical license at Hasanuddin University (No:0060/PL09/KEPK FKG-RSGM UNHAS/2023). Test animals were given standard laboratory food and drink and weighed at the end of acclimatization. All test

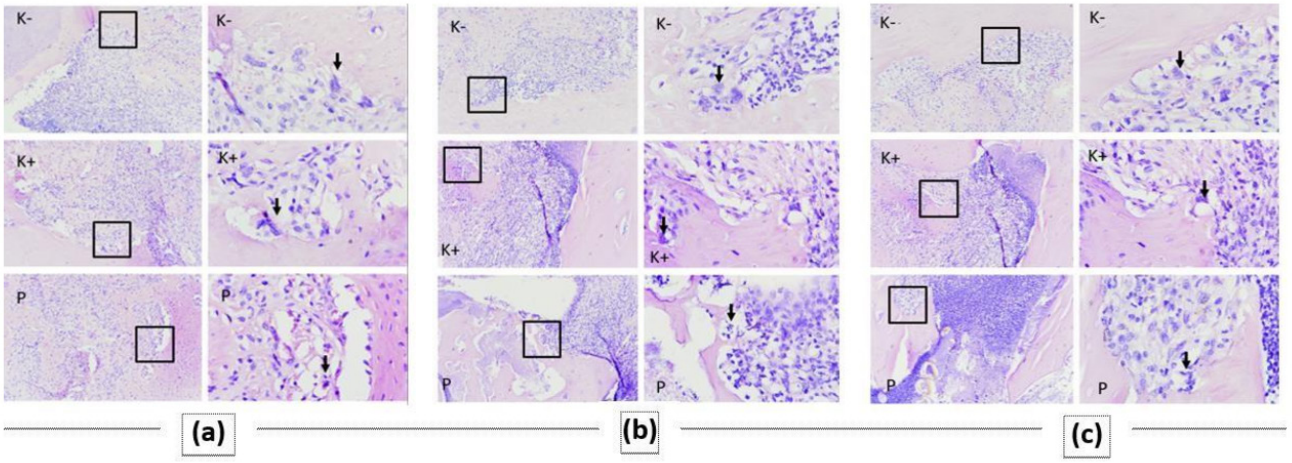
animals used as a subject in this research were treated according to international code of conduct for research animals.

Treatment

In group 1 (n=9), the extraction socket was given xenograft in combination with Metformin Gel 1% as the treatment group. In group 2 (n=9), the extraction socket was given a xenograft in the form of a deproteinized bovine bone matrix with a granule diameter of 0.25 – 1 mm as positive control. In group 3 the extraction socket was not treated, the blood was allowed to clot using cotton pellets as a negative control. After the socket is filled, suturing is performed. On the 7th, 14th, and 28th day after extraction, the rats were sacrificed. Mandible labial bone and alveoli were then separated and fixed in 10% formalin for histomorphological evaluation.

Histomorphological Evaluation

The bone specimen was brought to Hasanuddin University Anatomical Pathology Laboratory for testing and creation of histology slides, after which the slides were sent to the faculty's Biochemistry-Biomolecular Laboratory in Brawijaya University Faculty of Medicine for haematoxylin eosin (HE) staining test and imaging and Osteoclast number assessment. The preparations were assessed according to percentage with baseline group as an indicator of osteoclasts and osteoblasts quantity. Observation of histological preparations was carried out with the help of a Nikon E100 microscope with a Sony A7 camera. First, the area of the apical third of the socket was determined at 100x magnification; then the observed area is determined under 200x magnification.



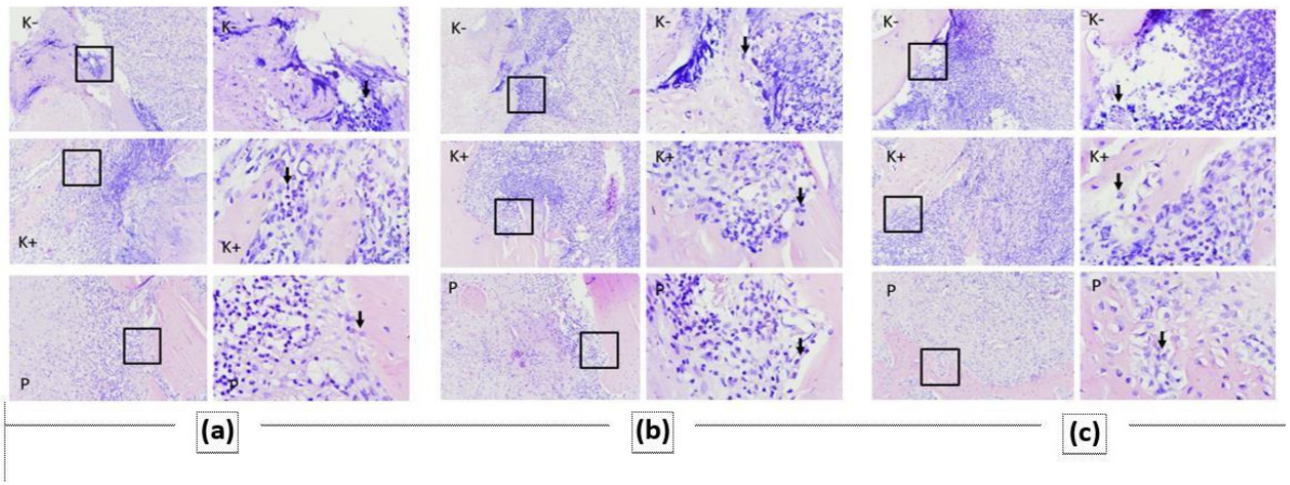


Fig. 1:(a) Haematoxylin eosin Imaging (HE) staining showing the number of osteoclast on day 7 at 200x and 400x magnification (b) Haematoxylin eosin Imaging (HE) staining showing the number of osteoclast on day 14 at 200x and 400x magnification (c) Haematoxylin eosin Imaging (HE) staining showing the number of osteoclast on day 28 at 200x and 400x magnification

Fig. 2:(a) Images of haematoxylin eosin (HE) staining showing the number of osteoblast on day 7 at 200x and 400x magnification (b) Images of haematoxylin eosin (HE) staining of the number of osteoblast on day 14 at 200x and 400x magnification (c) Images of haematoxylin eosin (HE) staining showing the number of osteoblast on day 28 at 200x and 400x magnification

Statistical analysis

Data retrieved during this study were analyzed using either ANOVA Test or Kruskal-Wallis Test. Both of these statistical tests use data with a ratio or interval scale. Before determining the statistical test to be used, the data was tested for normality using the Shapiro-Wilk test because the number of research subjects was less than 50 subjects. If the Normality Test results are > 0.05, then the data is normally distributed, and vice versa. Data that is normally distributed will then be tested using ANOVA, while data that is not normally distributed will be tested using Kruskal-Wallis. If the ANOVA test obtains a P-value of <0.05, the data will then be tested for variance using the Levene test. If the variances are the same, Tukey's Post-Hoc test is performed. If the variances are different, proceed with Tamhane's test. The type of data used is primary data, data processing were done using IBM SPSS Statistics V.21.

RESULTS

On day 7, the average osteoblast in the 1% metformin gel + bovine xenograft treatment group was 7.00. On the 14th day the average osteoblast increased by 11.33 and further increased on the 28th day by 13.67. One Way ANOVA statistical testing revealed a significant difference in osteoblast values on days 7, 14, and 28 between the metformin gel+bovine xenograft treatment group, with a value of p=0.006 (p<0.05).

The mean osteoblast of the positive control group (bovine xenograft) on day 7 was 6.33. On the 14th day the average osteoblast increased by 10.00 and further increased on the 28th day by 12.33. One Way ANOVA statistical testing revealed no significant change in osteoblast values on days 7, 14, and 28 in the positive control group (bovine xenograft), with a value of p=0.000 (p<0.05).

On day 7, the average Osteoclast in the 1% metformin gel + bovine xenograft treatment group was 6.33. On the 14th day the average osteoclast decreased by 3.33 and further decreased on the 28th day by 2.33. One Way ANOVA examination revealed a significant difference in osteoclast values on days 7, 14, and 28 between the metformin gel+bovine xenograft therapy group, with a value of p=0.004 (p<0.05).

The average Osteoclasts of the positive control group (bovine xenograft) on day 7 was 7.00. On the 14th day the average osteoclast was 4.00 and decreased further on the 28th day to only 2.33. One Way ANOVA statistical tests revealed a significant variance in osteoclast values on days 7, 14, and 28 between the positive control group (bovine xenograft), with a value of p=0.002 (p<0.05).

DISCUSSION

Recently, a substantial amount of information has come to light regarding the possible applications of the first-line antidiabetic biguanide metformin in promoting osteoblastic differentiation.[18]

Cortizo et al. (2006) initially reported the pro-osteogenic effects of metformin in MC3T3E1 mouse calvaria-derived osteoblast-like cells. Metformin stimulated calcium deposition and collagen type I production in a dose-dependent manner.[18] In MC3T3E1 cells, metformin also dramatically increases the expression of endothelial nitric oxide synthase and bone morphogenetic protein-2, both of which are drug- and dose-dependent.[19]

All the P-values on the tables showed the number of osteoclast cells decreased and the number of osteoblast cells increased after the application of 1% metformin gel and xenograft.

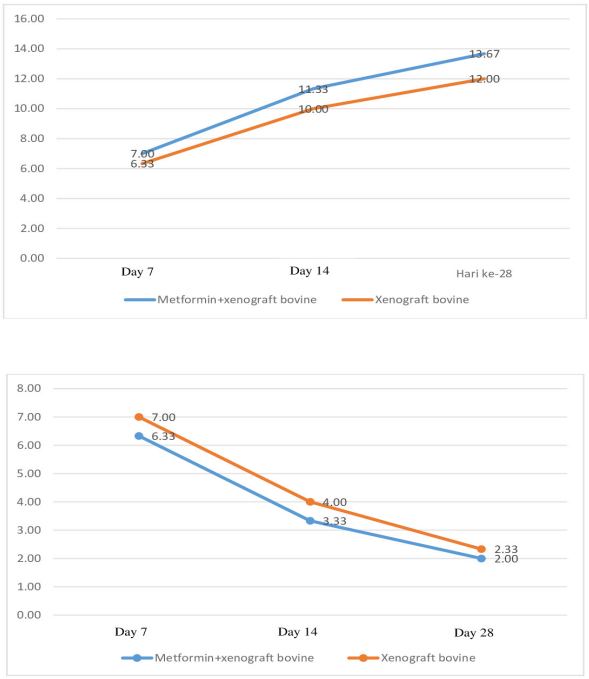


Fig. 3: Graph of dynamics of average values of Osteoblasts and Osteoclasts between Treatment Group (Metformin gel 1% & Xenograft bovine) and Positive Control Group (Xenograft) on day 7, 14 and 28. Metformin has also been shown to benefit bone in periodontitis models. [23, 24, 5, 23] Bak et al. looked into how metformin affected the rate at which rats with ligature-induced periodontitis developed the disease. In periodontal tissues, metformin decreased alveolar bone loss and inflammatory cell infiltration. [5] These and other results point to a reduction in bone loss with low doses of metformin. [23]

These are two graphs of the dynamics of changes in the mean values of osteoblasts and osteoclasts in the two treatment groups based on days of observation. In this graph we can see the difference in changes in the mean values between osteoblasts and osteoclasts from days 7, 14, and 28, this shows that the average value of osteoblasts in the treatment group (metformin gel 1% + xenograft bovine) is highest on day 28 and the lowest on day 7, as well as the graph of osteoblasts in the positive control group (xenograft bovine) shows that the mean value of osteoblasts was highest on day 28 and lowest on day 7. In contrast, in the osteoclast graphs of

the treatment and positive control groups, there was a decrease in the osteoclast mean graph from days 7, 14 and 28, showing the lowest osteoclast mean value on day 28.

Osteoblasts derived from mesenchymal stem cells play an important role in the maintenance and regeneration of bone mass. The main role of osteoblasts is to synthesize and secrete various proteins involved in bone formation and mineralization of the extracellular matrix into bone. In contrast, osteoclasts originate from Hematopoetic Stem Cells (HSCs), responsible for bone resorption which causes bone tissue destruction.[20]

This study shows that RUNX2 transactivation via an AMPK-driven regulatory cascade likely mediates the osteogenic activity of metformin.[20] Metformin not only affects osteoblasts, but new research has also demonstrated that it controls MSCs' osteogenic development. Metformin inhibited an adipogenic phenotype which encouraged osteoblastic development in rat-derived bone marrow MSCs.[21]

Using rat bone marrow MSCs, a different group demonstrates that metformin can counteract rosiglitazone's pro-adipogenic action in favor of an osteoblastic phenotype, which is partially mediated by activation of AMPK and RUNX2.[22] Similarly, metformin therapy significantly affected the osteogenic development of stem cells generated from adipose tissue, while inhibiting the differentiation of these cells into adipocytes.[18] Remarkably, our investigation revealed that the documented metformin-induced reactions were attenuated upon the administration of the antiviral drug adenine 9-β-D-arabinofuranoside, a particular AMPK inhibitor.[19] These findings revealed that metformin activated AMPK signaling, which in turn caused osteoblast-like cells to differentiate and mineralize. Similarly, a dominant negative version of AMPK genetically suppressed metformin-induced bone-forming gene transcription in MC3T3E1 cells, or Compound C, a commonly used antagonist of AMPK activation, chemically downregulated it.[21]

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In comparison to rats without diabetes, Sun et al. discovered that diabetic rats had more osteoclasts.[24] Metformin was found to reduce osteoclast counts in the same study when compared to diabetic rats that were not treated.[24]

An earlier study (Pradeep, 2013) looked at the effectiveness of metformin gel at 0.5%, 1%, and 1.5% in treating individuals with chronic periodontitis. In comparison to 0.5% and 1.5% metformin gels, it was discovered that 1% metformin gel had a better drug-release pattern and greater clinical efficacy. Metformin was selected for this investigation because to its correlation with elevated expression of osteogenic genes, which trigger the development of osteoblasts. There may be further advantages for periodontal and bone healing from this. Additionally, metformin can be used in conjunction with periodontal therapy to improve the outcomes of traditional periodontal treatment by increasing osteoblastic differentiation. Because it provides a superior medication release pattern and greater clinical efficacy, metformin gel 1% was utilized in this study.[17]

CONCLUSION

It seems intriguing to use the osteoanabolic effects of metformin as a treatment strategy to boost bone and periodontal tissue regeneration by promoting the osteogenic incorporation of autogenous iPSC-MSCs. It was discovered that using xenograft and 1% metformin gel will maximize the growth of osteoblast cells while limiting the amount of osteoclast cells, thereby opening up new possibilities for periodontal regeneration.

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