World Journal of Advanced Research and Reviews
eISSN: 2581-9615 CODEN (USA): WJARAI
Cross Ref DOI: 10.30574/wjarr
Journal homepage: https://wjarr.com/

(RESEARCH ARTICLE)

Administration of hydroxyapatite and ellagic acid combination to bone defects on Interleukin-6 Expression

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World Journal of Advanced Research and Reviews, 2025, 26(01), 1028-1034

Publication history: Received on 27 February 2025; revised on 06 April 2025; accepted on 08 April 2025

Article DOI: https://doi.org/10.30574/wjarr.2025.26.1.0978

Abstract

Background: Bone defect is the loss of some bone tissue which can be caused by several factors, namely trauma, infection, tumor, or congenital abnormalities. Efforts to repair bone defects can be done by giving bone grafts. This study used a xenograft derived from bovine bone, namely hydroxyapatite. Hydroxyapatite combined with Ellagic acid which is a polyphenol has antioxidant and anti-inflammatory properties which are expected to reduce Interleukin-6. Interleukin-6 is a marker that indicates the presence of an inflammatory process.

Objective: To analyze the administration of a combination of Hydroxyapatite and Ellagic Acid to bone defects on the expression of IL-6.

Methods: This study was a randomized post test only with control group design. The research sample used 30 Wistar rats with a defect in the left femur, and were divided into 3 groups, namely group K- with PEG, P1 with PEG and HA, and P2 with PEG, HA, and EA. On the 7th and 14th days, femoral bone tissue was taken to observe IL-6 expression using immunohistochemical techniques. Observations were made under a light microscope with a magnification of 400x.

Results: Statistical analysis showed there was a significant difference between groups ($p<0.05$).

Conclusion: Administration of a combination of HA and EA to bone defects of Wistar rats can reduce the expression of IL-6 for bone regeneration in the span of 7 and 14 days.

Keywords: Dentistry; Ellagic Acid; Hydroxyapatite; Inflammation; IL-6

1. Introduction

Bone defects are the loss of some bone tissue from where it should be which can be caused by several factors, namely trauma, infection, tumors, or congenital abnormalities. Optimal treatment of bone defects is still a major challenge in the medical world which has resulted in an increase in the burden of health care as well as the economy. Efforts to repair bone defects can be done by giving bone grafts [1,2]. The bone graft as gold standard is autograft because it comes from the patient's own tissue so it has good compatibility. However, autograft has drawbacks, namely the process of taking tissue creates new defects in patients [3]. This causes the need for an alternative bone graft that is more comfortable to use, namely xenograft derived from animals. Xenografts have structures and properties similar to human bones, are available in large quantities, are relatively affordable and have a fast-manufacturing procedure [4,5]. Types of xenografts derived from bovines such as hydroxyapatite (HA) have good biocompatibility and chemical stability in the implantation area [3].

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The use of HA as a bone substitute is widely accepted because it has a chemical composition that is almost identical to bone mineral. Hydroxyapatite consists of bioactive and bioresorbable calcium phosphate which makes up the bulk of the inorganic components of bone tissue [3]. Good biocompatibility makes HA widely applied biomedically, including as a bone filler and implant prosthetic coating [6,7]. However, the use of HA has an impact, namely triggering local inflammation due to the release of debris nanoparticles through activation of the innate immune system [7]. This can also be exacerbated by the administration of HA which comes from bovine bone because it includes foreign material outside the host which can trigger inflammation [8]. Based on this, the use of HA needs to be combined with anti-inflammatory ingredients such as Ellagic Acid (EA) compounds to accelerate the healing process [9].

Ellagic Acid is a polyphenol that is found in many fruits such as pomegranates [9]. This compound is known as a therapeutic agent because it exhibits anti-apoptotic, antioxidant, and anti-inflammatory properties. Ellagic Acid functions as an antioxidant because it can inhibit reactive oxygen species (ROS) or free radicals caused by inflammatory reactions [10]. Inhibition of ROS causes inhibition of Nuclear Factor kappa Beta (NF-κB) translocation to the nucleus so that the secretion of pro-inflammatory cytokines such as Tumor Necrosis Factor – α (TNF-α) and Interleukin-6 (IL-6) decreases [11]. Decreased expression of TNF-α and IL-6 will cause the rate of bone resorption to decrease due to inhibition of NF-κB activity which facilitates osteoclast differentiation resulting in decreased osteoclastogenesis and increased bone remodeling processes [12].

Therefore, the content of EA derived from pomegranate is expected to reduce the inflammatory reaction caused by HA (xenograft) so that it can accelerate the healing process of bone defects. Wardhana's research proved that the administration of hydroxyapatite and ellagic acid with a ratio of HA 97% and EA 3% showed an increase in the expression of osteocalcin (OCN), osteoprotegerin (OPG) and the number of osteoblasts as well as decreased expression of receptor activator of nuclear factor kappa beta (RANKL) during the healing process. bone defects [13].

The process of healing bone defects has complex stages consisting of an inflammatory phase, a repair phase, and a remodeling phase [14]. Acute inflammatory reaction is one of the factors that determine the success of bone healing. Several types of cytokines are released at the defect site during the inflammatory phase such as IL-6 and TNF-α expression which were found to increase in the first 24 to 72 hours after bone injury. If this is not controlled properly, there will be an increase in osteoclastogenesis, causing bone resorption [12,15,16].

Interleukin-6 (IL-6) is a proinflammatory cytokine. These cytokines also play a role in regulating the complex bone fracture healing cascade. Therefore, healing complications often occur in patients with inflammatory disorders which are often associated with increased IL-6 levels. The role of IL-6 in the process of healing bone defects occurs in the inflammatory and remodeling phases as evidenced by its activity in facilitating the formation of osteoclasts in the remodeling phase [16]. While research on the expression of IL-6 as a pro-inflammatory after being given a combination of HA and EA has never been done. Based on the explanation above, it is necessary to study the effect of application of HA and EA combination on IL-6 expression to accelerate healing of bone defects.

2. Materials and methods

2.1. Ethical Clearance

This research protocol was approved by the Ethics Committee of the Faculty of Dental Medicine, Airlangga University (488/HRECC.FODM/VIII/2021.).

2.2. Materials

HA powder (BATAN, Jakarta, Indonesia); EA (90%, Xi'an Biof Bio-Technology, Shaanxi, China); polyethylene glycol (PEG, 202398, Sigma-Aldrich); anti-IL-6 polyclonal antibody.

2.3. Preparation of HA and EA-HA

HA and EA are made in gel form to facilitate application to bone defects. HA gel is made by mixing HA with PEG in a ratio of 1:0.25. EA-HA is made by mixing HA powder and EA powder in a ratio of 97:3

2.4. Animals

Thirty healthy male Wistar rats (Rattus norvegicus) weighing 200 to 250 g each were obtained from the Biomedical Laboratory of the Faculty of Medicine, Airlangga University. The rats were divided into six groups of five animals and adapted, fed, and given water according to a standard diet and animal care protocol.

2.5. Bone Defect Model

Prior to bone defect creation, all animals were fasted for 12 hours but were still given drinking water. Anesthesia was performed using 100 mg/kg ketamine hydrochloride (Ketalar, Warner Lambert, Ireland) and 4 mg/kg xylazine (X1126, Sigma-Aldrich).

A bone defect was made lateral to the femur, 50 mm from the joint between the tibia and femur, as a 1 cm incision with a 0.84 mm diamond round bur (801G; 018, Mesinger, Germany). This creates a defect with dimensions of 2 mm in diameter and 2 mm in depth. The bone is irrigated with saline solution during the formation of the bone defect. The group division consisted of a control group of bone defects given PEG, treatment group 1 (P1) of bone defects given PEG and hydroxyapatite, treatment group 2 (P2) of bone defects given PEG, hydroxyapatite and ellagic acid. After treatment, the defects were sutured with nylon (Nylus nylon, nonabsorbable suture, Lotus surgical, India) and gentamicin sulfate topically applied to the wound at a dose of 2 to 4 mg/kg every 24 hours. o'clock. Animals were sacrificed after 7 or 14 days of treatment and the femurs were dissected for further analysis.

Expression of Interleukin-6 An indirect IHC examination was carried out to analyse the bone tissue. The animals' macrophages were assessed for their IL-6 expression using IL-6 monoclonal antibodies (ab6671, rabbit polyclonal antibody, Abcam).

2.6. Statistical Analysis

Data from research results were processed using SPSS version 25 (Windows). The data were tested for normality with the Shapiro-Wilk test, and the variance homogeneity test with the Levene's Test. The data were normally distributed and homogeneous ($p>0.05$), parametric One-way Anova analysis with a 95% confidence level ($\alpha=0.05$) showed that there were significant differences in the amount of IL-6 expression between groups, and the Tukey HSD (Honestly Significant Difference) test was continued to determine the differences between groups (Table 2)

3. Results

Table 1 The mean and standard deviation of IL-6 expression

Group	Day 7	Day 14
	$\bar{x}$ ± SD	$\bar{x}$ ± SD
K- (PEG)	12.8 ± 2.168	15.2 ± 1.924
P1 (PEG + HA)	1.4 ± 2,074	13.4 ± 2.608
P2 (PEG + HA + EA)	6 ± 1.414	8.6 ± 2.074

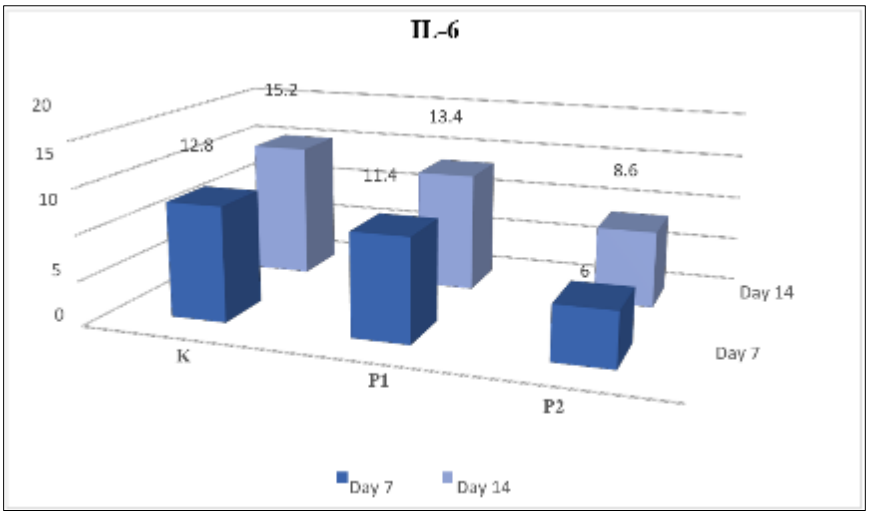

Figure 1 The mean diagram of IL-6 expression

Based on table 1 it can be seen that there was a decrease in the mean of IL-6 expression from day 7 to day 14. The lowest mean was found in treatment group 2 (P2) both on day 7 (6) and 14 (8,6). The negative control group (K0) had the highest mean number, both on day 7 (12,8) and on day 14 (15,2).

Table 2 Tukey HSD of IL-6 expression

	K0-7	**P1-7**	**P2-7**	**K0-14**	**P1-14**	**P2-14**
K0-7		0.889	0.000 *	0.467	0.997	0.039*
P1-7			0.005 *	0.994	0.652	0.304
P2-7				0.000 *	0.000 *	0.381
K0-14					0.742	0.000 *
P1-14						0.014 *
P2-14						

*= There is significant difference (Sig.<0.05)

- K0-7: Observation of the negative control group given PEG on the 7th day
- K0-14: Observation of the negative control group given PEG on the 14th day
- P1-7: Observation of the treatment group given PEG + HA on the 7th day
- P1-14: Observation of the treatment group given PEG + HA on the 14th day
- P2-7: Observation of the treatment group given PEG + HA + EA on the 7th day
- P2-14: Observation of the treatment group given PEG + HA + EA on the 14th day

Table 2 shows the results of Tukey HSD to determine the significance of the difference between groups, Group K-07 is significantly different (Sig. <0.05) compared to the Group P2-7 and Group P2-14. However, Group K0-7 was not significant (Sig.> 0.05) compared to the Group P1-7, Group K0-14 and Group P1-14. Furthermore, Group P1-7 is significant compared to the Group P2-7, but not significant compared to the Group K0-14, Group P1-14 and Group P2-14. The following data, Group P2-7 is significant compared to Group K0-14 and Group P1-14, but not significant compared to Group P2-14. While Group K0-14 is significant compared to Group P2-14 and not significant with Group P1-14. Furthermore, Group P1-14 is significant with Group P2-14.

4. Discussion

This study was conducted to observe the results of a combination of HA and EA applied to bone defects on the expression of Interleukin-6 in the process of inflammation and bone regeneration within 7 to 14 days. Hydroxyapatite as a bone filler or bone graft material has many biomedical applications because it is a calcium phosphate which forms most of the inorganic components of bone tissue so that it has good biocompatibility to bone [3,6,7]. The use of HA can trigger inflammation because it comes from bovine bone and includes foreign materials from outside the body [8]. The use of HA can also produce debris that triggers local inflammation through activation of the innate immune system [7,17]. Based on these impacts, the use of HA needs to be added to anti-inflammatory ingredients such as Ellagic Acid (EA) compounds to accelerate the healing process of bone defects [9].

When bone is damaged, immune cells including PMN cells, NK cells, mast cells and platelets are activated in the early stages of inflammation. Next, secreted cytokines recruit and activate monocytes or macrophages which then going to the defect site. Macrophages will secrete proinflammatory cytokines including IL-6 and TNF-α. Increased levels of TNF-α stimulate osteoclastogenesis and inhibit osteoblast function through increased activity of nuclear factor kappa-b (NF-kB) and mitogen activated protein kinase (MAPK)[14]. NF-kB, which is unstimulated, resides in the cytoplasm as an inactive heterodimer consisting of two subunits, p50 and p65. The heterodimer forms complexes with inhibitory proteins IkB-α or IkB-β, retaining NF-kB in the cytoplasm [11].

The results of this study indicated that the control group on day 7 (K0-7) which was given PEG had a higher mean of IL-6 expression compared to the P2 group which was given PEG + HA + EA. The high average yield of IL-6 was due to trauma on day 7 (K-07) in the control group which caused damage-associated molecular patterns (DAMPs) to activate, thereby activating signaling initiated by stimulation of adapter proteins in the cytoplasm such as Mitogen- activated protein kinase (MAPK) and IκB kinase which phosphorylates and degrades IkB-α. This will cause the release of the inhibitory protein IkB-α from the nuclear factor kappa beta (NF-κB) complex. Nuclear factor kappa beta then

translocates into the nucleus, binds to specific DNA and activates gene transcription to produce proinflammatory cytokines such as IL-6 and TNF-α which prolong the inflammatory process [11].

The results of the study in the control group on the 7th day (K0-7) were given PEG compared to the treatment group 1 on the 7th day (P1-7) which was given PEG + HA, as well as the K0-14 group compared to the P1-14 group show the insignificant expression of IL-6 caused by the administration of HA derived from bovine bone caused an intense inflammatory reaction even though HA has osteoconductive properties [8].

According to Wardhana's research (2021), the OCN expression of the group given PEG + HA showed lower OCN expression compared to those given PEG + HA + EA on the 7th and 14th days. This shows that HA has a smaller osteogenic potential compared to the PEG + HA + EA group, as well as a slower bone healing process. The application of HA which has osteoconductive properties will induce MSCs to proliferate and differentiate into osteoblasts. An increase in osteoblasts can be seen with an increase in OCN expression because it is one of the proteins produced by osteoblasts [18].

The results of the study in the treatment group 2 on day 7 (P2-7) which were given PEG + HA + EA had significant results compared to the control group on day 7 (K0-7) and treatment 1 (P1-7), namely having lower mean of IL-6 expression. Low IL-6 results will accelerate the resolution of inflammation, macrophages will polarize into an M2 phenotype which will produce anti-inflammatory cytokines such as IL-4, IL-10,19 which play a role in increasing OPG and decreasing NF-κB activator receptors, namely RANKL [20]. Increased expression of OPG causes RANKL to bind to OPG and suppresses osteoclastogenesis because OPG blocks the binding of RANK and RANKL [21]. This will reduce osteoclastogenesis and cause apoptosis of existing osteoclasts [22]. This significant decrease in IL-6 is in accordance with a study conducted by Wardhana (2021), proving that HA and EA administration showed an increase in the expression of osteocalcin (OCN), osteoprotegerin (OPG) and the number of osteoblasts as well as decreased expression of RANKL in the healing process of bone defects.

Furthermore, the results of the study showed that there was a significant decrease in IL-6 expression. IL-6 expression decreased in treatment group 2 on day 14 (P2-14) given PEG + HA + EA compared to the control group on day 14 (K0-14), and treatment group 1 day 14 (P1 -14). This shows that the combination of HA and EA has a significant effect on the regulation of the inflammatory phase. This proves that inflammation caused by HA administration can be regulated by EA. Ellagic Acid includes natural polyphenols that function as antioxidants that can inhibit reactive oxygen species (ROS) or free radicals caused by inflammatory reactions [10]. Inhibition of ROS causes inhibition of NF-κB translocation to the nucleus so that the secretion of pro-inflammatory cytokines such as TNF-α and IL-6 decreases [11]. Decreased expression of TNF-α and IL-6 will cause the rate of bone resorption to decrease due to inhibition of NF-κB activity which facilitates osteoclast differentiation resulting in decreased osteoclastogenesis and increased bone remodeling processes [12].

The results of the study on IL-6 expression in the 2nd treatment group on the 7th day (P2-7) were given PEG + HA + EA against the 14th day 2 treatment group (P2-14) showed that there was an increase in IL-6 expression on the 14th day. According to Prystaz (2018) an increase in IL-6 on day 7 shows a negative effect on bone repair, even though IL-6 is needed for ossification because it is in the inflammatory phase. On day 14 there was an increase in IL-6 produced by osteoblasts, because physiologically it is needed as a regulator of ossification in the process of bone repair [16]. Although expression of IL-6 showed higher results on day 14, expression of IL-6 in the P2-14 group was lower than K0-14. This suggests that giving a combination of HA and EA to bone defects can suppress the inflammatory process, reduce osteoclast activity and accelerate bone healing by reducing IL-6 expression.

5. Conclusion

Administration of a Hydroxyapatite and Ellagic Acid combination to bone defects reduce the expression of Interleukin-6.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

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