ORIGINAL ARTICLE

Effect of Chicken Eggshell Powder on Osteoblast, Osteocyte, and Osteoprotegerin (OPG) Expressions in Alveolar Bone Defect Healing of Wistar Rats

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ABSTRACT

Introduction: Alveolar bone defects are bone damage due to the extraction process, periodontal disease, avulsion tooth trauma, tumor recession, and other factors. Chicken eggshell (Gallus gallus domesticus) contains calcium carbonate which can be used in bone healing. Calcium carbonate stimulates increased osteoblast differentiation which results in an increase in OPG which then binds to RANKL to inhibit osteoclastogenesis and indirectly inhibits osteoclast formation. **Methods:** This study is true experimental with randomized post test only control group design. The study group numbered 3, the negative control group was not given any treatment, the positive control group was given alveolar bone defect, the treatment group was given an alveolar bone defect and continued giving chicken eggshell powder. The rat alveolar bone was taken and then given IHC and HE staining. The number of cells was then calculated from 10 visual fields with a magnification of 1000x using a microscope and then the mean was calculated. **Results:** Data were analyzed using the Post-Hoc statistical test showing a significant difference in the increase in osteoblasts, osteocytes, and OPG between E1 and E2 ($p = 0.05$). **Conclusion:** the application of chicken eggshell powder affects increasing osteoblast, osteocytes and OPG expression in healing the alveolar bone defects of wistar rats.

Keywords: Bone regeneration; Osteoblast; Osteocyte; Osteoprotegerin; Eggshell

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INTRODUCTION

Bone damage is one of the most challenging cases in dental practice (1). An example of bone damage is bone defect. Bone defects could be due to trauma, neoplasms, congenital defects, or periodontal disease (2,3). The bone healing process has three main phases, namely the inflammatory, proliferative, and remodeling phases (4,5). The inflammatory and proliferative phase lasts about 6-8 weeks, while the remodeling phase can last to months or years (6). The formation of fibrous tissue and cartilage is followed by bone formation and cartilage resorption, regulated by the expression of members of the transforming growth factor TGF-β superfamily (7).

In the bone remodeling phase, osteoblast cells will fuse with bone intercellular substances so that they will form new collagen fibers and osteoid cells. When osteoid is formed, osteoblast cells will be trapped inside the osteoid cell so that it will form new bone cells, namely osteocytes (8). Osteoblast cells and stromal cells produce OPG (Osteoprotegerin) which will bind to RANKL. The binding of OPG and RANKL inhibits the binding of RANKL to RANK, thereby inhibiting the activity of osteoclast cells (9-12).

The material that is often used in bone healing therapy is hydroxyapatite (HA). HA is an inorganic mineral that has a typical apatite lattice structure as

($Ca_{10}(PO4)_6(OH)_2$). HA contains 39.68% calcium and 18% phosphorus by weight. HA crystals cover 65 to 70% of the bone as a bioactive ceramic. The human bone comprises type-I collagen as an organic component and the HA as an inorganic component. HA is often used in biomedical applications as a bone substitute. Synthetic hydroxyapatite has biocompatible, osteoconductive properties and can blend with bone thereby increasing bone formation. Hydroxyapatite comes from two main sources, namely chemical sources in the form of synthetic materials and biological sources derived from natural materials (13,14).

Hydroxyapatite has been widely used in dentistry because of its good biocompatibility and osteoconductivity, but it is very difficult to decompose. That causes the graft material to remain in the defect for a long time and makes it difficult for new bone to form (15,16).

The eggshell is one of the hydroxyapatite sources because it contains high calcium carbonate (17,18). Eggshell consists of 96% calcium carbonate, 2% organic matrix, magnesium, phosphorus, and various other elements (19). The cuticle consists of glycoproteins, polysaccharides, lipids, and inorganic phosphorus including hydroxyapatite crystals. Eggshells also contain ovocleidin and ovocalyxin which are antibacterial (20).

Calcium carbonate is a calcium salt that contains the most elemental calcium compared to other calcium salts such as calcium citrate, calcium phosphate and calcium acetate. About 2.5 grams of egg shell contains enough calcium carbonate for an adult's daily calcium intake. The calcium carbonate in eggshells can reduce bone damage. Previous research on post-menopausal female rats with osteoporosis showed that the group of rats fed with eggshell powder had significantly higher bone mineral density compared to the group of rats that were not given treatment, the group with calcium carbonate from eggshells also showed better results than the group that was given pure calcium carbonate (21,22).

Poultry egg production in Indonesia in 2015 reached 1,795,711 tons. On average, Indonesians consume eggs and milk as much as 5.78% of their total food consumption every month, higher than fruits and beverages (23,24). The eggshell by weight is about 10% of the whole egg, so with the production mentioned, egg waste reaches 179,571 tons annually. Waste from eggshells currently has the potential to cause pollution due to microbial activity (25,26).

Eggshells have many benefits that have been felt by the community, but scientific research on the benefits of eggshells in the healing process of alveolar bone defects has yet to be found. Based on the described background, research was conducted on the effect of chicken eggshell powder on the number of osteoblasts, osteocytes, and OPG expressions in the healing of mandibular alveolar bone defects in male Wistar (Rattus Norvegicus) rats.

MATERIALS AND METHODS

Research Design

The type of this research is a true experimental laboratory in vivo with the Randomized Posttest Only Control Group Design method. This study was divided into 3 groups and each group consisted of 9 research samples. Group C was control group of healthy ratss without any alveolar bone defects or eggshell powder treatment. Group E2 is a group of rats that were given alveolar bone defects without eggshell powder treatment. Group E2 is a group of rats that were given alveolar bone defect and eggshell powder treatment.

The dose of eggshell powder given was calculated according to the method of Hunt et al., (2008), which was 60 mg/mL with an application of 10 mL/kg BW/day in rats weighing 250-350 grams or equivalent to 0.25-0.35 kg BW. The dose of calcium in male Wistar rats is 0.4 – 0.686 gr. Calcium content in chicken eggshells is 26.92% (27). So, the amount of eggshell powder given to experimental rats every day was 1.486 – 2.548 gr (rounded up to 1.49-2.55 gr).

Research Procedure

I. Eggshell Powder Manufacture

The eggshell powder manufacturing process is carried out at the Pharmacy Laboratory, Faculty of Medicine, Universitas Brawijaya. The eggshells were cleaned and dried in the sun. The dried eggshells were blended and mashed (28). The dose of eggshell used was following the dose of calcium for rats according to Hunt et al., (2008). According to research by Aziz, et al., (2018), the level of calcium in chicken eggshells is 26.92 (29).

II. Wistar Rat Treatment

Twenty-seven male Wistar (Rattus norvegicus) rats aged 3 months were divided into 3 groups, namely: negative control group (C-) which was not given any treatment, experimental group 1 (E1) which was only given mandibular bone defect, and experimental group 2 (E2). given the mandibular bone defect and eggshell powder.

Wistar rats were treated at the Animal House, Faculty of Dentistry, Brawijaya University. Wistar rats were placed in individual cages and adapted for at least 1 week. Rats were fed standard pellet feed and were given water ad libitum.

Wistar rats in groups E1 and E2 were anesthetized using an anesthetic injection of ketamine and xylazine until they fell asleep. An incision was made on the mucosa of the Wistar rats in the left buccal diastema area until the alveolar bone surface was visible. Bone defects were made. Drilling was carried out from the left buccal side of the mandible of Wistar rats as deep and as wide as the diameter of the bur. The bone defect formed should be located buccally to the incisor root and mesiobuccally to the molar root, without affecting the cementum of the tooth. The incision was returned to its original state and then the Wistar rats were injected with antibiotics and anti-inflammatories for 3 days.

Group E2 rats were given eggshell powder orally. Eggshell powder is mixed with food and administered once a day for 42 days. On day 43, Wistar rats were euthanized and underwent surgery and mandibular bone sampling. Furthermore, the samples were processed into preparations with hematoxylin-eosin (HE) and indirect Immunohistochemical (IHC) staining. Paraffin block making and HE staining were carried out at the Anatomica Pathology Laboratory, Faculty of Medicine, Universitas Brawijaya. Immunohistologic staining was carried out in the Laboratory of Biochemistry and Biomolecular, Faculty of Medicine, Universitas Brawijaya.

III. Cells Observation and Count

The preparations were observed under a microscope with a 1000x field of view as many as 10 fields of view. The field of view observed in the experimental group 1 (E1) and experiment 2 (E2) is the bone matrix edge area which is the location of the bone defect and in the negative control group (C-) is the bone matrix edge that is undergoing remodeling. The edges of the bone matrix are the site of newly mineralized osteoblasts, osteocytes, and OPG expression.

IV. Data Analysis

Data analysis included normality test and homogeneity test using the SPSS computer statistics program with a significance of 0.05 (p=0.05). The test used are the Saphiro-Wilk Normality test and the Levene Homogeneity test. If the data distribution is normal and homogeneous (p>0.05), then the One Way ANOVA hypothesis test is used and then followed by the Post-hoc Multiple Comparison Equal Variance by Tukey test and the Pearson correlation test. If the distribution of the data is not normal and not homogeneous (p<0.05), the Kruskal Wallis test is used, followed by the Mann-Whitney test to determine significant differences in each group.

RESULTS

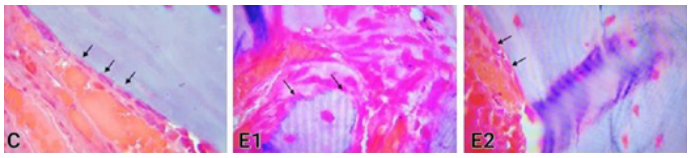

Fig. 1 : Histological image of osteoblas cells with HE staining in groups C, E1, and E2 under the microscope with 1000x magnification .

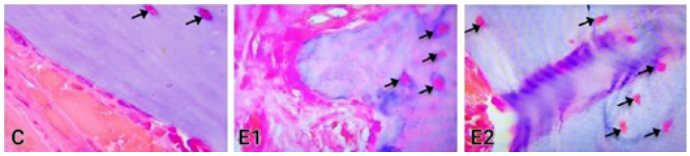

Fig. 2 : Histological image of osteocyte cells with HE staining in groups C, E1, and E2 under the microscope with 1000x magnification.

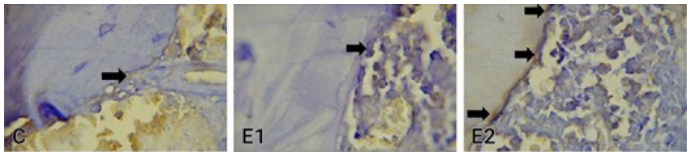

Fig. 3 : Histological image of osteocyte cells with IHC staining in groups C, E1, and E2 under the microscope with 1000x magnification .

Table I : Mean numbers of osteoblast and osteocyte cells, and OPG expression found in C group, E1 group, and E2 group (n=9)

Groups / Variables	(C)	(E1)	(E2)
Osteoblast	85.33	107.56	142
Osteocyte	44.44	55.44	65.55
OPG	68,22	80,11	108,78

Table II : Significance number results of Saphiro Wilk Normality Test, Levene Homogeneity Test, One Way Anova Parametric Test, Post-hoc Multiple Comparison Test, and Pearson Correlation Test

Variable	Saphiro -Wilk	Levene	One Way -Anova	Post-hoc Multiple Comparison		
				C & E1	C & E2	E1 & E2
Osteocyte	0.69	0.927	0.035	0.332	0.027	0.391
Osteoblast	0.069	0.121	0.000	0.036	0.000	0.001
OPG	0.917	0.702	0.000	0.096	0.000	0,000

Fig. 3 showed the results of Hematoxylin Eosin (H&E) staining on 27 preparations of alveolar bone tissue in Wistar rats and the result of OPG expression using immunohistochemical staining. The preparations from each group were observed using a light microscope with 1000x magnification and 10 fields of view to see the osteoblasts, osteoclasts, and osteocytes.

Osteoblast cells are located at the periphery of the bone, with spherical rounded nuclei and dark purple in color that almost fills the entire cell (12). While the osteocyte cells are pink-purple cells and are embedded in the bone matrix. OPG expression indicator using the DAB chromogen will appear brown in color around the osteoblast cells with purple nuclei. Table I showed that the lowest mean number of osteoblasts, osteocytes and OPG expression was in the control group, while the highest average number of osteoblasts, osteocytes, and OPG expression was in the experimental 2 (E2) group that was given eggshell powder.

Statistical Test Result

Osteoblast

Table II shows the Saphiro Wilk Normality Test result on osteoblasts showed that the significance value of the study sample was 0.069 (p>0.05), so it could be concluded that the data were normally distributed. The results of the Levene Homogeneity Test show that the significance value of the calculation of the average number of osteoblasts is 0.121 (p>0.05), so it can be concluded that the research data are homogeneous.

The results of the One-Way ANOVA Parametric Test showed a significance value of 0.000 (p<0.05), so it can be concluded that there was a significant difference in the number of osteoblasts between the C group, E1 group and E2 group which were treated with chicken eggshell powder for 42 days.

Furthermore, the Tukey HSD post hoc test with a 95% confidence level was conducted to find out which groups had significant differences. The results of the Post-Hoc test showed that there was a significant difference between each group. This is caused by the results of the significant value which is less than 0.05 (p <0.05).

The Pearson Correlation test was conducted to determine the relationship between the concentration of chicken eggshell powder and the number of osteoblasts. The results of the osteoblast cell correlation test showed a significant (2-tailed) value between the concentration of chicken eggshell powder and the number of osteoblasts of 0.000 (<0.05).

It can be concluded that there is a significant correlation between the concentration of chicken egg shell powder and the number of osteoblast cells. The r-value is positive (0.838) indicating that the relationship between the two variables is positive, so the greater the concentration of eggshell powder given, the number of osteoblasts will also increase.

Osteocyte

The results of the data normality test (Saphiro Wilk test) showed that the significance value of the research sample was 0.690 (p>0.05), so it can be concluded that the research sample data were normally distributed. The results of the homogeneity test (Levene's test) showed that the significance value of the calculation of the average number of osteoblasts was 0.927 (p>0.05), so it could be concluded that the research data were homogeneous.

The results of the one-way Anova test showed a significance value of 0.035 (p<0.05), so it could be concluded that there was a significant difference in the number of osteocytes between the negative control group, the positive control group and the treatment group that was given chicken eggshell powder for 42 days.

The results of the Post-hoc Multiple Comparison test on osteocytes showed that the difference between the C group and the E2 group was significant, while the difference between the E1 group and the C group, and the E1 group and the E2 group was not significant.

The results of the Pearson Correlation Test showed a significance value of 0.001 (<0.05) meaning that there was a correlation between the dose of eggshell powder and the degree of healing of bone defects. The significance value or r-value of 0.001 indicated that if the dose of eggshell powder increases, the healing of mandibular alveolar bone defects in Wistar rats will also increase. The degree of correlation was 0.705, which is included in the category of strong correlation. This shows that the number of osteocytes formed in the healing of mandibular alveolar bone defects in male Wistar (Rattus norvegicus) rats is strongly related to the dose of eggshell powder given.

Osteoprotegerin (OPG)

The results show that the average number of OPG expressions in the treatment group (P1) has higher results than the average number of OPG expressions in the positive control group (K1). The results of this study were the number of OPG expressions that were statistically analyzed using the Saphiro-Wilk normality test, and the Levene test for homogeneity. If the data obtained are normally distributed and the research data is homogeneous, then the statistical test can be continued using the one-way ANOVA test. Based on the Saphiropiro-Wilk test, the significance value of the normality test was 0.917 so the data were normally distributed. Based on the Levene test, the significance value of the data homogeneity test was 0.702, which indicated that the data was homogeneous. Calculations using the one-way ANOVA test between the positive control group and the treatment group have a significance value (p) of 0.000 or (p) <0.05 so the conclusion obtained is that there are differences in the amount of OPG expression in the healing process of bone defects. The results of the post hoc LSD test showed a significant difference in the average number of OPGs between the treatment group and the positive control group and the negative control group.

DISCUSSION

Osteoblast

The results of the One-Way Anova Parametric Test on osteoblast cells showed that there were differences in the number of osteoblasts between groups C, E1 and E2. It could be concluded that there was an effect of chicken eggshell powder on the number of osteoblasts in healing bone defects. Alveolar of male Wistar rats. When bone healing occurs due to bone defects, macrophages will be regulated so that it will increase the production of proinflammatory cytokines. The number of inflammatory mediators will increase in number during the inflammatory process (30). This will result in a decrease in the number of osteoblasts and an increase in the number of osteoclasts (31). A decrease in the number of osteoblasts and an increase in the number of osteoclasts results in an increase in bone damage due to degeneration of connective tissue (15).

The results of the Post-Hoc Multiple Comparison test showed that the significance value between the C and E1 groups was lower than the significance value between the C and E2 groups. This result occurred because in the bone healing process of the C group and E1 group, osteoblast cells produce BMP (Bone Morphogenic Proteins). BMP is a pivotal regulator of early MSC commitment to osteogenesis (32). BMP plays a role in inducing the differentiation of mesenchymal cells into osteoblast cells (33). This causes osteoblast cells to increase in number according to the normal bone healing process.

C group and E2 group have higher significance values. This is because the rats in E2 group were treated with chicken eggshell powder. One of the factors that affect bone healing is nutrition, that's why it is necessary to have sufficient calcium and vitamin D in the healing process. Egg shells contain large amounts of calcium carbonate and it can serve as a source of hydroxyapatite and promote the formation and differentiation of osteoblast cells. Thus, the number of osteoblasts in group P will be much more increased (34,35,36).

Hydroxyapatite has biocompatible properties that can downregulate and migrate macrophages. This will result in a decrease in the number of osteoclasts and an increase in the number of osteoblasts so that the bone healing process can occur faster (35,37). Eggshell powder containing hydroxyapatite was given to experimental animals by being mixed with food orally. Hydroxyapatite will enter the digestive tract and arrive in the small intestine. Calcium absorption occurs in the small intestine with the help of the hormone calcitriol. The amount of calcitriol hormone will increase, resulting in an increase in calcium absorption (38). The absorbed hydroxyapatite will enter the blood circulation and go to the bone defect area.

In the area of bone defects, hydroxyapatite will stimulate osteoprogenitor cells to become osteoblasts. Hydroxyapatite also makes it easier for osteoprogenitor cells to occupy a suitable medium with actual bone conditions so that they can proliferate and differentiate into osteoblast cells (39). This resulted in an increase in the number of osteoblasts. There has not been any study reporting on the effect of eggshell powder increasing the osteoblast cells.

Osteocyte

About 90–95% of the bone cells are comprised of

osteocyte cells. Osteocytes can feel mechanical stimuli and stress changes and regulate matrix remodeling directly. Osteocytes also regulate the activity of osteoclasts and osteoblasts such as bone resorption and bone formation to reach bone homeostasis (40). The average number of osteocytes from the lowest to the highest was in the C < E1 < E2 group. There was an increase in the number of osteocytes in groups with the bone defect and eggshell powder. However, the results of the Post-hoc test showed that a significant difference was only found between the C and E2 group but not significant between the E1 and E2 groups. This indicates that eggshell powder can increase osteocyte cells in bone defects, but not in a significant amount.

When damage or defects occur in the bone matrix, macrophages will degrade and deposit organic material and growth factors, resulting in a phase of bone deposition. Administration of eggshell powder with high calcium concentration will improve the bone deposition phase by increasing the formation of trabeculae and increasing the mineralization and proliferation of osteoblasts into osteoid and osteocytes. It explains how the number of osteocytes in the E2 group is more than the C and E1 groups (41,42,43).

According to Feng and McDonald (2013), osteocytes will detect an injury in the bone and will recruit osteoclast precursors and subsequently recruit osteoprogenitor (41,42,44). Osteoprogenitors will differentiate into osteoblasts. Then 10-20% of osteoblasts that were formed will be embedded in the bone matrix to become osteocytes. This resulted in a higher number of osteocytes in the area of bone defect such as in the E1 and E2 groups, than in healthy bones such as the C group. The remaining osteoblasts that did not become osteocytes will remain at the edges of the bone matrix to become inert-cell lining, and 65% of osteoblasts will undergo apoptosis. The process of osteoblast differentiation in rat alveolar bone takes 19 days and the age of osteocytes in rats alveolar bone is 10-20 days (15). Thus, on the day of euthanasia (day 43), some osteocyte cells might have undergone apoptosis. This resulted in a less significant increase in the number of osteocyte cells between the number of osteocytes from the untreated C group to the E1 group with bone defect, and between the E1 group and the E2 group treated with eggshell powder. There has not been any study reporting on the effect of eggshell powder increasing the osteocyte cell.

Osteoprotegerin (OPG)

The RANK, RANKL and OPG system plays an important role in regulating bone remodeling. OPG is expressed by osteoblast cells and bone marrow stromal cells. OPG binds to RANKL with 500x greater affinity than RANK, so that OPG can inhibit osteoclastogenesis and protect bone resorption mediated by osteoclasts (17,45,46). In this study, we believe one of the factors that contributes in bone healing process is nutrients, that's why it is necessary to have sufficient amount of Calcium and Vitamin D during the Rat's bone healing process (47). Calcium and Vitamin D play a role in bone mineralization and are part of the bone healing process (30,31). Osteoblast cells, stromal cells, macrophage cells, osteoclast cells and chondrocyte cells have calcium receptors or Calcium-sensing Receptors (CaR). By the administration of eggshell powder, the calcium contained within will bind to calcium receptors on preosteoblast cells, preosteoblast cells theses cells then migrate to the resorption area and differentiate and calcify the matrix (33). Calcium and vitamin D can also bind to the Vitamin D Receptor (VDR) on osteoblasts and promote osteoblast differentiation and mineralization (34). By the increasing the binding of calcium on pre-osteoblast, the amount of OPG is increasing as well, thus inhibiting RANK and RANKL binding and promotes bone healing process. This is shown on the positive control group and treatment group that shows a statistically significant results, in which the treatment group represents the increasing amount of OPG expressed by osteoblast. The increasing number of OPG inhibits osteoclast maturation and protects the physiological process of osteoclast in bone remodeling (35). In this study, we used Three-month old mice, because it had higher osteoblast activity. Bone mineralization and osteoblast activity began to active when the mice were one month old and reached their peak when the mice were three-months old which contributed to bone development. This is indicated by the Bone Longitudinal Growth Rate (LGR) and Bone Mineral Apposition Rate (MAR) which increased when the rats were three-months old (48). Systemic administration of egg shell powder to treated rats for 42 days aims to observe the development of bone formation cells and the bone healing process. The Bone growth on day 42 experienced an acceleration of callus formation and mineralization as well as an increase in the total volume of callus formed (49). There has not been any study reporting on the effect of eggshell powder increasing the osteoprotegerin count. But the study from Takeyama et al, 2000. Stated that OPG which is an inhibitory factor of osteoclast formation, expressed more decrease in low-calcium environment than in a normal calcium environment (50). Showing a relation between additional calcium and the number of OPG produced.

CONCLUSION

Oral administration of chicken eggshell powder has a potential to increase the number of osteoblasts, osteocytes, and OPG expression in the bone healing process of male Wistar rats (Rattus norvegicus) with

bone defects. Future study is expected to provide a better understanding of the effect of eggshell powder administration on the duration of bone healing.

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