Original Article

The Use Of Chitosan Milkfish (Chanos Chanos) Scales Waste As An Alternative Bone Regeneration Material In Socket Preservation

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Abstract

Introduction: Tooth extraction is frequently performed due to irreversible tooth decay. A well-directed tooth extraction will aid in the process of regenerating the bone around the extraction, commonly known as a preservation socket. Because chitosan acts as a regeneration substance, its usage in the preservation socket is predicted to speed up the bone healing process. Chitosan can be made from a variety of natural elements, one of which is milkfish scale waste, which is common in Makassar, South Sulawesi, Indonesia. The aim of this study is to assess the benefits of chitosan milkfish (chanos chanos) waste scale as an alternative bone regeneration material in socket preservation. This is a control group research with only a post-test. **Methods** : Cavia cobaya were divided into four groups of 32: (1) Socket preservation using milkfish scales chitosan, (2) milkfish scales chitosan + bovine xenograft, (3) bovine xenograft as a positive control, and (4) placebo as a negative control, then sacrificed on the third, seventh, fourteenth, and twenty-eighth days. The immunohistochemical analysis of the mandibular jaw samples was performed to assess the levels of OPG and RANKL. The Kolmogorov-Smirnov test, Levene's test, and one-way analysis of variance were used to examine the data. **Results**: On days 3, 7, 14, and 28, groups with chitosan added showed lower levels of RANKL and a faster increase in OPG expressions compared to those without chitosan. **Conclusion**: Milkfish scales can increase the production of OPG and decrease RANKL expression so that it can help the process of bone regeneration after tooth extraction.

Keywords: Bone Regeneration, Chitosan, Tooth extraction, Socket Preservation

INTRODUCTION

Tooth extraction is a common procedure in the majority of dental treatments that patients desire. According to Riskesdas data from 2018, 45.3 percent of the Indonesian population has caries or cavities, and tooth extraction is a common treatment option. After drug delivery, tooth extraction is the second most common operation used to treat dental and oral issues, accounting for 7.9 percent of all procedures.[1.] According to the findings of a study conducted by Devaraj et al (2012) in India, 54 percent of patients who visited the dentist had their teeth extracted. If tooth extraction is not followed by denture replacement, it will cause some problems surrounding the supporting tissue in the area where the tooth was pulled, so a preservation socket is required to avoid severe damage to the area where the tooth was extracted [2.]

When teeth are extracted, there is roughly 30% alveolar ridge resorption. Several studies have shown that two-thirds of hard and soft tissues resorb within three months of tooth extraction. The majority of bone loss happens in the first six months after tooth extraction, and the likelihood of resorption rate increases by an average of 0.5-1 percent annually, with the loss of alveolar bone width predicted to be up to 50% in the 12 months after extraction.[3] This loss of alveolar bone will have an impact on the stability, retention, and support of dental prostheses and dental implant placements, ultimately resulting in decreased patient comfort. As a result, the optimal moment to prepare the alveolar ridge is during extraction, also known as socket preservation.[4]

Socket preservation can be accomplished by inserting several graft materials, one of which is hydroxyapatite, into the socket. Because hydroxyapatite is biocompatible, bioactive, and osteoconductive in nature, it is frequently employed in hard tissue regeneration. Hydroxyapatite has advantages and downsides, including being brittle and relatively difficult to absorb by the body.[5,6] Several research have coupled chitosan and hydroxyapatite to increase the biocompatible qualities of hydroxyapatite with the goal of compensating for the weak mechanical properties of hydroxyapatite and boosting bioactivity and attachment of biomaterials to bone.[7,8]

Chitosan is a natural linear, semi-crystalline polysaccharide that is a derivative of N-deacetylated chitin and consists of -(14)-2-acetamido-2-deoxy-b-d-glucan (n-acetyl d-glucosamine) and -(14)-2-amino-2-deoxyb-d-glucan (n-acetyl d-glucosamine (d-glucosamine).[9] Chitosan is biodegradable and can be broken down into non-toxic residues by lysozyme or chitinase. Chitosan has been extensively researched in the biomedical, surgical, and tissue regeneration disciplines due to its biocompatibility, biodegradability, and mucosal adherence.[10] Chitosan has a variety of applications, including genetic treatment (drug / gene transfer), wound dressings, soft and hard tissue regeneration materials, hemostasis agents, hypocholesterolemic agents, anti-thrombogenic agents, bone regeneration biomaterials, and antibacterial agents.[11] Chitosan has several disadvantages, including low water solubility at neutral or high pH and low mechanical properties, so it is still combined with polymer materials or other natural materials, biomaterials, or bioactive molecules to increase mechanical resistance, protein absorption, and protein biomineralization.[12,13]

Several studies using chitosan and other substances have shown a beneficial effect on bone formation. Maryani et al found that the PRP and chitosan gel combination group had a significantly higher number of osteoblasts than the PRP, chitosan gel, and povidone iodine groups 14 days following tooth extraction in Wistar rats.[14] This is also consistent with the findings of Danilchenko et al., who discovered bone tissue production due to improved osteoconductive characteristics in a mix of hydroxyapatite and chitosan materials. Chitosan is a chitin derivative that can be derived from a variety of fisheries products, including shellfish, crabs, shrimp, and fish scales.[15]

Bone is a type of tissue made up of calcareous intercellular material known as the bone matrix and three types of cells known as osteocytes, osteoblasts, and osteoclasts. Because it is constantly remodeled, bone tissue is dynamic. The goal of bone remodeling is to regulate calcium homeostasis, repair tissue damage caused by physical movement, minor damage caused by stress factors, and skeletal development during growth.[16] In the bone remodeling process, an imbalance between bone resorption and bone creation can result in decreased bone density, which can lead to bone metabolic disorders. Reduced bone cell density can be caused by a decrease in the number of osteocytes or a shortage of minerals, although both can result in bone fragility.[17]

According to the above description, the aim of this study is to assess the benefits of chitosan milkfish (chanos chanos) waste scale as an alternative bone regeneration material in socket preservation.

METHODS

This study was according to the ARRIVE guidelines for animal pre-clinical research.

Animals

This was experimental laboratory research conducted on guinea pigs (Cavia cobaya). Male Cavia cobaya weighing 250–300 g and aged 2–3 months were utilized. Before treatment, Cavia cobaya were adapted to a 12-h light/12-h dark cycle and given free access to tap water and standard food for a week. Unhealthy Cavia cobaya were excluded if they lose more than 10% of their body weight after a week of adaption.

Preparation of chitosan gel from milkfish scales

One thousands grams of milkfish scales were washed under flowing water. Milkfish scales were wrapped in aluminum foil to ensure uniform drying, dried for seven days at 50°C–55°C, then homogenized to obtain up to 1.58 grams of fish scale powder.[18] The deproteinization procedure was carried out by mixing 3.5 N NaOH solution and fish scales at 90°C for 1 h at a speed of 50 rpm, followed by filtration. The obtained solids were washed with distilled water and dried for 24 h at 70°C. The products of deproteination were subsequently demineralized for 1 h by mixing 1.5 N HCl solution at 90°C.[18-20] After rinsing the solid with water, filtering, and cooling, chitin was obtained. Deacetylation was accomplished by soaking chitin in a 40% NaOH solution at 90°C for 1.5 h to provide white chitosan with no unpleasant odor, made to be gel.[21]

Experimental procedures

Following the adaptation period, male Cavia cobaya were randomly assigned to one of four groups (each with twelve Cavia cobaya): (1) Socket preservation using milkfish scales chitosan; (2) socket preservation using a combination of milkfish scales chitosan and bone graft (bovine xenograft); (3) socket preservation using bone graft (bovine xenograft) only as a positive control group; and (4) socket filled with placebo gel as a negative control group. The right mandibular incisor was carefully extracted without rotation using a needle holder after femoral anesthesia with 0.2 ml/50gr/BW ketamine. The socket was irrigated with solution saline, filled according to assigned groups, and sutured with 6-0 Vicryl absorbable suture. On the 3rd, 7th, 14th, and 28th days, three Cavia cobaya were sacrificed using ether. The mandibular jaw was removed and kept in 10% buffered formalin. The specimen was then sent to the Biochemistry Biomolecular Laboratory at Brawijaya University for immunohistochemistry analysis to measure OPG and RANKL levels. The data were analyzed with IBM Corp, Armonk, NY, USA. The Kolmogorov–Smirnov test was used to determine the normality of the data, and then Levene's test was used to determine the homogeneity of the data. One-way analysis of variance (ANOVA) was used to evaluate the differences between groups. A $p < 0.05$ indicates a significant result. The data were processed in SPSS 24.0 and displayed in tables and graphs.

RESULT

Characterization of milkfish scales derived chitosan We synthesized chitosan gel from milkfish scales based on previously described deproteinization, demineralization, and deacetylation techniques.[21,22] Functional group testing is carried out using FTIR spectrophotometry to demonstrate that the chitin deacetylation process has produced chitosan. The results of the absorption shift indicated that chitin had been deacetylated into chitosan.

OPG and RANKL responses to milkfish scales chitosan

Fourty-eight males Cavia cobaya weighing 250–300 g were used in this study. The Cavia cobaya were healthy throughout the research, and hence, none of them was excluded. Immunohistochemical analysis showed OPG and RANKL expressions in all experimental groups. Tables 1 and 2 show a description of OPG and RANKL results, respectively, for each group. The data were analyzed with IBM SPSS Statistics Program version 21. The Kolmogorov–Smirnov test showed normal distribution of the data ($p > 0.05$), and Levene's test showed the homogeneity of the data ($p > 0.05$). Parametric statistical test using One-way ANOVA was then used to determine the effect of the materials on OPG and RANKL expressions ($p < 0.05$), followed by Tukey HSD to study the differences between the variables.

Table 1. Descriptive statistics showing results of OPG expressions in each group within day 3, day 7, day 14, and day 28

Group	Sample Size	Mean ± SD			
		Day 3	Day 7	Day 14	Day 28
Chitosan	12	6.33±0.58	8.33±1.16	9.33±1.53	11.33±1.53
Chitosan and Bonegraft	12	6.00±1.00	9.00±2.00	11.33±1.53	12.67±2.52

Bonegraft	12	5.00±1.00	7.33±2.52	7.67±2.31	9.67±1.16
Placebo	12	3.00±1.00	4.67±1.53	5.67±2.08	5.67±1.53

Shapiro–Wilk P > 0.05; data are distributed normally. Levene Homogeneity Test P > 0.05; data are homogenic. One-way ANOVA P < 0.05. SD: Standard deviation, OPG: Osteoprotegerin

OPG is expressed differently in each group, as shown in Figure 1. In 3, 7, 14, and 28 days, all groups showed increase of OPG levels.

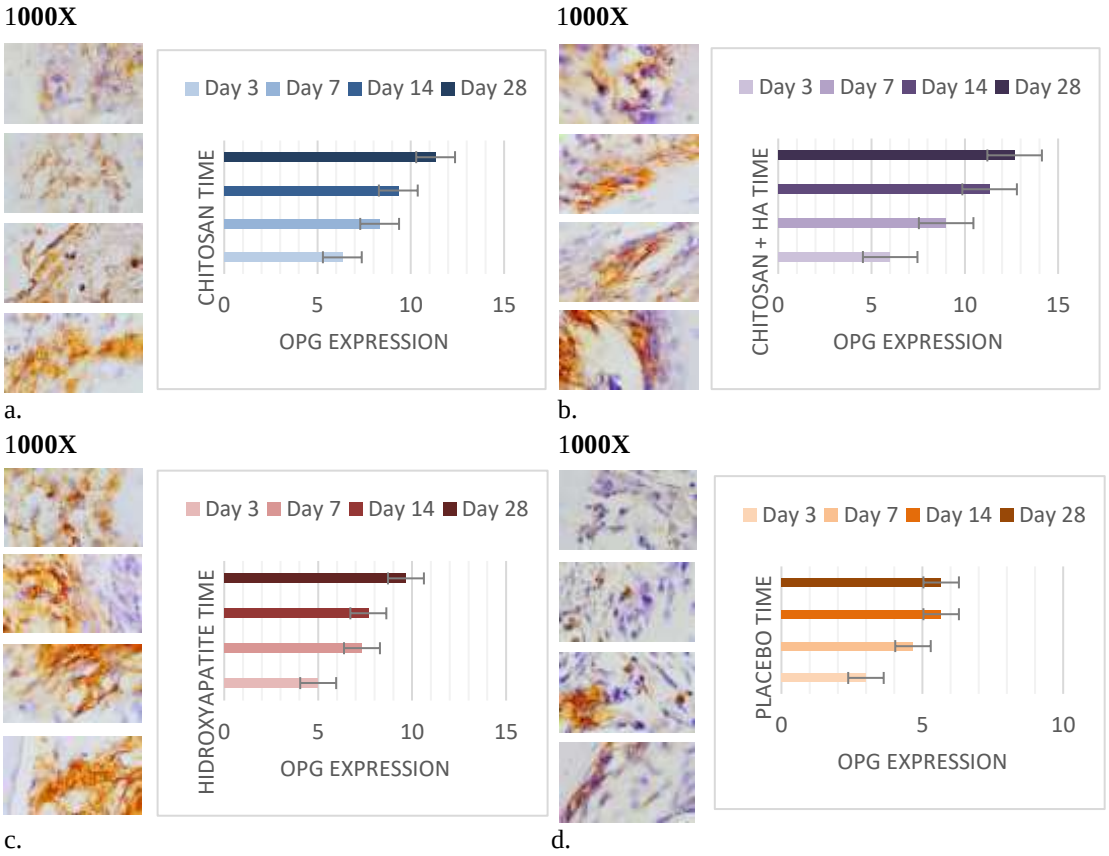

Figure 1. OPG expression within each group on day 3, day 7, day 14, and day 28. (a) OPG expression within the chitosan group. (b) OPG within the chitosan and bonegraft group. (c) OPG expression within the bonegraft group. (d) OPG expression within the placebo group

Table 2. Descriptive statistics showing results of RANKL expressions in each group within day 3, day 7, day 14, and day 28

Group	Sample Size	Mean ± SD			
		Day 3	Day 7	Day 14	Day 28
Chitosan	12	3.33±1.53	4.00±1.00	6.00±2.00	7.33±1.53
Chitosan and Bonegraft	12	3.33±1.53	5.00±1.00	5.00±2.00	7.67±1.53
Bonegraft	12	4.67±1.53	7.00±1.00	7.33±0.58	8.67±1.53
Placebo	12	7.67±1.53	8.67±1.53	9.00±2.00	10.33±1.53

Shapiro–Wilk P > 0.05; data are distributed normally. Levene Homogeneity Test P > 0.05; data are homogenic. One-way ANOVA P < 0.05. SD: Standard deviation, RANKL: Receptor activator of NF-kB ligand

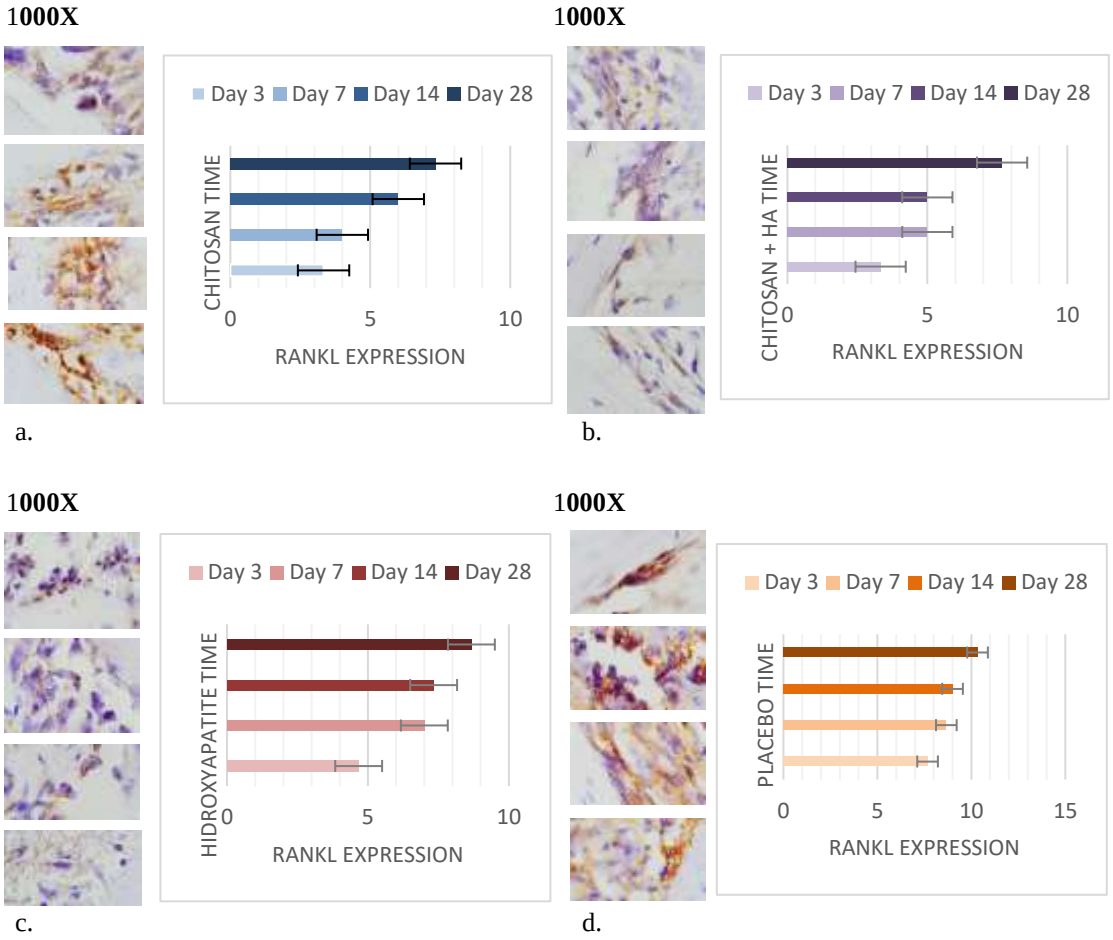

Figure 2. RANKL expression within each group on day 3, day 7, day 14, and day 28. (a) RANKL expression within the chitosan group. (b) RANKL within the chitosan and bonegraft group. (c) RANKL expression within the bonegraft group. (d) RANKL expression within the placebo group

Table 2 and Figure 2 illustrate the expression RANKL between the groups according to days 3, 7, 14, and 28.

Table 3. OPG expression levels on days 7, 14, and 28.

Groups	Samples	OPG			
		Mean ± SD	Minimal	Maximal	p
Day 7					
Chitosan	3	8.33±1.16	7	9	
Chitosan and Bonegraft	3	9.00±2.00	7	11	0.97
Chitosan	3	8.33±1.16	7	9	
Bonegraft	3	7.33±2.52	5	10	0.91
Chitosan	3	8.33±1.16	7	9	
Placebo	3	4.67±1.53	3	6	0.15
Chitosan and Bonegraft	3	9.00±2.00	7	11	
Bonegraft	3	7.33±2.52	5	10	0.70

Chitosan and Bonegraft	3	9.00±2.00	7	11	0.08
Placebo	3	4.67±1.53	3	6	
Bonegraft	3	7.33±2.52	5	10	0.36
Placebo	3	4.67±1.53	3	6	
Day 14					
Chitosan	3	9.33±1.53	8	11	
Chitosan and Bonegraft	3	11.33±1.53	10	13	0.59
Chitosan	3	9.33±1.53	8	11	0.71
Bonegraft	3	7.67±2.31	5	9	
Chitosan	3	9.33±1.53	8	11	0.16
Placebo	3	5.67±2.08	4	8	
Chitosan and Bonegraft	3	11.33±1.53	10	13	0.16
Bonegraft	3	7.67±2.31	5	9	
Chitosan and Bonegraft	3	11.33±1.53	10	13	0.03*
Placebo	3	5.67±2.08	4	8	
Bonegraft	3	7.67±2.31	5	9	0.59
Placebo	3	5.67±2.08	4	8	
Day 28					
Chitosan	3	11.33±1.53	10	13	
Chitosan and Bonegraft	3	12.67±2.52	10	15	0.79
Chitosan	3	11.33±1.53	10	13	0.67
Bonegraft	3	9.76±1.16	9	11	
Chitosan	3	11.33±1.53	10	13	0.02*
Placebo	3	5.67±1.53	4	7	
Chitosan and Bonegraft	3	12.67±2.52	10	15	0.23
Bonegraft	3	9.76±1.16	9	11	
Chitosan and Bonegraft	3	12.67±2.52	10	15	0.01*
Placebo	3	5.67±1.53	4	7	
Bonegraft	3	9.76±1.16	9	11	0.09
Placebo	3	5.67±1.53	4	7	

*p < 0.05 via ANOVA with Tukey's multiple comparison test. SD: Standard deviation, OPG: Osteoprotegerin

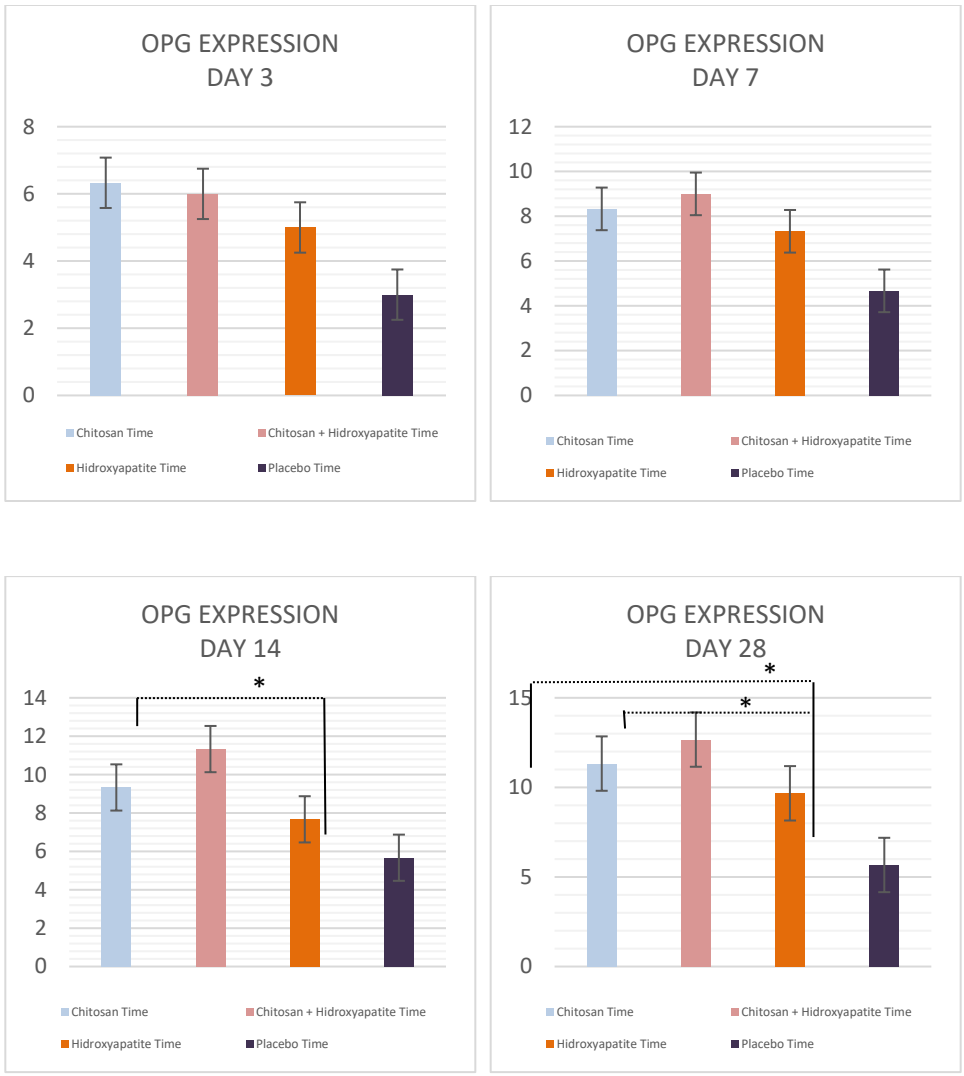

Figure 3. OPG expression levels were significantly different from the four groups on days 3, 7, 14, and 28. (a) On day 3, group with chitosan have a high level of OPG than three other grups though not significantly. (b) The differences in OPG expression across the four groups on day 7. (c) Differences in OPG expression across the four groups on day 14, Chitosan+HA groups have a significant differences than Placebo groups. (d) Differences in OPG expression across the four groups on day 28. In this day, there are two significant groups differences, Chitosan with Placebo groups and Chitosan+HA groups with Placebo. The asterisk (*) indicates a statistically significant difference between the chitosan group, the chitosan + bone graft combination group, and the bone graft group versus the placebo group. ($p < 0.05$ via analysis of variance with Tukey's multiple comparison test) Take note that the groups that received chitosan+HA have a high OPG expression than those that received just bone grafts or a placebo

The expressions of OPG in each group on days 7, 14, and 28 are presented in Table 3 and Figure 3. There was a statistically significant decrease ($p < 0.05$) in OPG expression in the chitosan group between day 14 (11.33 ± 1.33) and day 28 (12.67 ± 2.52).

Table 4. RANKL expression levels on days 7, 14, and 28.

Groups	Samples	RANKL			
		Mean ± SD	Minimal	Maximal	p

Day 7					
Chitosan	3	4.00±1.00	3	5	0.72
Chitosan and Bonegraft	3	5.00±1.00	4	6	
Chitosan	3	4.00±1.00	3	5	0.51
Bonegraft	3	7.00±1.00	6	8	
Chitosan	3	4.00±1.00	3	5	0.01*
Placebo	3	8.67±1.53	7	10	
Chitosan and Bonegraft	3	5.00±1.00	4	6	0.23
Bonegraft	3	7.00±1.00	6	8	
Chitosan and Bonegraft	3	5.00±1.00	4	6	0.02*
Placebo	3	8.67±1.53	7	10	
Bonegraft	3	7.00±1.00	6	8	0.35
Placebo	3	8.67±1.53	7	10	
Day 14					
Chitosan	3	6.00±2.00	4	8	0.89
Chitosan and Bonegraft	3	5.00±2.00	3	7	
Chitosan	3	6.00±2.00	4	8	0.79
Bonegraft	3	7.33±0.58	7	8	
Chitosan	3	6.00±2.00	4	8	0.23
Placebo	3	9.00±2.00	7	11	
Chitosan and Bonegraft	3	5.00±2.00	3	7	0.42
Bonegraft	3	7.33±0.58	7	8	
Chitosan and Bonegraft	3	5.00±2.00	3	7	0.08
Placebo	3	9.00±2.00	7	11	
Bonegraft	3	7.33±0.58	7	8	0.67
Placebo	3	9.00±2.00	7	11	
Day 28					
Chitosan	3	7.33±1.53	6	9	0.99
Chitosan and Bonegraft	3	7.67±1.53	6	9	
Chitosan	3	7.33±1.53	6	9	0.72
Bonegraft	3	8.67±1.53	7	10	
Chitosan	3	7.33±1.53	6	9	0.15
Placebo	3	10.33±1.53	9	12	
Chitosan and Bonegraft	3	7.67±1.53	6	9	0.85
Bonegraft	3	8.67±1.53	7	10	
Chitosan and Bonegraft	3	7.67±1.53	6	9	0.22
Placebo	3	10.33±1.53	9	12	
Bonegraft	3	8.67±1.53	7	10	0.57
Placebo	3	10.33±1.53	9	12	

*p < 0.05 via ANOVA with Tukey's multiple comparison test. SD: Standard deviation, RANKL: Receptor activator of NF-kB ligand

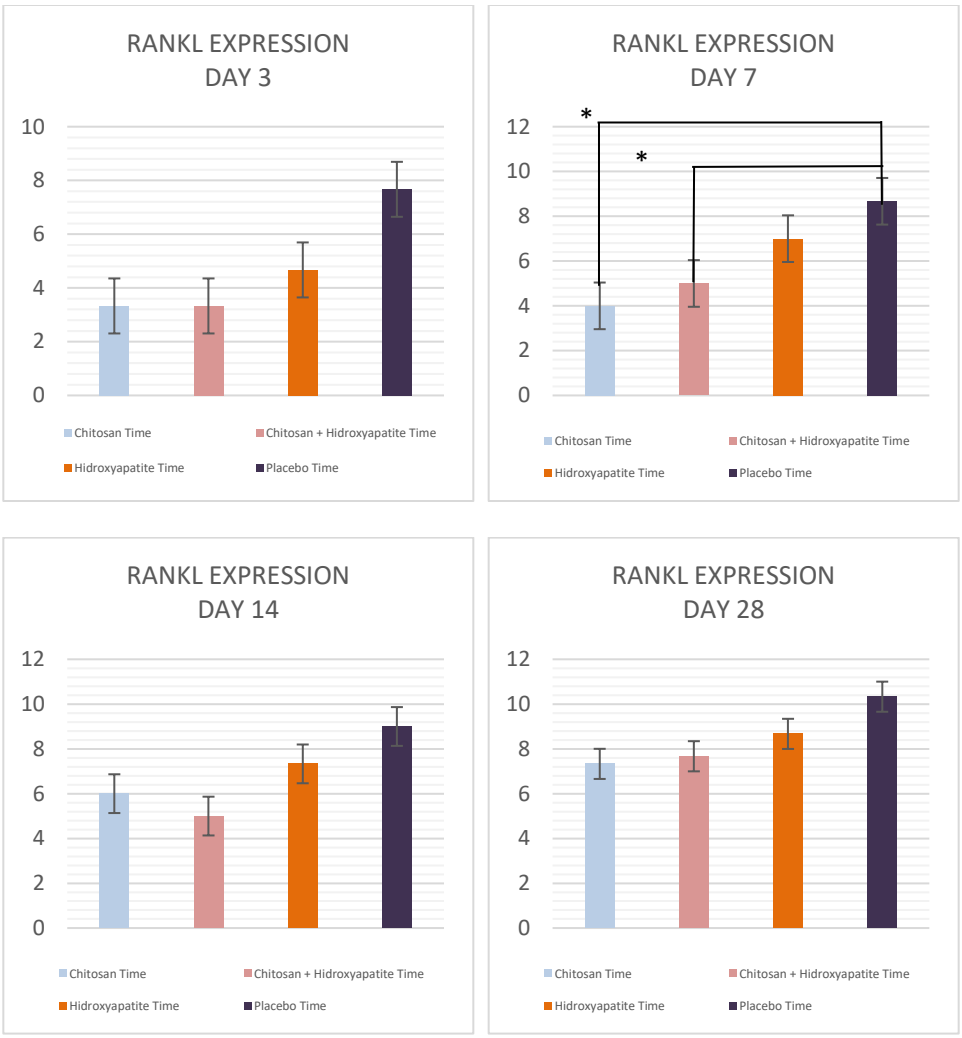

Figure 4. RANKL expression levels were significantly different from the four groups on days 3, 7, 14, and 28. (a) On day 3, group with placebo have a high level of RANKL than three other grups though not significantly. (b) The differences in RANKL expression across the four groups on day 7. (c) Differences in RANKL expression across the four groups on day 14, Chitosan with placebo groups and chitosan+HA with placebo groups have a significant differences. (d) Differences in OPG expression across the four groups on day 28. The asterisk (*) indicates a statistically significant difference between the chitosan group, the chitosan + bone graft combination group, and the bone graft group versus the placebo group. ($p < 0.05$ via analysis of variance with Tukey's multiple comparison test) Take note that the groups that received Placebo have a high RANKL expression than those that received chitosan or bonegraft.

Table 4 and Figure 4 show RANKL expression levels from the four groups on days 3, 7, 14, and 28. On day 7, The chitosan and chitosan bonegraft group exhibit significantly lower RANKL expressions compared to the placebo group ($p < 0.05$).

DISCUSSION

The RANKL/RANK/OPG system is known for its roles in osteoclasts maturation, bone modeling, and bone remodeling. Receptor activator of NF-kB (RANK), receptor activator of NF-kB ligand (RANKL), and osteoprotegerin (OPG) are the main components of this signaling system.[23] Chitosan, a hydrophilic polysaccharide with antibacterial[24-

[25], anti-inflammatory, immunostimulatory, hemostatic[26], wound-healing characteristics and bone regeneration[27-28], has various bio-dental uses. There was a significant increase in OPG parameters in groups with added chitosan and group chitosan + hidroxyapatite within 28 days. Groups with added chitosan showed significantly higher OPG values compared to the negative control. The chitosan and chitosan + hidroxyapatite combination groups exhibited higher OPG expression than the bone graft group. This shows that the addition of chitosan can help increase the amount of OPG that helps the bone remodeling process. Chitosan is a natural, biodegradable and biocompatible polymer that has a potential to act as a scaffold that promotes bone healing.[28] Chitosan is a linear polysaccharide comprising randomly distributed b-linked D-glucosamine and N-acetyl-D-glucosamine units. In vitro studies suggest that chitosan has osteogenic properties as it promotes differentiation of stem cells into bone-forming osteoblasts and promotes growth of bone colonies. In vivo studies suggest that chitosan alone is sufficient to stimulate osteogenesis. In some previous studies, other materials with osteogenic, osteoinductive and/or osteoconductive properties were added to chitosan-based composites to further stimulate the bone healing process.[29] Soher et al. investigated the effects of chitosan and OPG expression in bone regeneration. The obtained data demonstrate that the group with chitosan gel at 6 months were completely filled with thin, transparent soft tissue and at 12 weeks, the surgical defects of groups with chitosan gel were completely filled with hard tissue.[30]This result is consistent with the findings of this research, which indicate a significant increase in OPG expression on day 28.

RANKL (also known as OPGL, ODF, and TRANCE), as a homotrimeric protein, is produced by osteoblasts and some other cells like activated T cells.[29-31] The secreted type of RANKL is a result of proteolytic division or alternative splicing on the membrane form.[32] RANKL, which is a secretion of preosteoblasts, osteoblasts, osteocytes, and periosteal cells, make RANK activated, which is expressed by osteoclasts and its precursors.[33-36] RANKL has assignments for stimulating preosteoclasts' differentiation, adherence osteoclasts to bone tissue and their following activation and their maintenance.[23] Rahmitasari et al. conducted a study on the effect of the combination of chitosan on bone repair with the result that the combination of chitosan and collagen scaffold can improve the healing process of bone defects in Wistar rats through an decrease in RANKL expressions.[37] This study is in accordance with the results of our study, namely a significant decrease in the expression of RANKL in the chitosan group and the combination of chitosan + hydroxyapatite against the negative control group on day 7. Since the chitosan derived from milkfish scales is a novel product, we experimented with various periods and concentrations in previous experiments using other fish scales and shells to determine the DDA.[22,38-40] This might also explain why our outcomes were less significant in some variables. There is currently no published research detailing the bone remodelling benefits of milkfish scales chitosan. However, our study showed that this chitosan product could help the bone remodellig process. This is especially advantageous when combined with other biomaterials; in this example, chitosan increase OPG expression levels and decrease RANKL expression in xenografts. The disadvantage is that this research is the first in a series on milkfish chitosan, focusing only on the bone remodelling process through OPG and RANKL. This study shows an increase in OPG expression and reduction in RANKL expression, indicating that clinical trials will proceed. Another study team is conducting a histological investigation. In the future, we intend to use the vast quantity of fish scale waste to make chitosan with improved characteristics, shortening the inflammatory process and accelerating the proliferative and remodeling phases of socket bone formation.

CONCLUSION

This study demonstrates conclusively that chitosan from milkfish scales have an effects in the production of RANKL and OPG expression. However, since this is a new product, a more efficient process of deacetylation should be investigated. This may enhance accelerating the proliferation and remodeling stages.

ETHICS

All experimental protocols were approved by the University of Hasanuddin's Health Research Ethical Committee (No. No. 0055/ PL.09/KEPK FKG-RSGM UNHAS/2022).

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