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# South Sulawesi Milkfish (Chanos Chanos) Scale Waste as a New Anti-inflammatory Material in Socket Preservation

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## Abstract

**BACKGROUND:** One of South Sulawesi's huge brackish water fishery product is milkfish (Chanos chanos). Scales are wasted in milkfish processing. However, they are a good source of chitosan, which has been found to promote anti-inflammation, wound healing, and bone regeneration.

**AIM:** This study aims to determine the effect of milkfish scales waste on the inflammatory response of wound healing after tooth extraction by tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-6 analysis.

**METHODS:** This is a post-test-only control group design study. Thirty-two Cavia cobaya were divided into four groups: (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 were sacrificed on 3$^{rd}$, 7$^{th}$, 14$^{th}$, and 28$^{th}$ days. The mandible jaw specimen was taken for immunohistochemical analysis to determine the levels of TNF-$\alpha$ and IL-6. The data were analyzed using the Kolmogorov–Smirnov test, Levene's test, and one-way analysis of variance.

**RESULTS:** On days 3, 7, 14, and 28, groups with chitosan added showed lower levels of TNF-$\alpha$ and a faster decrease in IL-6 expressions compared to those without chitosan.

**CONCLUSION:** Milkfish scale chitosan suppresses TNF-$\alpha$ and IL-6 production, thus reducing inflammation in socket preservation.

## Introduction

Tooth extraction can cause buccolingual and apicocoronal alveolar bone resorption [1], [2]. This condition worsens denture stability and retention, causing patient discomfort. *Socket preservation* involves grafting material into the tooth socket to prevent alveolar bone loss after tooth extraction [3], [4]. These include autograft, allograft, xenograft, and alloplastic [5], [6], [7], [8], [9], [10]. However, these materials are limited by their high cost. As a result, consumers prefer natural alternatives because they are less expensive and have fewer side effects. Chitosan, a natural substance, has been shown to aid in wound healing and bone regeneration [11], [12].

Chitosan is a known natural cationic polysaccharide composed of (14)-2-amino-2-deoxy–d-glucan. It is produced by the deacetylation of chitin, the primary component of crustacean shells, fungal and algae cell walls, insect exoskeletons, and mollusks [13]. Chitosan is antibacterial, non-toxic, biodegradable, biocompatible, and promotes wound healing and osteogenesis [13], [14]. Chitosan has been shown to improve tissue healing, increase platelet growth factors, and reduce post-operative discomfort [14], [15]. Chitosan is an antibacterial cationic polymer that destabilizes Gram-negative bacteria's outer membranes and permeabilizes their plasma membranes. Inflammatory cells such as macrophages, polymorphonuclear leukocytes, fibroblasts, and osteoclasts can be stimulated by chitosan, promoting granulation tissue and angiogenesis [16].

After tooth extraction, the wound goes through the inflammatory, proliferative, and bone remodeling phases [1], [17]. Cytokines are quickly released during the inflammatory phase. Tumor necrosis factor-alpha (TNF-alpha) is one of the first cytokines made by activated monocytes and macrophages. Since these cytokines alter the capillaries, white blood cells can reach

the infection site faster. Interleukin 6 (IL-6) is another pro-inflammatory cytokine signaling molecule that is involved in the process of osteogenesis during bone repair [18]. According to Gupta *et al*., chitosan was efficient in accelerating the wound healing process and promoting osteogenesis in tooth extraction sockets by increasing the activity of inflammatory cells such as PMN leukocytes, macrophage cells fibroblasts, and osteoclasts [16].

Many studies are now being done on producing chitosan from chitin in fish scales [15], [19], [20], [21]. One of them is milkfish (*Chanos chanos*) from South Sulawesi's extensive brackish water fishing [22]. Milkfish processing frequently uses meat without scales, increasing the chance of wasting fish scales. Indonesia has massive production of milkfish, with huge fish scales waste produces every year. Ironically, chitosan for anti-inflammation and regeneration purposes in Indonesia is expensive. No study on the application of chitosan produced from milkfish scales in soft-tissue regeneration has ever been conducted so far. Therefore, the authors are interested in conducting this research.

This research was conducted on guinea pigs (*Cavia cobaya*) since they have a long history of being used as experimental animals for tooth extraction, and the process is relatively quick for an initial study. The purpose of this research is to study the influence of chitosan derived from milkfish scales on the inflammatory response of wound healing following tooth extraction using TNF- and IL-6 analyses.

## 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. All experimental protocols were approved by the Health Research Ethical Committee (No. 0051/PL.09/KEPK FKG-RSGM UNHAS/2021). 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*

Five hundred and thirty-three 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 59 grams of fish scale powder [19]. 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 [19], [23], [24]. 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 [25].

### *Experimental procedures*

Following the adaptation period, male *Cavia cobaya* were randomly assigned to one of four groups (each with eight *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 3$^{rd}$, 7$^{th}$, 14$^{th}$, and 28$^{th}$ 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 TNF-$\alpha$ and IL-6 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.

## Results

### *Characterization of milkfish scales derived chitosan*

We synthesized chitosan gel from milkfish scales based on previously described deproteinization, demineralization, and deacetylation techniques [13], [25]. 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. The degree of deacetylation (DDA) shows how much chitin is transformed into chitosan (measured by UV-Vis spectrophotometry) [13]. The DDA of milkfish scale chitosan was determined to be between 92.71% and 92.43%.

### *TNF-α and IL-6 pro-inflammatory responses to milkfish scales chitosan*

Thirty-two 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 TNF-α and IL-6 expressions in all experimental groups.

Tables 1 and 2 show a description of TNF-α and IL-6 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 TNF-α and IL-6 expressions ($p < 0.05$), followed by Tukey HSD to study the differences between the variables.

**Table 1: Descriptive statistics showing results of TNF-α 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 | 8 | 8.00 ± 1.00 | 4.33 ± 1.52 | 3.67 ± 1.52 | 3.00 ± 2.00 |
| Chitosan and bonegraft | 8 | 6.00 ± 2.00 | 4.00 ± 1.00 | 4.33 ± 2.08 | 3.00 ± 1.00 |
| Bonegraft | 8 | 9.33 ± 1.52 | 7.00 ± 2.64 | 5.67 ± 1.52 | 4.00 ± 1.00 |
| Placebo | 8 | 11.33 ± 2.51 | 12.33 ± 1.52 | 14.33 ± 1.52 | 14.33 ± 3.21 |

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, TNF-α: Tumor Necrosis Factor Alpha.

TNF-α is expressed differently in each group, as shown in Figure 1. In 3, 7, 14, and 28 days, all groups showed decrease of TNF-α expressions, except in the placebo group.

**Table 2: Descriptive statistics showing results of interleukin six 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 | 8 | 12.67 ± 1.16 | 6.67 ± 2.08 | 6.00 ± 2.00 | 3.33 ± 1.53 |
| Chitosan and bonegraft | 8 | 14.67 ± 2.52 | 5.67 ± 3.06 | 4.00 ± 1.00 | 3.33 ± 1.53 |
| Bonegraft | 8 | 6.33 ± 1.53 | 9.33 ± 4.04 | 10.00 ± 1.00 | 11.67 ± 3.06 |
| Placebo | 8 | 2.67 ± 0.58 | 6.33 ± 4.62 | 8.33 ± 3.22 | 7.67 ± 1.53 |

Shapiro-Wilk P > 0.05; data is distributed normally. Levene homogeneity test P > 0.05; data are homogenic. One-way ANOVA P < 0.05. SD: Standard deviation.

Table 3 and Figure 2 illustrate the expression of TNF -α between the groups according to days 3, 7, 14, and 28. On day 7, the chitosan (4.33 ± 1.52) and the chitosan and bonegraft group (4.00 ± 1.00) showed a significant decrease of TNF-α expressions ($p < 0.05$) compared to the placebo group (12.33 ± 1.52). There was also a significant difference in TNF-α expression

**Table 3: TNF-α expression levels on days 7, 14, and 28**

| Groups | Sample | TNF-α | | | |
|---|---|---|---|---|---|
| | | Mean ± SD | Minimal | Maximal | p |
| Day 7 | | | | | |
| Chitosan | 2 | 4.33 ± 1.53 | 3 | 6 | 1.000 |
| Chitosan and Bonegraft | 2 | 4.00 ± 1.00 | 3 | 5 | |
| Chitosan | 2 | 4.33 ± 1.53 | 3 | 6 | 0.907 |
| Bonegraft | 2 | 7.00 ± 2.65 | 4 | 9 | |
| Chitosan | 2 | 4.33 ± 1.53 | 3 | 6 | 0.001* |
| Placebo | 2 | 12.33 ± 1.53 | 11 | 14 | |
| Chitosan and bonegraft | 2 | 4.00 ± 1.00 | 3 | 5 | 0.808 |
| Bonegraft | 2 | 7.00 ± 2.65 | 4 | 9 | |
| Chitosan and bonegraft | 2 | 4.00 ± 1.00 | 3 | 5 | 0.000* |
| Placebo | 2 | 12.33 ± 1.53 | 11 | 14 | |
| Bonegraft | 2 | 7.00 ± 2.65 | 4 | 9 | 0.071 |
| Placebo | 2 | 12.33 ± 1.53 | 11 | 14 | |
| Day 14 | | | | | |
| Chitosan | 2 | 3.67 ± 1.53 | 2 | 5 | 1.000 |
| Chitosan and bonegraft | 2 | 4.33 ± 2.08 | 2 | 6 | |
| Chitosan | 2 | 3.67 ± 1.53 | 2 | 5 | 0.991 |
| Bonegraft | 2 | 5.67 ± 1.53 | 4 | 7 | |
| Chitosan | 2 | 3.67 ± 1.53 | 2 | 5 | 0.000* |
| Placebo | 2 | 14.33 ± 1.53 | 13 | 16 | |
| Chitosan and bonegraft | 2 | 4.33 ± 2.08 | 2 | 6 | 1.000 |
| Bonegraft | 2 | 5.67 ± 1.53 | 4 | 7 | |
| Chitosan and bonegraft | 2 | 4.33 ± 2.08 | 2 | 6 | 0.000* |
| Placebo | 2 | 14.33 ± 1.53 | 13 | 16 | |
| Bonegraft | 2 | 5.67 ± 1.53 | 4 | 7 | 0.000* |
| Placebo | 2 | 14.33 ± 1.53 | 13 | 16 | |
| Day 28 | | | | | |
| Chitosan | 2 | 3.00 ± 2.00 | 1 | 5 | 1.000 |
| Chitosan and bonegraft | 2 | 3.00 ± 1.00 | 2 | 4 | |
| Chitosan | 2 | 3.00 ± 2.00 | 1 | 5 | 1.000 |
| Bonegraft | 2 | 4.00 ± 1.00 | 3 | 5 | |
| Chitosan | 2 | 3.00 ± 2.00 | 1 | 5 | 0.000* |
| Placebo | 2 | 14.33 ± 3.22 | 12 | 18 | |
| Chitosan and bonegraft | 2 | 3.00 ± 1.00 | 2 | 4 | 1.000 |
| Bonegraft | 2 | 4.00 ± 1.00 | 3 | 5 | |
| Chitosan and bonegraft | 2 | 3.00 ± 1.00 | 2 | 4 | 0.000* |
| Placebo | 2 | 14.33 ± 3.22 | 12 | 18 | |
| Bonegraft | 2 | 4.00 ± 1.00 | 3 | 5 | 0.000* |
| Placebo | 2 | 14.33 ± 3.22 | 12 | 18 | |

*p < 0.05 via ANOVA with Tukey's multiple comparison test. SD: Standard deviation, Tumor Necrosis Factor Alpha.

in the chitosan, chitosan, and bonegraft, and bonegraft groups compared to placebo groups after 14 days and 28 days (p = 0.000).

The expressions of IL-6 in each group on days 3, 7, 14, and 28 are presented in Table 4 and Figure 3. There was a statistically significant decrease ($p < 0.05$) in IL-6 expression in the chitosan group between day 3 (12.67 ± 1.16) and day 28 (3.33 ± 1.53). In addition, there was a significant reduction ($p < 0.05$) in IL-6 expression on day 7 (p = 0.006), day 14 (p = 0.001), and day 28 (p = 0.000) compared to day 3 in the chitosan and bone graft combination group. Table 5 and Figure 4 show IL-6 expression levels from the four groups on days 3, 7, 14, and 28. On day 3, the chitosan bonegraft group and the chitosan group expressed significantly higher IL-6 levels compared to the bonegraft and the placebo group ($p < 0.05$). However, this was reversed on day 28. The chitosan group and chitosan bonegraft group exhibit significantly lower IL-6 expressions compared to the bonegraft group ($p < 0.05$).

## Discussion

Inflammation is the body's first defensive reaction to infection or damage initiated inside a tissue area by a specialized population of immune and

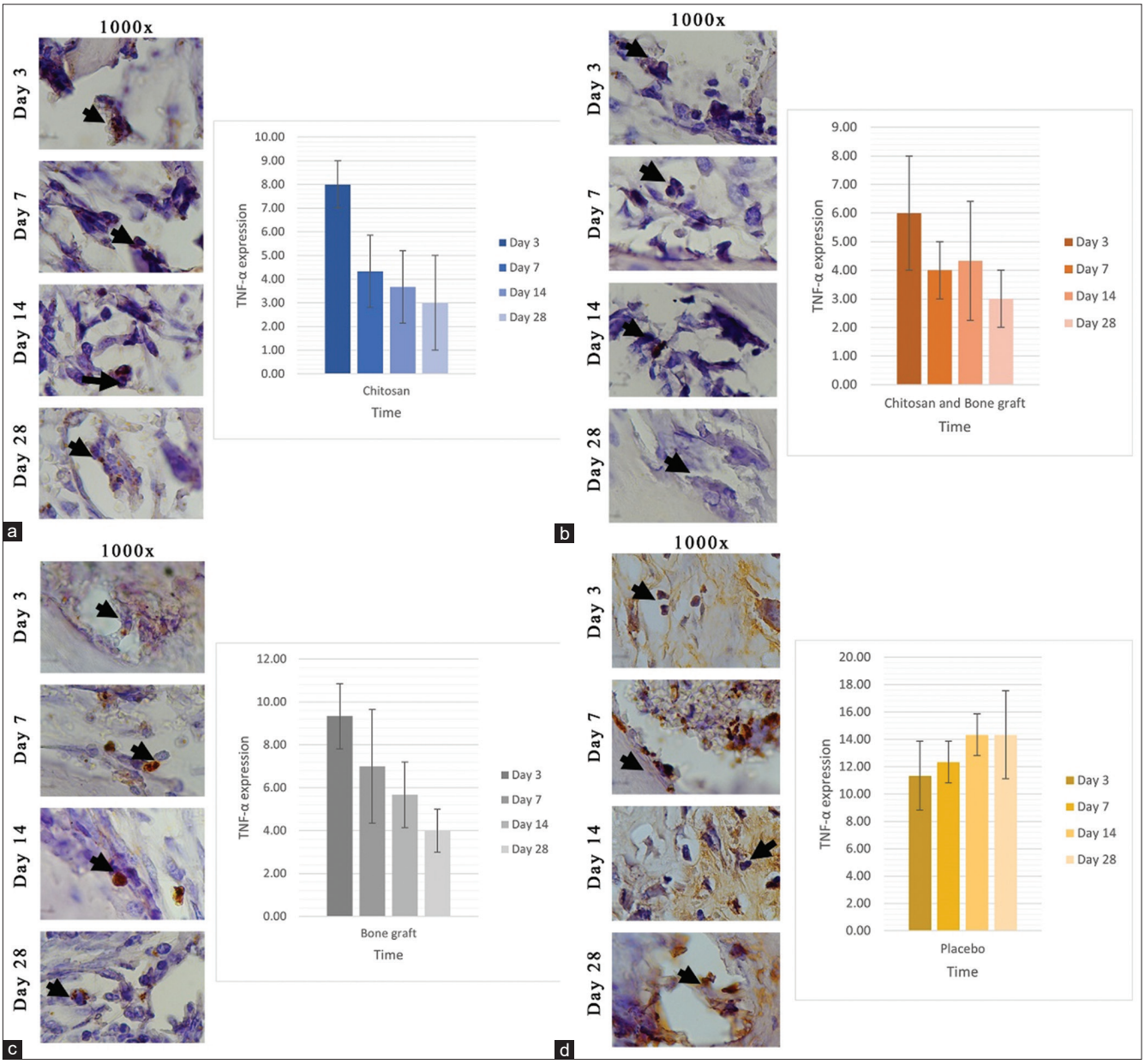


*Figure 1: Tumor necrosis factor-alpha expression within each group on day 3, day 7, day 14, and day 28. (a) Tumor necrosis factor-alpha expression within the chitosan group. (b) Tumor necrosis factor-alpha expression within the chitosan and bonegraft group. (c) Tumor necrosis factor-alpha expression within the bonegraft group. (d) Tumor necrosis factor-alpha expression within the placebo group*

inflammatory cells to re-establish the tissue's structural and functional integrity after exposure to negative stimuli [26]. TNF-α and IL-6 are the most prevalent pro-inflammatory cytokines observed. While both TNF- α and IL-6 may be generated by LPS to increase inflammation and protect against bacterial invasion, they have a synergistic effect. TNF-α may trigger the production of IL-6, which promotes inflammation, while IL-6 inhibits TNF-α synthesis by increasing the expression of the TNF receptor p55. However, IL-6 is a pleiotropic cytokine that plays a role in regulating immune responses and may act as an anti- or pro-inflammatory cytokine [27].

Chitosan, a hydrophilic polysaccharide with antibacterial [28], [29], anti-inflammatory [30], immunostimulatory, hemostatic [30], and wound-healing characteristics [31], [32], has various bio-dental uses. There was a significant decrease in IL-6 parameters in groups with added chitosan within 28 days. Groups with added chitosan showed significantly lower TNF-α values compared to the negative control. The chitosan and chitosan + bone graft combination groups exhibited significantly lower IL-6 expression than the bone graft group. Interestingly, on day 3, the groups that used chitosan had considerably increased IL-6 expression. On day 28, however, this was reversed. The groups that did not use chitosan exhibited an increase in IL-6 expression, whereas those that used chitosan had relatively low IL-6 levels. This shows that inflammation happens earlier and is rapidly suppressed by the added chitosan. The inflammation process is shortened in groups with added chitosan.

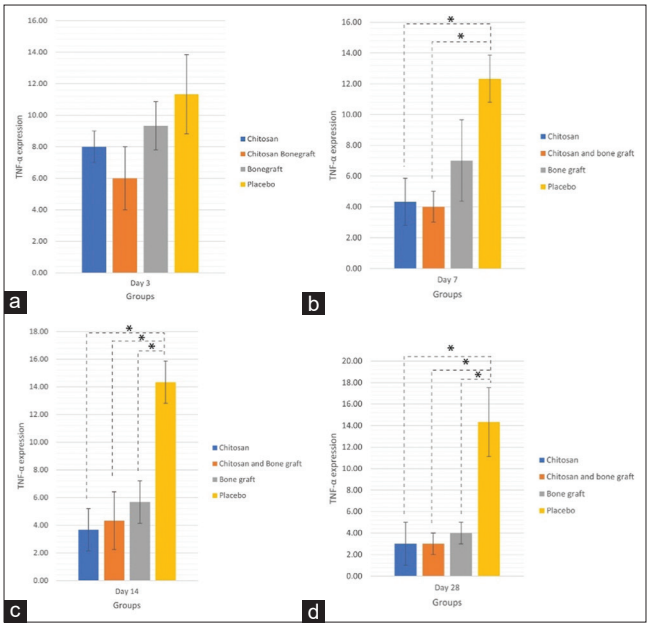


*Figure 2: Tumor necrosis factor-alpha expression levels were significantly different from the four groups on days 3, 7, 14, and 28. (a) On day 3, all groups had a reasonably high level of tumor necrosis factor-alpha expression, indicating an inflammatory response. However, the groups treated with chitosan had considerably reduced tumor necrosis factor-alpha expression than the other groups, though not significantly. (b) The differences in tumor necrosis factor-alpha expression across the four groups on day 7. (c) Differences in tumor necrosis factor expression across the four groups on day 14. (d) Differences in tumor necrosis factor expression across the four groups on day 14. 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 had lower tumor necrosis factor-alpha expression than those that received just bone grafts or a placebo*

**Table 4: Interleukin 6 expressions within groups between days 3, 7, 14, and 28**

| Groups | Day | IL-6 | | | |
|---|---|---|---|---|---|
| | | Mean ± SD | Minimal | Maximal | p |
| Chitosan | Day 3 | 12.670 ± 1.155 | 12.000 | 14.000 | 0.214 |
| | Day 7 | 6.670 ± 2.082 | 5.000 | 9.000 | |
| | Day 3 | 12.670 ± 1.155 | 12.000 | 14.000 | 0.109 |
| | Day 14 | 6.000 ± 2.000 | 4.000 | 8.000 | |
| | Day 3 | 12.670 ± 1.155 | 12.000 | 14.000 | 0.004 |
| | Day 28 | 3.330 ± 1.528 | 2.000 | 5.000 | |
| | Day 7 | 6.670 ± 2.082 | 5.000 | 9.000 | 1.000 |
| | Day 14 | 6.000 ± 2.000 | 4.000 | 8.000 | |
| | Day 7 | 6.670 ± 2.082 | 5.000 | 9.000 | 0.937 |
| | Day 28 | 3.330 ± 1.528 | 2.000 | 5.000 | |
| | Day 14 | 6.000 ± 2.000 | 4.000 | 8.000 | 0.990 |
| | Day 28 | 3.330 ± 1.528 | 2.000 | 5.000 | |
| Chitosan and bonegraft | Day 3 | 14.670 ± 2.517 | 12.000 | 17.000 | 0.006* |
| | Day 7 | 5.670 ± 3.055 | 3.000 | 9.000 | |
| | Day 3 | 14.670 ± 2.517 | 12.000 | 17.000 | 0.001* |
| | Day 14 | 4.000 ± 1.000 | 3.000 | 5.000 | |
| | Day 3 | 14.670 ± 2.517 | 12.000 | 17.000 | 0.000* |
| | Day 28 | 3.330 ± 1.528 | 2.000 | 5.000 | |
| | Day 7 | 5.670 ± 3.055 | 3.000 | 9.000 | 1.000 |
| | Day 14 | 4.000 ± 1.000 | 3.000 | 5.000 | |
| | Day 7 | 5.670 ± 3.055 | 3.000 | 9.000 | 0.997 |
| | Day 28 | 3.330 ± 1.528 | 2.000 | 5.000 | |
| | Day 14 | 4.000 ± 1.000 | 3.000 | 5.000 | 1.000 |
| | Day 28 | 3.330 ± 1.528 | 2.000 | 5.000 | |
| Bonegraft | Day 3 | 6.330 ± 1.528 | 5.000 | 8.000 | 0.972 |
| | Day 7 | 9.330 ± 4.041 | 5.000 | 13.000 | |
| | Day 3 | 6.330 ± 1.528 | 5.000 | 8.000 | 0.879 |
| | Day 14 | 10.000 ± 1.000 | 9.000 | 11.000 | |
| | Day 3 | 6.330 ± 1.528 | 5.000 | 8.000 | 0.378 |
| | Day 28 | 11.670 ± 3.055 | 9.000 | 15.000 | |
| | Day 7 | 9.330 ± 4.041 | 5.000 | 13.000 | 1.000 |
| | Day 14 | 10.000 ± 1.000 | 9.000 | 11.000 | |
| | Day 7 | 9.330 ± 4.041 | 5.000 | 13.000 | 0.997 |
| | Day 28 | 11.670 ± 3.055 | 9.000 | 15.000 | |
| | Day 14 | 10.000 ± 1.000 | 9.000 | 11.000 | 1.000 |
| | Day 28 | 11.670 ± 3.055 | 9.000 | 15.000 | |
| Placebo | Day 3 | 2.670 ± 0.577 | 2.000 | 3.000 | 0.879 |
| | Day 7 | 6.330 ± 4.619 | 1.000 | 9.000 | |
| | Day 3 | 2.670 ± 0.577 | 2.000 | 3.000 | 0.288 |
| | Day 14 | 8.330 ± 3.215 | 6.000 | 12.000 | |
| | Day 3 | 2.670 ± 0.577 | 2.000 | 3.000 | 0.481 |
| | Day 28 | 7.670 ± 1.528 | 6.000 | 9.000 | |
| | Day 7 | 6.330 ± 4.619 | 1.000 | 9.000 | 1.000 |
| | Day 14 | 8.330 ± 3.215 | 6.000 | 12.000 | |
| | Day 7 | 6.330 ± 4.619 | 1.000 | 9.000 | 1.000 |
| | Day 28 | 7.670 ± 1.528 | 6.000 | 9.000 | |
| | Day 14 | 8.330 ± 3.215 | 6.000 | 12.000 | 1.000 |
| | Day 28 | 7.670 ± 1.528 | 6.000 | 9.000 | |

*p < 0.05 via ANOVA with Tukey's multiple comparison test. IL-6: Interleukin 6, SD: Standard deviation.

Chitosan acts as an anti-inflammatory agent by decreasing the synthesis of the inflammatory cytokine interleukin (IL)-6 in human keratinocytes and IL-12 in human monocytes, as well as prostaglandin E2 levels. At the mRNA level, TNF- α and IL-6 are downregulated [33], [34], [35]. Oliveira *et al*. investigated the inhibitory and anti-inflammatory properties of chitosan film [36]. The obtained data demonstrate a decrease in TNF-α in cells cultivated on chitosan film for 3-10 days and a considerable rise in anti-inflammatory cytokines IL-10 and TGF-β1. This result is consistent with the findings of this research, which indicate a reduction in pro-inflammatory cytokines beginning on day 7.

Interestingly, Chang *et al*. [37] discovered that chitosan's molecular weight (MW) influences its anti-inflammatory activities. Chitosan with a higher MW (300, 156, 72 kDa) has an anti-inflammatory effect, while those with a lower MW (7.1, 3.3 kDa, COS) have pro-inflammatory activity. The 156 kDa and 72 kDa chitosan suppressed MAPK signaling proteins ERK, JNK, and p38 in macrophages by binding to CR3 receptors (for 156 kDa chitosan) or TLR4 and CR3 receptors (for 72 kDa chitosan), respectively. They also reduced LPS-induced NF-κB activation, TNF-α, and IL-6 production. By binding to the CD14, TLR4, and CR3 receptors in macrophages, the 7.1 kDa chitosan boosted the phosphorylation of MAPK signaling proteins JNK, NF-κB activation, and iNOS 174

**Table 5: Interleukin 6 expressions between groups on days 3 and 28**

| Groups | IL-6 | | | |
|---|---|---|---|---|
| | Mean ± SD | Minimal | Maximal | p |
| Day 3 | | | | |
| Chitosan | 12.670 ± 1.155 | 12.000 | 14.000 | 1.000 |
| Chitosan and bonegraft | 14.670 ± 2.517 | 12.000 | 17.000 | |
| Chitosan | 12.670 ± 1.155 | 12.000 | 14.000 | 0.154 |
| Bonegraft | 6.330 ± 1.528 | 5.000 | 8.000 | |
| Chitosan | 12.670 ± 1.155 | 12.000 | 14.000 | 0.002* |
| Placebo | 2.670 ± 0.577 | 2.000 | 3.000 | |
| Chitosan and bonegraft | 14.670 ± 2.517 | 12.000 | 17.000 | 0.015* |
| Bonegraft | 6.330 ± 1.528 | 5.000 | 8.000 | |
| Chitosan and bonegraft | 14.670 ± 2.517 | 12.000 | 17.000 | 0.000* |
| Placebo | 2.670 ± 0.577 | 2.000 | 3.000 | |
| Bonegraft | 6.330 ± 1.528 | 5.000 | 8.000 | 0.879 |
| Placebo | 2.670 ± 0.577 | 2.000 | 3.000 | |
| Day 28 | | | | |
| Chitosan | 3.330 ± 1.528 | 2.000 | 5.000 | 1.000 |
| Chitosan and bonegraft | 3.330 ± 1.528 | 2.000 | 5.000 | |
| Chitosan | 3.330 ± 1.528 | 2.000 | 5.000 | 0.015* |
| Bonegraft | 11.670 ± 3.055 | 9.000 | 15.000 | |
| Chitosan | 3.330 ± 1.528 | 2.000 | 5.000 | 0.700 |
| Placebo | 7.670 ± 1.528 | 6.000 | 9.000 | |
| Chitosan and bonegraft | 3.330 ± 1.528 | 2.000 | 5.000 | 0.015* |
| Bonegraft | 11.670 ± 3.055 | 9.000 | 15.000 | |
| Chitosan and bonegraft | 3.330 ± 1.528 | 2.000 | 5.000 | 0.700 |
| Placebo | 7.670 ± 1.528 | 6.000 | 9.000 | |
| Bonegraft | 11.670 ± 3.055 | 9.000 | 15.000 | 0.799 |
| Placebo | 7.670 ± 1.528 | 6.000 | 9.000 | |

*p < 0.05 via ANOVA with Tukey's multiple comparison test. IL-6: Interleukin 6, SD: Standard deviation.

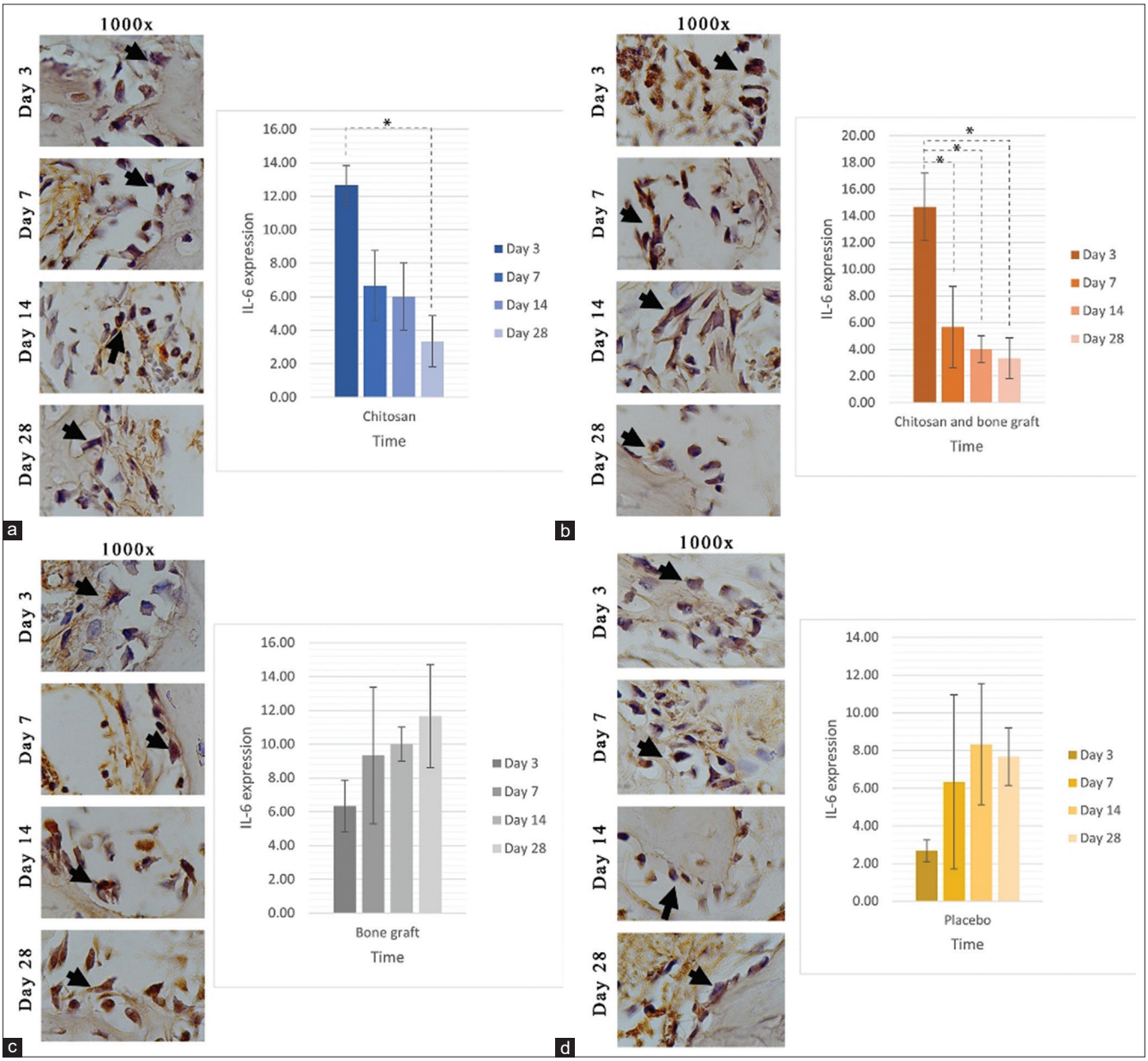


*Figure 3: Interleukin-6 expression within each group on day 3, day 7, day 14, and day 28. (a) Interleukin-6 expression within the chitosan group, which shows a significant decrease between day 3 and day 28. (b) Interleukin-6 expression within the chitosan and bonegraft group, which showed a significant decrease in days 7, 14, and 28, respectively compared to day 3. (c) Interleukin-6 expression within the bonegraft group. (d) Interleukin-6 expression within the placebo group. The asterisk (*) indicates a statistically significant difference between the groups. ($p < 0.05$ via analysis of variance with Tukey's multiple comparison test)*

expressions, and therefore increased the generation of NO and pro-inflammatory cytokines, TNF-α and IL-6. 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. This issue may influence the MW of the compound [13], [37]. This might also explain why our anti-inflammatory outcomes were less significant in some variables. In this situation, increasing its MW may decrease TNF- α and IL-6 expression, enhancing its anti-inflammatory activity.

There is currently no published research detailing the anti-inflammatory benefits of milkfish scales chitosan. However, our study showed that this chitosan product could reduce pro-inflammatory cytokines and shorten the inflammation process. This is especially advantageous when combined with other biomaterials; in this example, chitosan reduces IL-6 levels in xenografts. The disadvantage is that this research is the first in a series on milkfish chitosan, focusing only on the inflammatory response through TNF-α and IL-6. This study shows a reduction in inflammatory cytokines at the immunohistochemistry stage, 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.

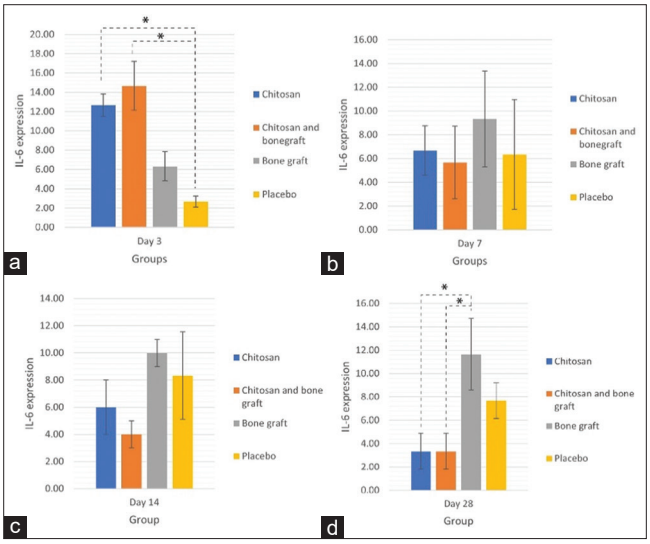


*Figure 4: On days 3, 7, 14, and 28, the four groups' interleukin-6 expression levels were compared. (a) On day 3, the chitosan group expresses significantly more interleukin-6 than the placebo group. When comparing the chitosan and bone graft combination group to the bone graft and placebo groups, the chitosan and bone graft combination group demonstrated a significant increase in interleukin-6 expression. (b) The differences in interleukin-6 expression amongst the four groups on day 7. (c) On day 14, differences in interleukin-6 expression were observed across the four groups. (d) Differences in the interleukin-6 expression on day 28 between the four groups. Both the chitosan group and the chitosan and bonegraft group showed a significant decrease of interleukin-6 expressions compared to the bonegraft group. The asterisk (*) indicates a statistically significant difference between the groups. ($p < 0.05$ via analysis of variance with Tukey's multiple comparison test)*

## Conclusion

This study demonstrates conclusively that chitosan from milkfish scales inhibits the production of pro-inflammatory cytokines TNF- and IL-6. However, since this is a new product, a more efficient process of deacetylation should be investigated, such that the chitosan retains its high MW but is less acetylated. This may enhance the anti-inflammatory impact by lowering early IL-6 levels, shortening the inflammation process, and accelerating the proliferation and remodeling stages.

## Ethics

All experimental protocols were approved by the University of Hasanuddin's Health Research Ethical Committee (No. 0051/PL.09/KEPK FKG-RSGM UNHAS/2021).

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## Authors Contribution

Arni Irawaty Djais: Conceptualization, Methodology, Writing - Review & Editing, Supervision Surijana Mappangara: Validation, Resources, Supervision Asdar Gani: Formal analysis, Resources, Data Curation, Supervision, Writing - Review & Editing Harun Achmad: Project administration, Funding acquisition, Writing - Review & Editing Sherly Endang: Investigation, Resources, Data Curation, Visualization, Writing - Original Draft Jennifer Tjokro: Investigation, Resources, Writing - Original Draft, Visualization Nurhadijah Raja: Investigation, Resources, Data Curation.

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