The Effect of Nanochitosane of Red Snapper Fish Scales (Lutjanus Sp.) on Pain and Pulp Inflammation

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ABSTRACT

Pulpitis, or pulp inflammation, is a type of dental disease that is commonly associated with tooth pain symptoms. The immune reaction, characterized by TNF-α biomarkers and leukocyte cells, plays a part in the prevalence of pulpitis. (PMN). The latest innovation in dentistry is nanochitosane, which is derived from red snapper scales and includes calcium and chitin. The nanochitosane found in red snapper fish scales (Lutjanus Sp.) has been shown to decrease tumor necrosis factor (TNF) and pain levels. Twenty-four Sprague Dawley rats were split into four groups. Healthy rodents comprised group 1. Rats were used in Group II as a reversible pulpitis model with no material change. Rats were used in Group III as a pulpitis model with calcium hydroxide addition. Group IV included rodents as model pulpitis with red snapper fish scale nanochitosane addition. The inclusion of chitosan therapy reduced leukocyte cells, TNF-α levels, and pain significantly in group IV compared to group II without therapy and group III with calcium hydroxide treatment. The results of this study showed that redfish scale nanochitosane can decrease inflammation by lowering TNF-α and pain levels.

KEYWORDS

Nanochitosane
TNF-α
Pain

GRAPHICAL ABSTRACT
Introduction

Pulp inflammation or pulpitis is a type of dental disease frequently experienced by the community with signs and symptoms of tooth pain. The reason patients visit the dentist is 90% because of pulpitis [1]. Pulpitis is classified into two categories: reversible and irreversible pulpitis. Reversible pulpitis is mild to moderate pulp inflammation, but the pulp can return to its normal state after removing the stimulus. Signs and symptoms of reversible pulpitis pain occur when exposed to stimulation. The etiology of reversible pulpitis includes bacteria, caries, trauma, and iatrogenic measures [2]. Reversible untreated or treated pulpitis can result in severe pain, causing pulp necrosis [3]. The immune response is the main factor that contributes to the occurrence of pulpitis. Several immune cells and inflammatory mediators significantly contribute to the occurrence of pulpitis, such as polymorphonuclear neutrophils (PMNs) and tumor necrosis factors-α (TNF-α) [4]. Neutrophil infiltration is a key marker of the severity of pulpitis [5]. Tumor necrosis factors (TNF) are inflammatory mediators that have biological activities, including stimulating and inhibiting several cell components in the immune system [6]. Tumor Necrosis Factor-α can activate cells and induce the synthesis of pro-inflammatory cytokines [7]. In addition, the TNF-α biomarker is from oxidative stress originating from the uncontrolled production of lipid peroxidase resulting in damage to pulp cell integrity [8]. The treatment for reversible pulpitis is through pulp capping. Pulp capping can restore inflammatory conditions to normal again by forming reparative dentine [9]. The frequently applied pulp capping material is calcium hydroxide, but several drawbacks include being very soluble in saliva, necrosis on the pulp surface, poor adhesion properties, forming tunnels, and defects in the dentinal bridge [10].

Indonesia has an extensive water area containing abundant natural resources (fisheries) that have not been optimally developed. Red snapper is a natural resource with many potentials and is easy to discover in Indonesian territorial waters. The utilization of red snapper meat is the main raw material. Therefore, red snapper scales will become waste and cause social and health problems [11]. Red snapper (Lutjanus Sp) scales have chemical constituents: water, protein, fat, carbohydrates, and calcium [12]. In addition, based on the Fourier-transform infrared spectroscopy (FTIR) test results, fish scales also contain chitin, which has the highest polysaccharide structure after cellulose [13]. Red snapper scales have a percentage degree of chitin deacetylation of 80%, more significant than the scales of other fish. Therefore, red fish scales contain chitosan, a carbohydrate biopolymer derived from chitin deacetylation.

Chitosan can trigger fibroblast cells to release anti-inflammatory cytokines. D-Glucosamine hydrochloride as a chitosan monomer [14]. Chitosan is antibacterial and easily absorbs ions due to its high reactivity [15]. Chitosan can further increase the synthesis of type I collagen and the number of odontoblast-like cells in pulp cap treatment [16]. The development of science is increasing, and chitosan is not only a large molecule, but also has become a very small molecule into nanochitosane. Nanochitosan is chitosan in nanometer size and making chitosan into nanoparticles aims to increase physical and mechanical strength and make the molecules more reactive and easily absorbed by the body. The primary component of the organic matrix derived from cuttlebone is b-chitin, a natural carbohydrate (polysaccharide) composed of parallel-arranged poly[2-acetamido-2-deoxy-(1,4)-b-D-glucopyranose] [17]. Chitin (-{(1-4)-poly-N-acetyl-d-glucosamine), a long-chain monopolysaccharide of N-acetyl glucosamine subunits linked by (14) glycosidic linkages, is found in the cell walls of crustaceans and marine invertebrates, and also contains cellulose as an important component [18].

Based on this background, researchers are interested in researching nanochitosane, which will be an alternative material in treating reversible pulpitis characterized by TNF-α and leukocyte cells (PMN) as signs of pulp inflammation which can cause pain.
Materials and Methods

This type of research was an in vivo laboratory (true experimental) method with the animal model of Sprague Dawley rats. The design of this study used a posttest only with a control group design, while research on red snapper fish scale nano chitosan manufacturing occurred at the Chemical Pharmacy Laboratory, Faculty of Pharmacy, Universitas Muhammadiyah Surakarta. This study conducted a reversible pulpitis experimental animals in Sprague Dawley rats at the Laboratory of Biomedical Integration, Faculty of Medicine, Universitas Sultan Agung, Semarang. Histological Examination occurred at the Anatomy Laboratory of the Universitas Muhammadiyah Surakarta.

The process of making red snapper nano chitosan occurred in several stages, including preparing red snapper scales (taking red snapper scales until a 60 mesh size powder is obtained). Isolation of chitosan from red snapper fish scales through the stages of deproteination, demineralization, and deacetylation to obtain chitosan products. Furthermore, it was continued with nano-chitosan production by the Ionic Glass Method by making a 0.2% chitosan solution in an acetic acid solution. The solution was added with 1% NaTPP solution with variations in the ratio of chitosan solution: NaTPP solution 3:1 and stirring speed of 900 rpm. Stirring was conducted for 1 hour. The obtained nanochitosane was in the form of dispersed solids.

The subjects or samples of this study used male Sprague Dawley rats with a body weight of 250-350 grams, no anatomical abnormalities, and normal activity and behavior. The number of research samples in this group was 24 rats. Samples were divided into four groups, each with six rats. Group I is a group of healthy mice, group II is a group of rats as a reversible pulpitis model without materials addition, group III is a group of rats as a pulpitis model with calcium hydroxide addition, and group IV is a group of rats as a pulpitis model with red snapper fish scales nanochitosane material addition.

An animal model of reversible pulpitis was made by administering intramuscular anesthesia to rats with 0.2 cc of ketamine. Subsequently, the preparation was conducted by low-speed drilling using a round bur with a diameter of 1 mm on the maxillary incisors with a depth of 3 mm. Furthermore, it was cut using a size 10 K-file until the pulp perforation. In group I, after the treatment was conducted, a temporary closure was immediately conducted. In group II, after the treatment was given calcium hydroxide, pulp capping material used a ball applicator. In group III, after the treatment, 1 mg of nanochitosane was given with a ball applicator. In addition, ether was applied to terminate the rats on the third day. Extraction of pulp tissue on incisor teeth using a barbed broach or extirpation. The extracted pulp was put into a microtube, which was subjected to an ELISA examination to determine the expression of TNF-α.

Pain measurement or scoring used the Mouse Grimace Scale method, while a video tool was employed to record the Action Unit (AU) performed by rats. Scoring is based on Action Unit (AU) or specific facial action units, including eyes, nose, cheeks, ears, and mustache. The intensity assessment score for each AU is Score 0: no AU, Score 1: AU looks moderate, and Score 2: AU looks real [19].

Data analysis was done by statistical tests. In discovering the normally distributed data, Shapiro-Wilk was applied; in testing hypotheses, one-way ANOVA on TNF-α and PMN data was employed, while Kruskal Wallis was used for pain calculations.

Results and Discussion

The subject of this study was the dental pulp of Sprague Dawley rats, which had been given pulp capping medicament by dividing the groups into four groups. The decrease in pain response in each rat can be discovered by focusing on the number of TNF-α, leukocyte cells, and decrease in pain from the MGS score. The data on the number of TNF-α, leukocyte cells, and MGS score assessment of four treatment groups can be shown as follows:

Table 1 indicates that the leukocyte cells in group I had a low average, and the leukocyte cells in group II had the highest average score among the other groups because the treatment was not
given therapeutic agents. Thus, after being given Ca(OH)\(_2\) addition, it decreased in group III, but in group IV, leukocyte cells had a higher decrease than in group III.

Likewise, the statistical results in groups I, II, III, and IV were normally distributed through the Shapiro Wilk test (p > 0.05) and the Kruskal Wallis test (p < 0.05), which showed a significant difference among group I and group II, III, and IV. It was continued for the Mann-Whitney test that leukocyte cells among groups I, II, III, and IV showed (p < 0.05), which means that there was a significant difference.

The data in Table 2 shows that TNF-α value was the lowest in group IV (with chitosan addition). The value of TNF-α was higher in the treatment of group II because no therapeutic agent was added. In addition, it decreased in group III which was given Ca(OH)\(_2\) addition, and in group IV, which was given chitosan addition.

Statistical results in Table 2 illustrate that groups I, II, III, and IV are normally distributed with the Shapiro-Wilk test (p < 0.05). The test is continued with the ANOVA test, which shows that the data in groups I, II, III, and IV have a value (p < 0.05), which means it has a significant difference. It is followed by the Post Hoc Multiple Comparisons tests to discover whether there were differences between one group and another, which had a value of (p < 0.05) in group I compared to group II, group II to III, and IV.

Mouse Grimace Scale results in Figure 1 show that group IV has decreased pain better than groups II and III. The data in Table 3 reveals that scoring pain in the healthy group that was not given treatment has very little value. Regarding group II, only treated with reversible pulpitis, the pain score was high. It decreased in group III with Ca(OH)\(_2\) addition. It had an increase in group IV, which was given chitosan addition. The average was significantly decreased as compared to group II.

### Table 1: Average leukocyte cell results

<table>
<thead>
<tr>
<th>No.</th>
<th>Group</th>
<th>Leukocyte Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I (Healthy)</td>
<td>4.83</td>
</tr>
<tr>
<td>2</td>
<td>II (Reversible Pulpitis)</td>
<td>18.25</td>
</tr>
<tr>
<td>3</td>
<td>III (Reversible Pulpitis + Ca(OH)(_2))</td>
<td>14.08</td>
</tr>
<tr>
<td>4</td>
<td>IV (Reversible Pulpitis + Nanochitosan)</td>
<td>12.83</td>
</tr>
</tbody>
</table>

### Table 2: Average TNF-α results

<table>
<thead>
<tr>
<th>No.</th>
<th>Group</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I (Healthy)</td>
<td>238.8</td>
</tr>
<tr>
<td>2</td>
<td>II (Reversible Pulpitis)</td>
<td>288.2</td>
</tr>
<tr>
<td>3</td>
<td>III (Reversible Pulpitis + Ca(OH)(_2))</td>
<td>238.3</td>
</tr>
<tr>
<td>4</td>
<td>IV (Reversible Pulpitis + Nanochitosan)</td>
<td>226.0</td>
</tr>
</tbody>
</table>

### Figure 1: Mouse Grimace scale observation
The statistical results are presented in Table 3 in groups I, II, III, and IV with normal distribution tested by Shapiro Wilk (p>0.05) and Kruskal Wallis test (p<0.05), which showed a significant difference among groups I and groups II, III, and IV. After these two tests, it is continued with the Mann-Whitney test, which compared group I to group II, obtaining p <0.05; group I to group III, obtaining p > 0.05; group I to group IV, obtaining p < 0.05; and group to group IV, obtaining p <0.05. From the results of Mann Whitney test, all groups are related significantly to group I, but group III is less significant.

The teeth treatment with reversible pulpitis treated with chitosan from Tables 1, 2, and 3 showed that the same phase of leukocyte cells, TNF-α, pain scores would decrease compared to teeth that only experienced pulpitis reversible without treatment.

The immune responses that influence pulpitis are polymorphonuclear neutrophils (PMNs) and tumor necrosis factors-α (TNF-α). Levels of pro-inflammatory cytokines and infiltration of PMN cells are responsible for the severity of inflammatory process [20]. These signs of inflammation can manifest as symptoms of severe pain in the oral cavity after it was proven that chitosan reduced pain indicators insignificantly and its ability can also reduce the number of PMN cells and levels of pro-inflammatory cytokines TNF-α [21].

Nanochitosan is an innovation from research on chitosan which has the size of a nanoparticle (nanotechnology) to protect the particles from degradation and facilitate their entry into the target location. Chitosan has the ability as an antioxidant, antitumor, immune cell stimulator, and antibacterial. Therefore, it is helpful as an alternative therapy for several disease complaints. Besides, chitosan has a silver-doped bioactive glass/chitosan hydrogel proven to downregulate inflammatory cytokines by signaling the p38 MAPK pathway [20].

The decreased levels of the cytokine TNF-α and activated PMN cells could be due to the ability of nano chitosan to reduce the IkB-α degradation in the cytoplasm and the levels of NF-κB p65 in the nucleus. Obstacles in the IkB-α degradation will cause the activation of NF-κB transcription factor to be inhibited. Decreased activation of the transcription factor NF-κB will inhibit DNA transcription of the gene that produces TNF-α. The decreased production of TNF-α will hamper the function of the chemoattractant, so fewer PMN leukocytes will be found [22].

Mechanical exposure to the pulp chamber has successfully induced inflammation, as indicated by high drilling speed and releasing CGRP in the dental pulp. CGRP is produced by trigeminal cell bodies and acts as a vasodilating agent that causes an increase in local blood flow [23].

Activation of nervous system in A delta releases proinflammatory neuropeptides, including SP (Substance P), which has an important role in the initial inflammation process by producing neurogenic inflammation in the dental pulp, causing vasodilation and contraction of endothelial cells, resulting in plasma extravasation and mast cell degranulation. These mast cells will release histamine and activate nociceptors. Hyperalgesia will cause macrophages and mast cells to release several inflammatory mediators, including bradykinin, PGE-2, thromboxane, and inflammatory cytokines IL 6, IL 1, and TNF-α [24]. This molecular event will increase the pain in the teeth if no pulp-capping medicaments are given.

Another mechanism that can reduce pain in chitosan lies in its antibacterial properties. Chitosan has a biological component of polysaccharides with cationic properties where the protonation of -NH2 group is at a low pH. In addition, it can interact with negatively charged

<table>
<thead>
<tr>
<th>No.</th>
<th>Group</th>
<th>Pain Scoring 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I (Healthy)</td>
<td>0.073</td>
</tr>
<tr>
<td>2</td>
<td>II (Reversibel Pulpitis)</td>
<td>0.380</td>
</tr>
<tr>
<td>3</td>
<td>III (Reversibel Pulpitis + Ca(OH)2)</td>
<td>0.373</td>
</tr>
<tr>
<td>4</td>
<td>IV (Reversibel Pulpitis + Chitosan)</td>
<td>0.200</td>
</tr>
</tbody>
</table>
components. Chitosan is antibacterial caused by its polycationic. Electrostatic interaction occurred between the chitosan structure and the anionic components of the surface of microorganisms. This interaction is caused by a lower pH than the pKa of chitosan [25].

Conclusion

Nanochitosan is a type of chitosan with nanoparticle-sized antioxidants, antitumors, immune system stimulators, and antibacterial properties. Pulp treated with nanochitosane derived from red snapper scales revealed decreased TNF-α levels, leukocyte cells, and pain response with reduced degradation of IkB-α in the cytoplasm and the NF-κB p65 levels in the nucleus more than pulp not treated derived from red snapper scales revealed increased TNF-α levels, leukocyte cells, and pain response. Thus, it has been demonstrated that redfish scale nanochitosane can decrease inflammation by lowering levels of TNF-α, leukocyte cells, and pain.

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Authors’ Contributions

All authors contributed to data analysis, drafting, and revising of the paper and agreed to be responsible for all the aspects of this work.

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