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POTENTIAL OF CHITOSAN BLACK SOLDIER FLIES (*Hermetia Illucens*) PUPAE ON POST-EXTRACTION WOUND HEALING PROCESS

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Introduction

Tooth extraction creates an empty socket in the alveolar process, which initiates a wound-healing mechanism. However, extraction wounds can result in complications and patient complaints, including tenderness, edema, hemorrhage, and dry sockets. A dry socket, one of the most common post-extraction complications, occurs when the bloodstream of a tooth socket is not formed, exposing the bone and tissues to the oral cavity or subjected to chemical stimulations and causing pain (1–3). As the wound heals, new tissue formation will accelerate the process (4) and becomes the primary determinant of healing through epithelialization, connective tissue formation, proliferation stage of fibroblasts, extracellular matrix formation, and activities of

Fibroblast Growth Factor (FGF) and Transforming Growth Factor-β1 (TGF- β1) released

by macrophages. Ideally, fibroblasts are seen in the wound area after the third day and should increase on the seventh and fourteenth day (5).

Several medicinal plants and animals are used as an alternative to accelerate wound healing, including the BSF (*Hermetia illucens*) (6,7). BSF (*Hermetia illucens*) originates from the United States, and its larvae have been used widely as a waste processor, especially organic waste, generally benefiting from the insect's chitin. BSF has 40-50% protein; during the prepupae phase into pupae, BSF acts as a potential chitin source through its exoskeleton, which contains 35% chitin when converted to chitosan through a deacetylation process. Chitosan, a natural polysaccharide (N-acetyl-D-glucosamine) potentially contributes to hard and soft tissue healing mediated by protein signals that regulate various intracellular and extracellular events (8–10). Chitosan contains a polymer structure similar to hyaluronic acid and contains glycosaminoglycan group (GAGs), an extracellular matrix macromolecule essential for wound healing. Chitosan could also act as an anti-inflammatory material and stimulate cell proliferation and remodeling in wound healing by accelerating the infiltration of inflammatory cells, promoting angiogenesis, inducing rapid vascular formation in tissue granulation, accelerating skin regeneration, minimizing scar tissue formation, preventing purulence, and has candidacidal and bactericidal effects. In addition, chitosan is used as a stimulant for fibroblast formation to trigger an increase in FGF-2 by activating production of cytokines that will activate fibroblasts in the wounds (5,11).

As chitosan is biodegradable, biocompatible and non-toxic, it is useful in the biomedical field (12). This study aims to assess the Potential of Chitosan BSF Pupae for the effect of accelerating wound healing in the Cavia Cobaya socket by analyzing macrophages and fibroblast activities on post-extraction sockets on the 2nd, 3rd, and 5th days.

Materials And Methods

Chitin and chitosan preparation

This study used the validated experimental method with a posttest in the control group design and received ethics clearance (reference: 041/KEPKG-FKULM/EC/III/2023). The first step involved the formation of BSF pupae chitosan through stages of demineralization by soaking the sample in HCl 3 m 1:10 (b/v) for 36 hours at room temperature (25-30°C) and then rinsed with aquadest on filter paper until a neutral pH and dry on the oven temperature of 60 ° C for 1 hour and produce residues. The next stage was deproteination, where the residue was soaked in NaOH 2 M 1:10 (B/V) for 36 hours at room temperature (25-30°C). This resulted in a change in color in the BSF pupae sample, from dark brown to yellowish brown. The next stage was depigmentation to obtain chitin by soaking residue in a 2% 1:10 (w/v) KMnO4 solution for 2 hours followed by oxalic acid 2% 1:10 (b/v) for 2 hours. Then, the mixture was filtered, washed until it reached a neutral pH, and continued with deacetylation to convert chitin to chitosan by soaking in a 50% NaOH solution (1:10) at 80°C for 12 hours using a magnetic stirrer. Lastly, the sample was filtered and rinsed with aquadest until it reached a neutral pH, then dried for 48 hours at 60°C in the

oven. The produced chitosan was available in powder form with an 80% degree of deacetylation, indicating purity of the chitosan. Finally, chitosan was converted into a gel preparation, which was applied on post-extraction sockets in *Cavia cobaya* by mixing the chitosan BSF pupae powder, PEG 400, and PEG 4000 until homogenous.

Grouping allocation and animal care

The Guinea pigs (*Cavia Cobaya*) can be used as a trial animal because their physiological and reproductive conditions are similar to mammals and have the same immunological and pathological responses as humans. Mandibula incisors were chosen because they are longer and bigger than teeth in the upper jaw, so they are expected to facilitate extraction. The *Cavia Cobaya* does not have canine or premolar teeth, so there is a diastema between the anterior tooth and posterior tooth. The diastema between the anterior and posterior tooth and the more compact jaw bone facilitates access to the anterior tooth (28).

The subjects were 18 healthy male Guinea pigs (*Cavia cobaya)* with weight 200-300 gram, age 2.5-3 months divided into two groups taken randomly (n-9 respectively): the treatment group (CBSF) that was given chitosan BSF (*Hermetia illucens*) pupae after tooth extraction, and the control group (CC) that was not given chitosan black soldier fly (*Hermetia illucens*) pupae after tooth extraction and then sacrificed on the 2nd, 3rd, and 5th days after treatment.

Macrophage cells appear first in 2-4 days after injury, polymorphonuclear cells (PMN) appear, and after 24-48 hours, PMN cells will be replaced with macrophages, which are the most dominant cells in inflammation with the highest amounts on the 2nd day and reach the peak on 3rd day. On the 5th day, there was a decrease in the number of macrophages because it had switched to the proliferation phase. The proliferation phase occurs from the 3rd day to the 14th day and is dominated by the formation of granulation tissue and epithelization. In this phase, the number of fibroblast cells is more than that of inflammatory cells(29).

The Guinea pigs (*Cavia cobaya)* were anesthetized with 50mg/kg bw of ketamine and 5mg/kg bw of intramuscular xylazine. Subsequently, the lower left incisor was extracted, and the socket was irrigated with sterile distilled water to remove debris. In the CC group, the wound was sutured immediately after extraction with a non-resorbable suture. In the CBSF group, BSF chitosan gel was applied with a sterile syringe to completely fill the post-extraction sockets, then sutured with a non-resorbable suture. The animals were euthanized on the 2nd, 3rd, and 5th days after treatment. The mandibular bone around the interdental area of the lower incisors was cut and inserted into a fixative 10% buffered formalin. The histopathology anatomy slide was made using (HE) hematoxylin-eosin staining. A light microscope equipped with a camera is used for calculating the number of macrophage cells.

Statistical analysis

The number of macrophages and fibroblasts is calculated using a light microscope with a magnification of 400x in a third of the apical dental socket and examined by anatomical pathology specialists in the anatomical pathology laboratory. Normally distributed and homogenous data were analyzed using the Oneway ANOVA test. Meanwhile, non-parametric analysis via the Kruskal-Wallis test was employed when the data were not normally distributed or not homogenous. Statistical significance was determined at a p-value < 0.05.

Results

The mean and standard deviation of macrophages and fibroblasts in both treatment and control groups on the 2nd, 3rd, and 5th days of observation are listed in Table 1 and Table 2. Table 1 showed that macrophages began to appear, and dominantly, after the 2nd day of the wound. They increased on the 3rd day but decreased on the 5th day. The highest number of macrophages on the 2nd day was in the chitosan black soldier fly pupae (CBSF) group, followed by the control group (CC). The highest number of macrophages on the 3rd day was in the CBSF group, followed by CC. The highest number of macrophages on the 5th day was in the control group (CC), followed by the CBSF group.

Table 1. Mean and standard deviation of macrophages on the 2nd, 3rd, and 5th days.

Treatment Group	2nd Day	3rd Day	5th Day
CC	48.30 ± 6.25	65.60 ± 1.31	41.40 ± 0.52
CBSF	71.93 ± 1.28	88.60 ± 4.15	33.53 ± 2.23

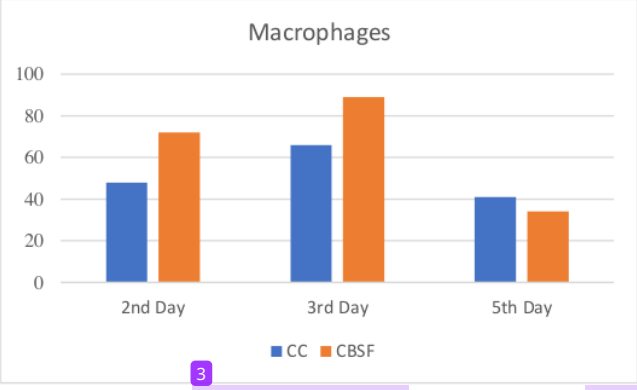
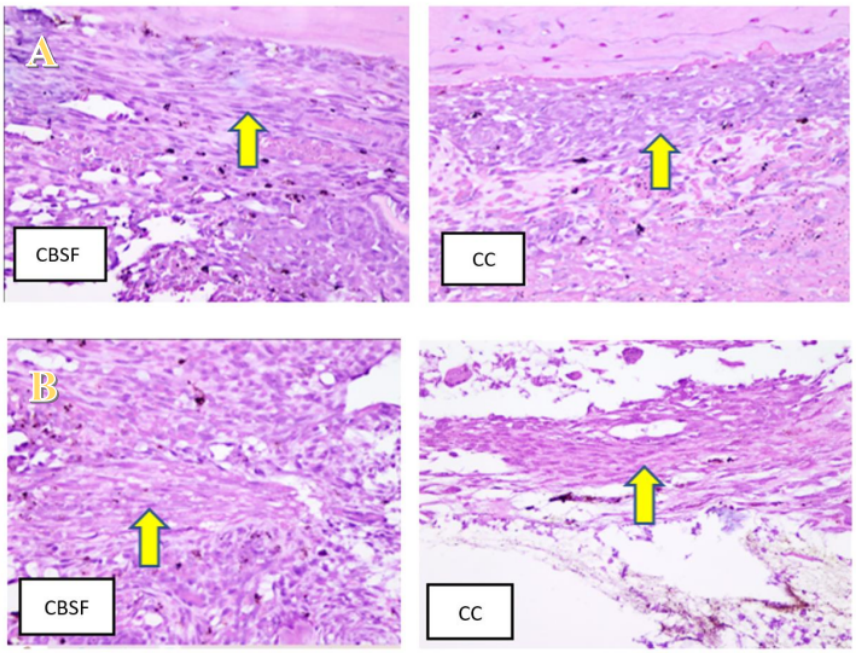


Figure 1. A bar chart depicting the mean number of macrophages in the control group (CC) and the black soldier fly pupae chitosan group (CBSF) on the 2nd, 3rd, and 5th days of observation.



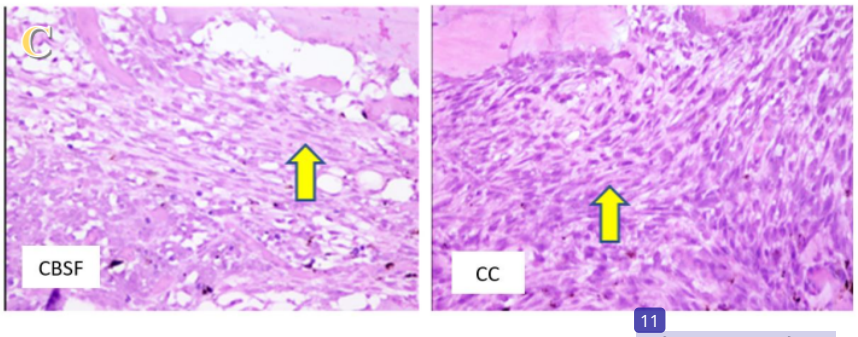


Figure 2. Histopathology anatomy image of fibroblasts on (A). the 2nd day, (B). 3rd day and (C). 5th day of observation at 400x magnification in the chitosan black soldier fly (*Hermetia illucens*) pupae group (CBSF) and control group (CC).

Figure 2 A&B indicates the highest macrophage count was found in the chitosan group compared to the control group on the 2nd and 3rd days of observation. Figure 2 C shows the macrophage count on the 5th day of the negative control group, which was not given higher treatment than the chitosan group.

The data normality test showed a significance of 0.000 ($p < 0.05$). Therefore, the data were not normally distributed or homogenous. Kruskal Wallis test was then used to determine the difference in macrophage count between the treatment group and the control group. The results showed that there was a significant difference between groups on the 2nd, 3rd, and 5th days with a p-value of 0.006 ($p < 0.05$). The Mann-Whitney test was used to determine the significance of differences between both groups. Statistical analysis revealed a significant difference in macrophage count between the chitosan group on the 3rd day and the chitosan group on the 5th day.

Table 2. Kriskal-Wallis statistical analysis test

Kruskal-Wallis	16.268
Df	5
Asymp.Sg	.006

Table 3. Mean and standard deviation of fibroblasts on the 2nd, 3rd, and 5th days.

Treatment Group	2nd Day	3rd Day	5th Day
CC	19.13 ± 1.17	25.27 ± 2.02	35.00 ± 1.11
CBSF	32.07 ± 1.40	51.33 ± 2.00	65.87 ± 1.94

Table 3 indicated that fibroblasts started to appear on the 2nd day after the wound, which increased on the 3rd and 5th days. The highest number of fibroblasts on the 2nd, 3rd, and 5th days was found in the chitosan group (CBSF), followed by the control group (CC).

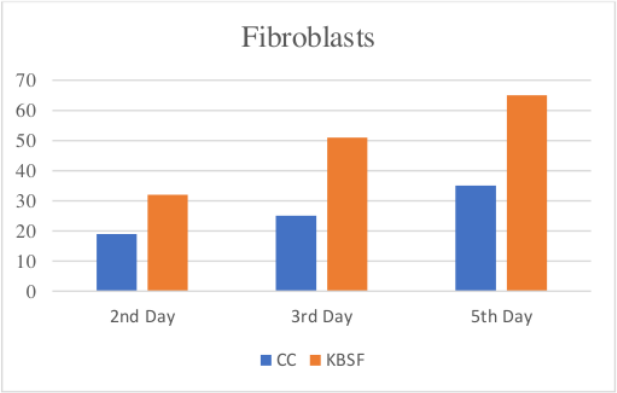


Figure 3. A bar chart depicting the mean number of fibroblasts in the control group (CC) and the black soldier fly pupae chitosan group (CBSF) on the 2nd, 3rd, and 5th days of observation.

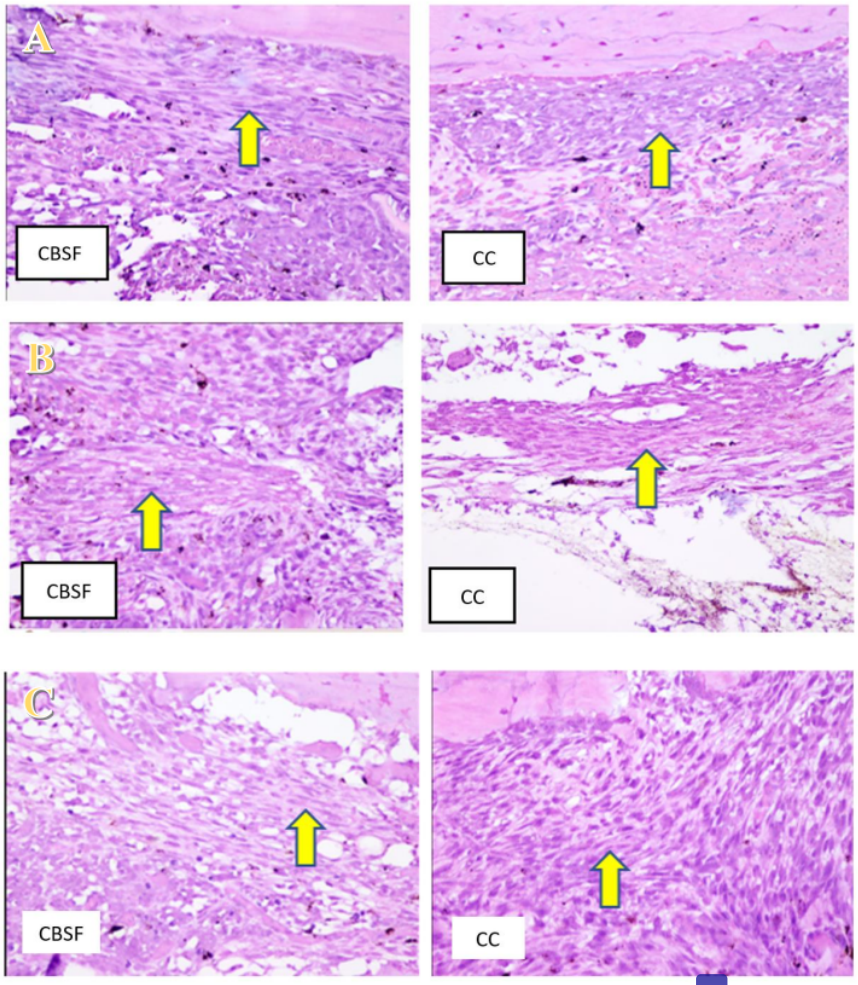


Figure 4. Histopathology anatomy image of fibroblasts on (A). the 2nd day, (B). 3rd day and (C). 5th day of observation at 400x magnification in the chitosan black soldier fly (*Hermetia illucens*) pupae group (CBSF) and control group (CC).

Fibroblast count was carried out at a 400x magnification on the apical third of the tooth socket and found that the greatest number of fibroblasts was found in the chitosan group compared to the control group on the 2nd, 3rd, and 5th day of observation.

The ANOVA test found a significant difference in fibroblasts between treatment groups on the 2nd, 3rd, and 5th days with a p-value of 0.000. The significance of each group was determined through the Post Hoc test.

Table 4. One-way ANOVA statistical analysis test

ANOVA	14.238
Df	5
Asymp.Sg	.000

The data normality test showed a significance level of 0.094 ($p > 0.05$), indicating normally distributed and homogenous data. One-way ANOVA was carried out to determine the difference in fibroblasts between groups. The results showed a significant difference in fibroblasts between treatment groups on the 2nd, 3rd, and 5th days with a p-value of 0.000 ($p < 0.05$). A Post Hoc test was used to determine the significance between each group. Statistical analysis found a significant difference between the control group on the 2nd and 3rd days and the chitosan group on the 2nd, 3rd, and 5th days. A significant difference was found between the control group on the 5th day and the chitosan group on the 3rd and 5th days. A significant difference was also found between the chitosan group on the 2nd day and the chitosan group on the 3rd and 5th day.

Discussion

Wound healing, in principle, consists of the inflammatory, proliferation, and maturation phases by forming new tissues or repairing existing tissues (13). The study showed the highest macrophage count was on the 3rd day in the chitosan group, followed by the control group. Afterward, the macrophages decreased on the 5th day.

The early stage of inflammation can occur within 24-48 hours and continue up to the 6th day. As the first line response to tissue injury, neutrophil accumulates at the wound site during the early inflammatory response phase. It will become the most abundant cells during the first 24 hours. Excessive neutrophil formation in the wound area can inhibit wound healing. Thus, neutrophil cleansing is required. Macrophages can phagocyte neutrophils through integrin β2 after being activated by DAMP (14). Other than that, CCN1 matricellular protein also plays a role in neutrophil cleansing. It acts as a molecule that bridges neutrophils and macrophages. CCN1 can bond with the phospholipid membrane of neutrophils known as phosphatidylserine, which is an "Eat Me" signal common in apoptotic cells, which is presented to macrophages' integrin, activating neutrophil efferocytosis. This leads to neutrophil cleansing from the wound site, contributing further to wound healing (15). When phagocyting neutrophils, the phenotype of the macrophage changes into a proinflammatory type-1 macrophage (M1) and an anti-inflammatory type-2 macrophage (M2), which is a process regulated through a mediator released by neutrophils (24).

Macrophages first appear within 2-4 days after an injury. Polymonuclear cells (PMN) appear after 48 hours, which will be substituted by macrophages, the most dominant cell during an inflammation from the 2nd day, peaking on the 3rd day. An increase in macrophage activities will stimulate growth factor release, such as the VEGF, TGF-β, and anti-inflammatory cytokines that can accelerate wound healing, such as IL-10. Macrophages actively phagocytes, repair tissues, and produce growth factors. Although decreasing, macrophages still linger in the wound to continue the healing process because they activate the proliferation phase (16-18).

Applying chitosan black soldier fly (*Hermetia illucens*) pupae on the socket area of *Cavia cobaya* is more effective than no chitosan application in improving the number of macrophages seen through histopathology on the 2nd and 3rd days.

In an injury, macrophages will appear 24 hours after a pro-inflammatory activity with a polarity of M1 phenotype (CD86+) that releases anti-inflammatory cytokines such as IL-1β, IL-6, TNFα, induced by nitric oxide synthase (iNOS), and involved in pathogen elimination, inflammatory cytokine release, and releases a Th1 type reaction. Within 24-48 hours after an injury, macrophage changes function into an M2 phenotype as an anti-inflammatory that promotes cell proliferation and tissue remodeling. M2 (CD206+) macrophage that releases anti-inflammatory cytokines such as IL-10 to reduce proinflammatory response and growth factors such as the vascular endothelial growth factors-α (VEGF-α), transforming growth factor-β (TGF-β), platelet-derived growth factors (PDGF), and insulin-like growth factor-1 (IGF-1) to

increase cell proliferation and angiogenesis (Figure 10). IL-10 also stimulates pre-osteoblast formation that will differentiate into osteoblasts by regulating runt-related transcription factor 2 (Runx2). Runx2 is the most upstream transcription factor essential for osteoblast differentiation. Increased IL-10 can cause increased osteoprotegerin (OPG). OPG prevents RANKL from binding with RANK as a sign of disrupted bone resorption and increased alveolar bone apposition. Macrophage, as a phagocytic cell, will clear out foreign bodies in the inflammation area and increase in number on the 3rd day due to a hypoxic environment caused by muscle injury for several days and peak on the 4th day. The increase of macrophages is also supported by the study of Sularsih and Soeprijanto (2016), who explained that during the inflammatory phase, inflammatory cells such as macrophages will migrate into areas with inflammation and increase in number (14, 19, 23).

Lymphocyte cells and macrophage cells interact in two ways. Macrophage produces cytokines such as the tumor necrosis factor-alpha (TNFα), IL-12, IL-6, and IL-23, introducing antigens to T lymphocytes to produce a response from lymphocytes. The activated T lymphocyte will produce lymphokine that activates more monocytes and macrophages in the form of macrophage aggregating factor (MAF)/IFN-γ and macrophage chemotactic factor (MCF). The lymphocytes will produce IL-2 cytokine and fibroblast activating factor which will affect fibroblasts to support the next stage of wound healing (24, 25).

Chitosan has a chemical formula of N-acetyl-Dglucosamine with a polymer structure similar to hyaluronic acid, which is the glycosaminoglycan group (GAGs), an important extracellular matrix macromolecule for wound healing. During the inflammatory phase, applying chitosan to the wound causes chitosan to be actively polymerized to secrete N-acetyl glucosamine by the lysosome enzyme. Then, the N-acetyl glucosamine monomer binds with the macrophage's primary receptor and triggers the macrophage's migration and proliferation. A higher number of macrophages in an injured tissue will accelerate the wound-healing process, especially during the inflammatory phase (23).

The findings on the 5th day showed a decrease in the number of macrophages with the lowest average of comparison between the chitosan and the control groups.

Applying black soldier fly (*Hermetia illucens*) pupae chitosan is more effective than the control group in reducing the number of macrophages seen in the histopathological findings on the 5th day. The decreased number of macrophages indicates that macrophages regulate the formation of scars to reduce scars after wound healing and the end of the inflammatory phase, which is marked by the reduced number of macrophages. This decrease began with the appearance of the lysosome enzyme, one of the enzymes released by phagocytic cells such as macrophages. The enzyme will degrade chitosan into active N-acetyl glucosamine dimers and smaller molecules (monomers). The monomer shape will bond with the primary receptor in the macrophage, which is the mannose receptor. After binding

with macrophages, chitosan can reduce the number of macrophages that form fibroblasts in the proliferation phase (5, 23, 30).

The application of black soldier fly chitosan also significantly increased fibroblasts on the 2nd, 3rd, and 5th days compared to the group that was not given chitosan. Chitosan will trigger macrophages to increase the production of TGF-β1, the most dominant cytokine released in trauma or wound sites. Chitosan applied on post-extraction wounds can stimulate the increase of TGF-β1 and FGFs. The growth factors will trigger the proliferation of fibroblasts to increase the number of fibroblasts in post-extraction wound healing (21, 22).

The proliferation stage begins on the 2nd and 3rd day. Epithelial proliferation occurs on the surface of blood clots. Fibroblasts derived from mesenchymal cells begin to proliferate and spread into blood clots. Jesus et al. (2023) stated that no patient was bleeding after chitosan application post-surgery. This concludes that there is an interaction between chitosan and blood cells. The negative outer layer of erythrocytes and platelets are pulled into the positive reactive amino group from chitosan, leading to platelet activity in producing thrombus. Okamoto et al. also supported this, who stated that applying chitosan can increase blood coagulation, thus accelerating bleeding cessation (24, 27).

Chitosan molecules stimulate fibroblasts to release IL-8 and other cytokines that can induce angiogenesis, fibrosis, and epithelization. Fibroblasts proliferate from the

connective tissue from the 5th to 7th day, and the maximum number of fibroblasts is found during this period. When tissues are inflamed, new fibroblasts migrate into the wounded area, proliferating, and producing collagen matrix to repair the damaged tissue. Fibroblasts are derived from undifferentiated mesenchymal cells, producing mucopolysaccharides, glycine amino acid, and proline, which are the main ingredients of collagen fibers that attach to the sides of the wound. Fibroblasts have a unique characteristic in wounds compared to fibroblasts in normal tissue, with myofibroblastic appearance and phenotype characteristics of abundant contractile filaments, dense intercellular junction, and a different nucleus membrane (23, 24, 27).

Several studies have supported the role of chitosan in tissue regeneration. The body's immune system recognizes the amino group from this substance, inducing inflammatory cells and fibroblasts to migrate into the wound site and activate them to produce many anti-inflammatory cytokines. Boynueğri et al. evaluated the effect of chitosan gel by comparing the combination of collagen membranes applied to intraosseous lesions for periodontal regeneration. They concluded that all treatments increase the clinical measurement of tissue regeneration. Using chitosan in the form of gel did not trigger an inflammatory reaction. The Antimicrobial activity of chitosan also promotes the repair of damaged tissue and prevents wound infection. This biopolymer increases inner and outer membrane permeability, disrupts the bacterial cell membrane, and releases its contents. This provides an antibacterial barrier to gram-positive and gram-negative organisms, including methicillin-resistant staphylococcus, vancomycin-resistant enterococcus, and *Acinetobacter baumannii*.

The unique biological properties and cost-effectiveness of chitosan are essential in dental practice for faster wound healing and as a cost-effective alternative for patients. This improved the quality of the patient's condition after surgery, enabling faster wound healing with fewer complications (8, 27).

In this study, there are constraints when conducting the extraction of Cavia Cobaya tooth, so when carrying out the extract, one must be careful so that the tooth is not fractured.

Conclusion

The application of chitosan from black soldier fly (*Hermetia illucens*) pupae accelerates wound healing process through an increase of macrophages on the 2nd and 3rd day, its decrease on the 5th days, and also an increase of fibroblasts after the extraction of *Cavia cobaya* teeth on the 2nd, 3rd and 5th days.

Acknowledgment

The authors big thanks to the Faculty of Medicine Science Laboratory, Lambung Mangkurat University, the Faculty of Biochemistry Medicine Science Laboratory, Airlangga University, Universiti Kebangsaan Malaysia, Hasanuddin University.

Competing interests

The authors declare that we have no competing interests.

Table 1: Mean and standard deviation of macrophages on the 2nd, 3rd, and 5th days.

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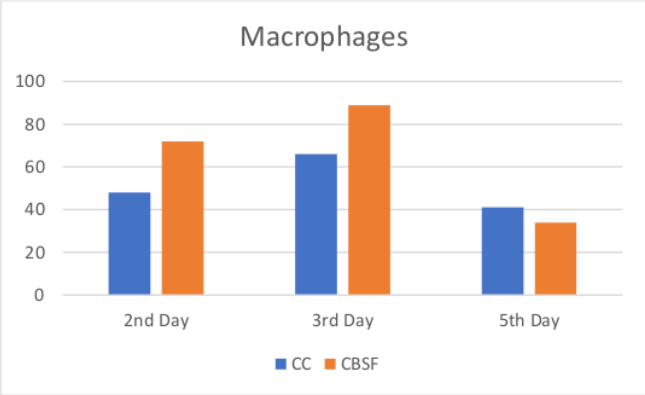

Figure 1: A bar chart depicting the mean number of macrophages in the control group (CC) and the chitosan black soldier fly (*Hermetia illucens*) pupae group (CBSF) on the observation's 2nd, 3rd, and 5th days.

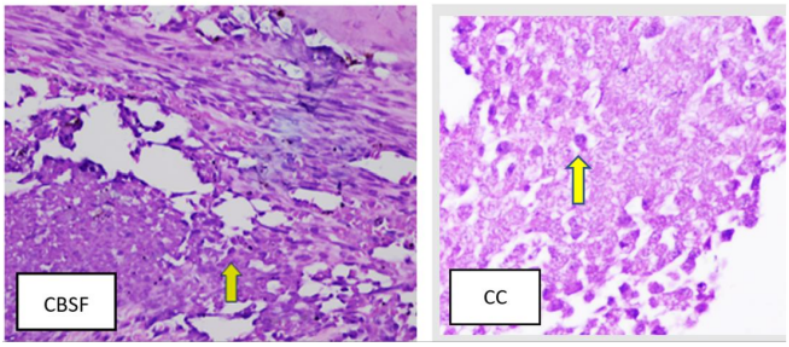

Figure 2: Histopathology anatomy image of macrophages on the 2nd day of observation at 400x magnification in the chitosan black soldier fly (*Hermetia illucens*) pupae group (CBSF) and control group (CC).

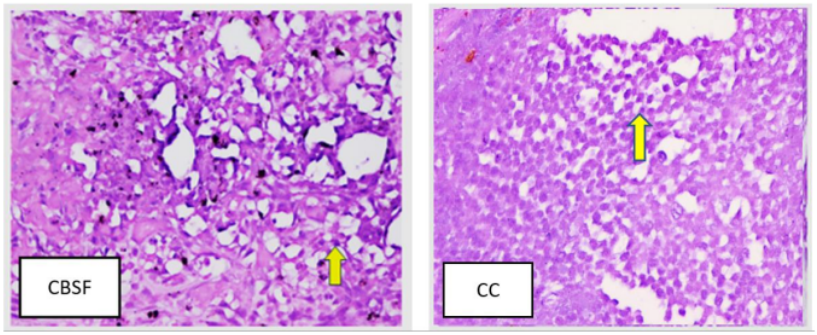

Figure 3: Histopathology anatomy image of macrophages on the 3rd day of observation at 400x magnification in the chitosan black soldier fly (*Hermetia illucens*) pupae group (CBSF) and control group (CC).

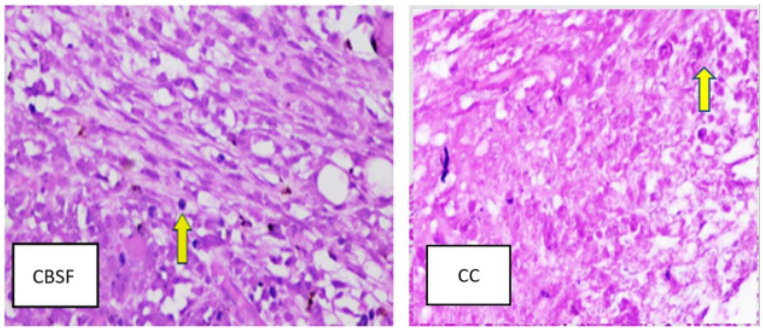

Figure 4: Histopathology anatomy image of macrophages on the 5th day of observation at 400x magnification in the chitosan black soldier fly (*Hermetia illucens*) pupae group (CBSF) and control group (CC).

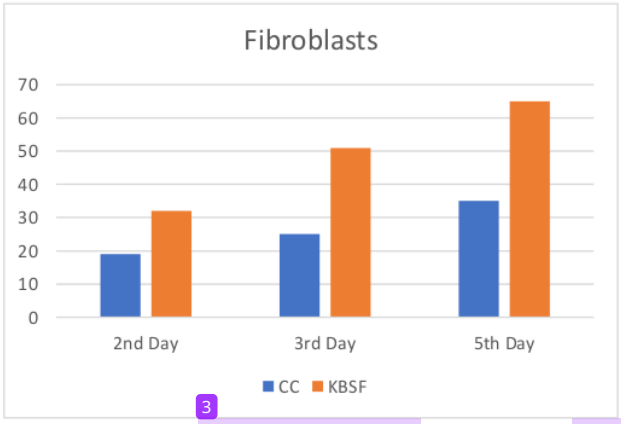

Figure 5: A bar chart depicting the mean number of fibroblasts in the control group (CC) and the chitosan black soldier fly (*Hermetia illucens*) pupae group (CBSF) on the observation's 2nd, 3rd, and 5th days.

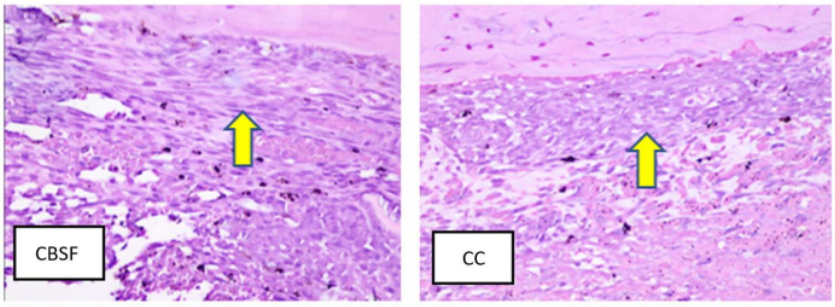

Figure 6: Histopathology anatomy image of fibroblasts on the 2nd day of observation at 400x magnification in the chitosan black soldier fly (*Hermetia illucens*) pupae group (CBSF) and control group (CC).

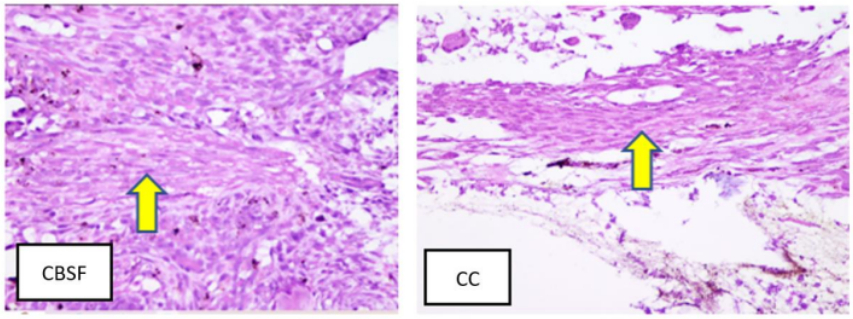

Figure 7: Histopathology anatomy image of fibroblasts on the 3rd day of observation at 400x magnification in the chitosan black soldier fly (*Hermetia illucens*) pupae group (CBSF) and control group (CC).

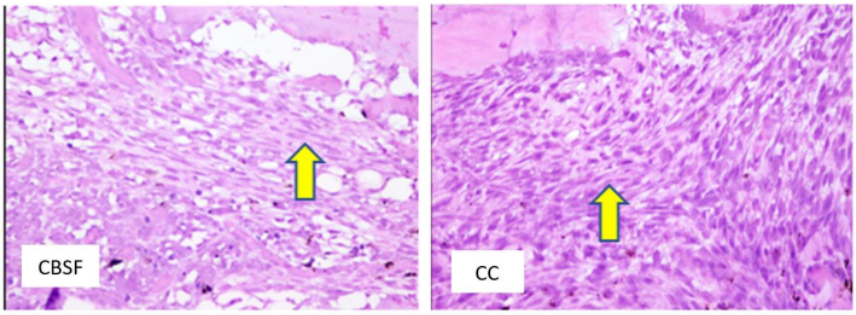

Figure 8: Histopathology anatomy image of fibroblasts on the 5th day of observation at 400x magnification in the chitosan black soldier fly (*Hermetia illucens*) pupae group (CBSF) and control group (CC).

Renie Kumala Dewi

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