ORIGINAL ARTICLE

The impact of chitosan derived from black soldier fly (*Hermetia illucens*) pupae on bone remodeling post-tooth extraction: an in vivo study

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ABSTRACT

Introduction: Bone defects or alveolar sockets commonly occur after tooth extraction. Black Soldier Fly (BSF) pupae contain 35% chitin, which can be converted into chitosan. This study aims to analyze the effect of BSF pupae chitosan gel on the number of osteoblasts and osteoclasts in post-extraction sockets. **Method:** This study employed a true experimental design. The left mandibular incisor of guinea pigs was extracted. In the control group (n=9), the socket was filled with polyethylene glycol (PEG) gel as a placebo, while in the treatment group (n=9), the socket was filled with BSF pupae chitosan gel. The gel was applied until the socket was full, followed by suturing with non-absorbable silk. Euthanasia was performed on days 7, 14, and 21 to evaluate the number of osteoblasts and osteoclasts. Data were analyzed using one-way Anova. **Results:** The osteoblast count in the treatment group increased on day 7 (52.20 ± 1.90), day 14 (91.53 ± 1.00), and day 21 (104.13 ± 5.33) compared to the control group: day 7 (39.80 ± 5.43), day 14 (61.13 ± 1.10), and day 21 (82.60 ± 2,11). The number of osteoclasts decreased in both groups: in the control group on day 7 (9.83 ± 0.35), day 14 (12.80 ± 0.72), and day 21 (2.46 ± 0.11); and in the treatment group on day 7 (4.86 ± 1.51), day 14 (9 ± 0.34), and day 21 (2.66 ± 0.11). Statistical analysis revealed significant differences in osteoblast and osteoclast counts between the treatment and control groups (p = 0.000). **Conclusion:** The application of chitosan BSF pupae gel can increase osteoblast numbers and decrease osteoclast numbers after tooth extraction, potentially accelerating bone formation and offering benefits for future bone regeneration.

KEYWORDS

Chitosan black soldier fly, osteoblasts, osteoclasts, bone remodeling

INTRODUCTION

Tooth extraction is a very common dental procedure. While it is often seen as simple, it's important for patients to understand that every extraction can have potential side effects that need to be managed.[1–3] Injuries after tooth extraction

include tissue injury and interruption of continuity.[4] Following extraction, wounds will go through a physiological healing process that includes hard tissues (alveolar bone) and soft tissues (connective tissue and gingival epithelium) healing.[5–7] Hemostasis, inflammation, proliferation, and remodeling are the four basic stages of wound healing. These four stages take place in overlapping temporal intervals.[3,8–10]

The process of bone remodeling involves osteoclasts resorbing older or damaged bone and osteoblasts forming new bone.[11] Alveolar bone resorption occurs when osteoclasts proliferate in the alveolar bone with abnormalities, but osteoblasts do not increase in proportion.[12–14] The ability of bones to heal themselves is not at its best when there is excessive bone injury. In this case, a bone transplant is required to act as a scaffold and to provide support for the formation of new bone.[15,16]

Through its exoskeleton, which contains 35% chitin when converted to chitosan by a deacetylation process, BSF functions as a possible supply of chitin throughout the prepupae phase into pupae.[17] Chitosan is a natural polysaccharide synthesized from chitin extracted from the shells of crustaceans, insects, mollusks, and some types of fungi. The extracted chitin can be converted into chitosan through deacetylation.[3,18–20] Black soldier fly (BSF) therapy has been around for several centuries, and during the prepupal phase. Chitin is a homopolymer of β (1,4)-N-acetyl-D-glucosamine, while chitosan is chitin that has lost its acetyl groups, forming a copolymer consisting of 2-acetamido-D-glucose and 2-amino-D (usually more than 80%) linked together by β(1 → 4) glycosidic bonds.[17,19–21]

Chitosan possesses several beneficial properties, including biodegradability, biocompatibility, antibacterial, antimicrobial, antifungal, analgesic, and antitumor effects. It also has high bioavailability, good water permeability selectivity, and high chemical resistance. Chitosan is not carcinogenic to animals or humans, but it is bioactive and has a rapid tissue healing effect.[3,17–22] High molecular weight chitosan gel has shown promise in stimulating osteoblast and fibroblast cell growth at tooth extraction sites over seven to fourteen days.[3] Chitosan also contains growth factors needed to stimulate progenitor cells, enabling them to fill defects or damage in bone remodeling.[23]

Although chitosan has been widely studied for its potential in wound healing and tissue regeneration, research specifically exploring chitosan derived from black soldier fly (BSF) pupae in bone remodeling remains limited, particularly regarding its influence on osteoblast and osteoclast activity following tooth extraction. Previous studies have primarily focused on other indicators of healing, such as fibroblast or macrophage proliferation, without examining the direct cellular processes involved in bone formation and resorption.

The novelty of this study lies in evaluating the biological activity of BSF pupae-derived chitosan on osteoblast and osteoclast cell dynamics in post-extraction alveolar bone, providing new insights into its potential role as a biomaterial for bone regeneration. This study aims to analyze the effect of the BSF Pupae chitosan gel application on the number of osteoblasts and osteoclasts after tooth extraction.

METHODS

This study employed a true experimental design with a control group. The research utilized 18 male guinea pigs (Cavia Cobaya) with specific characteristics: body weight of 200-300 grams, age 2-3 months, and active movement to ensure physical health. The sample was divided into two groups: 9 guinea pigs in the treatment group (T) and 9 in the control group (C).

The BSF pupae chitosan gel was prepared based on the method described by Dewi (2023). BSF pupae were obtained from farms in South Kalimantan, cleaned of debris, and processed through demineralization, deproteinization, and depigmentation to extract chitin. The chitin was then converted into chitosan via a deacetylation process, yielding chitosan powder with an 80% deacetylation rate.

The chitosan gel was prepared by 8 g of PEG 400 and 2 g of PEG 4000 in a porcelain cup container, heated in a water bath at 70°C, and stirred until homogeneous. The chitosan powder was gradually added to this mixture and stirred until homogeneous.[24] Polyethylene glycol (PEG) was chosen as a stabilizer due to its non-toxic, non-corrosive, ability to enhance solubility and improve the physical and chemical stability of the gel. The resulting BSF chitosan gel was transferred to plastic containers, ready for application to the guinea pig dental extraction sockets. The entire gel preparation process was completed within one day.

Guinea pigs were anesthetized using an intramuscular injection of ketamine (50 mg/kg body weight) and xylazine (5 mg/kg body weight). The left mandibular incisors were extracted, and the sockets were irrigated with 12% NaOCl to remove debris. In the control group (CC), after the teeth were extracted, the socket was filled with PEG gel and then sutured using non-absorbable silk. In the treatment group (CBSF), after the teeth were extracted, the socket was filled with BSF chitosan gel and then sutured using non-absorbable silk. The chitosan gel was applied using a sterile plastic syringe to ensure the socket was completely filled.

Euthanasia was performed on days 7, 14, and 21 to prepare histopathological samples. Histological analysis was conducted using Hematoxylin-Eosin (HE) staining and observed under a light microscope. Observations were made on the apical third of the mandibular socket at 400x magnification in five different fields of view, including the mesial, distal, and central regions of the tooth socket. The number of osteoblast and osteoclast cells per field of view was recorded, calculated, and analyzed.

The results of osteoblast cell calculations are processed and analyzed using the normality test, namely the Shapiro-Wilk test, and the variance homogeneity test using the Levene test. Data about the effect of the BSF Pupae chitosan gel application on the number of osteoblasts and osteoclasts effect were analyzed using One Way Anova.

RESULTS

The mean ± standard deviation (SD) of osteoblast counts in the Treatment Group with Chitosan BSF Pupae (CBSF) and Control Group (CC) on days 7, 14, and 21 are presented in Table 1.

Table 1. Average amount of osteoblasts in the CBSF group and CC group at observed on the 7th, - 14th, and -21st days.

Group	Number of each	Mean ± Standard Deviation of Osteoblast Cell Number		
		Day 7	Day 14	Day 21
CBSF	3	52.20±1.90	91.53±1.00	104.13±5.33
CC	3	39.80±5.43	61.13±1.10	82.60±2.11

Histopathological examination revealed osteoblast presence on days 7, 14, and 21, as shown below:

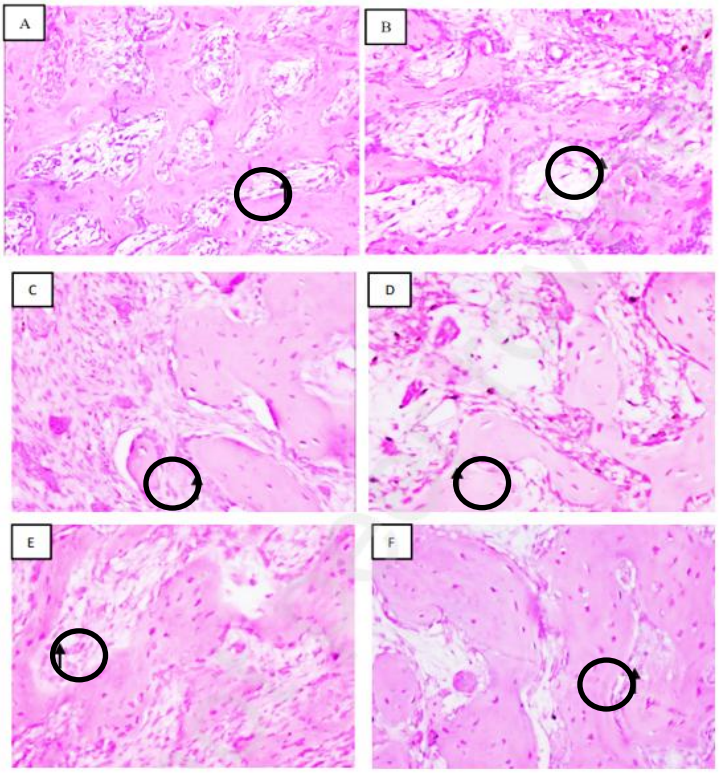

Figure 1. Histopathological features of *Cavia cobaya* osteoblast cells in the CBSF group and CC group: (A) CBSF group on day 7, (B) CC group on day 7; (C) CBSF group on day 14, (D) CC group on day 14; (E) CBSF group on day 21, (F) CC group on day 21 after tooth extraction.

An increase in osteoblast numbers accelerates bone repair and formation. The highest osteoblast expression was observed in the CBSF group on day 21 (104.13 ± 5.33), while the lowest was in the CC group on day 7 (39.80 ± 5.43) (Figure 1). Osteoblast expression was higher in the CBSF groups than in the CC group. These results demonstrate that chitosan derived from BSF pupae significantly increases osteoblast numbers on days 7, 14, and 21 compared to the control group without BSF pupae chitosan gel.

Osteoblast cell counts were analyzed for normality using the Shapiro-Wilk test and for homogeneity of variance using Levene's test. The normality test results showed P > 0.05 for all groups, indicating normally distributed data. The Levene test yielded a p-value of 0.027 (<0.05), indicating non-homogeneous variance.

Table 2. Normality test using the Shapiro-Wilk test

Data	Shapiro-Wilk Test	
	Sig	Result
CBSF on the 7th days	0.100	Normal
CBSF on the 14th days	0.780	Normal
CBSF on the 21st days	0.324	Normal
CC on the 7th days	0.570	Normal
CC on the 14th days	0.174	Normal
CC on the 21st days	0.363	Normal

Table 3. Levene's homogeneity test

Data	Levene's Test	
	Sig	Result
Osteoblast	0.027	Not Homogeneous

Given the normal distribution and non-homogeneous variance, the data were analyzed using a parametric one-way ANOVA test at a 95% confidence level. The ANOVA test revealed significant differences between the Black Soldier Fly Chitosan groups on days 7, 14, and 21 and the control groups on days 7, 14, and 21 (p = 0.000, p < 0.05).

Table 4. Post Hoc Games Howell Test

Group	CBSF 7th days	CBSF 14th days	CBSF 21st days	CC 7th days	CC 14th days	CC 21st days
CBSF 7th days		0.000	0.006	0.177	0.024	0.000
CBSF 14th days			0.177	0.011	0.000	0.037
CBSF 21st days				0.001	0.014	0.047
CC 7th days					0.066	0.009
CC 14th days						0.003
CC 21st days						

Table 5. Average amount of osteoclast in the CBSF group and CC group at observed on the 7th, 14th and 21st days.

Group	Number of Each	Mean ± Standart Deviation of Osteoclast Cell Number		
		Day 7	Day 14	Day 21
CBSF	3	4.86±1.51	9±0,34	2.66±0.11
CC	3	9.83±0.35	12.80±0.72	2.46±0.11

Histopathological examination revealed the presence of osteoclasts on days 7, 14, and 21, as shown below:

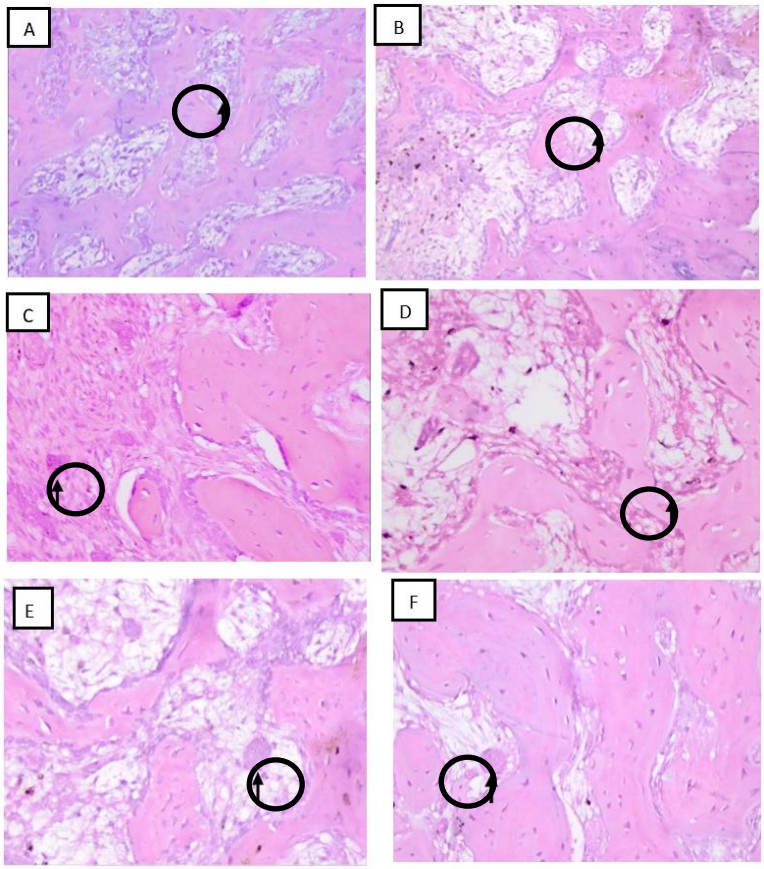

Figure 2. Histopathological features of *Cavia cobaya* osteoclast cells in the CBSF group and CC group: (A) CBSF group on day 7, (B) CC group on day 7; (C) CBSF group on day 14, (D) CC group on day 14; (E) CBSF group on day 21, (F) CC group on day 21 after tooth extraction.

The mean ± standard deviation (SD) of osteoclast counts in the CBSF and CC groups on days 7, 14, and 21 are presented in Table 3. On day 7, the CBSF group (4.8) showed a greater decrease in osteoclasts compared to the control group (9.8). On day 14, the CBSF group experienced an increase in osteoclasts (12.8) compared to the control group (9). On day 21, there was a decrease in the number of osteoclasts in the CBSF group (2.66) compared to the control group (2.46).

The results of the normality test for the CC group on day 7 (P=0.253), day 14 (p = 0,000), and day 21 (P=0,000), and for the CBSF group on day 7 (P=0.843), day 14 (P=0.537), and day 21 (P=0,000) indicated that some groups had normally distributed data ($p > 0.05$), while others did not ($p < 0.05$). Since the data did not meet the assumptions for a parametric one-way ANOVA test, the non-parametric Kruskal-Wallis test was used to determine significant differences between groups.

The Kruskal-Wallis test for the Black Soldier Fly Chitosan application yielded a significance value of p=0.006 ($p<0.05$), indicating significant differences between groups. These results support the hypothesis that the application of chitosan BSF pupae gel reduces osteoclast counts on days 7, 14, and 21 following tooth extraction in *Cavia cobaya.*

Table 6. Kruskal Wallis Test Results

Kruskal Wallis Test		
Data	**df**	**Sig**
Osteoclast	5	0.006

DISCUSSION

The research results indicate that the CBSF group and the CC group had an impact on the number of osteoblast and osteoclast cells on days 7, 14, and 21 in the tooth extraction socket of guinea pigs (*Cavia cobaya*). This effect was demonstrated by an increase in the number of osteoblast cells in the CBSF group and the CC group on days 7, 14, and 21. Meanwhile, the number of osteoclast cells in both the CBSF group and the CC group showed an increase on days 7 and 14, followed by a decrease on day 21.

Theoretically, it is expected that a socket or alveolar bone defect may develop following tooth extraction. The healing process of an injury starts as soon as it happens. [6,7] Tooth extraction causes tissue injury and bleeding from the socket. Hemostasis is the process by which the body acts to stop bleeding after it has started. Within the first 24 hours following the injury, a process known as hemostasis is carried out with the goal of preventing blood from continuously leaking out of the wound region.[10,25]

The analysis of these results from table 1 and figure 1 suggests that chitosan has already begun in this phase. When applied to the socket, chitosan containing amino groups, namely glycosaminoglycans (GAGs), will react with the cell surface. Chitosan binds to the cell surface through electrostatic bonds.[3]The percentage of amino groups in chitosan is commonly expressed as the degree of deacetylation (DD). The biocompatibility of chitosan is also influenced by the degree of deacetylation. Higher DD, for instance, enhances the contact and positive charge of chitosan with cells, leading to better biocompatibility.[3,18–20] With a deacetylation degree of 80%, this research's chitosan may be classified as having a high deacetylation level.

The degree of deacetylation of chitosan influences how well the coagulation process starts, with a higher degree of deacetylation leading to a more efficient coagulation process. However, the quantity of protonated amine groups is the main component influencing this ability. Chitosan is a naturally occurring cationic alkaline polysaccharide that adds NH3+ (positive charge) to its chain. Electrostatic contact is made possible by this positive charge with anions on red blood cell surfaces, causing strong aggregation at the location of the wound. This grouping quickly creates a blood clot, which stops the bleeding.[3]

Based on Figure and Table 1, the results of the study show that on day 7 post-tooth extraction, osteoblasts began to appear, with an average osteoblast count of 52.20 in the CBSF group and 39.80 in the CC group. This is consistent with the research by Sa'diyah et al, which states that on day 7 post-tooth extraction, there is still proliferation of newly differentiated osteoblast cells, and the formation of immature (woven bone) starts from the socket edges and spreads towards the center of the socket, moving toward the trabecular bone. These osteoblasts assist in the mineralization of the soft callus by secreting type I collagen matrix, ALP, and osteocalcin, which play a role in matrix maturation. The expression of ALP enzyme serves as an early marker of differentiation and mineralization during bone healing and also indicates the activity of osteoblasts, which will eventually form immature (woven) bone.[26-29]

The healing process continues after hemostasis is achieved, specifically with the final inflammatory phase that focuses on the elimination of pathogens. This phase begins 0–7 days after the injury. The inflammatory reaction causes blood vessels to become more permeable, which allows neutrophils and monocytes to move into the surrounding tissue.[30] Pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, are secreted by neutrophils, and they also release proteases to break down the extracellular matrix that remains.[3,17] Neutrophils will either die or be phagocytosed by macrophages after completing their phagocytosis task.[25]

The results of this study show that the number of osteoclasts on day 7 post-tooth extraction began to appear, with the number of osteoclasts observed on day 7 being 4.8 in the CBSF group and 9.83 in the CC group. This study is also supported by Khilar RC et al, who found that on day 7 post-tooth extraction, there was an increase in osteoclasts due to the elevation of certain cytokines caused by pro-inflammatory mechanisms such as IL-1, IL-6, and TNF-α. These cytokines trigger the expression of key mediators for osteoclast formation, which are expressed by osteoblasts binding to their receptor RANK. This binding between RANKL and RANK stimulates osteoclast differentiation.[31]

The analysis of the figure 1 and table 1 demonstrates that the increase in the average number of osteoblasts on day 14, with the comparison between the CBSF group showing an average of 91.53 and the CC group showing an average of 61.13, is in line with the research conducted by Chandra K et al. They stated that osteoblasts increase on day 14 due to the active osteoblasts entering the proliferative phase to repair the damaged bone by synthesizing type I collagen. This phase is also characterized by the activity of osteoblasts in transforming woven bone into lamellar bone.[32]

In contrast to osteoblasts, on day 14 post-tooth extraction, the average number of osteoclasts increased compared to day 7. This increase is attributed to the elevated levels of pro-inflammatory cytokines IL-1, IL-6, and TNF-α, which result in a higher number of osteoclasts and lead to more active bone resorption compared to normal conditions. This finding is supported by research conducted by Vieira et al, which states that, under normal conditions, the number of osteoclasts after tooth extraction increases during the first and second weeks, with day 14 being the peak of alveolar bone resorption carried out by osteoclasts.[29,33]

The goal of the remodeling phase is to optimize the structural integrity and strength of the newly formed wound filler tissue, epithelial growth, and scar formation. This phase lasts from the first day to about one year.[25] The tissue that will eventually be replaced by bone develops during this phase. Pluripotent mesenchymal cells will start to become fibroblasts, chondroblasts, and osteoblasts when tissue is reabsorbed. OPG will be stimulated by fibroblast tissue to block RANKL binding to RANK and initiate FGF2 growth. Additionally, OPG will express Runx2, a transcription factor that is the earliest marker of osteoblasts. Runx2 expression is modulated to aid in the production of osteoblasts by affecting both osteoblast cells and mesenchymal cells (MSCs). [12,34]

From the research results observed in the figure 1 and table 1, it was found that the CBSF group on day 21 showed an average osteoblast count of 104.13,

followed by the CC group with an average count of 82.60. The increase in osteoblasts on day 21 is due to osteoblasts becoming trapped within the bone trabeculae and transforming into osteocytes. This is consistent with the research by Sa'diyah et al, which states that when osteoblasts are trapped in the bone trabeculae, calcium salt deposition begins, starting with the formation of small islands or spicules, which later form osteons. When the osteoid is formed, some osteoblasts trapped within it are referred to as osteocytes.[28]

Based on the findings presented in the figure 2 and table 5, it was discovered that on day 21 post-tooth extraction, osteoclasts began to decrease, with the average osteoclast count in the CBSF group being 2.66 and the CC group showing a count of 2.46. This is consistent with the research conducted by Kurniawatik A et al, which found that the number of osteoclasts decreases on day 21. After tooth extraction, the bone begins to harden into trabecular bone, and at this stage, osteoblasts are trapped within the bone trabeculae, leading to a reduction in osteoclast activity compared to the previous days.[29,35]

The limitation of this study is that it does not provide information regarding the bone remodeling process beyond day 21, whereas the remodeling process could potentially continue for extended periods, such as 28 days, one month, or even longer.

CONCLUSION

The application of chitosan BSF pupae gel increases osteoblast numbers and decreases osteoclasts after tooth extraction, potentially accelerating bone formation and benefitting bone regeneration. The implications of this research suggest that innovative materials for bone growth could potentially offer significant advancements in enhancing bone healing and regeneration, opening up new possibilities for treating bone-related disorders, and could therefore be used as materials for bone remodeling in the future.

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Informed Consent Statement: Not applicable" for studies not involving humans

Data Availability Statement: The results reported support data can be found, including links to a data collection that is publicly archived that is analyzed or produced during the study.

Conflicts of Interest: The authors declare no conflict of interest to report.

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