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Efficacy of Green Mussel Shell-Derived Hydroxyapatite as a Bone Graft Substitute in Rabbit Femoral Defects: An In Vivo Study

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A B S T R A C T

Bone grafting is a standard procedure in fracture management to enhance bone healing using osteogenic, osteoinductive, and osteoconductive materials. Bovine hydroxyapatite (HA) is a commonly used bone substitute, but its production can be expensive. Green mussel shells have emerged as a potential alternative source of HA due to their abundance and lower cost, particularly in Indonesia. This study aimed to evaluate the efficacy of HA derived from green mussel shells on bone healing in rabbit femoral defects through radiological examination. A post-test only experimental study was conducted on male New Zealand white rabbits (n=36) with surgically created femoral defects. The rabbits were randomly divided into three groups: a control group (K) with no treatment, a group (P1) treated with bovine HA, and a group (P2) treated with HA derived from green mussel shells. Bone healing was assessed radiologically at weeks 2, 4, and 6 post-surgery using the Radiographic Union Score for Tibial Fractures (RUST) score on femoral X-rays. Statistical analysis was performed to compare the RUST scores between the groups and across the different time periods, with a p-value of <0.05 considered significant. Significant differences in RUST scores were observed between the groups at week 2 (K: 1.33 ± 0.50, P1: 2.67 ± 0.50, P2: 2.78 ± 0.44; p<0.001) and week 4 (K: 2.22 ± 0.44, P1: 3.11 ± 0.33, P2: 3.33 ± 0.50; p=0.003). However, no significant difference was found between the P1 (bovine HA) and P2 (green mussel shell HA) groups at these time points (p>0.05). At week 6, no significant differences in RUST scores were detected among all three groups (p=0.087). In conclusion, the findings of this study suggest that the efficacy of HA derived from green mussel shells in promoting bone healing in rabbit femoral defects is comparable to that of bovine HA, as assessed by the RUST score. This highlights the potential of green mussel shells as a cost-effective and readily available alternative source for HA in bone graft applications.

1. Introduction

Fractures, which are characterized by disruptions in the structural continuity of bone tissue, are a widespread health issue with substantial repercussions for both individuals and society. These injuries can significantly diminish an individual's quality of life, curtailing productivity and impeding the capacity to execute routine daily tasks, thereby imposing considerable burdens on those affected and their social environment. The healing process of bone following a fracture necessitates a complex interplay between biological mechanisms and biomechanical stability. Achieving this equilibrium can be particularly challenging in instances involving significant loss of bone tissue, a factor that can elevate the likelihood of delayed or non-union fractures. To mitigate the complexities inherent in bone healing, particularly in cases marked by extensive bone loss, the application of bone grafts has become a fundamental component of clinical practice. Bone grafts are biomaterials designed to foster the body's innate bone repair mechanisms through their osteogenic, osteoinductive, and osteoconductive properties. Autografts, which involve the transplantation of bone from one anatomical site to another within the same individual, are widely acknowledged as the preferred method for addressing bone defects, primarily due to their intrinsic osteogenic, osteoinductive, and osteoconductive attributes. Despite their efficacy, the utilization of autografts is associated with certain limitations. The procedure necessitates an additional surgical intervention to harvest the graft material, which can result in donor site morbidity, encompassing pain, the potential for infection, and a protracted recovery period. Furthermore, the quantity of bone tissue available for harvest is often limited, which may prove insufficient for the repair of larger bone defects.[1-3]

In light of the constraints associated with autografts, the exploration and development of synthetic materials capable of serving as effective bone substitutes have gained increasing importance, with the aim of achieving comparable or superior efficacy in promoting bone healing. Hydroxyapatite (HA), a naturally occurring calcium phosphate mineral that constitutes the primary inorganic component of bone, has emerged as a promising biomaterial in the realm of bone grafts. The inherent osteoconductive properties of HA render it a desirable material for utilization as a bone substitute, as it provides a structural matrix that facilitates the attachment, proliferation, and differentiation of cells responsible for new bone formation. Traditionally, HA employed in bone grafts has been derived from bovine sources (bovine HA). However, the production of bovine HA can be a complex and relatively expensive endeavor, necessitating rigorous quality control and safety protocols to minimize the potential risk of disease transmission. Consequently, there has been a substantial focus on the identification of alternative materials that offer enhanced cost-effectiveness and greater availability for the synthesis of HA. Previous research has explored the potential of utilizing green mussel shells as a viable alternative source for HA synthesis. In Indonesia, the cultivation of green mussels (Perna viridis) yields substantial harvests, estimated to be between 200 and 300 tons per hectare annually. Notably, the shell constitutes approximately seventy percent of the total weight of the green mussel, resulting in a considerable volume of shell waste, estimated at 140 to 210 tons per hectare annually within Indonesia alone. This abundance of green mussel shells represents a compelling opportunity to transform this waste material into a valuable resource for biomedical applications, including the synthesis of HA for bone grafts.[4-6]

Prior investigations have suggested that HA derived from green mussel shells demonstrates biochemical efficacy comparable to that of bovine HA, as evidenced by increased levels of osteocalcin, a key marker of bone formation, and an elevated number of osteoblast cells, which are instrumental in the synthesis of new bone tissue. Furthermore, the particle size of HA derived from green mussel shells has been reported to be smaller than that of bovine HA. This smaller particle size could potentially confer a larger surface area, thereby enhancing osteoinductive activity and positioning it as a promising alternative for HA synthesis. The evaluation of bone healing success in response to a bone graft necessitates the employment of appropriate assessment methodologies. Radiological examination plays a pivotal role in visualizing the condition of the bone defect and monitoring the progression of healing over time. Several factors are typically considered when analyzing fracture healing progress on radiographs, including the number of consolidated cortices, the presence and extent of bone callus formation, and the persistence or resolution of the fracture line. To facilitate a more standardized and reliable evaluation of fracture healing based on radiographic findings, the Radiographic Union Score for Tibial Fractures (RUST) was developed. The RUST score is a fracture assessment tool that has demonstrated favorable intra-observer and inter-observer agreement, underscoring its reliability and reproducibility in both clinical practice and research settings. This scoring system has proven effective in differentiating between varying degrees of fracture union. Study reported that the RUST score can be utilized to evaluate the degree and duration of tibial fracture healing and to identify instances of delayed union. Similarly, another study observed that lower RUST scores are associated with an elevated risk of nonunion, and that the results correlate with clinical outcomes, weight-bearing status, and functional recovery.[7-10] Given the promising preclinical findings concerning the efficacy of HA derived from green mussel shells and the utility of the RUST score in assessing bone healing, the present study was designed to investigate the effectiveness of green mussel shell-derived HA as a bone graft material in critical-sized femoral defects within a rabbit model.

2. Methods

This study employed a laboratory experimental research design utilizing a post-test only control group. The research was conducted in strict adherence to institutional guidelines governing animal treatment. Approval for the study was granted by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Diponegoro, Semarang, Indonesia. Furthermore, the study protocol was designed to be fully compliant with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. To ensure the creation of a standardized bone defect, a predetermined quantity of 300 milligrams of hydroxyapatite (HA) was deemed sufficient. This amount was calculated to be appropriate for implanting a tubular defect with specific dimensions: 5 mm in diameter and 5 mm in depth.

The animal model and the detailed surgical procedures implemented in this study were consistent with those described in our previous research endeavors. A total of 36 male New Zealand white rabbits (Oryctolagus cuniculus) were included in the study. The rabbits' ages ranged from 6 to 12 weeks, and their weight varied between 2.5 and 3 kilograms. The rabbits were randomly assigned to one of three experimental groups. Assessments were conducted at three distinct time periods following the surgical procedure: weeks 2, 4, and 6. This resulted in a total of nine subgroups, with each subgroup consisting of three rabbits. For the surgical procedure, each rabbit was anesthetized according to standard veterinary protocols. A standardized bone defect, measuring 5 mm in diameter and 5 mm in depth, was created on the lateral aspect of the distal femoral metaphysis of each rabbit using a surgical drill. Following the creation of the bone defect, the rabbits in the control group (K) did not receive any further treatment. In the first treatment group (P1), the bone defects were filled with commercially available bovine HA. In the second treatment group (P2), the bone defects were filled with HA derived from green mussel shells. The green mussel shell-derived HA was prepared in our laboratory using established protocols. Postoperative care was administered to all rabbits in accordance with standard veterinary practices. Any rabbit that died during or after the surgical procedure, regardless of the cause, was excluded from the study. This exclusion criterion was implemented to maintain the integrity of the final data analysis.

The primary outcome measure for this study was the evaluation of bone healing. This evaluation was conducted using the Radiographic Union Score for Tibial Fractures (RUST) scoring system. The RUST score, originally developed for tibial fractures, was adapted for use in assessing the healing of the surgically created femoral defects in this study. At each of the pre-determined evaluation periods (weeks 2, 4, and 6 post-surgery), antero-posterior (AP) and lateral radiographs of the femur were obtained for each rabbit. The RUST scoring system assigns a score to each of the four cortices of the bone. These scores are based on the extent of healing observed on the radiographs. A cortex receives a score of 1 if there is no callus formation and a visible fracture line is present. A cortex receives a score of 2 if a bridging callus is present, along with a visible fracture line. A cortex receives a score of 3 if there is callus formation without a visible fracture line. The scores for each of the four cortices are then summed to obtain a total RUST score for each set of radiographs. Higher total RUST scores indicate a greater degree of bone healing.

To minimize bias in the evaluation of the RUST scores, an experienced radiologist performed the evaluation. The radiologist was blinded to the study protocols and the randomization of the subjects to the different treatment groups.

Statistical analysis of the collected data was performed using SPSS version 25 software for Windows 10. Prior to conducting parametric statistical tests, the Shapiro-Wilk test was used to assess the distribution of the data for normality. For data that were determined to be normally distributed, a paired t-test was used. In cases where the data did not follow a normal distribution, the non-parametric Wilcoxon matched pairs test was applied for within-group comparisons over time. A one-way ANOVA test was used to compare the differences between the three experimental groups during each specific week of evaluation for normally distributed data. Following the ANOVA test, the post hoc Least Significant Difference (LSD) test was used to identify which specific groups differed significantly from each other. If the data were not normally distributed, the non-parametric Kruskal-Wallis test was utilized to assess overall differences between the groups at each time point. The Mann-Whitney test was subsequently used for pairwise comparisons between the groups. In all statistical analyses, a p-value of less than 0.05 was considered to indicate a statistically significant difference.

3. Results and Discussion

Table 1 presents the Radiographic Union Score for Tibial Fractures (RUST) data, comparing bone healing across three groups (K, P1, and P2) at three different time points (Week 2, Week 4, and Week 6). At Week 2, the control group (K) showed a mean RUST score of 1.33 with a standard deviation of 0.50. Groups P1 and P2, treated with bovine hydroxyapatite and green mussel shell-derived hydroxyapatite, respectively, exhibit higher mean RUST scores: 2.67 (SD 0.50) for P1 and 2.78 (SD 0.44) for P2. The p-value for this time point is less than 0.001, indicating a statistically significant difference between the groups. At Week 4, the mean RUST score for group K was 2.22 (SD 0.44). Groups P1 and P2 continue to demonstrate higher scores, with P1 at 3.11 (SD 0.33) and P2 at 3.33 (SD 0.50). The p-value at this time point is 0.003, also indicating a statistically significant difference. At Week 6, the mean RUST scores for all three groups are higher, suggesting continued healing. Group K has a mean RUST score of 3.33 (SD 0.50), while both P1 and P2 show a score of 3.78 (SD 0.44). However, the p-value at Week 6 is 0.087, which does not indicate a statistically significant difference between the groups at this later time point. The statistical analysis used was the Kruskal-Wallis test, as indicated by the footnote. Significant differences ($p < 0.05$) are noted at Week 2 and Week 4.

Table 1. Radiographic Union Score for Tibial Fractures (RUST) data.

RUST score	**Group**	**N**	**Mean ± SD**	**p**
Week 2	K	3	1.33 ± 0.50	<0.001*‡
	P1	3	2.67 ± 0.50	
	P2	3	2.78 ± 0.44	
Week 4	K	3	2.22 ± 0.44	0.003*‡
	P1	3	3.11 ± 0.33	
	P2	3	3.33 ± 0.50	
Week 6	K	3	3.33 ± 0.50	0.087‡
	P1	3	3.78 ± 0.44	
	P2	3	3.78 ± 0.44	

Notes: * significant differences (p<0.05); ‡ Kruskal-Wallis test.

Table 2 presents a comparison of the Radiographic Union Score for Tibial Fractures (RUST) between the different treatment groups. It shows the p-values resulting from statistical comparisons using the Mann-Whitney test at Week 2 and Week 4. At Week 2, the comparison between group K (control) and group P1 (bovine HA) yields a p-value of less than 0.001. Similarly, the comparison between group K and group P2 (green mussel shell HA) also results in a p-value of less than 0.001. These values indicate statistically significant differences between the control group and both treatment groups at Week 2. In contrast, the comparison between group P1 and group P2 at Week 2 results in a p-value of 0.730, showing no statistically significant difference between these two treatment groups. At Week 4, the comparison between group K and group P1 shows a p-value of 0.001, again indicating a statistically significant difference. The comparison between group K and group P2 at Week 4 shows a p-value of less than 0.001, also indicating a statistically significant difference. The comparison between group P1 and group P2 at Week 4 results in a p-value of 0.436, indicating no statistically significant difference between these two treatment groups at this time point.

Table 2. Comparison of RUST score in each group.

RUST score[a]		**P1**	**P2**
Week 2			
	K	<0,001	<0,001*
	P1	–	0,730
Week 4			
	K	0,001	<0,001*
	P1	–	0,436

Notes: * significant differences (p<0,05); [a]Mann-Whitney analysis.

In the evaluation of bone fracture healing, radiological imaging plays an indispensable role. Radiographs, or X-ray images, provide a non-invasive method to visualize the internal structure of bone, enabling clinicians and researchers to monitor the progression of healing over time. The analysis of these radiographic images involves a meticulous examination of several key indicators that collectively contribute to an understanding of the healing status of the bone. These indicators typically encompass the assessment of cortical consolidation, the evaluation of callus formation, and the analysis of the fracture line's presence or absence. Cortical bone, also known as compact bone, forms the dense outer layer of bones and provides significant structural support. In the context of fracture healing, the assessment of cortical consolidation is crucial. It involves evaluating the continuity and integrity of the bone cortex at the fracture site. A fracture disrupts this continuity, and the healing process aims to restore it. Radiographically, cortical consolidation is observed as the gradual disappearance of the fracture line and the bridging of the fracture gap by newly formed bone. The assessment includes determining the number of cortices that show evidence of healing, as this provides a measure of the extent of bone union. Callus is a specialized bone tissue that forms around a fracture site as part of the healing process. It acts as a bridge, stabilizing the fracture fragments and providing a framework for subsequent bone remodeling. Radiographically, callus appears as a region of increased density surrounding the bone at the fracture site. The evaluation of callus formation involves assessing its presence, extent, and maturity. The amount of callus, its shape, and its density on radiographs can provide insights into the stage of healing and the stability of the fracture. The fracture line is the radiolucent line that represents the discontinuity in the bone caused by the fracture. Monitoring the presence or absence of the fracture line on radiographs is a fundamental aspect of assessing fracture healing. In the early stages of healing, the fracture line is typically clearly visible. As healing progresses, the fracture line gradually becomes less distinct and eventually disappears as the bone fragments unite. The persistence of a visible fracture line over an extended period may indicate delayed healing or nonunion. While the observation and interpretation of individual radiographic features such as cortical consolidation, callus formation, and the fracture line provide valuable qualitative information about the healing process, the utilization of a standardized and reliable scoring system is of paramount importance for a more objective and quantitative assessment. Such systems offer several advantages. Firstly, they enhance objectivity by reducing subjective variability in the evaluation of radiographic images. Different observers may interpret the same radiographic image differently, leading to inconsistencies in assessment. A standardized scoring system provides clear criteria and guidelines for evaluating specific radiographic features, thereby minimizing inter-observer variability. Secondly, standardized scoring systems facilitate quantitative analysis and comparison of bone healing across different experimental groups or treatment conditions. By assigning numerical scores to different aspects of healing, these systems allow for statistical analysis and the determination of statistically significant differences in healing rates or outcomes. Thirdly, the use of standardized scoring systems improves the reproducibility of research findings. When studies employ a common scoring system, it becomes easier to compare the results of different studies and to synthesize the evidence in a meaningful way. The Radiographic Union Score for Tibial Fractures (RUST) was developed to address the need for a standardized and reliable tool for assessing fracture healing using radiographic images. While initially designed for evaluating tibial fractures, its principles and methodology can be adapted for the assessment of other long bones, as demonstrated in this study's application to femoral defects in rabbits. The RUST scoring system provides a relatively simple yet effective methodology for evaluating fracture healing. It achieves this by assigning scores to specific aspects of bone healing observed on radiographs. The core principle of the RUST system involves dividing the bone into four cortices and assigning a score to each cortex based on the degree of healing. The four cortices typically considered in long bones are the anterior, posterior, medial, and lateral cortices. For each of these cortices, a score is assigned based on predefined criteria that reflect the progression of healing. A cortex receives a score of 1 if there is no evidence of callus formation and the fracture line remains clearly visible. This indicates minimal healing at that particular cortex. A cortex receives a score of 2 if there is evidence of bridging callus formation, but the fracture line is still discernible. This suggests that some healing has occurred, but the fracture is not yet fully united. A cortex receives a score of 3 if there is callus formation and no visible fracture line. This indicates that the fracture has healed at that cortex. Once each of the four cortices has been assigned a score, these individual cortex scores are summed to obtain a total RUST score for the bone. This total score represents the overall degree of bone healing, with higher scores indicating a greater extent of union. An important aspect of any scoring system is its reliability and reproducibility. Reliability refers to the consistency of the measurements obtained with the scoring system. Reproducibility refers to the degree to which different observers obtain similar scores when evaluating the same radiographic images. The RUST scoring system has demonstrated good intra-observer and inter-observer agreement. Intra-observer agreement refers to the consistency of scores assigned by the same observer on different occasions. Inter-observer agreement refers to the consistency of scores assigned by different observers. The reported good agreement for the RUST system suggests that it provides a reliable and reproducible method for evaluating fracture healing. This is crucial for ensuring the validity of research findings and for facilitating comparisons across different studies and clinical settings. The RUST scoring system has found utility in both clinical practice and research investigations. In clinical settings, it can aid in the assessment of fracture healing progress, guiding clinical decision-making regarding patient management and rehabilitation. It can also be used to identify cases of delayed union or nonunion, allowing for timely intervention. In research, the RUST score provides a standardized outcome measure for evaluating the efficacy of different treatments or interventions on fracture healing. It allows researchers to quantify the degree of bone healing and to compare healing rates across different experimental groups. Previous research has demonstrated the effectiveness of the RUST scoring system in differentiating between varying degrees of fracture union. Studies have shown that the RUST score can be used to assess the degree and duration of tibial fracture healing and to identify cases of delayed union. Furthermore, research has indicated that lower RUST scores are associated with a higher risk of nonunion, and that the RUST scores correlate with clinical outcomes, weight-bearing status, and functional recovery. These findings highlight the clinical relevance and prognostic value of the RUST scoring system.[11-13]

The findings derived from this in-vivo study present robust evidence supporting the notion that the application of hydroxyapatite (HA), irrespective of its origin—whether sourced from bovine materials or green mussel shells—exerts a demonstrably positive influence on the process of bone healing within the experimentally induced rabbit femoral defects. This enhancement of bone healing is particularly evident when contrasted with the outcomes observed in the control group, which, notably, did not undergo any treatment intervention aimed at promoting bone repair. The assertion of HA's beneficial impact on bone healing is substantiated by the significantly elevated Radiographic Union Score for Tibial Fractures (RUST) scores that were consistently recorded in both the group treated with bovine HA (designated as P1) and the group treated with HA derived from green mussel shells (designated as P2). This pattern of higher RUST scores in the treatment groups was observed at the earlier time points of assessment, specifically at weeks 2 and 4 post-surgery, when compared to the scores recorded for the control group (K). This observed superiority in bone healing, as evidenced by the higher RUST scores in the treatment groups, strongly implies that both types of hydroxyapatite, whether of bovine or green mussel shell origin, possess a substantial degree of osteoconductive potential. Osteoconductivity, in the context of bone graft substitutes, refers to the inherent ability of a material to function as a structural scaffold. This scaffold facilitates the crucial process of new bone growth. In essence, an osteoconductive material provides a framework or template that supports the attachment, migration, proliferation, and subsequent differentiation of osteogenic cells. Osteogenic cells are those cells responsible for the formation of new bone tissue. The enhanced RUST scores observed in the treatment groups, P1 and P2, serve as a compelling indicator that both bovine HA and green mussel shell-derived HA effectively fulfill this osteoconductive role. By providing a suitable substrate for cellular activity, these HA materials create a microenvironment that is conducive to bone regeneration. This, in turn, fosters the overall healing process within the femoral defects that were surgically created in the rabbit model. The osteoconductive property of HA is crucial because it allows the material to integrate with the host bone tissue, promoting the ingrowth of blood vessels and cells necessary for bone repair. The significance of osteoconductivity in bone graft substitutes cannot be overstated. It is a fundamental requirement for any material intended to aid in bone regeneration. Without adequate osteoconductivity, a bone graft material may fail to effectively support the complex cellular and molecular events that are essential for successful bone healing. The findings of this study highlight that both bovine HA and green mussel shell-derived HA exhibit this critical property, making them potentially valuable tools in the treatment of bone defects. Moreover, the fact that both HA sources led to significantly improved bone healing compared to the control group underscores the importance of using bone graft substitutes in situations where the body's natural healing capacity may be insufficient. In cases of large bone defects, fractures with significant bone loss, or situations where there are other factors impairing bone healing, the application of a material with osteoconductive properties can be essential for achieving successful outcomes. It is also important to consider the implications of these findings in the context of different clinical scenarios. While this study focused on a rabbit model with surgically created femoral defects, the principles of osteoconductivity and the potential benefits of HA bone grafts are applicable to a wide range of situations in which bone regeneration is needed. This includes the treatment of traumatic fractures, the repair of bone defects resulting from surgery or disease, and the augmentation of bone in dental and craniofacial applications.[14,15]

A particularly noteworthy observation that arises from the analysis of this study's results is the absence of any statistically significant difference in the Radiographic Union Score for Tibial Fractures (RUST) scores. This lack of significant difference was consistently observed between the experimental group that received treatment with bovine hydroxyapatite (HA), designated as P1, and the experimental group that received treatment with HA derived from green mussel shells, designated as P2. This equivalence was maintained across all time points at which assessments of bone healing were conducted, including the evaluations performed at weeks 2, 4, and 6 post-surgery. This specific finding carries substantial implications for the potential application of green mussel shell-derived HA as a bone graft substitute. The fact that the RUST scores did not significantly differ between the bovine HA group and the green mussel shell HA group suggests that, within the specific parameters of this rabbit femoral defect model and based on the radiological evaluation employing the RUST scoring system, the efficacy of HA derived from green mussel shells in promoting bone healing is comparable to that of commercially available bovine HA. In simpler terms, the green mussel shell HA performed just as well as the conventional bovine HA in aiding bone repair in the context of this experiment. This observed equivalence in efficacy is of considerable importance for several reasons. Primarily, it opens up the possibility of utilizing a more sustainable and potentially cost-effective alternative to bovine HA in bone grafting procedures. While bovine HA has been a commonly used bone substitute, its production can be associated with certain drawbacks, including complexities and costs related to sourcing, processing, and safety considerations. The demonstration that green mussel shell-derived HA exhibits comparable efficacy to bovine HA alleviates concerns about compromising the quality of bone healing when considering a switch to this alternative source. When evaluating the potential of any biomaterial for medical applications, efficacy is paramount. The primary goal is to achieve the desired therapeutic outcome, in this case, the successful regeneration and healing of bone tissue. The finding that green mussel shell-derived HA performs equivalently to bovine HA in promoting bone healing, as assessed by the RUST scoring system, confirms that it meets this crucial criterion. This confirmation of efficacy is essential before considering any secondary benefits, such as cost-effectiveness or sustainability. However, the importance of this equivalence extends beyond just efficacy. It also brings into focus the potential advantages associated with the utilization of green mussel shells as a source for HA production. These advantages present a compelling case for further exploration and potential adoption of this alternative HA source. One of the most significant advantages lies in the potential for lower production costs. The process of producing bovine HA can be complex and involve multiple steps, including sourcing, processing, and purification, which can contribute to the overall cost. In contrast, the production of HA from green mussel shells may offer a more streamlined and less expensive route. This potential for cost reduction is particularly relevant in the context of healthcare economics, where efforts are continuously being made to reduce the costs of medical procedures and treatments without compromising quality. Another key advantage is the abundant availability of green mussel shells, particularly in certain geographical regions. In countries with a thriving aquaculture industry focused on green mussel cultivation, such as Indonesia, the availability of green mussel shells as a raw material is substantial. This readily available supply offers a reliable and sustainable source for HA production, reducing dependence on potentially limited or more expensive sources. The abundance of this resource also implies a potential for large-scale production, which is essential to meet the demands of clinical applications. In Indonesia, for example, the cultivation of green mussels (Perna viridis) yields substantial harvests, estimated to be between 200 and 300 tons per hectare annually. Notably, the shell constitutes approximately seventy percent of the total weight of the green mussel, resulting in a considerable volume of shell waste, estimated at 140 to 210 tons per hectare annually within Indonesia alone. This large volume of shell waste presents a significant opportunity to transform a byproduct into a valuable resource. The ability to utilize green mussel shell waste for HA production not only presents an economically viable alternative to the potentially more complex and expensive process of producing bovine HA but also contributes significantly to the principles of environmental sustainability. This is a crucial consideration in today's world, where environmental concerns are growing. By repurposing a material that would otherwise be considered waste, the production of green mussel shell-derived HA helps to reduce environmental burden and promotes resource efficiency. Instead of discarding green mussel shells, which can contribute to pollution and disposal problems, they can be utilized as a valuable raw material for the synthesis of HA. This process aligns with the principles of a circular economy, where waste is minimized, and resources are used more efficiently. The conversion of waste into a useful biomaterial not only reduces environmental impact but also adds value to a product that would otherwise have none. Moreover, the use of a waste material like green mussel shells can reduce the reliance on other resources that may have a greater environmental footprint. For example, the sourcing and processing of bovine bones for HA production can involve energy-intensive processes and may raise concerns about animal welfare and potential disease transmission. Utilizing green mussel shells can avoid these issues and provide a more sustainable alternative.[16,17]

The observation that no statistically significant difference in the Radiographic Union Score for Tibial Fractures (RUST) scores was detected among all three experimental groups—the control group (K), the bovine hydroxyapatite (HA) treated group (P1), and the green mussel shell HA-treated group (P2)—at the final assessment point, which was week 6 post-surgery, warrants further and more detailed consideration. This apparent convergence in RUST scores at this later stage of the healing process presents an interesting phenomenon that necessitates careful analysis and interpretation. Several potential factors could contribute to this finding, and understanding these factors is crucial for a comprehensive evaluation of the study's results. One possible explanation for this convergence in RUST scores across the groups is that, by this later stage in the healing process, the natural bone healing mechanisms in the control group may have progressed to such an extent that the degree of bone consolidation achieved was comparable to that observed in the treatment groups. In other words, even without the application of HA bone grafts, the inherent capacity of the rabbit's body to repair the surgically created femoral defects may have led to a level of healing by week 6 that was similar to the level of healing observed in the groups where HA bone grafts were used to augment the healing process. To fully appreciate this explanation, it is essential to consider the complex sequence of events that characterize the natural process of bone healing. Following a fracture or bone injury, the body initiates a cascade of biological responses aimed at restoring the structural integrity of the bone. This process involves a series of overlapping phases, including inflammation, soft callus formation, hard callus formation, and bone remodeling. In the early stages of healing, the inflammatory response plays a crucial role in initiating the repair process. Inflammatory cells migrate to the fracture site, removing debris and releasing signaling molecules that attract cells responsible for tissue repair. Subsequently, a soft callus, primarily composed of cartilage, forms at the fracture site, providing initial stability. Over time, this soft callus is replaced by a hard callus, which consists of woven bone. Finally, the woven bone is remodeled into lamellar bone, the mature and organized bone tissue that provides long-term structural support. In the context of this study, it is conceivable that, while the application of HA bone grafts in the treatment groups (P1 and P2) accelerated the earlier phases of bone healing, as evidenced by the higher RUST scores at weeks 2 and 4, the natural progression of healing in the control group (K) eventually caught up by week 6. The inherent healing capacity of the rabbits, driven by these natural biological processes, may have resulted in a degree of bone consolidation in the control group that was comparable to that achieved in the treatment groups by the final assessment time point. This interpretation highlights the importance of considering the time course of bone healing when evaluating the efficacy of bone graft substitutes. While these materials can undoubtedly play a significant role in enhancing and accelerating the healing process, the body's natural healing mechanisms should not be overlooked. In some cases, particularly in relatively small defects or in healthy individuals with good healing potential, the natural healing process may eventually lead to satisfactory outcomes, even without the use of bone grafts. Another potential factor that could contribute to the observation of no significant difference in RUST scores among all three groups at week 6 is that the RUST scoring system, while demonstrating effectiveness in evaluating the earlier phases of fracture healing, may exhibit a ceiling effect at later time points in the healing process. A "ceiling effect" in a measurement tool or scoring system refers to a situation where the instrument is no longer able to detect further improvements or changes beyond a certain point. In the context of bone healing assessment using the RUST score, it is possible that as bone consolidation progresses and the healing process advances, the RUST score becomes less sensitive in detecting more subtle differences in the degree of healing achieved. The RUST scoring system, as described earlier, assigns scores to each of the four cortices of the bone based on the presence and characteristics of callus formation and the visibility of the fracture line. While this system effectively differentiates between different stages of healing in the earlier phases, such as the presence of a visible fracture line with minimal callus formation versus the presence of bridging callus, it may become less discerning at later stages when the fracture line has largely disappeared, and substantial callus formation and bone consolidation have occurred. It is conceivable that by week 6, the majority of the defects in all groups, including the control group and the treatment groups, had reached a level of union that the RUST system could no longer effectively differentiate. At this stage, subtle variations in the quality or completeness of healing, such as differences in bone density, trabecular pattern, or the degree of cortical remodeling, may have existed between the groups, but these variations may not have been adequately captured by the RUST scoring system. If the RUST system indeed exhibits a ceiling effect at later time points, this could explain why no significant differences were observed between the groups at week 6, even if subtle differences in the healing process were still present. It is important to acknowledge that any measurement tool has its limitations, and the RUST system, while valuable for assessing fracture healing, may not be able to capture all aspects of the healing process with equal sensitivity at all time points. This potential limitation of the RUST scoring system has implications for the interpretation of the study's findings. While the RUST score provides a useful semi-quantitative measure of bone healing based on radiographic features, it is crucial to recognize that it may not provide a complete picture of the healing process, especially at later stages. Other assessment methods, such as histological analysis or biomechanical testing, may be needed to more comprehensively evaluate the quality and completeness of bone healing. In addition to the explanations related to the natural healing process and the potential limitations of the RUST scoring system, other factors could also contribute to the observed results. These factors may include individual variations in healing rates among the rabbits, subtle differences in the surgical procedure or postoperative care, or the specific characteristics of the bone defects created in this study. It is important to acknowledge that this study, like any research investigation, has certain limitations. While the rabbit model provides a valuable tool for studying bone healing, the results may not be directly generalizable to humans. Further research, including clinical studies in humans, is needed to confirm the findings and to evaluate the long-term efficacy of green mussel shell-derived HA in promoting bone healing.[18-20]

4. Conclusion

In conclusion, this study provides compelling evidence that hydroxyapatite (HA) derived from green mussel shells is a comparable alternative to bovine HA in promoting bone healing in rabbit femoral defects. The efficacy of green mussel shell-derived HA is supported by the observed improvements in radiographic healing, as assessed by RUST scores, which were statistically similar to those achieved with bovine HA. This equivalence in efficacy is particularly significant because it suggests that green mussel shells, an abundant and low-cost resource, especially in regions like Indonesia, can be effectively utilized for HA production. The use of this alternative source not only offers a potentially more sustainable and economically viable option for bone graft materials but also contributes to reducing waste and promoting resource efficiency. While the findings of this study are promising, it is important to acknowledge its limitations, including the use of an animal model. Further research, including clinical trials, will be necessary to fully validate the long-term efficacy and safety of green mussel shell-derived HA in human applications. However, the current evidence strongly supports the potential of this material as a valuable alternative in bone grafting procedures.

5. References

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