ORIGINAL ARTICLE

The Role of Crosslinked Collagen-Hydroxyapatite on the Properties of Tissue Graft Material

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ABSTRACT

Objective. This review article aims to determine the properties, uses, toxicity, and other side effects of crosslinking agents in tissue scaffolds when applied in vitro and in vivo.

Methods. A literature search was performed using the PubMed-NCBI (MEDLINE) database (https://pubmed.ncbi.nlm.nih.gov/) with keywords: *crosslinking reagent*, *collagen*, *hydroxyapatite*, and *bone regeneration*. GRADE criteria were used to assess the quality of evidence.

Results. A total of six articles were included in the study. Improved mechanical properties of collagen-hydroxyapatite scaffolds with high porosity can be achieved by employing crosslinking methods, including physical dehydrothermal (DHT) treatment, chemical treatment with glutaraldehyde (GA), Microbial Transglutaminase (mTGase), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC), or a combination of both DHT and EDAC. Furthermore, the crosslinking of EDAC and DHT can lead to forming ester bonds between activated carboxyl groups and hydroxyl groups.

Conclusion. The combination of DHT and EDAC crosslinking can increase mechanical strength, make the pore size appropriate, make the scaffold more stable, and support cell adhesion so that new cells can grow, and the process of osteogenesis can run more optimally.

Keywords: crosslinking reagent, collagen, hydroxyapatite, bone regeneration

INTRODUCTION

Cases of bone damage have increased significantly over the past few years worldwide and cases are expected to continue to increase over time.[1] Basically, bones can heal on their own if the damage is not extensive.[2] In cases of large bone defects, clinical intervention is required to promote the regeneration of tissue. The preferred method for repairing bone damage is through the utilization of autografts and allografts, recognized as the standard of excellence in promoting bone regeneration. The use of an autograft can result in new bone damage, while the use of an allograft can trigger disease transmission between donor and recipient.[3,4] The development of biomaterials is necessary to offer alternative solutions, creating materials with the ability to restore, heal, sustain, and enhance tissue function.[5] An optimal material for bone substitution should possess characteristics such as easy degradability, non-toxicity, biocompatibility, and the ability to promote the attachment, growth, and differentiation of cells. This, in turn, would stimulate the formation of new bone tissue, contributing to the process of bone repair.[6] The use of scaffold materials for bone tissue regeneration has been widely developed by researchers recently.

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Scaffolds can provide an optimal environment for bone tissue regeneration, these materials require mechanical properties, conducive architecture such as porosity, and good pore size to facilitate osteogenesis.[7] Materials that are being widely developed in the manufacture of scaffolds are collagen and hydroxyapatite (HA). The combination of these two materials in making scaffolds can optimize the properties of the material so that bone tissue regeneration will be optimal.[8] The fundamental components of the original bone include hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) and collagen fibrils, the combination of hydroxyapatite and collagen proves to be an optimal choice for materials used in the regeneration of bone tissue. Hydroxyapatite, constituting the primary inorganic component of the original bone, demonstrates remarkable biocompatibility with soft tissues. Meanwhile, collagen serves as the principal organic compound in the original bone, capable of initiating and stimulating the growth of apatite carbonate minerals within bone. Additionally, collagen stands as a key structural protein in the human body, providing strength and integrity to various tissues like skin, bones, tendons, and cartilage.[9,10] The incorporation of crosslinking materials plays a crucial role in advancing the creation of scaffolds for bone regeneration, enhancing mechanical properties through the establishment of robust bonds within a polymer matrix. The favorable mechanical characteristics of scaffolds composed of collagen and hydroxyapatite with high porosity can be improved by adding crosslink materials in the scaffold using crosslink materials physically or chemically and can also be a combination of physical and chemical.[7,11] The goal of this review is to discuss how different crosslinking agents work to make collagen-hydroxyapatite scaffolds better for bone regeneration.

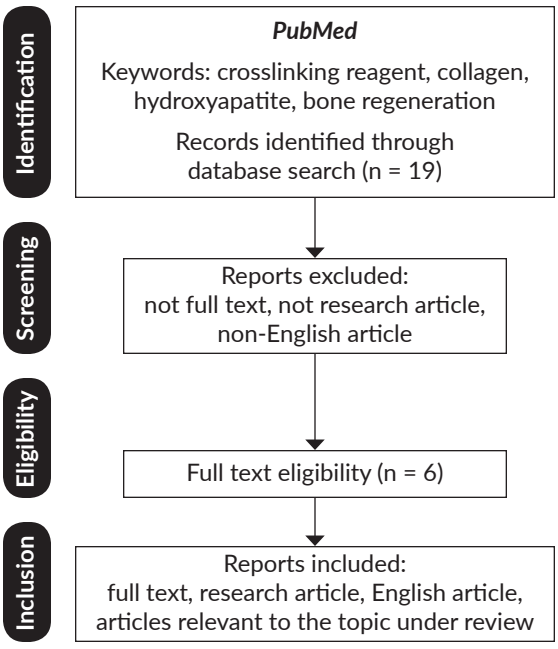

Figure 1. PRISMA diagram for literature search.

MATERIALS AND METHODS

A systematic review of available literature was performed using the PubMed-NCBI (MEDLINE) database (https://pubmed.ncbi.nlm.nih.gov/). The following search terms were used: *crosslinking reagent, collagen, hydroxyapatite,* and *bone regeneration*. Full text, research articles, written in English were included in the analysis. Electronic searches were performed between April to May 2024. Grading of Recommendations Assessment, Development and Evaluation (GRADE) approach was used to evaluate risk of bias. Data extraction was performed by a single reviewer due to limited resources. No independent verification or contact with study investigators was conducted, and no automation tools were used during the data collection process. This systematic review was exempted from ethics review by the Health Research Ethics Commission of University of Hang Tuah.

RESULTS

A total of 19 records were obtained from the search. After exclusion of non-full text, non-research/original article and non-English articles were removed; six articles were included in the review.

Several research articles in Table 1 describe the types and roles of crosslink types in the manufacture of collagen and hydroxyapatite grafts.

Potential Bias Grade

Four criteria are used in the following grading rubric to assess possible bias: (1) In Vivo Validation; (2) Experimental Rigor/Sample Size; (3) Limitations Disclosure; and (4) Cross-validation or Comparative Context. From A (lowest bias risk) to C (higher potential bias), grades are assigned (Table 2).

Based on the reporting that is available in each publication, these grades are interpretive and indicative. Lower grades (B, C) indicate areas where methodological or reporting clarity could be improved, while higher grades (A, A-) indicate a lower risk of bias.

DISCUSSION

Scaffolds play a crucial role in stimulating the growth and formation of new, more regular bone tissue by providing structural support and enhancing the healing process.[16,17] Similar to the natural extracellular matrix (ECM) of bone which facilitates cell adhesion where new cells grow, scaffolds must be made of materials with good biodegradation so that it can be metabolized by the body when new cells have begun to grow.[18] To aid regeneration, the ideal scaffold material should have the following properties:[19] **Biocompatibility** is a critical attribute that pertains to the potential inflammatory response or toxicity experienced by patients. Scaffold materials must possess non-toxic qualities to ensure patient safety and

Table 1. Overview of Included Studies

Author	Study design	Objectives	Variables studied	Crosslink method	Results
Ryan et al.[7]	Research articles	Evaluate 3 techniques for including HA in collagen scaffolds and its impact on mechanical characteristics, structural integrity, and cellular responsiveness	Examined the porosity, stiffness, and cell function of the fabricated CHA (micron-HA), CnHA (nano-HA), and CpHA (HA-coated) scaffolds	Physical (DHT) and chemical (EDAC) cross-linking.	• Although CpHA had poorer initial cell attachment, it had the best mechanical strength and calcium deposition. • This crosslinking maximizes the number of amide bonds formed between the collagen-free and free amine-free carboxyl groups, which are targeted by the DHT and EDAC cross-bonds. • While EDAC and DHT are cross-linked, an ester bond can also form between the active carboxyl group and the hydroxyl group. • The incorporation of hydroxyapatite (HA) into collagen-based scaffolding significantly affects its mechanical properties, porosity, pore size, and biological performance.
Zhang et al.[8]	Research articles	Utilizing a solvent-free dehydrothermal crosslinking technique, build a biocompatible, customizable collagen/HA scaffold	Assessed the mechanical and biological performance of freeze-dried collagen/HA composites both in vitro and in vivo after thermal crosslinking	Dehydrothermal	• Excellent biocompatibility without chemical agents, adjustable characteristics, and satisfactory bone repair in rabbits. • The dehydrothermal method used to prepare the composite improves its biocompatibility and simplifies the process, demonstrating strong potential for commercialization. • The method also ensures safety and effectiveness for in vivo applications by avoiding additional chemical reagents and allowing for customizable properties suitable for personalized medical care.
Teixeira et al.[12]	Research articles	Design HA scaffolds functionalized with collagen type I and evaluate their fit for use in bone tissue engineering	The researchers examined human bone marrow cells using macroporous scaffolds created by polymer replication, surface-coated with collagen, and crosslinked with EDC/NHS.	EDC/NHS	• Scaffolds promoted adhesion, proliferation, and ECM mineralization; cell clusters efficiently bridged pores. • Collagen cross-linking in hydroxyapatite scaffolds plays an important role in supporting cell adhesion, proliferation, and mineralization matrix formation. • Crosslinking with EDC/NHS methods has been noted to stabilize collagen-based materials and prevent rapid degradation, which is critical to maintaining the structural integrity of scaffolding during cell culture.
Itoh et al.[13]	Research articles	Make a bone-like HA/Coll mixture and test how it might be used for spinal fusion and fixing bone defects	Evaluated anterior spinal fusion and tibial defect models in dogs using either crosslinked or non-crosslinked HA/Coll implants, with or without rhBMP-2.	Glutaraldehyde	• Crosslinking increased mechanical strength; rhBMP-2 accelerated bone regeneration. Best results came from combining both. • Conducted through experiments in beagle dogs and tibial bone defects, this study showed that cross-linking improved mechanical strength and bioresorbability, while the addition of rhBMP-2 significantly improved bone remodeling and integration. The findings suggest that HA/Col composites, especially when combined with rhBMP-2, could be a viable alternative to current ceramic systems in orthopedic applications.
Kikuchi et al.[14]	Research articles	This study is to use glutaraldehyde (GA) crosslinking to regulate the mechanical characteristics and biodegradability of HA/Coll nanocomposites.	The researchers produced and evaluated GA crosslinked composites for strength, swelling, and biodegradation using rabbits.	Glutaraldehyde	• GA crosslinking delayed biodegradation and enhanced mechanical qualities, keeping osteoconductivity constant. • Cross-linking with glutaraldehyde (GA) successfully increases the mechanical strength of HAp/Col composites by forming inter- and intra-fibril cross-bonds between collagen fibrils. • Cross-linking reduces the rate of resorption in vivo without causing toxic reactions, such as inflammation.
Ciardelli[15]	Research articles	Using enzymatic crosslinking with microbial transglutaminase (mTGase), enhance the mechanical stability and biocompatibility of porous hydroxyapatite/collagen (HA/Coll) scaffolds.	Researchers crosslinked freeze-dried HA/Coll scaffolds using mTGase in different ratios and evaluated them in terms of mechanical, chemical, and biological aspects	Microbial Transglutaminase (mTGase)	• The enzymatic treatment helped MG63 and HUVEC cells stick better, stay alive longer, and develop properly; it also made the material stronger and more stable in water. • The HA/Coll sample exhibits a typical foam-like morphology with interconnected pores, and the pore walls increase in thickness with an increase in the amount of HA, giving the foam a more compact structure. • Higher ALP activity was detected in MG63/HUVEC cocultures compared to MG63 osteoblast-like cells, which may be associated with positive endothelial cell effects, especially for scaffolds with 30% and 50% HA. • The study also discovered that the swelling in the saturated atmosphere of cross-linked HA/Coll foam vapor was substantially less in each composition because it absorbed less water.

Table 2. Potential Bias Grade

Study	In vivo validation	Sample Rigor	Transparency	Cross-validation	Bias grade	Potential bias
Ciardelli et al.[15]	No	Moderate	High	Low	**B**	The lack of in vivo confirmation constrains the generalizability of the findings. Despite the methodology being thoroughly documented and the in vitro outcomes being favorable, the lack of genuine biological settings heightens the risk of overstating its efficacy
Itoh et al.[13]	Yes (dog models)	Moderate	Moderate	Moderate	**B+**	This work employed a robust animal model and incorporated pertinent biological parameters (rhBMP-2); nonetheless, minimal transparency and limited sample sizes provide a danger to reproducibility and generalizability to humans
Kikuchi et al.[14]	Yes (rabbits)	Limited	Moderate	Low	**B**	Potential bias stems from insufficient information concerning the long-term toxicity of glutaraldehyde and the limited scope of testing. Furthermore, diminished cross-validation among experimental groups weakened comparative robustness
Ryan et al.[7]	No	High	High	High	**B**	Despite being confined to in vitro experiments, the work demonstrated exceptional transparency, comparative methodology, and mechanical rigor, thus mitigating the potential for procedural bias
Teixeira et al.[12]	No	Moderate	High	Low	**B**	The in vitro emphasis and lack of mechanical testing under dynamic loads introduce a bias in forecasting in vivo performance. The explicit technique and utilization of human cells enhance trustworthiness
Zhang et al.[8]	Yes (rabbits)	High	High	Moderate	**A-**	This research exhibits substantial experimental rigor and incorporates animal testing, thereby mitigating potential bias. The preliminary development phases and continuous long-term safety assessments restrict the generalizability of the findings

compatibility. The scaffold should enable both cell adhesion and proliferation, with the morphology of its porous structure being a crucial factor in design. This morphology plays a key role in facilitating the supply of nutrients and essential body fluids to transplanted and regenerated cells. Scaffolds featuring interconnected pores can enhance the diffusion rates of nutrients and fluids, promoting vascularization for improved outcomes. Mechanical properties are equally vital, requiring sufficient strength and rigidity to support tissue growth until the newly formed tissue attains the strength needed to sustain itself. **Biodegradability** is a preferred property for many scaffold types, with careful attention paid to degradation characteristics that create space for new cell growth. The scaffold material should possess the ability to break down and diffuse in biological fluids. Moreover, chemical surfaces that are conducive to cell attachment, proliferation, and differentiation contribute to the success of scaffolds as prosthetic devices in biomedical applications. Cell adhesion assumes particular importance, triggering essential cellular functions such as dispersal, proliferation, migration, and biosynthetic activity.

Hydroxyapatite (HA), represented by the molecular formula $Ca_5(OH)(PO_4)_3$, is classified as a ceramic material that is bioactive, nontoxic, osteoinductive, and osteoconductive. HA is very important for bone scaffolds due to its resemblance to real bone minerals and teeth, forming chemical bonds directly through the formation of apatite layers with bone tissue and biologically through interface interactions. In medical applications, HA is widely used for bone grafting, bone repair, and bone replacement. HA can stimulate new bone growth. Hydroxyapatite is also directly bound to bone tissue chemically through the formation of an apatite layer.[20,21]

HA has good bioactive, biocompatibility, and bioresorption properties so it can be used for bone regeneration. Bioactive properties indicate that HA can bind to bone tissue and can provide a specific biological response. The nature of biocompatibility shows that HA can adjust to the body so that there is no immune rejection in the body. The nature of bioresorption suggests that HA has a porous structure through which bone cells grow.[22-24]

Collagen stands out as the predominant protein within the extracellular matrix, a collection of proteins characterized by a distinctive molecular structure, specifically a fibrillar arrangement. This structural organization plays a crucial role in preserving the biological and structural integrity of the ECM, providing essential physical support to various tissues. Collagen is abundantly present in bones, cartilage, tendons, ligaments, blood vessels, nerves, and skin, serving as the primary source of structural proteins for both hard and soft tissues.[25] Furthermore, collagen exhibits low immunogenicity and possesses a porous structure with excellent permeability. It demonstrates high biocompatibility and biodegradability. Additionally, collagen plays a pivotal role in regulating cell morphology, adhesion, migration, and differentiation. The benefits of this natural polymer position it as a promising biomaterial for use as a tissue scaffold. Nonetheless, collagen scaffolds face drawbacks such as low mechanical strength and limited structural stability, restricting their applications in certain tissues. To address these limitations, intermolecular

crosslinks in collagen scaffolds can be established through either physical or chemical methods, with the goal of enhancing mechanical strength and other properties. Moreover, a common approach involves combining collagen with other materials, including natural substances, synthetic polymers, and inorganic materials, to augment the mechanical strength of collagen scaffolds.[26]

Organic-inorganic composite materials have gained significant interest because of their ability to integrate the superior properties of each component. Hydroxyapatite (HA), a bioactive ceramic, is highly biocompatible and shares a similar chemical composition with natural bone tissue. Its strong osteoinductive properties make HA a widely used material in bone tissue engineering.[27,28]

Collagen and hydroxyapatite (HA) are the predominant proteins and key constituents of natural bone. These substances are frequently employed as composite or combination materials in tissue regeneration due to their biomimetic properties. Their utilization is based on the fact that collagen and HA possess outstanding biocompatibility and biodegradability, making them well-suited for applications in tissue engineering and regenerative medicine.[29] Original bone tissue comprises organic components, predominantly collagen, and inorganic crystalline mineral components like hydroxyapatite (HA). The organic elements contribute to flexibility, while the inorganic components impart strength and rigidity to the bone structure. Collagen-based scaffolds are extensively utilized in various applications due to the bioactivity of collagen, which triggers excellent biological performance.[30] These materials, while displaying high porosity and permeability, tend to have low mechanical properties and undergo rapid enzymatic biodegradation. This limitation restricts their use in applications where high mechanical strength is a crucial requirement.[31] Indeed, because of its favorable biological properties, collagen can be combined with hydroxyapatite (HA) to enhance mechanical properties and promote osseointegration in bones.[32]

Crosslinking is a crucial technique employed to optimize scaffolds for bone regeneration. This process involves creating strong bonds within the polymer matrix of the scaffold, thereby enhancing its biomechanical properties.[11] Certainly, the crosslinking technique involves creating bonds between molecules, strengthening hydrogel fibers and preventing the release of chains from one another under stress. This method is employed to induce interconnections between molecules, leading to improvements in various aspects such as mechanical properties, molecular weight, cellular activity, degrees of stability, and resistance to heat, light, and other physical properties.[33] There are two kinds of crosslink techniques, namely chemical crosslinking (the use of glutaraldehyde materials, carbodiimides group (ex: 1-ethyl 3-3 dimethyl aminopropyl carbodiimide (EDAC), N-hydroxysuccinimide (NHS), and genipin), and physical crosslinks that are not using cytotoxic agents (by irradiation using ultraviolet (UV) and dehydrothermal (DHT) light.[34]

Crosslink techniques chemically and physically have advantages and disadvantages. Excess crosslinks can chemically form very strong bonds, but they are cytotoxic, so post-crosslink residue removal is required. In addition, crosslinks are chemically more expensive than physical crosslinks. While crosslinks physically have the advantages of being safer than crosslinks chemically, toxicity and reaction to networks are low and relatively cheap. Physical disadvantages of crosslinks include weaker bond formation than chemical crosslinks, longer time to application, and less control of crosslink kinetic reactions.[35]

Scaffolds are designed to create an optimal environment for the regeneration of bone tissue necessitate a careful equilibrium between mechanical and architectural attributes, including high porosity and well-suited pore dimensions. Augmenting collagen scaffolds, which serve as analogs of the native extracellular matrix (ECM), with hydroxyapatite (HA) materials further improves their efficacy. This combination ensures the maintenance of adequate pore architecture, fostering effective cell infiltration, vascularization, and ultimately, bone regeneration.[7]

The relatively favorable mechanical properties of scaffolds with elevated porosity are achieved, in part, through crosslinking techniques. This involves employing both physical crosslinking methods, such as treatment with DHT, and chemical crosslinking methods, achieved by incorporating EDAC materials. The manipulation of the scaffold's microstructure is monitored by adjusting the freeze-drying temperature, allowing for precise control over the resulting scaffold characteristics.

The incorporation of hydroxyapatite (HA) before the freeze-drying process was observed to typically decrease pore size. The method of fabricating scaffolds through the combination of collagen and HA demonstrated an enhanced ability to regulate the microstructure of the resulting scaffold.[7] The scaffold pores should possess a size that permits cell migration, allowing cells to eventually attach to the ligands within the scaffold. However, these pores must still maintain a size that is sufficiently small to ensure a specific surface area conducive to effective cell adhesion.[35] Optimization of the scaffold's average pore size is very important to improve osteogenesis and subsequent mineralization. Scaffolds with physical (DHT) and chemical (EDAC) crosslinks maximize mechanical properties.[7] Following the freeze-drying process, all scaffolds undergo crosslinking with DHT at a temperature of 105˚C for a duration of 24 hours under a vacuum of 0.05 bar in a vacuum oven (Vacuum cell 22; MMM, Germany).[36] This crosslink technique can also sterilize scaffolds so that they can be used in cell culture.

Chemical crosslinking with EDAC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) is also employed to enhance the rigidity of the scaffold. EDAC functions as a chemical crosslinking agent, creating a 'zero-length' crosslink of about 1 nm between neighboring collagen molecules. The primary benefit of this chemical crosslinking method

is the small bond size, which maintains the scaffold's microstructure. In contrast, other chemical crosslinkers, such as glutaraldehyde (GA), have the potential to form long polymer chains and may carry cytotoxic effects.[37] Following the crosslinking process with EDAC, the scaffold undergoes a washing step in a Phosphate Buffer Saline Solution (PBS). This step is carried out to eliminate any remaining residual EDAC and urea, which are byproducts of the reaction.

The results of Ryan et al.'s research show that good mechanical properties and high porosity can be improved through collagen-HA scaffold crosslinks by both physical (DHT) and chemical (EDAC).[7] The utilization of this crosslinking combination aims to maximize the formation of amide bonds between carboxyl groups (specifically from glutamic or aspartic acid residues) and free amines (from lysine or hydroxylysine residues) within collagen. Both DHT and EDAC crosslinks are targeted to facilitate the establishment of these amide bonds.[38,39] In addition, DHT and EDAC crosslinks can also result in the formation of ester bonds between activated carboxyl groups and hydroxyl groups.[40] Limitations included conduct of the study in vitro and the trade-off between mechanical strength and biological contact.

Collagen-HA scaffolds that are not crosslinked will rapidly deteriorate in a cell culture environment. Crosslinks become important for adjusting the mechanical properties as well as the degradation period of the scaffold. However, from a practical terms, it is essential to construct scaffolds with a minimal reliance on solvents or additional chemicals, limiting the components to collagen materials and HA.[8] DHT which is heating in a vacuum proved efficient for crosslinking single-component collagen materials compared to crosslinking techniques of other collagen materials such as glutaraldehyde (GA), hexamethylene diisocyanate (HMDC), cyanamide, tannic acid, or EDAC.[41,42] The characteristics of materials, such as porosity, mechanical strength, molecular loading, and release, can be readily adjusted across a broad range. In Zhang et al.'s study, the preferred crosslinking method employed is DHT due to its high biocompatibility.[8] This technique stands out for not necessitating additional chemical reagents, offering advantages that not only enhance material biocompatibility but also streamline the preparation process. Animal trials demonstrate that the collagen-HA scaffold material can prompt successful regeneration of osteoid tissue, yielding results comparable to grafts derived from autogenous bone.[8] The investigation of long-term safety and scalability is still in its early stages.

Research by Teixeira et al. on HA-collagen scaffold crosslinked EDAC and NHS showed that cells can adhere, proliferate, and form a matrix on all material surfaces.[12] Cells can spread from one pore to another and form clusters of cells.[12] Limitations of this research included the conduct of the study totally in vitro with no dynamic mechanical strain.

According to the research conducted by Itoh et al. in 2004, the incorporation of glutaraldehyde (GA) crosslinks into hydroxyapatite (HA)-collagen scaffolds can be easily regulated.[13] The study revealed that the bending strength of the HA/Col composite increased by 1.8 times after undergoing crosslink treatment, albeit with a decrease in Young's modulus. Immersing the test piece in GA resulted in the scaffold absorbing a small amount of water, causing it to swell. While water-induced swelling can potentially soften the scaffold material and lead to a decrease in Young's modulus, GA was observed to enhance mechanical strength. The increase in mechanical strength of the HA-collagen scaffold is attributed to inter- and intrafibril cross-linkages among collagen fibrils within the scaffold, particularly at higher GA concentrations. This simultaneous increase in mechanical strength also correlates with a reduction in in vivo resorption rates without triggering toxic reactions, including inflammation.[13] Limitations included the size of the animal models, inadequate control over the degree of degradation, and the potential for immunological reactions.

Kikuchi et al. showed that the average particle size of HA-collagen scaffolds crosslinked with GA increased, but there was no refractive diffraction indicating the orientation of HA nanocrystals along collagen fibers.[14] The rate of resorption of HA-collagen scaffold decreases with increasing GA content observed by microscopic observation of light. This scaffold exhibits neither toxic nor inflammatory reactions.[14] Limitation of this research is that GA might inhibit collagen-HA self-organization; long-term toxicity is not known.

Ciardelli et al. showed that porous 3D scaffolds of HA-collagen were obtained by freeze-dry and crosslink.[15] The chosen method for crosslinking involved an enzymatic treatment utilizing microbial transglutaminase (mTGase). This approach led to a notable enhancement in the mechanical strength and thermal stability of the scaffold. The enzymatic action of mTGase facilitated the formation of isopeptide bonds between collagen chains, thereby increasing the resistance of collagen to biodegradation by collagenase. This particular scaffold exhibits promising attributes for bone tissue regeneration, as it supports the proliferation and differentiation of cells such as osteoblast MG63. Crosslinking with mTGase enables rapid cell infiltration into the HA-collagen scaffolds, contributing to enhanced osteoblast viability and alkaline phosphatase (ALP) activity.[15] The study lacks in vivo testing, and the mechanical strength of the scaffolds is modest.

Although the six studies show some impressive progress, major obstacles still exist. Much research still only provides in vitro results; hence, it lacks in vivo relevance.[7,8,12-15] Different approaches, such as GA, mTGase, EDC/NHS, and dehydrothermal, exhibit varied results, and there is no consensus on the optimum strategy that balances strength, biodegradation, and safety. High stiffness generally reduces early cell attachment; so, the ideal trade-off is continuous.[7] Biological functionality differs from mechanical performance. Future studies should seek to link lab innovation with surgical application by including optimum crosslinking, real-world mechanical testing, and long-term in vivo tests.

In essence, the combined information among all six investigations showed progress in bone tissue engineering through creative scaffold design. Each study utilizes either enzymatic, chemical, or thermal crosslinking methods to provide a unique perspective on balancing scaffold strength, biocompatibility, and degradation rate. While some methods showed better biological integration, others featured mechanical reinforcement.[7,8,12-15]

The best scaffolds seem to be those that deliberately mix biomimetic structure with crosslinking with physiologically active compounds. Still, the fundamental restriction is the move from the research laboratory to clinic. Few studies totally address scaling for practical use or long-term in vivo responses.

Useful information was obtained on how crosslinked collagen-hydroxyapatite scaffolds help bones heal while recognizing that there are also problems that should be noted. Only one database was used for the literature search, which may have limited the number of studies included in the review, most of which were done in vitro.-Future research should include larger database searches, in vivo and clinical investigations with standardized outcome measures, and consistent methods and reporting. This will make future studies on scaffold-based bone tissue manufacturing more reliable, easier to compare, and more likely to be useful in real life.

CONCLUSIONS

Crosslinking techniques in the development of collagen-HA scaffolds can be done chemically (EDAC, NHS, GA, enzymatic mTGase) or physically (DHT), and a combination of DHT and EDAC. DHT and EDAC for crosslinking can enhance mechanical strength, adjust pore size to suitable dimensions, stabilize the scaffold, and promote cell adhesion, thereby facilitating the growth of new cells and optimizing the osteogenesis process.

Future progress will rely on closing this gap—merging the long-term safety and strength achieved in the lab with practical use in real-life situations. Realizing the complete therapeutic potential of these scaffold systems depends on a cross-disciplinary strategy including translational medicine, cellular biology, and materials science.

Statement of Authorship

All authors certified fulfillment of ICMJE authorship criteria.

Author Disclosure

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