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BIOLOGICAL EFFECT OF COLLAGEN SCAFFOLDS ON RABBIT'S SOCKET HEALING

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ABSTRACT

BACKGROUND: Tooth extraction initiates a series of biological processes, including alveolar bone resorption driven by the inflammatory response; this eventually leads to alveolar bone resorption. Many researchers have focused on controlling this phenomenon and accelerating new bone formation following tooth extraction. Grafting biomaterials in the extraction socket is now the most dependable and successful strategy for reducing post-extraction resorption. Collagen plugs in the sockets might be an option with potentially desirable outcomes.

OBJECTIVES: To assess the biological effect of collagen scaffolds on the healing of extraction sockets

MATERIALS AND METHODS: 20 healthy male New Zealand rabbits weighing about 2-2.5 kg, aged 14-16 weeks, was used in this study; their right mandibular first premolar was extracted and divided into two groups with 10 each, control group: sockets were left for normal healing without scaffold administration, collagen group: collagen plugs were administered to the socket. All the rabbits' sockets were dissected and processed for histological analysis. A scanning electron microscope was used to observe the surface morphology of the socket, and Energy dispersed x-ray analysis [EDXA] was used to analyze the calcium and phosphate levels. The information gathered was recorded, and statistical analysis was performed.

RESULTS: The results (histology, scanning electron microscopy, and Energy dispersive X-ray analysis) revealed that the group with collagen plugs presented with more bone formation than the group with no scaffold placed in the socket.

CONCLUSION: According to the findings of this study, collagen plugs used post-extraction in rabbits increased bone formation compared to sockets without plugs.

KEYWORDS: Bone tissue engineering, Scaffolds, Socket preservation, Teeth extraction.

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INTRODUCTION

Tooth extraction triggers several biological events, including blood clotting, granulation tissue generation, woven bone formation, and eventually resolving with repair. Alveolar bone resorption is one of the sequelae, resulting in a considerable decrease in the height and width of the alveolar ridge(1). Alveolar ridge resorption is a physiological process that is chronic, progressive, permanent, and cumulative(2). The loss of bone mass may make it difficult to restore the removed site's aesthetics and function(3). In the first year, particularly the first three months, the rate of ridge resorption is at the highest levels. In addition, other studies have shown that without grafting the socket, 40–60 percent of the entire alveolar bone is lost in the first 2 - 3 years following tooth extraction.(4, 5) Other factors also influence the healing, such as the patient's age at the time of tooth loss and significant differences between the maxilla and the mandible, which influence bone loss(6).

Ridge preservation is a technique that focuses on placing a scaffolding biomaterial in the socket. The concept is founded on the premise that is using this grafting material assists in decreasing post-extraction alveolar bone resorption. (7)

A range of biomaterials for socket grafting has been documented for ridge preservation purposes, including autogenous, Allogeneic, xenogeneic, alloplastic, bone grafts, and other materials such as platelet-rich plasma, platelet-rich fibrin, bone morphogenetic protein(8).

The ideal grafting material has always been the target of research. (9-11) Studies have examined the grafting capability, inflammatory reaction, biodegradability, angiogenesis, and other characteristics in various collagen presentations, including gels, sponges, and meshes. All these studies have revealed positive results. (12-15)

Collagen is a class of nearly 29 polymeric proteins that make up the most prevalent extracellular matrix (ECM) protein component and account for around one-third of the weight of protein content in mammals(16). Three pro-collagen polypeptide chains produce a distinctive triple helix in all collagen proteins. Type I collagen is the most prevalent, accounting for 90% of the

organic mass of bone(14). Bone cells interface with exposed collagen fibers during the synthesis and degradation of the extracellular matrix of bone, which include interacting with the cell's integrin-binding motifs, which is the arginine-glycine-aspartic sequence (RGD) and DEGA (17) (18).

It was demonstrated that when collagen type 1 was employed as a scaffold, physiologically active osteoclast-like cells developed, invaded, and degraded the scaffold. Internalizing the scaffold as intracellular vesicles signals the osteoblasts to penetrate and generate a new extracellular mineralized matrix. (19) It was also shown that osteoblastic-specific genes OCN, BSP, and OPN were expressed by bone marrow cells grown with a type I collagen scaffold. These cells even presented with significant levels of ALP activity and collagen production. (20) This osteogenic behavior might be attributed to the interaction of amino acid sequence (DEGA) in collagen with integrin receptors found on the osteoblast's cell membrane (13, 18)

Studies utilizing clinical, radiographic, and histomorphometric assessment of the sockets augmented with collagen, have shown that collagen grafting resulted in significant alveolar ridge preservation compared to non-grafted sockets. These findings encouraged the use of collagen post-extraction, resulting in a more predictable implant placement site. (21, 22)

Due to the osteogenic nature of collagen, introducing it as a scaffold in the form of sponges or plugs in extraction sockets might enhance bone regeneration, thereby reducing the incidence of alveolar ridge resorption. The resulting alveolar bone provides a predictable implant site, thus avoiding secondary augmentation surgeries. The collagen scaffold might also produce a hemostatic effect by stabilizing the clot and reducing the chances of dry socket incidences. (23)

The study was conducted with the null hypothesis that there is no significant difference between the presence of collagen scaffolds and no scaffold used in bone formation throughout the healing of a rabbit's socket.

MATERIAL AND METHODS

Study type

An animal study following ARRIVE checklist

Sample size calculation

The minimal sample size is calculated based on a previous study to evaluate the efficacy of collagen compared to untreated sockets in dogs. Based on their results, adopting a power of 80% to detect a standardized effect size in the new bone formation (d=0.725) (medium-sized standardized effect size) and a level of significance of 95% (a=0.05), the minimum required sample size was found to be 10 extraction sockets per group (number of groups=2) (Total sample size=20 extraction sockets)

Study sample

Twenty healthy male New Zealand rabbits, aged 14-16 weeks, having all mandibular premolars free from caries and fracture, with an approximate weight of 2-2.5kg, were used. Animals were obtained from the animal's house of the Medical Research Institute, Alexandria University.

The experiment got the approval of the ethical committee on the guidelines of Alexandria University. Institutional Animal Care and Use Committee approval number (IORG0008839).

Random allocation

Rabbits were divided randomly by computer-assisted software into two equal groups with 10 rabbits in each:

Control group: sockets left without scaffold for normal healing.

Collagen group: collagen plugs were administered to the sockets.

Each rabbit in this study followed these criteria.

Materials

A collagen plug in the form of Collacone® was purchased from Straumann as a collagen plug for ridge preservation.

Xylazine in the form of Xyla-ject® by Adwia co. SAE, Egypt.

Ketamine10%® by Alfasan, Holland.

Methods

Surgical procedures

All surgical procedures, extractions, and experimental protocols were performed under general anesthesia. General anesthesia was induced in sterile conditions using intramuscular injection of xylazine (3 mg/kg) and (20 mg/kg) ketamine.

The mandibular right first premolar was extracted atraumatically. Following the extraction, in the control group, the sockets were left to heal spontaneously without applying any material, while in the collagen group, the sockets were loaded with collagen plugs. They were shaped to meet the requirement of bone repair and implantation. The soft tissue in all groups will be sutured to achieve site stability.

Euthanization

At the end of the experimental period, two weeks post-surgery, all animals from the two groups were euthanized. Mandibles were dissected out, and the right halves were separated buccolingually into mesial and distal halves. The obtained mandibles were divided according to the fixation method, where the mesial half was fixed in 4 % formaldehyde with 1% glutaraldehyde [4F1G] for the Scanning Electron Microscopic examinations and Energy Dispersed x-ray analysis [EDXA]. For histological processing, the distal half was fixed in 10% neutral–buffered formalin.

Histological Processing

Following fixation, the mandibles were washed and decalcified with 5% trichloroacetic acid, dehydrated with ascending concentrations (50%, 70%, 90%, and 95%) of ethanol, cleared with xylene, and

embedded in paraffin wax blocks. The blocks were cut with an average thickness of 5 µm. The cutting direction was buccolingual in serial sections until the socket area was reached. Numbers were given to the slides, and sections in the middle of the socket were chosen. Slides were stained with H&E to be examined by a light microscope for histological evaluation(24). All slides were assigned a random number allocated by a computer program; the specimens were then blindly interpreted by histology experts.

Ultrastructural evaluation of bone regeneration: (25)

SEM evaluated bone regeneration to assess the newly formed bone trabeculae in all groups. Samples from both groups were collected 6 weeks after extraction and were fixed in 4 % formaldehyde with 1% glutaraldehyde 4F1G. The socket areas were split longitudinally in a buccolingual direction at the mandibular right first premolar region, using a straight head handpiece with a double abrasive disc. A groove was made at the buccal, lingual plate of the bone, and basal bone at the center of the socket and then split into two halves. Each half was immersed in phosphate buffers, dehydrated through a 30–100 % alcohol series at room temperature (24°C), dried at the critical point, coated with gold in a vacuum, and observed by SEM.

Energy Dispersive x-ray Analysis (EDXA) (Faculty of Science, Alexandria University): EDXA is an analytical technique used for the elemental analysis of a sample. It was used to compare the amount of calcium and phosphate present in the socket area.

Statistical analysis

Statistical analysis Data were analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp). The Kolmogorov-Smirnov test was used to verify the normality of distribution. The significance of the obtained results was judged at the 5% level. Student t-test was used for normally distributed quantitative variables to compare the two groups.

RESULTS

Histological results:

Control group

The histological examination of the socket revealed newly formed bone trabeculae at the lateral border of the socket while showing randomly arranged thin bone spicules at the center of the socket. The bone marrow was fibrous with limited vascularity (Fig. 1)

Collagen group

Sockets revealed more active bone formation, with mature bone trabeculae extending from the lateral border to the center of the socket supported by highly cellular and vascular bone marrow (Figs.2-4). Higher magnifications showed osteoblasts lining the surface of the bone trabecula and active osteocytes residing within. (Fig. 4). Incremental lines could be noted (Fig. 3)

Scanning electron microscopy results:

Control group

The low power magnification revealed that most of the socket was filled with granulation tissue and small spicules of bone with relatively thin cortical bone. (Fig 5)

Collagen group

The low power magnification revealed new organized dense bone formation spanning the socket and extending from the relatively thickened cortical bone.

Energy Dispersive x-ray Analysis (EDXA):

Table (1) reveals the difference between both groups regarding calcium and phosphate levels. The mean calcium readings for the control group and collagen group were 49.67±5.7 and 68.56±4 respectively, with the difference being statistically significant. The phosphate readings had a statistically significant difference, with the control group having a mean of 50.33±5.7 while the collagen group had a mean of 31.44±4, all with a p-value of <0.001. These findings revealed that using the collagen scaffold significantly increased the calcium levels compared to unfilled sockets.

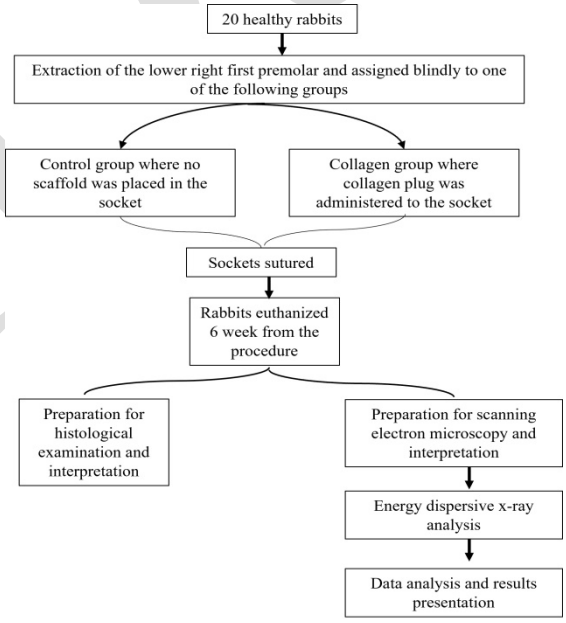

Figure 1: Study design flow chart

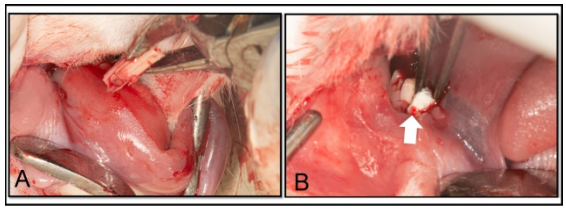

Figure 2: (A) atraumatic extraction of the right mandibular first premolar in rabbits, (B) administration of the collagen sponge (arrow) in the socket, while no graft was inserted in the control group

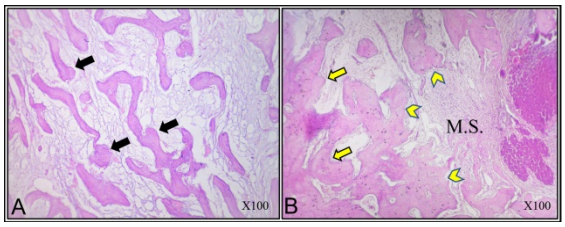

Figure 3: Light micrograph (LM) (6 weeks) showing: (A) control group with the center of the socket showing bony spicules (black arrows) (B) silk group with mature trabecular bone extending from the lateral border of the socket (yellow arrows) towards the center of the socket (yellow arrowheads) with highly cellular marrow spaces (M.S). (H&E stain; X100).

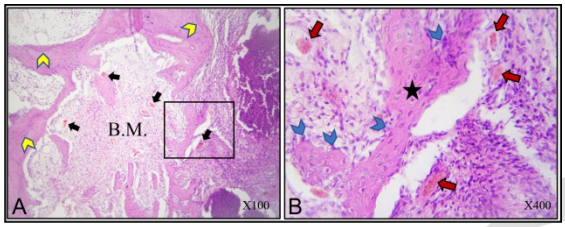

Figure 4: LM (silk group, 6 weeks) showing: (A) socket filled with trabecular bone supported by highly cellular bone marrow (B.M.) approximated by blood capillaries (black arrows). Note various reversal lines (yellow arrowheads) (H&E stain; X100), (B) higher magnification of the previous micrograph inset showing bone trabecula (star) packed with osteocytes and lined by osteoblasts (blue arrowheads) with numerous blood vessels in proximity (red arrows) (H&E stain; X400).

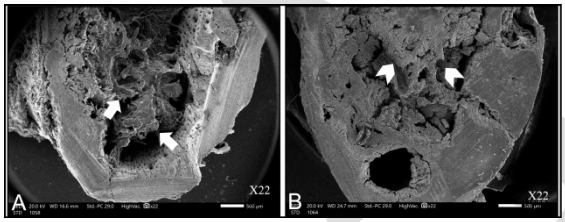

Figure 5: Scanning electron micrograph (SEM) at 6 weeks revealing: (A) control group with the majority of the socket's center filled with granulation tissue (white arrows), (B) study group with socket filled with well-organized trabecular bone (arrowheads). (x 22)

Table (1): Comparison between the control group and collagen group regarding calcium and phosphate levels obtained by EDXA

	Control	Collagen	t	p
Calcium				
Mean±SD	49.67±5.7	68.56±4	8.134*	<0.001
Median (Min-Max)	49 (42-58)	68 (62-75)		
Phosphate				
Mean±SD	50.33±5.7	31.44±4	8.134*	<0.001
Median (Min-Max)	51 (42-58)	32 (25-38)		

t: Student t-test
P: p value
*: Statistically significant at p≤ 0.05

DISCUSSION

Extraction socket preservation is essential for enhancing restorations' functional and esthetic outcomes. Grafting materials of various origins, such as autografts, allografts, xenografts, or synthetic origins, have been used and studied with or without barrier membranes. (26, 27) Due to the absence of cell adhesion signals in synthetic polymers and various drawbacks, the emphasis of current research has switched to natural polymers for uses in bone tissue engineering. (28)

Collagen, fibrinogen, and elastin are a few of the natural polymers that make up the extracellular matrix (29). Natural polymers, like collagen, possess a biological recognition characteristic that promotes cell attachment and differentiation, rendering them biocompatible (30). Collagen has also been proposed as a potential biomaterial for bone tissue engineering due to its availability, biocompatibility, pore structure, ease of processing, hydrophilicity, low antigenicity, and restorability. (13) This research aimed to compare the histological and ultrastructural results of using collagen cones as a socket preservation material in comparison to graft-free healing of extraction socket.

Rabbits were chosen as the target experimental animal; they are relatively economical, easy to handle and house, and available in large genetically homogenous (31). In addition, adult rabbits show Haversian remodeling and have a bone metabolism comparable to humans(32).

The 6-week experimental period was selected as the modeling and remodeling phase of the rabbit's sockets healing was demonstrated to be distinct at that interval. This time frame would allow both treatments to achieve significant healing and provide potential new bone for EDXA calcium and phosphate measurements. (33)

In the current research, histological analysis of all the sockets six weeks following extraction revealed newly formed bone, fibrous tissue, and supporting blood supply. These findings were concluded and documented by Kuboki et al. (34), who investigated the physiological healing of sockets following tooth extraction in rabbits. They established that the granulation tissue that formerly occupies the socket diminishes and is replaced by fibrous tissue, followed by blood supply penetration and proliferation and differentiation of osteogenic cells, resulting in the formation of woven bone.

The action of growth factors is responsible for bone repair during the socket healing process. These factors play a crucial role in the differentiation and proliferation of osteoprogenitor cells and

osteoblasts. Transforming growth factor- beta (TGF-β) and platelet-derived growth factor (PDGF), produced by platelets from a blood clot, cause osteoprogenitor cells to differentiate into osteoblasts in the early stages of bone healing. Growth factors from injured bone ends may contribute to sustained osteoblastic activity stimulation. The osteoblasts participating in the healing process are stimulated to express and synthesize certain growth factors produced by platelets and bone tissue (35). Two days post extraction, bone morphogenetic protein (BMP) is expressed by periosteal osteoblasts in the healing phase; its production signals mesenchymal stem cells to differentiate into osteoblasts. Other growth factors, including TGF-B, FGF, and PDGF, are generated 7 to 12 days post-extraction by osteoblasts to ensure a high proliferation and metabolic level of osteoblasts implicated in bone healing (36).

The sockets of the collagen administered with a collagen sponge reveal a highly arranged thicker bone trabecula filling the socket with high vascularity. This was in agreement with Cardaropoli et al. (37), who grafted the socket with a collagen sponge in dogs to study its efficacy. Their histological assessment demonstrated a more significant amount of newly developed bone in the collagen sponge grafted locations (61.4 percent). In a recent study, Tarun et al. (38) measured the bone formed after extraction in patients grafted with collagen aided by histomorphometry. The bone used in the measurement was obtained by removing the core for implant placement. Their study revealed a significant increase in mean bone formation in the sockets augmented by collagen, which is in conjunction with the current study.

Collagen fibers may aid progenitor cell migration and secretion of extracellular matrix to develop the bone matrix. The collagen matrix structure and fiber orientation presented in the graft might have encouraged cell migration in the direction of the matrix. (39) In an in-vitro investigation, collagen scaffold promotes human osteoblast cell adhesion, recruitment, and survival, indicating that it has great promise as a biomaterial for human bone tissue engineering. (40) The outstanding biocompatibility of collagen sponges was shown in an in vivo investigation, where collagen was placed in a critical defect created in rats. The results were obtained by histological and micro-computed tomography, uncovering a substantial degree of cellular infiltration during the early healing phase and eventually higher bone density 8 weeks from the grafting procedure. (41) In addition, collagen plugs possess a porous structure that enables them to absorb fluid and blood at the extraction site, thus enhancing their hemostatic properties, which help control bleeding(12).

In terms of vascularity, the histological findings of the control group indicated marrow gaps with limited blood supply, while the collagen sponge-treated socket exhibited an abundance of blood vessels throughout the developed bone, indicating new bone production. These findings agree with Khairy et al. (22); in their study, cores from the grafted and non-grafted sites were histologically evaluated and evidenced the positive angiogenetic effect of collagen sponges. In another experimental study by Calabrese et al. (42), and ectopically implanted collagen scaffold in mice resulted in the formation of well-organized blood vessels. The expression of CD31 proved this finding through fluorescence molecular tomography (FMT) analysis. CD31, also known as platelet endothelial cell adhesion molecule (PECAM-1) necessary for angiogenesis because it enables the formation of new blood vessels through cell-cell adhesion.

A recent study evaluating the efficacy of collagen scaffold vs. ungrafted sockets indicated the use of a collagen sponge (Collacone) with no significant bone and soft tissue healing compared to ungrafted sockets. It was speculated that their obtained results were due to impairment of the vascularity caused by the collagen scaffold. A primary limitation mentioned in that study was the limited sample size and brief follow-ups. (43)

The scanning electron micrographs revealed a qualitative increase in the formation of bone in the collagen group compared to the control group. This finding follows a recent study on the effect of scaffolding after teeth extraction(44), where the grafted sockets showed noticeably increased bone formation than the non-grafted sockets.

The EDXA results revealed that the calcium levels significantly increased in the control group than in the collagen group. The phosphate levels also showed a significant difference, with the control averaging 50.33 while the collagen group was averaging 31.44. The ratio between calcium and phosphate in mature healthy bone was shown to be 2.2, obtained by dividing the calcium and phosphate percentage (45). The collagen group accounted for a ratio of 2.18, closer to the normal value than the control group, which yielded a ratio of 0.98.

The understanding of the difference in the means can be explained through the knowledge that the inorganic phase is made up of hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), where the calcium and phosphate play an integral role in the activity of bone cells (46). Calcium ions induce osteoblast proliferation and differentiation, promoting the production of bone organic matrix and mineralization. On the other hand, increased extracellular calcium suppresses the proliferation of osteoclasts. This suppression is due to the interaction between the calcium receptors expressed

by cells of the osteoclast lineage (CaSR) and calcium ions, which increases osteoclast apoptosis (47). This combined effect of calcium ions on bone results in enhanced bone mass, which was evident and supported by the scanning electron micrographs of this study and the increased calcium levels in the collagen group.

CONCLUSION

Based on the study's findings, the enhancement of bone formation and the angiogenetic ability of collagen plugs encourages further research on its efficacy, biocompatibility, and degradability, thus evaluating its efficacy as a bone regenerative scaffold.

RECOMMENDATIONS

Further studies should be conducted on the biological properties of collagen sponges, assessed with the aid of biological markers for bone regeneration. In addition, studies on collagen composites, by including inorganic components, should be considered to enhance the overall mechanical and bone regenerative properties of collagen scaffolds. The healing process of the collagen grafted socket should also be studied for longer periods to evaluate the long-term effect of the collagen scaffold.

AUTHORS CONTRIBUTION

Karim M. Hafez conceived, planned, and carried out the experiments. Azza S. Koura, Khadiga Y. Kawana, and Nihal T. El Kazzaz interpreted the histology and verified the analytical and statistical methods. All authors discussed the results and contributed to the final manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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