REVIEW ARTICLE

In Vitro Study on Collagen Application in Wound Healing: A Systematic Review

Salleh F, Amid A, Nordin NFH
International Institute for Halal Research and Training (INHART), International Islamic University Malaysia (IIUM), Kuala Lumpur, Malaysia

ABSTRACT

Collagen is key component of extracellular matrix for human and animals. It can be extracted and applied for various field especially in tissue engineering as main component for wound healing. Collagen especially type I collagen is used in skin regeneration process due to its high compatibility with donor site and low antigenicity besides of having suitable properties for wound healing like high cell proliferation and adhesion. However, the usage of collagen alone would not give maximum wound healing since collagen degrades faster, has poor structure for a scaffold and attracts bacteria due to high moisture content. Therefore, to produce a good collagen scaffold, it must be incorporated with functional biomaterials to enhance the characteristic of the collagen and fabricated for a better scaffold structure. The objective of this systematic review is to summarize the relevant literature for *in vitro* study on collagen application in wound healing by focusing on the source of collagen, biomaterials and fabrication methods used in making collagen scaffold. Three databases were searched; PubMed, Scopus and Science Direct. Keywords used were: collagen, recombinant collagen, collagen scaffold, application and wound healing. A total of 1105 were articles screened but only 50 articles were suitable and were further reviewed. Collagen in wound healing study has versatility in terms of the source of collagen used, the biomaterials combined with the collagen to make an enhanced scaffold, and the fabrication methods to create a desirable structure of collagen scaffold.

Keywords
Wound healing, collagen scaffold, biomaterials, fabrication methods, in-vitro

Corresponding Author
Prof. Ts. Dr Azura Amid
International Institute for Halal Research and Training (INHART),
Level 3, KICT Building, International Islamic University Malaysia (IIUM),
Jalan Gombak 53100
Kuala Lumpur Malaysia
E-mail : azuraamid@iium.edu.my

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INTRODUCTION

Allograft, autograft, or xenograft are the gold standard of wound healing solutions by promoting cell migration and proliferation on patient's skin.[1] However, these types of surgeries come with huge disadvantages such as shortage of donor tissue and the probability of infection is always high.[2] Meanwhile, due to it being biocompatible with the native extracellular matrix (ECM), collagen is named as a promising biomaterial for wound healing study because of its high cell attachment, adhesion, and proliferation properties due to its specific molecular structure and bioactivities.[3,4]

Collagen is a powerful and resourceful biomaterial that can be extracted from land and sea. This biomaterial has been isolated from various animals on the land such as porcine, bovine, equine, avian, amphibians, and aquatic animals.[5] There are 29 different types of collagens that possess the typical triple helices such as Collagen Types I, II, III, V, and XI. Generally, collagen have a repeating sequence of Gly-X-Y where X and Y are proline and hydroxyproline.[6] Now, collagen can be found in different kinds of forms like sponges, spray, gels, and film. The structure, physicochemical and biological activities of the collagen make it different from each other in the medical industry, especially in wound healing studies. These attributes are important to treat different kinds of wounds such as burn injury, pressure sores, and ulcers by absorbing tissue exudates, preserve a moisturized environment and encourage tissue granulation on the wound site.[7] However, collagen alone does not have an anisotropic structure for wound healing. It needs to be enhanced by biomaterials such as polymers, functional chemicals and fabricated according to the specific need in the wound healing process.

Therefore, the objective of this review is to summarize the relevant literature for the application of collagen on wound healing for *in vitro* study where the sources of collagen, biomaterials, and fabrication methods of the collagen scaffold used for wound healing were discussed.

MATERIALS AND METHODS

The systematic review follows the guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analysis. Three databases were searched for the reviews: PubMed (https://pubmed.ncbi.nlm.nih.gov/), Scopus (https://www.scopus.com/), and Science Direct (https://www.sciencedirect.com). The search took place between 5–7th April 2021. The search keywords are generated by the database with specific results summarized in Table I. The databases were searched for the application of any form of collagen or recombinant collagen on any form of wound for wound healing purposes as mentioned in the title.

Table I. Summary of databases and search keywords.

Database	Keywords
PubMed	((((Collagen[Title]) OR (Recombinant Collagen[Title])) OR (Collagen Scaffold[Title])) AND (Application[Title/Abstract])) AND (Wound healing[Title/Abstract])
Scopus	TITLE (*collagen) OR TITLE (*recombinant AND collagen) OR TITLE (*collagen AND scaffold) AND TITLE-ABS-KEY (*application) AND TITLE-ABS-KEY (*wound AND healing)
Science Direct	Title, abstract, keywords: Application AND Wound healing Title: Collagen OR Recombinant Collagen OR Collagen Scaffold

Selection Criteria

The criteria for exclusion during the abstract screening mainly revolves around the year of the article is published (articles published before the year 2000 are excluded), the absence of abstract, articles that are not in English, no external collagen and wound healing involved in the articles. During the title and abstract screening, the words like "collagen deposition", "collagen synthesis", "collagen regeneration", "collagen organization", "collagen formation", "collagen fiber/fibril", "collagen production", "collagen synthesis", "collagen metabolism", "collagen stabilization" and "collagen expression" are not representing the external collagen incorporated into the experiment, therefore, it was omitted during pre-screening of title and abstract.

During the screening of full text, the exclusion criteria are the availability of the full text online, focus on *in vitro*, and only one type of wound study is chosen which is skin wound/external wound. The wound study aside from skin wounds such as bone repair, dental-related injury, and corneal repair are all excluded. The *in vivo* and clinical studies are excluded from this review. The articles that reported the following data are included: 1) Source of collagen, 2) Biomaterials combined with collagen scaffold, and 3) Fabrication method of collagen scaffold.

RESULTS AND DISCUSSION

By using the search keywords summarized in Table I, 237 articles were found from the PubMed database, 748 articles from the Scopus database, and 120 articles from the Science Direct database. In total, there are 1105 articles. From the 1105 articles, 380 articles were duplicates and 368 articles were excluded based on abstract screening and 307 articles were excluded based on full-text screening. Finally, 50 articles were selected to be reviewed as the result. The whole process is summarized in Figure 1.

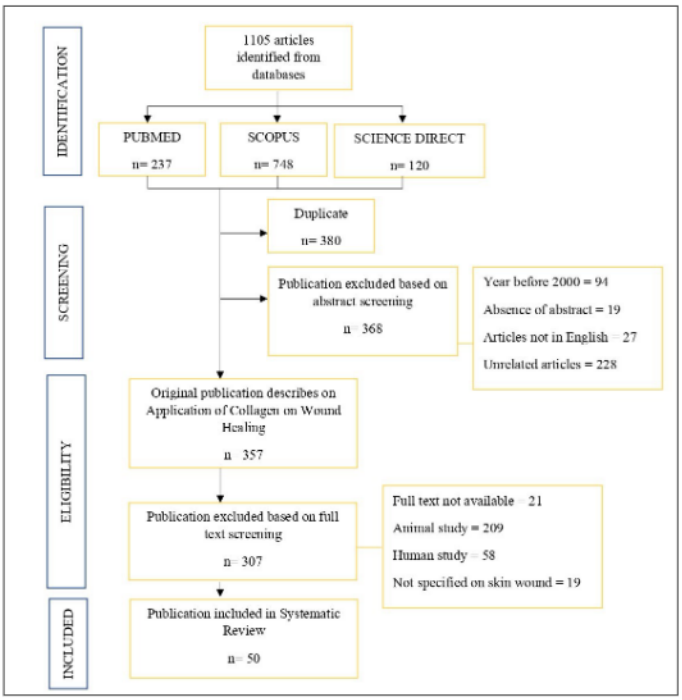

Figure 1. Flow diagram of the inclusion process according to PRISMA. Articles are assorted based on identification, screening, and eligibility.

Source of Collagen

Collagen can be extracted from various kinds of animals in the world from the land to the sea. This functional biomaterial is extracted abundantly in skins, hides, tendons, bones, and cartilage of animals like porcine, bovine, equine, and marine.[5] Twenty-nine types of collagen can be extracted from the animals and the most extracted and studied collagen is Type I collagen where it consists of more than 90% of all parts in organic mass.[6,8] Our review found that, from 50 articles selected, there are 48 articles reported on usage of the type I collagen from different kind of species as the medium of the scaffold. The type I collagen reported in this review mostly were extracted from bovine (9 articles), fish (13 articles), rat tails (6 articles), porcine (1 article), equine (2 articles), avian (1 article), and 18 articles with unmentioned sources. Only 1 article reported the use of collagen type IV collagen,[9] and 1 article reported the use of recombinant human collagen.[10] The review found that the collagen from bovine and fish have been recorded as the most used for the *in vitro* study. The articles are summarised in Table II.

Table II. Summary of review on application of collagen on wound healing for *in vitro* study

Type of collagen	Biomaterial	Fabrication method	References
Type I collagen fibrils	Transglutaminase 2 from guinea pig liver	Air-drying	59
Bovine Achilles tendon (native insoluble type I collagen)	Fibroblast growth factor (bFGF)-loaded chitosan-heparin nanoparticles	Crosslinking, freeze and air-drying	4
Type I collagen	Poly(epsilon-caprolactone)	Electrospinning	21
Fish	Silver sulfadiazine (AgSD)	Electrospinning	60
Type I collagen	Silk cocoons, Titania (TiO_2)	Freeze drying	32
Calf hides	Sericin silkworm	Freeze drying	33
Type I collagen	Schwann cell (SC)	Crosslinking, freeze-drying	57
Rat tail tendons	Silver nanoparticles (AgNA), pectin	Air-drying, crosslinking	61
Type IV collagen	Heparin sulfate	Freeze drying	9
Fish scales	Guar gum, ceftazidime	Crosslinking	36
Type I collagen	Pectin	Electrospray deposition	62
Collagen hydrolysate	Cellulose acetate	Electrospinning	63
Fish scale	*Macrotyloma uniflorum* plant extract	Crosslinking	7
Type I collagen (Rat)	Glycosaminoglycans (GaGs), hyaluronan, chondroitin sulfate	Vacuum drying	64
Type I collagen	Hyaluronan	Crosslinking with UV light	65
Unicorn leatherjacket collagen peptide	na	Enzymatic hydrolysis	14
Flatfish	Sodium alginate (brown algae), chitooligosaccharides	Crosslinking	15
Type I collagen	Recombinant fibroblast growth factor 2 protein	Crosslinking by formalin	66
Fish	Cellulose acetate	Electrospinning	67
Type I collagen	Sulfated xylorhamnoglycuronan (SXRGlu)	Crosslinking	35
Rat tail	Aloe vera gel, chitosan, nanofibrous poly(L-Lactic acid)	Electrospinning	47
Arothron stellatus (fish)	Poly(3-hydroxybutyric acid)	Electrospinning	16
Calfskin	Doxycycline, chitosan, sodium alginate	Electrospinning	39
Type I collagen	*Artemisia absinthium* plant extract (wormwood)	Freeze drying	43
Type I collagen	Heparin induces vascular endothelial growth factor (VEGF)	Freeze drying	68
Rat tail	Dodecenylsuccinic anhydride (DDSA), simvastation	Crosslinking	40
Type I collagen	Hyaluronic acid, epidermal growth factor (EGF)	Crosslinking & Freeze-drying	58
Calf hides	Indomethacin loaded polyvinyl alcohol	Crosslinking	69
Type I collagen (Fish)	PLA, silver sulphadiazine, *Azpalathus linearis* extract	Electrospinning	44
Rat tail tendons	Silica	Crosslinking	70
Fish scales	Curcumin	Freeze drying	45
Bovine sponge bones	heparin	Crosslinking	71
Type I collagen	Poly-L-lactic acid, poly-(α,β)-DL-aspartic acid	Electrospinning	72
Recombinant human collagen	Chitosan	Freeze-drying	10
Bovine, porcine, avian	Chitosan	Freeze drying	73
Rat tail tendons	Fucoidan	Air-drying	34
Type I collagen	Nanofibrous poly-caprolactone (PCL)	Electrospinning	74
Equine Achilles tendon	Nanostructured lipid carrier (NLC)	Freeze drying	75
Bigeye Tuna fish (*Thunnus obesus*)	na	Enzymatic hydrolysis	17
Type I collagen	Allantoin, lidocaine hydrochloride, chitosan	Freeze drying	42
Labeo rohita scale	Nano/microfibrous chitosan	Electrospinning	18
Bovine Achilles tendon	Chitosan, Gallic acid	Crosslinking by glutaraldehyde	76
Equine Achilles tendon	Curcumin	Freeze drying	46
Atelocollagen (Type I collagen)	Hyaluronic acid	Freeze drying	12
Scomberomorus lineolatus fish skin	na	Freeze drying	19
Fish collagen	Chitosan (shrimp shell)	Air-drying	77
Calf hides	Tripolymer polycaprolactone (PCL)	Electrospinning	78
Type I collagen	Polymer polycaprolactone (PCL)	Electrospinning	79
Type I collagen	S-nitrosoglutathione (GSNO)	Electrospinning	41
Bovine collagen type I	Chitosan	Electrospinning	80

This review observes that type I collagens from bovine extracted from calf hide/skin and bovine Achilles tendon, are preferred more as collagen scaffold than type I collagen from porcine. The extraction of calf hide/skin has been recorded in early 1960[11] and continuously extracted and fabricated into sponge, mesh and membrane to be applied in *in vitro* experiment until today. One of the articles reported the use of atelocollagen[12] as collagen sponge together with hyaluronic acid, although it is not specifically mentioned the species of origin, but based on Nimesh,[13] it is mainly prepared by pepsin treatment from calfskin.

According to the review, the collagen extracted from fish is originally from various species of marine and freshwater fishes such as *Aluterus monoceros* (Unicorn Leatherjacket),[14] *Paralichthys olivaceus* (Olive flounder),[15] *Arothron stellatus* (Starry pufferfish),[16] *Thunnus obesus* (Bigeye tuna),[17] *Labeo rohita* (Rohu carp),[18] and *Scomberomorus lineolatus* (Streaked Spanish mackerel).[19] The collagens of these fishes are extracted from the skin and scales since these are the part of fish that are rich with type I collagen.[20]

Biomaterial Combination with Collagen Scaffold

Collagen is abundantly used in *in vitro* study of wound healing because of the ability of collagen as a biomimetic microenvironment that is close to the extra cellular matrix (ECM), the native cell-growing environment.[21] However, collagen alone is not appropriate because it is a protein that easily degrade in the open air, thermally unstable, and attracts bacteria due to high moisture content and the bacteria can use it as the substrate, therefore, collagen alone will not facilitate the wound healing process.[22]

According to Butler, Goldstein, & Guilak,[23] a good collagen matrix structure should attract, anchor, and protect the cells and then degrades at a controlled rate. All these attributes are important to prevent any mechanical disruption during the healing and to ensure the biocompatibility happened between the native and synthetic ECM. To produce an excellent collagen matrix for wound healing, functional biomaterials such as polymers, enzymes, or specific drugs are needed to be cross-linked with collagen. A normal skin wound healing has overlapping phases of healing started with achieving haemostasis, inflammation, proliferation, and remodelling phases.[24] The mentioned healing phased should be the guideline on choosing the right biomaterials to identify the right materials suite to certain healing phases, and thus, fasten the wound healing process.

This review observed that several biomaterials have been combined and cross-linked with collagen to enhance and increase the value of the collagen matrix for wound healing study. The most biomaterial used for combination with collagen is chitosan (11 articles). Chitosan is a derivative of chitin that can be extracted from exoskeletons of arthropods such as crabs, horseshoe crab, shrimp, fish scales and from the cell wall of some fungi.[25–28] It is known to have a haemostatic property for controlling bleeding wounds and increase tissue granulation in the proliferation phase of wound healing.[29,30] However, this functional biopolymer is reported to have poor solubility, and to overcome this problem, the chitosan is hydrolyzed into chitooligosaccharide (COS) to make it soluble in water due to their shorter chain lengths and free amino groups in the D-glucosamine unit.[31] Chandika et al.[15] reported the used of COS fabricated with pure collagen to help the degradation of the scaffold structure and slow down the wound healing process. A controllable scaffold degradation is an important key to achieve an optimum level of wound healing.

Aside from chitosan, fibres from silkworms called silk fibroin[32] and silk sericin,[33] polysaccharides from brown algae called fucoidan[34] and from green algae called sulfated xylorhamnoglycuronan (SXRGlu),[35] and polysaccharides from plants such as guar gum[36] and pectin[37] are also used as the biomaterials that cross-linked with collagen. All these natural polymers exhibit the same property which enhance the structure of collagen scaffold, making it suitable for cell attachment, help controlling the releasing drugs to the donor site, and thus making the phases of wound healing as smooth as possible.[38]

Besides the biopolymers that help the collagen scaffold in terms of structure, the biological activity of the collagen scaffold as a wound healing medium may be improved by

the addition of suitable biochemicals that can improve the healing process. There are many biochemicals incorporated into the collagen scaffold for selective reasons such as for antimicrobial activity, anti-inflammatory effect, antibiotics such as ceftazidime,[36] and doxycycline,[39] antioxidant properties such as simvastatin[40] and s-nitroglutathione,[41] pain relief like lidocaine hydrochloride,[42] and moisturizing effect by allantoin.[42] The review also found out that four plant extracts were added to the scaffold formulation for their functional benefits in wound healing process. *Artemisia absinthium* (wormwood) known for its antimicrobial activity,[43] *Aspalathus linearis* (rooibos) for its antioxidant and anti-inflammatory properties,[44] *Mactrotyloma uniflorum* (horse gram leaves) for its antibacterial and antioxidant values,[7] curcumin for its known antibacterial properties[45,46] and aloe vera are applied on the wound for its soothing and antioxidant benefits.[47]

Despite all the enhancement from these biomaterials, collagen is still the main material for wound healing as it has excellent biocompatibility, is hydrophilic, has low immunogenicity, and can be produced in high quantities with minimum cost[48,49] especially the type I collagen.

Fabrication Method of Collagen Scaffold

The collagen scaffold itself has poor mechanical properties despite having low antigenicity and being biocompatible with native ECM.[6] To mimic the complex ECM microenvironment, the collagen scaffold must possess anisotropic structures like what has been demonstrated in different parts of organs such as tendons with criss-cross fibril patterns, bones with interstitial lamellae structure, and in corneas with crystalline layers.[50–52] Based on the finding by Leong et al,[53] a good scaffold should possess a suitable macro or nanostructure to promote cell proliferation, has a highly porous surface that allows cell ingrowth, manage to avoid pore occlusion with optimal pore size, good surface morphology to encourage intracellular signalling and recruitment of specific cells, and the ingredient is made from a non-toxic material (synthetic or natural) with a controllable degradation rate.

There are various methods of fabrication for collagen scaffold today but the most popular among the articles in this review are electrospinning, freeze-drying and crosslinking methods. The earliest electrospinning or electrostatic nanofiber processing activity recorded was in 1897.[54] This method needs high voltage to impart a charge on polymer solution (collagen with biomaterials) and the electrostatic forces applied produce fibres which diameter can be adjusted from nanometer to micrometer.[54] Giriprasath Ramanathan et al.[16] used the electrospinning method in their study where poly(3-hydroxybutyric acid) and gelatine are electrospun together forming a nanofibrous scaffold and the collagen solution extracted from *Arothron stellatus* fish coated the nanofibrous scaffold and used in wound healing study. The scaffold was tested with a swelling behaviour test where the electrospun nanofiber has better swelling ability compared to another scaffold. Good swelling behaviour of collagen scaffold is important because it will allow the scaffold to absorb the wound exudates and prevent the wound region from drying and unwanted infection. The electrospinning method can increase the porosity of the collagen scaffold which resulted in good oxygen permeability and thus improves wound healing efficiency.[16]

Next collagen scaffold fabrication method is freeze-drying. This method starts by removing water or any other solvent from the frozen collagen by sublimation process where the frozen liquid turned into a gaseous state due to extremely low temperature. The ice crystals that evaporated from the sublimation process shaped the pore structure in the collagen scaffold.[55] However, this method has a minor limitation where the size and distribution of the pores solely depends on the shape of the sublimated ice crystals.[53] To overcome this problem, Yeong et al.[55] fabricated a specific 3-D mould to get the scaffold the desired pore size and structure when it was freeze-dried.

Many articles reported on fabricating the collagen scaffold using crosslinking method. According to Maitra & Shukla,[56] crosslinking method mainly affects the physical properties of the collagen scaffolds by their elasticity, viscosity, insolubility, strength, and behaviour towards heat. There are two methods of crosslinking which are chemical and physical crosslinking. The crosslinking agents/treatment such as genipin, glutaraldehyde, citric

acid, chromium, or physical treatments such as UV and gamma irradiation are normally used to enhance the crosslinking process.[36] The review found that some of the articles reported on combining crosslinking and freeze-drying fabrication methods together and resulted in a better quality collagen scaffold.[4,57,58]

FUTURE DIRECTION

There are some limitations with the studies from this review in terms of the source of the collagen used for the scaffold. There will be a religious concern (for Islam and Judaism) and zoonotic and allergic issues when it comes to collagen extracted from mammalians. Recombinant collagen from bacteria should be added as a choice for source of collagen to be used in wound healing as it is sustainable, flexible, and does not contradict any religion. Notably, there is only one recombinant collagen which is from humans used in the study of wound healing for this review.

CONCLUSION

In conclusion, collagen in wound healing study has versatility in terms of the source of collagen used, the biomaterials combined with the collagen to make an enhanced scaffold, and the fabrication methods to create a desirable structure of collagen scaffold. In this review, the source of collagen, biomaterials and fabrication methods of collagen scaffold for wound healing application were discussed and summarized.

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