Validation of three different sterilization methods of Tilapia skin dressing: impact on microbiological enumeration and collagen content

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Methodology article

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Abstract

Background

Tilapia fish skin has demonstrated promise as a stable and practical biological dressing to be used in wound and burn management. However, the appropriate sterilization technique of the Tilapia fish skin is crucial before its clinical application. The standard sterilization technique must eliminate harmful pathogens but maintain the structural and biochemical properties that could compromise the dressing function. This study investigated and compared the efficiency of three sterilizing agents; chlorhexidine gluconate 4% (CHG), povidone iodine 10% (PVP-I), and silver nanoparticles (25 µg/mL) (AgNPs), at three different times (5, 10, and 15 min) on Tilapia fish skin based on the microbial count, histological and collagen properties.

Results

Among the sterilization procedures, AgNPs showed rapid and complete antimicrobial activity, with a 100% reduction in microbial growth of the fish skin throughout the treated times. Furthermore, AgNPs did not impair the cellular structure or collagen fibers content of the fish skin. However, CHG and PVP-I caused alterations in the collagen content.

Conclusion

This study demonstrated that the AgNPs treatment of Tilapia fish skin provided sterile skin while preserving the histological properties and structural integrity. These findings provide an efficient and quick sterilization method suitable for Tilapia fish skin that could be adopted as a biological dressing.

Background

Biological dressings, such as amnion, allografts, xenografts, bioengineered tissues, and cultured-wound healing cells, have been used recently for topical treatment of burns and wounds [1]. Of which, collagen dressings are widely used due to their beneficial properties as low antigenicity, enhanced inflammation and hemostasis, and accelerating fibroplasia and epithelization [2]. Collagen dressings derived from cattle and pig skin or chicken waste are inappropriate due to the risk of disease transmission or religious and cultural reasons [3, 4]. However, marine fish are a good source of collagen and unlikely to be a source of infectious diseases and have antibacterial and antioxidant properties [3, 5, 6].

The use of Nile Tilapia (Oreochromis niloticus) skin as a potential natural material in the management of burns and wounds arose because of its collagenous, histological, and mechanical comparability to human skin [7–9]. In addition, Tilapia skin is characterized by increased safety, low cost, easy to apply, readily available materials, and good quality [10].

The sterilization of wound dressings before use on a patient is critical to guarantee the wellbeing of the patient and the healing process of the skin wound. Ordinary methods used to sterilize skin grafts are ethylene oxide gas sterilization, irradiation, and chemical sterilization [11–13]. Ethylene oxide is a toxic gas which likewise generates harmful reaction products with potential hazards to the patient [14, 15]. However, sterilization of skin grafts with irradiation worsened the structure of the skin matrix as it can cause collagen matrix cross-linking or breakage of the peptide bonds within the collagen molecules [16]. Unlike mammalian skin grafts, which require harsh chemical sterilization, Tilapia skin grafts require a gentle processing methods because the colony forming units found in the tilapia skin samples suggested the existence of a normal, non-infectious microbiota [17]. However, the fish skin may be contaminated with different microorganisms from water environment or other sources of contamination [18].

Chlorhexidine and povidone iodine are routinely used for sterilization and disinfection of grafting procedures as they are effective against Gram-negative, Gram-positive bacteria and fungi and kills by disruption of the cell membrane [11, 19–22]. In addition, silver nanoparticles have long been known as an antimicrobial agent and are used as silver-containing wound dressings and for disinfection of the wound graft [13, 23].

Results

Microbiological Evaluation of the Treated Skin

The effectiveness of chlorhexidine gluconate 4%, povidone iodine 10%, and silver nanoparticles (25 µg/mL) on the microbial count of the fish skin at the different three contact time points was summarized in Table 1. The silver nanoparticles (AgNPs) achieved a 100% reduction percentage against aerobic bacterial, Staphylococcal, Coliform, and Yeast and Mold counts throughout the treatment time points (5, 10, 15 min). However, povidone-iodine achieved a 100% reduction against the Coliform and Yeast and Mold counts at the contact time of 10 min, and against the aerobic bacterial and Staphylococcal counts at the contact time of 15 min. Chlorhexidine gluconate achieved 100% reduction against Staphylococcal and Yeast and Mold counts after the treatment time of 15 min.
Histological Evaluation of the Treated Fish Skin

Histological analysis of the fish skin samples from the control group showed compactly arranged bundles of collagen fibers in the dermis and occasionally focal epithelial coating and superficial melanophores were seen. The collagen fibers were well organized and distributed in a parallel pattern with no evidence of disaggregation (Fig. 1a and b).

Fish skin treated with 4% CHG for 5 min showed well organized and parallel collagen fibers (Fig. 2a and b) with mild changes in the collagen fibers after 10 min treatment (Fig. 2e and f). However, disorganized and disaggregated collagen fibers were obviously seen after 15 min treatment (Fig. 2i and j).

In the 10% PVP-I-treated group, the structure of the collagen fibers has not been changed after 5 min treatment (Fig. 3a and b). Disorganization and disaggregation of the collagen fibers were observed after 10 min and 15 min treatment and were more prominent at 15 min treatment (Fig. 3e, f, i and j).

However, there was no change in the organization pattern of disaggregation of the collagen fibers in the AgNPs-treated group after 5-15 min treatment in comparing with control samples (Fig. 4a, b, e, f, i, and j).

Histochemical Evaluation of the Treated Fish Skin

We further examined the organization of the collagen fibers by staining the skin samples from the control and treated groups with Gomori's trichrome stain and measured the collagen intensity in the fish skin using ImageJ. The fish skin treated with CHG for 5 min (Fig. 2c and d) or PVP-I for 5 min (Fig. 3c and d) showed the well organization of the collagen fibers and the preservation of the collagen intensity in comparing with the control one (Fig. 1c and d). There was mild change in the disposition pattern of collagen fibers and the collagen intensity after sterilization for 10 min with CHG (Fig. 2g and h and Fig. 5) or PVP-I (Fig. 3g and h and Fig. 5). However, the reduction in intensity of the collagen in the fish skin was statistically significant after sterilization for 15 min with CHG or PVP-I (Fig. 5). On the other hand, the sterilization of the fish skin with AgNPs showed well organized collagen fibers (Fig. 4c, d, g, h, k, and i) with non-significant change in the intensity of the collagen (Fig. 5), regardless of the treated time.

Discussion

Biological dressing using Tilapia skin is a growing area that has been suggested as a potential xenograft for skin wounds and burns treatment [1]. The fish skin is characterized by large amounts of moisture and collagen proteins at levels comparable to human skin. This prevents scarring while promoting the healing of wounds. In addition, unlike the gauze bandages, the tilapia skin does not need to be changed every day [3, 5–9].

However, the critical attribute of using fish skin as a biological dressing is the sterilization process prior to clinical application to avoid the possibility of zoonotic diseases transmission. Therefore, the present study compared three different sterilization procedures; chlorhexidine gluconate (CHG), povidone iodine (PVP-I), and silver nanoparticles (AgNPs), and determined which method was more efficient in reducing the microbial counts and maintaining the structural components of the fish skin.

CHG has been used to disinfect the skin before surgery and sterilize surgical instruments and biological dressings [11, 22, 24]. In consistent with previous data [11], our results showed that CHG at concentration 4% was effective in the reduction of microbial load of tilapia fish skin in variable degrees and the bactericidal effect increased with increasing contact time. CHG is a bisbiguanide compound that has a broad spectrum of activity against gram-positive and gram-negative bacteria and fungi, even in the presence of interfering materials, such as blood or serum [19, 24]. It dissociates and releases the positively charged chlorhexidine cation. In turn, this cation binds to the negatively charged bacterial cell walls resulting in leakage of cellular contents, precipitation of intracellular compounds, and inhibition of adenosine triphosphate, which eventually inactivate or kill the bacteria [19, 24]. In this study, complete inhibition of microbial contamination of fish skin was not achieved even with increasing the contact time till 15 min. This may be attributed to the fact that CHG is a more potent antiseptic against gram-positive than gram-negative bacteria and the development of resistance to gram-negative bacteria such as Pseudomonas spp. [25, 26].

On the other hand, the arrangement and content of the collagen fibers were not affected after CHG treatment for 5 min. However, long exposure to CHG for 10 or 15 min resulted in dissociation and disintegration of collagen fibers with decrease in the collagen intensity. Our results are in accordance with that reported by Alomar et al. (2012) [19] and are contradictory to Alves et al. (2018) [11].

PVP-I is routinely used as an antiseptic agent with a broad spectrum activity against bacteria, fungi, and yeast. It is a complex of polyvinylpyrrolidone iodine that gradually releases iodine as a free iodine, which provides antimicrobial efficacy through lipid iodination and oxidation of cytoplasmic and membrane compounds. Additionally, polyvinylpyrrolidone has a high affinity for cell membranes, supplying free iodine directly to the target cell and contributing to rapid microbial kill [24, 27, 28].

The current study demonstrated that fish skin treated with PVP-I had significantly reduced microbial count up to 100% by increasing the contact time up to 15 min. These findings were in accordance with Mann-Salinas et al. (2015) [22]. Although PVP-I has a minimal toxic potential compared
to CHG, it also induced alterations in the collagen fibers (dissociation and disintegration of collagen fibers) of the tilapia fish skin treated with PVP-I for 10 or 15 min, while, these changes were not noted in skin samples submitted for 5 min treatment.

Silver has been widely used in chronic wounds and burns care. Moreover, a number of silver-impregnated dressings have been developed [29–32]. Silver has broad spectrum antimicrobial properties against a wide spectrum of gram-positive and gram-negative bacteria. It binds to negatively charged components in proteins and nucleic acids, thereby effecting structural changes in bacterial cell walls, membranes, and nucleic acids that affect viability. It is believed that it inhibits proteins by binding to and denaturing their thiol groups and preventing DNA replication by its condensation [30–32]. Therefore, silver impregnation of biological-derived wound dressings has been recently used and it has been reported that silver impregnation of amniotic membranes combats microbial infection without any change on the physical characteristics [13, 23].

Interestingly, the microbial growth was rapidly and completely vanished by using AgNPs as an antiseptic for fish skin at all used treatment times (5 to 15 min) compared to CHG or PVP-I. Furthermore, the rapid antimicrobial activity of AgNPs is an obvious option so that the operative time is not extended beyond the minimum necessary.

By controlling the bioburden, AgNPs facilitate less toxic metabolites production, which result in a better collagen fibers preservation in the Tilapia skin. Furthermore, eukaryotic cells are less prone to silver toxicity than prokaryotes, which is thought due to an increased degree of structural and functional stability in eukaryotes [33].

Conclusion
Sterilization of Tilapia fish skin, used as a biological dressing, using AgNPs is effective with a 100% reduction against microbial growth and without any change in their microscopical properties. This is the first study to report excellent performance of AgNPs in sterilization of the Tilapia fish skin as a biological dressing. Our findings could contribute towards to a quick and efficient sterilizer procedure to treat biological dressings relevant to clinical applications.

Methods

Fish Skin Collection
Tilapia skin was collected from fresh Nile Tilapia (*Oreochromis niloticus*) fish (700 ± 40 gm, 22 ± 3 cm standard length), which obtained from the tanks of the Aquatic Unit, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt. Immediately after the fish were euthanized physically by decapitation following the American Association of Zoo Veterinarians (AAZV) Guidelines [34], fresh skin samples were obtained by sharp and blunt dissection from the underlying muscles and then transected into strips 3 × 3 cm². Tilapia skin strips were subjected to three different sterilization procedures, each at three contact times (5, 10, and 15 min) as described below.

Sterilization Procedures
Sterilization procedures were carried out using three different sterilizing agents; the chlorhexidine gluconate 4% (CHG) (Hibiclens Mölnlycke Health Care, Norcross, GA), povidone iodine 10% (PVP-I) (BETADINE, El-Nile Co. for Pharmaceutical and Chemical Industries, Egypt), and silver nanoparticles (AgNPs) (25 µg/mL). The Tilapia skin strips were allocated randomly into four main groups (3 skin strips for each); CHG, PVP-I, and AgNPs groups. Each group was then incubated for different contact time points (5, 10, and 15 min). In addition to a control group (C) treated with normal saline. The tilapia skin strips were subjected to bacteriological and histological evaluation following each sterilization treatment at the indicated time points.

Preparation of Silver Nanoparticles
Stable silver nanoparticles (AgNPs) less than 100 nm were synthesized in a typical one-step protocol as described before [35]. Briefly, 1.0 gm of soluble starch was added to 100 mL of deionized water and heated until complete dissolution. One mL of 100 mM aqueous solution of silver nitrate (AgNO₃) crystal (ACS AgNO₃, F.W 169, 87 Gamma laboratory chemicals, Assay: Min 99.0%) was added and stirred well. This mixture was put into a dark glass bottle and autoclaved. The resulting solution was clear yellow in color indicating the silver nanoparticles formation.

The stock solution of AgNPs was kept in dark glass bottles away from direct sunlight and at room temperature (25°C). The concentration and size of the particles were measured before using. The morphology and size of AgNPs were measured by transmission electron microscopy (TEM) (JEOL-JEM-100CX II) at the Electron Microscopy Unit, Assiut University (S1 Fig.). The concentration of AgNPs stock was measured by Graphite Furnace Atomic Absorption Model 210VGP at the Faculty of Science, Assiut University.

Microbiological Evaluation
Microbiological evaluation of the fish skin strips was performed according to APHA (2005) [36] and Qazi et al. (2008) [37]. An area of 3 × 3 cm² was aseptically swabbed for microbial count. Each of Plate count agar “HMESIA-Ref-M091A”, Manitol Salt Agar “Oxoid CM0085”, MacConkey Agar
OMRI W/O Crystal violet “Biolife ™” and Difco™ Potato Dextrose Agar- Ref 213400 were used for Total viable bacteria, Staphylococci, Coliforms, Yeasts and Molds count, respectively.

**Sterilization Efficiency**

The efficiency of each sterilization procedure was calculated by comparing the bacterial count before and after each treatment for each contact time via the following equation:

\[
\text{Disinfection efficacy} \% = \frac{(C_0 - C)}{C_0} \times 100, \text{ where } (C_0) \text{ is the initial bacterial count (control negative) and (C) is the count of bacteria after a certain contact time of the sterilizing agent [38].}
\]

**Histological Evaluation**

Specimens (0.5 × 0.5 cm) of the Tilapia skin strips were collected from the groups following each sterilization treatment at different times and fixed in 10% neutral buffered formalin. The formalin-fixed skin samples were routinely processed and embedded in paraffin. They were then sectioned at 5 μm thickness and stained with Mayer's hematoxylin (Merck, Darmstadt, Germany) and eosin (Sigma, Missouri, USA). Afterwards, the slides were examined microscopically and the histological evaluation was performed in a blind fashion on coded samples, and a comparison was made with the sections from the control-treated group.

**Histochemical Staining of Collagen**

Gomori's trichrome stain was used for evaluation of the collagen content [39]. Paraffin-embedded sections were deparaffinized in xylene and rehydrated in a graded series of ethanol solutions (into 0.1 M phosphate-buffered saline, pH 7.2) to distilled water. The sections were then stained with Gomori's trichrome stain following the manufacturer's protocol, dehydrated in graded alcohol, made transparent with xylene, and mounted. Afterwards, slides were observed under the microscope to check for collagen staining with green color. The collagen content was evaluated according to the depth of the green color, the organization, and disintegration of the collagen.

Using threshold area fraction determination, the percentage of collagen positive area was calculated using ImageJ [11]. The amount of collagen was reported as a percentage from the total number of pixels in the optical view as a percentage and expressed as mean ± SEM.

**Negative Image Study Using CMEIAS Color Segmentation**

An improved computing technology has been used to process color images by segmenting background object pixels from the foreground object [40, 41], making this technique especially useful due to the complex color that existed for this analysis.

**Statistical Analyses and Software**

Statistical analyses were performed by one-way ANOVA using GraphPad Prism software version 5.03 (GraphPad Software Inc., La Jolla, CA, USA). \( p \) values of less than 0.05 were considered statistically significant. Figures were generated using Adobe Photoshop CS6 and Prism 5 version 5.03.

**Abbreviations**

CHG: Chlorhexidine gluconate; PVP-I: Povidone iodine; AgNPs: Silver nanoparticles; AAZV: American Association of Zoo Veterinarians; AgNO\(_3\): Silver nitrate; TEM: Transmission electron microscope; APHA: American Public Health Association.

**Declarations**

**Ethical approval:**

The present study was approved by the National Ethical Committee of the Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt. The study protocol was according to the OIE standards for the use of animals in research and teaching.

**Consent for publication:**

Not applicable

**Availability of data and material:**

From the corresponding author.
Competing interests:
The authors declare that they have no competing interests.

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Authors' contributions:
A.I., S.K., and M.S., designed the study; A.I., S.K., N.K., and M.S., performed the experiments; A.I., S.K., D.H., and M.S., analyzed the data; A.I., S.K., and M.S., helped data interpretation; A.I., S.K., and M.S., discussed the results; M.S., wrote the manuscript; A.I., S.K., and M.S., edited the manuscript.

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Tables

Table 1. Mean value of microbial count before and after application of antiseptics.
| Treatment                     | Time (min) | Aerobic bacterial count | Staphylococcal count | Coliform count | Yeast and mold count |
|-------------------------------|------------|-------------------------|----------------------|---------------|----------------------|
|                               |            | (Mean value ± S.E)      |                      |               |                      |
| Control                       |            | 5x10⁴±1.32              | 1.8x10⁴±0.89         | 2.2x10⁴±1.79  | 1.2x10⁴±1.29         |
| Chlorhexidine gluconate       | 5          | 2.4x10⁴±0.92            | 52%                  | 7x10³±1.86    | 61.11%               | 1x10⁴±0.45          | 54.54%              | 7x10³±0.11          | 41.66%              |
|                               | 10         | 6x10³±1.54              | 88%                  | 1x10³±1.02    | 94.44%               | 2x10³±2.32          | 90.90%              | 2x10³±1.92          | 83.33%              |
|                               | 15         | 0.1x10³±1.82            | 99.80%               | 0.0±0.0       | 100%                 | 0.1x10³±0.82        | 99.54%              | 0.0±0.0             | 100%                |
| Povidone-iodine               | 5          | 1x10³±0.92              | 80%                  | 6x10³±1.87    | 66.66%               | 4x10³±1.02          | 81.81%              | 2x10³±1.02          | 83.33%              |
|                               | 10         | 1x10³±0.52              | 98%                  | 1x10³±0.98    | 94.44%               | 0.0±0.0             | 100%                | 0.0±0.0             | 100%                |
|                               | 15         | 0.0±0.0                 | 100%                 | 0.0±0.0       | 100%                 | 0.0±0.0             | 100%                | 0.0±0.0             | 100%                |
| Silver nanoparticles          | 5          | 0.0±0.0                 | 100%                 | 0.0±0.0       | 100%                 | 0.0±0.0             | 100%                | 0.0±0.0             | 100%                |
|                               | 10         | 0.0±0.0                 | 100%                 | 0.0±0.0       | 100%                 | 0.0±0.0             | 100%                | 0.0±0.0             | 100%                |
|                               | 15         | 0.0±0.0                 | 100%                 | 0.0±0.0       | 100%                 | 0.0±0.0             | 100%                | 0.0±0.0             | 100%                |