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Nile tilapia skin (*Oreochromis niloticus*) for burn treatment: ultrastructural analysis and quantitative assessment of collagen

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ABSTRACT

Nile tilapia (*Oreochromis niloticus*) skin is a well-known biomaterial used as an occlusive dressing for burn treatment. It is also an inexpensive and important source of collagen. This study aims to describe the ultrastructural aspects of Nile tilapia skin, assess its collagen amount and organization, and compare quantitative methods of histochemical and immunohistochemical analysis (in all sterilization steps for use in burn dressings). One sample (0.5 × 0.5 cm) of ten different fish skins was divided in four groups: *in natura* skin (IN), chemical sterilization (CH), additional irradiation (30 kGy) (IR), and skins used in burn treatment (BT) to compare histochemical and immunohistochemical findings of collagen amount and describe ultrastructural aspects through scanning electron microscopy. The amount of type I collagen decreased during sterilization and clinical use owing to gradual reduction of immunostaining (anti-collagen-I) and decreasing fiber thickness of the collagen, when compared to type III (Picrosirius-red-polarized light). The collagen fibers were rearranged at each sterilization step, with a low collagen percentage and large structural disorganization in BT. The amount of type-I collagen was further reduced after BT ($p < 0.05$). Both the methods did not exhibit a quantified value difference ($p = 0.247$), and a positive correlation ($r = 0.927$; 95 % CI = 0.720–0.983) was observed between them, with concordance for collagen quantification in similar samples, presenting a low systematic error rate (Dalberg coefficient: 6.70). A significant amount of type-I collagen is still observed despite sterilization, although clinical application further reduces type I collagen. Its quantification can be performed both by immunohistochemistry and/or Picrosirius Red reliably.

1. Introduction

Collagen, as a primary component of the extracellular matrix, plays an important role in maintaining its biological and structural integrity. This molecule has a triple-helix structure (Cen et al., 2008), with more than 29 different types described. Type I collagen (COL-I) is most commonly found in humans in the bone, skin, teeth, fibrocartilage, and tendons and has the unique ability to form highly-resistant fibrils (Gómes-Guillén et al., 2011). The chemical composition and structure of this extracellular matrix component enables numerous applications, specifically in the composition of biomaterials used in tissue engineering. COL-I has been used in numerous applications, such as drug delivery, skin substitutes, suturing, and tissue engineering substrates (Tang and Saito, 2015).

The development of biomaterials, mostly containing collagen as the main constituent, has gained considerable support because it improves

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their biocompatibility. Therefore, collagen is used widely in different presentations as biological dressings, even in association with other products and regenerative techniques, most of which are heterogenous biomaterials of porcine or bovine origin (Guo et al., 2016; Mathangi et al., 2013; Norbury et al., 2016).

However, zoonotic infectious diseases and allergic problems may be considered when using bovine or porcine collagen. In addition, there are some religious restrictions in many countries on the application of certain mammalian isolates (Sun et al., 2017; Tang and Saito, 2015). Therefore, other sources of COL-I must be explored. In this context, tilapia skin has been studied, which could be an alternative source of collagen in addition to those already recommended.

Yamamoto et al. (2014) showed that COL-I extracted and purified from tilapia fish scales is easily adsorbed than bovine and porcine collagen (Yamamoto et al., 2014). Because of this effect, tilapia fish COL-I has gained popularity in the cosmetic industry (Tang and Saito, 2015) and has been described as a safe substance without adverse skin reactions following intracutaneous and topical application (Yamamoto et al., 2014). Furthermore, Matsumoto et al. (2015) demonstrated that tilapia fish COL-I is superior to porcine skin COL-I in inducing human mesenchymal stem cell differentiation (Matsumoto et al., 2015).

In addition, our group showed that tilapia skin has satisfactory histological and mechanical properties, consistent with those of human skin, which justifies the scientific investments in using it as a biomaterial (Alves et al., 2015, 2018).

Nile tilapia (*Oreochromis niloticus*) belongs to the Cichlid family and originates from the Nile basin in East Africa. This fish is found in the tropical and subtropical regions of the world. In particular, it is abundantly found in the state of Ceara, where it is an important commercial product because of its availability and low cost. It is known for its meat quality and mild taste and is an important product in regional gastronomy (Alves et al., 2015; Franco et al., 2013; Lineen and Namias, 2008).

In Ceara, there is a heavy consumption of Nile tilapia fish generating by-products, such as skin, intended for disposal. After the Ethics Committee approval, burns were treated successfully using tilapia skin as an occlusive dressing, which has gained worldwide attention (Lima Júnior et al., 2020, 2021). It allows tissue repair and serves as a physical barrier in addition to preventing dehydration, sepsis, and metabolic and functional complications (Boateng and Catanzano, 2015; Inoue et al., 2016; Lineen and Namias, 2008). The use of tilapia skin has transcended its application in burns and is also used in other soft tissue reconstructive surgeries, such as neovaginoplasty (Bezerra et al., 2018; Dias et al., 2020; Pinto Medeiros Dias et al., 2019) and male-to-female gender-affirming surgery (Rodríguez et al., 2020).

In tissue engineering, the discovery of products with promising properties often occurs empirically; for example, the amniotic tissues, which were first used in 1938 (Rötth De, 1940) in reconstructive eye surgery. Currently, the amniotic membrane is widely used in several medical fields, including burn treatment (Clare et al., 2012).

Currently, knowledge regarding the histological and micromorphological aspects of this naturally-derived biomaterial is limited (Lima-Verde et al., 2020). Therefore, this study aims at describing the ultrastructural aspects of Nile tilapia skin, assess its collagen amount and organization, and compare quantitative methods of histochemical and immunohistochemical analysis (in all sterilization steps for use in burn dressings).

2. Material and methods

2.1. Sample gathering, sterilization protocol, and study groups

Nile tilapia (*Oreochromis niloticus*) skin samples were obtained from fish farms in Castanhão (Jaguaribara-CE). Fish are raised and sacrificed at approximately 800–1000 g, through stunning by thermal shock and subsequent bleeding. The skins were removed, cleaned, and cut in a standardized manner for further processing.

For this study, 10 different fish skins were used with four groups established according to the evaluation phase (stages of the sterilization process or clinical application) (Fig. 1).

In the *natura* skin group (IN), samples were cleaned only with saline solution (natural fish skin). In the chemical sterilization (CH) group, skins were subjected to successive 30-minute baths in 2 % chlorhexidine. Sequentially, they were subjected to three baths in glycerol with crescent concentrations (50 %, 75 %, and 99 %), followed by 1% penicillin/streptomycin/fungizone; each step lasted 5 min. In irradiation group (IR), skins were, at first, individually packaged into double plastic (hermetically sealed) envelopes and submitted to additional sterilization by radiation (30 kGy) (Alves et al., 2018).

Irradiated Tilapia skins were stored under refrigeration (2–8 °C) until their clinical use for burn care in four different participants of a phase II randomized controlled trial (Lima Júnior et al., 2020). Prior to dressing application, rehydration was performed in a saline solution. After treatment was completed (approximately 15–21 days, depending on each patient's healing response and the extent of the wound), the skin that was in close contact with the wound was collected for analysis, comprising the last study group (BT) (case-control ratio = 2:1).

All collected samples (0.5 × 0.5 cm) were fixed in 10 % formaldehyde (pH = 7.4) or 0.05 M cacodylate buffer + 2.5 % glutaraldehyde + 4 % formaldehyde solution for optical and scanning electron microscopy, respectively.

2.2. Histochemical analysis – picrosirius red

Paraffin blocks were prepared by semi-automatic histological processing (LupeTec®, PT05) of 10 % formalin-fixed skins. After microtomy (4 μm), (Leica Microsystems®, RM2235) microscopic slides were deparaffinized, dehydrated, and stained by the conventional hematoxylin & eosin (H&E) method for histological analysis of sample cutting and representativity. Subsequently, new sections were submitted to Picrosirius Red's histochemical reaction (Easypath®) according to the manufacturer's instructions.

Histochemical reactions were performed with 3 μm paraffin block slices, using Picrosirius Red staining, and the collagen content of the skin samples was analyzed and typified in collagen I (yellowish-red-birefringence) and III (whitish-green birefringence), through light polarization (Leica DM 2000), as described in previous studies (Alves et al., 2018, 2015; Yuan et al., 2011).

Photomicrographs of three microscopic fields were captured (Leica Microsystems®, DFC295) from each sample at 400× magnification (comprising the entire length of the sample). The collagen I percentage was calculated using the color threshold command (Image > Adjust > Color Threshold) in ImageJ® software, regulating red and green colors on RGB color channels. By selecting the area of interest and isolating COL-I, the image was binarized (Process > Binary > Make Binary) and the fractional area percentage was calculated (Analyze > Analyze Particles). Thus, the COL-I percentage was calculated based on the percentage of total collagen in each photomicrograph (quantified without color threshold command) (Alves et al., 2018). The average of the three fields was considered as the sample unit.

2.3. Immunohistochemical analysis

Immunohistochemical reactions were performed with 3 μm paraffin block slices using the streptavidin-biotinylated technique (Hsu et al., 1981). Antigenic recuperation was performed by heating in citrate solution (pH 6.0), followed by incubation with a primary antibody for COL-I (ab23730, 1:500, overnight). Tuna skin was used as a positive control, while the negative control was the suppression of the primary antibody. The immunostained areas were visualized using the Universal Immune-peroxidase Polymer (Histofine®, for Abcam® primary antibodies; 30 min), followed by DAB (5,5-diaminobenzidine

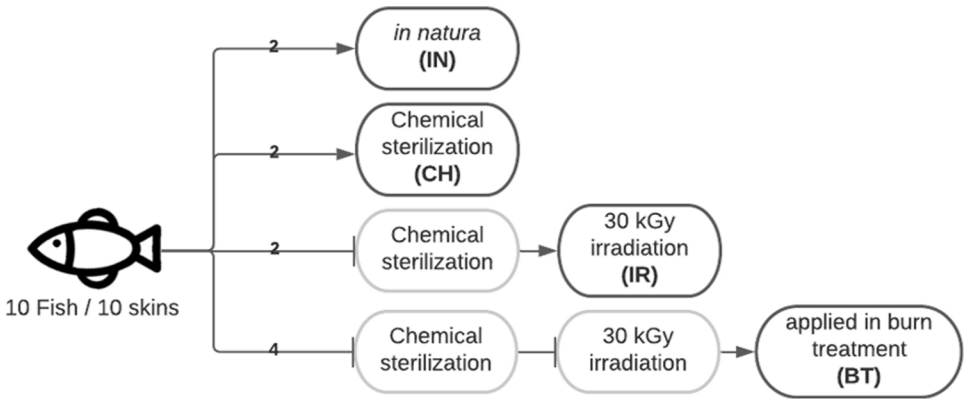

Fig. 1. Flowchart describing number of skins used in each of the four groups, according to evaluation phase.

tetrahydrochloride) and Harris' hematoxylin counterstaining. Entellan® was used as the mounting medium for all slides.

For microscopic evaluation (Leica Microsystems®, DM2000), photomicrographs of three microscopic fields were taken (Leica Microsystems®, DFC295) from each sample at 400x magnification (comprising the entire length of the sample). The marked area percentage was calculated by the color deconvolution command (Plugins > Color Function > Color Deconvolution) using ImageJ® software, by selecting DAB + Hematoxylin option (H DAB), and using color stratification compatible with DAB marking. Thus, the image was binarized (Process > Binary > Make Binary), and the fractional area percentage was calculated (Analyze > Analyze Particles). The COL-I percentage was calculated based on the percentage of the total area in each photomicrograph (quantified without deconvolution command), considering the average of the three fields as sample units (Zhou et al., 2015).

2.4. Scanning electron microscopy (SEM) analysis

Samples fixed in 2.5 % glutaraldehyde, 4 % formaldehyde, and 0.05 M sodium cacodylate buffer solution were washed three times with 0.05 M sodium cacodylate buffer, dehydrated in an acetone series, and critical point dried. After mounting in stubs, the samples were sputter covered with gold (Quorum QT-ES) for observation under a scanning electron microscope (Quanta® 450 FEG-FEI) for structural and descriptive analysis of collagen. One representative image of each group was performed at 2500× and 80000× magnification after analysis to allow visualization of the collagen fiber structure and its possible alterations. The analysis was performed at 20 kV.

2.5. Statistical analysis

Quantitative data were expressed as mean and standard error, submitted to the Kolmogorov–Smirnov normality test, and compared using paired Student's *t*-test for comparison between two evaluation methods (histochemical and immunohistochemical). In addition, measurements were validated using Pearson's correlation and Dahlberg's random systematic error measurement analysis through the calculation of interclass agreement coefficient. ANOVA/Bonferroni was employed for comparison between different groups (parametric data) (SPSS 20.0, $p < 0.05$).

3. Results

3.1. Histochemical and immunohistochemical descriptive analyses

The amount of type I collagen decreased during sterilization and clinical use owing to a gradual reduction in immunostaining (Fig. 2A, C, E, G) and decreasing fiber thickness (Fig. 2B, D, F, H) of the collagen, compared to the thickness of type III collagen fibers. *In natura* skin (IN) samples confirmed the morphological findings of previous studies performed by this group, showing the preservation of the collagen fiber morphology, with parallel and vertex shape disposition, and now evidencing an abundance of type I collagen (Fig. 2A and B), as shown by immunohistochemical staining and Picrosirius Red staining methods.

In contrast, the CH group showed a homogeneous decrease in immunostaining for type I collagen throughout the samples (Fig. 2C). In parallel, disorganization of the collagen fiber arrangement can be observed using both methods. Picrosirius Red, in turn, discriminates this disorganization by showing a decrease in the thickness of type I collagen fibers, when compared to type III collagen fibers (Fig. 2D).

After irradiation (IR group), tilapia skin samples exhibited a reestablishment of immunostaining for type I collagen, as well as a reorganization of collagen fibers (Fig. 2E), which can be exemplified by the restructuring of the thickness of these structures when compared to type III collagen fibers (Fig. 2F).

After clinical application of burn treatment (BT), skin samples did not undergo the same changes observed after chemical sterilization. Although no morphological disorganization of the fibers was observed, the immunostaining for type I collagen (Fig. 2G) and fiber thickness (observed by Picrosirius Red) (Fig. 2H) exhibited a prominent reduction. Residual inflammatory cells were also observed in these samples.

Both optical and electron microscopy observations showed an apparent rearrangement of collagen fibers at each sterilization step (Figs. 2 and 6).

3.2. Collagen quantification and correlation between methods

In both methods (immunohistochemical staining for anti-collagen I and Picrosirius Red histochemical reaction), there was a decrease in the amount of COL-I during sterilization and after clinical application. The IN skin (74.53 ± 1.47) presented a higher amount of COL-1 than CH, IR, and BT ($p < 0.05$). In addition, CH (63.08 ± 3.73) and IR (63.05 ± 2.07), despite being associated with a decrease in the amount of collagen compared to IN, present higher values in relation to BT (43.61 ± 1.49). This reinforces that factors related to clinical application likely interfere with the amount of COL-1 ($p < 0.005$) (Fig. 3).

Comparing the collagen values obtained between the COL-I immunohistochemistry technique and the histochemical staining of polarized Picrosirius for reddish yellow birefringence (suggestive of COL-I), no differences were found in the quantified values ($p = 0.247$/Student`s t test). Thus, both techniques were equally effective in identifying this type of collagen (Fig. 4).

In the error measurement analysis, the two methodologies were compared in different samples, and they were able to identify similar amounts of collagen, without differences between them ($r = 0.927$, ICC $= 0.938$). In linear regression, a large proximity was observed between

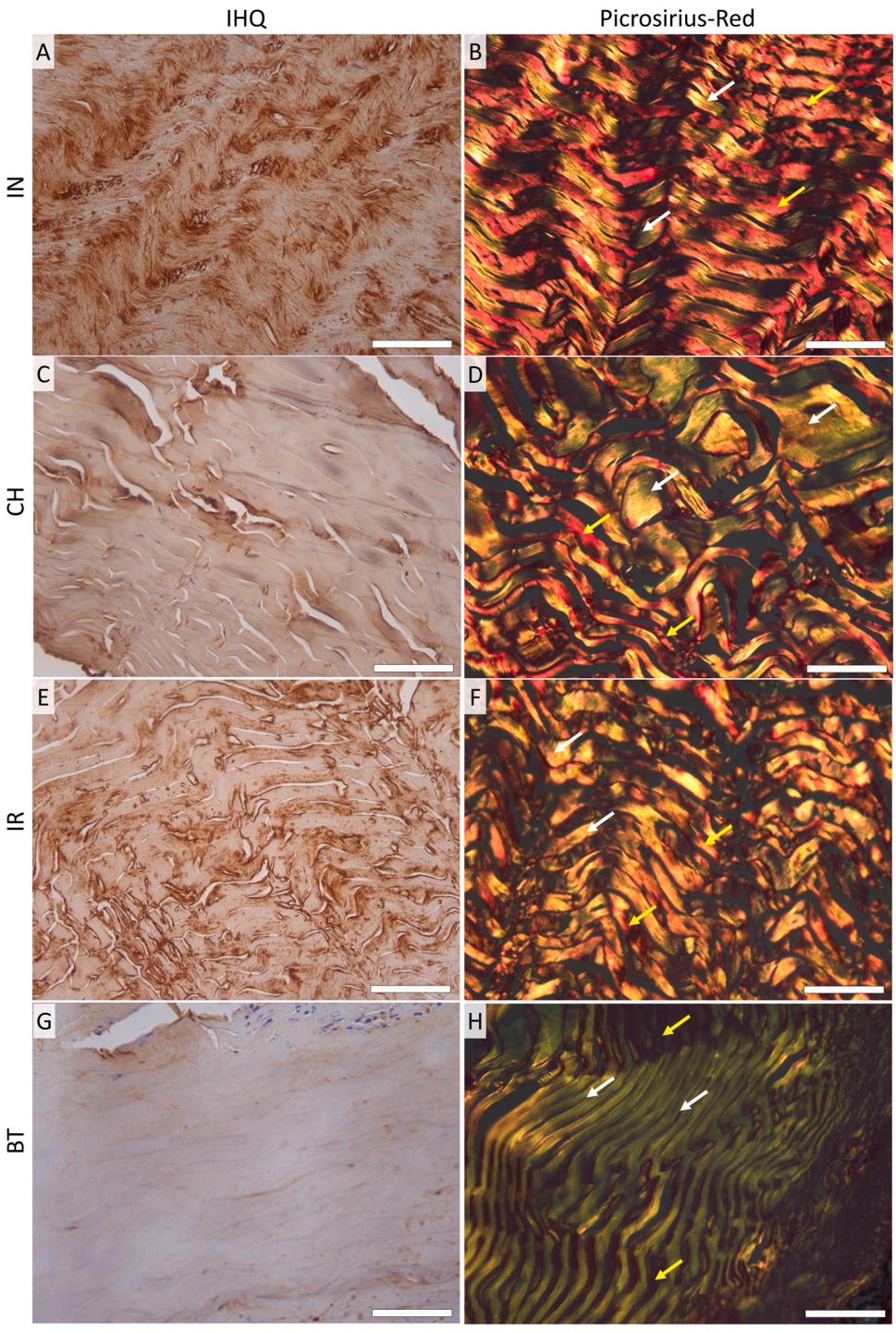

Fig. 2. Microscopical features of tilapia *in natura* skin (IN), after chemical (CH) and additional radiation (IR) sterilization, and after clinical use (BT) samples under immunohistochemical (IHC) and histochemical (Picrosirius Red) – polarized light – techniques perspective (400x), showing a reduction in the amount of type I collagen, during the steps of sterilization and clinical use, as observed in reducing immunostaining (A, C, E, G) and decreasing fiber thickness (B, D, F, H) of type I collagen (yellow arrows) compared to the thickness of type III collagen fibers (white arrows). Bars: 50 μm.

the points and the midline, by analyzing the correspondence between the two techniques in the different samples, with a low systematic error index (Dalberg's coefficient: 6.70) (Fig. 5).

3.3. SEM analysis

Structural and descriptive analysis of collagen revealed a sample (IN) composed in large part by the presence of a well-preserved fibrillar collagen structure (Fig. 6A).

The results of the methodologies using optical microscopy were validated by SEM analysis, because it was possible to perceive the presence of collagen fibrillar structure with overlap of material suggestive of chemical substance in CH group. Moreover, the helical structure that characterizes the collagen fibers was less evident (Fig. 6B).

After rehydration, the sterilization process was enhanced by additional irradiation (IR), leaving the sample free of any impurity and preserving the fibrillar structure of collagen by eliminating the debris (Fig. 6C), despite the modifications observed by optical microscopy.

Finally, the inflammatory process that characterizes the lesions to which this skin is intended for clinical use (BT) had a direct impact on the integrity of type I collagen fibers (evidenced by optical and electron microscopy). The SEM analysis results showed both the partial degradation of these fibers and presence of organic material and cellular content, including cell structures likely originated from the surgical

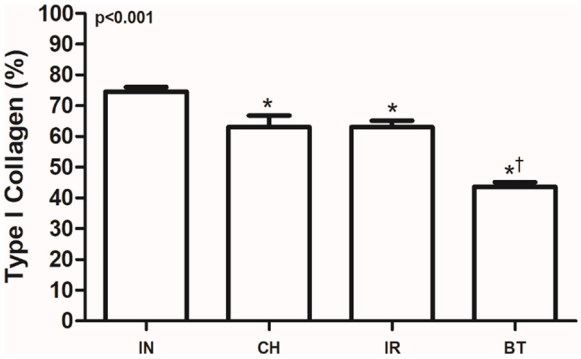

Fig. 3. Quantification and comparison of COL-1 amount between groups, showing that IN samples present a higher amount when compared to other groups. Additionally, burn treatment seems to further reduce COL-1 amount (p = 0.247/ANOVA/Bonferroni). Caption: IN: *in natura*; CH: chemical sterilized; IR: irradiated group; BT: burn treatment group.

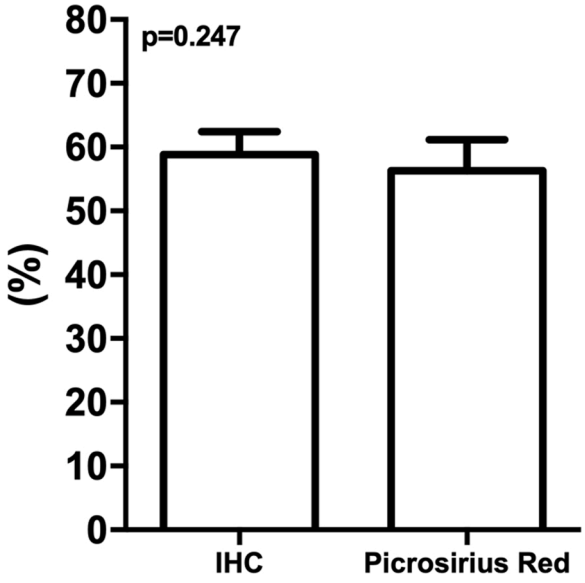

Fig. 4. Quantification of COL-I by immunohistochemical (IHC) and histochemical (Picrosirius Red) techniques (p = 0.247/Student's t test) for collagen quantification.

wound and by interspersion of the collagen fiber network. Most parts of the cell structures are still preserved (Fig. 6D).

4. Discussion

Morphological observation of tilapia skin collagen after different steps of the sterilization protocol and clinical use and its quantification (total and in type I and III) using different methods (immunohistochemistry and Picrosirius-Red) reinforced the potential of this byproduct as an abundant source of COL-I and raw material for tissue engineering.

Collagen constitutes approximately 30 % of the total protein content of animals. This protein accounts for triple-helix structures consisting of nearly identical polypeptide chains (Sell et al., 2009), which offers them with great tensile strength and thermal stability (Mogilner et al., 2002), thereby showing its potential use as a raw material for different clinical products.

The dermis structure, which is the primary constituent of the integument, has approximately 80 % of its dry weight composed of COL-I. It exists in the form of loosely interwoven, large, wavy, randomly oriented

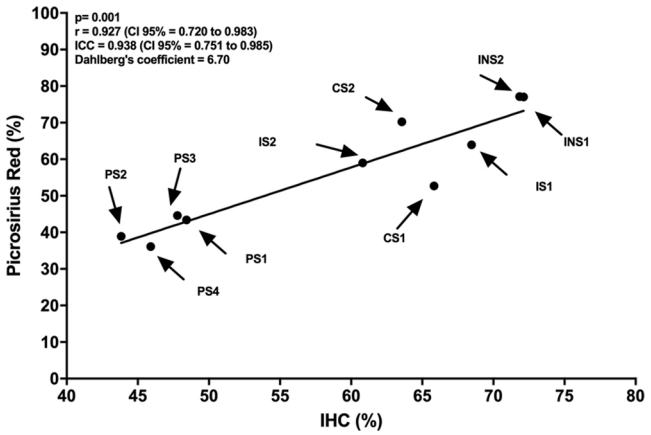

Fig. 5. Correlation plot for COL-I detection between histochemical (Picrosirius Red) and immunohistochemical (IHC) techniques in *in natura* skin (IN), after chemical (CH) and additional radiation (IR) sterilization, and after clinical use (BT) samples. Positive correlation (r = 0.927; 95 % CI = 0.720 to 0.983) could be observed between the two techniques performed, with cordage for collagen quantification in similar samples, presenting low systematic error rate (Dalberg coefficient: 6.70).

bundles, and provides skin protection and resistance properties (Waller and Maibach, 2006). Recently, this collagen has been preferentially used in regenerative medicine because of its predominance in the extracellular matrix. Therefore, it has high biocompatibility, low antigenicity, and can be extracted by different methods, using chemical and enzymatic processes (Song et al., 2019).

Collagen fibers have excellent tensile strength and thermal stability because of their three left-handed helices supercoiled right-handed around a common axis, constituting the triple helix structure (Mogilner et al., 2002). Nile tilapia has been studied for its potential use in biomaterials. Acid-soluble and pepsin-soluble collagen extracted from Nile tilapia skin have typical COL-I characteristics (Kittiphattanabawon et al., 2019).

Tilapia skin is a COL-I-rich biomaterial and the amount of this important component depends on the processing steps of the biomaterial (Mogilner et al., 2002)

In our study, however, tilapia skins were analyzed at different stages of sterilization and after their clinical application. This analysis revealed a decline in COL-1 levels after the skin underwent irradiation and chemical sterilization, as well as a remarkable reduction after clinical use.

This reduction is caused by the action of inflammatory cells in the wound bed. Inflammatory wound parameters in tilapia skin treatment were evaluated in previous experimental studies, demonstrating a mild-to-moderate mononuclear inflammatory phenotype in tilapia skin-covered wounds, which positively influenced the healing process, compared to the control group (in which acute inflammatory reactions were evidenced) (Lima-junior et al., 2017).

Despite all the inflammatory infiltrates, the collagen of tilapia skin was not completely damaged; superficial layers were maintained, which guaranteed the permanence of the occlusive dressing until complete healing of the patient's skin (Lima Júnior et al., 2020).

Thus, tilapia skin exerts a protective effect on the wound beyond the biological buffer and the water content reduction, and acute inflammatory reactions were softened in the wound bed and directed to the dressing (which, because of COL-1 abundance, was degraded by the inflammatory action, as observed in other conditions) (Kotaniemi et al., 2003).

This study also revealed that the Picrosirius-Red histochemical technique demonstrated a similar sensitivity, when compared with the immunohistochemical technique, in detecting COL-I in different tilapia skin substrates.

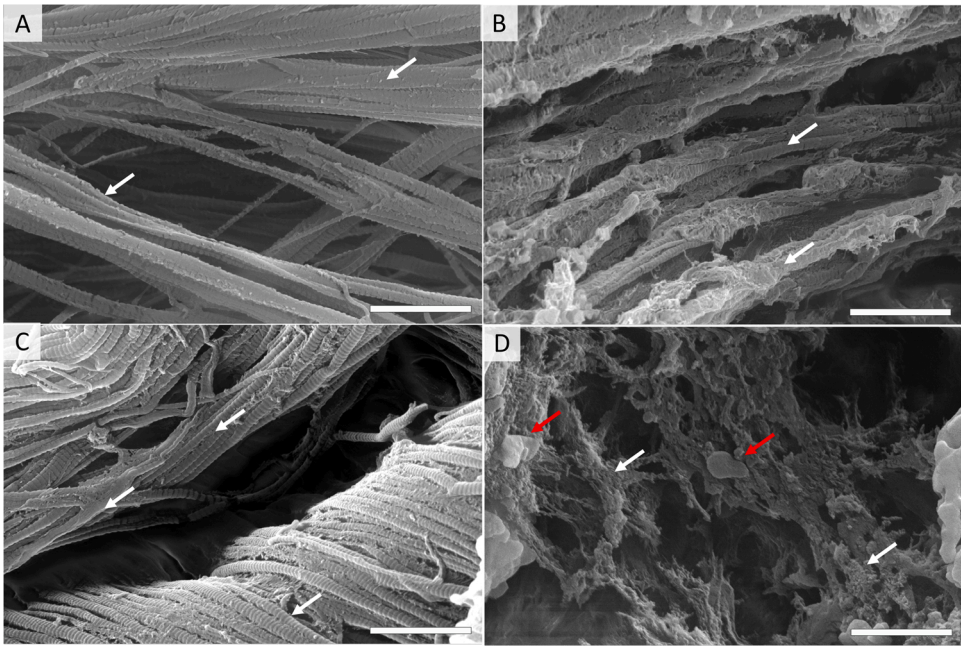

Fig. 6. Scanning electron microscopy of tilapia skin samples, showing the clear striated structure of collagen fibers (white arrows) in IN (A) and IR (C) samples, while CH (B) and BT (D) samples present areas suggestive of chemical or organic (red arrows) substances overlapping, respectively. Bars – 1 μm.

The different types of collagen have been visualized using various techniques. Scanning and transmission electron microscopy offer high-resolution visualization of individual collagen fibers but are expensive, involve complicated specimen preparation, and offer nanoscale detail that is excessive for many applications and do not differentiate the collagen (Starborg et al., 2013). In addition, second harmonic generation imaging is sensitive, specific for fibrillar collagen, and compatible with automated quantification methods but is relatively expensive as it requires multiphoton microscopy (Drifka et al., 2016).

Histochemical dyes, such as Picrosirius Red, are resistant to fading, are inexpensive, and provide reproducible staining (Junqueira et al., 1979). The Picrosirius staining method relies on the elongated, anionic structure of the Sirius red dye molecule, which binds parallel to cationic collagen fibers. This staining, under linear polarized light, enhances the natural birefringence of collagen bundles, which are revealed as red, orange, yellow, or green fibers. One limitation of this technique is that sample orientation under linearly polarized light substantially affects the Picrosirius hue and signal strength (Borges et al., 2005; Wegner et al., 2017).

The Picrosirius staining is a cheaper technique with the same ability to detect collagen, such as immunohistochemistry (Vogel et al., 2015). Lattouf et al. (2014) used both techniques for detecting type III collagen on different normal and pathological tissue substrates and suggested that the picrosirius polarization method is simple, sensitive, and specific for collagen staining (Lattouf et al., 2014). It is useful to reveal the molecular order, organization, and/or heterogeneity of collagen fiber orientation in different connective tissues. For this, however, the author compared only the raw values found in both techniques and a descriptive analysis of regions stained by immunohistochemistry and green refringence in Picrosirius. Therefore, a quantitative study is essential to validate the correlation of these analyses and comparatively evaluate their applicability in a more reliable manner.

To date, only one study, per our knowledge, recently published in 2017 reveals that Picrosirius red has similarity with Fluorescence staining and immunohistochemistry for different types of collagen by quantitative analysis (without performing statistical correlation analysis between methods). This study also suggests that each subtract should have its properties previously analyzed to use the Picrosirius technique as a safe tool for collagen quantification, as performed in the present study for tilapia skin (Wegner et al., 2017).

The literature shows conflicting results regarding different sterilization methods and modification of collagen properties in different extracts. Alomar et al. (2012) suggested that low chlorhexidine concentrations up to 2% did not alter the organizing properties of collagen or its mechanical characteristics in tendon-derived grafts based on large amounts of collagen; however, the same author stated that these characteristics could be modified at higher concentrations (4 %) (Alomar et al., 2012). Other studies analyzing the effect of various chemical protocols, including ethylene and peracetic acid, suggested that this type of sterilization could result in residue impregnation and collagen cross-link alteration (Faraj et al., 2011).

Delgado et al. (2014) demonstrated that chemical graft sterilization demonstrates the lower agglomeration of collagen fibers when compared to other techniques, a structural characteristic that may influence their mechanical properties.

In our study, skins submitted to chemical sterilization obtained smaller amounts of type I collagen and presented more disorganized fibers. Electron microscopy confirmed that the fibers also presented a large amount of material overlapping the fibrils, probably residual to the chemical process.

There are few noteworthy studies with chlorhexidine protocols similar to those performed in the present study, such as collagen type quantification. In addition, the literature indicates that different substrates respond differently to sterilization protocols, and prior analysis is important to select the best form for each biomaterial.

Regarding irradiation sterilization, it was evidenced in the present study that this mechanism resulted in smaller amounts of type I collagen and thinner fibrils in relation to fresh skin, but these characteristics were superior to chemically sterilized skin.

Irradiation at extremely low or high doses causes change in collagen organization, such as increased degradation and severe loss of cross-linking (Barth et al., 2011). However, intermediate doses (20–35 kGy) increase fiber condensation but do not interfere with mechanical properties (Delgado et al., 2014).

Therefore, the dose used in the present study, which was established in the previous studies of the same study group, provides improvement in collagen condensation (detected by higher type I collagen labeling) and removal of debris remaining during chemical sterilization, thereby

showing better characteristics as described in the SEM, where the irradiated skin was cleaner and had more organized fibrils.

5. Conclusions

Tilapia skin can be an important source of collagen, particularly COL-I, with no negative influence on the ultrastructural and morphological aspects of the sterilization protocol. After being used in burn treatment, tilapia skins exhibit structural disorganization and lower collagen content.

Immunohistochemistry and Picrosirius Red are two methods that can be used for collagen detection, and quantification using both methods has equal reliability.

Author statement

- Maria Elisa Quezado Lima-Verde: Conceptualization; Methodology; Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization.
- Antonio Ernando Carlos Ferreira Junior: Methodology; Investigation, Data Curation, Writing - Original Draft.
- Paulo Goberlânio de Barros Silva: Methodology; Formal analysis; Writing - Review & Editing.
- Emilio de Castro Miguel: Resources, Writing - Review & Editing; Supervision; Validation.
- Monica Beatriz Mathor: Writing - Review & Editing; Supervision.
- Edmar Maciel Lima Junior: Resources; Supervision; Project administration; Funding acquisition.
- Manoel Odorico de Moraes Filho: Resources; Supervision; Project administration; Funding acquisition.
- Ana Paula Negreiros Nunes Alves: Conceptualization; Methodology; Validation; Writing - Review & Editing, Project administration.

Declaration of Competing Interest

All authors declare no conflicts of interest. In addition, no funding was received for conducting this study.

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