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Comparative study of bullfrog (Rana catebeiana) skin preservation for use as a biomaterial

Estudo comparativo de meios de conservação de pele de rã-touro (Rana catesbeiana) para utilização como biomaterial

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Highlights

Bull frog skin has great potential for use as wound dressing.
Glycerin has characteristics that favor the preservation of tissues stored in it.
98% glycerin and freezing in 20% glycerin maintain the structure of frog skin.
Skins preserved in 98% glycerin showed darkened color and greater rigidity.
Both means of preservation are free of contaminating microorganisms up to 120 days.

Abstract

The use of biological membranes in wound dressings has increasingly become a reality. Accordingly, an ideal means of preservation is sought that can provide tissue maintenance for long periods without interfering with its quality or clinical applicability. Therefore, the objective of the present study was to evaluate and histologically and microbiologically compare frog skins subjected to two different preservation methods. Sixteen frog skins were evaluated and, depending on the preservation method, subdivided into two groups with eight skins each, namely, the Freezing Group, in which the skins were frozen at -4º in a 20% glycerin solution; and the Glycerin Group, whose skins were kept in 98% glycerin at room temperature (average of 28 ºC and average humidity of 78%). The skins were analyzed fresh (T0) and at 30 (T1), 60 (T2), 90 (T3), and 120 (T4) days of preservation. Data were analyzed comparatively. There was no bacterial or fungal growth, and the skin structure and collagen arrangement remained intact at all time points in both treatments. In

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conclusion, both preservation methods are efficient and capable of maintaining the tissue morphological structure and preventing the growth and proliferation of contaminants for up to 120 days.
Key words: Biological membranes. Glycerin. Skin.

Resumo

A utilização de membranas biológicas em curativos tem se tornado cada vez mais uma realidade. Concomitante, busca-se um meio de conservação ideal que possa proporcionar a manutenção do tecido por longos períodos de tempo sem interferir em sua qualidade e aplicabilidade clínica. Desta forma, o objetivo deste trabalho foi o de avaliar e comparar histologicamente e microbiologicamente peles de rã submetidas a dois diferentes métodos de conservação. Foram avaliadas 16 peles de rã-touro as quais foram, em função do método de conservação, subdivididas em dois grupos com 08 peles cada: O Grupo Congelamento (GC) no qual as peles foram submetidas ao congelamento a -4º, em solução de glicerina a 20%; e o grupo Glicerina (GG), no qual as peles foram conservadas em temperatura ambiente (média de 28ºC e umidade média de 78%) em glicerina a 98%. As peles foram analisadas a fresco (T0) e com 30 (T1), 60 (T2), 90 (T3) e 120 (T4) dias de conservação. Os dados foram analisados de forma comparativa. Em todos os tempos analisados e em ambos os tratamentos, não houve crescimento bacteriano ou fúngico e a estrutura da pele e o arranjo de colágeno mantiveram-se íntegros. Conclui-se que, ambos os métodos de conservação são eficientes e capazes de manter a estrutura morfológica tecidual, e impedem o crescimento e a proliferação de contaminantes por até 120 dias.
Palavras-chave: Membranas biológicas. Pele. Glicerina.

Introduction

The application of biological dressings in the treatment of extensive skin lesions constitutes an efficient method that aids the tissue healing process; hence its widespread use. In addition, biological dressings have been preferred over synthetic materials due to their low cost and ease of access. Thus, there is a growing search for alternative biological materials that meet the biodegradability and biocompatibility requirements (M. C. Ferreira et al., 2011; Lima et al., 2017; Machado et al., 2021).

Frog skin has been considered among the by-products researched for use as biological dressings due to specific characteristics such as high collagen concentration and the presence of antimicrobial and lipid peptides. These factors optimize the healing process and create a protective barrier against opportunistic microorganisms in the wound bed, when used as an occlusive dressing (Santos et al., 2020; Alves, 2022).

Despite the scarcity of informative content about the clinical use of frog skin as a biological dressing, results seem to converge, as success is always observed when monitoring the evolution of skin lesions treated with this therapy (Falcão et al., 2002; Piccolo et al., 2008; Bzaz et al., 2013, 2015).

Along with clinical trials to evaluate the behavior of frog skin as a biological dressing, it is important to stress the need for efficient preservation methods, which will enable its storage for further clinical use. These methods must meet the minimum requirements to be considered efficient in the preservation process, e.g. maintaining

tissue cell architecture and a tissue free of contaminating microorganisms; ease of acquisition and handling; and low cost (J. B. S. da Silva et al., 2022).

Freezing is one of the oldest preservation methods, which is able to maintain tissue characteristics for prolonged periods and inhibit the action of microorganisms. However, at the cellular level, this technique has the great disadvantage of leading to the formation of ice crystals that cause disarrangement of the cellular matrix, compromising the clinical use of the tissue (Schiozer, 2012; J. B. S. da Silva et al., 2022).

On the other hand, at high concentrations and at room temperature, glycerin constitutes a medium that does not result in the formation of intra- or extracellular crystals, being preferred for the maintenance of the cellular architecture of tissues preserved in it. In addition, it is a low-cost, easy-to-handle method widely used in various biological tissues, which possesses antiseptic properties that ensure safety in its clinical use (Oliveira et al., 2015; Steffen, 2018).

A proposed option to avoid the deleterious effects of freezing is inoculating the tissue to be preserved under low temperatures in a medium with low-concentration glycerin, which will act to protect the cells from crystal formation by being a hygroscopic component, thereby preserving the architecture of the cellular matrix (Lemos et al., 2007).

Therefore, the present study evaluated frog skins preserved in 98% glycerin at room temperature and frozen in a 20% glycerin solution, from the macroscopic, microbiological, and histological viewpoints, to determine whether or not the preservation methods interfere with cell structural characteristics and provide the maintenance of a biomaterial free of contaminating agents.

Material and Methods

Preparation of the skins

All procedures performed in this study were approved by the Animal Use Ethics Committee – CEUA/UENF (approval no. 525). The study involved skins of 16 female and male frogs with similar weight and age, acquired from a single farm. The skins were purchased fresh, immediately after slaughter and skinning (procedures performed by the farm), and kept refrigerated in Styrofoam with ice for approximately 3 h until arrival at the multidisciplinary laboratory of the Large Animals Section at the State University of Northern Rio de Janeiro. Immediately afterwards, the skins were cleaned, the muscle adhered to the tissue removed, and skin portions involving the hind and forelimbs discarded. The resulting portion frog back and belly had approximate dimensions of 15 × 10 cm. Next, the skins were washed with a 0.9% NaCl solution and, immediately after, with a 2% degerming chlorhexidine solution (2% Riohex, Rioquímica®). One by one, the samples were massaged and kept at rest in this same solution for 30 min. This procedure was repeated once again, and then the skins were washed abundantly in a 0.9% NaCl solution. The methodology applied in the skin washing procedures was based on an analysis of similar methodologies (Camargo et al., 2014; Lima et al., 2017; Santos et al., 2020) and subsequent technical adaptation developed by the research group.

Preservation

The skins were placed in glass bottles (500 mL) with sealing lids, containing the respective media. The bottles had been previously immersed in boiling water (100 ºC), for 15 min and then immersed in 70% ethyl alcohol solution.

Eight skins were immersed in a 98% glycerin solution (glycerin group, GG) and stored at room temperature (28 °C) for 120 days. Another eight skins (frozen group, FG) were immersed in a 20% glycerin solution and frozen in a domestic freezer (-4 °C) for the same period of time. The methodology applied in the preparation of the preservation media was based on an analysis of similar studies (Lemos et al., 2007; Camargo et al., 2014; Vivas et al., 2021) and subsequent technical adaptation by the research group.

Sample collection

At zero, 30, 60, 90, and 120 days (T0, T1, T2, T3, and T4), three skin fragments were collected: one for histological analysis (2 cm × 6 cm) and two for microbiological analysis (fungal and bacterial; 2 cm × 0.5 cm). The collection at T0 was performed before the skins were subjected to the preservation media. Collections were performed in a laminar flow hood with sterilized instruments and using personal protective equipment. The skins in the CG group were subjected to a thawing process in a water bath at 42 ºC for approximately 15 min before being handled at all time points evaluated, except T0.

Microbiological analyses

The fragments removed for microbiological analyses were packed in 15-mL conical tubes containing their respective enrichment means 2 mL of Broth Heart Infusion (BHI) and 2 mL Sabouraud Dextrose Broth (Acumedia, USA) and incubated in a laboratory oven at 37 °C for approximately 16 h. After this period, inocula from the BHI and Sabouraud Broth tubes were streaked with a platinum loop into Petri dishes with BHI agar (Himedia) and Sabouraud Dextrose Agar (Kasvi), respectively, to isolate the microorganisms. The dishes were incubated in a laboratory oven at 37 ºC for 24 h and colony growth was observed every 24 h for four days. The objective of this process was to identify and isolate possible contaminating microorganisms from the preparation process or common microorganisms of the natural frog skin flora, whether they are fungi or bacteria.

The analysis of bacterial and fungal samples followed the standards of conventional biochemical-physiological identification. Fungal analysis was achieved by microscopic observation of the fungus on a slide with panoptic staining, identifying its morphological structures. For bacterial analysis, the colonies were Gram-stained (Laborclin, Brazil) and subjected to catalase (3% hydrogen peroxide) and oxidase production (Fluka, Chemika, Switzerland) tests. Subsequently, the fungal and bacterial samples had their classification confirmed by the Vitek 2 instrument (Biomerieux, France), which allows the specific identification of isolated strains.

All microbiological processing was performed at the Animal Health Laboratory of the State University of Northern Rio de Janeiro.

Histological analyses

For histological analyses, frog skin fragments were fixed in neutral buffered formalin for at least 24 h. Subsequently, they were cleaved, identified for processing, stained with hematoxylin-eosin and later evaluated by light microscopy. Histological characterization was achieved through comparative analysis between frog skin described in the literature and the samples analyzed in the study, adopting the studies of D. S. Ferreira et al. (2006), Mangione et al. (2011), Haslam et al. (2014), and H. A. M. da Silva et al. (2017) as reference.

All histological processing took place at the Animal Pathology Laboratory of the State University of Northern Rio de Janeiro.

Results and Discussion

On macroscopic evaluation, the frozen group (FG) samples showed better tissue malleability than the glycerin group (GG) samples, which were rigid and difficult to handle. The GG skins showed a brownish color, whereas the FG samples were more similar to the original color (Figure 1).

The higher tissue malleability observed in FG was associated with the lower fluid loss of the skins compared with GG, which was caused by the immersion of the latter in the low-concentration glycerin solution. Lemos et al. (2007) described similar findings in the preservation of dog skin also by freezing, using a similar protocol.

Crypreservation is considered a method that interferes little with the macroscopic physical characteristics of the stored tissue after thawing (Santos, et al., 2020). This fact was confirmed in all analyzed skin samples that were frozen, albeit preserved in low glycerin concentrations, since neither their color nor malleability was changed.

On the other hand, the rigidity observed in the GG skins, also described in human skin preserved in 98% glycerin (Wood et al., 2014), is because glycerin is a hygroscopic chemical compound and, thus, the tissues immersed in it have a significant loss of fluids, displaying important viscoelastic changes (J. B. S. da Silva et al., 2022). These results also corroborate Bariani (2021), who reported handling difficulty in pericardia preserved in 98% glycerin.

Although most authors report lower malleability in membranes preserved in glycerin, Vivas et al. (2021) did not observe this difference in the preservation of bovine pericardium, which is conflicting with our results. Despite this, as also seen here, these authors identified significant alterations in the color of the preserved pericardium, which acquired a brownish appearance, like the frog skins evaluated in this study. These results differ from those of Oliveira et al. (2015), who observed a reddish color in tunica albuginea preserved in 98% glycerin. This fact may be related to the great vascularity present in the studied tissue, unlike frog skin and pericardium.

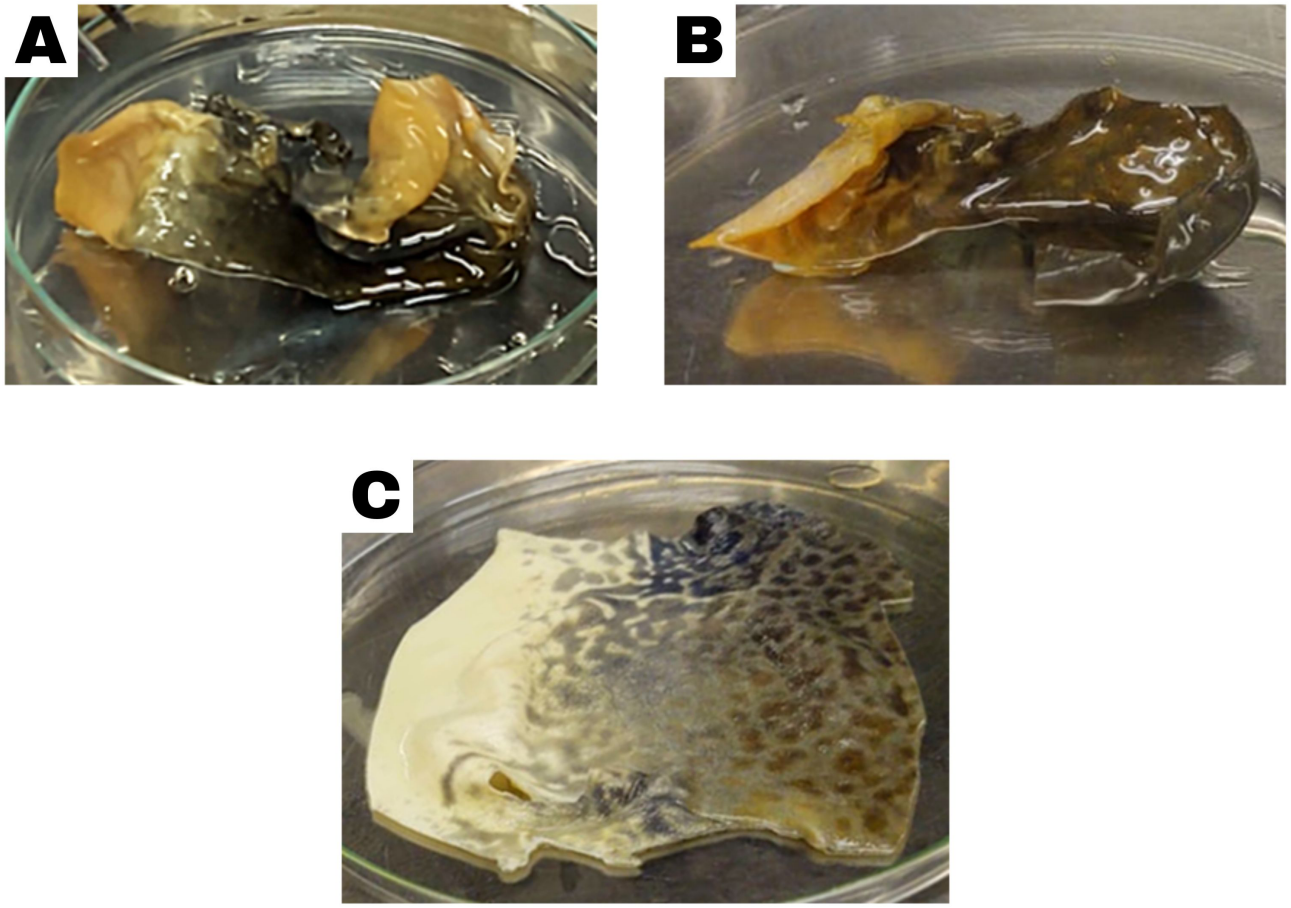

Figure 1. Image exhibiting frog skins in different preservation media. A and B: Frog skins preserved in 98% glycerin. Note the darkened/brown color and rigid appearance. C: Frog skin frozen in a 0.9% sodium chloride and 20% glycerin solution. Note that the skin is lighter compared with skins A and B and does not have a rigid appearance.

From a clinical point of view, the tissue rigidity observed in the frog skins preserved in 98% glycerin may represent a disadvantage, considering that this characteristic will make it difficult to apply the tissue to the wound bed. Therefore, it is recommended to previously hydrate tissues preserved in glycerin in a sterile 0.9% saline solution, which will restore the tissue's malleability, facilitating its clinical application (Oliveira et al., 2015; Campos et al., 2021). Nonetheless, Leal et al. (2013) observed that even after hydration, the peritoneum of lowland paca preserved in 98% glycerin remained little malleable, making it difficult to handle.

Despite being a widely disseminated method, for the cryopreservation technique, sophisticated equipment is used in most cases to allow freezing at lower temperatures (-80 ºC) (J. B. S. da Silva et al., 2022). In the present study, as well as in the experiment of Lemos et al. (2007), the material was frozen in a domestic freezer, at a temperature of approximately -4 °C. This process proved to be effective, considering that it was able to provide good preservation of the tissues,

in addition to dispensing with specific apparatus for this. However, the vulnerability of the electrical network can constitute an important disadvantage, compromising the quality of the stored material.

None of the analyzed samples showed bacterial or fungal growth at any of the evaluated time points. The nutrient solutions displayed no turbidity and no colonies were observed at any of the time points or treatments studied.

The natural microbiota of frog skin is vast and can vary according to their habitat and species. According to Proença et al. (2021) and Assis et al. (2017), members of the bacterial families *Bacillaceae, Enterobacteriaceae, Streptomycetaceae, Xanthomonadaceae, Aeromonadaceae, Pseudomonadaceae, Staphylococcaceae,* and *Moraxellaceae* have already been identified and isolated from frog skins and considered natural microorganisms of the microbiota of these individuals. According to K. L. da Silva (2021), fungi of the genus *Penicillium; Trichoderma, Fusarium,* and *Cladosporium* species; and some yeast-like fungi species have already been isolated from the natural microbiota of anurans.

We did not identify the growth of any of the microorganisms expected in the evaluations carried out, and this result may be related to the fact that the animals used in our experiment did not represent free-living individuals, i.e. their natural microbiota will be different from that reported in the literature.

In addition, it is known that by promoting cellular dehydration, glycerin prevents degeneration reactions by microorganisms that require the presence of water. Therefore, it consists of a broad-spectrum antiseptic, making it difficult for tissues preserved in it to manifest fungal or bacterial growth (J. B. S. da Silva et al., 2022).

This fact is reported in studies such as that of Bariani (2021), in the microbiological analysis of bovine pericardium preserved in 98% glycerin; and in clinical trials such as those by Menezes et al. (2004), Leal et al. (2014), Scorsato et al. (2019), and Campos et al. (2021), who tested dog skin, lowland paca perotonium, canine bone, and canine tunica vaginalis, respectively, preserved in the same medium. In all these clinical trials, the results were favorable from a microbiological standpoint, as the recipients did not show signs of infection or rejection to the clinical use of the biological membrane reactions, demonstrating the maintenance of a preserved tissue free of contaminating agents.

The non-growth of microorganisms observed in this study can also be related to the residual effect of the 2% chlorhexidine used in the previous washing of the skins and the 30-min immersion baths in the same solution. In a similar study, Menezes et al. (2004) performed fungal analyses on dog skin preserved in 98% glycerin and observed fungal growth in preserved samples at 15 days. However, in the previous preparation of the skins, these authors performed only one wash in a 2% chlorhexidine solution.

Our results corroborate Steffen (2018), who also reported the effectiveness of 98% glycerin and freezing in 0.9% sodium chloride solution in preserving human bone in a bone bank.

Similar to the present results for the FG group, Lemos et al. (2007) also described the absence of microorganisms in samples

preserved in a 20% glycerin solution under freezing. Because cellular activity is reduced in the freezing process, the metabolic activity of microorganisms is consequently inhibited. Additionally, the preservative solution included glycerin, which has antiseptic power (J. B. S. da Silva et al., 2022).

In the histological evaluations, the tissue morphological structures were maintained in all analyzed samples, both in GG and in FG, at all time points evaluated. The epidermis was covered by slightly keratinized stratified squamous epithelial tissue and the papillary dermis was divided into a spongy layer, made up of loose connective tissue, and a compact layer made up of dense regular connective tissue. Skin appendages such as granular glands and mucous glands were also observed, in addition to the acellular layer that divides the spongy and compact layers of the dermis, called the Eberth-Katschenko layer (Figure 2 and 3).

The identified cellular structures are in line with the histological characterizations of frog skin described by D. S. Ferreira et al. (2006), Mangione et al. (2011), Haslam et al. (2014), and H. A. M. da Silva et al. (2017).

Therefore, although glycerin provides preservation by cellular dehydration, in histological terms, it does not interfere with the architecture or morphology of cells, being able to maintain the cellular matrix preserved. Similar results were observed by Vivas et al. (2021) in the morphological evaluation of bovine pericardium preserved in 98% glycerin and by Oliveira et al. (2015) in the histological evaluation of tunica albuginea preserved in 98% glycerin.

Because it is a widely used preservation method, 98% glycerin has already been the subject of studies on the preservation of various biological tissues such as bovine pericardium, bovine tunica albuginea, canine tunica vaginalis, and canine and human skin. As reported, 98% glycerin has always provided good preservation and maintenance of cell architecture in tissues evaluated histologically (Menezes et al., 2004; Oliveira et al., 2015; Campos et al., 2021; Vivas et al., 2021; J. B. S. da Silva et al., 2022). The histological data analyzed in the present study corroborate this information.

Disagreeing with our findings, Santos et al. (2020) evaluated menisci frozen at -80 ºC and reported significant changes in the architecture of collagen fibers, which occurred due to the increase in water content in the tissue, caused by freezing. This divergence of results is believed to be due to how Santos et al. (2020) performed freezing in their experiment, as they did not keep the material in any cryoprotective solution, unlike in the present study, where frog skins were stored in a 20% glycerin solution.

As observed by Lemos et al. (2007) in dog skin, the preservation of frog skin in 20% glycerin was able to maintain stable cellular architecture, without the formation of ice crystals. Thanks to its hygroscopic function, the glycerin added to the freezing medium prevented the formation of ice crystals, removing water from the intracellular medium so that crystal formation did not occur, and consequently preventing the disarrangement of the cellular matrix (J. B. S. da Silva et al., 2022).

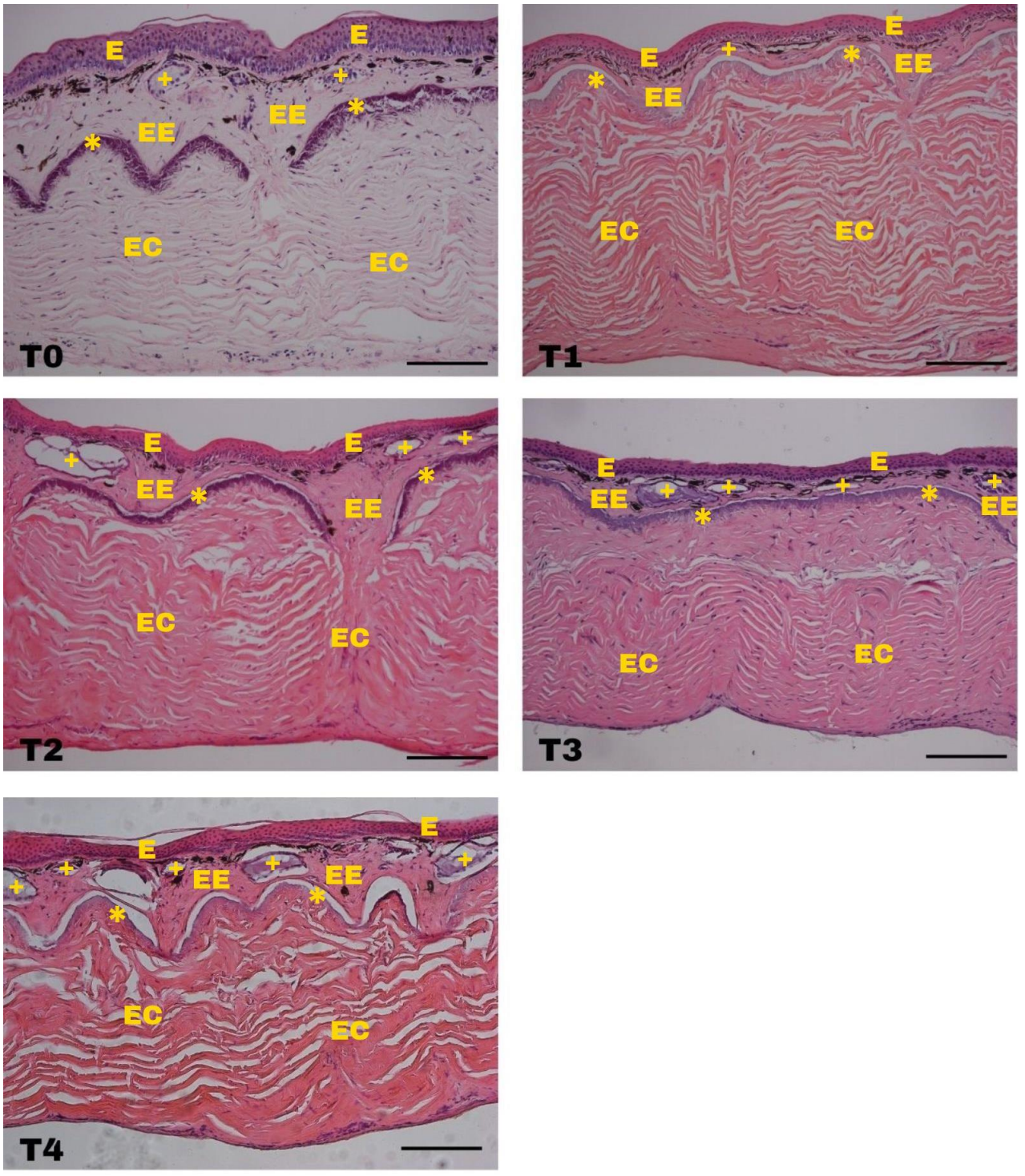

Figure 2. Photomicrographs of the histological analyses of the Glycerin Group, at the five preservation times, displaying maintenance of cell morphological structures at all analyzed time points. (Staining: hematoxylin-eosin, 10x). Identification of structures: Epidermis (E): Slightly keratinized stratified squamous epithelium, papillary dermis – spongy layer (EE): Loose connective tissue, papillary dermis – compact layer (EC): Dense regular connective tissue, granular gland/ mucous gland (+), Eberth-Katshenko layer (*).

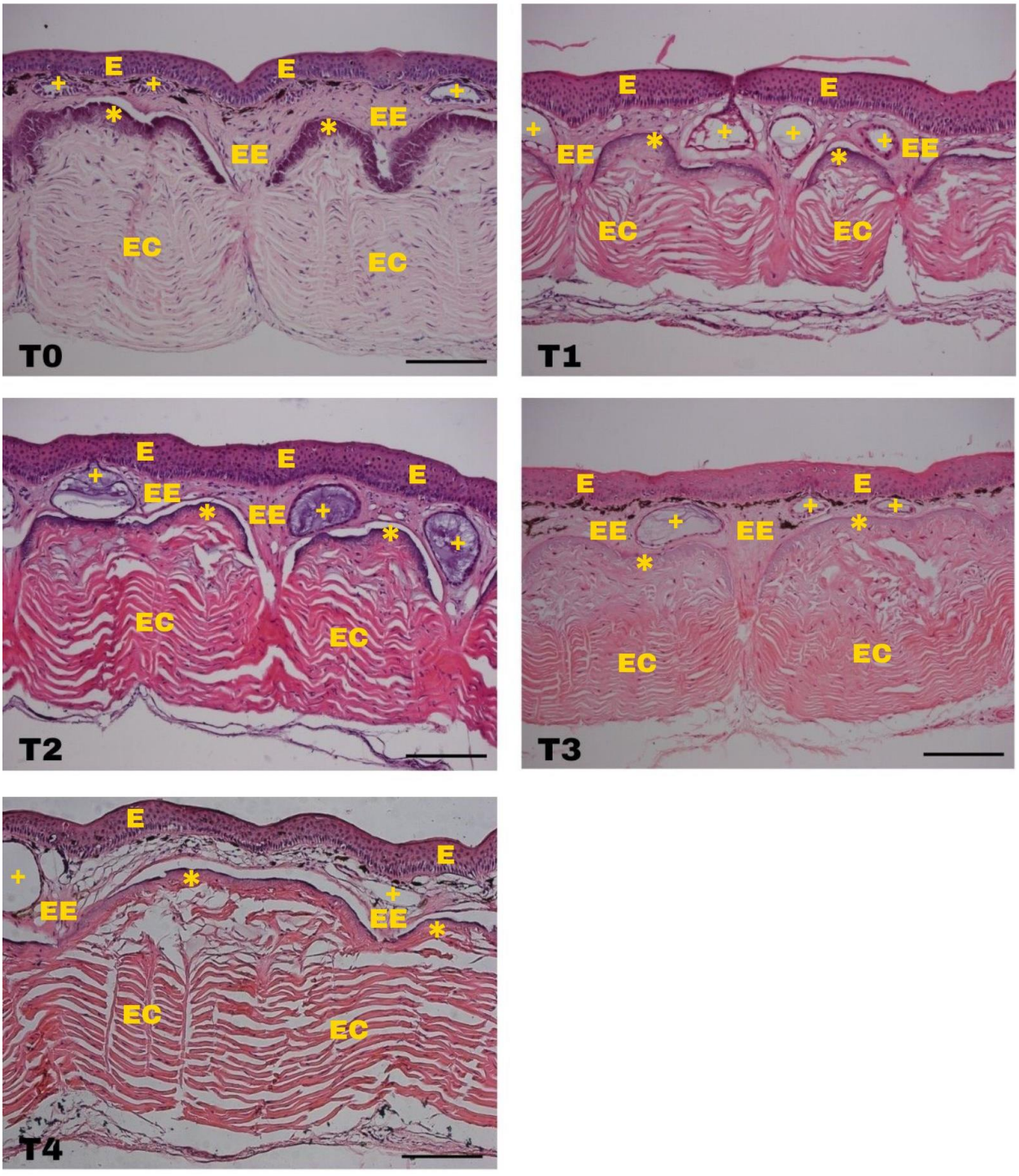

Figure 3. Photomicrographs of the histological analyses of the Frozen Group, at the five preservation times, displaying maintenance of cell morphological structures at all analyzed time points. (Staining: hematoxylin-eosin, 10x). Identification of structures: Epidermis (E): Slightly keratinized stratified squamous epithelium, papillary dermis – spongy layer (EE): Loose connective tissue, papillary dermis – compact layer (EC): Dense regular connective tissue, granular gland/ mucous gland (+), Eberth-Katshenko layer (*).

Conclusions

The preservation media studied were efficient from the microbiological and histological points of view, as they enabled the maintenance of the tissue architecture and protected the samples from contaminating microorganisms at all time points analyzed.

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