https://doi.org/10.48048/tis.2021.36

Fabrication of Sponges from Amnion Hydrogel to Apply as Wound Dressing

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Received: 17 March 2021, Revised: 17 May 2021, Accepted: 27 May 2021

Abstract

A burn is a type of injury to the skin or other tissues. It can give rise to skin defects or even death. In this study, sponges derived from amnion hydrogel were generated to apply as a wound dressing. The sponges were created by combining crosslinking and freeze-drying methods. There were three types of the obtained sponges: MGA-0 (only washed with PBS), MGA-1 (washed in glycine 1 % for one day) and MGA-2 (washed in glycine 1 % for two days). These sponges were evaluated by scanning electron microscope (SEM), mechanical test, swelling, and cytotoxicity. The tensile strength of the sponges was about 1.8 MPa, and the absorption increased during 24 h. The relative growth rates (%RGR) of MGA-0, MGA-1, and MGA-2 were 88.8, 58.2 and 67.2 %, respectively. The obtained results suggested that the MGA-0 sponge has potential for wound dressing application.

Keywords: Amnion hydrogel, Wound dressing, Freeze-drying, Glutaraldehyde, Sponge

Introduction

According to the World Health Organization (WHO) estimates, about 180000 deaths from burns are reported from the low and middle-income countries each year [1]. A burn is a significant medical problem in the world. Burn wound causes skin structure and function injuries, seriously impairing the body temperature, fluid balance, and physical obstacle to harmful environmental effects [2,3].

For burn treatment, autologous skin grafting is considered the gold standard. However, it still has some limitations when the patients have a large burn or the treatment time is quite long [3,4]. A current trend focuses on generating a membrane that can cover the burn wound to prevent outside infection, stimulate proliferation, scar minimizing, and regenerating skin to shorten cure time.

Many wound dressings, including a polymer containing human skin cells (fibroblasts and/or keratinocytes), have been commercialized. For instance, Dermagraft, Apligraf, and TransCyte have shown effectiveness in burn treatment. However, these products are costly, require complicated storage methods, and can increase the risk of immune reactions [5]. Hence, the creation of effective, long-time stored, affordable cost products to apply in wound management in low- and middle-income countries is in great demand.

For the fabrication of wound dressing, a suitable material on effectiveness and cost should be considered. There are various materials to make wound dressings, such as hydrogels, sodium alginate, collagen, foams, and hydrocolloids [6-10]. Hydrogels are considered the best materials in generating wound dressings because there are dominant features such as hydrophilic nature, soft tissue-like properties, a cooling and soothing effect on the skin [8,11-13]. In this study, human amniotic membrane hydrogel (amnion hydrogel) was used to create wound dressing. An amnion is a membrane on the inner side of the fetal placenta. Amnion consists of 3 layers: A thin epithelial layer, a thick basement membrane of extracellular matrix (ECM) proteins, and an avascular stroma. There are three layers of an avascular stroma: Compact, fibroblast, and intermediate (spongy) layers. Like the human skin, the amnion is composed of extracellular matrix components such as collagen I, III, IV, V, VI, fibronectin, laminin, which provide stiffness and elasticity for amnion [2,14,15]. Amnion has been demonstrated for its anti-

inflammatory characteristic, anti-bacterial and anti-viral features [2]. Based on these mentioned properties, the amnion can be applied to create products for wound healing purposes [5]. However, using the intact amnion to cover the wound directly has some difficulties in routine use. Limitations related to the difficulty of handling the thin membrane over the injury without folding or tearing, fixing in place on the wound requires the support of sutures or adhesives [16]. Besides, the transportation and storage of the living tissue are also complicated for routine clinical use [16-19]. In 2017, Murphy *et al*. [20] developed a new product for wound treatment from amnion-containing cell-free solution. That hydrogel was created by combining the solubilized amniotic membrane with hyaluronic acid. Its efficiency was proved in a mouse wound model. In 2020, another work of Murphy *et al.* [16] showed the wound-healing efficacy of amnion hydrogel and lyophilized amnion membrane powder in a full-thickness porcine skin wound model. These studies demonstrated that amnion hydrogel treatment contributes to improving wound healing. Nevertheless, the degradation of hydrogel could become much faster in aqueous environments such as body fluids [19]. Hence, to overcome these limitations and apply the advantages of the amnion, this study aims to create sponges from human amnion hydrogel using crosslinking and freeze-drying techniques for application as a wound dressing.

Materials and methods

Ethics statement

This study was approved by the Medical Ethics Committee of Hung Vuong Hospital, Ho Chi Minh City, Vietnam (the decision number of the permission from Ethical committee: 2395/GCN-BVHV, Hung Vuong Hospital, Ho Chi Minh City, Vietnam). The human amnion was obtained at Hung Vuong Hospital and were accepted by the donors.

The fabrication of sponges

The human amnion was decellularized, handled to make pre-hydrogel solution, and provided by the Laboratory of Tissue Engineering and Biomedical Materials, University of Science, Vietnam National University, Ho Chi Minh City (Vietnam). The amnion pre-hydrogel structure was white fluid gel. The hydrogel was formed by incubating the pre-hydrogel at 37 °C for 1 h. The hydrogel was then crosslinked by immersing in 0.625 % glutaraldehyde (GA, Merck, Germany) at 4 °C for 24 h. There are 3 ways to remove the residual GA: (1) rinse the samples with 1X phosphate-buffered saline (PBS, Gibco, USA) for 3 days, (2) wash with 1 % glycine solution (Himedia, India) for 1 day and rinse with 1X PBS for 2 days, and (3) wash with 1 % glycine for 2 days and rinse with 1X PBS for 1 day. After washing, the samples were kept at −86 °C for 24 h and underwent a drying process (BenchTop Pro 3L XL-75, SP Scientific, USA). The sponges were sterilized by gamma irradiation (25 kGy).

Based on the 3 removed ways for the residual GA, 3 types of sponges were created: MGA-0 (only washed with PBS), MGA-1 (washed in glycine 1 % for 1 day), MGA-2 (washed in glycine 1 % for 2 days). Moreover, a non-crosslinked sponge (M0) was used as a control.

Investigation of the sponge characterizations

Structure analysis

The surface of the sponges was observed by the scanning electron microscope (SEM, JSM-6510 JEOL, Japan).

Mechanical test

To examine the mechanical properties such as compression or expansion of the sponges, the tensile strength formula was used;

$$\text{Tensile strength} = F/S \text{ (MPa)},$$

where F is the maximum load that the sponge can withstand (N), and S is the sample area (mm^2).

Swelling ratio

To consider the water-absorbing ability of the sponges, the samples (1.5×2 cm^2) were immersed in 3 mL A solution (sodium chloride 2.298 g/L, calcium chloride dihydrate 0.368 g/L, and distilled water). The sponge mass was measured before and after immersing. The swelling ratio was calculated according to the following equation;

Swelling ratio (%) = $\frac{W_e - W_0}{W_0} \times 100$,

where W_0 is the initial mass of specimens, W_e is the mass of specimens at the time of 0, 24 and 48 h.

In vitro cytotoxicity

In vitro cytotoxicity was assessed using (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (ISO 10993-5:2009). Experimental groups include negative control (culture medium), positive control (the extraction of medical latex gloves), and sponges MGA-0, MGA-1, MGA-2.

A sample was cut into 1.5×2 cm^2 pieces. The liquid extract was obtained by incubating the pieces in 0.5 mL of medium DMEM/F12 (Sigma-Aldrich, USA) supplemented 10 % fetal bovine serum (FBS; Sigma-Aldrich, USA) and 1 % penicillin/streptomycin (Sigma-Aldrich, USA) at 37 °C for 24 h. Human fibroblasts, which were provided by the Laboratory of Tissue Engineering and Biomedical Materials, were seeded in a 96 well-plate at a cell density of 10^3 cells per well, and incubated at 37 °C, 5 % CO_2. When the cells reached 80 % of the confluence, they were treated with liquid extract. After 24 h, the liquid extract or medium was discarded; the cells were washed once with 1X PBS. 0.5 mg/mL MTT (Sigma-Aldrich, USA) solution was added into each well and incubated for 4 h at 37 °C. Formazan crystals were dissolved in Dimethylsulfoxide/Ethanol (1:1) (Dimethylsulfoxide, SigmaAldrich, USA; Ethanol, Merck, Germany). The number of active cells was estimated by measuring absorbance at 570 nm in the Biochrom EZ Read 400 Microplate Reader (Biochrom, United Kingdom).

Statistical analysis

Each experiment was performed 3 times. Statistical analyses were performed by 1-way analysis of variance (ANOVA). It has statistically significant when p-value < 0.05.

Results and discussion

Morphology and structure of the sponges

The amnion pre-hydrogel was a fluid gel (**Figure 1A**). It has built up a hydrogel phase after incubating at 37 °C for 1 h (**Figure 1B**). However, the amnion hydrogel was degraded after incubating for 5 min at room temperature (**Figure 1C**). Thus, the crosslinked method was used to improve the hydrogel structure at room temperature.

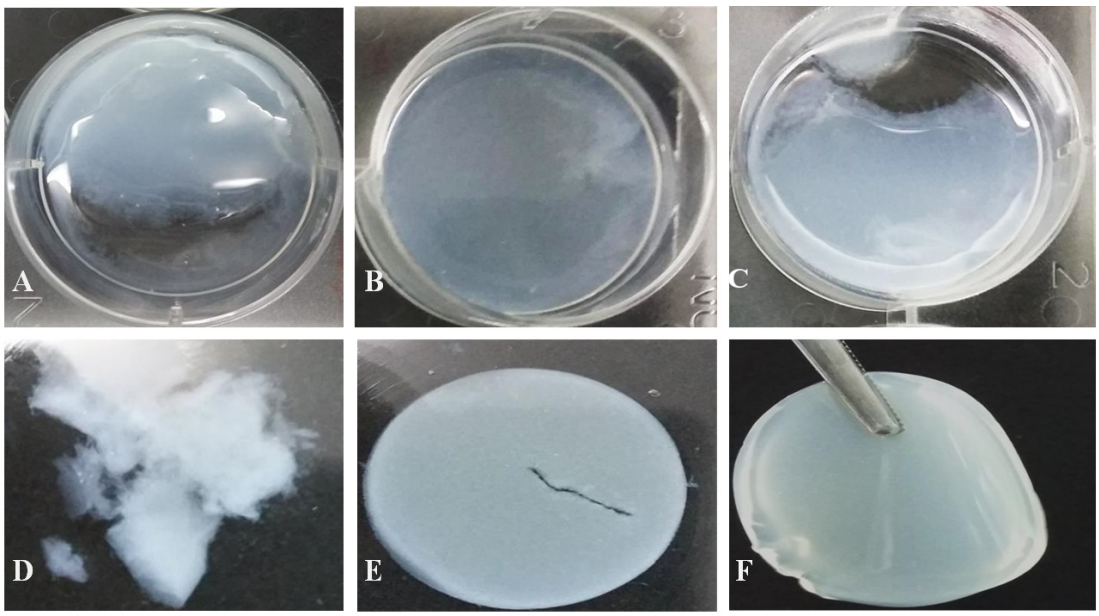

Figure 1 Amnion hydrogel before and after crosslinking; A) pre-hydrogel, B) pre-hydrogel after incubating at 37 °C for 1 h (hydrogel), C) hydrogel at room temperature after 5 min, D) hydrogel at room temperature after 24 h, E) and F) GA crosslinked hydrogel after 24 h.

The results showed the crosslinked samples could preserve the shape when immersed in distilled water (**Figure 1E**) or pick up out of a container (**Figure 1F**). After freeze-drying, there were 4 types of sponges: Non-crosslinked sponge (M0), GA crosslinked sponges (MGA: MGA-0, MGA-1, MGA-2) (**Figure 2**).

Figure 2 Sponges from amnion hydrogel after freeze-drying; A) M0, B) MGA-0, C) MGA-1 and D) MGA-2.

The M0 and MGA-0 sponge colors were white (**Figures 2A** and **2B**), while the MGA-1 and MGA-2 were yellowish (**Figures 2C** and **2D**). The borders of MGA-0, MGA-1, and MGA-2 were smooth and one was serrations in the border of the M0. A scanning electron microscopy was used to compare the surface structure of the sponges. SEM images displayed a generally flat surface texture of M0 and MGA sponges. A fibrous-like structure can be observed in M0, which become more visible in MGA. Some areas with visible fibrous structure can be detected on MGA surface (**Figure 3** B white arrows).

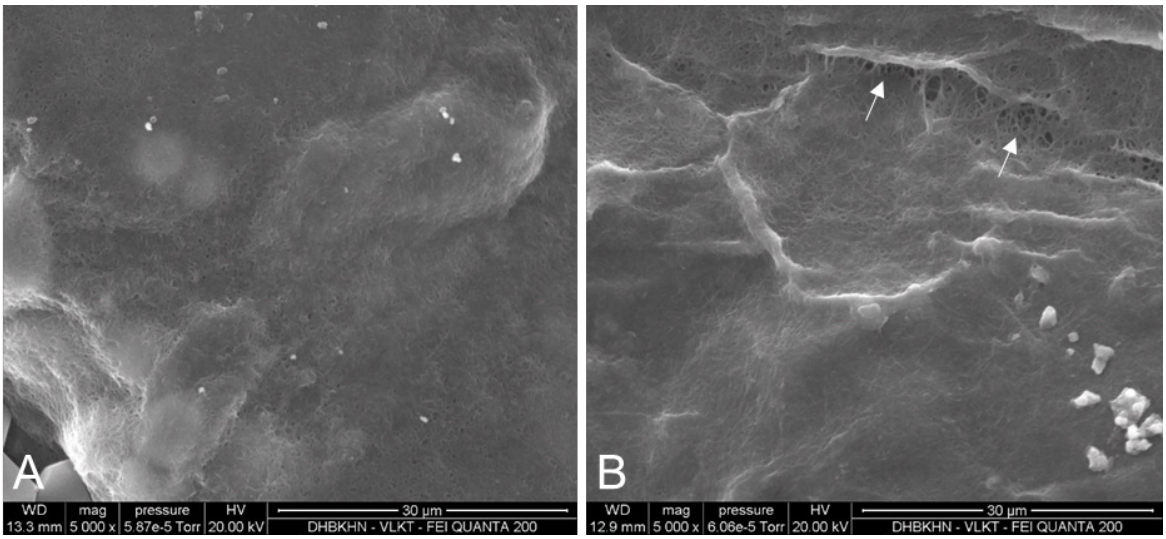

Figure 3 Surface structure of the different sponges; A) M0 sponge and B) MGA sponge.

Mechanical test

The M0 sponge could be ignored because it broke easily during the test processing. The tensile results of the MGA-0, MGA-1 and MGA-2 were 1.87 ± 0.37, 2.38 ± 0.25 and 1.85 ± 0.28, respectively (**Figure 4**). The tensile strength of the MGA-1 was the largest in three sponges, and it is statistically significant compared to the MGA-0 and the MGA-2 ($p < 0.05$). For the MGA-0 and the MGA-2, the tensile difference is nonsignificant ($p > 0.05$).

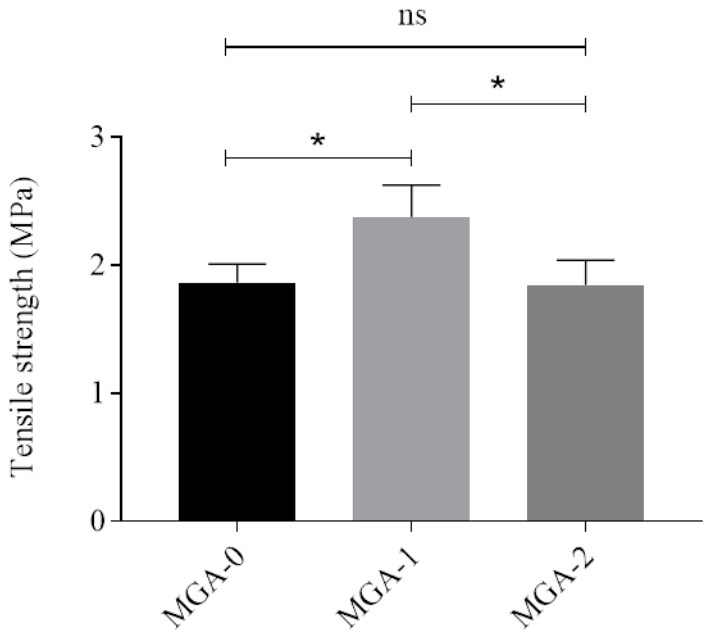

Figure 4 The tensile strength of the MGA-0, MGA-1 and MGA-2 sponges; ns: Not significant, *: *p*-value < 0.05.

Swelling ratio

The results of the swelling ratio of the MGA-0, MGA-1 and MGA-2 were shown in **Figures 5** and **6**. The M0 sponge was not investigated because it was disintegrated after immersing in A solution in **Figure 5**.

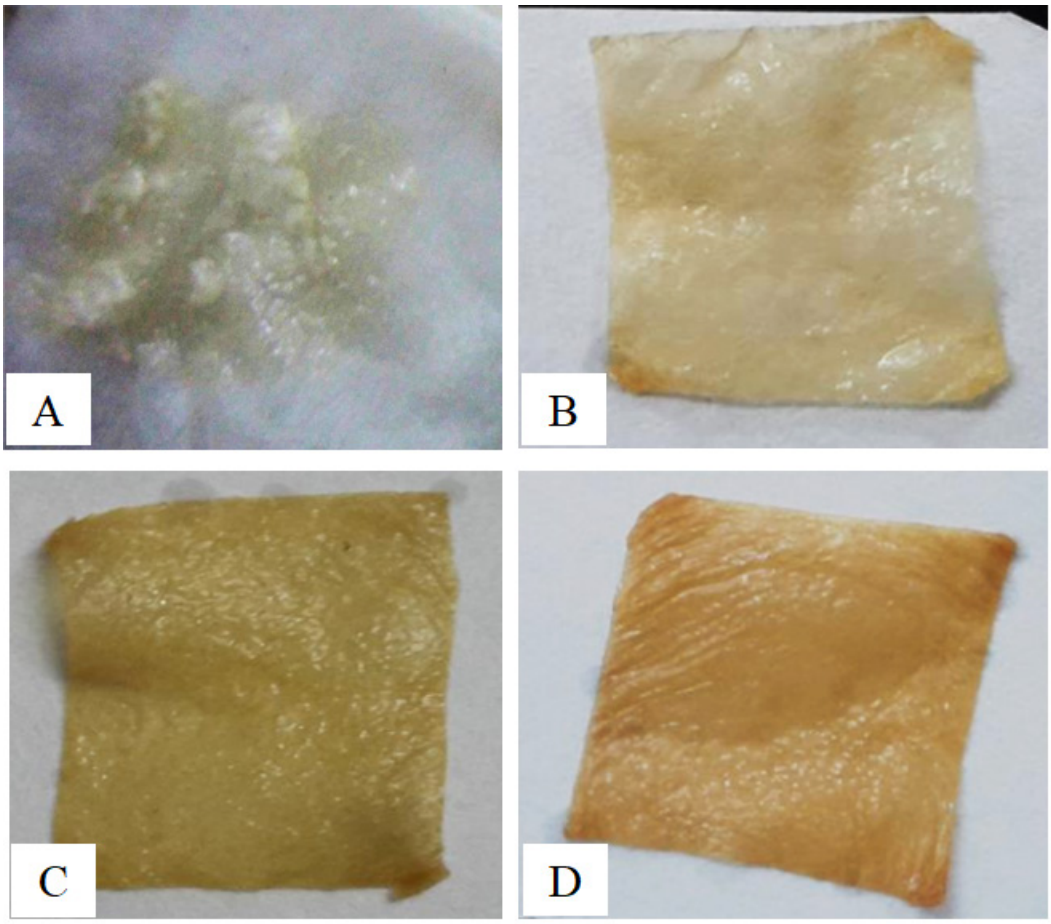

Figure 5 Snapshot of the M0, MGA-0, MGA-1 and MGA-2 sponges after immersing A solution; A) M0, B) MGA-0, C) MGA-1 and D) MGA-2.

The swelling ratio of the MGA-0, MGA-1 and MGA-2 had an increasing trend when the measured time was from 0 to 24 h. From 24 to 48 h, the swelling of MGA-0 increased linearly; however, for the MGA-1 and MGA-2, the obtained results were unchanged. The swelling ratio of the MGA-0, MGA-1 and MGA-2 had statistical significance between the observed time at 0 and 24 h, but they were nonsignificant between 24 and 48 h. At 48 h. The MGA-0 had the largest swelling, and there was a statistical significance comparing to the MGA-1 and MGA-2. However, there was nonsignificant MGA-1 comparing to MGA-2, as shown in **Figure 6**.

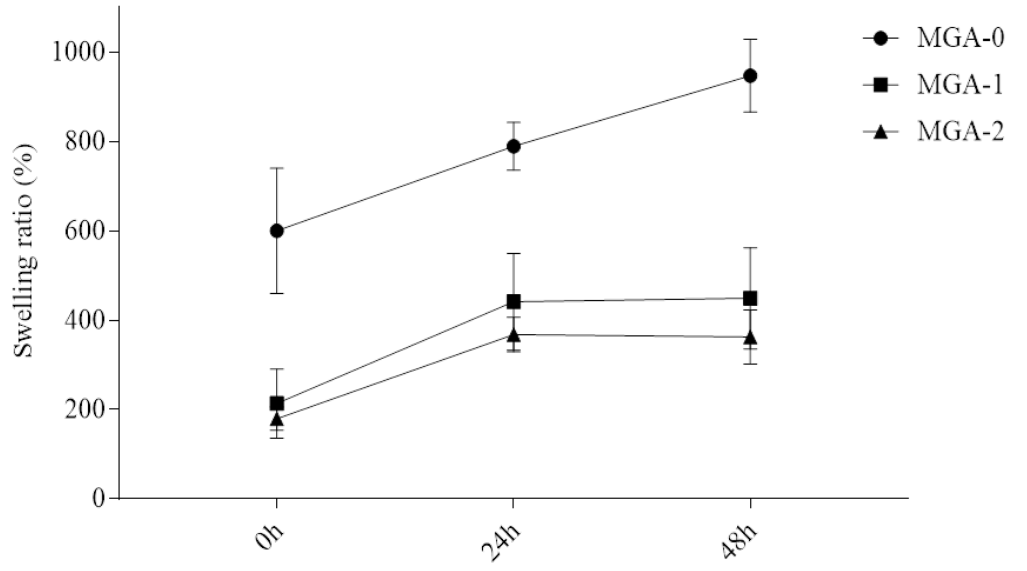

Figure 6 The swelling ratio of the sponges at different time points.

***In vitro* cytotoxicity**

The results showed that the sponges have cytotoxicity (**Figure 7**). The relative growth rates (RGR, %) of the MGA-0, MGA-1, MGA-2 sponges are lower than 70 %. Furthermore, it is not significant in comparison with the positive control (the extraction of medical latex gloves). Maybe, the reason for these sponges' cytotoxicity is the residual glutaraldehyde. To remove this residue glutaraldehyde, all sponges were washed by 1X PBS within 1 day and re-obtain the extract and again the progress.

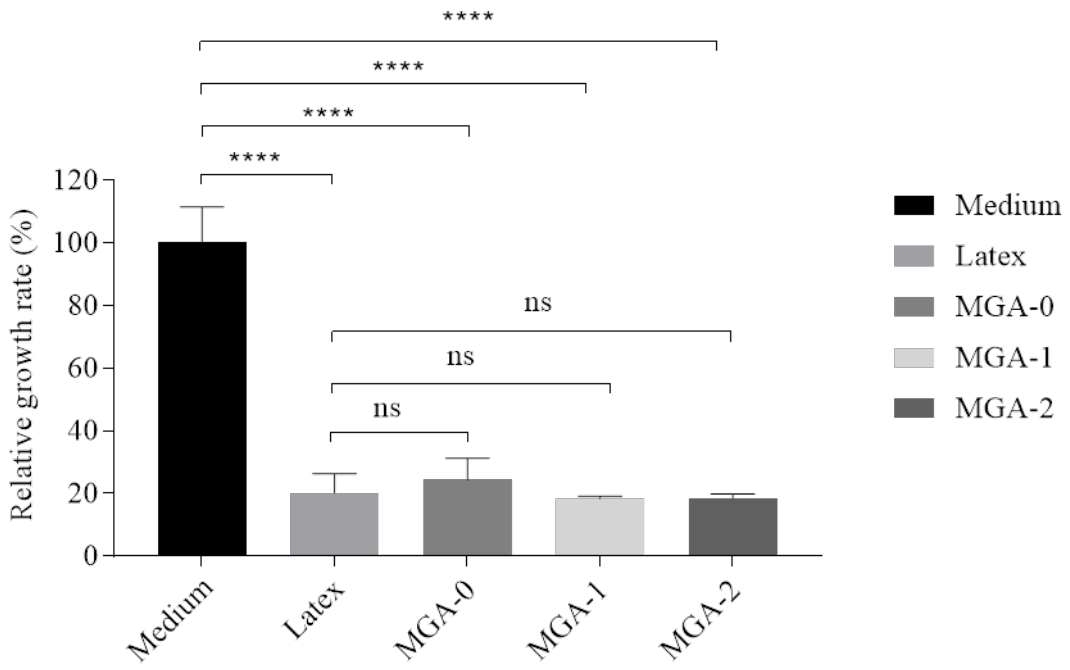

Figure 7 Relative growth rate (%RGR) of fibroblast of the MGA-0, MGA-1, MGA-2 sponges, and Latex and medium after 24 h by MTT assay; ns: Not significant, ****: p-value < 0.0001.

In vitro cytotoxicity of all sponges was considered after washing by the 1X PBS (**Figure 8**). The results showed that the relative growth rates are increasing. However, only MGA-0, %RGR is higher than 70 %, but for MGA-1 and MGA-2, one is still lower.

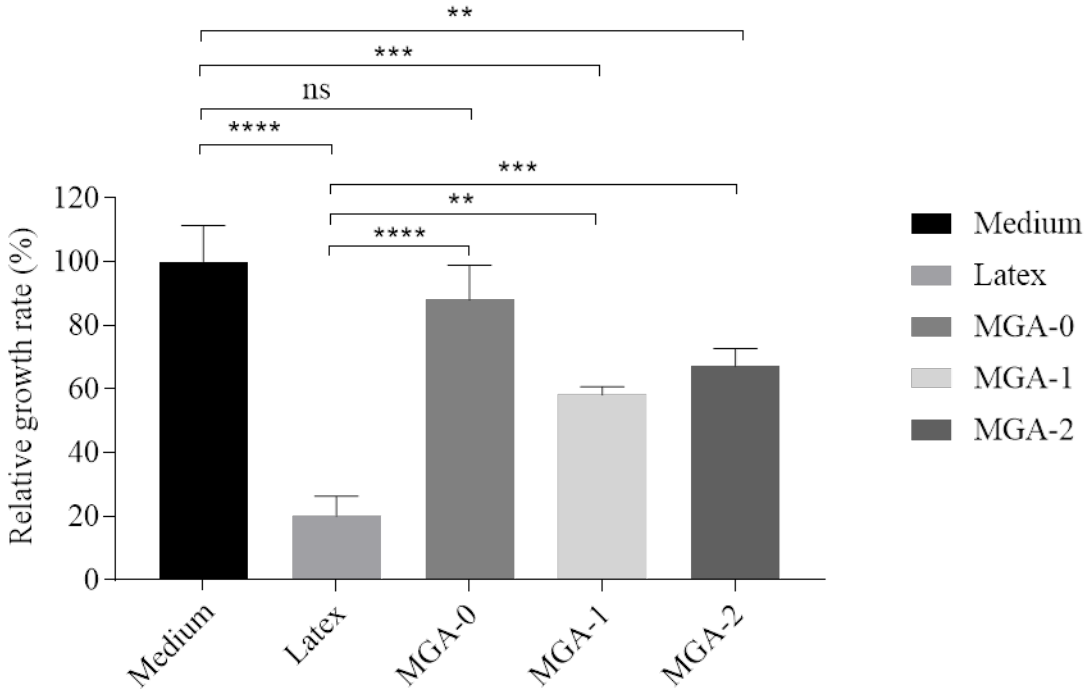

Figure 8 Relative growth rate of fibroblasts of the sponges, latex and medium after washing by 1X PBS; ns: Not significant, **: p-value < 0.01, ***: p-value < 0.001: ****: p-value < 0.0001.

Discussion

This work generated wound dressing with the sponge structure by the freeze-drying method. Non-crosslinked sponges are easy to break; hence, to improve structure stabilization, 0.625 % GA was applied as the crosslinker because it is the common chemical crosslinker and has not been disrupted by temperature [21,22]. The GA has 2 aldehyde groups in the structure; this will enhance the ability to crosslink. In addition, with a small size, it is easy to penetrate the sponge structure. The aldehyde groups of GA react with the amine groups of protein to form stable crosslinks [23,24]. The obtained results showed that the crosslinked hydrogel was more difficult to disintegrate. However, the residue GA can induce cytotoxicity [25], so it needs to be removed in the sponges. To remove residual GA, we washed the samples in 1X PBS solution or 1X PBS solution containing glycine. The %RGR of 3 sponges showed that they were cytotoxic (**Figure 7**). These results could be predicted that the sponges had residual GA; hence the sponges were again washed by 1X PBS for 24 h. As expected, the %RGR of these sponges was improved (**Figure 8**), and we obtained a non-cytotoxic sponge (MGA-0). Nevertheless, the MGA-1 and MGA-2 were still cytotoxic. This can be explained that the residual GA in the MGA-0 was almost eliminated, while the MGA-1 and MGA-2 might not have completely removed. The results showed that removing the residual GA with 1X PBS was effective compared with Gly.

In addition, some other criteria were also considered, including mechanical test, swelling ratio. For the mechanical test aspect, the aldehyde groups of GA react with the amine groups of protein to form a stable crosslink, enhancing the stiffness in the crosslinked sponges. The tensile strength of the obtained sponges is about 1.8 MPa, and it is similar to the tensile strength of human skin [25]. For the swelling ratio, the sponges can absorb much water quickly. Due to the sponges being fabricated by the freeze-drying method, the water in the structure was removed. In the work of Ngadaonye *et al.* [23], the swelling ratio was about 250 - 400 % after 24 h. In our study, the swelling ratio of MGA-0, MGA-1, MGA-2 was 790 ± 54, 441 ± 108 and 368 ± 40 %, respectively. These results showed that the swelling ratio was

suitable as supported by the study of Ngadaonye *et al*. [23]. In general, our work created the sponges from human amnion hydrogel by crosslinking and freeze-drying techniques. This study contributes to the use of extracellular matrix-based biomaterials for wound healing.

Conclusions

In this study, the MGA-0, MGA-1 and MGA-2 sponges were created by combining freeze-drying with 0.625 % glutaraldehyde. The results showed that the crosslinked sponges had the tensile strength and swelling ratio better than the uncrosslinked sponge (M0). In general, these sponges are cytotoxic. However, after washing again with 1X PSB, the MGA-0 sponge was non-cytotoxic. The obtained results suggested that the MGA-0 sponge is a potential candidate in wound dressing application.

Acknowledgments

This research is funded by University of Science, VNU-HCM under grant number T2020-15.

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