

Amniotic membrane applied to burns healing: Pre-clinical study

Membrana amniótica aplicada na cicatrização de queimaduras: Estudo pré-clínico

Membrana amniótica aplicada en la curación de quemaduras: Un estudio preclínico

Received: 03/25/2021 | Reviewed: 04/03/2021 | Accept: 04/04/2021 | Published: 04/15/2021

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Abstract

This preclinical study aimed to evaluate the tissue repair process of burns treated with human amniotic membrane (hAM) patches in rats. Twenty-four rats were subjected to superficial burns of partial thickness, and randomly allocated into two groups: Control and Treated Group, subdivided into two experimental periods of 7th and 14th days. The lesions were evaluated by digitalized images (macroscopy) and by the analysis of histological sections stained in H&E to quantify the number of inflammatory cells and fibroblasts present in the different experimental times (histomorphometry). The histomorphometric analyses were performed blindly. Statistical analysis employed Kolmogorov-Smirnov and Mann Whitney tests, with 95% confidence interval at 5% significance level ($p < 0.05$). Macroscopically, the lesions of Treated group presented a crust formation before Control Group, and there were no signs of infection in both groups. Microscopically, the qualitative analysis showed a faster evolution in the healing process of the Treated groups compared to the Control, with reduction of the inflammatory infiltrate, intense fibroblasts proliferation and better organization of the collagen fibers. The quantitative analysis showed statistically significant results regarding the reduction of inflammatory cells ($p < 0.0001$) at 7th and 14th day and increased proliferation of fibroblasts at 14th day ($p < 0.0001$) in lesions treated with hAM compared to Control group. The results of this preclinical study demonstrated that the application of hAM patches reduces the inflammatory process and accelerates the onset of the proliferative phase in burn injuries.

Keywords: Burns; Amnion; Wound healing; Skin; Rats; Biomedical engineering.

Resumo

Este estudo pré-clínico teve como objetivo avaliar o processo de reparo tecidual de queimaduras tratadas com fragmentos de membrana amniótica humana (MAH) em ratos. Vinte e quatro ratos foram submetidos a queimaduras de espessura parcial superficial, e alocados aleatoriamente em dois grupos: Grupo Controle e Tratado, subdivididos em dois períodos experimentais de 7º e 14º dias. As lesões foram avaliadas por imagens digitalizadas (macroscopia) e pela análise de cortes histológicos corados em H&E para quantificar o número de células inflamatórias e fibroblastos presentes nos diferentes tempos experimentais (histomorfometria). As análises histomorfométricas foram realizadas às

cegas. A análise estatística empregou os testes de Kolmogorov-Smirnov e Mann Whitney, com intervalo de confiança de 95% com nível de significância de 5% ($p < 0,05$). Macroscopicamente, as lesões do Grupo Tratado apresentaram formação de crosta antes do Grupo Controle, não havendo sinais de infecção em ambos os grupos. Microscopicamente, a análise qualitativa mostrou evolução mais rápida no processo de cicatrização do grupo Tratado em relação ao Controle, com redução do infiltrado inflamatório, intensa proliferação de fibroblastos e melhor organização das fibras colágenas. A análise quantitativa mostrou resultados estatisticamente significantes quanto à redução das células inflamatórias ($p < 0,0001$) no 7º e 14º dia e aumento da proliferação de fibroblastos no 14º dia ($p < 0,0001$) nas lesões tratadas com MAH em relação ao grupo Controle. Os resultados deste estudo pré-clínico demonstraram que a aplicação dos fragmentos de MAH reduz o processo inflamatório e acelera o início da fase proliferativa em queimaduras.

Palavras-chave: Queimaduras; Âmnio; Cicatrização; Pele; Ratos; Engenharia biomédica.

Resumen

Este estudio preclínico tuvo como objetivo evaluar el proceso de reparación tisular de quemaduras tratadas con fragmentos de membrana amniótica humana (MAH) en ratas. Veinticuatro ratas fueron sometidas a quemaduras de espesor superficial parcial y fueron distribuidas aleatoriamente en dos grupos: Grupos de Control y Tratados, subdivididos en dos períodos experimentales de 7º y 14º días. Las lesiones se evaluaron mediante imágenes digitalizadas (macroscopia) y mediante el análisis de cortes histológicos teñidos en H&E para cuantificar el número de células inflamatorias y fibroblastos presentes en los diferentes tiempos experimentales (histomorfometría). Los análisis histomorfométricos se realizaron a ciegas. El análisis estadístico empleó las pruebas de Kolmogorov-Smirnov y Mann Whitney, con un intervalo de confianza del 95% con un nivel de significancia del 5% ($p < 0,05$). Macroscópicamente, las lesiones en el grupo tratado mostraron costras antes que el grupo de control, sin signos de infección en ambos grupos. Microscópicamente, el análisis cualitativo mostró una evolución más rápida en el proceso de cicatrización del grupo tratado en comparación con el grupo control, con reducción del infiltrado inflamatorio, intensa proliferación de fibroblastos y mejor organización de las fibras de colágeno. El análisis cuantitativo mostró resultados estadísticamente significativos con respecto a la reducción de células inflamatorias ($p < 0,0001$) en los días 7 y 14 y aumento de la proliferación de fibroblastos el día 14 ($p < 0,0001$) en las lesiones tratadas con MAH en relación al grupo Control. Los resultados de este estudio preclínico demostraron que la aplicación de los fragmentos de MAH reduce el proceso inflamatorio y acelera el inicio de la fase proliferativa en las quemaduras.

Palabras clave: Quemaduras; Amnios; Cicatrización de heridas; Piel; Ratas; Ingeniería biomédica.

1. Introduction

Injuries resulting from burns demand complex and costly treatments, require assistance from specialized multidisciplinary teams, and present high morbidity and mortality rates, which makes them an important public health problem (Rowan et al., 2015; World Health Organization, 2018).

Burns, caused by thermal, chemical, electrical or radioactive agents, are traumatic injuries with varying levels of tissue loss. The specific cause of a burn, as well as the classification of the injury according to severity (depth and size), guides the choice of an appropriate treatment (Jeschke et al., 2020).

Partial-thickness burns are the most frequent ones, with their injuries reaching the dermis and epidermis, causing painful lesions, phlyctens, edema, erythema, and ulcerations (Lima et al., 2016). The treatment of burns has always been a challenge, both for its severity and for the multiplicity of complications that normally occur. The most frequently used drugs for the treatment of partial thickness burns are silver sulfadiazine, silver alginate, hydrogel, and wet dressings (Wasiak & Cleland, 2015). However, the traditional clinical protocols employed in the treatment of burns present moderate to high costs.

In light of that, one of the great challenges of researchers is to find new therapies that enable tissue repair, reducing the use of drugs and the need for long-term and high cost treatments. Several substances and therapeutic techniques have been developed and tested in research aiming to maintain, improve or restore tissue function impaired by burns. Some studies have clinically evaluated the potential benefit of the amniotic membrane as a biological dressing (Raza et al., 2020; Ahuja, Jin, Powers, Billi & Bass, 2020; Lashgari, Rostami & Eternad, 2019; Mohammadi, Eskandari, Johari & Rajabnejad, 2017; Reilly, Hickey, Glat, Lineaweaver & Gerverman, 2017; Eskandarlou, Azimi, Rabiee & Rabiee, 2016; Salehi, As'adi, Mousavi, & Shoar, 2015). However, studies that histologically evaluate the effects of the use of the Human Amniotic Membrane (HAM)

are still unknown or inexistent considering the initial stages, inflammation and proliferation, of the tissue repair process of burns.

Considering the phases of the healing process, it is understood that acute and self-limited inflammation is an important defensive response that plays a critical role in the regeneration of injured tissues. However, when the process becomes chronic and unresolved, it can lead to excessive tissue damage and delayed healing (Silini, Magatti, Cargnoni & Parolini, 2017). It is noteworthy that before the end of the inflammatory response, the phases of proliferation, cellular differentiation and deposition of connective tissue occur, enabling the healing process of the lesion (Kumar, Abbas, Fausto, & Mitchell, 2013).

Thus, the aim of our study was to evaluate the histological aspects of superficial partial thickness burns treated with HAM patches in rats.

2. Materials and Methods

This is an experimental research. Experimental studies consist of research in which the main objective is the cause and effect relationship, which must have as a parameter a control group and an experimental group and control the maximum of relevant factors for what one wishes to achieve (Lakatos & Marconi, 2019). In this type of research, manipulation in the quantity and quality of variables makes it possible to study the relationship between causes and effects of a given phenomenon, through the control and evaluation of the results of these relationships (Koche, 2011). Thus, an intervention or treatment that can be called a control test or clinical test is induced in order to test whether it causes changes in the dependent variable (Polit & Beck, 2011).

This study was approved by the Research Ethics Committee of the Universidade do Vale do Paraíba (2.077.418) and the Ethics Committee on the use of animals of the Centro Universitário UNINOVAFAPI (005P/V2/2017). The procedures were performed in the experimental surgery laboratories of the Centro Universitário UNINOVAFAPI, located in the city of Teresina (PI), Brazil.

The sample consisted of 24 Wistar rats (*Rattus norvegicus albinus*, males, 40 days old, 200±50g) housed in polypropylene cages, in a controlled environment (light/dark 12h, temperature 20±2 °C) and with feed and water ad libitum. The animals were randomized in two groups: Control Group (C, n =12) - composed of rats submitted to second-degree burning; and Treated Group (T, n =12) - rats submitted to experimental burn induction and treated with the application of HAM fragments. These groups were subdivided into two experimental periods of 7th and 14th days.

Experiment

The experiment was developed in four stages: collection of the human placenta, biomaterial processing, burn induction, and application of HAM fragments. Pregnant women who donated the placentas (two) were selected considering the following inclusion criteria: elective cesarean schedule; healthy clinical history; HIV-1, VDRL, HbsAg, and anti-HCV negative serologic tests, gestational age from 37 weeks to 41 weeks and 6 days (term placenta).

The placenta was obtained from elective cesarean section of a patient with term pregnancy, selected according to the criteria established in a previous study (Sant'Anna, Cargnoni, Ressel, Vanosi, & Parolini, 2011), after signing the written informed consent. Immediately after medical inspection, the placenta was placed in a sterile plastic bag, stored at approximately 10 °C, and transported to the experimental surgery laboratory for biomaterial processing under sterile conditions in a laminar flow hood.

The HAM was manually separated from the chorion, and was repeatedly washed with 0.9% physiological solution containing 100 U/mL of penicillin, 100 mg/mL of streptomycin and amphotericin B, until it acquired a transparent aspect. The

AM was then fractionated into 4 x 4 cm pieces that were stored at room temperature in 50ml vials containing 40ml of Dulbecco's Modified Eagle's medium (DMEM) culture medium without serum and phenol red in sterile conditions until application. The AM pieces were used within 24h according to protocols (Hennerbichler et al., 2007; Cargnoni et al., 2009; Sant'Anna et al., 2016).

In order to induce the superficial partial-thickness burns, the animals were weighed, the dorsal region epiled and the combination of Xylazine (2%, 0.01 mL / kg) and Ketamine (10%, 0.005 mL / kg) was administered intramuscularly. The experimental burn was induced by means of a 3 cm diameter tube, filled with 50 mL of water heated to 100° C, placed in direct contact with the skin of the epilated region, for 10 seconds. Subsequently, the lesions were evaluated considering the macroscopic aspects of the skin, such as color (red or pink) and the presence of phlyctenas, for characterization of the second-degree burns. In the animals of the Treated group, a fragment of HAM was applied with the mesenchymal face facing the area of the skin injury, exceeding by 1 cm the edges of the lesion, and fixed with topical adhesive.

Macroscopic and histological evaluation

Macroscopic evaluations were performed considering the appearance of the lesion, skin coloration, presence of phlyctens, superficial crust formation, and flogistic signs. Microscopic analysis evaluated the inflammatory cells, formation of blood vessels, young and/or mature fibroblasts, and organization of collagen fibers from scanned images.

The animals in the Control and Treated groups were euthanized at 7th or 14th days with the application of an excessive dose (100 mg/kg) of Thiopental via intraperitoneal route. The burned skin and the surrounding areas were excised and fixed in 4% formaldehyde, forwarded for routine histological processing, and the histological sections were stained with Hematoxylin & Eosin (H&E) for qualitative histological and morphometric analysis.

Statistical analysis of the data

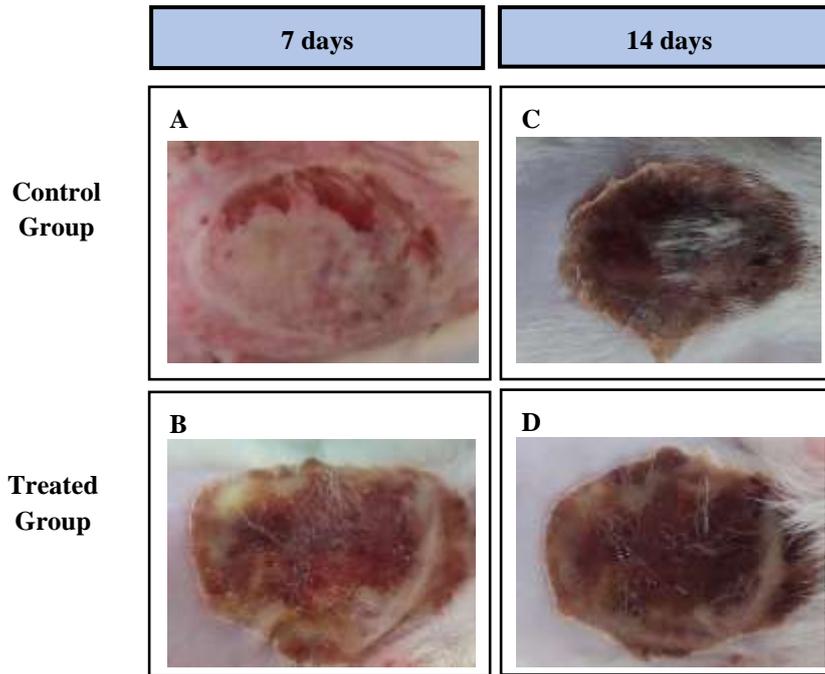
The images obtained from the cross sections of the four sequential fields of each glass slide (40X objective) were evaluated under a light microscope (final amplification \times 400), scanned and analyzed with the ImageJ software in order to quantify the number of inflammatory cells and fibroblasts. All the histomorphometric analyses were performed blindly.

The data were treated regarding the coefficient of variation and the sample distribution for the determination of the statistical test to be used. Statistical analysis employed GraphpadPrism V (GraphPad Software, California, USA), Kolmogorov-Smirnov, and Mann Whitney tests, with 95% confidence interval at 5% significance level ($p < 0.05$).

3. Results

The macroscopic analysis of the burn injuries was performed at 7th and 14th days (Figure 1), showing the early crust formation in the Treated Group compared to the Control Group. In both groups, no characteristics of infectious process were observed.

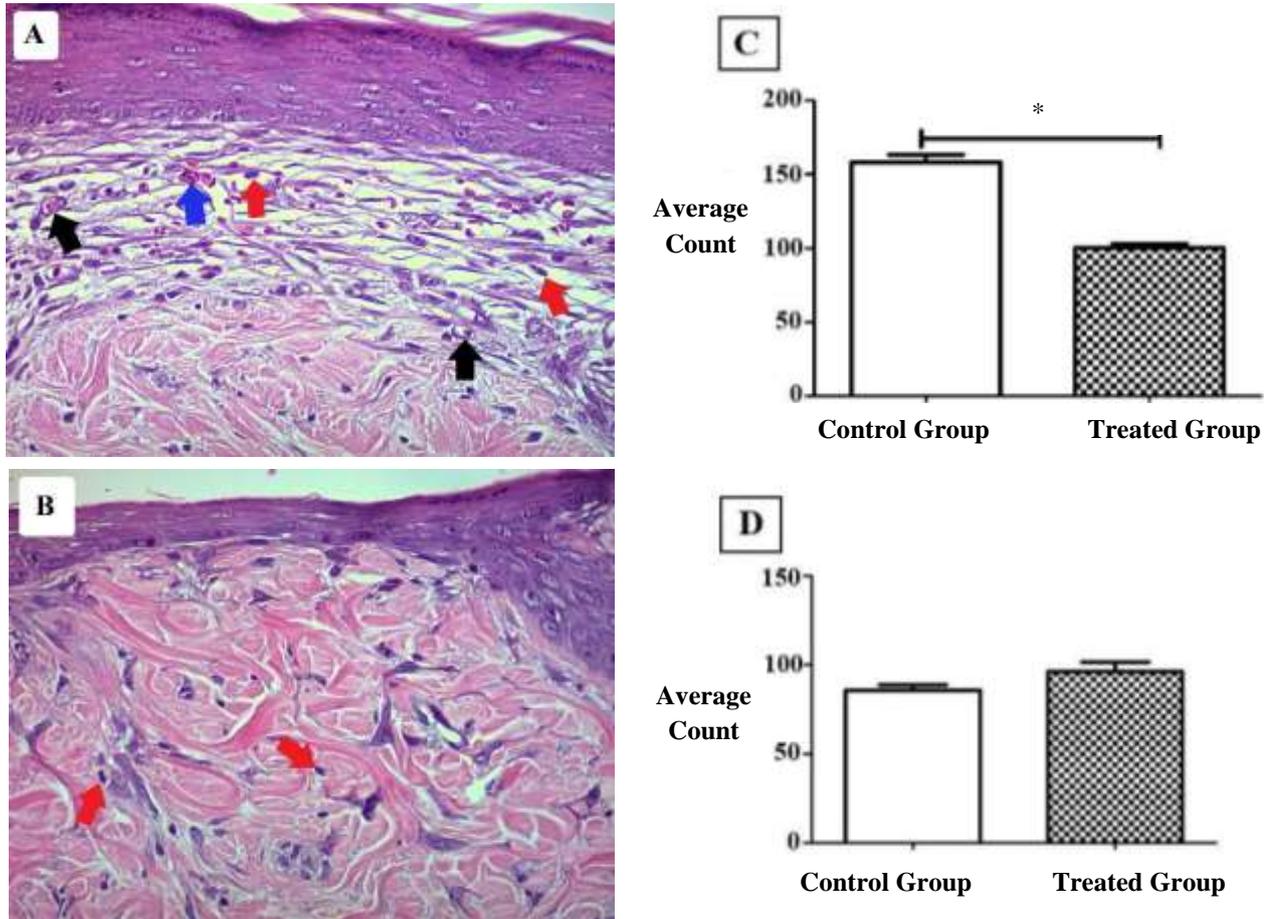
Figure 1. Macroscopic analysis of lesions in the control and treated groups at 7th and 14th days.



(A) Macroscopic analysis of lesions in the Control Group at 7th. (B) Macroscopic analysis of lesions in the Treated Group at 7th. (C) Macroscopic analysis of lesions in the Control Group at 14th. (D) Macroscopic analysis of lesions in the Treated Group at 14th. Source: Authors.

In the first experimental time (Figure 2), the results observed in the qualitative histological analysis showed that the Control Group presented inflammatory infiltrates, with a predominance of macrophages, young fibroblasts, rupture of blood vessels characterized by free red blood cells in the intracellular environment and disorganization of collagen fibers. In the Treated Group the inflammatory cells were barely visible, with the presence of young fibroblasts and proliferation of collagen fibers. The quantitative analysis of these cells showed a significant reduction ($p < 0.0001$) in the number of inflammatory cells, showing a slight increase of fibroblasts, although without statistical significance, in the Treated Group compared to the Control Group (H&E, 40X).

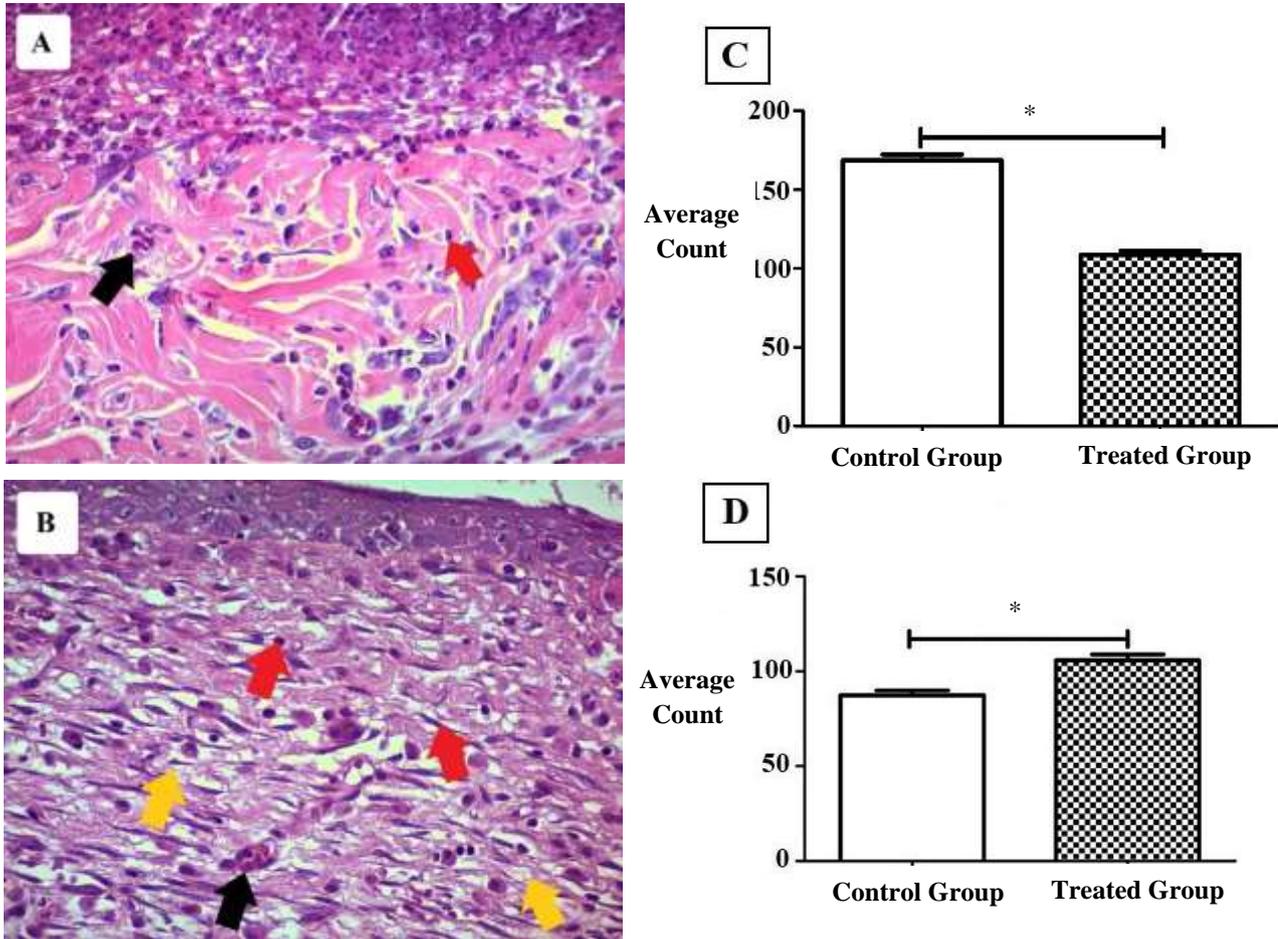
Figure 2. Histological and quantitative analysis of burn injuries in the Treated and Control groups at the 7th day, assessing inflammatory cells and fibroblasts (H&E, 40X).



(A) Histological analysis of the Control Group at 7th days. Inflammatory cells: neutrophils/macrophages (black arrows); Fibroblasts (red arrows); Free red blood cells (blue arrows). (B) Histological analysis of the Treated Group at 7th days; Fibroblasts (red arrows). (C) Quantitative analysis of inflammatory cells at 7th day. (D) Quantitative analysis of fibroblasts at 7th day. * $p < 0.0001$. Source: Authors.

In the second experimental time (Figure 3), the qualitative histological analysis, in the intergroup evaluation, showed that the inflammatory infiltrates still present, mainly in the Control Group. It was observed proliferation of fibroblasts, with a predominance of volumous fibroblasts (Control Group) while in Treated group the fibroblasts presented a fusiform appearance. Also, it was detected a better organization of collagen fibers at the Treated Group. The quantitative analysis showed a significant reduction ($p < 0.0001$) of inflammatory cells and an increase in statistically significant number of fibroblasts ($p < 0.0001$) in the Treated Group compared to the Control Group.

Figure 3. Histological and quantitative analysis of burn injuries in the Treated and Control groups at the 14th day, assessing inflammatory cells and fibroblasts (H&E, 40X).



(A) Histological analysis of the Control Group at 14th days. Inflammatory cells: neutrophils/macrophages (black arrows); Fibroblasts (red arrows). (B) Histological analysis of the Treated Group at 14th days; Inflammatory cells: neutrophils/macrophages (black arrows); Fibroblasts (red arrows); Collagen fibers (yellow arrows). (C) Quantitative analysis of inflammatory cells at 14th day. (D) Quantitative analysis of fibroblasts at 14th day. * $p < 0.0001$. Source: Authors.

In the intragroup analysis (Table 1), there was no statistically significant difference in the mean count of inflammatory cells in the Control and Treated groups. There was a slight increase in the mean number of fibroblasts in the Control Group, but there was no statistically significant difference. Nevertheless, there was a significant difference ($p < 0.05$) in the Treated Group on the 14th day.

Table 1. Intra-group evaluation of the mean inflammatory cells and fibroblast count (mean \pm standard error).

Cell types	Groups	Experimental Times (days)		<i>p</i>
		7 th	14 th	
Inflammatory cells	Control Group	158,4 \pm 4,92	168,8 \pm 3,64	<i>ns</i>
	Treated Group	100,5 \pm 2,53	108,8 \pm 2,40	<i>ns</i>
Fibroblasts	Control Group	85,8 \pm 3,05	87,5 \pm 2,42	<i>ns</i>
	Treated Group	96,2 \pm 6,23	105,8 \pm 5,31	*

p = *p* value; *= Significant difference ($p < 0.05$); *ns* = Nonsignificant difference. Source: Authors.

4. Discussion

The search for low-cost alternatives for the treatment of burns has been intense with a view to its long-term use in public health (Barbuto et al., 2015) and encouraging research about the implementation of new products, among which the amniotic membrane and its derivatives. Thus, *in vitro* and *in vivo* studies have showed the great potential of HAM in regenerative medicine as a treatment option for different types of wounds (Lashgari et al., 2019; Rahman et al., 2019; Kshersagar, Kshirsagar, Desai, Bohara & Joshi, 2018; Rana et al., 2020).

The present study is unprecedented for histologically evidencing the interaction of HAM with the injured tissue in the inflammatory and proliferative phases of tissue repair in experimentally induced superficial partial-thickness burns in rats.

The process of tissue repair lesions presents four overlapping stages: hemostasis, inflammatory, proliferative and maturation. The inflammatory phase is mainly characterized by phagocytosis of cells and tissue debris by defense cells, mainly neutrophils and macrophages, preparing an area for tissue repair. In the other phases, there is intense cell proliferation, formation of extracellular matrix and collagen fibers (Shakespeare, 2001).

In a study with acute wounds, the induced inflammation revealed that when this response is exacerbated and prolonged, it harms the reepithelialization process as it directly modifies the formation of granulation tissue, with an increase in the possibility of scar formation (Qian et al., 2016). In burns, the early inflammation in a controlled manner led to optimal wound healing, but prolonged inflammation was detrimental (Zhang, Lui, Chen, Lok & Wong, 2020).

Our findings showed that the results of the application of the amniotic membrane were previously therapeutic in this phase, since the treated lesions significantly decreased the inflammatory process in the two experimental times studied (7 and 14 days), reinforcing the antioxidant and anti-inflammatory properties of HAM already known in other contexts (Nicodemo et al., 2017; Campelo et al., 2018; Hana et al., 2019; Shu et al., 2018).

A study performed with mice showed that although the inflammatory stage is vital for tissue repair, the presence of inflammatory cells such as neutrophils may delay wound closure, making their depletion essential to accelerate the healing process (Dovi, He & DiPietro, 2003). In our study, the reduction in inflammatory cells characterized by histological findings showed that in the Treated group, the appearance of other cell types precipitated and contributed to the evolution of tissue repair phases.

In the present study, no flogistic signs were observed in the lesions in both groups at the experimental times evaluated. It is noteworthy that the application of the HAM fragment reduces the risk of infection by covering the injured area, acting as a physical and biological barrier against invasion by microorganisms (Baradaran-Rafii, Aghayan, Arjmand & Javadi, 2007).

The migratory phase of tissue repair is characterized by the formation of a crust from the clot that forms in the area of the lesion, enabling the migration of epithelial cells in the region (Shakespeare, 2001; Guo, Ali, Hamid, Zaini & Khaza'ai,

2017). Our results showed that, macroscopically, until the 7th day of the Treated Group, the crust covered the entire injured area, whereas in the lesions of the Control group, the complete formation of the crust was seen only on the 14th day.

Since amnion is able to promote moisture to the lesion, avoid irritation and dryness of the wound (Eskandarlou et al., 2016) and, in light of the fact that maintaining the wound surface moistened and oxygenated accelerates the migration process (Balbino, Pereira & Curi, 2005; Ravishanker, Bath & Roy, 2003), we found that in our study, the early formation of the crust in the treated lesions is due to these properties.

We also proved that this anticipation of the complete formation of the crust of the lesions treated for the 7th day, in fact, had a direct relationship with the histology observed in that phase. The microscopic characteristics observed were: activation of innate immune cells such as neutrophils and macrophages in the inflammation process acute, with adaptive inflammation without chronicity and, subsequently, intense and early cell proliferation of fibroblasts. These characteristics confirmed other studies results that emphasized that this newly deposited matrix confers the lesion a microenvironment that favors the restoration of the structural integrity of the tissue from cell movement, the performance of growth factors, cell proliferation and angiogenesis (Balbino, Pereira & Curi, 2005; Peacock & Winkle, 1984; Steen et al., 2020).

The proliferative stage of tissue repair is characterized by the multiplication of epithelial cells under the superficial crust, extracellular matrix-producing fibroblasts, and collagen fibers, besides intense vascular neof ormation (Shakespeare, 2001). In the present research, the amniotic membrane adhered and incorporated to the tissues in the lesion area, accelerating the proliferation of fibroblasts and inducing angiogenesis in the Treated Group, proving the action of HAM to potentiate cell multiplication in this stage (Shakespeare, 2001; Ravishanker et al., 2003), as well as its beneficial action when compared with other types of dressings (Duarte & Duval-Araujo, 2014).

We found that the Treated Group showed an extremely significant increase ($p < 0.0001$) in the number of fibroblasts at 14th days. Our results corroborate with studies that showed the proliferation of fibroblasts, as well as significant angiogenesis in rats treated with HAM (Rahman et al., 2019; Kshersagar, 2018).

HAM presents in its microenvironment several factors that actively act on cell proliferation, such as the fibroblast growth factor (FGF), platelet derived growth factor (PDGF), epidermal growth factor (EGF), and vascular endothelial growth factor (VEGF). Bioactive factors, the beta transforming growth factor (TGF), anti-inflammatory factors, and the presence of collagen II, III, IV, V, VI, and VIII have also been identified (Lashgari et al., 2019). These properties of HAM allowed dressings made with this biomaterial or associated with other drugs or therapeutic protocols to induce fibroblast proliferation and release of angiogenic factors (Sant'Anna et al., 2016; Zhang et al., 2020). An *in vitro* study confirmed the influence of amniotic membrane cells on the proliferation and cell cycle of fibroblasts and keratinocytes (Kitala et al., 2019).

The basic fibroblast growth factor (bFGF) has been indicated as fundamental in the tissue repair of burns in rats, for its effects on proliferation, differentiation, and migration of keratinocytes, recruitment of inflammatory cells, as well as for the neovascularization observed in the granulation tissue during the initial stage of the wound healing process (Kibe, Takenaka & Kishimoto, 2000). In our study, we found that inflammatory cells were recruited early and there was a stimulus for fibroblast proliferation, suggesting that such effects may be associated with the potentiation of the basic fibroblast growth factor, pointing to the need for further investigations that contemplate its participation in the repair of burns treated with HAM.

It is noteworthy the easy adhesion of HAM fragments to the bed of injured areas was verified in this study, this characteristic provides not only protection against the invasion of microorganisms, but also eliminates the need for dressing change, a common and mandatory procedure when using another therapeutic protocol, with the application of topical products. In clinical studies, the decrease in the frequency of dressing changes attributed to this property of HAM was responsible for reducing the pain of patients, for the protective action and for the reduction of local edema (Lashgari et al., 2019; Mohammadi et al., 2017).

The standard protocol for the treatment of burn injuries includes the application of drugs associated with the dressing change. This procedure may cause bleeding, with consequent delay in the process of tissue repair. Thus, we found that adhesion to the injured area maintained the integrity of the newly formed granulation tissue and, associated with the reduction in the manipulation of the injury, helped to accelerate the phases of the repair process.

We understand that our study can support the staging and monitoring of superficial partial-thickness burns, since we verified the efficiency of the use of fragments of human amniotic membrane, with emphasis on the initial stages of the repair process, from the investigation of microscopic structures of injuries.

Considering the complexity of the healing process in burns, we recommend the expansion of the study with HAM, in the field of tissue engineering, aiming to introduce this biomaterial in clinical protocols for the treatment of burn injuries.

5. Conclusion

In conclusion, we found that the use of the amniotic membrane to treat superficial partial-thickness burn injuries in rats reduced the inflammatory response and improved the proliferative phases of the healing process. In our findings, the absence of phlogistic signs reiterates the already known properties of the amniotic membrane that acts as a protective barrier in wounds. The early appearance of the crust in lesions treated with HAM strengthens the microscopic findings that suggest the faster onset of the tissue repair process. The application of this biomaterial, supported by histological and macroscopic analysis, allowed attest to its effectiveness in promoting a quick cure of the crucial steps to guarantee skin regeneration in this type of wound.

For future studies, we recommend the inclusion of more experimental times and other techniques and tests that assess the entire evolution of the tissue repair process of burn injuries treated with HAM.

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