Applicability of crystalline cellulose membrane in the treatment of skin wounds induced in Wistar rats

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ABSTRACT

PURPOSE: To evaluate the healing of skin wounds induced experimentally in rats using a crystalline cellulose membrane (Veloderm®).

METHODS: Thirty-two rats were divided into two groups: control group (CG) wounds treated with a solution of 0.9% sodium chloride and Veloderm® group (VG) wounds treated with a crystalline cellulose membrane. The rats were evaluated at different times over twenty-six days.

RESULTS: Weight loss was observed in the animals from both groups in the early stages, with greater weight in the VG animals at the end. Times of predominant hypothermia, pink color of the wound in both groups over all time points, increased granulation tissue in the CG animals, the presence of slight oozing from the wound and feature in the VG animals, more serous exudation of the bloody feature, greater wound contraction and pain in the CG animals and an absence of pain and earlier complete wound healing in the VG rats were also observed.

CONCLUSION: The crystalline cellulose membrane is effective in the treatment of wounds in rats, easy to use, protects and maintains the humidity of the wound, decreases pain, eases the visualization and control of the evolution of the lesion.

Key words: Biological Dressings. Wound Healing. Rats, Wistar.

Introduction

The skin consists of three layers: epidermis, dermis and subcutaneous tissue[1], and wound healing is a complex event that involves the organization of cells, chemical signals and the extracellular matrix to repair the skin and induce scar formation[2].

When there is no impediment, healing has three distinct and interrelated phases: the inflammatory, the exudative and the proliferative phase of regeneration and repair also known as tissue remodeling. The latter stage can last from months to years and includes reorganization of the collagen and increased resistance of the scar[3].

Cavazana *et al*.[4] report that the treatment of wounds is still under research due to the lack of consensus regarding the best treatment.

To be effective for the treatment of a lesion, a product should be able to keep moisture in the wound bed, remove excess exudate, allow gas exchange, provide thermal insulation, be waterproof and ensure that bacteria do not cause trauma[5].

A crystalline cellulose membrane (hemicellulose), sold in Brazil as a temporary skin replacement, is obtained from a cane sugar-based structure of hemicellulose microfibers made by a biotechnological process. Its application promotes rapid healing and is indicated for all cases where there is loss of the epidermis, such as abrasions, ulcers, and graft and donor sites for burns[5-8]. The crystalline cellulose membrane is contraindicated for infected and very exudative wounds[9]. A survey was developed by the Biotechnology and Pharmaceutical Technology departments of Federal University of Paraiba, and the product Veloderm® is registered as the result of continuous technological development and market research by a Brazilian company that is controlled by an Italian group. Veloderm® has been registered in Italy since 2001 as a sterile class IIA medical device and is registered with National Agency of Sanitary Surveillance (ANVISA) in Brazil.

The product results from a fermentation process with a mixture of *Acetobacter Xylinum*, *Saccharomyces Cerevisiae*, and *Saccharomyces Pombe*, producing a polymeric structure with a density of 0.02 mm to 0.08 mm, characterized by the following composition: 96.2% hemicellulose, 0.4% carbohydrates and 3.4% water. The product is a transparent, odorless film available in four different sizes: 6x6 cm, 6x9 cm, 9x12 cm and 12x18 cm. Studies report that when the crystalline cellulose membrane is used in healing wounds, it induces rapid healing, does not produce stretch marks and eliminates the pain resulting from the absence of the skin without the need for painkillers. When wet with a solution of 0.9% sodium chloride, the film becomes transparent, allowing direct visualization of the wound and providing mobility without being torn[5].

As the search for alternative treatments for cutaneous lesions has been intensified in recent years, a few studies were performed using Veloderm® in humans with excellent results. Because of the absence of studies with animals, this study was designed to histologically evaluate the use of the crystalline cellulose membrane (Veloderm®) in healing skin wounds induced in rats.

Methods

Research approved by the Ethics Committee (Protocol n. 44/09) of Oeste Paulista University (UNOESTE) and the study was conducted in accordance with the ethical principles.

Thirty-two males of the Wistar rat, weighing between 250 g and 60 days, respectively, were divided into two experimental groups of 16 animals per group. The groups were divided as follows: control group (CG) and Veloderm® group (VG). The animals were kept under controlled temperature (22 ± 2ºC) and light (12-hour light-dark cycle) with free access to food and water. The rats were restrained manually, and a shaving was performed in the dorsal region. Next, the animals were anesthetized with pentobarbital (Cristália Produtos Químicos e Farmacêuticos, Itapira-SP) at a dose of 30 mg/kg administered intraperitoneally and immobilized on operative surfboards. The skin was marked with a Pilot® black pen and a 3x2 cm plastic mold. The skin was removed using a scalpel blade number 22 and an anatomical clamp to preserve the muscle. Treatment of the animals in both groups was performed every monday, wednesday and friday. The wounds of the CG animals were always treated with a solution of 0.9% sodium chloride, followed by the placement of sterile gauze and a crepe bandage over the wound. For the VG animals, the wound was cleaned with a 0.9% sodium chloride solution, and a 6x6 cm crystalline cellulose membrane (Veloderm®) was placed over the wound and then covered with sterile gauze and a crepe bandage. In subsequent treatments, the film was not withdrawn, and only the gauze and bandage were restored. The animals were kept in individual boxes at different times and evaluated for 26 days.

Evaluations were performed at the following times: M0 (day of induction of the wound), M2 (2 days), M5 (5 days), M7 (7 days), M9 (9 days), M12 (12 days), M14 (14 days), M16 (16 days), M21 (21 days), M24 (24 days), and M26 (26 days). At all times, the wound was photographed with a Kodak® digital camera.

We assessed the weight in grams (g) using a digital scale, brand ELC-10®, and the rectal temperature with a Geratherm

Clinic® brand digital thermometer. The assessments of macroscopic the wound were scored as follows: the color of the wound: 1-pink, 2-yellow, 3-pale, 4-cyanotic; the wound edges: 1-without granulation tissue, 2 - little granulation tissue, 3-very granulation tissue; the exudate: 1- absence of exudate, 2-little exudate, 3-very exudate; exudate characteristic: 1-serous, 2-sanguineous, 3 -purulent; sensitivity: 1-no pain, 2-present pain. The wound area was measured using digital pachymeter DC-60® at the moments indicated above and we calculated the percentage of contraction of each lesion using the mathematical model proposed by Oliveira *et al*.[19] where the percentage of contraction (Pc) is equal to the final area (FA) minus the initial area (IA) times 100 (x100) divided by the initial area (AI): Pc = (FA - IA)/IA x 100.

At M7 (7 days), M14 (14 days), M21 (21 days) and M26 (26 days), four animals of each group were euthanized at each moment with sodium pentobarbital (Cristália Produtos Químicos e Farmacêuticos, Itapira-SP) at a dose of 100mg/kg for accomplishment of histopathological. We evaluated the epidermis for degeneration, necrosis and regeneration, dermal edema, hemorrhage, degree of neovascularization, fibrosis, and mononuclear and polymorphonuclear inflammatory infiltrate. For all these parameters, we applied the following scoring system: (0) absent, (1) mild alteration, (2) moderate alteration and (3) marked alteration.

After biopsy of the skin with a scalpel covering the central area and edge of the wound, the sample was referred to the Department of Pathology of the Veterinary Hospital of the institution.

The tissue of the lesion and a surrounding area was removed, keeping the pieces in 10% formalin for 48 hours, and then paraffin-fixed and subjected to transverse sections of 5 µm and stained with hematoxylin-eosin. The analysis was performed by a pathologist who was unaware of the treatments given to the different animals. The normality of the variables of body weight, temperature and the area of wound contraction were verified through a Komolgrov-Smirnov test, by which all variables were considered to be parametric. To detect significant differences between the means of the CG and VG for these variables in each time point, we used the unpaired t test. The other variables measured by scores were considered as non-parametric, and the median values of the CG and VG were compared within each time point using the non-parametric Mann-Whitney test. The temporal evolution of the data for the CG and VG was assessed graphically using the computer package Excel®. The histological variables were analyzed by the non-parametric Mann-Whitney and Kruskal-Wallis tests. All statistical analyses were performed using the computer package GraphPad InStat 4.0®. The level of significance for all comparisons was 5%.

Results

In the times M2 and M5 for the CG rats and M2, M5 and M7 for the VG rats, there was a loss of body weight in relation to the initial weight. In the moments M7, M9, M12 and M14, the animals of the CG had higher weight than the VG animals. For the moments M16, M21, M24 and M26, the weight was greater for the animals in the VG compared to CG.

In the evaluation of the rectal temperature, when comparing the groups in M2, a significant decrease ($p<0.05$) was observed in the VG animals that remained compared with the CG M5, which maintained the temperature within the normal for the species. At M7, the animals in the CG showed a small decrease in temperature. The temperature for the VG was within the normal range, and there was no significant difference ($p>0.05$) between the groups. At M9, the decreased temperature of the CG animals became significant compared with the VG. At M12, both groups had an average below the reference values that was significant in the comparison groups and more relevant compared with the VG. From the M14, the average temperature remained within the normal range. The color of the wound remained pink with no significant difference ($p> 0.05$) between the groups.

It was observed that by M5, the edges of the wound in the CG animals showed higher granulation tissue compared with the VG animals. At M7, M9, M12 and M14, the granulation tissue was similar among the groups. At M16, a gradual decrease in the granulation tissue began to occur for the VG animals, and the wound was completely closed at M24 in 50% of the animals and at M26 for 100% of the animals. In the CG, at this time, there had not yet been complete healing of the lesion, and there was still substantial granulation of the wound edges (Figure 1)

Exudate was present from M2 in small amounts in the animals from both groups, but for the CG, exudation was always higher compared with the VG and was significant up to M9. The predominant feature of the exudate was serous for the VG animals and bloody for the CG animals (Figure 2).

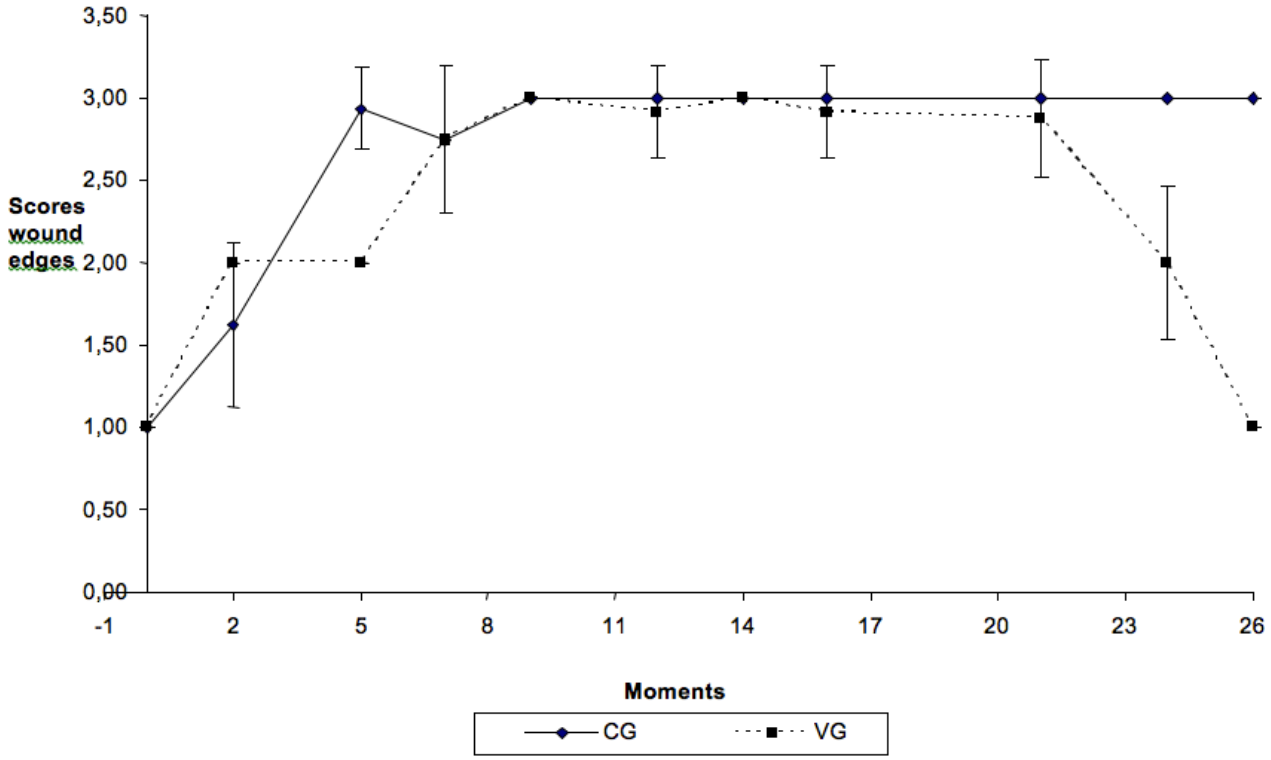

FIGURE 1 - Medians and percentiles of the wound edges (1-without granulation tissue, 2-little granulation tissue, 3-very granulation tissue) in animals of the control group **(CG)** and Veloderm group **(VG)** in different moments (days) of evaluation.

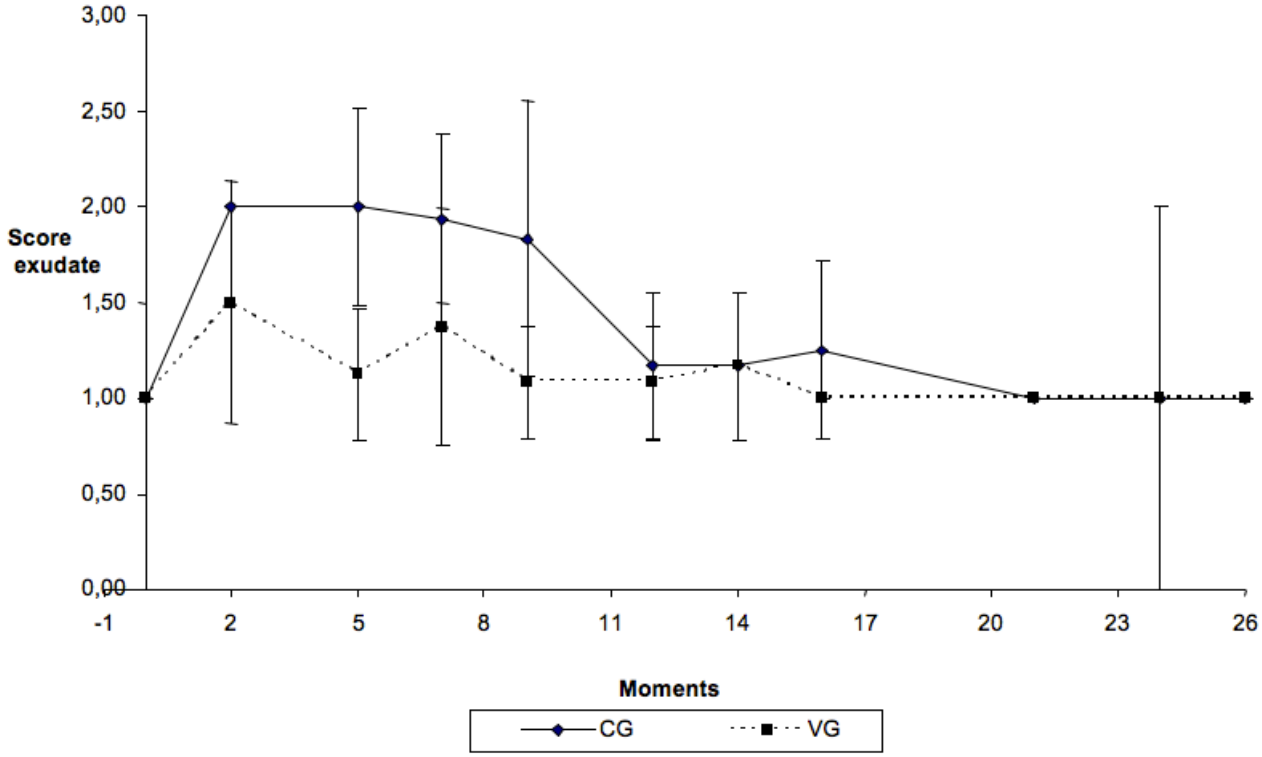

FIGURE 2 - Medians and percentiles of the exudates (1-absence of exudate, 2-little exudate, 3-very exudate) in animals of the control group **(CG)** and Veloderm group **(VG)** in different moments (days) of evaluation.

Regarding the presence or absence of pain sensitivity, 100% of the animals from the CG showed pain at M2, while only one VG animal showed pain, and this difference was significant. In all subsequent times, the VG animals no longer showed the presence of pain sensitivity, while 37.5%, 25%, 8.33%, 16%, 8.33% and 9% of the CG animals still had pain at moments M5, M7, M9, M12, M14 and M16, respectively.

In the evaluation of the wound area, the average area initially increased to M2 and slowly dwindled as the days passed. Comparing the groups at all the time periods evaluated showed no significant difference except at M24 and M26. The average percentage of wound contraction was higher in the CG animals than in the VG animals at M21 and M2 and higher for the VG animals at M24 and M26 compared with the CG animals (Figure 3).

In the histological evaluation of the skin, degeneration and necrosis in the CG were significantly higher at M7 than at M21. In the VG, necrosis was higher at M14 than at M21. Regeneration was more intense at M21, which differed from the M7 in the CG, and was greater in the VG at M26 than at M14.

In the dermis, there was light bleeding in the CG and VG animals at M7, which differed from the M21, and in the VG at M26. Fibrosis was greater at M26 than at M21 in the VG. In the comparison between the groups within the same time periods, swelling and fibrosis was higher in the control group at M21 and M26, respectively. Neovascularization and infiltrating polymorphonuclear and mononuclear cells showed no statistically significant difference between the groups at any time period but were present at all time points (Table 1) (Figures 4 and 5).

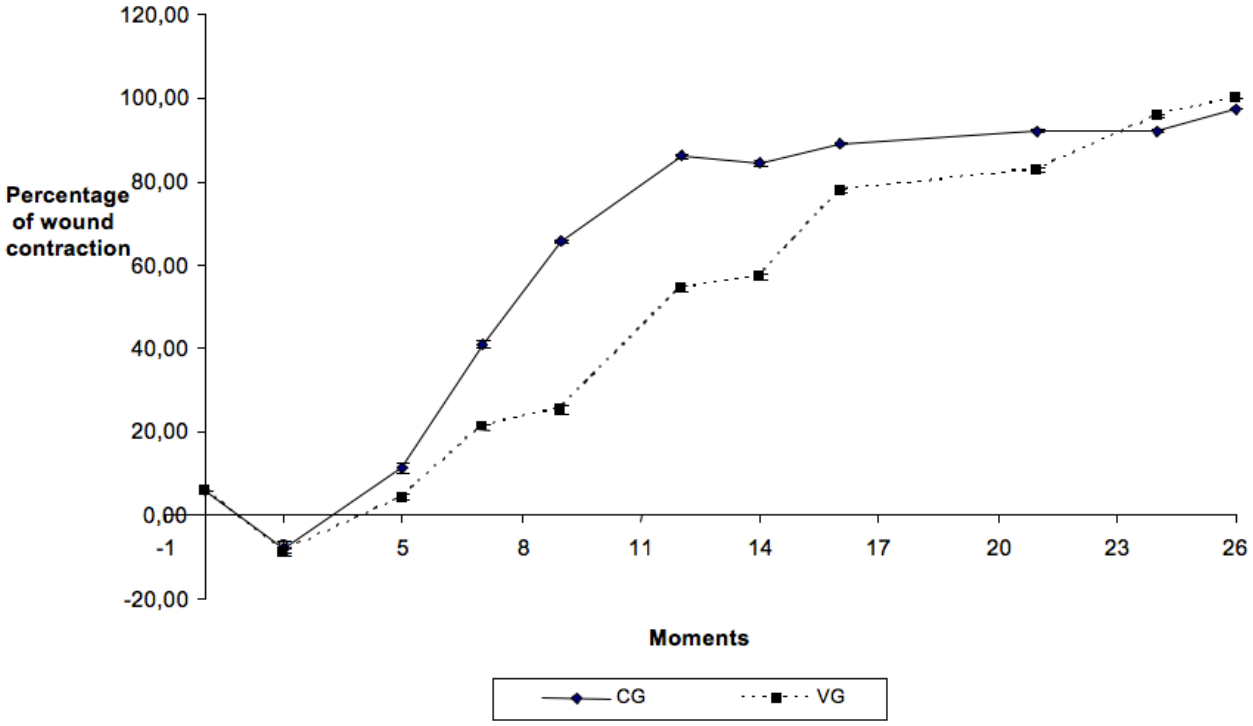

FIGURE 3 - Percentage of wound contraction in animals of the control group **(CG)** and Veloderm group **(VG)** in different moments (days) of evaluation.

TABLE 1 – Median values and percentiles of histologic comparison between the control group (CG) and Veloderm group (VG) during the evaluations.

	CG				VG			
	M7	M14	M21	M26	M7	M14	M21	M26
EPIDERMIS								
Degeneration	3.0**A** (2.0-3.0)	2.0 (1.0-2.0)	0.0**B** (0.0-2.0)	0.5 (0.0-3.0)	1.5 (1.0-2.0)	2.0 (1.0-2.0)	1.0 (1.0-1.0)	1.0 (0.0-3.0)
Necrosis	3.0**A** (3.0-3.0)	3.0 (2.0-3.0)	0.0**B** (0.0-2.0)	1.0 (0.0-3.0)	2.0 (2.0-2.0)	3.0**A** (2.0-3.0)	1.0**B** (1.0-1.0)	1.0 (0.0-2.0)
Regeneration	0.0**A** (0.0-0.0)	1.0 (1.0-1.0)	2.5**B** (1.0-3.0)	1.0 (0.0-3.0)	1.5 (1.0-2.0)	1.0**B** (1.0-1.0)	2.0 (2.0-2.0)	2.5**A** (2.0-3.0)
DERMIS								
Edema	2.0 (2.0-2.0)	2.0 (2.0-2.0)	1.0**a** (1.0-2.0)	1.0 (0.0-3.0)	2.0 (2.0-2.0)	2.0 (1.0-2.0)	0.0b (0.0-1.0)	1.0 (0.0-2.0)
Hemorrhage	2.0**A** (2.0-3.0)	2.0 (1.0-2.0)	0.0**B** (0.0-1.0)	0.0 (0.0-2.0)	2.5A (2.0-3.0)	1.0 (1.0-2.0)	0.0B (0.0-1.0)	0.5**B** (0.0-1.0)
Neovascularization	3.0 (3.0-3.0)	3.0 (3.0-3.0)	3.0 (2.0-3.0)	2.5 (2.0-3.0)	3.0 (2.0-3.0)	3.0 (2.0-3.0)	3.0 (2.0-3.0)	2.0 (2.0-2.0)
Fibrosis	3.0 (3.0-3.0)	3.0 (3.0-3.0)	2.5 (2.0-3.0)	3.0**a** (2.0-3.0)	3.0 (2.0-3.0)	2.0 (2.0-3.0)	3.0**A** (3.0-3.0)	1.5**Bb** (1.0-2.0)
Polymorphonuclear infiltrate	2.0 (2.0-3.0)	2.0 (1.0-3.0)	0.0 (0.0-2.0)	1.0 (0.0-2.0)	1.5 (1.0-3.0)	2.0 (1.0-2.0)	1.0 (1.0-1.0)	0.5 (0.0-1.0)
Mononuclear infiltrate	3.0 (3.0-3.0)	3.0 (3.0-3.0)	2.5 (2.0-3.0)	3.0 (2.0-3.0)	3.0 (2.0-3.0)	2.0 (2.0-3.0)	2.0 (2.0-2.0)	2.0 (2.0-2.0)

Capital letters compare moments in each group. Lowercase letters compare groups at every moment. Scores: 0-absent;1-light; 2-moderate; 3 accentuated.

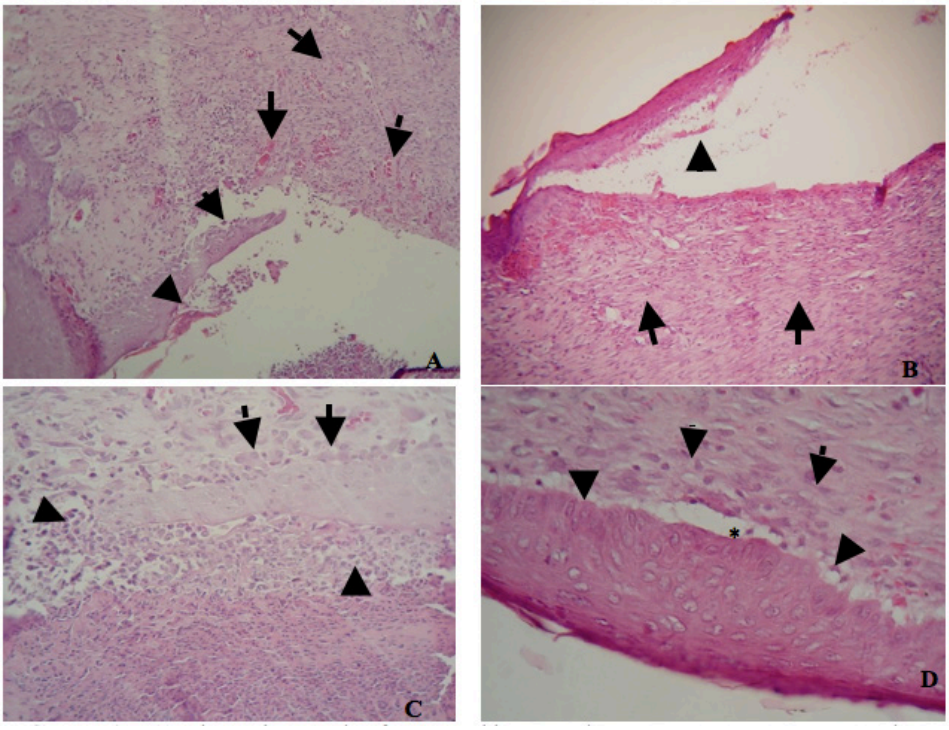

FIGURE 4 - A) Photomicrograph of mouse skin control group M7 moment. Image shows intense spongiosis in the epidermis (arrowheads) and mild regeneration of the epidermis (lower arrow). In dermis observed accentuated neovascularization and fibrosis (large arrow). H/E-x100. **B)** Photomicrograph of mouse skin control group when M14-Image shows epidermal spongiosis and mild regeneration hemorrhage in dermal-epidermal junction (arrowhead). In dermis is observed fibrosis and severe neovascularization (arrows) H/E-x40. **C)** Photomicrograph of mouse skin control group M21 - Picture shows intense neutrophilic inflammatory infiltrate (arrowheads) and mild regeneration of the epidermis (arrows). H/E-x400. **D)** Photomicrograph of mouse skin control group M26-Image shows accentuated regeneration of the epidermis (arrowheads) and cleft dermal-epidermal junction (*). In dermis is observed accentuated fibrosis (arrows). H/E-x400.

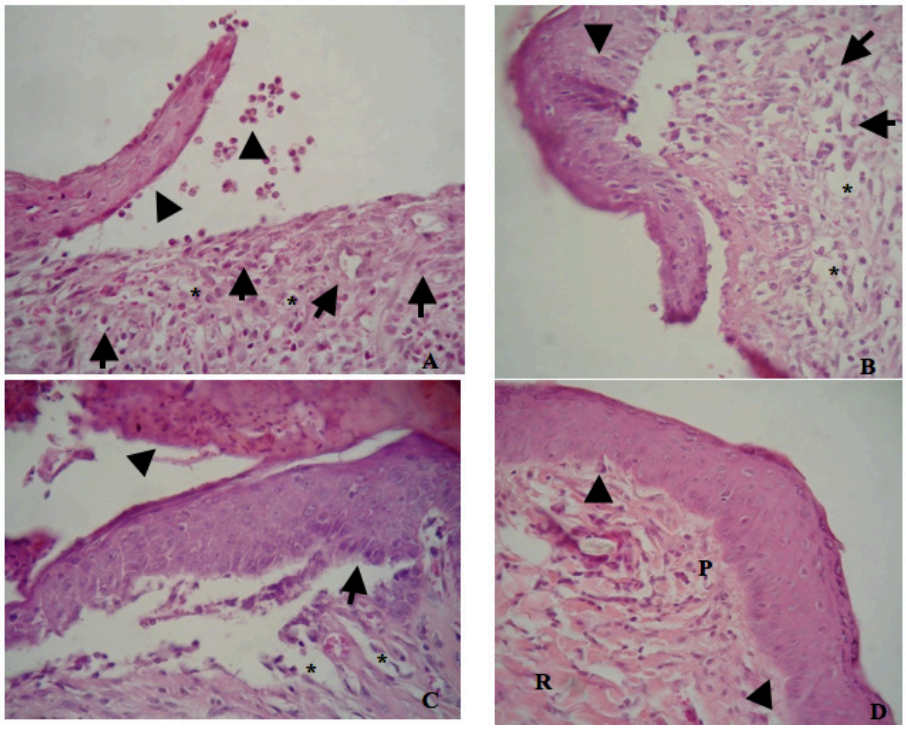

FIGURE 5 - A) Photomicrograph of mouse skin in group Veloderm® M7-Image shows mild regeneration of the epidermis (arrowhead), neutrophilic inflammatory infiltrate at the dermal-epidermal junction (lower arrow). In dermis is observed severe edema (*), fibrosis, and severe neovascularization (large arrow). H/E-x400. **B**) Photomicrograph of mouse skin group Veloderm®, M14-Image shows moderate spongiosis (arrowhead) and mild regeneration of the epidermis, epidermal cleft sub, mild inflammatory infiltrate of mixed type in the dermal-epidermal junction. It is also observed in dermal edema marked (*) and proliferation of fibroblasts and accentuated fibrosis (arrows). H/E-x100. **C**) Photomicrograph of mouse skin group Veloderm®, M21-Image shows crust under the epidermis (arrowhead), with moderate regeneration apparent proliferation of the basal layer (arrow). Moderate edema in the dermis (*) with the presence of slit epidermal-dermal junction. H/E-x400. **D**) Photomicrograph of mouse skin group Veloderm®, M26-Image shows fully regenerated epidermis (arrowheads). In dermis observed distribution of fibroblasts in both the papillary dermis (P) and reticular (R) in normal limits. H/E-x400.

Discussion

The initial weight loss may have occurred as a result of the stress and pain caused by the induction of the injury. The response mechanism in the face of new situations, known as stress, can lead to decreased intake of food and water. Pain is a sensory quality alert for individuals to understand the occurrence of tissue damage and to establish the mechanisms of defense or escape. The critical point is how to assess pain in animals[10]. The assessment of behavior, weight gain, and early recovery in animals has been demonstrated in many studies to be better in animals where the stress was minimized, for example, with the use of analgesics[10].

The temperature oscillations with a predominance of hypothermia observed at a few time points for the CG and VG animals may have been caused by the stress of trauma, the large area of skin removal and the use of the 0.9% sodium chloride solution at room temperature rather than heated.

The treatment of wounds using a solution of warm (37°C) 0.9% sodium chloride provides increased mitotic activity of the tissue and prevents the cooling of the wound bed[11].

Proliferation, cell migration and epithelialization occurred from the edges of the wounds and VG was along time, decreased granulation tissue and wound healing favoring early, in agreement with other authors[1,12]. In the two groups, there was no contamination of the wound site and humidity was maintained, probably by the continued use of the dressing and according to Mandelbaum *et al*.[11], this factor results in improvement of 35-45% in the rate of epithelialization of wounds. The phase of fibroplasia starts approximately 48 hours after injury with the appearance of fibroblasts to multiply and produce components such as ground substance, and collagen also occurs to intensify endothelial proliferation[13]. According to Mendonça and Coutinho-Neto[14], the exponential period (greater fibroplasia) occurs between six and fifteen days after injury, which is consistent with the findings of this study, since the period with the greatest presence of granulation tissue (activity of myofibroblasts) is proportional to the period of increased tissue repair.

These results are in agreement with Ferreira *et al*.[5] who used Veloderm® membrane in human patients. Some authors reported that sucrose from cane sugar reduces local edema and passive congestion, stimulates epithelialization has bactericidal effect, haemostatic properties and can positively influence wound healing[1,6,9,15].

The tissue repair process showed several phases with characteristics that developed together. After removing the piece of skin, a continuous flow is formed that is initially filled by a fibrin clot and inflammatory exudate, forming the crust covering the wound. The next phase is inflammation, with the presence of inflammatory exudate, vasodilation, increased vascular permeability, and extravasation of the plasma, erythrocytes, leukocytes, and particularly the monocytes and neutrophils, followed by macrophages that last for 48 to 72 hours, which justifies the presence of exudate at M2 in the animals from both groups in this study[13].

The presence of significant pain, agitation and issuing in animals at CG and absence in VG show efficiency of the membrane in minimizing patient discomfort. In human patients treated with Veloderm®, the presence of pain was not reported by the patients, and the product is probably a temporary skin substitute, agreeing with the observations of this study[5]. Exposure of the skin or any other body part to potentially harmful stimuli induces an unpleasant sensation[16]. According to Crissiuma and Almeida[17], the negative consequences of pain can be grouped under the title of stress response, where several physiological functions may be impaired. Adequate pain relief promotes the welfare of the animal and has a positive effect on the speed and quality of recovery from tissue damage. It is believed that animals have a nervous system similar to humans, so any procedure considered painful for humans is also painful for animals; the proof is that behavioral disorders due to painful stimuli disappear when painkillers are provided to the appropriate animal[10].

Although the percentage of contraction was greater than the CG animals at some time points, the VG animals showed a healing process of the wounds with the edges aligned more homogeneously, without edema or exudate and little granulation tissue, which may have contributed to an earlier macroscopic epithelialization compared with the CG animals. The permanence of the membrane over the wound during treatment greatly contributed to this repair process. In each wound in the CG animals, crusts were present, and each withdrawal and cleaning caused trauma, bleeding and a painful reaction. The area of the initial wound contraction increases compared with the template area because the edges suffer centrifugal retraction due to the elastic tension of the surrounding skin, a loss of adhesion to the deep fascia and the mobility of mouse skin[18]. This variation, does not interfere in the calculation of the wound area, whereas the contraction depends on the difference between the starting area and that measured on the day of assessment. This assessment is performed with the individual values, not the averages[19].

Works recommend maintaining the animals in the same position for the measurement of the area but does not clearly describe what position and how this could be accomplished with the animal awake. Repeated exposure of the animal to anesthetic procedures may be harmful to their health and the wound and cause damage during induction of anesthesia[20]. The current study used a manual physical restraint, thus avoiding the repeated exposure of the animal to anesthesia.

Yaguishita[21] reported that in their experiment using a porous cellulose membrane called Membracel® from the twenty-first day after surgery, there was a higher rate of contraction of the lesion area in the treated group compared with the control group, corroborating in part the results of the current study. This fact can be attributed to the humid conditions provided by the porous membrane, increasing chemotactic factors for defense at the injury site by attracting neutrophils, lymphocytes and macrophages.

The mechanism of contraction plays a fundamental role in reducing the area of wound healing by secondary intention, as in our experiment. The wound contraction is the centripetal movement of the wound margins to the center of the lesion, which begins during the proliferative phase and continues during the remodeling process and occurs due to the contractile activity of myofibroblasts present in the granulation tissue[22]. In a study with rats, Branco Neto *et al*.[12] and Amorim *et al*.[23], reported in the control group a high concentration of polymorphonuclear cells at the beginning of the inflammatory process with a significant decrease in its presence in the 14th and 21th days compared to the 7th day, consistent with the current study where a decrease was observed from M21 for both the CG and the VG.

Conclusion

The crystalline cellulose membrane is effective in the treatment of wounds in rats, easy to use, protects and maintains the humidity of the wound, eases the visualization and control of the evolution of the lesion.

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