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Research Article

Effect of Aloe vera Extract on the Wound Healing Process. Histological Study in Cyclophosphamide-Treated Mice

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Abstract

Wound healing is a fundamental response to tissue injury, resulting in restoration of the tissue's integrity. Aloe vera is a tropical cactus that has shown its therapeutic potential in a variety of soft-tissue injuries, and has been used in the treatment of wounds. In this study, wounds were created on the back of cyclophosphamide-treated mice which received treatment consisting in the topical application of Aloe vera gel on the surface of the wounds. Biopsy specimens were collected from all groups of mice at 4, 7 and 10 days after the beginning of the treatment. In mice treated with Aloe vera gel the wounds showed increased epidermal growth after 7 days of treatment. Therefore, Aloe vera extract enhanced the healing process of cutaneous wounds in the immunosuppressed mice.

Keywords: Aloe vera; Wound Healing; Cyclophosphamide; Immunosuppressed; Mice

Introduction

Wounds have taken part in men's life from the early beginning, mainly through disease, accident, warfare and surgery. Sumerian, with their cuneiform script, figures as the first nation to have written about wound care. Ancient civilizations such as Egypt, Greece and Rome expanded literature on wounds and their treatment [1].

Over time, humans seem to have tried everything within their immediate grasp in the laudable effort to promote wound healing. It would be impossible to reference all the substances and procedures which were employed over the centuries to provide effective treatment to wound diseases [2].

Wound healing is a natural and an essential process that involves a regulated series of biological events and is capable to repair and regenerate tissue structure and functions that have been disrupted, or wounded, by physical, chemical, bacterial or viral insults [3,4].

Wound healing is a highly complex, but orchestrated cascade of events which can be divided in four overlapping phases; the entire process may last for months: haemostasis, inflammation, epithelialization or granulation tissue formation and remodeling of the extracellular matrix [5, 6]. If after the repairing process the result is tissue that has grown structurally and functionally identical to the original tissue, then regeneration has taken place. However, if the tissue's integrity is replaced with the formation of proper fibrous connective tissue or scarring is observed, repair has occurred [6].

Immediately after injury and when this lesion reaches the dermis, damaging tissue and capillary vessels, there is clot formation and the earlier phases of wound repair involve inflammation and synthesis of ground substance [3]. The clot thus formed results in a temporary barrier and source of hemostatic signals, which attract several types of

inflammatory cells and involve many co-coordinated interactions between cells within both the dermis and the epidermis [5,6].

These events show a variety of cellular activity including phagocytosis, chemotaxis, mitogenesis and synthesis of matrix components [3]. Many soluble mediators, such as platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), fibroblast growth factor (FGF), and transforming growth factor-ß (TGF-ß) are released. Most of the substances involved in the early phase of wound repair are capable of stimulating the proliferation of wound components and direct the migration of target cells [5,6].

After tissue injury induced by trauma or by surgical procedures the inflammatory step is initiated by blood clotting and the mast cell/platelet degranulation process. Vasodilatation, capillary permeability, complementary activation, migration of polymorphonuclear leukocytes and macrophages has shown to be significant. The neutrophils and macrophages engulf and destroy bacteria and release protease, including elastase and collagenase, which can damage extracellular matrix [4,5].

In the process of connective tissue healing, a number of inflammatory cells in the wound decreases and fibroblasts, endothelial cells and keratinocytes take over synthesis which is followed by migration and proliferation of the keratinocytes, obtained from the intact remained tissues at the site of the wound. There is also the deposition of collagen, a tissue synthetized by the highly vascularized granulation tissue, and the contraction of myofibroblasts around the site, which change the wound's edges in order to close down the injured spot. This remodeling process continues for several weeks after initial wound closure[4-6].

Wounds may be occurring in patients without any systemic problems but tend to occur in normal or sick tissue in patients with particular infections and/or metabolic disturbances, like venous leg ulcers and pressure ulcers in diabetic patients, or who received a specific group of medicines, as for instance oncology patients [7-9]. In both groups the wound process can lead to infection and sepsis [7]. Wound infections might have an adverse prognosis, leading to significant morbidity, poor cosmetic results, prolonged hospitalization and sometimes death [7,9].

Such impaired healing inflicts a huge cost upon society, diminishing the quality of life for millions worldwide; thus, recent attention has been turned to the investigation of cost-effective, accessible, alternative therapeutic strategies [7]. Herbal treatments became popular and some surveys related an increase in the use of unconventional treatments among individuals in the general population, and when these therapeutic agents are used on dermatological conditions this rate grew 380% in the last few years [10]. Traditional forms of medicine practice and herbal remedies from medicinal plants are being scientifically investigated for their potential in the treatment of wound and related disturbances [11].

Aloe vera (Aloe vera Linn, synonym Aloe barbadenisi Miller) is a perennial succulent herb belonging to the Liliaceal family. It is a cactus-like plant that grows in hot, dry climates [12,13]. It has been used medicinally for over 5.000 years by Egyptian, Indian, Chinese and European cultures [14]. Perhaps its survival in a harsh environment has encouraged people to believe that Aloe vera contains some potential capacity to promote care and heal to conditions such as tissue injuries, dermatological problems and infections. It contains over 70 biologically active compounds and has been reported to display immunomodulatory, anti-inflammatory, UV protective, antiprotozoal, wound healing, burn healing, anti-oxidant, anti-cancer, anti-diabetic and anti-ageing properties, besides being used for cosmetic purposes in various physiological processes not yet properly described [14-16].

Topical Aloe vera is commonly used for the treatment of sunburn, scalding burns and frostbites [17,18]. Studies in vivo have reported the beneficial effects of Aloe gel in wound healing, [19] skin protection from radiation injury[20-22] and in the treatment of oral ulcers and oral lichen planus [23,24]. Among cancer patients the use of topical Aloe vera gel is frequent to prevent radiation-related dermatitis and mucositis [20,21].

Because of Aloe vera promising preclinical activity, low cost, and popularity as an alternative medicine among patients who presented problems associated with burns, plastic surgery, oncology treatment and dermatological lesions, the goal of this work is to evaluate the influence of Aloe vera on the wound healing process in cyclophosphamide-treated mice.

Materials and Methods

Animal treatment

Forty-two seven-week-old adult male Swiss mice weighing from 28g to 34g sent from the Clinical Research Unit & Institute of Biomedicine (at the Federal University of Ceará, Brazil) were used in this study. All of the animals were kept into individual boxes, on a 12h light/dark cycle, temperature of 24°C, with water and chow ad libitum. The animals were divided into seven groups of six animals each. The experimental procedure was approved by the Animal Ethics Committee of the Federal University of Ceará.

Drugs

Aloe vera (a formulated product of 10% Aloe vera gel plus carbopol; Ethical Pharmacology, Ceará, Brazil). Cyclophosphamide (Genuxal; Asta médica, São Paulo, Brazil) (1000 mg ampoule) was prepared in a saline solution (Sodium Chloride 0.9% w/v).

Cyclophosphamide treatment

The group of mice pretreated with cyclophosphamide became leukocytopenic by means of an intraperitoneal injection of cyclophosphamide in a single dose of 150mg per kg (body weight), 2 days before the surgery. Mean peripheral white blood cell count (n=6) was 1.016/mm^3 (control mice showed 4.900/mm^3).
Groups

The forty-two Swiss mice were divided in seven groups of six animals each. Four groups were pretreated with cyclophosphamide (CTX group), and three groups received saline in the pretreatment (Saline group). For all groups each experimental wound (left side) was treated twice a day with an ointment containing 10% Aloe vera gel (Saline + Aloe vera and CTX + Aloe vera). The right side of both groups on each animal was treated only with carbopol gel (Saline + carbopol and CTX + carbopol). The animals were euthanatized after 1, 4, 7 and 10 days (in the CTX group) and 4, 7 and 10 days (the groups that received only saline during the pretreatment).

Wound surgery

The animals were anesthetized by an intraperitoneal injection of Ketamina HCl (100 mg kg^{-1}, i.p.) (Pfizer, São Paulo, Brazil) and Xilazina (5 mg kg^{-1}, i.p.) (Virbac, São Paulo, Brazil). After shaved with a razor blade, and having undergone manual depilation and asepsis, two square cutaneous excisions were made on the back of each animal, close the cervical area, after demarcation with a stainless steel device, using a metal template measuring about 8 mm in diameter.

Macroscopic analysis

At the time determined for each experiment, animals were anesthetized with cloral hydrate (250 mg kg-1, i.p.), before the animal was sacrificed. For macroscopic analysis, inflammatory aspects such as erythema, hyperemia, hemorrhagic areas and abscesses were evaluated. This analysis was performed by an only examiner (FACV).

Biopsy specimens

Biopsy specimens were obtained from the skin lesions at 1, 4, 7 and 10 days after the wound surgery. Each specimen was spread flat on a piece of card paper, fixed in a Bouin solution for 24h and embedded in paraffin. Sections of 4µm in thickness were prepared. The slides were then stained with hematoxylin and eosin. These materials were analyzed using a light microscopy (x40). The parameters of inflammatory cell infiltration, vascular dilatation and ingurgitation, hemorrhagic areas, edema, ulcerations and abscesses were determined and graded on a score of 0-3, as follows[25]:

Score 0: normal epithelium and connective tissue without vasodilatation; absence of, or discreet cellular infiltration; absence of hemorrhagic areas, ulcerations or abscesses.
Score 1: discreet vascular ingurgitation, re-epithelization areas; discreet inflammatory infiltration with mononuclear prevalence; absence of hemorrhagic areas, edema, ulcerations or abscesses.
Score 2: moderate vascular ingurgitation, inflammatory infiltration with neutrophil prevalence, presence of hemorrhagic areas, edema and eventual ulcerations, absence of abscesses.
Score 3: severe vascular ingurgitation and dilatation, inflammatory infiltration with neutrophil prevalence, presence of hemorrhagic areas, edema and extensive ulceration and abscesses.

Morphometric analysis

The maximum length and width of each wound were measured to establish the baseline and at the deadline values. The area of the wounds was calculated from these measurements. The degree of the contraction observed in the wounds was determined from the differences between the initial and final areas observed; employing the elliptical formula [25]. The mean values of the change measured in the elliptical areas of the wounds were determined for the Saline and the CTX groups.

Statistical analysis

The results were analyzed by unpaired Student's t-test. The significance level adopted was $p < 0.05$ for the wound healing analysis.

Results

In all the experimental groups, 4-, 7- and 10-day-old ounds formed a dry, dark-red cell cover that evolutioned to a ark-brown crust which thickened with time. Exudates were observed in the control group until day 4, while absent in the other groups. No contamination was observed.

Histopathological analysis of the skin from the wounds in mice pretreated with cyclophosphamide showed a great score 3 (3-3) in both sides, on day 1 (Table 1).

Table 1. Microscopic analysis of skin wound in mice

	day 1	day 4	day 7	day 10
Cyclophosphamide – *Aloe vera*	-	0 (0-1)*	0 (0-1)	0 (0-1)
Cyclophosphamide – Carbopol	3 (3-3)	2 (2-3)	1 (0-1)	0 (0-0)
Saline – *Aloe vera*	-	1 (0-2)*	1 (0-1)*	0 (0-0)
Saline – Carbopol	-	1,5 (0-2)	1,5 (0-2)	0 (0-1)

Skin wounds (8mm in diameter) were induced by a stainless steel blade, using a metal template. Animals were examined at days 4, 7 and 10 after the surgery. Data represent the average values (and range) of microscopic scores in 6 animals per group. *P < 0.05 compared to the control group (CTX + Carbopol).

On day 4 all wounds in the control group and in the mice pretreated with cyclophosphamide showed moderate vascular ingurgitation, moderate cell infiltration with neutrophil prevalence, hemorrhagic areas, edema, ulceration, and the absence of abscesses. On day 10, the same scores were observed for all the groups.

Figure 1 compares the differences observed between the initial and the final measurements in wound areas employing the elliptical formula, concerning the initial and final areas of the wounds of the control group (right side) and the wounds treated with Aloe vera (left side), and between the group that received the cyclophosphamide pre-treatment and the Saline group.

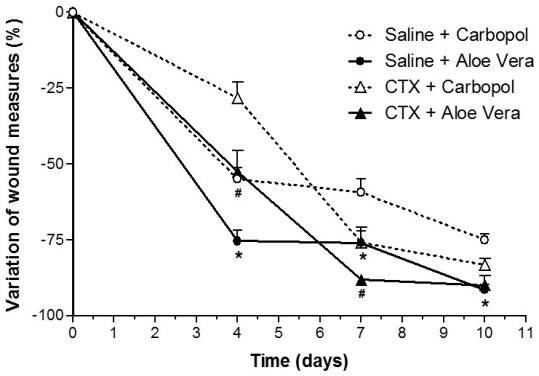

Figure 1. Differences between the initial and final areas (wound measure -%) of cutaneous wounds treated with aloe vera gel and an ointment base of carbopol into the Saline and CTX groups. Mean ± S.D. (n=6)* #P < 0.05.

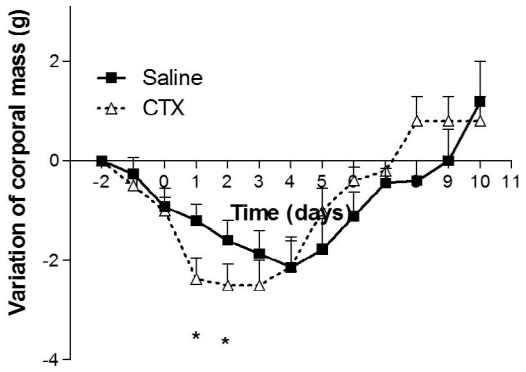

Figure 2. Cyclophosphamide treatment (150mg/kg) increases weight loss in mice with cutaneous wounds. Data are expressed as the mean value ± standard error of the mean (SEM) of body variation (g). *p<0.05 represents statistical difference between the cyclophosphamide and Saline groups.

In order to verify a possible systemic repercussion of the pre-treatment with cyclophosphamide and the skin wound surgery, body weight was measured and leukocyte counts were performed. Figure 2 shows the administration of cyclophosphamide (150mg/kg) plus induced wound surgery, which caused significant difference (P < 0,05) in body weight, on days 1 and 2, when compared to the Saline group. Leukopenia was observed on day 0 and leukocytosis on day 4 in the group of animals that received immunosupressed pre-treatment with antineoplasic agent (P < 0,05) (Figure 3).

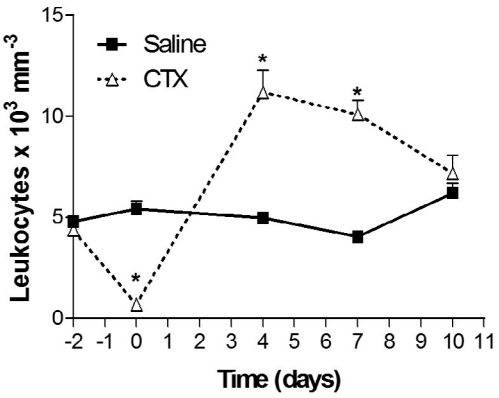

Figure 3. Cyclophosphamide treatment (150mg/kg) induces the decrease of leukocytes on the initial period of the experiment and increased values on total leukocytes at day 4. Blood was collected via orbital plexus puncture before the animals were euthanatized. Data are expressed as the mean value ± standard error of the mean (SEM) of total leukocytes x 10^3/mm^3 of at least 5 animals. * P < 0.05 represents a statistically significant difference among groups.

Discussion

In this study, Aloe vera gel utilized for the treatment of cutaneous wounds seemed to promote a better healing process in relation to carbopol, in animals with immunosuppressed and normal immune systems.

Cyclophosphamide (whose molecular formula is $C_7H_{15}C_{12}N_2O_2PH_2O$) is a potent drug used to treat some chronic diseases. The most frequently uses of cyclophosphamide include the killing of cells that are causing harm, usually cancer (Malignant lymphomas-Stages III and IV of the Ann Arbor staging system, Hodgkin's disease, lymphocytic lymphoma-nodular or diffuse, mixed-cell type lymphoma, histiocytic lymphoma, Burkitt's lymphoma; multiple myeloma; chronic lymphocytic leukemia, chronic granulocytic leukemia, acute myelogenous and monocytic leukemia, acute lymphoblastic - stem cells, leukemia, neuroblastoma) or inflammatory cells [7].

Classified as an "alkylating agent", cyclophosphamide works by binding to the DNA and interfering with normal cell function by disrupting cellular DNA. White cell blood lines show suppression in approximately 1-2 weeks following a dose of cyclophosphamide, turning the patients especially vulnerable to infections [26]. The application of cyclophosphamide (150 mg/kg) induces a stage of leukopenia such as when an immunosupressed individual has less than 1.000 granulocytes per mm3 of blood, and in danger of septis [7].

In this study, cyclophosphamide (150mg/kg) was used to simulate cancer treatment two days before the wound surgery. Animals that received this treatment have significantly depressed levels of leucocytes (mean peripheral white blood cell count 1016/mm^3 X control mice 4900/mm^3 at

days 0 and 1). It occurred at the same period that wounds had the most important macroscopic and microscopic findings, but Aloe vera was more efficient on wound regeneration in comparison to the carbopol group.

Surgery wounds showed erythema, hyperemia, hemorrhagic areas, and extensive ulcers and abscesses that appeared by day 0, and their degree was maximal on day 1. These signs of lesion diminished between days 4 and 7 for wounds treated with topical administration of Aloe vera gel and between days 7 and 10 for control animals. All these aspects were confirmed by histopathological analysis, revealing, on day 1, severe vascular ingurgitation and dilatation, accentuated inflammatory infiltration with neutrophil prevalence, hemorrhagic areas, edema, and extensive ulceration. These data are in accordance with previous studies. Furthermore, this model reproduces important signs and symptoms observed in wounds during chemotherapy treatment in cancer patients, and have not been previously used in a study of this kind dealing with the complications of cancer care.

Cutaneous injury is characterized by fibroplasias, angiogenesis and re-epithelization, observed on histopathological analysis on days 4, 7 and 10. The response to injury involves the migration and proliferation of cells such as fibroblasts, endothelial and epithelial cells, besides deposition of connective tissue and wound contraction [6]. The healing process proceeds naturally, as the damaged tissue (day 0) attempts to re-estabilish haemostasis (days 1-10). However, risk factors such as excessive inflammatory reaction, infections, and immunosuppressed system vulnerability may compromise the repair process.

Findings from in vitro and animal studies have suggested that Aloe vera can enhance wound healing by reducing vasoconstriction and platelet aggregation at the wound site, improving wound oxygenation, scavenging free radicals, increasing collagen formation, inhibiting collagenase and metalloproteinase and activating macrophages [26-30].

Other studies had shown that Aloe vera can act as an anticancer treatment by exerting antineoplastic effects through the anthraquinones contained in it. These molecules may improve the effects of the drugs used in cancer chemotherapy, thus representing a promising approach [31, 32].

Based upon the results of the present work and on previously published results [20, 30], Aloe vera gel represented a beneficial topical treatment to wound care in various phases. From the application of Aloe vera gel, the healing process was accelerated, in a complete and better way than in the Saline group.

Conclusion

In immunosuppressed mice, Aloe vera may enhance the healing process of cutaneous wounds.

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