

WOUND CARE WITH THE LEAF EXTRACT OF CECROPIN P1-PRODUCING TRANSGENIC KALANCHOE: HISTOLOGICAL FINDINGS

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Management of purulent wounds is a problem that requires particular attention: wounds are a common injury type for which suppurative complications are frequent, mortality rates are high and antimicrobial therapy may be ineffective due to the presence of drug-resistant bacteria in the wound. In this work we have studied the effectiveness of wound treatment with the leaf extract of transgenic *Kalanchoe pinnata* modified to produce antimicrobial peptide cecropin P1. Purulent wounds infected with *Staphylococcus aureus* were modeled in Wistar rats. Four groups of animals were formed, with 10 animals in each group. In all groups, the wounds were cleansed with 3 % hydrogen peroxide solution once a day; all groups except the controls received additional treatment. Group 2 received 10 % cefazolin solution, group 3 received kalanchoe juice, group 4 received the juice of cecropin P1-producing kalanchoe. Histologic stains of biopsy samples were performed after rats were sacrificed by anesthetic overdose on days 3, 10 and 14 after treatment onset. On day 3, wound dynamics was the same in all groups. On day 10 exudate was still observed in the controls; in group two exudation was almost finished and regeneration was about to begin; in groups 3 and 4 the wound defect was filled with granulation tissue. In spite of epidermal repair along the wound edges in groups 2 and 3, there still was some sloughing and granulation tissue was less mature than in group 4. We recommend conducting more extensive clinical research of the leaf extract of cecropin P1-containing transgenic *Kalanchoe pinnata*.

Keywords: wound healing, purulent wound, *Staphylococcus aureus*, *Kalanchoe pinnata*, cecropin P1, antimicrobial treatment

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ЛЕЧЕНИЕ РАН ЭКСТРАКТОМ ЛИСТЬЕВ ТРАНСГЕННОГО КАЛАНХОЭ С ЦЕКРОПИНОМ P1 (ГИСТОЛОГИЧЕСКОЕ ИССЛЕДОВАНИЕ)

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Проблема лечения гнойных ран актуальна в хирургии в связи с распространенностью ран различной этиологии, частотой гнойных осложнений, высокой летальностью, появлением антибиотикорезистентных штаммов бактерий. В работе исследована эффективность фармакотерапии раневого процесса экстрактом листьев трансгенного каланхоэ перистого с антимикробным пептидом цекропином P1. Гнойную рану моделировали на крысах линии Wistar с внесением в рану культуры *Staphylococcus aureus*. Сформировали 4 группы по 10 животных в каждой. Во всех группах раны обрабатывали ежедневно однократно 3 % раствором перекиси водорода и дополнительным препаратом, кроме группы 1 (контрольной). В группе 2 использовали 10 % раствор цефазолина, в группе 3 — сок каланхоэ, в группе 4 — сок каланхоэ с цекропином P1. Гистологическое исследование раневых биоптатов производили на 3, 10 и 14 сутки с начала лечения после выведения крыс из эксперимента путем передозировки наркоза. Результаты лечения через 3 сут были схожими во всех группах. Через 10 сут для ран крыс контрольной группы была отмечена незавершенность фазы экссудации, группы 2 — переход фазы экссудации в фазу регенерации, а групп 3 и 4 — покрытие грануляционной тканью. Несмотря на восстановление эпидермиса по краям ран в группах 2 и 3, кое-где сохранялся струп, а грануляционная ткань была менее зрелой, чем в группе 4. Результаты позволяют рекомендовать экстракт листьев трансгенного каланхоэ перистого с цекропином P1 для широкого клинического изучения.

Ключевые слова: раневой процесс, гнойная рана, *Staphylococcus aureus*, каланхоэ перистое, *Kalanchoe pinnata*, цекропин P1, антимикробная терапия

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Treatment of purulent wounds of various origins is complicated by frequent suppurative complications and associated with high fatality rates entailing considerable expenses [1, 2]. According to some authors, suppurative complications account for 30–35 % of all surgical conditions, causing death in 25 % of cases [3–5].

There are a lot of approaches to treating purulent wounds [6–10]; new methods are also being continuously developed and introduced into clinical routine. Among them are hyperbaric oxygen therapy, laser therapy, magnet therapy, wound treatment in the aseptic environment, etc. [11–15]. But the most common method relies on the use of dressings, since they are available, easy to use and cheap [16–18].

It should be noted that overuse or misuse of antibacterial drugs promotes antibiotic resistance in bacteria impeding treatment of complex chronic diseases, such as venous leg ulcers in diabetic patients who have to undergo a long-term antibacterial therapy [19, 20]. Promising alternatives to traditional antibiotics are antimicrobial peptides and biostimulators that promote healing, such as *kalanchoe*.

Many plants of the genus *Kalanchoe* are medicinal herbs: their juice is used to treat burns, dermal wounds, and ulcers; they can be used as biostimulators after skin grafting. *Kalanchoe* juice is rich in flavonoids, such as bufadienolides and lectins known to trigger mitosis in lymphocytes, vitamins, organic acids, polysaccharides, antioxidants, and micronutrients [21, 22]. So far, transgenic plants — “bioreactors” for producing active pharmaceutical ingredients — have been engineered based on *K. daigremontiana* [23, 24], *K. laciniata* [25] and *K. blossfeldiana* [26]. Of them all, *K. pinnata* has the most substantial pharmacological potential. In 2012 a new method was developed to obtain transgenic *K. pinnata* plants in which the gene of cecropin P1 is expressed [27].

Cecropin P1 is a secretory factor of a large roundworm of pigs, *Ascaris suum*; it belongs to the group of linear α -helical peptides that do not contain cysteine [28]. Unlike insect cecropins, porcine cecropin P1 consists of a long positively charged α -helix that carries a large number of amino acid residues. In the experiments *in vitro*, cecropin P1 has been shown to be highly active against gram-positive and gram-negative pathogenic bacteria [29], fungi [30] and some tumor cells [31], but its antimicrobial activity *in vivo* has not been reported so far.

In light of the above, our work aimed to assess the effect of the leaf extract of transgenic cecropin P1-producing *K. pinnata* on purulent wounds infected with *Staphylococcus aureus* in the rat model.

METHODS

The experiment was carried out in Wistar rats (age of 4 months, weight of 200–220 g) that had been quarantined prior to the experiment in the animal facility of the Research Institute for Environmental Medicine of Kursk State Medical University. Only healthy animals were chosen for the experiment. The animals were housed in a standard biologically clean room at 22–24 °C under 12/12 light cycle. All rats received pellet food and filtered tap water. Treatments were conducted in the afternoon at a fixed time. The rats were anaesthetized with intraperitoneal injections of the chloral hydrate aqueous solution, 300 mg/kg body weight, and sacrificed with its overdose. The experiment was conducted in compliance with the principles of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986).

The animals were stratified by body weight and the type of treatment applied. The rats with a modeled purulent wound infected with *S. aureus* were distributed into the following groups: group 1 (controls) consisted of animals treated with 3 % hydrogen peroxide (n = 10); group 2 included animals treated with 3 % hydrogen peroxide and 10 % cefazolin solution (n = 10); group 3 included animals treated with 3 % hydrogen peroxide and *kalanchoe* juice (n = 10); group 4 consisted of animals treated with 3 % hydrogen peroxide and transgenic *kalanchoe* juice with cecropin P1 (n = 10). Wounds were modeled on the anaesthetized animals under sterile conditions. A 20 × 20 mm area on the back was shaved to remove hair and treated with an aseptic followed by the excision of the skin and subcutaneous tissue. Then 1 ml of 10⁶ CFU/ml *S. aureus* 592 solution (a 24-h old culture) was introduced into the wound. To provide uniform treatment conditions, prevent the wound from deformation, drying, contamination or animal bites, a piece of gauze dressing was sewn onto the skin to cover the wound. In 48 h all animals showed typical signs of inflammation and suppuration. After sutures were removed, the dressing was taken off, puss was drained and wound care was performed once a day for 14 days in a row.

Biopsy was performed on days 3, 10 and 14 after treatment onset; the animals were sacrificed in threes. Soft tissue samples were excised from the wound bed and the adjacent edges using the razor. The samples were immediately fixed in 10 % neutral formalin solution, dehydrated through an ascending series of alcohols and embedded into paraffin according to the standard protocol. Paraffin slices were stained with hematoxylin and eosin.

Microscopy and microimaging were performed using the optical system consisting of Leica CM E microscope (Leica Microsystems, Germany) and Micromed DCM-510 SCOPE digital camera (Nablyudatelnie pribory, Russia) at magnifications of ×40, ×100, ×200 and ×400; images were captured using Future Win Joe software (Future Optics, China) supplied with the digital camera. In the course of the histological analysis, we assessed inflammation intensity, the onset of granulation, epithelialization at wound edges, and quality of the new epithelium. A cell profile of the tissue adjacent to the wound edges or of the newly formed tissue at later healing stages was also prepared. Fibrous tissue was differentiated from other cells karyologically. The proportion of various type cells was calculated after counting 100 cells in ≥ 10 non-overlapping fields of view.

Statistical analysis was performed using Microsoft Excel 10.0. Mean value (M) and standard error of mean (m) were computed for all parameters. The two-sample t-test with unequal variances was applied to compare the groups and establish differences between them. Differences were considered significant at p < 0.05

RESULTS

Treatment outcome in group 1 (controls)

Day 3

Purulent wounds showed signs of acute suppurative inflammation. The wound surface was covered with fibrinous and leukocyte detritus; underneath, degrading leukocytes were accumulating and hemorrhagic areas were observed. Tissues adjacent to the wound were edematous showing signs of leukocyte infiltration. Edema and infiltration were observed in deeper layers, down to the muscle tissue. The epithelium at the

wound edges was thickened and disorganized. In the dermis, collagen fibers were swollen and blood vessels were dilated and plethoric (Fig. 1, A).

Day 10

The wounds were filled with a purulent necrotic mass; the epidermis at the wound edges was thickened. Macrophages and mast cells were observed in the dermis which showed conspicuous leukocyte infiltration (see Table). Interstitial edema had spread into deeper dermal layers down to the muscle fibers (Fig. 1, B).

Day 14

Deep leukocyte infiltration was still present; the wounds were filled with necrotic tissue. The dermis was edematous at the wound edges, and the epidermis was thinned. Edema and infiltration persisted in the underlying muscle tissue. Granulation tissue started to develop in the wound bed; occasional microabscesses were observed filled with leukocytes (Fig. 1, C).

Treatment outcome in group 2 (10 % cefazolin solution)

Day 3

Histology revealed signs of acute suppurative inflammation. The wounds were filled with necrotic tissue; multiple leukocyte and fewer neutrophil infiltrates were observed. A large number of macrophages were spotted in a field of view (Fig. 1, D).

Day 10

Connective tissue was actively growing in the dermis to form the organized structure; fibroblastic cells and macrophages were abundant (see Table). Epithelial cells were vigorously proliferating and differentiating. (Fig. 1, E).

Day 14

Areas of the new epidermis with clearly differentiated layers were noticed, but its thickness exceeded that of the intact skin. Granulation tissue was mature. A few inflammatory microfoci were spotted in deeper dermal layers. On the whole, the wound granulated actively; granulation tissue was subsequently replaced by fibrous tissue. The skin defect was covered with multiple collagen fibers running in different directions (Fig. 1, F).

Treatment outcome in group 3 (kalanchoe juice)

Day 3

We observed a morphological pattern similar to that in the controls and group 2. Mast cells were actively involved in the reparative process indicated by the increased number of total cells near the wound. The wound retained residual purulent exudate and necrotic tissue. (Fig. 2, A).

Day 10

The epidermis at the edges started to advance to the wound bed where foci of granulation tissue had already appeared. Single collagen fibers were arranged chaotically surrounded predominantly by fibroblasts and macrophages (Table, Fig. 2, B).

Day 14

In some samples, complete epithelialization was observed. Variably mature granulation was observed in the derma. Collagen fibers were surrounded by fibroblasts and ran parallel to the skin surface (Fig. 2, C).

Treatment outcome in group 4 (juice of transgenic kalanchoe producing cecropin P1)

Day 3

The wounds were filled with necrotic tissue. The samples contained a lot of neutrophils. Marked edema and dilated capillaries were observed in the derma (Fig. 2, D).

Day 10

Regeneration was accompanied by a localized inflammatory response induced by the arrival of neutrophils at the wound site. Inflammatory infiltrates were polymorphic. The wound bed was granulating. Vigorous angiogenesis improved tissue vascularization in the area surrounding the wound; edema diminished, inflammatory infiltration decreased (Fig. 2, E).

Day 14

The wound was filled with multiple collagen fibers surrounded by fibroblasts. Fibers were arranged chaotically, though horizontal orientation prevailed. The epithelium was advancing growing over the granulation tissue. It was thicker than the intact skin (Fig. 2, F).

Comparison of treatment outcomes

Histological analysis conducted on day 3 of the experiment did not reveal any significant differences in treatment outcomes in different groups. However, on day 10 the situation was different. The exudative phase was still unfinished in the control group. In group 2 the exudative phase was giving way to the remodeling phase. In groups 3 and 4 the wounds were granulating.

On day 14 the number of fibroblasts significantly exceeded the number of granulocytes and macrophages in groups 3 and 4 (see Table) indicating an active regenerative process. Regeneration was the most successful in group 4 (transgenic kalanchoe with cecropin P1). By the end of the experiment the wounds in this group had been fully covered with the new epidermis.

DISCUSSION

Since 2007, a number of authors have described the process of obtaining a transgenic *K. pinnata* able to express the cecropin P1 gene and accumulate this peptide in the cytoplasm [30]. Accumulation of cecropin P1 and its effect on phytopathogens and traditional bacterial cultures have been assessed *in vitro*. However, neither of those works contain any information about the therapeutic effect of transgenic kalanchoe juice in the treatment of infections in animals. Still, healing, immunomodulatory and remodeling properties of *K. pinnata* [32] may enhance the antibacterial effect of cecropin P1 in the experiments with the transgenic plant *in vivo*.

Our study demonstrates that kalanchoe juice expedites transition from the first stage of the inflammatory process to remodeling. In comparison with the controls, kalanchoe

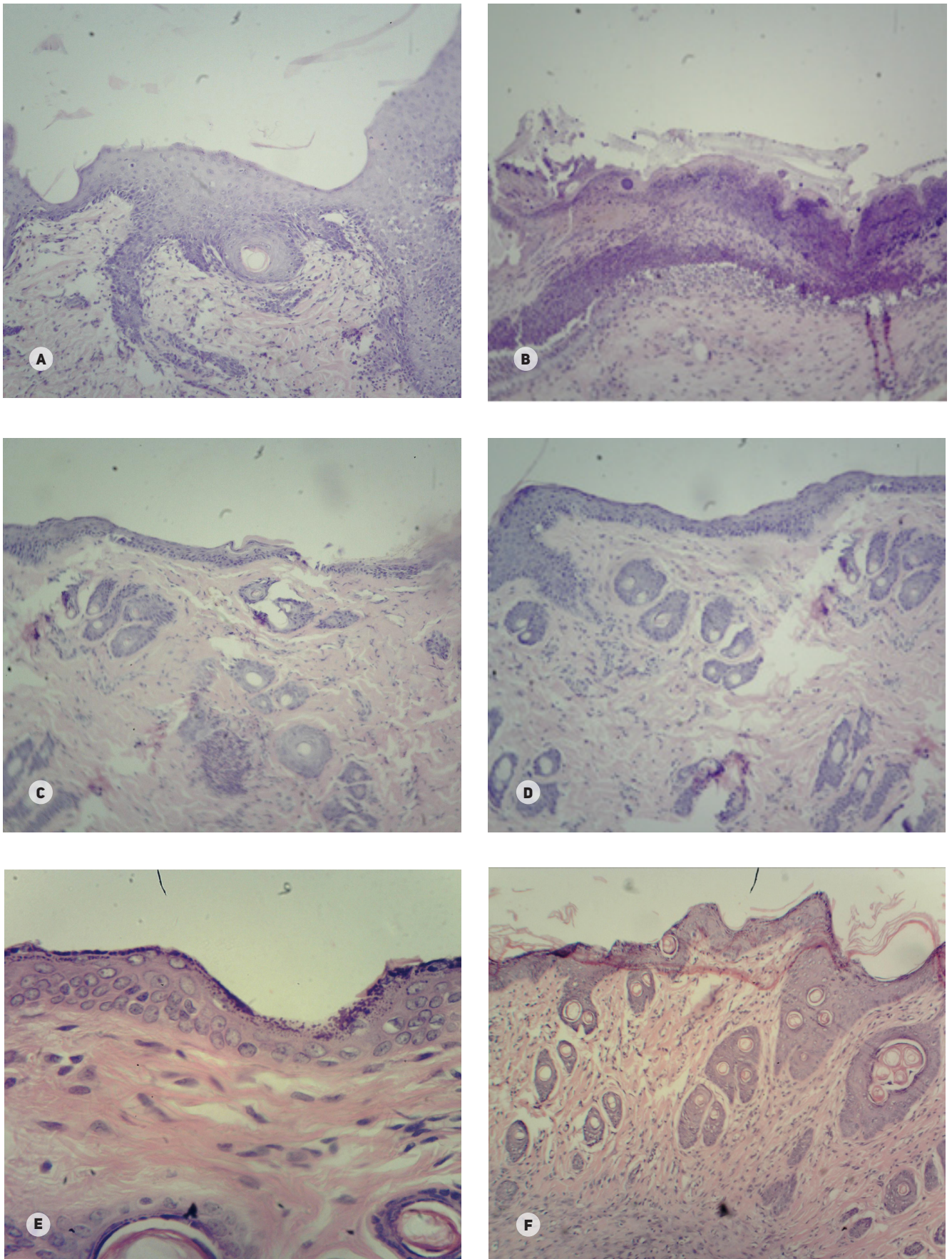


Fig. 1. Histological study of wounds modeled in rats. **(A–C)** Histological slices of animals in the control group (group 1) sacrificed at different stages of the experiment: **(A)** on day 3; **(B)** on day 10; **(C)** on day 14. **(D–F)** Histological slices of animals in group 2 (additional treatment with 10 % cefazolin) sacrificed at different stages of the experiment: **(D)** on day 3; **(E)** on day 10; **(F)** on day 14. Hematoxylin and eosin staining, $\times 280$

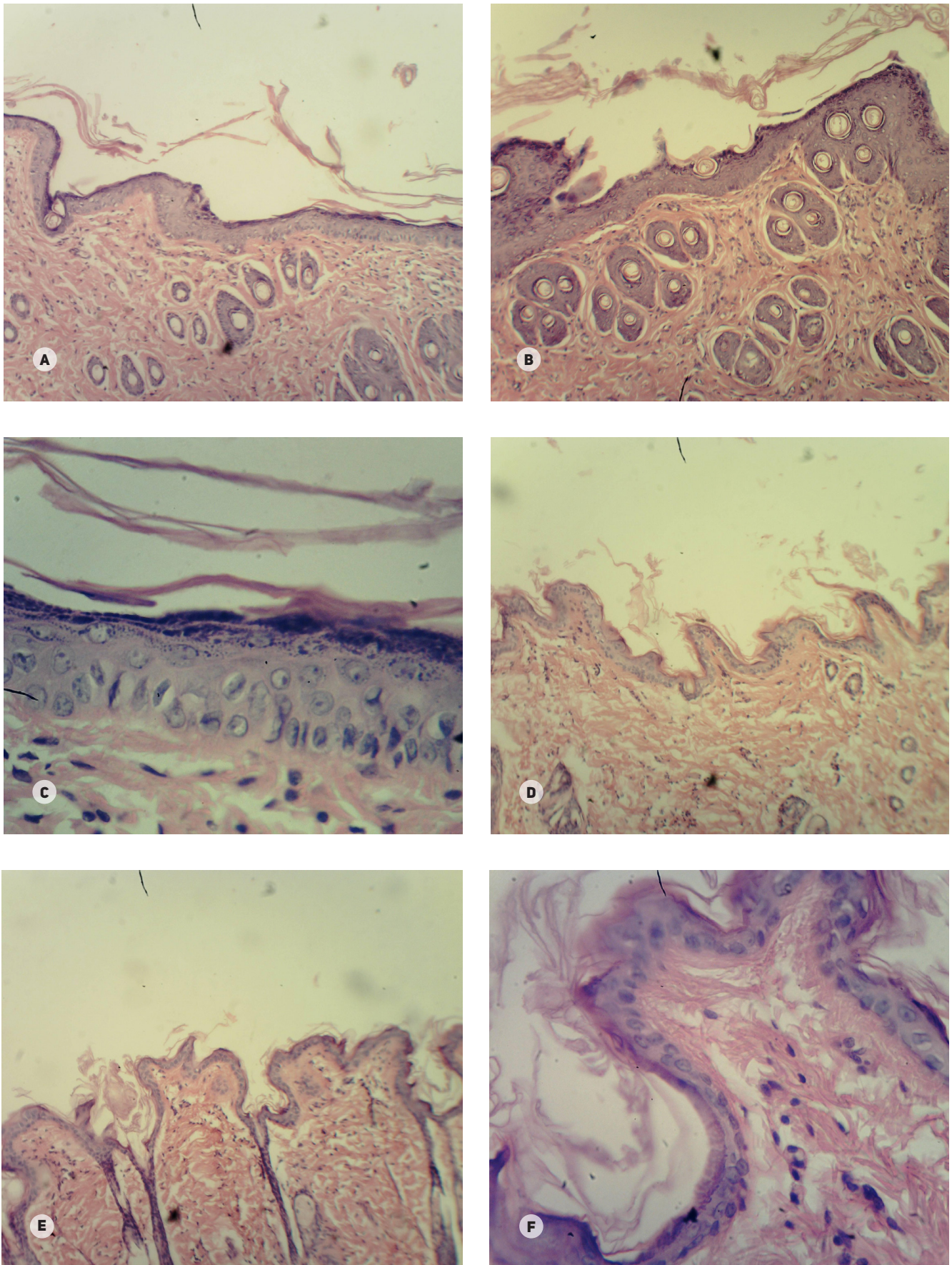


Fig. 2. Histological study of wounds modeled in rats. **(A–C)** Histological slices of animals in group 3 (additional treatment with kalanchoe juice) sacrificed at different stages of the experiment: **(A)** on day 3; **(B)** on day 10; **(C)** on day 14. **(D–F)** Histological slices of animals in group 4 (additional treatment with kalanchoe juice containing antimicrobial peptide cecropin P1) sacrificed at different stages of the experiment: **(D)** on day 3; **(E)** on day 10; **(F)** on day 14. Hematoxylin and eosin staining, $\times 280$

Proportion of various cells in the modeled wounds with regard to the treatment type and the day of sacrifice

Group	Day of sacrifice											
	3				10				14			
	Fibroblasts	Macrophages	Granulocytes	Lymphocytes	Fibroblasts	Macrophages	Granulocytes	Lymphocytes	Fibroblasts	Macrophages	Granulocytes	Lymphocytes
1 (controls)	14,7 ± 0,7	12,9 ± 0,3	49,9 ± 1,8	22,5 ± 0,6	17,0 ± 0,4	13,3 ± 0,2	48,0 ± 2,8	21,7 ± 0,3	24,9 ± 0,5	38,7 ± 1,7	15,6 ± 0,8	20,8 ± 0,3
2 (10 % cefazolin solution)	9,5 ± 0,2	59,3 ± 2,1	7,9 ± 0,2	23,3 ± 0,2	15,2 ± 0,1	62,4 ± 1,3	7,5 ± 0,4	14,9 ± 0,6	31,9 ± 0,7	51,7 ± 2,6	8,4 ± 0,1	8,0 ± 0,1
3 (kalanchoe juice)	12,9 ± 0,6	37,1 ± 0,7	30,3 ± 1,6	19,7 ± 0,4	17,7 ± 0,2	38,9 ± 1,6	15,6 ± 0,2	27,8 ± 0,4	24,0 ± 0,6	47,3 ± 2,5	14,4 ± 0,2	14,3 ± 0,2
4 (kalanchoe juice + cecropin P1)	12,1 ± 0,2	21,4 ± 0,4	44,0 ± 1,2	22,5 ± 0,1	20,5 ± 1,0	23,9 ± 0,4	24,2 ± 1,1	31,4 ± 2,6	30,1 ± 1,2	26,4 ± 1,6	18,4 ± 1,1	25,1 ± 1,2

treatment promoted faster elimination of edema and debridement of necrotic tissue, stimulated granulation and epithelialization. The polymorphic cell profile and the presence of variably mature fibroblastic cells indicate a stimulating effect of the transgenic kalanchoe on the proliferative and functional activity of granulation tissue. The therapeutic effect of transgenic kalanchoe juice is very marked at the last stage of wound healing in comparison with the controls. A combination treatment with 1 % cefazolin and kalanchoe juice was more effective than the treatment received by the controls. Still it was less effective than in the group treated with transgenic kalanchoe: in spite of complete regeneration of the epidermis at the wound edges in groups 2 and 3, sloughing was still observed, and the presence of various cell types in the granulation tissue indicated that it was less mature in comparison with group 4. Full epidermal closure in group 4 and integumentary structures indicated faster regeneration and complete skin restoration due to a more active migration and proliferation of endotheliocytes and vigorous angiogenesis.

Surprisingly, transgenic kalanchoe juice produced a stronger inhibitory effect on the growth of *S. aureus in vivo* than we had expected knowing about its antibacterial activity demonstrated in some experiments in bacterial cultures *in vitro* [30]. This might be due to the combined antimicrobial effects of P1 cecropin and endogenous bufadienolides of *K. pinnata*. Besides, the rate of pathogen elimination from the wound could be determined to a great extent by hemagglutination of *K. pinnata* lectins that have an immunostimulatory effect [33]. Similar to concanavalin and phytohemagglutinin, these lectins can promote proliferation of

lymphocytes, including those that recognize specific pathogen antigens accumulating near the infection site. Coupled with cecropin P1-induced suppression of bacterial functions and the antimicrobial factors of the juice, lectins can promote healing and stimulate production of antigens. Also, pathogen elimination from the wound can be expedited by vascularization and scar tissue remodeling induced by some kalanchoe juice components, yet unidentified. This can stimulate transport of immune system cells and soluble factors to the infection site promoting better healing.

CONCLUSIONS

We have demonstrated a strong therapeutic effect of transgenic kalanchoe juice (with cecropin P1) on purulent wounds infected with *S. aureus* in rats. The obtained results allow us to recommend the studied substance for use in the clinical setting. Transgenic kalanchoe juice is a promising agent for treating varicose leg ulcers in patients with diabetes. Their immunity is compromised and the ulcers often harbor mixed infections; however, long-term therapy is complicated by drug resistance that bacteria develop in the course of treatment.

In cases of external bacterial infections, the use of transgenic kalanchoe juice instead of the extracted antimicrobial peptide will reduce purification expenses and enhance the therapeutical effect of cecropin P1. Thus, *K. pinnata* may be recommended as a promising, cost-effective and available "bioreactor" for obtaining peptide- or protein-derived antimicrobial substances, including alpha-lytic protease, lysostaphin and hirudin.

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