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Comparative study of microvascular density in experimental third-degree skin burns treated with topical preparations containing herbal extracts

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Abstract

During the healing process of third-degree skin burns, a very complex response involves different cells and tissues linked together by intra- and extra-cellular mechanisms. For the restoration of damaged tissues, angiogenesis is the key point in the formation of new blood vessels. By their emollient, astringent, antiseptic, anti-inflammatory, biostimulator, epithelizing and cicatrizing effect, active principles from natural products contribute to the acceleration of the wound-healing process. In our study, we investigated the angiogenesis process in experimental model of third-degree skin burns treated with three topical preparations (cold-creams) containing 10% herbal extracts, comparing with 1% sulfadiazine cream and cold-cream base respectively. By their biostimulator, epithelizing and cicatrizing effect, cold-creams with herbal extracts are locally modulators of the cellular response and support the wound healing. The phytocomplex stimulates the favorable evolution of the burnt skin wounds and the development of neoangiogenesis capillaries.

Keywords: microvascular density, skin burns, topical preparations, herbal extracts.

Introduction

Worldwide, each year burns are a major health problem because of their high incidence and chronic lesions they produced regardless of age groups [1]. According to recent estimates, around the world various burns affect annually more than 6.6 million people, out of which about 265 000 are registered deaths [2].

In the United States, each year more than 1.2 million Americans suffer from burns and almost 450 000 of them require specialized treatment in medical care units [3]. In the United Kingdom, each year 500 000 people are affected by different types of burns, of which approximately 112 000 require emergency care and nearly 200 die because of serious injuries [4].

Primarily, burns affect the integrity of the skin as a barrier structure protecting the body to the external aggressions (*e.g.*, microbial invasion) [4, 5]. Taking into account the extent and depth of the skin lesions, the damage caused by burns could be more or less severe and their complications contribute to one of the most difficult pathologies [5, 6].

Both by the sufferings and by disabilities they cause, severe burns have a catastrophic influence on the patient's life. Thus, difficult healing, hormonal imbalances, kidney, liver or lung damages, the psychological impact and also the frequent infections continuously endanger the patient's life. Among the leading factors that influence the mortality rate by burns are the severity of injuries, the patient's age and the associated diseases [5–8].

Because of the extent and severity of lesions (the entire damage of epidermis and dermis, of muscles tendons and sometimes of underlying bone tissue), third-degree skin burns induce also a severe stress and mental disorders often requiring a specialized treatment [7–9]. In addition, third-degree skin burns affecting more than 20% of the body surface, causes alteration of vital functions by the fast and risky loss of fluids, electrolytes and proteins; in most cases, additional surgery is required for the improvement of unaesthetic scars and distortion of the surrounding tissues [1, 10].

Various cells and tissues linked together by intra- and extra-cellular mechanisms represent a very complex response (hemostasis, inflammation, proliferation, remodeling) involved in the healing process of third-degree skin burns. Thus, angiogenesis is a key point in the formation of new blood vessels for the restoration of damaged tissues through local supplying with nutrients, vitamins, enzymes and growth factors [11–15].

The purpose of our study is the evaluation of the angiogenesis process in experimental third-degree skin burns treated with some topical preparations containing herbal extracts.

Materials and Methods

Vegetal material

The plant material was harvested in May 2012 from the *Rubus caesius* L. (*Rosaceae*) and *Sambucus nigra* L. (*Caprifoliaceae*) species, from the Botanical Garden of the University of Craiova, Dolj County, Romania. Leaves were harvested from the first species (*Rubi caesii folium*) and flowers (*Sambuci flos*) and leaves (*Sambuci folium*) from the second species. The vegetal material was processed in optimal conditions of temperature and humidity. Voucher specimens are deposited in the Herbarium of Pharmacognosy and Phytotherapy Department, Faculty of Pharmacy, University of Medicine and Pharmacy of Craiova, Romania.

Reagents and solvents

All of the analytical grade solvents and reagents were purchased from Sigma–Aldrich (Seelze, Germany).

Preparation of tinctures

According to the Romanian Pharmacopoeia Xth edition, 20% tinctures from *Rubi caesii folium* (R), *Sambuci flos* (S1) and *Sambuci folium* (S2) were obtained by percolation, using 70^{c} ethanol as extraction solvent. Tinctures were filtered and then stored in brown-glass bottles, in the refrigerator, until use [16].

Obtaining of topical preparations with 10% herbal extracts

Using a Heidolph rotary evaporator, at 50–60^{0}C, under reduced pressure, the tinctures were slowly evaporated until soft extracts. The soft extracts were weighed and then embedded in a cold-cream H/L-type ointment base, prepared according to our patented previous researches. More accurate, the soft extracts were dissolved in a small amount of 70^{c} ethanol (extraction solvent) and then emulsified in the cold-cream base, at room temperature, by continuous grinding [17].

Experimental model of third-degree skin burns

Animals

The study was performed on five groups of common adult Wistar rats, each of 10 animals, weighing between 290 and 340 g. Both before and after the experiment, at the Animal Facility of the University of Medicine and Pharmacy of Craiova, animals were kept under standard conditions of light, temperature, humidity, food and water (*ad libitum*). The experimental protocol was approved by the Ethics Committee of the University of Medicine and Pharmacy of Craiova, according with the European Council Directive No. 86/609/November 24, 1986 (86/609/EEC), the European Convention on the Protection of Vertebrate Animals (2005) and the Romanian Government Ordinance No. 37/February 2, 2002 [14, 15, 18–21].

Procedures

By intramuscular injection of 85 mg/kg Ketamine hydrochloride (Ketalar®, Parke-Davis) and 6 mg/kg Xylazine hydrochloride (Rompun®, Bayer), general anesthesia was induced for the animals of our experiment. Then, on the higher dorsal region of the rats, the hair was removed on an area of approximately 5 cm^2. For the third-degree burns infliction on an area of 1.5 cm^2, we used a special cone-shaped stainless steel device (1 cm diameter and 350 g weight), equipped with a control thermometer. After the heating in boiling water (100^{0}C), the metallic device was applied locally on the dorsal region of each rat for five seconds [14, 15, 18–21].

Throughout the experiment, for the wound protection of each group of animals, thin films of five topical dressing preparations were applied daily, as follows: cold-creams with 10% herbal extracts for the first (R), second (S1) and third (S2) group, respectively; 1% silver sulfadiazine cream (SDA) for the fourth group, considered as reference; and cold-cream base (CCB) for the fifth group, regarded as control.

For three weeks, the evolution of the third-degree skin burns and the animal welfare were daily monitored. No animal died during the experiment.

Histological study

From each group of Wistar rats, the granulation tissue on the burn wound, with approximately 3 mm of perilesional area, was collected under general anesthesia at 7, 14 and 21 days from the skin burns infliction, for the dynamically assessment of the angiogenesis process. After this, the remaining wound of each rat was surgically sutured.

Directly after the sampling, the burnt-skin pieces were fixed in 10% buffered neutral formalin, for 72 hours, at room temperature, and then processed for histological paraffin inclusion technique. For the histological study, 4-µm thick serial cross-sections were cut using a Microm HM350 rotary microtome equipped with a water bath sections transfer system (STS, Microm). Classical stains with Hematoxylin–Eosin and trichromic Goldner–Szekely were used for the light microscopy assessment of cross-sections.

Immunohistochemical study

For the immunohistochemical study, 3-µm thick sections were retained on poly-L-Lysine coated slides and then stored at 37^{0}C, in the thermostat, for one day. In the next step, after dewaxing and hydration of the cross-sections, the histological material was incubated in 1% hydrogen peroxide solution for 30 minutes. Then, sections were washed in tap water and for antigen unmasking, were boiled in citrate buffer solution (pH 6) for 20 minutes. After boiling, sections were cooled for 15 minutes and washed in bisaline phosphate buffer solution (PBS).

Endogenous peroxidase blocking step was achieved using 2% skimmed milk for 30 minutes. After this stage, cross-sections were incubated with primary antibody, overnight, at 4^{0}C. Next day, the signal was amplified using peroxidase secondary antibody on polymer support (EnVision, Dako) for 30 minutes. Detection of the signal was made using 3,3'-diamino-benzidine (DAB, Dako). The slides were covered with

DPX (Fluka), after contrasting with Hematoxylin. For the evaluation of the angiogenesis process, we used anti-CD34 antibody (rabbit anti-rat, clone EP373Y, dilution 1:100, Epitomics, Medicalkit, Craiova, Romania) raised against a rat endothelium epitope.

Image acquisition and microscopic evaluation

For the assessment of angiogenesis vessel density, areas of maximum vascular density ("hot spot" method) were chosen and four microscopic images were captured. Vessel count was performed using the "manual tagging" feature in ImageProPlus software package. Acquired data were exported to an Excel spreadsheet for automatic calculation of vascular densities. Microscopic evaluation was performed by grabbing 40× images under the Nikon Eclipse 55i microscope equipped with a 5 Mp CCD color sensor (Apidrag, Romania). Images were captured, stored and analyzed utilizing the Image ProPlus 7 AMS package (Media Cybernetics, Inc., Buckinghamshire, UK).

Statistical analysis

For statistical analysis, we first plotted the means and standard errors for each day stage and each topical preparation, and next we used ANOVA testing to evaluate the differences between these groups.

Results

Initially, third-degree skin burns were inflicted for all the animals, with a peculiar macroscopic appearance: area of coagulation necrosis of epidermis, dermis and superficial muscles; alteration of the vascular network beneath the wound; hyperemia; edema.

After the application of topical preparations, the evolution of burnt skin wounds was variable, as follows: a good epithelization for R, S1, and S2 cold-cream groups, with an almost complete wound healing at 21 days; an incomplete epithelization for SDA (reference) and CC (control) groups, with a marked delay of wound healing at 21 days, compared with R, S1 or S2 groups.

For all topical preparations, starting with seventh day until 21st day, the number of angiogenesis vessels gradually decreased (Figure 1).

Seven days after the injury, the largest number of angiogenesis vessels was recorded for R group (32) comparing with S2 (30), S1 (29), CC (14), and SDA (11) groups. The inflammatory reaction was much reduced for R, S1 and S2 groups comparing with CC or SDA groups (Figure 1; Figure 2, a and b; Figure 3, a and b).

Fourteen days after the infliction of third-degree skin burns, a rich vascular network can be seen into the depth of the wound. A small number of neoformation vessels but with large area and perimeter was observed for SDA (8) and CC (11) groups comparing with S2 (19), R (16), and S1 (13) cold-cream groups (Figure 1; Figure 2, c and d; Figure 3, c and d).

At 21 days after burn, all topical preparations show a small number of angiogenesis vessels but with the largest area and perimeter comparing with the initial situation (at seventh day): S1 (7), S2 (6), R (6), CC (5), and SDA (4). For R, S1 and S2 cold-cream groups, the granulation tissue is relatively well restored and the inflammatory infiltrate is almost absent. In this stage, development of neoformation blood vessels highlights the intensity of remodeling process. For CC and SDA groups, the re-epithelization process was difficult; in this case, coagulation necrosis area and abundant inflammatory infiltrate still persist (Figure 1; Figure 2, e and f; Figure 3, e and f).

Our topical preparations with 10% herbal extracts (R, S1, and S2) stimulated the development of angiogenesis capillaries, comparing with SDA and CC groups where the healing was delayed and almost spontaneously. Development of granulation tissue was more rapidly and more intense for R, S2, and S1 cold-cream groups, in that order. The most active was the cold-cream with 10% *Rubi caesii folium* soft extract, which induced the apparition of the highest number of neoangiogenesis capillaries (Figure 1).

In case of all topical preparations, progressively decreasing in the number is inversely correlated with the diameter (lumen), area and perimeter of angiogenesis vessels, and directly correlated with the granulation tissue maturation (extracellular matrix, especially collagen fibers) and reducing of the inflammatory response. In fact, at the microscopic level, from the deep wound to the surface, the remodeling of the dermal connective tissue continues long after the macroscopic healing of the burnt wound.

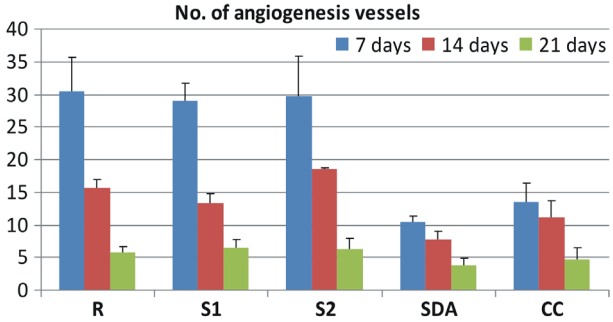

Figure 1 – ***Number of angiogenesis vessels gradually decreased starting with 7th day until 21st day, with a significant difference between the means of all five groups of experimental third-degree skin burns, after the application of topical preparations.***

Discussion

Protecting damaged skin from infections, reducing inflammation and stimulating cell proliferation for the recovering of cellular destructions are the three main requirements of an ideal wound dressing. Active herbal principles like heteroglycans, flavonoids, tannins, anthracene-derivatives, essential oils, vitamins, minerals contribute to the acceleration of the wound-healing process by their emollient, astringent, antiseptic, anti-inflammatory, diuretic, immunomodulatory, antioxidant, epithelizing and cicatrizing effect [19, 22].

In case of two cold-creams containing 10% soft extracts of elder flowers and leaves, respectively, our previous research showed astringent, antiseptic, anti-inflammatory, epithelizing and cicatrizing effect in experimental model of third-degree skin burns. Thus, flavonoids (rutoside, luteol-7-glucoside), catechic tannin,

polyphenolic acids and coumarins were the main compounds highlighted by thin layer chromatography analysis [18, 19].

Bioflavonoids and tannin from the composition of cold-creams with 10% herbal extracts are mainly responsible for the good evolution of burnt skin wounds. Flavonoids exhibit antioxidant, anti-inflammatory, epithelizing, wound healing, capillaroprotective and vasculotropic effects. Tannins are astringent, antiseptic, haemostatic, anti-inflammatory, epithelizing. Emollient, epithelizing, cicatrizing and biostimulator effect was revealed also for the beeswax from the composition of topical preparations [18, 19, 23, 24].

The skin exhibit a high capacity of autoregeneration, as a vital organ for maintaining the body homeostasis and mechanical barrier for the defense against external aggressive factors (viruses, bacteria, toxic compounds, UV irradiation, etc.). Third-degree burns developed on wide surface severely limit the skin functions and profoundly altered his ability of regeneration [7, 8, 25].

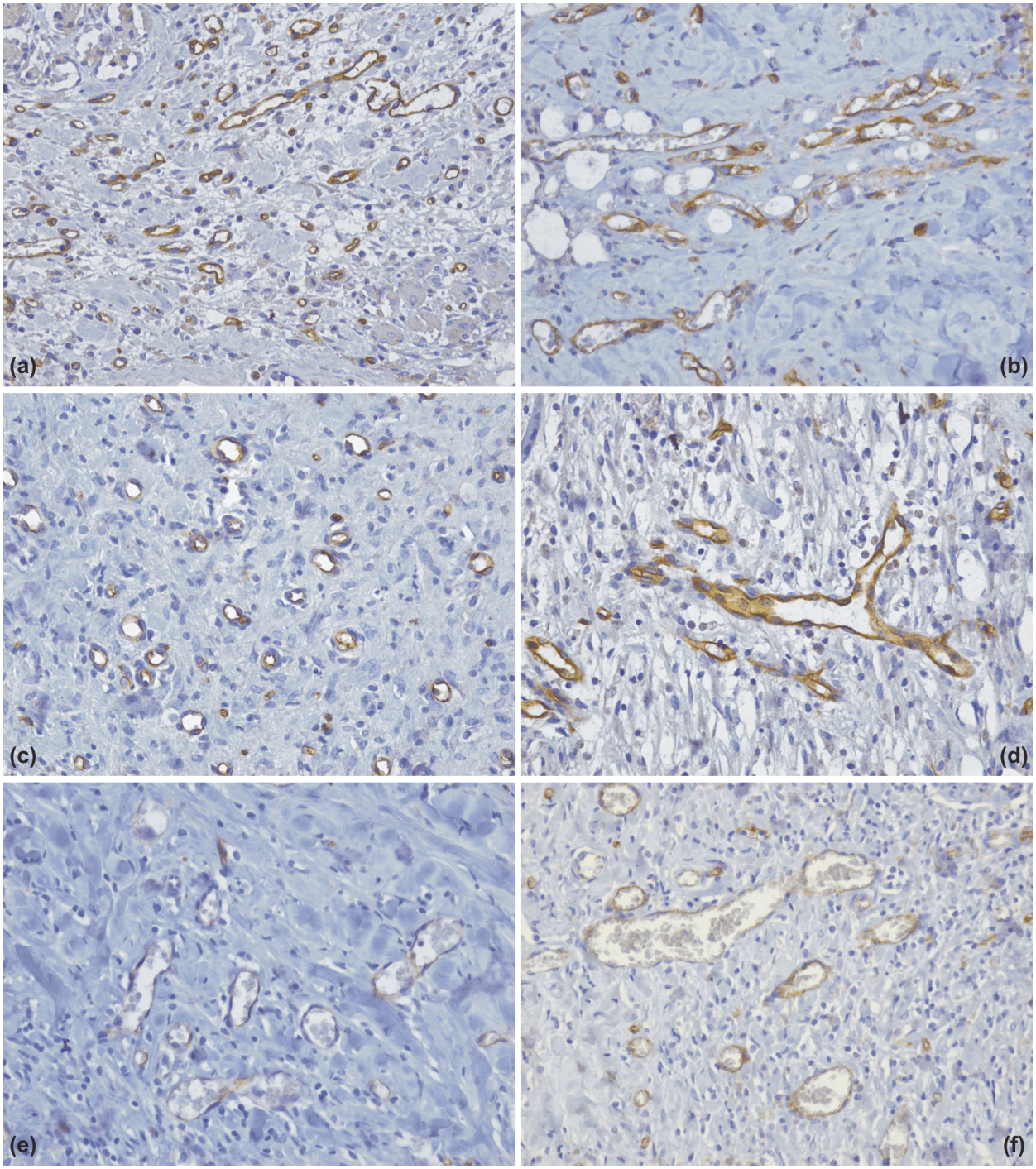

Figure 2 – ***Immunohistochemical aspects of angiogenesis vessels with positive immunostaining for CD34, after the application of topical preparations: (a) cold-cream with 10% Rubi caesii folium soft extract (R), at seven days; (b) 1% silver sulfadiazine cream – reference group (SDA), at seven days; (c) cold-cream with 10% Rubi caesii folium soft extract (R), at 14 days; (d) cold-cream with 10% Sambuci flos soft extract (S1), at 14 days; (e) cold-cream with 10% Rubi caesii folium soft extract (R), at 21 days; (f) cold-cream with 10% Sambuci folium soft extract (S2), at 21 days. LSAB technique, ×200.***

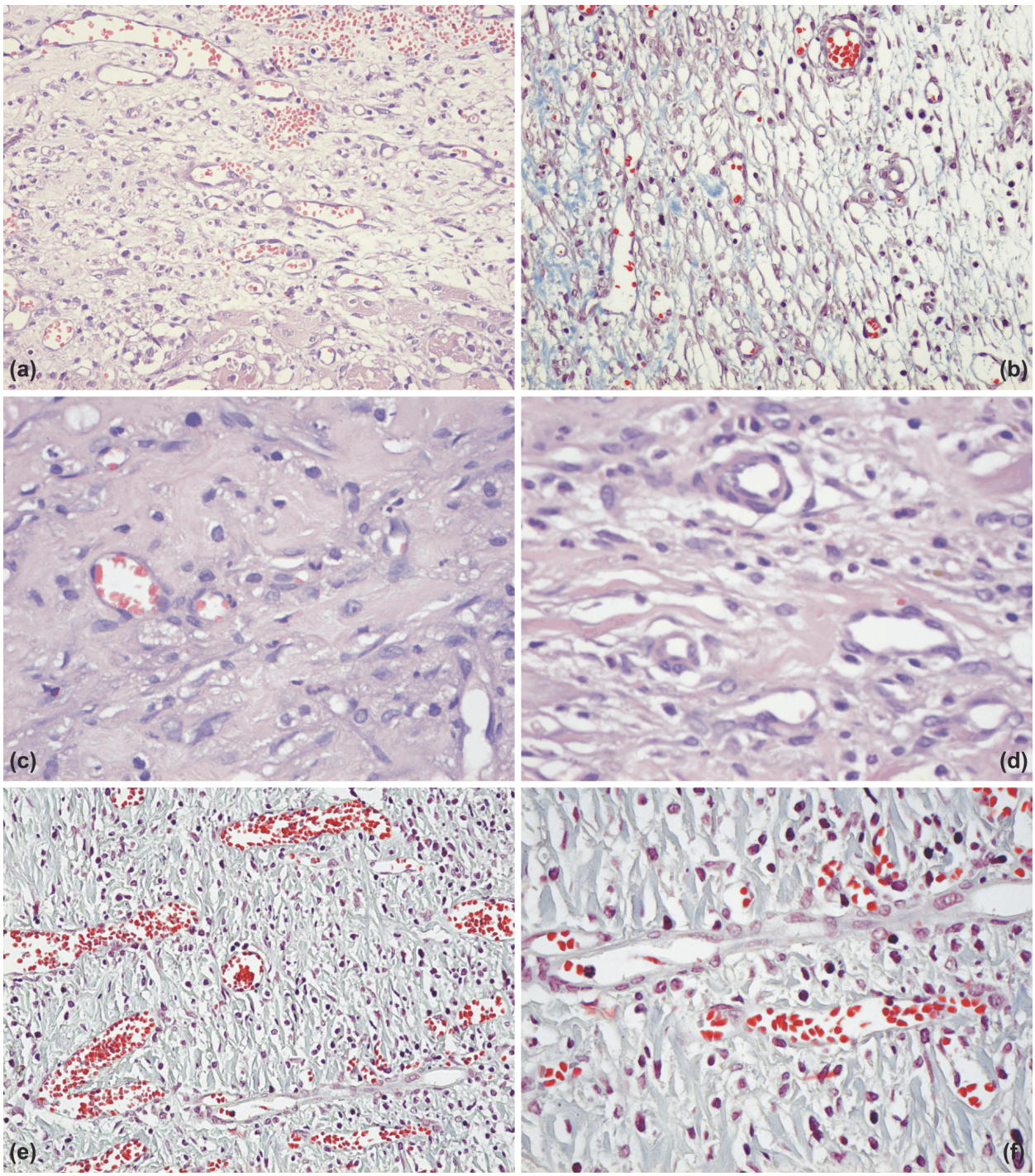

Figure 3 – ***Microscopic evolution of microvascular density in experimental third-degree skin burns, after the application of topical preparations: (a) cold-cream with 10% Sambuci flos soft extract (S1), at seven days; (b) cold-cream with 10% Sambuci folium soft extract (S2), at seven days; (c) cold-cream with 10% Rubi caesii folium soft extract (R), at 14 days; (d) 1% silver sulfadiazine cream – reference group (SDA), at 14 days; (e and f) cold-cream with 10% Sambuci folium soft extract (S2), at 21 days. HE stain: (a) ×200; (c) and (d), ×400. Trichromic Goldner–Szekely stain: (b) and (e), ×200; (f) ×400.***

Inflammation, proliferation and remodeling represent cascade-developed events because of skin burns. The host response and tissue repair are due mainly to monocytes/macrophages and fibroblasts acting to the level of the damaged area for the restoration of homeostasis. In this respect, recent studies have shown the effects on monocyte adhesion and the production of inflammatory, matrix remodeling, and growth factor proteins at the injury site [20, 21, 26–28].

The main stages of skin recovery process include: migration of blood cells from the depth of burnt wound, promotion of a local inflammation, removing of cellular debris and pathogens, proliferation of connective tissue and extracellular matrix, development of a new blood vessels network (angiogenesis), and tissue remodeling (granulation tissue) [11, 26, 27, 29]. In this respect, angiogenesis plays a major role in the skin regeneration by ensuring the recovery of a new blood vessels network from an existing one. An insufficient development of the angiogenesis stage is closely related to the delay

of the wound-healing process: extent of coagulation necrosis area, aggression of microbes, appearance of scarring [9].

In our study, stimulation of the angiogenesis process by the topical preparations containing 10% herbal extracts (flavonoids, tannins) was evidentiated by the presence of CD34-positive cells. In fact, the angiogenesis vessels are strongly related to healthy blood vessels from the burned wound periphery [14, 15]. Expressed by endothelial cells of small blood vessels and lymphatic [30], hematopoietic stem cells, and progenitor cells [31], CD34 is a highly glycosylated transmembrane glycoprotein.

The existence of bone marrow-derived circulating endothelial precursor cells with a significant contribution to the wound-healing process by the formation of new angiogenesis vessels was highlighted by some authors [32–34]. Proangiogenic factors are released by various cells including macrophages involved in different stages of wound repair, especially for the stimulation of angiogenesis. In addition, recent studies identified two types of macrophages: M1 for proinflammatory mediators and M2 for the stimulation of angiogenesis and wound-healing process [35–37].

For the treatment of subcutaneous and chronic inflammations of the skin, due to their easily application, patients often prefer cold-creams instead of greasy ointments. Because of their special composition (waxes, liquid paraffin, sodium tetraborate, water), cold-creams produce a local cooling effect useful in the treatment of skin burns [18]. The effectiveness of those topical preparations is even better when herbal extracts are added in the base composition.

Conclusions

The effects of three topical preparations (cold-creams) with 10% herbal extracts were evaluated in experimental model of third-degree skin burns, comparing with 1% sulfadiazine cream and cold-cream base respectively. Mainly due to the content of flavonoids and tannins, cold-creams with herbal extracts are locally modulators of the cellular response, supporting the wound-healing process by their biostimulator, epithelizing and cicatrizing effect. The phytocomplex stimulates the formation and development of neoangiogenesis capillaries, and the favorable evolution of the burnt skin wounds.

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Contribution Note

All authors have equally contributed to the manuscript.

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