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Liposomal Form of Dihydroquercetin Contributes to Skin Regeneration after Thermal Burns

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Abstract—It was found that dihydroquercetin, a flavonoid of plant origin, localized in lecitin nanoparticles with glycine amino acid, reduced inflammatory reactions in wound zones after thermal burns. The application of the liposomal complex to burn trauma stabilized the endogenous antioxidant system and minimized the area of secondary necrosis in the wound. The intensification of skin regeneration and repair of hair follicles and sebaceous glands were also observed.

Key words: antioxidants, oxidative stress, thermal burn, skin structure, regeneration.

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It is known that many traumas, including burns, are accompanied by an imbalance between the intensity of free-radical processes and the functional activity of the antioxidant system (Aruoma, 1998; Halliwell and Gutteridge, 1990; Potselueva et al., 1999). Under normal conditions, the stimulation of lipid free-radical oxidation is compensated for by adequately increased lipid peroxidation and the rapid depletion of the antioxidant system (Scandalios, 1997). In the case of burns the process of peroxide oxidation of lipids occurs and enough fast depletion of the antioxidant potential (Samoi-lenko et al., 2000). However, in the case of burns, it is advantageous to use antioxidant substances for their treatment. Medicines containing antioxidants must contact damaged surfaces, be nontoxic, and exert a prolonged effect. Therefore, it is reasonable to use antioxidants in lyposomal form to prevent lipid peroxidation and facilitate the regeneration processes. As a preparation with these properties, we used Flamena, a drug that contains the natural flavonoid dihydroquercetin (DHQ) and has five hydroxyl groups, which makes it a highly active antioxidant. The preparation is enclosed in a phospholipid container in the presence of glycine.

The purpose of the work was to study the efficiency of the liposomal form of DHQ in the local treatment of wounds after thermal burns in animals. Planimetric, biochemical, and histological examinations were applied to evaluate the efficiency of the drug.

MATERIALS AND METHODS

The efficiency of Flamena in the treatment of thermal burns was tested on Wistar rat males with weights of 220–250 g. Manipulations with animals were performed according to the "Guide to Performing Experiments with Experimental Animals." Animals were divided into two groups, i.e., thermal burns (control group) and thermal burns (experimental group) smeared daily with Flamena.

Skin and hair covering the upper scapula region were removed by a blade and depillator. A thermal burn was induced on skin for 1 min by applying a heated ($110 \pm 5°C$) 1-cm^2-thick metallic rod. In animals, this produced second-degree burns.

The natural flavonoid DHQ is a component of the lyposomal preparation Flamena and its concentration in the preparation is 4 mg per 1 ml of liposomes. The preparation also includes a membrane-forming phospholipid (MFL) that consists of 10% lecitin and 5% glycin in 0.1% water-alcohol solution. The size of a liposome obtained by a two-phase separation method is 20–300 nm.

The process of wound healing was registered by the following methods: a planimetric evaluation of wound area and a morphological analysis of longitudinal histological sections of the wound and adjacent skin regions in experimental and control animals. For histological analysis, animal skin specimens (wound and near-wound regions) were fixed in 10% formalin. Five- to seven-micrometer skin sections were subjected to the standard technique of preparing histological preparations and stained with hematoxylin–eosin according to Ehrlich to identify cellular membrane and cytoplasm structures (Semchenko et al., 2006). Histological preparations were visualized under an Axiovert 200 microscope (Germany). The relative rate

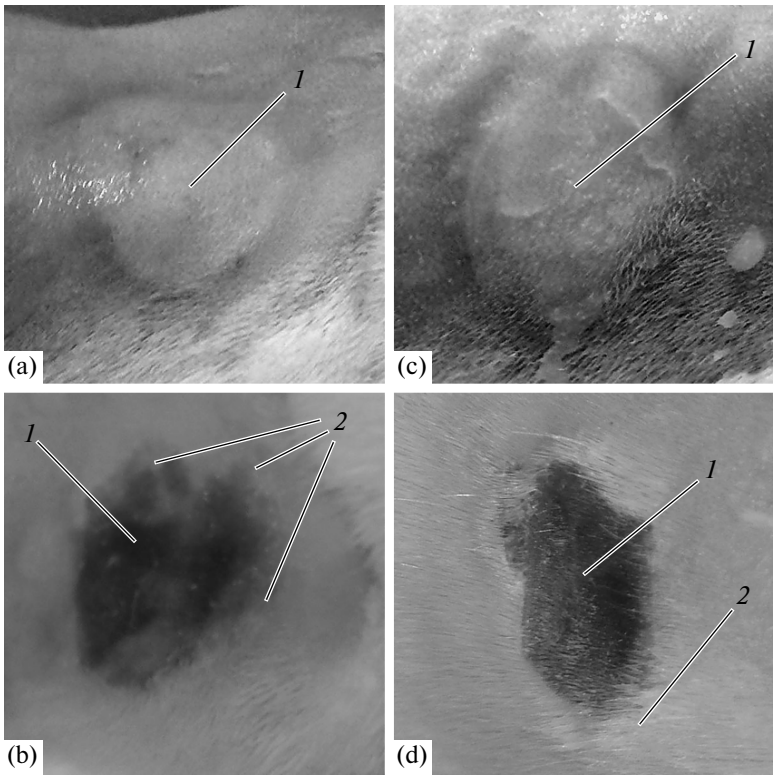

Fig. 1. Appearance of thermal burn wound in the control (a, b) and of a similar burn after treatment with the preparation Flamena at various time intervals. (a) Wound in control animals immediately after the burn: *1*, wound center, S = 1.16 cm^2; (b) wound in control animals in 72 h: *1*, inflammation zone, *2*, paranecrosis zone, S = 1.5 cm^2; (c) wound in experimental animals immediately after the burn: *1*, wound center, S = 1.32 cm^2; (d) wound treated with Flamena (four times a day) in 72 h: *1*, wound center, *2*, recovered hair, S = 1.05 cm^2.

of epithelialization in burn wounds was estimated by the planimetric method. Measurements were performed every day. We determined the area of wound and its daily reduction relative to the area calculated for the preceding measurement according to the following formula:

$$Sp = (S - Sn) \times 100/St,$$

where Sp is the area of wound recovery during the day, %; S is the wound area for the preceding measurement; Sn is the wound area for the given measurement; and t is the number of days between measurements.

Blood plasma was isolated by differential centrifugation at 500 rpm for 10 min followed by centrifugation at 1200 rpm for 10 min. The antioxidant concentration in blood plasma after the thermal burn was determined by the routine technique. The amount of SH groups in low- and high-molecular substances (of protein and nonprotein origin) was determined spectrophotometrically by the level of products stained with 5-ditiobis-2-nitrobenzoic acid (DTNB) (Sokolovskii et al., 1997; Karpishchenko et al., 1999). 100 μl of blood plasma was mixed with 875 μl 0.1 M Tris-HCl buffer (pH 8.0) supplemented with 25 μl

The influence of the liposomal nanocomplex Flamena on the antioxidants level in rat blood plasma 15 days after thermal burn

Animals	Endogenous antioxidants, μM, Xep ± S_{Xep}			
	Uric acid	α-tocopherol	low-molecular SH-groups	protein SH-groups
Intact animals	58.8 ± 9	10.9 ± 0.8	2.4 ± 0.6	35 ± 7
Burn (control)	34.7 ± 7	8.6 ± 0.65	2.0 ± 0.8	26 ± 5
Burn + Flamena	48.1 ± 8	10.3 ± 0.7	2.3 ± 9	30 ± 7

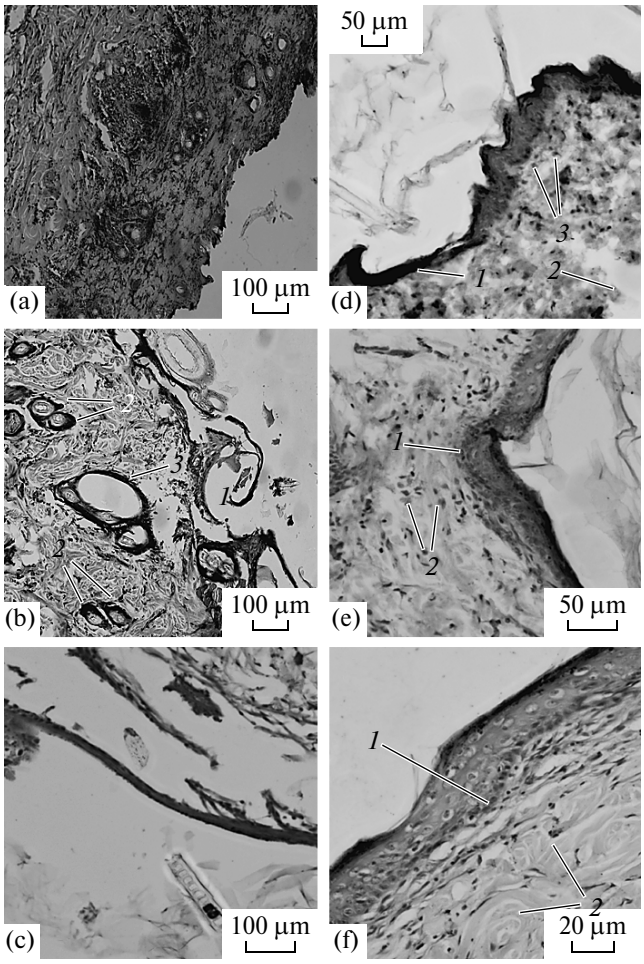

Fig. 2. Dynamics of skin regeneration in a thermal burn of a rat scapular area in 5, 11, and 15 days after the burn in the control (a–c) and in animals treated with Flamena (d–f). (a) Damaged epidermis and destructive process in the derma in 5 days; (b) epidermis (*1*), hair follicles (*2*), sebaceous glands (*3*) and adjacent connective tissue are destroyed; (c) the epidermis area with lost cellular structure 15 days after the burn; (d) destroyed epidermis (*1*), derma regions with damaged structure (*2*), fibroblast-like cells (*3*); (e) cells of the basal layer (*1*), islets with cells in various mitotic stages (*2*); (f) well-defined epidermis and mitotic cells in the basal layer (*1*), connective tissue fibers (*2*). Hematoxylin–eosin staining.

0.4% DTNB in ethanol. The optical density was measured at 412 nm. The concentration was calculated by a calibration curve with the application of standard solutions of reduced glutathione. To determine the content of thiol of nonprotein origin, a sample of blood plasma was mixed with 20% sulfosalicylic acid (1 : 3) and centrifuged at 3000 rpm for 10 min. The concentration of SH groups in the supernatant was determined as described above. The content of uric acid in the blood plasma was determined by the uricase method (Menshikov et al., 1987). To determine the content of α-tocopherol, it was extracted from the blood plasma with heptane and subjected to the spectrophotometric analysis (Arkhipova et al., 1998; Esterbauer et al.,1989).

RESULTS AND DICUSSION

Anatomic and functional changes, as well as reactive-inflammatory and regeneration processes, were observed in burn areas. Figure 1 shows a wound produced in the skin by a thermal burn at 110 ± 5°C in the control immediately after the burn (a) and after 72 h (b), as well as with the application of Flamena, the lyposomal form of the DHQ antioxidant, just after the burn (c) and after 72 h (d). It was seen that, in control animals, the area of thermal damage has clear edges and the formation of burn blisters and significant skin redness are notable. Over the course of time, the damaged area with evidence of inflammation and necrosis increased 1.2–1.3 times (Fig. 1b). In the experimental group (Figs. 1c, 1d), the damaged region and the paranectrotic area did not increase. The wound scab had a smooth shape and uniform healing was apparent underneath it; inflammation was not observed. The burned area reduced by 15 ± 3% over 3 days.

To study the action of the Flamena preparation in more detail, skin histological sections were prepared at various times intervals after the thermal burn (Fig. 2). Figure 2a demonstrates a skin histological section prepared 5 days after the thermal burn in the control group. Disrupted epidermis and dermal destruction are clearly seen. In wounds treated with the Flamena preparation (Fig. 2d), a disrupted region of epidermis is apparent under the remains of necrotic tissue; however, a regenerating area with all layers is visible close to it. Randomly located fibroblast-like cells are seen under the epidermis in the derma layer. An increased number of proliferating cells is observed both in the basal layer of the epidermis and papilla and in the reticular layers of the derma close to the damage edge. In the control group, 10 days after the start of the experiment (Fig. 2b), destructive areas are still visible in the skin in the thermal burns, i.e., the epidermis is disrupted and the hair follicles, sebaceous glands, and adjacent connective tissue are damaged. In animals with wounds regularly treated with Flamena (Figs. 2e, 2f), 10 and 15 days after the burn formation, a demarcation line between the disrupted and regenerating layers is well-defined; the activation of basal layer cells is evident. Cells in various stages of mitosis are seen in the derma layer. Figure 3 shows the morphology of hair follicles and sebaceous glands in histological sections of skin surface in 5, 10, and 15 days after the thermal burn. In the control group (Figs. 3a–3c), the disordered epidermis and undifferentiated cell clusters point to the occurrence of destruction processes in the area. After 15 days, the degradation of hair follicles and disordered structure of the underlying derma layers were observed. In experimental animals treated

with the Flamena preparation (Figs. 3d–3f), the formation of new hair follicles is observed (Figs. 3d, 3e) in the area of regenerating epidermis; the structure of sebaceous glands capable of recovery is retained better (Fig. 3f).

To summarize, Flamena proved to be an effective preparation for the treatment of local wounds after a thermal burn. The drug inhibits factors that induce the development of necrosis, tissue ischemia, and wound infections, as well as facilitates tissue regeneration. The positive effect of Flamena is due to its optimal composition. Dihydroquercetin, the major component, is a highly effective antioxidant capable of binding free-radicals and preventing lipids from peroxidation. When applied to a burn, Flamena stabilizes the endogenous antioxidant system, inhibits the free-radical peroxidation of biomembrane lipids and other cellular structures and restricts the area of secondary necrosis in wounds. Besides, the preparation has anti-inflammatory properties, which are apparent in the absence of wound suppuration and reduced damage to the skin surface. The absence of inflammation in the burn treated with Flamena may be due to the capacity of flavonoids to form complexes in the presence of metals with variable valence, particularly iron, which may exert both antioxidant and prooxidant activity (Sugihara et al., 1999) and, therefore, prevent the dissemination of bacteria on thesurface of the wound. Another cause of the reduced inflammatory reaction may be decreased histamine secretion by mast cells (Bronner and Landry, 1985) in the presence of DHQ.

Pathological processes that take place in burns are accompanied by significant disorders in the structural and functional properties of biomembranes. Antioxidants may improve the elastic and mechanical, as well as functional, properties of various cells. Therefore, we examined the dynamics of antioxidant capacity of blood plasma after a thermal burn. It was found (table) that the lyposomal form of DHQ supports levels of protein and low-molecular SH-groups, α-tocopherol, and uric acid that are nearly unchanged compared to the control. The capacity of the nanocomplex to stabilize the level of endogenous antioxidants in the plasma prevents the development of oxidative stress to minimize its harmful consequences.

In addition to the highly active antioxidant dihydroquercetin, the Flamena preparation also includes glycine, which supports the respiratory functions of mitochondria and inhibits apoptosis in damaged cells (Tonshin et al., 2007), as well as lecithin, which facilitates the recovery of cellular membranes. All Flamena components normalize and maintain tissue homeostasis and, therefore, as the experiments demonstrated, facilitate the intensive regeneration of cutaneous integument and the recovery of skin derivates after chemical (Naumov et al., 2009) and thermal burns.

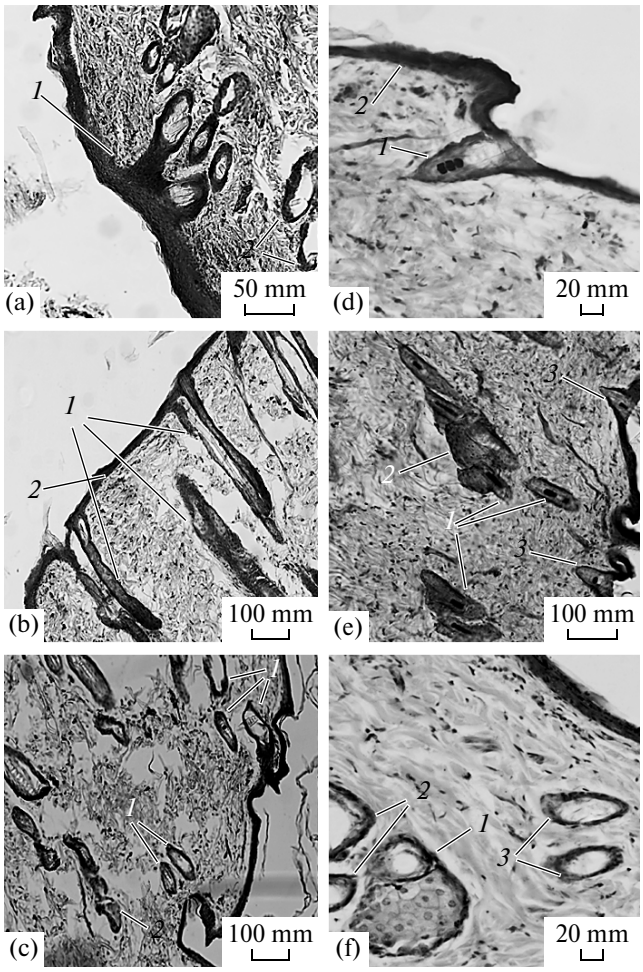

Fig. 3. Dynamics of hair follicle formation during skin regeneration in 5, 11, and 15 days after thermal burn in control (a–c) and in animals treated with Flamena preparation (d–f). (a) Damaged epidermis (*1*), destroyed hair follicles and clusters of undifferentiated cells (*2*); (b) degeneration of hair follicles (*1*), epidermis destruction (*2*), disordered structure of underneath derma layers; (c) destruction in the surface layer, hair follicles (*1*) and sebaceous glands (*2*); (d) formation of hair follicle (*1*), area of regenerating epidermis (*2*); (e) group of hair follicles (*1*), sebaceous gland (*2*), hair that appeared at skin surface (*3*); (f) sebaceous gland (*1*), cells of peripheral areas of the sebaceous gland capable of cell division (*2*), areas of hair follicles (*3*). Hematoxylin–eosin staining.

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