Comparative Ultrastructural Study of the Effect of Imidazo-Benzimidazole Derivatives RU-185 and RU-254 on the Development of Microcirculatory Disturbances in the Skin under Conditions of Reduced Blood Flow

V. V. Banin, V. P. Galenko-Yaroshevskii*, A. V. Tegai*, T. N. Khropova*, and M. G. Kostyaeva

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In rats with experimental partial ischemia of the skin fold the state of microvessels and surrounding skin was studied by electron microscopy under control conditions and after treatment with imidazo-benzimidazole derivatives RU-185 and RU-254. RU-185 was more potent than RU-254 in preventing the development of microcirculatory disturbances and altered permeability of capillary walls.

Key Words: *skin; ischemia; microcirculation; permeability; imidazo-benzimidazole derivatives*

Microcirculatory disorders and disturbances in vascular wall permeability play a major role in the pathogenesis of ischemic injury during circulatory hypoxia [5,7,8]. However, cell damage results not only from the direct effect of oxygen deficiency in the ischemic area. The development of tissue injury at the early stages of ischemia and, particularly, during reperfusion is related to the cytotoxic effect of reactive oxygen species (ROS) [13]. It should be emphasized that cells in capillary walls are involved in ROS generation [10]. It contributes to the damage to microvascular endothelium, which determines progression of circulatory disturbances and changes in vascular permeability [7,8]. The search for new drugs protecting capillaries and surrounding tissues is an urgent problem. Our previous studies on rats showed that imidazo-benzimidazole derivatives RU-185 and RU-254 produce a protective effect under conditions of reduced blood flow in isolated skin flap [2-4]. Administration of these preparations during ischemia reduced the severity of necrotic changes in the skin flap and increased survival of keratinocytes. The protective effect of preparations was considered to be associated with activation of glycolysis and mitochondrial respiratory chain, modulation of arachidonic acid and protein metabolism, and stimulation of the antioxidant system. The test preparations produced different effects on biological and biochemical parameters.

Here we performed a comparative ultrastructural study of the protective effect produced by RU-185 and RU-254 on microcirculation and cell response in rat ischemic skin.

MATERIALS AND METHODS

Experiments were performed on 16 male rats weighing 120-200 g. Ischemia (skin fold) and tissue sampling were performed under hexenal anesthesia (60 mg/kg

Russian State Medical University, Moscow; *Krasnodar Research Center, Russian Academy of Medical Sciences, Krasnodar Krai Administration. ***Address for correspondence:*** banin@rsmu.ru. Banin V. V.

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intraperitoneally). The animals were euthanized with hexenal overdose.

The rats were divided into 4 groups (intact, control, and 2 experimental groups). In group 1 intact rats the only procedure was hair removal on the back under hexenal anesthesia (control). A skin fold (length 4.5-5.0 cm, thickness 1 cm) was formed in animals of groups 2-4 to model partial ischemia. A single-thread interrupted suture with continuous stitches was placed on the base of the fold. Group 2 control rats intraperitoneally received physiological saline in a daily dose of 0.2 ml for 3 days. The animals of groups 3 and 4 received an equivalent volume of RU-185 and RU-254, respectively (final concentration 10 mg/kg). For electron microscopy narrow skin strips (1.5×4.0 mm) was obtained from the upper middle area of the fold. Strips were fixed in 2.5% glutaraldehyde in phosphate buffer (pH 7.4) for 2 h, cut into pieces, postfixed in 1% OsO_4, treated routinely, and embedded in paraffin. Semithin sections were stained with 1% methylene blue. Thin sections (30-50 nm) were oriented perpendicular to the surface of the epidermis and covered the area corresponding to the papillary and reticular (in part) layer of the dermis and first 2-4 cell layers of the epidermis. Sections were stained with uranyl acetate and lead citrate and examined under a Hitachi HU-12A electron microscope.

RESULTS

The skin fold gained a mulberry-blue color and thickened 1-1.5 h after suturing. External signs of circulatory disturbances and edema became less pronounced, but did not disappear 1 day after surgery. Morphological study showed that changes in the skin fold resemble the "re-flow" state (complete or partial recovery of blood flow after severe ischemia).

Ultrastructural signs of circulatory disturbances and cell damage were similar in experimental rats of groups 2-4. It should be emphasized that these disorders varied in the degree and incidence and were absent in some rats. Although the observed changes reflect the reaction of various tissue components in the skin, they characterize blood supply, alteration of permeability, and state of cells and extracellular matrix in the connective tissue and epidermis. For convenience sake, we compiled a list of signs that appeared regularly and episodically or were absent under various experimental conditions (Table 1).

A specific feature of blood supply to the skin was that examined zones included only capillaries. Therefore, the observed changes were related to disturbances in blood supply and transport function of capillary walls, but not of other vessels differing in permeability of the endothelial layer (*e.g.*, venules) [1]. Standardization of the results allowed us to use the comparative descriptive method, since quantitative ultrastructural study is not convenient for evaluating the effects of drugs.

Obstructed capillaries were sometimes revealed in intact animals (Table 1). This is normal for skin, because changes in blood volume and number of active capillaries in the skin serve as the regulatory mechanism. The skin belongs to hypoxia-resistant tissues [11,14]. However, hydration of the interstitium ob-

TABLE 1. Microcirculatory Disturbances and Cell Damage in Rats during Experimental Ischemia and Administration of RU-185 and RU-254

Sign	Group			
	1 (intact)	2 (control)	3 (RU-185)	4 (RU-254)
Obstructed capillaries	±	+	±	+
Exfoliated endothelium	—	+	—	±
Destructed endothelium	—	+	—	±
Transformed endothelium	—	+	—	±
Edematous pericytes	—	+	—	+
Pericapillary edema	—	+	±	+
Hydratation of matrix	±	+	+	+
Erythrocytes in tissues	—	+	±	+
Edema of tissue cells	—	+	—	+
Cell destruction, phagocytosis	—	+	—	+
Degranulated mast cells	—	—	—	+
Edema and damage to epidermis	—	+	—	±
Edema and damage to nerve fibers	±	+	±	±

Note. +, regularly observed; ±, episodically observed; —, absent.

served in the papillary layer of the dermis indicates that even a short interruption of circulation during sampling and immersion fixation is sufficient for accumulation of excess water in the tissue.

Electron microscopy showed that most pronounced disturbances develop in control rats of group 2 (Table 1). We revealed obstructed or sharply narrowed capillaries and vessels (Fig. 1, *a*). The ratio of these capillaries was 50% of the control level. Obstructed capillaries were often seen in group 4 animals. Perivascular edema was characteristic of obstructed capillaries and vessels with normal lumen. The capillary wall and surrounding tissue were infiltrated with the follicular precipitate, which illustrates high protein content in the exudate. Perivascular protein edema was observed in some rats of group 3. The development of edema and presence of erythrocytes in the interstitium at a considerable distance from the vascular wall do not seem to be related to hypoxia. As differentiated from group 4 and 2 rats, the endothelial layer in group 2 animals was undamaged (Fig. 1, *b*). Interestingly, dense contacts in the endothelium were preserved in rats with pronounced perivascular edema and serious damage to this layer (Fig. 1, *c*). Ischemic transformation of dense contacts can be related to rearrangement of intramembrane particles not visualized during a transmission microscopy [12]. Therefore, other approaches are required for more complete analysis (*e.g.*, cryofractography). Signs of hydration were rarely observed in endothelial cells. Destruction of mitochondrial cristae was a common sign. Thinning of peripheral zones in endothelial cells, increase in the volume of the cytoplasmic fraction in plasmalemmal vesicles, and formation of transendothelial channels were revealed in group 4 rats and, particularly, in control animals (physiological saline and ischemia). These changes are not typical of capillaries with the somatic endothelium. Transformation of the endothelial layer probably reflects high-intensity passage of fluid and relatively large molecules in the endothelium. Contours of light edematous pericytes not containing membrane organelles were visualized in dense and protein-infiltrated spaces. Partial exfoliation of the endothelial layer from the basal membrane was characteristic of group 2 and 4 rats (Fig. 1, *d*). Confined fluid-filled spaces appeared in these regions. Large fluid-contai-

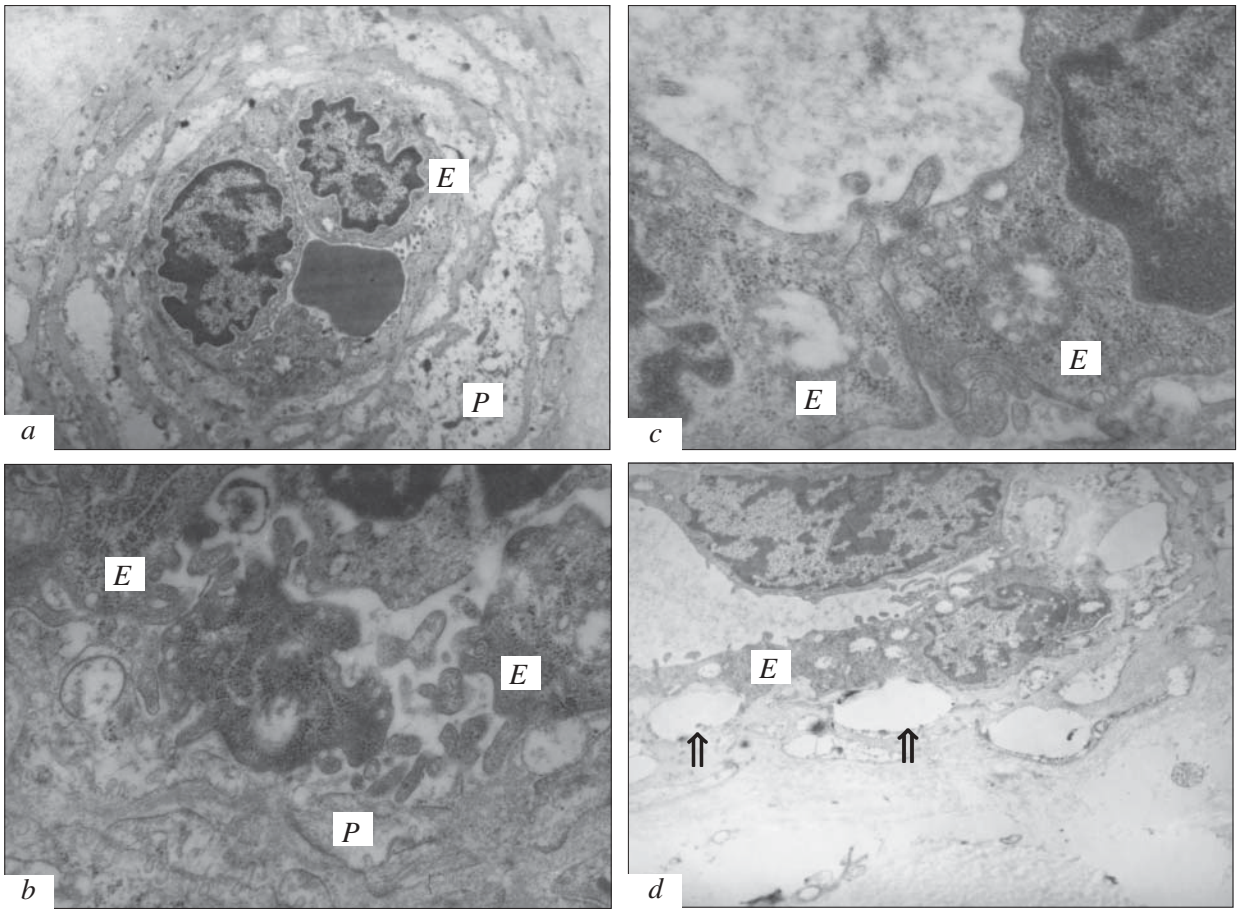

Fig. 1. Damage to blood capillaries and surrounding tissues in the skin of rats with partial ischemia. Sharply narrowed capillary, perivascular edema (*a*, ×6000); damage to the endothelial layer of obstructed capillaries in group 2 rats (*b*, ×18,000); dense contact between endothelial cells (*c*, ×25,200); "hollow" subendothelial spaces (arrows, *d*, ×6000). *E*, endothelium; *P*, pericyte.

ning spaces in the extracellular matrix were present in various areas of the dermis. Connective tissue cells localized at a considerable distance from the vascular wall were characterized by hydration of the cytoplasm and membrane injury. However, these changes were less pronounced than in cells adjacent to capillaries. This is surprising, since oxygen tension in tissues decreases exponentially with increasing the distance from the vascular wall [9] and pO_2 gradient further increases under hypoxic conditions. Previous studies showed that the xanthine oxidase system is the major source of free oxygen radicals during ischemia [10]. These data explain the development of most pronounced damage in cells adjacent to the capillary. To some extent it contributes to injury in nonmyelinated nerve fibers that run with capillary loops in the papillary layer of the dermis.

Clear symptoms of ischemic damage or complete destruction of cells were revealed in the dermis. However, necrotic changes were absent in the epithelial layer of the skin (even in control rats). Keratinocytes were hydrated and contained mitochondria with partially destructed cristae. Hydration of the epidermal layer was especially pronounced in basal cells and observed in group 2 rats and, more rarely, in group 4 animals. Widened intercellular spaces gained a moniliform shape, since contacts (desmosomes) between cell processes were preserved in the basal and spinous layer of the epidermis. We revealed no signs of exfoliation of the epithelial layer from the basal membrane or papillary layer of the dermis.

Our results indicate that RU-185 and RU-254 produce different effects. RU-185 was more potent than RU-254 in preventing circulatory disturbances and ischemic cell injury. The observed changes in group 3 rats reflect altered permeability of the capillary wall for fluid and, to a lesser degree, for proteins. The protective antihypoxic effect of RU-254 in group 4 animals was insignificant. These animals were characterized by the development of damage to mast cells. Degranulation, destruction, and apoptosis of these cells were often seen. Pronounced circulatory disorders and altered permeability in group 4 rats were probably associated with changes in mast cells. Previous studies showed that degranulation of these cells results in the release of vasoactive substances into the interstitium surrounded by microvessels [6].

Different effectiveness of the test preparations in preventing ischemic damage is consistent with the results of our previous experiments [2]. We showed that as distinct from RU-254, RU-185 markedly decreases the degree of necrotic changes in the isolated skin flap [2]. A lower effectiveness of RU-254 is probably associated with stimulation of mast cells in the dermis. Allergic reaction accompanied by microcirculatory disturbances can complicate tissue reaction to ischemia.

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