NEW MEDICINAL PREPARATIONS

Pine Resin and Biopin Ointment: Effects on Repair Processes in Tissues

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The effects of Biopin (ointment containing pine resin) used in the treatment of burns, wounds, and pyoinflammatory processes on cell composition of the wound surface (smears) and granulation tissue were studied on rats. *In vitro* antibacterial activity of the drug was also evaluated. At the early stage of inflammation Biopin modulated the nonspecific and inhibited specific cell immune response, normalized hemodynamics in the inflammation focus, activated synthetic processes in the wound, and was effective towards anaerobes and bacilli.

Key Words: *pine resin; Biopin ointment; composition of wound smears; composition of granulation tissue; antibacterial effect*

Wound healing is a multistage process requiring cooperation of different types of cells. Keratinocytes, endothelial cells, and fibroblasts in damaged skin interact through cytokines and molecules of extracellular matrix formed from initial blood clot (containing mainly plasma fibrin and fibronectin). The formation of granulation tissue including production of extracellular matrix components (fibroblasts and macrophages produce fibronectin, fibroblasts produce collagen and proteoglycans) and proliferation and migration of mesenchymal (fibroblasts and myocytes) and epidermal (keratinocytes) cells is regulated by many cell factors [3-7]. Inflammatory and regenerative reactions are associated with functioning of immune cells (neutrophils, macrophages, and lymphocytes). The role of these cells in local inflammation is well established. In particular, lymphocytes produce factors modulating proliferation and growth of fibroblasts and production of fibroblast-derived factors. Increased RNA content (against the background of unchanged DNA content) and reduced content of free amino acids in tissues during wound healing can serve as a marker of intensive synthetic processes.

Here we studied the effects of a pine resin (PR) preparation, Biopin ointment (BO) — highly effective in the treatment of wounds and burns during phase I of wound process [2], on cell composition of wound surface and biochemical composition of young granulation tissue. Antibacterial activity of PR and BO was evaluated *in vitro* (these parameters are less informative, because they do not include the reaction of the organism, but they help to evaluate the direct antibacterial effect of the preparations).

MATERIALS AND METHODS

Antibacterial activity of PR and BO *in vitro* was studied on standard test strains (gram-positive *St. aureus* and gram-negative *E. coli* and *Ps. aueroginosa*) by the diffusion method. BO, PR (in DMSO), and reference drug gentamicin were put into wells (cylindrical, 2 mm in diameter) in agar (Petri dishes) inoculated

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with bacterial culture. The dishes were incubated at 37°C for 24 h. The zones of inhibition of microorganism growth were measured. The minimum suppressing concentrations in liquid culture for PR (BO can not be dispersed in water) were evaluated by titration. To this end, 10^6 cells of the test strain were incubated with PR (2-fold dilutions starting from 100 μg/ml) at 37°C for 24 h.

The effects of BO on reparative processes *in vivo* were studied on 20 random-bred male rats weighing 180-200 g (Rappolovo Breeding Center, Russian Academy of Sciences). The animals were divided into 5 equal groups. The reference drugs were levomekol, 5% synthomicin liniment (SL), and Shostakovskii balm (SB). The animals were narcotized with 70 mg/kg ketamine and 1 mg/kg droperidol intramuscularly, the skin and fascia on the back were removed, and a 2×2 cm wound was modeled. In 4 of 5 groups the defects were covered with gauze soaked with test drugs, dressed with 6-fold sterile gauze, and the dressing was sutured to the skin. After 3 days the dressing was removed, the wounds were examined, smear impressions from wound surface were collected, and specimens of immature granulation tissue (thin surface layer) were cut with fine scissors; the quality of the specimen (absence of connective tissue) was controlled by the RNA/DNA ratio.

Cytological analysis of smears was carried out using a 5-point semiquantitative scale [1]; cell elements were counted after azur-eosin staining.

The content of DNA and RNA in biopsy specimens was evaluated by alkaline hydrolysis. The samples were homogenized in 300 mM KOH, incubated at 37°C for 1 h (RNA hydrolysis), 1 N $HClO_4$ was added to a concentration of 0.2 N, acid-insoluble material (proteins, DNA) was separated by centrifugation (3000*g*, 20 min), and absorption of the supernatant was measured at 260 and 284 nm. RNA concentration was evaluated by the formula:

$$[\text{RNA, } \mu\text{g/ml}]=75\times(A_{260}-A_{284}).$$

Then acid-insoluble sediment was incubated with 1 N $HClO_4$ at 95°C for 30 min (DNA hydrolysis), $HClO_4$ concentration was adjusted to 0.2 N with KOH, the samples were centrifuged at 3000*g* for 20 min, and absorption was measured at 268 and 283 nm. DNA concentration was evaluated by the formula:

$$[\text{DNA, } \mu\text{g/ml}]=93\times(A_{268}-A_{283}).$$

The content of amino acids in biopsy specimens was evaluated by chromatography of DNS derivatives on a Nucleosil-SC fluoroplast microcolumn (0.5×300 mm) (elution with acetonitrile linear step gradient in Na-formalin buffer).

Coefficients of stimulation of synthesis (CSS) of RNA and DNA were estimated as a ratio of RNA and DNA synthesis in experiment to RNA and DNA synthesis in the control, CSS for nucleic acids (CSS_{NA}) as the product of CSS_{RNA} and CSS_{DNA}.

The means and dispersion were calculated for each group and the differences were analyzed by Student's *t* test.

RESULTS

Study of antibacterial activity *in vitro* showed that BO had no effect on microorganism growth, while the reference drug gentamicin formed extensive fields of microflora inhibition. PR is characterized by pronounced bactericidal effects (Table 1), therefore the absence of this activity in BO can be explained by inhibition of its diffusion due to the presence of some other components (specifically, beeswax ester macromolecules). The minimum suppressive concentrations of PR in liquid culture were: bacteriostatic — 50 μg/ml for *St. aureus* and 100 μg/ml for *E. coli* and *Ps. auroginosa* and bactericidal — 100 μg/ml for *St. aureus* and *Ps. auroginosa* (no effect on *E. coli*).

Visual examination after removal of dressing with BO showed fibrin deposition on the wound surface. After removal of fibrin bright red muscle tissue with forming granulations was seen. Treatment with other drugs produced similar results (status of the wounds and fibrin deposition). In controls the wounds dried and looked dim (a thin crust formed in 1 of 4 rats). No abundant suppurative discharge was observed.

TABLE 1. Antibacterial Activity of PR in DMSO *In Vitro* (Diffusion from Wells in Agar, mm; *M*±*m*, *n*=5)

Test strains, 110 cells/ml	Control (DMSO)	PR dose, mg/ml			Gentamicin, 80 μg/ml
		1	10	100	
St. aureus	0	14±3*	16±3*	19±4*	40±4*
E. coli	8±2	8±2	12±3	15±4*	35±4*
Ps. auerogenosa	10±2	15±3	19±4*	25±5*	30±3*

Note. Here and in Tables 2 and 3: *$p<0.05$ compared to the control.

TABLE 2. Content of Cells in Smears from Wound Surface (%) and Content of Nucleic Acids in Granulation Tissue (μg/g) in Rats after 3 Days (Hematoxylin-Eosin/Azur-Eosin; *M*±*m*, *n*=4)

Preparations	Control	BO	Levomekol	SL	SB
Neutrophils	+/67±9	+++/92±8*	++/60±7	++/59±7	+/32±5*
Macrophages	-/10±3	+/2±1*	++/32±5*	++/29±5*	+/19±4*
Epithelium	+/12±3	+/1±1*	+/3±1*	+/1±1*	-/0*
Plasmocytes	-/0	-/0	+/2±2	+/2±1	+/3±1*
Lymphocytes	+/11±3	±/1±1*	±/2±2*	++/9±2	+++/46±5*
Erythrocytes	+++/+++	++/+	++/++	+++/+++	+++/+++
Bacterial flora					
cocci	++	++	+	++	++
bacilli	++	—	+	+	—
anaerobes	++	+	+	+++	—
RNA	24.0±1.5	44.3±2.7*	36.6±2.8*	32.7±2.9*	84.6±3.1*
DNA	25.5±1.6	20.1±1.7*	12.3±1.9*	11.4±1.6*	31.3±1.7*
RNA/DNA	0.94±0.09	2.20±0.10	2.98±0.17	2.87±0.17	2.70±0.07
CSS_{RNA}	1	1.85±0.09	1.53±0.10	1.57±0.10	3.53±0.07
CSS_{DNA}	1	0.79±0.11	0.48±0.17	0.45±0.15	1.23±0.08
CSS_{NA}	1	1.46±0.15	0.73±0.36	0.71±0.34	4.34±0.07

Note. "—" no elements, "±" solitary, "+" little, "++" moderate quantity, "+++" abundant.

TABLE 3. Content of Free Amino Acids in Granulation Tissue (10^2 nmol/mg) of Rats after 3 Days (*M*±*m*, *n*=4)

Preparations	Control	BO	Levomekol	SL	SB
Hydroxyproline	36±4	89±11*	102±14*	87±10*	103±16*
Proline	56±11	41±8	31±7*	50±12	45±8
Tyrosine	31±9	7±2*	25±7	23±7	30±8
Histidine	171±28	73±11*	123±19	147±22	140±31
Lysine	83±13	41±7*	48±9*	48±8*	32±7*
Ornithine	43±9	38±8	62±12	70±15*	34±7
Valine	12±3	7±2*	7±2*	9±2	16±4
Tryptophan	34±5	23±4*	31±4	26±4*	30±5
Phenylalanine	48±10	37±6*	41±9	43±10	39±9
Alanine	168±37	170±41	183±35	173±40	175±37
Threonine	73±12	47±9*	69±16	28±9*	49±11*
Glutamine	231±62	100±24*	91±20*	115±23*	109±29*
Glutamic acid	980±220	451±103*	473±119*	718±166	429±91*
Serine	61±14	70±17	98±23*	127±29*	57±14
Arginine	98±26	85±17	88±21	110±27	50±13*

Cytological analysis of the wounds (Table 2) showed that at the early stage of inflammatory process the effects of BO on nonspecific resistance were different. BO stimulated migration of neutrophilic granulocytes into the inflammatory focus and inhibited migration of macrophages. At the same time BO inhibited specific cell immune response (migration of lymphocytes and plasmocytes). BO more intensely limited erythrocyte release through endothelium compared to reference agents, *i.e.* normalized hemodynamics in the inflammation focus. As for *in vivo* antibacterial activity, BO was little effective towards micrococci, but effectively cleansed the wound from anaerobes and bacilli (in comparison with *in vitro* activity, Table 1).

BO increased RNA content in the granulation tissue, CSS_{NA} was above 1 (despite inhibition of DNA

synthesis), which attested to activation of synthetic processes (Table 2). The content of free amino acids decreased (as during treatment with other agents), which could be a result of their incorporation into proteins (Table 3).

Hence, during the early stage of inflammatory process BO modulated nonspecific and inhibited specific cellular immune response, normalized hemodynamics in the inflammation focus, activated synthetic processes in tissue, and was effective towards anaerobes and bacilli.

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