EXPERIMENTAL METHODS FOR CLINICAL PRACTICE

Experimental and Clinical Validation of Plasmadynamic Therapy of Wounds with Nitric Oxide

A. B. Shekhter, R. K. Kabisov, A. V. Pekshev, N. P. Kozlov, and Yu. L. Perov

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny,* Vol. 126, No. 8, pp. 210-215, August, 1998
Original article submitted April 30, 1997

Pronounced wound-healing effect of exogenous nitric oxide obtained by the plasma chemical method was confirmed in rats with aseptic and infected wounds and in patients with complicated stubborn wounds, postradiation and trophic ulcers, necroses of transplanted skin flaps, etc. Morphological study revealed normalization of microcirculation, decreased inflammation, enhanced phagocytosis, activation of macrophages, and accelerated proliferation of fibroblasts.

Key Words: *nitric oxide; wound healing; microcirculation; inflammation; aerial plasma*

The discovery of the properties of endogenous nitric oxide (NO) as a polyfunctional regulator of the "new signal molecule" is one of important achievements in biology and medicine in the last decade. By activating the guanylate cyclase and other cellular mechanisms, NO in health and, even more so, in disease promotes vasodilatation, inhibits intravascular platelet and erythrocyte aggregation, leukocyte adhesion and blood coagulation, modulates the immunity, macrophage and neutrophil activities, antibacterial and antitumor activities, nerve pulse conductivity, etc. [1,4,5]. Inhalations of gaseous NO are used for treating pulmonary hypertension and acute respiratory distress syndrome [3,6]. Disorders in microcirculation, phagocytosis, macrophagal functions, fibroblast proliferation, vascular and nervous trophics play the key role in the wound process, particularly in suppurative complications and in chronic wounds (stubborn wounds, trophic and radiation ulcers, bed sores, etc.) [2,7]. The regulatory functions of endogenous NO prompted us to use endogenous NO obtained by the plasma chemical method for treating wounds.

I. M. Setchenov Moscow Medical Academy; P. A. Herzen Moscow Oncological Institute, Ministry of Health of Russia; N. E. Baumann Moscow State Technological University; Medical Center, Administration of the President of Russia, Moscow

MATERIALS AND METHODS

Plasmatron of a medical air-plasma device Gemoplaz was the source of NO. Analysis of the axial chemical and thermal characteristics of gaseous flow using a KM 9006 Quintox gas analyzer yielded the following parameters of tissue therapeutic exposure: distance from the outlet 17-20 cm, flow diameter 25-30 mm, t=35-45°C. The concentration of gaseous compounds of the flow in the focus was (mg/m^3): 400-500 NO, 70-90 NO_2, and 8-9 CO. The concentrations of O_2, N_2, and CO_2 were virtually the same as in atmosphere.

Two series of experiments were carried out on 90 male rats. In the first series, conditioned aseptic

0007-4888/98/0008-0829$20.00

skin wounds (300 mm^2) were inflicted; a ring was inserted into wound edges and covered with cellophane to prevent drying. After 2, 3, and 4 days wound surface and edges in experimental group (25 rats) were exposed to NO-containing flow for 60 sec (distance 20 cm, t=40°C). In the control (25 rats) the wounds were exposed to heat ventilators at the same temperature. In the second series (40 rats), wounds were infected with *Staphylococcus aureus* culture (1×10^9 bacterial bodies) for simulating an infected suppurative wound. The experiment and the control were as in the first series. After 4 days, the rings were removed and the wounds healed under crusts. On days 4, 7, 10, 14, and 21 animals were sacrificed under narcosis, 2-3 animals per term. Histological and histochemical analysis of wound tissues included staining with hematoxylin and eosin, Van Gieson staining with picrofuchsin, staining with toluidine blue for proteoglycans, silver impregnation according to Gomori, PAS reaction for glycogen and glycoproteins, Brasche's RNA test, and Feulgen's DNA test. Other rats were left until complete healing of the wound. Planimetry of wounds was carried out every 3 days, the results were statistically processed.

Eighty-six patients were treated at P. A. Herzen Moscow Oncological Institute: 36 patients with complicated postoperative wounds, 7 patients with post-radiation trophic ulcers, 11 patients with trophic ulcers in the presence of venous insufficiency, atherosclerosis, and diabetes mellitus, 27 patients with necroses of transplanted skin and musculo-cutaneous grafts, and 5 patients with postinjection necrosis in patients treated by chemotherapy. Daily or every other day the wound surface and adjacent tissues were exposed to NO flow at a distance of 17-22 cm from the plasmatron outlet, so that the patient felt no thermal discomfort. The duration of exposure of one site was 5-12 sec, total duration depended on the wound size. The treatment was determined by the type and extent of the pathological process. Wound tissue biopsy specimens from 8 patients were studied by histological and histochemical methods before and during treatment. In 27 patients prophylactic treatment (3-5 sessions) of postoperative wounds with NO was carried out in order to decrease the risk of postoperative complications.

RESULTS

In the first series of experiments, aseptic regulations were neglected in order to assess the preventive effect of NO therapy on wound complications. In the control, the signs of suppuration appeared in 42.3% animals on days 3 and 5. No complications of this kind were observed in experimental animals. Planimetric examination showed that the wounds became closed much sooner in experimental animals than in the controls (Fig. 1, *a*). The mean term of complete healing was 7.5 days (24.6%) shorter in experiment.

Histological and histochemical studies showed that starting from day 4, wound healing was markedly accelerated in experimental animals; by this term the inflammatory phase of the process transformed into the reparative phase. Granulation tissue was well developed at the wound bottom (in comparison with the foci in inflamed infiltrated subcutaneous fat), fibroblasts actively proliferated (numerous mitoses), and new capillaries were actively forming; collagen fibrogenesis and accumulation of proteoglycanes and PAS-positive glycoproteins were observed. Macrophagal reaction was more pronounced in experiment, as well as the phagocytic activity of macrophages. Phagocytosis of bacteria by macrophages and neutrophils was enhanced; bacterial colonies on the wound surface were observed only in the controls. The microcirculatory reaction is of

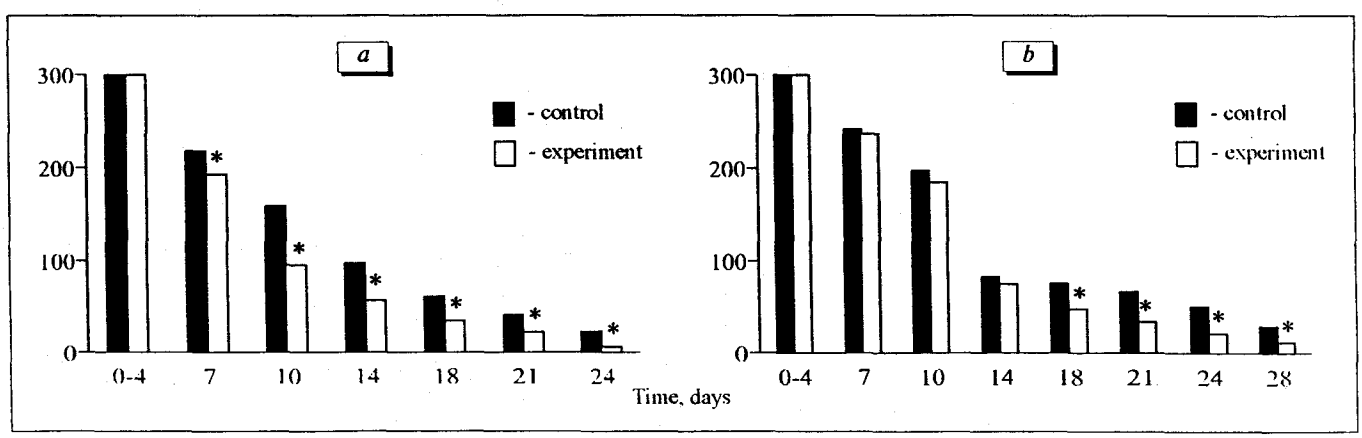

Fig. 1. Time course of healing of experimental conditioned aseptic (*a*) and infected (*b*) wounds. Ordinate: size of wound, mm^2. *$p<0.01$ *vs.* the control.

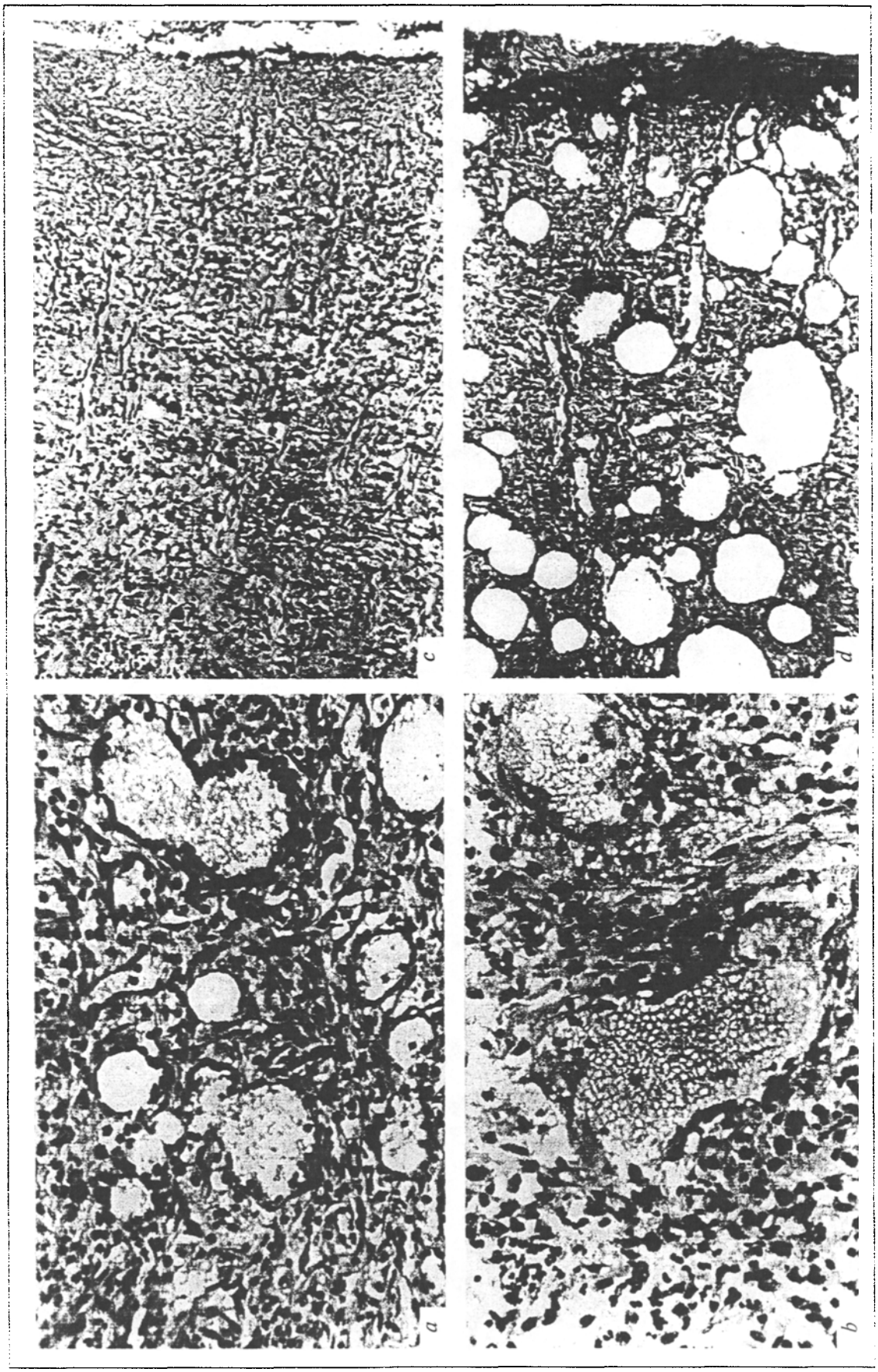

Fig. 2. Morphology of conditioned aseptic wounds in experimental group on days 4 (*a*) and 7 (*c*) and in control group on days 4 (*b*) and 10 (*d*). *a*) well-developed granulation tissue, vasodilatation of new capillaries, fibroblast proliferation, macrophagal reaction, weak inflammatory infiltration, ×250; *b*) no granulation tissue, erythrocyte sludge, and platelet aggregation in capillary lumen, edema, fibrin exudation, and neutrophilic infiltration of tissue, slight fibroblast proliferation, ×400; *c*) mature granulation tissue consisting of fibrin layers, capillary loops, vertical capillaries, horizontal fibroblasts, fibrous tissue, and increased count of mast cells, ×100; *d*) less mature granulation tissue which does not completely replace subcutaneous fat, ×100. Staining: *a*, *b*, *d*: hematoxylin and eosin, *c*: toluidine blue.

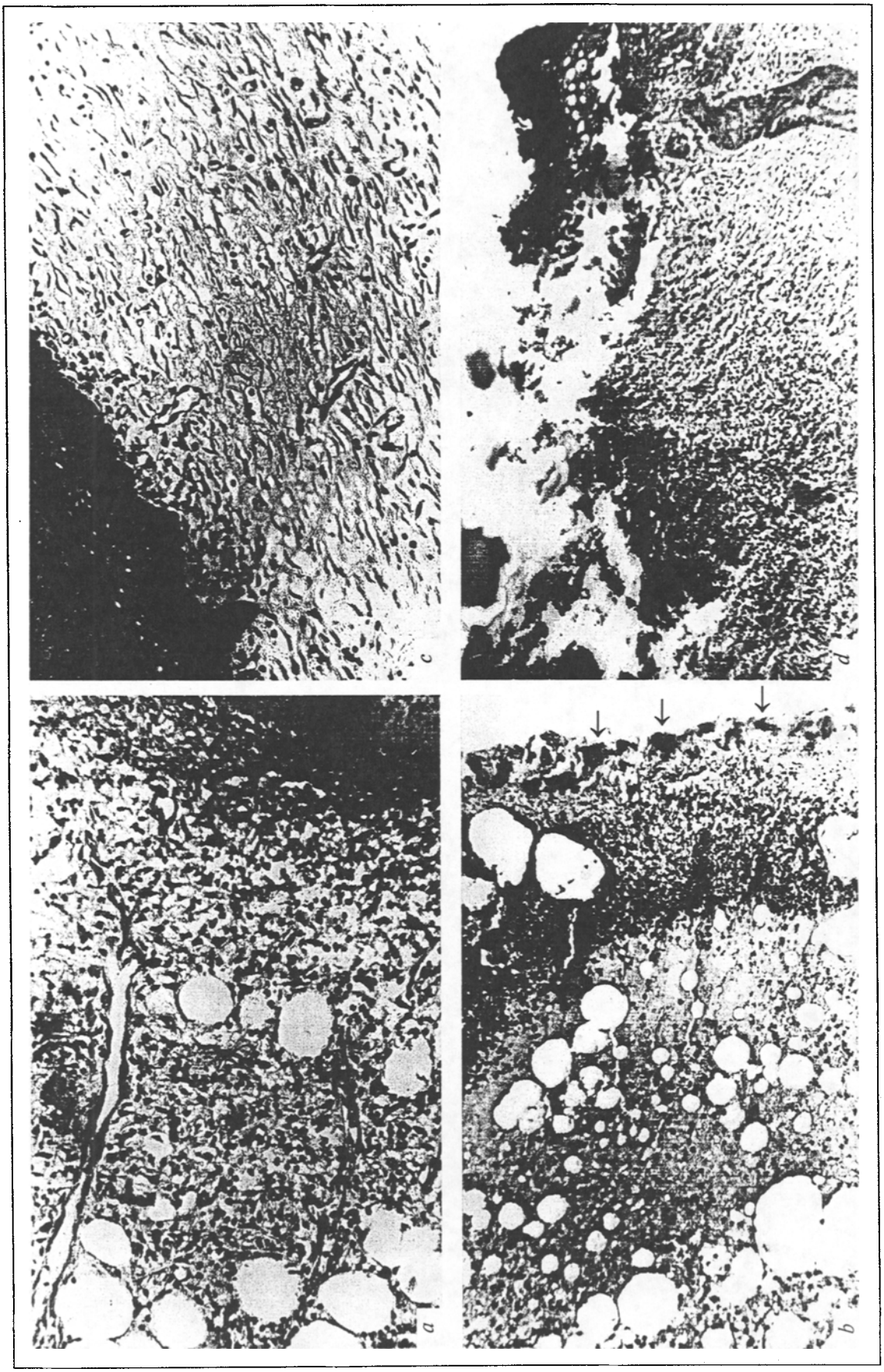

Fig. 3. Morphology of infected wounds in experimental group on days 4 (*a*) and 14 (*c*) and in control group on days 4 (*b*) and 21 (*d*). *a*) granulation tissue with vertical vessels, fibroblast proliferation, lymphocytic and weak neutrophilic infiltration under the leukocytic fibrous layer, ×200; *b*) leukocytic necrotic layer with bacterial colonies on the surface (arrows), no granulation tissue and fibroblasts, fibrin exudate and neutrophilic infiltration in subcutaneous fat, ×200; *c*) fibrous cicatricial transformation of granulation tissue and epithelial regeneration, ×200; *d*) leukocytic infiltration (suppurative inflammation) of granulation tissue, epithelial destruction at wound edges, ×100. Hematoxylin and eosin staining.

special interest. Vasodilatation of new capillaries was observed in the experiment (Fig. 2, *a*), while endothelial destruction, erythrocyte slugging, platelet aggregation, microthrombosis, neutrophil adhesion, and increased migration were much less expressed than in the control (Fig. 2, *b*) and then disappeared. Edema, neutrophilic infiltration, fibrin exudation, and sites of tissue necrosis were less pronounced in experimental animals. After 7-10 days, granulation tissue in experimental animals rapidly matured and divided into layers (Fig. 2, *c*), while in the control it was far less mature (Fig. 2, *d*). After 14 days, granulation tissue in experimental group underwent fibrous cicatricial transformation, capillaries were reduced, and marginal epithelialization and differentiation of the epithelium were observed. By day 21, epithelialization was complete in the majority of animals, and remodulation of cicatricial tissue started; no symptoms of inflammation were observed. In the control cicatrization and epithelialization lagged behind, with signs of inflammation still observed.

In the second series of experiments, when the wounds were infected with *St. aureus,* macroscopic signs of suppuration were observed in all animals, being more pronounced in the controls. On day 17, the wounds in experimental animals shrank more (Fig. 1, *b*) than in control animals; the mean period of complete wound healing was shorter by 31.6%. In the controls, morphological studies revealed microcirculatory disorders, neutrophilic infiltration, edema, vasculitis, formation of primary and secondary necroses, microabscesses, a high level of wound infection, inhibition of bacterial phagocytosis and necrotic detritus, inhibition of macrophagal reaction, fibroblast proliferation, capillary growth, mature granulation tissue, and epithelialization. In experimental group all these signs were less pronounced starting from day 4 and soon disappeared. Granulation tissue started developing on day 4, and fibrosis, contraction, and epithelialization were observed starting from day 14 (Fig. 3).

The extent of the above-listed tissue injuries was: <10 cm^2 in 25.1%, <30 cm^2 in 48.2%, and <200 cm^2 in 26.7%. Before NO therapy, complicated and chronic wounds were treated traditionally (by low-energy laser exposure, etc.). Sluggish granulations in the wounds and ulcers were observed in 32.8% patients, poor marginal epithelialization in 9.4% patients. Inflammatory signs in the wound and adjacent tissues (hyperemia, edema, serous purulent discharge, pain, etc.) were observed in the majority of patients. Tissue hemodynamics improved after the first sessions of NO therapy, as shown by ultrasonic dopplerography. After 3-5 sessions, inflammatory symptoms decreased, focal marginal epithelialization appeared or progressed. In general, this accelerated the healing of complicated wounds by 3-4 times, of trophic ulcers and postinjection and radiation necroses by 2-3 times in comparison with traditional or laser treatment. Prophylactic NO therapy of postoperative wounds in cancer patients at a high risk of postoperative complications (radio- and chemotherapy, weak and senile patients) decreased the incidence of complications to 6.2%, in comparison with 64.3% in the reference group.

Morphological studies of biopsy specimens from complicated and chronic wounds and ulcers showed a marked decrease in the severity of microcirculatory disorders (microthrombosis, endothelial destruction, sludge syndrome, vasculitis, contraction of capillary lumen, etc.), inflammatory symptoms, bacterial contamination; macrophagal reaction and phagocytosis were pronounced, elimination of necrotic detritus, fibroblast proliferation, growth and maturation of granulation tissue and normalization of its structure were accelerated, and the defect rapidly shrank and epithelialized.

Thus, experimental, clinical, and morphological studies have shown that air-plasma flow in the therapeutic mode (t=40°C) stimulates wound healing, particularly in patients with complicated and chronic wounds, and prevents suppuration and wound complications. NO is a specific component of the gas flow and its function of an active cell regulator and messenger is well-known; therefore, we can assert that NO is the key active factor in the procedure described above. The mechanisms of NO effect on the wound healing process may be as follows: 1) vasodilatation and normalization of microcirculatory disorders, improvement of vascular trophics and tissue metabolism; 2) direct bactericidal effect, including that during reaction with superoxide anion; 3) stimulation of phagocytosis of bacteria and necrotic detritus by neutrophils and macrophages; 4) probable inhibition of free oxygen radicals; 5) secretion of cytokines, stimulating fibroblast and vascular growth, by activated macrophages; and 6) direct effect on fibroblast proliferation (in our previous experiments with Dr. O. Yu. Abakumova, a 15-sec exposure to NO flow increased DNA production in human fibroblast culture by 261%). NO therapy is promising treatment and prevention of inflammatory, ulcerative, necrotic, and sclerotic processes involving pathogenetic disorders similar to those observed in wounds [2]. Further studies should examine the potential toxicity of high NO doses.

A patent on the method of NO therapy is applied for. The authors thank Drs. Z. P. Milovanova, T. G. Rudenko, A. V. Gavril'chak, and V. N. Malikov for assistance.

REFERENCES

1. Kh. M. Markov, *Pat. Fiziol.*, No. 1, 34-39 (1996).
2. A. B. Shekhter and V. V. Serov, *Arkh. Pat.*, No. 7, 7-14 (1991).
3. S. Adnot and B. Raffestin, *Thorax*, **51**, 762-764 (1996).
4. M. G. Davies, G. J. Fulton, and P. O. Hagen, *Br. J. Surg.*, **82**, 1598-1610 (1995).
5. S. Monkada and E. A. Higgs, *FASEB J.*, **9**, 1319-1330 (1995).
6. R. Rossaint, U. Pison, H. Gerlach, and K. J. Falke, *Eur. Heart J.*, **14**, Suppl. 1, 113-140 (1993).
7. S. Sarin, T. R. Cheatl, and S. Colleridge, *Br. J. Hosp. Med.*, **45**, 303-309 (1991).