PHARMACOLOGY AND TOXICOLOGY

Regeneratory Characteristics of Complex Extract and Isolated Diterpene Alkaloids of *Aconitum baikalense*

Yu. V. Nesterova, T. N. Povetieva, N. I. Suslov, V. V. Zhdanov, T. Yu. Hrichkova, E. V. Udut, A. S. Chaykovskiy, N. N. Gaydamovich, T. I. Andreeva, and A. M. Dygai

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 152, No. 10, pp. 420-424, October, 2011
Original article submitted May 27, 2010

The effects of complex extract from *Aconitum baikalense* on reparative regeneration of a plane dorsal skin wound were studied. Treatment with *Aconitum baikalense* tincture stimulated reparation and skin regeneration. The effects of the *Aconitum baikalense* alkaloids on functional activity of fibroblast precursors were studied *in vitro* by cultural methods. Mesaconitine, hypaconitine, songorine, napelline, and 12-epinapelline N-oxide significantly stimulated the growth of colonies from fibroblast precursors. This indicated direct stimulation of fibroblasts by aconite alkaloids, which could be a mechanism of reparative activity of the complex extract.

Key Words: *plane skin wound; regeneration; fibroblast precursors; diterpene alkaloids*

Inflammatory reaction is an essential component in the pathogenesis of the wound process. This fact necessitates studies of the efficiency of potential wound-healing means on standard models of inflammation [10]. Profound studies of specific activity are carried out on several experimental models in order to evaluate the drug effects on various components of the pathological process in the wound: exudation, proliferation, pain, capillary status, concomitant infection [12].

We studied diterpene alkaloids isolated from *Aconitum baikalense* (AB): napelline, songorine, mesaconitine, hypaconitine, 12-epinapelline N-oxide. The alkaloids exhibited anti-inflammatory and analgesic effects [6], stress-protective effect [9], antihypoxic and antibacterial activities on various models.

We previously demonstrated stimulation of reparative regeneration of the skin in mice by complex extracts and alkaloid fraction isolated from another representative of the *Ranunculaceae* family, tall delphinium [7]. Treatment with a sum of delphinium alkaloids produced maximum wound-healing effect: more rapid wound healing, increase in the number of animals with complete epithelialization of the wound defect, and formation of better skin regenerate. Importantly, all the delphinium components contained alkaloids of diterpene structure, the predominating of which was elatine [5]. In addition, thin layer chromatography showed the presence of napelline and songorine alkaloids in the aerial part of delphinium. All these data suggested reparative activity of complex extracts and isolated diterpene alkaloids of AB, because of similar alkaloid composition of delphinium and aconite.

Reparative regeneration is a complex process including accumulation of fibroblasts and synthesis of scleroproteins in the focus of dermal injury, wound shrinkage, and epithelialization [3]. That is why fibroblasts and bioactive substances they produce are es-

Institute of Pharmacology, Siberian Division of the Russian Academy of Medical Sciences, Tomsk, Russia. ***Address for correspondence:*** nes-yuliya@yandex.ru. Yu. V. Nesterova

0007-4888/12/1524-0439

sential for tissue reparation after inflammatory process of any kind [11]. Fibroblasts are cells of mesenchymal origin. They are the main component of connective tissue; they produce procollagen, fibronectin, glycosaminoglycanes, and proelastin, which serve as the fibrous carcass for connective tissue and are involved in the formation of extracellular matrix. Fibroblasts produce enzymes and proteins playing an important role in the regulation of local homeostatic processes and cell-cell interactions [2]. It is therefore interesting to evaluate the direct effects of isolated AB diterpene alkaloids on functional activity of fibroblast precursors. This cell population includes, in addition to committed stromal precursors, mesenchymal stem cells (MSC) [13-15].

Hence, we studied regeneratory activity of AB complex extract and separately of isolated diterpene alkaloids on the standard skin flap model of reparative regeneration and *in vitro* by the fibroblast precursor colony formation method.

MATERIALS AND METHODS

The study was carried out on outbred female mice and CBA/CaLac mice weighing 20-25 g (first-category animals from Breeding Center of Institute of Pharmacology). The objects of the study were complex water-ethanol extract from AB in a dose of 0.2 ml/kg and AB alkaloids isolated separately: mesaconitine, hypaconitine, songorine, napelline, 12-epinapelline N-oxide, obtained at Institute of Organic Chemistry, East Siberian Division of the Russian Academy of Sciences (Irkutsk). The alkaloids were isolated by chloroform extraction from raw (aerial) AB plant in the form of free bases, separated, and identified by the standard method [8]. The AB tincture is a complex of active and concomitant substances extracted from fragmented plant by ethanol solution. The alkaloid sum in this tincture is 0.007-0.018%, dry residue at least 1%, ethanol at least 25% [8]. The data on AB tincture as a drug for the treatment of proliferative inflammatory diseases have been presented to the Pharmacological Committee of the Russian Federation.

The effects of phytopreparations on wound healing were studied on the model of a plane skin wound. A skin fragment (10×10 mm) was cut from the skin on an area free from hair on the back in mice under slight ether narcosis. In order to prolong the healing, the crust was regularly (every 24 h) removed from the experimental wound. The animals received the studied preparations intragastrically through a tube starting from day 1 after wound infliction throughout the entire period of the wound healing. The criteria of pathological process development were the common status of animals, visual evaluation of the defect and adjacent tissue status, mean diameter of the wound. The effect of phytopreparations was evaluated by comparing the parameters in experimental and control animals.

The objects of histological studies were skin biopsy specimens from mice collected on day 15 from the wound edge. Biopsy specimens of the skin wound defect were stained with hematoxylin and eosin. Skin regenerate relief, degree and type of infiltration, presence and severity of edema, number of new vessels, hair follicles, and sweat glands were evaluated. The following pathomorphological processes of the mouse skin were studied: acanthosis, hyperkeratosis, dyskeratosis [4].

The content of fibroblast CFU (CFU-F) in the bone marrow was studied by cultural methods [1]. All diterpene alkaloids were added into the culture in concentrations of 1, 10, 50, and 100 nM. The resultant fibroblast colonies (cell aggregations of more than 50 cells) were counted.

The results were processed by ANOVA using Student's *t* test and nonparametric Mann–Whitney *U* test. The significance of differences in the results of experiments with the parameters expressed in fractions was evaluated by Fisher's angular transformation. The differences were considered significant at Pt, Pu, $P\varphi \leq 0.05$.

RESULTS

Treatment of animals with AB tincture (0.2 ml/kg) stimulated significantly the regeneration process. Starting from day 9 the size of the wounds in experimental group decreased by 1.4 times in comparison with the control (Fig. 1). By day 11 complete healing of wound surface was seen in 50% animals treated with the tincture *vs.* none in the control group. By day 15 of the experiment, complete regeneration of the wounds was recorded in all experimental animals (Table 1).

Analysis of morphological processes in the skin wound defect in control mice showed that surface epithelium in some specimens was incomplete, in some places the epidermis was detached from the derma, forming subepidermal fissures; in other words, epi-

TABLE 1. Effects of 10% AB Tincture on the Number of Animals with Healed Wounds (%) in Mice ($X \pm m$)

Group	*n*	Day of observation		
		11	13	15
Control	23	0	43.5	69.6
Tincture, 0.2 ml/kg	8	50*	75*	100

Note. *$p<0.05$ in comparison with the control.

thelialization of the wound was incomplete (Fig. 2, *a*). The epithelial layer was thick, its layers were poorly differentiated, the basal, granular, and horny layers were thickened. Neutrophilic leukocytes with the formation of microabscesses were found in the epithelium; slight acanthosis, hyper- and dyskeratosis were seen. The sublying tissue was represented by maturing granulations with moderate histiocytic and leukocytic infiltration, edema, and hemorrhagic foci in the upper layers of the derma. Hair follicles and sweat glands in the zone adjacent to the defect zone were scanty in all specimens. Skin regenerate in the controls was characterized by moderate inflammation, which led to intense proliferation of the epithelium, characterized by atopic growth and changes in the epithelium (acanthosis, hyper- and dyskeratosis). The regenerative process in the skin in this group of mice eventuated in the formation of inadequate regenerate.

Study of the histostructure of the wound defect in experimental animals showed the formation of a proper connective tissue cicatrix covered with the epithelium (Fig. 2, *b*). In the rest specimens the epithelial layer was sharply uneven with thickening in the defect zone at the expense of basal layer cells. The sublying tissue was granulation tissue, somewhere with focal moderate neutrophilic infiltration. A moderate number of hair follicles and sweat glands was seen in the areas adjacent to the defect in all tissue specimens. Epithelialization was more complete in this group in comparison with the control, the intensity of inflammatory processes and proliferative activity of the epithelium were lower (Fig. 2, *b*). Hence, AB tincture stimulated the regenerative processes in the skin of mice, accelerating the healing of wound defect, increasing the number of animals with complete epithelialization of the defect, and causing the formation of better skin regenerate in comparison with the control.

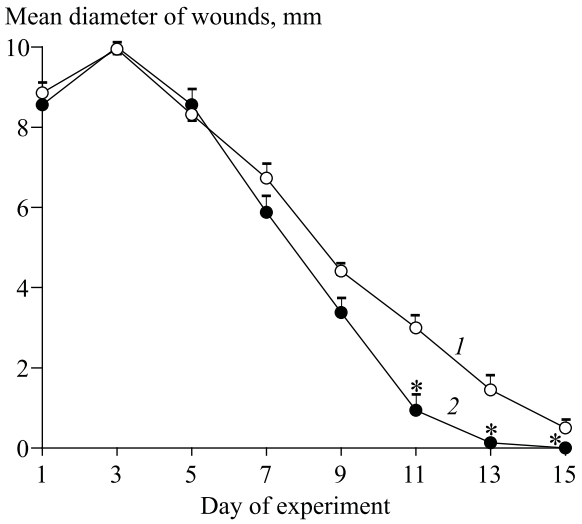

Fig. 1. Effect of a course of AB tincture on the mean diameters of wounds under conditions of a skin flap model. *1*) control; *2*) AB herb tincture, 0.2 ml/kg. **p*<0.05 in comparison with the control.

The efficiency of CFU-F formation from bone marrow cells of intact animals changed significantly in response to addition of AB alkaloids into the culture (Fig. 3). The use of various alkaloids in a concen-

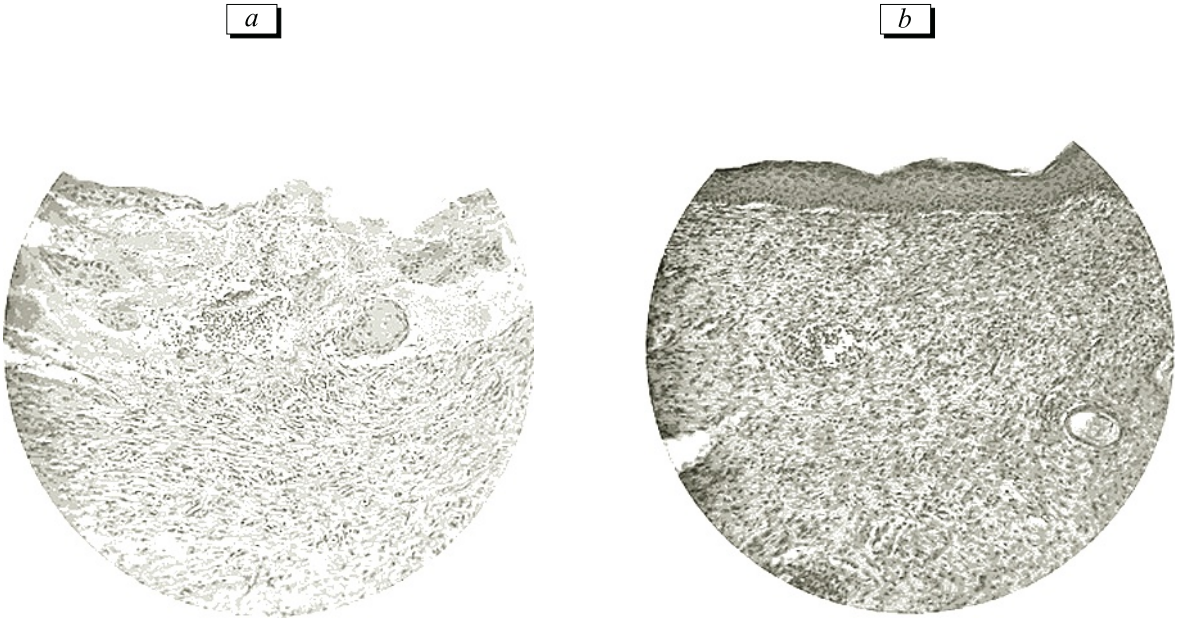

Fig. 2. Effects of a course of AB tincture on reparative regeneration of the skin in mice under conditions of the skin flap model. Hematoxylin and eosin staining, ×400. *a*) incomplete epithelialization of skin defect in mouse (control group); *b*) complete epithelialization of skin wound defect in mouse (AB herb tincture).

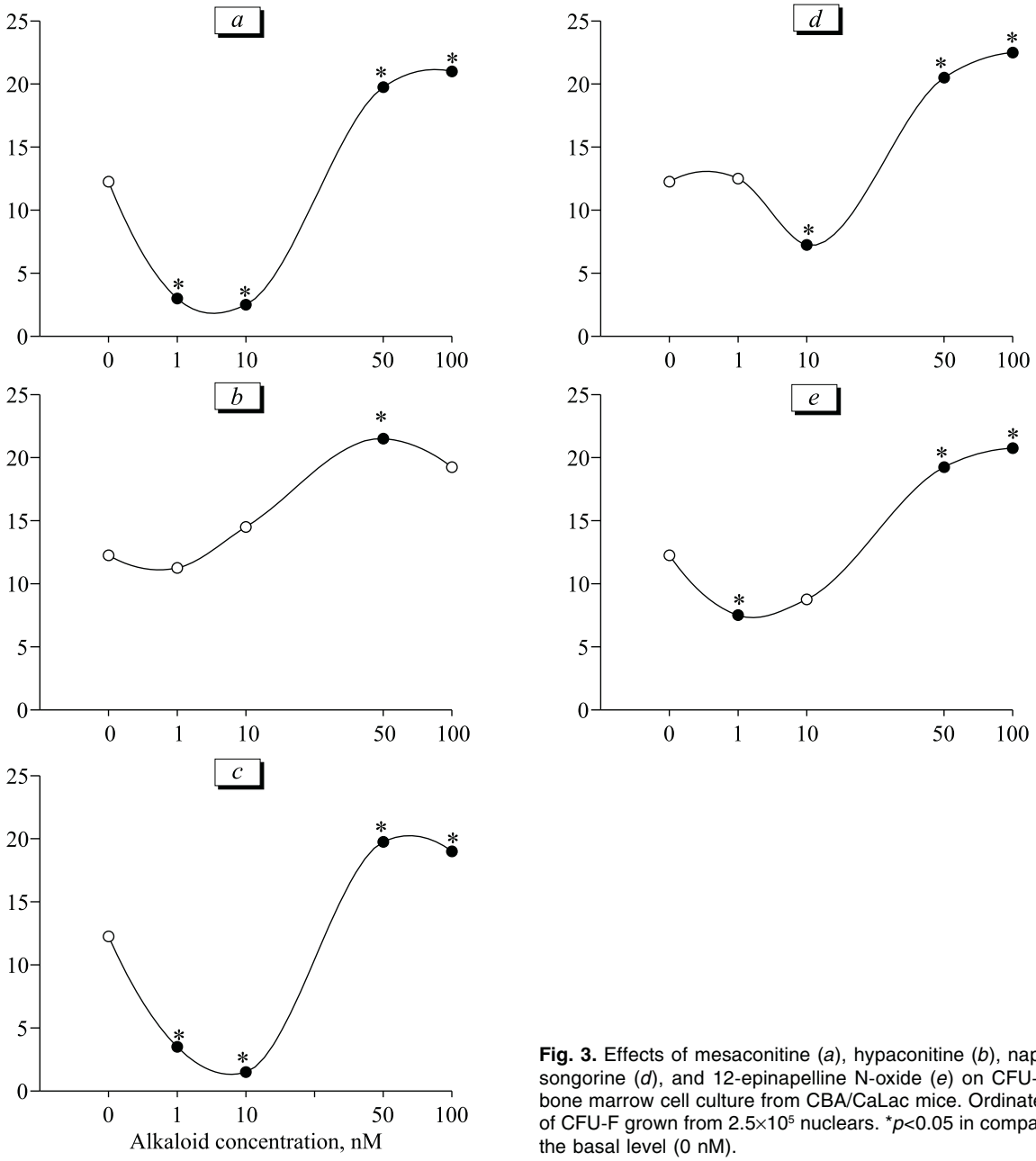

Fig. 3. Effects of mesaconitine (*a*), hypaconitine (*b*), napelline (*c*), songorine (*d*), and 12-epinapelline N-oxide (*e*) on CFU-F level in bone marrow cell culture from CBA/CaLac mice. Ordinate: number of CFU-F grown from 2.5×10^5 nuclears. **p*<0.05 in comparison with the basal level (0 nM).

tration of 50 nM *in vitro* significantly increased the number of CFU-F in comparison with the basal level (by 57-75.5%); hypaconitine exhibited the most pronounced effect (Fig. 3, *b*). AB diterpene alkaloids in a concentration of 100 nM stimulated colony growth by 55-83.7% in comparison with the basal level; the most effective in this case was songorine (Fig. 3, *d*). These findings attest to direct dose-dependent stimulation of CFU-F activity by AB alkaloids, which, no doubt, indicated one of the mechanisms of reparative activity of the complex extract.

Hence, the data indicate that complex AB extract exhibited the regeneratory effects *in vivo* not only due to its previously detected anti-inflammatory, analgesic, antibacterial, and antihypoxic activities, but also due to a direct stimulatory effects of AB alkaloids present in the tincture, on fibroblast precursors immediately involved in reparative regeneration processes. As CFU-F contain MSC, polypotent parental cells [13,14], more complete cell regeneration under the effect of alkaloids can be due to stimulation of tissue-specific differentiation of MSC.

REFERENCES

1. E. D. Goldberg, A. M. Dygai, and V. V. Zhdanov, *Tissue Culture Methods in Hematology* [in Russian], Tomsk (1992).
2. V. P. Goncharov, *Fiziol. Zh.*, **80**, No. 9, 163-173 (1994).

3. K. A. Lukmanova, Z. Z. Yangirova, N. Kh. Salikhova, *et al.*, *Eksp. Klin. Farmakol.*, **67**, No. 3, 73-75 (2004).
4. *Methods of Microscopy*, Ed. D. S. Sarkisov [in Russian], Moscow (1996).
5. D. A. Muravyova, I. A. Samylina, and G. P. Yakovlev, *Pharmacognosia: A Manual* [in Russian], 3rd Edition, Moscow (2002).
6. Yu. V. Nesterova, T. N. Povetieva, N. I. Suslov, *et al.*, *Byull. Eksp. Biol. Med.*, Suppl. 2, 30-36 (2008).
7. Yu. V. Nesterova, T. N. Povetieva, Yu. G. Nagornyak, *et al.*, *Eksp. Klin. Farmakol.*, **72**, No. 3, 40-43 (2009).
8. N. N. Pogodaeva, Ts. Zhapova, A. L. Vereshchagin, *et al.*, *Rast. Resursy*, **36**, No. 2, 79-84 (2000).
9. S. V. Pushkarskii, V. G. Pashinskii, T. N. Povetieva, *et al.*, *Ibid.*, No. 2, 115-119 (2006).
10. *Manual of Experimental (Preclinical) Studies of New Drugs*, Ed. R. U. Khabriev [in Russian], Moscow (2005).
11. I. I. Stepanova, *Klet. Tekhnol. Biol. Med.*, No. 3, 165-168 (2007).
12. N. Yu. Frolova, T. I. Melnikov, A. V. Buryakina, *et al.*, *Eksp. Klin. Farmakol.*, **72**, No. 5, 56-60 (2009).
13. P. S. In't Anker, W. A. Noort, S. A. Scherjon, *et al.*, *Hematologica*, **88**, No. 8, 845-852 (2003).
14. J. J. Minguell, A. Erices, and P. Conget, *Exp. Biol. Med.* (Maywood), **226**, No. 6, 507-520 (2001).
15. M. Owen and A. J. Friedenstein, *Ciba Found. Symp.*, **136**, 42-60 (1988).