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Low concentrations of bacterial melanin prevent secondary changes after cns lesions in rats and significantly accelerate motor recvery

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ABSTRACT

Bacterial melanin has been tested in various models of CNS lesion as a neuroprotector. Different concentrations of have been used in vivo to show its ability to stimulate regeneration, nerve sprouting and protective action. In the present study 6 different concentrations of BM were used to evaluate their effects on recovery processes. Bacterial melanin, injected intramuscularly on the day after the unilateral ablation of sensorimotor cortex, was demonstrated in rats on the model of the recovery of instrumental conditioned reflexes (ICR) and movements of paralyzed hindlimb. The obvious difference between the recovery times of the instrumental conditioned reflex and limb movements in the control and experimental groups provided evidence of an apparent favourable influence of low concentations of bacterial melanin in rats after CNS lesion. Best results with faster recovery rate was obtained in the group treated with 6mg/ml solution of bacterial melanin. This concentration didn't have any toxic effects or secondary complications after neurotrauma. The concentration at the rate of 6mg/ml is the most optimal to be used in vitro experiments to test the antioxidant, anticancer and mitogenic activity of bacterial melanin.

KEYWORDS

Instrumental conditioned reflex;
Bacterial melanin;p
Sensorimotor cortex;
Secondary changes;
Acceleration of compensatory recovery.

INTRODUCTION

Bacterial melanin (BM) has been tested in various models of CNS lesion as a neuroprotector. Different concentrations of have been used in vivo to show its ability to stimulate regeneration, nerve sprouting and protective action. BM enhances plasticity in the CNS, supporting the acceleration of recovery processes after lesions of various structures of CNS. According to the current concepts[1]the plasticity can arise after the lesions located in various parts of CNS, not only in young but also in adult animals, promoting the recovery of impaired function. The nervous system of adult organism contains much more growth inhibitory than growth associated factors. However, trophic changes in the organism can be favorable for the blockade of growth inhibitory factors and contribute in that way to the recovery of impaired functions, stimulating sprouting and formation of new synapses[2], or activation of stem cell divisions[3]. Thus the application of neurotrophic factors can completely prevent the atrophy of axotomized rubrospinal and corticospinal neurons[4, 5]. Neuroprotectors also have a definite role in these processes, particularly for the mini-

mization of secondary damage and for maintaining the integrity of nerve cells. Other researchers have shown that after unilateral labyrinthectomy and injections of adrenocorticotropic and melanocyte-stimulating hormones in frogs the vestibular compensation was accelerated, and the deflection of head gradually decreased[6].

Studies have shown also that neuromelanin containing dopaminergic neurons of the SN are more subject to degeneration in patients with Parkinson's disease than dopaminergic neurons that do not contain melanin. The authors have shown that the free extracellular neuromelanin and microgliosis are the main causes of Parkinson's disease. The latest data show that the human extracellular neuromelanin in the absence of microglia itself is not toxic for neurons. But release of neuromelanin from destructed neurons causes the activation of microglia and subsequent neurodegeneration, proving that melanin containing neurons of Substantia Nigra are targeted in Parkinson's disease. The question is whether bacterial melanin has the same toxic effect on CNS neurons or not.

Based on the above mentioned observations, experiments were conducted to study the effects of various concentrations of water soluble bacterial melanin, obtained by the researchers of the institute of "Biotechnology" from the mutant Bacillus Thuringiensis[7], on the recovery process after unilateral ablation of sensorimotor cortex (model of cerebral stroke).

The aim of present work was to study the effects of different concentrations of water-soluble bacterial melanin (BM) on the recovery of an instrumental conditioned reflex and movements of a paralyzed limb in rats after unilateral ablation of sensorimotor cortex. The study aims to identify the optimal concentration of BM which is non toxic, prevents secondary changes in the lesion area and has the most pronounced protective effects. The identification of BM optimal concentration is required to test the effects of the substance in vitro on cell cultures.

MATERIAL AND METHODS

Study was performed on 42 adult white mongrel male rats weighing 180-250g. Animals of control group (n=6, no BM solution) and experimental groups (n=6, in each group) were initially trained to the instrumental conditioned reflex[8,9] and were then subjected to unilateral ablation of sensorimotor cortex on the left side[10]. The rats underwent craniotomy, during which a surface 2 mm rostral, 3 mm caudal, and 3 mm lateral to the "0" line of the coronal suture (Bregma) was exposed and the cortex of this area was ablated by suction through a fine glass pipette to the level of the white matter. Animals of six experimental groups (n=6), were injected intramuscularly on the day after surgery with BM. Initial concentration of melanin (94mg/ml) was diluted 1, 2, 5, 10, 15, 20 times (94, 47, 18, 9, 6, 4.5 mg/ml, correspondingly). The volume of injected solution for each rat was calculated, taking into account its weight and with the optimally tolerable dose at the rate of 0,17gr/kg.

The instrumental conditioned reflex of balancing on the rotating bar for 250 seconds, with the interrupted intervals of 60 seconds was elaborated in all animals, repeating the testing ten times daily. After completing the experiments all experimental animals were decapitated under deep anesthesia (Nembutal 50mg/kg), brains were removed and fixed in 5% neutral formalin (in phosphate buffer pH=7.4). 50-60μm sections were prepared for microscopy. A histological method was used to identify the microcirculatory bed and a modified histochemical method was used to identify acid phosphatase activity [11] providing not only a rich morphological picture, but also an assessment of the morphofunctional state the structures. The significance of differences on recovery of the instrumental conditioned reflex and morphometric data was assessed using Student's *t* test [12].

RESULTS AND DISCUSSION

As it was revealed by statistical analysis of obtained data (see TABLE 1) the recovery period of motor functions in rats, dosed with various concentrations of BM, was different. In general the recovery of ICR occurred earlier than the recovery of hindlimb movements, impaired after the operation. When injected with three low concentrations of BM (9.4, 6, 4.5mg/ml) the recovery period for ICR and balancing limb movements was significantly shorter. Recovery period for the

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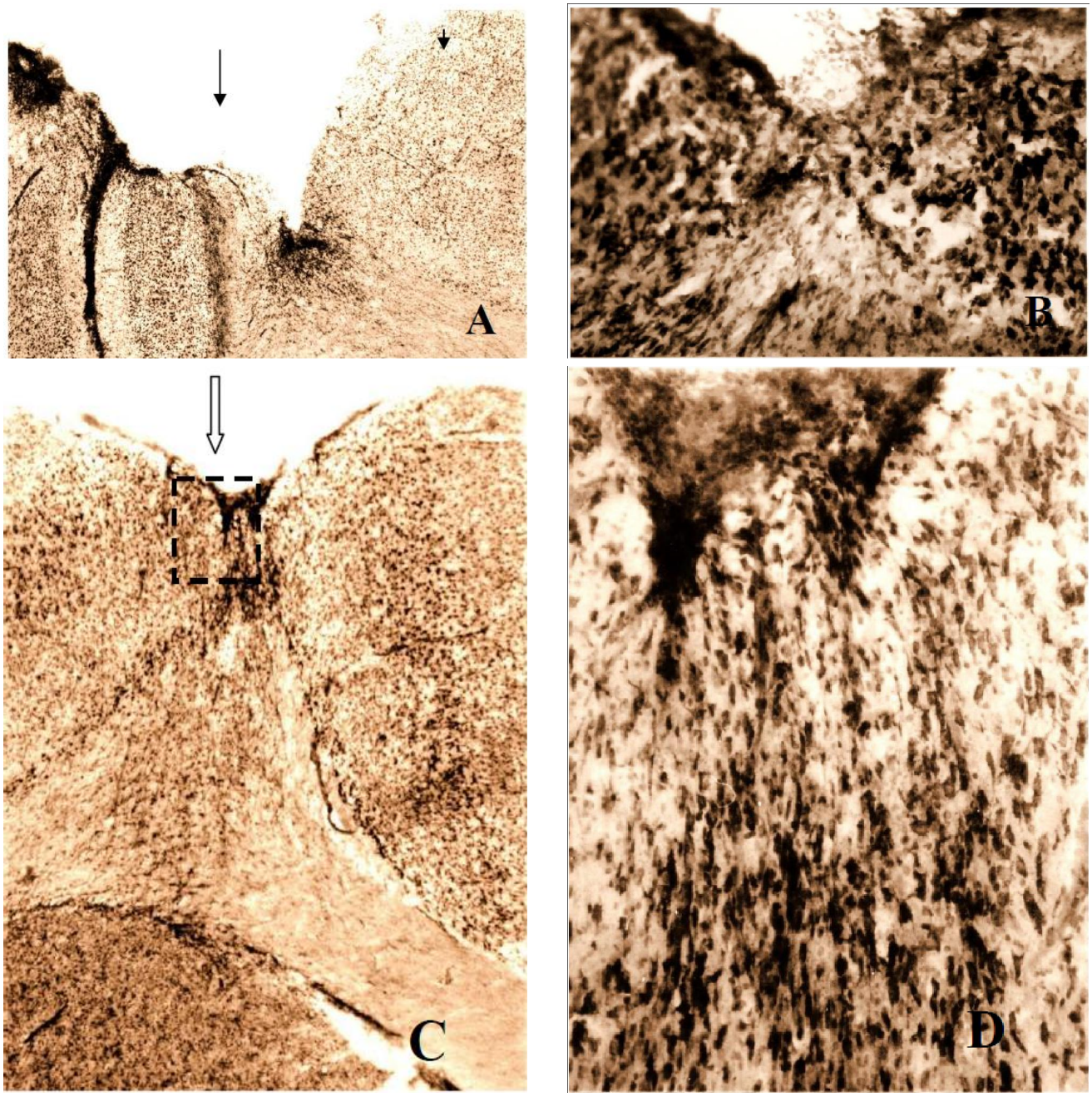

Figure 1 : The ablation area of sensorimotor cortex is indicated by an arrow. A-D are sections from animals treated with bacterial melanin (6mg/ml). The ablation area is filled with cell elements. Magnification: ocular - 10; objective - 2,5 (À, Ñ); 6,3 (Â); 10 (D).

concentration 6mg/ml was 6 days and for the control group 16 days ($p<0.01$). The acceleration of recovery was significant also for the concentration 4.5mg/ml ($p<0.05$). The recovery period for limb movements in these two groups was rapid, complete and clinical picture during the neurological testing was similar with that of normal rats. The only difference was the rate of paralyzed hindlimb recovery, which recovered later in the group injected with 4.5mg/ml.

The morphofunctional state of cellular structures in the brain sections was assessed by performing histochemical and histoangiological studies. The margins of ablated area in the brains of experimental animals dosed with BM at the rate of 6mg/ml and 4.5mg/ml (Figure. 1), were not demarcated by cicatrical tissue. In brain tissue surrounding the lesion moderate nuclear reaction of glial cells was revealed and cica-

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TABLE 1 : Mean Data for periods of elaboration of the ICR and Its Recovery in Control Rats Subjected to Unilateral Ablation of the Sensorimotor Cortex (A) and in Rats Treated with Different Concentrations of bacterial melanin solution (B-G)

	Group of animals	Time period for the ICR elaboration, experimental days	Recovery period of the ICR after ablation of the sensorimotor cortex, experimental days	Recovery period of hindlimb movements after ablation of the sensorimotor cortex, experimental days
A	Control group (n=6)	2.1 ± 0.75	16 ± 2.2	Complete recovery has not been achieved
B	Experimental, given BM at a rate of 94mg/ml (n=6)	2,4±1,5	11,6±7,4	21,4±2,8
C	Experimental, given BM at a rate of 47mg/ml (n=6)	2,83±1,03	13,7±2,9	23,5±2,4
D	Experimental, given BM at a rate of 18.8mg/ml (n=6)	1,83±0,4	7,8±1,2	20,5±0,5
E	Experimental, given BM at a rate of 9.4mg/ml (n=6)	3,5±2,1	12,8±4,8	19±4,4
F	Experimental, given BM at a rate of 6 mg/ml (n=6)	2,8±1,3	5,8±1,03*	10,2±2,3*
G	Experimental, given BM at a rate of 4.5mg/ml (n=6)	2,1±1,1	9,2±1,8	19,6±7,6

trix, which is a strong blocking factor for axonal growth, was absent.

The examination of sections from surrounding brain tissue carried out after the injections with low concentrations, showed that nerve cells were almost completely preserved. The injections of low concentrations of BM being an attempt of induced clinical recovery after CNS lesion, possibly entails a large number of trophic support mechanisms as a favorable stimulus for regeneration [1, 2, 13]. When injected with three high concentrations (94, 47, 18.8 mg/ml) the recovery period of ICR was significantly different from control group results (see TABLE 1), but the recovery period for the limb movements occurred later than in rats dosed with lower concentrations of melanin, though the clinical rehabilitation compared with control group was more complete. After the injection with initial concentration of BM (94mg/ml) in brain sections dilation of capillaries was clearly visible, especially in areas surrounding the lesion. Analysis of morphometric data revealed that 70% of brain capillaries in animals injected with BM were dilated for more than 1μ, which made up 8.5%, as compared with control samples ($p<0.001$). Thus according to the data obtained from histochemical examination the lowest concentration of BM is considered to be the most acceptable.

Injections of high concentrations of bacterial melanin cause an increase in brain volume, due to increased vascularization and edema of neurons. Expressed chromatolysis was revealed in neurons, up to the complete destruction of cellular elements of brain tissue (Figure. 2). Moreover, the closer were the brain cells to the cortex lesion, the more pronounced were changes and the faster they occurred.

In nuclei of thalamus (VL, CM) and hypothalamus (SO and PV) expressed intensity of staining was revealed, due to the activity of acid phosphatase. After the injections with the initial concentration of BM (94 mg/ml), brain were extremely dilated, especially in areas surrounding the lesion. In all frontal sections of brain, on the side of ablated area, in its thalamic part a round, gangliform formation, filed with intensively stained glial nuclei, was revealed (Figure. 2). Therefore, high concentrations of BM, most likely, have toxic influence and restrict the regeneration, vessels because of the demarcating cicatrix, which forms a barrier for the regenerating fibers.

DISCUSSION

Of special interest is evaluation of various concentrations of bacterial melanin on the CNS. In different

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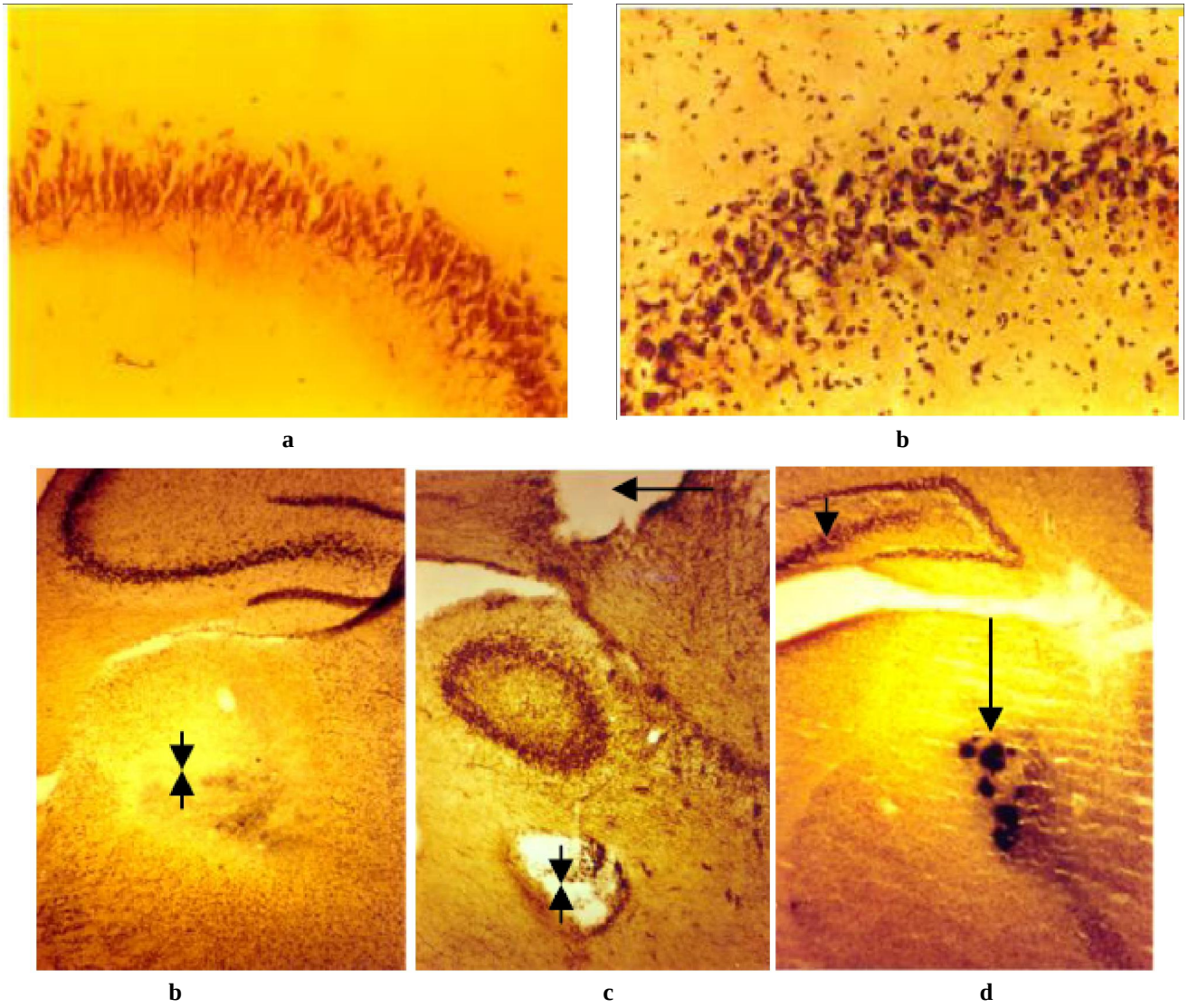

Figure 2 : (a) hippocampus in control rats;(b) hippocampus in rats, injected by BM solution of 18.8 mg/ml;(c) the ablation area of the SMC in animals injected by BM solution of 9.4mg/ml (indicated by arrow). The cross shows gangliform formation. (d) the cross shows accumulation of melanin pigment in the form of dust in thalamus. (e) the cross shows accumulations of melanin pigment in the form of lumps in ventrolateral thalamic nucleus. Magnification Figure.2. A-B - ok.10×ob.6,3; C-E - ok.10×ob.2,5

series of experiments BM was tested as a neuroprotector to support recovery processes after lesions of various CNS structures. In experiments with induced motor tract lesion[17], BM accelerates motor recovery in rats by stimulating the axonal growth and restoring the conductance. BM favors regeneration, enhances motor and behavioral recovery after Substantia Nigra destruction in rats[18]. Bacterial melanin has proved to be non toxic, and it does not cause activation of microglia when applied directly to brain tissue or after injections. It is proved that inflammatory factors may lead to the death of DA neurons in SNc. Bacterial melanin supports the survival of neurons in SNc after induced destruction and preserves dopaminergic cell bodies. BM causes dilation of capillaries in the lesion area, which increases the blood flow in the brain tissue[16]. In the pathogenesis of the Parkinson's disease the activation of microglia is a key factor which supports further destruction of dopaminergic neurons. After neuronal destruction the neuromelanin accumulates in the Substantia Nigra, which in turn supports the activation of microglia. In the electrophysiological study performed to test effects of BM on neuronal activity of

SN neurons in intact rats bacterial melanin showed not to have any activating effect on microglia or any general toxic effect on the organism.

The data obtained on the recovery times of the instrumental conditioned reflex and paralyzed limb movements after sensorimotor cortex lesion in control rats and animals of the seven groups given three i/m of bacterial melanin solution revealed that melanin plays a clear protective role, accelerating compensatory recovery in the central nervous system after trauma. Other studies have confirmed the similar effects of melanocyte-stimulating hormone[6]. The effects of BM in the brains of experimental animals include enhancement of trophic processes due to increased vascularization, and considerable dilation of brain cappilaries. The obtained results show that in control animals limb movements recovered either partially or very late. The main reason for recovery of movement deficit is believed [8,9] to be the ability of the corticorubrospinal tract to take over the functions of the lesioned corticospinal tract. These two motor tracts interact via numerous branches at the cortical and stem levels and also have projections to several spinal cord levels. In the present study ablation of the sensorimotor cortex was followed by removal of the inhibitory influence of the pyramidal tract on the corticorubrospinal pathway at the level of the cerebral cortex and red nucleus, and the rubrospinal system, by means of rubroolivary projections, assumed control of voluntary motor function and supported the completion of initially elaborated instrumental conditioned reflex.

Thus, the rapid and compete elimination of motor deficit revealed in rats given BM at the rate of 6 mg/ml shows the neuroprotective action of this substance. No activation of glial cells was observed in this group. In animals treated with BM at the rate of 4.5mg/ml (lower rate), the recovey of ICR was faster but the paralyzed movements recovered significantly later. Based on the accumulated data [14,15] the concentration of bacterial melanin at the rate of 6mg/ml has the most favorable action on regeneration, plasticity and motor recovery after induced CNS lesion. This concentration is the most optimal to be used in vitro experiments to test the antioxidant, anticancer and mitogenic activity of bacterial melanin on cell cultures.

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