DOI 10.1007/s10517-015-3096-1
Bulletin of Experimental Biology and Medicine, Vol. 160, No. 1, November, 2015 PHARMACOLOGY AND TOXICOLOGY

Study of Subchronic Toxicity of Relatox on Sexually Immature Animals

A. A. Churin, T. I. Fomina, L. A. Ermolaeva, T. V. Vetoshkina, T. Yu. Dubskaya, T. Yu. Lamzina, E. P. Fedorova, O. V. Neupokoeva, and A. N. Nikiforova*

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 160, No. 7, pp. 63-66, July, 2015
Original article submitted March 31, 2014

Intramuscular injections of Relatox in therapeutic and toxic doses to young outbred laboratory rats for 14 days caused no changes in the peripheral blood and bone marrow parameters, serum biochemical parameters, and morphology of the major viscera. In the toxic dose, the drug caused local irritation (inflammation, atrophy, and sclerosis in muscle tissue). Regeneration processes started in muscle tissue 7 days after Relatox withdrawal.

Key Words: *Relatox; subchronic toxicity; young rats; botulinum toxin A; preclinical studies*

Botulinum toxin produced by *Clostridium botulinum* cells causes severe toxic injuries in humans: peripheral muscle palsy and autonomic disorders resulting from failure of cholinergic mediation. Clinical manifestations of the effects of botulinum toxin are pronounced muscular relaxation at the site of injection and significant analgesia of muscles. For this reason, botulinum toxin preparations are widely used in neurology, urology, dentistry, and esthetic medicine for the treatment of patients with myofascial syndromes, stress headaches, mimic muscle contractures, trismus, bruxism, hyperkinetic facial folds, sphincter dyssynergia, and local hyperhydrosis. Botulinum toxin A is used in pediatrics for the treatment of spastic forms of cerebral palsy [1,2,4,7].

The following botulinum toxin-containing drugs manufactured in foreign countries are approved for use in the Russian Federation: Botox (USA), Disport (France), Xeomin (Germany), and Lantox (China). Relatox, botulinum toxin A complex with hemagglutinin, is developed at Microgen Center. Systemic and local reactions to Relatox are rare and slight. Relatox proved to be highly effective in the treatment of blepharospasm and correction of hyperkinetic folds (mimic wrinkles) in adult patients, its efficiency comparable to that of botox and its relaxing effect more pronounced and longer [5].

We study the subchronic toxicity of Relatox on sexually immature animals in order to extend the range of the drug application in pediatric neurology.

MATERIALS AND METHODS

Subchronic toxicity of Relatox 100 U (in flasks) was studied in sexually immature male and female outbred rats receiving daily intramuscular injections for 2 weeks [6].

The study was carried out on outbred rats (stock CD; 30 females and 30 males) from Department of Experimental Biological Models of E. D. Goldberg Research Institute of Pharmacology and Regenerative Medicine. All manipulations on animals were approved by the Institute Committee for Care and Use of Animals (IACUC Application No. 49042013, April 19, 2013). The rats were kept in accordance with the regulations of the European Convention for Protection of Vertebrates Used for Experimental and Other Research Purposes (Strasbourg, 1986). Before and during the experiments, the animals were kept in vivarium at 20-23°C, humidity no higher than 50%, 8:10

E. D. Goldberg Research Institute of Pharmacology and Regenerative Medicine, Tomsk; *Microgen Research-and-Production Center for Medical and Immunobiological Drugs, Ministry of Health of the Russian Federation, Moscow, Russia. ***Address for correspondence:*** toxicology_lab@mail.ru. A. A. Churin

0007-4888/15/1601-0053 © 2015 Springer Science+Business Media New York

(vent:inflow) air exchange, 1:1 day:night regimen, in Velaz standard plastic cages (57.5×35.0×18.5 cm) with fine wood chips, 5 animals of the same gender per cage, in an open system, on standard ration.

Relatox was injected for 14 days in the therapeutic (4.3 U/kg) and toxic (43 U/kg) doses. Controls received 0.9% NaCl. The animals were observed for 7 days after drug withdrawal. The animals were sacrificed on days 14 and 21, 5 males and 5 females per term.

Relatox effects on individual behavior of animals and time course of body weight, on the peripheral blood and bone marrow parameters, and on liver function were studied. Histological studies of the following organs were carried out: brain, heart, liver, lung, kidney, spleen, thymus, adrenal, stomach, small intestine, large intestine, and musculus quadriceps from the site of injection. Histological preparations were made by the standard methods, stained by hematoxylin and eosin; histological preparations of the skeletal muscles were additionally stained by van Gieson method [3]. The main parenchymatous organs were weighed at necropsy.

The data were statistically processed using Stat-Plus 2009 software. Differences between the groups were evaluated by Mann–Whitney nonparametric test at 5% significance.

RESULTS

Clinical examinations of rats receiving Relatox injections for 14 days showed limited mobility of their hind limbs. The mobility was slightly limited in rats receiving Relatox in a dose of 4.3 U/kg, while animals receiving the dose of 43 U/kg developed pareses of the hind limbs. Movements of the hind limbs were restored in animals receiving Relatox in a dose of 4.3 U/kg 7 days after drug withdrawal. Limited mobility of the hind limbs in animals receiving Relatox in a dose of 43 U/kg persisted until the end of observation.

A course of Relatox in a dose of 43 U/kg led to body weight loss and reduction of body weight increment. These parameters did not normalize 7 days after drug withdrawal. Body weight loss seemed to be caused by disorders in motor activity, impeding the access to fodder. Rats receiving Relatox in a dose of 4.3 U/kg did not lose weight throughout the entire study.

Studies in the open-field and elevated plus-maze tests showed that Relatox in a dose of 43 U/kg suppressed locomotor activity and orientation and exploratory behavior of rats. The hole reflex, horizontal and vertical activities were reduced, these shifts were more manifest in males, though females also exhibited a trend to a decrease in these parameters. The parameters did not return to normal over 7 days after drug

TABLE 1. Orientation and Exploratory Behavior of Rats in the Open-Field Test after 2-Week Relatox Course and in 7 Days after Withdrawal ($X \pm m$)

Parameter	Control (0.9% NaCl)		Relatox, 4.3 U/kg		Relatox, 43 U/kg	
	males	females	males	females	males	females
Day 14						
Spontaneous motor activity	73.2±8.0	55.8±15.5	50.0±2.8	61.6±12.0	22.0±7.4*	44.2±5.1
Hole reflex	17.0±3.8	10.0±1.9	9.8±1.9	14.4±3.2	3.8±0.9*	11.6±2.0
Horizontal activity	49.2±4.1	36.2±11.9	36.0±1.4	42.2±8.6	18.2±7.4*	29.2±3.6
Vertical activity	6.6±0.6	8.8±2.3	3.8±0.9	4.4±1.3	0.8±0.8*	3.2±0.9
Grooming	0.4±0.2	0.8±0.2	0.4±0.4	0.6±0.4	0.2±0.2	0.2±0.2
Defecation	2.4±0.9	2.2±0.7	2.4±1.1	3.4±0.7	3.0±0.9	1.4±0.7
Day 21						
Spontaneous motor activity	79.4±3.4	67.2±5.2	57.0±4.6	70.6±6.6	24.2±2.7*	42.6±2.8*
Hole reflex	20.8±1.2	14.8±2.4	11.2±2.3	17.0±2.7	3.8±0.8*	9.8±1.3
Horizontal activity	51.8±2.8	42.2±7.7	39.6±3.3	48.8±6.3	18.8±4.1*	29.6±2.6
Vertical activity	6.6±0.9	9.6±0.8	5.6±0.6	4.2±0.7	1.2±0.6*	2.6±0.7*
Grooming	0.2±0.2	0.6±0.4	0.6±0.2	0.6±0.4	0.4±0.4	0.6±0.4
Defecation	2.0±0.8	0.8±0.4	0.8±0.5	2.2±0.9	1.8±0.9	1.2±0.6

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

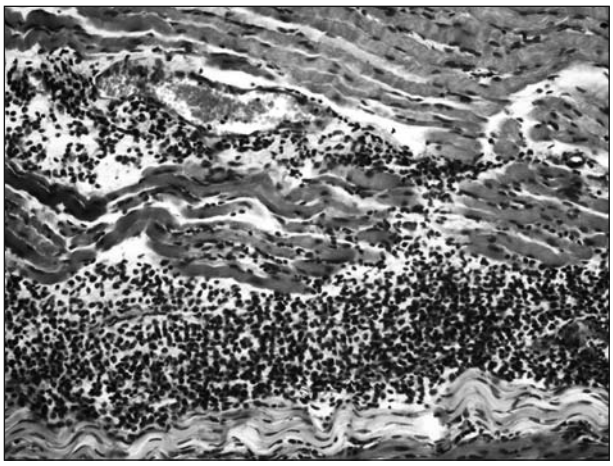

Fig. 1. Lymphocyte-macrophage infiltration destroying cross-striated myofibrils. Hyperemia, interstitial edema against the background of Relatox course treatment (43 U/kg). Hematoxylin and eosin staining, ×200.

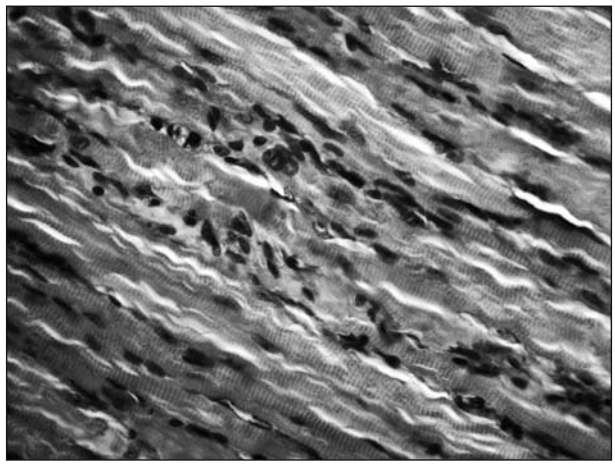

Fig. 2. Regeneration of cross-striated myofibril (muscle bud) 7 days after discontinuation of 4.3 U/kg Relatox. Hematoxylin and eosin staining, ×400.

withdrawal. A course of Relatox in a dose of 4.3 U/kg caused no changes in the locomotor activity and orientation and exploratory behavior of animals (Table 1).

Weighing the viscera and calculation of their weight coefficients in rats treated with Relatox in a dose of 43 U/kg showed a significant reduction of these values for the majority of the organs, which was due to a significant body weight loss in this group. The weights and weight coefficients of the majority of organs normalized 7 days after Relatox (43 U/kg) was discontinued. Injections of Relatox in a dose of 4.3 U/kg was inessential for visceral weights and weight coefficients in the males and females.

Courses of Relatox in doses of 4.3 and 43 U/kg caused no changes in the peripheral blood, bone marrow, and serum biochemical parameters.

Macro- and microscopic studies of organs and tissues of experimental animals on days 14 and 21 (7 days after the drug discontinuation) showed no visceral abnormalities, except the hip muscle status. Macroscopic examination of the muscle of rats, treated with Relatox in a dose of 4.3 U/kg, showed no apparent changes. In rats, treated with the drug in a dose of 43 U/kg, the muscle shrank and was pale on cross-section.

Microscopic examination of the hip muscle in rats treated with Relatox in a dose of 43 U/kg showed hyperemia and intermuscular edema on day 14. Some muscle fibrils were twisted, some sites in the fibrils were swollen and lost cross striation. Massive focal accumulations of lymphocytes, destroying the myofibrils, were found in intermuscular lamina (Fig. 1). These changes were less pronounced in the rats treated with Relatox in a dose of 4.3 U/kg.

Edema and infiltration in muscle tissue decreased 7 days after Relatox (4.3 U/kg) was discontinued, muscle buds (bulb-like swelling of the sarcoplasm

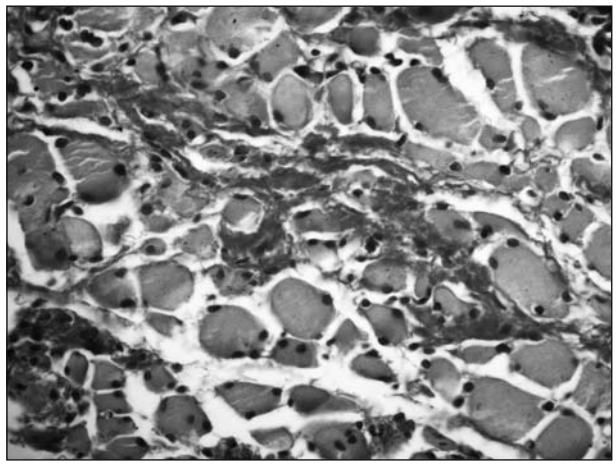

Fig. 3. Atrophy and sclerosis of skeletal muscle 7 days after discontinuation of 43 U/kg Relatox. van Gieson staining, ×400.

containing myoblast nuclei) emerged, which indicated myofibril regeneration (Fig. 2). Atrophic processes in the muscles were found 7 days after the treatment discontinuation in the rats which received the drug in a dose of 43 U/kg: part of myofibrils were thinned, and connective tissue growth was unfolding in the intermuscular lamina (Fig. 3). On the other hand, sites of myofibril regeneration were found in some animals.

The development of peripheral pareses and paralyses of the skeletal muscles under conditions of Relatox treatment were explained primarily by the main mechanism of the drug activity – impairment of nerve pulse transmission, as botulinum toxin blocked acetylcholine release in cholinergic synapses. However, the morphologic picture of muscle tissue (foci of lymphocyte accumulations, destroying the myofibrils) could not rule out the contribution of immunological mechanisms of muscle damage [8]

Hence, repeated intramuscular injections of Relatox in the therapeutic and toxic doses to young rats

caused no systemic effects. The drug injected daily for 2 weeks in doses surpassing the minimum therapeutic dose for humans 7-fold (4.3 U/kg) and 70-fold (43 U/kg) caused dose-dependent local irritating effects – inflammation (4.3 U/kg) and atrophy and sclerosis of muscle tissue (43 U/kg) in the site of injection. Regenerative processes developed in the muscle tissue 7 days after drug withdrawal.

REFERENCES

1. A. Kurenkov, S. Nikitin, A. Artemenko, and O. Orlova, *Vrach*, No. 7, 8-12 (2009).
2. S. A. Likhachev, Yu. N. Rushkevich, T. N. Chernukha, and E. V. Veevnik, *Zh. Nevrol.*, **9**, No. 3, 27-31 (2009).
3. *Microscopic Methods* [in Russian], Eds. D. S. Sarkisov and Yu. L. Petrov, Moscow (1996).
4. O. R. Orlova, *Ross. Med. Zh.*, **14**, No. 23, 1700-1707 (2006).
5. E. V. Plotnikova and V. D. Elkin, *Eksp. Klin. Dermatokosmetol.*, No. 6, 3-6 (2013).
6. *Manual of Preclinical Studies of Drugs* [in Russian], Ed. A. N. Mironov, Part 1, Moscow (2012).
7. V. N. Salkov, *Det. Podrostkov. Reabilitats.*, No. 2, 33-36 (2004).
8. *Supplement: Spasticity: Etiology, Evaluation, Management, and the Role of Botulinum Toxin type A. A Training Syllabus Developed by the Spasticity Study Group*, *Muscle Nerve*, **20**, No. S6, 146-168 (1997).