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SHORT COMMUNICATION

REDUCTION OF CROTOXIN-INDUCED NEUROMUSCULAR BLOCKADE BY GAMMA RADIATION

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M. Gallacci, E. C. Nunes, E. G. Moreira, N. Nascimento, J. R. Rogero and V. S. Vassilieff. Reduction of crotoxin-induced neuromuscular blockade by gamma radiation. *Toxicon* **36**, 941–945, 1998.—A comparative study between crotoxin and gamma irradiated crotoxin was performed on the indirectly evoked twitches and tetani of sciatic nerve-extensor digitorum longus muscle of rats. Crotoxin (3 to 14 μg/ml) decreased the amplitude of twitches and induced a slight tetanic fade, and irradiated crotoxin did not significantly affect either twitch amplitude or tetanic tension. Since gamma radiation reduced the neurotoxicity of crotoxin it may be useful for the production of anticrotalic serum. © 1998 Elsevier Science Ltd. All rights reserved

It has been demonstrated that gamma radiation is able to detoxify snake venoms without affecting significantly their antigenic and immunogenic properties (Salafranca, 1973; Kankonkar *et al.*, 1975; Herrera *et al.*, 1986; Murata *et al.*, 1990). Since serotherapy is the treatment of choice in snake-bite accidents, ionizing radiation could be a promising method to improve the antisera production as it extends the useful life of immunized horses.

The present study was undertaken to evaluate the ability of gamma radiation to detoxify crotoxin, the main toxin of *Crotalus durissus terrificus*. The major neurotoxic effect of crotoxin is the peripheral respiratory paralysis induced by its inhibitory action on the neuromuscular transmission (Vital Brazil, 1966). Crotoxin is a protein composed of two non-covalently associated subunits, one which presents a phospholipase A_2 activity, and another, crotapotin with no demonstrable enzymatic activity (Habermann

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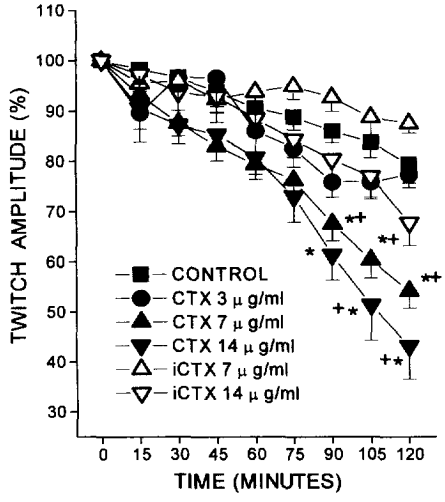

Fig. 1. Effect of crotoxin (CTX) and irradiated crotoxin (iCTX) on the indirectly evoked twitch amplitude in rat extensor digitorum longus muscle. The ordinate represents the % amplitude of twitches relative to the initial amplitude. The abscissa indicates the time (min) after addition of CTX or iCTX to the organ bath. Each point is the mean ± SEM of at least four muscles. *, $P < 0.05$ vs control; +, $P < 0.05$ vs the respective iCTX concentration.

and Breithaupt, 1978; Bon *et al.*, 1986). Individually the two subunits have little neurotoxic activity, but in combination they have a potent neuromuscular blocking action (Chang and Su, 1978). Evidences suggest that the phospholipase A_2 subunit binds to specific sites at the motor nerve terminal and crotapotin reduces the non-specific binding by chaperoning the phospholipase A_2 component to these sites (Bon *et al.*, 1989). The neuromuscular blockade induced by crotoxin is due to both pre- and postsynaptic mechanisms. At the presynaptic level crotoxin induces a typical triphasic change (depression, facilitation and final blockade) of the acetylcholine released by the motor nerve terminal (Vital Brazil and Excell, 1971; Hawgood and Smith, 1977; Chang and Lee, 1977). Postsynaptically crotoxin induces the stabilization of the acetylcholine receptor in an inactive state similar to the desensitized state (Vital Brazil, 1972; Bon *et al.*, 1979). Additionally, it has also been described a direct myotoxic effect of crotoxin in mammals (Breithaupt, 1976).

Previous work has shown that gamma radiation reduces the acute toxicity of crotoxin in mice and rats (Nascimento *et al.*, 1996; Moreira *et al.*, 1997). In the present work, a comparative study between crotoxin and gamma irradiated crotoxin on the rat neuromuscular junction "*in vitro*" was performed in order to evaluate if gamma radiation attenuates the neurotoxic activity of crotoxin.

Crotoxin was purified from *Crotalus durissus terrificus* crude venom by gel filtration on Sephadex G-75 (Pharmacia) followed by isoelectric pH precipitation. The Bradford method was used for protein determination and purity was assessed by SDS-PAGE (Souza-Filho *et al.*, 1992). Gamma radiation with ^{60}Co was assessed with a GAMMACELL 220 source (produced by the Atomic Energy Commission of Canada). A dose of 2000 Gy was applied at the rate of 400 Gy/h, using 2 mg/ml of crotoxin in 0.15 M of NaCl adjusted to pH 3.0 with 0.1 M HCl for solubility purposes (Nascimento *et al.*, 1996).

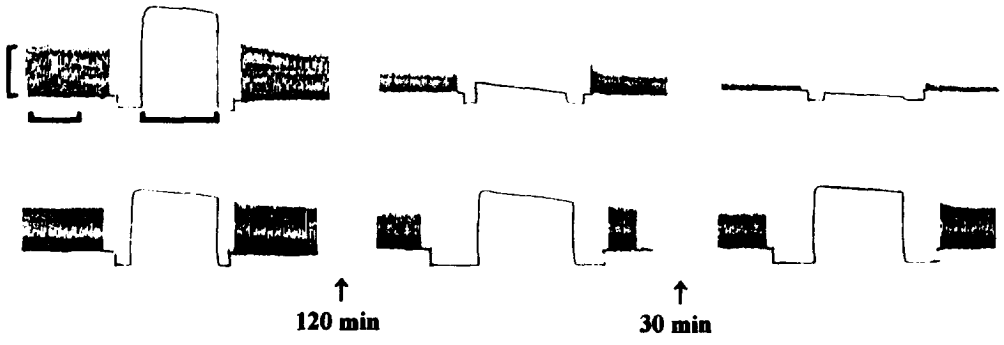

Fig. 2. Indirectly evoked twitches and tetani in rat extensor digitorum longus muscle *in vitro*. Recordings on the left represent controls. Those on the center were obtained 120 min after addition to the bath of 14 μg/ml of crotoxin (top) and irradiated crotoxin (bottom). The recordings on the right were obtained 30 min after washing the tissue with toxin-free physiological solution. The horizontal line under the twitches corresponds to 50 s and that under the tetanus indicates the duration of the tetanic contraction (5 s). Vertical calibration: 3 g for twitches and 6 g for tetani. Arrows indicate interruption of recording.

The sciatic nerve-extensor digitorum longus muscle preparations of male Wistar rats (200 to 250 g) were mounted for the myographic recording "*in vitro*", according to Gallacci and Oliveira, 1994. Briefly, each preparation was mounted vertically in a conventional isolated organ bath chamber containing 50 ml of physiological solution of following composition (mmol/l): NaCl, 135; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 2; $NaHCO_3$, 15; NaH_2PO_4, 1, glucose, 11. This solution was gassed with O_2 (95%) + CO_2 (5%), which kept the pH at 7.4 to 7.5, and was maintained at 27°C. The polygraphic recording of muscle tension was performed by means of an isometric transducer. Indirect twitches were evoked by supramaximal strength pulses with a duration of 0.5 ms and frequency of 0.5 Hz. Tetani (5 s long) were evoked by increasing the pulse frequency to 50–60 Hz. Data were analysed by ANOVA for one-way classification and *post hoc* tests were subsequently performed using Tukey–Kramer test with significance level set at $P < 0.05$.

Within 120 min of contact with the preparation, crotoxin (3 to 14 μg/ml) induced a concentration-dependent decrease of indirectly evoked twitches (Fig. 1), whereas it promoted a slight fade of the tetanic contraction (Fig. 2). The depression of muscle contraction still progressed 30 min after washing the preparation with toxin-free solution (Fig. 2). On the other hand, irradiated crotoxin at 7 or 14 μg/ml did not significantly affect the indirectly evoked muscle contraction (Figs 1 and 2).

The crotoxin-induced depression of the indirectly evoked muscle contraction was most probably due to its well described neuromuscular blocking action (Vital Brazil and Excell, 1971; Chang and Lee, 1977; Hawgood and Smith, 1977; Vital Brazil, 1972; Bon *et al.*, 1979). A direct effect of crotoxin on the muscle fiber might be discarded since it was only observed (Breithaupt, 1976) in a concentration range 9 to 15 times higher than that used in this work. Based on these findings we can conclude that gamma radiation reduces the neurotoxic activity of crotoxin.

Irradiation of crotoxin with 2000 Gy leads to aggregation and generation of lower molecular weight breakdown products (Nascimento *et al.*, 1996). The aggregation products retain at least part of the higher-ordered structure of crotoxin and many of its original antigenic and immunological properties. However, compared with crotoxin, the

aggregates are less myotoxic and largely devoided of phospholipase activity (Nascimento *et al.*, 1996). The lower molecular weight breakdown products require further characterization. The structural changes of crotoxin induced by gamma radiation are due to the formation of intermolecular covalent bonds and to the rupture of disulfide bridges (Souza-Filho *et al.*, 1992). Since the integrity of all disulfide bonds is essential for the toxicity of crobotoxin, a potent toxin of *Naja naja atra* venom (Yang, 1967), we suggest that the rupture of the disulfide bonds of crotoxin by gamma radiation might play an important role in the loss of its neuromuscular blocking action. Moreover, it should be taken into account that gamma radiation also reduces the protein solubility causing crotoxin precipitation (Souza-Filho *et al.*, 1992) that might lead to a partial loss of its biological activity.

The present findings support the premise that gamma radiation can be useful for the production of toxoids of *Crotalus durissus terrificus* venom, since they demonstrated the reduction of the neurotoxic activity of crotoxin by gamma radiation in the neuromuscular junction.

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