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Study of gamma-radiation effects on crotamine and crotoxin

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Crotoxin is a 23 kDa neurotoxin from *Crotalus durissus terrificus* and is composed of a 9 kDa acidic subunit (crotapotin) and a 14 kDa basic subunit (phospholipase A2). Crotamine is 4882 Da, basic polypeptide with myotoxic activity. These toxins, when submitted to gamma-rays, in aqueous solution, present structural modifications, preserving their immunogenic properties. In the present work, we investigated some structural modifications on both crotoxin and crotamine after gamma-radiation using various doses, in the presence or not of "scavenger" substances. Our results indicate that irradiation leads to progressive changes in the structure of the toxin, which could explain the lower toxicity observed.

Introduction

Ionizing radiation is a potential process to modify biomolecules. This can occur by two forms: direct process – ionizing radiation interacts directly on target biomolecules and indirect process – the products generated by water radiolysis interact with target molecules.

Indirect process is the main form of action, representing approximately 80%. In addition, the main species formed, with yields respectively, for 100 eV of absorbed energy (*G*-value), are:

$$H_2O \rightarrow 2.7\ e^-_{aq.} + 0.45\ H_3O^+ + 3.2\ OH^\bullet + \\ + 0.6\ H^+ + 0.45\ H_2 + 0.7\ H_2O_2 \qquad (1)$$

Thus, the two main products are: hydrated electron and hydroxyl radical. Hydroxyl radical acts in proteins promoting hydrogen abstraction of alpha carbon and also reacts with aromatics amino acids forming strongly reactive products.[1] Hydrated electrons react with amino acids, in the same way as the hydroxyl radical, besides promoting the removal of amino groups.

Considering these facts, irradiation of proteins in aqueous solution has been used, aiming to cause chemical changes and physico-chemical alterations on primary, secondary, and tertiary proteins structure. These changes are related with lower toxicity while preserving immunological properties,[2] a rather interesting combination for antisera and vaccine production. The extension of radiation damage can be studied and modified through the addition of "scavengers", molecules that act selectively removing free radicals, allowing a better understanding of the role of each reactive species on the detoxification process.[3]

A great number of substances can be used as "scavengers". Inorganic salts in N_2O saturated solutions, convert the hydrated electron into hydroxyl radical and this radical reacts with the salt to form anion of its respective salt.[4]

Other substances can be used as "scavengers", one example is ascorbic acid that reacts with radiolysis products formed. Organic compounds are frequently used as "scavenger" of hydroxyl radical.[5] The most frequently employed scavengers which displays high specificity for hydrated electron are nitrate ions, while for hydroxyl radicals, alcohols are usually used, more specifically t-butylic alcohol.[6]

Crotoxin is a β-neurotoxin that possesses highly specific toxicity towards neuromuscular junction. At this level, the crotoxin acts primarily by causing a triphasic change (depression, facilitation and final blockage) of acetylcholine release at the motor nerve terminal. Secondarily, crotoxin induces a postsynaptic blockage of neuromuscular by stabilizing a desensitized state of the nicotinic receptor.[7] This toxin is composed by two subunits, a basic protein (phospholipase A_2) with 14 kDa and an acidic protein (crotapotin) with 9 kDa.[8]

Crotamine is a highly basic polypeptide (pI-10.3), with miotoxic activity and a molecular weight of 4882 Da. It is composed of 42 amino acids residues, without free sulphydryl groups and reticulated by three disulfide bonds.[9]

This toxin affects the functioning of voltage-sensitive sodium channels of skeletal muscle sarcolemma inducing a sodium influx, resulting in a depolarization and contraction of skeletal muscle. These effects induce necrosis of muscle fibers characterized by extensive vacuolization of sarcoplasmatic reticulum and disruption of actin and myosin filaments.[10] In the present paper, crotoxin and crotamine were used as models, the first presenting high toxicity as well as catalytic activity, while the second has a known structure, enabling further structural characterizations.

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Experimental

Irradiation of toxins

Toxins were dissolved in a 0.15M NaCl solution to a final concentration of 2 mg/ml, with or without "scavengers" and irradiated with 400, 2,000 and 10,000 Gy doses with a 5.17 kGy/h dose rate using gamma-rays emitted by a ^{60}Co source (Gammacell 220 Canada). The "scavengers" used were t-butanol and sodium nitrate.

Mass spectrometry of crotamine

Aliquots of native and irradiated (400, 2,000 and 10,000 Gy) were injected into an electron spray ion source fitted to a Micromass Q-Tof mass spectrometer in positive mode. The mass was obtained by scanning the instrument from 800 to 2000 *m/z* at rate of 2 seconds per scan.

Solvent mediated fluorescence quenching of crotamine

Aliquots of 5 μg/ml in 50mM ammonium phosphate pH 7.8 of the native and irradiated crotamine were analyzed using a Hitachi F-2000 fluorescence spectrophotometer at 25 °C. The excitation wavelength was fixed at 275 nm and the emission was scanned from 300 to 500 nm.

Enzymatic activities of crotoxin

Enzymatic activity of crotoxin was analyzed according to HOLZER and MACKESSY.[11] This method uses an organic synthetic substrate [4-nitro-3-(octanoyloxy) benzoic acid] that, upon catalysis, releases a 425 nm absorbing chromophore.

Enzymatic activity was estimated using a single concentration of toxin, and varying the radiation doses as well as the presence or absence of "scavengers" substances.

Toxicity assays

To verify the toxic activity from native and irradiated crotoxin, with or without "scavengers", an LD_{50} assay was carried out. The lethal dose used was 0.06 μg/g of animal as described by AIRD et al.[12] And to evaluate the decrease of toxicity, we used 1, 5 or 10 LD_{50}. The animal mortality was observed for 48 hours after injection.

The animals employed were 20 g male outbred mice from IPEN. All animals were maintained in cages with pinewood, with commercial food and water ad libitum.

Results and discussion

In order to evaluate the conformational alterations of proteins after irradiation, the crotoxin and crotamine (toxins from *Crotalus durissus terrificus*) were used as models. Mass spectrometry and solvent mediated fluorescence quenching assays were carried out for crotamine. Crotoxin was submitted to enzymatic activities and toxicity assays.

Figure 1 shows the mass spectro of native and 400 Gy irradiated crotamine. Figure 2 presents the mass spectro of 2,000 and 10,000 Gy irradiated crotamine. These results suggest a mass increase of 2000 and 10,000 Gy.

Figure 3 shows the solvent mediated fluorescence quenching spectra of native as well as 400, 2,000 Gy and 10,000 Gy irradiated crotamine. These data suggest a dose-dependent decrease of relative intensity.

Results from enzymatic activity of native and irradiated (400, 2,000 and 10,000 Gy) crotoxin, with or without "scavengers", are presented in Fig. 4. Our data suggest a decrease of activity with the increase of radiation doses.

Toxic activity data of native and irradiated crotoxin, with or without "scavenger", are presented in Figs 5, 6 and 7. This data showed a decreasing of mortality of animals, suggesting the effectiveness of radiation to abolish the toxicity of toxins.

Analyzes of mass spectrometry of native and 400 Gy irradiated crotamine showed a mass of 4881.2 Da (Fig. 1) On the other hand, the 2,000 and 10,000 Gy irradiated crotamine (Fig. 2) indicate an increase of the complexity of the mass envelopes, which may be ascribed to the oxidation of the toxin. Also, multimeric forms of crotamine were detected in all spectra, corroborating the observations of HAMPE[13] about the spontaneous oligomerization of this peptide. Furthermore, considering the conditions in which the spectra were obtained (low pH and presence of CH_3CN), these oligomers are likely to be linked by covalent bonds.

The solvent mediated fluorescence quenching of native crotamine indicated a dose-dependent exposition of fluorophores in the solvent. These results suggest a gradual alteration of tertiary structure with the dose increase.

The enzymatic activity (Fig. 4) showed a dose-dependent decrease of activity. The native crotoxin, in the presence of "scavenger", did not show significant modifications. When irradiated with 400 Gy, with and without "scavengers", crotoxin had its enzymatic activity diminished. Curiously, with the 2,000 Gy dose, the "scavenger" protected the catalytic activity.

This data suggest that high radiation doses can promote unfolding of protein.

For native and 400 Gy irradiated crotoxin (without any "scavenger" or with t-butanol) the obtained LD_{50} was of 0.06 μg crotoxin/animal. When irradiated with 2,000 or 10,000 Gy, no toxic effects were observed. With 5 LD_{50} (0.30 μg crotoxin/g/animal) (Fig. 4), all the animals died except those that received native crotoxin with sodium nitrate and part of those treated with 400 Gy irradiated crotoxin, without "scavengers" or in the presence of t-butanol. The toxins irradiated with radiation doses higher than 2,000 Gy were not able to induce any toxic effects.

Finally, with 10 LD50 (0.60 μg crotoxin/g/animal) (Fig. 5) all the animals died either with native or 400 Gy irradiated toxin, either without "scavengers" or in the presence of t-butanol. On the other hand, no mortality was observed with 2000 and 10,000 Gy radiation doses. These data confirm the loss of the toxic activity of toxin in agreement with results of BARIDE et al.[14]

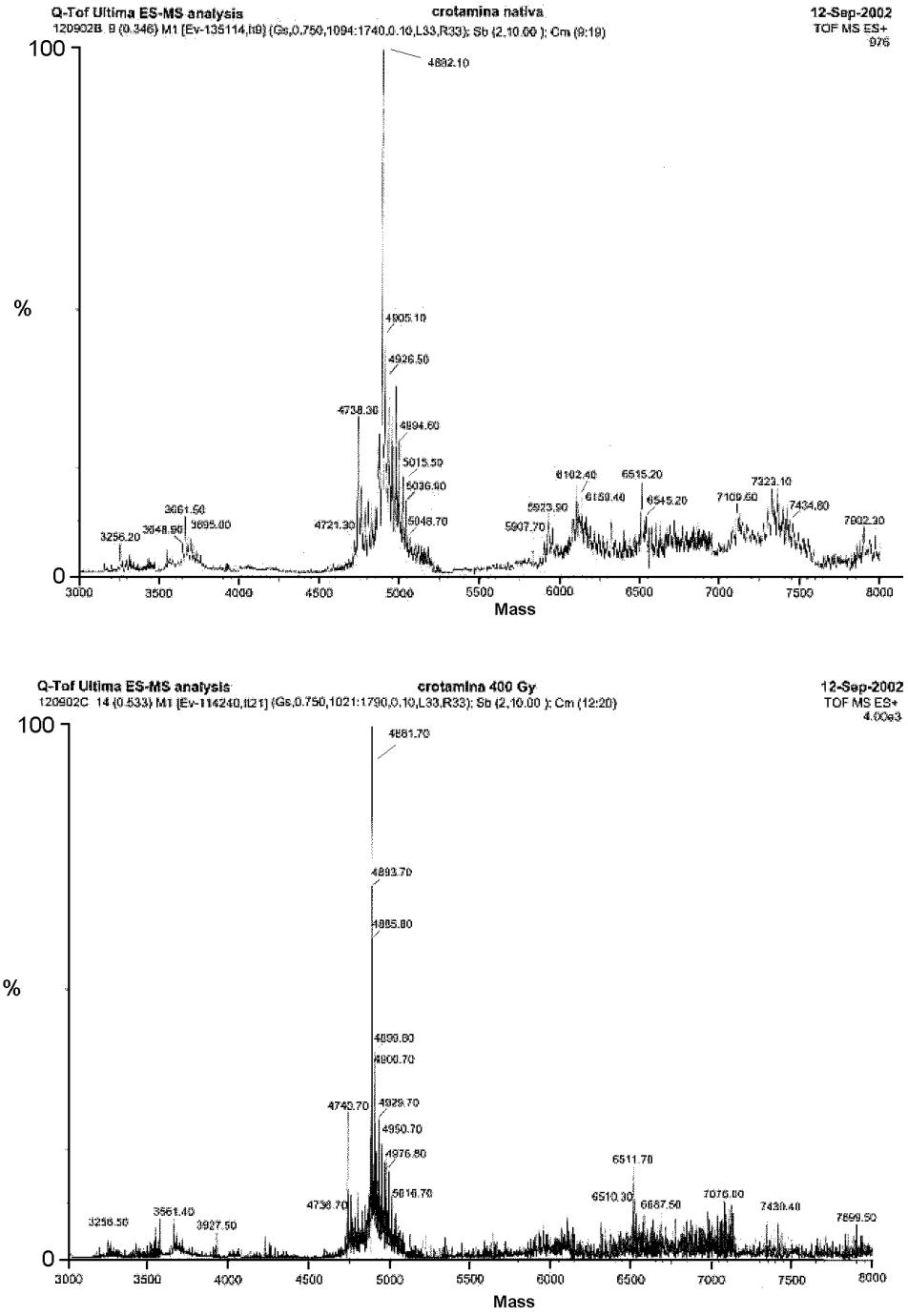

Fig. 1. Mass spectrometry of native crotamine and 400 Gy irradiated crotamine

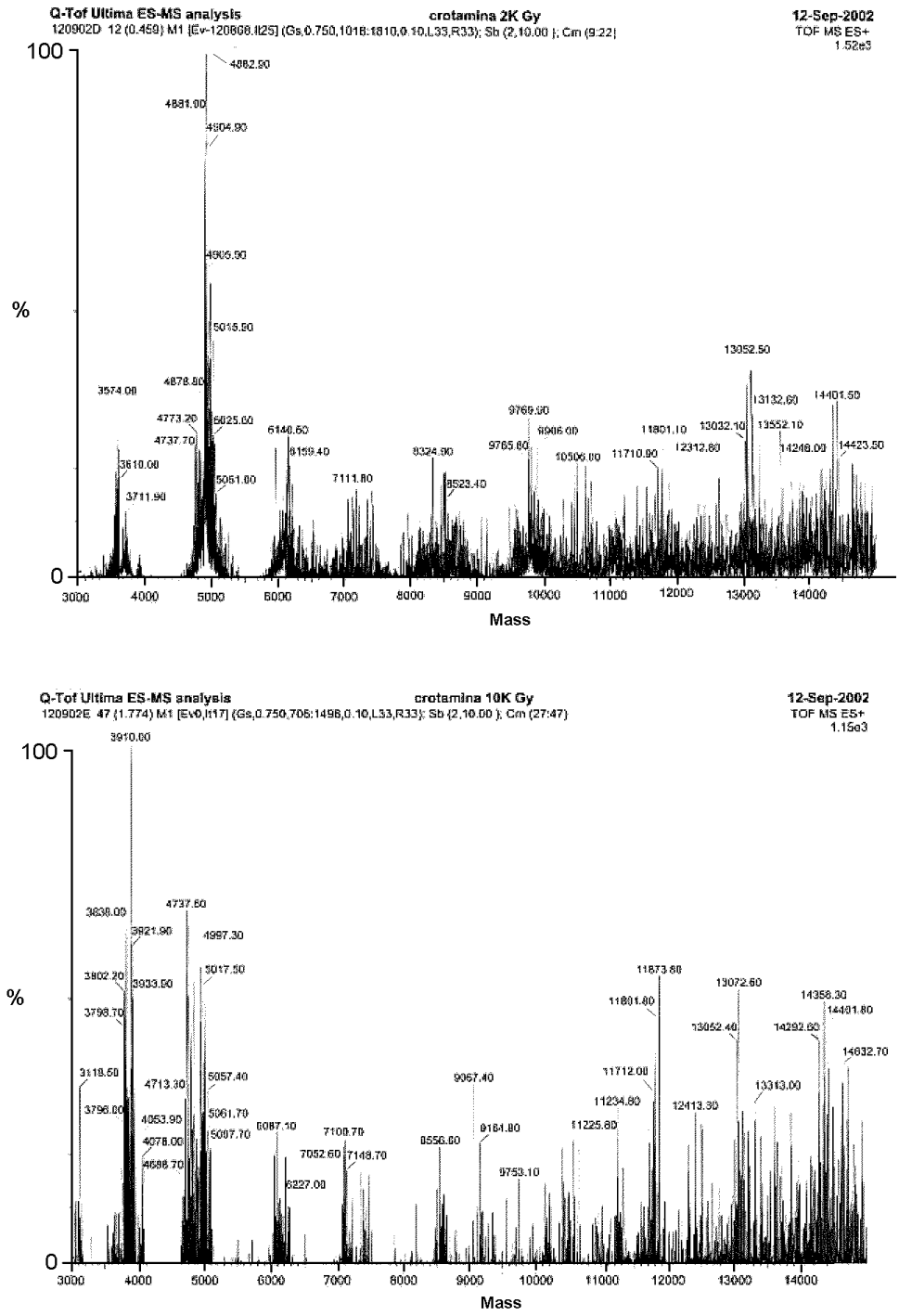

Fig. 2. Mass spectrometry of 2,000 Gy and 10,000 Gy irradiated crotamine

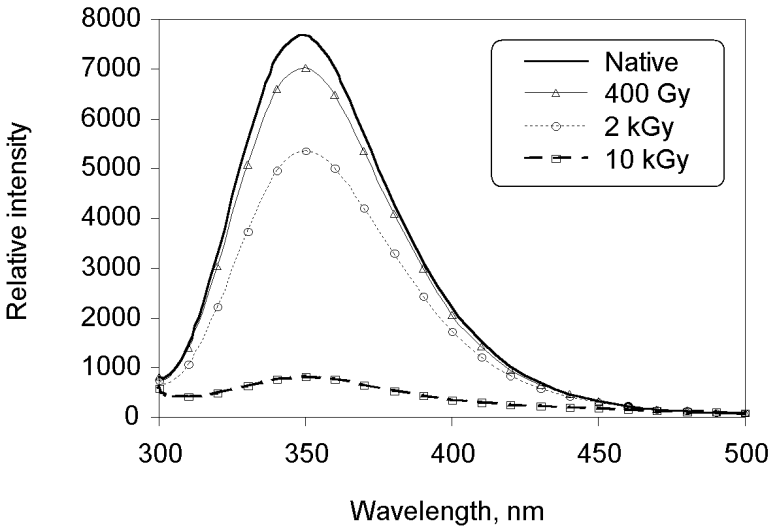

Fig. 3. Analysis of fluorescence quenching of native and irradiated crotamine

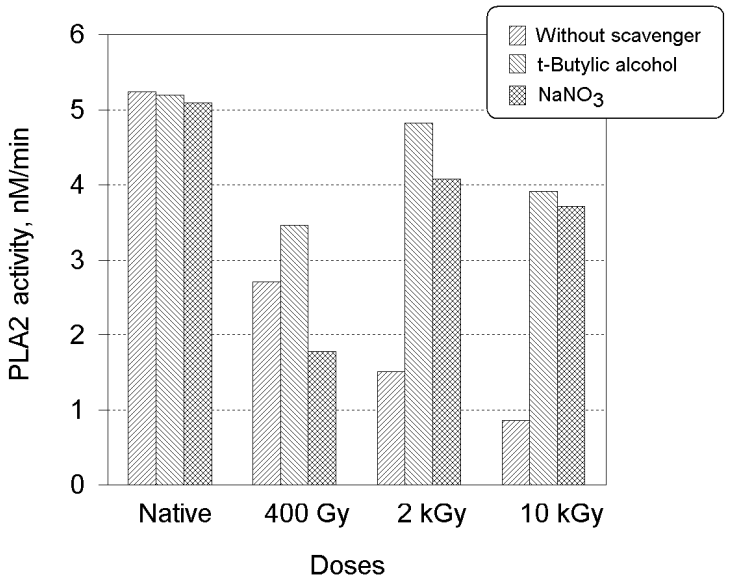

Fig. 4. Enzymatic activities of native and irradiated crotoxin with or without "scavenger"

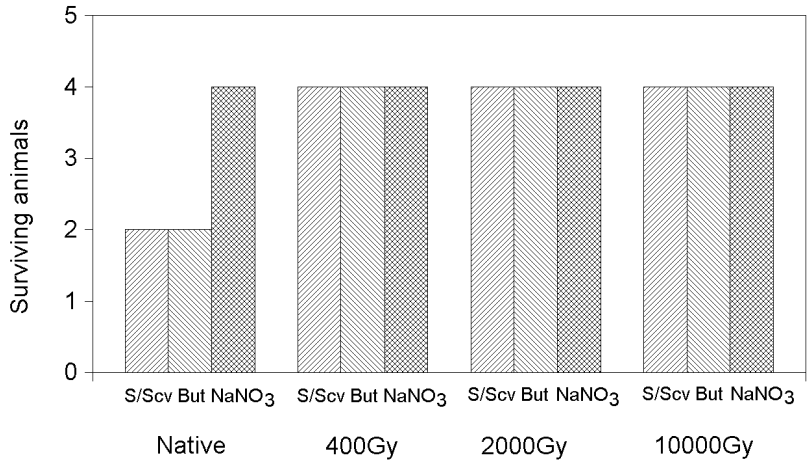

Fig. 5. Toxic activities of native and irradiated crotoxin, with or without "scavenger", 1 LD_{50}

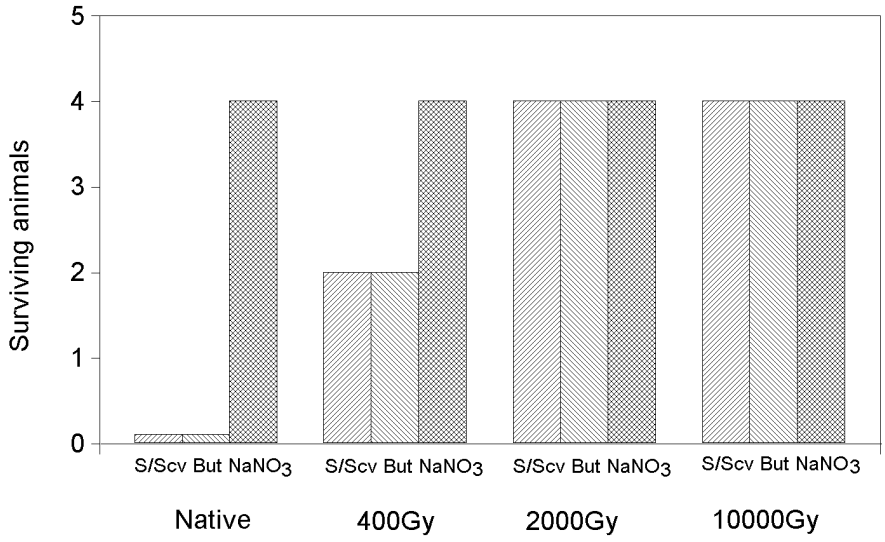

Fig. 6. Toxic activities of native and irradiated crotoxin, with or without "scavenger", 5 LD_{50}

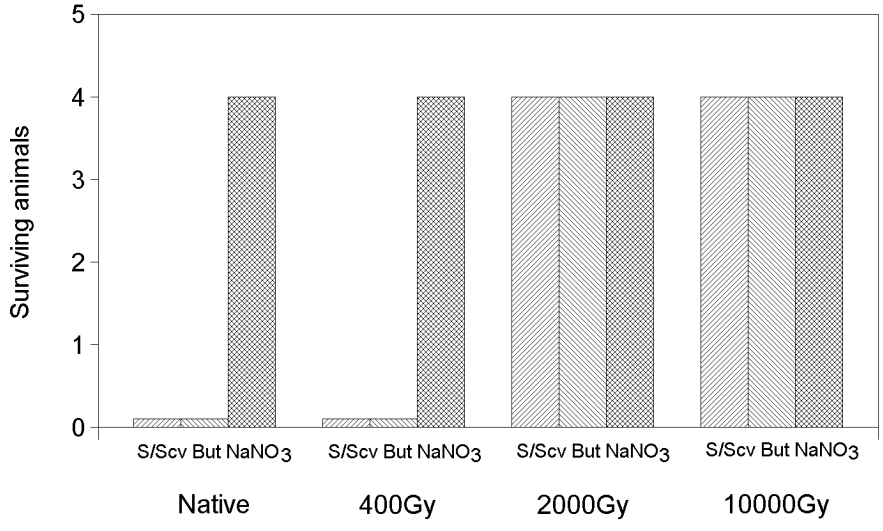

Fig. 7. Toxic activities of native and irradiated crotoxin, with or without "scavenger", 10 LD_{50}

Conclusions

In the present report were verified that gamma-radiation promoted crotoxin and crotamine structural alterations. The major effects observed were aggregation and oxidation of molecules. The "scavengers" demonstrated to be effective to protect the molecules, mainly t-butanol. No mortality was observed when the animals received 2,000 and 10,000 Gy irradiated crotoxin. In conclusion, the gamma-radiation has demonstrated effectiveness in abolishing toxic activity and could be useful to produce toxoids and vaccines.

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