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# CHARACTERIZATION AND EVALUATION OF THE ENZYMATIC ACTIVITY OF TETANUS TOXIN SUBMITTED TO GAMMA RADIATION BY COBALT 60

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## ABSTRACT

Tetanus is a neurological disease which blocks the inhibitory neurotransmitters liberation. Also treatment does not cure the disease, and its main form of prevention is through vaccination. The vaccine is produced by the inactivation of tetanic toxin (TeNT) with formaldehyde, which may cause side effects. An alternative way is the use of ionizing radiation for inactivation of the toxin and also to improve the potential immunogenic response and to reduce the pos-vaccination side effects. Therefore, the aim of this study was to characterize the TeNT structure after different doses of ionizing radiation of Cobalt 60, and also to assess the enzymatic activity after the radiation. SDS PAGE and MALDI-TOF analysis revealed gradual modification on the TeNT structure according to doses increase. Also, fragmentation and possible aggregations of the protein fragments were observed in higher doses. In the analysis of peptide preservation by enzymatic digestion and mass spectrometry, there was a slight modification in the recognition up to the dose of 4 kGy at subsequent doses, recognition was minimal. The analysis of the enzymatic activity by fluorescence showed a 35 % attenuation in the activity even at higher doses. In the antigenic evaluation performed by ELISA and Western Blot, anti-TeNT antibodies were detected against the irradiated toxins at the different doses, with a gradual decrease as the dose increased, but remaining at satisfactory levels, indicating the possibility of possible use as an immunogen, however, studies of enzymatic activity on higher should be further analyzed.

## 1. INTRODUCTION

Clostridium genus are gram-positive, anaerobic bacilli and are distributed worldwide [1]. Among these species is *Clostridium tetani*, a etiologic agent of tetanus [2], which produces the tetanus toxin (TeNT), which is released in the tissue and reached the nerve terminals [3], once there, it prevents the exocytosis of neurotransmitters [4] which is responsible for the onset of the disease [5]. Today, the best wat to prevent tetanus is through vaccination [6].

TeNT is used to produce tetanus vaccine through detoxification with formaldehyde, which promote modifications in the structure without destroying the antigenic sites of the molecule [7]. Although the vaccine is effective, the same process of detoxification is used since 1920 [8] and remains the same until now [9]. However, new technologies for vaccine production have been developed, such as the use of ionizing radiation for poison detoxification and possible vaccine candidates [10], and the inactivation of microorganisms for the use in vaccines [11].

Ionizing radiation consists of high-energy electromagnetic waves [12] that act through direct and indirect mechanisms [13]. Due to the knowledge of these properties, its use has contributed to continuous scientific and technological advances [14]. The ionizing radiation presents itself as a great tool for the production of vaccine antigens, since in addition to diminishing its toxicity, the method also shows the advantage of not adding new molecules to the sample of interest, as might happen in other methodologies such as incubation with formaldehyde [15,16].

## 2. METHODS

All salts and reagents used presents a pro-analytical quality and the deionized water is purified in Milli-Q® system (Millipore®), presenting resistivity of 18.2 mega Ω.

### 2.1. Tetanus toxin

Concentrated tetanus toxin was kindly provided by the Department of Bacteriology - Anaerobic Vaccines Section: Tetanus and Botulinum of the Butantan Institute, by production coordinator Fernanda Lucio dos Santos Macarini. The toxin was stored in its original flask at 4 °C until it was irradiated.

### 2.2. TeNT irradiation

Tetanus toxin aliquots were submitted to 1 kGy, 2 kGy, 3 kGy, 4 kGy, 5 kGy, 6 kGy, 7 kGy and 8 kGy gamma rays from a Gamma Cell of Cobalt 60 (Gammacel® Atomic Energy from Canada, Pinawa, Canada). A sample remained in its native state and remained on the outer side of the source during the time of radiation. The radiation was distributed homogeneously, without shielding, at room temperature and in the presence of O2. After irradiation, the samples were stored in the refrigerator at approximately 4 °C until use.

### 2.3. Characterization of native and irradiated tetanus toxin by polyacrylamide gel electrophoresis in the presence of SDS.

The native and irradiated samples were submitted to electrophoretic mobility analysis using 7.5 % SDS-PAGE in a discontinuous and denaturing system [17] using the Mini-Protean IV system (BIO-RAD®). Five µL of each tetanus toxin aliquot was diluted in 15 µL of reducing and non-reducing sample buffer and heated at 100 °C for 5 minutes. After denaturation, 20 µl of each sample was applied per well to the gel. The electrophoretic run was performed in the presence of running buffer (Tris 0.025M-Glycine 0.192 M pH 8.3). After the run, the gel was incubated in fixative solution and stained in a Coomassie Blue R 250 solution.

## 2.4. Enzymatic digestion with trypsin

The native and irradiated tetanus toxin bands were trimmed, discolored, reduced with dithiothreitol (DTT) and incubated with iodoacetamide. After incubation, the supernatant was discarded and the spots were washed with 25 mM ammonium bicarbonate. The samples were dehydrated with acetonitrile and rehydrated with a solution of 50 mM ammonium bicarbonate containing 100 ng of trypsin. Then, trypsin solution was removed and 50 mM ammonium bicarbonate added to cover each spot and was stored for a period of 18 hours at a temperature of 30 °C. For the peptide's extraction from the bands, a solution of acetonitrile, 5 % trifluoroacetic acid (1: 1) was used, and placed in an ultrasonic bath for 10 minutes. The supernatants were removed and stored until use [18].

## 2.5. Proteomic analysis of peptides of native tetanus toxin and irradiated fractions

The identification of peptides was carried out in collaboration with the laboratory of Biochemistry and Biophysics of the Butantan Institute by Dr. Douglas Mariano under the supervision of Dr. Daniel Carvalho Pimenta. The materials acquired by in-gel digestion were analyzed by liquid chromatography coupled to mass spectrometry (LC-MS) using a binary UFLC system (20A Prominence, Shimadzu Co, Japan) interconnected to Electrospray-Ion Trap mass spectrometer - Time of Flight - ESI-IT-TOF (Shimadzu Co®, Japan). The samples were resuspended in water / 0.1 % acetic acid and analyzed on the C18 (Discovery C18, 5 µm, 50 mm x 2.1 mm) column. MS spectra were collected in the positive mode and collected in the range of 350-1400 m/z (charge mass ratio) and the MS / MS spectra were collected in the 500 m/z - 1950 m/z range.

## 2.6. Mass spectrometry of native and irradiated TeNT samples

Filtered native and irradiated tetanus toxin aliquots were lyophilized and resuspended with 5 µl of 0.1 % trifluoroacetic acid (TFA) solution. Samples (1 µL) were co-crystallized with a matrix solution of sinapinic acid (ACN / water saturated solution / 0.1 % TFA). After drying at room temperature, they were analyzed in a Matrix Associated Laser Desorption Ionization Time of Flight type MALDI-TOF/TOF (Aimed Performance, Shimadzu®) mass spectrometer. The spectra were obtained using the positive linear mode, in a range of masses between 10000 m/z and 200000 m/z.

## 2.7. ELISA antigenicity analysis of native TeNT and irradiated at different doses

High-binding 96-well depolytic plates (Costar® 3590) were sensitized with native and irradiated toxins (1 µg / ml) diluted in 0.1M Sodium Carbonate buffer for 18 hours in a humid chamber at 4 °C. After this period, samples were washed with PBS 0.02 % Tween. Blocking was performed with PBS Tween + 0.3 % skim milk powder (Molico®) for one hour in the stove at 34 °C. After washing, 20 µl of serum from mice immunized with dual vaccine (tetanus and diphtheria toxoid) at a 1/400 dilution and taken in the stove at 34 °C for another hour was added. Then, 20 µl per well of anti-mouse IgG diluted conjugate (1/10000) Peroxidase (SIGMA®) was incubated in the stove at 34 °C. Reaction revealing was carried out by the addition of OPD (orthophenyl diamine) and interrupted after 30 minutes by the addition of 4N HCl. The reading was performed on a Multi-mode Microplate Reader FilterMax F5 Spectrofluorimeter (Molecular Devices®, California, USA) on a 492 nm absorbance filter[19]**.**

### 2.8Analysis of western blot reactivity of native TeNT and irradiated at different doses

SDS-PAGE was performed as described above. After the run, the proteins were transferred to nitrocellulose membrane in Trans-Blot RD semi-dry transfer system (BIO-RAD®), soaked in Towbin transfer buffer. The membranes were blocked by immersion in 5 % PBS Tween Milk solution for 1 hour under shaking. The membrane was then incubated overnight with the mouse serum immunized with the double vaccine at a 1/400 dilution. The antigen-antibody binding was performed by incubation with peroxidase conjugated mouse anti-IgG (SIGMA®) for 1 hour. Revealing was made using the DAB solution (10mg DAB, 10ml PBS, 10µl 30 % H2O2) [20]. Among the steps, the membrane washes were performed with PBS 0.05 % Tween.

### 2.9. Enzymatic activity analysis

The enzymatic activity was performed according to the protocol described by Perpetuo et al. [21]. A total of 167.52 µl / well of 150 mM Tris-NaCl buffer pH 7.5 was added to a black microplate (Costar®). Then, 27.48 µg/well of native TeNT and irradiated were added as follows: The first batch was performed with the native toxin and the irradiated fractions of 1 kGy to 3 kGy and the second batch with the native batch corresponding to and with proteins irradiated from 5 kGy to 8 kGy. And, finally, 5 mM/well of the FRET substrate (Fluorescence Resonance Energy Transfer) was added. Fluorescence values were measured every 30 seconds during 5 minutes, fluorescence was determined at 320 nm excitation and 420 nm emission. The FRET substrate was kindly provided by Dr. Ivo Lebrun of the Biochemistry and Biophysics laboratory of the Butantan Institute.

### 2.10. Statistics analysis

Student's t-tests for antigenicity analysis and linear regression analysis for the analysis of residual enzyme activity were performed in GraphPad Prism 6.0 statistical package.

## 3. RESULTS

### 3.1. Characterization of the electrophoretic profile of the native and irradiated TeNT

The analysis of the proteins not treated with reducing agent revealed a heterogeneous profile, with higher number of high molecular weight proteins and greater evidence in the 100 kDa and 150 kDa bands (Figure 1A). Comparing the fractions of proteins irradiated with the proteins in their native state, we observed a discrete change in the intensity of the bands up to the dose of 4 kGy. At doses of 5 KGy to 8 kGy, we noticed a smear and disappearance of the bands up to the weight of 100 kDa. From the molecular mass of 75 kDa, we observed profiles of poorly defined bands and the presence of a band of low molecular weight below 37 kDa, persisting up to the dose of 8 kGy. No formation of aggregates weighing above 150 kDa was observed (Figure 1).

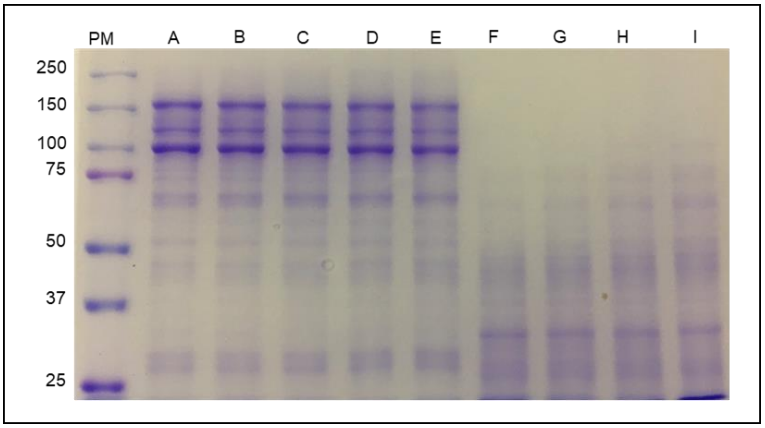


**Figure 1: Electrophoretic analysis in the absence of reducing agent of native and irradiated TeNT: (MW) molecular weight; (A) native; (B) 1 kGy; (C) 2 kGy; (D) 3 kGy; (E) 4 kGy; (F) 5 kGy; (G) 6 kGy, (H) 7 kGy and (I) 8 kGy.**

In the electrophoretic profile obtained under reducing conditions, we observed a change in the alignment of the proteins when compared to the samples without the use of reducing agent. In this profile, the tetanus toxin corresponds to the 100 kDa bands (heavy chain) and another 50 kDa band (light chain) (Figure 2).

In the aliquots of the proteins submitted to radiation, we noticed a gradual modification in the profiles as the dose increased, however, the bands remained defined up to the dose of 8 kGy. When comparing aliquots of irradiated and native proteins, the dose of 1 kGy showed no significant changes. Between the doses of 2 kGy to 4 kGy, the profile remained similar. From the 5 kGy dose, the bands are less expressive and the appearance of bands of lower molecular mass not observed in previous doses. Between the doses of 7 kGy and 8 kGy, there was a widening of the band of 100 kDa. There is no evidence of aggregate formation of molecular weight above 100 kDa (Figure 2).

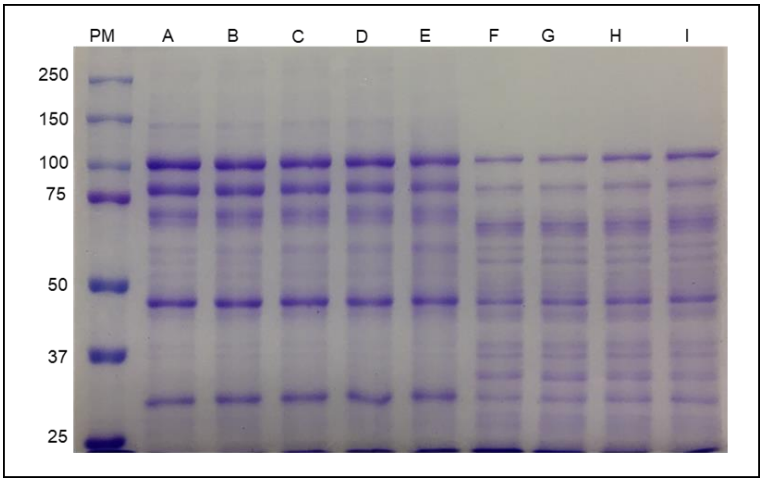


**Figure 2: Electrophoretic analysis in the presence of reducing agent, native and irradiated TeNT: (MW) molecular weight; (A) native; (B) 1 kGy; (C) 2 kGy; (D) 3 kGy; (E) 4 kGy; (F) 5 kGy; (G) 6 kGy, (H) 7 kGy and (I) 8 kGy.**

### 3.2. Identification of TeNT peptides by gel digestion and mass spectrometry

TeNT was identified in all samples, but recognition of the number of peptides varied as the dose increased. A pattern was observed in the number of peptides identified in the native sample, 1 kGy and 3 kGy, similarity was also observed at doses of 2 kGy and 4 kGy. From the 5 kGy dose the number of peptides identified decreased significantly without disparity up to the 8 kGy dose (Figure 3).

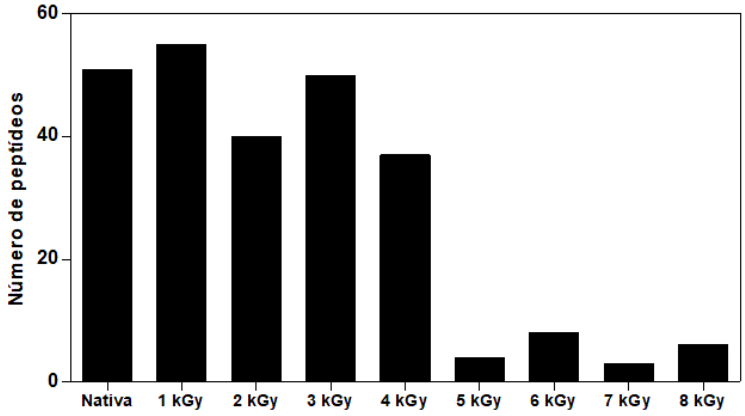


**Figure 3: Identification of native TeNt peptides and irradiated by ESI-IT-TOF mass spectrometry.**

### 3.3. Structural analysis of native and irradiated protein samples by mass spectrometry - MALDI-TOF

In the native toxin spectrum, two main mass loading ratios were found: 175261 m/z and 153846.6 m/z (Figure 4A). In the spectra of the irradiated proteins, the major peaks were not observed. Smaller molecular weight fragments formed gradually up to the 4 kGy dose (Figure 4A, 4B, 4C, 4D and 4E). More significant changes occurred from the dose of 5 kGy in which the presence of greater number of peaks was observed. Peaks of high masses greater than 170000 m/z were also noted from this dose (Figure 4F, 4G, 4H and 4I).

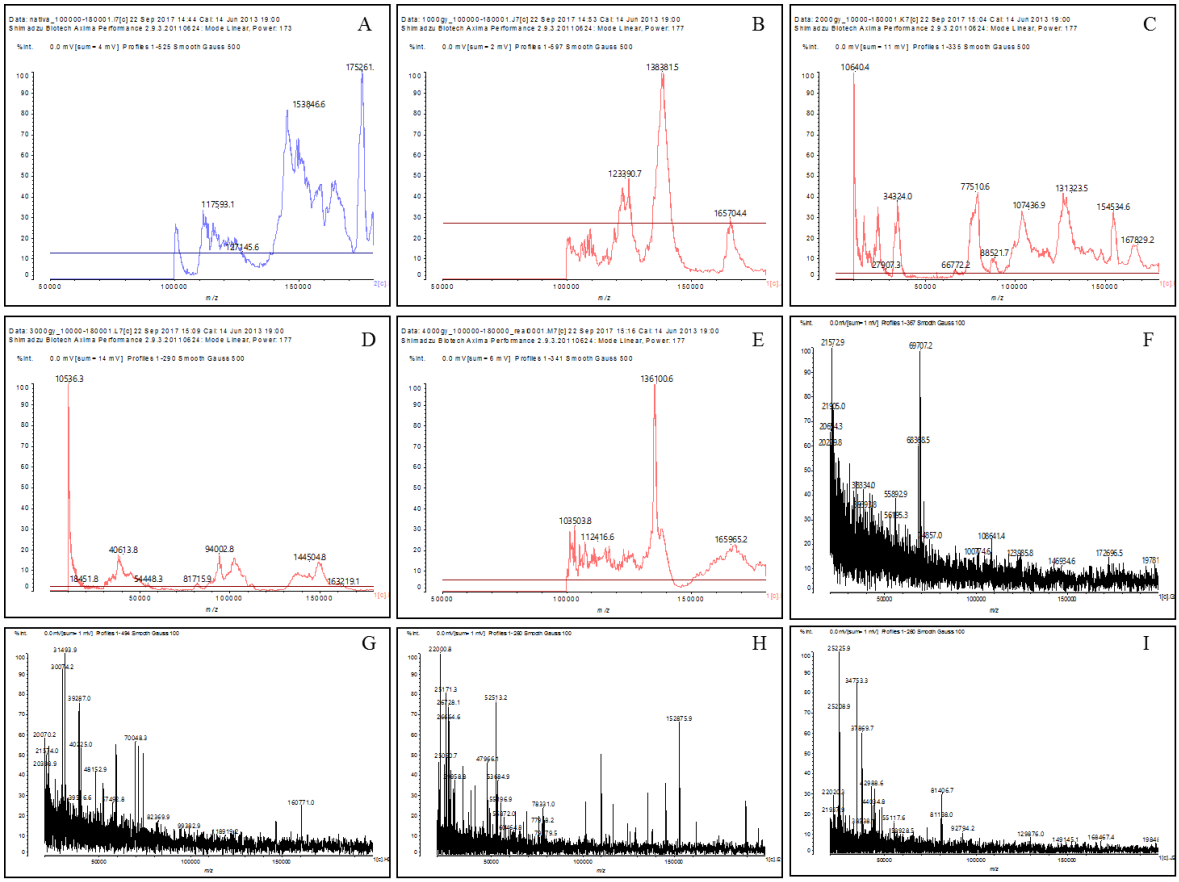


**Figure 4: Spectrum of native TeNT and irradiated fractions. (A) native; (B) 1 kGy; (C) 2 kGy; (D) 3 kGy; (E) 4 kGy; (F) 5 kGy; (G) 6 kGy; (H) 7 kGy; (I) 8 kGy.**

### 3.4. Evaluation of the recognition of native or irradiated TeNT at different doses by antibodies of C57B1 / 6j mice immunized with dual vaccine by ELISA and Western Blot

In the antigenicity analysis, IgG antibodies from C57B1/6j mice immunized with the double dose vaccine that the toxin was subjected were recognized. When comparing the antigenicity of the native toxin regarding to the irradiated toxins, we observed a significant difference of all the doses. Among the irradiated samples, there was a gradual reduction in the recognition of the antibodies by the antigen. In the antigens irradiated at 1 kGy, 2 kGy and 4 KGy, the significant difference was similar, followed by a greater loss of recognition in 3 KGy, and in the samples from 5 kGy to 8 kGy (Figure 5).

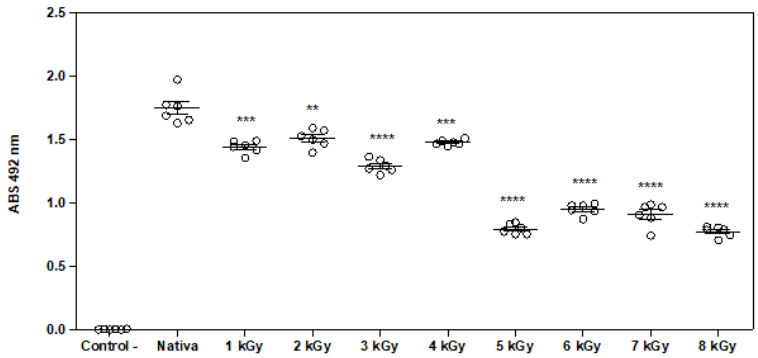


**Figure 5: Evaluation of the antigenicity, by immunoenzymatic assay, of the native TeNT and irradiated at different doses.**

Western blot analysis of the bands corresponding to the heavy chain (100kD) and the light chain (50kDa) of the TeNT demonstrated that there was recognition of the two chains, showing that there are antigenic epitopes in the two polypeptides (Figure 6A). In the fractions of the irradiated samples, the antibodies were recognized in all profiles, as the dose increased, the recognition decreased gradually, with the 8 kGy band demonstrating lower reactivity (Figure 6).

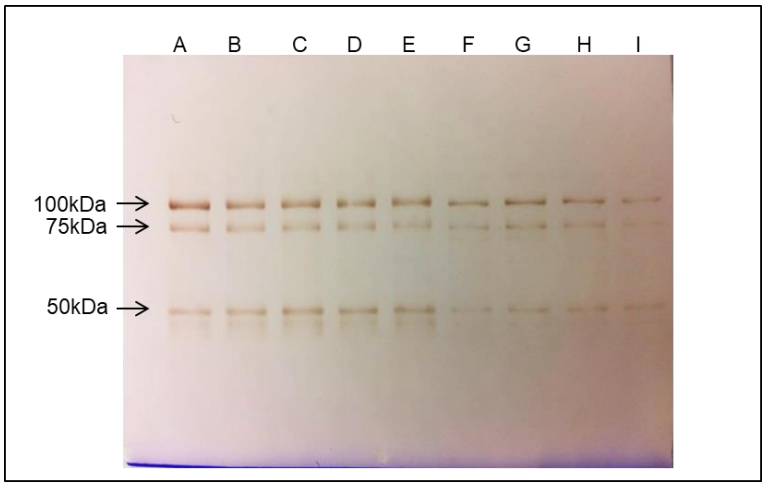


**Figure 6: Antigenicity of native and irradiated TeNT at different doses by anti-TeNT antibodies: (MW) molecular weight; (A) native; (B) 1 kGy; (C) 2 kGy; (D) 3 kGy; (E) 4 kGy; (F) 5 kGy; (G) 6 kGy; (H) 7 kGy; (I) and 8 kGy**

### 3.5. Enzymatic activity of the native and irradiated tetanus toxin on the FRET substrate

There was a reduction of the enzymatic activity as the dose increased at batch 1, the largest loss of activity of this lot was of 37.2 % related to the toxin irradiated to 3 kGy. In the second batch, we also observed the attenuation of the enzymatic activity as the radiation dose increased. The aliquot of toxin irradiated at 4 kGy remained with residual enzyme activity of 94.32 % and the sample irradiated at 8 kGy preserved 65 % of activity (Table 1).

**Table 1: Enzymatic activity of the native and irradiated tetanus toxin at different doses**

| Batch 1 | | Batch 2 | |
|---|---|---|---|
| **Sample** | **Enzymatic Activity (%)** | **Sample** | **Enzymatic Activity (%)** |
| Nativa | 100 | Native 2 | 100 |
| 1 kGy | 94,4 | 4 kGy | 94,32 |
| 2 kGy | 97,2 | 5 kGy | 81,7 |
| 3 kGy | 62,8 | 6 kGy | 75,66 |
| | | 7 kGy | 68,22 |
| | | 8 kGy | 65,0 |

## 4. DISCUSSION AND CONCLUSIONS

The characterization of the irradiated samples showed that the ionizing radiation caused a change in the molecular mass of the toxin according to the increase of the radiation dose, and from the dose of 5 kGy this modification was more representative. When considering the

effects produced in the proteins up to the dose of 5 kGy, previous works without the use of reducing agent present results similar to ours, however, in smaller doses. In the findings of Clissa et al. [22], the components with higher molecular weight of the venom of *Crotalus durissus terrificus* were destroyed after radiation doses of 2, 3 and 5 kGy. This work was carried out with crude venus irradiated in solution of five species of the genus Echis and Bitis and six species of the genus Naja and Dendroaspis, showed different profiles regarding the use of the reductant, but in both groups, aggregation was observed in doses higher than 4.5 kGy [23]. On the other hand, Caproni et al. [24] showed that the radiation promoted structural alterations characterized by proteins of higher molecular weight, but did not show dissociation of the subunits even in the presence of reducing agent, this finding suggests that the radiation resulted in the formation of resistant intermolecular bonds to the agent. In our experiment, changes in molecular mass such as aggregate formation and possible cross-linking at higher doses are consistent with the results demonstrated by other authors, such as those found in papain and [25] β-Galactoglobulin [26]. However, we did not observe if this change occurred, since the authors report such observation on products irradiated from 10 kGy.

MALDI-TOF mass spectrometry confirmed the fragments observed in the gel were due to the use of radiation in all fractions of proteins exposed at different doses. In general, the literature shows similar observations to our MALDI-TOF analyzes. Studies with crotamine demonstrated an increase in the mass at doses of 2 kGy and 10 kGy that can be attributed to poison oxidation [27]. They also observed, through this analysis, the formation of smaller fragments in the irradiated sample in comparison to the native protein.

Ionizing radiation has been studied over the years for causing changes in the structure of proteins and for contributing to decrease the toxicity of molecules [28–30]. This conformational change and fragmentation occur by degradation and other actions caused by the direct and indirect action of ionizing radiation, where direct effects of radiation are caused when gamma rays and high energy electrons interact directly with molecules. On the other hand, the greatest damage can be indirectly caused by the ionization of water radiolysis, free radicals that interact with amino acids through addition or reduction of the ions by oxidation [31]. After irradiation of molecules in solution, the produced free radicals react very efficiently with the proteins, favoring modifications such as dimerization and fragmentation [32]. After generation of the free radicals, the amino acids exposed to solvents are more likely to be oxidized by hydroxyl radical [23]. Thus, the biological effects of ionizing radiation on proteins result in amino acid oxidation, oxidative cleavage of the protein backbone, and modification of the amino acid side chains [33].

In all doses analyzed, it was possible to observe the maintenance of the antigenic epitopes of the TeNT, with recognition by anti-TeNT IgG immunoglobulins. Despite the structural changes caused by the radiation process, it was possible to observe the recognition of anti-TeNT antibodies against the irradiated toxins at different doses, with a gradual decrease in proportion to the increase in dose, but remaining with satisfactory levels up to the highest dose. Test of antigenicity of the formaldehyde-treated tetanus toxin for its detoxification was also performed by Metz et al. [34] and showed similar results. This may have occurred due to structural modifications in specific epitopes in the toxin molecule. Similar results were also found in crotoxin [35] and total protein extract of *Toxoplasma gondii* [36] in which these antigens show antigenic and immunological properties conserved after the radiation process.

The enzyme activity test demonstrated attenuation of tetanus toxin activity as the radiation dose increased, except for the 3 kGy dose which had the lowest residual activity. Probably, the decrease in activity in this aliquot was contributed by environmental factors such as the degradation of the sample along with the absorbed radiation dose. The results obtained from this analysis are in agreement with previous studies demonstrating the attenuation of the enzymatic activity by radiation, for example the loss of 52 % of the enzymatic activity of the total venom of the snake Echis coloratus irradiated at 3 kGy [37].

Although gamma radiation has caused the same effects in these experiments, the radiation dose used is lower than ours, showing some resistance by the tetanic toxin to the radiation. Such resistance may have been provided by some type of antioxidant molecule present in the culture medium Sample. The IB culture medium produced by the Butantan Institute, in addition to other molecules, is composed of B vitamins [38] , which are evidenced in the literature as potent antioxidants [39, 40]. Another fact that may have contributed to the resistance of the toxin to radiation is the presence of other proteins, conferring mutual protection and competition for the radicals formed by the indirect action of radiation. On the other hand, enterotoxin study of enteric *Salmonella* var Typhimurium demonstrated inactivation from the dose of 25 kGy, but remained with a slight enterotoxicity at the dose of 10 kGy, which suggests that some proteins may need higher doses for the attenuation of their activity [41].

The data produced in this work showed that the irradiation of tetanus toxin by gamma rays of Cobalt 60, at different doses, changed the molecular structure as the dose increased, maintaining its antigenic capacity, but not presenting satisfactory efficiency in the loss of the enzymatic activity. Although the tetanus toxin remained with enzymatic activity above 50 %, the data suggest the possibility of inactivation at higher doses and the use of ionizing radiation as an alternative method of detoxification of the tetanus toxin for use as an immunogen. The use of ionizing radiation will also contribute to the improvement of the production process, optimizing the incubation time for detoxification, reduction of the chemical residues resulting from the process and the possibility of developing a vaccine without the need for adjuvant.

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