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INFLUENCE OF RADIOLYSIS PRODUCTS OF WATER IN THE CYTOTOXICITY ASSAY

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ABSTRACT

The ionizing radiation has shown to be an useful tool in the improvement of immunogens such as toxins; venoms; protozoa among others. A recent study has demonstrated that hydroxyl radical is the main radiolysis product of water involved in the protein irradiation. It promotes important structural modifications in the toxins meanwhile the immunogenic properties are preserved. Tests of biological activity are essential to determine the detoxification of the samples and the safety of the inoculums. The *in vitro* cytotoxicity assay, determining the cellular viability, was chosen for this analysis. This test has good reproducibility; it can be carried out with little amount of sample and has been useful for biological evaluation of biomaterials. Herein are presented the results gotten with gyroxin (a serine protease, glycoprotein with 26 kDa) isolated from the rattlesnake *Crotalus durissus terrificus* venom. The toxin was irradiated with 2 kGy of gamma ray of cobalt-60 in saline solution. The evaluation of the biological activity was carried out with different cell lines, using MTS dye or ^{3}H-thymidine incorporation as indicators of cells viability. The best correlation between the cellular viability assay and lethality assay *in vivo* was obtained with endothelial cells (HUVECs) and ^{3}H-thymidine incorporation. The MTS evaluation presented a false-positive result of cytotoxicity, probably due to the reaction with free radicals formed by radiolysis of water during the irradiation process.

1. INTRODUCTION

The ionizing radiation interacts with the substances transferring energy in direct or indirect form, and producing chemical reactions that can modify the molecular properties. The dose of 2kGy in aqueous solution has shown to be the ideal dose for the attainment of attenuated venoms or isolated toxins [1,2]. The indirect effect prevails in the cited conditions and oxidative reactions with proteins molecules and free radicals (OH*, H*, e-aq., O^{*}_{2}, H_2O_2 and HO^{*}_{2}) are frequent [3].

The cytotoxicity assays are, among the *in vitro* methods, the first choice to analyze the toxicity of substances in several tissues. It is one of praised assays as referred by ISO10993 for toxicological test of biomaterials destined to medical applications. The high sensitivity of the cytotoxicity test is decurrent of the cells isolation in culture and the absence of immunological mechanisms of the body of *in vivo* test [4].

The present study aims to check the accuracy of the cytotoxicity assay with native and irradiated toxins. Specifically, this work compares the response of two cellular viability indicators (MTS dye and ^{3}H-thymidine incorporation) and two cells lines (HUVEC and CHO-K1) for *in vitro* evaluation of the biological activities of the native and irradiated gyroxin.
Gyroxin is a glycosilated multi-functional serine protease present in rattlesnake venom (*Crotalus durissus terrificus*) with molecular weigh 26585 Da.

2. MATERIALS AND METHODS

2.1. Isolation of the gyroxin

The gyroxin was isolated from the crude venom using gel filtration and affinity chromatographies [5]. The venom of *Crotalus durissus terrificus* was gently granted by Institute Butantan.

2.2. Irradiation.

The purified toxin was dissolved in sodium chloride solution 150 mM, 1 mg/mL and filtered in 0,2 μM. This solution was irradiated with 2 kGy of gamma rays from ^{60}Co (GammaCell 220; CTR-IPEN) and the dose rate was 3,75 kGy/h.

2.3. Cytotoxicity assay.

The cells line CHO.K1 and HUVEC were purchased from ATCC.

2.3.1. Cell culture.

For the culture cells CHO-K1 was used RPMI 1640 medium supplemented with 10% of bovine fetal serum (SFB), 1% of antibiotics (penicillin 100 UI, streptomycin 10 μg/mL, fungizona 0,25 μg/mL)and 1% of L-glutamine. For HUVEC cells, Hams F-12K medium was used supplemented with L-glutamine 2 mM, 1% of antibiotics (penicillin 100 UI/mL, streptomycin 100 μg/mL and anfomicine 0,025 μg/mL); heparin 45 ug/mL, 2-mercaptoethanol 50 μg/mL and mouse brain extract.

All the cells cultures were maintained at 37^{o}C in a humidified 5%CO2 atmosphere.

2.3.2. Colorimetric method

The procedure is a colorimetric method which uses a tetrazolium compound MTS (Promega) for determining the number of viable cells in proliferation. A suspension of CHO-K1 with 3.000 cells /50μL was pipetted in the 96 wells microplates and incubated for 72 hours at 37^{o}C in a humidified 5% CO_2 atmosphere. The native and irradiated gyroxin was diluted (1:2) in RPMI medium without SFB and were assayed in the interval from 25 to 0,05 μM. The test was compared with a negative control without addition of the toxin and a positive control of phenol 0.3% in saline 0.9% solution. The blank was made without cells or toxin. The cellular viability was measured by adding 20μL of MTS/PMS (20:1) solution and incubated for 2 hours at 37^{o}C in the humidified 5% CO_2 incubator. The microplates were read in a spectrophotometer reader at 495 nm.

The same procedure was carried out with HUVEC cells using about 4.000 cells/well.
The effective concentration 50% based on the cellular viability (EC_{50}) was determined with nonlinear regression-sigmoidal dose-response, using GraphPad Prism software.

2.3.3. Radioactive method.

The same procedure described above was used for the ^{3}H-thymidine incorporation assay (0,074 MBq/well). However, in this in case, ^{3}H-thymidine were added at the beginning of the experiment.

After 72 hours, the incubation was interrupted placing the culture plates at -80ºC, until the measurement of radioactivity. For this, it was filtered (Filtermat of the Wallac mark), washed with buffer two times, the filter was transferred to a plastic bag with 9 mL of scintilation liquid (Fision Chemicals); sealed and counted.

3. RESULTS

COHEN & QUISTAD, 1998 [6] and OLIVEIRA et al., 2002 [7] studying crude venoms demonstrated a positive correlation between the lethal dose (LD50), determined by the classic *in vivo* assay [8] and the effective dose (EC50) gotten in cytotoxicity test. However, our results showed to be different; in the assays that compare native and irradiated gyroxin this equivalence was not observed.

The results are presented in Figure 1 and in Table 1.

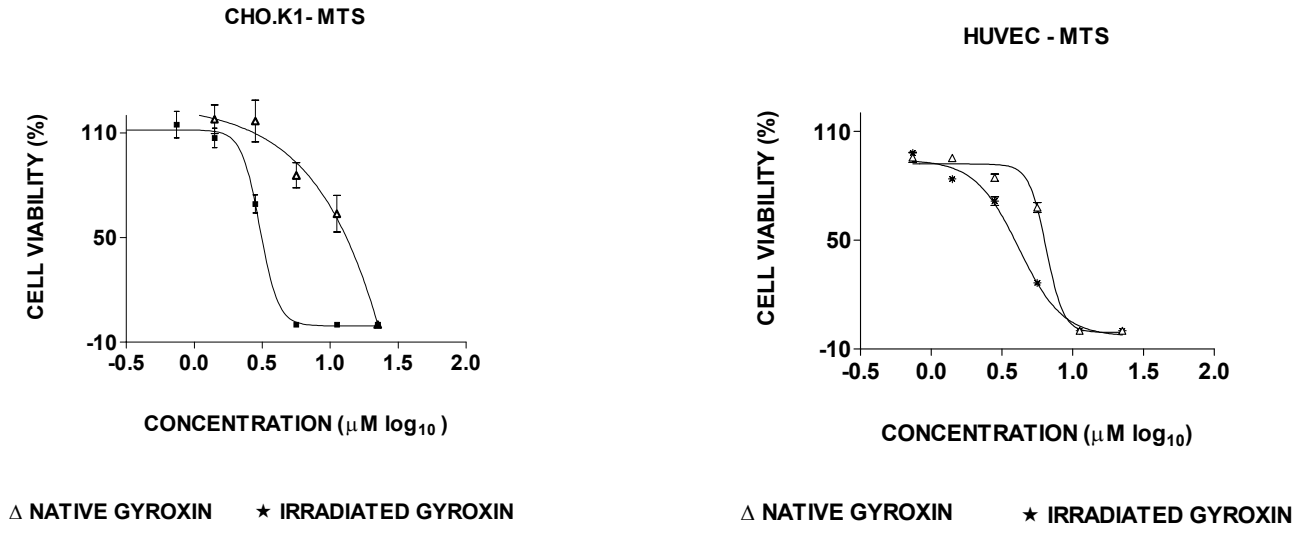

Figure 1. Curves of the cytotoxicity assay using MTS dye as cellular viability indicator. The assay was developed with CHO.K1 or HUVEC cells and several concentrations of native or irradiated gyroxin. The data were adjusted with nonlinear regression.

The initial results of EC_{50} with the native and irradiated gyroxin with CHO-K1 cells and MTS dye, suggest that the irradiated one is more cytotoxic.
This was an unexpected result, since the opposite was gotten with the *in vivo* assays as observed in Table 2. It was seen that the LD50 are always bigger after the irradiation, proving the reduction of toxicity of the total venom and isolated toxins.

Comparing Table 1 and 2, there is a lack of correlation between the methods *in vivo* and *in vitro*. This observation can be associated with false positive results of the cytotoxicity assay. Thus, two possibilities of interferences had been suggested:

- The first possibility was that the CHO-K1 cell line was inadequate for the assay with this toxin because these cells are not the target of gyroxin action. HASHIZUME & CAMILLO (2005) [10] had demonstrated the action of the gyroxin in endothelial cells in culture, so HUVEC cells are more appropriate for this assay.

- The second possibility was that free radicals and/or products of radiolysis of the water react with the MTS dye. The formed free radicals in the irradiation of the toxin in solution can "attack" aromatic rings of MTS (^{3}H-thymidine does not had the same reactivity with the products of radiolysis of the water).

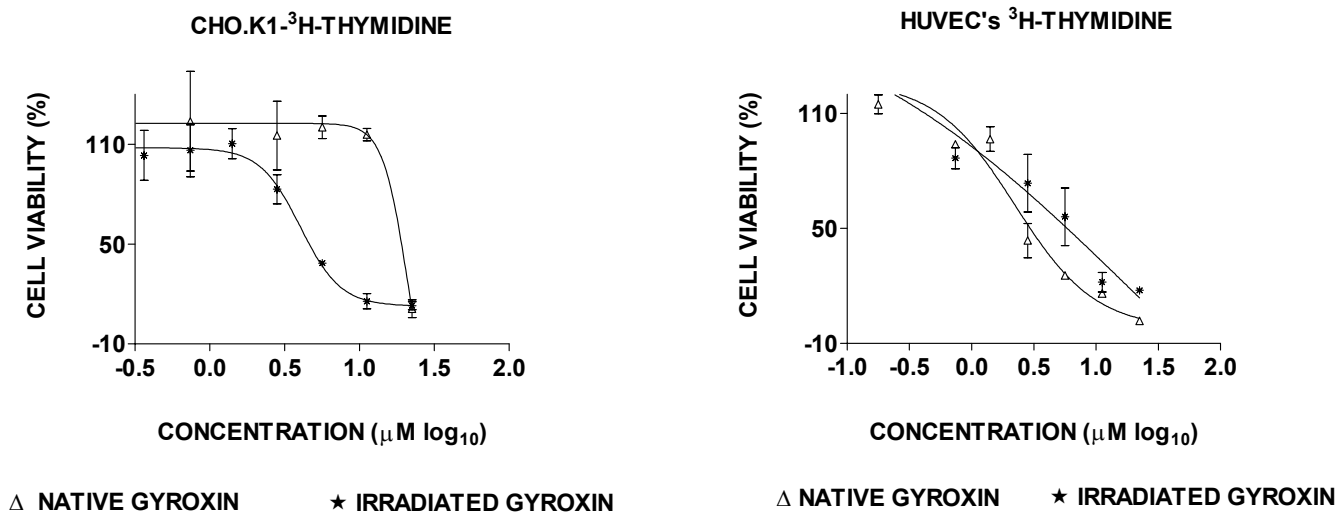

Figure 2. Curves of the cytotoxicity assay using 3H-thymidine as cellular viability indicator. The assay was developed with CHO.K1 or HUVEC cells and several concentrations of native or irradiated gyroxin. The data were adjusted with nonlinear regression.

Table 1. Values of EC_{50} gotten in the tests of cytotoxicity assay for gyroxin native and irradiated with 2.000 Gy of gamma rays of cobalto-60.

GYROXIN				
	EC_{50} NATIVE	*EC_{50} IRRADIATED*	*EC_{50} NATIVE*	*EC_{50} IRRADIATED*
	MTS	*MTS*	*^{3}H-THYMIDINE*	*^{3}H-THYMIDINE*
HUVEC	6,50 ± 0,03 μM	4,17± 0,02 μM	2,23 ± 0,12μM	18,96 ± 3,14 μM
CHO-K1	321,36±11,39μM	3,04± 0,02 μM	20,99 ± 1,71μM	4,03 ± 0,07 μM

Table 2. Values of DL_{50} gotten in the *in vivo* assay for crude venom and toxins native and irradiated with 2.000 Gy of gamma rays of cobalto-60.

	DL_{50} NATIVE	*DL_{50} IRRADIATED*	*REFERENCES*
CRUDE VENOM	0,15 (μg/g)	0,40 (μg/g)	[1]
CROTOXIN	0,06 (μg/g)	0,11 (μg/g)	[9]
CROTAMINE	5,98 (μg/g)	11,55 (μg/g)	[2]

In the comparison of native and irradiated toxins HUVECs with ^{3}H-thymidine incorporation assay, had presented the lesser EC_{50} for native gyroxin, showing that the native toxin is more cytotoxic. Also, this result corroborates the data of the *in vivo* assays; showing that in this condition the *in vitro* assay reproduced the relation described *in vivo*.

4. CONCLUSIONS

Concluding, the adequate conditions of the cytotoxicity test to compare activity of native and irradiated gyroxin is the use of endothelial cells and ^{3}H-thymidine incorporation as indicator of cellular viability. This conclusion could be extended for others irradiated toxins and is an important exception to the use of the cytotoxicity assay with MTS dye.

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