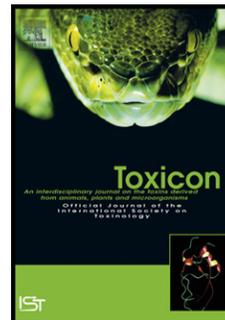


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Title:

Tityus zulianus venom induces massive catecholamine release from PC12 cells and in a mouse envenomation model

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Running title: Massive autonomic stimulation by Venezuelan scorpion venoms.

Key words: Epinephrine, catecholamine release, *Tityus zulianus*, *Tityus discrepans*, scorpion venoms, PC12 cells, Dopamine release.

Abbreviations: BSA: Bovine Serum Albumin, $[Ca^{2+}]_i$: Intracellular Ca^{2+} concentration, CC: Carbamylcholine, COMT, Catechol-Orto-Methyl Transferase, Epi: Epinephrine, IpTxA: Imperatoxin A, RyRs: Ryanodine receptors, SDS: Sodium Dodecyl Sulphate, TD: *Tityus discrepans*, TTX: Tetrodotoxin, TZ: *Tityus zulianus*, Tz1: main β -toxin from *Tityus zulianus*, nAChR: nicotinic acetylcholine receptor (Na^+ channel), (Nav): Voltage-dependent sodium channel, NE: Nor-Epinephrine, MAO: Mono Amino-Oxidase, MCa: Maurocalcine, MHPG: 3-Methoxy-4-Hydroxy-Phenyl-Glycol.

1.- Introduction.

Scorpion envenomation is a public health problem in tropical countries including Venezuela. The most serious clinical cases are produced by species belonging to the genus *Tityus* (Buthidae), particularly *Tityus discrepans* (TD, north-central range) and *Tityus zulianus* (TZ, western range), both species being responsible for a high percentage of the accidents. Recently, a molecular phylogeography approach, using mitochondrial sequences derived from cytochrome oxidase subunit I and 16S rRNA genes from most Venezuelan *Tityus* species, allowed to establish that TD and TZ cluster

in different mitochondrial DNA haplotypes, with TD grouping with northcentral species whereas TZ clustered with species prevalent in the western Andean range (Borges et al., 2010a). Such strong biogeographic structuring of *Tityus* species in Venezuela underlies the existence of structural and functional differences amongst the toxins produced by distinctive species in the country. Thus, combining phylogenetic, clinical as well as immunological cross-reactivity data, Borges et al. (2010b) suggested a partitioning of the Venezuelan territory into “toxinological provinces” wherein TD and TZ belong to distinct groups.

Notably, scorpion accidents involving TZ are associated with high mortality rate at children under eight years of age (Borges et al., 2002). TZ-mediated cardiopulmonary clinical disorders, as respiratory arrest and death by pulmonary edema, have been associated with high levels of plasma catecholamines (Mazzei de Davila et al., 2002). This last research group described a significant increase in nor-epinephrine (NE) plasma levels determined by HPLC, in TZ-envenomed patients. These authors also claimed that catecholamine plasma levels correlated positively with the severity of cardiopulmonary disorders.

Borges et al. (2004a) have reported that TZ venom induce intense pancreatic alterations related to an acute pancreatitis in TZ venom-injected mice. Also, Borges et al. (2004b) isolated, cloned and characterized a novel β -toxin, Tz1, from TZ venom. Leipold et al. (2006) have shown that Tz1 specifically modify the gating properties of the voltage-dependent sodium channel (Na_v) isoform ($\text{Na}_v1.4$) expressed in skeletal muscle. Borges et al. (2006) have shown that the toxin transcriptome of TD and TZ vary in composition and that Tz1 is also expressed in TD but its transcript is present at a lower abundance. These authors have also shown that TZ venom reactivity against the anti-TD antivenom is lower compared to TD. These evidences underlie the diversity of long-chain toxins in Venezuelan *Tityus* both in structural and functional terms.

Recently, Borges et al. (2011) have reported that TZ venom induce a significantly increase in reactive oxygen species (ROS) production mainly in neutrophils compared to TD venom. Apparently, TZ venom specifically up-regulates neutrophil intracellular ROS production, which may be an important *in vivo* target and could have a role to play in the cardio-respiratory complications elicited after envenomation by TZ as described by Mazzei de Davila et al. (2002). The toxins responsible for this TZ venom effect remains to be identified.

The main components of scorpion venoms are single-chain polypeptides that

interfere with the voltage-dependent sodium channels (Na_v) of the mammalian excitable tissue (Gordon et al, 1996). Toxicity of *Tityus* venoms is mainly due to the action of Na_v -active toxins, which have been classified as alpha- and beta-toxins depending on whether they alter the kinetics of Na_v inactivation or activation respectively (Catterall et al., 2007). These Na_v -active toxins induce the release of autonomic neurotransmitters, mainly catecholamines such as nor-epinephrine (NE) and epinephrine (Epi) (sympathetic system), and also acetylcholine (parasympathetic system), which are responsible for several of the clinical manifestations elicited by scorpion envenomation (Amitai, 1998; Gueron et al. 2000). Some of these manifestations are similar to those described by Rodríguez et al. (2008) for patients presenting pheochromocytoma, a catecholamine-releasing tumor.

The purpose of this work was to investigate the nature and chronology of the autonomic nervous system (sympathetic) TZ-mediated stimulation, using *in vivo* and *in vitro* models, trying to understand the pathophysiological mechanisms linked to the TZ envenomation. Whole scorpion venoms were used for modeling these effects as the final envenomation outcome is most likely the result of the combined action of low and high molecular weight components acting synergistically and also in conjunction with non-toxic components as described by Cohen et al. (2006).

2.- Materials and Methods.

2.1.- Scorpions and Venoms

Adult *Tityus zulianus* scorpions were collected near Mesa Bolivar, Mérida State, western Venezuela. *Tityus discrepans* scorpions were collected near San Antonio de Los Altos, Miranda State, north-central Venezuela. Scorpions were classified according to the criteria of González-Sponga (1996). Venoms were obtained by manual stimulation of the telson according to Zlotkin and Shulov (1969) and subsequently lyophilized. Pools of venoms obtained from 50-60 scorpions were reconstituted in the same volume with 0.9% (w/v) NaCl, centrifuged at 12,000xg for 10 min, and protein concentration (Lowry et al., 1951) determined in the supernatant using bovine serum albumin (BSA) as protein standard.

2.2. Animals

All experiments were performed according to protocols approved by the Bioethical Animal Care guidelines of Instituto de Medicina Experimental, Universidad

Central de Venezuela. Male BALB/C (20-22 g) mice were used throughout. All rodents were bred and housed in standard cages in a room at an ambient temperature of $23^{\circ}\text{C} \pm 2^{\circ}\text{C}$ and a 12-h light-dark cycle. They were fed using standard laboratory chow and had *ad libitum* access to filtered water. Mice were randomly assigned to control or experimental groups.

2.3. Preparation of serum samples

Mice were injected (0.2 ml) intraperitoneally (i.p.) with TZ and TD venoms at a dose of 0.5 mg protein/kg body weight. Such venom concentration is known to produce pancreas structural alterations in the case of TD (Blanco et al., 1999). This dose is below the LD_{50} for TD (2.51 mg/kg; Borges et al., 1990) and TZ (1.54 mg/kg; Borges et al., 2006) venoms. Control animals were injected with 0.9% (w/v) NaCl. Venom-injected mice were divided into four groups ($n = 6$ per group) and blood removed by cardiac puncture at 0, 1, 6 and 24 h after venom injection. Whole blood was centrifuged at $3,000\times g$ for 10 min at 4°C and the plasma was removed and stored at -80°C .

2.4. Determination of epinephrine (Epi) and MHPG plasma concentrations by high pressure liquid chromatography (HPLC).

Epinephrine and MHPG (3-methoxy-4-hydroxyphenylglycol) plasma levels were estimated as described by Lima et al. (1994) using a Waters Breeze 464 employing a reverse phase HPLC system equipped with an electrochemical detector. Samples were thawed and diluted 1:6 in 20% sulphosalicylic acid, vortexed and centrifuged at $17,000\times g$ for 20 min. The supernatant was diluted to 1:2 in mobile phase (1mM EDTA, 1 mM citric acid, 0.65 mM Sodium Octyl Sulphate, 7% (v/v) acetonitrile, 128 mM formic acid, pH 3.4). Subsequently, samples were injected automatically in volumes between 10 and 100 μl . The epinephrine and MHPG were separated on a Supercosyl LC18 column (250 x 4 mm) at a flow of 1 ml/min using a Waters 600[®] isocratic pump. Potential between reference and work electrode was +700 mV. The results were analyzed using the external standard method provided by Waters Breeze Program[®].

2.5. [³H]dopamine releasing activity in PC12 cells.

PC12 cells were obtained from Dr. Gloria M. Villegas, Instituto de Estudios Avanzados (IDEA) (Caracas, Venezuela) and were sub-cultured in DMEM culture medium containing penicillin (100 UI/ml), streptomycin (0.1 mg/ml) and Amphotericin

B (2 µg/ml) (Gibco, U.K.) as described by Nakasawa et al. (1994). Medium was supplemented with 5% (v/v) fetal calf serum and 5% (v/v) horse serum in a humidified atmosphere of 5% CO₂/95% air. Cells were cultured on six-well chambers (1 ml/well) until they reached 90% cell confluence. Before assays, cells were washed twice with BSS buffer (150 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1.2 mM MgCl₂, 1.2 mM NaH₂PO₄·2H₂O, 10 mM D-Glucose, 0.1mM EDTA, 25 mM HEPES-NaOH, pH 7.4). Cells were loaded with 250 nM [³H]dopamine ([³H]DA) (0.3 µCi per well). After, 90 min of incubation, media was removed and cells were washed three times with BSS buffer. The [³H]DA release was induced during 1 min with the cholinergic agonist Carbamylcholine and a depolarizing solution of 20 mM KCl. After cholinergic or venom incubation, the supernatants were taken and placed in scintillation vials. Cells in the wells were lysed with 0.5% (w/v) SDS and transferred to vials containing aqueous-scintillation cocktail (73% (v/v) Xylene, Triton 22% (v/v) X-100, 5% (v/v) Liquifluor). The [³H]radioactivity was determined with 40% efficiency in a Beta LKB 1219 scintillation counter. [³H]DA release was calculated in nmoles of [³H]DA/mg protein and later each percentage was estimated using the 100% of total [³H]DA at 0 time (t = 0 condition).

Protein content in these cell culture lysates was determined using a procedure described by Lowry et al. (1951) with the modification of Bensadoun and Weinstein (1976) to remove interfering substances. BSA was used throughout as protein standard.

2.6. Statistical analysis

All results are presented as the mean ± standard error. Data analysis were performed using ANOVA and the Tukey-Kramer *post hoc* test implemented in the programs Excel[®] (Microsoft, USA), Graph Pad In Stat[®] and Prizma[®] (Science, USA).

3. Results.

3.1. Plasma catecholamine levels during TZ and TD envenomation

The circulating catecholamine concentrations were determined in blood samples from envenomed mice at different times in the Control, TD and TZ groups. A significant and dramatic rise was found in the Epi plasma concentrations (2000 ng/ml) at 1 h in the TZ group as shown in Figure 1. In contrast, a significant but much smaller increment in Epi was induced by TD venom. This massive rise in Epi plasma concentrations remained higher at 6 h for the TZ group but decreased to basal levels in

the TD group. At 24 h, both venoms showed similar behavior, exhibiting a decrease in Epi concentrations, but remained significantly higher in comparison with the Control group ($p < 0.01$). The Epi data are expressed in log scale with the aim of showing these statistical differences.

These remarkable Epi plasma elevations induced by both venoms should correlate with the plasma levels of its catabolic products, mainly the (MHPG). Thus, MPHG values exhibited a significant elevation ($p < 0.01$) at 1 h, after venom injection in all envenomed-mice groups as shown in Figure 2. Noticeably, at 6 h the TZ group displayed higher MHPG plasma levels than the TD group. The MHPG plasma levels remained elevated until 24 h post-injection in both groups of envenomed mice being the levels in TD and TZ groups significantly higher than the control group.

3.2. [^3H]dopamine release induced by TZ and TD venoms in PC12 cells.

The remarkable TZ venom-elicited increments in Epi plasma levels in mice and those induced to a lesser extent by TD venom could be related to an intense stimulation of the adrenal medulla activity. Consequently, we decided to evaluate the effects of these venoms in a cellular model of sympathetic neurons, the chromaffin tumor-derived PC12 cells from rat pheochromocytoma, originally established by Green and Tischler (1976), an excellent catecholamine releasing cell system via nicotinic cholinergic receptor, in which dopamine (DA) is the predominant catecholamine being released (Green and Rein, 1977). The [^3H]DA release was induced during 1 min with the cholinergic agonist carbamylcholine (CC) and a depolarizing solution of 20 mM KCl (the latter experimental condition was used as control and data are not shown). Several dose-response curves for CC in the presence of TD and TZ venoms (15 $\mu\text{g}/\text{ml}$) were generated. Additionally, in order to identify the cholinergic receptor involved in this [^3H]DA releasing activity, we evaluated the effect of the muscarinic antagonist atropine (10 μM) and the ganglionic blocker hexamethonium (1 mM) on these venom-mediated effects.

PC12 cells were loaded with [^3H]DA and later exposed to CC in the presence of TD and TZ venoms (15 $\mu\text{g}/\text{ml}$). These scorpions venoms induced a leftward shift in the CC dose-response curves as shown in Figure 3, reaching the maximal [^3H]DA releasing activity at 100 μM CC. Both venoms increased significantly this CC-dependent [^3H]DA release from PC12 cells. Thus, venom-mediated significant increments ($p < 0.01$) in

E_{\max} (expressed as a percentage of the basal release) were found: TD = 64.85 ± 8.54 ; TZ = 95.38 ± 10.16 ; Control = 38.14 ± 2.16 . However, only TZ venom decreased significantly ($p < 0.01$) the EC_{50} for carbamylcholine (TZ = 29.73 ± 0.03 ; Control = 36.94 ± 0.03) as shown in Table 1. In addition, when the venoms were incubated in the presence of 100 μ M CC, the [3 H]DA release increased dramatically upon TZ venom incubation and reaching almost 100%, whereas TD venom-induced release was about 60% (Figure 4). In order to identify pharmacologically the cholinergic receptor associated with this [3 H]DA release activity, atropine (a muscarinic antagonist) and hexamethonium (a nicotinic blocker) were used. As shown in Figure 5, CC induced a 2.3-fold increase in [3 H]DA release, which was inhibited in 66% by 1 mM hexamethonium, indicating the involvement of nAChRs. In contrast, TZ venom induced a 2.2-fold increase in [3 H]DA release, of which only 30% was inhibited by hexamethonium. Interestingly, when CC and TZ venom were added simultaneously, the [3 H]DA release increased 3-fold over the basal rate of which was inhibited (46%) by hexamethonium.

Discussion.

The clinical consequences of human envenomation by Venezuelan scorpions belonging to the genus *Tityus* have been described previously. Sequera et al. (1993) have reported clinical findings in TD-envenomed patients, whom develop mainly gastrointestinal disorders. In the case of accidents by TZ in the western state of Mérida, Mazzei de Dávila et al. (2002) have reported that the clinical outcome seems to be the result of an autonomic activation associated with a significant rise in catecholamine plasma levels.

In this work, a massive catecholamine release induced by TZ venom in intact mice (*in vivo*) and PC12 cells (*in vitro*) models here described, can be explained by a combination of the following molecular mechanisms, which produce a [Ca^{2+}]_i rise leading to neurotransmitter release from endocrine cells and neurons:

- 1.- The presence of TZ-specific α - and β -toxins capable of affecting voltage-dependent sodium channels (Na_v).
- 2.- The existence in TZ venom of iberiotoxin-like toxins, which is a specific inhibitor of the large-conductance Ca^{2+} -activated K^+ channels.
- 3.- The existence in TZ venom of similar toxins displaying a depolarizing activity on neuronal nAChRs as present in *Buthus occitanus tunetanus* venom.

4.- The existence of putative TZ toxins resembling imperatoxin A and/or maurocalcine affecting the RyR-activated Ca^{2+} -release channel activity in neuronal or non-neuronal cells.

To identify the mechanisms underlying these differential venom effects, it was endeavored to reproduce the venom-mediated catecholaminergic discharge in a biomodel (mice) through a systematic evaluation of the plasma Epi concentrations during a 24-h period after the injection of sub-lethal doses of TD and TZ venoms.

In addition, an *in vitro* assay to evaluate the TZ and TD venoms effects on the ability of rat pheochromocytoma-derived PC12 cells to release dopamine (DA), established by Green and Tischler (1976), which is model for catecholamine release via nicotinic cholinergic (nAChR) stimulation as described by Greene and Rein (1977).

A significant increment in plasma concentrations of Epi and MHPG as a result of intense autonomic stimulation induced by TZ and TD venoms in mice was observed. In TZ-envenomed patients, high catecholamine plasma levels have been reported by Mazzei de Davila, et al. (2002). Our *in vivo* results showing 4000% increments in the BALB/c plasma Epi levels demonstrate that it is possible to reproduce in biomodels (mice), at least some of the clinical findings described in TZ-stung humans. Similar findings reporting a rise of 3000% in Epi plasma levels have been published by Zeghal et al. (2000) in studies performed in *Buthus occitanus* envenomed-rats.

The Epi plasma elevations should correlate with a corresponding rise in MHPG plasma levels, which is the main Epi metabolite (Westfall, and Westfall, 2006). Accordingly, MHPG plasma levels increased significantly from 1 h post-injection and remained elevated until 24 h in all envenomed groups. However, there is an apparent paradox concerning the MHPG and Epi plasma concentrations since the elevation of Epi was exponential, which does not correlate well with the initial linear increase and reaching plateau in MHPG plasma levels. This difference in kinetics may be explained due to the saturation of the enzymatic systems involved in the metabolic processing of catecholamines, formed by COMT, MAO and aldehyde reductase (Westfall and Westfall, 2006), which is responsible for MHPG synthesis, and taking into account that Epi is first oxidatively deaminated by MAO-A, the main catecholamine metabolizing enzyme (Son et al., 2008). MAO inhibition by putative peptides present in these Venezuelan scorpion venoms, similar to those described for *Mesobuthus gibbosus*, (Ucar et al., 2005), which might account for this MHPG behavior.

The massive release of catecholamines mainly from adrenal gland and sympathetic nerve terminals or PC cells elicited by TD and TZ venoms is probably due to the fact that these scorpions produce toxins that can affect, directly or indirectly, the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in these neuroendocrine cells. Initially, it was postulated that these scorpion venoms contain α - and β -toxins capable of slowing inactivation or enhancing activation, respectively, of voltage-dependent sodium channels (Na_v) (Thomsen et al. 1995, Gordon et al. 1996; Borges et al. 2004b, 2006; Leipold et al., 2006). These toxins should reduce the triggering threshold of the $\text{Na}_v1.7$ isoform expressed in these cells, producing, in turn, depolarization and elevation of cytoplasmic $[\text{Ca}^{2+}]_i$ and leading to neurotransmitter release from the adrenal gland (mainly Epi) with the subsequent augmented catecholamines release to blood stream or the DA release in PC12 cells.

To elucidate the effects of TD and TZ venoms on adrenal cells, it was decided to use tumor chromaffin PC12 cells originally derived from rat adrenal cells as an *in vitro* model of neurotransmitter release. However, the $\text{Na}_v1.7$ expressed in adrenal medullary chromaffin cells are not the main molecular target responsible for the secretion of catecholamines from chromaffin cells induced by some scorpion toxins as reported by Conceição et al. (1998). These authors suggested that in cell depolarization, the opening of voltage-dependent Ca^{2+} channels, and Ca^{2+} entry are more relevant events for catecholamine secretion than the Na^+ movements via Na_v channels in chromaffin cells. In this sense, several lines of experimental evidence suggest that $[\text{Ca}^{2+}]_i$ regulation is crucial for catecholamine secretion from adrenal chromaffin cells as reviewed by Garcia, et al. (2006). Consequently, in this chromaffin PC12 cells, Ritchie (1979) established the existence of two independent pathways for Ca^{2+} entry to activate the DA release, i.e. a voltage-dependent Ca^{2+} channel and the Ca^{2+} channel-linked acetylcholine (ACh) receptor, via the activation of TTX-insensitive nicotinic ACh receptors (nAChRS) (Macdonald et al.2005). In our studies, a nAChR should be involved in TD- and TZ-dependent DA release since their effects were partially abolished by hexamethonium. In this sense, PC12 cells express neuronal nAChRs, mainly alpha7 nAChR, which are ligand-gated cation channels that can alter intracellular Ca^{2+} levels inducing neurotransmitter release as described by Dickinson et al. (2007). Similar depolarizing activity on nAChRs skeletal muscle as reported by Cheikh et al. (2007) for the scorpion *Buthus occitanus tunetanus* (Bot) venom. Thus, after, nAChR stimulation, Ca^{2+} can enter cells directly, via the intrinsic ion channel associated with this receptor,

or indirectly following voltage-operated Ca^{2+} channel (VOCC) activation. As a result, Ca^{2+} levels can subsequently be amplified via Ca^{2+} -induced Ca^{2+} release linked to the Ryanodine receptors system (RyR) at intracellular Ca^{2+} stores as reviewed by Hidalgo et al. (2005).

Another way to augment catecholamine secretion from adrenal chromaffin cells and to explain the synergistic effect of TZ venom on the CC-induced DA releasing activity in PC 12 cells has been postulated by Wada et al. (1995) in cultured bovine adrenal chromaffin cells, suggesting that the stimulation of nAChR eventually opens large-conductance $\text{Ca}^{(2+)}$ -activated K^+ channels, blockade by iberiotoxin, a toxin purified from the scorpion *Buthus tamulus*.

It was found that there is a significant independent DA release via nAChRs (resistant to hexamethonium) induced by TZ venom, which could contain species-specific toxins that may affect the mechanism of DA release associated with the voltage-operated Ca^{2+} channel (VOCC) activation, which has been established in nerve terminals and neuron cells (De Crescenzo et al., 2006) and the calcium-induced calcium release described in PC12 cells, via the activation of Ryanodine receptors (RyR) (Hidalgo et al., 2005). In relation to the last mechanism, imperatoxin A from the scorpion *Pandinus imperator* (El-Hayek et al., 1995) and maurocalcine from the Tunisian scorpion *Scorpio maurus palmatus* venom (Esteve et al., 2003) have been shown to strongly modify the properties of RyR-activated Ca^{2+} channels associated to intracellular Ca^{2+} stores.

As above discussed, these Venezuelan scorpion venoms, particularly TZ, might contain novel toxins, which remain to be identified and whose biological activities are yet to be established, in order to fully understand the molecular mechanisms underlying the pathophysiology of human envenomation by these species.

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Table 1: Effect of TD and TZ venoms on [³H]Dopamine release parameters from PC12 cells induced by Carbamylcholine dose-response curves.

Treatment	E _{max} (% over basal release)	E _{max} Ratio (over Control)	EC ₅₀ (μM)
Control	38.14 ± 2.16	1.00	36.94 ± 0.03
TD	64.85 ± 8.54*	1.70	34.20 ± 0.04 ns.
TZ	95.38 ± 10.16*	2.50	29.73 ± 0.03**

Each value represents the mean ± SEM of three independent experiments performed in triplicate. E_{max} ANOVA F_(2,24) = 13.61(*) p < 0.01 TD and TZ vs Control.

EC₅₀ ANOVA F_(2,24) = 11.69. (***) TZ vs Control (p < 0.01).

EC₅₀ = Carbamylcholine concentration produces a 50 % of [³H]Dopamine release.

E_{max} = Maximal release of [³H]Dopamine in each condition.

ns: not significant. TD : *Tityus discrepans*. TZ : *Tityus zulianus*.

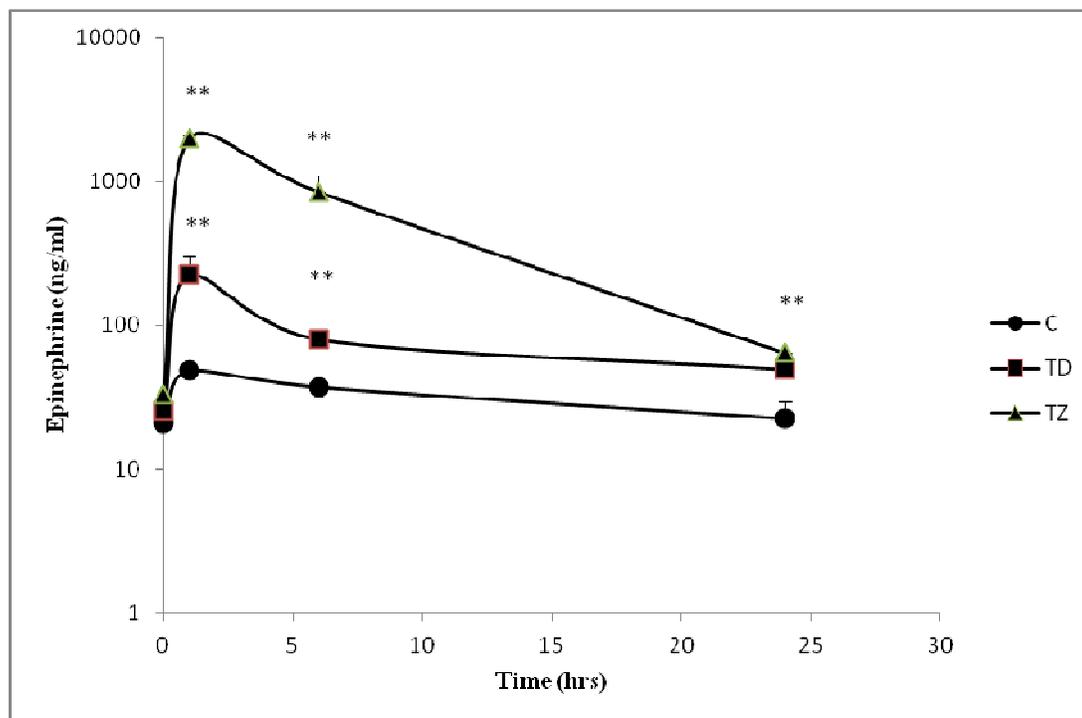


Figure 1: Time course of Epinephrine plasma levels (ng/ml) in mice injected with TD and TZ venom (0.5 mg/kg). Epinephrine was determined by HPLC coupled to an electrochemical detector. Each point represents the mean \pm SEM from samples from 6 animals. ANOVA 1 hr. $F_{(2,15)} = 1979.70$; 6 hr $F_{(2,15)} = 57.71$; 24 hr $F_{(2,15)} = 153.75$. (***) $p < 0.01$ vs Control.

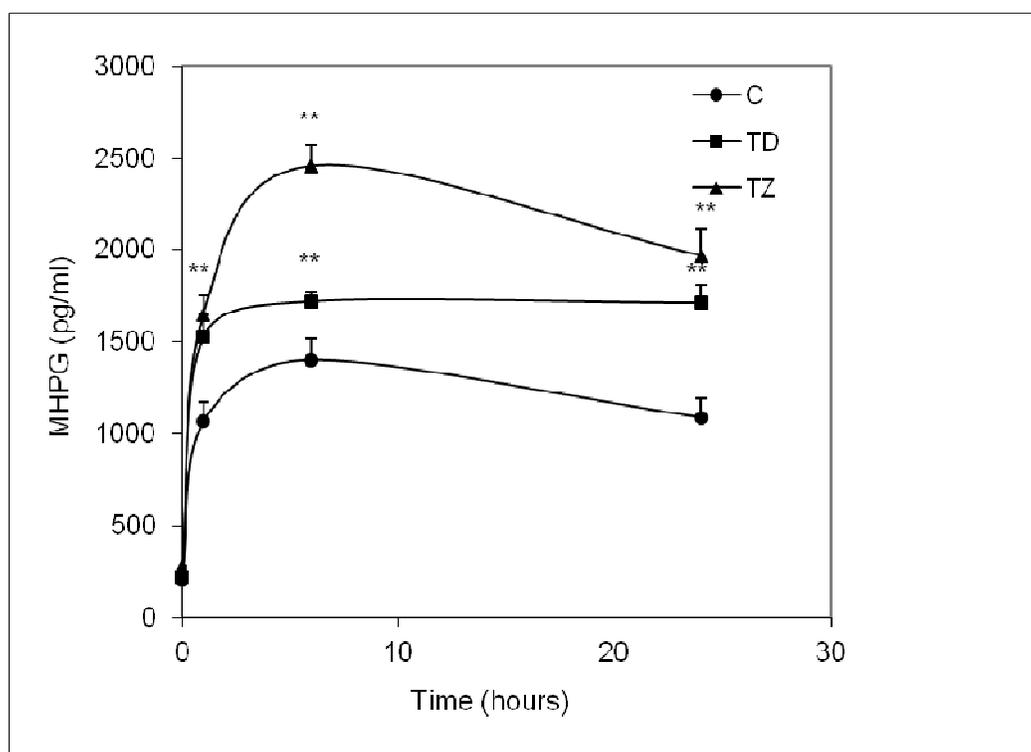


Figure 2: Time course of plasma 3-Methoxy-4-hydroxyphenylethylene glycol (MHPG) levels (pg/ml) in mice injected with TD and TZ venom (0.5 mg/kg). MHPG was determined by HPLC coupled to an electrochemical detector. Each point represents the mean \pm SEM from samples of six separated animals. ANOVA 1 h $F_{(2,15)} = 48.38$; 6 hr $F_{(2,15)} = 179.35$; 24 hr $F_{(2,15)} = 88.35$ (** $p < 0.01$ vs Control).

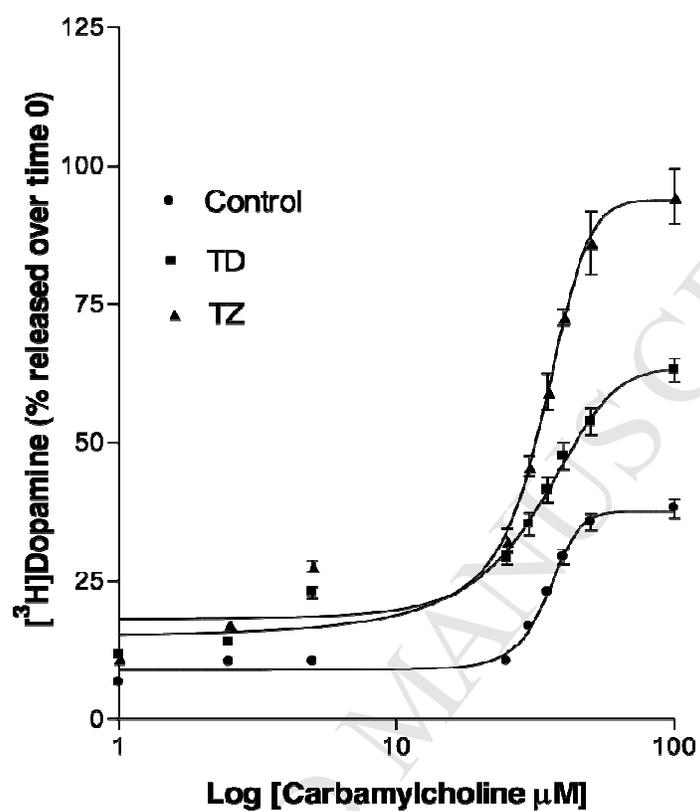


Figure 3: Curves of dose-dependent effects of cholinergic agonist (carbamylcholine) on [³H]Dopamine release from PC12 culture cells. Each point represents the mean \pm SEM of three independent experiments performed by triplicate. The concentration of TD and TZ scorpion venoms was 15 μ g /ml. Percentage (%) was estimated using the total amount of uptake [³H]-Dopamine at 0T as 100.

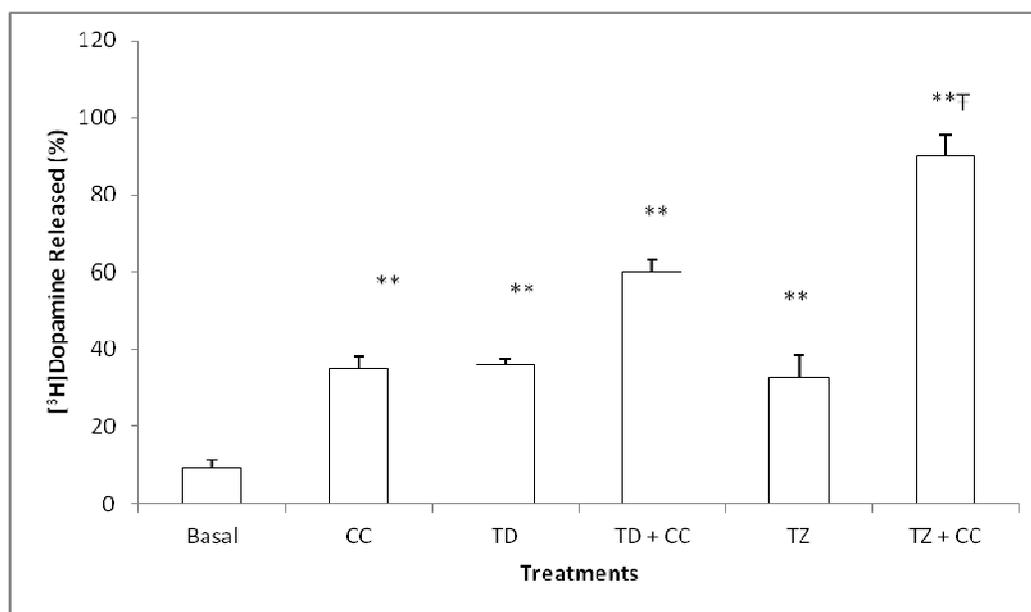


Figure 4: The basal [³H]dopamine release from PC12 culture cells and in the presence of carbamylcholine (CC) 100 μ M, TD venom (15 μ g/ml), TZ venom (15 μ g/ml) and the different combinations of CC + TD venom and CC + TZ venom. Each bar represents the mean \pm SEM of three independent assays performed in triplicate. ANOVA $F_{(6,48)} = 29.34^{**}$ $p < 0.0001$ vs Control. F : ($p < 0.01$ vs CC).

Percentage (%) was estimated using the total amount of uptake [³H]-Dopamine at 0T as 100.

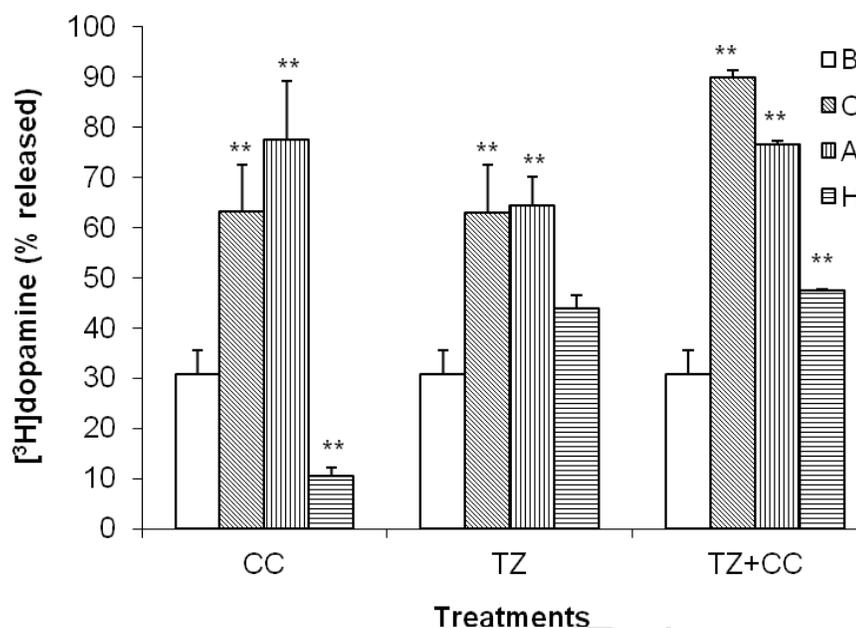


Figure 5: The [^3H]dopamine release from PC12 culture cells in the presence of carbamylcholine (CC) 100 μM , TZ venom (15 $\mu\text{g}/\text{ml}$) and the combination of CC + TZ venom with Atropine (muscarinic receptor antagonist) or hexamethonium (Ganglionic N_n blocker). Each bar represents the mean \pm SEM of three independent assays in triplicate. ANOVA $F(9.80) = 7.632$. ** $p < 0.01$ vs Control.

B = [^3H]Dopamine Basal release.

C = [^3H]Dopamine release in the presence of each condition.

A = [^3H]dopamine release with CC 100 μM + 10 μM Atropine.

H = [^3H]dopamine release with CC 100 μM + 1 mM hexamethonium.

Highlights.

We simulated in mice, catecholamine rise of *Tityus zuliaanus* (TZ)-envenomed patients > TZ venom increased in 4000%, the plasma catecholamine (Epinephrine) levels in mice> Plasma levels of MHPG (main Epi-catabolite) were also increased in TZ-envenomed mice> TZ venom potentiated the dopamine release from PC12 cells (PC12) by carbamylcholine > Dopamine release by TZ at PC12 is hexamethonium-insensitive suggesting novel toxins.