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Major Article

Local and systemic effects caused by *Crotalus durissus terrificus, Crotalus durissus collilineatus*, and *Crotalus durissus cascavella* snake venoms in swiss mice

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Abstract

Introduction: *Crotalus* envenomations cause serious complications and can be fatal without appropriate treatment. Venom isoforms present and inter/intraspecific variations in the venom composition can result in different symptoms presented by bites by snakes from the same species but from different geographical regions. We comparatively evaluated the local and systemic effects caused by *Crotalus durissus terrificus* (*Cdt*), *C.d. collilineatus* (*Cdcolli*), and *C.d. cascavella* (*Cdcasc*) envenomation. **Methods:** Venom chromatography was performed. Proteolytic, phospholipase, and LAAO activities were analyzed. Edema, myotoxicity, hepatotoxicity, nephrotoxicity, and coagulation alterations were evaluated. **Results:** The venom SDS-PAGE analyses found the presence of convulxin, gyroxin, crotoxin, and crotamine in *Cdt* and *Cdcolli* venoms. Crotamine was not present in the *Cdcasc* venom. *Cdt*, *Cdcollli*, and *Cdcasc* venoms had no proteolytic activity. Only *Cdcasc* and *Cdt* venoms had phospholipase activity. LAAO activity was observed in *Cdcolli* and *Cdcasc* venoms. *Cdcolli* and *Cdcasc* venoms caused 36.7% and 13.3% edema increases, respectively. *Cdt* venom caused a 10% edema induction compared to those by other venoms. All venoms increased TOTAL-CK, MB-CK, and LDH levels (indicating muscle injury) and ALT, AST, GGT, and ALP levels (markers of liver damage) and were able to induce a neuromuscular blockade. Urea and creatinine levels were also altered in both plasma and urine, indicating kidney damage. Only *Cdcolli* and *Cdcasc* venoms increased TAPP and TAP. **Conclusions:** Together, these results allow us to draw a distinction between local and systemic effects caused by *Crotalus* subspecies, highlighting the clinical and biochemical effects produced by their respective venoms.

Keywords: *Crotalus durissus*. Myotoxicity. Nephrotoxicity. Hepatotoxicity. Neurotoxicity. Edema.

INTRODUCTION

Crotalus snakebites cause serious problems and can be fatal unless adequately treated. The high lethality occurs due to the frequency with which *Crotalus* envenomation causes acute renal failure, one of the major causes of death due to snake bites[1]. In addition to neurotoxicity, myotoxic action and clotting disorders may cause micro-bleeding, which characterizes the systemic effect of envenomation[2,3]. The local effects that can be observed are mild pain and local or regional paresthesia that may persist for different periods of time, slight edema, and erythema at the bite site[4].

Protein isoforms present in *C. durissus* venom are also of great clinical importance, due to the fact that the venom pool used for antivenom production may be inadequate. This leads to reduced immunogenicity in animals and results in a product

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that cannot adequately neutralize the clinical manifestations of patients bitten by these snakes[5,6].

This study aims to compare the toxic effects (local and systemic) induced by the venom of *Crotalus* subspecies, *C. durissus terrificus*, *C. durissus cascavella*, and *C. durissus collilineatus,* in individuals bitten by *Crotalus* snakes. Moreover, this study demonstrates that the variations found can be related to differences in neutralization rates by antivenom actions against the venoms from different subspecies, which is consistent with the results published by Boldrini-França et al.[6] and Oliveira et al.[7].

METHODS

Venoms and Animals

Cdt, Cdcolli, and *Cdcasc* venoms were acquired from Instituto Butantan, São Paulo-SP and were maintained at -20ºC in the Amazon Venom Bank at CEBio-UNIR-FIOCRUZ-RO (licenses: CGEN/CNPq 010627/2011-1 and IBAMA 27131-2). All *Crotalus* sp. venoms used were from a pool of venoms from adults male and female snakes from different parts of Brazil (carried by IBAMA, ONGs, firemen, or physical people).

Male Swiss mice (18-20 g) were housed in temperature-controlled rooms and received water and food *ad libitum* until used (approved protocol number 2012/09 from the FIOCRUZ-RO Animals' Ethics Committee).

Venom biochemical characterization

Cdt, *Cdcasc*, and *Cdcolli* venoms' chromatographic profile was produced by molecular exclusion chromatography (Sephadex G75) in an FPLC (Akta Purifier System/GE Healthcare®). The method was performed according to Bercovici et al.[8] in which about 35 mg of venom was suspended in 1 mL of 0.05 M ammonium formate buffer, pH 3.5. The peaks obtained were analyzed by SDS-PAGE on 12.5% and/or 15% polyacrylamide gels. The proteins' concentrations were measured using the Bio-Rad DC Protein Assay method (BIO-RAD).

Proteolytic activity

150 µL of 2% azocasein solution (substrate) was added to 7 µL of each venom (20 µg of *Cdt*, *Cdcolli*, or *Cdcasc*) in a 96-well plate (substrate) and incubated for 1 h at 37ºC. Subsequently, the reaction (Azocasein+venom) was stopped by adding 150 µL of 20% trichloroacetic acid (TCA). After 30 min, the samples were centrifuged at 180 x*g* for 10 min. Next, 100 µL of the supernatant was added to a 96-well plate, and 100 µL of 500 mM NaOH were added. The absorbance at 440 nm was monitored using spectrophotometer (Biotek)[9]. *Bothrops'* venom (from Amazon Venom Bank at CEBio-UNIR-FIOCRUZ-RO) was used as a positive control.

Phospholipase activity

Phospholipase activity was determined using the method described by Holzer and Mackessy[10] adapted to 96-well plates. Buffer containing the chromogenic substrate 4-nitrophenyl (3-octanoyloxy) benzoic acid (4N3OAB) was added to 10 µL of each venom (20 µg *Cdt*, *Cdcolli*, or *Cdcasc*) or water (negative control). The solution was analyzed in a spectrophotometer at 425 nm over 30 min for 30 s intervals[10].

LAAO Activity

For LAAO activity, the method described by Torii[11] adapted to a 96-well plate was followed according to Pontes et al.[12]. In brief, each venom (10 µg) was added separately to the reaction mixture containing horseradish peroxidase (50 µg/mL), 100 µM l-leucine, and 10 µM 3'3'diaminobenzidine in 100 mM Tris-HCl buffer (pH 7.8). The reaction was incubated at 37°C for 30 min. The reaction was stopped using 10% citric acid and the absorbance was measured on a spectrophotometer at 490 nm.

Paw edema assay

*G*roups of 5 mice were inoculated with a subplantar injection of 30 µL of 100 µg/kg of *Cdt*, *Cdcolli*, or *Cdcasc* diluted in 150 mM sterile physiological saline in the right hind paw. As a control, sterile physiological saline (30 µL) was injected into the contralateral paw. Paw volume was measured immediately before sample injection (basal time 0 h) and at different time intervals thereafter (0.5, 1, 3, 6, 9, 12, and 24 h). Paw volume was measured using a hydroplethysmometer (Ugo Basile). Results were expressed as percentage paw volume increase in relation to the control paw[13].

Myotoxic, Hepatotoxic, and Nephrotoxic activities

Groups of 5 mice received an intramuscular (i.m.) injection of 30 µL of *Cdt, Cdcolli*, or *Cdcasc* venom (100 µg/kg of venom diluted in 150 mM sterile physiological saline) in the gastrocnemius muscle. Control group animals received 30 µL (150 mM sterile physiological saline) in the same conditions. After 3, 6, 9, 12, and 24 h, mouse blood was withdrawn from their orbital plexus, dispensed into heparinized tubes, and centrifuged at 2205 x*g* for 5 min according to Teixeira et al. (2018)[13].

Myotoxicity activity was evaluated by measuring creatine kinase (CK), creatine kinase MB isoenzyme (CK-MB), and lactate dehydrogenase (LDH). Hepatotoxic activity was evaluated by measuring alanine transaminase (ALT), aspartate transaminase (AST), gamma-glutamyl transferase (GGT), and alkaline phosphatase (AP) activity. Kidney function was evaluated by measuring plasma creatinine and urea biochemical parameters and total urine proteins and calcium. All determinations were conducted using commercial diagnostic kits purchased from Labtest Diagnostica SA (Brazil).

Coagulation tests *in vivo*

Groups of 5 mice received in the gastrocnemius muscle an i.m. injection of 30 µL of *Cdt, Cdcolli*, and *Cdcasc* (100 µg/kg of venom diluted in 150 mM sterile physiological saline). Control group animals received 30 µL (150 mM sterile physiological saline) in the same conditions. After 3 h, mouse blood was drawn from the inferior vena cava, dispensed into citrated tubes, and centrifuged at 2205 x*g* for 5 min in order to obtain platelet-poor plasma. Prothrombin time (TAP) and activated partial thromboplastin time (TAPP) were determined using Hemostasis (Labtest, Brazil).

Myographic Study

Cdt, Cdcolli, and *Cdcasc* venoms neuromuscular activities were assessed in mice phrenic-diaphragm preparations in accordance with the method previously described by Gallacci and Cavalcante[14]. The amplitudes of indirect and direct twitches were measured at 150 and 420 min.

Statistical analysis

Results were analyzed by ANOVA complemented by the Tukey Kramer test (*GraphPad Prism 5.0*). Values were expressed as mean ± S.E.M with $P<0.05$ considered significant.

RESULTS

Biochemical Characterization

Venoms were fractioned by molecular exclusion chromatography using the Sephadex G-75 (GE healthcare®, 10 x 60 cm). The *C. durissus* venoms chromatographic profile analysis showed three peaks, *Cdt*-I, *Cdt*-II, and *Cdt*-III, for *Cdt* (**Figure 1A**); three peaks, *Cdcolli*-I, *Cdcolli*-II, and *Cdcolli*-III, for *Cdcolli* (**Figure 1B**); and two peaks, *Cdcasc*-I and *Cdcasc*-II, for *Cdcasc* (**Figure 1C**).

After electrophoresis, convulxin (Cvx) and gyroxin (Grx) were visualized in the lanes associated with *Cdt*-I, *Cdcolli*-I, and *Cdcasc*-I with approximate molecular weights (MW) of ~72 kDa for Cvx and ~30 kDa for Grx. Moreover, crotoxin (Ctx) was found in the *Cdt*-II, *Cdcolli*-II, and *Cdcasc*-II lanes at ~24 kDa. However, crotamine (Ctm) alone was visualized in the *Cdt*-III and *Cdcolli*-III lanes at ~4.8 kDa, but was not observed in *Cdcasc* venom (Ctm-negative) (**Figures A2**, **B1,** and **C1**). It was observed that the estimated protein content for *Cdt*-I, *Cdcolli*-I, and *Cdcasc*-I peaks were 2%, 6%, and 18%, respectively. For *Cdt*-II, *Cdcolli*-II, and *Cdcasc*-II the protein contents were 81%, 67%, and 82%, respectively. Additionally, the estimated protein contents for *Cdt*-III and *Cdcolli*-III were 16% and 27%, respectively. The results above apply only to *Cdt* and *Cdcolli* because *Cdcasc* was found to be devoid of crotamine.

Enzymatic Characterization

Results suggested that *Cdt, Cdcolli*, and *Cdcasc* venoms do not exert a proteolytic effect on azocasein compared to the negative control (**Figure 2A).**

Cdcolli and *Cdcasc* venoms (20 µg) had significant LAAO activity compared to that of the control. This result was determined by the peroxidase reaction, wherein H_2O_2 was produced. However, *Cdt* venom showed no LAAO activity (**Figure 2B**).

Cdcolli venom (20 µg) demonstrated low phospholipase activity on the substrate 4N3OAB at the concentration used. However, *Cdcasc* and *Cdt* venoms, using the same substrate and the same concentration, displayed significant catalytic activity compared to control (**Figure 2C**).

Local and Systemic Effect Characterization

Cdcolli and *Cdcasc* venoms induced paw edema increases of 76.7% and 40%, respectively, after 1 h. Additionally, *Cdt* venom induced a paw edema increase of 33.3% after 3 h (**Figure 3**).

Mice blood CK levels are depicted in **Figure 4A**. *Cdcolli* and *Cdcasc* induced a significant increase in total-CK liberation compared to control animals 3 h after inoculation. After 6, 9, and 12 h, all venoms induced a significant total-CK increase compared to that of the control (182.3 U/L). Total-CK liberation induced by *Cdcolli* was statistically different from *Cdt* from 3 to 12 h after inoculation. However, total-CK liberation induced by *Cdcasc* was statistically different from *Cdt* just after 12 h of inoculation. In **Figure 4B**, animals inoculated with venom had significantly higher MB-CK levels compared to control animals after 3 h of inoculation. At this time, animals inoculated with *Cdcolli* differed statistically from those inoculated with *Cdcasc*. This was not observed in mice envenomated with *Cdt* venom after 6 h of inoculation. After 9 h, all envenomated animals had basal MB-CK levels. However, after 12 h, *Cdcolli* and *Cdt* venoms injected in mice induced a significant increase in MB-CK levels compared to that in control animals. Additionally, 24 h after inoculation, all venoms induced a significant MB-CK increase compared to the controls.

Figure 4C shows that all venoms studied increased LDH levels 3 and 6 h after inoculation compared to control animals. However, the animals inoculated with *Cdt* alone differed statistically from those inoculated with *Cdcasc*. Moreover, *Cdcolli* and *Cdt* venoms increased LDH levels after 12 h compared to controls.

As shown in **Figure 4D**, none of the venoms changed ALT levels after 3 h compared to control animals. However, *Cdcasc* and *Cdcolli* venoms increased ALT levels compared to the controls 6 and 9 h after inoculation, respectively. After 9 h, animals envenomated with *Cdcolli* differed statistically from those with *Cdt* and *Cdcasc.* After 12 h past *Cdcolli* and *Cdt* inoculation, ALT levels significantly increased compared to *Cdcasc* and the control group. Additionally, 24 h after *Cdcolli, Cdcasc*, or *Cdt* injection, ALT levels significantly increased compared to controls.

In **Figure 4E**, significantly higher levels of AST can be seen in animals injected with *Cdcasc*, *Cdcolli*, or *Cdt* compared to those in control animals after 3, 6, 9, and 24 h. However, only animals injected with *Cdcolli* venom did not display an increase in AST levels 12 h after inoculation. Animals inoculated with *Cdcasc* differed statistically from those inoculated with *Cdcolli* and *Cdt* 3 and 12 h after inoculation. At 9 h after *Cdcasc* inoculation, *Cdcasc* action differed from animals inoculated with *Cdcasc* and *Cdt*. However, animals inoculated with *Cdt* and *Cdcasc* differed from those inoculated with *Cdcolli* 24 h after inoculation.

The animals injected with *Cdt* venom showed an increase in ALP levels after 6 h (**Figure 4F**). Animals injected with *Cdcolli* venom induced higher ALP levels compared to control animals 9, 12, and 24 h after inoculation. ALP levels in animals inoculated with *Cdcolli* differed statistically from those inoculated with *Cdt* and *Cdcasc* 12 and 24 h after injections.

Figure 4G shows that 3 h after inoculation, only animals injected with *Cdcolli* venom induced a GGT increase compared to the control and *Cdt* animals. Moreover, after 6 h, all animals injected with the *Crotalus* subspecies venoms presented a

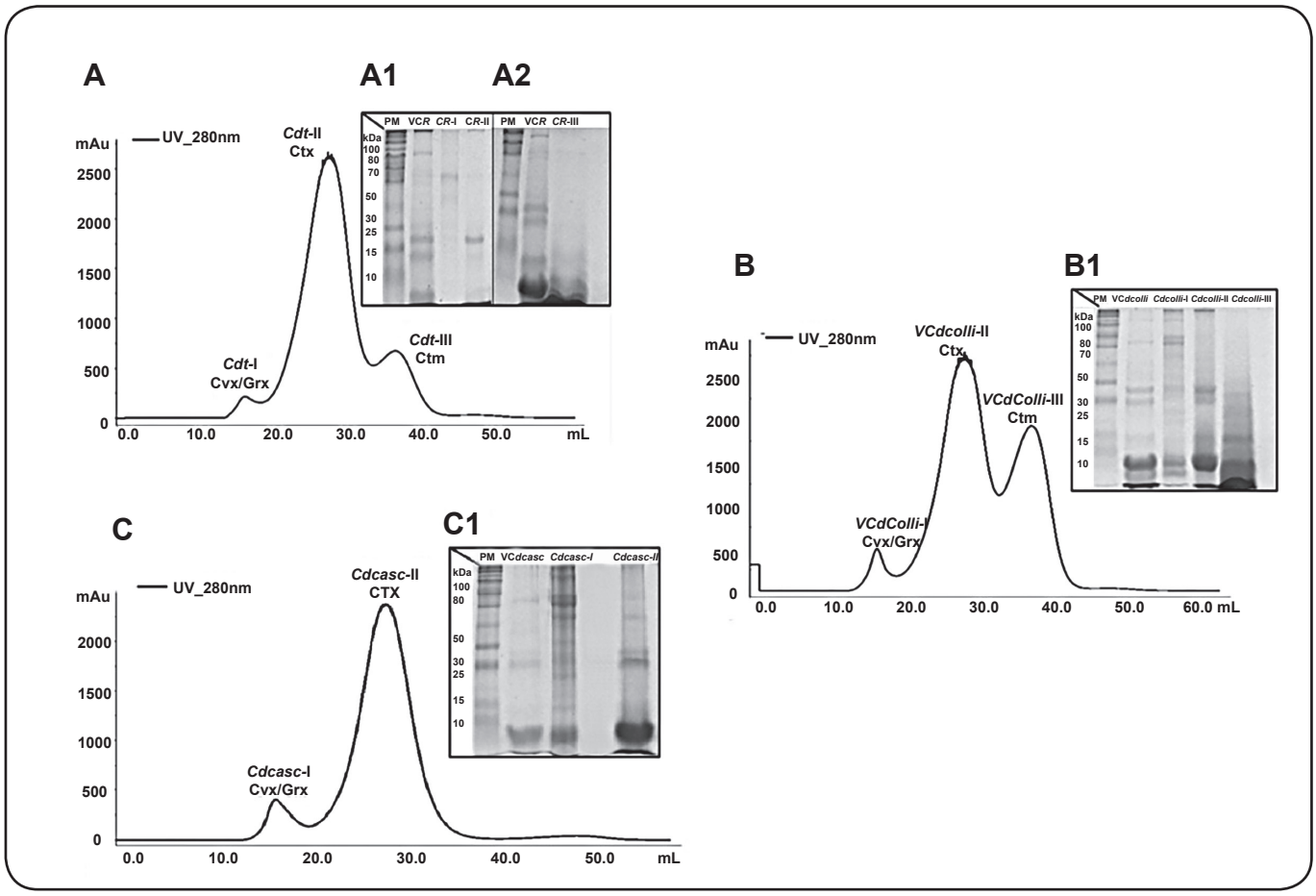

FIGURE 1: (A) Chromatographic profile of *Cdt* venom by a size exclusion column. Electrophoresis profile of the fractionation of *Cdt* venom. **A1) A 12.5% and A2) 15% SDS-PAGE in denaturing conditions**. PM corresponds to the molecular weight standard; *Crotalus durissus terrificus* venom (V*Cdt*); and *Cdt*-I, *Cdt*-II, and *Cdt*-III to PI, PII, and PIII, respectively. **(B) Chromatographic profile of *Cdcolli* venom by size exclusion column**. Electrophoresis profile of the fractionation of *Cdcolli* venom. Peaks I, II, and III correspond to DI convulxin (Cvx)/gyroxin (Grx), DII crotoxin (Ctx), and DIII crotamine (Ctm), respectively. **B1) 15% SDS-PAGE in denaturing conditions**. PM corresponds to molecular weight standard; *Crotalus durissus collilineatus* venom (V*Cdcolli)*; and *Cdcolli*-I, *Cdcolli*-II, and *Cdcolli*-III to PI, PII and PIII, respectively. **(C) Chromatographic profile of *Cdcasc* venom by a size exclusion column**. Electrophoresis profile of the fractionation of *Cdcasc* venom. Peaks I and II correspond to BI convulxin (Cvx)/gyroxin (Grx) and BII crotoxin (Ctx), respectively. **C1) 15% SDS-PAGE in denaturing conditions**. PM corresponds to the molecular weight standard; *Crotalus durissus cascavella* venom (V*Ccasc)*; and *Cdcasc*-I and *Cdcasc*-II to PI and PII, respectively.

significant increase in GGT levels. At this time, the GGT levels of animals inoculated with *Cdt* differs from those animals inoculated with *Cdcolli* and *Cdcasc*. After 9 h, mice injected with *Cdcolli* and *Cdcasc* venoms had a reduced GGT levels compared to control and *Cdt* mice.

Figure 4H shows that 3 h after *Cdcasc* and *Cdcolli* mice envenomation there was a significant increase in urea levels compared to control animals. Only *Cdcolli* venom induced a significant increase in urea levels 6 h after envenomation. Additionally, 9 h after *Cdt* and *Cdcolli* injection there was a significant increase in urea levels compared to control animals.

For urine urea levels at 3, 6, 12, and 24 h, all *Crotalus* venoms did not induce urea liberation in mouse urine. However, *Cdcolli* induced a significant increase in urine urea levels at 9 h after envenomation (**Figure 4I**). *Cdcolli* and *Cdt* induced a significant increase in serum creatinine compared to *Cdcasc* animals 9 h after envenomation (**Figure 4J**).

Results showed that 3 h after *Crotalus* venom injection there was no increase in urine creatinine levels (**Figure 4K**). In contrast, after 6 h, mice injected with *Cdcasc* venom had significant increases in urine creatinine levels. At 9 h post injection, increased urine creatinine levels were significant in mice injected with *Cdcolli* or *Cdt* venom. Additionally, a significant increase in urine protein levels was observed 3, 6, 9, and 12 h after venom injection (**Figure 4L**).

Cdcolli venom induced an increase in prothrombin time of 120 sec, which is significantly different from the control group's prothrombin time of 28 sec. However, *Cdt* and *Cdcasc* venom did not increase coagulation time. With respect to activated partial thromboplastin time, *Cdcolli* and *Cdcasc* venom induced an increase of 240 sec, statistically different from the control group (92.5 sec, **Figure 4N**).

Cdt, *Cdcolli*, and *Cdcasc* venoms induced a time- and concentration-dependent blockade of indirectly evoked twitches

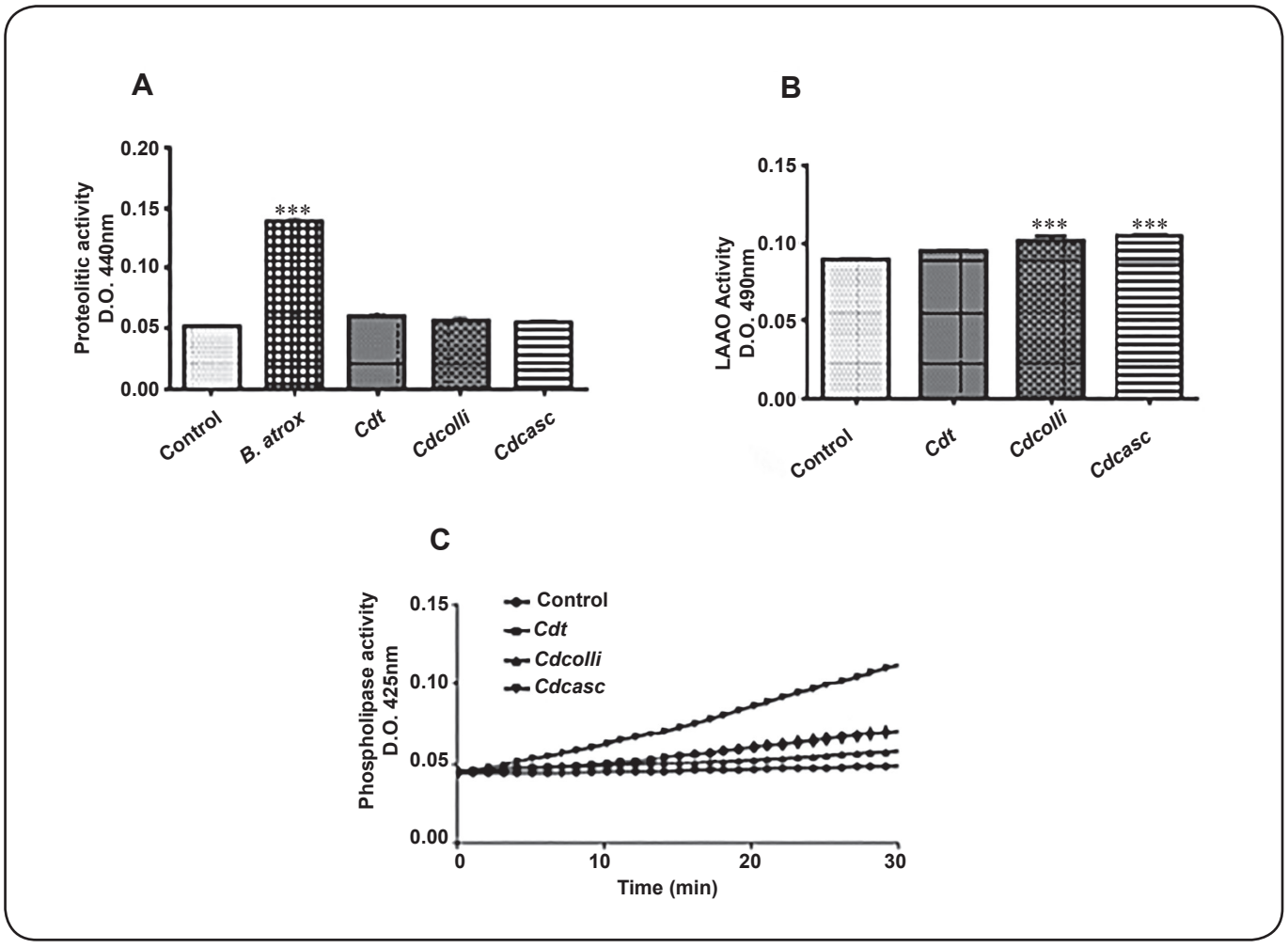

FIGURE 2: Biochemical characterization (A, B, C) and local effect (D) of *Cdt*, *Cdcolli*, and *Cdcasc* venoms. A) Proteolytic activity of *Cdt*, *Cdcolli*, and *Cdcasc* venoms on azocasein. B) Determination of L-amino acid oxidase activity of *Cdt*, *Cdcolli*, and *Cdcasc* venoms. C) Determination of phospholipase activity of *Cdt*, *Cdcolli*, and *Cdcasc* venoms. Results represent the mean ± SEM (n = 5). ***Values significantly different from control (p<0,001) (ANOVA).

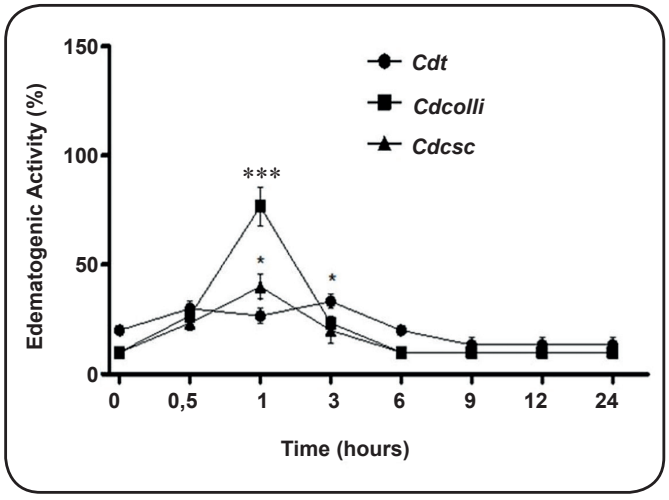

FIGURE 3: Edematogenic effect induced by *Cdt*, *Cdcolli*, and *Cdcasc* venoms in mice. The graph shows a time course of paw edema induced by *C. durissus* subspecies venoms (100 µg/kg) or sterile physiological saline. Results are expressed as means ± SEM (*** p <0.001 and * p <0.05) of percentage increase of paw volume compared to control (n=5) (ANOVA).

(**Figures 5A** and **5B**), and at the higher concentration studied (5 µg/mL), they also paralyzed the directly evoked twitches (**Figure 5C**). As shown in **Table 1**, the times required for 50% blockade (t_{50}) of indirect twitches were significantly lower than those necessary to blockade the direct contractions. While the blockade of indirect contraction is an unequivocal indicator of a neurotoxic activity, the blockade of direct contractions frequently denotes a myotoxic activity affecting muscle contractility.

DISCUSSION

Molecular exclusion chromatography of *Crotalus durissus* venom identified four major toxins: Cvx[15], Gvx[16], Ctx[17] and Ctm[18]. Therefore, *C. durissus* venom has a variable composition, and Ctm may or may not be present in it[19]. Venom composition is directly associated to age, sex, captivity, and the individual glands of a snake[20-22]. Ontogenetic and seasonal variations also contribute to molecular diversity and venom complexity[7,23].

Oliveira et al.[7] used proteomic and functional analyses of 22 *Cdcolli* individuals' venoms to explore their qualitative and

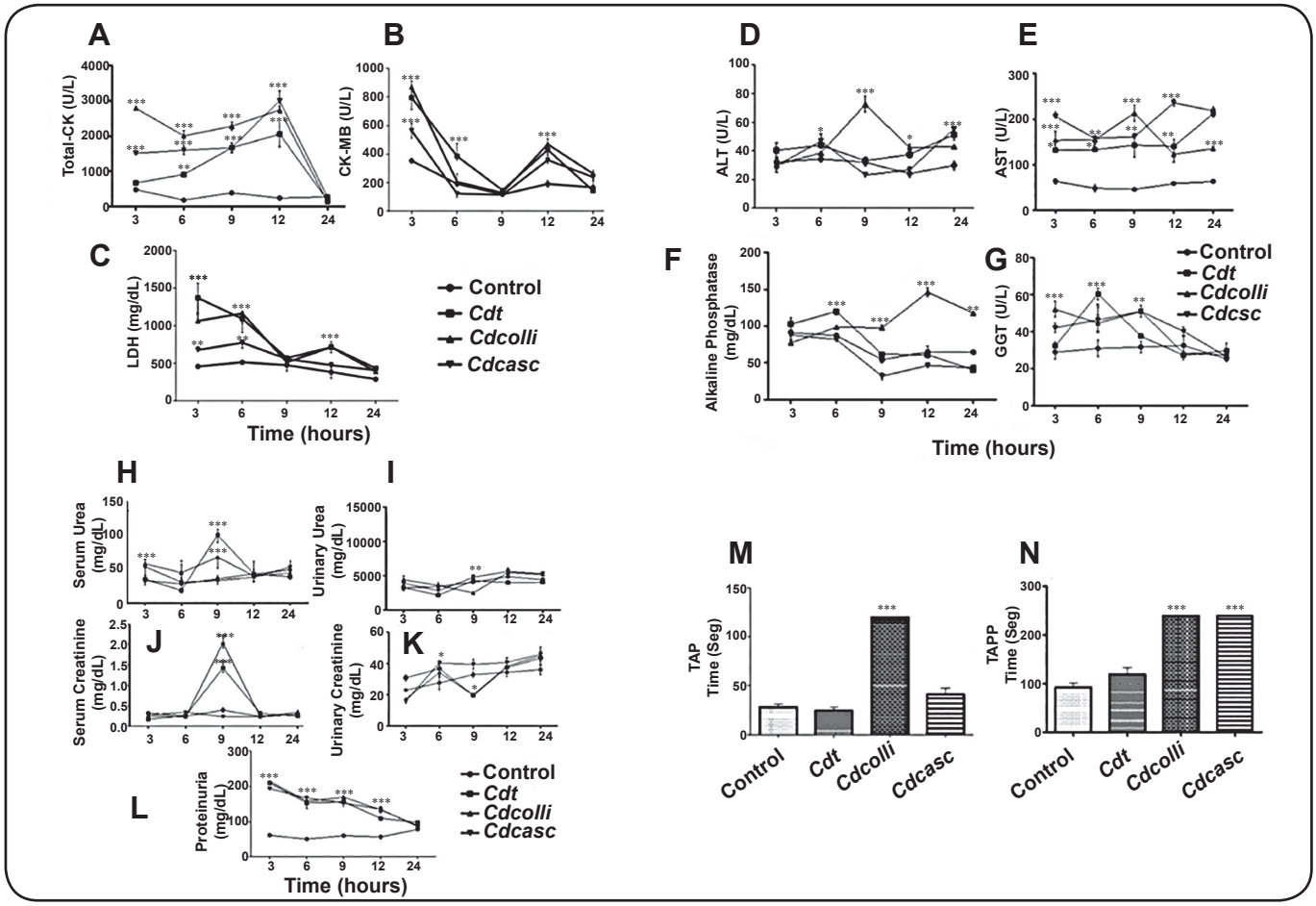

FIGURE 4: Myotoxic (A, B, C), hepatotoxic (D, E, F, G), nephrotoxic (H, I, J, K, L), and coagulation (M, N) effects of *Cdt*, *Cdcolli*, and *Cdcasc* venoms in Swiss mice. A) Total creatine kinase (CK) (Total CK); **B)** MB fraction CK (MB-CK); **C)** lactate dehydrogenase (LDH); **D)** alanine aminotransferase (ALT); **E)** aspartate aminotransferase (AST); **F)** alkaline phosphatase (ALP); **G)** *gamma*-glutamyl transferase (GGT); **H)** Serum Urea; **I)** Urinary Urea; **J)** Serum Creatinine; **K)** Urinary Creatinine; and **L)** Proteinuria. **M)** Prothrombin time (TAP) and **N)** activated partial thromboplastin time (TAPP). The results are expressed as the means ± SEM of 5 mice in each group. *p<0.05, **p<0.01, ***p<0.001 compared to the corresponding control group for each interval (ANOVA).

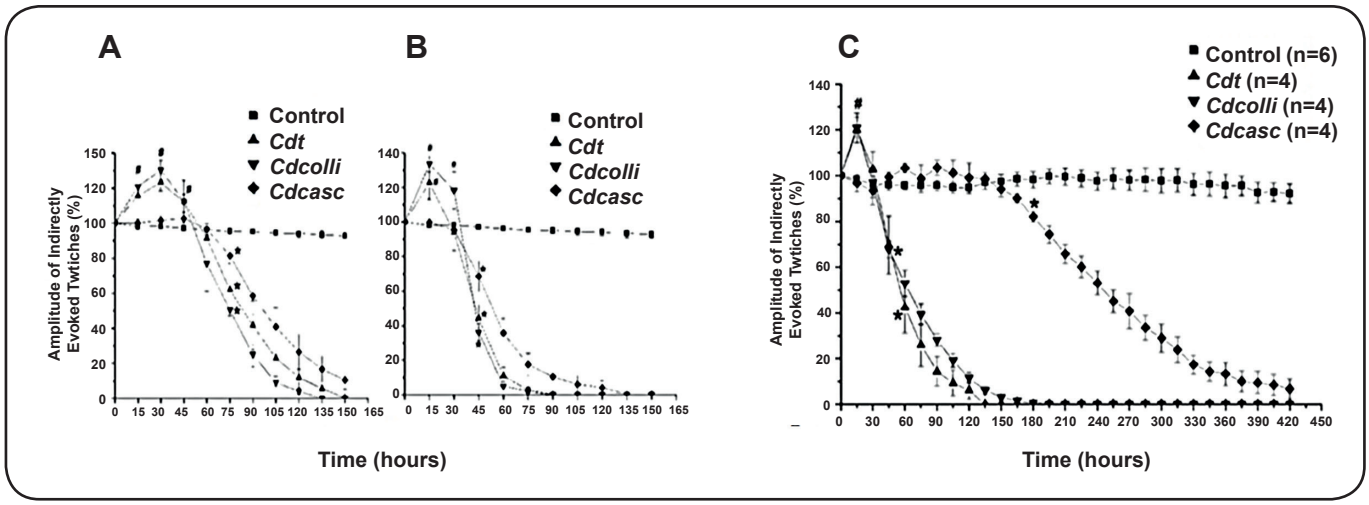

FIGURE 5: Effect of *Cdt*, *Cdcolli*, and *Cdcasc* venoms, at concentrations of 1 µg/mL (A) and 5 µg/mL (B) on indirectly and (C) directly evoked twitches in mice phrenic-diaphragm preparations. The ordinate represents the % amplitude of twitches relative to the initial amplitude.

TABLE 1: The time (min) required for 50% blockade (t_{50}) of indirectly and directly evoked twitches.

Experimental Group	Indirect		Direct
	1 µg/mL	5 µg/mL	5 µg/mL
C.d. terrificus Venom	84.36 ± 8.48 (n=4)	42.70 ± 2.3 (n=5) #	56.80 ± 6.43 (n=4) *
C.d. collilineatus Venom	71.20 ± 6.35 (n=6)	42.24 ± 0.88 (n=4) #	64.26 ± 4.01 (n=4) *
C.d. cascavella Venom	100 ± 7.79 (n=4)	57.96 ± 4.43 (n=6) #	249.6 ± 11.7 (n=4) *

#Indicates differences in t_{50} of indirectly evoked preparations exposed to the same venom at different concentrations (1 µg/mL vs 5 µg/mL). *Indicates differences in t_{50} between directly and indirectly evoked preparations exposed to the same venom and concentration (5 µg/mL).

quantitative variations. Moreover, these authors found that different *Cdcolli* venoms caused envenomings with different changes in biochemical and immunological parameters.

Lourenço et al.[24] found Ctm venom heterogeneity but did not observe a statistical difference in *C. durissus* venom proteolytic activity. The findings of this study are in accordance with our data.

Phospholipase A_2 (PLA_2) can be responsible for edema induction, since it is directly related to envenoming pathophysiology that accounts for various local and systemic disorders[25-27]. Furthermore, *Cdt*, *Cdcolli*, and *Cdcasc* venoms were uniform in relation to phospholipase activity with the substrate 4N3OBA. The capacity of some PLA_2s to recognize and act on specific targets can explain these differences. The same results were obtained by Santoro et al.[28] with *Cdcasc* and *Cdcolli*.

Unlike other viperid venoms, *Crotalus* venoms do not induce significant inflammatory reactions at the bite site in animals or humans[29,30]. However, there was a report of an edematogenic response induced by *Cdt* venom that was not dose-dependent and had a fast and transient course[41]. Some studies have reported that PLA_2 induced edema, an effect that in some cases are dependent on PLA_2s binding to specific membrane proteins[31].

Other than the association with edema induction, other studies have emphasized the participation of PLA_2s in myotoxicity caused by crotalic envenoming. A large part of this action at least is due to two components: Ctm and Ctx[32-34]. The toxicity induced by Ctx is generated by the CB subunit, which is a PLA_2[35].

Several biological activities including cytotoxicity; mild myonecrosis; apoptosis induction; platelet aggregation, induction, and/or inhibition; as well as hemorrhagic, hemolytic, edematogenic, antibacterial, antiproliferative, antiparasitic, and anti-HIV activities[36] have been attributed to LAAO. In 2015[37], a very small amount (1.8% of the total venom) of the first LAAO from *Cdt* yellow venom, Bordenein-L, was isolated. Few studies have been conducted to determine the mechanisms of action of LAAOs in the induction of edema compared to other classes of snake toxins. Studies investigating different toxins revealed that the action of these proteins is related to the release of inflammatory mediators such as histamine, prostaglandin, kinins, and serotonin[38]. However, the edematogenic activity of LAAOs does not seem to be mediated by the same mechanisms described for other toxins, since these enzymes do not lose their edematogenic activity in the presence of antihistamines.

There are essentially two clinical models for myotoxicity: local and systemic. For instance, rhabdomyolysis constitutes a generalized muscle breakdown and causes myoglobin and CK increases in circulation that may lead to renal dysfunction, which is one of the envenoming characteristics of crotalics[2,28,39].

Results showed that all *Crotalus* subspecies can induce alterations in CK (Total and MB) levels in different ways. The variance between the extravasation of Total-CK induced by crotalic venoms can be explained by the venom's PLA_2[40] and LAAO activity. *Cdcolli* had the highest activity in the muscular system, which differs from results found by Santoro et al.[28] that showed that *Cdt* had its highest activity in muscles. This could be the result of the two toxins involved in myotoxicity, Ctm and Ctx, working synergistically. Both are found in high concentration in *Cdcolli* venom.

The significant increase in MB-CK circulation caused by *Cdt* and *Cdcolli* venoms can be explained by PLA_2 action on the myocardium muscle, the occurrence of early lesions, and the release of mediators that contribute to delayed injury. However, Cupo et al.,[41] observed an absence of clinical signs of myocardiotoxicity and the presence of normal serial ECG and echocardiography. Siqueira et al.[42] reported myocardium damage in a victim bitten by a *C.d. terrific* snake. *C. durissus* venom's toxicity in heart muscle is controversial and poorly understood. It was observed by electrocardiogram changes[43] and acute myocardial infarction[41] in *Cdt* bitten patients. TOTAL-CK and MB-CK results in the present study are in agreement with the works mentioned above in which CK increases were verified in the first 2 to 3 h after venom inoculation. Additionally, the myotoxic activity results of our work are in line with the data of Saraiva et al.[44]. They measured mice plasma CK levels 3 h after *Cdt* venom intramuscular injection in the right gastrocnemius muscle. According to Barraviera et al.[45] in their retrospective study of *Crotalus* bitten victims, 100% of the analyzed patients presented increased CK levels.

Cupo et al.[41,43] showed that the LDH (heart fraction) concentrations in *Cdt* bitten patients were higher than those of other LDH isoforms. Rowlands et al.[46] performed autopsies on *Pseudechis australis* envenoming victims and observed rhabdomyolysis with necrosis foci in the myocardium. De Siqueira et al.[42] reported damage to the myocardium after *Cdt* envenoming. Autopsies showed diffuse edema myocytolysis and rare micro-infarcts foci.

Accordingly, it was suggested that the *Cdcasc* venom induced liver injury 6 h after inoculation, whereas *Cdcolli*

and *Cdt* venoms induced the same reaction after 9 h and 12 h, respectively. AST levels also changed after individual inoculation of these three venoms. These differences in activities triggered by the venoms can be attributed to Ctx actions and its PLA_2 subunit and/or LAAO action on specific sites in the hepatocytes membranes[47].

Elevation of ALT and AST may be related to side effects caused by biological factors released by tissue injury. ALP and GGT alterations are shown here reinforcing the hepatotoxicity induced by *Crotalus* envenoming. Barraviera et al.[48] showed a positive correlation between bromsulphalein retention and ALT serum levels as well as AST and ALT serum increase in *Cdt* bitten patients. The authors proposed that these alterations were associated with liver dysfunction. In other research, Barraviera et al.[45] performed an anatomopathological exam on a patient who died and were diagnosed with extensive hepatic necrosis. However, França et al.[47] measured rat serum levels of ALT, AST, ALP, and GGT after *Cdt* venom inoculation and showed acute hepatotoxicity. Snake venom from other families, such as Elapidae *Nana naja* venom, also induces liver changes[49]. However, the mechanisms involved in hepatic injury are still not understood, which makes them an important question in the field.

Given the creatinine excretion reduction caused by envenoming, it can be inferred that it is related to bloodstream retention. However, the presence of albumin in urine indicates kidney injury because this protein has a high molecular weight and is not able to cross the glomerular membrane in physiological conditions. Reduction or loss of kidney function, known as acute renal failure, is the main complication of crotalic envenoming. This condition can be attributed to systemic manifestations, dehydration, and especially myotoxicity. Furthermore, it can develop into a severe renal ischemia, leading to kidney function loss[2,39,50]. However, Amora et al.[51] used isolated rat kidney to find that Ctx and PLA_2 were involved in this process, since the renal effects observed would be due to the venom components' synergistic action.

Another systemic manifestation of crotalic envenoming is the alteration of the blood coagulation system[52]. These characteristics may be attributed to the enzymes present (serineproteases and metalloproteinases) that primarily affect the hemostatic system[53]. The difference in these enzymes expression patterns in these three subspecies might explain the different results shown in coagulation.

De Oliveira et al.[54] reported on the activity of fibrinolytic enzymes, mainly in the fibrinogen Bβ and Aα subunits in the *Cdt* and *Cdcolli* venoms. This is considered a characteristic of the serineproteases present in these venoms. Accordingly, the blood incoagulability observed in severe cases of *C. durissus* bites are derived from fibrinogen consumption[52].

The studied venoms were able to induce a contraction blockade. The blockade of indirect contraction is an unequivocal indicator of a neurotoxic activity, the blockade of direct contractions usually denotes myotoxic activity affecting muscle contractility[55].

C. durissus subspecies venoms contain large amounts of Ctx[6,28,56-58], a potent β-neurotoxin that induces neuromuscular transmission blockade and progressive muscle paralysis[59]. In addition, Ctx also induces *in vivo* and *in vitro* myotoxic activity, which may be underlying the blockade of direct muscle contractions induced by crotalic venoms[60-62]. Prior to the establishment of the neuromuscular blockade, *Cdt* and *Cdcolli* venoms induce initial facilitation of muscle contractions. This effect can be attributed to the presence of Ctm in both venoms[28,58]. This myonecrotic toxin binds to voltage-sensitive Na^+ channels on the skeletal muscle sarcolemma, leading to a large influx of Na^+ ions, which causes depolarization, strong contraction, injury, and myonecrosis[63-65]. In contrast, the absence of this molecule in *Cdcasc* venom may explain the t_{50} for direct evoked twitches, which was six times higher than those observed for the other venoms (**Table 1)**.

Taken together, the data obtained in this study created a new view of the intraspecific variation of local and systemic effects caused by *Cdt*, *Cdcolli*, and *Cdcasc* venoms. Based on this evidence and the changes in the liver, kidney, muscle systems, and coagulation induced by these envenoming processes, besides variations in protein and enzymatic composition, we can evaluate the differences between local and systemic effects caused by subspecies of *C. durissus*. This highlights the clinical and biochemical effects produced by their respective venoms. The differences in some pharmacological activities observed in our study are in accordance with published data[6,66,44].

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Conflict of Interest

The authors declare that they have no competing interests.

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