Histochemical analyses of muscle injury induced by venom from Argentine *Bothrops alternatus* (víbora de la cruz)*

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Abstract

Garcia Denegri, M.E.; Rey, L.; Leiva, L.; Acosta de Pérez, O.: ***Histochemical analyses of muscle injury induced by venom from Argentine Bothrops alternatus (víbora de la cruz).*** *Rev. vet. 17: 2, 67–71, 2006.* Histochemical methods were used to study necrosis of skeletal muscle fibers induced by *Bothrops alternatus* snake venom from Argentina. Rats with a body weight between 220–270 g, were used. Animals received an i.m. venom injection (800 µg) in the gastrocnemius. To determine creatinphosphokinase activity (CPK), blood samples were taken from the tail 60 min, 3, 6, 12 and 24 h after the envenoming. About 24 h later, rats received chloral hydrate anesthesia for histological analysis with Hematoxilin–Eosin (H–E) stain, and histochemical studies such as lipid peroxidation (Schiff's reaction), and calcium precipitation (alizarin red stain). Results showed an increment in plasma CPK level, with its major peak at 3 h. Histochemical analyses revealed an intense destruction of muscular fibers as a consequence of a significant lipid peroxidation and calcium precipitation as well. Histochemical methods can be considered as a valuable tool in applied research regarding toxicological problems such as snake venom intoxication. It can be concluded that *B. alternatus* snake venom leads to a lipid peroxidation accompanied by citoplasmatic calcium precipitation. In addition, it was demonstrated that H–E stain made on frozen cuts (histochemical technique) is effective to evidence a panoramic tissular view of muscular lesion caused by *B. alternatus* venom, with the advantage of demanding a shorter execution lapse (few hours) in relationship to classic H–E histological technique, which requires several days of procesing.

Key words: *Bothrops alternatus,* muscle injury, histochemical analyses.

Resumen

Garcia Denegri, M.E.; Rey, L.; Leiva, L.; Acosta de Pérez, O.: ***Análisis histoquímicos del daño muscular inducido por veneno de Bothrops alternatus (víbora de la cruz) de Argentina.*** *Rev. vet. 17: 2, 67–71, 2006.* Se emplearon métodos histoquímicos para estudiar la necrosis de fibras musculares esqueléticas inducida por veneno de *Bothrops alternatus* de Argentina. Se utilizaron ratas con peso corporal de 220–270 g. Estos animales recibieron 800 µg de veneno vía i.m. en el músculo grastrocnemio. Las muestras de sangre fueron tomadas de la cola a los 60 min, 3, 6, 12 y 24 horas luego del envenenamiento, para realizar determinaciones de creatinfosfoquinasa (CPK). Luego de 24 horas, las ratas recibieron anestesia con hidrato de cloral para realizar análisis histológicos con tinción Hematoxilina–Eosina (H–E) y también estudios histoquímicos tales como peroxidación de lípidos (reacción de Schiff) y precipitación de calcio (tinción rojo de alizarina). Los resultados mostraron un incremento de los niveles de CPK, cuyo acmé se registró a las 3 horas post–envenenamiento. Las pruebas revelaron una intensa destrucción de fibras musculares como consecuencia de una significativa peroxidación de lípidos y también por precipitación de calcio. Se concluye que el veneno de *B. alternatus* conduce a peroxidación de lípidos acompañada de precipitación citoplasmática de calcio. Además, se demuestra que la tinción H–E efectuada en cortes por congelación (técnica histoquímica) es eficaz para evidenciar una vista tisular panorámica de los cambios musculares causados por el veneno de *B. alternatus*, con la ventaja de exigir un lapso de ejecución más corto (pocas horas), con relación al método H–E clásico (técnica histológica), la cual exige varios días de procesamiento.

Palabras clave: *Bothrops alternatus,* daño muscular, análisis histoquímicos.

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INTRODUCTION

Local tissue damage induced by *Bothrops alternatus* snake venom includes edema, myonecrosis, hemorrhage, and an inflammatory response associated with a prominent cellular infiltration [1, 2, 19]. Myonecrosis is the most prominent short–term effect following the envenoming by *B. alternatus* snake in the Northeast area of Argentina, and the loss of muscle mass with a consequent muscle deficit is a common sequela [2]. Venom of *B. alternatus* from Argentina has myotoxins that induce necrosis in the inoculated site [17] and in cardiac muscle fibers [21].

Other researchers observed pathological changes in muscle cells during early hours after the injection of toxins of snake venom in rats. Earliest injuries consisted in local disruptions at the plasma membrane of muscular cells and hypercontraction of the myofilaments. About 3 to 6 hours after injection the necrotic cells were invaded by phagocytic cellular infiltration [12].

In previous trials, it was demonstrated that the presence of lisolecitin in extract of injected muscle exposed to notexin had effects on phospholipids not only i*n vitro* but also *in vivo,* where notexin was capable of hydrolysis of phospholipids [13]. On the other hand, it was proposed that the primary action site of phospholipase on the muscle is the plasma membrane, and certainly this action is related to hydrolysis of phospholipids [10].

In addition, disruption of intracellular calcium homeostasis leading to a sustained increase in cytosolic Ca^{2+} level, has been associated with cell killing in a variety of studies [8, 9]. Xenobiotics induce a lipid peroxidation which is an oxidative disarrangement producing lipid peroxides or hydroperoxydes and their respective radicals [5]; the same mechanism is evidenced in the skeletal muscle of the injected mice with snake venom [3].

The objective of this trial was to evaluate muscular fiber destruction caused by *B. alternatus* venom, with histochemical methods. It was also foreseen to evaluate the utility of Hematoxilin–Eosin (H–E) stain in frozen cuts, to shorten time of the whole process, and to allow a complete observation of the tissular changes.

MATERIAL AND METHODS

B. alternatus venom was purchased from the serpentarium of a local Zoo, in Corrientes, Argentina. Crude venom was lyophilized, then kept frozen at –20ºC. When required, venom was diluted with phosphate buffered saline solution (PBS, pH 7.2). The small amount of insoluble material was centrifuged and the supernatant was used.

Sixteen Wistar male rats (220–270 g body weight) were used. Food (chow rat diet) was withdrawn 12–14 h before the experiment, but animals had free access to water. Animal room temperature was 23±2ºC, and the relative humidity ranged between 35 and 65%. Lights were on from 6 a.m to 6 p.m.

Twelve rats were i.m. injected in the right gastrocnemius muscle with 800 µg of venom dissolved in 0.1 ml of phosphate buffered saline solution (PBS, pH 7.2). Four rats were used as control, each receiving 0.1 ml of PBS. After 30 min, 3, 6, 12 y 24 hours blood samples were taken from the caudal vein without anticoagulant. Serum was obtained to analyze the activity of creatinphosphokinase (CPK), using the UV–kinetic method (Randox).

All animals were euthanized at 24 h; previously, cloral hidrate was administered (300 mg/kg i.p.) for anaesthesia. Tissue samples of the injected muscle were taken, and then frozen at –80°C. Then, they were cut using a cryostat and mounted on glasses for H–E staining (n = 4).

Histochemical techniques for detection of lipid peroxidation and calcium precipitation were performed. Lipid peroxidation (n = 4) was based on the direct Schiff's reaction [18], and calcium detection (n = 4) was based on alizarin red [15].

RESULTS AND DISCUSSION

Snake envenomation of the genus *Bothrops* (Viperidae) causes severe local pain, swelling, hemorrhage and necrosis of the bitten limb, which may cause permanent disability, or require amputation [7]. Non–clotting blood and systemic hemorrhage are usually associated with viperid envenomation [1]. Hemorrhagic metalloproteinases from *B. alternatus* venom induce bleeding due to proteolytic degradation of extracellular matrix components, in addition to degradation and rupture of endothelial cells in capillary blood vessels [11].

Present study states that *B. alternatus* venom causes histopathological changes in the gastrocnemius muscle after an i.m. administration. Light microscopy and H–E stain showed that gastrocnemius muscle from rats injected with PBS had a normal morphology (Figure 1). Twenty four hours after administration of 800 µg of *B. alternatus* venom, histologic observation showed necrosis of musclar fibers in the gastrocnemius, evidenced by nuclear pyknosis and fragmentation of the myofibers into homogeneous eosinophilic masses, separated by blank segments using the same staining

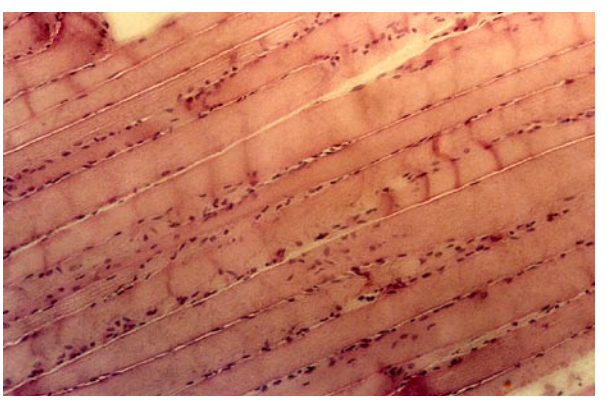

Figure 1. Gastrocnemius muscle section from a rat 24 h after saline solution administration. Sections were cut with a cryostat. Note that muscular fibers are in their original extended position. H–E 140x.

method. Hemorrhage was observed at the periphery of necrotic muscle, neutrophils and macrophages were seen around and inside of necrotic muscle fibers (Figure 2), associated with abundant edema. These results were coincident with the increase of CPK levels, which highest peak was at 3 h (Figure 3).

Local tissue damage induced by *Bothrops* snake venom includes an inflammatory response associated with a prominent cellular infiltrate, as it can be observed in this work; neutrophils play a significant role in the phagocytosis of necrotic material as well as in the recruitment of other inflammatory cells; both events are associated with a successful muscle regenerative response [20]. Similar effects were observed with the same venom on previous assays [2, 14] as well as with *B. jararaccussu* venom [16] and *B. asper* [3] venoms.

Cell injury is considered as a perturbation that aparts cell form its normal homeostasis. If a cell is able to restore its equilibrium, not necessarily the most favorable but compatible with cellular integrated activity, it may result reversibly damaged and will survive. If it does not succeed, the cell will die and damage will be lethal. Cell injury caused by *B. alternatus* venom was evident because of the increment in the activity of CPK, with its major peak at 3 h (874 ± 14 U/L) after injection.

Free radicals are thought to be involved in many toxic processes, among them the intoxications [6]. Free radicals are atoms or molecular structures having an impaired electron (in their outermost orbital); they are usually very reactive. The latter is due to the electronic deficiency they have in the outermost orbital. They are electrophilic, thereby during chemical reactions they tend to restore the vacant bond [4].

Their exceptional reactivity is responsible for the ability to interact with a variety of molecules which might be in their immediate neighborhood, or with other free radicals. The more relevant reactions of free radicals are: hydrogen abstraction, addition, disproportionation and cancellation. The most frequently phenomenon involved with pharmacological or toxicological properties of free radicals or free radical–forming xenobiotics are the addition and abstraction reactions of hydrogen. Schiff positive areas presented well circumscribed foci, as shown in Figure 4. The direct Schiff's reaction detects cellular aldehydes in a sensitive, rapid, histologically and topographically estimable way. The

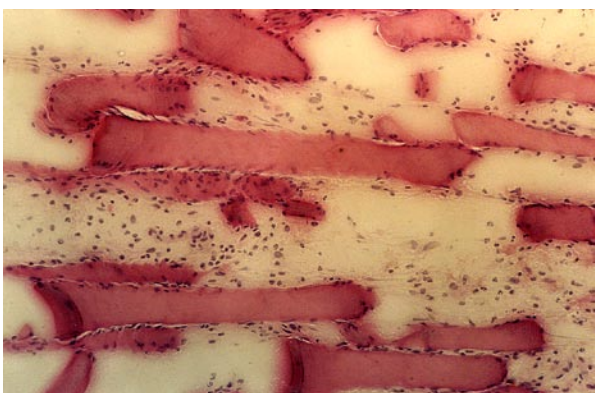

Figure 2. Gastrocnemius muscle section from a rat 24 h after i.m. injection of *B. alternatus* venom. Note the presence of several necrotic muscle cells with evident disorganization of myofibrillar material. Polymorphonuclear infiltrate and edema are indicated by the increased spaces between muscle cells. H–E 140x.

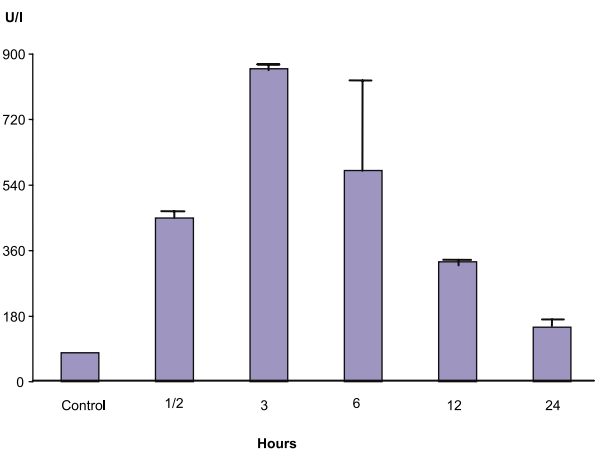

Figure 3. CPK activities from injected rats with 800 µg of *Bothrops alternatus* venom versus controls. Values are expressed in $\bar{x} \pm$ DE.

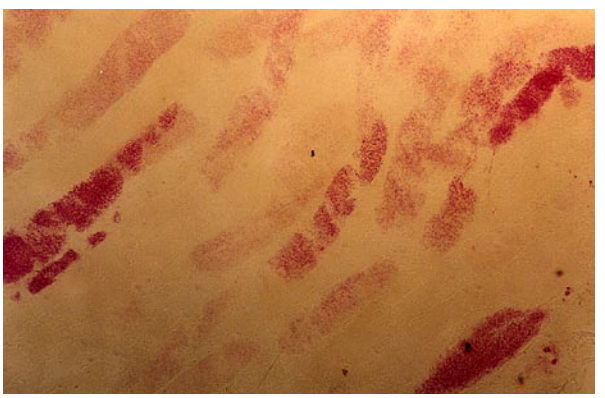

Figure 4. Gastrocnemius muscle section from a rat 24 h after i.m. injection of *B. alternatus* venom. Direct Schiff´s reaction detects cellular aldehydes in a sensitive, rapid, histologically and topographically estimable way. A variation of shade in muscle sections is evident. 140x.

Figure 5. Gastrocnemius muscle section of a rat 24 h after saline solution administration. Note the normal disposition of calcium. Alizarin red staining. 140x.

appearance of these aldehydes precedes distinctly morphological alterations detectable by histochemical techniques. No positive results were obtained in controls. Histochemical detection of aldehydes may give useful information on different aspects of tissue and organ intoxication, such as their topography, appearance, evolution, extension, consequences, and effects of treatment [18].

The cells are destroyed by a process that involves at least two steps. In each type of injury, disruption of the integrity of the plasma membrane occurred, as lipid peroxidation mentioned before. Another different mechanism is followed by a common functional consequence involving extracellular calcium, and most likely represented an influx of calcium across the damaged plasma membrane and down steep concentration gradient. The latter represents, or at least initiates, a final common pathway for the toxic death of these cells [17]. In this work, the distribution of calcium in a control animal section (Figure 5) was homogenous. Histological sections of muscle fibers affected by *B. alternatus* venom showed calcium accumulations (Figure 6), that significantly altered the normal muscular architecture pattern.

We conclude that histochemical methods provided a rapid evaluation for specific muscular lesions, and H–E stain performed on frozen cuts (histochemical technique) was effective to evidence a panoramic tissular view of muscular lesion caused by *B. alternatus* venom, with the advantage of demanding a shorter execution lapse (few hours) in relationship to classic H–E histological technique, which requires several days of work.

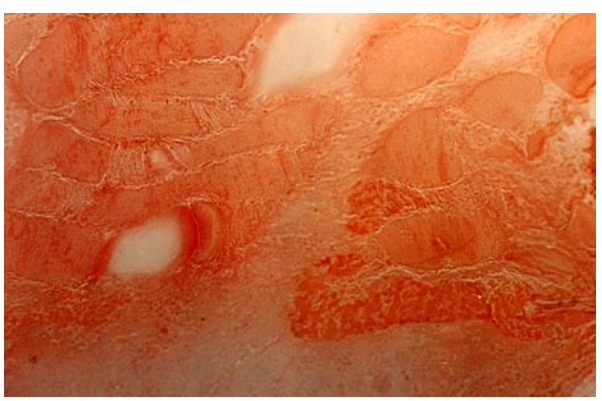

Figure 6. Gastrocnemius muscle section of a rat 24 h after i.m. injection of *B. alternatus* venom. Note intense calcium accumulation in muscular fiber. Alizarin red staining. 140x.

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