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# Changes in basement membrane components in an experimental model of skeletal muscle degeneration and regeneration induced by snake venom and myotoxic phospholipase $A_2$

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ABSTRACT

Skeletal muscle regeneration is impaired after myonecrosis induced by viperid snake venoms, but the mechanisms behind such poor regenerative outcome are not fully understood. This study compared the changes in basement membrane (BM) components in mouse skeletal muscle in two different scenarios of muscle injury: (a) injection of *Bothrops asper* venom, as a model of poor regeneration, and (b) injection of a myotoxic fraction (Mtx) isolated from this venom, as a model of successful regeneration. The degradation and reposition of laminin, type IV collagen and fibronectin were assessed over time by a combination of immunohistochemistry, Western blot, and real time polymerase chain reaction. Both treatments induced degradation of laminin and type IV collagen in areas of muscle necrosis since day one, however, there were differences in the pattern of degradation and reposition of these proteins along time. Overall, Mtx induced a higher synthesis of fibronectin and higher degradation of laminin at intermediate time points, together with higher levels of transcripts for the chains of the three proteins. Instead, venom induced a higher degradation of laminin and type IV collagen at early time intervals, followed by a reduced recovery of type IV collagen by 15 days. These differences in extracellular matrix degradation and remodeling between the two models could be associated to the poor muscle regeneration after myonecrosis induced by *B. asper* venom.

## 1. Introduction

Skeletal muscle regeneration is a complex and finely regulated process, which occurs in a variety of circumstances associated with acute and chronic muscle damage. It involves the activation, proliferation and fusion of myogenic cells in muscle tissue, in close coordination with an inflammatory process involving the recruitment of neutrophils and macrophages, and the synthesis of cytokines, chemokines and growth factors (Chargé and Rudnicki, 2004). Along this process, the extracellular matrix (ECM) plays a key role in demarcating cellular niches, storing growth factors, regulating the migration of myoblasts and inflammatory cells, and releasing fragments with paracrine activities (Grounds, 2008; Pozzi et al., 2017). The turnover of ECM during muscle regeneration is a finely regulated process involving endogenous proteinases and synthesis of ECM proteins and proteoglycans by various cell types (Goetsch et al., 2003; Grounds, 2008). In addition to changes occurring in the interstitial matrix, the basement membrane (BM) plays a key role in the regenerative process, serving as a scaffold which demarcates the space where myogenic cells replicate and fuse (Grounds, 2008; Mackey and Kjaer, 2017; Vracko and Benditt, 1972).

In a variety of diseases and experimental models involving acute muscle damage, the regenerative process proceeds successfully and, after several weeks, muscle tissue regains its basic structural and functional properties. However, in other disease conditions, such as

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muscular dystrophies, ischemic muscle damage, inflammatory myopathies or muscle strain lesion, muscle regeneration is severely limited, and muscle is replaced by fibrotic tissue, with the consequent deleterious implications in muscle function (Ciciliot and Schiaffino, 2010; Harris, 2003; Karpati and Molnar, 2008). Several requirements should be met for a successful regenerative process, such as (a) an adequate blood supply, (b) an intact nerve supply, (c) an inflammatory reaction that ensures the removal of necrotic muscle fibers and the synthesis of cytokines and growth factors, and (d) the conservation of the structure of the BM around damaged muscle fibers, which serves as a scaffold for orderly regeneration (Ciciliot and Schiaffino, 2010; Gutiérrez et al., 2018; Slater and Shiaffino, 2008; Vracko and Benditt, 1972).

Snakebite envenoming is a neglected tropical disease of high impact on a global level, particularly in impoverished rural settings of sub-Saharan Africa, Asia and Latin America (Gutiérrez et al., 2017). Envenomings caused by snakes classified in the family Viperidae (vipers and pit vipers) often cause a pronounced skeletal muscle necrosis at the anatomical site of venom injection (Gutiérrez et al, 2009, 2017; Warrell, 2004, 2010). In many cases, an incomplete process of muscle regeneration ensues, ending up in permanent muscle tissue loss, fibrosis, and functional disabilities (Gutiérrez et al., 2017; Jayawardana et al., 2016; Otero et al., 2002; Waiddyanatha et al., 2019). Viperid venoms contain toxins, generally phospholipases $A_2$ ($PLA_2$s) or $PLA_2$ homologues devoid of enzymatic activity, which cause direct muscle damage by disrupting the integrity of the plasma membrane of skeletal muscle fibers (Gutiérrez et al., 2009; Montecucco et al., 2008). In addition, these venoms are rich in zinc-dependent metalloproteinases (SVMPs) (Lomonte et al., 2014), which cause hemorrhage due to hydrolysis of key structural components in the BM of capillary blood vessels (Escalante et al., 2011b).

Previous studies have shown that muscle regeneration after injection of purified venom myotoxic components, such as $PLA_2$s and cardiotoxins/cytotoxins, occurs successfully and ends up in the generation of a population of regenerative muscle fibers that restores form and function of the affected muscle (Gutiérrez et al, 1984a, 1991; Harris et al., 2003; Hernández et al., 2011; Williams et al., 2019). Noteworthy, these myotoxins do not affect the blood supply and nerves, nor do they degrade BM structure. In contrast, after necrosis induced by injection of crude viperid venoms, such as that of *Bothrops asper*, muscle regeneration is impaired, in association with disruption of the capillary network, damage to intramuscular nerves and degradation of the BM, together with an altered inflammatory process in which cells arrive late and removal of necrotic material is limited (Arce et al., 1991; Gutiérrez et al., 1984a; Hernández et al., 2011; Queiróz et al., 1984). It has been proposed that such impaired regenerative process has to do with the action of hemorrhagic SVMPs which, by disrupting the microvasculature, cause ischemia and preclude the timely arrival of inflammatory cells (Arce et al., 1991; Gutiérrez et al, 1984a, 2018; Hernández et al., 2011). The comparative analysis of these two different experimental models of muscle regeneration, i.e. after myonecrosis induced by crude *Bothrops asper* venom and by myotoxic $PLA_2$s, constitutes a useful platform to investigate why regeneration is successful in one case and incomplete in the other.

One particular aspect that has received relatively little attention has to do with the alterations in the ECM, particularly the BM, in muscle necrosis and regeneration caused by snake venoms and toxins. In this study we have compared the alterations occurring in several proteins of the BM of muscle injected with crude *B. asper* venom and a myotoxic $PLA_2$ fraction, using three experimental approaches, i.e. immunochemical analysis of changes in BM proteins, immunohistochemical analysis of the changes occurring in the BM surrounding necrotic muscle fibers, and analysis of the expression of type IV collagen, laminin and fibronectin in muscle injected with venom or $PLA_2$. Results highlight differences in the degradation and regeneration of these proteins, which could be relevant for the explanation of the drastically different regenerative outcomes in these two models of myonecrosis.

## 2. Materials and methods

### 2.1. B. asper *venom and myotoxin*

The venom used was a pool obtained from more than 40 adult specimens of *B. asper* collected in the Pacific region of Costa Rica and kept at the serpentarium of Instituto Clodomiro Picado (Universidad de Costa Rica, San José, Costa Rica). Once obtained, the venom was lyophilized and stored at −20 °C until used. A myotoxic fraction (myotoxin, Mtx) was isolated from the venom by ion-exchange chromatography (CM-Sephadex C-50) as described (Gutiérrez et al., 1984b). This fraction contained a mixture of the two types of basic myotoxins present in the venom (Gutiérrez and Lomonte, 1995), i.e. catalytically-active Asp49 $PLA_2$ and catalytically-inactive Lys49 $PLA_2$ homologue, in a 1:2 approximate molar ratio. This myotoxic fraction was used to reproduce the combined action of these molecules in the crude venom, owing to their synergistic effect (Cintra-Francischinelli et al., 2009; Mora-Obando et al., 2014). Venom and myotoxin solutions were prepared immediately before injection in sterile 0.12 M NaCl, 0.04 M phosphate, pH 7.2 (PBS), and filtered through 0.22 μm membranes.

### 2.2. *Ethical statement*

Experimental protocols involving the use of animals meet the International Guiding Principles for Biomedical Research Involving Animals (CIOMS). These experiments were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUA) of the University of Costa Rica (approval number CICUA 008–16). Experiments were performed in CD-1 female mice of 6–8 weeks (18–20 g).

### 2.3. *Model of acute muscle injury*

A model of venom and toxin-inducedmuscle injury on mouse gastrocnemius muscle was used to study the changes on ECM proteins during the process of muscle regeneration (Hernández et al., 2011). Groups of four CD-1 mice were injected in the right gastrocnemius with either crude *B. asper* venom (75 μg) or Mtx (75 μg), dissolved in 50 μL PBS. A group of mice injected only with PBS was included as a control. Animals were sacrificed by $CO_2$ inhalation at different times after injection (1, 5 and 15 days for histology and immunohistochemistry, 1, 3, 5, 7, and 15 days for Western blot analysis, and 1 and 5 days for qPCR). Injected muscles were dissected out and processed as described in the following sections.

### 2.4. *Histology and immunohistochemistry*

Resected muscles were placed in zinc fixative solution (BD Biosciences, San Jose, CA) for 48 h, followed by routine processing protocols for embedding in paraffin. Several transversal non-sequential sections of 4 μm were prepared for each sample and placed in positively charged slides (ThermoScientific, Waltham, MA). In order to evaluate the histopathological changes at the different time intervals, a set of slides was stained with hematoxylin-eosin for light microscopic observation. To assess the alterations in ECM proteins, another set of slides was immunostained with anti-type IV collagen antibody or anti-laminin α2 chain antibody. Briefly, the sections were subjected to antigen retrieval with proteinase K for 3 min followed by blocking solution for 30 min. Next, tissues were incubated with anti-type IV collagen antibody at a dilution of 1:3000 (Abcam ab6586; Cambridge, MA) or anti-Laminin α2 chain antibody at a concentration of 3.75 μg/mL (Abcam ab11576). Primary antibodies were detected with biotinylated secondary antibodies (Dako, Glostrup, Denmark), enhanced by a Tyramide signal amplification kit (PerkinElmer, Waltham, MA) and visualized with Alexa Fluor 555-Streptavidin conjugate (ThermoFisher, Waltham, MA). Images were captured with a Cool SNAP-Pro camera (Media Cybernetics, Rockville, MD).

### 2.5. Immunodetection of fibronectin, collagen IV and laminin in muscle homogenates by Western blot

Resected muscles of animals treated with venom, Mtx or PBS were frozen and stored at −70 °C until used. Homogenization was performed as previously described (Saravia-Otten et al., 2013). In short, muscles were placed in 1 mL of cold extraction buffer (25 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.1% SDS and 20 mM EDTA [pH 7.6]) supplemented with complete protease inhibitor cocktail tablets (Roche Diagnostic, Mannheim, Germany) and incubated 1 h at 4 °C with mild stirring. After centrifugation at 6000*g*, supernatants were immediately pooled and stored at −70 °C until analysis. Total protein concentration was quantified with Dc Protein Assay (Bio-Rad, Hercules, CA).

The presence of fibronectin, collagen IV, and laminin α2 in muscle homogenates was analyzed by Western blot. Briefly, samples containing either 75 μg total protein for collagen IV; 50 μg for fibronectin; or 15 μg for laminin α2 were separated under reducing conditions on 6–15% Tris-HCl polyacrylamide gradient gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Burlington, MA). Immunodetection was performed by incubating the membranes with either rabbit polyclonal anti-type IV collagen at a dilution of 1:500 (Abcam, ab6586), rabbit polyclonal anti-fibronectin at a dilution 1: 3000 (Abcam, ab23750) or rabbit polyclonal anti-laminin α2 at a dilution 1: 1000 (Abcam, ab140482). A monoclonal mouse anti-GAPDH at a dilution of 1:5000 (Calbiochem, San Diego, CA) was used as loading control. Following incubation with primary antibodies, anti-rabbit (Abcam) or anti-mouse (Invitrogen, Carlsbad, CA) HRP-conjugated secondary antibodies were added. The protein bands were visualized using a chemiluminescent detection kit (Lumi-Light$^{plus}$ Western Blotting Substrate; Roche, Basel, Switzerland) and exposed to X-ray film. The intensity of immunoreactive bands representing the full-length forms of fibronectin, type IV collagen and laminin α2 was analyzed by densitometry and a relative quantification was performed adjusting each sample to the corresponding control.

### 2.6. Quantification of ECM proteins transcripts on muscle homogenates by real time PCR (qPCR)

The matrix component transcripts were assayed by qPCR using primers listed in Table 1 and previously designed with PRIMER3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Gastrocnemius muscles from mice injected with PBS, venom or Mtx were dissected out and immediately placed in RNAlater solution (ThermoFisher Scientific, Waltham, MA). RNAs extraction was performed following the instructions included in GeneJET RNA purification Kit (K0731, ThermoFisher Scientific, Lithuania) for tissues. Total RNA was purified from DNA contamination with DNase I treatment (EN0521, ThermoFisher Scientific, Lithuania). The correspondent cDNAs were produced using both oligo (dT)18 and random primers by following the instruction of RevertAID H Minus First strand cDNA synthesis Kit (K1632, ThermoFisher Scientific, Lithuania). qPCR reactions were carried out with 100 ng of cDNA template using SYBR Green Master Mix (A25741, Life Techniologies, Carlsbad, CA, USA) in a 20 μl final volume. The analysis was performed with QuantStudio 3 Real-Time PCR Systems, with the following cycling program: initial 50 °C 2 min, denaturation 95 °C 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A final step of melting curve between 65 °C and 90 °C, 1 °C/s temperature speed was incorporated. Relative fold change relative to housekeeping control gene (HPRT1-hypoxanthine phosphoribosyltransferase 1) was calculated as $2^{-\Delta Ct}$ where: $\Delta C_t = C_t$ (target) - $C_t$ (HPRT1), according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guideline 54. All samples were analyzed in triplicate from three independent animals, which underwent the same treatment.

### 2.7. Statistical analysis

One-way analyses of variance (ANOVA) and Tukey test were used to compare multiple groups; $p < 0.05$ was considered statistically significant. Data are presented as average ± standard deviations (SDs).

## 3. Results

### 3.1. Histopathological observations

Sections of control muscle injected with PBS showed a normal tissue structure, with homogeneous bundles of muscle fibers with peripheral nuclei within a normal connective tissue matrix in endomysium, epimysium and perimysium. Arteries, veins and nerves also had a normal structure (Fig. 1). Mtx treatment induced extensive necrosis of muscle fibers, together with an inflammatory infiltrate, which was evident one day after injection (Fig. 1). At this time point, venom-treated muscle showed hemorrhagic lesions with groups of erythrocytes in the interstitial space, in addition to necrotic muscle fibers and inflammatory cells. By day five, Mtx-injected tissue had extensive areas of immature regenerating muscle fibers characterized by a basophilic cytoplasm and centrally-located nuclei. Few remaining necrotic fibers were also observed, together with limited areas of fibrosis. In contrast, sections of venom-treated muscle showed a higher extent of remaining necrotic fibers, as compared to Mtx-injected tissue, and regenerating muscle fibers of varying sizes surrounded by abundant connective tissue (Fig. 1). After 15 days, sections from Mtx-injected muscle presented extensive areas of regenerated muscle fibers homogeneously distributed and of similar sizes of those of control tissue. These cells were characterized by

**Table 1**
Primers used for the quantification of transcripts of ECM proteins fibronectin (Fn), laminin (Lam) and type IV collagen (Col 4 A).

| | Forward | Reverse | Size (bp) | mRNA accession Nº |
|---|---|---|---|---|
| Fn1 Nt | AGCCTGGCTGTTTTGACAAT | GCTTCCTCCATAGCAGGTACA | 101 | NM_010233.2 |
| Fn1 EDA | CCCAGCTCACTGACCTAAGC | TGCCGCAACTACTGTGATTC | 113 | NM_010233.2 |
| Fn1 EDB | ACTGGCATTCACTGATGTGG | CATCCTCAGGGCTCGAGTAG | 104 | NM_010233.2 |
| Fn1 IIICS | TTCCACACCCCAATCTTCAT | CCATTTTCGGTGTCATACCC | 100 | NM_010233.2 |
| Fn1 Ct | TGACAACTGCCGTAGACCTG | TCAATGGGGCAATTTACGTT | 123 | NM_010233.2 |
| Lam alpha1 | GAGTCCCCCAGTGTGACTGT | ATGTTCCTGGCCCTGTAGTG | 105 | NM_008480.2 |
| Lam alpha 2 | ACTTGACCATCGACGACTCC | CTGGCTTTGGGTCCATCTAA | 100 | NM_008481.2 |
| Lam beta1 | AGGCCTGAACTGTGAGCTGT | CCAGTAGCCAGGAAGACTGC | 105 | NM_008482.2 |
| Lam beta2 | TCGTGGACTCAACTGTGAGC | GTGTTGTGCTGACACCCATC | 105 | NM_008483.3 |
| Lam gamma 1 | GAGTCCATGAGGAAGCCAAG | GTCAATCAGACGGTCCAGGT | 110 | NM_010683.2 |
| Col4Aalpha1 | GGTGAAAGGGGAGAAAAAGG | TCCAGGTTGACACTCCACAA | 105 | NM_009931.2 |
| Col4Aalpha2 | AGTGTGAGCATCGGCTACCT | TAGCAGGCTGTACCCACTCC | 102 | NM_009932.4 |
| Col4Aalpha3 | AGGATGGGAAACCAGGAACT | TTTAAGCCTGGCATTCCATC | 103 | NM_007734.2 |
| Coil4Aalpha4 | AGATCCAGGCATCAAAGGTG | CTCCTGGAAGACCCCTCTCT | 105 | NM_007735.2 |
| Col4Aalpha5 | CAGATTTCCTGGGATCGAAA | TGATCCTGGTTGTCCACTCA | 110 | NM_001163155.1 |
| HPRT1 | TGCTGACCTGCTTGGATTACA | TATGTCCCCCGTTGACTGAT | 105 | NM_013556.2 |

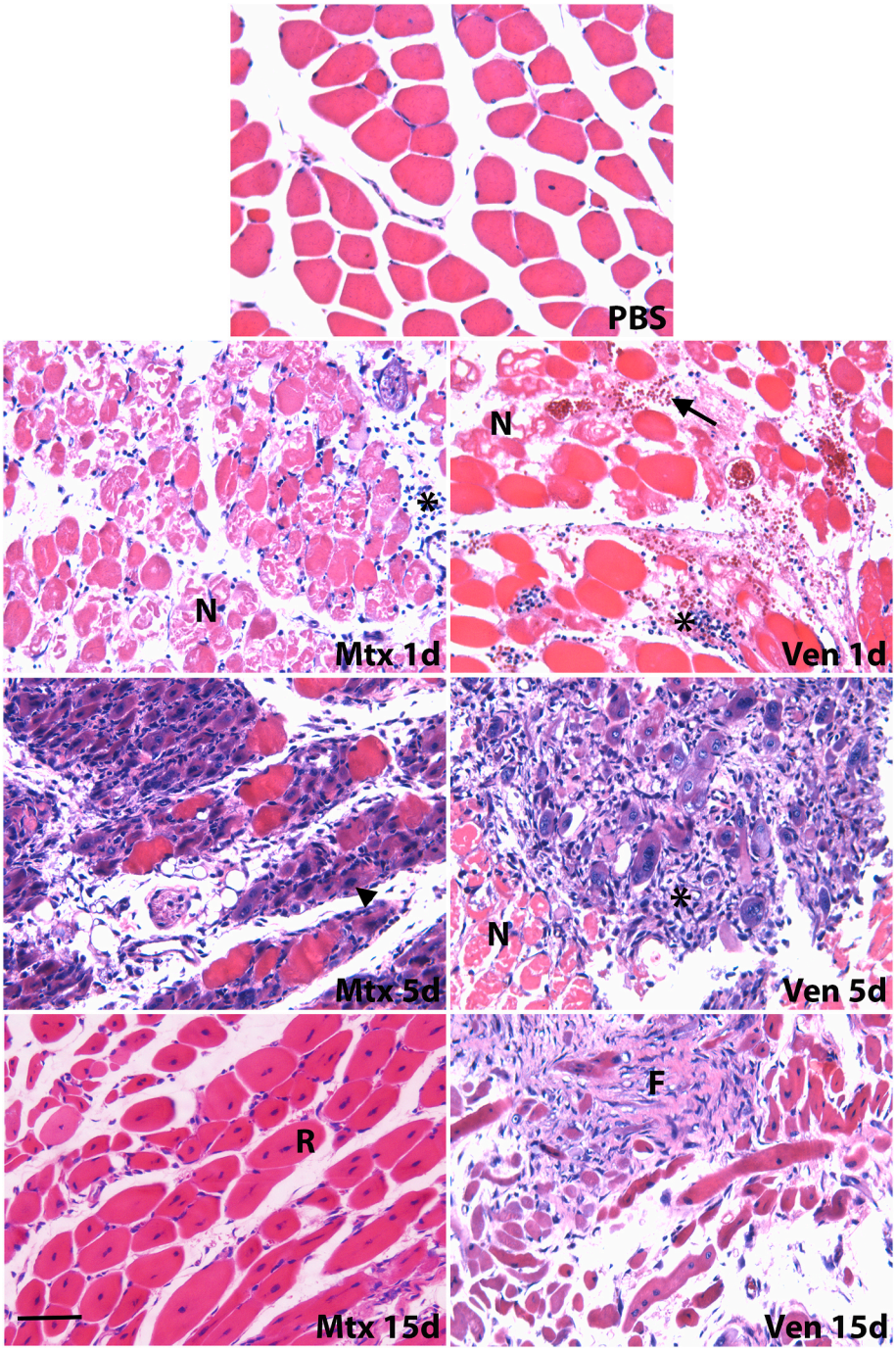


**Fig. 1. Light micrographs of sections of mouse gastrocnemius muscle at various time intervals after venom or Mtx injection.** Groups of four CD-1 mice were injected in the right gastrocnemius with either crude *B. asper* venom, Mtx or PBS as control. Mice were sacrificed at various time intervals and a sample of injected muscle was collected and processed for histological observation. Muscle injected with PBS show a normal morphology of muscle fibers. By day one, both treatments induced necrosis of muscle fibers (N) and an inflammatory infiltrate (*), in addition, venom-injected tissue has hemorrhagic areas (arrow). By Day 5, Mtx-treated muscle presents abundant immature regenerating muscle fibers (arrow head); venom-treated muscle presents remnants of necrotic material (N), areas with inflammatory cells (*) and fewer regenerating fibers. By day 15, Mtx-treated tissue shows an effective regeneration process, with abundant regenerated fibers (R); venom-treated tissue shows a dispersed population of regenerating fibers and a fibrotic area (F), thus revealing an incomplete process of regeneration. Hematoxylin-eosin staining. Bar represents 100 μm.

centrally-located nuclei. There were also limited areas of fibrosis (Fig. 1). In contrast, venom-treated samples had fewer regenerative muscle fibers. These fibers were surrounded by fibrotic tissue and showed variations in size and distribution, generating a heterogeneous morphological pattern (Fig. 1).

### 3.2. Immunohistochemistry of type IV collagen and laminin α2

*Type IV collagen.* Control muscle stained with anti-type IV collagen antibody showed a normal immunostaining of this protein in the BM of muscle fibers, nerves, capillaries and larger vessels (Fig. 2). The immunostaining of type IV collagen in muscle sections of Mtx-treated animals evidenced interruptions in the continuity around some muscle fibers in affected areas. These alterations were observed in samples at one and five days after treatment. By day 15, the immunostaining in Mtx-treated samples was similar to that of control muscle, characterized by a homogeneous pattern in all muscle fibers, including regenerative fibers (Fig. 2). In the case of venom-treated muscles, an evident decrease in the staining of type IV collagen around capillaries was observed one day after injection. At days one and five, an overall reduction of the staining intensity for type IV collagen around muscle fibers occurred, with interruptions in the staining in some necrotic muscle fibers (Fig. 2). By day 15, the staining intensity around muscle fibers was similar to that of control tissue, both in regenerative fibers and in unaffected fibers. However, in some areas with extensive fibrosis in venom-injected tissue there were less regenerative muscle fibers in comparison to PBS and Mtx-treated samples.

*Laminin α2.* Immunostaining of laminin α2 in PBS-treated animals

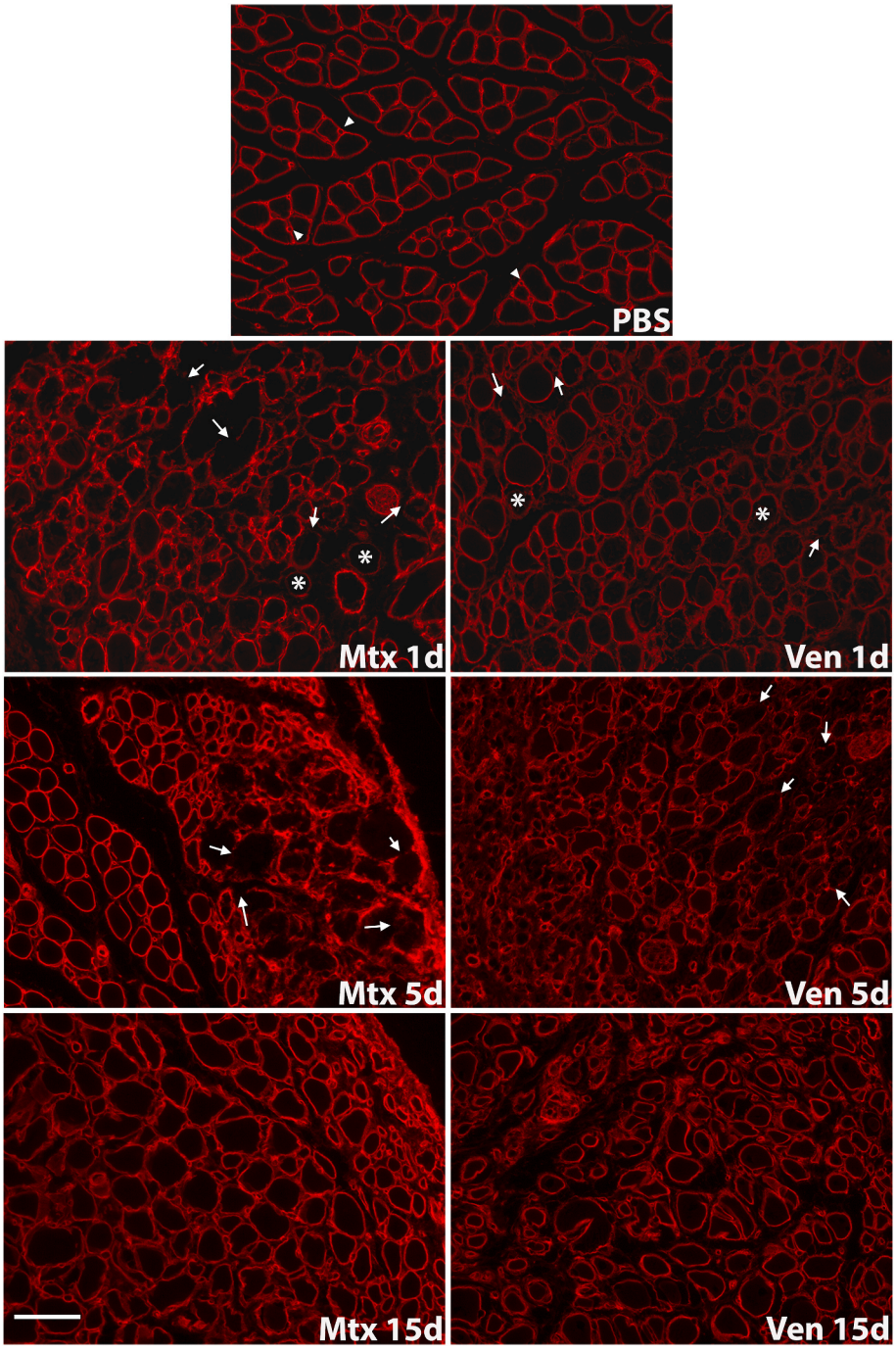


**Fig. 2. Immunostaining of type IV collagen in sections of mouse gastrocnemius muscle at various time intervals after venom or Mtx injection.** Sections of mouse gastrocnemius were stained with anti-type IV collagen rabbit polyclonal antibody followed by a biotinylated secondary antibody. Reactions were detected with a tyramide signal amplification kit using and a streptavidin–Alexa 555 conjugate. Control tissue injected with PBS shows continuous staining around muscle fibers and capillaries (arrow head). In contrast, tissues injected with Mtx at day 1 and 5 present interruptions (arrows) and decrease in immunostaining (*) around some affected muscle fibers. Venom treated muscle, at day 1 and 5, shows interruptions (arrows) and a generalized decrease in immunostaining around muscle fibers and around capillary vessels. By day 15, Mtx-treated tissue shows an immunostaining similar to the control whereas venom-treated tissue presents an irregular distribution of regenerating fibers with positive staining for type IV collagen. Bar represents 100 μm.

evidenced a pattern of distribution similar to that of type IV collagen, with a homogeneous and strong signal around muscle fibers, whereas in capillary vessels the staining was less intense (Fig. 3). Treatment by Mtx or venom induced a loss of the staining around some damaged muscle fibers, an alteration that was more evident at five days. By day 15 the intensity of immunostaining around regenerative muscle fibers was similar for both treatments and comparable to the staining of normal fibers in control muscle injected with PBS, but venom treated samples showed a more irregular distribution of regenerating fibers (Fig. 3).

### 3.3. *Immunoblotting analysis of changes in fibronectin, type IV collagen and α2 laminin*

*Fibronectin.* Immunodetection of fibronectin in muscle homogenates from control mice injected with PBS demonstrated the presence of a band of apparent molecular mass of 230–238 kDa, corresponding to the full-length form of the protein (Fig. 4A and B). Both treatments induced an increase in fibronectin since day one; however, this increment was higher in Mtx-treated samples, reaching a maximum level at days five and seven (Fig. 4A). In contrast, the increment of fibronectin induced by venom was less prominent and reached a peak at day three (Fig. 4B). In addition, several bands of lower molecular mass were observed for both types of samples, probably revealing degradation products of fibronectin (Fig. 4A and B).

*Type IV collagen.* Western blot analysis of muscle homogenates obtained from control mice revealed an immunoreactive band of approximately 171 kDa, corresponding to the reduced chains of type IV collagen (Fig. 5A and B). Samples from venom-injected mice collected at

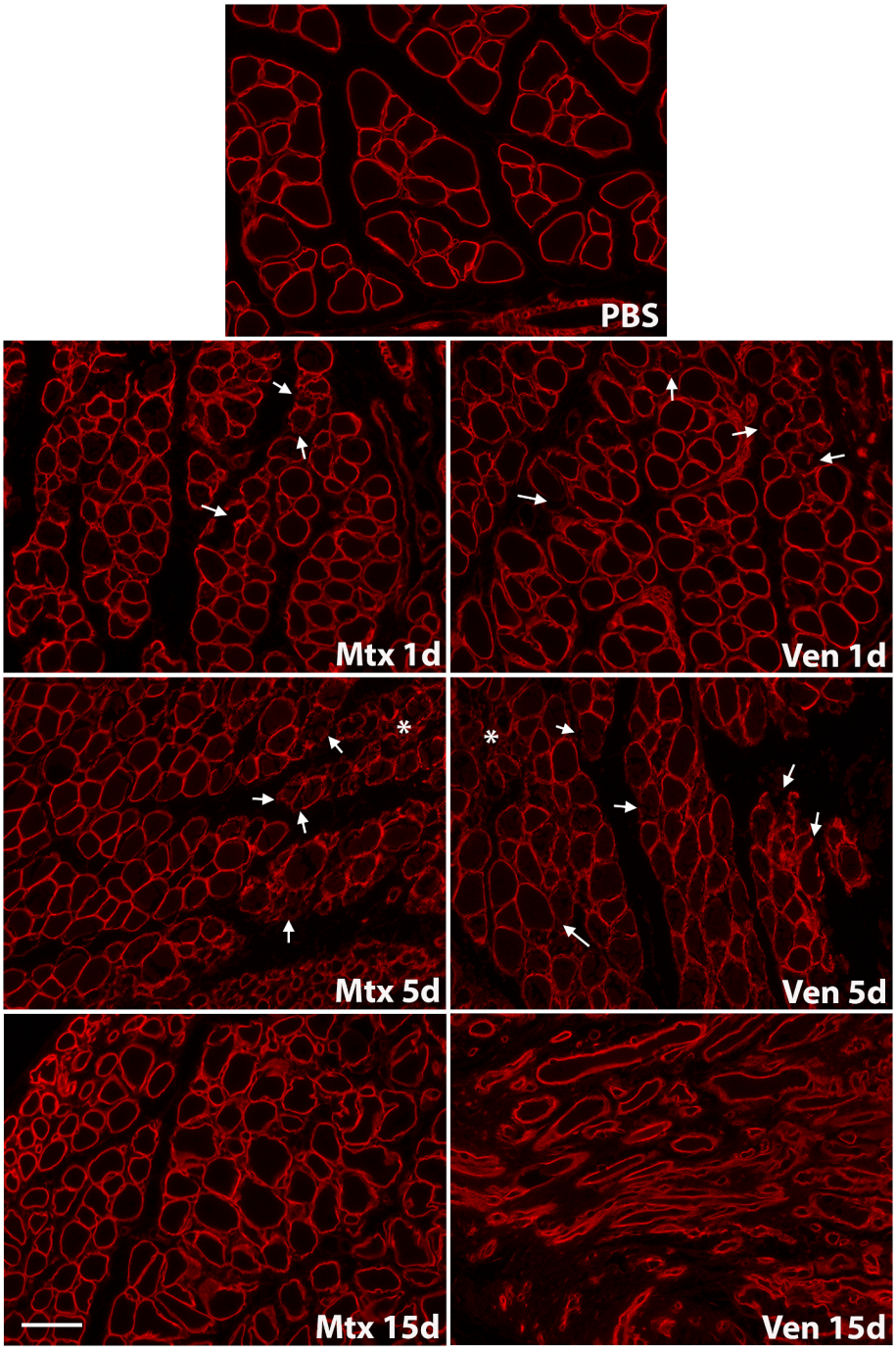


**Fig. 3. Immunostaining of laminin α2 in sections of mouse gastrocnemius muscle at various time intervals after venom or Mtx injection.** Sections of mouse gastrocnemius were stained with anti-laminin α2 rat monoclonal antibody followed by a biotinylated secondary antibody. Reactions were detected with a tyramide signal amplification kit using and a streptavidin–Alexa 555 conjugate. Control tissue injected with PBS shows continuous staining around muscle fibers. At days 1 and 5 there were interruptions around some affected muscle fibers (arrows) in samples treated with Mtx and venom. At day 5 there were areas with extensive degradation of laminin around muscle cells (*). By day 15, Mtx-treated tissue shows an immunostaining similar to the control whereas venom-treated tissue presents an irregular distribution of regenerating fibers with positive staining for laminin. Bar represents 100 μm.

days one, three and five showed a drastic decrease in this band. In addition, in samples of days one and three a slight band of ~120 kDa was observed, probably a degradation product (Fig. 5B). Muscle homogenates from samples collected at day 15 after venom injection showed an increment in type IV collagen band, although still being of lower intensity as compared to PBS-injected muscle (Fig. 5B). In the case of Mtx-treated muscle, a slight decrease in the intensity of type IV collagen band was observed at days one and three; this reduction was more pronounced at day five and reached a maximum at day seven. By day 15, the intensity of this band had increased significantly, showing an immunostaining similar to that of control muscle (Fig. 5A).

*Laminin α2*. An immunoreactive band of approximately 340 kDa, representing the full-length laminin α2 chain, was detected with the anti-α2 laminin antibody in samples from mice injected with PBS (Fig. 6A and B). Mtx-treated samples showed a prominent decrease in the intensity of laminin between days three to seven together with the appearance of inmunoreactive bands of lower molecular mass, interpreted as degradation products of laminin α2 (Fig. 6A). At day 15 the intensity of laminin band had reached a similar intensity as in the control muscle, and the bands of lower molecular mass were not detected (Fig. 6A). In contrast, in venom-treated samples the intensity of the 340 kDa band decreased significantly only in samples collected at day one, and a band of ~70 kDa was observed, probably corresponding to a degradation product (Fig. 6B). By day three and until day seven the intensity of the 340 kDa immunoreactive band increased almost to the same intensity as the control, however, lower molecular mass bands were also detected. At day 15, the intensity of laminin reached the basal level observed in control mice and no degradation products were

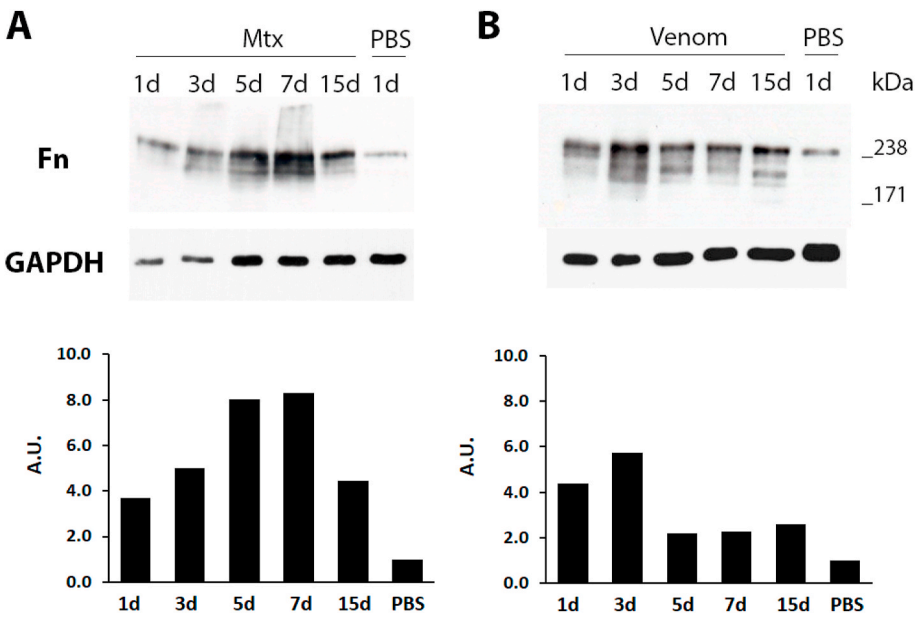


**Fig. 4. Immunodetection of fibronectin in homogenates of mouse gastrocnemius muscle at various time intervals after Mtx or venom injection.** Groups of mice were injected in the right gastrocnemius muscle with solutions of either Mtx (A) or *B. asper* venom (B). After 1, 3, 5, 7 and 15 days, mice were sacrificed, and muscles were removed and homogenized. Muscle homogenates from mice injected with PBS and sacrificed after 1 day were used as controls. Samples were separated by SDS-PAGE and immunodetection was done as described in materials and methods. The intensity of immunoreactive bands representing the full-length form of fibronectin was analyzed by densitometry and a relative quantification was performed adjusting each sample to the corresponding control. Results are representative of three independent experiments. Both treatments induced an increase in fibronectin immunoreactive bands in comparison to control samples and the appearance of lower molecular mass bands; Mtx treatment induced a higher increase mainly at 5 and 7 days. Fn; fibronectin.

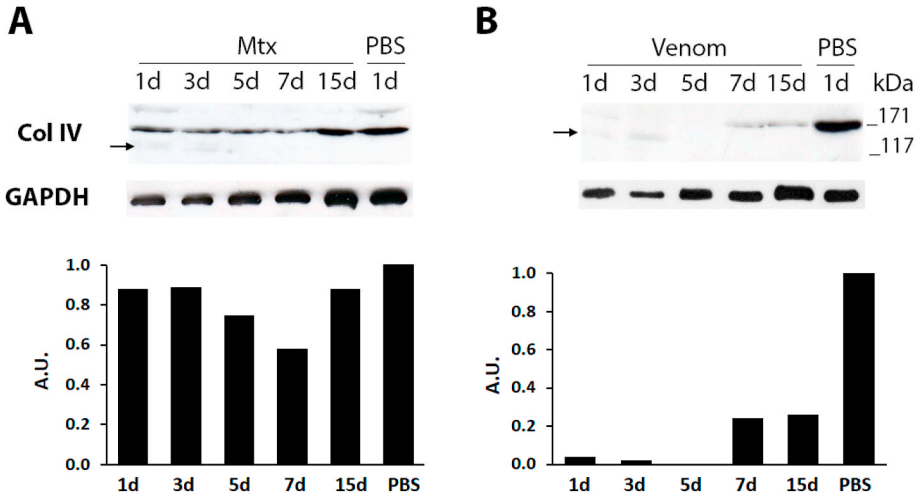


**Fig. 5. Immunodetection of type IV collagen in homogenates of mouse gastrocnemius muscle at various time intervals after Mtx or venom injection.** Groups of mice were injected in the right gastrocnemius muscle with solutions of either Mtx (A) or *B. asper* venom (B). After 1, 3, 5, 7 and 15 days, mice were sacrificed and muscles were removed and homogenized as described in materials and methods. Muscle homogenates from mice injected with PBS and sacrificed after one day were used as controls. Samples were separated by SDS-PAGE and immunodetection was done as described in materials and methods. The intensity of immunoreactive bands representing the full-lenght form of type IV collagen was analyzed by densitometry and a relative quantification was performed adjusting each sample to the corresponding control. Results are representative of three independent experiments. Mtx treatment induced a moderate decrease in the immunostaining of type IV collagen which was recovered by 15 d; in contrast venom treatment induced an important degradation of this protein at all time intervals. Both treatments induced the appearance of degradation products (arrows). Col IV: type IV collagen.

detected (Fig. 6B).

*3.3.1. Expression of transcripts of fibronectin, collagen 4 A and laminin isoforms*

In order to understand the changes in the extracellular matrix during skeletal muscle regeneration process, we quantified the levels of transcripts of fibronectin, laminin and collagen 4 A isoforms in muscle tissue at one and five days after venom and Mtx injection (Fig. 7). Tissue injected with PBS was used as control.

*Fibronectin.* Five couples of primers were chosen to determine the abundance of the different transcripts of fibronectin domains. After one and five days after venom injection all the analyzed fibronectin domains were significantly upregulated 3–4 times as compared to the PBS treatment (Fig. 7A). Interestingly, after one day of Mtx treatment, two domains (EDA and EDB) showed a higher fold increase than the other three domains. This difference tended to decrease after five days.

*Collagen 4A.* We analyzed the transcripts of five collagen 4 A isoforms: Collagen 4 A α1, -2, -3, -4, and -5, on the same samples. After one day of venom and Mtx injection, the isoforms α1 and α2 were highly over expressed, especially in samples from Mtx-injected mice (Fig. 7B). Moreover, in Mtx-injected tissue, the other three collagen isoforms transcripts were also upregulated, whereas in samples from venom-treated muscle those transcripts were slightly downregulated. After five days, the only significant change observed was a reduction in the transcripts of collagen 4 A isoforms α1 and -2 in both treatments, as compared to day one, but a stable and high transcription of collagen 4 A α3, -4, and -5 was observed only in samples treated with Mtx (Fig. 7B).

*Laminin:* Five different laminin isoform transcripts were analyzed. Transcripts related to laminin α1, β1 and γ1 were upregulated in samples treated with venom but were highly expressed after Mtx treatment one day after injection (Fig. 7C). However, laminin α2 and β2 showed a mild increase only in samples injected with Mtx. In contrast, after five days these transcripts showed a similar increase to the other three laminin chains in both venom and Mxt treatments.

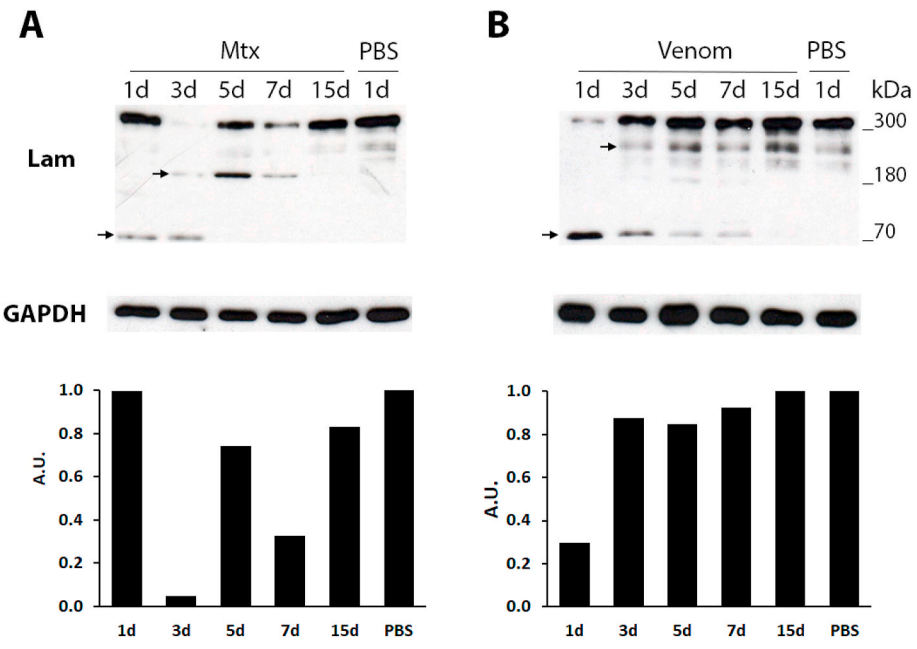


**Fig. 6. Immunodetection of laminin α2 in homogenates of mouse gastrocnemius muscle at various time intervals after Mtx or venom injection.** Groups of mice were injected in the right gastrocnemius muscle with solutions of either Mtx (A) or *B. asper* venom (B). After 1, 3, 5, 7 and 15 days, mice were sacrificed, and muscles were removed and homogenized as described in materials and methods. Muscle homogenates from mice injected with PBS and sacrificed after one day were used as controls. Samples were separated by SDS-PAGE and immunodetection was done as described in materials and methods. The intensity of immunoreactive bands representing the full-length form of laminin α2 was analyzed by densitometry and a relative quantification was performed adjusting each sample to the corresponding control. Results are representative of three independent experiments. Venom treatment induced degradation of laminin α2 at the first time intervals, whereas Mtx treatment induced a more pronounced degradation of this protein between days 3 and 7. Both treatments induced the appearance of degradation products (arrows). Lam: laminin α2.

## 4. Discussion

Experimental observations in this and previous studies demonstrate that skeletal muscle regeneration is impaired after experimental myonecrosis induced by viperid snake venoms containing hemorrhagic SVMPs (Arce et al., 1991; Gutiérrez et al, 1984a, 2018; Hernández et al., 2011; Queiróz et al., 1984). This agrees with clinical reports describing musculoskeletal sequelae in snake-bitten patients (Jayawardana et al., 2016; Otero et al., 2002; Waiddyanatha et al., 2019), which generate multiple physical, psychological, economic and social consequences (Gutiérrez et al., 2017). Since viperid venoms induce drastic alterations in the microvasculature and nerves (Hernández et al., 2011; Queiróz et al., 2002), it has been proposed that the basis of poor regeneration in these models is related to impairment of blood flow, with the consequent generation of tissue hypoxia and the delay on the arrival of inflammatory cells (Gutiérrez et al., 2018; Hernández et al., 2011). This hypothesis is supported by the observation that injection of hemorrhagic SVMPs also results in a deficient regeneration (Gutiérrez et al., 1995; Hernández et al., 2011; Williams et al., 2019). In agreement, when muscle damage is induced by myotoxic components that do not affect the vasculature, the regenerative process is effective and leads to an almost complete muscle regeneration (Gutiérrez et al., 1984a; Harris et al., 2003; Hernández et al., 2011; Williams et al., 2019). Our histological observations agree with previous investigations in that regeneration is successful after necrosis induced by a Mtx but is impaired after *B. asper* venom-induced muscle injury.

In addition to adequate vascular and nerve supply, and effective removal of necrotic material by inflammatory cells, the presence of BM around necrotic fibers is another requirement for a successful muscle regeneration process (Grounds, 2008). BM plays a role of a structural scaffold and a niche where myoblast replication and fusion take place (Calve and Simon, 2012; Mackey and Kjaer, 2017; Vracko and Benditt, 1972). But the involvement of BM goes beyond this structural role, as it is a storage site for growth factors and cytokines, and the hydrolysis of its components releases bioactive fragments with multiple physiological roles (Pozzi et al., 2017). This study explored the changes in BM components in muscle injected with myotoxin and crude venom of *B. asper*.

Immunohistochemical analysis of laminin in sections from muscle treated with Mtx or venom revealed focal interruptions in the staining in muscle cell BM in some regions of the tissue, especially in areas of muscle fiber necrosis, at days one and five after injection. The antibody used is specific for the α2 chain of laminin, characteristic of BM of muscle fibers (Durbeej, 2010). Thus, no staining with this antibody was observed in capillary vessels. In the case of type IV collagen, a similar focal interruption of staining in the periphery of muscle cells was observed with both treatments. In addition, in venom-injected muscle, a drastic reduction occurred in capillaries as well, as a consequence of SVMP-induced microvessel damage (Escalante et al., 2011a). In the case of venom, the partial loss of staining of these proteins in muscle cell BM is likely to depend on the hydrolytic action of SVMPs (Escalante et al., 2011a; Herrera et al., 2015; Williams et al., 2019).

The loss of staining of type IV collagen in capillaries, also described in previous studies (Baldo et al., 2010; Escalante et al., 2011a; Herrera et al., 2015), depends not only on the enzymatic hydrolysis of this protein, but also on the action of biophysical forces operating in the circulation, which distend and disrupt the integrity of the capillary wall once the mechanical stability of this ECM structure is weakened after hydrolysis of type IV collagen (Escalante et al., 2011a, 2011b; Gutiérrez et al., 2005). This idea is supported by our unpublished observations showing that immunostaining of type IV collagen is not lost in capillaries treated with SVMPs in conditions of lack of blood flow. Hence, the apparently higher degradation of capillary BM over muscle cell BM after venom injection might be due to this combination of biochemical and biophysical phenomena in the case of capillaries, whereas in muscle BM there is only the hydrolysis of type IV collagen. The higher degradation of capillary BM by venom may be also related to the different set of isoforms in muscle and capillary BMs, which may have variable susceptibility to SVMPs.

Despite the lack of proteinase activity of Mtx, there was a reduction in the immunostaining of type IV collagen and laminin in muscle injected with this toxin. This agrees with earlier studies demonstrating a similar loss of immunostaining in the BM of muscle fibers in another type of muscle injury (Gulati et al., 1983). It is suggested that, in these circumstances, degradation of BM components is due to proteinases synthesized as part of the inflammatory reaction that follows muscle damage. Endogenous matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9, play a role in the remodeling of muscle ECM in diverse pathological and physiological settings, in a delicate interplay of synthesis, activation and inhibition by tissue inhibitors of metalloproteinases (TIMPs) (Bellayr et al., 2009; Davis et al., 2013). Remodeling of ECM proteins by MMPs is a crucial step in skeletal muscle regeneration process (Mahdy, 2019). MMP-2 is constitutively expressed

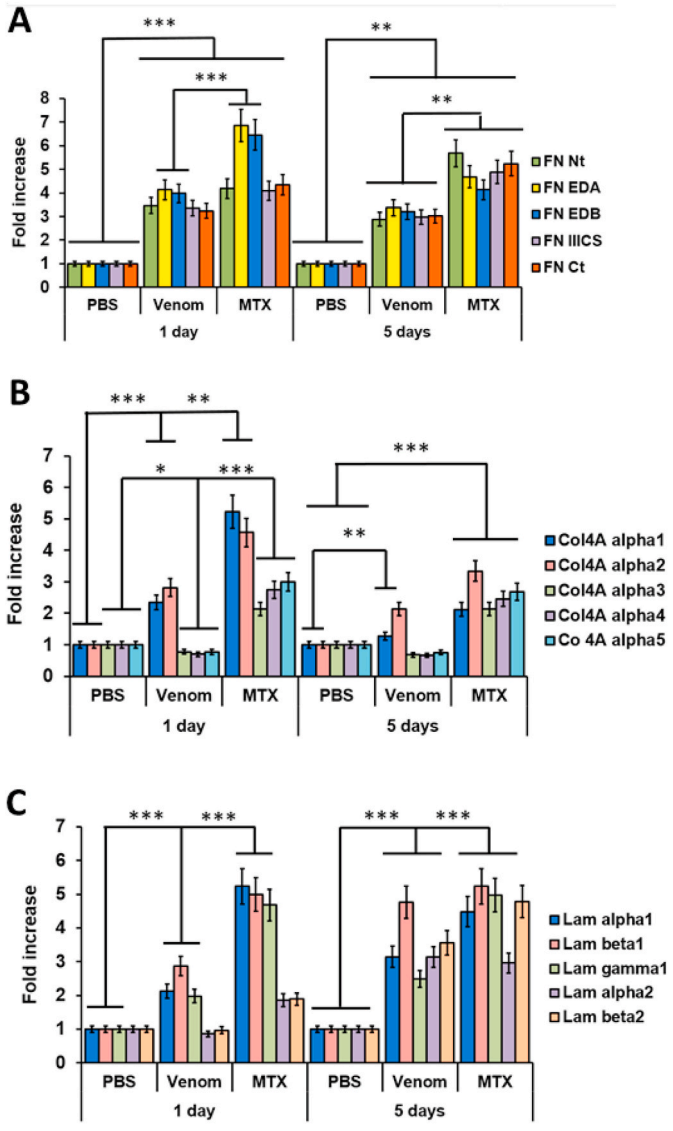


**Fig. 7. Quantification of transcripts by quantitative PCRs of ECM proteins in samples of gastrocnemius mouse muscles one and five days after injection of either PBS, venom or Mtx.** The corresponding abundances are expressed as fold change compared to the PBS treatment (fold increase = 1) and normalized to the housekeep gene HPRT. Average and ±SD are referred to qPCR measurements done in quadruplicate from three independent muscles for each treatment. **(A)** Fibronectin splice variants transcripts are differently regulated after venom and Mtx treatments. Fibronectin (FN) domains are indicated by different colors and described in figure. **(B)** Embryonic collagen transcripts are overexpressed during early events after venom and Mtx injection in mouse skeletal muscle. Collagen 4 A α-1 and -2 transcripts were up-regulated after venom and Mtx treatment one day after injection. In contrast, a down-regulation of collagen 4 A α-3, -4 and -5 transcripts were observed at both time intervals after venom treatment. All collagen 4 A isoforms RNAs were upregulated at both time intervals after Mtx treatment. **(C)** Laminin transcripts isoforms −1 are highly expressed in venom and Mtx-injected samples after one day of injection. A significant increase of α, β and γ-1 laminin isoforms was detected in samples collected one day after treatments. In the three figures, average and ±SD are fold increases values for venom and Mtx treatments referred to PBS control samples and normalized to the housekeep gene, HPRT1. The identity of the transcript quantified is indicated with different colors in the figures. *** = $p < 0.005$; ** = $p < 0.01$; * = $p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

in muscle, whereas MMP-9 is an inducible enzyme synthesized by a variety of inflammatory cells and regenerating muscle fibers (Kherif et al., 1999). An abundant inflammatory infiltrate has been described in muscle injected with Mtx and *B. asper* venom (Hernández et al., 2011). MMP-2 has a similar level of expression during the 15 days after injection of *B. asper* venom and Mtx, whereas MMP-9 is overexpressed during the first days, as part of the inflammatory response, and decays later on (Saravia-Otten et al., 2013). It is suggested that the reduction in the intensity of immunostaining of type IV collagen and laminin during the first five days, in muscle treated with Mtx, is the consequence of the action of these endogenous MMPs. Since these enzymes are also present in tissue injected with venom, it is likely that both venom proteinases and endogenous MMPs play a role in BM protein hydrolysis in this model in the case of crude venom.

Western blot analysis confirmed the degradation of type IV collagen and laminin in muscle injected with venom or Mtx. However, the differences, especially at day one, were more marked than in the case of immunohistochemistry in the samples from venom-injected muscle. This underscores a higher degradation of these proteins in venom-treated samples due to the action of SVMPs, as reported in previous studies (Escalante et al., 2009). Noteworthy, even at 15 days, the intensity of the immunoreactive band of type IV collagen was lower in samples treated with venom as compared to controls, suggesting defects also in the synthesis of this protein. The more pronounced degradation of BM proteins detected by Western blot in venom-treated samples at day one can be explained since, in this procedure, tissue was homogenized and centrifuged. In these conditions, fragments of BM proteins from muscle cells and capillaries can be present in the supernatant. Since venom, but not Mtx, has proteinase activity able to hydrolyze BM components from both muscle fibers and capillaries, it is expected that the degradation of BM proteins will be higher in venom-treated tissue at the first day, when venom concentration in the tissue is still high (Saravia-Otten et al., 2013). Another possible explanation is that the BM components preferentially present in the supernatant are derived from capillaries, whereby there is a higher degradation in venom-treated muscle. Interestingly, in the case of laminin α2, Western blot analysis revealed a higher degradation of this protein in Mtx-treated samples between days three and seven in comparison to venom-treated samples. This finding suggests that the normal ECM remodeling required for tissue repair could be altered in venom-injured muscle.

The changes in ECM during muscle regeneration involve not only BM proteins, but also other ECM components that contribute to the microenvironment of muscle fibers; one such protein is fibronectin (Grounds, 2008). In this work we demonstrated that both treatments induced an increase of fibronectin. Such increment may be related to an increased synthesis of this protein or to the release of this protein after hydrolysis of other ECM components that bind fibronectin. In addition, the presence of immunoreactive bands of lower molecular mass in both types of samples may reflect hydrolysis of fibronectin by venom proteinases and/or MMPs. It is noteworthy, however, that although both treatments increased the amount of fibronectin in the tissue, there were differences in the pattern of increment along time. Mtx treatment induced a marked increase between days three and seven, when important regenerative events are taking place, whereas venom induced a more conspicuous increment at the initial stage of tissue injury, between days one and three. Since fibronectin plays an important role in muscle regeneration (Lukjanenko et al., 2016), these differences could have an impact in the regenerative process. In general, key decisions in the muscle regeneration process take place, in a highly coordinated fashion, within the first days after injury. Hence, the differences observed in the time-course of ECM protein degradation between venom and Mtx may have implications for the outcome of the regenerative process.

The analysis of transcripts of fibronectin, laminin and type IV collagen revealed differences between treatments. At day one, transcription of mRNA for fibronectin domains and several laminin and type IV collagen chains was higher for both treatments as compared to

control muscle, underscoring the onset of biosynthetic processes. The upregulation of laminin α1, β1 and γ1, observed in both treatments after one day of injection, suggests an early response of matrix regeneration by producing laminin 111, as observed in other repair matrix models in skeletal muscle. After five days, the expression of a mature and muscle-specific laminin (laminin 221) starts, as evidenced by the increase of the corresponding transcripts laminin α2, β2 and γ1 (Gullberg et al., 1999; Riederer et al., 2015). However, higher levels were observed in Mtx-injected than in venom-injected tissue. In the case of fibronectin, for both venom and Mtx there was a significant increment of fibronectin transcripts after one day, and it continued after five days, in agreement with Western blot observations. As a sign of ECM regeneration, the domains EDA and EDB of cellular fibronectin were slightly over expressed in venom-injected tissue but were more abundant after Mtx treatment. This clearly reflects a higher transcriptional activity for these proteins after Mtx injection, a factor that could contribute to a better regenerative scenario due to the crucial role of laminin and fibronectin in tissue repair (Midwood et al., 2006; Riederer et al., 2015). The mechanisms behind the higher levels of transcripts of ECM proteins in Mtx-treated samples remain unknown. $PLA_2$s induce many intracellular events in their target cells and one possible outcome of this activation could be the upregulation of the Akt/mTOR pathway, which has been associated to the synthesis of ECM proteins (Favier et al., 2010).

The differences in mRNA transcription between the two treatments were more evident in the case of collagen 4 A isoforms. Muscle treated with Mtx showed, since the first day, an increment in the transcription of all chains of type IV collagen. This elevation was higher for α1 y α2 chains, the most abundant type IV collagen isoforms (Pozzi et al., 2017), reflecting the synthesis of new BM in damaged muscle. It was nevertheless of interest that also transcripts for collagen chains α3, α4, and α5, which are characteristic of neuro-muscular junctions (Sanes et al., 1990), were increased. In contrast, in venom-injected muscle there was a lower increment in transcripts for type IV collagen chains, occurring only for chains α1 and α2. This agrees with the observation of lower intensity of type IV collagen bands in Western blot in venom-injected samples.

Taken together, our findings shed light on a hitherto poorly studied aspect of muscle regeneration after necrosis induced by snake venoms and myotoxins, i. e, the degradation and synthesis of BM components. Our results show that differences in the degradation and synthesis of BM components by venom or Mtx may provide another explanation for the poor regenerative outcome after necrosis induced by venom. In the centerpiece of this scenario are SVMPs, which cause widespread hydrolysis of BM components in capillaries and muscle fibers. In addition, for reasons that remain to be investigated, the synthesis of BM components, mainly type IV collagen, is also hampered in venom-injected tissue, hence affecting the niche for proliferation and fusion of myogenic cells in the regenerative process. Tissue hypoxia down-regulates Akt/mTOR (Favier et al., 2010), and it is known that Akt (protein kinase B) regulates the expression of laminin and type IV collagen (Li et al., 2001). Since hemorrhagic SVMPs induce tissue ischemia secondary to microvessel damage, it is suggested that the impairment in the synthesis of BM components may be another consequence of the tissue ischemia resultant from SVMP-induced microvessel damage. On the other hand, the possible upregulation of Akt/mTOR pathway by myotoxic $PLA_2$ could explain the higher amount of transcripts of ECM proteins found in tissues treated with Mtx, an hypothesis that deserves further investigation.

Finally, our results suggest three potential therapeutic avenues to improve regeneration after viperid venom-induced myonecrosis: (a) inhibition of SVMPs by the use of natural or synthetic metalloproteinase inhibitors (Albulescu et al., 2020; Gutierrez et al., 2007; Rucavado et al., 2000), hence reducing BM hydrolysis, (b) promotion of neovascularization in order to reduce the hypoxic environment in regenerating tissue, and (c) enrichment of the niche provided by the BM, by the exogenous provision of ECM components, such as laminin (Riederer et al., 2015). Exploring the potential of these and other strategies for improving tissue regeneration is an urgent task that will contribute to reduce the sequelae in people suffering snakebite envenoming.

## Credit author statement

Patricia Saravia-Otten: Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Funding acquisition. Stefano Gastaldello: Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Funding acquisition. Rosario Hernández: Conceptualization, Investigation, Formal analysis, Writing – review & editing, Funding acquisition. Alexa Marín: Investigation, Formal analysis, Gabriela García: Investigation, Formal analysis, Lourdes García: Investigation, Formal analysis. Erick Estrada: Investigation, Formal analysis. Alexandra Rucavado: Formal analysis, Writing – review & editing, Funding acquisition. José María Gutiérrez: Conceptualization, Formal analysis, Writing – original draft, Writing – review & editing. Teresa Escalante: Conceptualization, Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Funding acquisition.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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