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"EFEITO DA L-ARGININA E DEFLAZACORT NA REGENERAÇÃO MUSCULAR APÓS O ENVENENAMENTO EXPERIMENTAL POR *BOTHROPS JARARACUSSU*"

Tese apresentada ao Instituto de Biologia para obtenção do Título de Doutor em Biologia Celular e Estrutural, na área de Anatomia.

Orientador: Prof. Dr. Humberto Santo Neto

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Dedicatória

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Resumo

As serpentes do gênero *Bothrops* são responsáveis por 90% dos acidentes ofídicos. Dentre elas se destaca a *B. jararacussu* pela capacidade de inoculação de grande quantidade de veneno. O veneno desta espécie se caracteriza pela ação miotóxica, e esta ação causam importantes alterações locais, como a necrose das fibras musculares e conseqüentemente perda da massa muscular. No presente trabalho, estudamos os efeitos do deflazacort (glicocorticóide derivado da predinizona) e da L-Arginina (precursor do óxido nítrico) na regeneração das fibras musculares frente aos efeitos mionecróticos do veneno. Para tanto, utilizamos camundongos Swiss adultos jovens do sexo masculino. O músculo tibial anterior foi injetado com 80 μg do veneno bruto de *B. jararacussu* diluídos em 0,1 ml de solução fisiológica. Após as injeções dos venenos iniciaram-se a administração das drogas, com injeções intraperitoneais diárias de deflazacort na dosagem de 1,2 mg/kg por 5 e 20 dias. O segundo grupo recebeu a L-arginina junto à água de beber na concentração de 3,75 mg/ml desde o momento da injeção do veneno por 2 meses. O grupo controle foi composto por animais injetados com o veneno sem a realização de tratamento. A regeneração muscular foi avaliada no período de 2 meses e 5 dias através de cortes transversos do terço médio dos músculos, sendo corados com H&E e Tricrômico de Masson, para contagem da população total das fibras musculares e de células com núcleos centrais para análise da regeneração muscular. Quantificações da área muscular e da fibrose tecidual também foram realizadas. Identificou-se que com a L-Arginina ocorreu um aumento na regeneração muscular com a presença de maior número de fibras musculares regeneradas (2.230 ± 478) em relação ao animal injetado não tratado (1.005 ± 134). E no grupo tratado com Deflazacort observou-se um aumento da fibrose tecidual (1.077.051 ± 466.658,2; versus 777.107,3 ± 356.804,8 pixels quadrado) com número menor de fibras musculares (783,5 ± 134) em relação ao grupo não tratado. Desta forma, identificou-se o estímulo da regeneração muscular promovida pela L-arginina e o aumento da fibrose tecidual e diminuição da área muscular causado pela administração do antiinflamatório Deflazacort após o envenenamento pelo veneno bruto de *B. jararacussu*.

Palavras-chave: regeneração muscular, *Bothrops jararacussu,* L-arginina, Deflazacort.

Índice

CAPÍTULO I

1. Introdução

No Brasil os acidentes ofídicos são tidos como problema de saúde pública. As serpentes do gênero *Bothrops* são responsáveis por cerca de 90% destes acidentes, e isto se deve a grande variabilidade do seu habitat que inclui campos, florestas e até áreas mais povoadas; e a natureza mais agressiva destas serpentes (ROSENFELD, 1971; CARDOSO, 1984; BARRAVIERA e PEREIRA, 1994). Some-se a isto o fato que o envenenamento por serpentes do gênero *Bothrops* causa importantes alterações locais como edema, hemorragia e necrose podendo, em determinados casos, levar a perda da função e até amputação do membro atingido (ROSENFELD, 1971; NISHIOKA e SILVEIRA, 1992; BARRAVIERA e PEREIRA, 1994; MILANI et al., 1997).

Dentre estas serpentes destaca-se a *Bothrops jararacussu.* Popularmente conhecida como jararacuçu, esta serpente pode alcançar 2 metros de comprimento e secretar até 4 ml de veneno, o que corresponde a 1g de veneno seco. Frente a esta grande capacidade de inoculação do veneno em uma única ocasião, sua picada produz lesões locais dramáticas e de grande extensão (MILANI et al., 1997; JORGE et al., 1999).

A necrose das fibras musculares é, na fase aguda do envenenamento, uma característica deste veneno. Trata-se de uma lesão importante sob o ponto de vista clínico. A precária regeneração muscular que se segue produz atrofia muscular permanente, o que confere ao acidentado, limitação ou perda da capacidade motora do membro picado (HOMMA e TU, 1971; JORGE et al., 1999; TEIBLER e PEREZ, 2001).

No envenenamento pelo *B. jararacussu* a mionecrose ocorre rapidamente, identificou-se a ação direta do veneno sobre o sarcolema, onde produz lesões focais dois minutos após a inoculação do veneno (QUEIRÓZ et al., 1984). Isto devido à ação catalítica do veneno sobre a

bicamada lipídica do sarcolema. Através destas lesões, haveria o influxo do próprio veneno e de íons Ca^{+2} do meio extracelular para o interior da fibra muscular. O aumento destes íons cálcio resulta em hipercontração das miofibrilas que se soma à ação direta do veneno nestas e nas organelas com subseqüente necrose celular. Outra possibilidade é que íons cálcio atuariam ativando as proteases cálcio-dependentes que produziriam necrose da fibra muscular (QUEIRÓZ et al., 1984; SANTO NETO, 1987; MOURA-DA-SILVA et al., 1991; GUTIERREZ e LOMONTE, 1995; SOUZA et al., 2002; GUTIERREZ e OWNBY, 2003).

Por isto, a patogênese da mionecrose tem sido extensivamente estudada nas últimas décadas e está razoavelmente bem estabelecida. Entretanto, meios para combater a perda da massa muscular, e a promoção da regeneração tecidual do local afetado após o envenenamento ainda mostram-se incipientes.

A soroterapia, como o procedimento para o tratamento dos acidentados, tem sido realizada com sucesso na prevenção de óbitos e nos efeitos sistêmicos, mas relativamente pouco efetivo na eliminação das lesões locais, em especial no caso do *B. jararacussu*. (MILANI et al., 1997; JORGE et al., 1999). Assim sendo, os estudos atuais tem como objetivo principal à identificação de produtos ou procedimentos que possam antagonizar os efeitos mionecróticos do veneno. Estes variam desde a administração de plasma sanguíneo de animais como gambá, substâncias como heparina, bromofenacil, poliânions em geral incluindo até emprego de laser de baixa energia (MELO e SUAREZ KURTZ, 1988; MELO et al., 1993; MELO e OWNBY, 1999; OSHIMA-FRANCO et al., 2001; SOUZA et al., 2002; ALMEIDA-SILVA et al., 2003). Embora alguns destes tenham apresentado resultados experimentais promissores, eles ainda não foram suficientemente encorajadores para que iniciasse os testes em humanos.

No contexto acima, e com o intuito de contribuir na regeneração tecidual após o envenenamento, procuramos investigar a ação de fármacos atualmente estudados na promoção

da regeneração muscular, tais como a L-arginina, precursor de óxido nítrico e do Deflazacort, glicocorticóide derivado da prednisona.

No capítulo II será apresentado o artigo publicado "*L-arginine enhances muscle regeneration after experimental envenomation by B.jararacussu: A future nitric oxide-based therapy?"*. Nesta etapa foi estudado a utilização e efeito da L-arginina (2-amino-5-ácido guanidinovalerico), um precursor do óxido nítrico na regeneração muscular após ao envenenamento.

A oxidação realizada pelas enzimas óxido nítrico sintases do grupo guanidina da L-Arginina no meio intracelular forma citrulina e o óxido nítrico (NO) em quantidades equivalentes (BÖGER & BODE-BÖGER, 2001; STUEHR, 2004). A óxido nítrico sintase (*nitric oxide synthase* – NOS) enzima responsável pela produção de NO, possui isoformas como a óxido nítrico sintase neuronal (*neuronal nitric oxide synthase*- nNOS) encontrada em maior quantidade no músculo esquelético e sistema nervoso, e em menores quantidades nas isoformas endotelial (eNOS) e induzível (iNOS) produzida apenas após estímulos imunológicos em macrófagos e hepatócitos (STUEHR, 1996). A nNOS localiza-se próximo ao sarcolema ligada às sintrofinas e no citosol das fibras musculares (RANDO, 2001).

O óxido nítrico está envolvido com a manutenção do fluxo sanguíneo adequado para o tecido muscular durante a contração, com a angiogênese, ativação de células inflamatórias, com a liberação dos íons Ca^{+2} do retículo sarcoplasmático, com o metabolismo da glicose (KAMINSKI e ANDRADE, 2001; LOWENSTEIN et al., 1994; MUROHARA et al., 1998; RANDO, 2001), bem como, na ativação das células satélites, responsáveis pela regeneração muscular (ANDERSON, 2000).

Nas células satélites o óxido nítrico induz a expressão da folistatina, molécula que ativa a miogênese. A folistatina,entretanto, inibe a ação da miostatina, um potente regulador negativo da miogênese expresso pelas células satélites quiescentes, possibilitando o início da miogênese (PISCONTI et al., 2006; LE GRAND e RUDNICKI, 2007).

A utilização de L-arginina como doador exógeno de NO em camundongos distróficos, obteve resultados positivos na regeneração muscular, agindo possivelmente na ativação das células satélites (ANDERSON e VARGAS, 2003).

Assim sendo, após uma lesão das fibras musculares a reconstituição do tecido muscular inicia-se em seguida, concomitantemente ao processo inflamatório e de fagocitose das células necróticas. Sabe-se que a ativação das células satélites representa a primeira etapa dos eventos celulares, e parte fundamental para o sucesso da regeneração muscular (BISCHOFF, 1994; ANDERSON, 2000). Entretanto, para que ocorra esta cascata de eventos é necessário um estímulo precedente do óxido nítrico (NO), o mensageiro endógeno responsável pelo início deste ciclo (ANDERSON, 2000; STAMLER e MEISSNER, 2001; ANDERSON e VARGAS, 2003).

Desta forma, nesta etapa do projeto procurou-se verificar a aplicabilidade e benefícios locais na regeneração das fibras musculares causados pela administração da L-arginina após o envenenamento por *B.jararacussu*.

O capítulo III refere-se ao manuscrito no formato da revista a ser submetido, Toxicon: "*Loss of muscle regeneration following Bothrops jararacussu envenoming is aggravates by anti-inflammatory: experimental study"*. O efeito do glicocorticóide deflazacort foi analisado no processo de regeneração das fibras musculares após a inoculação do veneno de *B.jararacussu*.

Na Distrofia Muscular de Duchenne (DMD) a degeneração progressiva dos músculos esqueléticos e cardíacos ocorrida nesta doença é resultante de mutações do gene que codifica uma proteína do sarcolema chamada distrofina (HOFFMAN et al., 1987). Esta falha no sarcolema promove ciclos de necrose, com posterior perda da capacidade regenerativa (BULFIELD et al., 1984; ENGEL et al., 1994). Tem-se observado que o deflazacort melhora substancialmente o quadro clínico dos pacientes com DMD atenuando a necrose das fibras musculares (ANGELINI et al., 1994; REITTER, 1995; BIGGAR et al., 2001). O deflazacort é um glicocorticóide derivado da prednisona, possui efeitos antiinflamatórios e imunossupressores,

e é utilizado no tratamento de várias doenças sendo comparável a outros antiinflamatórios corticoesteróides. Interessante é que, experimentalmente, observou-se que além de proteger a fibra muscular distrófica (ST-PIERRE et al., 2004) o deflazacort reduziu o número de núcleos centrais e ocorreu um aumento no diâmetro das fibras musculares (ANDERSON e VARGAS, 2003).

Embora a utilização do deflazacort na distrofia muscular tenha sido positiva, os efeitos do tratamento de lesões musculares com antiinflamatórios ainda é um assunto controverso.

Alguns estudos indicam efeitos positivos na diminuição da resposta inflamatória (MENDIAS et al., 2004; RAHUSEN et al., 2004; JARVINEN et al., 2005). Entretanto, outros demonstram um possível efeito prejudicial no retardo da fagocitose tecidual, devido à importância das células inflamatórias na regeneração muscular em fatores como a fagocitose das células necróticas, a produção de fatores tróficos na ativação de células satélites, como as citocinas, e na angiogênese (CANTINI et al., 2002; CHARGE e RUDNICKI, 2004; TIDBALL, 2005).

Apesar do tratamento com soroterapia ser o mais eficaz e utilizado atualmente, alguns dados epidemiológicos mostram que a administração de antiinflamatórios também é utilizada nos acidentes ofídicos (RIBEIRO et al., 1995; DA SILVA et al., 2003).

A depleção induzida de células inflamatórias e posterior envenenamento de *B.asper* causou redução significativa do número de fibras regeneradas (TEIXEIRA et al, 2003). Nos estudos de Noirez et al. (1999), observou-se que, 25 dias após a injeção do veneno de *Notechis scutatus scutatus* e tratamento com dexamethasona (glicocorticóide), houve um decréscimo da massa muscular em comparação ao grupo controle, com diminuição no diâmetro das fibras musculares e hipoplasia. Em contra partida, a inibição da reação inflamatória não afetou a regeneração muscular após a inoculação do veneno hemorrágico de *B. jararaca* (TEIXEIRA et al., 2005).

Com isto, objetivamos analisar os efeitos deste glicocorticóide na regeneração muscular no envenenamento botrópico experimental. Com o estudo da ação do Deflazacort visamos

compreender melhor a regeneração muscular e tratamento dos efeitos locais do veneno da *Bothrops jararacussu*.

No capítulo IV apresentaremos nossas considerações finais deste projeto de doutorado e o capítulo V refere-se a trabalhos adjacentes publicados realizados em outros projetos e grupos de pesquisa durante o período do curso de doutorado: “Intrinsic laryngeal muscles are spared from myonecrosis in the *mdx* mouse model of Duchenne Muscular Dystrophy” e “Pharmacological and local toxicity studies of a liposomal formulation for the novel local anaesthetic ropivocaine”.

2. Objetivos

Os objetivos do presente trabalho foram:

i) Analisar o efeito da L-arginina, precursor do óxido nítrico, na regeneração das fibras musculares após o envenenamento experimental pelo *Bothrops jararacussu.*

ii) Avaliar o efeito do antiinflamatório Deflazacort e a importância do processo inflamatório na regeneração muscular após a inoculação do veneno *B.jararacussu*.

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CAPÍTULO II

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Short communication

L-arginine enhances muscle regeneration after experimental envenomation by *B. jararacussu*: A future for nitric oxide-based therapy?

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Abstract

We investigated whether muscle fiber regeneration would be rescued by exogenous administration of L-arginine, the precursor of endogenous synthesis of nitric oxide. The right tibialis anterioris muscle of adult mice ($n = 20$) was injected with 80 μg of venom. One group of mice ($n = 10$) received drinking water containing L-arginine (3.75 mg/ml) and another group ($n = 10$) did not receive any pharmacological treatment. Two months later, muscle regeneration was evaluated by counting the total number of muscle fibers. We found that in L-arginine-treated mice, muscle regeneration was significantly higher ($p < 0.05$) than in saline-treated (2.230 ± 478 muscle fibers versus 1.005 ± 134, respectively) although the level of muscle fiber population of uninjured tibialis anterioris muscle (3.121 ± 102) was not attained. These results show that muscle regeneration was significantly facilitated by L-arginine and suggest that pharmacological activators of the NO pathway may be potentially useful for improving muscle regeneration in human envenomation by *B. jararacussu*.

Keywords: *Bothrops jararacussu*; L-arginine; Muscle regeneration; Nitric oxide

Muscle dysfunction due to loss of muscle mass, consequent to poor muscle fiber regeneration, is the main sequella following envenomation by the South America *Viperidae* snake, *Bothrops jararacussu* (Jorge et al., 1999). Myogenic resident satellite cells seem to be resistant to snake toxins (Harris, 2003) being suggested that their death is not the cause of poor muscle fiber regeneration after *B. jararacussu* envenomation (Santo Neto and Marques, 2005).

The activation of satellite cells is a fundamental step to the success of muscle regeneration. Following muscle fiber injury, satellite cells become activated, proliferate and differentiate into myotubes, which fuse to form new muscle fibers (Chargé and Rudnicki, 2004; Tidball, 2005). The activation of satellite cells is mediated by endogenous nitric oxide (NO), a freely diffusible molecule released by injured muscle fiber and inflammatory cells (Anderson, 2000; Chargé and Rudnicki, 2004). Recently, it had been shown that muscle regeneration in dystrophic mice is improved by administration of an exogenous source of NO, which acts possibly by

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activating satellite cells (Anderson and Vargas, 2003; Barton et al., 2005; Marques et al., 2005; Voisin et al., 2005). This suggests that NO-based therapy would be a useful strategy for rescuing muscle fiber regeneration in *B. jararacussu* envenomation. To examine this possibility we evaluated muscle regeneration in tibialis anterioris muscles of mice injected with *B. jararacussu* venom and treated with L-arginine, an amino acid precursor for the endogenous synthesis of NO.

Twenty adult male Swiss mice were used. All of the experiments described here were done within the guidelines established by the Brazilian College of Animal Experimentation (COBEA). The mice were anesthetized with a mixture (1:1) of ketamine hydrochloride (Ketalar®, Parke Davies) and thiazine hydrochloride (Rompum®, Bayer) at a dose of 0.02 mg/kg i.p. and the skin of the antero-lateral aspect of the right leg was opened. The right tibialis anterioris (RTA) muscle of all mice was then injected with 80 μg of *B. jararacussu* venom diluted in 0.3 ml of physiological saline solution through several injections in the distal, proximal and middle belly regions of the muscle. This procedure ensured the degeneration of all muscle fibers in the injected muscle (Santo Neto, 1987).

After the injection of *B. jararacussu* venom, animals were divided into two groups. One group ($n = 10$) received drinking water containing L-arginine (3.75 mg/ml; Sigma, St. Louis, MO) and it is referred as L-arginine group. The second group of mice ($n = 10$) did not receive treatment and was referred to as untreated group. After 60 days of pharmacological treatment, mice were again anesthetized and the RTA and left tibialis anterioris (LTA) muscle, which is referred as uninjured control muscle, were excised and immersed in formol-calcium fixative (40% formaldehyde; 10 ml distilled water, 90 ml calcium acetate 1 g) for 24 h. The mice were killed with an overdose of anesthetic after removal of the muscles. After fixation, the muscles were processed for embedding in hydroxymethyl methacrylate and transverse sections (7 μm thick) were obtained from the middle part of the muscle belly and stained with hematoxylin and eosin.

Muscle regeneration was evaluated as previously described (Luz et al., 2002). Briefly, low-power video images (10 × objective) of the entire cross-section were taken with a highly sensitive video camera linked to a light microscope and enhanced with an image processor system. The total number of muscle fibers was counted on a video monitor. The quantitative assessments were double blinded and five sections 35 μm apart were obtained from each muscle evaluated.

The mean and standard deviation of the number of muscle fibers were determined and compared among groups and the differences were determined post hoc using Duncan's multiple range tests. A probability of less than 0.05 indicated significance.

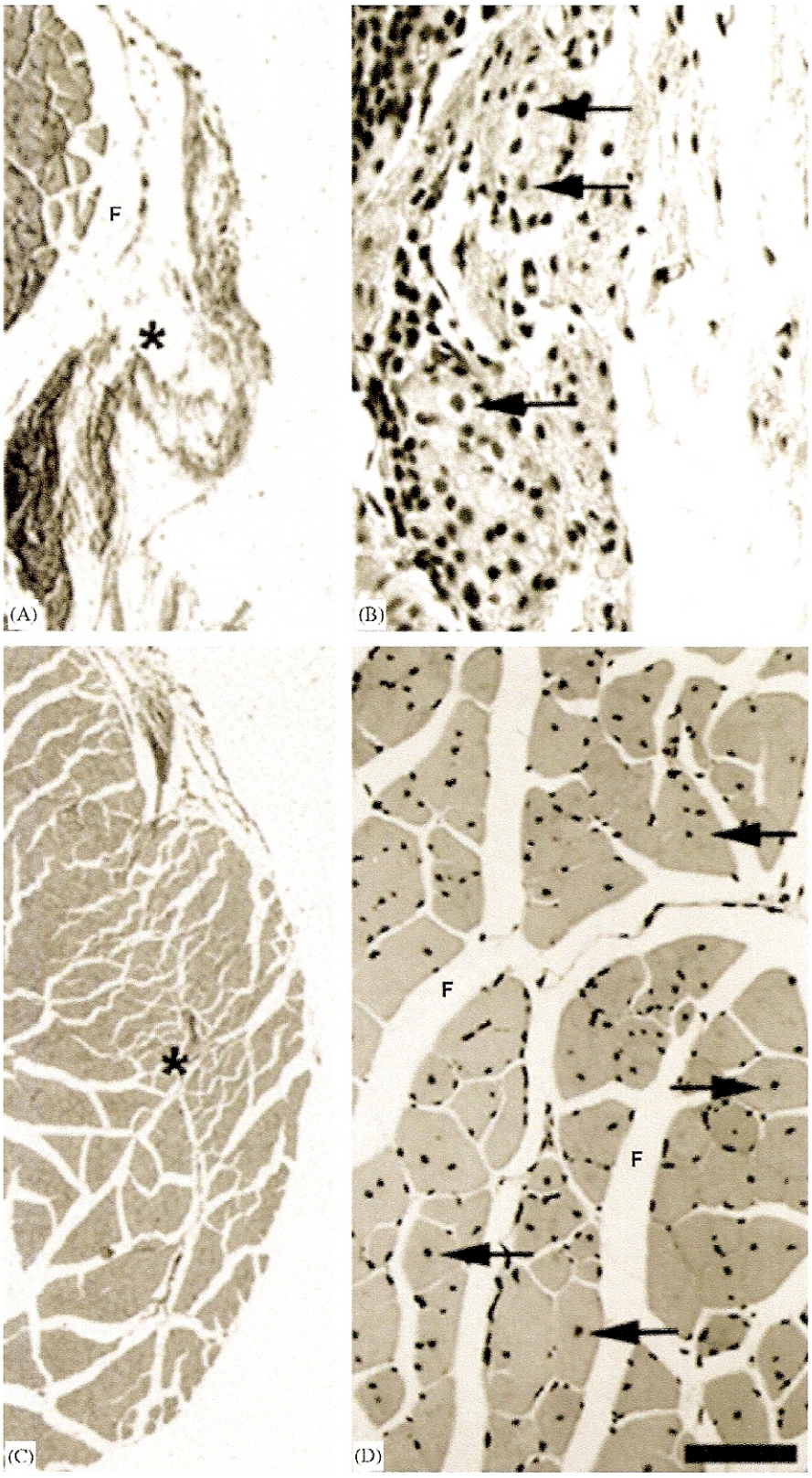
The histopathological features of the untreated group were essentially similar to those previously described for *B. jararacussu* envenomation (Queiróz et al., 1984). Briefly, we found poor muscle regeneration characterized by the presence of scattered small regenerated muscle fibers surrounded by great amount of fibrosis and fat tissue (Fig. 1A, B). Conversely, in the L-arginine group there were numerous, evenly distributed, regenerated muscle fibers of larger diameter, although fibrosis was still present (Fig. 1C, D).

The total number of muscle fibers in RTA muscles of the untreated group (1005 ± 134 muscle fibers) was decreased by 69%, when compared to uninjured control (3121 ± 102 muscle fibers). The total number of muscle fibers in L-arginine group (2230 ± 478) was significantly higher ($p < 0.05$) than in untreated group although it was reduced by 29% in comparison to uninjured control (LTA) (Fig. 2).

Our results demonstrate that treatment with L-arginine enhanced muscle regeneration after *B. jararacussu* envenomation. Whether enhanced muscle regeneration is in fact due to activation of resident satellite cells by L-arginine-derived NO cannot be demonstrated here.

Activation of satellite cells depends on several myogenic factors, such as those secreted by macrophages, including NO, and hepatocyte growth factor (HGF) which is released from the extracellular matrix (Sheehan et al., 2000; Peek et al., 2002; Tatsumi et al., 2002). Recruitment and

Fig. 1. Right tibialis anterioris muscle (*) after *B. jararacussu* venom in untreated (A, B) and L-arginine-treated mice (C, D). In untreated mice (A), the muscle is affected by the venom, with loss of muscle fibers and replacement by fibrous tissue (F). In (B), detail of (A) to show small regenerating muscle fibers with central nuclei (arrows). In (C), regenerated muscle has essentially normal volume and appearance at this magnification. In (D), detail showing regenerated fibers (arrows) with large diameter and homogenously distributed. Scale bar: 150 μm (A); 12 μm (B); 300 μm (C); 40 μm (D).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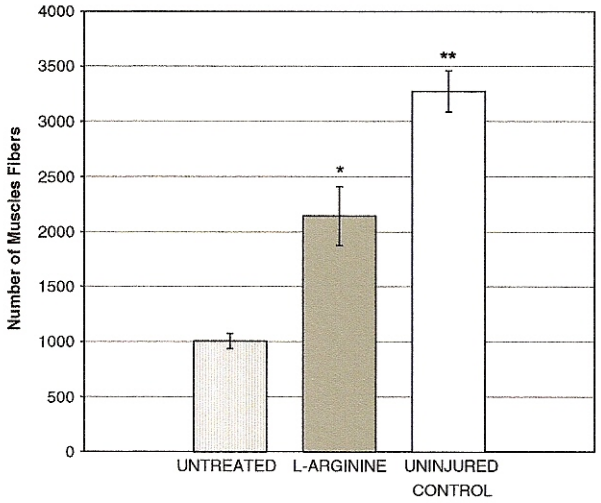

Fig. 2. Quantitative analysis of the effects of L-arginine treatment on muscle regeneration, 60 days after *B. jararacussu* envenomation. *Significantly different from untreated mice. **Significantly different from untreated and L-arginine-treated mice. ($*P<0.05$).

activation of macrophages are dependent on the presence of neutrophils (Cantini et al., 2002) and NO is a vasodilator that can promote leukocyte migration (Cirino et al., 2003). Thus, the activation of macrophages depends on intact local microcirculation and the activation of the HGF precursor also requires serum (Sheehan et al., 2000; Peek et al., 2002; Tatsumi et al., 2002). Particularly in case of snake venoms, neutrophils play a role in muscle regeneration (Teixeira et al., 2003). Since *B. jararacussu* venom damages local microcirculation leading to a lack of inflammatory cells within few hours after envenomation (Queiróz et al., 1984, 2002), it seems unlikely that the positive effects of L-arginine observed here are mediated by serum factors and inflammatory cells.

How L-arginine-derived NO could activate resident satellite cells in the absence of local microcirculation is an intriguing question. One possibility is that L-arginine diffuses first from the neighborhood tissues, such as extensor digitorium longus, to the periphery of RTA muscle. Once the revascularization is reestablished, L-arginine reaches deeper areas of RTA muscle. In this scenario, satellite cells located at the periphery would be activated first, as observed in free muscle grafts, where muscle regeneration occurs centripetally (Carlson et al., 1979). If the satellite cells located at the periphery were activated first, then muscle fiber diameter in peripheral layers would be larger than in deeper layers. However, our finding that the diameter of regenerating fibers was homogenous instead of being larger in the periphery of muscles does not support this possibility. Another explanation is that L-arginine reaches the damaged muscle only after microcirculation is reestablished. In this context, resident satellite cells would be activated by L-arginine-derived NO only by 3 weeks after envenomation, when microcirculation seems to be reestablished (Santo Neto and Marques, 2005). The fact that the activation of resident satellite cells by NO is time dependent, starting a few minutes after muscle fiber injury (Chargé and Rudnicki, 2004; Pisconti et al., 2006), argues against this hypothesis. Under normal conditions, satellite cells from undamaged muscles can migrate to damaged ones, promoting their regeneration (Chargé and Rudnicki, 2004; Tidball, 2005) and it would be interesting to see whether this also happens after *B. jararacussu* envenomation and L-arginine treatment.

Although nitrates are the most widely used NO donors (Parker and Parker, 1998; Boger and Bode-Boger, 2001), in the case of snake bites, the clinical use of nitrates could be disadvantageous because it can produce postural hypotension particularly in immobile standing patients (Parker and Parker, 1998; Boger and Bode-Boger, 2001). This side effect is potentially inconvenient for patients of *B. jararacussu* envenomation since they can already suffer a dramatic decrease in blood pressure and usually need to stay on the bed for a few days; nevertheless, the clinical use of L-arginine for the treatment of several human pathological conditions has been growing and seems to be well tolerated (Bode-Boger et al., 2003; Tousoulis et al., 2005; Wierema et al., 2005; Qian et al., 2005).

In conclusion, we have demonstrated that administration of L-arginine enhances muscle regeneration after experimental *B. jararacussu* envenomation. This result suggests that pharmacological activators of the NO pathway may constitute a realistic treatment to avoid loss of muscle mass and function in human envenomation.

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CAPÍTULO III

Loss of muscle regeneration following *Bothrops jararacussu* envenoming is aggravates by anti-inflammatory: experimental study

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Running title: Rescuing muscle regeneration at *B. jararacussu* envenomation

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Abstract

The study evaluates the effect of deflazacort (DFZ), an anti-inflammatory oxalazine derivative of prednisone, on muscle regeneration following myonecrosis experimentally induced by *B. jararacussu* venom. Mice (*n*=15) right tibialis anterioris muscle was injected with 80 µg of venom. Two groups of animals (*n=10)* was treated during 5 and 20 days with a daily intraperitoneal injection of deflazacort, an anti-inflammatory oxalazine derivative of prednisone (1,2 mg/kg). The third group was injected with crude venom and did not receive any pharmacological treatment. The animals were killed 5 and 60 days after envenoming and muscle regeneration was evaluated by counting the number of muscle fibers and measuring the fibrosis area. We found that in deflazacort-treated group the area of fibrosis was higher ($p < 0.05$) than in injected muscles without treatment (1.077.051 ± 466.658,2; versus 777.107,3 ± 356.804,8 pixels square), and the number of muscle fiber was significant different too (755 ± 84; versus 1.221 ± 102). Although it does not reach the level of muscle fiber population of uninjured tibialis anterioris muscle (3.257 ± 478). We conclude that deflazacort treatment is detrimental to muscle fiber regeneration aggravating the loss of muscle mass after *B. jararacussu* envenoming.

Key words: *Bothrops jararacussu*; Deflazacort; muscle regeneration; fibrosis.

1. INTRODUCTION

Bites by snakes of the genus *Bothrops* account for nearly 90% of the human cases of ophidism in Brazil and *Bothrops jararacussu* is largely distributed in the southern regions of this country (Rosenfeld, 1971; Watt, 1989). Necrosis of muscle fibers is the most prominent local effects and poor muscle regeneration leading loss of muscle mass represents the main sequella following poisoning by *Bothrops jararacussu* (Milani et al., 1997; Jorge et al., 1999; da Silva et al., 2003).

Although serum therapy is the only effective treatment a few epidemiological studies demonstrate that anti-inflammatories have sometimes been used in the treatment of snakebites, (Ribeiro et al., 1995; da Silva et al., 2003). Inflammatory cells are known to play a positive role on muscle regeneration (Harris 2003; Chargé and Rudnick, 2004; Tidball, 2005) but the effects of anti-inflammatories remains primary controversial after muscle injury. While some studies indicate it promotes healing by reducing inflammation others have demonstrated detrimental effect possible consequent to retard phagocyte (Mendias et al., 2004; Rahusen et al., 2004; Shen et al. 2005; Jarvinen et al., 2005). Accord, it is of interest to examine whether anti-inflammatory could affect muscle regeneration following *B. jararacussu* envenomation. Here we examine the effect of deflazacort (DFZ), an anti-inflammatory prednisone derivative on muscle regeneration after experimental envenoming by *B. jararacussu* in mice.

2. MATERIAL AND METHODS

2.1. Animals and Venom

Fifteen adult male Swiss white mice (25-30g) were supplied by the animal services unit of the State University of Campinas. The mice were maintained in the animal house of the Department of Anatomy on a 12 h light/dark cycle at 22 °C with free access to water and standard rodent chow (Purina®, Campinas, SP, Brazil). *Bothrops jararacussu* venom obtained from adult snakes of both sexes was purchased from the CETA (Centro de Extração de Toxinas Animais, Morungaba, SP, Brazil).

2.2. Venom inoculation and animal's treatment

*E*xperiments described here were done within the guidelines established by the Brazilian College of Animal Experimentation (COBEA, protocol n°.1708-1). The mice were anesthetized with a mixture (1:1) of ketamine hydrochloride (Ketalar®, Parke Davies) and thiazine hydrochloride (Rompum®, Bayer) at a dose of 0.02 mg/kg *i.p.* and the skin of the antero-lateral aspect of the right leg was opened. The right tibialis anterioris muscle (RTA) of all mice was then injected with 80 μg of *B. jararacussu* venom diluted in 0.1 ml of physiological saline solution through several injections in the distal, proximal and middle belly regions of the muscle.

After the injection of *B. jararacussu* venom, animals were divided into two groups. In one group, mice (*n*=10) received a diary intraperitoneal injection of deflazacort (LIBBS, Brazil, Sigma, St. Louis, MO; 1,2mg/kg). Treatment was discontinued after 5 (n=5) and 20 (n=5) days which mice were with food and water *ad libitum*. The second group of mice (n=5) did not receive any pharmacological treatment and is referred as untreated group.

2.3. Histological methods and qualitative assays

At 5 and 60 days post-envenomation, mice were anesthetized as above described and the RTA muscles were excised and immersed in formol-calcium fixative (40% formaldehyde; 10 ml-distilled water 90 ml -calcium acetate 1g) for 24 h. After fixation, the muscles were processed for embedding in hydroxymethyl methacrylate and transversal sections (7 µm thick) were obtained from the middle part of the muscle belly and stained with Masson's trichrome and hematoxilin-eosin (HE). The left tibialis anterioris (LTA) of untreated group (animals of 60 days post-envenoming only) were also processed and stained with HE and was referred as control-uninjected muscles. Qualitative analyses were made on a light microscope (Nikon® Eclipse E400).

2.4. Quantitative analysis

Muscle regeneration (60 days after envenoming) was evaluated as previously described (Santo Neto et al., 2004). Briefly, a low-power video images (10X objective) of the entire TA muscle cross-section were taken with a highly sensitive video-camera (Nikon® Express Series) linked to a light microscope (Nikon® Eclipse E400) and enhanced with an image processor system (Image Pró-Express®). Muscle fibers were directly counted on a video-monitor.

Evaluation of fibrosis (60 days after envenoming) was made on Masson's trichrome-stained sections and the Masson's trichrome-positive area was used as a measurement of the fibrosis area. For each sample in untreated and DFZ-treated group the entire muscle fields were used for each sample, and images were collected by using the equipment above described. The images were digitized through the use of Image Pró-Express® frame grabber board. Features were then extracted, and the absolute area of trichrome-positive staining was measured for each image and expressed in terms of square pixels. The results were compared among experimental groups.

Analysis in short time of the muscles (5 days) was performed by observation of the inflammatory process and tissue debris, directly on the monitor video as above described on HE-stained sections.

2.4. Statistical analysis

The mean and standard deviation of the number of muscle fibers were determined and compared among groups and the differences were determined post hoc using Duncan's multiple range tests. A probability of less than 0.05 indicated significance.

3. RESULTS

3.1 Histological analyses

In the histopathological basis the effects of *B. jararacussu* were similar in appearance to that previously described (Queiróz et al., 1984), although conspicuous differences between control and DFZ treated mice were observed. At 5 days post-envenoming the upper two thirds of TA show coagulative necrosis and some blood vessels contained fibrin thrombi, although most are empty. Inflammatory cells were barely observed within of the injured area and they were present in the periphery of muscles from untreated mice (Fig. 3, A, C, E) but they were practically absent on muscles of the DFZ-treated mice (Fig. 3, B, D, F).

At the longest survival time (60 days) the necrotic fibers had been replaced by regenerating muscle fibers in both control and DFZ-treated group. In the basic histological observations no gross differences between those groups could be detected; muscle fibers displayed central nuclei of both groups were similar did not they. Some areas of tibialis anterioris muscle mainly on the DFZ treated mice were replaced by fibrosis (Fig. 4, B, C, D).

3.1 Quantitative findings

Statistically differences were observed between untreated and DFZ-treated mice in all parameters we examined. Figure 1 shows that at the end of 60 days the total of muscle fibers

in DFZ group (755 ± 84) was 73.4% lower than untreated group (1.221 ± 102) which in turn was significantly lower than those of control group (3.257 ± 478). In mice of the DFZ group the amount of fibrosis area showed to be larger than untreated group (1.077.051 ± 466.658,2; versus 777.107,3 ± 356.804,8 square pixels), (Figure 2).

4. DISCUSSION

Muscle regeneration was worsened when envenoming by *B. jararacussu* was treating with DFZ since the population of muscle fibers decreased and fibrosis increased significantly in comparison to untreated group, in this study. It may be of clinical interest because snake bites have sometimes been treated with anti-inflammatory (Ribeiro et al., 1995; da Silva et al., 2003) and muscular dysfunction consequent to poor muscle regeneration is already the main sequella following enevenoming by *B. jararacussu* in humans (Milani et al., 1997; Jorge et al., 1999; da Silva et al., 2003).

Muscle regeneration is not affected by inhibition of inflammation reaction after *B. jararaca* envenoming (Teixeira et al., 2005a) but it was impaired following envenoming by *B. asper* (Teixeira et al., 2003). Such difference has been attributable to the fact that vascular damage with dysfunction on microcirculation precedes necrosis of muscle fibers making irrelevant for muscle regeneration whether inflammatory cells cannot readily reach the area of necrotic fibers at *B. jararaca* envenoming (Teixeira et al., 2005). The time-course and pathogenesis of local lesions induced by *B. jararacussu* (Queiróz et al., 1984; Santo Neto et al., 2004) share many features with that one described at *B. asper* envenoming (Gutierrez et al., 1984, 1986; Arce et al., 1991). Neutrophils predominate earlier after *B. jararacussu* envenoming in untreated mice in previous (Queiróz et al., 1984) and in this study. At 5 days the density of inflammatory cells was accentually reduced meaning that phagocytosis may be delayed in DFZ-treated mice. Because remotion of necrotic debris plays a role on muscle regeneration (Harris, 2003; Chargé

and Rudnick, 2004; Tidball, 2005) it seems that impairing muscle regeneration should be consequent to retarding paghocytosis in DFZ-treated mice. However, to better of our knowledge a causal relationship between slowing phagocytosis and decreasing number of muscle fibers, as we observed here, has not been reported.

Satellite cells, the resident myogenic cell lineage play a central role in muscle regeneration. Following muscle injury they become activated, proliferate and differentiate into myotubes, which fuse to form new muscle fibers (Chargé and Rudnicki, 2004; Tidball, 2005). Satellite cells also migrate from the neighbor's muscles to reach the site of injury and migration can be impairing by interstitial fibrosis since it can act as a physical barrier (Schultz and McCormick, 1994; Orchard and Best, 2002). In this study we found that The fact that interstitial muscular fibrosis was significantly increased in DFZ treated mice suggest that that increasing fibrosis may contribute directly to decreasing population of regenerated muscle fibers in this experimental group.

Metalloproteinases from *Bothrops* snake venoms degrades extracellular matrix in skeletal muscles (Rucavado et al., 2002; Teixeira et al., 2005b). The activation of plasminogen system including urokinase-type plasminogen activator (uPA) and its primary inhibitor the plasminogen activator inhibitor-1 (PAI-1) depends on the integrity of extracellular matrix in skeletal muscles. Deficiency in PAI-1 increases the expression of uPA enhancing muscle regeneration, possible by facilitating myoblast proliferation. DFZ increases the expression of PAI-1 in diseases of extracelular matrix but not in healthy subjects (Lluís et al., 2001; Suelves et al., 2002; 2005; Del Rosso et al., 2005). These findings suggest that disintegration of extracellular matrix after *B. jararacussu* envenomation may also account for decreasing population of regenerating muscle fibers in DFZ group.

In sum we demonstrated that administration of DFZ impair muscle regeneration after experimental *B. jararacussu* envenomation. It suggests that the use of anti-inflammatory drugs

to treat human envenomation warrants caution because it may interfere with muscle healing aggravating the loss of muscle mass as the main sequella at *B. jararacussu* envenomation.

Acknowledgements

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, grants 95/6110-2 and 01/00570-4). H.S.N. and M.J.M. are supported by research fellowships from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (302880/04-6 and 301286/03-5).

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Captions

Figure 1. The total of muscle fibers in DFZ group (755 ± 84) was lower than untreated group (1.221 ± 102) which in turn was significantly lower than those of control group (3.257 ± 478) at the end of 60 days after *B.jararacussu* envenomation. *Significantly different from untreated mice. **Significantly different from untreated and DFZ-treated mice. (*P<0.05).

Figure 2. Area of total tissue, muscle fibers and fibrosis in TA muscle 60 days after *B. jararacussu* envenomation. *Significantly different from untreated (Bj) mice. **Significantly different from control and Deflazacort treated mice (Dfz). (*P<0.05).

Figure 3. Transverse sections of tibialis anterioris stained with HE muscle from control and DFZ-treated mice 5 days after the inoculation of *B. jararacussu* venom. The inflammatory cells are present in the periphery of untreated muscles (A; arrows) but they were practically absent on muscles of the DFZ-treated mice in the local of envenomation (B; arrows). In untreated group, there is an amount of small regenerating muscle fibers (C, E central nucleated fibers; (arrows) whereas necrotic debris persists in DFZ group with poor presence of inflammatory cells (D, F; arrows). Scale bar: 300 µm (A, B); 30 µm (C, D); 10 µm (E, F).

Figure 4. Transverse sections of tibialis anterioris stained with Masson's trichrome 60 days after *B. jararacussu* envenomation. Note that areas of intersticial fibrosis is larger in DFZ-treated mice (B, C, D) than untreated group (A) where the muscle fibers were replaced by fibrotic tissue (arrows); Scale bar: 300 µm. The details (arrow head) show smalls regenerating muscle fibers between the fibrotic tissue; Scale bar: 15 µm.

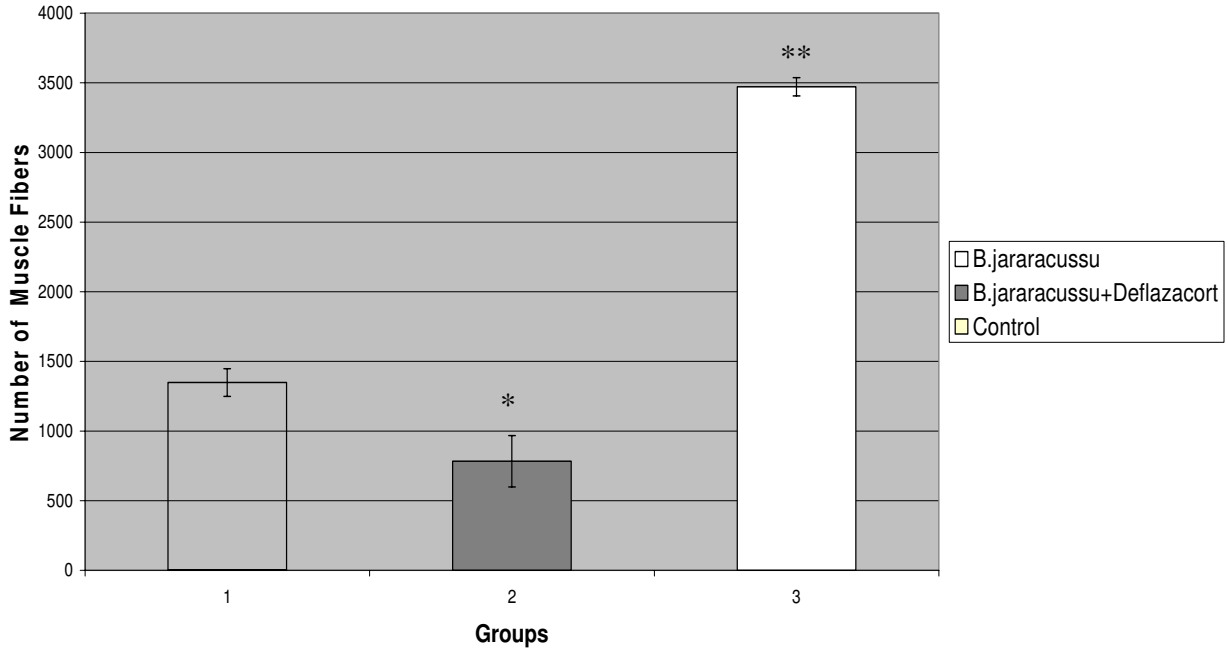

Figure 1.

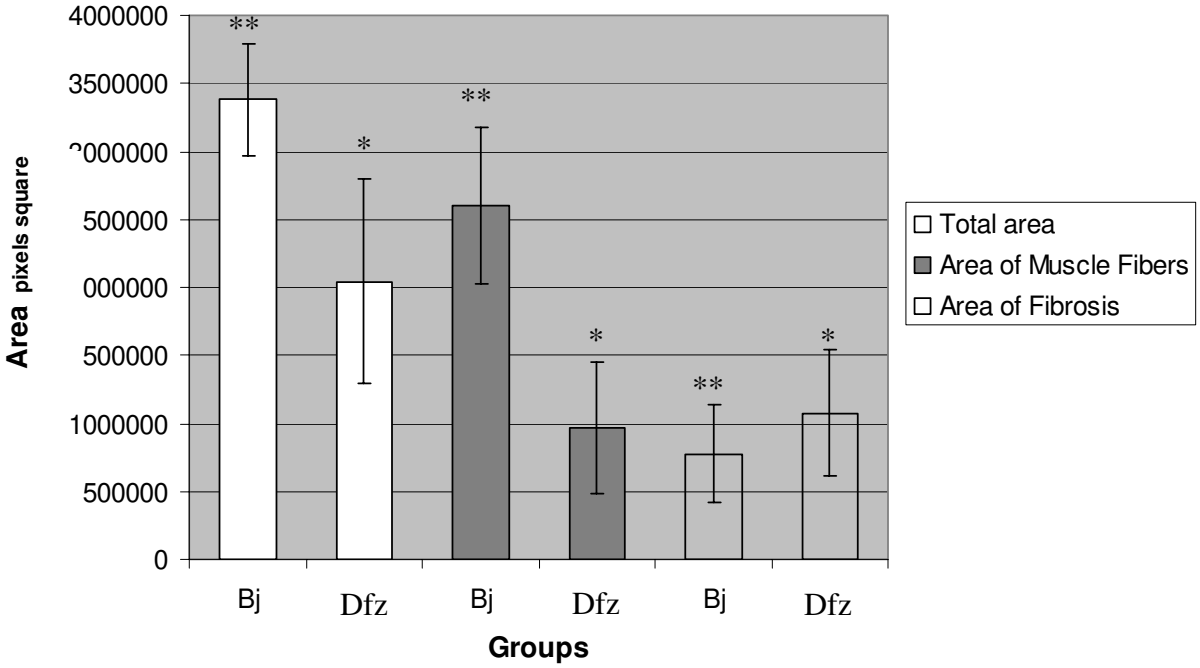

Figure 2.

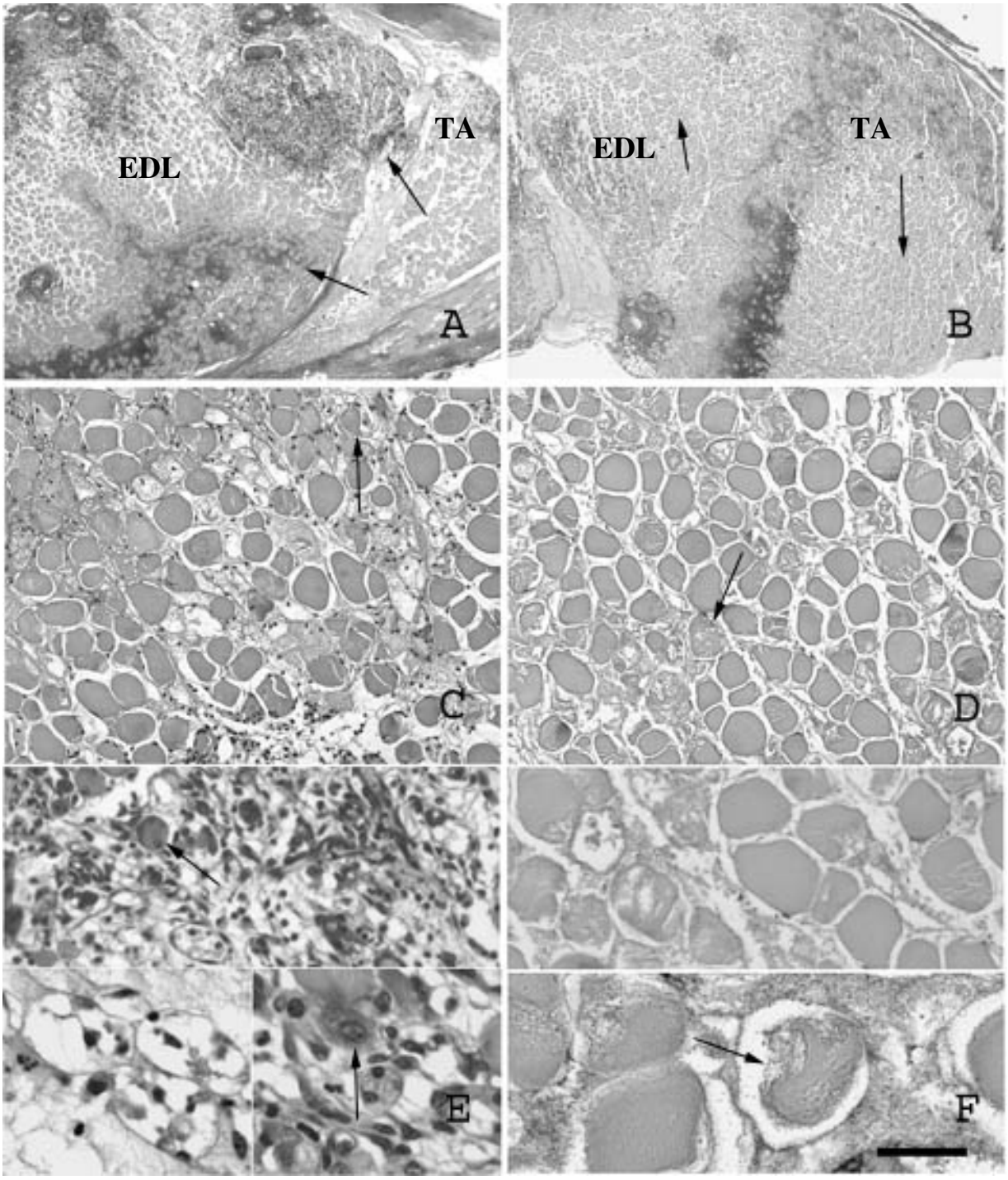

Figure 3.

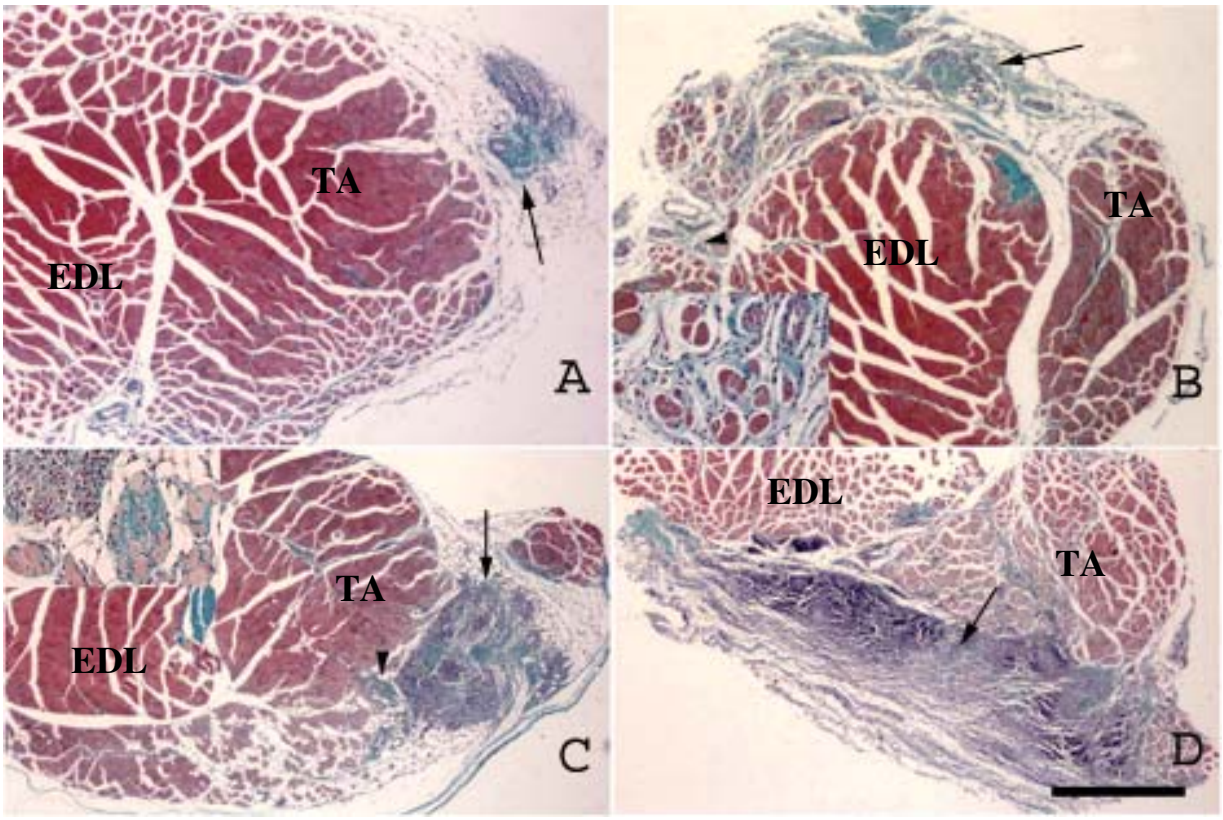

Figure 4.

CAPÍTULO IV

4. Considerações Finais

No presente trabalho demonstramos morfologicamente os efeitos dos fármacos L-arginina e Deflazacort no tratamento dos danos locais decorrentes pelo envenenamento por *B. jararacussu.*

A L-arginina, o nitrato mais utilizado como precursor de óxido nítrico, apresentou resultados promissores na análise do tecido muscular após o envenenamento. Observou-se que, após dois meses de administração da L-arginina posterior à inoculação do veneno, as fibras musculares regeneradas apresentaram-se em maior número, diâmetro e homogeneamente distribuídas, em relação ao grupo não tratado, embora a fibrose ainda esteja presente neste grupo.

No grupo não tratado nossos resultados foram similares aos observados em estudos anteriores (Queiroz et al, 1984, Santo Neto, 1987), notou-se a presença de fibras em regeneração de pequeno diâmetro, grande quantidade de tecido fibrótico e adiposo no local das fibras musculares.

A diferença no total das fibras musculares entre o grupo inoculado com o veneno e tratado com a L-arginina, grupo injetado não tratado e controle demonstrou um resultado significativo, com presença de maior número de fibras musculares no grupo tratado em relação ao grupo não tratado. Observou-se uma diminuição na defasagem da população das fibras musculares do grupo injetado tratado com a L-arginina em comparação com o grupo controle não injetado. Provavelmente o aumento da regeneração das fibras musculares ocorreu pela ativação de células satélites de áreas vizinhas não atingidas pelo veneno, causando a migração destas e promovendo a regeneração tecidual, como observado nos estudos de Tidball (2005).

Desta forma demonstramos que o tratamento com a L-arginina promoveu um aumento significativo na regeneração muscular após o envenenamento experimental por *B. jararacussu*. Estes resultados sugerem que a administração de um precursor exógeno de óxido nítrico mostra-se um tratamento promissor no combate à atrofia muscular encontrada posterior ao envenenamento.

Entretanto, os efeitos locais decorrentes da utilização do deflazacort, glicocorticóide derivado da prednisona, demonstraram agravar a diminuição da regeneração muscular após o envenenamento por *B. jararacussu.*

Cinco dias após o envenenamento observou-se escassa presença de células inflamatórias no local da injeção do veneno no grupo não tratado, com maior população destas na periferia da área de lesão tecidual. No grupo injetado tratado com deflazacort a presença de células inflamatórias era praticamente ausente nas áreas de mionecrose.

No período de 2 meses de vida após a injeção de veneno e tratamento de 20 dias com o antiinflamatório notou-se a presença de fibras musculares regeneradas com núcleo central e de pequeno diâmetro, semelhante ao grupo não tratado. Contudo, observou-se que no grupo tratado com deflazacort ocorreu um aumento da reposição tecidual do músculo tibial anterior por fibrose.

O total de número de fibras musculares demonstrou-se significativamente menor no grupo tratado com deflazacort em relação ao grupo não tratado. Assim como a diferença na área de fibrose tecidual, que no grupo tratado mostrou-se maior que o grupo não tratado.

Desta forma, nossos resultados indicam que o retardo no debridamento do tecido necrótico poderia causar o aumento da fibrose tecidual, e esta, contribuindo diretamente no déficit da regeneração muscular. Além das alterações ocorridas no meio extracelular promovidas pelo veneno, uma possível ação do deflazacort no aumento da expressão de inibidor-1 do ativador de plasminogênio poderia influenciar na diminuição da proliferação de mioblastos (Lluís et al., 2001; Suelves et al., 2002; Del Rosso et al., 2005).

Em conclusão, demonstramos que a administração do antiinflamatório deflazacort prejudica a regeneração muscular após o envenenamento experimental por *B. jararacussu.* O que sugere que a utilização de antiinflamatórios no tratamento após os acidentes ofídicos requer cautela com relação ao agravamento da seqüela local, como o que ocorre pelo veneno da *B. jararacussu*.

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CAPÍTULO V

ABSTRACT: Intrinsic laryngeal muscles share many anatomical and physiological properties with extraocular muscles, which are unaffected in both Duchenne muscular dystrophy and *mdx* mice. We hypothesized that intrinsic laryngeal muscles are spared from myonecrosis in *mdx* mice and may serve as an additional tool to understand the mechanisms of muscle sparing in dystrophinopathy. Intrinsic laryngeal muscles and tibialis anterior (TA) muscle of adult and aged *mdx* and control C57Bl/10 mice were investigated. The percentage of central nucleated fibers, as a sign of muscle fibers that had undergone injury and regeneration, and myofiber labeling with Evans blue dye, as a marker of myofiber damage, were studied. Except for the cricothyroid muscle, none of the intrinsic laryngeal muscles from adult and old *mdx* mice showed signs of myofiber damage or Evans blue dye labeling, and all appeared to be normal. Central nucleation was readily visible in the TA of the same *mdx* mice. A significant increase in the percentage of central nucleated fibers was observed in adult cricothyroid muscle compared to the other intrinsic laryngeal muscles, which worsened with age. Thus, we have shown that the intrinsic laryngeal muscles are spared from the lack of dystrophin and may serve as a useful model to study the mechanisms of muscle sparing in dystrophinopathy.

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INTRINSIC LARYNGEAL MUSCLES ARE SPARED FROM MYONECROSIS IN THE *mdx* MOUSE MODEL OF DUCHENNE MUSCULAR DYSTROPHY

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Duchenne muscular dystrophy (DMD) is a severe X-linked recessive disorder characterized by progressive loss of muscular strength that affects 1 in 3500 live male births.[9,10] DMD is caused by a lack of dystrophin, a cytoskeletal protein localized on the inner surface of the muscle cell membrane. Lack of dystrophin predisposes the cell membrane to breakdown, leading to muscle-fiber necrosis.[13] In the *mdx* mouse, an experimental model for DMD, skeletal muscle fibers exhibit a drastic reduction in the expression of dystrophin, which results in myonecrosis.[6,26]

The extraocular muscles (EOMs) of both DMD patients and *mdx* mice remain unaffected during the course of the disease. Because necrosis of muscle fibers is central in the pathophysiology of DMD, understanding the mechanisms that allow EOMs to escape from myonecrosis is of interest and has been examined extensively in *mdx* mice.[1,3,5,16,20,22–24,27]

The intrinsic laryngeal muscles (ILMs) share many anatomical and physiological properties with the EOMs.[2,7,11,14] The ILMs are innervated by cranial nerves, express extraocular myosin heavy chain, and present short contraction times and continuous muscle-fiber remodeling.[11,14,25] Hence, we hypothesized that the ILMs are spared from myonecrosis in the *mdx* mouse model of DMD and may serve as an additional tool to study the mechanisms of muscle sparing in dystrophinopathy. The present study was undertaken to investigate this hypothesis.

Abbreviations: CT, cricothyroid; DMD, Duchenne muscular dystrophy; EBD, Evans blue dye; EOMs, extraocular muscles; ILMs, intrinsic laryngeal muscles; PBS, phosphate-buffered saline; TA, tibialis anterior

Key words: aging; dystrophin; intrinsic laryngeal muscles; *mdx* mouse; muscle regeneration

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MATERIALS AND METHODS

Male *mdx* and C57Bl/10 mice obtained from the mouse breeding colony at our institution were housed under controlled conditions of 12/12-h light/dark cycle and temperature, with free access to food and water. All experiments were performed in

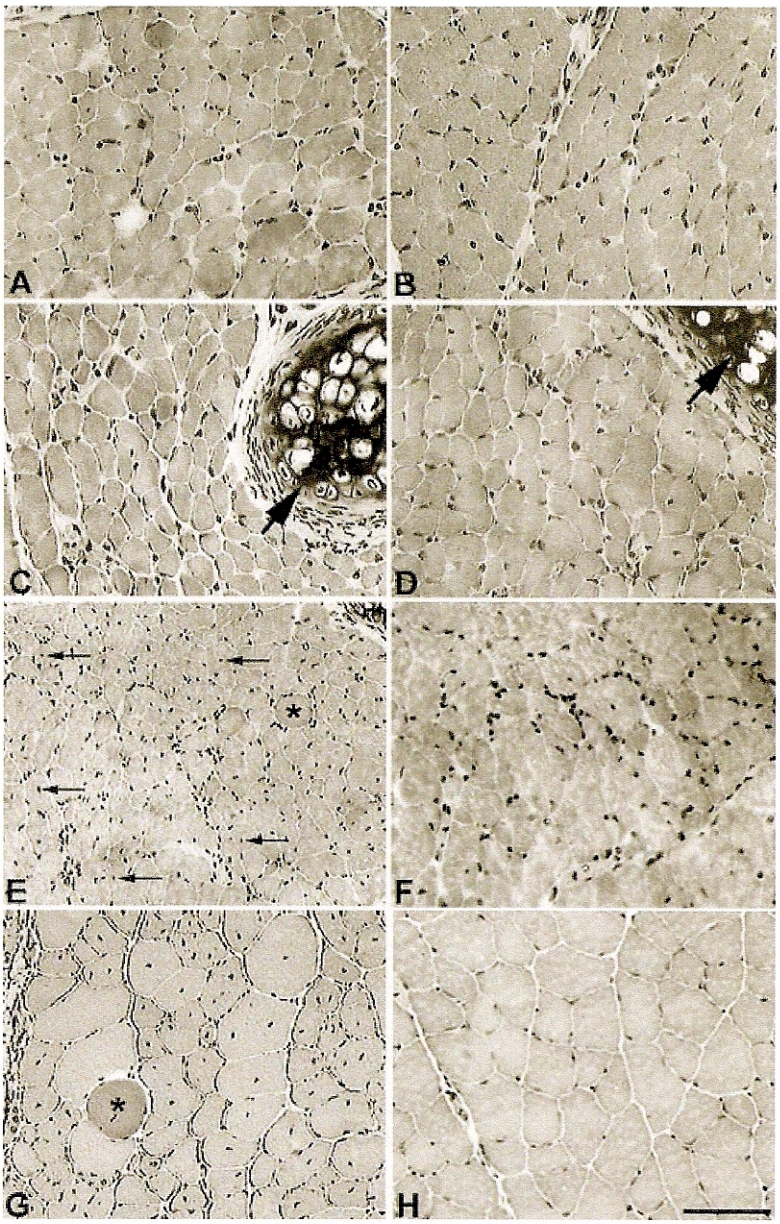

FIGURE 1. Transverse sections of intrinsic laryngeal muscles stained with hematoxylin–eosin. Left column: *mdx* muscles. Right column: counterpart controls; note the presence of peripheral cell nuclei. No differences were observed between old *mdx* lateral thyroarytenoid **(A)**, adult control lateral thyroarytenoid **(B)**, adult *mdx* lateral cricoarytenoid **(C)**, and adult control lateral cricoarytenoid **(D)** muscles. Central nucleated fibers were present in old *mdx* cricothyroid **(E)** (arrows) muscle. **(F)** Old control cricothyroid muscle. Tibialis anterior muscle from *mdx* **(G)** and control **(H)** mice. Asterisk in **E** and **G**: hypercontracted fibers. Arrow in **C** and **D**: cartilage. Scale bar, 140 μm.

accordance with the guidelines for the use of animals set forth by our institution.

For visualization of muscle-fiber damage, adult (4 months of age) *mdx* ($n = 5$) and C57Bl/10 (control; $n = 5$) and old (18 months of age) *mdx* ($n = 5$) and C57Bl/10 ($n = 5$) mice were injected with Evans blue dye (EBD; Sigma, St. Louis, Missouri), a marker of sarcolemmal lesions.[12] The animals received an intraperitoneal injection of 1% EBD in phosphate-buffered saline (PBS) at a dose of 100 μl per 10 g body weight. Twenty-four hours later, the mice were killed with an overdose of chloral hydrate and the larynx and right tibialis anterior (TA) muscle were dissected out and snap frozen in isopentane cooled in liquid nitrogen.

Cryostat cross-sections of the larynx (transverse and longitudinal 7-μm-thick sections) and TA (trans-

Table 1. Percentage of central nucleated fibers in lateral cricoarytenoid (LCA), posterior cricoarytenoid (PCA), lateral thyroarytenoid (LTA), medial thyroarytenoid (MTA), cricothyroid (CT) and tibialis anterior muscles from adult (4-month-old) and aged (18-month-old) C57B1/10 (control) and *mdx* mice.

	LCA		PCA		LTA		MTA		CT		Tibialis anterior	
	Adult	Aged	Adult	Aged	Adult	Aged	Adult	Aged	Adult	Aged	Adult	Aged
Control	2.0 ± 1.3 (n = 7)	2.2 ± 0.4 (n = 3)	0.8 ± 0.6 (n = 6)	1.1 ± 0.2 (n = 3)	1.1 ± 0.7 (n = 7)	1.2 ± 0.8 (n = 3)	1.2 ± 0.7 (n = 6)	1.4 ± 0.9 (n = 3)	4.8 ± 1.1* (n = 7)	5.3 ± 1.1* (n = 3)	1.0 ± 0.1 (n = 5)	0.9 ± 0.1 (n = 5)
mdx	2.4 ± 1.1 (n = 10)	2.5 ± 0.3 (n = 6)	1.0 ± 0.5 (n = 10)	1.1 ± 0.3 (n = 5)	1.3 ± 0.6 (n = 10)	1.4 ± 0.6 (n = 5)	1.2 ± 0.6 (n = 5)	1.3 ± 0.5 (n = 6)	9.3 ± 4.0* (n = 8)	18.0 ± 1.5*† (n = 5)	50.0 ± 1.0* (n = 5)	96 ± 2.0 (n = 5)

Values represent the mean ± standard deviation; n = number of muscles examined.
** $P < 0.05$: significantly different from all groups (Duncan's multiple comparisons of means).*
† $P < 0.05$: significantly different from adult animals of the same strain (Duncan's multiple comparisons of means).

verse 7-μm-thick sections) were stained with hematoxylin–eosin (H&E) for quantification of the total number of fibers and the number of fibers with central nucleation, indicative of muscle regeneration. The number of fibers and of central nucleated fibers was counted by a blinded observer. The ILMs studied were the lateral thyroarytenoid, medial thyroarytenoid (vocalis muscle), lateral cricoarytenoid, posterior cricoarytenoid, and cricothyroid (CT).

Some sections were labeled for dystrophin. Sections were air dried, hydrated for 30 min with PBS, incubated with 0.3% Triton X-100 for 10 min, and then blocked with blocking solution (15% glycine, 3% bovine serum albumin, and 0.6% Triton X-100 in PBS; Sigma) for 3 h. The sections were incubated with dystrophin antibody (NCL-DYS1 mouse monoclonal, Novacastra, Newcastle upon Tyne, UK) at 1:500 overnight at 4°C, washed with PBS, and incubated with secondary anti-mouse immunoglobulin G–flourescein isothiocyanate (IgG-FITC; Sigma, St. Louis, Missouri) at 1:500 for 1 h at room temperature. Sections were washed again in PBS, coverslipped with 1,4-diazabicyclo [2.2.2]octane (DABCO; Sigma) mounting medium, and observed under a confocal microscope (MRC 1024; BioRad Laboratories, Hercules, California).

EBD staining appears as a bright red emission under a fluorescence microscope. Fiber counts of EBD-positive muscle fibers and H&E observation were done for all sections and photographed under a Nikon fluorescence microscope connected to a Hamamatsu video camera. Statistical analysis was performed using the ProcGLM (general linear models) of the SAS statistical program; mean comparisons were done using the average multiple comparison test (SAS Institute, Cary, North Carolina).

RESULTS

In adult and old *mdx* mice, no signs of myofiber damage were observed in any of the ILMs (Fig. 1A, C), except for the CT muscle (Fig. 1E). The lateral thyroarytenoid, medial thyroarytenoid (vocalis muscle), lateral CT, and posterior CT appeared to be normal, with muscle fibers round or roughly polygonal with rounded angles. In cross-sections, their nuclei were randomly placed, always found in a peripheral location directly under the sarcolemma,

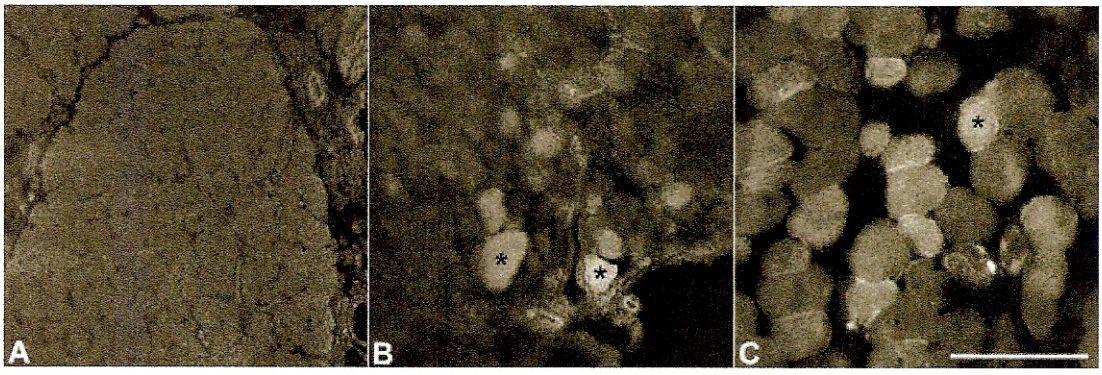

FIGURE 2. Transverse sections of Evans blue dye (EBD)–labeled muscles. Adult *mdx* lateral thyroarytenoid muscle **(A)** showing no EBD-positive fibers. EBD-positive fibers were observed in adult *mdx* cricothyroid **(B)** (asterisk) and tibialis anterior **(C)** (asterisk) muscles. Scale bar, **(A)** 166 μm; **(B)** 144 μm; **(C)** 183 μm.

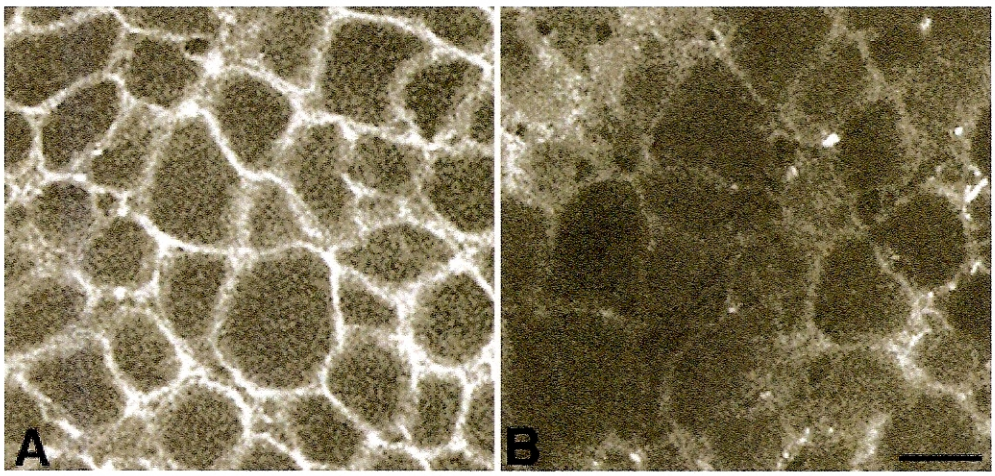

FIGURE 3. Transverse sections of dystrophin-labeled posterior cricoarytenoid muscles. In controls **(A)**, dystrophin was seen in the sarcolemma as a bright outline of each fiber. In *mdx* mice **(B)**, the lack of dystrophin was evident. Scale bar, 75 μm.

similar to control muscles (Fig. 1B, D). Muscle fibers had a relatively uniform diameter, and no degenerating myofibers or extensive areas of inflammatory reaction were observed (Fig. 1A, C). In these muscles, the percentage of central nucleated fibers, the morphological indicator of fibers having undergone damage, did not differ from control (Table 1). No myofibers containing EBD, an earlier marker of sarcolemmal disruption, were seen (Fig. 2A).

The adult *mdx* CT muscle displayed evidence of myopathy, represented by an increased percentage of central nucleated fibers when compared to the other ILMs (Fig. 1E). Compared to the TA muscle, this increase was not significant. The dystrophic phenotype of the CT was more evident in aged mice, which showed a twofold increase in the percentage of central nucleated fibers (Table 1). Both inflammatory reaction and fiber injury, as demonstrated by EBD-positive fibers, were observed more frequently (Fig. 2B). In the TA of adult and old *mdx* mice, EBD-positive fibers were present (Fig. 2C) and central nucleated fibers were readily visible (Fig. 1G and Table 1).

Control posterior CT muscles exhibited a normal pattern of dystrophin distribution, with dystrophin labeling associated with the sarcolemma (Fig. 3A). In *mdx* mice, posterior CT muscles were negative for dystrophin (Fig. 3B). This shows that, despite the lack of dystrophin, there was no muscle-fiber degeneration in ILMs.

DISCUSSION

In the present study, we evaluated whether ILMs from *mdx* mice show signs of muscle-fiber degeneration. Usually, myonecrosis in *mdx* mice starts at about 3–4 weeks of age, with degeneration of limb muscles having occurred by 10 weeks of age.[8,26] Except for the CT muscles, we did not observe any signs of muscle-fiber damage in the ILMs of adult or aged *mdx* mice. Central nucleation was significantly lower in ILMs than in the TA muscles, as is described also for the EOMs, where myonecrosis is not observed.[1,3,16,22–24,27] Therefore, the ILMs do not exhibit the pattern of muscle necrosis and regeneration seen in most *mdx* skeletal muscles, demonstrating that these muscles are protected from the lack of dystrophin.

Loss of calcium homeostasis has been suggested to play a role in the mechanism of muscle necrosis in DMD and *mdx* mice.[16] In the EOMs, proteins involved in calcium reuptake, such as parvalbumin and sarcoplasmic reticulum calcium ATPase, are increased, and this may explain their escape from myonecrosis.[7] Preliminary observations have shown that calcium reuptake and release systems are both amplified in laryngeal muscles,[4] suggesting that, similar to the EOMs, dystrophic laryngeal muscles may be spared from myonecrosis by a better capacity to maintain calcium homeostasis.

Continuous myofiber remodeling has been reported in non-dystrophic EOMs as a result of fusion of satellite cells into existing myofibers, and this may account for the sparing of dystrophic EOMs in Duchenne dystrophy.[20] Continuous remodeling of muscle fibers has also been reported for non-dystrophic ILMs,[11] and this may explain the lack of muscle degeneration in these muscles.

We found that the CT muscle in *mdx* mice is affected significantly compared to the other laryngeal muscles. The EOMs also show non-spared mus-

cles, such as the retractor bulbi,[24] for unclear reasons. Possibly, the lack of dystrophin itself associated with lower levels of calcium-handling proteins may explain this finding, but further studies are needed on this topic. The CT muscles also showed muscle regeneration over time, similar to that in human thyroarytenoid muscles.[15,19] Alternatively, the CT muscles may be more susceptible to damage over time than the other ILMs, possibly in response to eccentric contractions or oxidative stress.[14]

The CT myosin heavy chain shows components typical of limb muscles,[25] and CT contraction times are closer to values of fast limb muscles.[14] Conversely, the other ILMs share myosin heavy chain components with the EOMs[11,18] and their contraction times are in the range of those observed for EOMs,[11] which are protected from dystrophy. Although the CT muscles were less affected than *mdx* TA, the biochemical and structural properties of CT muscles might explain why they were affected while the other ILMs were spared. In agreement with this suggestion is the fact that aging worsens the CT myopathy, the same being observed in limb muscles.[17,21]

In conclusion, we have shown that ILMs are protected from the lack of dystrophin in adult and old *mdx* mice, whereas no sparing occurs of the CT muscle. Further studies of dystrophic laryngeal muscles will be needed to better understand the mechanisms of sparing and its relation to aging, and also to develop new therapeutic strategies for the treatment of dystrophinopathies.

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Pharmacological and local toxicity studies of a liposomal formulation for the novel local anaesthetic ropivacaine

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Abstract

This study reports an investigation of the pharmacological activity, cytotoxic and local effects of a liposomal formulation for the novel local anaesthetic ropivacaine (RVC) compared with its plain solution. RVC was encapsulated into large unilamellar vesicles (LUVs) composed of egg phosphatidylcholine, cholesterol and α-tocopherol (4:3:0.07, mole %). Particle size, partition coefficient determination and in-vitro release studies were used to characterize the encapsulation process. Cytotoxicity was evaluated by the tetrazolium reduction test using sciatic nerve Schwann cells in culture. Local anaesthetic activity was assessed by mouse sciatic and rat infraorbital nerve blockades. Histological analysis was performed to verify the myotoxic effects evoked by RVC formulations. Plain (RVC_{PLAIN}) and liposomal RVC (RVC_{LUV}) samples were tested at 0.125%, 0.25% and 0.5% concentrations. Vesicles size distribution showed liposomal populations of 370 and 130 nm (85 and 15%, respectively), without changes after RVC encapsulation. The partition coefficient value was 132 ± 26 and in-vitro release assays revealed a decrease in RVC release rate (1.5 fold, $P < 0.001$) from liposomes. RVC_{LUV} presented reduced cytotoxicity ($P < 0.001$) when compared with RVC_{PLAIN}. Treatment with RVC_{LUV} increased the duration ($P < 0.001$) and intensity of the analgesic effects either on sciatic nerve blockade (1.4–1.6 fold) and infraorbital nerve blockade tests (1.5 fold), in relation to RVC_{PLAIN}. Regarding histological analysis, no morphological tissue changes were detected on the area of injection and sparse inflammatory cells were observed in only one of the animals treated with RVC_{PLAIN} or RVC_{LUV} at 0.5%. Despite the differences between these preclinical studies and clinical conditions, we suggest RVC_{LUV} as a potential new formulation, since RVC is a new and safe local anaesthetic agent.

Introduction

Ropivacaine (RVC) is an amino-amide, enantiomerically pure (S-isomer), novel local anaesthetic largely used in surgical procedures. RVC presents physico-chemical and therapeutic properties similar to those of bupivacaine, but with lower toxicity to the cardiovascular and the central nervous systems. In addition, a slight decrease in the lipid solubility of RVC confers to it a greater selectivity or differential block for sensory over motor function in isolated nerve preparations or epidural administration, in relation to bupivacaine (Rosenberg & Heinonen 1983; Bader et al 1989; Brockway et al 1991). These features point out RVC as an important option for regional anaesthesia and management of postoperative pain (McClure 1996; Simpson et al 2005).

Commercially available local anaesthetics formulations are used in a variety of doses and routes of administration. Despite the advances, the relatively short duration of analgesia (due to the transfer and redistribution from the site of injection) (Grant & Bansinath 2001; Grant 2002) and the severe side effects (evoked by large doses or inadvertent intravascular injections) restrict their clinical use (McClure 1996; McLure & Rubin 2005). Then, the use of drug delivery systems, such as liposomes, would be highly desirable for the clinical use of local anaesthetics, offering the possibility to control the release of these drugs, to prolong the duration of action, specially for the newer and safer agents such as RVC.

Liposomes consist of one or more concentrically organized assemblies of phospholipid bilayers where the fatty acid tails are in the core of the bilayer while the hydrophilic heads are oriented to the aqueous phase. Because of their amphiphilic nature, local anaesthetics interact with these model membrane systems, sitting mainly in the bilayer (lipid) region and they also retain a fraction of molecules in the aqueous phase (de Paula & Schreier 1995, 1996). In fact, works in the literature show that the sustained release of local anaesthetics in liposomes has advantages such as biocompatibility, low toxicity and biodistribution controlled by their size (Grant & Bansinath 2001). Studies with bupivacaine, for instance, report prolonged effect (Boogaerts et al 1993, 1994, 1995; Malinovsky et al 1999; Yu et al 2002; Grant et al 2003, 2004), changes in biodistribution (Boogaerts et al 1995), decreased plasma concentrations and low systemic toxicity (Boogaerts et al 1993; Malinovsky et al 1999) after encapsulation in large multilamellar liposomes (MLV), when compared to plain bupivacaine solution.

Our research group observed that the duration and intensity of sensory blockade induced by mepivacaine (de Araujo et al 2004), prilocaine and lidocaine (Cereda et al 2004, 2006) were enhanced by encapsulation in large unilamellar liposomes (LUV) even though these effects were not observed with bupivacaine. In another recent study, a liposomal-encapsulated RVC topical gel effectively reduced pain during needle insertion and increased the duration of soft-tissue anaesthesia in dentistry (Franz-Montan et al 2007); however, the efficacy of a liposomal system for RVC was not studied considering an infiltrative route and its possible local toxic effect. Thus, the purpose of this preclinical study was to investigate the pharmacological activity of a parenteral liposomal formulation for RVC, using the sciatic and infraorbital nerve blockade models, as well as to assess the cytotoxic and the myotoxic local effects in comparison with its plain solution.

Materials and Methods

Drugs

RVC hydrochloride and sodium thiopental were donated by Cristália Prod. Quím. Farm. Ltda (Itapira, SP, Brazil). Egg phosphatidylcholine (EPC), cholesterol (Ch), α-tocopherol (α-T), bovine serum albumin (BSA) and HEPES buffer were purchased from Sigma Chemical Company (St Louis, MO). 3,4,5-Dimethylthiazol-2-yl-2,3-diphenyltetrazolium bromide (MTT) was obtained from Calbiochem Corp. (La Jolla, CA) and antibody anti-S-100 polyclonal from DAKO (Glostrup, Denmark). Dulbecco's Modified Eagle's Medium (DMEM), collagenase and trypsin were obtained from Nutricell (Campinas, SP, Brazil).

Animals

Male adults Swiss mice, Wistar rats (30–35 g and 250–350 g, respectively) and newborn Sprague-Dawley rats were obtained from CEMIB-UNICAMP (Centro de Bioterismo, State University of Campinas – UNICAMP, Campinas, São Paulo). Protocols were approved by the UNICAMP Institutional Animal Care and Use Committee, which follows the recommendations of the Guide for the Care and Use of Laboratory Animals. Rats or mice, divided in groups of 6 or 7 animals each, were randomly selected for the pharmacological assays and treated by infiltration (0.1 mL) with $LUV_{ropivacaine\text{-}free}$ (5 mM) or with RVC_{PLAIN} or RVC_{luv} at 0.125%, 0.25% or 0.5% concentrations.

Liposomal ropivacaine

EPC-Ch-α-T (4:3:0.07, mole %) films were obtained by evaporating stock chloroform solutions under a stream of wet nitrogen followed by vacuum, for 2 h. Films were suspended in HEPES buffer (20 mM, 154 mM NaCl, pH 7.4) and MLVs were obtained after vortexing at ambient temperature (5 min, 25°C). LUVs were prepared by repeated extrusion (15 cycles) of the MLVs within 0.4-μm membrane filters (25°C), in a Lipex Biomembranes Inc. (Vancouver, Canada) extruder. The total phospholipid concentration, determined by inorganic phosphate quantification was 5 mM (de Paula & Schreier 1995). RVC was added directly to the liposomes after extrusion at the same final concentrations of RVC_{PLAIN}: 0.125% (4.02 mM), 0.25% (8.04 mM) and 0.5% (16.08 mM).

The mean diameter and size distribution of the LUV, stored at 4°C were analysed by laser light-scattering (Malvern Mastersizer-Malvern Instruments, France) before and after RVC encapsulation. The polydispersity index was also evaluated as a measurement of the homogeneity of the dispersion (ranging from 0 to 1, representing a homogeneous or a heterogeneous distribution, respectively) (Barth & Flippen 1995).

The partition coefficient (P) was determined by ultracentrifugation (120 000 *g* for 2 h at 10°C) of samples containing 2 mM RVC and 4 mM liposomal suspensions. Four repetitions of duplicate tests were used for each P determination. The amount of RVC incorporated into the vesicles was optically determined at 260 nm (de Araujo et al 2008), by subtracting the supernatant concentration from the total RVC concentration, measured previous to phase mixing. P values were calculated using equation 1 (de Paula & Schreier 1995, 1996).

$$P = (n_m/V_m)/(n_w/V_w) \quad (1)$$

where n is the number of moles of RVC, V denotes volume (L), m refers to the membrane phase and w to the aqueous phase.

In-vitro release experiments were conducted at 37°C in a two-compartment dialysis system where the donor compartment (1 mL capacity, containing RVC_{PLAIN} or RVC_{luv} sample) was separated from the acceptor compartment (100 mL, containing 20 mM HEPES buffer pH 7.4) by a cellulose membrane (Spectrapore, MWCO 12 000–14 000 Da). Samples were withdrawn from the acceptor compartment at regular intervals and drug concentration was determined by UV absorption (260 nm).

Cell culture and cytotoxicity assay: sciatic nerve Schwann cells

Schwann cells were isolated from the sciatic nerve of newborn Sprague-Dawley rats and purified as previously

described with minor modifications (Brockes et al 1979; Assouline et al 1983). Nerves segments were aseptically removed and dissected out from the epineurium and surrounding tissue, incubated in 0.05% collagenase for 30 min at 37°C and then in 0.15% trypsin for 20 min. The cell mixture was recovered by centrifugation in BSA 3% (300 *g*, 10 min) and resuspended in DMEM with 10% fetal calf serum supplement with glucose, insulin-like nerve growth factor (NGF), pituitary extract, forskolin and antibiotic (penicillin and streptomycin). Cells were seeded (15×10^4 cells/well) into a plastic cell culture dish with 48 wells (Corning-Costar Co., Cambridge, MA) and cultured for 4 days (37°C, 5% CO_2). Cultures were fixed in 4% paraformaldehyde in phosphate buffer for 10 min (pH 7.4, 37°C). To avoid non-specific staining, the specimens were incubated for 45 min with 1% BSA in phosphate buffer containing 0.25% Triton X-100. The purity of the culture was evaluated by the antibody anti-S-100. Finally, the cells were rinsed in PBS (37°C), incubated with Cy3 antibody anti-rabbit conjugate for 45 min and observed under a Nikon eclipse TS100 microscope equipped for fluorescence analysis.

Purified Schwann cells were incubated for 2 h with the vehicle ($LUV_{ropivacaine\text{-}free}$, 5 mM) or with RVC ($RVC_{PLAIN}$ or RVC_{LUV}) at three different concentrations 0.125% (4.02 mM), 0.25% (8.04 mM) and 0.5% (16.08 mM). Cell viability was assessed by the tetrazolium reduction (MTT test). MTT (1 mg mL^{-1}) was incubated for 2 h with the treated Schwann cells (37°C). The number of viable cells was determined by measuring the amount of MTT converted to formazan by mitochondrial dehydrogenases. The produced formazan crystals were dissolved in 1 N HCl-isopropyl alcohol (1:24 v/v) and were shaken for 20 min. After that, the dye-containing solution was removed and the sample absorbance was determined at 570 nm (Denizot & Lang 1986; Park et al 2005).

Infraorbital nerve blockade

Rat infraorbital nerve blockade was performed as previously described (Fink et al 1975) and was used with minor adaptations. The rats were anaesthetized with 25 mg kg^{-1} sodium thiopental (intraperitoneal route) and an unilateral injection of the vehicle ($LUV_{ROPIVACAINE\text{-}FREE}$) or RVC formulations was performed into the infraorbital notch. The intact left side served as control. The analgesic effect was assessed by observation of aversive response to rat upper lip pinching according to the scores: 0 (aversive response) or 1 (no aversive response). The rats were tested every 5 min up to the time when the first aversive sign in the injected side was detected. Score values were expressed as percent of rats with analgesia. The efficacy of infraorbital nerve block was analysed by the time for sensory function recovery and the total local anaesthetic effect. Local anaesthetic effect was estimated by the area under the time curve (AUC) expressed as score/hour (Cereda et al 2006) and calculated using Origin 6.0 Software (Microcal TM Software Inc., Northampton, MA).

Sciatic nerve blockade

Before the experiment, the ability of each mouse to walk normally with four limbs on both the top and inverted side of a wire mesh screen (1 mm diameter wire, 5 mm mesh) was evaluated. Mice showing this behaviour were selected for the experiment. Vehicle, plain solution or liposomal formulations were injected by inserting a needle into the popliteal space on the posterior surface of the knee, in the area of the sciatic nerve. Motor blockade intensity was assessed by the loss of motor control in the injected limb according to the scores: 0 (normal movement), 1 (unable to flex the limb completely) and 2 (total paralysis). The efficacy of motor blockade was evaluated every minute, from 1 to 5 min, and thereafter every 10 min up to at least 1 h following the injection. Latency (time between injection and the loss of motor function), time to reach the maximum score (Tmax), time for motor function recovery and the total local anaesthetic effect (Area Under the effect Curve vs Time, expressed as score/h) were evaluated (Leszczynska & Kau 1992; Gantenbein et al 1997).

Sensory blockade evaluation was performed by the paw pressure test (Randall & Selitto 1957) using an analgesymeter (Ugo Basile, Varese, Italy), which exerts a force (in grams) on the paw. The withdrawal reflex was considered representative of the pain threshold or Paw Withdrawal Threshold to Pressure (PWTP). The baseline of the PWTP test was measured before vehicle or drug injection, to determine the pain threshold of the mouse. Baseline values of 30–50 g were selected as the pain threshold and mice that presented lower or higher values than that baseline were excluded. The established antinociception cut-off value was 150 g, considered to be representative of the anaesthetic state (de Araujo et al 2004, 2008). After drug or vehicle administration, measurements were carried out at intervals of 15 min during the first hour, 30 min in the second and third hour and finally 60 min up to 5 h after treatment.

Histological assays

Mice were sacrificed by cervical dislocation 3 days after injection of $LUV_{ROPIVACAINE\text{-}FREE}$ (5 mM), RVC_{PLAIN} or RVC_{LUV} at 0.125, 0.25 and 0.5%. To evaluate the surroundings of the site of injection, gastrocnemius and soleus muscles were dissected out, fixed in 10% paraformaldehyde (pH 7.6) for 24 h and transferred to 70% ethanol solution. Samples were embedded in paraffin and transverse sections (6 μm) were obtained from the muscle bellies adjacent to the popliteal space and stained with hematoxylin and eosin (H&E). The presence of inflammatory cells, degenerating and regenerating myofibres was analysed using light microscopy. Low-power video-images (10 × objective) of the entire cross-section were taken with a highly sensitive video camera (Sony CCD) linked to a light microsce and enhanced with an image processor system (CoolSnap, Media Cybernetics, USA).

Statistical analysis

Size distribution of liposomes and in-vitro release tests were analysed by two-tailed unpaired *t*-test. Sciatic (motor function) and infraorbital nerve blockade data (latency, Tmax, time for recovery and AUC) were analysed by the Kruskall-Wallis test and expressed as medians (minimum and maximum limits). Sciatic nerve blockade (sensory function) and cytotoxic assays

data were analysed by one-way analysis of variance with Tukey-Kramer as a post-hoc test (Zar 1996). Statistical significance was defined as $P < 0.05$. Data were analysed using Origin 6.0 Software (Microcal TM Software Inc., USA) and Graph Pad Instat (Graph Pad Software Inc., USA) programs.

Results

Liposomal ropivacaine

Size distribution analysis by laser light-scattering assays showed two different populations of liposomes. The main population, representing 85%, had an average size of 371 ± 7.9 nm while a smaller fraction (15%) included liposomes with diameters of 128 ± 6.3 nm; the size did not change after RVC encapsulation (356 ± 8.3 nm and 138 ± 7.8 nm, respectively). All the measurements presented a polydispersity index of 0.12–0.17, reflecting the homogeneous distribution of the liposomal population obtained. The partition coefficient of RVC into the liposomes was 132 ± 26, corresponding to an encapsulation efficiency of 23.8 ± 3.5% (mean ± s.d.). Encapsulation of RVC significantly reduced (1.3 fold, $P < 0.001$) its rate of release from one dialysis compartment to another, as compared with RVC_{PLAIN}. One hour after dialysis, 58.1 ± 0.88% of RVC was released from the liposomes against 76.1 ± 6.2 % from the plain solution. At the time noted for total release (100%) of RVC_{PLAIN}, which was observed at 180 min of dialysis, only 68.2 ± 0.72% of RVC was released from the liposomal system (Figure 1).

Cell culture and cytotoxicity assay: sciatic nerve Schwann cells

The treatment with the vehicle ($LUV_{ROPIVACAINE\text{-}FREE}$) did not reduced the cell viability. On the other hand, RVC_{PLAIN} reduced the cell viability by 70% (0.5% RVC), while RVC_{LUV} induced similar effects to $LUV_{ROPIVACAINE\text{-}FREE}$, with no effect on cell viability up to 2 h after treatment (100% cell viability, at 4.02 mM), compared to RVC_{PLAIN} ($P < 0.001$) (Figure 2).

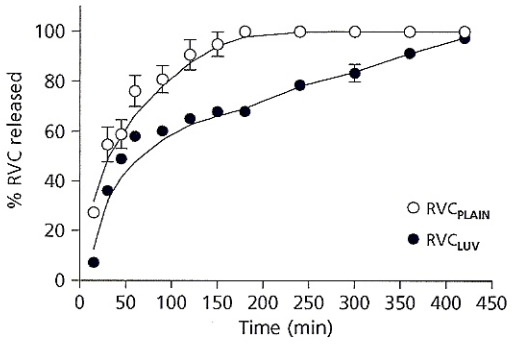

Figure 1 In-vitro release experiments for plain (RVC_{PLAIN}) and liposomal (RVC_{LUV}) ropivacaine in Hepes buffer, pH 7.4 at 37°C. Data are expressed as mean ± s.d., n = 4 experiments.

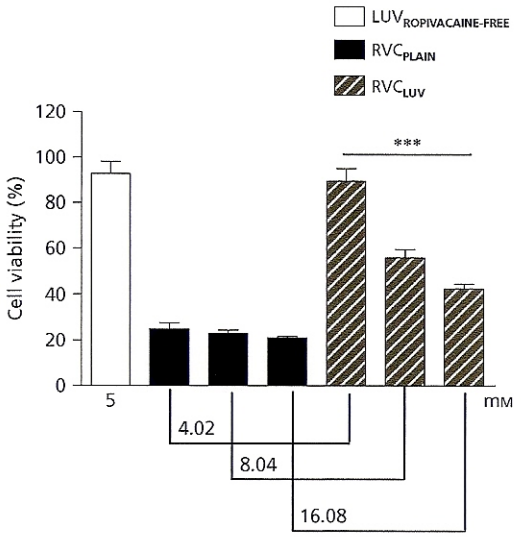

Figure 2 Cytotoxic effects of $LUV_{ROPIVACAINE\text{-}FREE}$ (ropivacaine-free large unilamellar liposomes, 5 mM) and RVC_{PLAIN} (plain ropivacaine) or RVC_{LUV} (liposomal ropivacaine) at 0.125, 0.25 and 0.5%, on a primary sciatic nerve Swann cells culture incubated for 2 h at 37°C and 5% CO_2 as evaluated by MTT reduction test. Data are expressed as % cell viability (mean ± sd., n = 3 experiments). ***$P < 0.001$, RVC_{LUV} vs RVC (one-way analysis of variance with Tukey-Kramer post-hoc test).

Infraorbital nerve blockade

Table 1 summarizes the infraorbital test results for RVC_{PLAIN} and RVC_{LUV} regarding the local anaesthetic total effect (expressed as AUC) and time for recovery.

No signs of sensory blockade were observed on the intact left side of rats in any of the groups, punctuated with score 0 (i.e., aversive response to pinch) (data not shown). $LUV_{ROPIVACAINE\text{-}FREE}$, used as controls, presented no analgesic effect, whereas RVC_{LUV} induced an improvement on intensity of total local anaesthetic effect (27.5, 42.5 and 107.5 score/h for 0.125, 0.25 and 0.5% RVC_{LUV}, respectively) associated with prolonged times for recovery (duration of sensory blockade), since analgesia was observed until 37, 50 and 122 min after treatment with 0.125, 0.25 and 0.5%, as compared with RVC_{PLAIN}. Statistical analysis showed that RVC_{LUV} prolonged the time for recovery and enhanced the total effect of RVC at 0.125% ($P < 0.01$), 0.25% ($P < 0.05$) and 0.5% ($P < 0.01$).

Sciatic nerve blockade

The injection of $LUV_{ROPIVACAINE\text{-}FREE}$ into the mouse sciatic nerve did not cause any effect on motor blockade. However, even if the overall motor function was not significantly different between RVC_{PLAIN} and RVC_{LUV} formulations, dose-dependent effects were observed on latency, motor blockade duration and total effect of the local anaesthetic for each experimental group.

In this manner, statistical differences were observed after comparisons within the same groups (intra-group) for the

Table 1 Total local anaesthetic effect (AUC) and time for recovery for plain (RVC_{PLAIN}) and liposomal ropivacaine (RVC_{LUV}) in rat infraorbital nerve blockade test

Group	Concentration (%)	Time for recovery (min)	AUC (score/h)
RVC_{PLAIN}	0.125	22.0 (22.0–27.0)	17.5 (17.5–22.5)
	0.25	38.0 (32.0–40.0)	32.5 (27.5–32.5)
	0.5	81.0 (80.0–96.0)	72.5 (67.5–82.5)
RVC_{LUV}	0.125	32.0 (28.0–37.0)**	27.5 (22.5–332.5)**
	0.25	50.0 (39.0–50.0)*	42.5 (32.5–42.5)*
	0.5	115.0 (97.0–122.0)**	107.5 (92.5–117.5)**

Data expressed as median (minimum–maximum limits) (n = 6 or 7/group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, RVC_{LUV} vs RVC_{PLAIN} (Kruskall-Wallis test). Q1

three different concentrations used. Then, comparisons among RVC concentrations showed statistical differences in relation to time for recovery and AUC for RVC_{PLAIN} (0.5% vs 0.125% and 0.25% vs 0.125% with $P < 0.001$ and $P < 0.01$, respectively) and RVC_{LUV} (0.5% vs 0.25% with $P < 0.05$; 0.5% vs 0.125%, $p < 0.01$) (Table 2).

On the other hand, comparisons between RVC_{PLAIN} and RVC_{luv} (inter-group) did not reveal statistical differences.

The sensory blockade results showed that the RVC formulations were statistically different from the $LUV_{ROPIVACAINE-FREE}$ group ($P < 0.001$). On the other hand, RVC_{LUV} had an increased duration and intensity of anti-nociceptive effect, when compared with the plain solution. Assessing individual time values, 0.125% RVC_{LUV} (Figure 3A) was different from RVC_{PLAIN} treatment from 45 up to 150 min ($P < 0.001$), increasing the intensity of analgesia (1.6 fold). The analgesia was observed until 180 min after injection of 0.125% RVC_{LUV}, when compared with RVC_{PLAIN}. The treatment of the mice with 0.25% RVC_{LUV} (Figure 3B) showed similar results to those at 0.125% concentration. Statistical differences in anti-nociceptive effects between 0.25% RVC_{LUV} and 0.25% RVC_{PLAIN} were observed from 45 up to 180 min ($P < 0.001$). At that time interval, the intensity of analgesia using RVC_{LUV} was 1.6 times higher than that with RVC_{PLAIN} and was observed up to 240 min after injection of 0.25% RVC_{LUV}. The group treated with 0.5% RVC_{LUV} (Figure 3C) presented different responses from 30 up to 240 min after infiltration, when compared with 0.5% RVC_{PLAIN} ($P < 0.001$). The intensity of analgesia reached with 0.5% RVC_{LUV} was 1.4- to 1.6-times higher than that with RVC_{PLAIN} and the duration was seen until 300 min.

Histological assays

Histological analysis of the mouse gastrocnemius and soleus muscles after the treatment with $LUV_{ROPIVACAINE-FREE}$, RVC_{PLAIN} or RVC_{LUV} at 0.125%, 0.25% or 0.5% revealed that these muscles appeared to be normal, with fibres round or roughly polygonal with rounded angles. No morphological tissue changes were detected in control mice, since the muscle fibres underlying the area of injection remained visibly unaffected and normal in all morphological aspects. Muscle fibres with peripheral nuclei location similar to control muscles were found and regenerated muscle fibres, characterized by central nuclei, were detected only on the needle track for all experimental groups. Sparse inflammatory cells were observed in only one of the mice treated with $LUV_{ROPIVACAINE-FREE}$ (5 mM) and RVC_{PLAIN} or RVC_{luv} at the higher concentration (0.5%) (Figure 4).

Discussion

Despite being also a long-acting local anaesthetic, RVC is a relatively new drug with a similar clinical profile to bupivacaine but is associated with lower motor blockade and cardiotoxicity (Markham & Faulds 1996). The development of a liposomal system would be of interest to solve problems relating to the fast clearance of those molecules from the site of injection (Grant & Bansinath 2001; Grant 2002). In the development of a drug delivery system for local anaesthetics, aspects must be considered that determine the concentration and effect of the formulation on the nervous tissue: firstly, the

Table 2 Latency, T_{MAX}, time for recovery and total effect (AUC) from the motor blockade evaluation for plain (RVC_{PLAIN}) and liposomal ropivacaine (RVC_{LUV}) in sciatic nerve blockade test in mice

Group	Concentration (%)	Latency (s)	Tmax (min)	Time for recovery (min)	AUC (score/h)
RVC_{PLAIN}	0.125	50.0 (25.0–55.0)	1 (1–2)	30.0 (20.0–40.0)	25.0 (15.5–45.0)
	0.25	40.0 (30.0–50.0)	1 (1–3)	40.0 (30.0–50.0)	35.0 (15.0–50.0)
	0.5	30.0 (25.0–55.0)	1 (1–2)	55.0 (45.0–65.0)[a]***,[b]**	66.0 (50.0–80.0)[a]***,[b]**
RVC_{LUV}	0.125	45.0 (24.0–60.0)	1 (1–3)	30.0 (20.0–40.0)	25.0 (14.0–46.0)
	0.25	37.0 (20.0–60.0)	1 (1–4)	50.0 (40.0–80.0)[d]**	44.0 (33.5–108.0)[d]*
	0.5	34.0 (26.0–48.0)	1 (1–3)	60.0 (40.0–70.0)[c,e]**	57.0 (34.0–108.0)[c]**

Data are expressed as median (minimum–maximum limits) (n = 6 or 7/group). [a]RVC 0.5% and RVC 0.125%; [b]RVC 0.5% and RVC 0.25%; [c]RVC_{LUV} 0.5% and RVC_{LUV}0.25%; [d]RVC_{LUV}0.25% and RVC_{LUV} 0.125%; [e]RVC_{LUV}0.5% and RVC_{LUV} 0.125%; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (Kruskall-Wallis Test).

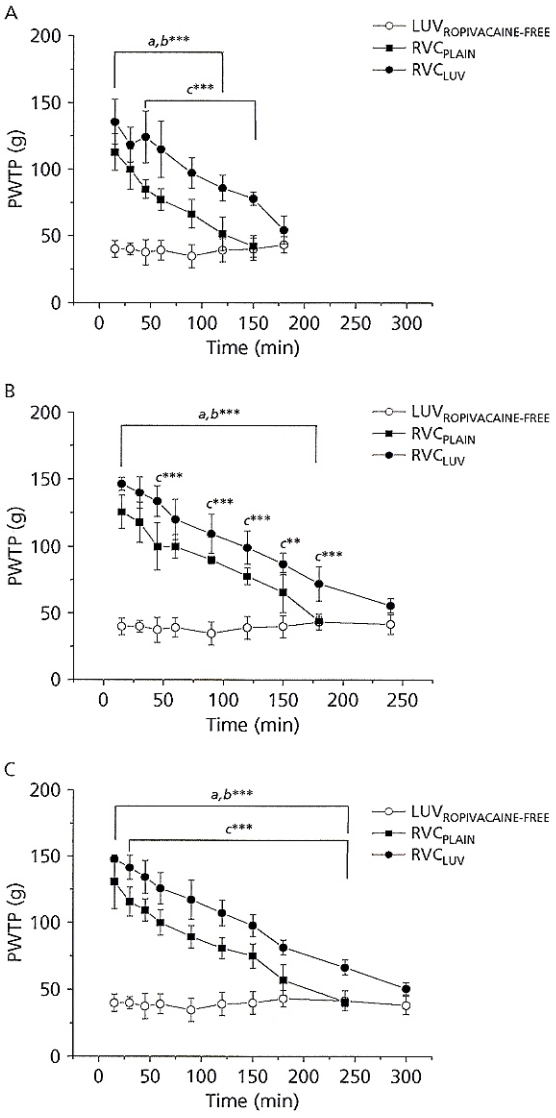

Figure 3 Time course (min) × PWTP (g) of sensory function evaluated by sciatic nerve blockade test in mice (n = 6 or 7/group). 5 mM ropivacaine-free large unilamellar liposomes ($LUV_{ROPIVACAINE-FREE}$), plain ropivacaine (RVC_{PLAIN}) and liposomal ropivacaine (RVC_{LUV}) at 0.125% (A), 0.25% (B) or 0.5% (C). Data are expressed as mean ± s.d. Statistical differences are shown between: [a]RVC and LUV; [b]RVC_{luv} and LUV; [c]RVC_{luv} and RVC_{PLAIN}. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (one-way analysis of variance with Tukey-Kramer post-hoc test).

drug must be sufficiently encapsulated to maintain the therapeutic concentration; secondly, the diffusion of the drug from the injected solution into the extracellular fluid and its uptake by nerve fibres must be slow; thirdly, clearance of the drug and the carrier should be sustained to allow a prolonged effect. Regarding local anaesthetics, features such as local concentration, diffusion and uptake by the nervous tissue determine the latency, spread and intensity of the blockade; however, the clearance rate influences the duration of action (Grant & Bansinath 2001).

Liposomes have been effectively used as slow-release systems for local anaesthetics. Encapsulation of these drugs has led to reduced systemic toxicity (Boogaerts et al 1993, 1995; Malinovsky et al 1997) and to longer duration of action (Booagerts et al 1994; Malinovsky et al 1999; Grant et al 2003, 2004; de Araujo et al 2004; Cereda et al 2004, 2006).

As shown here, the RVC_{LUV} system was not able to modify the motor blockade duration (since comparisons between RVC_{PLAIN} and RVC_{luv} did not reveal statistical differences), but improved the intensity ($P < 0.001$) and duration of the analgesic effect ($P < 0.001$ at the final point of analgesic effect induced by RVC_{PLAIN}) in a mouse sciatic nerve blockade model at the three concentrations used.

Considering this, we here provided an initial assessment of the drug distribution and the pharmacological effects of a liposomal system for the novel local anaesthetic RVC, since the results showed a decrease in drug release rate and a slow delivery of RVC in-situ caused by its encapsulation into the liposomes. In fact, this slower release rate of RVC_{LUV} was confirmed by the increase in intensity and duration of sensory nerve blockade observed in rats and mice. The gradual drug release changes the rate of distribution of local anaesthetic molecules among membrane/water compartments, with possible therapeutic advantages such as prolonged effects, low plasmatic levels and reduced systemic toxicity (Grant & Bansinath 2001). Thus, we postulate that by changing the drug-membrane equilibrium, it is possible to reduce the latency without prolonging the duration, neither enhancing the intensity of motor blockade, but remaining at the site of action at sufficiently low concentration to improve the duration of analgesia. To explain this and the other factors involved in this purpose, pharmacokinetic and bioavailability studies are underway with this formulation.

Additionally, the rat infraorbital nerve blockade test provides information about intensity and duration of sensory blockade induced by local anaesthetic agents used as plain solutions (Fink et al 1975) or as pharmaceutical associations (vasoconstrictors, dextrans) (Hassan et al 1985 a, b), being an important experimental model, especially in dentistry for orofacial surgeries.

We have reported that the encapsulation in LUV intensified the analgesic effects of prilocaine (Cereda et al 2004, 2006), mepivacaine and lidocaine (Cereda et al 2006), showing that mepivacaine was affected to the greatest extent of analgesic effect and that liposomes provided effective carriers for intermediate-duration local anaesthetics. In this study, our results also showed that RVC_{LUV} induced an improvement on intensity of total local anaesthetic effect associated with prolonged times for recovery at 0.125% ($P < 0.01$), 0.25% ($P < 0.05$) and 0.5% ($P < 0.01$), pointing out this liposomal system as a great advantage for the possible future use of RVC in dentistry.

Another important consideration is that comparisons among the partition coefficient values previously determined for other local anaesthetics molecules, such as bupivacaine (136 ± 32), lidocaine (114 ± 16), mepivacaine (93 ± 7) and prilocaine (57 ± 6) (de Araujo et al 2004; Cereda et al 2004, 2006),

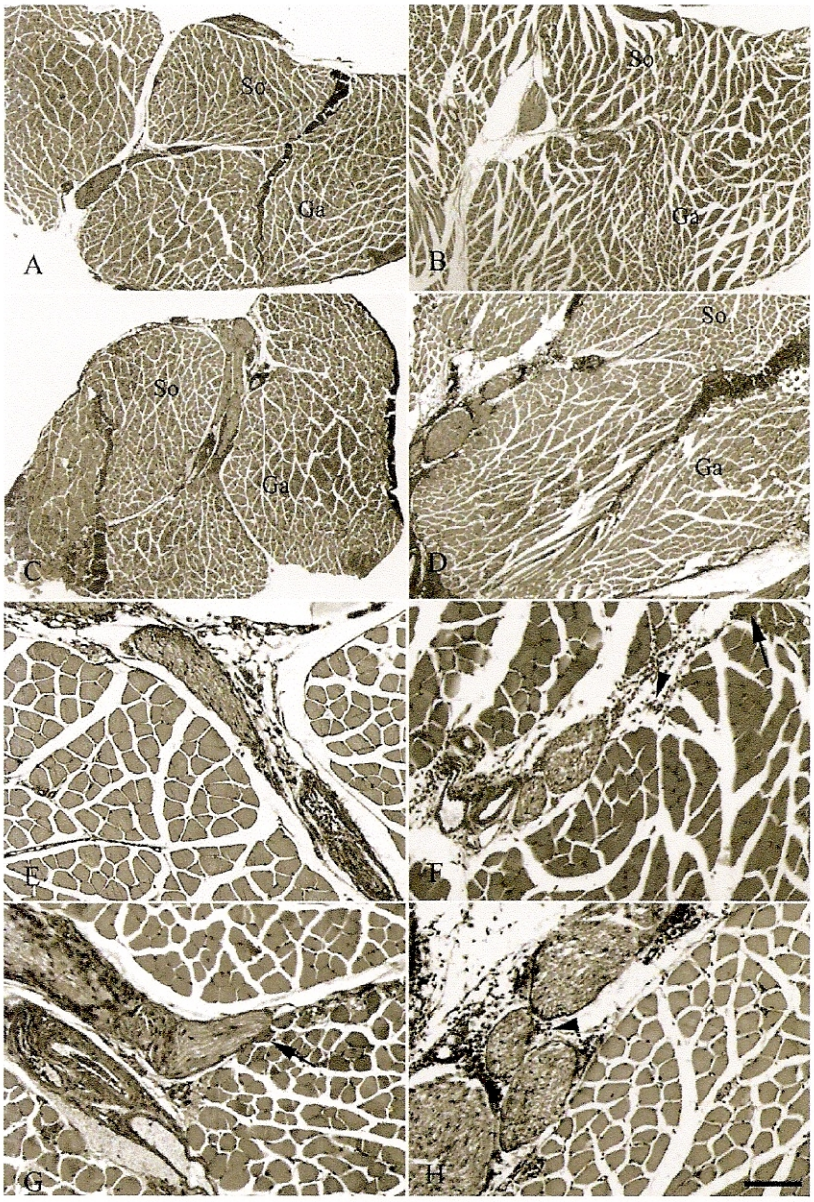

Figure 4 Transverse sections of mouse gastrocnemius (Ga) and soleus (So) muscles, in controls (A and E) and mice treated with LUV 5 mM (B and F), RVC 0.5% (C and G) or RVC_{LUV} 0.5% (D and H), showing normal muscle fibres and a few cells with central nuclei (arrows). Some inflammatory cells were also observed (arrowhead). Scale bar, 100 μm (A, B, C, D); 400 μm (E, F, G, H) (n = 6 or 7/group).

revealed that RVC (partition coefficient value 132 ± 26) has a relatively high hydrophobic character in relation to the other linear (lidocaine, prilocaine) and cyclic (mepivacaine) amino-amides, as expected by the length of the alkyl chain substitution (propyl) at its piperidine ring. Then, the lipophilicity also justifies the differences in terms of anaesthesia (potency, toxicity and duration of action) since RVC is known to produce a sensory blockade profile similar that of bupivacaine (Mizogami et al 2002).

The partition coefficient value of RVC corresponded to an encapsulation efficiency of ca. 24%, obtained from the liposome preparation method used here. Reports in the literature have described different procedures, such as freeze-thaw and dehydration-rehydration, showing increased

encapsulation efficiency (Grant et al 2001, 2003, 2004). In fact, to increase the encapsulation efficiency, to improve the liposomal stability and to extend the duration of this formulation, we have tested the liposome preparation by spray-drying in a pilot scale, showing promising results for other local anaesthetics, such as lidocaine (Almeida 2008). Besides, we also focused our attention on the interaction with membrane lipid components, since this formulation presents Ca. 40% mol cholesterol, reflecting the membrane lipid composition of nerve cells (Mizogami et al 2002). In this manner, we believe that by enhancing the encapsulation efficiency or changing the lipid composition or the molar ratios of the liposomal constituents and controlling the liposomal size (to avoid fast clearance or delayed onset) as well as the release rate, it will be possible to obtain a prolonged analgesic effect associated with lower cytotoxicity.

Concerning the in-vitro assay, a previous study reported that among the amino-amide local anaesthetics, bupivacaine significantly induced Schwann cell death, but this effect was not evoked by RVC (ranging from 0.001 to 1 mM concentration) (Park et al 2005). In our study, the cytotoxicity of RVC was assessed at higher concentrations (4.02, 8.04 and 16.08 mM) than those, to attain the clinical concentrations. One of the most important considerations about this toxicity model is that RVC induced cytotoxic effects in a dose-dependent manner, when used at these higher concentrations and the cellular protective effects were observed after RVC was encapsulated into liposomes.

Regarding myotoxicity, it is well-described that intramuscular injections of local anaesthetics regularly result in striated muscle damage and myonecrosis, with a drug-specific and dose-dependent rate of toxicity (Zink & Graf 2004). Although a variety of local anaesthetics used in clinical practice have been studied, few data are available about the recently introduced local anaesthetic ropivacaine.

Previous studies showed that the acute myotoxicity evoked by ropivacaine is less severe than that seen with bupivacaine after continuous peripheral nerve block (Zink et al 2003, 2005). In addition, another study evaluated the influence of two different concentrations of ropivacaine used in clinical practice, reporting that the muscle damage was reversible and also occurred in a dose-dependent manner after single intramuscular injection (Amaniti et al 2006).

The histopathologic changes and time course of skeletal muscle injury after local anaesthetic administration appear to be rather uniform and non-specific. In general, these morphologic alterations are characterized by hypercontracted myofibrils, followed by lytic degeneration of sarcoplasmic reticulum and by myocyte oedema and necrosis over the next 1–2 days. The Ca^{2+} released from the sarcoplasmic reticulum of skeletal muscle fibres and simultaneous inhibition of the Ca^{2+} reuptake is considered the major pathomechanism in local anaesthetic myotoxicity, especially for ropivacaine and bupivacaine (Zink et al 2005).

In this study, no obvious signs of cell damage were observed and there was no evidence of necrotic material around the area of the injection. This three days post-injection study revealed only the presence of sparse inflammatory cells in one of the mice treated with the vehicle, plain and liposomal RVC at the higher local anaesthetic concentration (0.5%), suggesting no adverse reaction upon application of the formulations, at this condition.

Even the skeletal muscle injuries are reversible within a few weeks and frequently remain clinically inapparent, limiting the detection of their clinical impact; all local anaesthetics that have been examined are myotoxic in clinical concentrations, with a drug-specific and dose-dependent rate of toxicity (Foster & Carlson 1980; Zink & Graf 2004). For this reason, further studies are necessary to evaluate the consequences of long-term exposure to this liposomal system and the reversibility of its effects on skeletal muscle.

Conclusions

Despite the differences between this study and the real clinical conditions, we showed an in-vitro-in-vivo evaluation of a liposomal system for RVC. Encapsulation into the liposomes provided an improvement in analgesic effect and a decrease in cytotoxicity of RVC in comparison with its plain solution. Thus, we suggest the liposomal system RVC_{LUV} as a potential new formulation, since RVC is a new and safe long-acting local anaesthetic agent.

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DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha dissertação/tese de Doutorado intitulada "Efeito da L-arginina e Deflazacort na regeneração muscular após o envenenamento experimental por *Bothrops jararacussu*":

( ) não se enquadra no § 3° do Artigo 1° da Informação CCPG 01/08, referente a bioética e biossegurança.

( ) está inserido no **Projeto CIBio/IB/UNICAMP** (Protocolo n°_____), intitulado _____;

(X) tem autorização da **Comissão de Ética em Experimentação Animal/IB/UNICAMP** (Protocolo n°1708-1) Aprov. 18/11/08

( ) tem autorização do **Comitê de Ética para Pesquisa com Seres Humanos/FCM/UNICAMP** (Protocolo n°_____);

( ) tem autorização de comissão de bioética ou biossegurança externa à UNICAMP.
Especificar: _____

_____
Aluno: Viviane Urbini Vomero

_____
Orientador: Prof. Dr. Humberto Santo Neto

Para uso da Comissão ou Comitê pertinente:
(X) Deferido ( ) Indeferido

Ana Maria Gonçalves Guaraldo

Nome:
Função:

Profa. Dra. ANA MARIA A. GUARALDO
Presidente
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