UNIVERSIDADE ESTADUAL PAULISTA
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Avaliação da miotoxicidade e neurotoxicidade (com foco na junção neuromuscular) após aplicação de bupivacaina seguida de laser terapia

Tese apresentada à Faculdade de Medicina, Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Botucatu, para obtenção do título de Doutora em Bases Gerais de Cirurgia.

Orientadora: Profa. Dra. Selma Maria Michelin Matheus

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Botucatu, 20 de Outubro de 2016.

Dedicatória

Eu dedico a Deus que não me permitiu fraquejar, chorar, adoecer, sem que antes me fortalecesse.

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Epígrafe

A vereda do justo é como a luz da alvorada, que brilha cada vez mais até a plena claridade do dia.

Provérbios 4:18

Resumo

Avaliação da Miotoxicidade e neurotoxicidade (com foco na junção neuromuscular) após aplicação de bupivacaina seguida de laser terapia

A bupivacaina é um anestésico utilizado na prática médica e odontológica para bloqueio do nervo periférico e alívio da dor pré e pós-operatória devido à sua ação analgésica de longa duração. Suas principais limitações são a miotoxicidade, a neurotoxicidade e a inflamação. A laserterapia de baixa potência (LBP) tem sido utilizada para várias propostas terapêuticas, apresentando ação anti-inflamatória, regenerativa e analgésica. O objetivo do estudo foi avaliar o efeito do laser de Arseneto de Gálio (AsGa) sobre a morfologia das junções neuromusculares, fibras musculares e nervo associados ao músculo esternomastóideo de ratos após injeção de bupivacaina. 32 ratos Wistar machos adultos foram divididos em 2 grupos: Grupo Controle (C: n=16) e Grupo Laser (L: n= 16). Os grupos foram subdivididos seguindo os antímeros e substâncias injetadas: direito (bupivacaina 0,5%), esquerdo (Cloreto de sódio 0,9%). Após 24 horas houve aplicação de LBP (AsGa 904nm, 50mW, 4,8J) durante 5 dias consecutivos. A seguir, os animais foram eutanasiados, o sangue foi coletado para determinação da creatina kinase (CK); a porção superficial dos músculos esternomastóideos e os nervos associados foram dissecados, removidos e submetidos às seguintes análises: análise histopatológica e ultraestrutural; análises morfológica e morfométrica das JNMs (reação Esterase inespecífica), microscopia confocal de varredura a laser e análise ultraestrutural; e os nAChRs (alpha, beta e gama) e os níveis de TNFα foram quantificados pelo Western Blotting; foi ainda realizada morfometria dos nervos e quantificação da CK muscular. Não foram observadas alterações no antímero que recebeu o cloreto de sódio com ou sem aplicação de laser. Os músculos que receberam bupivacaina apresentaram maiores níveis de inflamação, atrofia e necrose e dos valores de CK muscular; maior número de núcleos central e porcentagem de colágeno, o diâmetro máximo das junções foram menores quando comparado aos músculos que receberam cloreto de sódio, não havendo efeito neurotóxico. Após aplicação do laser terapia houve redução nos scores histopatológicos, no número de núcleos centrais, na porcentagem de colágeno, havendo aumento no diâmetro máximo das JNM. Ultraestruturalmente foi observada redução da mionecrose, havendo recuperação nas dobras juncionais e zona ativa. Através da microscopia confocal houve aumento no perímetro dos nAChR, bem como aumento na área relativa planar. A análise da expressão proteica do nAChRα1 mostrou similaridade nos grupos estudados. Houve aumento da expressão proteica da subunidade ε após aplicação de LLLT. Os valores de TNFα mantiveram-se constantes. A LBP, na dose utilizada, reduziu a fibrose e mionecrose no músculo esternomastóideo desencadeada pela bupivacaina, acelerando o processo de regeneração muscular. Também reduziu as alterações estruturais da JNM e molecular dos nAChRs desencadeadas pela bupivacaina, fornecendo dados importantes para indicação da LBP em protocolos terapêuticos de lesões desencadeadas pelos anestésicos locais.

Palavras chaves: Bupivacaina, Laserterapia com Luz de Baixa Intensidade, Regeneração Muscular, Fibrose, Junção Neuromuscular, Receptores de Acetilcolina.

Abstract

Evaluation of myotoxicity and neurotoxicity (focusing on the neuromuscular junction) after application of bupivacaine followed by laser therapy

Bupivacaine is an anesthetic used in medical and dental practice as a peripheral nerve block and for relief of pre and postoperative pain due to its long duration analgesic action. Its principle limitations are myotoxicity, neurotoxicity, and inflammation. Low-level laser therapy (LBP) has been used for various therapeutic approaches, presenting an anti-inflammatory, regenerative, and analgesic action. The aim of the study was to evaluate the effects of the Arsenide Gallium laser (GaAs) on the morphology of neuromuscular junctions, muscle fibers, and the nerve associated with the sternomastoid muscle of rats after injection with bupivacaine. In total, 32 adult male Wistar rats were divided into 2 groups: Control group (C: n = 16) and Laser Group (L: n = 16). The groups were subdivided according to the antimeres and injected substances: right (0.5% bupivacaine), left (sodium chloride 0.9%). Twenty-four hours after the injection, LBP was applied (GaAs 904nm, 50mW, 4.8J) for 5 consecutive days. Subsequently, the animals were euthanized; blood was collected for determination of creatine kinase (CK); the surface portion of the sternomastoid muscles and associated nerves were dissected, removed, and submitted to the following tests: histopathological and ultrastructural analysis; morphological and morphometric analysis of the JNMs (nonspecific esterase reaction), confocal laser scanning microscopy and ultrastructural analysis; and the nAChRs (alpha, beta, and gamma) and TNFα levels were quantified by Western blotting; in addition, nerve morphometry and quantification of muscle CK were performed. No alterations were observed in the antimere which received the sodium chloride, with or without laser application. The muscles receiving bupivacaine presented higher levels of inflammation, atrophy and necrosis, and muscle CK values; a greater number of central nuclei and percentage of collagen; and the maximum diameter of the junctions was lower when compared to the muscles that received sodium chloride, without a neurotoxic effect. After application of laser therapy there was a reduction in histopathology scores, number of central nuclei, and percentage of collagen, with an increase in the maximum diameter of the JNM. Ultrastructurally, a reduction in myonecrosis was observed, with recovery in the junctional folds and active zone. Through the confocal microscopy, there was an increase in the perimeter of nAChR, as well as a relative increase in planar area. Analysis of nAChRα1 protein expression demonstrated similarity in the groups studied. There was increased protein expression of the subunit ε after application of LBP. The TNFα values remained constant. LBP, at the dose used, reduced fibrosis and myonecrosis triggered by bupivacaine in the sternomastoid muscle, accelerating muscle regeneration. It also reduced structural alterations in the JNM and molecular alterations in nAChRs triggered by bupivacaine, providing important data for the indication of LBP in therapeutic protocols for injuries triggered by local anesthetics.

Keywords: Bupivacaine, Low-Level Light Therapy, Muscle Regeneration, Fibrosis, neuromuscular junction, Nicotinic acetylcholine Receptor

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Paper 2

LISTA DE ABREVIATURAS, SIGLAS e SÍMBOLOS

Acetylcholine (ACh)

Acetylcholinesterase (AChE)

Adenosine triphosphate (ATP)

Alpha (α)

Cytochrome c oxidase (COX)

Creatine Kinase (CK)

Diameter (ϕ)

Duchenne muscular dystrophy (mdx)

Epsilon (ε)

Extracellular signal-regulated kinase (ERK2)

GAllium Arsenide (GaAs)

Gallium Arsenide Aluminum (GaAsAl)

Gamma (γ)

Gliceraldeído-3-fosfato desidrogenase (GAPDH)

Helium-Neon (HeNe)

Hematoxylin-Eosin (HE)

Indium-gallium-aluminum-phosphide (InGaAIP)

Joules (J)

Kilogram (Kg)

Laser Therapy (LT)

Light Emitting Diode Therapy (LEDT)

Low-Level laser therapy (LLLT)

Meter (m)

Microgram (μg)

Microliter (μl)

Micrometer (μm)

Milligram (mg)

Milliliter (ml)

Milliwatts (mW)

Myogenic regulatory factors (MRFs)

Nanometer (nm)

Neuromuscular junction (NMJ)

Nicotinamide adenine nucleotide (NADH)

Nicotinic acetylcholine receptor (nAChR)

Ribonucleic acid (RNA)

Transmission Electron Microscopy (TEM)

Tumor Necrosis Factor (TNFα)

World Association for Laser Therapy (WALT)

Sumário

1. Introdução

Os anestésicos locais são drogas que atuam como um bloqueador reversível da condução nervosa, impedindo a transmissão da informação de dores, sensoriais, motoras e autonômicas para o sistema nervoso central e a partir dele (Carvalho, 1994). São utilizados em procedimentos cirúrgicos médicos e odontológicos, dando ao paciente mais segurança e conforto (Cobb et al., 2016; Kishore et al., 2016; Balga et al., 2013; Horn et al., 2015; Jaichandran et al., 2015; Ilfeld et al., 2015; Shapiro & Schroeck, 2016). Têm ação no bloqueio da transmissão de estímulos em nervos periféricos no alívio da dor pós-operatória (Nouette-Gaulainet al., 2011; Abdolhossein-Davoodabadi, 2015; Yang et al., 2015; Beck et al., 2015; Kalchofner Guerrero et al., 2016; Yu et al.,2016) e na terapêutica da dor crônica.

Os anestésicos locais são substâncias que podem interferir na transmissão neuromuscular e seus efeitos são potencializados quando associados aos bloqueadores neuromusculares (Wang et al., 2010; Locks et al., 2015).

Os anestésicos locais atuam tanto nos canais de sódio voltagem-dependentes (axônio), quanto nos receptores nicotínicos de acetilcolina (nAChRs) da junção neuromuscular (JNM) (Rossman, 2011; Goodman & Gilman, 2012).

O tipo de nAChR localizado na junção neuromuscular tem um papel importante durante a administração da anestesia local. Os anestésicos locais agem como antagonistas não competitivos nos receptores nicotínicos de acetilcolina (nAChRs), e assim, previnem a contração muscular. Sendo assim, os nAChRs devem ser considerados, pois muitos objetivos anestésicos são alcançados por estímulo nestes receptores (Rossman, 2011).

A JNM é uma sinapse química, anatômica e funcionalmente especializada por transferir as informações de um neurônio motor para a fibra muscular (Engel, 2003; Malomouzh, 2012). Utiliza a acetilcolina como neurotransmissor, sendo envolvida diretamente com o processo de contração muscular (Willmann & Fuhrer, 2002). O funcionamento, a manutenção e a regeneração do músculo estriado esquelético dependem da integridade da JNM (Koirala et al., 2003).

A morfologia da JNM contempla três compartimentos: o compartimento pré-sináptico, com as terminações nervosas e a célula de Schwann; o compartimento extracelular, com a lâmina basal; e o compartimento pós-sináptico, com o sarcolema juncional com as dobras juncionais e o sarcoplasma que propicia apoio estrutural e metabólico para a região pós-sináptica (Engel,2003; Malomouzh, 2012) (Figura 01).

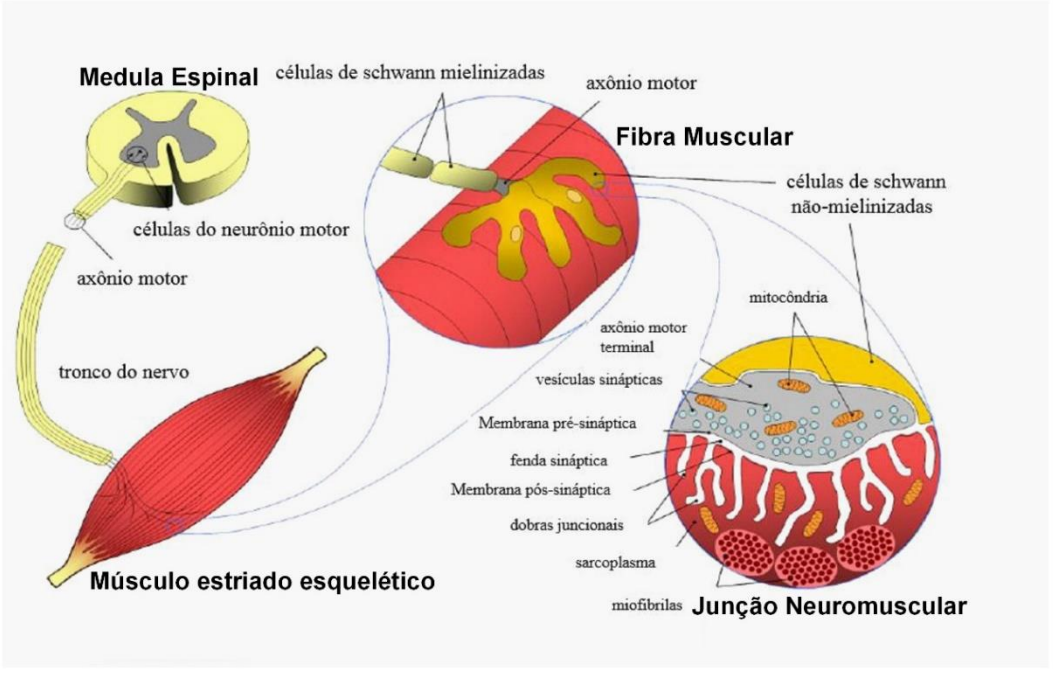

Figura 01 - Morfologia da junção neuromuscular. Desde a origem do neurônio motor na medula espinhal até a inervação na fibra muscular com as especializações dos 3 compartimentos da junção neuromuscular. **Fonte:** adaptado de Malomouzh (2012).

É, portanto, no compartimento pós-sináptico que estão localizadas as dobras juncionais. Nelas, estão presentes os nAChRs, que são proteínas integrais de membrana heteroligoméricas, com peso molecular de 290KDa (Lindstrom, 2000).

De morfologia rosácea, possui uma estrutura pentamérica em formato de canal iônico (Rossman, 2011). Existem dois tipos de receptores do tipo muscular, sendo o primeiro presente na fibra muscular embrionária ou desnervada com as subunidades α1 (número de 2), β1, δ e γ. O segundo receptor está na forma juncional madura, localizado na fibra muscular inervada, composto pelas subunidades α1 (número de 2), β1, δ e ε (Shuetze & Role, 1987; Rossman, 2011).

O sítio de ligação para a Acetilcolina (ACh) ocorre na interface das subunidades $\alpha\delta$ e $\alpha\varepsilon$ (Rossman, 2011) (Figura 2A e 2B). A duração do estado aberto do receptor depende da duração da ocupação da ACh no local (Naguib et al., 2002).

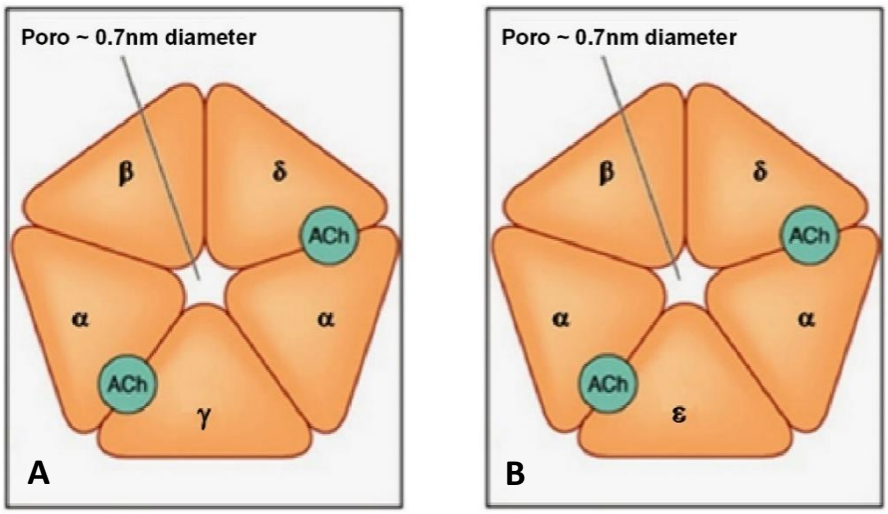

Figura 02- Imagem do receptor nicotínico de acetilcolina (nAChR). Adaptado de Unwin, 1995

A bupivacaina, por exemplo, é um anestésico local que age como antagonista não competitivo, e pode bloquear a abertura do canal iônico

ou de um sítio alostérico dos nAChRs, inibindo-o (Rossman, 2011).

A maioria dos estudos utilizam abordagem com protocolos mistos e seguem uma hierarquia de potência dos anestésicos locais que são: bupivacaina ≥ levobupivacaína > ropivacaína > lidocaína = mepivacaína ≥ prilocaína> procaína (Mather, 2010).

A maior limitação dos anestésicos locais são as reações adversas incluindo, dentre outras, a inflamação, a neurotoxicidade (McAlvin et al, 2013) e a miotoxicidade (McAlvin et al, 2013; Plank et al., 2016).

A avaliação da miotoxicidade de um anestésico local de uso clínico é de fundamental importância, pois o dano muscular pode ser considerado uma complicação em potencial na anestesia local (Plank et al., 2016). No entanto, o tecido muscular apresenta uma capacidade de se adaptar a diversas ocorrências (alta plasticidade), inclusive às lesões decorrentes dos anestésicos locais (Harridge, 2007).

A bupivacaina tem sido utilizada em protocolos experimentais, como modelo de estudo para mionecrose (Danieli-Betto et al., 2010; Wen et al., 2013; Otrocka-Domagala et al., 2015) ou mesmo para caracterização da desnervação (Çalgüner et al., 2003). Este anestésico acarreta mudanças nas características morfológica e estrutural das JNMs durante os processos de degeneração e regeneração da fibra muscular, processo este semelhante à diferenciação que ocorre na fibra muscular normal (Nishizawa et al., 2003).

Foster & Carlson, em 1980, relataram que a lidocaína produz uma menor lesão muscular quando comparada à bupivacaina, que promove mionecrose (Peraz-Castro et al., 2009).

As soluções anestésicas, bupivacaina e a ropivacaína, são consideradas miotóxicas e formadoras de tecido cicatricial quando administradas em longo prazo. Estes efeitos são mais acentuados na administração da bupivacaina (Zink et al., 2005).

A bupivacaina age com excelência na analgesia pós-operatória, mas induz à mionecrose e alterações no metabolismo e estrutura do retículo sarcoplasmático e mitocondrial, ocasionando dor muscular e disfunções musculares. As miopatias induzidas pela bupivacaina são tempo e concentração-dependentes, devendo ter mudanças adaptativas no protocolo de forma individualizada, para cada paciente (Nouette-Gaulain, et al., 2011).

Sua miotoxicidade foi avaliada na concentração-dependente de 0,75%, 0,38% e 0,19% em músculos extrínsecos do olho de coelho. Foi observado que, após cinco dias na concentração de 0,75%, ocorreram mionecrose e degeneração aguda com estágio inicial de regeneração da fibra muscular e, em estágios avançados, o aparecimento de cicatrizes. Já, nas concentrações de 0.38% e 0.19% não foram observados efeito a longo prazo e aparecimento de cicatrizes (Zhang et al, 2010).

Quando o processo de inflamação é excessivo e prolongado, ele interfere na etapa de regeneração, resultando até mesmo na formação de fibrose muscular (Porter et al., 2002; Liu et al., 2015).

As soluções de anestésicos locais podem estar associadas à inflamação local, e o músculo estriado esquelético parece ser muito sensível a esta reação. Renton et al. (2010) investigaram as lesões decorrentes de injeções de anestesias locais, e observaram grandes lesões iatrogênicas após administração destas soluções no bloqueio do nervo alveolar inferior e lingual, tendo como consequência para o paciente, sintomas de dor e manifestações importantes na função do aparelho estomatognático, como a presença de trismo (rigidez muscular), limitando assim a abertura da boca (Sanchez, Takara & Alonso, 2010).

Na prática clínica, os anestésicos locais comerciais, por causar miotoxicidade, inflamação e neurotoxicidade têm desafiado pesquisadores a desenvolverem novas formulações. Estas novas formulações

anestésicas em estudo é um desafio atual nos procedimentos cirúrgicos médico e odontológico, a fim de aumentar o tempo de bloqueio e diminuir o processo inflamatório e a toxicidade local, como na associação de carreadores como lipossomas, ciclodextrinas, géis e polímeros (Cereda, et al., 2012; McAlvin et al., 2013).

Segundo Yang et al., 2011, as lesões no nervo após anestésicos locais é uma complicação, podendo levar à dormência local ou fraqueza muscular. Hinton, Dechow & Carlson, em 1985, já relatavam efeitos miotóxicos como a presença de fraqueza muscular com redução de 30 a 40% da força de mordida após injeção de anestésico local, com efeitos persistentes por até duas semanas.

Em experimentos, Ji et al. (2015) observaram lesões neurológicas na raiz e coluna posterior da medula após anestesia intratecal com o anestésico bupivacaina. Na ultraestrutura identificaram edema, vacuolização e descontinuidade da bainha de mielina e apoptose de neurônios. Alterações morfológicas semelhantes à atrofia muscular é uma alteração evidenciada também após injeção de bupivacaina (Scott, Miller & Shieh, 2009) como verificadas em estudos de desnervação (Benoit e Belt, 1970; Carlson, 1976; Çalgüner et al., 2003).

Diversos recursos terapêuticos são utilizados para a saúde funcional do tecido muscular esquelético. Dentre esses destacamos, a Laserterapia de Baixa Intensidade (LBI), que tem sido empregada como ação anti-inflamatória, regenerativa e analgésica (Genovese, 2007).

Há muito tempo, a luz é utilizada com finalidades terapêuticas. Como mostra a história, os gregos usavam a helioterapia; os chineses tratavam com a luz solar algumas doenças cutâneas, câncer e até alguns casos de psicoses. Os povos indianos utilizavam a luz em fotoquimioterapia, empregando fotossensibilizador exógeno extraído de plantas por volta de 1400 a.C. (Brugnera-JR & Pinheiro, 1998).

Todas essas modalidades de tratamento estão respaldadas na relação que a luz tem com as funções biológicas e com a saúde dos seres vivos. É bem conhecida a importância da luz na síntese de substâncias vitais para os animais, e na fotossíntese dos vegetais, da qual provém grande parte da energia orgânica existente no planeta. A luz tem a capacidade de destruir microrganismos patogênicos, e por outro lado pode induzir doenças como o câncer de pele e as queimaduras. O laser com todas as suas similaridades e diferenças da luz convencional, também tem o efeito de modificar funções biológicas, positiva ou negativamente (Goodman & Kaufman, 1997).

É bem estabelecido que a laserterapia atua na cadeia de transporte de elétrons mitocondrial, aumentando o espaço entre as membranas externa e interna da mitocôndria e também dilatando a crista mitocondrial (Iyomasa et al., 2013).

Os lasers são formados por uma energia de fótons com propriedades específicas, com o mesmo comprimento de onda eletromagnética, direção, frequência e cor (Maiman, 1960). São capazes de levar grande quantidade de energia aos tecidos, com precisão. Essas características conferem a essa luz as propriedades de monocromaticidade, colimação e coerência (temporal e espacial) que acrescentam benefícios aos procedimentos em que é associado (Goodman & Kaulfman, 1997; Robertson et al., 2009).

Bem tolerada pelo organismo, a laserterapia é uma forma de energia não-invasiva, de fácil aplicação, baixo custo, não possui efeito mutagênico e pode ser utilizada sem risco (Brugnera-JR & Pinheiro, 1998; Ferraresi, Hamblin & Parizotto, 2012; Alves et al., 2014).

A laserterapia é um procedimento que precisa ser estudado e compreendido na prática clínica (Miyashita & Fonseca, 2004). Devido às variações nos parâmetros e protocolos de tratamentos, a laserterapia não

é uma modalidade terapêutica estabelecida. Estes parâmetros incluem: tipo de aparelho, meio semicondutor, comprimento de onda, densidade de energia, potência, tempo de exposição, número de aplicações e duração total do tratamento (Vieira, 2004; Bjordal, 2012).

Baseado na teoria proposta por Albert Einstein em 1916 sobre “os princípios da amplificação da luz por emissão estimulada de radiação” (Takac & Stojanovic, 1999; Geiges, 2011), o primeiro laser foi desenvolvido na década de 1960, tendo sido utilizado para clínica somente em 1978 por uma escola Alemã, no Departamento de Dermatologia da Luawing, em Munique.

A primeira diretriz da World Association for Laser Therapy (WALT) sobre a dosagem do laser terapia de baixa intensidade (LBI) para as desordens musculoesqueléticas foi publicada em agosto de 2005. A Walt recomenda a utilização de energia final de 2 a 4 Joules (J) no tratamento de diversos tecidos humanos, dentre eles, o músculo estriado esquelético (WALT, 2006).

Estudos laboratoriais relatam que o efeito positivo do LBI pode ser perdido, quando se faz uma overdose do laser, alterando a morfologia dos fibroblastos, danificando e diminuindo a atividade mitocondrial, e alteração do DNA e da membrana plasmática. Portanto, a fototerapia é capaz de alterar células normais e lesionadas (Hawkins & Abrahamse, 2006). Freitas et al. (2015) não observaram alterações na porcentagem de colágeno, mesmo utilizando alta dosagem da LBI (4,8J).

Iyomasa et al. (2013) observaram que 6 sessões de laserterapia de AsGaAl (780nm), potência de 40mW, utilizada com energia final de 20J/cm^2, é o melhor custo-benefício ao paciente, com melhor efetividade e segurança da aplicação da laserterapia. Verificaram que após 10 sessões da LBI ocorreram danos indesejáveis à morfologia e ultraestrutura mitocondrial, prejudicando a atividade metabólica da fibra muscular.

Apenas para a analgesia e reparação do nervo periférico, WALT recomenda que a dose seja maior que a recomendada para o músculo esquelético (Bjordal, 2012).

Vários são os lasers utilizados na prática clínica nas lesões teciduais, sendo mais frequentes os de Hélio-Neon (HeNe), Indio-Gálio-Alumínio-Fosforeto (InGaAIP), Arseneto de Gálio (AsGa) e Arseneto de Gálio Alumínio (AsGaAl) (Silva et al., 2010). A faixa de radiação eletromagnética do LBI está entre 1mW a 500mW, e o comprimento de onda (penetração no tecido) situa-se entre 660 a 1000 nanômetros (nm) (Huang et al., 2011).

As radiações vermelhas apresentam comprimento de onda abaixo de 700nm, enquanto a infravermelha, acima de 700nm. Quanto maior o comprimento de onda, maior é a penetração da radiação no tecido. No tecido muscular, por exemplo, o infravermelho tem maior penetração, e por este motivo, é utilizado com maior frequência nas práticas clínicas fisioterápicas (Turner & Hode, 2003; Freitas et al., 2015) (Figura 04)**.**

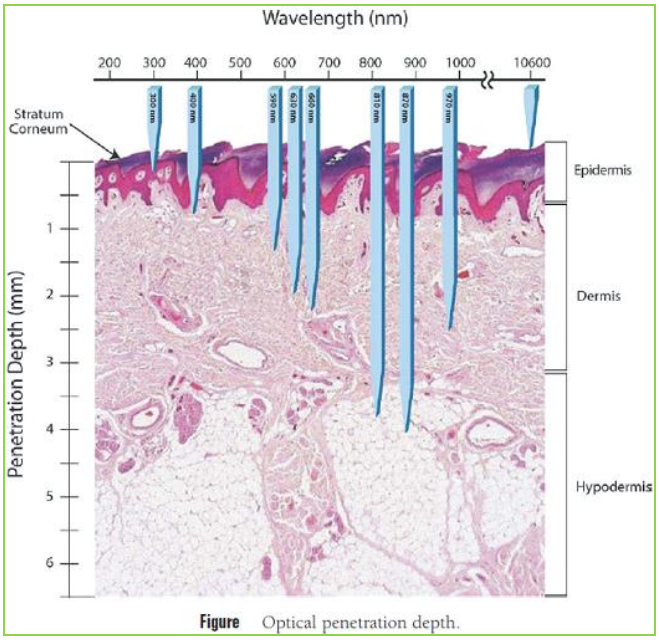

Figura 03: Esquema dos comprimentos de onda e profundida. Fonte: adaptado de Barolet, 2008.

Muitas são as respostas biológicas relatadas pela literatura. É benéfica na redução de processos inflamatórios após lesões (Freitas et al., 2015; Otrocka-Domagala et al., 2015; Zagatto et al., 2016), têm ação analgésica nas disfunções temporomandibulares e dores miofaciais (Shirani et al., 2009; Arduino et al., 2016; Pires de Sousa et al., 2016), agem como miorrelaxantes (Núñez et al., 2006), atuam na redução da fadiga muscular (Lopes-Martins et al., 2006; Borsa et al., 2013; Zagatto et al., 2016). A LBI também melhora a parestesia pós-cirúrgica (Simões, 2007), promovendo a regeneração muscular (de Sousa et al., 2011; Silva et al., 2012; Aranha de Sousa et al., 2013; Freitas et al., 2015), e prevenindo a formação da fibrose muscular (de Sousa et al., 2011). A LBI de 808nm obteve uma resposta efetiva, quando aplicada extra oral para trismo, edema pós-exodontia e reparo tecidual (Aras & Güngörmüs, 2010).

Segundo Silva et al. (2012), as aplicações de laser de Arseneto de Gálio (AsGa) 904nm, potência de 5mW, a uma dose de 3J, mostrou-se eficaz na melhora da regeneração muscular de ratos. Dias et al. em 2012, verificou que após a laserterapia no músculo masseter, houve modificação no fenótipo da fibra muscular, melhorando sua capacidade oxidativa.

Em 2011. Mandic & Rancié, utilizando uma energia final de 15J por 10 sessões, 60 mW potência, laser AsGa 904nm, observaram diminuição da dor e o espasmo muscular, quando comparado ao grupo controle.

Após criolesão no músculo tibial anterior de ratos, Souza et al. (2011), aplicaram laser InGaAIP (660nm), potência 20mW, 1,6J de dose final e observaram aumento da síntese de colágeno do tipo I e III e diminuição da área de mionecrose.

Silveira et al. (2013) após protocolo de trauma no músculo gastrocnêmio, observaram aceleração da regeneração muscular com a laserterapia de AsGa 904nm, potência de 40mW, com energia irradiada de 5J para cada ponto. Utilizando-se o mesmo comprimento de onda de

904nm, Leal-Júnior et al. (2014) verificaram a diminuição do processo inflamatório usando uma energia final de 1J.

Na avaliação do potencial eletrofisiológico da junção neuromuscular do músculo diafragma de camundongos, a irradiação do LBI de 830nm, com energia de 12 j/cm² nos elementos da junção neuromuscular, provocou uma diminuição da liberação de acetilcolina, mas apenas a nível fisiológico da liberação dos neurotransmissores. Já, o LBI de 655nm, com energia de 1 – 12 j/cm² não teve efeitos eletrofisiológicos detectados na liberação de neurotransmissores da junção neuromuscular de camundongos (Nicolau et al., 2004).

O LBI de AsGa é um laser infravermelho pulsado (904nm) capaz de penetrar alguns centímetros (0,5 a 2,5 cm) a mais, quando comparado ao laser HeNe (624nm), sendo, portanto, mais efetivo em tecidos mais profundos, como o músculo esquelético, além de proporcionar maior analgesia, enquanto que o laser HeNe age com mais eficácia no tratamento de úlceras da pele (Basford, 1993; Carvalho et al., 2006).Os lasers infravermelhos reduzem o espasmo muscular e aumentam a mobilidade muscular (Nicolau, 2004).

Na clínica, pacientes têm experimentado o uso do laser após anestesia local para alívio da dor, sem o uso de drogas (Aras et al., 2010; Yang et al. 2011). Clockie et al., 1991, aplicaram a LBI na dose de 0.97 j/cm², 3 minutos após a extração do 3˚ molar e obtiveram redução da dor pós-operatória. Já, Markovic & Todorovic, 2006, relataram que doses menores de 4 j/cm² não obtiveram resultados significativos.

Segundo os achados de Alcântara et al. (2013), é recomendável que a LBI deve ser iniciada o mais rapidamente possível após a lesão do nervo periférico, pois sua aplicação aumenta a atividade das Metaloproteinases, especialmente de MMP-9, e o nível da proteína TNFα durante a fase aguda de lesão do nervo, modulando a inflamação. A

atenuação do processo inflamatório, minimiza a formação de fibrose e estimula a regeneração muscular (Assis et al., 2012). Segundo França et al (2013), a LBI pode ter um efeito positivo no músculo esquelético durante seu processo de reparo muscular ao promover uma melhora na qualidade da reorganização das miofibrilas e no perimísio, reduzindo assim a fibrose (França et. al., 2013).

Na regeneração muscular, a LBI atua na analgesia, estimulando a proliferação das células musculares e satélites, intensificando, desta forma, a síntese de proteínas de mioblastos, aumentando a área da fibramuscular e a densidade mitocondrial (Genovese, 2007; Shirani et al., 2009; Dias et al., 2012; Silva et al., 2012; Aranha de Sousa et al., 2013; Borsa et al., 2013; Mantineo, Pinheiro & Morgado, 2014; Freitas et al., 2015). A irradiação com o laser ainda atua como terapêutica complementar em lesões neuronais (Liang et al., 2008), alívio da dor crônica (Masoumipoor et al., 2014; Erthal & Nohama,2015), e redução da apoptose de mionúcleos e células miogênicas decorrentes de desordens músculo esqueléticas (Sergio et al., 2016).

Há uma grande controvérsia que rodeia a laserterapia, devido à variação nos parâmetros envolvidos, tais como: tipo de aparelho, meio semicondutor, comprimento de onda, densidade de energia, potência, tempo de exposição, número de aplicações e duração total. Portanto, os resultados deste estudo têm a finalidade de contribuir no que se refere ao custo benefício desta terapia, na determinação da segurança para aplicação do laser e a compreensão dos seus efeitos sobre a interação neuromuscular. Além disso, a literatura frente a essa terapia em relação aos músculos da cabeça e pescoço e as junções neuromusculares é bastante escassa.

2. Objetivo

Avaliar os efeitos da laserterapia sobre as junções neuromusculares, fibras musculares e nervo associados ao músculo esternomastóideo após injeção de bupivacaina.

Objetivos Específicos:

a) Níveis de inflamação: Análise morfopatológica das fibras musculares e quantificação de TNFα e da área de colágeno;
b) Miotoxicidade: Análise da Quantificação da Creatina kinase (CK) sérica e muscular, Análise morfológica e ultraestrutural das fibras musculares;
c) Neurotoxicidade: Análises morfológicas, morfométricas e ultraestruturais do nervo esternomastóideo e das junções neuromusculares associadas; Análise da distribuição dos receptores de acetilcolina (nAChRs) por meio de microscopia confocal, e quantificação proteica dos nAChRs.

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Paper 1

LOW-LEVEL LASER THERAPY (LLLT) ACCELERATES THE STERNOMASTOID MUSCLE REGENERATION PROCESS AFTER MYONECROSIS DUE TO BUPIVACAINE

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Keywords: Bupivacaine, Low-Level Light Therapy, Muscle Regeneration, Fibrosis.

Highlights: LLLT decreased fibrosis and myonecrosis (sternomastoid muscle) caused by bupivacaine.

Competing Interests
The authors declare no competing interests.

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Abstract

Background: Because of its long-lasting analgesic action, bupivacaine is an anesthetic used for peripheral nerve block and relief of postoperative pain. Muscle degeneration and neurotoxicity are its main limitations. There is strong evidence that low-level laser therapy (LLLT) assists in muscle and nerve repair. The authors evaluated the effects of a Gallium Arsenide laser (GaAs), on the regeneration of muscle fibers of the sternomastoid muscle and accessory nerve after injection of bupivacaine. **Methods:** In total, 30 Wistar adult rats were divided into 2 groups:control group (C: n = 15) and laser group (L: n = 15). The groups were subdivided by antimere, with 0.5% bupivacaine injected on the right and 0.9% sodium chloride on the left. LLLT (GaAs 904nm, 50mW, 4.8J) was administered for 5 consecutive days, starting 24 hours after injection of the solutions. Seven days after the trial period, blood samples were collected for determination of creatine kinase (CK).The sternomastoid nerve was removed for morphological and morphometric analyses; the surface portion of the sternomastoid muscle was used for histopathological and ultrastructural analyses. Muscle CK and TNFα protein levels were measured. **Results**:The anesthetic promoted myonecrosis and increased muscle CK without neurotoxic effects. The LLLT reduced myonecrosis, characterized by a decrease in muscle CK levels, inflammation, necrosis, and atrophy, as well as the number of central nuclei in the muscle fibers and the percentage of collagen.TNFα values remained constant. **Conclusions**:LLLT, at the dose used, reduced fibrosis and myonecrosis in the sternomastoid muscle triggered by bupivacaine, accelerating the muscle regeneration process.

1. Introduction

Local anesthetics are generally used in clinical, medical and dental practices to reduce operative pain[1-7]and as co-adjuncts in postoperative analgesia[8-13].

Many adverse effects have been reported as a consequence of the injection of local anesthetics; these include numbness, muscle weakness[14,15], lockjaw[16,17], inflammation[18], paresthesia[15] and muscle degeneration[19-22].

In the potentiality hierarchy of local anesthetics, bupivacaine is believed to be neurotoxic[21,23,24], and the most myotoxic[25], leading to the formation of scar tissue, multiple calcifications in muscle tissue[26], myonecrosis and acute degeneration[19,22,27-29].

Considering these alterations, bupivacaine has also been used in experimental protocols as a model for myonecrosis[20-22]as well as for the characterization of denervation[19].

Used for epidural and spinal anesthesia, bupivacaine is considered a long-lasting local anesthetic, having the potential to trigger medical complications such as cauda equina syndrome, permanent spinal nerve injury, edema, vacuolization and rupture in the myelin sheath, degeneration of neurons and nuclei fragmentation[21,23,24,30], transient neurologic syndrome[31], and neuronal apoptosis[21,23,24,30,32-34].

Laser therapy (LT) has been reported to play a positive role in muscle regeneration. LT acts as an analgesic, stimulating the proliferation of muscle and satellite cells, thus enhancing protein synthesis in myoblasts and increasing the area of muscle fibers and mitochondrial density[35-42]. Laser irradiation also acts as an adjunctive therapy in neuronal injury[43] and, chronic pain relief[44-45], and reduces myonuclear apoptosis and myogenic cells resulting from musculoskeletal disorders[46].

Because local anesthetics are of fundamental importance in clinical practice and the deleterious effects of bupivacaine on muscle and nerve tissue are well described in the literature, LLLT emerges as a non-invasive co-adjunctive procedure to minimize tissue damage, which is a potential complication in clinical practice.

Thus, the objective of the present study was to investigate the effects of low-level laser therapy on the healing process of muscle fibers of the sternomastoid muscle and associated nerve after injection of bupivacaine.

2. Methodology

Thirty adult male Wistar rats were kept in individual cages with food and water ad libitum in an environment with controlled temperature (24 ± 2°C) and photoperiod (12h:12h). All experiments and procedures were approved by the Animal Use Ethics Committee (São Paulo State University, UNESP, CEUA- Protocol 509).

The animals were anesthetized with ketamine/xylazine (90 mg/kg and 10 mg/kg, respectively) intraperitoneally. A midline incision was performed on the ventral side of the neck to expose the sternomastoid muscles. The injections were then administered as follows: 0.05ml bupivacaine hydrochloride (5mg/ml) (HypoFarma) in the right antimere and sodium chloride 0.9% in the left antimeres; the skin was sutured with no.

3.0 black nylon (Brasutura®). The solutions were deposited in the open muscle field, subfascially in the middle third[47] and distal muscle belly[48].

After 24 hours, the animals were randomly divided into two groups: control and laser.

The laser group received laser treatment with a GaAs diode laser (Endofthoton, KLD Biosystems, Amparo, Brazil) with a pulsed emission wavelength of 904nm, output power (average) of 50 mW, and 0.035 cm² emitting area of the beam for 5 consecutive days. The previously calibrated laser was directly applied to the skin (direct contact) in the injection areas in both antimeres. The application was performed with the laser pen held at an angle of 90° to the irradiated surface. The laser treatment lasted48 seconds with an energy of 2.4J per point, for a final total energy 4.8J.

Four groups were formed based on the antimere and treatment: right antimeres- CBupi (without LLLT), LBupi (with LLLT); left antimeres CCl (without LLLT), LCl (with LLLT).

Seven days after application of the solutions, the animals were anesthetized with ketamine/xylazine (90 mg/kg and 10 mg/kg, respectively) intraperitoneally and then decapitated. Blood samples were collected, centrifuged at 936RCF, and the serum was frozen.

The sternomastoid muscle used in this study consisted of two macroscopically clearly distinguishable even portions, white and red[49];based on the bupivacaine diffusion, only the superficial muscle portions were removed with the associated nerve and processed for analyses.

2.1- Creatine Kinase (CK)

Serum samples and approximately 100 mg of muscle tissue were used from each group (n = 5 in each group) for the determination of creatine kinase activity (Kit Labtest – CK-nac liquiform 117-1/60)[50].

The absorbance of the samples was read at 25°C using a UV spectrophotometer with a wavelength of 340nm and quartz cells with a 1-cm optical path length.

2.2- Histopathological analysis of muscle tissue and collagen quantification

Fragments of the surface portion of the sternomastoid muscle (n = 5) after freezing in liquid nitrogen (-170°C) were sectioned (6µm) in a Leica

CM1800 cryostat (-25°C). Two slides were prepared; the first was stained with Hematoxylin-Eosin (HE) and the second with Picrosirius Red[51]. The slides stained with HE were analyzed using a photomicroscope (BX 41-2) with a digital camera (model SIS-SC30) and objectives of 20x and 40x for image capture. Double-blind semi-quantitative analysis of the degree of tissue components (inflammatory infiltrate, atrophy and necrosis) was described as absent (grade 0), mild (grade 1), moderate (grade 2) and high (grade 3)[52]. These images were also used to count the central and peripheral nuclei with the "ImageJ" software program (http://rsbweb.nih.gov/ij/).

To quantify intramuscular collagen, 5 random images of each slide were obtained (20X). The percentage of collagen was calculated using Leica QWin software (Wetzlar, Germany).

2.3- Transmission Electron Microscopy (TEM)

Muscle fragments (n = 5 in each group), fixed in Karnovsky solution, were processed according to the routine for Transmission Electron Microscopy analysis by the Electron Microscopy Center IB/Unesp/Botucatu. Ultra-thin sections were obtained from longitudinal cuts in muscle fragments using an ultramicrotome (Ultratome 880 LKB III) and stained with uranyl acetate solution in 50% alcohol and subsequently with lead citrate. The material was analyzed and photographed with a transmission electron microscope (TECNAI Spirit Fei Company).

2.4- Morphological and Morphometric Analyses of the Sternomastoid Nerve

Sternomastoid nerve fragments (n = 5 in each group), after fixation in Karnovsky solution, were post-fixed in osmium tetroxide 1%. Twenty-four hours later, the histological routine was performed, during which cross sections were obtained (6µm) and histological slides were prepared. For each experimental group, 100x images were obtained with immersion oil in the photomicroscope (Axiophot– 2) using a digital camera (model AxioCam HR, Zeiss).The following variables were measured: number of axons, axonal diameters (Σ axon diameter/number of axons) and nerve fibers (Σ fiber diameter/number of fibers);the following calculations were made with the free software "ImageJ" (http://rsbweb.nih.gov/ij/): thickness of the myelin sheath (fiber diameter - diameter of axon/2) and G-ratio (average diameter of axons/average diameter of nerve fibers.

2.5- Western Blotting

Frozen muscle samples (third medium)from 5 animals from each group were homogenized with a tissue homogenizer (IKA UltraTurrax/T-25) in 0.5 ml of lysis buffer (1% Triton X-100, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, aprotinin 10 µg/ml, 1 mMPMSF, sodium orthovanadate - 0.25 mMNa_3VO_4,150 mM NaCl and 50 mMTris-HCl, pH 7.5). The samples were centrifuged at 11,000 rpm for 20 min and the supernatant collected. A 100-µL aliquot of the homogenate was treated with 100µL of Laemmli sample buffer (2% SDS, 20% glycerol, bromophenol blue 0.04 mg/ml, 0.12 M Tris-HCl, pH 6.8, and 0.28 M β-mercaptoethanol).The samples were then incubated at 97°C for 5 minutes and stored in a freezer at -20°C until use.

One aliquot of the pure extract from each sample (not treated with Laemmli) was used to quantify total protein using the Bradford method (Bradford, 1976). Exact quantities of the total protein from each sample (70 µg) were subjected to electrophoresis in 4-15% polyacrylamide gel (SDS-PAGE) and then transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, California) in the wet system.

The proteins transferred to the membranes were blocked with 5% skimmed milk diluted in TBS-Tween for 1h at room temperature and incubated with different TNFαprimary antibodies (abcam – ab66579; 1 mg/ml) (Gapdh – Cell Signaling - 14C10; 1:1000), overnight at 4ºC. After incubation with the primary antibody, the membranes were washed in TBS-Tween and incubated with specific anti-rabbit secondary antibody (Cell Signaling - 7074s) 1:5000 for 1 h at room temperature.Again, the membranes were washed, and the ECLTM Selected Western Blotting Detection Reagent (GE Healthcare, Uppsala, Sweden)detection system was used. After image capture in a transilluminator G-Box, densitometric quantification of the bands was performed using ImageJ software (version 1.71, 2006, Austria). The protein expression values were normalized to the values obtained for the GAPDH protein, which was used as a reference.

3. Results

Macroscopically, 7 days after the application of bupivacaine, a decrease in muscle volume was observed in the control group that received bupivacaine, compared to the control group that received sodium chloride (Figure 1A). After LLLT treatment, the muscle volume approached normal, suggesting regeneration (Figure 1B).

3.1- Creatine Kinase (CK)

The results of the quantification of CK serum levels revealed no statistically significant differences between the groups: Control (216.69 ± 30.54) and Laser (205.63 ± 40.50), p> 0.05.

We observed that muscle CK levels were higher in the control group that received bupivacaine (401.55 ± 40.41) compared to the group that received sodium chloride (331.90 ± 41.51), p <0.01.After application of laser therapy, there was a statistically significant decrease in CK levels (310.20 ± 38.51) (p <0.01).

3.2 - Histopathological, Electron Micrograph, and Quantification of Collagen and Western Blot Analysis

The general morphological analysis of the sternomastoid muscles stained with HE demonstrated the presence of inflammatory cells with basophilic nuclei (mononuclear cell infiltration), blood vessels with hyperemia, edema in the connective tissue, muscle fibers in a degenerative and necrotic process and total loss of its polygonal characteristic in the control group that received bupivacaine (Figure 2Aa).When it was possible to identify the muscle fibers, central nuclei were associated. After laser application, a lower quantity of inflammatory infiltrate was observed; areas in the process of regeneration were present, characterized by the presence of muscle fibers with basophilic cytoplasm containing central and peripheral nuclei (Figure 2Ab).

In the control group that received sodium chloride, there was loss of the polygonal characteristic in only a few muscle fibers; the mononuclear cell infiltration was slight when compared to the group that received bupivacaine (Figure 2Ae). After application of LLLT, the presence of inflammatory infiltrate was not detected and the histological architecture of the muscle fibers was preserved (Figure 2Af). The presence of fibroblasts in the endomysium of the animals that received LLLT in both antimeres was common.

The blind semi-quantitative analysis confirmed the histological observations that the muscles of the control group that received bupivacaine had significantly higher scores related to inflammation, atrophy and necrosis. When the anesthetic was associated with laser treatment, an attenuation of general pathological processes was observed. In the control group that received sodium chloride, only minor alterations were observed with regard to inflammation; after application of LLLT, there

were no alterations (Table 1) (values in scores).

There was an increase in the quantification of central nuclei in the control group that received bupivacaine (277) ($p<0.01$) and a decrease in the number of peripheral nuclei (53) ($p<0.05$) compared to the groups that received sodium chloride (Figures 2B e C). In the group that received bupivacaine and LLLT, there was a statistically significant increase in the number of peripheral nuclei (287) ($p<0.05$) (Figure 2C).

The evaluation of TNFα protein expression in all experimental groups revealed no statistically significant differences, although a decrease in TNFα expression was observed in the group receiving bupivacaine and LLLT application (Figure 2D).

The ultrastructural analysis found comparatively normal myofibrils, without signs of alteration, Z rectilinear line, inter-myofibrillar mitochondria of varying sizes and T tubules associated with the cisternae of the sarcoplasmic reticulum forming triads in the group that received sodium chloride (Figures 2Ag and 2Ah).

In the group that received bupivacaine, degeneration regions and myonecrosis were present, containing disorganized myofibrils with fragmented and discontinuous Z line and abundant central nuclei. Inflammatory cells, areas of fibrosis, and signs of edema were present (Figure 2Ae).

After application of LLLT, organized sarcomeres and scarce degeneration regions were detected. The nuclei were for the most part peripheral, and blood vessels and central nuclei were present (Figure 2Af).

The Picrosirius Red coloring enabled analysis of the collagen fibers present in the endomysium and perimysium, which were marked in red, and the muscle fibers in yellow. This analysis was performed using conventional optical light microscopy and optical polarized light microscopy (Figure 3A). In the group that received sodium chloride, an increase in red tone staining was identified in polarized light, suggesting the presence of mature collagen - type I. In the bupivacaine group, the birefringence of collagen fibers tended towards yellow to green, suggestive of the presence of type III collagen fibers (newly synthesized) (Figure 3A).

The analysis revealed that the percentage of collagen increased in the area of fibrosis in the group that received bupivacaine (53.3 ± 8.2) compared to the group that received sodium chloride (17.08 ± 6.0) ($p <0.05$). After the laser treatment, there was a reduction in the area of fibrosis in both the groups that received bupivacaine (24.19 ± 8.66) and

those that received sodium chloride (14.11 ± 1.16) ($p < 0.01$) (Figure 3B).

3.3- Morphological and Morphometric Analyses of the Sternomastoid Nerve

For the doses of 0.5% bupivacaine and 0.9% sodium chloride, in all groups, the histological images of the sternomastoid nerves demonstrated axon and intact myelin sheath, and axoplasm and endoneurium with morphology within the normal range.The ultrastructural analysis revealed bundles of organized myelinated axons containing neurofilaments and integral and concentric myelin sheaths without signs of demyelination. The axolemma and Schwann cells (represented by the nucleus) were well preserved. The endoneurium contained collagen cells oriented longitudinally (Figure 4 A).

The morphometric analyses of the number of axons and diameters of axons and nerve fibers, as well as calculations of the thickness of the myelin sheath and the G ratio, showed no statistically significant differences between the study groups ($p < 0.05$) (Figures A, B, C, D). The G-ratio demonstrated values of approximately 0.5, consistent with normal nerves (Figure 4 E).

4. Discussion

The results of the present study suggest that the bupivacaine dose used (0.5%) caused myonecrosis. Increases were observed in muscle CK ($p<0.05$) and scores related to inflammation, atrophy and necrosis. No morphological alterations related to the neurotoxic effect of the anesthetic were observed, and the G-ratio values were within the normal range (0.5), although several studies in the literature have described the presence of neurotoxic effects associated with the anesthetic bupivacaine[15,23,54].

The lower G-ratio values (near 0.4) indicate degeneration of the axon, while high values (near 0.7) indicate myelin degeneration or regeneration[55]. Some neurological lesions was observed in the root and posterior column of the spinal cord after intrathecal anesthesia with bupivacaine, along with ultrastructurally identified edema, vacuolation of the myelin sheath, neuronal apoptosis and disruption of the myelin sheath[23].

Long-acting bupivacaine produces morphological alterations similar to muscular atrophy [56], as demonstrated in studies on denervation [19,27,57].

Myonecrosis caused by bupivacaine is due to excessive intake of

intracellular Ca^{2+} ions, which promotes the activation of proteases, resulting in sarcomere disruption[56]. This framework is observed ultrastructurally through disorganized myofibrils, a fragmented and discontinued Z line, abundant central nuclei, the presence of inflammatory cells, and areas of fibrosis and edema. Due to the excessive accumulation of Ca^{2+}, there are alterations in the morphology and function of the mitochondria, which trigger inhibition of enzymatic activity and block the synthesis of ATPase, resulting in myonecrosis[58],[59].

Among the parameters used to verify myonecrosis, the CK level is commonly used in the diagnosis of myopathies[60-63]. In the experimental control and laser groups ($p>0.05$), no changes were found in serum CK levels. The increased muscle CK levels after administration of bupivacaine suggested a local and non-systemic effect of the anesthetic.

A 5.5-fold increase in serum CK levels was observed after injection with bupivacaine[64]. A reduction in serum CK was present after 5 sessions of laser therapy (810 nm) with a final energy of 4.8J in water polo athletes[65].

After LLLT, we observed a decrease in muscle CK ($p <0.01$). Decreases in CK values are directly related to the reduction in myonecrosis described after laser therapy[64,66], which has been used for its anti-inflammatory, regenerative and analgesic actions[35,42,65]. CK is an enzyme associated with the formation of ATP, found primarily in skeletal and cardiac muscle and the brain. It catalyzes the reversible phosphorylation of creatine into phosphocreatine with the release of adenosine triphosphate (ATP) from high energy phosphate. In muscle tissue, phosphocreatine acts as an energy storage molecule, and in cases of energy needs, a cleavage is performed to supply ATP for muscle activity[60]. After LLLT, the mitochondrial metabolism is modulated, promoting an increase in ATP synthesis as a secondary response[67].

The treatment with the GaAs laser used in this study (904 nm) with high final energy of 4.8J, applied for 5 consecutive days, was also able to reduce inflammation and atrophy, as well as the number of central nuclei and the percentage of collagen. Ultrastructurally, after the LLLT, the sarcomeres were for the most part arranged, and the majority of the nuclei were peripheral. The TNFα values remained constant.

TNFα is a pro-inflammatory cytokine that stimulates other cytokines, acting as a chemotactic agent of inflammatory cells, principally activated by macrophages in the lesion process, collagen synthesis and production

of extracellular matrix proteins[68]. In our experimental protocol, we found no alteration in tissue TNFα before and after LLLT. The fact that the levels of inflammatory cytokines represented here by TNFα were analyzed after 7 days of myonecrosis, and that LLLT acts by inhibiting factors involved in inflammation, such as pro-inflammatory cytokines[69], explains the similarity found in TNFα values.

No alterations in TNFα levels were present after application of LEDT (Light Emitting Diode Therapy) during persistent inflammatory pain[70], although a reduction in TNFα gene expression was observed in the injured group treated with a laser [42].

The quantification of collagen in the present study showed that the area of fibrosis observed in the LBupi subgroup was significantly smaller than in the CBupi group. These data was confirmed by the others studies, in which irradiation with the AsGaAl laser (635 nm, 7 mW) prevented the formation of fibrotic tissue[71]. Laser therapy inhibits fibrosis and accelerates muscle regeneration [72].

A non-significant reduction was observed in the percentage of collagen using the GaAs laser (904 nm, 50mW) with final energy of 4.8J at 7 days after cryoinjury of the tibialis anterior muscle[42]. Several authors have described an improvement in collagen synthesis and organization after laser therapy[71,73-76].

The integrity of the extracellular matrix promotes support, protection and stabilization of muscle fibers[77]. LLLT remodels the extracellular matrix[76] and TNF-α protein levels during the acute phase of injury, which modulates inflammation[65,78].

After GaAs laser therapy (904 nm), we observed a macroscopic reduction in atrophy in the group that received bupivacaine.

Laser therapy used in muscle regeneration promotes the differentiation of new myofibrils, causing recovery in necrotic tissue. After LLLT application (HeNe- 603 nm, 4mW, 5 or 10j/cm^2) was observed the regeneration of muscle fibers with preserved myofibrils and well-defined T tubules and sarcoplasmic reticulum[79].

It was Identified a reduction in myonecrosis and edema after LLLT treatment with an AsGaAl (780 nm, power 40mW, 3.2 J) in the tibialis anterior muscle after cryoinjury[65]. This muscle recovery was also observed after 5 applications using a GaAs laser (904 nm, 50mW) with a final dose of 4.8J[42]. These data suggest that LLLT is a co-adjuvant in muscle improvement at low or high final energy by optimizing the tissue repair

process[38].Moreover, LLLT with diodes emitting at infrared promotes positive responses in skeletal muscle because, they reach a skin depth of 2 to 4 cm[40] with a similar response to the laser used in the present study.

As infrared wavelength was used in the application of LLLT, the absorption occurs through the cytochrome c oxidase (COX) [67,80], which is the principal chromophore in cells, increasing mitochondrial products such as ATP, NADH, proteins and RNA and a reciprocal increase in oxygen consumption occurs[81]. All of these physiologic modifications induced by LLLT could have stimulated mitochondrial physiology due to lesions caused by the anesthetic, reducing the levels of muscle catabolism and loss of proteins and promoting regeneration and organization of muscle tissue[82], which may explain the increased number of nuclei observed in the LBupi subgroup in the present study.

It has been reported that the use of LLLT, even before musculoskeletal injury, promotes better, faster and more efficient muscle repair, reducing myonecrosis, inflammatory infiltrate and collagen synthesis [77].

Laser therapy acts through the absorption of light, and light absorption by non-specialized photoreceptor molecules can absorb light at certain wavelengths, but they are not integrated with any specialized light receiver; however, this is used extensively in medicine. The molecule that is absorbed can be transferred to another molecule or cell, which can activate or promote chemical reactions in the surrounding tissue[80].

The biological response to laser therapy depends on the choice of the treatment and irradiation parameters, such as the wavelength, device power, beam area, final energy, irradiation time, treatment frequency, application mode and the start of the treatment. One scientific review, reported that the results are often conflicting, due to the choice of when to start treatment, considering that the intervention can act at different stages of tissue repair[76]. All of these parameters are important to obtain a positive effect of laser therapy, although many studies do not report the complete data, which makes replication and reliability of the findings difficult.

Because LLLT is a low-cost, non-invasive therapy that is easy to administer, it can be introduced both before and after surgery, with beneficial effects similar or superior to oral medications given to reduce inflammation and pain.

5. Conclusion

The results support the use of GaAs laser therapy as an easy-to-use and low-cost co-adjuvant therapy, reducing the side effects associated with bupivacaine, accelerating the process of muscle regeneration and promoting reductions in myonecrosis and muscle fibrosis, according to the experimental model used

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Table 1- Morphological comparison of general pathological process in experimental models exposed to bupivacaine and the laser.

Subgroups	N (%)	General pathological processes - Med (Vmin – Vmáx)		
		Inflammation	Atrophy	Necrosis
CBupi	5,0 (25)	2,0 (2,0 – 3,0)[1]	2,0 (1,0– 2,0)[2]	2,0 (2,0– 3,0)[3]
CCl	5,0 (25)	1,0 (0,0 – 1,0)	1,0 (0,0 – 1,0)	0,0 (0,0 – 0,0)
LBupi	5,0 (25)	1,0 (0,0 – 2,0)	1,0 (0,0 – 1,0)	0,0 (0,0 – 0,0)
LCl	5,0 (25)	1,0 (1,0 – 1,0)	0,0 (0,0 – 0,0)	0,0 (0,0 – 0,0)
TOTAL	20,0 (100)			

Legends: Scores semiquantitative general disease processes in experimental models. Med = median; Vmin= minimum value; Vmax =maximum value. Significant values:1p=0.009; 2p =0.008; 3p =0.008, 52.

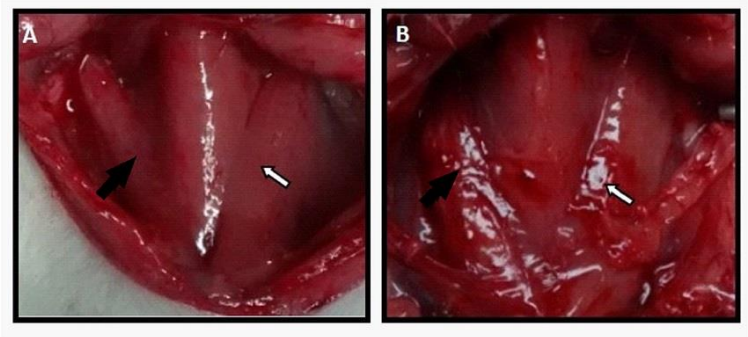

Figure 1.Ventral view of the right and left sternomastoid muscles. After 7 days of bupivacaine and chloride injections (A) CBupi (➡) and CCl (⇨); and applying LLLT, (B) LBupi (➡) and CCl (⇨).

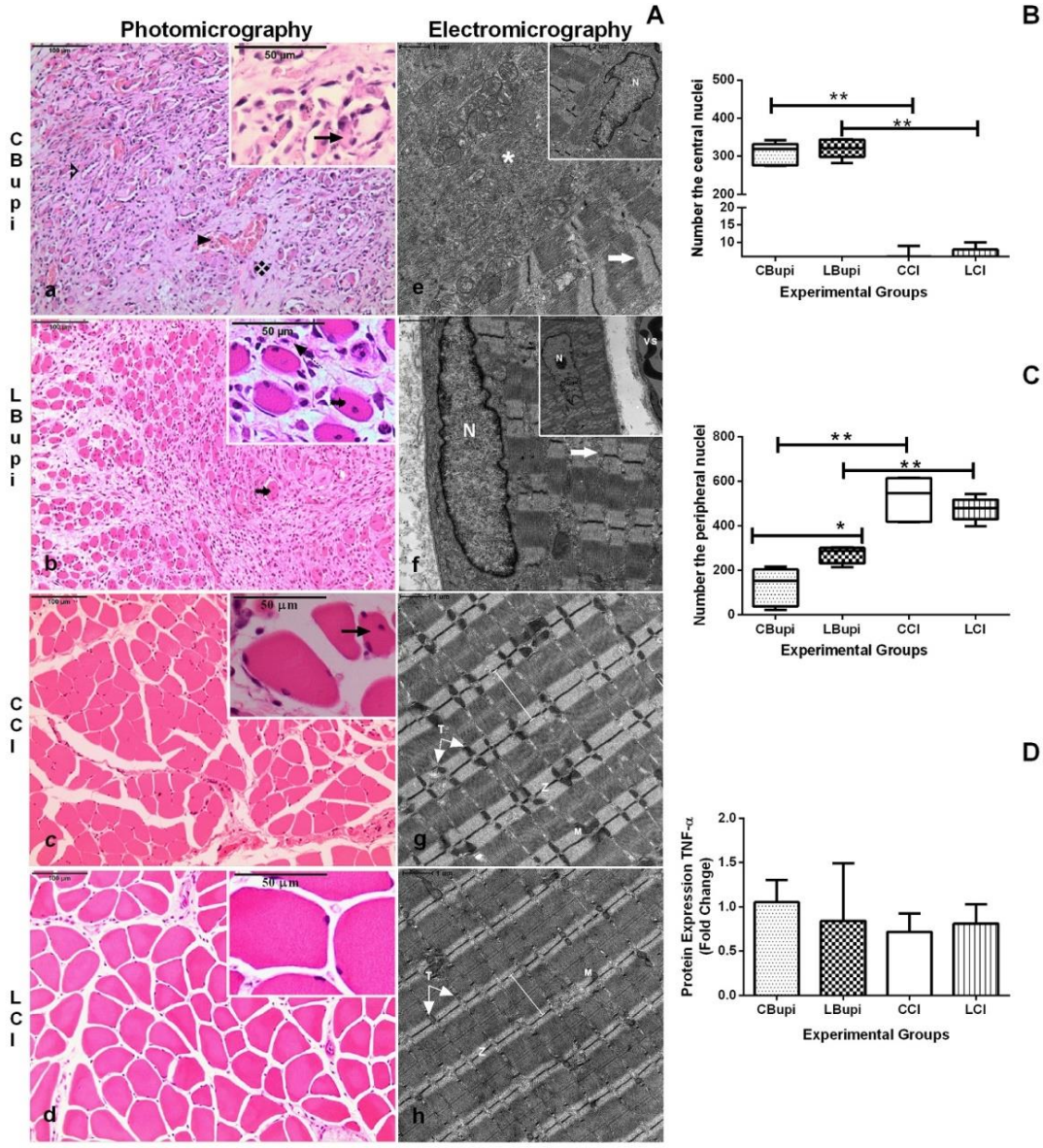

Figure 2. A- Photomicrography and electromicrography of cross sections of the sternomastoid muscle from the Control and Laser groups (HE). (a) The presence of mononuclear cell infiltration (➢), edema (❖), blood vessels with hyperemia (▶), muscle fibers in a degenerative process, necrotic and with total loss of polygonal characteristic (➞). (b) Muscle cells in the process of regeneration (➧), and fibroblasts (⇢). (c) Partial loss of the polygonal characteristic of the muscle fibers (➞). (d) Muscle fibers with preserved histological architecture. Electromicrography: Mitochondria (m), Z line (Z), triads (T), sarcomeres ([). e: areas in regeneration (*),Nuclei (N), areas of myonecrosis (*), normal myofibrils (white arrows). f:blood vessels (VS). B- Quantification of the number of central and peripheral nuclei in the subgroups, nonparametric analysis of variance for repeated measures model in the independent groups complemented with the Dunn test [53],* p<0.05; ** p< 0.01. C- Protein expression of TNFαthrough western blot of the CBupi, CCl, LBupi and LCL subgroups, analysis of variance complemented by the Bonferroni test [53].

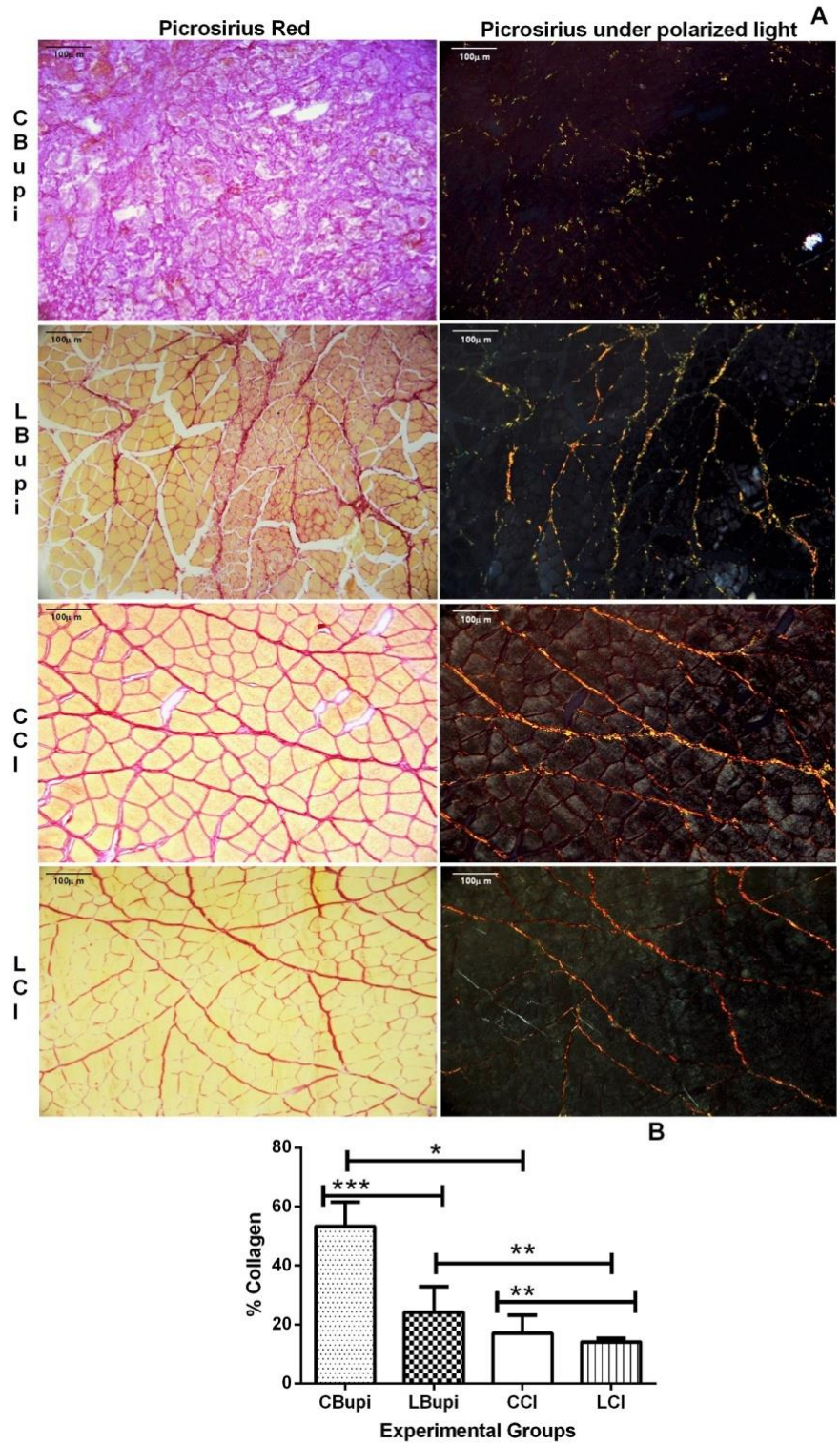

Figure 3. A- Photomicrographs of cross sections of the sternomastoid muscle (Picrosirius Red). B- Percentage of collagen area (analysis of variance, complemented by the Bonferroni test) [53].* p<0.05; ** p< 0.01;*** p< 0.001.

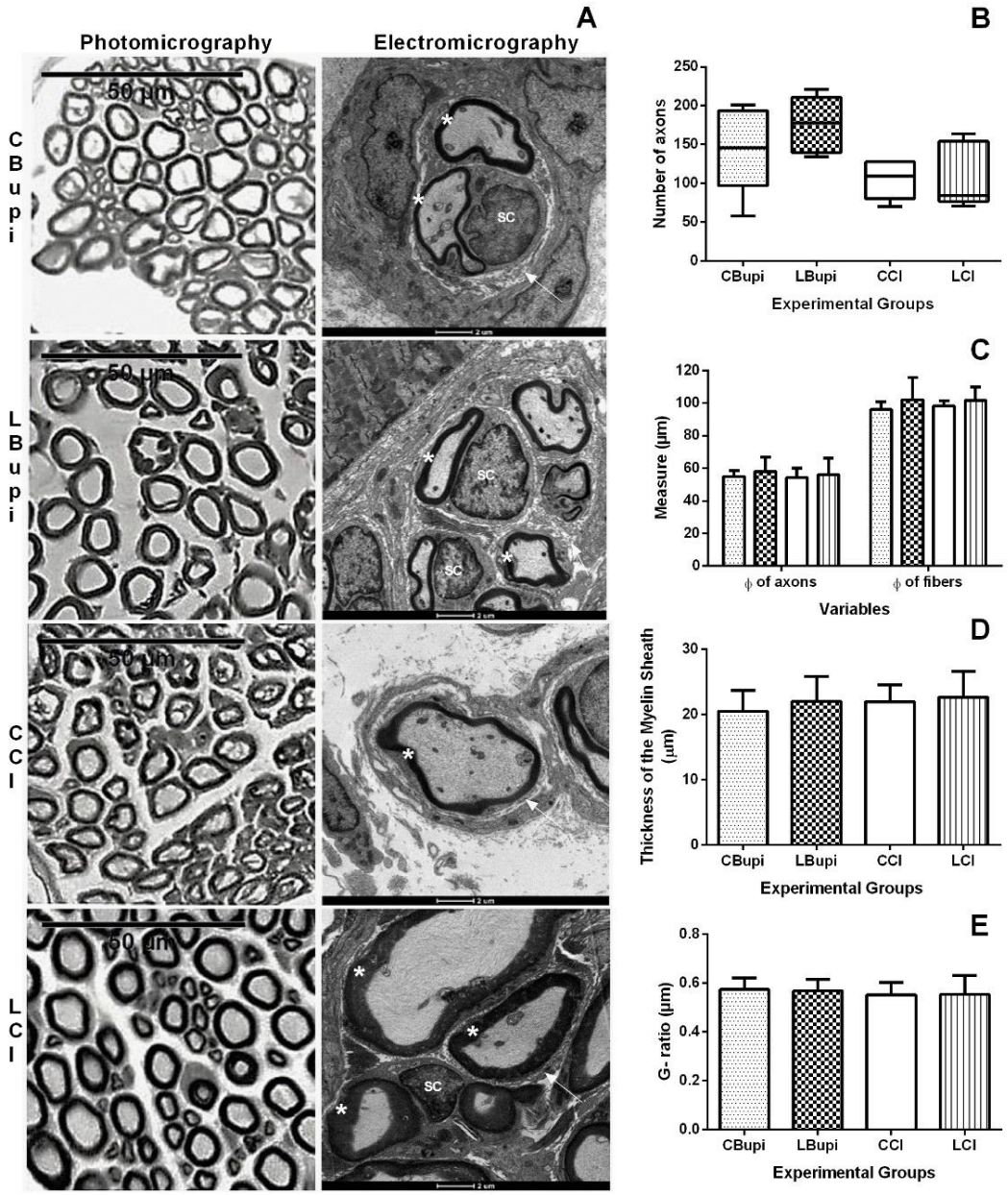

Figure 4. A- Photomicrographs of cross sections of the sternomastoid nerves . Schawnn Cell Nucleus (SC), myelin sheaths (*), endoneuium (arrow). Analysis of variance no parametric complemented by the Dunn tes[53]t(B), complemented by the Bonferroni test) [53]. Analysis of variance for two models factors complemented by the Tuckey test[53] (C,D,E.)

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Paper 2

GaAs laser therapy reestablishes the morphology of the NMJ and nAChRs after injury due to bupivacaine

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Highlights: LLLT reduces alterations in NMJ and in nAChRs triggered by bupivacaine.

Competing Interests

The authors declare no competing interests.

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Abstract

Background: Local anesthetics are used to relieve pre- and postoperative pain, acting on both sodium channels and nicotinic acetylcholine receptors (nAChR) at the neuromuscular junction (NMJ). Bupivacaine acts as a non-competitive antagonist and has limitations, such as myotoxicity, neurotoxicity, and inflammation. Low-level laser therapy (LLLT) has anti-inflammatory, regenerative, and analgesic effects.The aim of the present study was to evaluate the effects of a gallium arsenide laser (GaAs) on the morphology of the NMJ and nAChRs after application of bupivacaine in the sternomastoid muscle. **Methods:** Thirty-two adult male Wistar rats received injections of bupivacaine 0.5% (Bupi: right antimere) and 0.9% sodium chloride (Cl: left antimere). Next, the animals were divided into a Control group (C) and a Laser group (LLLT).The laser group received LLLT (GaAs 904nm, 50mW, 4,8J) in both antimeres for five consecutive days. After seven days, the animals were euthanized and the surface portion of the sternomastoid muscle was removed, frozen, and subjected to morphological and morphometric analyses of the NMJs (nonspecific esterase reaction), confocal laser scanning, and an ultrastructural analysis. The nAChRs were quantified by Western blotting. **Results:** In the chloride group, the morphology and morphometry of the NMJs remained stable. The maximum diameters of the NMJs were lower in the Bupi (15.048 ± 1.985) and LLLT/Bupi subgroups (15.456 ± 1.983) compared to the Cl (18.502 ± 2.058) and LLLT/Cl subgroups (19.356 ± 2.522) ($p < 0.05$).Ultrastructurally, LLLT reduced myonecrosis observed after application of bupivacaine, with recovery in the junctional folds and active zone. There was an increase in the perimeter of the LLLT/Bupi subgroup (150.33) compared to the Bupi subgroup (74.69) ($p < 0.01$) observed by confocal microscopy. There was also an increase in the relative planar area of the NMJ after LBI (8.75) compared to CBupi (4.80) ($p < 0.01$).An analysis of the protein expression of nAChRα1 showed no major differences in the groups studied. There was an increase in protein expression of the εsubunit after application of LLLT (13.055) compared to Bupi (0.251) ($p < 0.01$). Taken together,the present experiments indicate that there was a positive association of the $\alpha$and γsubunits ($p<0.05$). **Conclusions:** These results demonstrate that LLLT at the dose used in this study reduced structural alterations in the NMJ and molecular changes in nAChRs triggered by bupivacaine, providing important data supporting the use of LLLT in therapeutic protocols for injuries triggered by local anesthetics.

1. Introduction

Local anesthetics are chemical agents that act by reversibly blocking peripheral nerve conduction[1-3.]

These substances are widely used during medical and dental surgical procedures with the aim of achieving a safer and more comfortable procedure for patients[4-10], as well as the relief of postoperative pain. They are also used to treat chronic pain[11-16].

The structural and physicochemical characteristics of local anesthetics can be determining factors for their different effects[17]. These drugs act on both the voltage-dependent sodium channels (in axons) and nAChRs (acetylcholine receptors) of the NMJ[18-20]. The muscular acetylcholine receptor (AChR) is a heteropentamer composed of four polypeptide chains containing two alpha (α1), one beta (β), one delta (δ) and one gamma subunit (γ). The latter subunit, present in embryonic or denervated fibers, is replaced in adult muscle fibers and innervated by the epsilon subunit (ε). These five subunits are organized around a central ion-conducting pore that is permeable to cations[21].

NAChRs play a key role in the peripheral nervous system, are concentrated in the apex of the junctional folds of the postsynaptic membrane of the neuromuscular junction (NMJ)[22], and have been the target of interest for the development of new drugs, for example, for disorders of the peripheral nervous system[23-26]. There are numerous pharmacological substances that selectively alter the conformation of nAChRs[21]. Bupivacaine is a chemical substance that acts as a noncompetitive antagonist that can block the opening of the ion channel or an allosteric site of the nAChRs of the NMJ, inhibiting it, thus blocking muscle contraction[19]. This anesthetic is associated with adverse reactions leading to a rapid necrosis of muscle fibers[27-28], as well as alterations in the morphological and structural characteristics of the NMJs[28-29].

One of the principle obstacles to the development of new drugs is the presence of side effects. In this context, Low-level Laser Therapy (LLLT) has been shown to be a non-invasive clinical procedure that is easy to apply, low cost, lacks mutagenic effects, and risk-free[30-32]. In addition, it has anti-inflammatory, regenerative, and analgesic actions[33-36]. The electrophysiological effects of an LLLT 830 nm laser diode were examined on neuromuscular synaptic transmission and a reduction in ACh release at the physiological level was found [37]. This modulation of synaptic transmission may be related to the muscle relaxant effect of this type of laser. The therapeutic potential of LLLT was described in preventing an increase in nAChRs resulting from muscle atrophy after denervation[38].

Bupivacaine is highly lipophilic, and its neuro- and myotoxic effects are directly related to the uncoupling of oxidative phosphorylation, with inhibition of ATP synthesis of the mitochondrial respiratory chain[39]. However, there is evidence that LLLT increases the synthesis of ATP [39-40], with cytochrome *c* oxidase being a photo target[41-42]. LLLT also leads to a reciprocal increase in oxygen consumption[40].

Considering that bupivacaine is an anesthetic that is used on a large scale and that its action directly involves the components of muscle synapses, the purpose of this study was to investigate the possibility that LLLT might preserve or protect the morphology of the NMJ and nAChRs following apllication of bupivacaine.

2. Methodology

2.1- Animals

This study was approved by the Ethics Committee on Animal. Use of the Institute of Biosciences, UNESP, Botucatu (protocol no. 509-CEUA / IBB).

Thirty-two adult male Wistar rats, with an average weight of 300g, were obtained from the Central Animal Laboratory of Unesp in Botucatu. The animals were maintained in individual cages in a controlled environment, with feed and water provided *ad libitum*, and the room temperature maintained at 24 ± 2°C, with a 12 h:12 h photoperiod.

2.2- Experimental Protocol and Treatment Parameters

Initially, the animals were intraperitoneally (i.p.) anesthetized with ketamine/xylazine (90mg/kg and 10mg/kg). After trichotomy, a median incision was made on the ventral surface of the neck and the sternomastoid muscles were exposed. Subfascial injections of 0.5% bupivacaine and 0.9% sodium chloride were applied to the middle[43] and distal thirds of muscles pertaining to the right and left antimeres, respectively. This protocol followed the method used for induction of degeneration-regeneration of muscle fibers by local anesthetics[23,44-46].

Next, the skin was sutured with black Nylon no. 3.0 (Brasutura®). The animals were kept under heaters until they recovered; then, they received a single 100µl dose of sodium dipyrone solution.

Twenty-four hours after treatment, the animals were randomly divided into two groups, a Control group: right antimere (Bupi) and left antimere (Cl), and a Laser group: right antimere (LLLT/Bupi) and left antimere (LLLT/Cl).

A previously calibrated laser diode composed of gallium arsenide (GaAs) with a pulsed emission at904 nm (50mW)was applied directly to the skin (direct contact) with a beam emission area of 0.035 cm^2,corresponding to the areas of injections, in both antimeres, for five consecutive days. The pen was held at an angle 90° to the irradiated surface to prevent leakage of the emitted light. The applications lasted 48 seconds, with an energy of 2.4J per point, providing a total final energy of

4.8J. Twenty-four hours after the final laser application (day 7), the animals were euthanized by i.p. injection of excess anesthetic (ketamine/xylazine).

2.2.1- Morphological and Morphometric Analysis of the Neuromuscular Junction (NMJ) Based on the Nonspecific Esterase Technique

After euthanasia of the animals, the superficial portions of the sternomastoid muscles were trimmed to the middle third (containing the motor point), which was cut length wise into three or four slices. The resulting material was subjected to the nonspecific esterase reaction[47] to characterize the NMJ.

- Morphometric Analysis

Maximum diameter measurements were performed on 50 junctions from a total of five animals from each experimental group (Control and Laser). The measures were analyzed with the help of Image J software (http://rsbweb.nih.gov/ij/).

2.2.2- Morphological Analysis of the NMJ by Transmission Electron Microscopy (TEM) and Morphology and Morphometry Analyses by Confocal Microscopy

After anesthesia and trichotomy, the skin in the pectoral region was dissected and the sternal plastron was folded down. The thoracic viscera were exposed, and perfusion was performed via the left ventricle. First, the animals were perfused with PBS, followed by fixation with 2.0% paraformaldehyde in 0.1M sodium phosphate buffer, pH 7.4. The caudal vena cava was sectioned near the right atrium to drain blood and excess liquid from the perfusion.

- Transmission Electron Microscopy (TEM)

The muscular portion containing the motor point was reduced into fragments, which were then immersed in Karnovsky fixative solution and

subjected to the routine technique for TEM. The tissues were sliced so that longitudinal sections of muscles were obtained to allow identification of the NMJ. Ultrafine slices were obtained and examined, and the findings were documented using a Philips transmission electron microscope (model CM100 FEI).

- Confocal Microscopy

The muscles were reduced in the region containing the motor point, fixed with 2% paraformaldehyde and subjected to a protocol to mark acetylcholine receptors and nerve terminals. The fragments were kept in fixative for 15 minutes and then washed with PBS three times for 5 min each to inactivate the fixative. Next, they were incubated with 0.1M glycine for 30 min on an orbital shaker and subsequently washed with PBS and incubated with 1% collagenase (Sigma Type I C-0130) for 30 min on the shaker to assist with connective tissue removal. Following this, they were washed with PBS, and the nAChRs were labeled with α-bungarotoxin conjugated to rhodamine (Rh-BTX,Molecular Probes T1175,1: 1000 in PBS) for 40 min on the shaker. Then, the muscles were washed with PBS four times (for 5 min per wash) and incubated with 1% Triton X-100 (Sigma T9284) for 1 hour to permeabilize the muscle fibers. Subsequently, the samples were left overnight in blocking solution (60µl of Triton X-100, 0.3 g of 3% bovine serum albumin, 0.2 g 2% skimmed milk powder, 3.75mg glycine (1%), 800µl 8% fetal bovine serum, diluted in 10 ml PBS) on the shaker to block or decrease nonspecific binding of the primary antibody. After this period, the fragments were incubated with the primaryanti-neurofilament antibody (Ab) (Anti-neurofilament 200, Sigma N-5389, 1µl:1 ml in blocking solution) at 4°C for 12 hours. On the following day, they were washed with PBS (changed every 20 min for 1 hour and 30 minutes) and incubated with secondary anti-mouse-IgG-FITC (Sigma® F-0257, 1µl:1 ml in blocking solution) for three hours at 4°C on the shaker. Then,

the samples were washed again in PBS for one hour and mounted on slides under a cover slip in Vectashield® mounting medium for fluorescence staining with DAPI (Vector Laboratories).

The images were acquired using a Laser Scanning Confocal Microscope (Leica TCS model - SP5) belonging to the Electron Microscopy Center of IBB/UNESP/Botucatu and were analyzed using Image J software (http://rsbweb.nih.gov/ij/).

Morphological analysis was performed on the nerve terminals and acetylcholine receptors, and morphometric analysis was performed on approximately 30 axon terminals from five animals from each experimental subgroup. Measurements were taken of the area, perimeter, largest orthogonal axis of each nAChR and relative planar area (area of the nAChR/largest orthogonal axis of each nAChR)[48].

2.2.3- Western Blotting

After euthanasia, samples from the middle third of the muscles were frozen and then homogenized in tissue homogenizer (IKA UltraTurrax/T-25) with 0.5mL of lysis buffer (1% Triton X-100, 10 mM sodium pyrophosphate, 100mM sodium fluoride, aprotinin, 10 µg/ml,1 mM PMSF,0.25 mM sodium orthovanadate (Na_3VO_4),150 mM NaCl and 50 mM Tris-HClat pH 7.5). The samples were centrifuged at 11,000 rpm for 20 min, and the supernatant was collected. One 100-µL aliquot of the homogenate was treated with 100 µL of Laemmli sample buffer (2% SDS, 20% glycerol, 0.04 mg/mL bromophenol blue,0.12 MTris-HCl at pH 6.8 and 0.28M β-mercaptoethanol).The samples were then incubated at 97°C for five minutes and stored in a freezer at -20°C until use.

One aliquot of the pure extract of each sample (not treated with Laemmli) was used to quantify the total protein by the Bradford method [49]. Exact quantities of the total protein in each sample (70µg) were subjected

to electrophoresis in 4-15% polyacrylamide gels (SDS-PAGE) and then transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA) in a humidified system. The proteins transferred to membranes were blocked with 5% skimmed milk diluted in TBS-Tween for 1h at room temperature and incubated with different primary antibodies (AChRα1 (Santa Cruz Biotechnology - sc-136130) 1:100; AChRγ (Santa Cruz Biotechnology - sc-13998) 1:150; AChRε (Santa Cruz Biotechnology- sc-13999) 1:150; GAPDH (Cell Signaling - 14C10)1:1000, *overnight* at 4ºC. After incubation with primary antibodies, the membranes were washed in TBS-Tween and incubated with specific secondary antibodies (anti-rabbit (Cell Signaling - 7074s) 1:5000 or anti-mouse (Santa Cruz Biotechnology - sc-2005) 1:2000) for 1h at room temperature. The membranes were washed again, and the *ECL™ Selected Western Blotting Detection Reagent* (GE Healthcare, Uppsala, Sweden) was used. After image capture in a transilluminator *G-Box*, densitometric quantification of the bands was performed using Image J software (version 1.71, 2006, Austria). The protein expression values were normalized to the values obtained for the GAPDH protein, which were used as a reference.

3. Results

3.1- Neuromuscular junction: morphology and morphometry

The nonspecific esterase technique allowed for visualization of the distribution of NMJs in the surface portion of the sternomastoid muscle. NMJs are characterized by a wide and highly branched synaptic gutter, with transverse striations corresponding to junctional folds (Figure 1Ac). Some forms have fewer branches and small terminal projections. In this analysis, there were clear differences in the size and appearance of the NMJs in the Cl subgroup (Figure 1Ac) compared to those in the Bupi subgroup (Figure 1Aa). The Cl and LLLT/Cl subgroups presented greater

complexity of the sites marked by the nonspecific esterase technique.

Morphometrically, there were no significant differences in the NMJs between the Bupi and LLLT/Bupi subgroups or between the Cl and LLLT/Cl subgroups. However, the maximum diameters were smaller in the Bupi (15.048 ± 1.985) and LLLT/Bupi subgroups (15.456 ± 1.983) compared to the Cl (18.502 ± 2.058) and LLLT/Cl subgroups (19.356 ± 2.522) ($p < 0.05$) (Figure 1C).

The ultrastructure of the neuromuscular junctions in the Cl subgroup presented standard morphological characteristics, with several synaptic buttons, and were arranged in synaptic gutters with varied shapes and depths. Synaptic vesicles, mitochondria, and multivesicular bodies were present in the synaptic buttons. Characteristically, the presynaptic membrane presented more electron-dense regions corresponding to active zones, counter posed to the apex of the junctional folds of the postsynaptic membrane. The postsynaptic membrane was intact and contained numerous and long junctional folds, containing a more electron-dense apex corresponding to the local concentration of the acetylcholine receptors. These morphological patterns were also observed in the LLLT/Cl subgroup (Figure 1B).

In the Bupi subgroup, the synaptic vesicles and other organelles present in the synaptic buttons exhibited different stages of degeneration, and it was not possible to identify the region of the active zones. Junctional folds were not evident in the postsynaptic membrane, and the electron density was observed along the entire length of the postsynaptic membrane. In the LLLT/Bupi subgroup, although signs of degeneration of the organelles of the synaptic buttons were still observed, junctional folds were present with electron dense areas along them, primarily at the apex.

The synaptic clefts in the groups studied presented no morphological changes and displayed a basal lamina with normal

characteristics in the interior (Figure 1B).

3.2- Confocal Microscopy

In the CI subgroup, the acetylcholine receptors and nerve terminals showed a normal distribution, where the responses of the fluorophores were homogeneous and the nAChRs had auto fluorescence.

The analysis revealed a large number of nuclei (blue) present in the laser group (Figures 2Ab and 2Ad) compared to the control group (Figures 2Aa and 2Ac). With regard to the nerve terminals (green), no alterations were observed among the groups, and the nerve terminal were intact, preserved, and seamless in all subgroups.

In relation to the acetylcholine receptors (red), the chromogen dispersion was clear in the Bupi subgroup, demonstrating discontinuous marking. In the LLLT/Bupi subgroup, there was less dispersion of chromogen, and in the LLLT/Cl subgroup, there was greater complexity, with nAChR staining.

In the CI subgroup, the NMJs were characterized by the presence of continuous arms that ran in various directions along the muscle fibers. After application of laser therapy, the presence of larger and longer branches was observed in the LLLT/Cl subgroup.

Regarding the morphometry performed in the area of the nAChRs of the motor plate, the area in the Cl (759.43) and LLLT/Cl subgroups (803.70) was higher than that of the Bupi (76.30) and LLLT/Bupi subgroups (186.30) ($p < 0.01$) (Figure 2B).This physiological response was also observed in the morphology of the perimeter. However, the LLLT/Bupi subgroup (150.33) had significantly higher values compared to the Bupi subgroup (74.69) ($p < 0.01$).The relative planar area of the NMJ was significantly higher in the LLLT/Bupi subgroup (8.75) compared to the CBupi subgroup (4.80) ($p < 0.01$) (Figure 2B).

The percentage of nerve terminals present in the experimental

groups was not evaluated.

3.3- Western Blotting

The analysis of nAChRα1protein expression showed that the values were not significantly different between the control and laser groups, maintaining similar values in all experimental subgroups (Figure 2E). There was a decrease in the protein expression of nAChRγ in the LLLT/Cl subgroup compared to the CI subgroup ($p < 0.05$) (Figure 2D).There was no increase in the protein expression of nAChRε in the LBI/Bupi subgroup compared to the Bupi subgroup ($p < 0.01$). However, in the LLLT/CI subgroup, the values were lower compared to those in the LLLT/Bupi subgroup ($p < 0.01$) (Figure 2C).

When all of the experimental groups were considered together (control and laser) for all nAChR subunits, there was a positive association of subunits α and γ ($p < 0.05$). It was not possible to demonstrate significance when the groups were considered separately; however, it is clear that the association remained positive in the laser group (high values in both the α and γ subunits). The control group showed a negative relationship (high values for subunit γ occurring in conjunction with low values for subunit α and vice versa).In summary, there was a direct relationship between subunits α and γ in the laser group, and the reverse relationship was found for the control group (Figure 3).

4- Discussion

The aim of this study was to investigate the effects of LLLT on the morphology of the NMJ and nAChRs after the induction of myonecrosis by the local anesthetic bupivacaine. The results point to a positive effect of

laser therapy on the structural recovery of the NMJs and molecular recovery of nAChRs.

Based on the morphometry of the NMJs determined by the nonspecific esterase technique, there was a clear decrease in the maximum diameter of NMJs associated with the muscles injected with bupivacaine. Ultrastructurally, signs of degeneration were present in the pre- and postsynaptic regions, with characterization of the active zone and loss of junctional folds in the Bupi subgroup. Disaggregation of nAChRs was detected by confocal microscopy.

Significant changes in the ultrastructural architecture of the NMJs was also observed four hours after bupivacaine injection, with an increase in the space of the synaptic cleft, loss of junctional folds, and discontinuity or absence of post-junctional plasmatic membranes[28]. Disaggregation of the nAChR (an "islands" pattern) was present in the regenerated fibers of mdx mice[24,52].

In a study on the effects of another local anesthetic, lidocaine, the authors observed the same morphology 21 days after application of the anesthetic[23]. A similar alteration was observed in mdx mice, which was described how a secondary consequence of the regeneration of muscle fibers and not the absence of dystrophin[24].

This postsynaptic disaggregation in the Bupi subgroup was not accompanied by alterations in protein expression of the γ or α1 receptors, but there was a decrease in the expression of the nAChRε subunit. A decrease in this subunit has been associated with structural destabilization of the NMJ. Severe muscle weakness and premature death were observed in experimental animals in the absence or deficiency of nAChRε[25]. A decrease in the gene expression of the epsilon subunit has been associated with a loss in ERK2 (extracellular signal-regulated kinase), which is involved in the activation of agrin, a key synaptogenic factor

involved in the formation and maintenance of the NMJ[53]. Furthermore, the area, perimeter, and relative planer area determined by confocal microscopy were smaller in the Bupi subgroup. These data are consistent with the degenerative effects of the local anesthetic on NMJs.

Regarding the nerve terminals (green chromogen), no significant alterations were evident in relation to the experimental group, with all groups showing whole and preserved nerve terminals, without signs of discontinuity. Although confocal microscopy was not used in their studies, some researchers have been evaluated the nerve terminals after the application of bupivacaine and did not observe any alterations[27-29].

It is possible that the myonecrosis arising from bupivacaine promotes sarcolemmal injury, allowing excessive entry of calcium. This ion leads to hypercontraction of myofibrils[27,54,55], increasing the quantity of intracellular free radicals due to the generation of oxidative stress, culminating in mitochondrial degeneration[39]. Considering that proteins in the synaptic region are transmembranic, alterations in the plasma membrane of the fiber and its sarcoplasm directly affect the muscle synapse[21], such that alterations in the NMJs can be considered responses to the pathological process of myonecrosis[29].

After LLLT application, there was an increase in the relative planar area of the NMJ. Considering that this measurement refers to the degree of branching and fragmentation of the NMJ, indicating its complexity index, it is clear that there was recovery in the NMJ after LLLT, due to the fact that a higher relative planar area indicates lower fragmentation of the NMJ, and vice versa[48].

There was also a clear approximation of the ultrastructural morphological aspect of the NMJ in the LLLT/CI and LBI/Bupi groups, with the active zones showing a clear orientation of synaptic vesicles and increase in the number of junctional folds. A loss or reduction of the

number of junctional folds is characteristic of degenerate fibers, demonstrating that muscle regeneration leads to remodeling of pre- and postsynaptic components[23,56].

Even in the initial stages of regeneration, the active zone is present in specific regions of the presynaptic membrane as opposed to the junctional folds. The active zone is a neurotransmitter release site and is disorganized during the regeneration process, facilitating exocytosis from synaptic vesicles, releasing neurotransmitters[57].

Changes in junctional folds can lead to a loss of nAChRs and remodeling of the NMJ[58]. The efficiency of electrical transmission is enhanced by the formulation of the junctional folds and by the expression of adult AChRs and voltage-dependent sodium channels[59]. Regeneration of the NMJ follows the steps of myogenesis, with the process including the formation of new junctional folds [28].

In our confocal microscopy study, a lower dispersion of nAChRs (red chromogens) was evident in the groups that received LLLT, suggesting that there was a stabilization of nAChRs. The stabilization of nAChRs is dependent on the calcium influx during electrical activity[60-61].

After laser therapy, there was also a significant increase in protein expression of the ε receptor. The ε subunit is associated with a small opening of nAChR ion channels, regulating the influx of calcium, thus ensuring the stabilization of the initial interaction between nerve and muscle and synaptic maturation[25,62-63]. It is an essential subunit that is required for the maintenance of the organization and protection of the synaptic region against adverse effects resulting from excessive intake of calcium in adult muscle tissue[25,63].

When the subgroups are considered together, there was a strong positive association between the $\alpha 1$ and γ subunits. However, the α and γ subunits are regulated independently, and an increase in the $\alpha 1$

subunit is not directly associated with alterations in the γ and ε subunits [25].

In experiments in which subunit γ was replaced by a subunit with similar functional properties to the ε subunit, a pattern of altered muscle innervation was present, suggesting that during muscle development, nAChRγ ensures an orderly innervation pattern for skeletal muscle[64]. The expression of fetal nAChR is crucial to maintain the neuromuscular transmission in individuals with deficiencies caused by alterations in the nAChRε subunit and is associated with upregulation of the mRNA expression of the γ subunit [25].

The use of LLLT increases neovascularization[65] and the expression of growth factors[66] and decreases oxidative stress, inflammation[67] and myonecrosis, activating precursor cells of myogenesis (satellite cells)[32]. Studies involving muscle regeneration of elderly rats undergoing laser therapy showed an increase in the maturation of satellite cells into myoblasts and myotubes[68]. In the same way, the confocal microscopy experiment in the present study revealed a large number of nuclei (blue) in the group that received laser therapy. The marker used for the nucleus was not able to differentiate among cells, so the nuclei may have corresponded to inflammatory cells and muscle fibers or may have even indicated an increased number of satellite cells.

Considering that the muscle regeneration capacity primarily depends on the survival of satellite cells[69], it is well established that these cells are resistant to local anesthetics[70]. In addition, following injury, the muscles and NMJs pass through a regeneration process, which is partly regulated by myogenic regulatory factors (MRFs)[71]. MRFs are also targets of the laser, which promotes muscle regeneration[72], accelerating the processes of proliferation and differentiation[32,66].

During the remodeling of denervated/regenerated fibers, type IV collagen and non-neural agrin contribute to a new conformation of the pre- and postsynaptic components. This new distribution follows a pretzel or continuous arm pattern that is characteristic of the adult NMJ (mature). Therefore, the nerve terminal influences the spatial organization of the nAChRs. Thus, it can be inferred that changes in the distribution pattern of nAChRs are part of the processes of degeneration and regeneration of the muscle fiber[73].

It is well established that the mitochondrial electron transport chain is photosensitive to red and infrared light, and when activated by LLLT, there is an increase in the space between the inner and outer membranes of the mitochondria, which dilates the mitochondrial crest[74]. Exposure to laser light also increases the synthesis of adenosine triphosphate (ATP) and O_2 consumption[41].

Cytochrome *c* oxidase (complex IV of the mitochondrial respiratory chain) is a key light photoreceptor with a near-infrared spectral range. After application of laser therapy with GaAs (904nm), the activity levels of complexes I, II, III and IV of the mitochondrial respiratory chain were increased in the injured muscle[75].

Biostimulation produces primary effects during cell proliferation in the muscle healing process. At the cellular level, low power photo-irradiation promotes significant biological effects, such as stimulation of macrophages and lymphocytes and release of growth factors from cells[76].

The presence of myopathy induced by bupivacaine used for nerve blocks and other local anesthetic injections has been increasingly described[43,77-78]. However, the available literature suggests that the myotoxicity that developed after local anesthetic use is subclinical and reversible, most likely due to the short exposure time[77-81]. Continuous infusion pumps are now being used for pain, and it is still unknown whether

local anesthetics may cause more significant (i.e., irreversible) muscle injury in these individuals due to the prolonged exposure time[82].

5. Conclusion

Considering that LLLT in the dose used in the present study reduced the structural alterations of the NMJ and molecular alterations of nAChRs triggered by bupivacaine, this approach appears to represent a therapeutic protocol that may be indicated after injuries triggered by exposure to local anesthetics. Further studies should be conducted to fully clarify the mechanism(s) of action.

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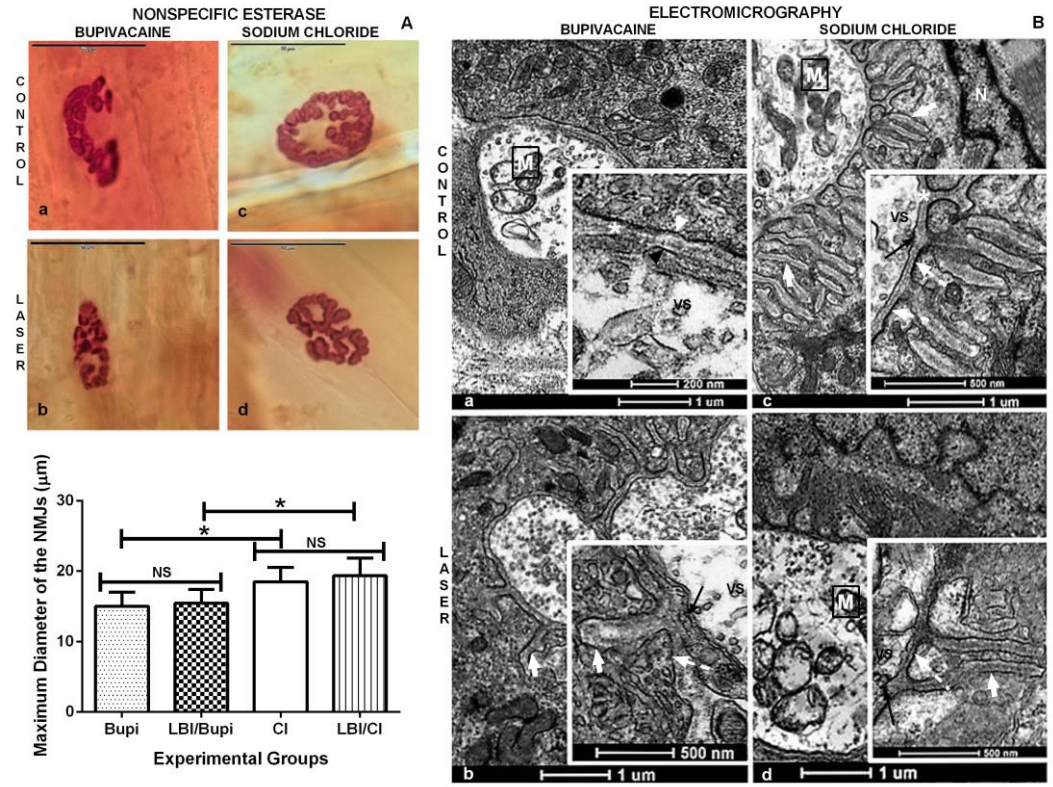

Figure1. **A-** Light microscopy findings following the nonspecific esterase reaction (Full preparation) **B-** Electromicrography of the NMJs of the various groups: (→) active zone; (▸) presynaptic membrane; (*) synaptic cleft; (▷) postsynaptic membrane; (⇨) junctional folds; (⇪) apex of a junctional fold; (M) mitochondria (vs) synaptic vesicles, (N) nucleus **C-** The maximum diameter (µm) of the NMJs of the subgroups studied. The significance of differences was determined using analysis of variance for the model with two factors, together with the multiple comparison Tukey test, considering a 5% level of significance, (*) $p<0.05$[50].

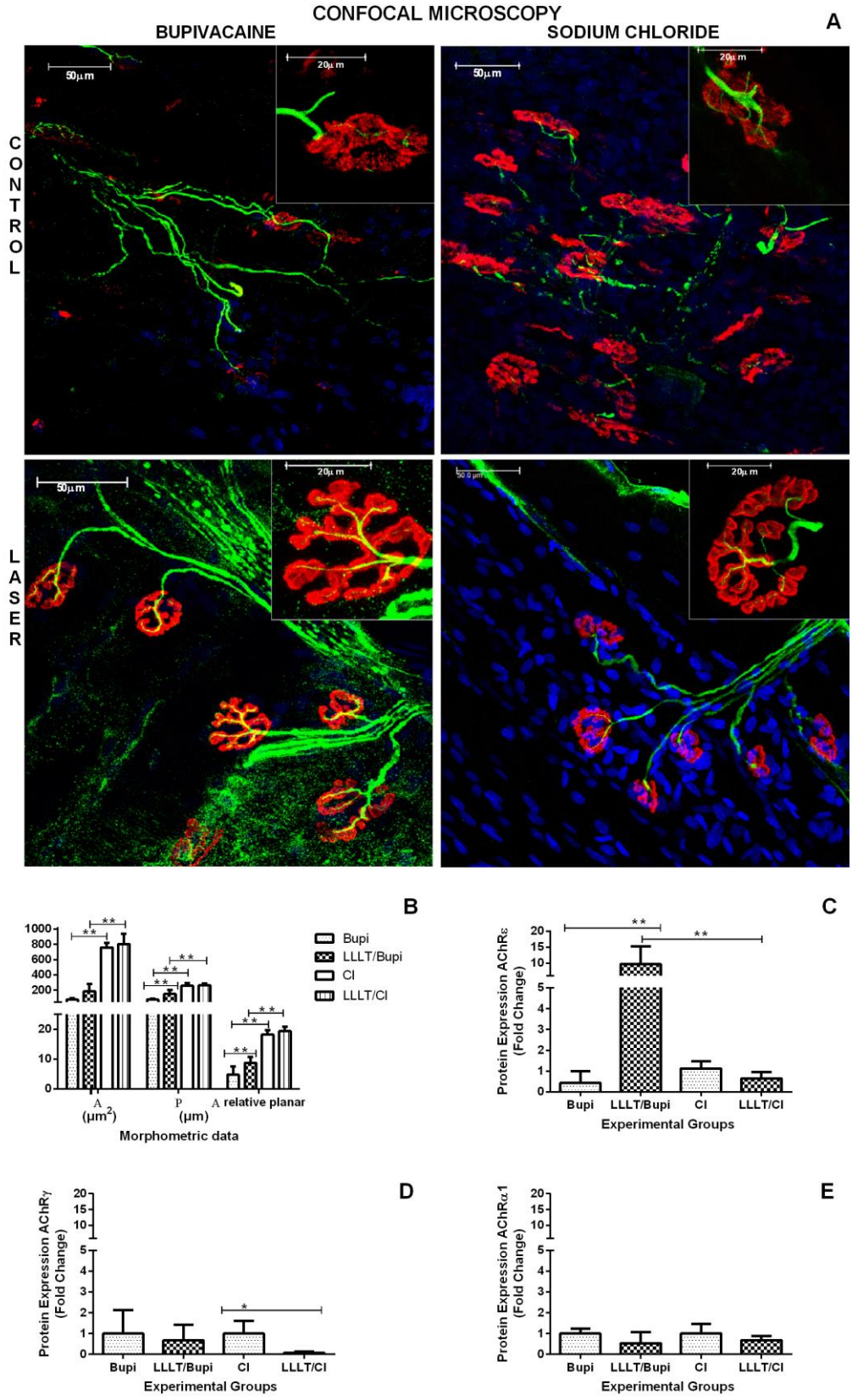
CONFOCAL MICROSCOPY
A
BUPIVACAINE
SODIUM CHLORIDE
CONTROL
LASER
50µm
20µm
B
Bupi
LLLT/Bupi
CI
LLLT/CI
A (µm²)
P (µm)
A relative planar
Morphometric data
C
Protein Expression AChRε (Fold Change)
Bupi
LLLT/Bupi
CI
LLLT/CI
Experimental Groups
D
Protein Expression AChRγ (Fold Change)
Experimental Groups
E
Protein Expression AChRα1 (Fold Change)
Experimental Groups

Figure 2. A- Confocal microscopy findingsof the surface portion of the sternomastoid muscle of the experimental group. The acetylcholine receptors (red), nerve terminals (green), and nuclei (blue) are shown. B- Morphometry of the area (A), perimeter (P), and relative planar area.The significance of differences was analyzed using an analysis of variance for repeated measures model for independent groups complemented with the Bonferroni test (**) p< 0.01,[50]. The protein expression of nicotinic acetylcholine receptors AChRε (C), AChRγ (D), AChRα1 (E). The significance of differences was determined using nonparametric analysis of variance for repeated measures model for independent groups complemented with the Dunn test[50]. (*) p<0.05, and (**) p<0.01.

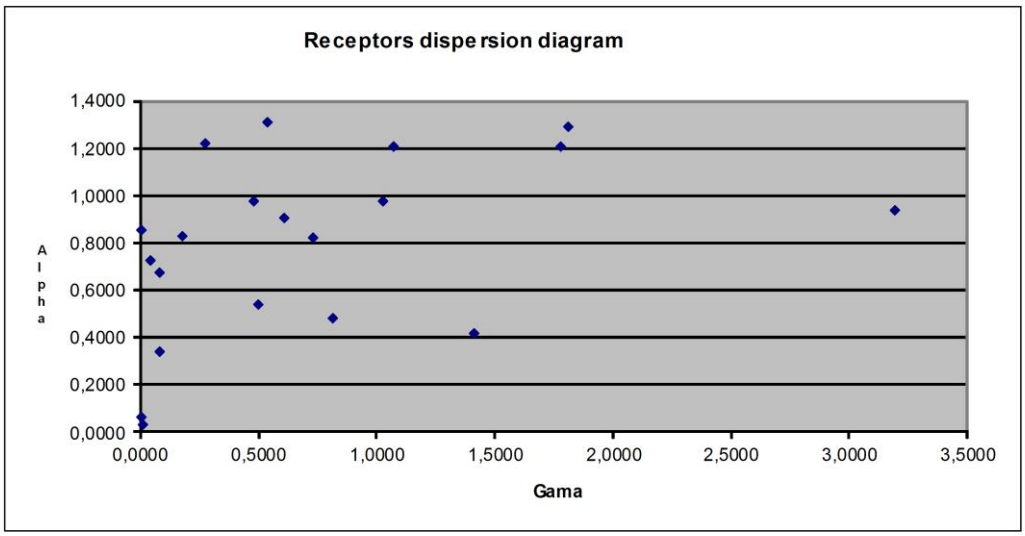

Figure 3. Dispersion data for the α and γ subunits of the nAChRs. The data were analyzed using the Spearman correlation[51].

Anexo I

unesp UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" Campus de Botucatu

Instituto de Biociências de Botucatu - Unesp

Certificado

Certificamos que o Protocolo nº **509-CEUA**, sobre "Avaliação da miotoxicidade e neurotoxicidade (com foco na junção neuromuscular) após aplicação de bupivacaína seguida de laser terapia", sob a responsabilidade de **Selma Maria Michelin Matheus**, está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Colégio Brasileiro de Experimentação Animal (COBEA) e foi aprovado "*Ad referendum*" da ***COMISSÃO DE ÉTICA NO USO DE ANIMAIS*** (CEUA), nesta data.

Botucatu, 26 de agosto de 2013.

Prof. Dr. Wellerson Rodrigo Scarano
Presidente da CEUA

Biociências – Diretoria Técnica Acadêmica