UNIVERSIDADE FEDERAL DE SÃO CARLOS

CENTRO DE CIÊNCIAS BIOLÓGICAS E DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM FISIOTERAPIA

ADAPTAÇÕES NEUROMUSCULARES E FUNCIONAIS EM DECORRÊNCIA DA ISQUEMIA CEREBRAL E DO NÃO USO APRENDIDO

FERNANDA MARIA FATURI

SÃO CARLOS

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RESUMO

Uma alta porcentagem (50% a 70%) dos indivíduos pós-Acidente Vascular Cerebral (AVC) apresentam perda de função do membro superior (MS). A hemiparesia que ocorre após o AVC gera inúmeras alterações tais como, atrofia e fraqueza muscular prejudicando a função e as atividades de vida diária destes indivíduos. Estas alterações podem estar relacionadas ao sistema nervoso central (SNC) e também ao musculoesquelético. Fatores como o não uso aprendido podem também auxiliar nas mudanças tanto neurais como musculoesqueléticas, gerando um ciclo mal adaptativo e exacerbando a disfunção do membro. Embora se conheça sobre as adaptações neurais, pouco é conhecido sobre as musculoesqueléticas principalmente em membros superiores. Grande parte dos estudos avaliam membro inferior e a heterogeneidade de comorbidades encontradas nestes pacientes dificultam as conclusões dos resultados. **Objetivos:** Os dois estudos tiveram por objetivo avaliar as alterações neuromusculares e funcionais no membro superior após o AVC. **Métodos:** No estudo 1 foi realizada uma revisão sistemática com busca em Dezembro de 2017 utilizando as bases Medline, PubMed, Scopus, Cinahl e Web of Science. Foram investigados estudos que realizaram análise de exame de imagem no intuito de avaliar adaptações musculoesqueléticas no MS após o AVC. No estudo 2 foi realizado um modelo de lesão isquêmica induzida por endotelina-1, bem como treino de alcance da pata anterior menos afetada de ratos. Foram realizados testes de desempenho funcional e análises morfométricas dos músculos da pata dianteira parética. **Resultados:** Para a revisão sistemática foram incluídos 7 estudos com uma ampla variedade de músculos distais avaliados, que obtiveram redução na área de secção transversa, densidade, comprimento de fascículo e aumento de ângulo de penação do músculo e variáveis responsáveis pela quantidade de tecido conjuntivo do lado parético comparado ao não parético. No estudo 2 a isquemia induzida por endotelina-1 prejudicou o desempenho do membro anterior parético durante a tarefa de alcance sem alteração musculoesquelética. Treino do membro anterior não parético acentuou o não uso aprendido e induziu atrofia dos extensores dos dedos no membro parético. **Conclusão:** estudo 1 mostrou evidência de qualidades moderada com alterações musculoesqueléticas em membro parético após o AVC, porém as comparações do lado parético e não parético podem ser inapropriadas e induzir a erros, assim estudos bem desenhados abordando esta questão são necessários. O estudo 2, concluiu que lesão isquêmica induzida por endotelina-1 causa disfunção das ´pata anterior de rato sem alteração muscular e recuperação tardia da função é associado a movimentos compensatórios, porém, sem atrofia. Treino do membro não parético prejudica a recuperação e causa atrofia seletiva do membro parético.

Palavras-chaves: Acidente vascular cerebral, isquemia cerebral, reabilitação, extremidade superior, paresia, não uso aprendido, alteração musculoesquelética, atrofia.

ABSTRACT

A high percentage (50% to 70%) of post-stroke patients have loss of upper limb function (MS). The hemiparesis that occurs after stroke generates a range of alterations such as atrophy and muscle weakness impairing the function and daily life activities of these individuals. These changes may be related to the central nervous system (CNS) and also to skeletal muscle. Factors such as learned non-use may also aid in both neural and musculoskeletal changes leading to a maladaptive cycle and exacerbating limb function. Although it is known about the neural adaptations, little is known about the musculoskeletal mainly in upper limbs. A large part of the studies evaluate lower limb and the heterogeneity of comorbidities found in these patients hinder the conclusions of the results. Thus, the thesis studies were performed in order to respond if there is a musculoskeletal alteration after the stroke and if through learned non-use we can generate a model of atrophy or worsen functional deficits and musculoskeletal atrophy. **Objectives**: Both studies aimed to evaluate the neuromuscular and functional alterations after stroke. **Methods:** In study 1 a systematic review was conducted with search in December 2017 using the Medline, PubMed, Scopus, Cinahl and Web of Science databases. We investigated studies that performed image analysis in order to evaluate musculoskeletal adaptations after stroke. In study 2, a model of ischemic injury induced by endothelin-1 was performed, as well as training of the reach of the less affected forelimb of rats. Functional performance tests and morphometric analysis of the muscles were performed. **Results:** For the systematic review we included 7 studies with a wide variety of distal muscles evaluated, which obtained a reduction in the cross-sectional area, density, fascicle length and increase of pennation angle and variables responsible by the amount of connective tissue on the paretic side compared to non-paretic. In study 2, endothelin-1 induced ischemia impaired the paretic upper limb performance during the reach task without musculoskeletal alteration. Training of the non-paretic upper limb accentuated the learning non-use and induced atrophy of the finger extensors in the paretic upper limb. **Conclusion:** study 1 showed studies with poor to fair quality of evidence showing musculoskeletal changes in a paretic limb after stroke, however, the paretic and non-paretic side comparisons may be inappropriate and error-inducing, so well-designed studies addressing this issue are necessary. In study 2, it was concluded that ischemic injury induced by endothelin-1 causes dysfunction of the forelimbs without muscular alteration and late recovery of function is associated with compensatory movements and without atrophy. Non-paretic upper limb training impairs the recovery of the paretic limb and causes selective atrophy of the paretic side.

Key words: Stroke, cerebral ischemia, rehabilitation, upper extremity, paresis, learning non-use, skeletal muscle alteration, atrophy.

LISTA DE FIGURAS

endothelin-1 15 days (ET-1 15d) groups. No differences were observed between groups for any muscle ($p>0.05$). 61

Figure 3. Percentage of successful on single-pellet retrieved task. *$p<0.05$ when ET-1 15d and ET-1 15d + T are compared to their own values on day -1 (pre) and also to control group; †$p<0.05$ compare to ET-1 15d + T; #$p<0.05$ when ET-1 15d compared to its values on day 3; ‡$p<0.05$ when ET-1 15d + T is compared to control. 62

Figure 4. Minor muscle fiber diameter of control, endothelin-1 15 days (ET-1 15d) and endothelin-1 15 days and non-paretic forelimb training (ET-1 15d + T) groups. *$p<0.05$ compared to ET-1 15d. No differences were observed between groups for biceps, triceps and fingers flexors muscles ($p>0.05$). However, nonparetic limb training reduced muscle fiber diameter on fingers extensors muscles. 63

Figure 5S. Muscle fiber cross-sectional area distribution. An increase in the percentage of muscle fibers of ET-1 15d + T around 50 and 100 extracts compared to ET-1 15d can be observed. 64

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

AVC – Acidente vascular cerebral

AVDs - Atividades de vida diária

B - Brachialis

BB - Biceps brachii

CNS - Central nervous system

CSA - Cross-sectional area

CT - Computerized tomography

DEXA - Dual Energy X-Ray Absorptiometry

EDB - Extensor digitorum brevis

EDC - Extensor digitorum communis

EI - Extensor indicis

ET-1 - Endothelin-1

ET-1 15d - Endothelin-1 15 days

ET-1 15d + T - Endothelin-1 for 15 days and non-paretic forelimb training

ET-1 4d - Endothelin-1 4 days

FDI - First dorsal interosseous

FDP - Flexor digitorum profundus

FDS - Flexor digitorum superficialis

FFA - Forearm flexors

FIM - Functional Independence Measure

FMA - Fugl-Meyer Assessment

FPI - First palmar interosseous

LaFiN - Laboratório de Pesquisa em Fisioterapia Neurológica

LUM - Lumbrical

M - Man

M1 - Primary motor cortex area

M2 - Secondary motor cortex area

MAS - Modified Ashworth Scale

MCAO - Middle cerebral artery occlusion

MI - Motricity index

MRI - Magnetic resonance imaging

mTardieu - Modified Tardieu

NPar - Nonparetic limb

NR - Not reported

Par - Paretic limb

Par-Dom - Paretic side was the dominant side before the injury

Par- NDom - Paretic side was the non-dominant side before the injury

RMI - Rivermead Motor Assessment

S1 - Primary somatosensory cortex

SD - Standard deviation

TB - Toluidine Blue/1% Borax

TB - Triceps brachii

UL - Upper limb

US - Ultrasonography

W - Woman

SUMÁRIO

APRESENTAÇÃO

Esta tese foi estruturada de acordo com as normas do Programa de Pós-Graduação em Fisioterapia da UFSCar e faz parte de uma linha de pesquisa do Laboratório de Pesquisa em Fisioterapia Neurológica (LaFiN), que investiga as alterações neuromusculares pós-Acidente Vascular Cerebral (AVC) e suas implicações para a Fisioterapia..

Será apresentada uma breve contextualização sobre a problemática investigada na tese, seguida por um objetivo geral, os manuscritos e uma conclusão geral. O primeiro estudo foi submetido à revista Topics in Stroke Rehabilitation e o segundo a Muscle & Nerve. Além disso, as atividades desenvolvidas durante o doutorado são apresentadas ao final do documento.

CONTEXTUALIZAÇÃO

O AVC é atualmente a principal causa de incapacidades na população adulta (MURRAY et al., 2012; FEIGIN et al., 2014). Após o AVC, mais de 50% dos sujeitos apresentam uma hemiparesia que afeta principalmente o membro superior contralateral à lesão (PELICIONI et al., 2016), gerando déficits na função e prejudicando as atividades de vida diária (AVDs) destes indivíduos, como alimentação e higiene pessoal (VAN VLIET e SHERIDAN, 2007; FREITAS et al., 2011). Espasticidade, alterações da sensibilidade, contraturas e fraqueza muscular são comuns nos quadros de hemiparesia (CHO et al., 2014; LEE et al., 2015).

A fraqueza muscular após o AVC pode ocorrer tanto por fatores neurais, como por alterações musculares (MCNULTY et al., 2014; SILVA-COUTO et al., 2014). As modificações intrínsecas dos músculos são, por exemplo, mudanças no fenótipo da fibra muscular, sarcômeros hiperalongados, proliferação do tecido conjuntivo, mudanças no ângulo de penação, no comprimento do músculo e atrofia muscular (LIEBER et al., 2004; LIEBER, 2010; RAMSAY et al., 2011; SMITH et al., 2011; GRAY et al., 2012; MCNULTY et al., 2014).

Além disso, no pós-AVC, o aumento da imobilidade em decorrência das alterações sensório motoras podem contribuir para acentuar a perda de massa muscular (GRAY et al., 2012) e da força, (CLARK, 2009). Além das adaptações já mencionadas, indivíduos pós AVC também passam a apresentar comportamentos compensatórios principalmente em relação ao membro superior, diminuindo o uso do membro afetado e aumentando o do membro menos afetado, um comportamento chamado, não uso aprendido (JONES e SCHARLLERT, 1992; TAUB et al., 2003, 2006).

O não uso aprendido é uma consequência que se inicia com o desequilíbrio interhemisférico que ocorre após uma lesão unilateral focal, como o AVC. Na inibição

interhemisférica, o hemisfério intacto aumenta a sua atividade em relação ao hemisfério que sofreu o dano cerebral, alterando áreas representativas corticais do movimento e isto restringe a função motora (TAKEUCHI et al.,2012; MURASE et al., 2004). Ao longo do tempo, outras alterações como, por exemplo, o fato do indivíduo não conseguir utilizar o lado afetado, auxiliam no processo de fixação deste comportamento (TAUB et al., 2002).

Já está bem documentado na literatura que as lesões do córtex motor responsável pela área dos membros anterior de ratos, associados ao treino de alcance do membro menos afetado, conduz a alterações plásticas no córtex de peri-lesão, e altera o comportamento destes animais, isto é, eles passam a utilizar mais a pata não afetada, piorando seu desempenho de alcance (JONES e SCHALLERT, 1989, 1994; ALLRED e JONES, 2004, 2008). Estes estudos também demonstraram que o treino de alcance com a pata não afetada atrasa a reabilitação do membro afetado (ADKINS et al., 2004; ALLRED e JONES, 2004; ALLRED et al., 2005; ALLRED e JONES, 2008). Em resumo, estes estudos mostram que não só a lesão, mas também que o reforço do comportamento do não uso aprendido, auxilia no processo de uma plasticidade mal adapatativa.

Em humanos, os estudo sobre as adaptações na arquitetura muscular ou sobre os efeitos do não uso aprendido no pós-AVC, em grande parte, são realizados em membros inferiores ou sintetizam conjuntamente os resultados de membro superior e inferior (ENGLISH et al., 2010 , 2012; SCHERBAKOV e DOEHNER, 2011). Outro problema encontrado são os resultados controversos em relação as adaptações musculares, variando desde ausência de atrofia muscular no membro parético (CARIN-LEVY et al., 2006), até diferenças significativas entre lado parético e não parético (RYAN et al., 2002; METOKI et al., 2003) e entre indivíduos hemiparéticos e controles saudáveis (SILVA-COUTO et al., 2014).

Apesar destes achados ser clinicamente relevantes, fatores como diferentes graus de espasticidade e fraqueza, aliados à variabilidade de idades dos sujeitos, intervalos pós-AVC e comorbidades existentes, atrapalham a homogeneidade dos grupos (SNOW et al., 2012), dificultam a generalização das conclusões sobre a adaptação muscular. Assim, observamos a importância de estudos que realizem sistematização das informações relacionadas às alterações musculares em membro superior, bem como a realização de estudos controlados com modelos animais.

Estudos já compararam o movimento de alcance entre roedores e em seres humanos saudáveis, e constataram diversas semelhanças no movimento de alcance entre as duas espécies (WHISHAW et al., 1992), e que após a lesão cerebral, há comprometimento das funções e das habilidades na pata anterior em animais de forma semelhante ao que ocorre em membros superiores de humanos (MURPHY e COBBERTT, 2009), possibilitando comparações.

OBJETIVOS

Esta tese teve dois objetivos principais. O primeiro foi revisar a literatura e sistematizar as informações encontradas sobre as adaptações musculares do membro superior frente ao AVC em seres humanos (manuscrito I). Já o segundo, a partir de um modelo animal de lesão isquêmica cortical com endotelina-1, verificar seus efeitos sobre a adaptação muscular da pata dianteira. Além disso, também visa investigar se o treinamento do membro não parético, simulando o não uso aprendido, geraria qualquer alteração na musculatura da pata parética (manuscrito II).

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MANUSCRITO I

Structural muscular adaptations in upper limb after stroke: a systematic review

Artigo submetido a Topics in Stroke Rehabilitation

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Abstract

Background: Stroke is the leading cause of disability in the adult population, impairing upper limb movements affecting activities of daily living. Muscle weakness has been associated to disabilities in this population, but much attention is given to central nervous system alterations and less to skeletal muscles.

Objective: To carry out a systematic literature review to identify structural muscle alterations in the upper limb of post-stroke individuals.

Method: The search was performed in December, 2017. Medline, PubMed, Scopus, Cinahl and Science Direct were used as electronic databases. There was no restriction regarding language and publication dates. Studies conducted on post-stroke subjects and results on upper limb skeletal muscle alterations identified by imaging tests were included.

Results: Seven studies were included. The sample size and the variables varied among the studies. All the studies compared the paretic upper limb with the non-paretic upper limb and one of the studies also compared healthy subjects. Ultrasonography was the most used measurement tool to assess muscle adaptation. Most of the studies showed poor quality.

Conclusions: This review demonstrated little evidence with poor to fair quality on the structural muscle adaptations in the post-stroke subjects, showing muscle atrophy, a higher stiffness and amount of fibrous and fat tissue without alterations in lean tissue of distal muscles of the paretic upper limb compared to the nonparetic limb. However, the nonparetic side also presented alterations, which makes it an inappropriate comparison. Thus, well-designed studies addressing this issue are required.

Keywords: stroke; upper extremity; skeletal muscle; muscular atrophy; muscle spasticity.

Introduction

Stroke is the main cause of neurologic disability in adult populations worldwide[1-3]. Most stroke survivors experience some loss of motor function and disuse of the upper limb (UL)[4], which can contribute to a reduction in functional independence and social participation[5]. These alterations in the UL may be related to motor and sensory impairments[6], such as spasticity, sensibility alterations and muscle weakness[7,8], which may, over time, alter the muscle architecture gradually, generating contractures and impairing the range of motion and force generation during activities of daily living[8-10].

According to the literature, muscle structural alterations can be related to those of muscle fiber size, volume, fiber type distribution, amount of intramuscular fat, sarcomeres and connective tissue proliferation[11-18]. Recently, imaging techniques have been used to evaluate these muscle properties within research practice. These non-invasive techniques present a high reliability of measurement[19] and the most common resources are magnetic resonance imaging (MRI)[20], computerized tomography (CT)[21], Dual Energy X-Ray Absorptiometry (DEXA)[22] and ultrasonography (US)[23].

Based on these imaging technologies, studies have observed important alterations in the elderly population, such as lower muscle thickness and mass, as well as higher muscle echo intensity and the amount of intramuscular fat[24-26], which can be related to impairments in functional performance[27]. However, in post-stroke subjects, most of the studies carried out an analysis on the lower limb[28] and none of them controlled comorbidities[27] or the effects of aging[17], making generalizations about the effects of stroke on UL skeletal muscles difficult to understand. Thus, the aim of this review is to identify the muscle alterations in the UL in

survivors after stroke in order to provide a better understanding of structural muscle adaptations in the post-stroke population.

Methods

This review was written using the Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) statement[29]. The State of Art through Systematic Review software (StArt - http://lapes.dc.ufscar.br/tools/start_tool) was used to systematize and organize search and data extraction[30].

Data Sources and Search Strategy

A systematic search was conducted on December 15, 2017 using five different databases (beginning of the search): PubMed (1972), MEDLINE (1946), CINAHL (1937), SCOPUS (1823), and Science Direct (1823). The final search was performed using the following MeSH headings or predefined keywords: ("stroke") AND ("upper extremity" OR "upper limb" OR arm OR hand) AND ("skeletal muscle" OR "muscle mass" OR "muscle architecture" OR "muscle atrophy"). All the databases were limited to humans and were not limited to the date and language of publications.

Eligibility Criteria

Full-text articles were included if studies were conducted in post-stroke individuals and outcomes included upper limb skeletal muscle alterations identified by imaging tests, such as computerized tomography and ultrasound, which are considered as gold standard for muscle morphology analysis[31]. Ultrasonography provided information related to tissue muscle tissue composition and stiffness measured by echo intensity and shear wave, respectively[8]. Articles

were excluded if other neurological diseases or health conditions were evaluated, if they focused on muscle adaptations of lower limbs or if they used only biopsies because they did not reflect the whole muscle. Studies using botulin toxin were not included. Systematic reviews, dissertations, theses, letters, meta-analyses, guidelines, scientific congress abstracts, case-reports and qualitative studies were also excluded.

Study Selection

Two independent researchers (GLS and GNO) screened the titles and abstracts according to the selection criteria of this review to identify potentially eligible studies. Afterwards, the full texts were read independently by the same two reviewers. In case of ambiguities or disagreement, a third researcher (FMF) was required to reach a consensus during the deliberation session. Additional articles were verified by screening the reference list of selected studies.

Data Extraction

Relevant data were extracted as follows: first author, publication year, study design, groups (sample size), baseline sample characteristics (time post-stroke, age, gender, UL deficits), measurement tool, muscle evaluated, comparisons and main outcomes involving structural muscle adaptations.

Methodological Quality Assessment

The methodological quality was evaluated using the Downs and Black assessment tool recommended by Cochrane[32]. This checklist has 27 items and assesses the following components

that evaluate: the reporting quality; methodological design; external validity and internal validity (bias and confounding) and power. All items were scored as yes (=1) or no/unable to determine (=0), except item 5, which can be scored as 0, 1 or 2 (no, partially, or yes, respectively). The total score was grouped into four categories of quality: excellent (26-28), good (20-25), fair (15-19) and poor (<14)[33]. Two researchers (GLS and GNO) classified the articles independently and the inter-rater reliability (kappa statistic) was 0.70, a substantial agreement between the investigators[34]. When there was any disagreement, the researchers discussed to reach a consensus for the final score.

Results

A total of 2,278 studies were found in the databases (PubMed = 806, MEDLINE = 87, CINAHL = 125, SCOPUS = 907, and Science Direct = 353). After removing the duplicates, the titles and abstracts of 1,489 articles were screened (Figure 1). Thereafter, 12 articles were read in full; however, only seven articles fulfilled all the inclusion criteria. The date of publication included studies ranging from 2002 to 2017 (Table 1). Out of these, six studies presented a cross-sectional design [8,9,12,35-37] and one longitudinal[22] (Table 1).

Figure 1. flowchart of the review steps

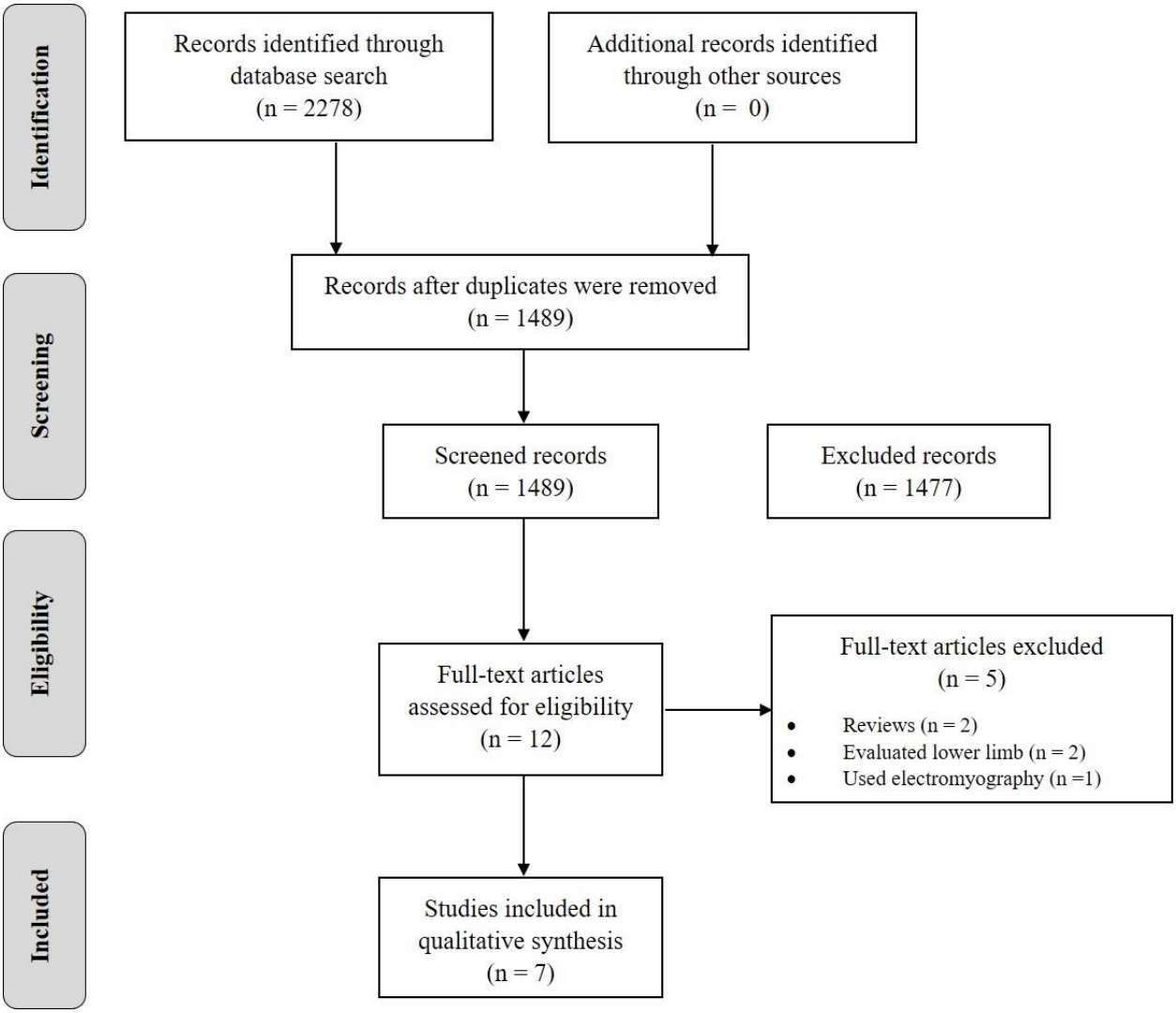

Table 1 - Main characteristics of included studies (n=7).

Baseline sample characteristics										
Author (year)	**Study design**	**Groups (n)**	**Time post-stroke**	**Age (years) Mean ±SD**	**Gender (M/W)**	**UL deficits**	**Measurement tool**	**Muscle evaluated**	**Comparisons**	**Main results**
Ryan *et al.* (2002)[12]	Cross-sectional	Stroke (n=60)	Chronic (> 6 months)	65 ±9.00	47/13	NR	DEXA	Arm*	Par x Npar	No differences in fat mass < lean tissue mass of Par.
Carin-Levy *et al.* (2006)[22]	Longitudinal	Stroke (n=18)	Acute (< 72 hours)	66 ±11.5	11/7	FIM=107** (32-125) RMI=4** (0-14)	DEXA (n=11)	Arm*	Par x Npar	No differences in lean mass at 3 weeks and week 24 post-stroke.
Ploutz-Snyder *et al.* (2006)[35]	Cross-sectional	Stroke (n=6)	Chronic 65 (10 - 125) moths**	55.93 ±3.96	5/1	MAS BB = 0 to 3 TB = 1 to 1+	MRI	BB and TB	Par x Npar	Lower muscle CSA in BB (5%) e TB (25%) for Par
Li *et al.* (2007)[9]	Cross-sectional	Stroke (n=7)	Chronic 4 (2 - 7) years**	48.57 (36-63)**	4/3	MAS B = 1+ to 4	US	B	Par *vs* NPar	> pennation angles and < muscle fascicule lengths for Par
Triandafilou & Kamper (2012)[36]	Cross-sectional	Stroke (n=25) Healthy (n=10)	Chronic (2-4 years)	45-65 years	16/9	Chedoke-McMaster (Stage 2 and 3 of hand)	US	FDS, FDP, EDC, EI, FDI, FPI and LUM	Par *vs* NPar	< CSA and muscle thickness for all muscles of Par
									Par-Dom *vs* Par-NDom	< CSA and muscle thickness for all muscles of Par-NDom
Lee *et al.* (2015)[8]	Cross-sectional	Stroke (n=16)	Chronic 11.6 (1.9 - 42.2) years**	60.7 ±8.00	6/10	FMA: 19 (4-48)** MAS: 0-3 mTardieu: 1-3 (62°-145°)	US	BB	Par *vs* NPar	> shear wave speed and echo intensity in BB of Par
Berenpas *et al.* (2017)[37]	Cross-sectional	Stroke (n=28)	Chronic 5.2 ±4.4 years	58.7 ±10.00	20/8	Brunnstrom stage 4 (3 - 5) MI 67 (33 - 91)	DEXA CT	BB, FFA, EDB	Par *vs* NPar	> echogenicity of BB, FFA and EDB for Par < muscle thickness for BB and FFA for Par
									Par *vs* Ref	> echogenicity of BB and FFA and < for EDB for Par

		< muscle thickness of EDB for Par
	NPar *vs* Ref	> echogenicity of BB and FFA and < for EDB for NPar
		< muscle thickness of BB, FFA and EDB for NPar

n: sample size. SD: standard deviation. M: man. W: woman. UL: upper limb. NR: not reported. FIM: Functional Independence Measure. RMI: Rivermead Motor Assessment. MAS: Modified Ashworth Scale. FMA: Fugl-Meyer Assessment. mTardieu: modified Tardieu. MI: Motricity index. DEXA: Dual Energy x-ray absorptiometry. MRI: magnetic resonance imaging. CT: Computer tomography. US: ultrasonography. BB: biceps brachii. B: brachialis. EDC: Extensor digitorum communis. EDB: Extensor digitorum brevis. EI: Extensor indicis. FDS: Flexor digitorum superficialis. FDP: Flexor digitorum profundus. FDI: First dorsal interosseous. FPI: First palmar interosseous. FFA: Forearm flexors. LUM: Lumbrical. TB: triceps brachii. Par: paretic limb. NPar: nonparetic limb. Par-Dom: paretic side was the dominant side before the injury. Par-NDom: paretic side was the non-dominant side before the injury. CSA: cross-sectional area. *Not reported which specific muscle or group of muscles assessed.**Data presented as mean and range. Ref: reference values based on studies with healthy subjects (Arts et al, 2010 and Verhulst et al, 2011). <: reduced; >: increased.

The sample size ranged from 6 to 60 (average = 23.29, standard deviation = 19.42), the majority of the subjects were male, and the age ranged from 45 to 65 years old. Only one study assessed the acute and subacute phases after stroke[22], the others 6 studies were performed in the chronic phase[8,9,12,36,37]. Sensorimotor impairments and/or independence were used for sample characterization in chronic post-stroke populations[8,9,22,35-37] and the Modified Ashworth Scale was the most common used among the studies[8,9,35.]

Only one study had two groups (post-stroke patients and healthy subjects)[36] for comparisons. The others presented only one group and compared the paretic to the non-paretic limbs[8,9,12,22,35,37]. Moreover, one study performed a comparison between reference values from the literature and the paretic or non-paretic limbs[37]. The most used method of analysis was US[8,9,36,37], followed by DEXA[12,22].

The muscles evaluated varied among the studies, however the most common muscle evaluated was the biceps brachii[8,9,35,37]. Furthermore, elbow flexors (EF), extensor digitorum brevis (EDB), flexor digitorum superficialis (FDS), flexor digitorum profundus (FDP), extensor digitorum communis (EDC), extensor indicis (EI), first dorsal interosseous (FDI), first palmar interosseous (FPI) and lumbrical (LUM) muscles were also assessed among studies[8,9,35-37].

Regarding the main results, one study compared the paretic and nonparetic UL to reference values of subjects after stroke, and showed that the paretic limb increased the echogenicity in BB and FFA muscles, whereas a decrease in echogenicity and muscle thickness in EDB muscles was observed[37]. Another study compared the paretic side, which before the stroke was the dominant upper limb, with the paretic side which before the stroke was non-dominant and also with healthy subjects, and found a decrease in the maximum thickness and the cross sectional area (CSA) of FDS, FDP, EDC, EI, FDI, FPI and LUM muscles[36].

The comparison between paretic and non-paretic UL showed differences in many variables. Three studies showed an increase in echogenicity/echo-intensity, variables related with connective tissue, intramuscular fat and contractures in the BB muscle[8,37] and the EDF[37] of the paretic side. They also showed a decrease in the thickness for the BB and EDB muscles[37] and in the maximum thickness of FDS, EDC, EI, FDI, FPI and LUM[36] and also an increase in shear wave speed in the BB muscle[8]. The CSA was evaluated in three studies. Two of these studies showed a decrease on the paretic side in CSA of EE, EF, FDS, EDC, EI, FDI, FPI and LUM[35,36] and in the other study no difference was found between the sides, even over time[22].

The other variables such as lean tissue mass and fat mass were evaluated in two studies[12,22]. One of them found a decrease in lean tissue mass on the paretic side[12], whereas the other showed a reduction in fat mass over time, but with no difference between the limbs (paretic vs non-paretic)[22]. An increase in the pennation angle with a decrease in muscle fascicule lengths in the paretic limb was also reported by one study[9]. No difference was found in appendicular skeletal muscle and total skeletal muscle, analyzed in one study[22].

Quality assessment

The total score and scores by each component (reporting, external and internal validity and power) were presented in Table 2. One study was classified as fair[34] and six as poor[8,9,12,22,36,37]. The main items not reported were those related to the external and internal validity (confounding – selection bias), since all the articles did not involve any type of intervention.

Table 2 - Downs and Black total score and score by each component (reporting, external validity, and internal validity and power) for all selected articles.

Authors	**Reporting**	**External validity**	**Internal validity**		**Power**	**Quality of evidence (Total score)**
			Bias	**Confounding**		
Ryan *et al* [12]	9	0	4	0	0	Poor (13)
Carin-Levy *et al*[22]	7	1	3	2	0	Poor (13)
Ploutz-Snyder *et al*[35]	9	1	4	1	0	Fair (15)
Li *et al*[9]	9	0	5	0	0	Poor (14)
Triandafilou *et al*[36]	9	0	4	1	0	Poor (14)
Lee *et al*[8]	8	0	4	0	0	Poor (12)
Berenpas *et* al[37]	8	0	4	1	0	Poor (13)

Discussion

This review investigated the evidence related to structural muscle alterations in the UL after stroke and identified only seven studies that evaluated different distal muscles of UL, which demonstrated little evidence of this issue. Overall, the studies observed a reduction in muscle CSA[35,36], thickness[36,37] and fascicule length[9], and an increase in pennation angles[9], shear wave[8], echo intensity[8] and echogenicity[37] in the paretic limb compared to the nonparetic side. These variables clinically represent atrophy[35-37], a lower muscle tension with respect to the tendon and functional range[9] and a higher stiffness[8] and amount of fibrous and fat tissue[8,37] without alterations in the lean tissue of the paretic limb[12,22].

According to the literature, these muscle changes after stroke can be attributed to abnormal central neural innervation and disuse[12,27]. However, disuse is considered the primary factor of the structural changes, especially to muscle atrophy[12,38,39]. Moreover, muscles post-stroke show many similarities with age-related muscle changes, such as lower muscle CSA and

fiber size[27,40,41]. Besides age, other factors such as gender, level activity, other comorbidities (i.e. diabetes mellitus) and dominance side can influence these muscle structural adaptations[24,26,42-45], which was not considered in most of the selected studies, except by Triandafilou and Kamper's study that considered the UL dominance before stroke. In this study, subjects with hemiparesis of the non-dominant side showed more atrophy (lower muscle CSA and thickness) compared to subjects with hemiparesis of the dominant side, which confirms the influence of the dominant side.

In addition to these comorbidities, aspects related to the UL deficits such as muscle tone and motor function need to be considered. Only one study showed that there was a correlation between the Fulg-Meyer scale with the shear wave speed for the biceps brachii muscle and no correlation with the MAS[8], however the authors did not discuss what the significance is regarding the muscle alterations inferred in the study for the motor disabilities. Studies that also used a clinical scale did not make comparisons between the muscle adaptation and these scales. It can be observed that the studies selected subjects with moderate to severe muscle tonus alterations and motor functional disabilities[9]. Thus, the sample selection with spasticity and hypertonia can influence the change on muscle fascicule length. Nevertheless, there is a lack of correlation with the clinical scales responsible for motor disabilities of the upper limb in the selected studies.

One important aspect not considered among studies is that sensorimotor changes may occur bilaterally in post-stroke subjects[27,37]. Changes in the non-paretic muscle can be related to the fact that 10% of the corticospinal connections to the motoneurons in the spinal cord do not undergo decussation[46,47]. However, other factors can also contributed to muscle alterations in nonparetic UL, such as sedentary lifestyle[38,48], metabolic disorder or nutrition[49]. Thus, besides

the comparison between paretic and nonparetic UL, it is important to compare both limbs with limbs of healthy subjects matched by age and gender.

Moreover, it is important to highlight that the selected studies focused on the evaluation of distal musculature, such as finger muscles, wrist and elbow flexors and extensors. However, proximal muscles are fundamental to providing stability to UL motions[50,51] and any alteration in these muscles can impact distal motions, and consequently activities of daily living. According to the literature, post-stroke subjects showed postural proximal alterations that can impair UL performance during drinking tasks, such as inadequate scapula and shoulder angles at static positions[52]. Thus, investigating possible structural alterations in the proximal musculature could help in the rehabilitation process of post-stroke patients as these alterations can lead to limitations of range of motion and UL performance.

Thus, faced with these difficulties to control the aspects that could influence structural muscular adaptations (i.e. activity level, age, gender, UL impairment), determining the appropriate evaluated comparison group and muscles, as well as the small sample size, the studies presented a poor to fair quality, which requires caution when extrapolating the results found. However, even considering these limitations and the small number of selected articles, the results point to an important clinical implication, which involves including techniques that aim to minimize these alterations, especially those that reduce disuse. Therefore, future studies with a larger sample size and sample more adequately matched in terms of age, gender and activity level are needed. In addition, studies that correlate the UL impairment, motor function and use with these structural muscle changes are necessary.

Conclusion

This review has shown little evidence of poor to moderate quality in structural muscle adaptations in post-stroke subjects, which shows muscle atrophy, increased stiffness and an amount of fibrosis and fatty tissue (fat) without changes in the lean body mass of the distal muscles of the paretic UL compared to the non-paretic UL. However, as the non-paretic side also presented changes, it makes the comparisons inappropriate. Thus, well-designed studies addressing this issue are required.

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MANUSCRITO II

Focal cortical ischemia and learned nonuse effects on muscle atrophy in rat

Artigo submetido a Muscle & Nerve

Faturi FM, Gianlourenço ACL, Jones TA, Zambetta RM[1], PT, Salvini TF, Moriscot AS, Russo TL. Focal cortical ischemia and learned nonuse effects on muscle atrophy in rat.

Abstract

Introduction: This study verified the effect of the endothelin-1 (ET-1) brain ischemia on early and late functional recovery and muscular adaptation of paretic forelimb in rats, as well as the effects of skill learning of non-paretic forelimb association to ET-1. **Methods:** Thirty 4-month-old Wistar rats were used. Groups: ET-1 4- or 15-days, ET-1 brain ischemia and euthanized 4 or 15 days after injury; Control; and ET-1 15-days and non-paretic forelimb training. Minor muscle fiber diameters from paretic forelimb were measured. **Results:** ET-1 impaired forelimb performance, but not altered muscle fiber diameter. The association between non-paretic training and ET-1 accentuated the functional impairment and induced selective atrophy of fingers extensors in paretic forelimb. **Discussion:** The ET-1 ischemia leads to early dysfunction, and late recovery associated to compensatory movements, but does not cause muscle atrophy. Nevertheless, increased non-paretic forelimb activity impaired function recovery and induced selective muscle atrophy on paretic side.

Key-words: rehabilitation, cerebrovascular disease, skeletal muscle, stroke, disability.

Introduction

Stroke is considered the second cause of death and the main cause of disability in adults in the world[1-3]. More than 50% of post-stroke individuals present some degree of disability and weakness on the upper limb contralateral to the injured brain hemisphere[4], affecting individual's daily life activities and participation[5,6]. Muscle weakness has been associated with compensatory strategies of movement due to the lesion of the motor cortex and its descending projections pathways[7], reduced muscle activation and incoordination[8-10], and anatomic modifications on skeletal muscle, such as muscle fiber phenotype shift, fibrosis and atrophy [6,11-14].

Furthermore, compensatory behavior strategies of decreasing the use of the paretic limb (*learning non-use*) generate maladaptive plasticity on the central nervous system (CNS), and impair the functional recovery of the paretic limb[15-19]. Preclinical studies showed that the skill learning of reaching pellets with non-paretic limb (mimicking "learning non-use") in rodents after brain ischemia impaired the functional recovery of paretic limb[15,18,20-22]. The neural mechanisms involved in the maladaptative plasticity due to training of the non paretic limb has been associated with the increase of somatossensorial area related to nonparetic limb and also with addition of multisynaptic boutons in peri-infarct primary motor cortex (M1) cortex area, affecting synapse addition and maturation. Moreover, they showed that alterations are persistent, making the rehabilitative process more difficult[7].

Although certain studies have shown neural adaptations and motor performance alterations in the paretic limb due the ischemic injury and skill learning with non paretic limb in animals, little information is available about its effects on muscular adaptation. A recent study described muscle atrophy in hindlimb 3 days after cerebral ischemia (60 minutes of the middle cerebral artery occlusion, MCAO) in paretic limb, whereas muscle mass remained unchanged in nonparetic limb in mice. Animals also presented severe sensorimotor deficits during walking[23]. Paretic hindlimb muscle atrophy was also observed using the same model of brain ischemia at 7 days post-injury[24]. Nevertheless, muscle adaptation in different models of brain ischemia must be characterized to verify clinical translation to post-stroke patients.

Recently, the translational working group of the Stroke Recovery and Rehabilitation Roundtable provided important directions to develop a set of guidelines and recommendations for preclinical stroke recovery research and to maximize clinical translation[25]. Despite of no "gold standard" stroke model in rodents, endothelin-1 (ET-1), a potent vasoconstrictor, is highly targetable and generates cortical strokes with excellent behavioral readouts[25].

Thus, this study was divided in two experiments and had two aims: (1) to verify the effect of the cerebral ischemia model of endetholin-1 on the functional deficits and muscular adaptation in the early and late phase in the rats paretic forelimb and (2) to verify whether skill learning of non-paretic forelimb can accentuate functional deficits and induce muscle atrophy in paretic forelimb in ET-1 model of brain ischemia. We hypothesized for experiment 1 that the cerebral ischemic induced by ET-1 would generate early- and long-term deficits in reach and muscle atrophy in the rat paretic forelimb. Furthermore, for experiment 2, we hypothesized that the non-paretic forelimb training would accentuate the functional deficits and induce atrophy in the paretic forelimb.

Methods

The study was conducted according to the international standard of animal experimentation after the approval by the Ethics Committee on the use of animals (ECUA) of the Federal University of Sao Carlos (UFSCar).

Animals and experimental design

Thirty-three 4-month-old male Wistar rats were pair housed in cages in the Department of Physical Therapy at the Federal University of São Carlos (UFSCar). A 12/12 h light/dark cycle was performed with water access *ad libitum*. Animals were daily handled 2 to 3 weeks prior to the experiment,

and all behavioral procedures were performed in the same room. Prior to the start of behavioral methods, animals were placed on scheduled feeding of 15g of rat chow given once per day (to ensure rats will not sat at the time of training). Weights were monitored throughout the study.

Animals were submitted to the training chamber during 3 consecutive days (habituation period), then more 2-10 days of procedures of shaping on the single-pellet retrieval task to determine forelimb preference (dominance). After that, animals were trained in the retrieval pellet task for 10 days. After training, at -1 day (pre-surgery), animals underwent measurement of forelimb asymmetry and functional tests (single-pellet retrieval test**)** and submitted to the ET-1 lesion. Four days after surgery, animals were randomly assigned into four different groups according to the experiments. The cylinder test was repeated 3 days after surgery, whilst the single-pellet retrieval test was repeated 3 and 14 days (day 14) after surgery according to experiments.

Groups were defined as follow:

*For Experiment 1***:** 1) Endothelin-1 4 days (ET-1 4d; n = 7): animals submitted to all procedures, including brain ischemia with ET-1 and euthanized 4 days after injury (early/acute phase post-injury); 2) Endothelin-1 15 days (ET-1 15d; n=6): animals submitted to all procedures, including brain ischemia with ET-1 and euthanized 15 days after injury (late phase post-injury); and 3) Control (n=5): animals submitted to all procedures except brain surgery. Animals were euthanized on day 15, similar to ET-1 15d.

For Experiment 2: 1) Endothelin-1 for 15 days (ET-1 15d; n = 6): the same group used on experiment 1; and 2) Endothelin-1 for 15 days and non-paretic forelimb training (ET-1 15d + T; n = 9): The ET-1 15d + T group performed reach training for 10 consecutive days after surgery.

Schematic illustration of experimental groups and procedures is presented as supplementary data (Fig. 1S).

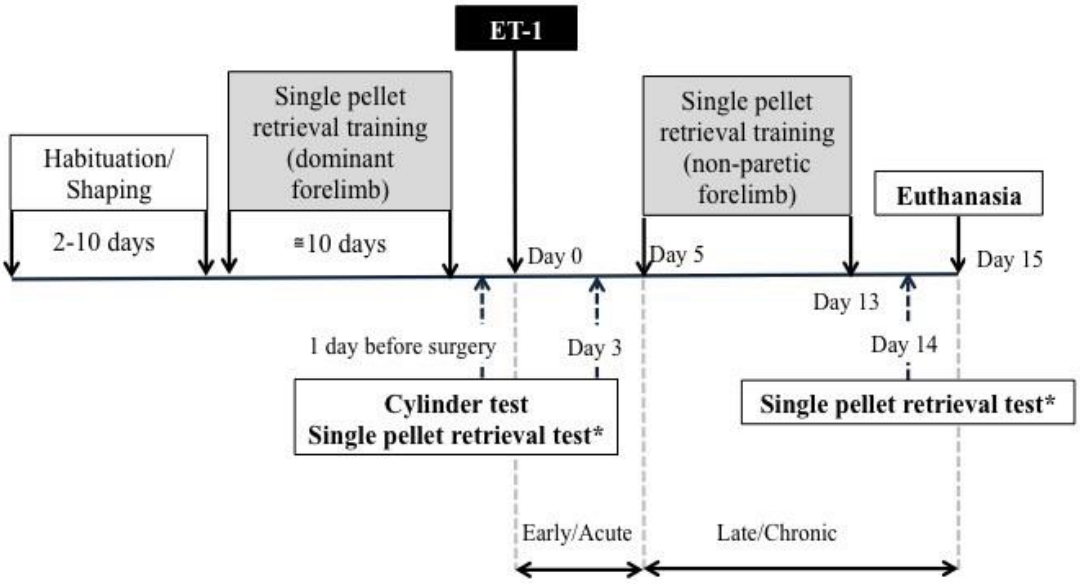


Figure 1S. Schematic representation of animals' procedures in a timeline. ET-1: endothelin-1. *the dominante/paretic forelimb was used in the single pellet retrieval test. Note all groups were submitted to single pellet retrieval training with dominant forelimb to teach animals how to perform the task

Habituation and shaping

All animals were submitted to a habituation period (10 minutes/day) for 3 consecutive days in a Plexiglas reaching chamber (30 cm long by 35 cm high by 15 cm wide) with a tall narrow window (1 cm wide and 3 cm high) in the center of the 15 cm wide wall (Fig. 2S).

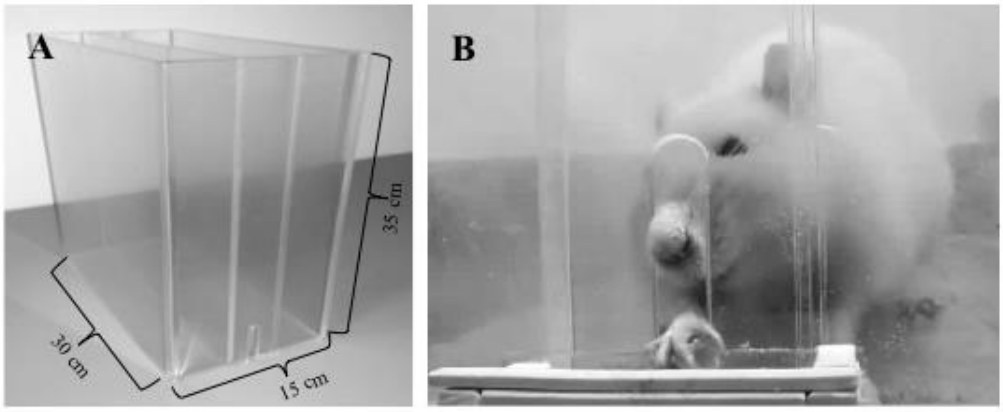


Figure 2S. Reaching chamber. Plexiglas reaching chamber (30 cm long by 35 cm high by 15 cm wide) with a tall narrow window (1 cm wide and 3 cm high) in the center of the 15 cm wide wall was used in this study. A small Plexiglas rod approximately 2 mm in diameter adhered to the base of the reaching window creating a barrier that prevented animals from scraping the pellets into the chamber, and also reduced attempts to use the tongue to retrieve pellets

For shaping, animals were placed in the same reaching chamber for 10 min. Animals reached with a forelimb through a small window for Froot loops (Kellogg's), which were placed in front of a block, approximately 3 cm in height. The wells were centered with the left and right edges of the window at a distance of 1 cm from the window. A small Plexiglas rod approximately 2 mm in diameter adhered to the base of the reaching window created a barrier that were prevent animals from scraping the pellets into the chamber and also reduced attempts to use the tongue to retrieve pellets (Fig. 2S). When 20 consecutive reach attempts were performed with one limb during a 20-minute session, this limb was identified as the preferred limb. Once animals reach these criteria, pre-operative shaping was ceased and the reach training before surgery started.

Training on the single-pellet retrieval task

Training on the single pellet-retrieval task was carried out in the reaching chamber During training, a Plexiglas wall were inserted into the reaching chamber ipsilateral or contralateral to the animal's trained limb and pellets were placed in the wells opposite the reaching limb. This wall effectively forces the animals to use the forelimb chosen by the experimenter for the reaching task, e.g., paretic or non-paretic limbs. In the initial design of the apparatus, the inner chamber wall was placed at a distance of 1.5 cm from the reaching window (Fig. 2S).

The training with the preferred limb was performed in all groups 10 days before the assessment of forelimb asymmetry and reach performance by single-pellet retrieval tests. Animals were trained for 60 trials or a cutoff time (20 min), which ever come first. A reaching trial were consisted of the animal either successfully grabbing the pellet and bringing it directly to its mouth (success), dropping the pellet before bringing it to its mouth, failing to grasp the pellet after five reaches or knocking the pellet out of its well. At the end of each reaching trial, a pellet was dropped into either the front or the back of the reaching chamber to “re-set” the animals and so that a new pellet was placed into its appropriate well.

After surgery, the ET-15d + T underwent to a new set of training, now using the non-paretic forelimb during 9 days. No-training controls (control and ET-15 d groups) were yoked to the trained animals on each day of training and were placed in a reaching chamber with a Plexiglas wall ipsilateral to what would be their trained limb. The no-training control animals had pellets dropped into the reaching chamber at approximately the same rate as the trained

animals received pellets (Fig. 1S). All trainings took place during the animals' light cycle. The reach training before surgery was performed with preference limb.

Testing on the single-pellet retrieval task

Testing on the single-pellet retrieval task was performed on day -1 (before surgery), day 3 and 14 after surgery. Reaching performance were calculated by dividing the total number of successful reaches by the total number of reach attempts with paretic limb [(total success/ total reach attempts) x 100], which corresponds to percent successful reaches. Test was performed during 20 minutes[26].

Measurement of forelimb asymmetry

The Schallert cylinder test[15] was used as an inclusion criterion to confirm lesion-induced asymmetries in forelimb postural-motor behavior. Animals were placed in a cylinder (19 cm diameter), which encourages upright exploratory movements and allow to identify an asymmetrical forelimb behavior. Animals were filmed in the Plexiglas cylinder for 2 min and after one measurer watched the movie and observed the exploratory standard of these rats in the cylinder wall. Analyses were performed one day before (day -1) and three days after surgery (Fig. 1S). Figure 3S shows a typical exploratory behavior pre- and post-ET-1 brain ischemia.

Surgery

A focal unilateral lesion of the forelimb representation area of the sensorimotor cortex (SMC) contralateral to the preferred limb was created. Animals were anesthetized using

intraperitoneal (i.p.) injections of xylazin (12 mg/Kg) and ketamine (95 mg/Kg), and placed in a stereotaxic apparatus. A midsagittal incision and a craniotomy were made between 2.5 mm anterior, 0.5 mm posterior, and 3.0 to 4.5 mm lateral to Bregma. Pia mater was exposed by removal of dura in the area underlying the craniotomy. Endothelin-1 (ET-1, 80 μM, 0.2 μg/μl; 8 μl total volume administered – American Peptide Company), a vasospasm-inducing peptide, was topically administered with a 10μl volume pipette for 10 minutes before the skin was sutured. During all surgery animal's temperature was controlled using a heat pad. Rats were allowed 4 days of recovery before postoperative behavioral manipulation starts (training)[20].

Muscle and brain analyses and euthanasia

At the 4 or 15-day post-injury, paretic triceps and biceps brachii, fingers extensors and fingers flexors muscles were dissected and removed. The muscles were frozen in isopentane in liquid nitrogen, stored at -86ºC and used to measure muscle fiber diameter. After muscle removal, the animals were euthanized with an overdose of anesthesia (ketamine and xylazine).

The brain was removed and placed to paraphormaldeyde fixative solution for 24 hours and them placed in a sucrose solution and kept refrigerated. Histological cross-sections (30 μm) from the motor cortex area injured were made and stained with a Nissl stain (cresyl violet) and were used to confirm the injury. Only animals that presented brain damage in morphology were included to analyses (Fig 4S).

Analysis muscle fiber diameter

Histological cross-sections (10 μm) from the middle belly of each muscle were obtained using a cryostat (Micron HE 505, Jena, Germany). Sections were stained with Toluidine Blue/1% Borax (TB), and analyzed by light microscopy (Axiolab, Carl Zeiss, Jena, Germany) equipped with a digital camera (AxioCam HRc, Carl Zeiss). One histological cross-section of each muscle located in the central region, with contiguous muscle fibers, was chosen for measurement. One image from this area was taken at 20x low magnification. The minor diameter of 70 randomly chosen muscle fibers was measured using Axiovision 3.0.6 SP4 software (Carl Zeiss, Jena, Germany). This variable seems to present less variability and to reflect force better than muscle fiber cross-sectional area[27]. A blinded evaluator made all the measurements.

Statistical Analyses

All variables (reaching performance and minor muscle fiber diameter) showed a normal and homogeneity distribution according to Shapiro-Wilk e Levene tests, respectively. For reaching performance assessment (percent successful reaches), two-way analysis of variance (group and evaluation time) with repeated measurements (evaluation time: day -1, day 3, and day 14) and Bonferroni's correction was used to examine the effect of group-by-evaluation time interaction, group (control, ET-1 4d, and ET-1 15d – experiment 1; ET-1 15d, ET-1 15d + T– experiment 2), and evaluation time. One-way analysis of variance followed of post-hoc Tukey was used to compare muscle fiber diameter between control, ET-1 4d and ET-1 15d (experiment 1). On the other hand, for the experiment 2 independent T-test to compare ET-1 15d to ET-1 + T was used. All statistical tests were carried out using SPSS software version 17.0 (SPSS Inc, Chicago, IL, USA), and a significance level was set at 0.05.

Results

All animals submitted to ET-1 brain ischemia presented forelimb asymmetry (Fig. 3S) and neural death in brain morphology (Fig. 4S). Detailed information is supplied as supplementary data.

Schallert Cylinder test

All the rats included in the analysis showed contralesional forelimb asymmetry as shown by the arrows in Figure 3S. In Figure 1, it can be observed that before the lesion (- 1 day), the animals used both paws to explore the environment. After the ischemic lesion in the contralateral dominant paw (3 day), an asymmetry (nonuse) of the dominant paw to explore the environment can be observed.

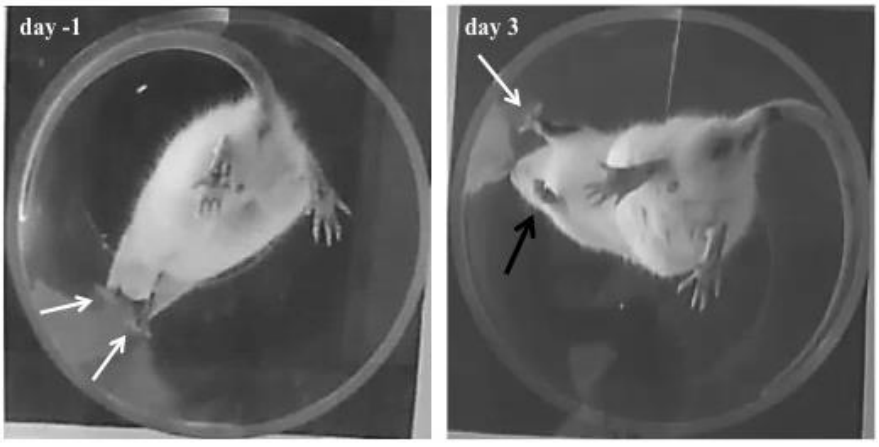
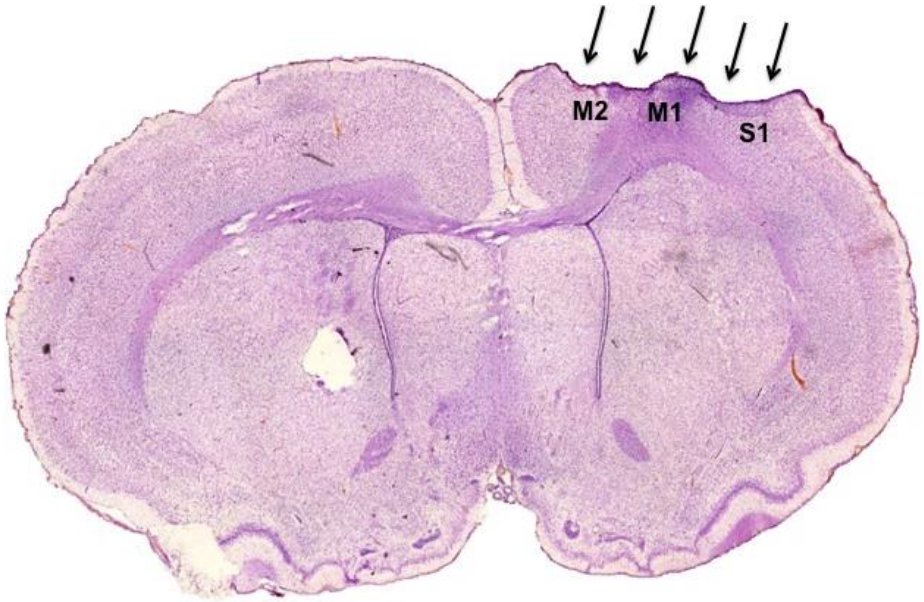


Figure 3S. Representative photographs showing the forelimb asymmetry. One day before the surgery, the rats were exposed to a cylinder for 2 min (day -1, pre-ET-1), and again 3 days after the surgery (3rd day). Note the animals presented exploratory behavior using both forelimbs pre-

ischemia (white arrows). After brain ischemia (day 3), the animals avoided using the paretic forelimb (white arrow: non-paretic forelimb; black arrow: paretic forelimb).

Brain morphology

Endothelin-1 produced reliable focal infarcts with neuronal death on primary (M1) and secondary (M2) motor cortices, and primary somatosensory cortex (Fig. 4S).



1.28 mm from bregma

Figure 4S. Representative photomicrography of rat brain injury after endothelin-1. Arrows showed the damaged place on the brain. Primary (M1) and secondary (M2) motor cortices, and primary somatosensory (S1) cortex were affected.

Experiment 1: *ET-1 brain ischemia effects on functional performance and muscle adaptation.*

Single-pellet retrieval task

Regarding the percentage of success, control and ET-1 15d groups were compared, and

interaction between the evaluation time and groups was observed ($F_{2,15}$=3.812, p=0.004; Fig. 1A). For the control group, it was not observed differences on the different time points investigated (p=1.000 for all comparations). On the other hand, for the ET-1 15d success percentage reduced on the day 3 compared to day -1 (pre) (p<0.001), but it increased on the day 14 compared to day 3 (p=0.026) (Figure 1A). There was a difference between the control and ET-1 15d groups ($F_{2,15}$= 13.609, p=0.001) only on day 3 (Fig. 1A).

Qualitative analysis of videos provided information about compensatory movements due to ET-1 model. Control images from pre-surgery moment (-1 day) were represented in the figure 1 B, C, D and E. Different phases of the movement were observed as reaching (1B), when rat took the paw to the food, grasping the food (C), supination (D) and taking the food to the mouth (E). Movements are smoothly performed. Although an improvement in the reach performance on day 14 was observed in ET-1 15 d group, the quality of reaching, grasping, supination and taking the food to the mouth is impaired. Compensatory strategies are observed on 15 days post-ET-1 (Fig. 1F-I). The animal failed to properly grasp the food (Fig. 1F-G). Because, supination was impaired, animals crew the hand attempting to pull the food into the box (1 G). In addition, the difficult of taking the food to mouth provoked compensatory movements such as using the non-paretic hand to help paretic one or taking the head into the hand with food using trunk inclination and rotation (H and I).

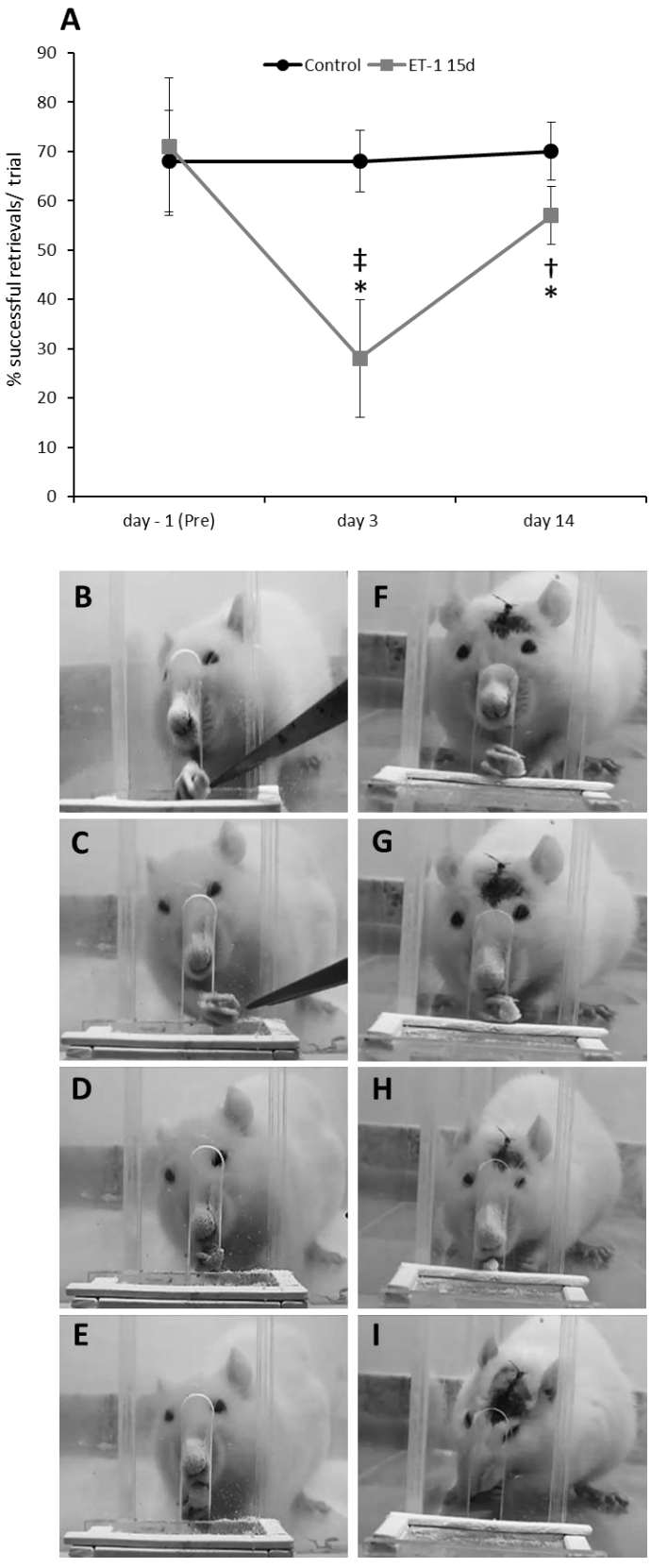

A
Control
ET-1 15d
% successful retrievals/ trial
90
80
70
60
50
40
30
20
10
0
day - 1 (Pre)
day 3
day 14
‡
*
†
*
B
C
D
E
F
G
H
I

Figure 1. (A) Graphic representing the percentage of successful reaches on the acute and chronic phase for control group and the group submitted to an ischemic lesion with endothelin-1 for 15 days (ET-1 15 d). *p<0.05 compared to day -1 (pre). † p<0.05 compared to day 3. ‡ p<0.05 compared to control group. (B to E) Sequential photographs of reaching, grasping and taking the food to mouth movements before surgery (day-1) are presented. (F to I) Compensatory strategies of the same approach 14 days post-ET-1. Note animals used trunk strategies to eat to compensate forelimb paresis (I).

Minor muscle fiber diameter

No difference in the minor muscle fiber diameter among control, ET-1 4d and ET-1 15d groups was observed for the biceps ($F_{2,13}$=0.435, p=0.657), triceps ($F_{2,12}$ =1.364, p=0.295), fingers flexors ($F_{2,11}$=2.307 p=0.146), and fingers extensors ($F_{2,10}$ =2.233, p=0.158) muscles (Figure 2).

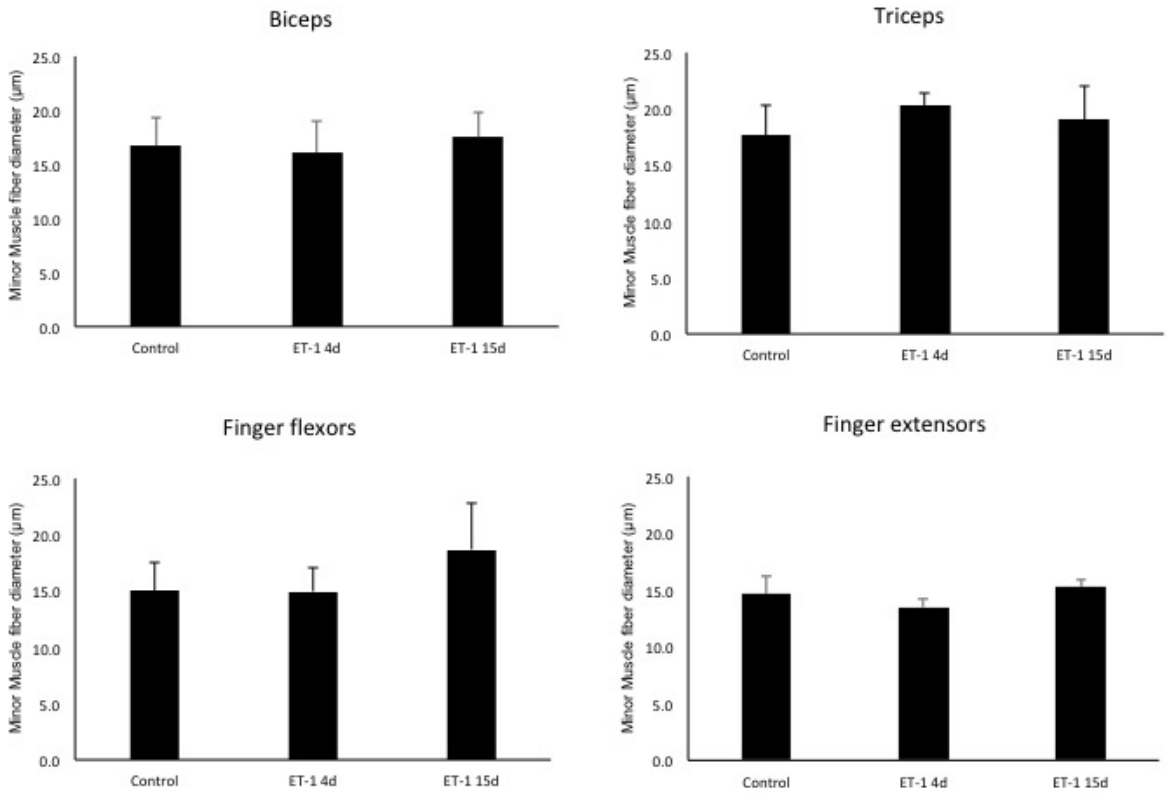


Figure 2. Minor muscle fiber diameter of control, endothelin-1 4 days (ET-1 4d) and endothelin-1 15 days (ET-1 15d) groups. No differences were observed between groups for any muscle ($p>0.05$).

Experiment 2: *Effects of non-paretic forelimb training on paretic functional performance and muscle adaptation*

Single-pellet retrieval task

Time-group interaction effect was observed ($F_{2,15}=4.704$, $p=0.004$; Fig. 3). All groups (control, ET-1 15d and ET-1 15d + T) presented similar performance on single-pellet retrieval task before surgery ($p>0.05$). The ET-1 15d and ET-1 15d +T decreased success rate on task at day 3 after injury compared to their values on day -1 and control ($p=0.001$ for both; Fig. 3).

However, ET-1 groups were different from each other on day 3 (p=0.05; Fig. 3). After 14 days, ET-1 15d recovery success rates similar to control (p=0.09; Fig. 3), whilst ET-1 15d +T remained impaired (p=0.01 vs control; Fig. 3).

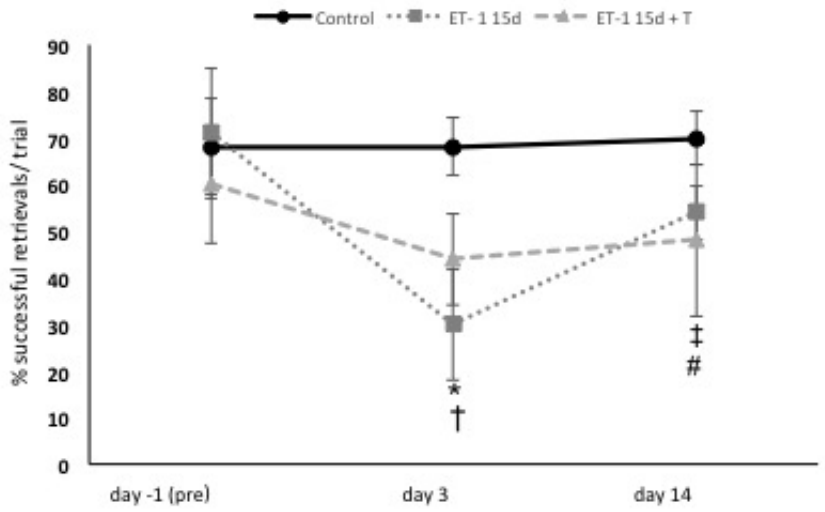


Figure 3. Percentage of successful on single-pellet retrieved task. *p<0.05 when ET-1 15d and ET-1 15d + T are compared to their own values on day -1 (pre) and also to control group; †p<0.05 compare to ET-1 15d + T; #p<0.05 when ET-1 15d compared to its values on day 3; ‡p<0.05 when ET-1 15d + T is compared to control.

Minor muscle fiber diameter

No difference beetwen ET-1 15d and ET-1 15d + T groups in muscle fiber diameter was observed in biceps (t= 1.217=, p= 0.251), triceps (t= 0.213, p= 0.836), and fingers flexors (t=1.870, p= 0.086) muscles (Fig. 4). Nevertheless, fingers extensors muscles presented reduced muscle diameter in ET-1 15d +T group compared to ET-1 15 days one (t= 3.791, p= 0.004; Fig. 4). Muscle fiber cross-sectional area confirmed this findings (Fig. 5S).

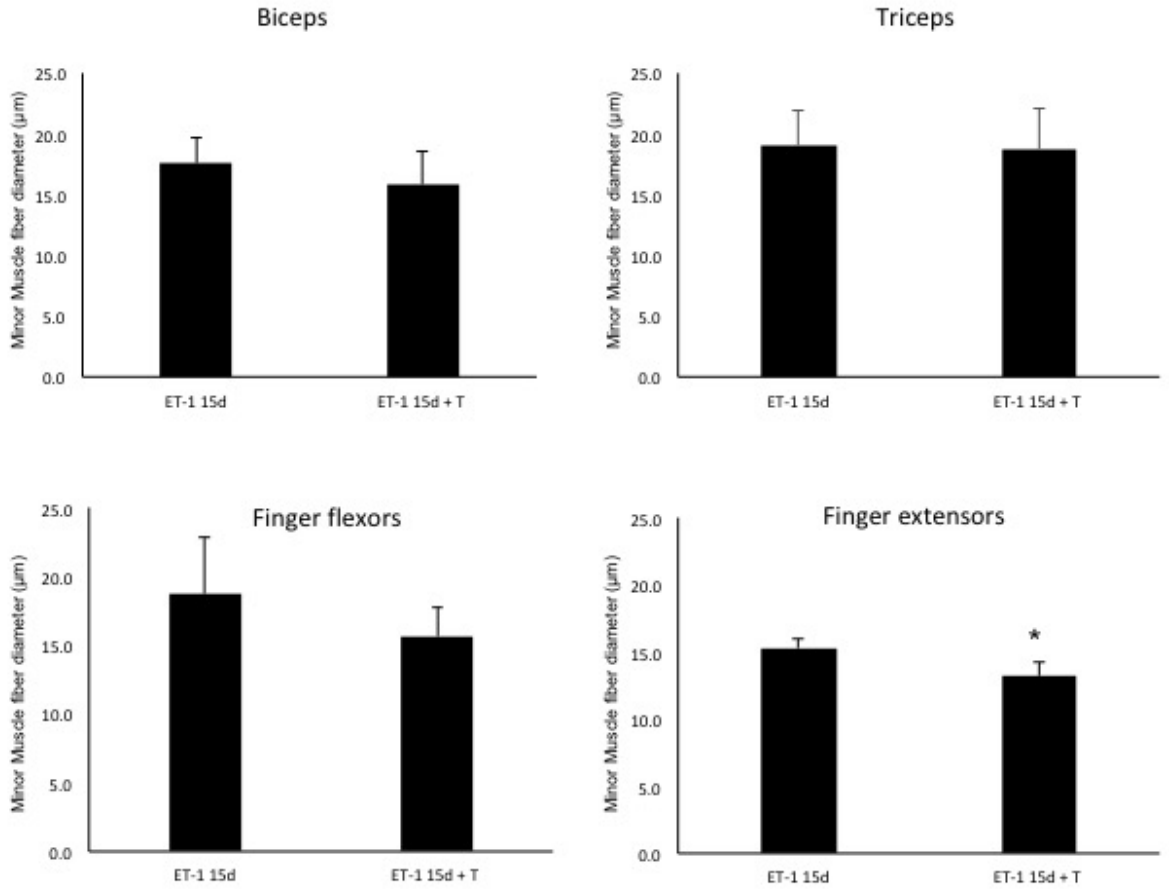


Figure 4. Minor muscle fiber diameter of control, endothelin-1 15 days (ET-1 15d) and endothelin-1 15 days and non-paretic forelimb training (ET-1 15d + T) groups. *$p<0.05$ compared to ET-1 15d. No differences were observed between groups for biceps, triceps and fingers flexors muscles ($p>0.05$). However, nonparetic limb training reduced muscle fiber diameter on fingers extensors muscles.

Muscle fiber cross-sectional area distribution

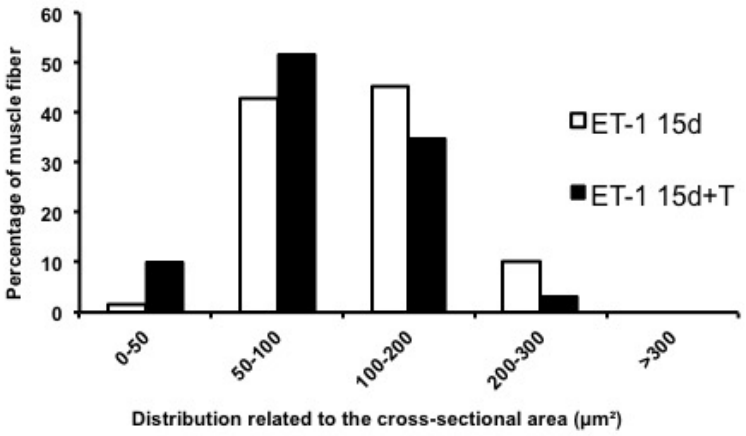


Figure 5S. Muscle fiber cross-sectional area distribution. An increase in the percentage of muscle fibers of ET-1 15d + T around 50 and 100 extracts compared to ET-1 15d can be observed.

Discussion

The present study showed that the model of brain ischemia in rats using ET-1 was able to impair forelimb performance during skilled tasks, although did not cause alterations in muscle fiber diameter of the paretic forelimb. On the other hand, the association of non-paretic forelimb training and brain ischemia, mimicking the "learn nonuse" behavior, accentuated the functional impairment and induced selective atrophy in fingers extensors muscles of the paretic forelimb.

Regarding results from experiment 1, impaired reach performance on acute phase post-brain ischemia followed by functional recovery corroborate with previous studies[28,29]. According to these studies the decrease of performance can be justified by injury of the motor cortex[28], depression behavioral state and diaschisis[29]. The functional recovery may be due to the presence of non-affected neurons in the peri-infarct region and by function restoration of the lost neural tissue[29-31]. However, in the present study late functional recovery was not followed by complete

restitution of the movements, but by compensatory movements including trunk rotation and inclination, evidencing the weakness of paretic forelimb[7].

Alterations of reach performance were neither associated to acute nor to late muscular atrophy. These results lead us to suppose that neural factors related to muscle weakness, such as, deficits in motor units activation[32,33], changes in the neural central drive[34] and damages to the structural integrity of the corticospinal tract[35] are affecting functional activities rather then intrinsic muscular factors. Considering muscle adaptations during acute phase post-stroke, one study in animals showed atrophy in quadriceps, soleus and tibialis anterior muscles of paretic paw, 3 days after ischemic lesion in mice[23]. The discrepancies between studies findings can be explained by the difference of limbs (forelimb *vs* hindlimb), models of brain ischemia (ET-1 *vs* 60 min of MCAO), and species (rat *vs* mouse) investigated. In addition, it has already been demonstrated in the literature that the degree of muscle atrophy depends on muscular groups analyzed, being more susceptible the antigravity muscles[36]. In humans, little is known about acute muscle adaptations post-stroke[37]. Previous studies showed that post-stroke hemiparetics in acute phase exhibited a decrease in the lean mass of upper and lower paretic limb compared to non-paretic side[38,39].

Regarding late responses of paretic muscle atrophy in humans, little information is available about upper extremity in chronic hemiparetics. Some studies showed a smaller regional muscular mass in the paretic limb compared to non-paretic limb[37], for example, atrophy on index fingers muscle[40] and triceps[41]. In animals, mice showed atrophy in paretic and non-paretic hindlimb 7 days after MCAO[42]. Another study with animals showed atrophy of type II muscle fibers of tibialis anterior of paretic hindlimb, 2 weeks after motor cortex hemorrhagic lesion[43]. Thus, the present study brings important information about acute and late forelimb muscle

adaptation in post-ET-1 brain ischemia model, whilst the literature focused mainly on hindlimb muscle changes in different models.

An important result about the present study involves the detrimental effect of non-paretic limb training on the paretic limb functional recovery in experiment 2. This effect had already been described in previous studies[21,22,44-47]. MacLellan and colls[22] (2013) showed that intense non-paretic reach training (2h/day) might contribute to deficits in long term of rats' paretic limb submitted to motor cortex ischemic lesion by ET-1. Another study that assessed the non-paretic limb training effect, starting 6 and 20 days after ischemic lesion induced by ET-1 in rats, showed smaller representation area of paretic limb on peri-lesion cortex, with a positive correlation with the performance of these limbs. This study indicates that the non-paretic limb training altered cortical map reorganization decreasing the paretic limb function[48].

An interesting result about the present study is that despite the small amount of non-paretic forelimb training, it was enough to impair functional recovery of paretic one and to provoke selective atrophy of distal muscles (fingers extensors). Clinical studies showed that the rates of change of force development in wrist extensor and handgrip strength are good predictors of upper limb function[49]. It can be supposed that the non-paretic upper limb training can harm spontaneous recovery of contralateral corticofugal projections and worsen the weakness in the fingers extensors, which can be associated with muscle atrophy.

This study presents some limitations. For example, the activity of the animals was not controlled in the cage, which. This measure could be important since the animals can explore the environment and bearing weight with all limbs[50]. Moreover, the current study quantified only the minor muscle fiber diameter without distinctions in fiber typing. Certain studies in humans and

in animals after stroke showed a preference for atrophy in type II fibers, and a shift from I to II muscle fibers[43, 51,52].

On the other hand, the study brings important information to rehabilitation, in our best knowledge, it is the first study which established relations among the lesion model, functional deficits and the muscle alterations that occur on the rat's paretic forelimb submitted to a ischemic lesion with ET-1 and learned nonuse. Future studies should try to increase the amount of non-paretic training in attempt to improve translation to post-stroke people.

In conclusion, the ET-1 brain ischemia model provokes early dysfunction of forelimb in rats, and late recovery of function is associated to compensatory movements, but not to muscle atrophy. Nevertheless, the increase of non-paretic forelimb activity impaired function recovery and induced selective muscle atrophy on paretic side.

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CONSIDERAÇÕES FINAIS

Esta tese demonstrou que existe pobre evidência e de qualidade razoável para as adaptações das estruturas musculares em indivíduos pós AVC, os trabalhos apontam para uma atrofia muscular, maior rigidez, quantidade de fibrose e tecido adiposo sem alterações no tecido magro dos músculos distais do membro superior parético em relação ao membro não parético. No entanto, uma vez que o lado não parético também apresentou alterações, o que torna a comparação inadequada, mostrando a necessidade de realizar estudos melhor desenhados abordando esta questão.

Além disso, foi demonstrado que o modelo de isquemia cerebral induzido por endotelina-1 provoca disfunção motora precoce da pata anterior parética de ratos e a recuperação tardia da função está associada a movimentos de compensação motora mas não à atrofia muscular. Além disso, o treino do membro anterior não afetado (simulando o não uso aprendido) prejudica a função da atividade de alcance e induz uma atrofia seletiva do membro parético. Este resultado mostra que o modelo, tanto de lesão isquêmica quanto a simulação do não uso aprendido através do treino de alcance do membro não afetado, parece ser um bom modelo de investigação para o entendimento da alteração muscular após o AVC.

ATIVIDADES NO PERÍODO

No ano de 2017, durante os meses de Abril a Outubro realizei o doutorado sanduíche no Laboratório da Profa. Dra. Theresa A. Jones, na Universidade do Texas em Austin, Estados Unidos, onde desenvolvi um novo projeto. A Profa. Theresa é referência mundial nos mecanismos de recuperação e compensação em modelos de isquemia cerebral de animais. Seus estudos embasaram nossos trabalhos e foi possível estabelecer uma parceria.

Também participei do projeto de extensão "Grupo Terapêutico para indivíduos hemiparéticos crônicos" sob a coordenação do Professor Dr. Thiago Luiz de Russo na Unidade de Saúde Escola (USE) da Universidade Federal de São Carlos em 2015/2016.

Além dos artigos produzidos nessa tese, um artigo foi produzido e aceito na Muscle & Nerve (ANEXO 1), o qual abordou os efeitos do alongamento como tratamento para atrofia após lesão nervosa periférica. Pude ainda co-orientar alunos de graduação e participar de projetos do laboratório na mesma temática.

ANEXOS

INTERMITTENT STRETCHING INDUCES FIBROSIS IN DENERVATED RAT MUSCLE

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ABSTRACT: *Introduction:* Stretching (St) has been used for treating denervated muscles. However, its effectiveness and safety claims require further study. *Methods:* Rats were divided into: (1) those with denervated (D) muscles, evaluated 7 or 15 days after sciatic nerve crush injury; (2) those with D muscles submitted to St during 7 or 15 days; and (3) those with normal muscles. Muscle fiber cross-sectional area, serial sarcomere number, sarcomere length, and connective tissue density were measured. MMP-2, MMP-9, TIMP-1, TGF-β1, and myostatin mRNAs were determined by real-time polymerase chain reaction. MMP-2 and MMP-9 activity was evaluated by zymography. Collagen I was localized using immunofluorescence. *Results:* St did not prevent muscle atrophy due to denervation, but it increased fibrosis and collagen I deposition at day 15. St also upregulated MMP-9 and TGF-β1 gene expressions at day 7, and myostatin at day 15. *Conclusions:* Stretching denervated muscle does not prevent atrophy, but it increases fibrosis via temporal modulation of TGF-β1/myostatin and MMP-9 cascades.

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Peripheral nerve injuries (PNIs) are a challenge to any rehabilitation team. After denervation, skeletal muscle undergoes numerous deleterious changes, including muscle atrophy,[1–3] decreased capacity to generate force,[4] increased proliferation of connective tissue,[5] and consequent loss of flexibility. Other muscular alterations include a shift of myosin heavy chain from type I to II.[6–8]

Morphological/functional changes in denervated muscles are due to molecular alterations of the muscle trophism regulatory pathways, such as hypertrophy,[9,10] atrophy,[1,9] and mass regulation,[1,11] in addition to the control and remodeling pathways of the extracellular matrix (ECM).[5] In this sense, the tumor growth factor-beta (TGF-β1)/myostatin pathway has an important role in mediating mass regulation, as well as ECM modifications.[12] During denervation, TGF-β1 stimulates myostatin. Myostatin, also known as growth differentiation factor-8, is a negative factor in muscle mass control. Increased myostatin gene expression restricts muscle growth and hypertrophy and also stimulates rigidity collagen (I and III) production in the ECM, generating fibrosis.[12]

Among the main agents of ECM remodeling, a family of zinc-dependent enzymes, matrix metalloproteinases (MMPs) stands out; these include MMP-2 and MMP-9 (collagenase A and B, respectively), which are rather common and important for the ECM of skeletal muscle and for collagen turnover, such as type IV.[13] MMP-2 and MMP-9 are regulated by an enzyme known as tissue inhibitor of metalloproteinase-1 (TIMP-1). Such regulation is essential for maintenance of structural tissue integrity.[14]

Early studies showed that there is a lack of data on efficacy and safety of therapeutic resources used in the clinic for denervated muscle treatment. Further study is needed of the resources that stimulate nerve growth and reduce or prevent muscle atrophy. Gigo-Benato *et al.*[15] showed that electrical stimulation seems to impair neuromuscular recovery after nerve crush injury. Furthermore, electrical stimulation associated with or without stretching does not prevent muscle atrophy, although it regulates muscle pathways such as ubiquitin-proteasome, transcription factors (such as the myogenic regulatory factors), myostatin, and the MMPs.[1] On the other hand, studies focused on the use of intermittent stretching showed that it attenuated muscle atrophy and phenotype changes.[8] Therefore, preclinical studies are necessary to investigate parameters for the success or failure of rehabilitation interventions.

Among the main tools used for treatment of denervated muscle, stretching stands out, or is at least noteworthy. The most common form of stretching used in the clinic and sports is intermittent stretching. Most of the findings about the effects of stretching were made in immobilization

Abbreviations: BSA, bovine serum albumin; CSA, cross-sectional area; D, denervated; ECM, extracellular matrix; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MMP, matrix metalloproteinase; N, normal; PB, phosphate buffer; PNI, peripheral nerve injury; St, stretching; TGF-β1, transforming growth factor-beta1; TIMP, tissue inhibitor of metalloproteinases

Key words: muscle atrophy; neurorehabilitation; physiotherapy; skeletal muscle; stretching

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