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Mini invasive skeletal muscle biopsy technique with a tri-axial end cut needle

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Abstract. – OBJECTIVE: Skeletal Muscle Biopsy is a minor surgical procedure for the diagnosis of different neuromuscular pathological conditions and has recently gained popularity also in the research field of age-related muscular modifications and sarcopenia. Few studies focused on the application of mini-invasive muscular biopsy in both normal and pathological conditions. The aim of our study was to describe a mini invasive ultrasound-guided skeletal muscular biopsy technique in complete spinal cord injured (SCI) patients and healthy controls with a tri-axial end-cut needle.

PATIENTS AND METHODS: Skeletal muscle biopsies were collected from 6 chronic SCI patients and 3 healthy controls vastus lateralis muscle with a tri-axial end cut needle (Biopince© – Angiotech). Muscle samples were stained for ATPase to determine fibers composition, moreover, gene expression of cyclooxygenase-1 (COX-1) and prostaglandin E2 receptor has been analyzed by Real Time RT-PCR.

RESULTS: All the procedures were perfomed easily without failures and complications. Control tissue was macroscopically thicker than SCI one. Control specimen displayed an equal distribution of type I and type II fibers, while SCI sample displayed a prevalence of type II fibers SCI specimen displayed a significant reduction in COX-1 gene expression. This mini-invasive approach was easy, accurate and with low complication rate in performing skeletal muscle biopsy in both SCI patients and controls.

CONCLUSIONS: This technique could be useful in conditions in which the overall quantity of specimen required is small like for molecular biology analysis. For histological diagnostic purposes and/or conditions in which the original tissue is already pathologically modified, this technique should be integrated with more invasive techniques.

Key Words:
Skeletal muscle, Biopsy needle, Image-guided biopsy, Molecular biology.

Introduction

Skeletal muscle biopsy is a minor surgical procedure that has been conducted over several decades for the diagnosis of different neuromuscular pathological conditions. This technique has recently gained popularity also in the research field of age-related muscular modifications and sarcopenia[1] evolving from surgical "open approach" to less invasive techniques, in order to be easier to perform and to be more tolerable for the patient[2].

Skeletal muscle contains several fibers deeply differing in their chemical composition and consequently in their twitch properties. It is generally accepted that muscle fibers can be divided into two main types: slow twitch (type I) and fast twitch (type II a,b,c) muscle fibers. Slow twitch fibers fire more slowly than fast ones and can sustain contraction for long periods of time before they fatigue. On the other hand, fast twitch fibers generate faster strenght and speed but they fatigue more quickly. These distinctions seem to influence how muscles respond to training and physical activity, and each fiber type has unique contraction ability[3-5]. On average, human muscles contain an equal percentage of slow and fast twitch fibers, however it has been reported a switch in this ratio in some pathological conditions like chronic neurological diseases and in

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particular spinal cord injury (SCI)[6,7]. Thus, fiber type discrimination and their relative distribution are essential to distinguish between normal and pathological skeletal muscle tissue.

Muscle tissue biology is regulated by a wide array of bioactive compounds among which prostaglandins (PG), and in particular prostaglandin E_2 (PGE_2) play a pivotal role[8]. PG are a family of active compounds produced by the ubiquitous cyclooxygenase (COX) enzymes (COX 1 and 2) and elicit an array of tissue-specific functions[9-10]. In skeletal muscle, PGE_2, produced by both isoforms of COX[11,12], regulate protein synthesis and degradation after exercise[13-15], along with being involved in muscle tissue injury recovery[16,17]. PGE_2 receptors are classified into four subtypes, Ep1, Ep2, Ep3, and Ep4, all of which interact with different G-proteins and their subsequent signaling pathways[18,19].

At present time, few studies focused on the application of mini-invasive biopsy techniques on skeletal muscle tissue in both normal and pathological conditions. In light of these considerations the aim of our study was to describe a mini invasive ultrasound-guided skeletal muscular biopsy technique in complete SCI patients and healthy controls with a tri-axial end-cut needle (Biopince© – Angiotech, Stenlose, Denmark). Moreover, this study focused on morphological (fiber type discrimination), molecular (COX-1 and EPs gene expression) and practical procedure related aspects (feasibility, complication rate and accuracy).

Patients and Methods

Subjects

Six chronic SCI patients (age: 39.8 ± 5.7 years, AIS score: A 66%, B 33%) were consecutively enrolled from the Physical and Rehabilitation Unit of the AOU "Maggiore della Carità" in Novara (Italy). Inclusion criteria were the following: spinal cord injury graded A or B on the American Spinal Injury Association (AIS) Impairment Scale[20]. Exclusion criteria were: diabetes mellitus, oral anticoagulation, osteosynthesis of the femur, and history of hepatitis B, C, or D or human immunodeficiency virus infection. All patients were male and had a complete AIS impairment scale score of A or B (complete motor lesion), and their level of neurological spine injury ranged from C5 to L1. The time since injury ranged from 3 to 11 years (mean time from injury: 6 ± 2.5 years).

Muscle specimens of 3 healthy controls (age: 34 ± 6.9 years) were obtained during knee surgery for cruciate ligament reconstruction in operatory room under sedation. The inclusion criteria for controls were the following: age > 18. The exclusion criteria were the same as for SCI group.

Written informed consent was obtained from each patient before biopsy after a full explanation of the procedure which was conducted according to the Declaration of Helsinki guidelines.

Muscle Biopsies

Skeletal muscle samples were obtained using a percutaneous tri-axial end cut needle (Biopince© – Angiotech, Stenlose, Denmark) of 18-gauge. Biopsies were collected from vastus lateralis muscle at two-third of the distance from trochanter as previously described by Leger et al[21,22]. Correct needle positioning in the target muscle was confirmed by ultrasound (US) (Hitachi – EUB-5500 Digital Ultrasound Scanner®), performed by a physician with specific experience in muscular sonography with a linear transducer (AL53L – scanning frequence 10 MHz). All biopsies were performed under local anesthesia (lidocaine 1% in 5 ml). A single incision was made in the skin, and two different muscle samples were consecutively taken for each patient.

The stroke length of the biopsy was adjusted according to the muscle size considering the presence of blood vessels and other vulnerable structures proximity; 33 mm stroke length was chosen whenever possible. All specimen were immediately frozen in liquid nitrogen until use.

Data about procedure related complications, easiness of needle US imaging and any other technical issue have been recorded by the operator.

ATPase Staining

Frozen sections of 10 μm thick were cut at –20°C and stored at –20°C until staining procedure. Fiber types were determined by the differential staining resulting from incubation at pH 9.6. This staining allows to distinguish type I (light staining) from type II (dark staining) fibers. Briefly, samples were incubated with 0.5 mg/mL ATP dissolved in 0.1 M glycine/0.1 M NaCl buffer with 0.75 M $CaCl_2$, for 11 minutes at 37 °C. Samples were then rinsed well in distilled water and incubated for 5 minutes at room temperature with 2% $CoCl_2$. Samples were extensively washed in both distilled and tap water.

Specimens were, then, incubated for 30 seconds in 2% ammonium sulphide solution, rinsed well in tap water and observed with an optical microscope (Leica DMIL, Leica Microsystems Wetzlar GmbH, Wetzlar, Germany).

Real-time Reverse Transcription-Polymerase Chain Reaction (Real Time RT-PCR)

Total RNA was isolated from tissue specimen using the Ribozol RNA extraction reagent (Amresco LLC, Solon, OH, USA). mRNA was converted into complementary DNA (cDNA) using the high capacity cDNA reverse transcription kit (Applied Biosystems,Carlsbad, CA, USA). cDNA mixtures were subjected to real-time PCR using SYBR® Green I dye (Applied Biosystems). Reactions were performed using the following primer sequences[23]: COX-1 forward: 5′-ACCTTGAAGGAGTCAGGCATGAG-3′; COX-1 reverse: 5′-TGTTCGGTGTCCAGTTCCAATA-3′; EP2 forward: 5′-TGAAGTTGCAGGCGAGCA-3′; EP2 reverse: 5′-GACCGCTTACCTGCAGCTGTAC-3′; GAPDH forward: 5′-GAGTCAACGGATTTGGTCGT-3′; GAPDH reverse: 5′-GACAAGCTTCCCGTTCTCAG-3′. All real-time PCR reactions were performed in triplicate using a Biorad CFX96 instrument. Gene expression levels were normalized using GAPDH as housekeeping gene and gene expression was calculated as $2^{-\Delta Ct}$, where $^{\Delta}Ct$ = Ct gene – Ct housekeeping[24].

Statistical Analisys

Mann-Whitney U test was utilized for statistical analysis. Statistical procedures were performed with Prism 5.0 Statistical Software (GraphPad Software Inc, CA, USA). Probabiliy of $p < 0.05$ were considered statistically significant.

Results

A total of 18 specimens were collected (12 from SCI and 6 from healthy controls). All the procedures were perfomed easily without failures and in all cases specimen were easily removed. US needle visibility was good and required in five cases only a small correction of needle insertion angle to visualize it. No biopsy had to be abandoned because of insufficient visibility of the needle. There were no cases of inadequate/insufficient material collection, however SCI specimen were macroscopically thinner and brittler compared to those of healthy subjects.

Complications were observed in 40% of the procedures and were all represented by self-limiting small hemorrages requiring no further medical treatment.

From a macroscopic point of view, control tissue was thicker than SCI one, which, from a miscroscopical point of view also displayed some structural degradation (vacuolization).

From an histological point of view the quantity of specimen collected was sufficient to perform ATPase staining. As shown in Figure 1 control specimen displayed an equal distribution of type I (light staining) and type II (dark staining) fibers, while the pathological sample displayed a prevalence of type II fibers.

To maximize information gain from biopsy specimen obtained during this study further molecular analysis were performed in order to evaluate COX-1 and EP2 gene expression. As shown in Figure 2, EP2 expression did not display any significant difference between SCI patients and healthy controls. Interestingly COX-1 expression reduction was statistically significant in SCI patients compared to healthy controls ($p < 0.05$).

Discussion

This study results highlighted the feasibility and accuracy of the tri-axial end-cut needle (Biopince© – Angiotech) biopsy in discriminating type I and II muscle fibers in complete SCI pa-

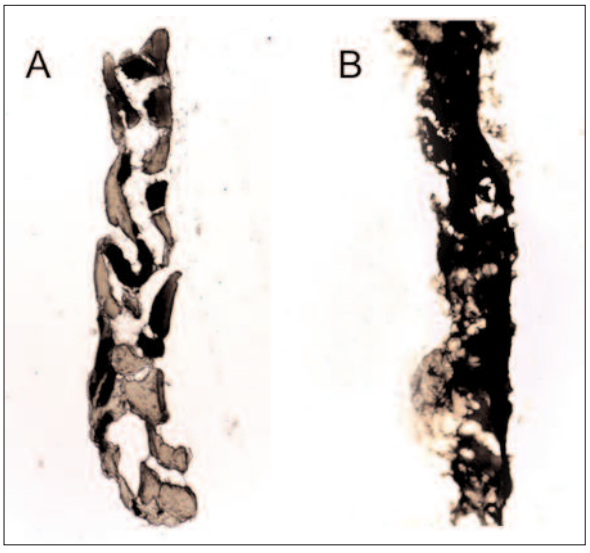

Figure 1. Atpase staining of an healthy control ***(A)*** and a SCI patient ***(B)***. Type I fiber stain light and Type II fiber stain dark. Magnification 10×.

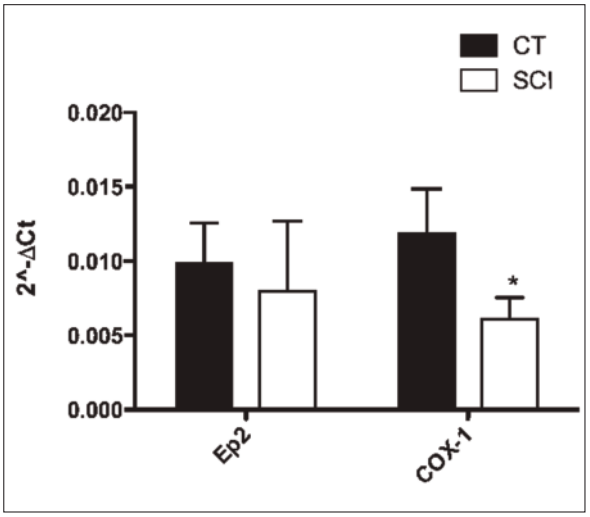

Figure 2. Gene expression quantification by real time RT-PCR. EP2: Prostaglandin E2 receptor; COX-1: Cyclooxygenase I; CT: healthy controls; SCI: Spinal Cord Injured patients. *$p < 0.05$.

tients and healthy controls. Moreover, the complication rate was also evaluated, showing only self-limiting hemorrages requiring no additional treatment.

Skeletal muscle biopsy is often essential in clinical setting to confirm a previous diagnosis emerged from imaging or circulating biomarkers. Thus, this approach should be a compromise between expected definitive diagnosis and complications and discomfort for the patient. For this reason image-guided percutaneous biopsy performed with a fine needle represent an interesting tool to solve this issue. A recent paper investigated the feasibility, dignostic accuracy and complication rate of biopsies performed in different soft tissues with a tri-axial end cut needle (Biopince© – Angiotech). Authors showed that this 18-gauge biopsy needle was easy to handle allowing both ultrasound and computed tomography-guided percutaneous biopsies of various organs. Moreover, the accuracy of biopsies was high, without crush artifacts and with low complication rate. However, even if this study was performed on a sample of 100 cases, only one biopsy was performed on skeletal muscle tissue[25]. Furthermore, previous studies, even with ultra thin (21-25 g) needles were limited to citologic diagnosis in malignancies and were not applicable in benign lesions or conditions in which larger samples for histological evaluations are needed[26]. Thus, in these circumstances, more invasive and technical demanding approaches, like core biopsies, are recommended, providing a larger specimen for histological evaluation. However, the quality of the specimen does not only depend on the tissue amount but also on the eventual presence of crush artifacts and pathological modifications[27]. A good example of pathological modifications that could be observed in muscle specimen is represented by SCI, where it has been previously described a fiber atrophy, myosteatosis and a shift from type I to type II fiber prevalence[6] and this last pathological feature, called "fiber shift" has been previosly described also in other chronic neurological conditions like stroke[6,7].

The results obtained in SCI specimen were consistent with these macroscopic pathological modifications, being thinner and more fragile compared to those of healthy controls. For a long time, fiber type determination in muscle specimens has been based on the classical myosin ATPase staining method developed by Padykula and Herman in 1955[28,29]. This method underwent several modifications, including the use of glycine buffer instead of sodium barbital buffer[30] to incubate tissue specimen maintaining a constant pH, providing in the end better histochemical results. Regarding fiber type identification, after alkaline ATPase staining, the results obtained in SCI patients were consistent with those previously described in literature[6], showing the expected shift from type I to type II fibers.

In this study the expression of some genes involved in muscle tissue biology like COX-1 and EP2 has been investigated. It is known that COX genes are involved in PGE_2 metabolism. COX-1 is traditionally viewed as a constitutively expressed protein primarily displaying homeostatic functions[9,11]. To the best of our knowledge, there are no studies focused on these genes expression modification in SCI patients. Thus, the results obtained seem to be promising, as they highlight that, like in previous animal studies[16], in an atrophic muscle an altered expression of COX genes is observed.

Conclusions

Results obtained herein support the easiness, accuracy and low complication rate of a mini-invasive percutaneous tri-axial end cut needle biopsy in skeletal muscle of both SCI patients and healthy controls. This technique could be a useful tool, even for definitive diagnosis, in conditions, like malignancies, in which the

overall quantity of specimen to perform a citological or molecular analysis is really small. Similarly, this technique could be suitable for research purposes, to perform molecular analysis, even in benign conditions. However, for histological diagnostic purposes and/or conditions in which the original tissue is already pathologically modified, like skeletal muscle in SCI, this technique could not be the most appropriate and should be integrated with more invasive core biopsy techniques.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

References

1) Lang T, Streeper T, Cawthon P, Baldwin K, Kaaffe DRT, Harris TB. Sarcopenia: etiology, clinical consequences, intervention, and assessment. Osteoporos Int 2010; 21: 543-559.
2) Goutman SA, Prayson RA. Role of Repeat Skeletal Muscle Biopsy: How useful is it? Muscle Nerve 2013; 47: 835-839.
3) Brooke MH, Kaiser KK. Muscle Fiber types: How many and what kind? Arch Neurol 1970; 23: 369-379.
4) Brooke MH, Williamson T, Kaiser KK. The behaviour of four fiber types in developing and reinnervated muscle. Arch Neurol 1971; 25: 360-366.
5) Essen B, Janson E, Henriksson J, Taylor AW, Saltin B. Metabolic characteristics of fibre types in human skeletal muscle. Acta Physiol Scand 1975; 95: 153-165.
6) Grimby G, Broberg C, Krotkiewska I, Krotkiewski M. Muscle fiber composition in patients with traumatic cord lesion. Scand J Rehab Med 1976; 8: 37-42.
7) Carda S, Invernizzi M, Cisari C. Sarcopenia or muscle modifications in neurologic diseases: a lexical or patophysiological difference? Eur J Phys Rehabil Med 2013; 49: 119-130.
8) Mo C, Romero-Suarez S, Bonewald L, Johnson M, Brotto M. Prostaglandin E2: from clinical applications to its potential role in bone- muscle crosstalk and myogenic differentiation. Recent Pat Biotechnol 2012; 6: 223-229.
9) Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LB, Lipsky PE. Cyclooxygenase in biology and disease. FASEB J 1998; 12: 1063-1073.
10) Sudbo J, Reith A, Florenes VA, Nesland JM, Ristimaki A, Bryne M. COX-2 expression in striated muscle under physiological conditions. Oral Dis 2003; 9: 313-316.
11) Testa M, Rocca B, Spath L, Ranelletti FO, Petrucci G, Ciabattoni G, Naro F, Schiaffino S, Volpe M, Reggiani C. Expression and activity of cyclooxygenase isoforms in skeletal muscles and myocardium of humans and rodents. J Appl Physiol 2007; 103: 1412-1418.
12) Vane JR, Bakhle YS, Botting RM. Cyclooxygenases 1 and 2. Annu Rev Pharmacol Toxicol 1998; 38: 97-120.
13) Burd NA, Dickinson JM, Lemoine JK, Carroll CC, Sullivan BE, Haus JM, Jemiolo B, Trappe SW, Hughes GM, Sanders CE Jr, Trappe TA. Effect of a cyclooxygenase-2 inhibitor on postexercise muscle protein synthesis in humans. Am J Physiol Endocrinol Metab 2010; 298: E354-E361.
14) Palmer RM. Prostaglandins and the control of muscle protein synthesis and degradation. Prostaglandins Leukot Essent Fatty Acids 1990; 39: 95-104.
15) Reeds PJ, Palmer RM, Wahle KW. The role of metabolites of arachidonic acid in the physiology and pathophysiology of muscle protein metabolism. Biochem Soc Trans 1987; 15: 328-331.
16) Bondesen BA, Mills ST, Kegley KM, Pavlath GK. The COX-2 pathway is essential during early stages of skeletal muscle regeneration. Am J Physiol Cell Physiol 2004; 287: C475-C483.
17) Shen W, Li Y, Zhu J, Schwendener R, Huard J. Interaction between macrophages, TGF-1, and the COX-2 pathway during the inflammatory phase of skeletal muscle healing after injury. J Cell Physiol 2008; 214: 405-412.
18) Watabe A, Sugimoto Y, Honda A, Irie A, Namba T, Negishi M, Ito S, Narumiya S, Ichikawa A. Cloning and expression of cDNA for a mouse EP1 subtype of prostaglandin E receptor. J Biol Chem 1993; 268: 20175-20178.
19) Hasegawa H, Katoh H, Yamaguchi Y, Nakamura K, Futakawa S, Negishi M. Different membrane targeting of prostaglandin EP3 receptor isoforms dependent on their carboxy-terminal tail structures. FEBS Letters 2000; 473: 76-80.
20) Ditunno JF Jr, Young W, Donovan WH, Creasey G. The international standards booklet for neurological and functional classification of spinal cord injury. Paraplegia 1994; 32: 70-80.
21) Leger B, Senese S, Al-Khodairy AW, De Riaz O, Gobelet C, Giacobino JP, Russell AP. Atrogin-1, MuRF1 and FoXO, as well as phosphorilated GSK-3and 4E-BP1 are reduced in skeletal muscle of chronic spinal cord-injured patients. Muscle Nerve 2009: 40: 69-78.

22) Ditor DS, Hamilton S, Tarnopolsky MA, Green HJ, Craven BC, Parise G, Hicks AL. Na,K -ATPase concentration and fiber type distribution after spinal cord injury. Muscle Nerve 2004; 29: 38-45.

23) Shoji M, Tanabe N, Mitsui N, Tanaka H, Suzuki N, Takeichi O, Sugaya A, Maeno M. Lipopolysaccharide stimulates the production of prostaglandin E2 and the receptor Ep4 in osteoblasts. Life Sci 2006; 78: 2012-2018.

24) Panariti A, Miserocchi G, Rivolta I. mRNA expression profile of selected oxygene sensing genes in the lung during recovery from chronic hypoxia. Biol Res 2013; 46: 169-176.

25) Diederich S, Padge B, Vossas U, Hake R, Eidt S. Application of a single needle type for all image-guided biopsies: results of 100 consecutive core biopsies in various organs using a novel tri-axial, end-cut needle. Cancer Imaging 2006; 6: 43-50.

26) Oikonomou A, Matzinger FR, Seely JM, Dennie JC, Macleod PJ. Ultrathin needle (25 G) aspiration lung biopsy: diagnostic accuracy and complication rates. Eur Radiol 2004; 14: 375-382.

27) Moore EH. Technical aspects of needle aspiration lung biopsy: a personal perspective. Radiology 1998; 208: 303-318.

28) Padykula HA, Herman E. Factors affecting the activity of adenosine triphosphatase and other phosphatases as measured by histochemical techniques. J Histochem Cytochem 1955; 3: 161-169.

29) Padykula HA, Herman E. The specificity of the histochemical method for adenosine triphosphatase. J Histochem Cytochem 1955; 3: 170-195.

30) Round JM, Matthews Y, Jones DA. A quick, simple and reliable histochemical method for ATPase in human muscle preparations. Histochem J 1980; 12: 707-710.