European Review for Medical and Pharmacological Sciences 2014; 18: 416-420

Histological and ultra-structural effects of rapid muscle expansion through intramuscular administration of carbon dioxide: an intra-operative study in an animal model

E. PACELLA, G. NISI[1], M. CAMPANA[1], F. PACELLA, F. MAZZEO, C. BRANDI[1], M.M. DE SANTI[2], V. MALAGNINO[2], P. TANGANELLI[2], C. D'ANIELLO[1]

Department of Sense Organs, Faculty of Medicine and Dentistry Sapienza University of Rome, Italy
[1]Department of General and Specialistic Surgery; Plastic and Reconstructive Surgery Unit; "Santa Maria alle Scotte" Hospital, University of Siena, Italy
[2]Department of Human Pathology and Oncology; "Santa Maria alle Scotte" Hospital, University of Siena, Italy

Abstract. – INTRODUCTION: Carbon dioxide (CO_2) therapy refers to trans-cutaneous or subcutaneous administration of CO_2 for therapeutic purposes, and recent studies have pointed out that it produces a vasodilation effect after it is locally injected, which helps amplify the reconstructive potentiality of an expanded-muscle flap.

MATERIALS AND METHODS: Thirty male Wistar rats, weighting between 350 and 400 g, were randomly divided into three groups of 10. In the first group, single intra-operative rapid expansion was carried out under the right latissimus dorsi muscle. In the second group, for five days prior to surgery, a pre-treatment with intramuscular injections of CO_2 was performed. The third group served as controls. For each group, the latissimus dorsi muscle was fixed as soon possible after mice died, and ultrathin sections of it examined with transmission electron microscope.

RESULTS: In the treated group, the majority of expanded muscles showed a normal striation pattern, whereas a few fibers showed mild disorganization of the myo-filaments in the sarcomeres, which appeared overstretched (average 2.37 µm).

CONCLUSIONS: This evidence could demonstrate a greater capacity of muscle recovery after treatment by CO_2 expansion.

Key Words:
Carbon dioxide therapy, Tissue expander, Ischemia, Neoangiogenesis.

Introduction

Intra-operatory acute tissue expansion is a reliable technique that helps amplify the reconstructive potentialities of a muscle flap normally used for the coverage of wider tissue defects. Although clinical research has confirmed its usefulness, the histological and biochemical mechanisms governing muscular compliance to acute expansion are still not completely understood[1-4]. In addition, recent studies have pointed out a vasodilatation effect after local injections of carbon dioxide[5] which can be of help when a muscular flap needs to be harvested. In this study, we executed a tissue expansion of the dorsal muscle of male Wistar rats. In order to achieve this tissue expansion, Carbomed® equipment was used for the controlled release of medical CO_2, and a daily 5 ml dose was injected, Successively, muscle samples of treated an non-treated mice were examined with the aim of evaluating the biochemical and histological muscular modifications after intra-operatory acute expansion. By examining the latissimus dorsi muscle of rats which had been pre-treated with local injections of CO_2, the results suggested that a controlled infusion of CO_2 might improve muscle trophism, thus, facilitating the quality of tissue recovery and ultimately improve therapeutic prospects overall.

Materials and Methods

Surgical Procedures and CO_2 Administration Protocol on an Animal Model

Thirty male Wistar rats, weighting from 350 to 400 g, were randomly divided into three groups of 10 each (group A, B and C). All the animals were kept in cages, and fed with food and water according to their needs. Independently from the

Corresponding Author: Fernanda Pacella, MD; e-mail: fernanda.pacella@gmail.com

group to which they belonged, every rat was submitted to surgery under general anaesthesia with intra-peritoneal ketamine.

Group A was used as control. Group B, after hair removal and disinfection of the dorsal area, underwent single intra-operative rapid expansion under the right latissimus dorsi muscle, through a lateral access and insertion of a Foley-type 10 Fr catheter, whose extremity was inflated with water (in the quantity of [(weight/50) x cc]), in order to compensate for possible weight differences among individual rats (Figure 1). The device was left in site for 45 minutes, and subsequently deflated and removed. Group C underwent intramuscular injections of CO_2 to right latissimus dorsi muscle during the five days prior to surgery. Carbomed® equipment was used for the controlled release of medical CO_2, and a daily 5 cc dose was injected. These rats did not undergo any pre-treatment. All the rats were sacrificed seven days after surgery. Removal of both latissimus dorsi muscles was performed from every animal. It was possible to obtain 5 series of muscular specimens as follows: (a) Expanded muscles with pre-treatment: 10 units; (b) Expanded muscles without pre-treatment: 10 units; (c) Non expanded muscles of non pre-treated rats: 10 units; (d) Non expanded muscles of non pre-treated rats: 20 units; (e) Muscles not expanded, modified only by insertion of the catheter: 10 units. The muscles were subjected to measurements (length, weight), fixed in formalin 10% and sent to laboratory for histological analysis. All data was processed for statistical analysis.

Transmission Electron Microscopy

For each group, the latissimus dorsi muscle was fixed as soon as is possible after mice died.

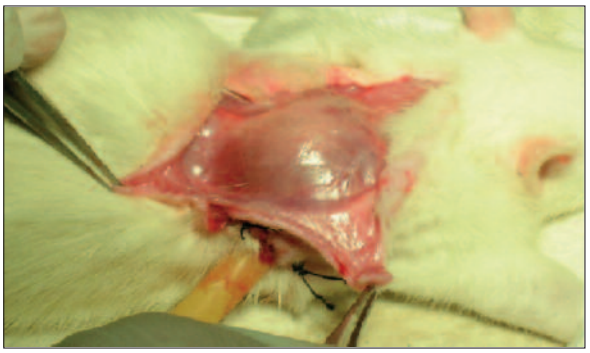

Figure 1. An example of single intraoperative rapid expansion carried out under the right latissimus dorsi muscle through a lateral access and insertion of a Foley-type 10 Fr catheter.

The muscle was isolated and dissected, and the tissue was quickly skinned and pinned to a piece of cork in the fully extended position and immediately fixed in 2.5% cacodylate-buffered glutaraldehyde, pH 7.3, at 4°C. After 1 hour, the muscle was minced into approximately 2 mm^3 pieces and replaced in the same fixative for a further 3 hours. The specimens were washed overnight in the same buffer, post-fixed in buffered, 1% osmium tetroxide for 2 hours, washed, dehydrated through a graded series of ethanol, and then cleared in propylene-oxide and embedded in Epoxy resin (Araldite).

Semithin sections 1 μm thick were cut with glass knives on an LKB V Ultratome (Washington, DC, USA), stained with 1% toluidine blue and examined under the light microscope for a general evaluation of tissue morphology. Ultrathin sections from selected areas were cut with a diamond knife using the same ultra-microtome. They were then retrieved and placed onto uncoated copper grids, double-stained with uranyl acetate/lead citrate and examined at 100 kV with a Philips 208 S transmission electron microscope.

Statistical Analysis

For each group we measured 75 sarcomeres. The length of sarcomeres was measured using Image J. The differences among groups were analyzed by analysis of variance (Kruskall-Wallis test for non parametric data, $p < 0.05$). The Kruskall-Wallis test was followed by Dunn's post hoc test.

Results

Transmission Electron Microscopy

Group A (Control group). The muscle fibers of control mice displayed a normal architectural order, with regularly arranged, uniformly sized and shaped myofibrils and peripherally located nuclei. The ultra-structural appearance of the sarcomeres, ranged from 1.5-1.8 μm in length (average 1.60 μm), and were homogenous. Small mitochondria were situated at both sides of Z lines and transverse tubules formed well structured triads with two cisterns of sarcoplasmic reticulum.

The vessels were normally shaped, exhibiting thin endothelial cells encircled by a continuous basement membrane (Figure 2).

Group B (Expansion). The majority of expanded muscles showed a normal striation pattern, whereas few fibers showed a mild disorganiza-

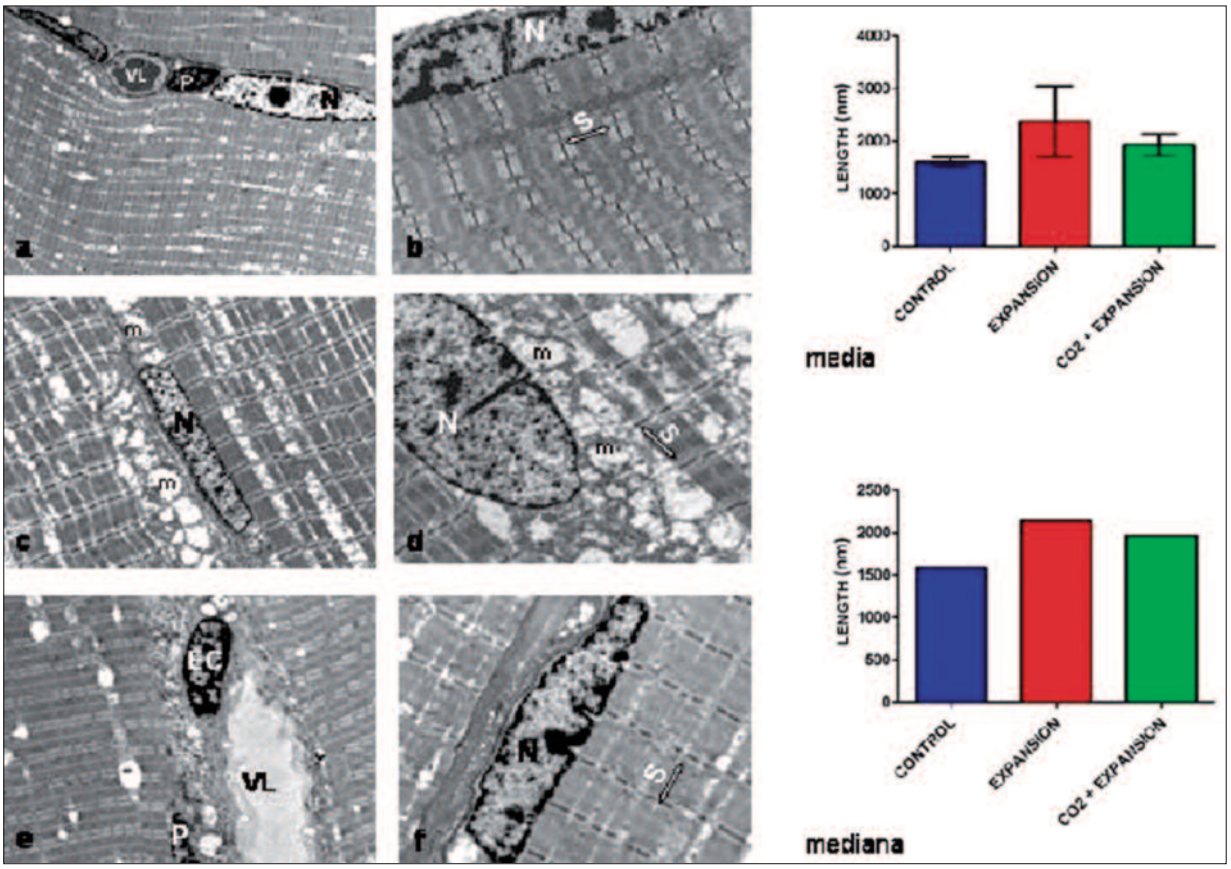

Figure 2. Control group. ***a,*** Low magnification (× 3.500) of muscular tissue, exhibiting a remarkable architectural order; a normal shaped blood vessel is intercalated among two myofibres (N: nucleus of a myofibre; VL: vascular lumen; P: pericyte). ***b,*** Higher magnification (× 7.100), showing a peripherally located, flattened nucleus (N) and regularly arranged, running lengthwise sarcomeres (S). Expansion alone. ***c,*** Low magnification (× 3.500), showing an increment of intermyofibrillar network and swollen mithocondria (m). ***d,*** At higher magnification (× 7.100), the sarcomeres appears overstretched. A nucleleus (N) is centrally located; the mitochondria (m) are swollen. (CO_2+ expansion). ***e,*** Low magnification (× 3.500), showing conserved myofibrillar organization. An enlarged blood vessel (EC: endothelial cell; P: pericyte; VL: vascular lumen) is intercalated among two myofibres. ***f,*** Higher magnification (× 7.100) of two adjacent myofibres showing organized, uniformly sized sarcomeres. The nucleus (N) is close to sarcolemma The intermyofibrillar spaces appear slightly incremented.

tion of the myo-filaments in their sarcomeres, which appeared overstretched (average 2.37 µm). In these altered areas, empty vacuoles and mitochondria with a swollen appearance were observed interspersed among myofibrils, and mainly in the peri-nuclear region where they formed small clusters. No signs of interstitial fibrosis, necrosis or inflammation were observed. Vascularization had a normal morphology (Figure 2).

Group C (CO_2+ expansion). All the examined specimens exhibited a normal appearance, with sarcomeres organized in regular arrays and peripheral nuclei. The mean length of the sarcomere was 1,9 µm. Most mitochondria were homogeneous in size and shape, but a few showed evidence of swelling, especially the ones adjacent to the nucleus, where some empty peri-nuclear vacuoles were also seen. Micro-vessel density appeared increased and demonstrated a vascular network characterized by an alternation of thick and thin endothelial cells filled with Weibel-Palade bodies. Both basal membrane and intima appeared thickened whilst the pericytes were evident (Figure 2).

Discussion

The use of tissue flaps to cover a loss of substance is one of the main principles of reconstructive surgery. Flaps are tissue transplants carried out maintaining the vascular connections with the donor site. Radovan, in 1982[6], first used

mechanical expansion of tissues for reconstructions, creating a silicone balloon that could be implanted subcutaneously(or even under muscles) and which could be slowly and progressively inflated from the outside by means of water injections. The mechanical push, due to the volumetric increment of the device, was found to be effective in expanding the overlying tissues, thus amplifying their reconstructive potentialities. Subsequently, several measures and shapes of tissue expanders were produced[7], and they are still successfully used in reconstructive surgery now. Tissue expansion is a safe technique, but it requires two surgical steps, a relatively long expansion time , and has some limits due to the parenteral implant of a foreign body. With regard to the progressive slow expansion of muscular tissues, Kim et al[8], in a histological analysis of the gracile muscle of rats, found that, after three-weeks of expansions, the normal structural features did not modify, but an increase in the number of sarcomeres in expanded muscular fibres resulted. Conversely, Gur et al[9], in 1998. found evidence that tissue expansion can lead to mechanical and ischemic injuries. Rapid intra-operative tissue expansion is an acute mechanical expansion of the tissues during the reconstructive act. Similar to slow expansion, it amplifies the reconstructive potentialities of a general muscular flap, in that the coverage of tissue losses are more extended if compared to the flap itself. Although clinical evidence confirms the usefulness of this technique, the histological and biochemical rules of the phenomena leading to muscular adaptability and rapid expansion are still poorly known. Hauben et al[10] in 2010 created a mechanical device for fast expansions in muscular-cutaneous reconstructions. However, it is important to emphasize that rapid expansion can lead to mechanical damage due to muscle stretching. Furthermore, ischemia may result, caused by traction of tissues in the acute phase of intervention, and may compromise the vitality of the expanded flap. It is well known that tissue expansion always provokes ischemia of variable duration. Prevention of the ischemic effects on acutely expanded tissues can be achieved by some ischemic preconditioning.

The histological analyses of the prepared muscles investigated revealed and quantified the possible tissue injuries and ultra-structural modifications of the muscular compound after expansion. This was demonstrated by evaluating the following parameters: muscular trophism, myofibril aspect, myofibril degeneration and/or regeneration, inflammatory phenomena, increase or lessening of blood vessels, anatomic abnormalities of blood vessels, necrosis.

Clinical studies have pointed out that local administration of CO_2 has positive effects on tissue microcirculation, increasing oxygen partial pressure and vascular wall motility in the treated areas. It is well known that CO_2 provokes vasodilatation and increases oxygen release in tissues (Bohr effect)[11-13].

In fact, our electron microscopy study of the treated areas has shown that the controlled administration of CO_2 seems to have a positive effect on microcirculation, causing an increased density of the vascular network. as characterized by the alternation of thick and thin endothelial cells filled with Weibel-Palade bodies. Furthermore, both basal membrane and intima appeared thicker than in the two groups where CO_2 was not delivered. These structural changes could contribute to improved tissue oxygenation and, thus, to lower the occurrence of ischemic events during rapid expansion.

The increase in the local microcirculation might explain the better preservation of the structures observed in sarcomeres. After the rapid expansion, in fact, an organization into regular arrays and peripheral nuclei, a lower overstretching, as well as a better preservation of mitochondria was noted in the treated group respect to the other two groups.

This evidence could demonstrate a better tolerance or a greater capacity of recovery after CO_2 expansion.

Conclusions

The controlled administration of carbon dioxide in an animal model, thanks to its proven ability to improve tissue trophism, has a favorable effect on the muscles undergoing a rapid expansion. This feature, no doubt, requires more detailed studies in humans, bur could be of major utility if it were to be applied in the surgical field, improving the characteristics of the expanded tissue and whilst reducing the time of expansion required.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References

1) Crosby MA, Dong W, Feng L, Kronowitz SJ. Effect of intraoperative saline fill volume on perioperative outcomes in tissue expander breast reconstruction. Plast Reconstr Surg 2011; 127: 1065-1072.
2) Pusic AL, Cordeiro PG. An accelerated approach to tissue expansion for breast reconstruction: experience with intraoperative and rapid postoperative expansion in 370 reconstructions. Plast Reconstr Surg 2003; 111: 1871-1875.
3) Shor G, Regev D, Amir A, Halperin M, Giler S, Hauben DJ. Balloon expansion of the pectoralis major muscle flap in sternoplasty: a biomechanical and histologic study in a rat model. Plast Reconstr Surg 2004; 113: 942-948.
4) Iwahira Y, Maruyama Y. Combined tissue expansion: clinical attempt to decrease pain and shorten placement time. Plast Reconstr Surg 1993; 91: 408-415.
5) Savin E, Bailliart O, Bonnin P, Bedu M, Cheynel J, Coudert J, Jean-Paul Martine JP. Vasomotor effects of transcutaneous CO_2 stage II peripheral occlusive arterial disease. Angiology 1995; 46: 785-791.
6) Radovan C. Breast reconstruction after mastectomy using the temporary expander. Plast Reconstr Surg 1982; 69: 195-208.
7) McGeorge DD, Mahdi S, Tsekouras A. Breast reconstruction with anatomical expanders and implants: our early experience. Br J Plast Surg 1996; 49: 352-357.
8) Kim KH, Hong C, Futrell JW. Histomorphologic changes in expanded skeletal muscle in rats. Plast Reconstr Surg 1993; 92: 710-716.
9) Gur E, Hanna W, Andrighetti L, Semple JL. Light and electron microscopic evaluation of the pectoralis major muscle following tissue expansion for breast reconstruction. Plast Reconstr Surg 1998; 102: 1046-1051.
10) Hauben DJ, Shulman O, Levi Y, Sulkes J, Amir A, Silfen R. Use of the SpaceMaker balloon in sternal wound closure: comparison with other techniques. Plast Reconstr Surg 2001; 108: 1582-1588.
11) Hartmann BR, Bassenge E, Pittler M. Effect of carbon dioxide-enriched water and fresh water on the cutaneous microcirculation and oxygen tension in the skin of the foot. Angiology 1997; 48: 337-343.
12) Nach R, Zandifar H, Gupta R, Hamilton JS. Subcutaneous carboxytherapy injection for aesthetic improvement of scars. Ear Nose Throat J 2010; 89: 64-66.
13) Brandi C, Grimaldi L, Nisi G, Brafa A, Campa A, Calabrò M, Campana M, D'Aniello C. The role of carbon dioxide therapy in the treatment of chronic wounds. *In vivo* 2010; 24: 223-226.