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# Skeletal Muscle Mitochondrial Function in Peripheral Arterial Disease: Usefulness of Muscle Biopsy

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## 1. Introduction

### 1.1 Peripheral arterial disease

Peripheral arterial disease (PAD) is a manifestation of atherosclerosis which produces stenoses and occlusions in lower limbs arteries. PAD was commonly divided in four stages, introduced by Rene Fontaine in 1954 (Fontaine *et al.*, 1954): stage 1 defined an asymptomatic patient, stage 2 defined a patient presenting with a significant impairment of his ability to walk (intermittent claudication). Then claudication worsens and the patient develops rest pain in stage 3, and non-healing ulcers or gangrene in stage 4.

Recently, other criteria have been proposed for the diagnosis of PAD. Stage 2 of Leriche is now called « functional ischemia », and stages 3 and 4 are now called « critical ischemia » (Norgren *et al.*, 2007). Critical ischemia is called this way because of its poor prognosis. With this new classification, the diagnosis of critical limb ischemia requires both clinical criteria, but also hemodynamic criteria (ankle-brachial index, toe pressure). Normal values of ankle-brachial index are between 0,9 and 1,3. PAD is characterized by ankle-brachial values under 0,9 (0,4-0,9: functional ischemia, <0,4: critical ischemia). The normal value of the toe pressure is 60-65 mm Hg, it can be normal or within the limits of the normal in functional ischemia, but it is commonly under 10 mm Hg in critical ischemia. These hemodynamic criteria objectify the arterial etiology of the lesions, because it is sometimes difficult to define the exact origin of rest pain or tissue loss (diabetes, venous insufficiency…).

Insufficient oxygen supply secondary to reduced blood flow is presumed to be the main physiologic cause for the manifestations of peripheral arterial disease, but more recently the presence of mitochondriopathy in chronically ischemic skeletal muscle has been proposed. Suboptimal energy production from defective mitochondria participates in PAD pathogenesis in addition to reduced oxygen supply (Marbini *et al.*, 1986; Lundgren *et al.*, 1989; Bhat *et al.*, 1999; Brass *et al.*, 2001; Pipinos *et al.*, 2008a)(Figure 1).

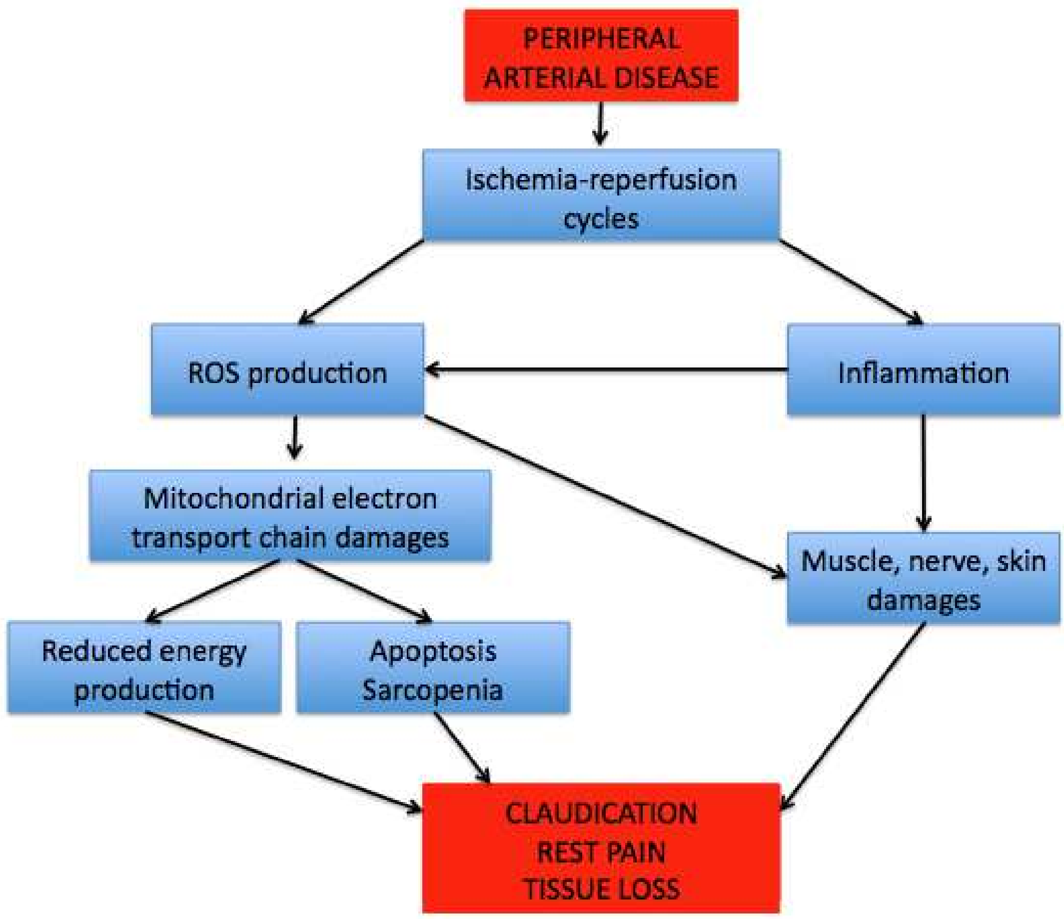



Fig. 1. Pathogenesis of peripheral arterial disease

### 1.2 Mitochondrial function and oxidative stress

Every action requires energy, and this energy is stored in adenosine triphosphate (ATP) molecules that are produced in the mitochondria by the process of oxidative phosphorylation. Mitochondria are present in every cell, but there are in high concentrations in muscle cells because high energetic requirements of muscles.

#### 1.2.1 Structure of mitochondria

Mitochondria are enclosed within two membranes: the outer membrane and the inner membrane. The outer membrane is a relatively simple phospholipid bilayer, containing protein structures called porins which allow molecules of 10 kilodaltons in weight to pass through it. This explains why the outer membrane is completely permeable to nutrient molecules, ions, ATP and ADP molecules. The inner membrane is more complex in structure than the outer membrane because it contains electron transport chain, ATP synthetase, and transport proteins. It is freely permeable only to oxygen, carbon dioxide and water. The wrinkles, or folds, are organized into layers called cristae, which increase total surface area of the inner membrane (figure2).

Outer and inner membranes delineate two compartments: the intermembrane space, and the cytoplasmic matrix. The intermembrane space is located between the inner and the outer membranes. It has an important role in oxidative phosphorylation. The cytoplasmic matrix contains the enzymes that are responsible for citric acid cycle reactions. The matrix also contains dissolved oxygen, water, and carbon dioxide.

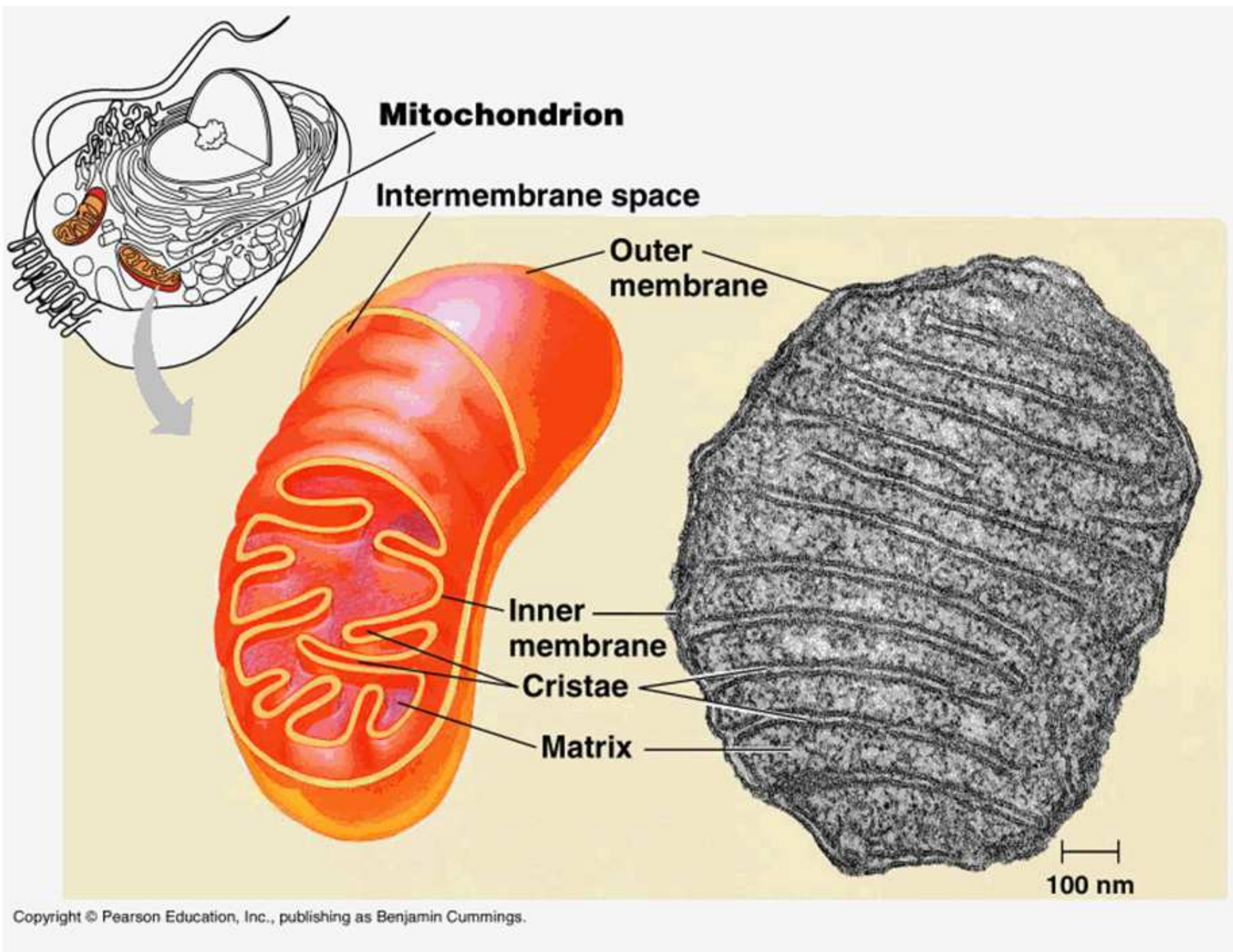



Fig. 2. Mitochondrial structure. (source: Pearson education, Inc., publishing as Benjamin Cummings).

### 1.2.2 Functions of mitochondria

One of the major mitochondrial functions is cellular respiration. It is a chemical process of releasing energy stored in glucose. The energy utilized in breaking down glucose is supplied by ATP molecules, and ATP molecules are produced by mitochondria. The entire process of aerobic cellular respiration is a three step process:

- Glycolysis: Glucose is a six carbon sugar. The enzymes in the cytoplasmic matrix initiate glycolysis in which a glucose molecule is oxidized to two molecules of three carbon sugars. Products of glycolysis are two molecules of ATP, two molecules of pyruvic acid and two NADH (Nicotinamide Adenine Dinucleotide) molecules (which are electron carrying molecules)

- Citric Acid Cycle (Krebs cycle): This is the second phase of cellular respiration. The three carbon molecules which have been produced as a result of glycolysis are converted into acetyl compounds. However, the intermediary reactions of this process yield ATP molecules of energy, NAD and FAD molecules too. NAD and FAD molecules are further reduced in the Citric Acid Cycle to high energy electrons
- Electron Transport: The electron transport chain is constituted of a series of electron carriers generated in the membrane of the mitochondria from Citric Acid Cycle. The ATP molecules are further produced by the chemical reactions of these electron carrier molecules. A eukaryotic cell produces about 36 ATP molecules after cellular respiration.

In fact, mitochondrial function in cellular energy metabolism is concerned with the processes of fatty acid and pyruvate oxidation, resulting in the formation of acetyl-CoA, which is subsequently oxidized in the Citric Acid Cycle. When combined, these processes generate reduced coenzymes, which deliver electrons to oxygen to form water, through the respiratory chain of the inner membrane. The whole process of fat and carbohydrate oxidation is strongly exergonic and the normal mitochondrion conserves the major part of this energy in the form of ADP phosphorylation to ATP. This dependence on oxygen is critical in skeletal muscle. Under normal circumstances, skeletal muscle has the capacity to increase its energy turnover, and this makes the transition from rest to exercise. Efficient oxygen delivery is very important for normal mitochondrial function, and patients suffering from peripheral arterial disease have a decreased blood flow to the legs due to arteriosclerosis, making less oxygen available to the mitochondria.

Other main mitochondrial functions are control of cell cycle, management of apoptosis, monitoring of cell differentiation, growth and development and reactive oxygen species production and clearance.

### 1.2.3 Mitochondria and reactive oxygen species

Mitochondria, main energy sources of the cells, are causes and targets of increased oxidative stress. Thus, the role of mitochondria extends far beyond energy production, as they are important generators of reactive oxygen species (ROS), which can act either as second messengers or as a source of cellular damage, depending on the produced amount. ROS are a double-edged sword: they are beneficial by playing an important role in cell signaling involved in antioxidant defense network, but could be harmful by inducing excessive oxidative stress resulting in protein carboxylation, lipids peroxydation and DNA damage. These free radicals have oxidizing properties, and they react in the environment where they are produced with a variety of biological substrates: fats, carbohydrates, proteins and DNA. There are also environmental factors that generate free radicals: pollution, sun exposure, smoking, consumption of alcohol or drugs, physical exercise. These situations induce an overproduction of reactive oxygen species. There are also defense systems that can regulate the production of theses species: free radicals are neutralized by enzymatic systems (superoxide dismutase, catalase and glutathione peroxidase), elements (copper, zinc, iron, selenium), as well as antioxidants such as vitamins A, C and E (Figure 3).

ROS include radical species such as primary superoxide $O_2^{\bullet-}$, and its conjugated acid hydroperoxyl radical $HO_2^{\bullet}$. Also included are the hydroxyl ($^{\bullet}OH$), carbonate ($CO_3^{\bullet\,-}$), peroxyl ($RO_2\bullet$), and alkoxyl ($RO^{\bullet}$) radical. Also some non-radical species are ascribed to

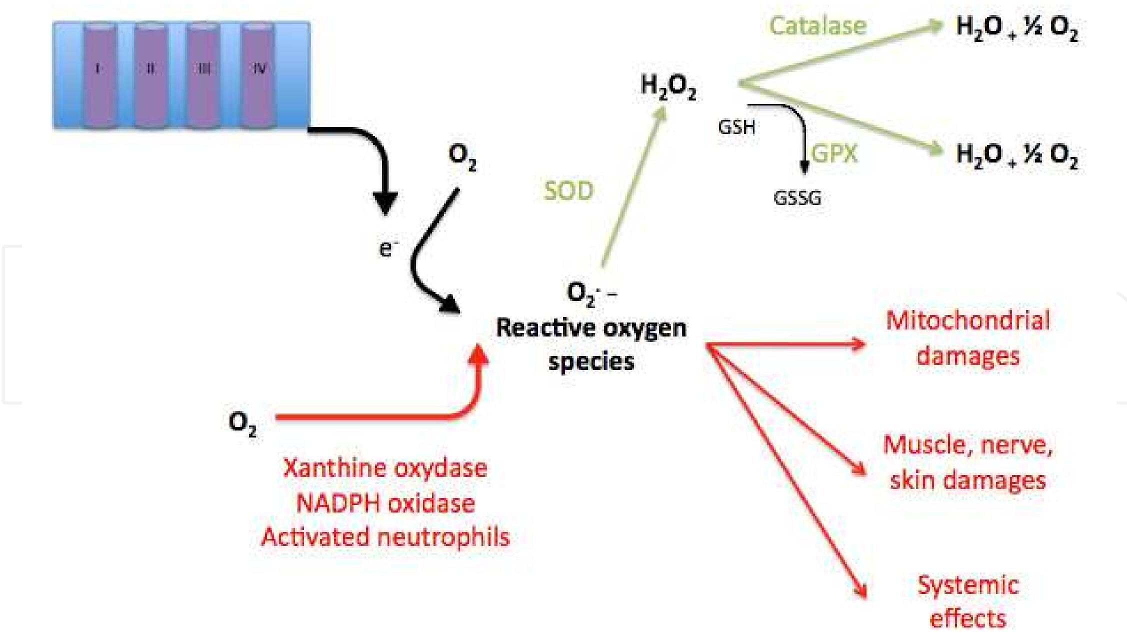



Fig. 3. Reactive oxygen species in peripheral arterial disease. SOD: superoxide dismutase; GPX: gluthatione peroxidase

ROS, namely $H_2O_2$, HOCl, fatty acid hydroperoxides (FAOOH), reactive aldehydes, singlet oxygen and other compounds (Chance *et al.*, 1979). Superoxide anion $O_2^{\bullet-}$ is the most important, it is a fairly stable compound, especially in an aqueous environment at neutral pH. Its toxicity is principally based on generation of further reactive species, called "downstream products" of $O_2^{\bullet-}$, which are then able to attack intracellular biomolecules.

There are currently seven separate sites of mitochondrial ROS production that have been identified (Figure 4) (Brand *et al.*, 2004).

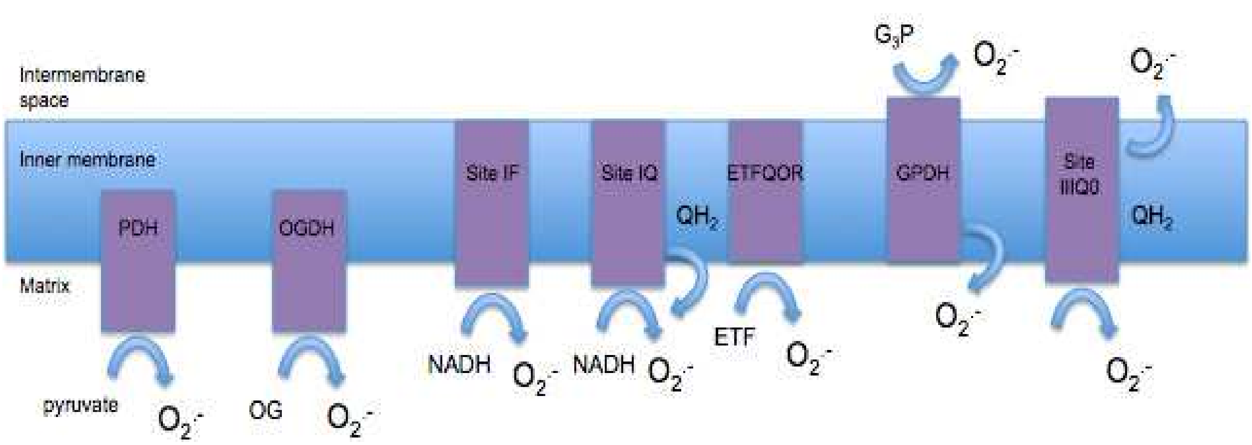



Fig. 4. Sites and topology of mitochondrial superoxide production. PDH: pyruvate dehydrogenase ; OGDH: 2-oxoglutarate dehydrogenase ; Site IF: NADH binding site of complex I ; Site IQ: uniquinine reduction site of complex I
ETFQOR: electron transferring flavoprotein ubiquinone oxidoreductase ; GPDH: glycerol 3-phosphate dehydrogenase
Site IIIQO: quinone binding site of the Q-cycle in complex III.

The relative importance of each site to total superoxide production in isolated mitochondria is contentious, partly because of different assays, different substrates and different sources of mitochondria. Most assays of superoxide production from defined sites measure maximal capacities for superoxide production, and the actual rate from each site in the absence of inhibitors is not known. During reverse electron transport from succinate to NAD+, complex I can produce superoxide at high rates (Han *et al.*, 2001; Votyakova & Reynolds, 2001; Kushnareva *et al.*, 2002; Liu *et al.*, 2002; Han *et al.*, 2003; Turrens, 2003; Lambert & Brand, 2004), although the physiological relevance is unclear (Votyakova & Reynolds, 2001). During forward electron transport from NAD-linked substrates (which may be more physiological), most mitochondria produce superoxide at high rates after addition of inhibitors such as rotenone (for complex I) (Han *et al.*, 2001; Liu *et al.*, 2002; St-Pierre *et al.*, 2002; Han *et al.*, 2003; Lambert & Brand, 2004) or antimycin A (for complex III)(Liu *et al.*, 2002; St-Pierre *et al.*, 2002; Muller *et al.*, 2004). Other physiologically relevant substrates, such as fatty acids and glycerol 3-phosphate, may cause superoxide production from sites that are less active during pyruvate oxidation, such as ETF-Q oxidoreductase and glycerol 3-phosphate dehydrogenase.

$O_2^{\bullet-}$ in the matrix is converted to $H_2O_2$ by matrix MnSOD, while $O_2^{\bullet-}$ released to the intramembrane space is partly dismuted by intermembrane space CuZnSOD (Inoue *et al.*, 2003). Any residual $O_2^{\bullet-}$ which diffuses into the cytosol is similarly converted by the cytosolic CuZnSOD. If any mitochondrial $O_2^{\bullet-}$ can reach the extracellular space, it is then detoxified by extracellular CuZnSOD ($SOD_3$) (Brand, 2010). Non-enzymatic lipoperoxidation is also a detoxification reaction. It can be considered not only as a detoxification reaction, but, due to its self-propagating nature, also as a new radical source initiated by the highly reactive radicals. Glutathione-based systems, including glutathione S transferase and the thioredoxin system, including peroxiredoxins, constitute the major redox buffer in the cytosol. Other detoxification systems (degrading $H_2O_2$ and ROS) are proteins of thioredoxin family, acting in concert with the thioredoxin-dependent peroxide reductase, and a family glutathione-S- transferase. $H_2O_2$ can be reduced to water by catalase or glutathione peroxidase, or alternatively to the hydroxyl radical in the presence of reduced copper or iron (Camello-Almaraz *et al.*, 2006).

Increased oxidative stress plays a key role in PAD and IR-induced muscular impairments. Both increased ROS secondary to mitochondrial dysfunction and decreased ROS catabolism are involved (Figures 1 and 3).

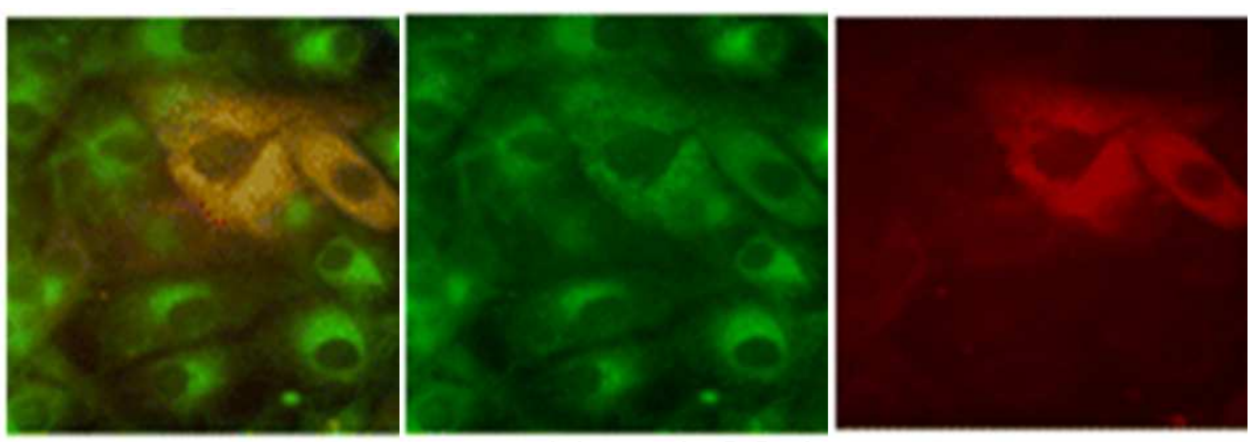
## 2. Mitochondrial and oxidative stress analysis of muscle biopsies

### 2.1 Histological methods

#### 2.1.1 Histological analysis of skeletal muscle mitochondria

Mitochondria can be detected in confocal microscopy by conventional fluorescent stains, such as rhodamine 123 and tetramethylrosamine. These stains are readily sequestered by functioning mitochondria, but they are subsequently washed out of the cells once the mitochondrion's membrane potential is lost. This characteristic limits their use in experiments in which cells must be treated with aldehyde-based fixatives or other agents that affect the energetic state of the mitochondria. To overcome this limitation, it is possible to use a serie of mitochondrion selective stains (MitoTracker probes®) that are concentrated by active mitochondria and well retained during cell fixation. Because these mitochondrion selective stains are also retained following permeabilization, the sample retains the fluorescent staining pattern characteristic of live cells during subsequent processing steps for immunocytochemistry, in situ hybridization or electron microscopy.

MitoSOX® Red mitochondrial superoxide indicator is a fluorogenic dye for highly selective detection of superoxide in the mitochondria of live cells. It is live-cell permeant and is rapidly and selectively targeted to the mitochondria. Once in the mitochondria, it is oxidized by superoxide (but not by other reactive oxygen species) and exhibits red fluorescence. Oxidation of the probe is prevented by superoxide dismutase. The oxidation product becomes highly fluorescent (excitation/emission maxima of approximately 510/580 nm) upon binding to nucleic acids. Cells adhering to coverslips have to be covered by 1 or 2 mL of 5 μM of MitoSOX® reagent working solution, and incubated for 10 minutes at 37°C, protected from light. They are then washed gently three times with warm buffer, and mounted in warm buffer for confocal microscopy imaging (Mukhopadhyay *et al.*, 2007) (figure 5).



Merge MitoTracker MitoSOX

Fig. 5 Assessment of superoxide generation. MitoSOX Red stain (top panel) revealed the presence of superoxide anion MitoSOX Red colocalized with MitoTracker Green (middle panel) in merged images (bottom panels), indicating that the excess superoxide anion was concentrated in mitochondria (Quinzii *et al.*, 2008).

In transmission electron microscopy, mitochondrial ultrastructure can be studied. The material is fixed, embedded, sectioned, and then examined. It is important to note that the sections must be less than 0.1 μm in thickness, even less than 0.05 μm, in order to show the structural details described with enough clarity for profitable study of the mitochondria (Frey *et al.*, 2002) (figure 6).

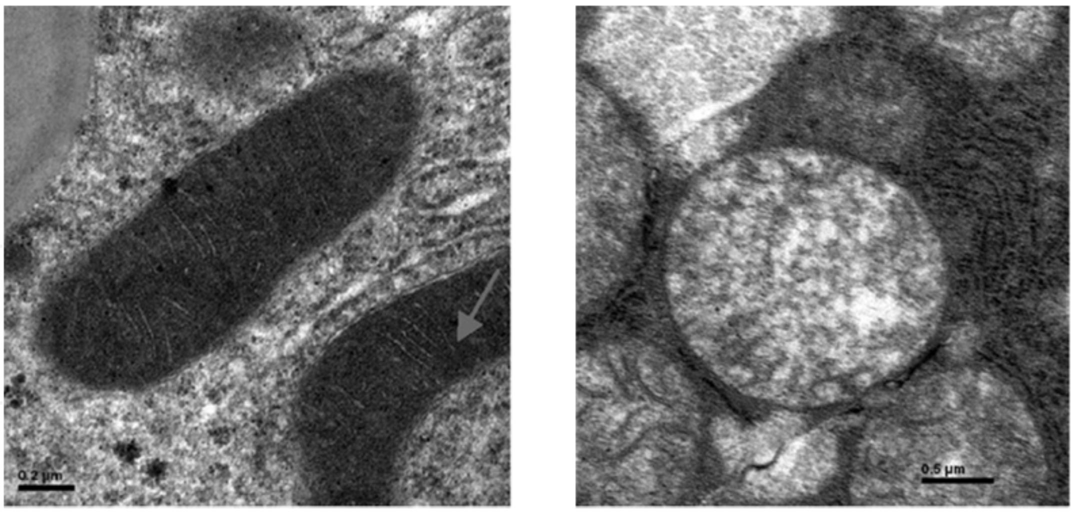


Fig. 6. Micrographs of transmission electron microscopy sections of mitochondria, TEM ×60K. The left photo represent a healthy mitochondrion the arrow indicates the cristae; the right photo represents a swelling mitochondrion (Li *et al.*, 2010).

#### 2.1.2 Microscopy fluorescence: Dihydroethidium staining

To detect the presence of ROS in skeletal muscles, serial sections (10 µm-thick) are cut on a cryostat microtome, mounted into glass slides and incubated with 2.5 µM dihydroethidium (DHE). DHE produces red fluorescence when oxidized to ethidium bromide (EtBr), mainly by superoxide anion. After staining, sections are examined under an epifluorescence microscope (Nikon Eclipse E800) and emission signal are recorded with a Zeiss filter (Dikalov *et al.*, 2007) (figure 7).

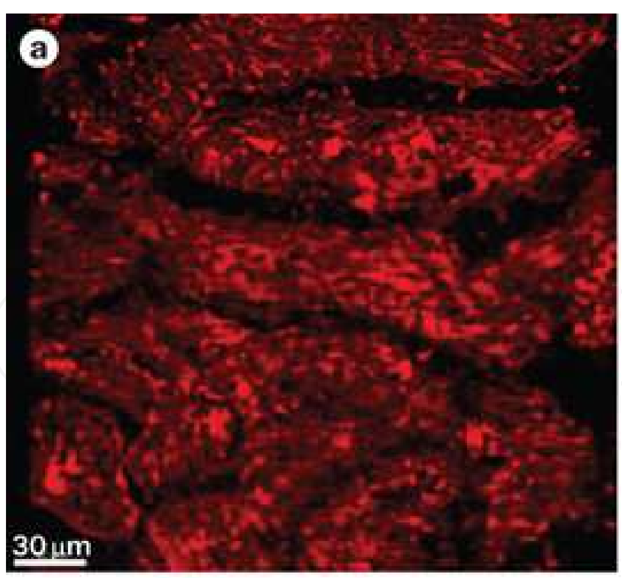



Fig. 7. A representative photo of Superoxide production by dihydroethidium staining of tissue in the acute phase of stress cardiomyopathy (Nef et al., 2008)

Inhibitors of NADPH oxidase (diphenylene iodonium) and xanthine oxidase are known to reduce mitochondrial superoxide production through inhibiting NADH ubiquinone oxidoreductase (complex I) (Riganti *et al.*, 2004).

## 2.2 Functional methods

### 2.2.1 Mitochondrial respiratory chain complexes activities using saponin skinned fibres

The mitochondrial respiratory chain complexes activities study is described in an other chapter. This technique is based on the measure of oxygen consumption in skinned fibres in order to determine the functional oxidative capacity of the skeletal muscle in its cellular environment (Veksler *et al.*, 1987; Riganti *et al.*, 2004) (see the chapter from Charles et al. for much more explanations and the description of the methods).

### 2.2.2 $H_2O_2$ Production in permeabilized fibres

$H_2O_2$ production is assessed in permeabilized fibres (Kuznetsov *et al.*, 2008) in response to sequential addition of substrates and inhibitors (Anderson & Neufer, 2006). $H_2O_2$ production is measured with Amplex Red reagent (Invitrogen), which reacts with $H_2O_2$ in a 1:1 stoichiometry catalyzed by HRP (Horse Radish Peroxidase; Fluka Biochemika) to yield the fluorescent compound resorufin and molar equivalent O2. Resorufin has excitation/emission characteristics of 563/587 nm and is extremely stable once formed. Fluorescence is measured continuously [change in fluorescence ($\Delta$F)/sec] with a spectrofluorometer with temperature control and magnetic stirring. After a baseline, $\Delta$F (reactants only) is established; the reaction is initiated by addition of a permeabilized fibre bundle to 600 µl of buffer Z with glutamate (5µM) and malate (2.5µM) as substrates for complex I and succinate (5mM) for complex II. ADP (2mM) is injected in the reaction buffer and led to a reduction in $H_2O_2$ release, which is expected when electron flow through the respiratory chain is stimulated. Finally, addition of the complex I inhibitor amytal (2mM) and the complex III inhibitor antimycin (8µM) led to interruption of normal electron flow and induced an increase in $H_2O_2$ release.

## 3. Mitochondrial dysfunctions during peripheral arterial disease

During PAD, significant muscles ischemia/reperfusion is well known to induce skeletal muscles alterations (Figure 1). The pathogenesis of PAD manifestations is lead to the development of athero-occlusive disease in the lower limb arteries. Arterial stenoses usually do not affect the blood supply at rest, but at the time of walking or other exercise, they make the leg ischemic and painful forcing the patient to rest. At rest, perfusion returns again to normal levels. These cycles of ischemia and reperfusion launch a cascade of inflammatory changes and induce the production of ROS in the skeletal muscle. Multiple daily ischemia/reperfusion events initiated by simple activities such as walking result, over time, in morphological and ultrastructural changes in both the contractile element of the muscle and its mitochondria. Dysfunctional mitochondria then further lower the already decreased (by compromised blood supply) energy levels in the pathologic muscle and become sources of ever increasing levels of ROS and possibly inducers of apoptosis. A vicious cycle is thus initiated gradually leading to deteriorating mitochondrial function and escalating ROS production with ongoing damage of every structure in the myocytes. Apoptosis, along with cellular necrosis (from ischemia, reactive oxygen species, and low energy levels), may then be induced, eventually leading to a severe myopathy that significantly affects the function and performance of PAD limbs. In addition, nerves, skin, and subcutaneous tissues damages are formed, ultimately leading to the characteristically atrophic legs of patients with advanced PAD having thin muscles; brittle, hairless, and thin skin with shiny texture; and impaired sensorimotor function. On the basis of these concepts, it is easy to understand how claudication, rest pain, and tissue loss find their place in the heart of this continuum of events, coming into view as the external manifestations of ongoing tissue injury and deterioration (Blaisdell, 2002; Pipinos *et al.*, 2008a).

### 3.1 Selected experimental data (Table 1)

<table>
<tr><th>Author</th><th>Journal</th><th>Year</th><th>Histology</th><th>Oxidative stress</th><th>Respirometry</th></tr>
<tr><td>Makris</td><td>Vascular</td><td>2007</td><td>Myopathic features<br>Drop in total protein content<br>Increased mitochondrial content</td><td>Increased oxidative stress</td><td>Bioenergetic decline<br><br>Inadequate oxidative phosphorylation<br><br>Decreased ATP energy production</td></tr>
<tr><td>Pipinos II</td><td>J Vasc Surg</td><td>2000</td><td rowspan="3">Myopathic features<br><br>Increased mitochondrial content, more oxidative type fibres.</td><td rowspan="3">- Increased oxidative stress : xanthine oxidase and activated neutrophils are source of ROS<br>- Alteration of activity and expression of MnSOD.<br>- Damage to mtDNA</td><td rowspan="3">Decreased activities of complexes I, III, and IV.</td></tr>
<tr><td>Pipinos II</td><td>Vasc Endovasc Surg</td><td>2008b</td></tr>
<tr><td>Brass</td><td>Vasc Med</td><td>1996<br>2000</td></tr>
<tr><td>Wallace</td><td>Am Heart J</td><td>2000</td><td>Increased mitochondrial content</td><td>Increased oxidative stress</td><td>-</td></tr>
<tr><td>Levak-Frank</td><td>J Clin Invest</td><td>1996</td><td rowspan="2">Increased mitochondrial content</td><td rowspan="2">-</td><td rowspan="2">-</td></tr>
<tr><td>Wredenberg</td><td>Proc Nath Acad Sci USA</td><td>1999</td></tr>
</table>

Table 1. Experimental data

Peripheral arterial disease is a consequence of compromised blood supply to the ischemic limb (Brass, 1996; Brass & Hiatt, 2000). Experimental data show that skeletal muscle responds to inflow arterial occlusion with the development of myopathic histological changes, a drop in total protein content, and a trend toward decreased wet weight (Makris, et al., 2007 ; Pipinos, et al., 2008b). Peripheral arterial disease is characterized by a significant increase in the mitochondrial content of skeletal muscle, and mitochondrial proliferation is characteristic of mitochondrial diseases and aging (Levak-Frank *et al.*, 1995; Wallace, 2000; Wredenberg *et al.*, 2002; Makris *et al.*, 2007; Pipinos *et al.*, 2008b). In skeletal muscle, an upregulation of mitochondrial biogenesis may be associated with and alteration of muscle fibre type toward the more oxidative type I and IIa fibres (Pipinos *et al.*, 2008b).

Defective mitochondria are central to this myopathy, through compromised performance as primary energy producers and regulators of oxygen radical species. Thus, PAD myopathy is characterized by an increased content of dysfunctional mitochondria having significant defects in electron transport chain complexes I, III, and IV (Pipinos *et al.*, 2000; Pipinos *et al.*, 2008b). These defects are associated with a bioenergetic decline, characterized by inadequate oxidative phosphorylation, decreased ATP energy production, and increased oxidative stress (Makris *et al.*, 2007; Pipinos *et al.*, 2008b). Ischemic skeletal muscle sustains substantial oxidative injuries indicated by an increase in protein carbonylation and lipid peroxidation adducts. Under resting conditions, a large proportion of cellular reactive oxygen species is produced in the mitochondria (Wallace, 2000).

Thus, ischemia/reperfusion is the central problem in animals with inflow arterial occlusion (Brevetti *et al.*, 2001). Ischemia/reperfusion increases oxidative stress, triggers inflammation and oxidative damage to the tissues, and initiates mitochondrial injury and dysfunction. Mitochondrial dysfunction can then be perpetuated by repeated destructive cycles of ischemia/reperfusion, causing amplification of respiratory chain defects, compromised bioenergetics, increased reactive oxygen species production, diminished MnSOD antioxidant activity. The combination of compromised bioenergetics and worsening oxidative stress may then lead to progressive oxidative damage of structures in the myocytes (Brass, 1996; Brass & Hiatt, 2000; Makris *et al.*, 2007; Pipinos *et al.*, 2008a).

There are non-invasive techniques that can evaluate *in vivo* the mitochondrial energy transformation by the monitoring of the tissue oxygen level: either directly with the $^{31}$phosphorous magnetic resonance spectroscopy (Hands *et al.*, 1990; Greiner *et al.*, 2006), or indirectly with infrared spectroscopy (Hands *et al.*, 1986; Watanabe *et al.*, 2004; Ubbink & Koopman, 2006). The $^{31}$phosphorous magnetic resonance spectroscopy is used to determine the concentrations of metabolites involved in muscle energy metabolism (phosphocreatin, inorganic phosphate, and ATP). From these data, free ADP and pH may be calculated (Quistorff *et al.*, 1993). The infrared spectroscopy is used to measure the state of oxygen saturation in hemoglobin and myoglobin in blood and muscle at a given time and a given location. It can be considered as an indirect measure of the muscle perfusion versus oxygen consumption (Comerota *et al.*, 2003).

Mitochondrial function may also be evaluated by respirometry on muscle biopsies. The feasibility, indications, contra-indications are now well known and such a technique become usual in specialized centers. Thus, skeletal muscle biopsies can be obtained during surgery, or they can be obtained under local anesthesia. Biopsy sites can be anesthetized with a 2%

lidocaine solution, and 1.0 cm incisions can be made through the skin and gastrocnemius fascia. A modified 5 mm Bergstrom biopsy needle can then be inserted 10-15 mm and used to obtain 40 to 50 mg of skeletal muscle. Contraindications are essentially represented by bleeding disorders, infection at biopsy sites, or allergy to local anesthetics.

### 3.2 Selected clinical data (Table 2)

<table>
<tr><th>Author</th><th>Journal</th><th>Year</th><th>Histology</th><th>Oxidative stress</th><th>Respirometry</th></tr>
<tr><td>Makris</td><td>Vascular</td><td>2007</td><td rowspan="4">Myopathic features<br><br>Increased mitochondrial content<br><br>More type I fibres</td><td>Increased oxidative stress</td><td>Bioenergetic decline</td></tr>
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<tr><td>Brass</td><td>Vasc Med</td><td>2000</td><td>Increased oxidative stress</td></tr>
</table>

Table 2. Clinical data.

Previous studies have shown that PAD is associated with alterations in skeletal muscle histology (Brass & Hiatt, 2000; Makris *et al.*, 2007; Pipinos *et al.*, 2008b). Necrotic and regenerating fibres as well as inflammation have been seen in the diseased legs in comparison to contralateral legs of patients with unilateral peripheral arterial disease hospitalized for surgical evaluation. Furthermore, in the setting of aortic aneurysm repair in human, light microscopy revealed a consistent granulocyte infiltration in the ischemic and reperfused skeletal muscle. Ultrastructural damage to the muscle fibers was seen during ischemia and became more severe upon reperfusion. The recruitment of granulocytes into the muscle tissue paralleled the activation of the blood complement system and an increase in circulating neutrophils (Formigli L *et al.*, 1992).

#### 3.2.1 Patients with functional ischemia

The 31phosphorous magnetic resonance spectroscopy examination shows a higher inorganic phosphate/phosphocreatin ratio compared to control patients (Hands *et al.*, 1986; Zatina *et al.*, 1986; Hands *et al.*, 1990). The infrared spectroscopy examination shows a large drop in the oxygen saturation in the muscle and an increased oxygenation recovery time after exercise when compared to control patients (Kemp *et al.*, 2001; Comerota *et al.*, 2003). Histological examination shows more type I muscle fibres containing a high amount of mitochondria in the gastrocnemius muscle of patients with functional ischemia. In addition, the severity of the peripheral arterial disease was correlated with the increased percentage of type I fibres (Makitie & Teravainen, 1977).

### 3.2.2 Patients with critical limb ischemia

The 31phosphorous magnetic resonance spectroscopy examination shows a higher intracellular pH, and a higher inorganic phosphate/phosphocreatin ratio compared to control patients (Hands *et al.*, 1986; Zatina *et al.*, 1986; Hands *et al.*, 1990). The infrared spectroscopy examination shows a large decrease in the oxygen saturation in the muscle and an increased oxygenation recovery after surgery. Respirometry shows a reduced mitochondrial respiratory rate in the gastrocnemius muscle compared to control patients (Pipinos *et al.*, 2003; Pipinos *et al.*, 2006). The reduced respiratory rate is specifically located to complexes I, III and IV enzymes of the respiratory chain; probably due to reactive oxygen species generated damage (Sjostrom *et al.*, 1980; Pipinos *et al.*, 2003).

## 4. Improving mitochondrial function and reducing oxidative stress: selected experimental and clinical results (Table 3)

| Author | Journal | Year | Type | Histology | Oxidative stress | Respiro-metry | Necrosis |
|---|---|---|---|---|---|---|---|
| Tran | Eur J Pharmacol | 2011 | Pre | - | Decreased superoxide production | C I, III and IV activities normalized | Reduced infarct size |
| Andreadou | Mini Rev Med Chem | 2008a,b | Pre Post | - | - | - | |
| Martou | J Appli Physiol | 2006 | Pre | Normal morphology | - | - | - |
| Addison | Am J Physiol Heart Circ Physiol | 2003 | Pre | Attenuation of neutrophil accumulation | Decreased oxidative stress | No bioenergetic decline | - |
| Thaveau | J Vasc Surg | 2007 | Pre | - | - | Restoration of complexes I and II activities | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Okorie | Eur Heart J | 2011 | Post | - | Decreased oxidative stress by inihibition of the opening of mPTP | - | - |
| McAllister | Am J Physiol Regul Integr Comp Physiol | 2008 | Post | | | | |
| Eberlin | Plast Reconstr Surg | 2009 | Post | Decreased of injured fibres | - | - | Reduced infarct size |
| Charles | Br J Surg | 2011 | Post | - | Decreased oxidative stress Preserved antioxydant defense | Increased complexes I, II, III, and IV activities | - |
| Tsubota | Eur J Vasc Endovasc Surg | 2010 | Post | Attenuation of neutrophil accumulation | - | - | Reduced tissue necrosis |

Table 3. Effects of pre- and post-conditioning. This table summarizes clinical studies realized on pre- or postconditioning.

### 4.1 Ischemic pre- and post-conditioning

Besides reducing preoperative ischemic time and surgery duration, ischemic preconditioning -defined as brief episodes of ischemia/reperfusion applied before sustained ischemia- decreases skeletal muscle mitochondrial dysfunction, enhances limb and remotes organ protections. Furthermore, remote and local ischemic preconditioning equivalently protects skeletal muscle mitochondrial function during experimental aortic cross-clamping (Mansour *et al.*, in Press). Nevertheless, ischemia occurrence is difficult to predict and might limit a broader use of ischemic preconditioning. Controlled reperfusion appears thus as a valuable therapeutic approach after limb ischemia and ischemic post-conditioning, characterized by repeated cycles of IR performed at the onset of reperfusion, appeared safe and easy to perform.

Ischemic preconditioning has been mainly elucidated in experimental cardiac ischemia. Ischemic preconditioning utilizes endogenous as well as distant mechanisms in skeletal muscle, liver, lung, kidney, intestine and brain in animal models to convey varying degrees of protection from ischemia/reperfusion injury (Ambros *et al.*, 2007). Specifically, preconditioned tissues exhibit altered energy metabolism, better electrolyte homeostasis and genetic reorganization, as well as less oxygen-free radicals and activated neutrophils release, reduced apoptosis and better microcirculatory perfusion. To date, there are few human studies, but trials suggest that different organ in human such as heart, liver, lung and skeletal muscle acquire protection after ischemia/reperfusion (Sjostrom *et al.*, 1980; Ali *et al.*,2007; Cheung M *et al.*, 1996; Kharbanda *et al.*, 2002). It has been showed that ischemic preconditioning positively influenced muscle metabolism during reperfusion, and this, results in an increase in phosphocreatin production and higher oxygen consumption (Andreas *et al.*, 2011).

Experimental data showed that ischemic postconditioning confers protection against different organ injuries caused by longer circulatory occlusions during elective major vascular surgeries, because it causes a significant reduction in systemic inflammatory response (TNF-alpha, oxygen-derived free radicals) (Eberlin *et al.*, 2009). Besides the heart (Skyschally *et al.*,2009), postconditioning is also effective in salvage of ischemic skeletal muscle from reperfusion injury and the mechanism likely involves inhibition of opening of the mPTP and/or reduced oxidative stress (Szijarto *et al.*,2009; Tsubota *et al.*, 2010; Park *et al.*, 2010; Guyrkovic *et al.*, 2011; Mc Allister *et al.*, 2008; Charles *et al.*, 2011). There are few human studies, but it has been showed that postconditioning by intermittent early reperfusion reduces ischemia/reperfusion injury, that might depend on K(ATP) channel activation, and is mimicked by inhibition of the mPTP at reperfusion (Okorie *et al.*, 2011).

### 4.2 Pharmacological protection of skeletal muscle in the setting of ischemia/reperfusion

Although preconditioning is a powerful form of protection, its clinical application is limited because of practical reasons. In fact, the short ischemic insults in preconditioning have to be applied before the onset of sustained period of ischemia which cannot be precisely anticipated. On the contrary, the very brief insults in postconditioning have to be applied immediately after the end of the long ischemia thus making the intervention more easily applicable. Both mechanisms limit the reperfusion injury but easier approaches deserve to be studied.

Pharmacological preconditioning and postconditioning represent ideal alternatives that may substitute the short ischemic insults for pharmaceuticals means. The components of preconditioning share two main pathways, one that involves the mitochondrial K(ATP) channels- free radicals and PKC and another one that involves adenosine and PKC. Reperfusion injury salvage kinases (RISK) prevent the mitochondrial permeability transition pores (mPTP) opening which destroy the mitochondria and cause cell death. PC *via* PKC and postconditioning *via* gradual restoration of pH at reperfusion up-regulate RISK and preserve viable part of the ischemic region. In order to confer pharmacological protection, novel therapeutic strategies, based on the knowledge of the ligands, of the receptors and of the intracellular signaling pathways have emerged (Addison *et al.*, 2003; Gamboa *et al.*, 2003; Martou *et al.*, 2006; Andreadou *et al.*, 2008b). Adenosine, nicorandil, tempol, coenzyme Q and other agents (Addison *et al.*, 2003; Gamboa *et al.*, 2003; Martou *et al.*, 2006; Thaveau *et al.*, 2010) have been already used as pharmacological mimetics of ischemic preconditioning. Furthermore, agents that increase RISK or directly prevent mPTP are also under investigation as postconditioning analogues (Andreadou *et al.*, 2008a; Tsubota *et al.*, 2010; Tran *et al.*, 2011). Antioxidant systems are also important: several endogenous antioxidant systems are found in muscle tissue, these include alpha-tocopherol, histidine-containing dipeptides, and antioxidant enzymes such as glutathione peroxidase, superoxide dismutase, and catalase. The contribution of alpha-tocopherol to the oxidative stability of skeletal muscle is largely influenced by diet. Dietary supplementation of tocopherol has been shown to increase muscle alpha-tocopherol concentrations and to inhibit lipid oxidation. Dietary selenium supplementation has also been shown to increase the oxidative stability of muscle presumably by increasing the activity of glutathione peroxidase, and dietary restriction improves systemic and muscular oxidative stress (Rodrigues *et al.*, 2011). The oxidative stability of skeletal muscle is also influenced by the histidine-containing dipeptides, carnosine and anserine (Chan & Decker, 1994).

## 5. Conclusions / Perspectives

Mitochondria are the main energy source of the cells and mitochondrial dysfunction is associated with cell and organ impairment. Consistently, IR has been shown to induce skeletal muscle mitochondrial dysfunctions in animals and humans and improving skeletal muscle mitochondrial function is an interesting and clinically pertinent therapeutic goal. Indeed, improving skeletal muscle mitochondrial function enhances walking capacities in patients suffering from peripheral arterial disease.

Muscle biopsy allows to precisely determine the deleterious effects of IR on skeletal muscle and can be used to better stratify patient's risk and to guide therapy. Ischemic pre- and post-conditioning and pharmacologic conditioning allows protection of skeletal muscle in the setting of ischemia/reperfusion, decreasing mitochondrial respiratory chain injury, reducing reactive oxygen species (ROS) production and enhancing muscles antioxidant defence.

Future work will be useful to determine whether even smaller biopsies, analyzed after being frozen, might yield the same information.

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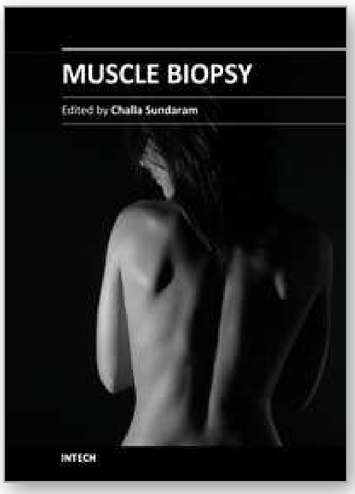


**Muscle Biopsy**

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Investigation of muscle diseases has changed dramatically with the understanding of genetic basis of a wide range of muscle diseases. Muscle biopsy has become a powerful tool not only to provide diagnosis but to make tissue available for genetic studies and to basic scientists for biomedical research. Accurate interpretation of muscle biopsy to detect cell dysfunction/ damage/death or absence / abnormality of a protein or genetic defect by the sophisticated technologies is important to guide treatment of various muscle diseases. In this book on muscle biopsy various chapters deal with the procedure and interpretation of muscle biopsy, its use in the culture of myotubes and membrane transport studies.Muscle biopsy is an important technique to investigate mitochondrial dysfunction and the mitochondrial DNA integrity in oxidation. Phosphorylation in various metabolic diseases like obesity, type 2 diabetes mellitus and peripheral vascular disease is explored in the other chapters with detailed descriptions on methodology. This book provides the advances in the basic techniques of muscle biopsy for a neuroscientist.

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