PHD COURSE IN LIFE AND ENVIRONMENTAL SCIENCES

Report Form for PhD student annual evaluation (XXXVII and XXXVIII cycles)

Name of PhD student: Loredana Rao

Title of PhD research: Morpho-functional evaluation of skeletal muscle after electric pulse stimulation. A potential beneficial role of mitochondrial nutrients on physical performance.

Name of PhD supervisor: Andrea Frontini

Research lab name: Laboratory of Morphological and functional Biology, DISVA

Cycle: XXXVII

PhD Curriculum: Biomolecular Sciences

DISVA instrumentation labs/infrastructure eventually involved in the project:
Advanced Instrumentation lab

ABSTRACT (1000 characters, including spaces):
Regular physical exercise is able to counteract adverse health conditions, including non-communicable health diseases, and the aging-related decline of skeletal muscle, process known as sarcopenia [1]. In fact, exercise can mitigate most of the molecular and metabolic processes involved in these processes and, by inducing a mild oxidative stress, triggers adaptation of the organism; however, in overtraining conditions, the effects of exercise can be deleterious [5]. Supplementation with antioxidants did not show consistent results on improving detrimental events. Thus, mitochondrial nutrients might provide an alternative strategy to prevent the effect of overtraining and promote mitochondrial adaptation to stress signals. Among all mitochondrial nutrients, coenzyme Q_{10} plays a major role: it is an endogenous cofactor exerting both an antioxidant and bioenergetic activity, it is also acquired through the diet. Although our body can synthetize CoQ_{10}, secondary deficits are quite common since biosynthesis is known to decrease with age [3]. Moreover, increased demand might be experienced during high intensity exercise, therefore dietary supplementation might have a role in improving physical performance (8).
The aim of this study is to evaluate the effects of supplementation with Ubiqsome (CoQ_{10} conjugated with phytosomes), a formula showing enhanced bioavailability in the muscle tissue, on reduction of mitochondrial disfunction, oxidative stress and cytotoxicity induced by high intensity exercise, and on amelioration of beneficial effects of moderate exercise. An in-vitro electric pulse stimulation system (EPS) has been developed to simulate physical exercise on myotubes.

Part 1. Scientific case of the PhD Research (2 to 3 pages, including figures)

- BACKGROUND

Physical inactivity and sedentary behaviour increase the risk of developing adverse health conditions, including non-communicable health diseases, and reduce life expectancy (1).

On the contrary, a regular physical activity is well known to have many beneficial effects on health. One of the pioneering works was published in 1953 from Jerry Morris, who undertook the first rigorous, epidemiological study investigating physical inactivity and chronic disease risk. Since then, much evidence has clearly documented the many health benefits of physical activity.
In fact, besides increasing muscle hypertrophy and number and quality of muscle fibres, exercise reduces the rates of mortality, coronary heart disease, stroke, metabolic syndrome, type 2 diabetes, as well as several other clinical conditions (2). Moreover, exercise is also known to induce physiological and metabolic adaptation, stimulates mitochondrial oxidative capacity and lipid oxidation, increases number of mitochondria and mitochondrial enzymes activity, also improving insulin sensitivity and glucose tolerance (3). Following the evidence that physical activity is clear involved in mitochondrial health, the benefits are best understood in skeletal muscle, where mitochondria regulate the mass and function of skeletal muscle and their function in turn is modulated by exercise.
Accordingly, it is possible to state that the most important effect of exercise on the body is the induction of adaptative processes. In fact, acting as a mild stressor, a single bout of exercise has the capability to induce adaptation. Therefore, physical activity can be considered as an oxidative stress to which the organism is able to adapt. An important aspect to promote adaptation is the intensity of the exercise training and to provide an optimal resting phase between exercise bouts. Indeed, while during exercise, metabolic and mechanical loading results in transient stress exposure to muscle as well as several other organs, during rest the body recovers and implements adaptive responses (4).
However, when the duration or the intensity of the exercise outsource the adaptive capacity of the organism, there is the risk of "overtraining" leading to oxidative stress and deleterious short and long-term effects (5).
One of the possible reasons of this phenomenon could be an imbalance in the intramuscular redox state that triggers inflammatory signalling, resulting in impaired force production and exercise performance.
Although there has been considerable controversy in the literature regarding whether or not oral antioxidant supplementation can inhibit the adaptive response of skeletal muscle (6,7), supplementation with nutritional factors characterized also, but not exclusively, by antioxidant activities, such as mitochondrial nutrients, might provide a successful strategy to prevent the effect of overtraining and promote mitochondrial adaptation to stress signals.
Among these type of nutrients, coenzyme Q_{10} plays a major role: it is a lipophilic cofactor exerting both an antioxidant and bioenergetic activity, endogenous to our organism but also acquired through the diet, since several foods provide an exogenous source of CoQ_{10}. Despite this, secondary deficit is not uncommon since biosynthesis is known to decrease with age and an increased demand might be experienced in condition of high intensity physical exercise, therefore dietary supplementation might have a role in improving physical performance (8).

- SCIENTIFIC AIMS

The aim of this study is to evaluate the effects of supplementation with CoQ_{10} conjugated with phytosome (Ubiqsome ®), a formula showing enhanced bioavailability in muscle tissue that results otherwise particularly refractory to exogenous CoQ_{10} uptake, on reduction of mitochondrial disfunction, oxidative stress and cytotoxicity induced by high intensity exercise, and on amelioration of beneficial effects of moderate exercise. The effects of Ubiqsome in terms of muscle bioavailability and bioenergetic and antioxidant properties could support its efficacy on sport nutritional supplementation.

- WORKPLAN AND RESEARCH ACTIVITIES

WP 1. Objective. It's known that CoQ_{10} shows limited gastrointestinal absorption and bioavailability due to its chemical-physical properties, but also a tissue specific uptake pattern, with organs highly relying on aerobic metabolism (muscle, heart, brain) showing the lowest.
A recent study by Drobnic et al. showed that CoQ_{10} formulated in phytosomes as Ubiqsome, led to a significant increase in quinone in skeletal muscle tissue *in-vivo* (8).
The objective of this project is to verify if the increased bioavailability of Ubiqsome (CoQ_{10} conjugated with phytosomes) compared to crystalline CoQ_{10} is associated with a higher functionality and mitochondrial health. Through the development of an Electric Pulse Stimulation (EPS) system that allows to implement different protocols of stimulation on an *in-vitro* model of myotubes, it will be possible to define the potential beneficial

effects of Ubiqsome supplementation on reduction of mitochondrial dysfunction, oxidative stress, and cytotoxicity typical of high intensity exercise and to burb deleterious effects of overtraining. In order to mimic the *in-vivo* absorption processes as much as possible, both CoQ_{10} forms are given in enriched lipoproteins obtained from the plasma of supplemented volunteers.

Methods. To obtain the lipoprotein that will be used for myotubes supplementation, we used the randomized crossover experimental design of a previous study (8): two formulations, one with crystalline CoQ_{10} and one with Ubiqsome both provided by INDENA, were given randomly to two arms of 10 subjects for 15 days. The two arms were inverted following two weeks washout phase. Blood samples were collected in heparinized vacutainer under fasting condition at base line and at the end of each treatment. Plasma was obtained by centrifugation, an aliquot was stored to evaluate CoQ_{10} amount and oxidative status, the remaining plasma was pooled for the extraction of LDL.
In-vitro experiments were conducted on C2C12 murine muscle cell line, differentiated for seven days with a specific culture medium to mature myotubes.
In order to simulate physical activity, the IonOptics EPS system and experimental procedures described in the literature (Nikolić N et al.) were taken as a model and a similar home-made EPS system has been developed in collaboration with the Department of Industrial Engineering and Mathematical Sciences (DIISM): it consists of a generator, a voltage amplifier, an oscilloscope and the 6-well plate, the lid of which was refitted with pairs of graphite electrodes, one per well (Fig.1 and Fig.2). The cells are kept for the entire duration of the stimulation in a Lionheart FX Automated Microscope, which also serves as an incubator and allows the contraction of the cells to be observed in real time.
LabVIEW software was used to fine-tune the waves for the different protocols.
Two stimulation protocols have been designed, a *low frequency* one (single pulses of 1 Hz 30 V, for 6-18 h) that simulates a chronic low intensity exercise, and a *high frequency* one (pulse trains of bipolar pulses 100 Hz for 200 ms given every 5th second, 30 V, for 5-60 min) to simulate a high intensity acute exercise (Fig.3).
Viability parameters for stimulated versus unstimulated cells have been assessed by evaluating LDH released from damaged myotubes in the culture medium, as well as by resazurin assay to evaluate the percentage of vital cells (PrestoBlue, Thermofisher). Moreover, a protocol to measure ROS produced after stimulation is in development: MitoSOX red and H2DCF-DA, two fluorescent probes used to measure respectively mitochondrial and cytoplasmatic ROS, will be used to for this purpose and optimized for live imaging.
Fluorescent markers such as Phalloidin or Mitotimer are being developed on the EPS model to advance a morphological and functional characterisation of myotubes following contraction (Fig.4).

Expected/Obtained Results. The most critical aspect of this project was setting up the electrostimulation system; with the help of the DIISM engineers we were able to build the EPS and two protocols of high and low frequency were developed to contract efficiently the myotubes. The low frequency one, in which the contraction was induced for up to seven consecutive hours, was taken as a model for a chronic exercise. For the high frequency one, several trials were carried out to find a sub-lethal condition that mimicked acute high intensity exercise. We are currently developing control experiments with hydrogen peroxide as a positive control to measure viability and LDH production in cells following EPS, the same for ROS production.
At the same time, we also started the human *in-vivo* supplementation with the two formulations mentioned, crystalline CoQ_{10} and Ubiqsome, given randomly to the two arms of subjects and the LDLs that will be used for treating the cells have been extracted.
In the coming months, research will proceed toward the validation of the exercise protocol measuring the levels of viability and oxidative stress. The aim is to identify sublethal doses of exercise that produce high level of oxidative stress and damage, possibly exceeding adaptive response of myotubes. Chronic exercise will be used as a positive control of adaptation processes.
Subsequently, once defined these experimental conditions, we will proceed with the treatment with CoQ_{10} enriched LDL in order to ascertain whether this mitochondrial nutrient is able to protect toward oxidative stress and increased viability of exercise-challenged cells.
Differential bioavailability of the two types of CoQ-enriched LDL is at the basis of expected differential outcomes in protection. In particular, once these aspects have been defined, a broader investigation on mitochondrial health will be conducted taking into account also mitochondrial biogenesis, turnover and dynamic.

Fig 1. EPS work station

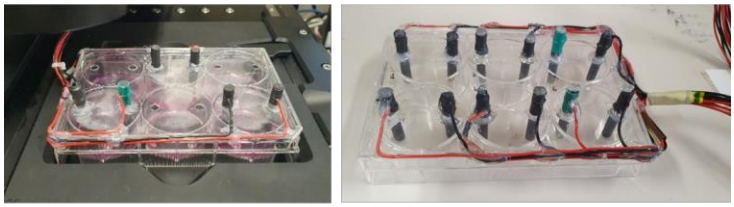

Fig 2. The 6-well with electrodes used for EPS

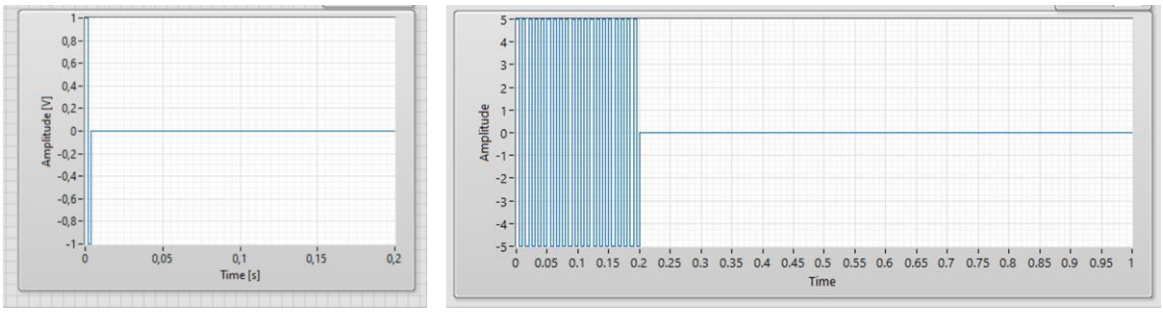

Fig 3. LabVIEW, low frequency protocol (single pulses of 1 Hz 30 V) on the left, high frequency protocol (zoom on bipolar pulses trains of 100 Hz for 200 ms) on the right.

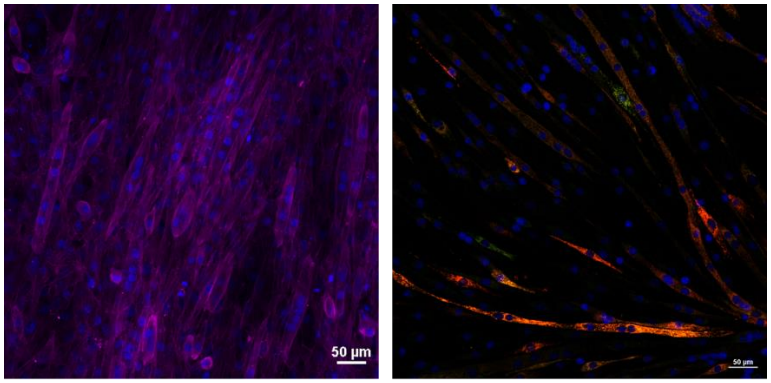

Fig 4. Confocal microscope fluorescence image of differentiated myotubes stained for F-actin filaments using Phalloidin on the left and Mitotimer on the right.

- REFERENCES

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2) Lee IM, Shiroma EJ, Lobelo F, Puska P, Blair SN, Katzmarzyk PT; Lancet Physical Activity Series Working Group. Effect of physical inactivity on major non-communicable diseases worldwide: an analysis of burden of disease and life expectancy. Lancet. 2012 Jul 21;380(9838):219-29
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5) Cheng AJ, Jude B, Lanner JT. Intramuscular mechanisms of overtraining. Redox Biol. 2020 Aug;35:101480.
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8) Marcheggiani F, Orlando P, Silvestri S, Cirilli I, Riva A, Petrangolini G, Orsini F, Tiano L. CoQ10Phytosomes Improve Cellular Ubiquinone Uptake in Skeletal Muscle Cells: An Ex Vivo Study Using CoQ10-Enriched Low-Density Lipoproteins Obtained in a Randomized Crossover Study. Antioxidants (Basel). 2023 Apr 20;12(4):964.
9) Nikolić N, Bakke SS, Kase ET, Rudberg I, Flo Halle I, Rustan AC, Thoresen GH, Aas V. Electrical pulse stimulation of cultured human skeletal muscle cells as an in vitro model of exercise. PLoS One. 2012;7(3):e33203.

Part 2. PhD student information on the overall year activity (courses/seminars/schools, mobility periods, participation to conferences)

List of attended courses/seminars/schools

1. Shot of Science 25/10/2022: Sonia Silvestri
2. Shot of Science 29/11/2022: Cristina Maracci
3. Shot of Science 28/02/2023: Alessia Pepe
4. Shot of Science 18/04/2023: Giorgia Giorgini
5. Shot of Science 30/05/2023: Marta Lombò
6. Seminar Steen Larsen 04/04/2023
7. Seminar Mattioli Belmonte 13/06/2023
8. PhD Course: Complex Networks
9. PhD Course: Design of Research
10. PhD Course: Latex
11. PhD Course: IR-Raman

List of mobility periods

1. Elettra, Trieste 15/11/2022-19/11/2022
2. Elettra, Trieste 08/12/2022-13/12/2022

List of conferences/workshops attended and of contributions eventually presented

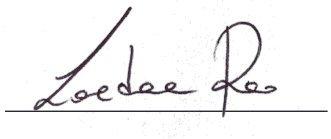
1. Poster presentation and participation in the Workshop “Mitochondrial Physiology- from Organelle to Organism summer school” in Copenhagen.
2. Poster presentation to 3rd DiSVA Masbic Symposium.

Part 3. PhD student information on publications

Sahu BS, Razzoli M, McGonigle S, Pallais JP, Nguyen ME, Sadahiro M, Jiang C, Lin WJ, Kelley KA, Rodriguez P,Mansk R, Cero C, Caviola G, Palanza P, Rao L, Beetch M, Alejandro E, Sham YY, Frontini A, Salton SR, Bartolomucci A. Targeted and selective knockout of the TLQP-21 neuropeptide unmasks its unique role in energy homeostasis. Mol Metab. 2023 Oct;76:101781.

[Date] 15/10/2023

Student signature



Supervisor signature