STUDY PROTOCOL AND STATISTICAL ANALYSIS PLAN

OFFICIAL TITLE: Effects of a Single Maximal Exercise Session on the Metabolic Function of Physically Inactive Young Adults

BRIEF TITLE: Single Maximal Exercise Session and the Metabolic Response of Physically Inactive Young Adults (EASY-Study)

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STUDY PROTOCOL

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ABSTRACT

Background: Physical exercise has been proposed as an effective intervention for the normalization of metabolic alterations caused by long-term physical inactivity. In this sense, one of the potential mediators of this response is Fibroblast Growth Factor (FGF) 21, due to its actions as an insulin sensitizer in various target tissues (e.g. skeletal muscle and white adipose tissue), promoter of lipid oxidation and anti-inflammatory. Recently, it has been described that a potential mediator of FGF21 production is lactate, since, in vitro studies have shown that treatment with increasing concentrations of lactate, both in adipocytes and muscle cells, promotes greater production of FGF21. These findings suggest that this metabolite would play a relevant role in the regulation of circulating concentrations of FGF21. However, the evidence in this regard is based mainly on findings in cell cultures, highlighting the absence of clinical studies focused on solving this problem.

Aim: To investigate the influence of exercise-induced changes in blood lactate on plasma FGF21 concentration in physically inactive subjects.

Methodology: This is a quasi-experimental study with pre- and post-intervention measurements. 20 adults (10 women and 10 men), aged between 18 and 30 years, physically inactive (performing less than 150 minutes/week of moderate physical activity and/or 75 minutes/week of vigorous physical activity) and without medical contraindications to physical activity will be included. Additionally, those individuals who are on medications that influence blood glucose or insulin levels and/or with anti-inflammatory effects will be excluded.

To induce elevations in blood lactate, an incremental load test will be applied on a stationary bicycle, in which the pedaling load will be increased every 3 minutes (30W; maintaining a pedaling speed of 60-70 revolutions per minute) until reaching the point of fatigue. Before starting the test, resting variables such as heart rate, subjective sensation of effort (SSE), blood pressure, pulse oximetry and muscle (vastus lateralis of quadriceps) will be recorded. In addition, a venous blood sample will be taken in a 2 ml tube with EDTA and another tube without anticoagulant for the subsequent plasma measurement of FGF21 and complementary biochemical tests (glycemia, insulin, lipid profile, transaminases and renal function markers), in addition to the measurement of lactate from a finger prick with a sterile lancet. In addition, urine samples will be taken both before and after exercise to measure renal function markers (proteinuria, creatinuria, albuminuria, glucosuria).

SPSS version 22 and GraphPad Prism version 8 software for Windows will be used. Quantitative variables will be described in terms of median and interquartile range and qualitative variables in terms of absolute frequencies. To make comparisons before and after the training program, the Wilcoxon test or Student t test for related samples will be used, depending on the distribution of the variables. To investigate the presence of correlations between the variables of interest, the Spearman coefficient will be used. The sample size calculation was carried out considering the following parameters: a power of 95%, an alpha value of 0.05, a difference in pre- and post-

intervention means of 50 (100 vs 150) pg/ml, with a standard deviation for both groups of 50 pg/ml. With these data, the sample size is 16 participants, and considering a 20% drop-off, the final size is 20 participants. A p value < 0.05 will be considered statistically significant for all analyses.

Expected results: It is expected to observe that the variations in blood lactate induced by exercise will be associated with changes in plasma concentrations of FGF21, in order to postulate lactate as one of the factors that modulate the production of this factor, and that, therefore, it could play a relevant role in the normalization of the metabolic function associated with exercise.

BACKGROUND

Physical inactivity is defined as not reaching the minimum levels of spontaneous physical activity required to maintain a healthy life. In this sense, this parameter is defined by the World Health Organization as performing less than 150 minutes/week of moderate-intensity physical activity and/or less than 75 minutes/week of vigorous physical activity (1). Therefore, maintaining a physically inactive life leads to the development of a range of cardiorespiratory and metabolic diseases, among which arterial hypertension, dyslipidemia, obesity, insulin resistance and type 2 diabetes mellitus stand out (2, 3).

However, even when these alterations manifest and develop, it has been described that physical activity and exercise have the capacity to counteract and treat these dysfunctions (4). In this regard, it has been postulated that these effects are mediated by factors whose release from endocrine organs (e.g., liver, white adipose tissue, and skeletal muscle) are induced by physical effort derived from exercise (5), recently being called exerkines (6). In this context, one of the mediators that has attracted scientific attention is fibroblast growth factor 21 (FGF21), a protein mainly produced in the liver, adipose tissue, and muscle (7). This interest is related to the multiplicity of functions in which this factor participates, where the following stand out: the promotion of glucose uptake, lipid oxidation, increased energy expenditure, and anti-inflammatory effects; all of these functions favor the prevention or treatment of metabolic dysfunctions associated with obesity (8), so the manipulation of FGF21 has been the subject of research in the field of management of metabolic dysfunctions associated with physical inactivity and obesity. This protein (~20 kDa) exerts its function by binding to FGFR 1 or 2 receptors, in addition to the binding of a co-receptor known as β-klotho to form a 1:2 hetero-complex (7, 9). Since the presence of these receptors and co-receptor is found in different tissues, such as white adipose tissue, skeletal muscle, heart, pancreas and brown adipose tissue (8), it is hypothesized that FGF21 can exert its metabolic functions in all of them, so that its manipulation would have rather systemic effects (Figure 1).

In this regard, recent literature has shown that circulatory levels of FGF21 change after physical exercise. Thus, in the face of a session of exercise at moderate intensities of effort, circulatory levels of FGF21 tend to increase by 20% compared to baseline values in sedentary people with overweight or obesity (10, 11). However, other authors have not found significant variations, despite subjecting participants to exercise sessions with similar characteristics (12, 13). This highlights the fact that it is unknown, to date, whether there are intermediate stimuli between physical exercise and the increase in FGF21 from its endocrine origins.

On the other hand, lactate has traditionally been described as a waste metabolite resulting from anaerobic glycolysis that develops, among others, during processes of high intensity and short duration physical effort (14). However, recent studies show that lactate has multiple cell signaling functions which, in part, have implications in the regulation of the metabolism of different tissues such as inhibiting lipolysis in white adipose tissue, regulating redox balance in skeletal muscle and promoting gluconeogenesis in the liver and kidney (15). Thus, investigating potential associations between circulating lactate during exercise and metabolism in skeletal muscle, Hojman et al. observed in a mixed model (humans, mice and myocyte cell culture) that elevations in blood lactate were related to elevations of the well-known myokine interleukin (IL) 6, and that the release of IL6 from muscle cells was from vesicles that required the activity of matrix metalloproteinases, which in

turn were activated by lactate signaling (16). Studies such as this have opened the door to the study of the metabolic mediating function of lactate, especially during physical exercise.

Considering the above, lactate has recently been proposed as a potential mediator of FGF21 release. Thus, Jeanson et al. described rapid elevations of FGF21 from mouse adipocytes after treatment with increasing concentrations of lactate, where the authors hypothesized that these elevations of FGF21 could have both autocrine and endocrine characteristics (17). In addition, similar results were described by Villarroya et al. in myotubes, in which increasing concentrations of lactate correspondingly and autocrinely increased the concentrations of FGF21, both in terms of messenger RNA and protein (18). The authors detail that the lactate concentrations necessary to produce these effects are in the order of >3 mM, concentrations that can be physiologically found in the circulation during physical exercise, particularly at moderate to high intensities (19). In addition, it has been described that blood lactate is also related to markers of function of other organs of high metabolic relevance, such as the kidney and liver. Thus, in people with type 2 diabetes mellitus (DM2), serum lactate levels were independently and positively related to alanine aminotransferase (GPT) levels (20), so it would be interesting to observe whether high-intensity physical efforts, which generate significant elevations of lactate, influence these markers of liver damage. From a renal point of view, similar results were found in a similar sample of more than a thousand people with DM2 in which, even in the absence of renal dysfunction, positive relationships were observed between blood lactate and creatinine, urea nitrogen, as well as with the transaminase GPT (21). Therefore, it would be relevant to observe whether high-intensity physical efforts have an impact on these markers of renal function acutely (after a session) in order to manage safety parameters when implementing these intervention strategies in physically inactive people.

Taken together, these findings suggest that lactate would play a relevant role in the regulation of circulating concentrations of FGF21, as well as markers of tissue stress in the kidney and liver. However, the evidence in this regard is based mainly on findings in cell cultures or observational clinical studies, highlighting the absence of quasi-experimental or experimental clinical studies focused on solving this problem, in order to explore the potential relationships between transient elevations of circulating lactate during exercise with those of other metabolic mediators such as FGF21 and tissue function markers (e.g. liver and kidney) (Figure 2). Therefore, and taking advantage of the widely described elevations of lactate concentrations after high-intensity exercise and/or close to fatigue levels (14), the present study aims to investigate the influence of variations in lactate concentrations on circulating FGF21 after incremental physical exercise until fatigue, in a clinical context.

Working hypothesis

The expected increase in blood lactate following incremental exercise to fatigue will increase plasma levels of FGF21 in physically inactive adults.

General aim

To investigate the influence of exercise-influenced changes in blood lactate on plasma concentration of FGF21 in physically inactive individuals.

Specific aims

• To characterize the phenotype of the study sample.

• To compare blood lactate levels pre and post incremental exercise.

• To compare plasma FGF21 levels pre and post incremental exercise.

• To relate changes in blood lactate to changes in plasma FGF21 pre and post incremental exercise.

Secondary aims

• To compare blood levels of kidney function markers in blood (creatinine, urea nitrogen, urea acid and urea) and urine pre and post incremental exercise.

• To compare blood levels of transaminases (GPT and GOT) pre and post incremental exercise.

• To relate changes in blood lactate to changes in renal function markers and blood transaminases pre and post incremental exercise.

METHODOLOGY

Design: This is a quasi-experimental study comparing pre- and post-intervention. The research question is associated with investigating the influence of changes in blood lactate influenced by exercise on the plasma concentration of FGF21 in physically inactive people. This project has the approval of the Scientific Ethics Committee of the Valdivia Health Service (Ord. 222/2022) and has Biosafety certification by the Institutional Biosafety Committee of the Austral University of Chile (No. 36/23).

Sample: People over 18 years of age (and up to 30 years of age) enrolled in the Kinesiology program at the Austral University of Chile will be included. They must be physically inactive (perform less than 150 minutes/week of moderate physical activity and/or 75 minutes/week of vigorous physical activity) and without medical contraindications to perform physical activity. People who are taking medications that influence blood glucose or insulin levels and/or with anti-inflammatory effects will be excluded. The sample size calculation was performed considering the following parameters: a power of 95%, an alpha value of 0.05, a difference in pre- and post-intervention means of FGF21 of 50 pg/ml (100 vs 150 pg/ml), with a standard deviation for both groups of 50 pg/ml (according to the studies by Sabaratnam et al. and Slusher et al.: references 10 and 11 of point 3.1). With these data, the sample size is 16 participants, and considering a 20% drop-off, the final size is 20 participants, where recruitment will be by convenience and maintaining gender parity.

Variables: At the time of entering the study, the age in years and sex of the participants will be recorded. Anthropometric variables such as weight in kilograms and height in meters will be

recorded using a scale (InBody® 270) and a height rod (SECA) respectively. From these measurements, the body mass index (BMI) will be calculated in kilograms/meters2. For the calculations of the waist-hip ratio (WHR) and waist-height ratio (WHR), the waist circumference will be measured in centimetres using the navel area as a reference point. The hip circumference will be measured as the largest circumference obtained at the trochanteric femoral level. The levels of total and segmental muscle and fat mass will be measured using the InBody® 270 bioimpedance meter. The level of spontaneous physical activity will be measured using the International Physical Activity Questionnaire (IPAQ) in its short version. To induce elevations in blood lactate, an incremental load test will be applied on a cycle ergometer or stationary bicycle, in which the pedalling load will be increased by 30W every 3 minutes, starting from an initial load of 30W until reaching the point of fatigue. Before starting the test, participants will remain seated for 10 minutes, during which time heart rate, subjective sense of exertion (SSE) with the modified Borg Scale, blood pressure, pulse oximetry and muscle (vastus lateralis of quadriceps) will be recorded at rest. In addition, a venous blood sample will be taken in a 2 ml tube with EDTA and another tube without anticoagulant for the subsequent plasma measurement of FGF21 with a specific ELISA kit for this (RayBio® catalog ELH-FGF21) and complementary biochemical tests (glycemia, insulinemia, total cholesterol, triglycerides, HDL, LDL, VLDL, non-HDL cholesterol, transaminases (GOT and GPT), creatinine, uric acid, urea and urea nitrogen). In addition, lactate measurement will be performed from a digital prick with a sterile lancet. For this, a specific reactive strip will be used, which will be measured with the AccuTrend Plus ® device. In parallel, the participant will be asked to bring a urine sample from the same day on an empty stomach for biochemical analysis (proteinuria, creatinine clearance, albuminuria, glucosuria). Once these measurements have been taken at rest, the incremental stress test will begin. Thus, on a cycle ergometer each participant will begin the test with a pedalling cadence of 60-70 RPM at 30W load. Every 3 minutes this load will increase by 30W, always maintaining the same pedalling cadence. During the execution of the test, participants will always be supervised and guided by a professional kinesiologist. Heart rate, pulse and muscle oximetry, and SSE will be assessed minute by minute, while blood pressure will be measured every 3 minutes. At the end of the test, the test execution time, the distance travelled in meters, heart rate, blood pressure, SSE and final pulse and muscle oximetry will be recorded. The percentage of frequency reserve used during the test will be calculated with the Karvonen formula, which depends on the heart rates at rest and post-test. Fatigue at the end of the test will be understood as or the fulfillment of one or more of the following criteria: use of heart rate reserve ≥ 90%, subjective feeling of effort ≥ 18/20 according to the original Borg scale, blood pressure ≥ 200/100 mg of Hg, desire of the participant to finish the test (Figure 3).

Immediately after the test, a new venous blood sample will be taken in a 2 ml EDTA tube and in another tube without anticoagulant. In addition, the post-exercise blood lactate concentration will be measured by finger prick using a specific reagent strip and with the AccuTrend Plus ® device. In addition, a post-exercise urine sample will be requested for biochemical analysis previously described. Subsequently, the participant's vital signs will be monitored for 10 minutes after the test to ensure proper recovery.

It is reiterated that, as an assessment of potential confounding/intermediate variables, the following biochemical parameters will be measured from blood samples taken pre- and post-exercise: Total cholesterol, triglycerides, HDL, LDL, VLDL, non-HDL cholesterol, insulin, glucose and transaminases

(GOT, GPT), creatinine, uric acid, urea and urea nitrogen. While, for urine samples, the levels of proteinuria, albuminuria, creatinine clearance, and glucosuria will be assessed.

Statistical analysis

Quantitative variables will be expressed as median and interquartile range, while qualitative variables will be described in terms of absolute frequencies. To calculate potential differences between pre- and post-stress test values, particularly lactate and FGF21, as well as transaminase levels and kidney function markers, the Wilcoxon test or Student t test for related samples will be used, depending on how the data distribution behaves (normal or nonparametric). In addition, associations between lactate and FGF21 concentrations, both pre- and post-exercise, will be explored using the Spearman correlation index and bivariate regression models. In addition, other potential associations of interest will be explored using the Spearman correlation index. For all analyses, a p value equal to or less than 0.05 will be considered statistically significant, using SPSS version 20 and GraphPad version 8 software.

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