

VO₂ Kinetics in Supra-Anaerobic Threshold Constant Tests Allow the Visualisation and Quantification of the O₂ Saving after Cytochrome C Oxidase Inhibition by Aerobic Training or Nitrate Administration

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Summary

We tested whether the known cytochrome c oxidase (COX) inhibition by nitric oxide (NO) could be quantified by VO_2 kinetics during constant load supra-Anaerobic Threshold (AT) exercises in healthy trained or untrained subjects following aerobic training or nitrate administration. In cycle ergometer constant load exercises supra-AT, identified in previous incremental tests, VO_2 kinetics describe a double exponential curve, one rapid and one appreciably slower, allowing the **area** between them to be calculate in O_2 L. After training, with increased NO availability, this **area** decreases in inverse ratio to treatment efficacy. In fact, in 11 healthy subjects after aerobic training for 6-7 weeks, **area** was decreased on average by 51%. In 11 untrained subjects, following the assumption of an NO donor, 20 mg isosorbide 5 mononitrate, **area** was decreased on average by 53%. In conclusion, supra-AT VO_2 kinetics in constant load exercises permit the quantification of the inhibitory effect NO-dependent on COX after either physical training or nitrate assumption.

Key-words

Oxygen consumption, anaerobic threshold, VO_2 kinetics, cytochrome c oxidase inhibition, nitrate, training

Introduction

Nitric oxide (NO), in appropriate concentrations, interacts with cytochrome c oxidase (COX), and causes an “early reduction” in the mitochondrial respiratory chain and a backlog of electrons in the upstream respiratory chain cytochromes because it partially and reversibly inhibits COX. Such an effect is modulated by both an increase in electrons turnover (ET) and by the normally high capacity of COX for processing electrons (Leterlier *et al.* 1994, Sharpe and Cooper 1998), allowing both cellular respiration and energy supply to be maintained. Experimentally, if NO production is strongly increased in cells, compensation will be gradually weakened, because the ability of COX to oxidize to water electrons, coming from cytochrome c, will approximate its saturation. This consequently decreases the O₂ consumption rate, i.e. mitochondrial respiration (Brown and Cooper, 1994, Brunori *et al.* 2005, Cleeter *et al.* 1994, Cooper and Giulivi, 2007, Erusalimsky and Moncada, 2007, Mason *et al.* 2006, Moncada and Erusalimsky, 2002, Palacios-Callender *et al.* 2007, Schweizer and Richter, 1994). This mechanism can predict that cells with low energy demand, with normal O₂ concentration [O₂], are respiring with a low ET rate. In this way, the COX catalytic sites, richer in oxidized species, will “consume” NO, oxidizing it to nitrate. In contrast, cells with high ATP demand, such as muscle fibers during intense physical effort, will respire with a high ET rate, and COX catalytic sites, richer in reduced species, will not consume NO, which, synthesized in quantity, will bind to and inhibit the COX catalytic sites with high affinity (Giuffré *et al.* 2000, Palacios-Callender *et al.* 2007).

Such a mechanism has been also reported by other authors to explain the VO₂ cost reduction during high-intensity exercises after dietary nitrate (Bailey *et al.* 2009, Lansley *et al.* 2010; Larsen *et al.* 2010) or L-arginine supplementations (Bailey *et al.* 2010), and to explain the opposite effect after infusion by L-NAME (Jones *et al.* 2003). We also

previously described this effect (Maione *et al.* 1998; Maione *et al.* 2000; Maione *et al.* 2001a; Maione *et al.* 2001b).

In this context, the present study aims to test whether it is possible visualize and quantify the interaction between NO and COX by recording VO₂ kinetics during cycle ergometer constant-load exercises (or square wave) supra-Anaerobic Threshold (AT) in a population of 11 healthy subjects after aerobic training and in 11 healthy subjects after administration of an organic nitrate acting as a NO donor.

Methods

Two groups of healthy volunteers were enrolled for the study. The first group included 11 healthy subjects: 9 young students and 2 amateur cyclists 68 and 72 old years (8 males, 3 females; mean age = 30.2 ± 20.1 years; BMI = 24.9 kg/m²); the second group included 11 healthy untrained subjects (6 males, 5 females; mean age = 36.5 ± 8.9 years; BMI = 25.6 kg/m²) from the Dept of Medical and Surgery Sciences, University of Bologna.

All subjects underwent a maximal cardiopulmonary test with a ramp protocol, preceded by a 3-minute rest period, and a 3-minute warm-up at the load of the first step ramp, which therefore began at the second step. Load progression during the ramp was adapted to the expected functional capacity of each subject, and loads of square wave tests were selected on the basis of previous incremental tests. Nevertheless, ramp test durations were significantly different for subjects in the second group, who were dissimilar in physical characteristics and functional capabilities. However, the objective of this study was only to demonstrate the ability of our method to illustrate and quantify the consequences of the interaction between NO and COX.

Subjects in the first group carried out two similar tests at constant load before and after a 7-weeks aerobic training program, performed on cycle ergometer. Four subjects also underwent a subsequent detraining of equal duration. Therefore, each subject in this group

performed a cardiopulmonary test before and after training, at constant intensity, and the four detraining subjects performed a third square wave test, with the same procedure and the same load. Each test was executed with the load at the point of "respiratory compensation to metabolic acidosis" (RC) (Wasserman *et al.* 1967; Wasserman *et al.* 1999), which was identified during the previous incremental tests.

The subjects of the second group carried out two similar square wave tests: before and after 1 dose of 20 mg of isosorbide-5-mononitrate, at the RC load, which was reduced by an average of 10.6% (range 4-40%). This large range is the result of attempts to identify the most effective load intensity in highlighting the interaction NO-COX. In any case, the first test was performed as a control, and the second, one day later, after nitrate at timing of its maximum effect: 1 to 1.5 hour after its assumption.

For all tests, a Medifit 1000 cycle ergometer (Holland), calibrated before each session and an ULTIMA-CPX (Medical Graphics Corporation - MGC) ergospirometer, calibrated before each test, were used and interfaced with a CASE 16 (Marquette, USA) electrocardiograph. We used a Rudolph mask (7930-7940 series, USA) and, only in the second group, we sealed the space between the mask and the face with a special gel, modelled on the internal geometry of the mask (Ultimate Seal, Rudolph, USA). To further improve adhesion between face and mask, again only in the second group, we replaced the cap and fastening straps of the Rudolph mask with PVC ones. A constant pedalling frequency during each test was set at 60 rpm and maintained with the help of a digital metronome. The raw breath-by-breath VO_2 data were averaged over 5 on 7 breaths. In addition, VO_2 data derived from the constant load tests were treated with a moving average filter in order to reduce the sampling noise, temporally aligned to the exercise beginning, and interpolated to 0.01 s.

A biexponential mathematical model was fitted to the data, using custom-built software, Cardio Pulmonary Exercise Parameters Estimator (CPEPE). This iteratively optimizes the

model parameters to fit to the VO_2 experimental data. The mathematical model was biexponential because in this kind of tests the VO_2 time-course presents two exponential kinetic components: a virtual one, extrapolated via software, which is earlier and rapid, and one corresponding to the actual VO_2 during the test, which is appreciably slower [the so called “slow component” (SLC)] and overlaps to the previous one (Fig. 1, above to left) (Barstow and Molé 1991; Casaburi *et al.* 1987; Pool *et al.* 1994; Whipp and Ward 1990, Whipp 1994). The integral of the **area** between two exponentials was calculated, expressed in litres (l) of O_2 . All square wave tests were run to enable the computing of the **area** during 6 minutes from the point of separation of two exponentials, except for a shorter duration in a single subject of the second group.

The trial was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) on volunteers, after informed consent had been confirmed in writing. The protocol was approved by the local ethical committee.

The normality of the distribution of the tested parameters was evaluated by the Kolmogorov-Smirnov test. Differences in **area** within every group, respectively before and after training, and before and after nitrate, were computed by the t-test for paired data, while in the 4 detrained subjects of the first group, differences were tested by the ANOVA test for repeated measures with Bonferroni correction. In all statistical analyses the significance level was set at $p < 0.05$. All summary statistics are presented as means \pm standard deviation (SD).

Results

After training, in the first group of subjects the **area** between the two exponential was reduced by the treatment, while it increased after detraining (Fig. 1). In these subjects the mean amplitude of the **area** before the training was 2.92 ± 0.96 l, while post-training it was 1.44 ± 0.90 l, with a difference of 1.47 ± 0.41 l (- 51%; $p < 0.001$); in the four detrained

subjects the mean amplitude of the area before and after training, and after the detraining was, respectively, 3.69 ± 0.57 , 2.11 ± 0.65 , and 3.04 ± 0.34 l. The mean difference between first and second tests was 1.59 l (- 42.8%; $p=0.006$), between the first and third tests 0.65 l (- 17.6%; $p=0.020$), between second and third tests 0.93 l (+ 44.1%; $p=0.014$). In subjects of the second group (Fig. 2), and the mean area between the two exponentials before nitrate assumption was 0.76 ± 0.42 l, and after nitrate 0.36 ± 0.28 l, with a difference of 0.40 ± 0.25 l (- 53%; $p<0.001$).

Discussion

The exercise at a constant load supra-AT, executed in subjects of the first group after aerobic training effectively illustrates the consequences of NO-COX interaction (Fig. 1). The training increased vascular capacity to synthesize NO, which the square wave exercise produced, decreasing VO_2 thus sparing O_2 , and inhibiting the COX. Oxygen sparing also reduces both metabolic and respiratory stress, and hence the fatigue, induced by exercise, promoting O_2 diffusion out of muscle fibres mitochondria towards other fibres far away from vessels (Thomas DD *et al.* 2001, Victor MV *et al.* 2009). So, it is not surprising if in one individual subjects of both group, independently of age, sex and the stimulus used (training/nitrate), the inhibitory action on COX was so effective as to completely null the **area** between the two exponentials in the square wave test, flattening the VO_2 curve to assume a mono-exponential course, typical of square wave exercises sub-AT (Fig. 3). Due to this mechanism, and the evidence that O_2 sparing is not compensated by an equal increase in anaerobic metabolism, O_2 sparing retards the critical moment when short term effort is no longer bearable by the cardiovascular and pulmonary systems. Such an effect is very probably due to the activation of AMP-activated protein kinase (AMPK) (Dzeja and Terzic 2009; Steinberg and Kemp 2009), which occurs when the AMP/ATP ratio increases beyond a certain threshold, mainly as a consequence of

accelerated ATP lysis, as during exercise, making this ratio a sensitive indicator of reduced cellular energy state (Hardie and Sakamoto 2006; Jorgensen *et al.* 2006). Such muscle metabolic changes produced by AMPK activation are smaller after a single effort than after endurance training, and lie in increased insulin sensitivity (Etgen *et al.* 1997; Goodyear and Kahn 1998). In fact, translocation of the glucose carrier GLUT4 from intracellular stores into myocyte plasma membrane is increased (Kurt-Kraczek *et al.* 1999), causing both greater glucose uptake, and hexokinase activation (Holmes *et al.* 1999), which promotes glycolysis, supplying more substrates to the Krebs cycle. At the same time, there is an inhibition of glycogen synthesis (Carling and Hardie, 1989), without affecting glycogenolysis, during muscular effort (Mu *et al.* 2003). Furthermore, plasma fatty acids uptake is increased, largely through increased AMPK-dependent expression of carrier proteins on the plasma membranes (Bonen *et al.* 2007). Plasma fatty acids and the fatty acids stored in muscle fibers are then oxidized (Kiens 2006). All these mechanisms, together with increased concentration of the Krebs cycle enzymes in the muscle fibers mitochondria (Holloszy *et al.* 1970), lead to both increased aerobic metabolism and reduced recourse to anaerobic metabolism. Consequently, both the peak of the respiratory exchange ratio at the mouth (RER or R) and the area under the RER curve decrease after training and increase after detraining (Fig. 1). In fact, the effect of AMPK in increasing GLUT4 and plasma fatty acids carriers is relatively short-lasting, and regular muscle recruitment is essential to maintain their activity (Host *et al.* 1998; Koonen *et al.* 2004). Taken together, these considerations show that both the O₂ sparing and the RER decrease depend not only on the NO-COX interaction, but also on the consequential, complex and effective, metabolic changes, reported above.

Yet, other mechanisms link NO-COX interaction to AMPK activation. As a result of such interaction the electrons leak from complexes I and III increases, and one electron is transferred from NO to molecular O₂ to produce superoxide anion (O₂^{•-}) and then hydrogen

peroxide (H_2O_2), causing rapid depletion of ATP and an accumulation of AMP (Almeida et al. 2004). This activates AMPK, indicating H_2O_2 to be a major signal for AMPK regulation under oxidative stress (Choi et al. 2001). Again, during both NO-COX interaction (Woods et al. 2005), and muscular exercise (Rose and Hargreaves 2003) Ca^{2+} is released and, linked to calmodulin kinase kinase, activates AMPK. Besides, endothelial NO-synthase is also a downstream target of AMPK that is therefore able to influence the availability of NO. So, the pathway NO-cytochrome c oxidase-AMPK is a real signalling cascade and could represent a survival pathway during various pathophysiological conditions (Fig. 4).

In the second group, after administration of nitrate, we confirmed that NO-dependent COX inhibition is unambiguously direct, and that it can be quantified through this clinical test using VO_2 kinetics during constant intensity exercises supra-AT (Fig. 2). Before and after treatment, the remarkable differences in **area** between the two groups are due in part to lower loads of the square wave tests in second group. In fact, the workload selection used in the first group is preferable, because the greater **area** developed allows for easier inter-subject and inter-group comparison. A reduction in the contribution of anaerobic metabolism after nitrate assumption was also observed in square wave tests of the second group, albeit less pronounced compared to those of the first group, due to the loads, all selected below the RC point of the incremental test. However, the results of the square wave tests in the two groups were qualitatively quite similar, despite the different load intensities: both higher NO concentrations and metabolic changes improved tolerance to stress, reduced VO_2 and decreased RER during muscular effort, after physical training and nitrate administration.

The quantification, by our test, of the variable **area**, could be used to monitor the benefits of physical aerobic training, because we believe that more effective training should be followed by a greater decrease in **area**. In clinical and experimental pharmacology and in cases of pathology, drugs either directly activating AMP-kinase or acting in general on

mitochondria can also affect the main, respiratory, mitochondrial function, and the method, that we described, can provide information about this.

This study is a starting point to implement this technique in clinical and experimental use, as well as in sports medicine.

In conclusion, on the basis of our preliminary data, the VO_2 kinetics supra-AT during constant load exercise allow the visualization and quantification of the NO inhibitory effect on COX after both physical training and nitrate assumption.

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Fig. 1. In a young student of the first group, VO_2 kinetics during square wave test supra-AT with the **area** between the two exponentials: training and detraining effects. Light gray line: VO_2 , continuous black line: fitting curve, dashed black line: first exponential extrapolated curve. Bottom unaveraged data: VO_2 in light grey line, R in black line.

Fig. 2. Supra-AT VO_2 kinetics in a 49-years old subject of the second group. Before (left) and 1 hour after (right) 20 mg of isosorbide-5-mononitrate: during 6 min **area** is decreased by 50%; bottom VO_2 in light gray, R in black.

Fig. 3. A 72-years old cyclo-amateur of the first group: pre-traing (left), and after training (right). The **area** between the two exponential disappeared after training; bottom unaveraged data; VO_2 in light grey, R in black.

Fig. 4. The activating pathways AMPK and its effects on glucose and fatty acids metabolisms and on RER. Explanation in the test.

Fig.1

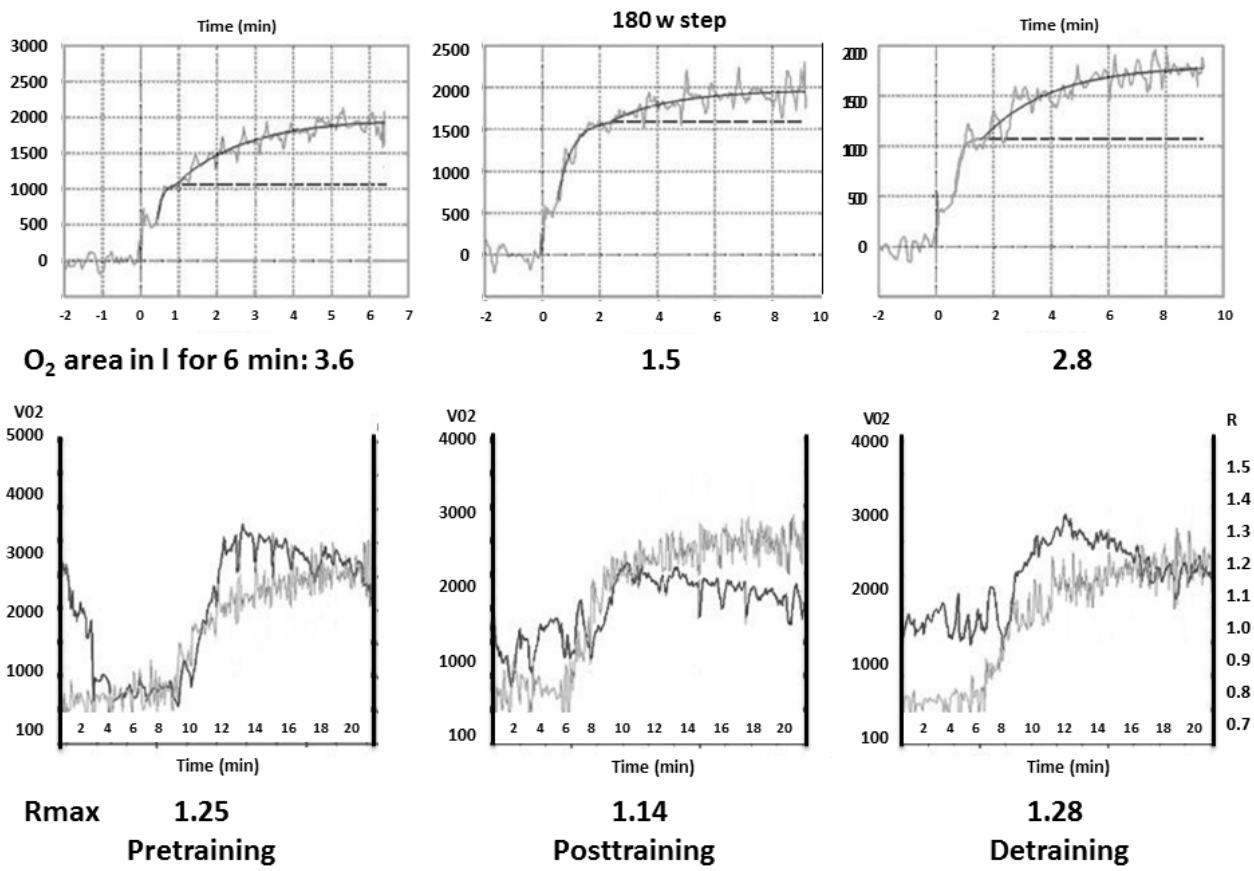


Fig.2

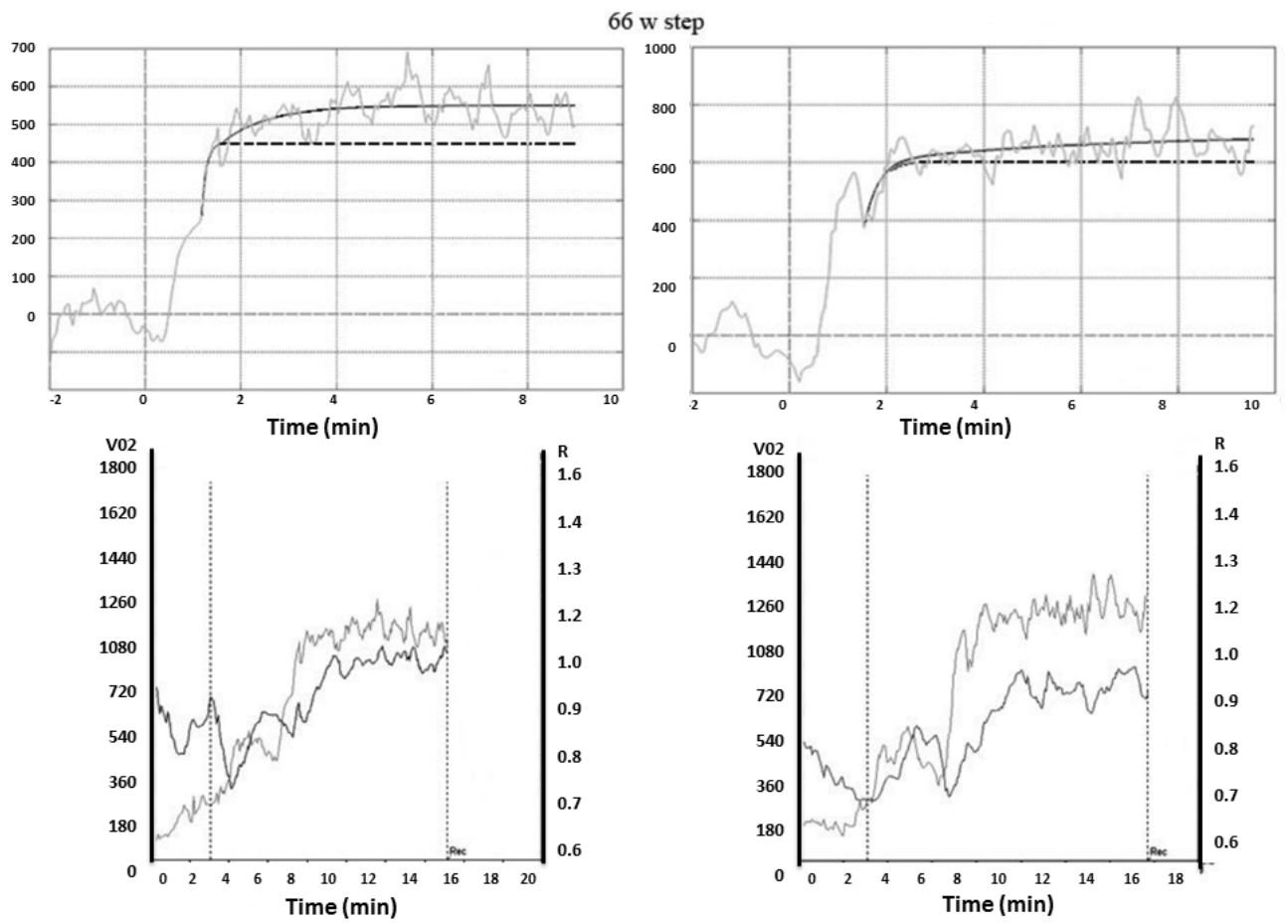


Fig.3

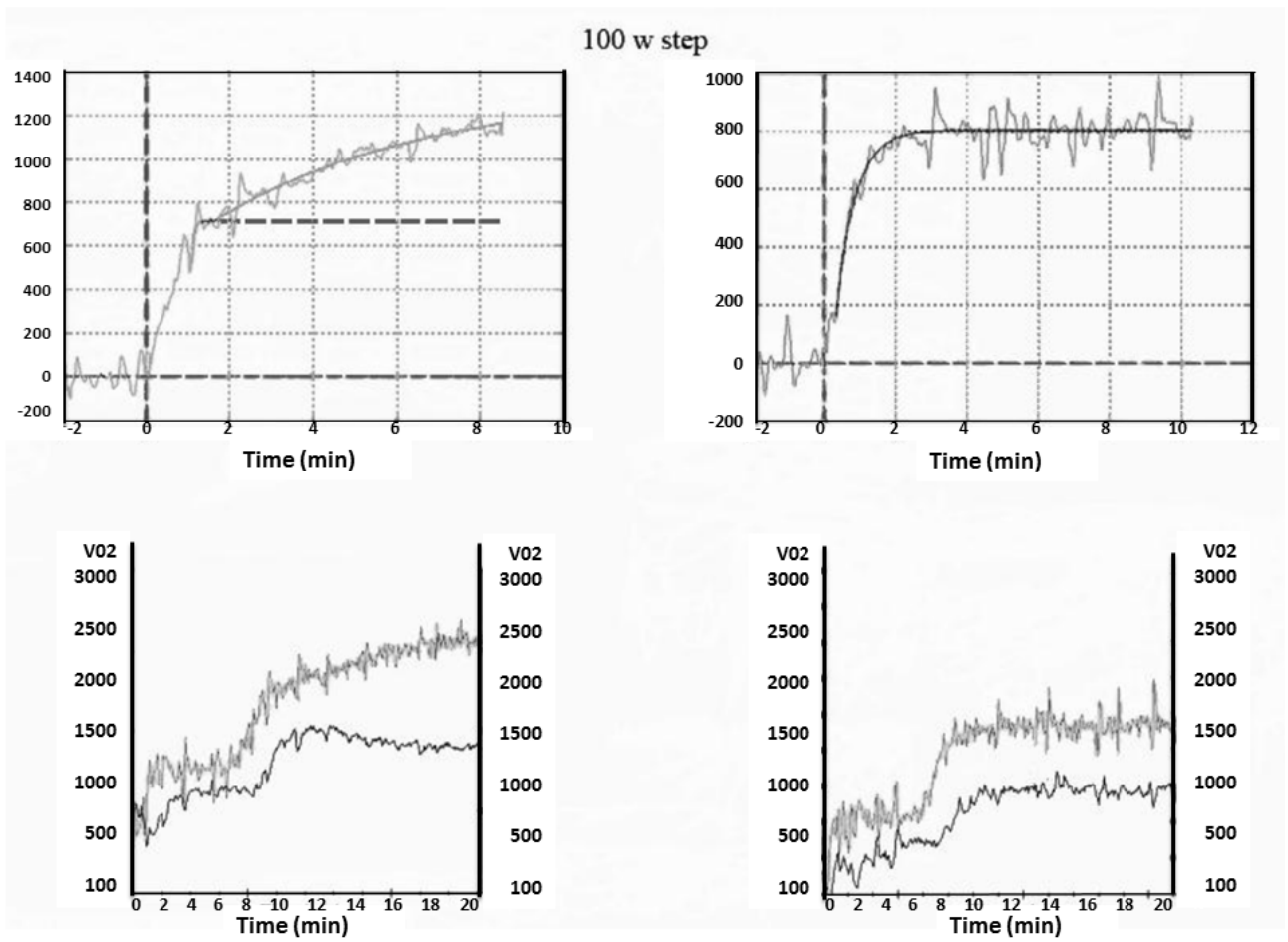


Fig.4

