Abstract:

Objectives: Dietary nitrate (NO_3^-) supplementation and ischaemic preconditioning (IPC) can independently improve exercise performance. The purpose of this study was to explore whether NO_3^- supplementation, ingested prior to an IPC protocol, could synergistically enhance parameters of exercise.

Design: Double-blind randomized crossover trial.

Methods: Ten competitive male cyclists (age 34 ± 6 years, body mass 78.9 ± 4.9 kg, $\dot{V}O_{2peak}$ 55 ± 4 mL·kg·min^{-1}) completed an incremental exercise test followed by three cycling trials comprising a square-wave submaximal component and a 16.1 km time-trial. Oxygen uptake ($\dot{V}O_2$) and muscle oxygenation kinetics were measured throughout. The baseline (BASE) trial was conducted without any dietary intervention or IPC. In the remaining two trials, participants received 3 × 5 min bouts of lower limb bilateral IPC prior to exercise. Participants ingested NO_3^--rich gel (NIT+IPC) 90 min prior to testing in one trial and a low NO_3^- placebo in the other (PLA+IPC). Plasma NO_3^- and nitrite (NO_2^-) were measured immediately before and after application of IPC.

Results: Plasma [NO_3^-] and [NO_2^-] were higher before and after IPC in NIT+IPC compared to BASE (P<0.001) but did not differ between BASE and PLA+IPC. There were no differences in $\dot{V}O_2$ kinetics or muscle oxygenation parameters between trials (all P>0.4). Performance in the time-trial was similar between trials (BASE 1343 ± 72 s, PLA+IPC 1350 ± 75 s, NIT+IPC 1346 ± 83 s, P=0.98).

Conclusions: Pre-exercise IPC did not improve sub-maximal exercise or performance measures, either alone or in combination with dietary NO_3^- supplementation.

Keywords: Nitric oxide; blood flow; hyperaemia; nitrite; exercise

Introduction

Ischemic preconditioning (IPC) typically consists of blood flow occlusion followed by a period of reperfusion which is repeated over 2 – 4 cycles. Whilst originally utilized to suppress the damaging effects of prolonged ischemia to an organ or skeletal muscle, IPC has recently been adopted as a preparation tool for performance enhancement[1]. Although the precise mechanism(s) by which IPC can improve exercise performance are not fully understood, recent evidence demonstrates that IPC causes an increase in circulating nitrite (NO_2^-) via shear stress activation of nitric oxide (NO) by endothelial NO synthase (eNOS), resulting in subsequent physiological effects[2,3]. For example, remote limb IPC provides systemic whole-body protection beyond the site of ischemia and when applied to either the upper or lower limbs, can lead to enhanced muscle blood flow and thus oxygen (O_2) delivery, and an improved efficiency during aerobic respiration[4–6]. These physiological factors may account for the purported ergogenic effects of IPC on exercise performance[7,8].

Dietary nitrate (NO_3^-) supplementation can also increase circulating plasma NO_2^- via the enterosalivary NO_3^- – NO_2^- – NO pathway[9]. During this process, facultative anaerobic bacteria residing in the oral cavity reduce NO_3^- to NO_2^- which can be further reduced to NO in hypoxic or acidic conditions[10]. Studies have demonstrated that dietary NO_3^- supplementation can induce vasodilation, reduce the O_2 cost ($\dot{V}O_2$) of exercise and, in some cases, improve exercise performance[10,11]. Importantly, these effects appear more pronounced in hypoxic or acidic conditions[12], such as during high-intensity exercise or at altitude[13]. Another potential synergetic interaction between IPC and NO_3^- is the time course of their effects. It has been shown that plasma NO metabolites reach peak levels 1-3 h following ingestion of NO_3^-, with levels returning to baseline levels after 6-8 hours[14]. Similarly, IPC has been shown to offer an early window of protection 1-2 h post-IPC for ischemic reperfusion injury[15] and influence exercise performance up until 8 h after administration[16]. Given that IPC and ingestion of NO_3^- can both independently increase NO_2^- and improve exercise performance, it is conceivable that a

combination of these interventions may lead to a more pronounced increase in NO availability and improvement in exercise performance.

The purpose of this study therefore, was to determine the combined effects of dietary NO_3^- supplementation and pre-exercise IPC of the lower limbs on the physiological responses to sub-maximal exercise and time-trial performance. We hypothesized that IPC combined with dietary NO_3^- supplementation would result in a cumulative rise in plasma NO_2^- and improve muscle oxygenation, $\dot{V}O_2$ kinetics, and exercise performance compared to a control or IPC alone.

Methodology

Ten competitive, trained male cyclists (age 34 ± 6 years, body mass 78.9 ± 4.9 kg, $\dot{V}O_{2peak}$: 55 ± 4 mL·kg·min^{-1}, ventilatory threshold: 272 ± 30 W, maximum work rate: 424 ± 42 W) volunteered and provided written informed consent to participate in the study. The participants had all previously participated in exercise testing in a laboratory. All participants met the following inclusion criteria: cycling training for a minimum of two years, training at least three days per week, and racing on a regular basis including time-trials[17]. The study was granted ethical approval by the School of Science and Sport Ethics Committee at the University of the West of Scotland, and all procedures were conducted in accordance with the Declaration of Helsinki.

The experimental design is outlined in **Figure 1**. Each participant visited the laboratory on four separate occasions over a 4–6 week period and all visits were interspersed with a minimum 1-week recovery period. Participants arrived at the laboratory at least 3 h post-prandial and completed each of their trials at the same time of day (± 2 h) in a temperature-controlled environment (20.5 ± 1.6°C). During visit 1, standard anthropometric measures were assessed prior to completion of a continuous graded incremental exercise test to exhaustion at a rate of 30 W·min^{-1} on an electronically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands) for determination of ventilatory threshold and VO_{2peak}. The second visit was a

baseline performance trial (BASE) which was followed by two further experimental performance trials.

The BASE trial followed a similar protocol to the experimental trials but was not preceded by any intervention. The experimental performance trials were preceded by ingestion of either 2 × NO_3^- gels (NIT+IPC; Science in Sport Go+ Nitrates, Lancashire, UK, ~ 500 mg NO_3^-) or a low NO_3^- placebo gel matched for taste and texture (PLA+IPC; Science in Sport bespoke gel, ~0.001 mg NO_3^-) 90 min before arrival at the laboratory[18]. This dose of NO_3^- has been previously shown to improve cycling performance[13]. The supplementation regimen was conducted using a double-blind randomized crossover design. The allocation of supplementation order was arranged using a random sequence generation and this was not revealed to the researchers until after analyses had been completed. Participants were asked to refrain from the consumption of alcohol and caffeine and to avoid any strenuous exercise for 24 h before each trial. In addition, they were requested not to use anti-bacterial mouthwash for the entire duration of the study.

During the NIT and PLA trials, each participant received four cycles of IPC. The IPC protocol for each cycle comprised 5 min bilateral occlusion of the lower-limbs at a pressure of 180 mmHg (E20 Rapid Cuff Inflator, Hokanson, Bellevue, WA) followed by 5 min reperfusion[4,19]. The pressure applied was >50 mmHg above resting systolic blood pressure (122 ± 6 mmHg), a stimulus which has been shown previously to improve exercise performance[8]. During the first cycle of IPC, visual confirmation of arterial occlusion was assessed using color Doppler imaging duplex with a L12 linear array transducer (Vivid 7 ultrasound machine, GE Electronics, Germany). During BASE, participants lay supine for 30 min to match the duration of IPC in the experimental trials. In each trial, participants initially lay supine for 15 min prior to obtaining a venous blood sample by venepuncture to ensure values were not influenced by postural changes[20]. Samples were collected in a vacutainer containing EDTA and spun immediately in a centrifuge for 10 min at 4000 rpm and 4ºC before the plasma was extracted and frozen at -80ºC. Plasma samples were later analysed for plasma [NO_3^-] and [NO_2^-] via gas-

112 phase chemiluminescence using methods previously described in detail[21]. A second venous
113 blood sample was obtained immediately after completion of the IPC protocol in the PLA+IPC
114 and NIT+IPC trials to determine the effects of IPC on NO_2^- and NO_3^- concentration.
115
116 Participants then performed a 12 min square-wave bout of submaximal cycling exercise
117 followed by a 16.1 km time-trial. The square-wave protocol consisted of 3 min rest in a seated
118 position followed by 6 min cycling at an intensity of 80% ventilatory threshold and cadence of
119 80 rpm followed by 3 min of seated recovery. The square-wave test was completed on an
120 electronically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands) and
121 enabled a standardized comparison of muscle oxygenation and $\dot{V}O_2$ kinetics between trials.
122 Pulmonary gas exchange and ventilation were continuously measured breath-by-breath for the
123 full duration of the square wave bout (Medgraphics Ultima, MGC Diagnostics, MN, USA) but
124 not during the time-trial. The coefficient of variation (CoV) for the measurement of $\dot{V}O_2$ during
125 moderate intensity cycling exercise in our lab is 2.4%. Near infrared spectroscopy (NIRS) was
126 used to monitor local muscle oxygenation of the right *vastus lateralis* (NIRO 200NX,
127 Hamamatsu Photonics KK, Hamamatsu, Japan). The NIRO uses three different wavelengths of
128 near-infrared light (735, 810 and 850 nm) transmitted via a light emitting diode. The receiving
129 diode measures the returning light from the tissue. The probes were placed in a manufacturer-
130 supplied black rubber holder (with a fixed emitter-detectors distance of 4 cm) and attached to
131 the muscle with tape then secured using a transparent film dressing. The modified Beer-
132 Lambert method was used to detect changes in the concentration of oxygenated (HBO_2) and
133 deoxygenated (HHb) haemoglobin and total tissue haemoglobin and myoglobin (tHB = HBO_2
134 + HHb). All NIRS data are expressed as arbitrary units based on the change from the baseline
135 value. Tissue oxygenation index (TOI) was assessed using the spatially resolved spectroscopy
136 technique. TOI is presented as a percentage and denotes the percentage ratio of HBO_2 to tHB.
137 The NIRS data were sampled at 5 Hz and then average for final minute of the resting phase and
138 for the last 3 min of the exercise phase were analysed.

The cycling time-trial was completed on an air and magnetically braked cycle ergometer (Wattbike Pro, Wattbike Ltd, Nottingham UK). Participants were instructed to cycle at a freely chosen cadence against an adjustable resistance in order to complete the time-trial in the fastest time possible. The Wattbike Pro cycle ergometer has been shown to have good reliability when used for repeated trials among trained participants[22]. The CoV for the measurement of 16.1 km time-trial performance in trained cyclists on the Wattbike cycle ergometer in our lab is 0.9%. Participants received verbal feedback on the distance covered upon completion of each kilometre and every 250 m for the final kilometre.

Breath by breath $\dot{V}O_2$ data from the square-wave test were filtered to remove values lying 4 standard deviations (SD) from the local 5 breath mean. A non-linear least squares monoexponential model was fitted to the data from 0 s to 540 s to characterise the $\dot{V}O_2$ responses to sub-maximal exercise using the following equation:

$$\dot{V}O_2(t) = \dot{V}O_2\text{rest} + A_p\,[1 - e^{-(t/\tau)}]$$

Where $\dot{V}O_2(t)$ is the $\dot{V}O_2$ at a given time point (t); $\dot{V}O_2$rest is the mean $\dot{V}O_2$ during rest; A_p is the amplitude (steady state $\dot{V}O_2$ - $\dot{V}O_2$rest) and τ the time constant.

The reported mean response time (MRT) was calculated as the τ of the exponential function describing the rate of $\dot{V}O_2$ and represents the time elapsed for a 63% increase in $\dot{V}O_2$. The functional "gain" was also calculated by dividing the A_p by the work rate of the submaximal exercise.

All analyses were carried out using RStudio Team (2016) Version (RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/) **(see Supplementary methods)** and Graph Pad Prism 7 (GraphPad Software Inc., San Diego, USA) for graph figures. One-way (condition) and two-way (condition and time) repeated-measures

analyses of variance were used to analyse the differences in plasma NO_3^- and NO_2^- concentrations, respiratory variables, muscle oxygenation, and time-trial outcomes. Post-hoc analyses of significant within-subject effects were performed with adjustments for multiple comparisons using the Bonferroni correction. Statistical significance was accepted when $P<0.05$. Results are expressed as mean ± SD and Δmean ± 95% confidence intervals (95% CI) where appropriate.

Results

Plasma NO_2^- and NO_3^-

The effect of NO_3^- supplementation and IPC on plasma NO metabolites are presented in **Figure 2A and Figure 2B**. There was a significant effect of NO_3^- supplementation on plasma [NO_3^-] and [NO_2^-] (both $P<0.001$). Prior to the administration of IPC, plasma [NO_3^-] and [NO_2^-] were significantly higher in the NIT+IPC condition compared to BASE (NO_3^- $P<0.001$, mean difference 375 μM, 95%CI 306–444 μM; NO_2^- $P<0.001$, mean difference 225 nM, 95%CI 85–366 nM). There was no difference between the PLA+IPC and BASE conditions for either measure (NO_3^- $P=0.991$; NO_2^- $P=0.991$). Following the administration of IPC in the NIT+IPC condition, plasma [NO_3^-] and [NO_2^-] remained elevated compared to BASE (NO_3^- $P<0.001$, mean difference 342 μM, 95%CI 280–404 μM; NO_2^- $P<0.001$, mean difference 250 nM, 95%CI 113–387 nM). Plasma [NO_3^-] and [NO_2^-] did not change from pre- to post-administration of IPC in the NIT+IPC condition ($P=0.991$, $P=0.995$, respectively). There were no differences in plasma [NO_3^-] and [NO_2^-] between PLA+IPC and BASE following IPC administration ($P=1.00$). These measures did not change from pre- to post-administration of IPC in the PLA+IPC trial (NO_3^- $P=0.991$; NO_2^- $P=0.999$).

$\dot{V}O_2$ kinetics

The pulmonary gas exchange data at rest and during submaximal exercise are presented in **Table 1**. The $\dot{V}O_2$ at rest and during steady state exercise was not different between conditions ($P=0.400$, $P=0.401$, respectively). There were also no differences in the MRT ($P=0.400$),

amplitude of the $\dot{V}O_2$ response (P=0.400), or the functional gain (decrease in $\dot{V}O_2$ relative to the increase in work rate) between trials (P=0.104).

Muscle oxygenation

The [HbO_2], [HHb], and [TOI] data are presented in **Table 1.** There were no significant differences between the three trials at rest or during exercise in any of the NIRS variables (all P>0.9).

Time-trial performance

The time-trial completion time was not different between trials (BASE 1342.8 ± 72.3 s, PLA+IPC 1350 ± 74.5 s, NIT+IPC 1346.2 ± 83.3 s, P=0.978, **Figure 2C**).

Discussion

To our knowledge, this is the first study to investigate the influence of dietary NO_3^- supplementation combined with bilateral lower limb IPC on the physiological responses to submaximal cycling and exercise performance. In contrast to our hypothesis, IPC combined with NO_3^- supplementation increased the availability of plasma [NO_2^-] from baseline but did not improve $\dot{V}O_2$ kinetics or muscle oxygenation during submaximal exercise or enhance cycling time-trial performance.

Whilst IPC has been previously shown to improve some physiological responses to exercise [4-6], there are conflicting findings[19] suggesting IPC does not alter $\dot{V}O_2$ or $\dot{V}O_2$ kinetics. Cocking and colleagues[23] recently reported that $\dot{V}O_2$ was lower during a cycling time-trial following the administration of IPC on the lower limbs. The authors suggested that local IPC may increase metabolic efficiency although this is likely task and/or intensity specific. The present study demonstrates further that pre-exercise administration of IPC does not improve muscle oxygen or reduce $\dot{V}O_2$ during sub-maximal exercise in well-trained cyclists. Moreover, the addition of an acute NO_3^- supplement to IPC also failed to alter these parameters. This finding is at odds

223 with the majority of studies investigating dietary NO_3^- supplementation, although the lack of
224 effect on $\dot{V}O_2$ is not entirely unprecedented[24].
225
226 The previously reported reductions in $\dot{V}O_2$ that result from either IPC or NO_3^- administration
227 may be underpinned by an increased NO availability[13] although the precise mechanism(s)
228 remain unconfirmed. Whereas dietary NO_3^- is believed to augment NO availability via the
229 enterosalivary NO_3^-–NO_2^-–NO pathway[9], IPC may increase endogenous production of NO via
230 eNOS stimulation[2]. Previous data suggests that an increased availability of NO may improve
231 the efficiency of mitochondrial respiration[25] and/or, reduce the energy cost of muscle force
232 production[26]. It is also well-established that NO availability plays a role in the regulation of
233 skeletal muscle blood flow and oxygenation during exercise[27]. In the present study, IPC did not
234 increase plasma [NO_2^-] or [NO_3^-], which may explain the null effect on the outcome parameters
235 assessed in this arm of the study. Conversely, the concentration of circulating NO metabolites
236 did substantially increase during the NIT+IPC protocol but $\dot{V}O_2$ and muscle oxygenation did
237 not differ from BASE. Whilst it can be argued that plasma [NO_2^-] and [NO_3^-] do not necessarily
238 reflect whole body NO production, plasma [NO_2^-] is generally accepted to be the best marker
239 of regional eNOS activity[28]. Whilst these findings are not readily explainable, a recent clinical
240 study by Hauerslev and colleagues[29] may shed some light on this discrepancy. These authors
241 reported that IPC and treatment with glyceryl tri-nitrate (an NO donor) each independently
242 protected against endothelial ischemic reperfusion injury. When combined, however, the
243 protection was lost. Others have speculated that excess NO generated by NO donors can inhibit
244 the neural signaling cascade that follows repeated bouts of ischemia and reperfusion[30]. This
245 neural stimulation causes unidentified low-molecular-mass circulating hydrophobic factor(s) to
246 be released into the blood stream which are suggested to underpin the cardioprotective effects
247 of IPC[31].
248
249 In line with the absence of any alteration in muscle oxygenation and $\dot{V}O_2$ kinetics parameters,
250 the application of IPC, either alone or in combination with dietary NO_3^- ingestion, did not have

any impact on cycling time-trial performance. Although previous research has shown that IPC can improve running[7], rowing[32] and swimming performance[8] these ergogenic benefits are not always observed[33]. Dietary NO_3^- supplementation has also been shown to improve cycling performance in some trials[13] but a recent meta-analysis suggests that the effects are trivial and non-significant[11]. The failure of either NIT+IPC or PLA-IPC to improve exercise performance may be explained by a number of factors. One cannot rule out that the beneficial effects of NO_3^- may have been abolished by co-administration of IPC[29] as previously discussed. Alternatively, studies have noted a profound inter-individual variability in response to NO_3^- supplementation[14] which may be influenced by multiple factors. For example, Porcelli and colleagues[34] have demonstrated that well-trained individuals, such as those used in the present study, have a blunted ergogenic response to NO_3^- supplementation. We have also demonstrated that the abundance of oral NO_3^--reducing bacteria can influence NO_3^-/NO_2^- pharmacokinetics[9]. However, the oral microbiome was not assessed in the present study and further research is required to determine how the abundance of these bacteria may influence the physiological responses to NO_3^- supplementation.

One potential limitation of our study is that we did not include a sham condition for IPC. Indeed, a recurring issue in the field is the lack of an appropriate control measure for IPC research studies. In some studies, cuff inflation pressures of 20-50 mmHg were used as a sham treatment or cuffs were applied but not inflated[1]. However, the pressure differences are easily identifiable making it impossible to adequately blind participants to the treatment. This raises the possibility that IPC may exert either placebo or nocebo effects on exercise performance. One recent study demonstrated similar ergogenic effects were obtained using both IPC (occlusion at 220 mmHg) and a sham treatment (pressure of 20 mmHg)[33]. Moreover, IPC has been shown to improve exercise tolerance (as measured by time to exhaustion at 0.5 km/h above peak velocity) but this improvement is no greater than that obtained through a placebo intervention of therapeutic ultrasound[35]. On the whole this highlights the need for a better understanding of the mechanisms of IPC action and the potential mediators involved.

279 Based upon our findings, future studies may wish to examine different exercise intensities when
280 combining IPC and dietary NO_3^- given that NO appears to best utilized in conditions of hypoxia,
281 at a low pH, and in non-oxidative fast twitch fibers. Given IPC causes complete arterial
282 occlusion it could prime muscle for exercise at extreme intensities where oxygen availability is
283 significantly decreased. Griffin et al.[36] have reported that IPC enhanced critical power (CP) in
284 recreationally active males, building upon the rationale that CP has been shown to be improved
285 when O_2 delivery is enhanced via exposure to hyperoxia (FiO_2 = 70%)[37]. If IPC can indeed
286 improve CP, this should theoretically translate to an improvement during exercise intensities
287 between the heavy and severe domains.
288
289 **Conclusions**
290 This is the first study to investigate the effects of IPC in combination with dietary NO_3^-
291 supplementation on the responses to submaximal cycling exercise and time-trial performance.
292 While previous research has reported that IPC and NO_3^- can each independently have ergogenic
293 effects, we found that IPC alone or in combination with NO_3^- did not alter $\dot{V}O_2$ kinetics, muscle
294 oxygenation, or performance. Of note, there was no improvement in these outcomes in the
295 NIT+IPC trial despite the protocol significantly increasing the availability of plasma NO_2^-
296 metabolites. While further research is required to unravel the interactions between responses to
297 IPC and NO_3^- supplementation, the present research study suggests that a combination of these
298 interventions is not an efficacious method to improve 16.1 km cycling performance in well-
299 trained cyclists.
300
301

Practical Implications

- Acute ingestion of dietary nitrate in combination with ischemic preconditioning does not influence oxygen kinetics, muscle oxygenation, or cycling performance
- A combination of acute dietary nitrate and ischemic preconditioning is not an effective method of improving exercise performance.
- Nitrate and nitrite bioavailability do not appear to be mediators of the physiological responses to ischemic preconditioning

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Figure Legends

Figure 1: Study design schematic outlining the three experimental conditions: Baseline (BASE), placebo plus ischemic preconditioning (PLA+IPC) and nitrate supplementation plus ischemic preconditioning (NIT+IPC). The BASE trial was completed first with the remaining two conditions completed in a randomized order.

Figure 2: (**A**) Plasma nitrite and (**B**) plasma nitrate concentration before (PRE) and after (POST) application of the ischaemic preconditioning protocol during each performance trial. (**C**) 16.1km time-trial completion time, including individual completion times. Data are presented as mean ± SD. *denotes significant difference from BASE condition ($P < 0.001$).

453 **Table 1.** Oxygen Kinetics and NIRS variables during submaximal exercise test

Variable	BASE	PLA + IPC		NIT + IPC	
		Difference	95 % CI	Difference	95 % CI
Oxygen Kinetics					
VO_2 rest ($ml \cdot min^{-1}$)	313	-24	-74, 25	-34	-83, 16
VO_2 exercise ($ml \cdot min^{-1}$)	2999	-201	-490, 88	-123	-412, 166
MRT (s)	41.9	0.6	-5.3, 6.4	0.5	-5.4, 6.4
Amplitude ($ml \cdot min^{-1}$)	2682	-177	-451, 97	-89	-363, 184
Functional gain ($ml \cdot min \cdot W^{-1}$)	12.6	-0.8	-1.5, -0.1	-0.4	-1.1, 0.3
NIRS (Arbitrary units)					
[HHb] rest	1.38	2.09	-4.32, 8.49	0.18	-6.23, 6.59
[HHb] exercise	7.52	-0.08	-6.48, 6.33	-1.08	-7.49, 5.33
[HbO_2] rest	0.89	0.93	-3.91, 5.77	1.45	-3.39, 6.29
[HbO_2] exercise	-4.84	-0.59	-5.43, 4.24	-0.57	-5.41, 4.27
[TOI] rest	65.62	-2.11	-10.83, 6.62	-0.74	-9.47, 7.99
[TOI] exercise	57.23	0.79	-7.94, 9.52	1.09	-7.64, 9.81

454

455 MRT = Mean response Time,

456 NIRS = Near-infrared spectroscopy

457 HHb = deoxyhaemoglobin

458 HBO2 = oxyhaemoglobin

459 TOI = Tissue Oxygenation Index

460

461

Figure 1

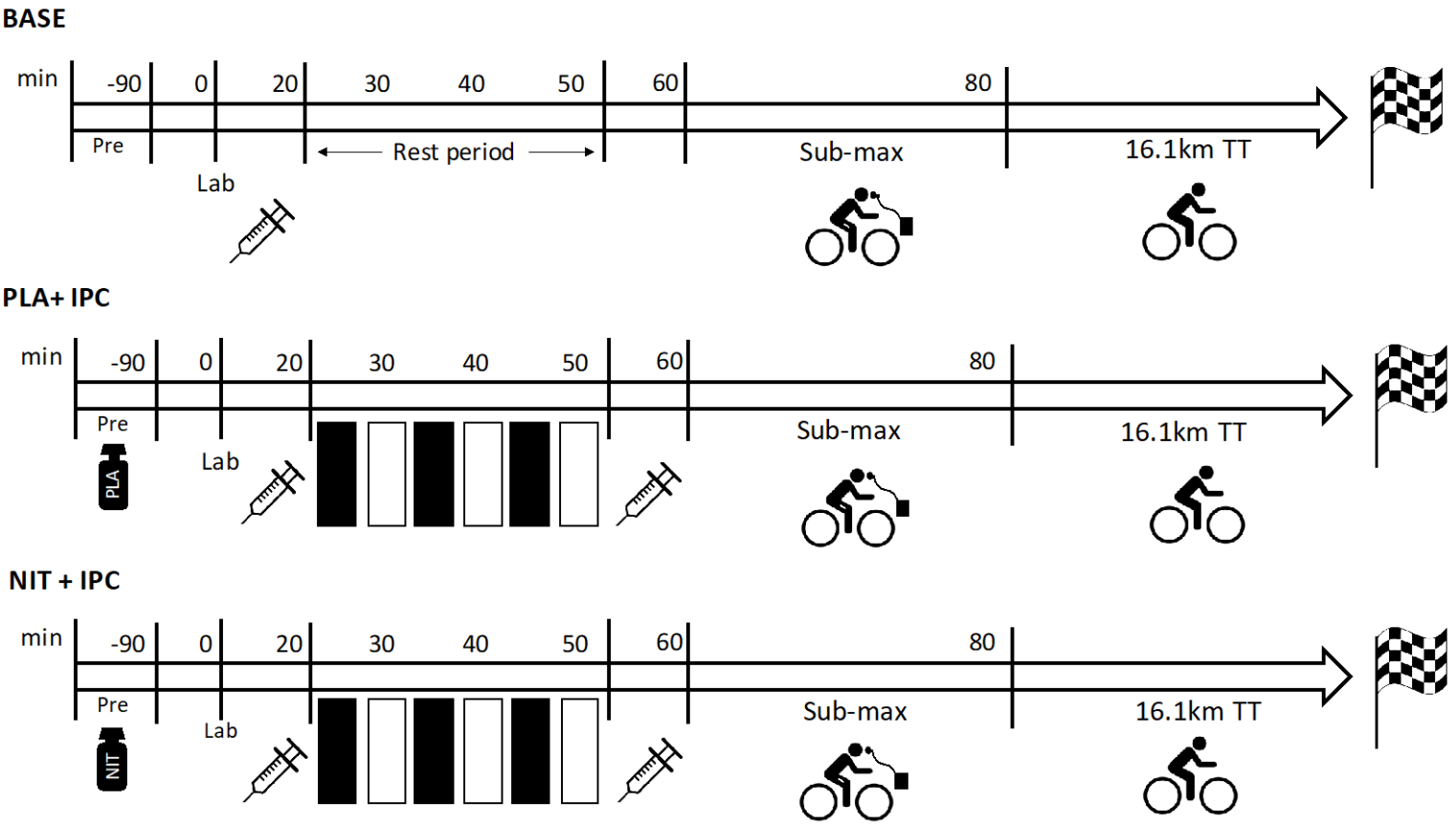
BASE
min
-90
0
20
30
40
50
60
80
Pre
Rest period
Sub-max
16.1km TT
Lab
PLA+ IPC
min
-90
0
20
30
40
50
60
80
Pre
Sub-max
16.1km TT
PLA
Lab
NIT + IPC
min
-90
0
20
30
40
50
60
80
Pre
Sub-max
16.1km TT
NIT
Lab

Figure 2