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Exercise performance increase in smokeless tobacco-user athletes after overnight nicotine abstinence

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ABSTRACT

The use of nicotine administered through smokeless tobacco (snus) has increased among athletes. The purpose of this study was to investigate the ergogenic effects of snus on aerobic performance during exercise until exhaustion in athletes after abstinence or satiety nicotine conditions.

The study utilised a randomised, controlled, within-subject design experiment. Sixteen male snus-user athletes completed an exercise until exhaustion at a constant load of their 80% of $_{max}$ (calculated by a maximal incremental test) in two separate sessions, corresponding to nicotine conditions: 12-hour overnight abstinence and satiety. A portion of 1 g of snus (~ 8 mg/g of nicotine) was administered 25 minutes before each experimental test. In each session, time to exhaustion (TTE), global rating of perceived exertion, cardiovascular and metabolic responses, and muscle and cerebral oxygenation were measured.

Nicotine and cotinine analysis confirmed session conditions (abstinence or satiety). Snus induced a significant increase (+13.1%) of TTE following abstinence (24.1 ± 10.7 min) compared to satiety condition (20.9 ± 8.0 min; P=0.0131). The baseline values revealed that abstinence of snus induced significant increase in the oxygenation of the muscular tissues (+4%), in metabolic values and in cardiovascular parameters, when compared to satiety condition.

Our results indicate an increase of exercise performance (+13.1% TTE) due to snus administration in an abstinence condition. Considering that twelve hours of abstinence from snus-contained nicotine affected metabolic, cardiovascular and muscular tissue oxygenation, we suggest that snus administration at test time might relieve these withdrawal changes and yield an increase in time to exhaustion.

INTRODUCTION

Currently, there are almost a billion (933.1 million) daily smokers in the world[1] with more than 300 million estimated to use smokeless tobacco, with a high prevalence in the South-East Asia Region[2]. In Europe, the use of smokeless tobacco is not commonly reported; however it has been a widespread habit in the Scandinavian countries for a long time[3]. The most famous Scandinavian smokeless tobacco product, snus, is legally sold in Sweden in small sachets that are typically positioned between the gum and the lips[4]. Similar to smoked tobacco, snus use has also been shown to be addictive due to its nicotine content[5].

Several studies have recently documented the use of nicotine in athletes[6–9]. Nicotine is not banned in sport competitions, although the World Anti-doping Agency (WADA) has inserted nicotine in its Monitoring Program[10] since 2012[11]. It is interesting to note that the use of supplements and non-banned substances, like nicotine, is a common practice among athletes who seek to improve their performance This is in spite of the fact that evidence-based research has not clarified the effect of nicotine on exercise [6,9]. For example, some studies suggest an enhancement[12–14], others no effect[15] and others a decreased[16] of exercise performance levels. The consumption of these substances, however, may be considered a potential health risk and a possible danger of transition toward doping[17].

Nicotine binds to nicotinic cholinergic receptors (nAChR) promoting the release of neurotransmitters that produce reward. In addition, regular daily nicotine intake warrants near-complete nicotine saturation, resulting in the desensitization of specific nicotinic cholinergic receptors[18]. These receptors remain largely desensitized as long as the threshold level of nicotine is maintained. However, when nicotine users abstain from use these receptors become re-sensitized and activated, resulting in the characteristic symptoms of withdrawal. This is noted even after abstinence overnight[19]. According to Benowitz[19] the first nicotine dose, for instance the first cigarette in the morning for smokers, has evident pharmacological effects on arousal, subsequently promoting tolerance to nicotine.

To the best of our knowledge, only few studies have investigated the effects of abstinence from nicotine in exercise. Escher et al.[20] showed that leg extensor force and rate of force development were lower when using nicotine compared to abstaining from tobacco. In another study, conducted by Baldini et al.,[21], 12 regular smokeless tobacco users

performed a Wingate tests after taking six different doses of nicotine (vs. placebo) in order to determine the influence of varying doses on exercise. No change in 5-sec peak and 30-sec mean power output were reported[21]. Other investigators[22] showed that maximal oxygen consumption ($\dot{V}O_{2max}$) and lactate accumulation remained unchanged between the two conditions of nicotine satiety and abstinence. Furthermore, it was noted that 12 hours of abstinence did not cause changes in aerobic capacity or anaerobic threshold[23]. Importantly, short-term nicotine abstinence produced negatively influenced mood and enhanced systolic blood pressure responses[24,25]. A recent review on cardiovascular effects of nicotine reported that exercise decreases blood flow to the splanchnic region during endurance performance and suggested that the use of nicotine can have potential detrimental effects on exercise capacity[26]. Additionally, Wang et al.[27] reported that nicotine abstinence correlates significantly with regional cerebral blood flow, underlining that this blood flow change was higher during abstinence (vs. satiety). Indeed, snus administration in non-tobacco users induces a better cerebral and muscular tissue oxygenation during an exercise until exhaustion[15]. However, only few studies, have utilized cerebral[28–30] and muscular[31] near-infrared spectroscopy (NIRS) to investigate nicotine effects.

On the basis of these premises, we aimed to investigate the effect of nicotine delivered via snus, on exercise performance during a time to exhaustion (TTE) task at a fixed intensity corresponding to 80% $\dot{V}O_{2max}$.. We tested regular snus-user athletes under nicotine abstinence or satiety conditions. The perceptions of effort ratings, muscle and cerebral NIRS, metabolic and cardiovascular responses were collected. It was hypothesised that the snus administration in the abstinence condition in nicotine-addicted athletes could increase exercise performance as it may relieve the withdrawal symptoms induced by overnight abstinence.

METHODS

Participants

Participants were recruited by local advertisements in the winter sport area of Northern Italy. All participants were deemed to meet the criteria regular snus use, as they were regularly using one or more snus sachets once a day. The Ethics Committee for Clinical Trials, University of Verona, Italy (n. Prot. 9922, 55CESC) approved the study, in accordance with

the 1964 Declaration of Helsinki. Before being recruited, participants read and signed the informed consent.

Male participant characteristics were: age = 21.1 ± 2.7 years; height = 177.3 ± 5.8 cm; body mass = 76.3 ± 6.1 Kg; VO_{2max}, = 48.1 ± 6.2 ml kg^{-1} min^{-1}; maximal workload at exhaustion, $\dot{W}_{max}$ = 356.3 ± 40.1 W; critical power, *CP* = 226.6 ± 28.6 W; workload at 80% $_{2max}$, $W(80\%max)$ = 232.4 ± 24.6 W; maximal heart rate, HR_{max} = 191 ± 8 beats min^{-1} (mean ± SD). Sixteen participants were required for a power of 0.8 and an effect size equal to 0.8 f (large)[32] considering the variable characterized by greater variance, i.e. exhaustion time. The sample size was calculated using G * Power software[32]. We took into consideration possible drop-outs recruiting 18 participants. One athlete withdrew from the study for epigastric pain after 10 min of the exercise during both experimental sessions. Another participant was forced to withdraw from the study because a leg injury at the end of the first experimental session. Fifteen out of sixteen of the participants were competitive athletes who had a high-level sport activity, inserted into nationally and internationally ski federation (for instance, they participated at Europe and World Cup of Alpine Ski races). The only one no competitive athlete was a ski instructor with a medium-high level of physical fitness.

Study design

The study design consisted of a randomised, controlled, within-subject design. Participants visited the lab on 3 different occasions. On the first day, a maximal incremental exercise test to determine $\dot{V}O_{2max}$ was performed. In the experimental session 1 and 2 participants pedalled at 80% $\dot{V}O_{2max}$ until exhaustion under nicotine satiety condition (SA) or under 12-hour overnight nicotine abstinence condition (AB). The order with which each participant completed the experimental conditions was randomised, and counterbalanced, using online software (random.org). In accordance with Lunell and Lunell[33], twenty-five minutes before starting the exercise a dose of snus was administered. No less than 7 days were allowed between experimental sessions in order to allow for sufficient washout and recovery.

Procedures

On the first visit, anthropometric measurements (body mass was recorded on an electronic scale (Tanita BWB-800, MA, USA) and height was measured with a Harpenden stadiometer (Holtain Ltd., Crymych Pembs, U.K.)) were collected and the Fagerstrom Test for Nicotine Dependence-Smokeless Tobacco (FTND-ST) was carried out. The FTND-ST, a modified 6-item scale from FTND/FTQ[34] is a questionnaire to determine the dependence to smokeless tobacco that allows the classification of nicotine dependence into five levels: very low (0 to 2 points); low (3 to 4 points); moderate (5 points); high (6 to 7 points); and very high (8 to 10 points)[35].

Participants performed a maximal incremental exercise test on an electronically braked cycle ergometer (Excalibur Sport, Lode, Groningen, The Netherlands) to assess $_{2max}$. The workload started at 50 W and was subsequently increased by 30 W every 1 min until voluntary exhaustion. Exhaustion was defined as the inability to maintain the pre-determined pedalling frequency (60 – 80 revolutions/min (rpm)), despite vigorous encouragement by the experimenters. Global rating of perceived exertion (RPE) was recorded in the last 15 s of each exercise step using the Borg 6-20 RPE scale[36]. A metabolic cart (Quark CPET, Cosmed, Rome, Italy) continuously monitored: heart rate (*HR)*, expiratory minute ventilation ($\dot{V}_E$), oxygen consumption ($\dot{V}O_{2)}$, carbon dioxide production ($\dot{V}CO_2$) and respiratory exchange ratio (*RER*). All following exercise test criteria were used for the achievement of $\dot{V}O_{2max}$**:** (1) final *HR* within 10% of predicted maximum; (2) a clear plateau of $\dot{}_2$ or (3) *RER* equal or greater than 1.10 and high post-exercise blood lactate levels (≥8 mM). $\dot{V}O_{2max}$ was calculated as the average of the breath-by-breath $\dot{V}O_2$ values occurring in the last 30 sec of exercise during maximal exercise[37]. The workload corresponding to 80% $_{2max}$ was calculated as: $W(80\%max) = \left(\dot{V}O_{2max} - \dot{V}O_{2rest}\right) * 0.8 * 0.348 * \Delta\eta$. Where $_{2rest}$ was measured at rest before pedalling with the participant sitting on the cycle ergometer, $\Delta\eta$ were the individual values of delta efficiency, defined as the ratio between meccanical power and Δ metabolic power measured during the incremental exercise, 0.348 is a constant used convert power from mL/min to watt. After 20 min of recovery from the maximal incremental exercise test, participants underwent a test to assess Critical power ( ) (for details see Supporting information: Critical Power) to confirm the calculation of the 80% VO_{2max}.

Experimental sessions 1 and 2

Twenty-four hour before the tests, participants followed a prescribed diet and abstained from strenuous physical activity, alcohol and caffeine consumption.

Only two tests were performed in the morning of each experimental day, the first at 8.00am and the second at 10.00am and each participant was tested at the same time of day in each of the two experimental sessions. In the hours preceding each test, we sent standard phone messages to remind the participant to comply with the requirements to ensue a correct experimental session. The standard text was: “Hello. We remind you that from now (8.00pm or 10.00 pm) until the beginning of the test scheduled for tomorrow you may use (SA) (or you cannot use – for AB) nicotine-containing products such as snus, cigarettes, patches or chewing gum. You will receive messages similar to this in the coming hours. Thanks for your collaboration. See you tomorrow”.

When participants arrived at the lab, 5 mL of blood was collected to confirm the condition (SA or AB). The carbon monoxide (CO) test in the exhaled air was also performed. Expired CO concentration was measured using the EC50 Smokerlyser (Bedfont Instruments; Kent, UK). This is a non-invasive assessment of smoking status that closely correlates with blood carboxy-haemoglobin concentration. Values defined a participant as non-smokers when CO value is < 6.0 ppm[38].

Afterwards, participants were instrumented and remained sitting on the cycle-ergometer while the baseline measurements were carried out. Blood samples were also taken 3 min prior to starting the exercise in order to determine blood lactate concentration (Lactate, mM) and blood glucose concentration (Glucose, mM) (Biosen C line, EKF Diagnostic, Barleben, Germany). Subsequently, participants took a portion of snus and they placed it in the anterior part of mouth between the upper gingiva and the lips (Time zero = T0). We administrated to all participants 1 g portion of a commercial Catch White Eucalyptus Portion Snus (Swedish Match). For more detailed on snus dose, readers are kindly referred to Zandonai et al. studies[8,15,16].

Eighteen min after snus administration a personalized warm up of 7 min was performed with intensity equivalent to 50% $\dot{V}O_{2max}$ (116.2 ± 13.3 W; mean ± SD). After that (25 min from TO) participants started pedalling at the individual workload corresponding to 80% VO_{2max} until exhaustion (defined as a the inability to maintain for at least 5 sec the pre-determined pedalling revolution 60 revolutions/min (rpm) despite strong verbal encouragement).

During exercise the following parameters were continuously measured: i) beat-by-beat *HR*, stroke volume *SV*, and cardiac output $\dot{Q}$ (Portapres®, FMS, Amsterdam, The Netherlands); ii) breath-by-breath gas exchanges and ventilation ($\dot{V}O_2$, $\dot{V}CO_2$,); iii) muscle and cerebral deoxhaemoglobyn (HHb), oxyhaemoglobin (HbO_2), total haemoglobyn (THb) and tissue oxygenation index (TOI) (OxiplexTS, ISS, Champaign, IL, USA) (For details see: Supplementary information: materials).

Also during this test, the *RPE* was recorded in the last 15 s of each 5-min stage[36]. Blood samples, obtained from the ear lobe, to determine Lactate and Glucose were taken each 5 min from the start of the exercise until cessation of the test. At the end of the exercise (i.e. at exhaustion), athletes spat out snus and they were immediately interviewed about adverse events and, if present, about their intensity (mild, moderate or serious). Finally, 5 mL of venous blood was obtained to measure the absorption of nicotine and metabolized cotinine. (For details see Supplementary information: chromatographic analysis for nicotine and cotinine analysis; materials for material characteristics). Figure 1 shows the schematic diagram of the protocol during experimental sessions

Data analysis and statistics

Data were tested for normal distribution by using Shapiro-Wilk test. A Wilcoxon signed-rank test has been used for the data that did not have a normally distributions. This test was performed to study the effect of snus on the time to exhaustion (min) under the two conditions (AB vs. SA).

Two-way ANOVAs for factors Time and Condition (AB, SA) were performed to determine differences in RPE, cardio-respiratory, Lactate, Glucose and NIRS values. Post-hoc analyses correction were performed by using the Bonferroni's multiple comparison test. Average values of: i) breath-by-breath $\dot{V}O_2$, $\dot{V}CO_2$; ii) beat-by-beat HR, SV and $\dot{Q}$; iii) TOI, HbO_2, HHb and THb were reported at baseline, warm-up, 25%, 50%, 75% and 100% of TTE. In order to get the relative exercise time, the data concerning the 25-50-75-100% of TTE were obtained by multiplying the individual TTE (100% of TTE) by 0.25, 0.50, 0.75, respectively. Statistical significance was always accepted at $P < 0.05$. All the data are reported as mean ± standard deviation (SD). Analysis was performed by Prism 6 statistical software (GraphPad, La Jolla, CA, USA).

RESULTS

Smoking status assessment and nicotine/cotinine levels

Average CO level was 1.3 ± 0.5 ppm prior to starting the test under AB and 1.8 ± 1.5 ppm before starting the test under SA. These values confirmed that our participants were non-smokers and they were not exposed to passive smoking prior to testing[38]. The mean age of the first snus experience of the participants was 16.6 ± 2.6 years**.** Participants reported that mean snus portion per day was 8.1 ± 4.1 sachets. The FTND-ST average value was 6.0 ± 1.7 suggesting a high nicotine dependence level by participants[35]. A significant difference was found in nicotine and cotinine values pre vs. post snus administration under AB ($P < 0.001$) (see Table 1). Moreover, our findings showed a significant difference between AB ($P < 0.001$) and SA ($P < 0.001$) conditions in nicotine and cotinine values at baseline (pre vs. pre). No significant differences were found under SA. No adverse events were reported from any participants.

Time to exhaustion (TTE) and Perception of effort (RPE)

Mean (± SD) TTE (24.1 ± 10.7 min) during AB session was 13.1 % longer than during SA (20.9 ± 8.0 min; $P = 0.0131$) (A Wilcoxon signed-rank test) (see Figure 2 panel A). Thirteen out of sixteen athletes improved TTE during the AB condition.

RPE (Borg's scale values) (mean ± SD) increased during exercise, but score values were not significantly different between conditions (see Figure 2 panel B).

Near-infrared spectroscopy (NIRS)

At baseline, cerebral oxygenation values TOI (C) (percentage ± SD) evidenced no significant difference between the two conditions (Figure 3A). No difference was also found also in muscular oxygenation values TOI (M) (Figure 3B). Furthermore, during exercise, no significant differences were found in TOI (C) between two conditions (AB vs. SA) (Figure 3A).

Differences in TOI (M) (percentage ± SD) between AB and SA were present at warm-up (WUP), 25%, 50 and 75% of TTE (Figure 3B). The details concerning HHb and HbO_2 values are presented in the Table 2.

Physiological variables

Average values at baseline were significantly different between conditions for $\dot{V}O_2$: AB = 415.1 ± 68.2 ml/min and SA 307.9 ± 50.5 ml/min; P = 0.042; $\dot{V}CO_2$: AB = 359.6 ± 72.5 ml/min and SA = 272.4 ± 54.3 ml/min; P = 0.031; HR was significantly lower in AB in comparison with SA (P = 0.003) (Figure 4 panel A). SV in AB was larger that in SA (P = 0.016) (Figure 4 panel B). Lactate in AB was significant lower in comparison with SA (P = 0.046 (Figure 4 panel C). Ejection time (EJT) in AB was larger than in and SA (P < 0.001) (Figure 4 panel D).

During exercise, $\dot{V}O_2$, $\dot{V}CO_2$, *HR* (Figure 4 panel A), *SV* (Figure 4 panel B), EJT (Figure 4 panel C) and Lactate (Figure 4 panel D) no differences between conditions were evident.

There were no significant differences between AB and SA conditions in $\dot{Q}$ and glucose values, at baseline, WU, 25%, 50%, 75% and TTE.

DISCUSSION

The main purpose of this study was to investigate the effects of the use of snus on time to exhaustion (TTE) and on the fatigue perception during endurance exercise at 80% $\dot{V}O_{2max}$ in regular snus-user athletes following abstinence or satiation. The plasma nicotine and cotinine values confirmed that all participants were tested in abstinence or satiety condition as the protocol required. Our findings showed a significant increase in TTE after snus administration in the abstinence condition. However, the consumption of snus following 12-hour period of abstinence did not influence the perception of fatigue during exercise.

In this study we found an improvement in exercise performance after the administration of nicotine (via snus) following a 12-hour abstinence and this finding confirmed our initial hypothesis. This novel finding has not been reported in previous studies. For instance, Escher et al.[20] demonstrated that nicotine induced an improvement in physical performance among smokeless tobacco users in a satiety condition. However, they did not administer nicotine prior to testing in the abstinence condition, as we did in the current study. Therefore, they could not test if acute administration of smokeless tobacco relieved the symptoms induced by abstinence and altered the performance. Furthermore, other studies have failed to demonstrate an increase of exercise performance after nicotine intake when users were abstinent[21,23] or showed conflicting and inconclusive results[22]. In our view, different reasons might explain the mixed results present in literature.

Firstly, in the present study a decrease in muscular TOI, from WUP until 75% of the time of exercise, was evident in both conditions. However, acute administration of snus following abstinence induced a muscle tissue oxygenation of ~4% higher than in satiety. The increase of TOI values indicates superior tissue oxygenation, which might in turn suggest a better muscular perfusion. These findings are somewhat in agreement with the results of Siafaka et al.[31] who found that vascular reactivity declined during the smoking session in smokers, leading to a greater peripheral vasoconstriction. Indeed, a condition of vasoconstriction in tissue promotes a condition of hypo-perfusion that may lead to a larger inhomogeneity of the ratio between O_2 delivery to muscular O_2 uptake. Consequently, an impairment of peripheral gas exchanges is evident in the less perfused zones and after abstinence, snus seems to promote a better tissue oxygenation and compensate for this possible disadvantage. Nicotine has been described to induce a biphasic response mediated by muscarinic receptors: it first elicits vasoconstriction, which is then followed by vasodilation

and this response occurs with blood concentrations of nicotine close to the ones reached in the smokeless tobacco conditions[39]. Nevertheless, it is worth noting that controversial effects of nicotine use are reported on peripheral vascular resistance, spanning from the absence of any effect to evident vasoconstriction[40]. Lastly, data on cerebral TOI denoted no differences both at baseline and during exercise between the two conditions (AB and SA) (see Figure 3). In contrast, Zandonai & Tam et al.[15] found in the prefrontal cortical areas a marked increase of TOI after snus intake (vs. placebo condition) in a sample of non-snus users.

Secondly, the results of our study could be explained due to a wide variation in individuals' tolerance and sensitivities to nicotine[6] and also through the differences in nicotinic acetylcholine receptors' (nAChR) availability[18]. Muscular nAChRs are localized in the neuromuscular junction of somatic muscle[41] and according to a recent review[6] nicotine can show a biphasic effect at the neuromuscular junction whereby it exerts excitatory (acutely, after withdrawal) followed by inhibitory (chronically, when tolerant) effects.. Moreover, Pesta et al.[42] reported that a mechanism called Ca2+-induced Ca2+ release could further boost intracellular calcium upon activation of nAChR. In addition, the review by Zheng highlighted how a dopaminergic release, through different drugs, can improve exercise performance[43].

Thirdly, the significant differences in cardiovascular and metabolic measurements at baseline may explain the observed increase in TTE. First, our data confirmed other studies that reported lower *HR* during abstinence[24,44]. Indeed, the abstinence condition is characterised by an altered sympatho-vagal drive at rest, in comparison with satiety. Also, alterations in EJT values at baseline confirmed this different sympatho-vagal drive. An increase of resting *HR* and changed hemodynamic variables were indeed shown in nicotine users and non-users at rest[45,46]. These findings were related to a higher sympathetic stimulation in a satiety condition[22,47]. Therefore, nicotine mediated sympathetic outflow and the acute attenuation of endothelium-dependent vasodilation seems to cooperate and induce a persistent increase of *HR* and vascular resistances[48]. With the respect to high intensity exercise performance, we have to consider that it is essential to have the highest possible oxygen delivery to the muscles and an homogenous distribution of the O2 delivery-to-muscular uptake ratio. The increase in sympathetic outflow may improve the central cardiac response, yet it may somehow make peripheral oxygen delivery less efficient. Therefore, a reduction of tissue oxygenation may impair performance. It is very likely that the improvement of the performance is to be related to the previous nicotine abstinence, which

reduces the sympathetic outflow favouring the transport of oxygen. Indeed, the condition of abstinence plus acute administration could play a biphasic role. i) Initially (under abstinence condition) the factors concerning the delivery of oxygen to the periphery prevail. ii) Afterwards (following the administration of snus), the psychostimulant effects have a larger role. The increase in sympathetic outflow mediated by nicotine would still favour the central cardio-circulatory response, but will not limit the oxygen flow at the peripheral level.

Our results are not without methodological limitations. One of these is the lack of an experimental session with regular snus users with the same 12-hour period of abstinence and without snus administration. Moreover, the participants were all males. According to Mundel[6] this is a typical limitation of studies on the effects of nicotine in sport. Further studies should consider a female sample in order to evaluate tolerance to nicotine in gender difference.

In conclusion, our study confirmed the hypothesis that the snus administration after 12-hour abstinence condition in nicotine-addicted athletes could increase exercise performance. This appears to be due to the relief of the symptoms induced by overnight abstinence. We showed that snus administration increased TTE following abstinence and this was the first study to investigate the responses to submaximal exercise (80% of Vo2max) in regular smokeless tobacco user athletes.

The significant increase (+13.1%) of time to exhaustion, does not exempt that further studies are needed to support the hypothesis that the nicotine and tobacco products may have an ergogenic effect on aerobic exercise performance. In addition, for non-tobacco users that use snus for the first time, there may be detrimental effects on both cognitive and physical performance due to possible adverse events by nicotine[8,15,16]. However, in compliance with the literature published until now[14,49] (including this study), the sport authorities should begin to take in account the possible effects of nicotine on exercise and foster investigations on this topic.

PERSPECTIVE

In recent years the use of smokeless tobacco in the sport environment is a sharply rising phenomenon. For this reason, nicotine is placed on the World Anti-Doping Agency (WADA) monitoring list to detect potential patterns of abuse of this drug in sport. In our study, snus administration, after overnight nicotine abstinence, was followed by the improvement of exercise performance in smokeless tobacco-user athletes. These findings raise important questions regarding the ergogenic effect of nicotine, and its possible role in enhancing performance. The results of the present study are novel and relevant, and could lead to research in other sport contexts and on other performance-related processes. Nicotine addiction sustains the prolonged use of smokeless tobacco products and, therefore, also the health of the athlete may be strongly jeopardized. Moreover, the sport authorities should educate athletes of the potential danger through educational projects.

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DECLARATION OF INTERESTS

Authors declare to have no financial or other interest in the production or distribution of the snus.

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Table 1.Nicotine and cotinine levels

	AB			SA		
	Pre	Post	P values	Pre	Post	P values
Nicotine (ng/ml)	0.9 ± 0.4	7.9 ± 0.8	< 0.001***	9.6 ± 2.8	8.1 ± 1.9	0.3484
Cotinine (ng/ml)	59.9 ± 25.1	134.2 ± 25.5	0.0134*	256.9 ± 64.4	168.1 ± 53.5	0.0564

Note: AB = Abstinence condition; SA = Satiety condition. *P < 0.05, ***P < 0.001 (n=16).

Table 2. NIRS Cerebral and Muscular values

Cerebral				**AB**						**SA**		
(µM)	Baseline	WUP	25%	50%	75%	TTE	Baseline	WUP	25%	50%	75%	TTE
HbO	39.5 ± 10.2	41.6 ± 10.5	42.6 ± 11.3	43.6 ± 11.7	44.1 ± 12.5	44.3 ± 12.0	37.9 ± 9.4	40.7 ± 9.6	41.6 ± 9.2	42.4 ± 9.4	42.7 ± 9.5	42.2 ± 10.1*
HHb	18.9 ± 4.3	19.5 ± 4.5	19.8 ± 4.6	19.9 ± 4.6	20.6 ± 4.9	21.7 ± 5.2	18.0 ± 5.1	18.0 ± 5.3**	18.8 ± 4.8*	18.7 ± 4.7*	19.0 ± 4.9**	19.7 ± 5.4***

Muscular				**AB**						**SA**		
(µM)	Baseline	WUP	25%	50%	75%	TTE	Baseline	WUP	25%	50%	75%	TTE
HbO	56.7 ± 21.1	57.8 ± 23.1	55.1 ± 21.2	55.4 ± 20.9	55.8 ± 21.5	56.7 ± 23.1	49.5 ± 10.6***	48.3 ± 9.7***	46.6 ± 8.7***	46.7 ± 9.2***	47.1 ± 9.9***	48.2 ± 11.5***
HHb	22.2 ± 9.6	24.5 ± 10.1	31.5 ± 15.8	32.4 ± 16.7	32.7 ± 17.0	32.7 ± 17.2	22.1 ± 9.5	25.7 ± 10.6	31.1 ± 14.9	31.6 ± 14.9	31.7 ± 14.7	31.0 ± 14.9

Note: values are mean ± SD and are expressed as [µM]. HbO_2: oxyhaemoglobin concentration; HHb: de-oxyemoglobin concentration; Cerebral: signals from frontal cortex cerebral probe; Muscular: signals from vastus lateralis muscular probe; AB: Abstinence condition; SA: Satiety condition; Baseline: average of last 30 sec at baseline; WUP: last 30 sec at the end of warm up (WUP) phase; 25%, 50%, 75%, TTE: are quartiles of time at exhaustion (TTE). * = P < 0.05, ** = P < 0.01, *** = P < 0.001; n = 16

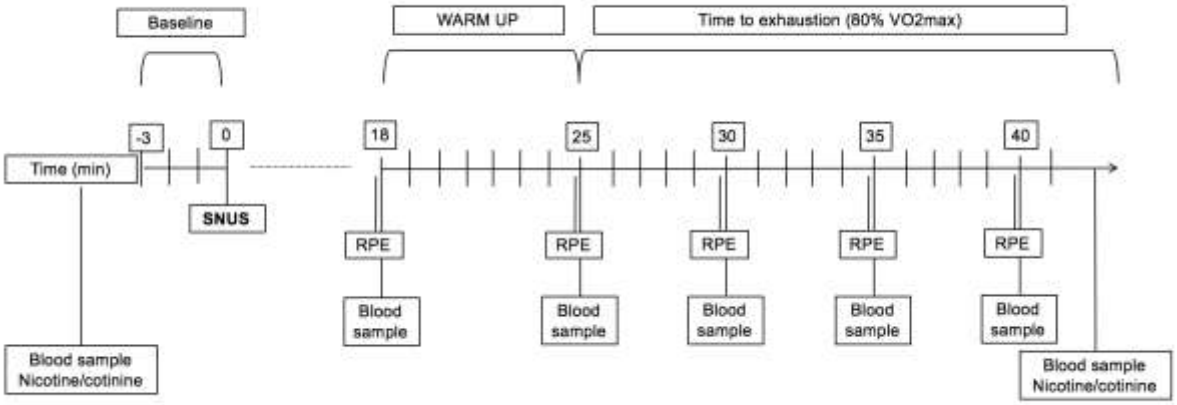
Figure captions

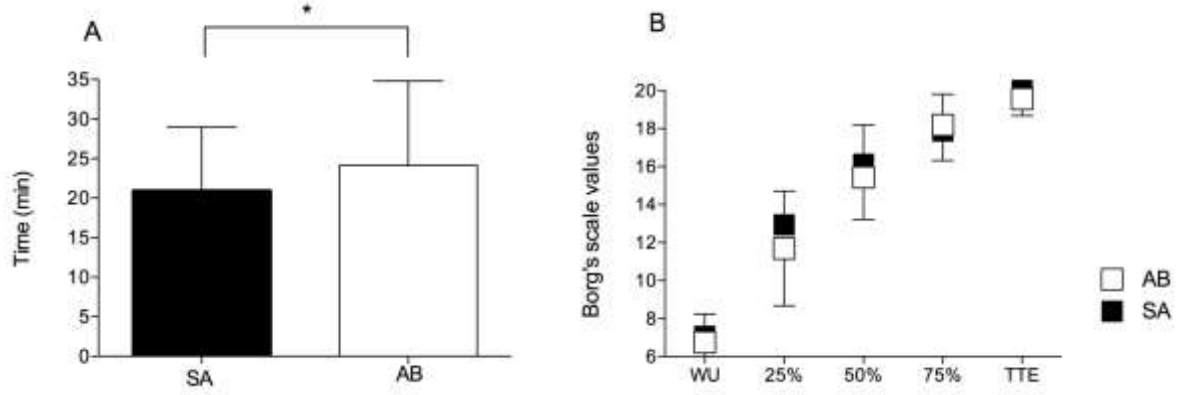
Figure 1. A schematic diagram of temporal progression of the experimental session. Timeline (from left to right) is expressed in minutes (numbers in box above the horizontal scale). *Abbreviations*: RPE: Rating perception of effort (Borg's scale).

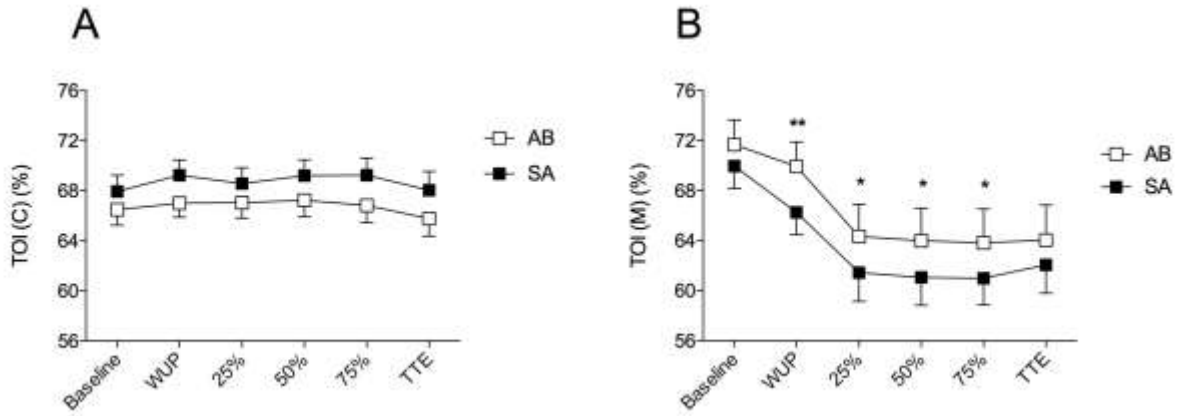
Figure 2. Time to exhaustion between satiety and abstinence conditions and RPE ues. Panel A: average of the time to exhaustion (TTE) in minutes (mean ± SD) during satiety (SA, black column) and abstinence (AB, white column); *P < 0.05; a paired Wilcoxon test; n = 16. Panel B: Borg's scale values at warm-up (WUP), 25%, 50%, 75% and 100% of time exhaustion (TTE) during Abstinence (AB) and Satiety (SA); n = 16.

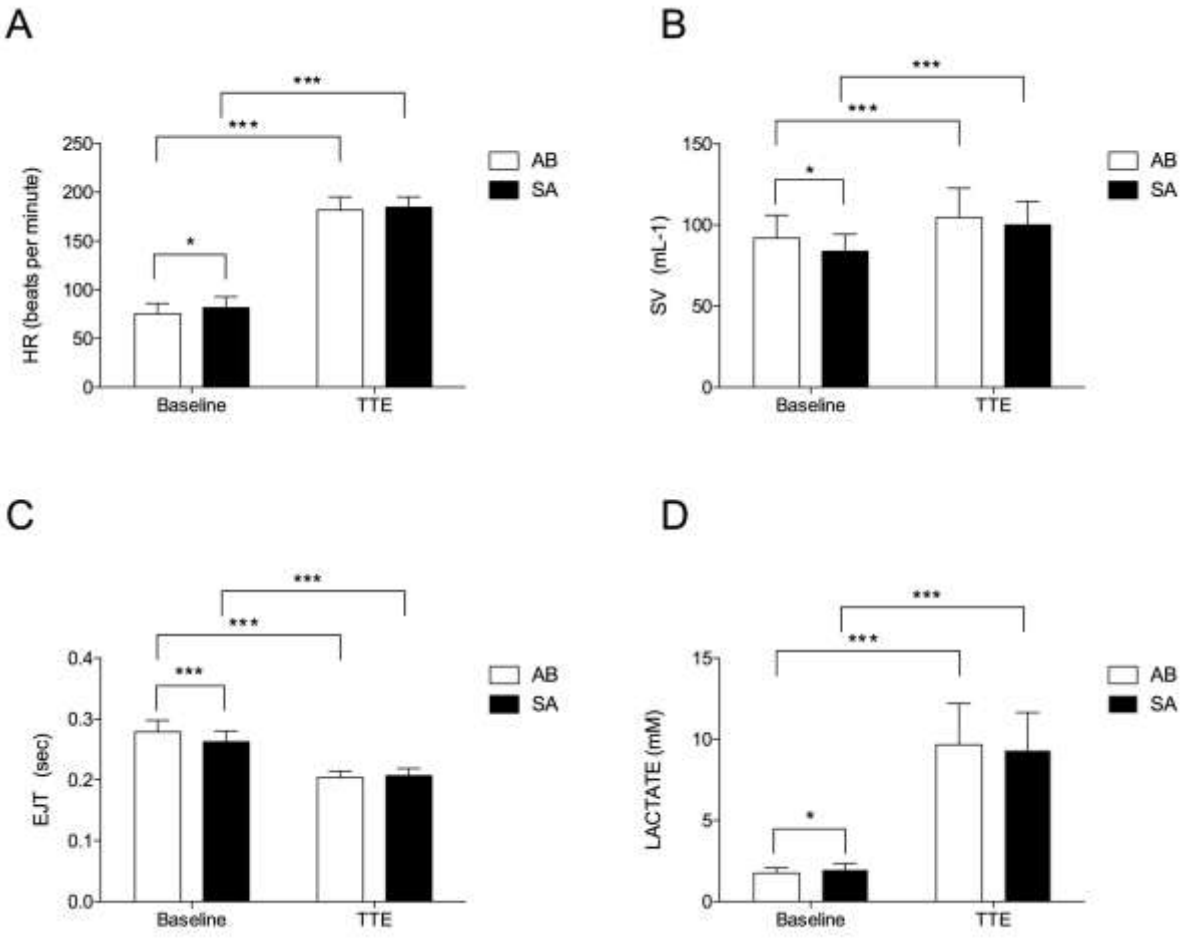
Figure 3. NIRS muscular and cerebral values at baseline and during exercise until e to exhaustion. Values of tissue oxygenation index (TOI) (panels A, Cerebral and B, Muscular), at baseline, warm-up (WUP), 25%, 50%, 75% and 100% of time exhaustion (TTE) during Abstinence (AB) and Satiety (SA) measured in the prefrontal cortex (C) and on vastus lateralis muscle (M) (mean ± SD). * = P < 0.05, ** = P < 0.01, n = 16

ure 4. Physiological responses at baseline and at time to exhaustion. Physiological responses values are represented at baseline and 100% of time to exhaustion (TTE) during AB (Abstinence) and SA (Satiety) conditions (mean ± SD). Abbreviations: HR = Heart Rate (panel A); SV = Stroke volume (Panel B); EJT = Ejection time (Panel C); Lactate (Panel D). *P < 0.05, *** = P < 0.001 (n=16)









Exercise performance increase in smokeless tobacco-user athletes after overnight nicotine abstinence

Zandonai T., Tam E., Bruseghini P., Capelli C., Baraldo M., Chiamulera C.

Supporting information: Critical power (CP)

After 20 min of recovery from maximal incremental exercise test, subject underwent to CP test. Briefly, critical power represents the highest rate of muscular work at which a metabolic 'steady-state' can be achieved[1,2]. Therefore, after the maximal incremental exercise test, workload was immediately decreased to 50 W and subjects pedalled for a further 5 min (active recovery period). The active recovery was followed by 15 min of passive recovery (subject sat on bike). CP test starts with 3 min of unloaded cycling using the cadence selected during the MIT. The operating mode of the cycle-ergometer during the CP test was set to "linear". The resistance applied to the ergometer flywheel was set proportional to the product of pedal cadence and a linear factor (alpha) (alpha=power/r.p.m.^{2}). During the last 5 s of the 3-min unloaded cycling period, subjects were instructed to increase their pedal cadence to ~110–120 rev min−1. At the start of the 3-min CP test, subjects rapidly increased their pedal cadence as much as they could (~160–180 rev min−1). To ensure maximal effort, subjects were instructed to maintain their cadence as high as possible throughout the 3-min duration of the test. The investigators provided strong verbal encouragement during the entire CP test. CP was calculated as the average power output attained during the final 30 s of the 3 min CP test (ref test CP 3 min).

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after overnight nicotine abstinence

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Supporting Information: Chromatographic analysis

Chemicals and reagents

Nicotine, cotinine and acetanilide (internal standard: I.S.) were purchased from Sigma-Aldrich (Milan, Italy). Acetonitrile (Applichem, Germany), dichloromethane and sodium hydroxide and formic acid (Sigma-Aldrich, Italy), and ammonium acetate (Carlo Erba, Italy), and hydrochlorid acid (AppliChem, Germany) were analytical-reagent grade; milliQ water was filtered and deionised with Ultra Pure Water System, MilliQ-plus (Millipore, USA).

Chromatographic analysis

Nicotine, cotinine and I.S. were determined by mean of a High Performance Liquid Cromatography (HPLC) technique coupled with a mass spectrometer double quadrupole detector (MS/MS). Analytes were separated on a Restek Ultra-C8 column (150 mm X 2.1 mm - 5 µm) (Milano, Italy) at a flow-rate of 0.2 ml min^{-1}. Separation was carried out in isocratic conditions with a solution of acetonitrile/water/formic acid + 2 mmol l^{-1} ammonium acetate (78/22/0.1 v/v). Chromatographic equipment consists of HPLC LC-200 pump; samples were detected in *Selected Ion Monitoring (SIM)* (m/z nicotine m/z 163.1, cotinine 177.1 and I.S. 136.0) with positive electrospray ionisation (ESI), by a Q-trap LC/MS/MS Systems (MDS Sciex - Ontario, Canada). Data were acquired and processed with *Analyst* 1.4 (Applied Biosystems package, MDS Sciex - Ontario, Canada). The system operated at room temperature. In these conditions nicotine, cotinine and acetanilide retention times were 2.38, 2.45 and 2.78 minutes respectively.

Calibrators and quality control samples

Calibrators and control samples containing nicotine and cotinine were prepared adding known amounts of analytes to blank serum. They were included in each batch of volunteers' samples. Calibration curves and quality control samples ranged respectively, from 5 to 50 ng mL^{-1} and 50 to 1000 ng mL^{-1} for nicotine and cotinine, respectively. Limit of detection (LOD) for nicotine and cotinine were 2,5 and 25 ng mL^{-1}, respectively.

Sample preparation

To purify nicotine and cotinine from serum samples, we modified the extraction procedure proposed by Nakajima et al.[1] extraction was a simple two-step procedure: alkalinisation and organic extraction. One mL of serum was transferred to a polypropylene tube, followed by addition of 50 µL of I.S. (10 ng μL^{-1}) and 50 µL of sodium hydroxide (10M). The tubes were vortexed and samples extracted with 8 mL of dichloromethane by rotation for 10 minutes and centrifuged at 3000 RPM for 10 minutes. The organic layer was transferred to a glass tube containing 50 µL of hydrochloride acid (37%). After evaporation of the organic phase, under a nitrogen stream at 40°C, the residue was dissolved with 75 µL of mobile phase and 20 µL was injected into the HPLC system.

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Sample preparation

To purify nicotine and cotinine from serum samples, we modified the extraction procedure proposed by Nakajima et al.[1] extraction was a simple two-step procedure: alkalinisation and organic extraction. One mL of serum was transferred to a polypropylene tube, followed by addition of 50 µL of I.S. (10 ng μL^{-1}) and 50 µL of sodium hydroxide (10M). The tubes were vortexed and samples extracted with 8 mL of dichloromethane by rotation for 10 minutes and centrifuged at 3000 RPM for 10 minutes. The organic layer was transferred to a glass tube containing 50 µL of hydrochloride acid (37%). After evaporation of the organic phase, under a nitrogen stream at 40°C, the residue was dissolved with 75 µL of mobile phase and 20 µL was injected into the HPLC system.

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