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ORIGINAL ARTICLE

Concurrent evaluation of salivary and urinary α-amylase activity following prolonged exercise with or without carbohydrate solution in aerobically active men

Nobuo YASUDA[1], Kohei YAMAMOTO[1] , Naoki IWASHITA[1]

1 Research Center in Sports Medicine and Science, Department of Physical Education, International Pacific University, Okayama 709-0863, Japan

Correspondence to: Nobuo Yasuda, Ph.D.
Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan
TEL.: +81-3-5454-6133; E-MAIL: nobuoyasuda17@gmail.com

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Abstract

OBJECTIVE: The purpose of this study was to determine the effects of 2-h moderately prolonged exercise with carbohydrate intake or water placebo on salivary and urinary α-amylase isoenzyme activity in trained men.

MATERIALS AND METHODS: Eleven aerobically fit men participated in this study. On two different occasions, participants performed 2-h cycling corresponding to a constant power output at 60% peak oxygen uptake. The study design involved a random order, placebo-controlled and cross-over assignment. Participants consumed either 6.2% carbohydrate solution or water placebo every twenty minutes thereafter (2 ml/kg body mass) over 2-h endurance exercise. Unstimulated whole salivary samples were collected using the passive drooling method at the 10-min period before and after exercise for the quantification of salivary α-amylase, immunoglobulin A (IgA) and total protein. Two-hour urinary samples were obtained at three time points before (-2-0h), immediately (0-2 h) after and 24-26 h after exercise for the analysis of α-amylase isoenzyme activity (pancreas- and saliva-derived types).

RESULTS: The activity of α-amylase in saliva and urine was significantly increased in connect with salivary total protein concentration immediately after moderately long-lasting exercise, but salivary IgA concentration was not statistically significant with or without exogenous carbohydrate intake.

CONCLUSION: These findings suggest that 2-h moderate exercise appears to lead to the enhanced α-amylase activity in saliva and urine regardless of exogenous carbohydrate availability, demonstrating enhanced mucosal immune defense.

INTRODUCTION

It has been known that α-amylase (1.4-alpha-D-glucan-glucanohydrolase, EC 3.2.1.1) plays a pivotal role in the digestion of dietary starches in humans, hydrolyzing α-1, 4-glucoside bonds in polymers of glucose such as starch and glycogen producing maltose, maltriose, and other dextrins (Hariharan *et al.* 2021). This amylolytic digestion in the oral cavity is initiated during mastication, accomplished by salivary α-amylase, continuing within the stomach (Mandel *et al.* 2010). Eventually, pancreatic α-amylase completes starch hydrolysis after the mixture passes into the small intestine (Mandel *et al.* 2010).

Urinary α-amylase isoenzymes are derived mainly from the two forms of pancreas- and saliva-derived types (Bakońska-Pacoń & Sobiech, 1989). While both loci lead to the production of complex families of isoenzymes, various biochemical methods (e.g., electrophoresis, chromatography, isofocusing and inhibition method) for quantifying pancreatic and salivary α-amylase activity have been explored and developed in human biological specimens (Bakońska-Pacoń & Sobiech, 1989). Under resting conditions, past investigations have represented that pancreas-derived type is higher than saliva-derived type in urine in healthy adults (Bank *et al.* 1992; Kamarýt *et al.* 1993).

There is evidence that salivary α-amylase in saliva and urine adapts primarily to the amount of dietary carbohydrate (Brannon, 1990) and its expression is influenced by endogenous and exogenous nutritional components (Mandel *et al.* 2010). In connection with that information, carbohydrate ingestion during endurance exercise is critically important to maintain glucose homeostasis and prevent dehydration (Cermak & van Loon, 2013). Specifically, carbohydrate intake during endurance exercise has been shown to enhance performance in lasting longer than 2-h at intensities of 50 to 70% of maximal oxygen consumption, attributing to maintenance of blood glucose levels and high rates of carbohydrate oxidation (Baker & Jeukendrup, 2014). Jeukendrup *et al.* (1997) have also reported improved performance as a positive effect of 1-h high intensity (75% of peak power output) cycling exercise with carbohydrate solution. Taking into consideration intensity and duration of exercise, carbohydrate ingestion during moderately prolonged exercise (< 2-h) may have a subtle effect on muscle glycogen degradation as the rate of glycogenolysis can exceed the rate of glucose transport with the maintenance of ATP levels (Greenberg *et al.* 2006).

In line with those phenomena, it has been shown that acute carbohydrate intake can elevate salivary α-amylase activity (Harthoon *et al.* 2009). Some investigations have been shown the effects of moderately prolonged exercise with or without carbohydrate intake on salivary α-amylase activity. For instance, a previous study dealing with saliva samples demonstrated augmented salivary α-amylase activity following 2-h moderate exercise at 60%VO_2peak without carbohydrate ingestion (Li & Gleeson, 2004). Contrastingly, Bishop *et al.* (2000) showed no changes of salivary α-amylase activity after 60% peak oxygen uptake (VO_2peak) of 2-h endurance exercise.

Only a limited number of studies have examined the effects of exercise on urinary α-amylase activity in healthy individuals (Bakońska-Pacoń & Sobiech, 1989; Cucinell *et al.* 1984; Poortmans, 1972). Bakońska-Pacoń and Sobiech (1989) have observed significantly enhanced activity of total α-amylase (pancreas- and saliva-derived types, described as U/l) assessed with enzyme-substrate reaction in urine immediately after the marathon race in runners, including a decreased tendency for the percentage of urinary pancreas- and saliva-derived types 22-24 h after the race.

It has been proposed that increased fitness level accompanied with regular training can evoke an adaptive and potentially pre-emptive effect on the mucosal immune response to a single bout of exercise, which leads to greatly elevated sympathetic nervous system activation, while exercise training may enhance parasympathetic activation under resting conditions (Kunz *et al.* 2015). In this respect, there are several studies investigating the relationship between fitness level and salivary α-amylase activity in healthy individuals. Some studies demonstrated that salivary α-amylase activity has a strong correlation with the anaerobic threshold (lactate threshold and/or ventilatory threshold) (Bocanegra *et al.* 2012; Calvo *et al.* 1997; de Oliveira *et al.* 2010), along with VO_2peak, which is an indicator of aerobic fitness level by training adaptation (Sales *et al.* 2019). On the contrary, one study did not obtain a significant relationship between the aforementioned dependent variables (Strahler *et al.* 2016). Thus, the precise cause-effect relationship between physical fitness and salivary α-amylase activity remains to be clarified.

In addition to those exercise training responses, previous animal study dealing with rats has represented increased basal pancreatic protein content and α-amylase activity at the pancreatic tissue level after endurance training on a treadmill (Minato, 1997). Moreover, Kondo *et al.* (2019) has recently observed that total pancreatic α-amylase activity transiently decreased immediately after exercise at the pancreatic tissue of trained rats, but increased after 24-h recovery. These findings in tissue-based research indicate that pancreatic enzyme synthesis could be augmented with endurance training due to an alteration of vagal nervous activity and gastrointestinal absorptive capacity.

Taken collectively with previous studies, α-amylase prominently interacts with both adrenergic regulation of sympathetic nervous system and carbohydrate metabolism, whereas it remains incompletely defined how prolonged exercise with or without carbohydrate solution concomitantly influences α-amylase activity

in saliva and urine in endurance-trained individuals. Given a systemic study of how water and/or carbohydrate beverage intake during endurance exercise affects salivary and urinary α-amylase activities, it would clarify the complexity and interconnection of salivary and urinary α-amylase activities in response to exercise and their potential contributions to digestive function on the basis of physical fitness.

Accordingly, the purpose of this study was to determine the effects of 2-h prolonged cycling exercise with or without carbohydrate solution on α-amylase levels in saliva and urine (α-amylase isoenzymes derived from pancreas and saliva) in endurance-trained men. It was hypothesized that α-amylase activity in saliva and urine would be elevated following 2-h prolonged exercise irrespective of exogenous carbohydrate feeding in aerobically fit men. Furthermore, the magnitude of α-amylase response in saliva and urine after endurance exercise would be dependent on participants' aerobic fitness levels.

MATERIAL AND METHODS

Experimental approach to the problem

All testing was executed in the laboratory of exercise physiology. At least one week prior to the experimental trials, each participant reported to the laboratory for a familiarization session. Following the anthropometric measurements and the determination of ventilatory threshold (Tvent) and VO_2peak, all participants performed 2-h cycling exercise corresponding to a constant power output at 60%VO_2peak with carbohydrate solution or water placebo on a stationary cycle ergometer on two different occasions. The study design involved a random order, placebo-controlled, crossover assignment. The order of trials was randomly assigned to eliminate any order or training effect.

Participants

Eleven aerobically well-conditioned men participated in this study. The physical characteristics of the participants are shown in Table 1. This study was reviewed and approved by the University Human Ethics Committee conforming to the Code of Ethics of the World Medical Association (Declaration of Helsinki). All participants provided informed consent prior to study participation. None of the participants had a history of medical or gastrointestinal diseases and use of medication and nutritional supplements. Each participant in this study maintained a daily regimen of training activities (2-3 h/day and 5-6 days/week) for more than one year.

Prior to the exercise trials, all participants were required to complete a Physical Activity Readiness Questionnaire (PAR-Q)(©Canadian Society for Exercise Physiology).

Tab. 1. Physical characteristics of the participants

Variable	Men (n=11)
Age (Yrs)	19.5±1.3
Height (cm)	171.1±3.9
Body mass (kg)	68.2±13.5
Body mass index (kg/m^2)	23.2±3.9
Body surface area (m^2)	1.792±0.163
Body fat (%)	14.2±6.0
VO_2peak (ml/kg/min)	62.2±9.4
Tvent (ml/kg/min)	37.1±6.3
%$V0_2$peak at Tvent	59.9±7.2

VO_2peak=peak oxygen uptake, Tvent=Ventilatory threshold, All data are shown as mean±SD.

Preliminary measurements

All participants had a physical examination, followed by assessment of their body composition during a preliminary visit on the laboratory. After an overnight fast of 12 hours (ad libitum water intake was allowed), all participants reported to the lab (7:00-7:30), followed by anthropometric measurements (height, body mass and percent body fat). Body mass and percent body fat at the basis of bioelectrical impedance analysis (InBody 270, Fujitex Corporation, Tokyo, Japan) were digitally measured on a calibrated scale with the participants wearing underwear and barefoot. Subsequently, all participants performed an incremental exercise test to exhaustion in order to determine VO_2peak conducted on an electromagnetically braked cycle ergometer (Excalibur 600™, Lode B.V., Groningen, Netherlands). Each participant completed a 5 min warm up before the testing. The participants performed a cycling exercise at an initial power output of 0 W for 3 min, which was increased by 25 W every 1 min until exhaustion. Pedaling frequency was 60 rpm for all subsequent tests. Expired gas samples were measured with an automated breath by breath gas analysis system (Oxycon Pro™, Erich Jaeger GmbH, Hoechberg, Germany). Heart rate was also continuously measured using electrocardiograph (BSM- 2401 ECG monitor, Nihon Kohden, Tokyo, Japan). The criteria for VO_2peak included achieving at least two of the following three criteria: a plateau in VO_2, Respiratory exchange ratio ≥ 1.1 and volitional exhaustion (ACSM guideline, 2009).

Determination of ventilatory threshold (Tvent)

In this study, Tvent was used as an index of anaerobic threshold as described previously (Yasuda *et al.* 2008). Three methods were simultaneously utilized to quantify Tvent in this study: (i) ventilatory equivalent method (VEQ method) (Shimizu *et al.* 1991), (ii) excess carbon dioxide ($ExCO_2$ method) (Anderson & Rhodes, 1989) and (iii) modified V-slope method using 15-s averaged data (Beaver *et al.* 1986). Two investigators separately

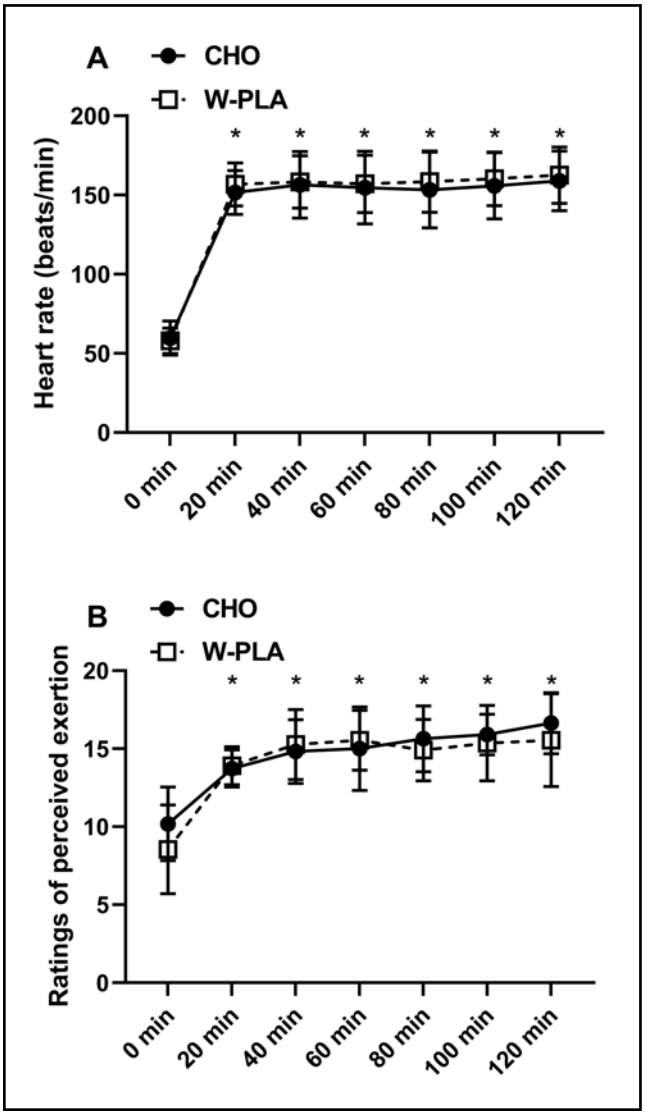

Fig. 1. (A) Heart rate (beats/min) and (B) ratings of perceived exertion over 2-h moderately prolonged exercise with fluid intake (carbohydrate or water placebo). *: Significantly different from baseline (0 min). CHO: carbohydrate, W-PLA: water placebo.

evaluated each testing record in the present study concomitantly using the three detection methods per procedure by Gaskill *et al.* (2001).

Experimental procedures

Two experimental trials were carried out on two different occasions separated by 7 days to ensure recovery period. Following an overnight fast of 12 hours, all participants arrived at the lab in the morning on the day of each experimental trial. The time of day for testing each participant was matched for all experimental days. The participants were required to refrain from exhaustive exercise for 2 days or not to consume alcohol for 5 days preceding each trial. Furthermore, each participant was instructed to record and maintain their normal diet in energy balance with a macronutrient composition of 60% carbohydrate, 25% fat and 15% protein for 5 days (long enough for the pancreas to adapt the diet, based on the concept manifested by Allan & White, 1976) prior to the first trial, exactly replicating the identical diet for the same period of time before the second trial.

Each participant performed 2-h cycling exercise corresponding to a constant power output at 60%VO_2peak, wearing garments made from an evaporative polyester fabric (Gavin *et al.* 2001), on a stationary cycle ergometer. Heart rate and ratings of perceived exertion were recorded every 20 min during the exercise. On each occasion, participants consumed either a 6.2% commercially available carbohydrate drink (Pocari Sweat®, Otsuka Pharmaceutical Co., Ltd.; energy: 104.6 kJ/100 ml, carbohydrate: 6.2%, Na^+:21 mEq/l, K^+:5 mEq/l, Cl^-:17 mEq/l, osmolality: 340 mOsm/l) or a water placebo immediately before the start (0 min) of cycling exercise (3 ml/kg body mass) and every 20 mins up to 100 min during 2-h exercise (2 ml kg/body mass) as described by Nassis *et al.* (1998). At 120 min (the completion of the exercise), fluid intake (carbohydrate or water) was not taken. The laboratory temperature and relative humidity were constant between two submaximal trials (21.3-21.5°C and 42-44%, respectively).

Salivary sample collection

All participants inserted a tip for a saliva amylase biosensor under the tongue for the first 30 seconds (those were taken for another study). Immediately after that, unstimulated whole saliva was collected for 10 min into 15 ml centrifuge tubes with eyes open, head tilted slightly forward and making minimal orofacial movement based on the passive drool technique as represented previously (Ben-Aryeh *et al.* 1989; Shaw *et al.* 2021). Saliva specimens were frozen at -80°C for later biochemical analysis.

Urinary sample collection

Two-hour urinary samples were obtained at three time points [before- (-2 to 0 h), immediately after- (0 to 2 h) and 24-26 h after exercise] since collecting 24-hour urine was difficult over a long period of time in participants from a practical point of view (Waller *et al.* 1971a, 1971b). All participants took their urine specimen with a sterile urine collection cup and then transferred it to a 15 ml tube. The urinary samples were stored at -80°C for the later analysis of the urinary α-amylase isoenzyme activity and its distribution.

Salivary α-amylase activity

Salivary α-amylase activity was determined with a biochemical assay as demonstrated previously (Wallenfels *et al.* 1978). In this assay, a chromagenic substrate, 2-chloro-p- nitrophenol bound to maltotriose was used. The enzymatic action of α-amylase on this substrate produced 2-chloro-p-nitrophenol, which was spectrophotometrically measured at 405 nm. The amount of α-amylase activity present in the salivary specimens was directly proportional to the increase in

absorbance at 405 nm. Salivary α-amylase activity was signified in units per milliliter.

Salivary IgA concentration

Salivary IgA concentration was quantified with immunoturbidimetry based on a protocol represented by (Whicher *et al.* 1984). With respect to the principle of immunoturbidimetry, when the antigen-antibody complex formed in the solution was irradiated with light, the light was scattered and then the transmitted light was reduced. The amount of this decrease in light was measured as the rate of change in absorbance, and was subsequently quantified using an automatic analyzer (JCA-BM8000, JEOL Ltd., Tokyo, Japan) according to Lambert-Beer's law. Salivary IgA concentration was expressed as milligram per deciliter.

Salivary total protein concentration

The total amount of protein in saliva was quantified using the biuret method as shown previously (Shaila *et al.* 2013). Concisely, the peptide-bonded protein was reacted with copper II ions in an alkaline solution to form a blue-violet complex (biuret reaction). The color formed was proportional to the protein concentration, which was subsequently measured at 545 nm. Salivary total protein concentration was described as milligram per deciliter.

Salivary flow rate

The saliva flow rate in microliter per min was determined from measurement of saliva divided by the collection time. Saliva was weighed to the nearest mg and saliva density was presumed to be 1.00 gram per milliliter (Allgrove *et al.* 2014; Shaw *et al.* 2021).

Urinary α-amylase isoenzyme activity

The quantification of the α-amylase isozymes derived from pancreas and saliva in the urine sample was carried out with an automatic device (JCA-BM8000, JEOL Ltd., Tokyo, Japan) based on a protocol demonstrated previously (Lorentz, 1998). Briefly, in this protocol, 4,6-O-ethylidene p-nitrophenyl-α-D-maltoheptaoside (Et-G7-pNP) was used with modified and protected non-reducing ends as a substrate. The conjugated enzymes (α-glucosidase: α-GH and glucoamylase: GA) were chemically reacted by p-nitrophenyl-α-D-maltoside (G2pNP)~4-nitrophenyl-α-D-maltotetraoside (G4pNP) produced by the action of α-amylase, and then para-nitrophenol (pNP) was released. Subsequently, the amylase activity of pancreatic and salivary origin was determined by measuring the rate of increase in absorbance accompanying the production of pNP. Pancreas- and saliva-derived α-amylase activities in urine were demonstrated as units per liter.

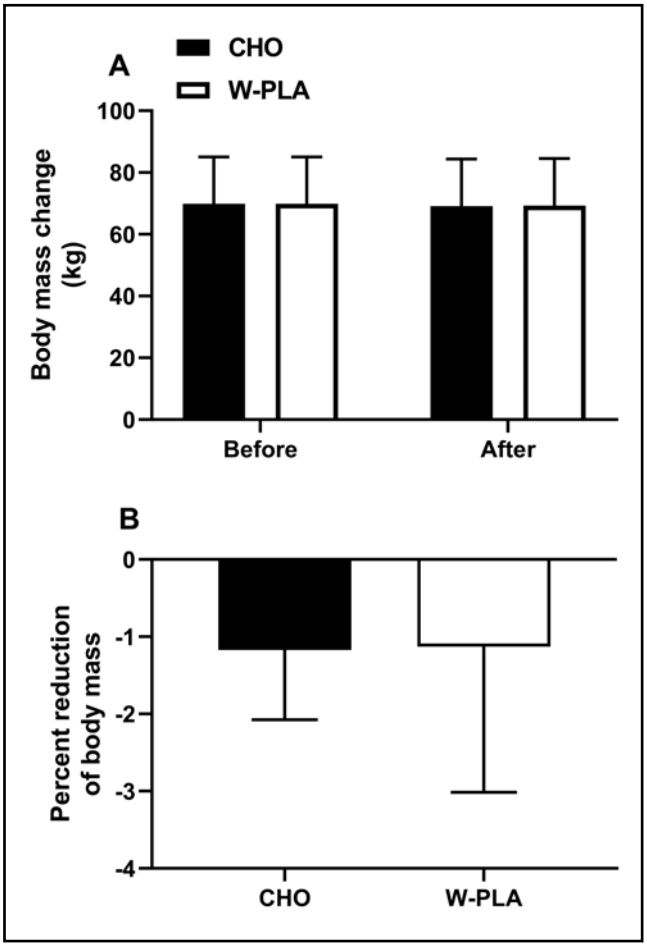

Fig. 2. (A) Body mass change (kg) and (B) percent reduction of body mass before and immediately after 2-h long-lasting exercise with fluid ingestion (carbohydrate or water placebo). CHO: carbohydrate, W-PLA: water placebo.

Urinary α-amylase isoenzyme proportion

The percentage of urinary α-amylase isoenzymes stemmed from pancreas and saliva was determined with cellulose acetate membrane electrophoresis as described by Takeuchi *et al.* (1974). In summary, the membranes were placed in 0.02 M phosphate buffer solution (pH 7.4) and de-aerated at low pressure for 10 min. After the membranes were blotted between filter papers in order to get rid of surface droplets, 3 µl of sample were applied to the middle. Urinary samples were diluted with inactivated urine (urine boiled for 1 h) (Irie *et al.* 1972). With an electrophoretic apparatus, electrophoresis was conducted at 2°C in 0.02 M phosphate buffer (pH 7.4) containing 0.02% NaCl and 0.1% bovine serum albumin at a constant voltage of 300 V for 3-h (Hammerton & Messer, 1971).

Statistical analysis

Normality among dependent variables were checked by the D'Agostino-Pearson omnibus test. A two-way analysis of variance (ANOVA) for repeated measures on two factors (sampling time x treatment) was used with a statistical package (Statistica V5.1 for Windows,

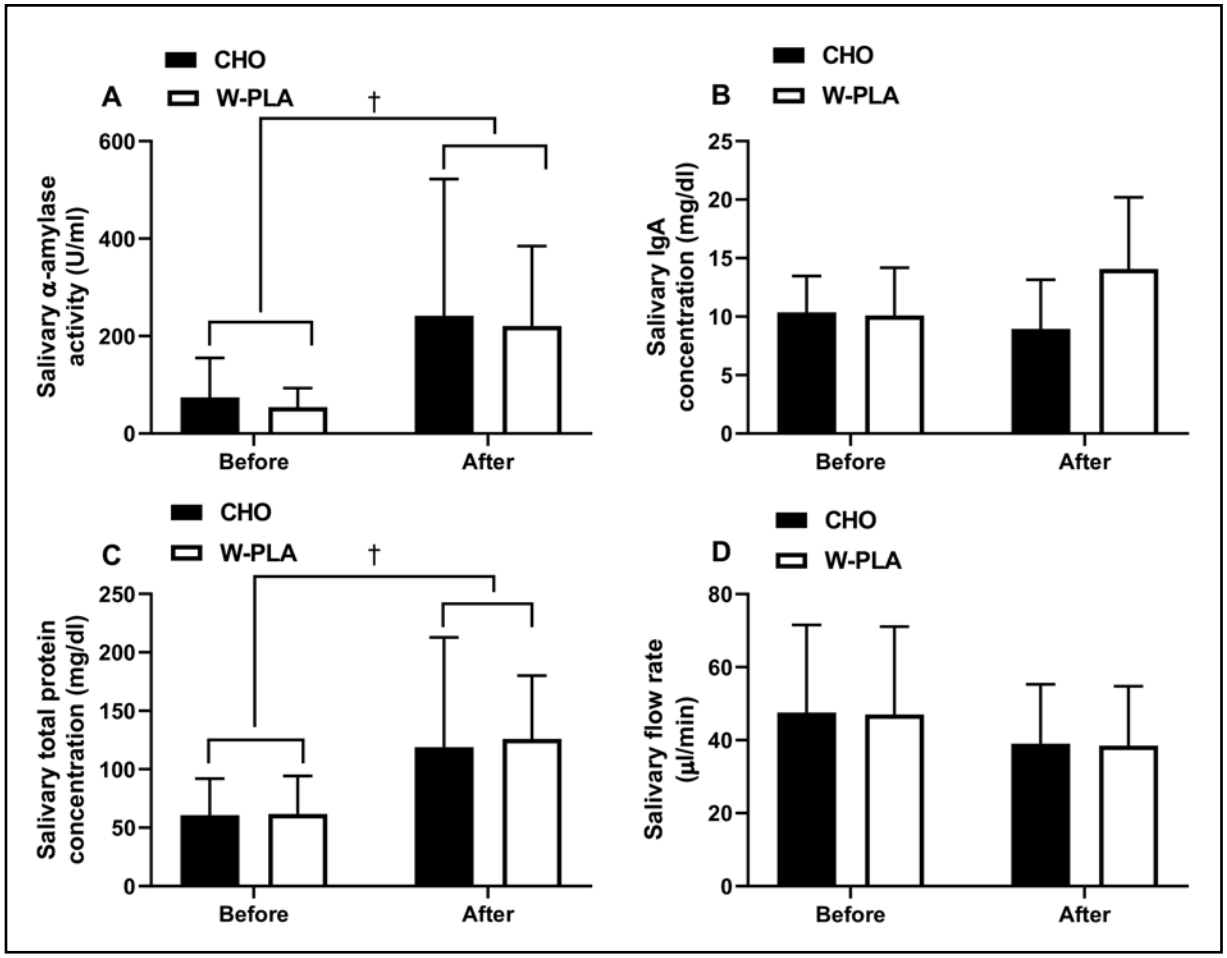

Fig. 3. Salivary (A) α-amylase activity (U/ml), (B) IgA concentration (mg/dl), (C) total protein concentration (mg/dl), and (D) flow rate (µl/min) before and immediately after 2-h moderate endurance exercise with liquid intake (carbohydrate or water placebo). CHO: carbohydrate, W-PLA: water placebo, †: main effect for time.

Statsoft, Tulsa, OK, USA). When significance was obtained, the location of the difference was identified using a Tukey's post-hoc test. Effect sizes for t-test and ANOVA were computed with Cohen's d and eta squared (η^2), respectively (Kirk, 2007). Interpretation of the effect size Cohen's d and η^2 was as follows: $0.2 < d < 0.5$ = small effect, $0.5 < d < 0.8$ = medium effect, and $d > 0.8$ = large effect; $0.01 \leq \eta^2 < 0.06$ = small effect, $0.06 \leq \eta^2 < 0.14$ = moderate effect, $\eta^2 \geq 0.14$ = large effect, respectively (Kirk, 2007). Pearson's correlation analysis was carried out to elucidate the relation of aerobic fitness and percent change in α-amylase activity in saliva and urine. Similarly, the association between aerobic fitness and salivary IgA concentration was also analyzed. Statistical significance was accepted at the 0.05 level. All values are described as mean±SD.

RESULTS

Physiological measurements

A two-way ANOVA revealed significant differences in heart rate (beats/min, main effect for time, $p<0.001$, $\eta^2=0.80$), but for treatment ($p=0.301$, $\eta^2=0.01$) or interaction ($p=0.996$, $\eta^2=0.01$) following 2-h moderately prolonged exercise (Figure 1A). Similarly, there were significant changes in ratings of perceived exertion (main effect for time, $p<0.001$, $\eta^2=0.52$), though no significant alterations were found for treatment ($p=0.249$, $\eta^2=0.01$) or interaction ($p=0.567$, $\eta^2=0.03$, Figure 1B). Moreover, no significant loss of body mass (kg) was identified (main effect for time, $p=0.895$, $\eta^2=0.01$), in association with the lack of significances for treatment ($p=0.991$, $\eta^2=0.01$) or interaction ($p=0.983$, $\eta^2=0.01$, Figure 2A). Additionally, no significance was noted in percent reduction of body mass ($p=0.190$, Cohen's d=-0.07, Figure 2B) before and after moderate endurance exercise.

Salivary biomarkers

As to salivary α-amylase activity (U/ml), significant main effects were recognized for time ($p=0.006$, $\eta^2=0.22$), but for treatment ($p=0.712$, $\eta^2=0.01$) or interaction ($p=0.993$, $\eta^2=0.01$, Figure 3A) after moderately prolonged exercise. Furthermore, there were no significant main effects in salivary IgA concentration (mg/dl) for time ($p=0.353$, $\eta^2=0.02$) and treatment ($p=0.083$,

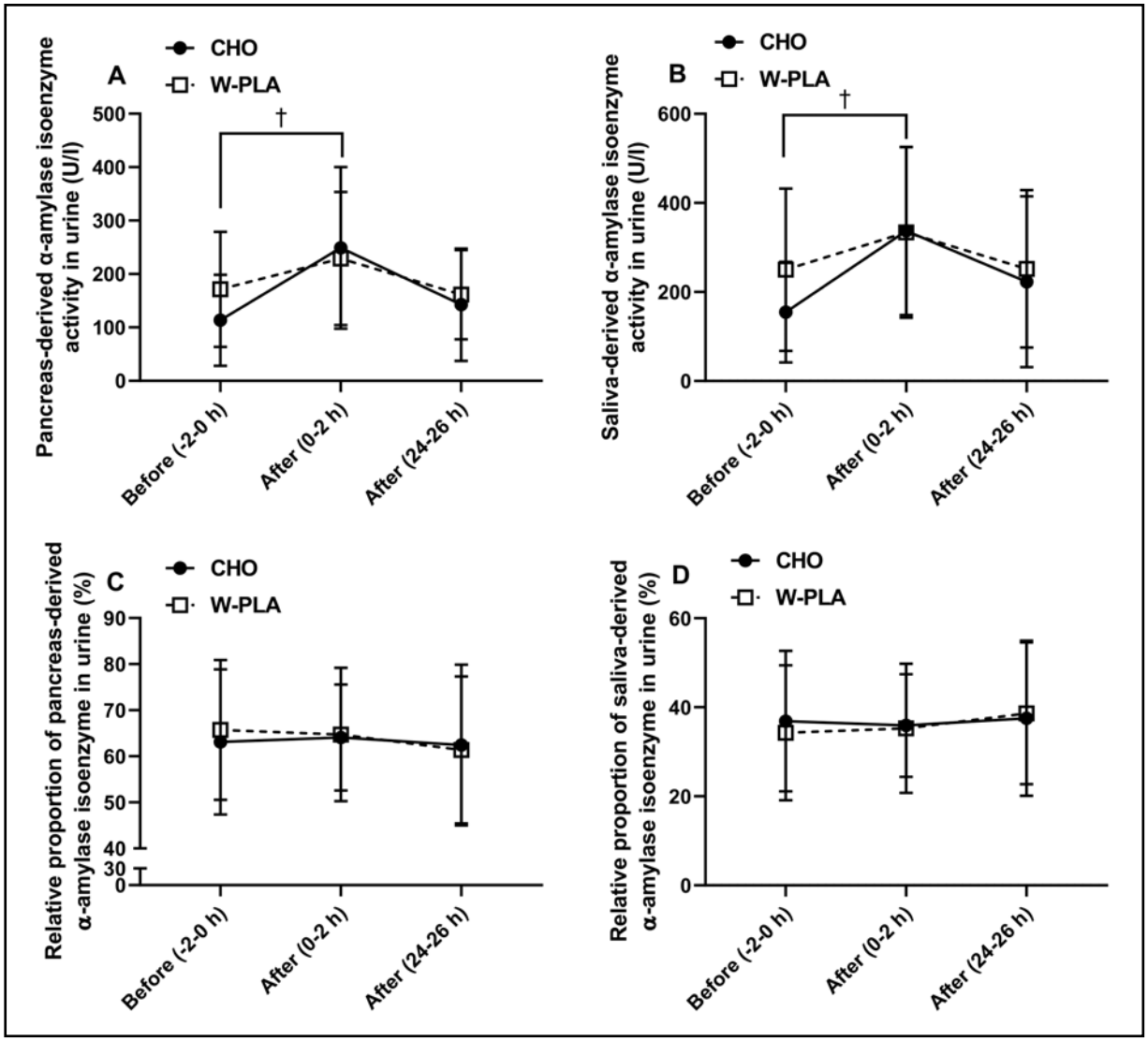

Fig. 4. (A) Pancreas-derived and (B) saliva-derived α-amylase isoenzyme activities (U/l) in urine based on enzyme-substrate reaction assay and relative percentage of (C) pancreas-derived and (D) saliva-derived α-amylase isoenzyme distribution quantified with cellulose acetate membrane electrophoresis before (-2-0 h), immediately after (0-2 h), and 24-26 h after 2-h moderately long-lasting cycling exercise with liquid ingestion (carbohydrate or water placebo). CHO: carbohydrate, W-PLA: water placebo, †: main effect for time.

η^2=0.07) or interaction (p=0.055, η^2=0.09, Figure 3B). Regarding salivary total protein concentration (mg/dl), significant main effects were noted for time (p=0.001, η^2=0.23), notwithstanding the lack of significances for treatment (p=0.818, η^2=0.01) or interaction (p=0.869, η^2=0.01, Figure 3C) after endurance exercise. Besides those salivary biomarkers, there were no significant main effects in salivary flow rate (μ/min) for time (p=0.176, η^2=0.05) and treatment (p=0.936, η^2=0.01) or interaction (p=0.999, η^2=0.01) following 2-h moderately long-lasting exercise (Figure 3D).

Urinary biomarkers

In relation to urinary α-amylase isoenzyme activity (U/l) assessed with enzyme-substrate reaction assay, there was significant elevation in both pancreas-derived (main effect for time, p=0.01, η^2=0.14) and saliva-derived type (main effect for time, p=0.041, η^2=0.10) immediately after prolonged cycling exercise, whereas no main effects for treatment (pancreas-derived type: p=0.494, η^2=0.01; saliva-derived type: p=0.356, η^2=0.01) or interaction (pancreas-derived type: p=0.52, η^2=0.02; saliva-derived type: p=0.64, η^2=0.01) were demonstrated (Figure 4A and 4B). Moreover, no significant main effects for time (pancreas-derived type: p=0.821, η^2=0.03; saliva-derived type: p=0.821, η^2=0.01) and treatment (pancreas-derived type: p=0.848, η^2=0.01; saliva-derived type: p=0.848, η^2=0.01) or interactions (pancreas-derived type: p=0.922, η^2=0.01; saliva-derived type: p=0.922, η^2=0.01) were observed after moderate endurance exercise concerning the percentage of urinary α-amylase isoenzyme distribution on the basis of cellulose acetate membrane electrophoresis (Figure 4C and 4D).

Tab. 2. Correlation analysis between indexes of aerobic fitness (Tvent and VO_2peak) and α-amylase activity under resting condition and the percent change in α-amylase activity in saliva and urine before and after exercise with carbohydrate intake or water placebo (n=11).

		Tvent		Significance	VO_2peak		Significance
		r	p		r	p	
Exercise with CHO							
Saliva							
	R-sAA	-0.314	0.411	ns	-0.498	0.172	ns
	PC-sAA	-0.506	0.165	ns	-0.401	0.284	ns
Urine							
	R-uP-AA	-0.302	0.368	ns	-0.107	0.755	ns
	R-uS-AA	-0.242	0.474	ns	-0.108	0.752	ns
	PC-uP-AA	0.425	0.192	ns	0.372	0.260	ns
	PC-uS-AA	0.485	0.131	ns	0.446	0.169	ns
Exercise with W-PLA							
Saliva							
	R-sAA	-0.394	0.295	ns	-0.265	0.490	ns
	PC-sAA	-0.138	0.724	ns	-0.261	0.498	ns
Urine							
	R-uP-AA	0.266	0.429	ns	0.398	0.225	ns
	R-uS-AA	0.532	0.092	ns	0.574	0.065	ns
	PC-uP-AA	-0.118	0.729	ns	-0.463	0.151	ns
	PC-uS-AA	-0.125	0.714	ns	-0.475	0.140	ns

Tvent: ventilatory threshold, VO_2peak: peak oxygen uptake, r: correlation coefficient, p: probability value, ns: no significance, CHO: carbohydrate, W-PLA: water placebo, R-sAA: resting salivary α-amylase activity, PC-sAA: percent change in salivary α-amylase activity before and after exercise, R-uP-AA: resting urinary pancreas-derived α-amylase isoenzyme activity, R-uS-AA: resting urinary saliva-derived α-amylase isoenzyme activity, PC-uP-AA: percent change in urinary pancreas-derived α-amylase isoenzyme activity before and after exercise, PC-uS-AA: percent change in urinary saliva-derived α-amylase isoenzyme activity before and after exercise.

Correlation analysis

Pearson's correlation analysis represented no significant relationship between aerobic fitness values (ventilatory threshold and VO_2peak) and percent change in saliva and urinary α-amylase activity before and after 2-h moderate endurance exercise (Table 2). In contrast, significant association were noted between aerobic fitness levels and resting IgA concentration irrespective of treatment (carbohydrate or water placebo intake) (Table 3).

DISCUSSION

The principal findings of this study were that α-amylase activity in saliva and urine were elevated after 2-h moderate exercise despite exogenous carbohydrate intake. To our knowledge, this was the first study to simultaneously analyze α-amylase activity in saliva and urine after moderately long-lasting cycling exercise with or without carbohydrate solution.

Regarding the effects of exogenous carbohydrate availability on α-amylase activity in saliva and urine at the resting state, previous studies have demonstrated the reduction of the levels of amylase activity in tears, whole saliva or urine in malnourished children compared to controls (Watson *et al.* 1977) in conjunction with decreased pancreatic function resulting in lower pancreatic isoenzyme activity (Townes *et al.* 1976). It has also been shown that fasting lowers pancreatic amylase activity in human and animal models (Minato, 1997). In contrast, some study has shown that a high carbohydrate diet could stimulate salivary α-amylase secretion with elevated amylolytic activity in healthy individuals (Harthoon *et al.* 2009). However, some investigation has represented no adaptational increase of stimulated human pancreatic α-amylase secretion after a long-term (14 days) diet with high-carbohydrate (Liehr, 1989). Collectively, it is plausible that the degree of α-amylase secretion in saliva and urine could be attributed to synthesis rates of α-amylase associated with carbohydrate intake.

Along with the aforementioned remark, it has generally been accepted that carbohydrate intake during prolonged (>2-h) moderate to high-intensity exercise is important for endurance athletes to maintain and

Tab. 3. Correlation analysis between indexes of aerobic fitness (Tvent and VO2peak) and salivary IgA concentration under resting condition and the percent change in salivary IgA concentration before and after exercise with carbohydrate ingestion or water placebo (n=11).

	Tvent		Significance	VO_2peak		Significance
	r	p		r	p	
Exercise with CHO						
Saliva						
R-sIgA	-0.673	0.023	s	-0.672	0.023	s
PC-sIgA	0.168	0.622	ns	0.122	0.721	ns
Exercise with W-PLA						
R-sIgA	-0.627	0.039	s	-0.772	0.005	s
PC-sIgA	0.069	0.840	ns	0.040	0.908	ns

Tvent: ventilatory threshold, VO_2peak: peak oxygen uptake, r: correlation coefficient, p: probability value, s: significance, ns: no significance, CHO: carbohydrate, W-PLA: water placebo, R-sIgA: resting salivary IgA concentration, PC-sIgA: percent change in salivary IgA concentration before and after exercise.

enhance their performance (Cermak & van Loon, 2013). For instance, carbohydrate feedings during prolonged exercise have been shown to increase performance in lasting longer than 2-h at intensities of 50 to 70% maximal oxygen consumption, attributing to maintenance of blood glucose levels, high rates of carbohydrate oxidation and possibly sparing endogenous glycogen stores (Baker & Jeukendrup, 2014). On the other hand, carbohydrate feedings during moderately prolonged exercise within 2-h appear not to have a great influence on muscle glycogen degradation as the rate of glycogenolysis can exceed the rate of glucose transport, implicating a greater contribution of glycogen breakdown to maintenance of ATP levels (Greenberg *et al.* 2006). In the current study, no significant differences were found in heart rate and ratings of perceived exertion between treatments, which implies that constantly ingested fluid intake during 2-h of moderately prolonged exercise could be indicative of a similar catecholamine response regardless of exogenous carbohydrate feeding, masking any response evoked by esophageal and/or gastric distension.

Previous data have been introduced as to the effects of endurance exercise with or without carbohydrate ingestion on salivary α-amylase activity. For example, some studies dealing with saliva samples demonstrated increased salivary α-amylase activity following 2-h moderate exercise at 60%VO_2peak without carbohydrate ingestion (Li & Gleeson, 2004). Conversely, Bishop *et al.* (2000) showed no alterations of salivary α-amylase activity after 60%VO_2peak of 2-h prolonged exercise with carbohydrate intake in healthy individuals. Taken together, these outcomes can partially be attributed to the amount of carbohydrate availability to maintain glucose homeostasis and hydration status in concord with the rate of glycogenolysis/gluconeogenesis, including intensity and duration of the exercise and training status associated with higher glucose uptake and muscle oxidative capacity at the muscle tissue level.

As to the relative proportion of α-amylase isoenzymes (pancreas- and saliva-derived types) under basal conditions (pre-exercise), past studies have reported that pancreas-derived type is higher than saliva-derived type in urine in healthy individuals (Bank *et al.* 1992; Kamarýt *et al.* 1993), which was similar in two trials of the present study, quantified with cellulose acetate electrophoresis. Furthermore, some research has shown a relatively wide range of urinary pancreas-derived type (17-63%) in healthy adults using the chromatographic separation on diethylaminoethyl-cellulose (Stěpán & Skrha, 1979). Regarding patients with pancreatic diseases, several studies have demonstrated that the percentage of pancreas-derived type increased to 70 to 100% (Tomaszewski *et al.* 1984). Additionally, Stěpán and Skrha (1979) reported that the percent of pancreas-derived type in the urine decreased to approximately 41% in cases of parotitis. Extrapolating from those facts, it is feasible that baseline urinary α-amylase isozymes in healthy individuals tend to be more pancreas-derived type, though there are some differences in the quantification of urinary amylase isozymes depending on various methods.

Few studies have demonstrated the effects of exercise on urinary α-amylase activity in healthy individuals (Bakońska-Pacoń & Sobiech, 1989; Cucinell *et al.* 1984; Poortmans, 1972). Bakońska-Pacoń and Sobiech (1989) have found significantly augmented activity of total α-amylase (both pancreatic and salivary origin, expressed as U/l) determined with selective inhibitors in urine immediately after the marathon race in all runners, which was consistent with our outcomes of the current investigation based on enzyme-substrate reaction, inclusive of a decreased tendency for the urinary pancreas- and saliva-derived types 22-24 h after the race. In contrast to it, the percentage of both pancreas- and saliva-derived types determined with cellulose acetate membrane electrophoresis in urine was constant between two exercise trials in this study. To sum up, the

reasons for discrepancies in aforementioned findings could in part be explained by the differences in type, intensity and duration of the exercise, training status, and carbohydrate availability in association with hydration status, with the addition of the differences of quantifying methods. Another possible reason could also be due to glomerular filtration rate and some degree of tubular absorption which regulates the urinary α-amylase excretion (Søling *et al.* 1979).

It remains indecisive whether endurance exercise training (aerobic conditioning) has an influence on salivary and/or urinary α-amylase isoenzyme activity. Long-term endurance exercise training appears to have the potential to attenuate sympathetic drive and augment parasympathetic activity with a concomitant decreased concentration of plasma catecholamines (Smith *et al.* 1989). Salivary α-amylase activity activated by sympathetic-adrenomedullary system and hypothalamic-pituitary-adrenal axis has been suggested as a biomarker to determine mental/physical stress levels (Rohleder & Nater, 2009). Specifically, it has been shown that increased sympathetic drive results in elevated α-amylase activity (Diaz *et al.* 2012). Thus, a series of repeated exercise training demonstrated by previous human research can lead to the lowered levels of peripheral resistance, resting blood pressure and resting heart rate (O'Sullivan & Bell, 2001). Consequently, it has been proposed that long-term endurance exercise training may lead to decreased salivary α-amylase activity (Diaz *et al.* 2013).

In parallel with those "training adaptation," some study in rats has shown increased basal pancreatic protein content and amylase activity at the pancreatic tissue level after endurance training on a treadmill (Minato, 1997). Recently, one study has represented that long-term endurance exercise training for a relatively longer duration (~6 h) transiently attenuated total pancreatic amylase activity immediately after exercise, but also remarkably enhanced total pancreatic amylase activity after 24-h recovery (Kondo *et al.* 2019). These phenomena in animal tissue-based study indicate that pancreatic enzyme synthesis could be enhanced with endurance training due to an alteration of vagal nervous activity and gastrointestinal digestive and absorptive capacity (Kondo *et al.* 2019).

In the current study, no correlation was observed between the physical fitness levels and the percent change in salivary and urinary α-amylase activity, which was in line with some study dealing with saliva sample (Strahler *et al.* 2016) but not for others (Filaire *et al.* 2013). As additional data, significant association was found between the fitness levels and resting salivary IgA concentration in the current research, which was in opposition to previous data (Blannin *et al.* 1998). One of the potential reasons for such inconsistencies concerning training adaptation could have been due to inadequate verification of endurance training volume as well as absence of objectively indexed physical fitness (Sanchis-Gomar *et al.* 2017), albeit it is difficult to prove the exact cause-effect relationship. In addition to those data related to training adaptation, the similar proportion of urinary α-amylase isoenzymes (pancreas- and saliva-derived types) in the present study may have been attributed to some degree of splanchnic blood flow as represented by Brouns *et al.* (1987). During moderately long-lasting exercise, splanchnic blood flow in the trained could be better than in the untrained, which may be explained by metabolic adaptation of the body in such a way that the same submaximal prolonged exercise results in less stress (Brouns *et al.* 1987). In this regard, no conclusion can be drawn because there are no comparative findings on splanchnic blood flow in this study. Thus, detailed examination is expected in this area.

Previous studies have reported decreased (Nieman *et al.* 2002) or increased salivary IgA concentration (Allgrove *et al.* 2014; Li & Gleeson, 2004) after prolonged exercise, whereas others have represented no change (McDowell *et al.* 1991), which has coincided with our findings of the current study. These discrepant findings may in part be attributed to hydration status after exercise since dehydration can lead to decreased salivary flow rate, which may artificially have an influence on salivary IgA concentration (Allgrove *et al.* 2014). In this regard, constant fluid intake during exercise in this study may have prevented the significant decline of salivary flow rate ascribed to the small percent reduction of body mass (less than 1.2%) despite each treatment. Moreover, the fluid intake may have led to an improved protective effect of saliva as a consequence of enhanced mucosal immune defense, in synchronization with elevated salivary α-amylase activity and total protein concentration.

Limitations of the study

Determination of body mass and percent body fat in the current study was performed by bioelectrical impedance analysis, which is low cost, convenient and ease for athletes and researchers but is inherently prone to estimation errors compared with other devices such as dual-energy X-ray absorptiometry (Moon, 2013). Additionally, all participants who did not take any stimulants, diuretics and drugs were recruited in this study in order to reduce the preanalytical influences by collecting urinary samples. However, there was still a limitation of the study concerning the effect of preanalytical influences on observed within-subject variation, which includes each participant's preparation for the experiment (e.g., physical fitness level, fasting state) in association with salivary and urinary sample collection and handling. Especially on the technical side, the optimal saliva collection time before and after exercise has been proposed as approximately 5 min according to Beltzer *et al.* (2010). With it in mind, the saliva collection was executed for 5 min in our preliminary experiment on strenuous exercise, but the amount of saliva

from several participants was insufficient for later biochemical analysis. Consequently, we set it to 10 min in order to secure a sufficient amount of saliva for the present study as demonstrated by Ben-Aryeh *et al.* (1989). Nonetheless, those procedures may have had an influence on the amount of substance secreted into saliva after exercise as the longer saliva collection time, the more parasympathetic nervous system dominates, which can result in the secretion of diluted saliva (Kunz *et al.* 2015). In addition to the above, although electrolytes were not measured in this study, the alterations of salivary ionic concentration can be affected by exercise intensity and duration, which is associated with changes in saliva flow rate, reabsorption and secretion of electrolytes in the secretory cells (Chicharro *et al.* 1998). Thus, a detailed study is needed to clarify how exercise with the consumption of carbohydrate beverages affect electrolyte secretion in conjunction with other salivary biomarkers.

In conclusion, the main outcomes of the current investigation indicate that α-amylase activity in saliva and urine is enhanced with constant fluid intake in spite of exogenous carbohydrate availability over 2-h moderately prolonged exercise in aerobically fit men, well-balanced immune defense being maintained.

Practical application

The results of the current study may provide valuable insights and information for monitoring the effects of exogenous carbohydrate availability on salivary and urinary α-amylase activity in order to prevent exercise-induced pancreatitis. Moreover, concomitant assessment of salivary and urinary α-amylase activity will bring multidirectional perspectives to the field of clinical and exercise physiology concerning optimal nutritional strategies. Further research is required to quantify α-amylase activity in saliva and urine for the comprehensive evaluation of endogenous and exogenous carbohydrate availability related to endurance exercise, taking into consideration glucose transporter and digestive enzymes in conjunction with insulin and glucagon levels.

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REFERENCES

1 Allan BJ, White TT. (1976). A demonstration that the ratios between human pancreatic enzymes can change in 4 days. Digestion. **14**(3): 232–241.
2 Allgrove JE, Oliveira M, Gleeson M. (2014). Stimulating whole saliva affects the response of antimicrobial proteins to exercise. Scand J Med Sci Sports. **24**(4): 649–655.
3 American College of Sports Medicine (ACSM). (2009). ACSM's Guidelines for exercise testing and prescription. 8th ed. Philadelphia: Lippincott Williams & Wilkins.
4 Anderson GS, Rhodes EC. (1989). A review of blood lactate and ventilatory methods of detecting transition thresholds. Sports Med. **8**(1): 43–55.
5 Baker LB, Jeukendrup AE. (2014). Optimal composition of fluid-replacement beverages. Compr Physiol. **4**(2): 575–620.
6 Bakońska-Pacoń E, Sobiech KA. (1989). Activity of urinary alpha-amylase isoenzymes in marathon runners. Acta Med Pol. **30**(1-2): 51–57.
7 Bank S, Abrol RP, Greenberg R, Blumstein M, Kranz V. (1992). P amylase is always greater than S in spot urine of normal subjects. Diagnostic implications. Int J Pancreatol. **11**(3): 191–194.
8 Beaver WL, Wasserman K, Whipp BJ. (1986). A new method for detecting anaerobic threshold by gas exchange. J Appl Physiol (1985). **60**(6): 2020–2027.
9 Ben-Aryeh H, Roll N, Lahav M, Dlin R, Hanne-Paparo N, Szargel R, et al. (1989). Effect of exercise on salivary composition and cortisol in serum and saliva in man. J Dent Res. **68**(11): 1495–1497.
10 Beltzer EK, Fortunato CK, Guaderrama MM, Peckins MK, Garramone BM, Granger DA. (2010). Salivary flow and alpha-amylase: collection technique, duration, and oral fluid type. Physiol Behav. **101**(2): 289–296.
11 Bishop NC, Blannin AK, Armstrong E, Rickman M, Gleeson M. (2000). Carbohydrate and fluid intake affect the saliva flow rate and IgA response to cycling. Med Sci Sports Exerc. **32**(12): 2046–2051.
12 Blannin AK, Robson PJ, Walsh NP, Clark AM, Glennon L, Gleeson M. (1998). The effect of exercising to exhaustion at different intensities on saliva immunoglobulin A, protein and electrolyte secretion. Int J Sports Med. **19**(8): 547–552.
13 Bocanegra OL, Diaz MM, Teixeira RR, Soares SS, Espindola FS. (2012). Determination of the lactate threshold by means of salivary biomarkers: chromogranin A as novel marker of exercise intensity. Eur J Appl Physiol. **112**(9): 3195–3203.
14 Brannon PM. (1990). Adaptation of the exocrine pancreas to diet. Annu Rev Nutr.**10**: 85–105.
15 Brouns F, Saris WH, Rehrer NJ. (1987). Abdominal complaints and gastrointestinal function during long-lasting exercise. Int J Sports Med. **8**(3): 175–189.
16 Calvo F, Chicharro JL, Bandrés F, Lucía A, Pérez M, Alvarez J, et al. (1997). Anaerobic threshold determination with analysis of salivary amylase. Can J Appl Physiol. **22**(6): 553–561.
17 Cermak NM, van Loon LJ. (2013). The use of carbohydrates during exercise as an ergogenic aid. Sports Med. **43**(11): 1139–1155.
18 Chicharro JL, Lucía A, Pérez M, Vaquero AF, Ureña R. (1998). Saliva composition and exercise. Sports Med. **26**(1): 17–27.
19 Cucinell SA, Wan S, O'Brien J, Wade C. (1984). Amylase excretion after exercise. Am J Gastroenterol. **79**(8): 619–622.
20 de Oliveira VN, Bessa A, Lamounier RP, de Santana MG, de Mello MT, Espindola FS. (2010). Changes in the salivary biomarkers induced by an effort test. Int J Sports Med. **31**(6): 377–381.
21 Diaz MM, Bocanegra OL, Teixeira RR, Soares SS, Espindola FS. (2012). Response of salivary markers of autonomic activity to elite competition. Int J Sports Med. **33**(9): 763–768.
22 Diaz MM, Bocanegra OL, Teixeira RR, Soares SS, Espindola FS. (2013). Salivary nitric oxide and alpha-amylase as indexes of training intensity and load. Int J Sports Med. **34**(1): 8–13.
23 Filaire E, Ferreira JP, Oliveira M, Massart A. (2013). Diurnal patterns of salivary alpha-amylase and cortisol secretion in female adolescent tennis players after 16 weeks of training. Psychoneuroendocrinology. **38**(7): 1122–1132.
24 Gaskill SE, Ruby BC, Walker AJ, Sanchez OA, Serfass RC, Leon AS. (2001). Validity and reliability of combining three methods to determine ventilatory threshold. Med Sci Sports Exerc. **33**(11): 1841–1848.
25 Gavin TP, Babington JP, Harms CA, Ardelt ME, Tanner DA, Stager JM. (2001). Clothing fabric does not affect thermoregulation during exercise in moderate heat. Med Sci Sports Exerc. **33**(12): 2124–2130.

26 Greenberg CC, Jurczak MJ, Danos AM, Brady MJ. (2006). Glycogen branches out: new perspectives on the role of glycogen metabolism in the integration of metabolic pathways. Am J Physiol Endocrinol Metab. **291**(1): E1–E8.
27 Hammerton K, Messer M. (1971). The origin of serum amylase. Electrophoretic studies of isoamylases of the serum, liver and other tissues of adult and infant rats. Biochim Biophys Acta. **244**(2): 441–451.
28 Hariharan R, Mousa A, de Courten B. (2021). Influence of AMY1A copy number variations on obesity and other cardiometabolic risk factors: A review of the evidence. Obes Rev doi: 10.1111/obr.13205. Epub ahead of print.
29 Harthoorn LF, Brattinga C, Van Kekem K, Neyraud E, Dransfield E. (2009). Effects of sucrose on salivary flow and composition: differences between real and sham intake. Int J Food Sci Nutr. **60**(8): 637–646.
30 Irie A, Hunaki M, Bando K, Kawai K. (1972). Determination of amylase activity in serum and urine using blue starch substrate. Clin Chim Acta. **42**(1): 63–66.
31 Jeukendrup A, Brouns F, Wagenmakers AJ, Saris WH. (1997). Carbohydrate-electrolyte feedings improve 1 h time trial cycling performance. Int J Sports Med. **18**(2): 125–129.
32 Kamarýt J, Stejskal J, Osicková L, Mrskos A, Pribylová H, Coupek P. (1993). Urinary amylase isoenzymes and amylase polymorphism variants in families with diabetes mellitus type 1. Eur J Clin Chem Clin Biochem. **31**(11): 739–742.
33 Kirk RE. (2007). Effect magnitude: a different focus. J Stat Plan Inference. **137**(5): 1634–1646.
34 Kondo S, Fukazawa A, Karasawa T, Terada S. (2019). Effects of long-term exercise training for different durations on pancreatic amylase activity and intestinal glucose transporter content in rats. Physiol Rep. **7**(20): e14255.
35 Kunz H, Bishop NC, Spielmann G, Pistillo M, Reed J, Ograjsek T, et al. (2015). Fitness level impacts salivary antimicrobial protein responses to a single bout of cycling exercise. Eur J Appl Physiol. **115**(5): 1015–1027.
36 Li TL, Gleeson M. (2004). The effect of single and repeated bouts of prolonged cycling and circadian variation on saliva flow rate, immunoglobulin A and alpha-amylase responses. J Sports Sci. **22**(11–12): 1015–1024.
37 Liehr RM, Herrmann ME, Emde C, Hopert R, Riecken EO. (1989). High amount of dietary carbohydrate does not cause an adaptational increase of stimulated human pancreatic amylase secretion. Int J Pancreatol. **4**(4): 419–430.
38 Lorentz K. (1998). Approved recommendation on IFCC methods for the measurement of catalytic concentration of enzymes. Part 9. IFCC method for alpha-amylase (1,4-alpha-D-glucan 4-glucanohydrolase, EC 3.2.1.1). International Federation of Clinical Chemistry and Laboratory Medicine (IFCC). Committee on Enzymes. Clin Chem Lab Med. **36**(3): 185–203.
39 McDowell SL, Chaloa K, Housh TJ, Tharp GD, Johnson GO. (1991). The effect of exercise intensity and duration on salivary immunoglobulin A. Eur J Appl Physiol Occup Physiol. **63**(2): 108–111.
40 Mandel AL, Peyrot des Gachons C, Plank KL, Alarcon S, Breslin PA. (2010). Individual differences in AMY1 gene copy number, salivary α-amylase levels, and the perception of oral starch. PLoS One. **5**(10): e13352.
41 Minato K. (1997). Effect of endurance training on pancreatic enzyme activity in rats. Eur J Appl Physiol Occup Physiol. **76**(6): 491–494.
42 Moon JR. (2013). Body composition in athletes and sports nutrition: an examination of the bioimpedance analysis technique. Eur J Clin Nutr. **67** Suppl 1: S54–S59.
43 Nassis GP, Williams C, Chisnall P. (1998). Effect of a carbohydrate-electrolyte drink on endurance capacity during prolonged intermittent high intensity running. Br J Sports Med. **32**(3): 248–252.
44 Nieman DC, Henson DA, Fagoaga OR, Utter AC, Vinci DM, Davis JM, et al. (2002). Change in salivary IgA following a competitive marathon race. Int J Sports Med. **23**(1): 69–75.
45 O'Sullivan SE, Bell C. (2001). Training reduces autonomic cardiovascular responses to both exercise-dependent and -independent stimuli in humans. Auton Neurosci. **91**(1–2): 76–84.
46 Poortmans JR. (1972). Effect of exercise on the renal clearance of amylase and lysozyme in humans. Clin Sci. **43**(1): 115–120.
47 Rohleder N, Nater UM. (2009). Determinants of salivary alpha-amylase in humans and methodological considerations. Psychoneuroendocrinology. **34**(4): 469–485.
48 Sales MM, Sousa CV, da Silva Aguiar S, Knechtle B, Nikolaidis PT, Alves PM, et al. (2019). An integrative perspective of the anaerobic threshold. Physiol Behav. **205**: 29–32.
49 Sanchis-Gomar F, Alis R, Rampinini E, Bosio A, Romagnoli M, Lombardi G, et al. (2017). Alpha-amylase serum levels in professional soccer players are not related with physical fitness. J Sports Med Phys Fitness. **57**(3): 214–218.
50 Shaila M, Pai GP, Shetty P. (2013). Salivary protein concentration, flow rate, buffer capacity and pH estimation: A comparative study among young and elderly subjects, both normal and with gingivitis and periodontitis. J Indian Soc Periodontol. **17**(1): 42–46.
51 Shaw DM, Merien F, Braakhuis A, Keaney L, Dulson DK. (2021). Adaptation to a ketogenic diet modulates adaptive and mucosal immune markers in trained male endurance athletes. Scand J Med Sci Sports. **31**(1): 140–152.
52 Shimizu M, Myers J, Buchanan N, Walsh D, Kraemer M, McAuley P, et al. (1991). The ventilatory threshold: method, protocol, and evaluator agreement. Am Heart J. **122**(2): 509–516.
53 Smith ML, Hudson DL, Graitzer HM, Raven PB. (1989). Exercise training bradycardia: the role of autonomic balance. Med Sci Sports Exerc. **21**(1): 40–44.
54 Søling K, Mogensen CE, Vittinghus E, Brock A. (1979). The renal handling of amylase in normal man. Nephron. **23**(6): 282–286.
55 Stěpán J, Skrha J. (1979). Measurement of amylase isoenzymes in human sera and urine using a DEAE-cellulose mini-column method. Clin Chim Acta. **91**(3): 263–271.
56 Strahler J, Fuchs R, Nater UM, Klaperski S. (2016). Impact of physical fitness on salivary stress markers in sedentary to low-active young to middle-aged men. Psychoneuroendocrinology. **68**: 14–19.
57 Takeuchi T, Matsushima T, Sugimura T, Kozu T, Takeuchi T. (1974). A rapid, new method for quantitative analysis of human amylase isozymes. Clin Chim Acta. **54**(2): 137–144.
58 Tomaszewski L, Umińska H, Konarska L, Szmajda D, Kulesza A, Oledzki J. (1984). Przydatność pomiaru izoenzymów amylazy we krwi i w moczu w ostrym zapaleniu trzustki [(Usefulness of determining amylase isoenzymes in the blood and urine in acute pancreatitis.)(In Polish with English abstract.)]. Pol Tyg Lek. **39**(19): 629–633.
59 Townes PL, Moore WD, White MR. (1976). Amylase polymorphism: studies of sera and duodenal aspirates in normal individuals and in cystic fibrosis. Am J Hum Genet. **28**(4): 378–389.
60 Wallenfels K, Foldi P, Niermann H, Bender H, Linder D. (1978). The enzymic synthesis, by transglucosylation of a homologous series of glycosidically substituted malto-oligosaccharides, and their use as amylase substrates. Carbohyd Res. **61**(1): 359–368.
61 Waller SL, Ralston AJ. (1971a). The hourly rate of urinary amylase excretion, serum amylase, and serum lipase. I. In control subjects and patients with renal disease. Gut. **12**(11): 878–883.
62 Waller SL, Ralston AJ. (1971b). The hourly rate of urinary amylase excretion, serum amylase, and serum lipase. II. Patients with gastrointestinal and pancreatic disorders. Gut. **12**(11): 884–890.
63 Watson RR, Tye JG, McMurray DN, Reyes MA. (1977). Pancreatic and salivary amylase activity in undernourished Colombian children. Am J Clin Nutr. **30**(4): 599–604.
64 Whicher JT, Warren C, Chambers RE. (1984). Immunochemical assays for immunoglobulins. Ann Clin Biochem. **21**(Pt 2): 78–91.
65 Yasuda N, Gaskill SE, Ruby BC. (2008). No gender-specific differences in mechanical efficiency during arm or leg exercise relative to ventilatory threshold. Scand J Med Sci Sports. **18**(2): 205–212.