UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE GENÉTICA E BIOQUÍMICA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

DETERMINAÇÃO DO LIMIAR ANAERÓBIO POR MEIO DE BIOMARCADORES SALIVARES: CROMOGRANINA A COMO NOVO MARCADOR DE INTENSIDADE EM NATAÇÃO.

Aluno: Olga Lucia Bocanegra Jaramillo

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Orientador: Prof. Dr. Foued Salmen Espindola

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(Foued Salmen Espindola)

A las personas que me enseñaron con o sin intención

porque me dieron la satisfacción de aprender

o me dejaron con más voluntad para hacerlo.

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Sumário

Apresentação

O treinamento de alto rendimento é um processo sistemático que envolve variações das cargas de trabalho (volume vs. intensidade). A variação destas cargas é determinada segundo o grau de adaptação e o nível de recuperação ao treinamento. Um melhor desempenho e tolerância às grandes cargas de trabalho refletem uma apropriada adaptação ao treinamento. Uma das medidas mais utilizadas para a avaliação de tal adaptação é a resposta do lactato sanguíneo durante uma prova de esforço incremental. A cinética do lactato pode ser utilizada para determinar a intensidade de trabalho em que os substratos utilizados são priorizados durante o exercício. O presente estudo investigou a viabilidade de biomarcadores salivares para a determinação do limiar anaeróbio, utilizando o lactato sanguíneo como referência, em sujeitos altamente treinados. Nesse trabalho, demonstraremos a relação existente entre a intensidade do exercício e a resposta da cromogranina A salivar, uma proteína co-liberada pela glândula submandibular e que possui propriedades anti-microbianas e anti-fungais. Doze sujeitos, atletas profissionais da natação, foram submetidos a um teste de esforço de velocidade incremental. O teste foi realizado em uma piscina de 50 metros e consistiu de oito estágios de 100 metros. Amostras de sangue e saliva foram coletadas simultaneamente antes do aquecimento, a cada estágio, cinco e dez minutos após o teste. A produção de saliva foi estimulada de forma mecânica utilizando *Salivettes*. O acúmulo de lactato durante a prova foi determinado no sangue e na saliva. A concentração de proteína total (TP), alfa-amilase (sAA), lactato (sLac) e cromogranina A (sCgA) foi determinada na saliva e comparada com o acúmulo de lactato no sangue (bLac). O limiar anaeróbio (LA) foi determinado de forma individual utilizando um modelo de regressão bi-segmentada. Uma alta correlação entre o lactato sanguíneo e o lactato salivar foi encontrada em todos os sujeitos. Da mesma forma, altas correlações entre o lactato sanguíneo sAA e sCgA foram observadas. Os resultados deste estudo são discutidos considerando o transporte do lactato do sangue para a saliva e os motivos pelos quais a secreção de proteínas salivares durante o exercício permite

a determinação do LA. Além disso, sugerimos cuidados especiais na coleta de saliva para evitar a contaminação e diluição da amostra, padronizando o uso do *Salivette* para provas realizadas na piscina. Finalmente, os resultados aqui apresentados corroboram a utilidade da saliva, em substituição ao sangue, em um teste incremental máximo no esporte da natação.

Capítulo 1

Fundamentação Teórica

Bioquímica do lactato

O ácido pirúvico resultante da glicólise é incorporado ao metabolismo oxidativo através do ciclo de Krebs ou é convertido em ácido láctico. A conversão do ácido pirúvico em ácido láctico pela enzima lactato desidrogenase (LDH) é um passo importante onde o NADH citoplasmático é oxidado. Isso garante um fornecimento contínuo de NAD^+ para a glicólise. Portanto, ao invés de inibir a glicólise, a formação de ácido láctico permite a continuidade desse processo (Hogan et al. 1998).

Além disso, o lactato pode ser oxidado dentro da fibra muscular na qual ele é produzido em uma fibra muscular adjacente, que esteja sob condições de estresse menores ou em repouso, como por exemplo, músculos menos ativos durante o exercício, o coração, o fígado e o cérebro (Brooks 2000). O acúmulo de lactato ou outros intermediários da via glicolítica, não são simplesmente evidência de produção de lactato, mas são considerados para representar a taxa metabólica acima do LA. Esse acúmulo pode ser no tecido muscular e / ou no sangue e representa a situação em que a produção glicolítica de ácido pirúvico e ácido lático supera a taxa de incorporação destas moléculas no ciclo de Krebs (Donovan & Pagliassotti 2000).

Durante exercício submáximo e máximo o lactato produzido é removido das fibras musculares e/ou transportado para dentro das mesmas através dos transportadores monocarboxilados (MCTs) ligados á membrana (Bonen et al. 1998). No músculo esquelético, existem duas isoformas de MCT: o MCT1 e o MCT4. Sendo que, nas fibras musculares oxidativos há prevalência de MCT1 e pequenas quantidades nas fibras glicolíticas, enquanto MCT4 é de densidade variável nos dois tipos de fibra. Após treinamento de alta intensidade, o conteúdo de MCT1 e MCT4 é elevado no músculo esquelético. Embora, MCT1 e MCT4 são co-expressos na membrana mitocôndrial e no sarcolema, apenas MCT1 parece participar no aumento da oxidação de lactato após o treinamento facilitando o transporte de lactato intramuscular (Thomas et al. 2005).

O limiar anaeróbio

O limiar anaeróbio (LA) é definido como a mais alta intensidade de exercício sustentada a partir da qual o fornecimento energético se dá por fonte anaeróbia para conseguir suprir a demanda energética, iniciando-se o acúmulo de lactato (Svedahl & MacIntosh 2003). No LA o nível de lactato se encontra em equilíbrio, sendo que a taxa de aparecimento de lactato sanguíneo é igual à taxa de remoção. Entretanto, ainda não há evidências de que a produção de ácido láctico acima do LA esteja relacionada ao fornecimento inadequado de oxigênio. Presumivelmente, o metabolismo oxidativo é o mecanismo dominante para a produção de ATP, em quase todas as celulas. Em exercicio de alta intencidade o metabolismo glicolítico se inclina para a produção de lactato, e a fosfocreatina (PCr) e a glicólise fornecem a reposição necesaria de ATP, enquanto o metabolismo oxidativo é diminuido (Hogan et al. 1998). Assim, se produz uma diminuição de PCr e no pH e um aumento de ADP, lactato, amônio e íons fosfato que caracterizam o estado de fatiga. A glicólise que resulta na formação de ácido lático deve ser interpretada como um processo que ocorre sem o uso de oxigênio, mas não necessariamente na ausência do mesmo (Svedahl & MacIntosh 2003).

A quantificação da intensidade do exercício no LA tem sido utilizada para a avaliação da saúde cardiovascular ou pulmonar a partir de sua determinação por parâmetros ventilatórios (Scharhag-Rosenberger et al. 2010), assim como para a prescrição e avaliação do treinamento (A. M. Jones et al. 2003). Vários testes foram desenvolvidos para determinar a intensidade do exercício associado ao LA. Dentre eles, destacam-se o teste de limiar ventilatório, teste de lactato mínimo, teste da máxima fase estável de lactato (MFEL), OBLA ou inicio de acumulo de lactato sanguíneo, o teste de limiar anaeróbio individual e o teste de limiar de lactato. Cada abordagem permite uma estimativa da intensidade do exercício relacionada ao LA. Da mesma forma, cada protocolo possui critérios específicos e erros previsíveis na identificação da intensidade de exercício do LA (Oliver Faude et al. 2009).

Teste de limiar de lactato

O teste de limiar de lactato busca identificar a intensidade de exercício associada a um aumento substancial de lactato sanguíneo durante o exercício incremental. O limiar é determinado por modelos matemáticos que permitem identificar um aumento exponencial no acúmulo de lactato durante o exercício (HINKLEY 1969). Assim, quanto maior a intensidade do exercício, maior a produção de lactato e maior seu acúmulo na musculatura e no sangue. Por esse motivo a concentração de lactato é comumente utilizada como marcador de intensidade do exercício físico e indicador da alteração da predominância entre os metabolismos aeróbio e anaeróbio.

Em esportes com um alto componente anaeróbio, como a natação, a determinação do LA é essencial na prescrição de cargas de trabalho, no controle da intensidade e na avaliação do treinamento (Ralph Beneke et al. 2011). O treinamento de rendimento é um processo sistemático que envolve variação nas cargas de trabalho (volume vs. Intensidade). Uma determinação da intensidade de treinamento deve necessariamente envolver medidas sobre o estado e a capacidade do metabolismo oxidativo de cada indivíduo (O. Faude et al. 2008).

O acúmulo de lactato sanguíneo dependem da quantidade de massa muscular envolvida na execução do padrão motor do movimento e como mencionado anteriormente na capacidade do metabolismo anaeróbio (R Beneke & von Duvillard 1996). Assim, é fundamental para atletas profissionais de natação que a determinação do LA seja feita de forma individual periodicamente e utilizando um teste na propia modalidade esportiva . Alguns testes, como o OBLA sugerem a concentração plasmática de lactato de 4 mmol/L durante o exercício como ponto de referência para a determinação do LA (Karlsson et al. 1981) Porém, este valor não considera o protocolo de exercício utilizado ou as características morfofisiológicas (tipo de fibra muscular, capacidade cardiovascular, entre outras) de cada atleta, fatores essências na produção de lactato durante o exercício.

Por outro lado, coletas de sangue para a determinação da concentração de lactato durante um teste em natação podem não ser apropriadas por questões de

higiene. Em muitas ocasiões durante o teste de esforço, após a coleta de sangue, os atletas precisam voltar á piscina para continuar a prova sem o que sangue do dedo (ou lobo da orelha) de onde foi coletada a amostra esteja coagulado. Dessa forma, a mensuração de biomarcadores de intensidade na saliva constitui uma alternativa atraente, segura e simples (Humphrey & Williamson 2001).

Saliva

A saliva é uma complexa mistura formada pelas secreções das três glândulas principais, as glândulas parótida, submandibular e sublingual. Alem de outras centenas de glândulas espalhadas na mucosa oral (Humphrey & Williamson 2001). A contribuição das diferentes glândulas salivares para a produção de saliva total depende do ritmo circadiano e do tipo de estimulação. As glândulas salivares recebem inervação do sistema nervoso autônomo. Em geral, a inervação parassimpática via ação colinérgica, provoca vasodilatação, o que aumenta a quantidade e fluidez da saliva contendo baixos níveis de compostos orgânicos e inorgânicos. A inervação simpática via ação adrenérgica provoca vasoconstrição, diminuindo o volume do fluxo salivar; porém com níveis elevados de proteínas e compostos inorgânicos, aumentando a viscosidade da saliva (Proctor & Carpenter 2007).

A glândula parótida é a maior glândula salivar e possui dois ductos, o ducto Stensen, que se abre para a cavidade oral ao nível do segundo molar superior e o ducto parotídeo localizado entre o arco zigomático e o canto da boca entre o lábio superior e o filtro do tragus. A glândula submandibular tem a metade do peso da parótida e está posicionada na base posterior da mandíbula. Essa glândula libera a secreção salivar no assoalho da boca, próximo ao frênulo da língua, através do ducto de Wharton. A glândula sublingual é a menor das glândulas salivares maiores e, ao contrário das glândulas parótida e submandibular, esta é drenada por cerca de 10 pequenos ductos, denominados de ductos de Rivinus, os quais desembocam no assoalho da boca.

As glândulas menores, diferentemente das glândulas salivares maiores, não possuem um ducto salivar próprio e estão concentradas em varias regiões na parte bucal, labial, palatina e lingual. A maioria das glândulas menores recebem somente inervação parassimpática (Proctor 2006)

Todas as glândulas salivares possuem células acinares, responsáveis pela produção e secreção salivar. Essas células contêm grânulos de secreção, sendo que nas glândulas serosas, esses grânulos contém amilase, e nas glândulas mucosas, esses grânulos contêm mucina. Alguns ácinos produzem secreções mistas. A glândula parótida é uma glândula salivar puramente serosa, enquanto a submandibular e sublingual são mistas, sendo que a primeira é predominantemente serosa e a segunda, predominantemente mucosa (D. Castle & A. Castle 1998).

O processo de secreção salivar ocorre em dois estágios; primero é produzida uma secreção inicial aquosa de um plasma líquido isotônico secretado pelas células acinares. Em seguida ocorre uma modificação de esta secreção, para um fluido hipotônico produzido pelas celulas epiteliais durante a passagem pelo sistema de ductos, nos quais outras substâncias provenientes do sangue são difundidas à saliva (Humphrey & Williamson 2001; Lee & Wong 2009).

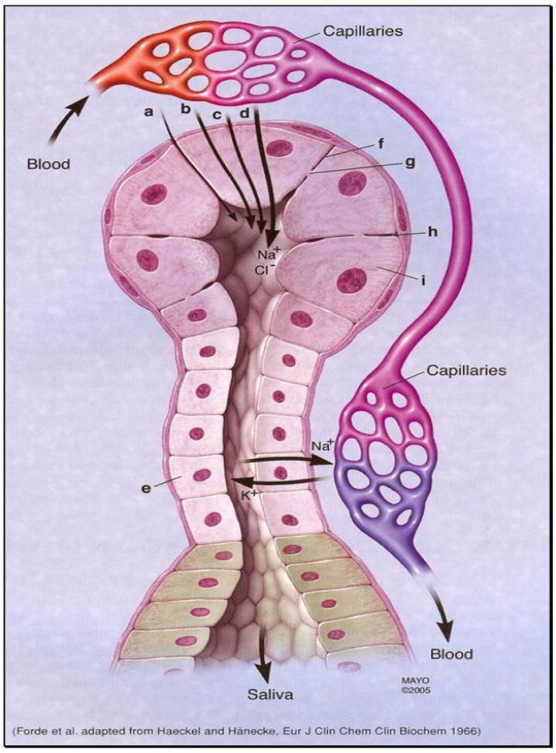

Figura 1. Mecanismo de transporte molecular nos dutos das glândulas salivares.

a. Transporte ativo. b. Difusão passiva. c. Filtração simples. d.Células acinares, ativamente pela bomba de íons de sódio Na+ dentro do duto.

Adaptado de: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2860957/?tool=pubmed.

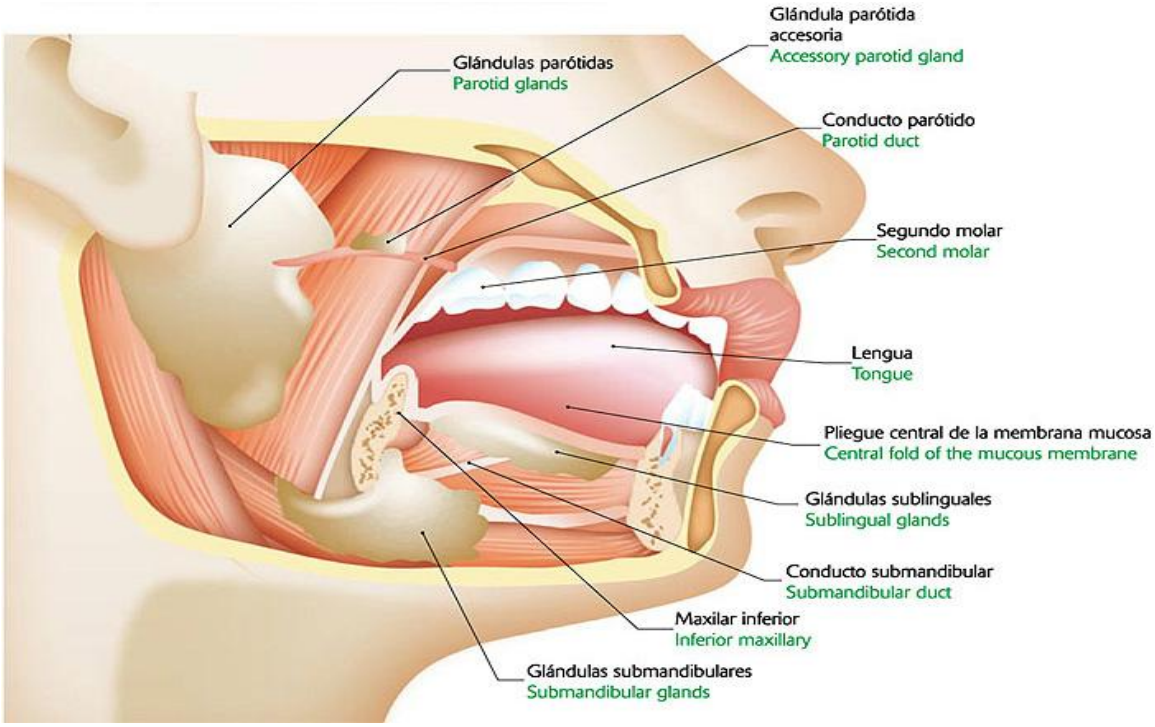

Figura 2. Glândulas Salivares

Adaptado de http://www.icarito.cl/herramientas/laminas/2009/glandulas-salivales .shtml.

Amilase salivar

A alfa-amilase salivar (sAA) é uma das proteínas mais abundantes na saliva e possui propriedades digestivas e anti-microbianas (Scannapieco et al. 1993). Estudos têm demonstrado a viabilidade da determinação do LA na saliva pela quantificação da sAA (Calvo et al. 1997; de Oliveira et al. 2010; Bortolini et al. 2009; Chicharro et al. 1994); já que o padrão de secreção da sAA está relacionado indiretamente às alterações nos níveis de catecolaminas que ocorrem devido ao aumento da atividade simpático-adrenal durante o exercício incremental submáximo e maximo (Chicharro et al. 1994; Chicharro et al. 1999; Chatterton et al. 1996). Chatterton et al (1996) verificaram altas correlações entre sAA e catecolaminas. Desde então, a sAA tem sido sugerida como um marcador indireto da atividade do sistema simpático-adrenal-medular (SAM) em quadros de exercício e estresse (Nater & Rohleder 2009).

Cromogranina A

A cromogranina A (CgA) no homem, é principalmente prodizida na medula supra-renal. Outros locais neuroendócrinos como axônios simpáticos e várias glândulas endócrinas parecem influenciar a concentração basal circulante da CgA (Takiyyuddin et al. 1991). A secreção da CgA por exocitose ocorre a partir dos terminais nervosos simpáticos e as células cromafins, quando as catecolaminas são liberadas (Dimsdale et al. 1992). Trabalhos anteriores demonstraram que perturbações no funcionamento do sistema nervoso simpático resultam em alterações correspondentes nos níveis de CgA no plasma (Kanno et al. 1999; Kanno et al. 2000). Takiyyuddin et al (1990), utilizando exercício intenso em cicloergômetro como estímulo neuronal simpático, demostraram um incremento significativo de CgA no plasma. Dessa forma, durante exercícios de alta intensidade, as concentrações de norepinefrina plasmática e CgA estiveram correlacionadas, sugerindo a CgA como um marcador da atividade simpática periférica.

No fluido salivar, a cromogranina A (sCgA) é co-liberada pela glândula submandibular e possui funções anti-fúngicas e anti-microbianas. Alguns estudos mostraram uma dinâmica similar na secreção de sCgA em comparação à sAA durante situações estressantes como na apresentação de aulas para cursos de graduação (Filaire et al. 2009), exercício moderado (Allgrove et al. 2008) e provas de desempenho cognitivo (Kanamaru et al. 2006). Ainda que considerada um marcador de atividade simpática, não existem relatos na literatura sobre a utilidade da sCgA como indicador do LA em um teste de exercício incremental de esforço máximo.

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Capítulo 2

Determination of the Anaerobic Threshold by means of Salivary Biomarkers: Chromogranin A as Novel Marker of Exercise Intensity

Resumo

Este estudo investigou a variação intra-individual do lactato (sLac), alfa-amilase (sAA) e cromogranina A (sCgA) salivares com relação ao acúmulo de lactato sanguíneo (bLac) durante um teste de exercício incremental máximo em natação. Amostras de sangue e saliva foram coletadas de doze atletas profissionais da natação durante um teste de esforço que consistiu de oito séries de 100 metros com incremento de velocidade a cada série no estilo *crawl*. A concentração de lactato no sangue e na saliva foi determinada por meio de um ensaio eletro-enzimático enquanto que as concentrações de sAA e sCgA foram determinadas por *Western blotting*. O acúmulo de lactato na saliva apresentou uma dinâmica similar à observda no sangue (r=0.92). Resultados similares foram encontrados para as concentrações de sAA (r=0.81) e sCgA (r=0.82) quando comparadas ao bLac. Estes resultados corroboram a utilidade da saliva para a determinação do limiar anaeróbio e fornecem a primeira demostração de que sCgA pode ser utilizada como um marcador de intensidade do exercício em sujeitos altamente treinados.

Palavras Chave: Lactato; limiar anaeróbio; Saliva; Exercício; Alfa-amilase salivar; Cromogranina A salivar; Natação.

Abstract

This study examined intra-individual variations in salivary lactate (sLac), alpha-amylase (sAA) and chromogranin A (sCgA) with reference to the accumulation of blood lactate (bLac) during progressive maximal exercise in swimmers. Samples of blood and saliva were collected simultaneously from 12 professional athletes during an incremental test that consisted of eight series of 100 m in front crawl with increasing velocity (0.03 m x s^{-1} each) and 70-sec intervals. The concentration of blood and salivary lactate was determined by an electro-enzymatic assay whereas sAA and CgA were analyzed by western blotting. Inflection point in the concentration of bLAc, sLac, sAA and CgA were found in all subjects. The accumulation of lactate in saliva followed the same pattern observed in blood with a high correlation between the two (r = 0.92). Similar results were observed between the dynamics of sAA (r = 0.81) and sCgA (r = 0.82) when compared to bLac. These findings support the usefulness of saliva for the determination of the anaerobic threshold and provide the first demonstration of sCgA as a novel marker of exercise intensity in well-trained men.

Keywords: Lactate; Anaerobic Threshold; Saliva; Exercise; Salivary Alpha-Amylase; Salivary Chromogranin A; Swimming.

Introduction

It is well documented that sympathetic adrenal activity increases during progressive maximal exercise (Chicharro et al. 1994), and that in response to such increase, catecholamines are released from the adrenal medulla (Takiyyuddin, Cervenka, Sullivan, Pandian, Parmer, Barbosa & O'Connor 1990b). Epinephrine is responsible to stimulate muscle glycogenolysis, increasing glycolytic flux and enhancing lactate production (Siciliano et al. 2003). Similar to lactate, catecholamines also show an inflection point during submaximal exercise. Such threshold in the concentration of catecholamines is highly correlated with the accumulation of blood lactate (bLac) and occurs at equivalent work rates (A. Weltman et al. 2000). Traditionally, this threshold has been referred to the anaerobic threshold and may be defined as an abrupt transition, from a slowly increasing phase to a rapidly accelerating phase in the accumulation of bLac during incremental exercise(Myers & Ashley 1997; Svedahl & MacIntosh 2003). Tests for the detection of the anaerobic threshold by means of bLac have gained a widespread use in clinical settings for the assessment of exercise capacity in cardiopulmonary disease (Binder et al. 2008) and in sports training to determine exercise intensity and recovery (Oliver Faude et al. 2009).

Although widely used and accepted, measuring of bLac is still invasive and requires special care regarding collection and manipulation of samples. The last decade has seen a considerable growth in the number of studies introducing saliva in preference to blood to assess the response to exercise (Papacosta & Nassis [s.d.]). Saliva offers clear advantages over blood to monitor exercise since it is readily available and can be easily collected, handled and stored. Interestingly, to date only a handful of studies have examined the kinetics of salivary lactate (sLac) during exercise (Segura et al. 1996; Pérez et al. 1999; Chicharro et al. 1995). Although sLac was highly correlated with bLac, the exercise protocols in these studies used moderate increases in work rate leading the authors to conclude that more rapid changes in intensity are necessary in order to determine whether sLac accurately reflects changes in bLac during exercise.

Because protein secretion into saliva is mainly regulated by sympathetic nerves, some salivary proteins have been suggested as surrogate markers of autonomic activity and blood catecholamines during exercise (Chatterton et al. 1996). Salivary alpha-amylase (sAA), for instance, has been successfully correlated with the accumulation of bLac during incremental tests in well-trained subjects (Calvo et al. 1997; de Oliveira et al. 2010). sAA is a digestive enzyme, released from parotid gland and is one of the most abundant proteins in saliva (Scannapieco et al. 1993). Since the early work of Chatterton and colleagues (1996), several studies have underscored the usefulness of variations in sAA to reflect changes in autonomic activity in sports and exercise (Kivlighan & Granger 2006; Nater & Rohleder 2009).

Salivary chromogranin A (sCgA) is a protein with anti-fungal and anti-bacterial properties secreted into saliva from the submandibular gland upon autonomic stimulation (Kanno et al. 2000). Variation in the concentrations of blood and salivary CgA after exercise has also been attributed to a higher sympathetic drive and activation of the adrenal medulla (Takiyyuddin, Cervenka, Sullivan, Pandian, Parmer, Barbosa & O'Connor 1990a; Allgrove et al. 2008). Considering that both sAA and sCgA are responsive to changes in autonomic activity, one could speculate that increases in the concentration of sCgA, as with sAA, would also reflect the kinetics of blood lactate during submaximal exercise.

Thus, we investigated the variation in sLac, sAA and sCgA during an incremental test in swimmers and compared their dynamics with the accumulation rate of bLac. We hypothesized that 1) blood and salivary lactate would show a similar pattern of variation both during and after exercise and 2) the anaerobic threshold would be represented also by the variation in sAA and CgA.

Methods

Subjects

Subjects were 12 male professional swimmers (age 21.2 ± 1.8 years, BMI 21.4 ± 2.6, VO_2max 52.7 ± 2.4 ml/kg^{-1}.min^{-1}). None of the subjects smoked, had significant medical or oral health history or were taking regular or incidental medication. The experimental design, aim of the study, procedures and the character of voluntariness of participation were explained to each subject before signed consent forms were obtained. All experimental protocols were approved by the Institutional Review Board.

Exercise Protocol

Subjects were asked to refrain from exercise, alcohol or caffeinated beverages 48h prior to the test and were instructed to refrain from eating, drinking (anything but water) or tooth brushing at least one hour before the test. Subjects were given approximately 500 mL of water 30 min before the test in order to ensure adequate hydration (Walsh et al. 2004).

The incremental test was conducted in a 50-m pool. Subjects performed the swimming test using the front crawl stroke. A warm-up of 500 m at 0.60 m x s^{-1} was standardized for each subject. The test consisted of eight 100-m swims with increments of 0.03 m x s^{-1} each and 70-second intervals. The initial velocity was established 16 - 24 s above the best mark of each athlete in a 100-m contest. Oral feedback was constantly provided to the subjects regarding velocity of swimming. Only one deviation of > 1.5 s from the required mark at each stage was allowed in order to validate the test. The exercise protocol was chosen based on a pilot study that allowed us to observe a gradual accumulation of lactate throughout the test on a relatively short period of swimming (± 12 min). The test was conducted at 15:00h with water temperature between 22-25° C.

Collection of Samples

Samples of blood and saliva were collected before warming-up, at each stage, and five (R5) and ten minutes (R10) after the test. Capillary blood (±25µL) was collected by finger stick with a disposable needle. The finger was wiped dry, pricked and then a blood sample was collected into a heparin-coated capillary tube. The sample was then placed in a pre-cooled microtube containing 50µL of 1%NaF. Whole saliva was stimulated by chewing a sterile cotton swab (Salimetrics, State College, PA, USA) at a frequency of 60/60 s. Subjects were given approximately 70 mL of distilled water at the end of each stage to rinse their mouths. Subjects were asked to spit the water and then to swallow in order to empty the mouth before saliva sample was collected. After collection, samples were placed on ice, transported to the laboratory and stored frozen at -20°C until analysis.

Measures

Aerobic capacity

One week before the swimming test, VO_{2max} was determined during an incremental exercise test on an electronic treadmill ergometer (MicroMed Biotechnology C200) with simultaneous measurements of respiratory gas exchanges (Cortex, Metasoft 3.1) until voluntary exhaustion. Criteria to validate the test included at least two of the following parameters: plateau in VO_2, respiratory exchange ratio > 1.1, and predicted maximal heart rate.

Lactate

On the day of analysis all samples were thawed and samples of saliva centrifuged at 1500 x g for 5 minutes. Blood and salivary lactate was analyzed on the Biochemistry Analyzer YSI 2700 SELECT (Yellow Springs, Ohio, USA). The same volume of samples (25µL) was used for lactate determination in blood and saliva. Samples were analyzed in duplicate.

Western blotting for sAA and CgA

Concentration of total protein in samples was determined using the Bradford method (Bradford 1976) with bovine serum albumin as standard. All samples from one subject were assayed on the same plate and in duplicate. In order to avoid possible effects of salivary flow rate on the concentration of proteins, ten micrograms of total protein from each sample were denatured under reducing conditions and applied on 5–20% SDS–polyacrylamide gradient gels. Proteins were separated and then transferred onto nitrocellulose membranes in transfer buffer (25 mM Tris, 190 mM glycine, 20% MeOH, pH 7.8-8.4) for two hours at 200mA and 4°C. Protein transfer was confirmed by visualization with Ponceau S. Membranes were blocked for 4 hours at 4°C in blocking buffer (5% non-fat dry milk in PBS w/v). Membranes were then incubated overnight at 4°C with an affinity purified polyclonal rabbit anti-human sAA (dilution 1:5000) produced in our laboratory (Santos et al. 2011) and mouse monoclonal anti-human CgA (dilution 1:1000) (Millipore, Temecula, CA), respectively, and subsequently incubated with secondary antibodies for two hours. After incubations with specific primary and then secondary antibodies, labeled proteins were detected using ECL reagents and exposing the developed blots to GE Healthcare films. Densitometrical analysis of the spots was performed using ImageJ (U.S. National Institutes of Health, Bethesda, Maryland, USA). The area in pixels of each spot was determined in triplicate and means were used for statistical analyses.

Statistical Analyses

Determination of thresholds for each variable was done using a two-phase regression model (HINKLEY 1969). Data were tested for normality using the Kolmogorov–Smirnov test prior to analyses. No transformations were necessary for any of the variables. All variables were compared by one-way analysis of variance (ANOVA) followed by the Tukey test for multiple comparisons. Pearson's correlation coefficient was used among variables. For all analyses, significance level was $\alpha = 0.05$. Statistical Analyses were conducted using Graph Pad Prism Version 4.0 (GraphPad Software, Inc., San Diego, CA).

Results

Dynamics of lactate during the incremental test

We found inflection points in the accumulation of blood and salivary lactate in all 12 subjects (Figure 1). Furthermore, the stage at which the anaerobic threshold was reached in saliva also corresponded to that in blood. Table 1 shows individual times throughout the incremental test.

Agreement between blood and salivary lactate

We observed a very high correlation [r (12) = 0.92, $p < 0.001$] between the concentration of blood and salivary lactate during exercise. In general, the concentration of lactate in blood was twice as high as in saliva (Figure 2). Differences in the concentration of blood and salivary lactate throughout the test were also observed [bLac = F (10, 121) = 62.9, $p < 0.001$] [sLac = F (10, 121) = 26.07 $p < 0.001$].

Effects of exercise on salivary flow

We noted a significant reduction in salivary flow especially during the last stages of the incremental test [F (10, 121) = 10.15, p = 0.0013] (Figure 3).

Correlation between blood lactate and salivary proteins

Here, we analyzed whether the concentration of sAA and especially sCgA, showed a similar pattern of bLac during the incremental test (figure 1).

Western blotting analysis revealed an increasing concentration of sAA (~57KDa) and sCgA (~49KDa) throughout the test (Figure 4-B). As expected, both sAA [r (12) = 0.81, $p <0.05$] and CgA [r (12) = 0.82, $p <0.05$] showed a high correlation with bLac during the incremental test (Figure 1). Inflection points in the concentration of sAA and sCgA were found in all 12. However, in two subjects the inflection point in bLac did not match the increase in the concentrantion of sCgA. Salivary total protein also displayed a high correlation [r (12) = 0.76, $p<0.05$] with bLac (data not shown). Figure 4-C shows representative values of the electrophoretic profile of salivary proteins and the dynamics of both sAA and CgA during the incremental test.

Discussion

The main finding of this study was the high correlation between bLac and salivary proteins, particularly CgA during progressive maximal exercise. Also of interest is the parallel in the dynamics of blood and salivary lactate. It has been previously suggested that exercise protocols in which the work rate increased rapidly were necessary in order to corroborate the usefulness of sLac to predict the anaerobic threshold (Segura et al. 1996). Here, we demonstrated that sLac highly correlates with bLac even during short periods (±1min) of incremental exercise.

In the last few years, the number of studies monitoring sports training by means on salivary markers has increased significantly (Papacosta & Nassis [s.d.]). As discussed elsewhere, saliva offers clear advantages over blood because it is simple to collect, non-invasive and sampling and handling does not require trained personnel. Safety regarding blood collection might be a delicate issue particularly in water sports where athletes are regularly requested to continue exercising immediately after a blood sample has been collected and blood on the finger is not allowed to clot.

We were able to detect anaerobic thresholds in all 12 subjects employing variations in the concentration of sLac. In all the subjects, the work rate defining the anaerobic threshold was the same in blood and saliva. Overall, a difference of 50% was observed between the concentration of bLac and sLac. This is in agreement with previous findings that show differences of 20% between bLac and sLac during maximal exercise (Segura et al. 1996) and of 20-50% during a long-distance race (30-km) (Santos et al. 2006). Such differences between the concentration of bLac and sLac might be attributed to the fact that we included mostly sprinters, who are known to produce more lactate during short periods of intense exercise than middle or long distance athletes. Lower concentrations of lactate might also diffuse at a lower rate into saliva. Interestingly, Segura and colleagues found equivalent concentrations in blood and salivary lactate in the last stages of the test and during the period of recovery, which means that lactate might have diffused more profusely into saliva once the concentration reached certain limit. Although it has been suggested that sLac might be influenced by production of lactate by oral

bacteria (Iwami et al. 2000; de Oliveira et al. 2010); the high correlation between bLac and sLac in this and previous studies implies that bacteria-derived lactate would have little, if any, effect on the accumulation of sLac during maximal exercise.

The argument behind the utilization of sAA to detect the anaerobic threshold (Calvo et al. 1997) comes from the fact that the salivary glands release proteins into saliva upon stimulation by sympathetic nerves (Proctor & Carpenter 2007). Due to the similarity of an inflection point in the concentration of blood catecholamines and lactate during maximal exercise, Calvo and colleagues (1997) explored the correspondence of sAA, as a surrogate marker of blood catecholamines, and bLac during an incremental test and found a high correlation between the two (r=0.93). In fact, other studies have also reported moderate and high correlations between sAA and noradrenaline (r=0.63) (Chatterton et al. 1996) and between sAA and bLac (r= 0.88) (de Oliveira et al. 2010) during incremental tests.

CgA on the other hand, has received little attention as a marker of intensity during exercise even though increases in the concentration of blood and salivary CgA have been previously described after different stimulus for sympathoadrenal discharge including low and high-intensity exercise itself (Allgrove et al. 2008; Takiyyuddin, Cervenka, Sullivan, Pandian, Parmer, Barbosa & O'Connor 1990a; Kanno et al. 1999). CgA is co-released with catecholamines into the circulation from the sympathetic nerves and adrenal medulla (Kanno et al. 1999). It has been demonstrated that sCgA is secreted into saliva from the granular convoluted tubule of the submandibular gland (Kanno et al. 2000; Saruta et al. 2005). To our knowledge, only one study has examined the variation in salivary CgA after exercise (Allgrove et al. 2008). The authors reported a three-fold increase in sCgA after cycling trials above 75% of VO_2max reaching baseline values one hour afterwards. However, results are still controversial regarding variations in sCgA after stressful stimuli different from exercise. Whereas there is evidence than sCgA increases after in response to stress (Kanamaru et al. 2006; Filaire et al. 2009) not all studies have been able to reproduce such results (Wagner et al. 2010; T. Yamakoshi et al. 2009). It has been proposed that catestain, which is co-secreted

with CgA, inhibits further CgA release acting as a feedback mechanism for sympathoadrenal activity (T. Yamakoshi et al. 2009). However, the authors based their suggestion on evidence for the release of catecholamines from the adrenal medulla and to date there is no evidence for such pattern within the submandibular gland.

We found correlations between bLac and sAA and CgA of 0.82 and 0.81, respectively. Although speculative, it seems that the dynamics of salivary proteins during exercise depend more on the mechanisms that stimulate salivary glands than on particular properties for the release of each protein since both sAA and CgA showed a corresponding behavior than bLac. This conclusion is reinforced by the fact that salivary total protein was also highly correlated with bLac in this (r=0.76) and another study (r= 0.93) (Bortolini et al. 2009).

Different from bLac, the concentration of both sAA and sCgA was higher at rest (before the test) than during the initial stages of the test. In this respect, it has been shown that a higher reactivity of the cardiovascular and monoaminergic systems occurs in preparation to challenge, i.e., exercise (Ferrari et al. 2003) which means that organism prepare physiologically in anticipation of expected events (Koolhaas et al. 2011). Thus, a higher sympathetic activity in anticipation to the maximal test might have resulted in also higher concentrations of sAA and CgA before the test. Such reactivity, however, might no be enough to produce increments in the concentration in bLac or sLac.

Saliva is an ideal matrix in which to determine the accumulation of lactate during exercise because of the absence of cells in the sample. This means that it is not necessary to separate the liquid from other solid constituents in order to avoid further production of lactate once a sample is collected (Segura et al. 1996). However, the analysis of salivary constituents, especially proteins, demands certain care is taken regarding collection of samples. In order to control the influence of mechanical stimulation on the secretion rate of sAA and CgA, the subjects in this study were instructed to chew the cotton swab at a frequency of 60/60 s and this was monitored by one of the researchers during the incremental test. Furthermore, we used the same concentration of protein (10µg) and not volume of saliva for western blotting analysis of sAA and CgA, as previously

suggested (de Oliveira et al. 2010). Other kinetic assays for the determination of sAA activity use a fixed volume of saliva and not protein (Granger et al. 2007) and as such might be influenced by secretion and salivary flow rates.

Conclusions

The concentration of bLac is highly correlated with sLac during maximal exercise even with rapid increases in work rate. The concentration of CgA, as with sAA, shows an inflection point during incremental exercise. This might indicate that the dynamics of salivary proteins during exercise depend on the stimulus responsible for their secretion into saliva and not on particular properties of specific proteins. The inflection points reported for CgA and sAA occurred at the same work rate that bLac and were proven useful for the determination of the anaerobic threshold. These findings provide novel evidence for the value of CgA as a marker of exercise intensity in well-trained men.

Conflict of interest

None declared.

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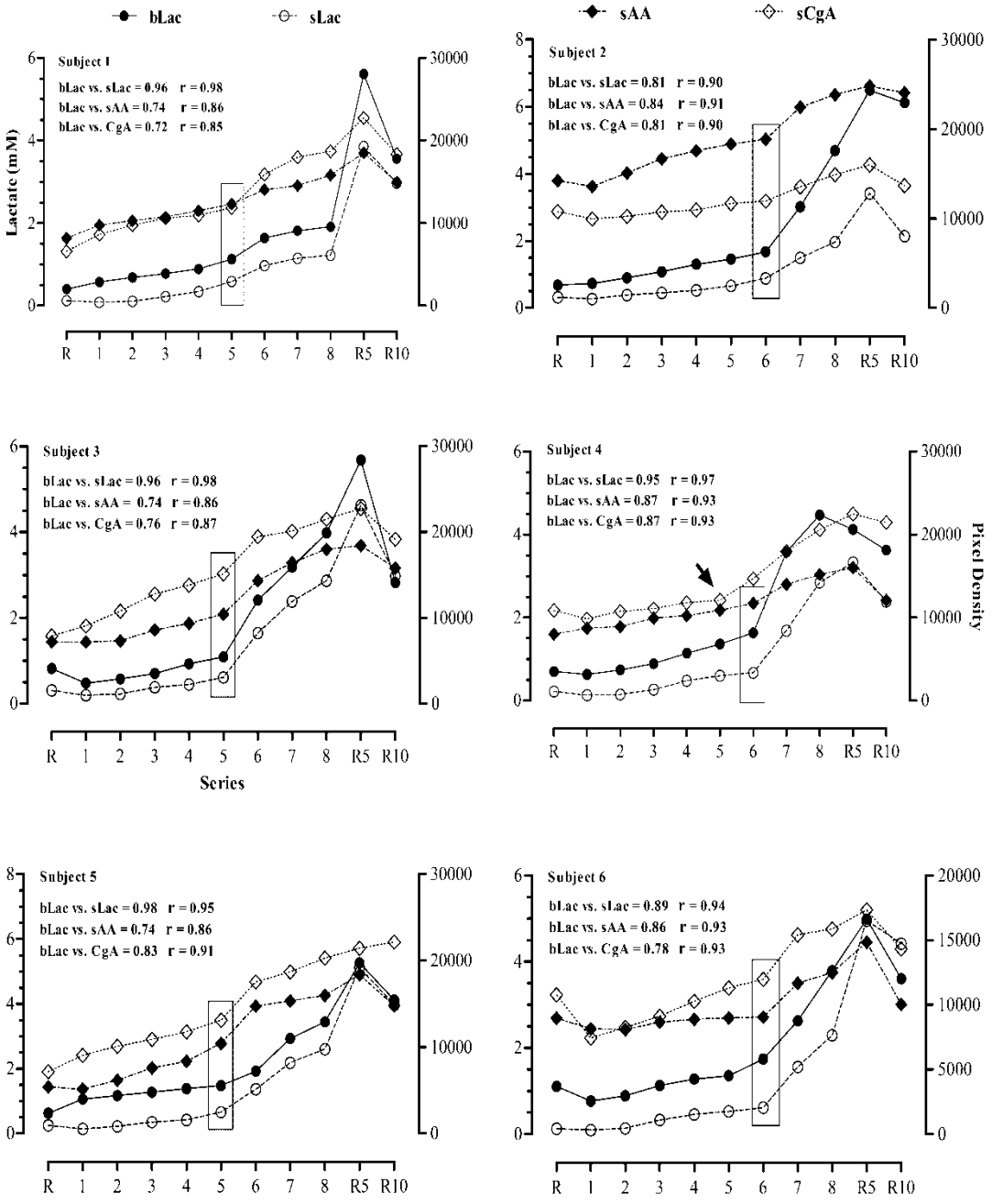
Figures

Figure 1. Dynamics of bLac, sLac, sAA and CgA of each of the 12 subjects during the incremental test. Bars represent exercise intensity at LA. Arrows represent an exponential rise in sCgA different from bLac.



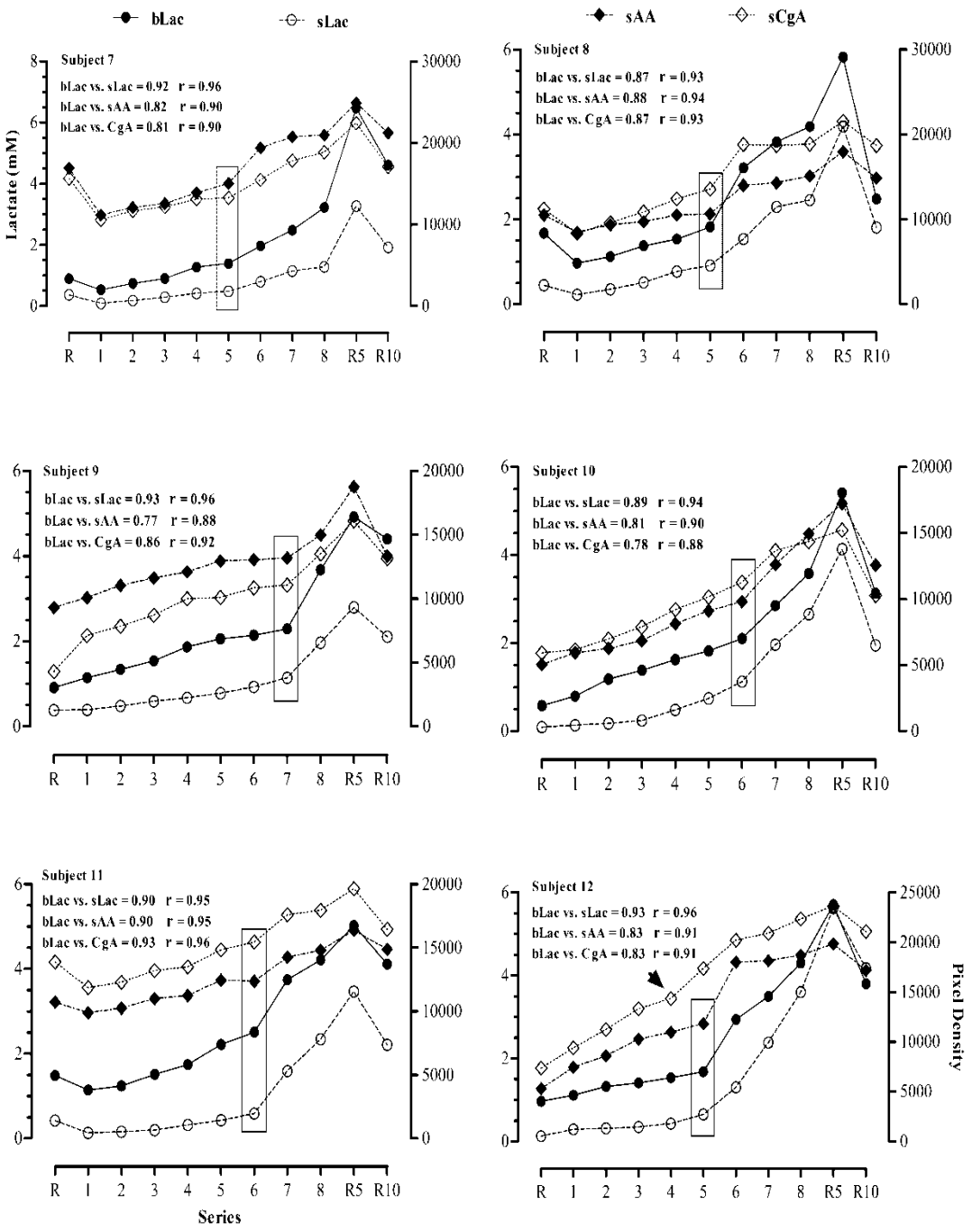

bLac
sLac
sAA
sCgA
Subject 7
bLac vs. sLac = 0.92 r = 0.96
bLac vs. sAA = 0.82 r = 0.90
bLac vs. CgA = 0.81 r = 0.90
Subject 8
bLac vs. sLac = 0.87 r = 0.93
bLac vs. sAA = 0.88 r = 0.94
bLac vs. CgA = 0.87 r = 0.93
Subject 9
bLac vs. sLac = 0.93 r = 0.96
bLac vs. sAA = 0.77 r = 0.88
bLac vs. CgA = 0.86 r = 0.92
Subject 10
bLac vs. sLac = 0.89 r = 0.94
bLac vs. sAA = 0.81 r = 0.90
bLac vs. CgA = 0.78 r = 0.88
Subject 11
bLac vs. sLac = 0.90 r = 0.95
bLac vs. sAA = 0.90 r = 0.95
bLac vs. CgA = 0.93 r = 0.96
Subject 12
bLac vs. sLac = 0.93 r = 0.96
bLac vs. sAA = 0.83 r = 0.91
bLac vs. CgA = 0.83 r = 0.91
Lactate (mM)
Pixel Density
Series
R 1 2 3 4 5 6 7 8 R5 R10

Figure 2. A- Mean values of the accumulation of blood and salivary lactate (A) and sAA and sCgA (B) during the incremental test. Error bars indicate SD.

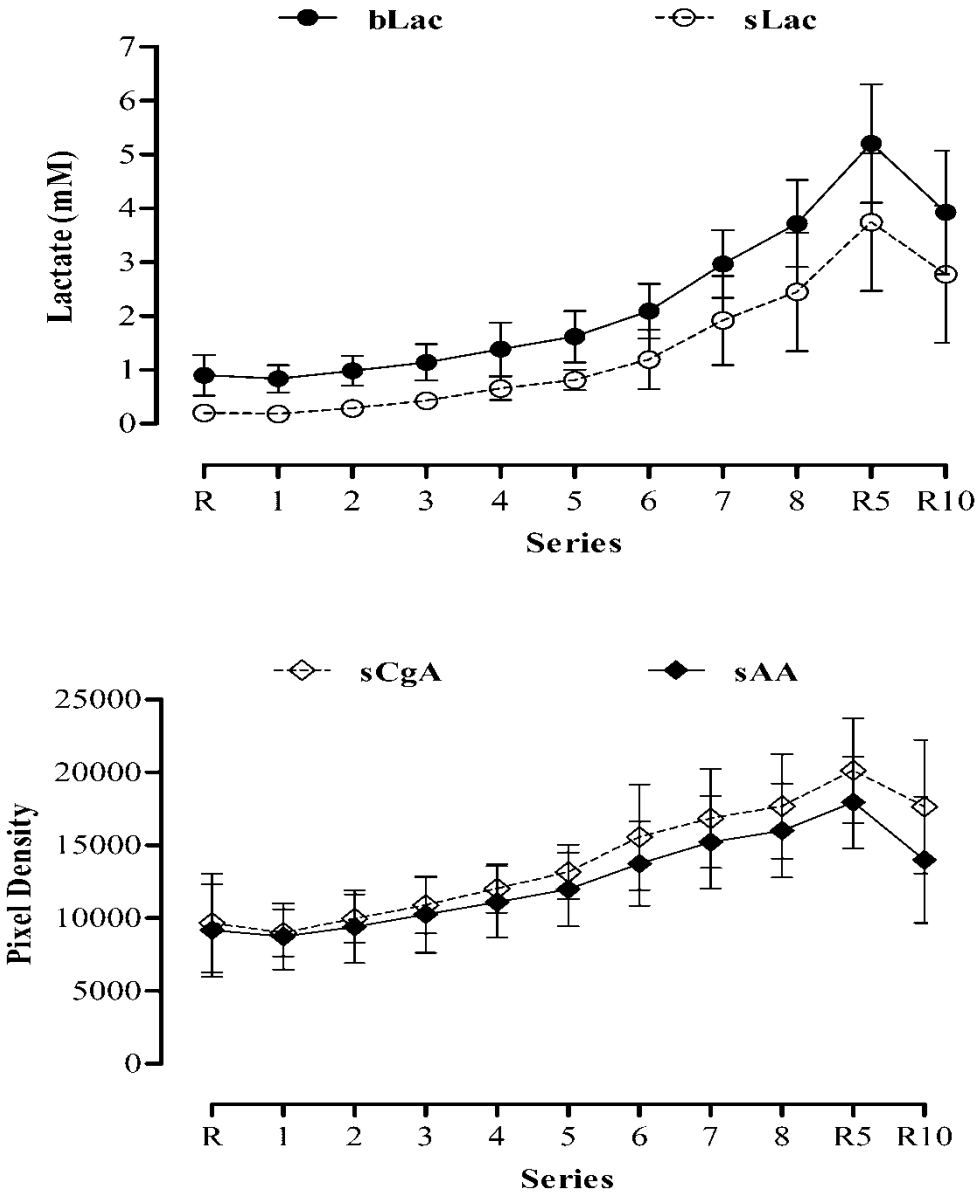


Figure 3. Variation in the salivary flow rate of subjects throughout the incremental test. (*) significantly different from R at $p<0.05$.

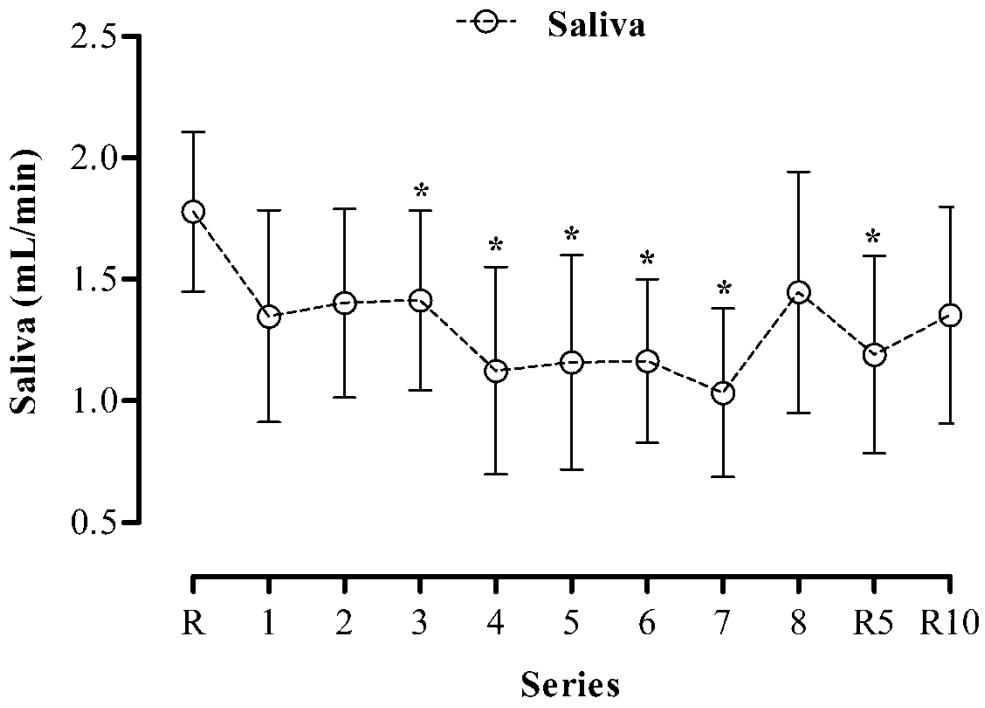


Figure 4. Variation in sAA and sCgA and salivary proteins during the incremental test. A shows a representative electrophoretic profile of saliva. B shows representative blots of the concentration of sAA and sCgA. C shows pixel density of blots of sAA and sCg during the incremental test.

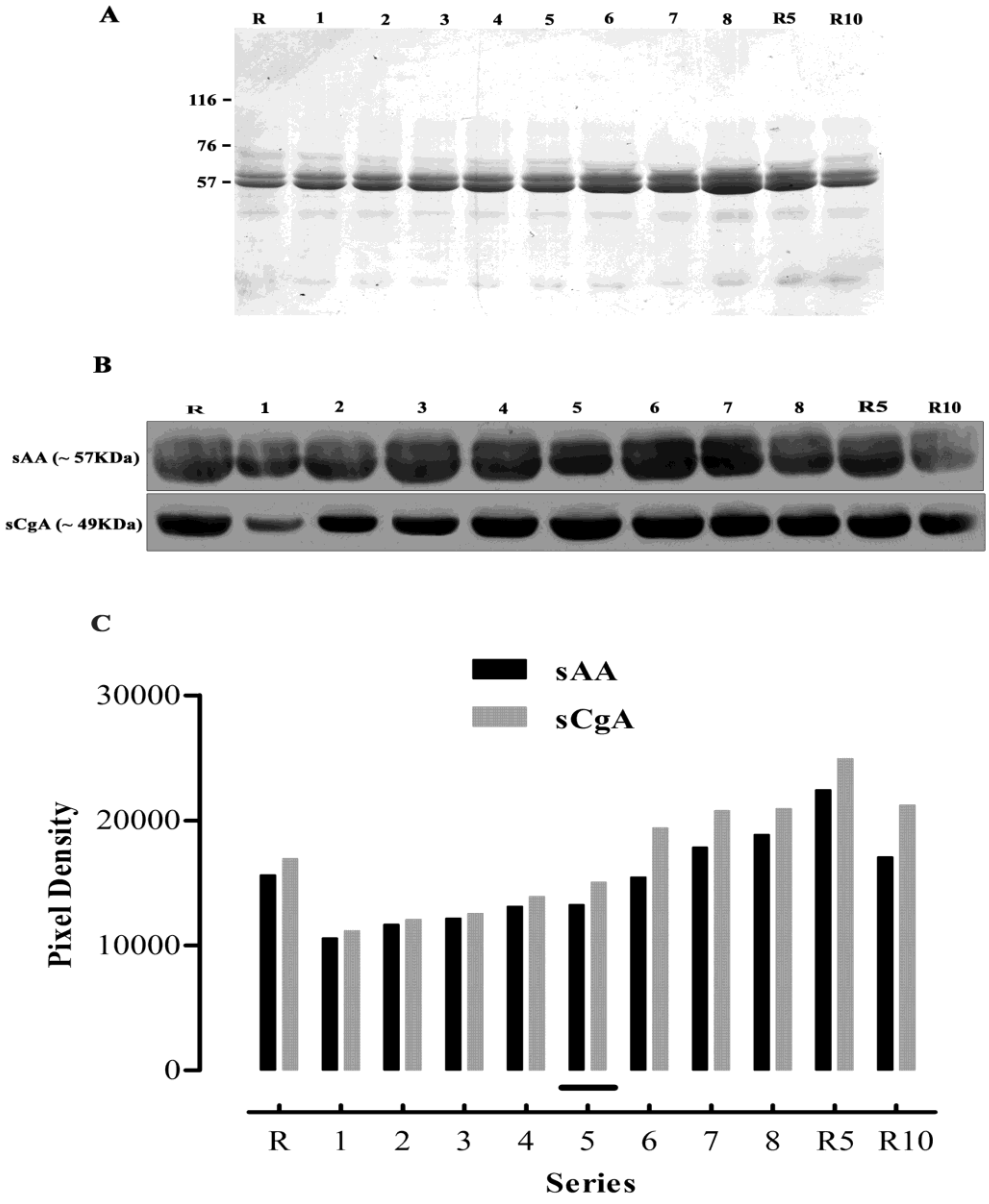


Table 1. Individual times of each swimmer during the eight stages of the incremental test.

Subject	**Series**							
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
1	1’17”36	1’14”89	1’12”36	1’09”83	1’07”45	1’05”47	1’02”23	59”00
2	1’15”34	1’13”69	1’10”54	1’07”69	1’04”76	1’02”46	58”42	56”90
3	1’15”22	1’12”70	1’09”49	1’06”57	1’03”15	1’00”63	57”34	54”00
4	1’14”88	1’11”38	1’08”56	1’05”24	1’02”60	59”00	56”04	54”12
5	1’12”89	1’09”56	1’06”75	1’03”47	1’00”35	57”19	54”21	52”35
6	1’12”63	1’08”07	1’05”51	1’02”12	59”25	57”59	54”74	51”08
8	1’11”04	1’08”81	1’05”42	1’02”53	59”78	56”02	53”90	51”51
9	1’12”80	1’09”04	1’07”72	1’04”00	1’01”90	58”59	56”24	53”46
10	1’17”71	1’15”56	1’13”34	1’11”72	1’09”10	1’06”47	1’03”94	1’01”56
11	1’15”96	1’12”58	1’09”35	1’06”27	1’04”59	1’02”04	1’01”28	1’00”14
12	1’18”87	1’17”39	1’15”70	1’12”26	1’10”59	1’07”19	1’04”47	1’02”34

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