A NEW APPROACH TO INTERPRETATION OF SALIVARY ALFA AMYLASE ACTIVITY CHANGES AS A STRESS INDICATOR

Lubomir Petrov[1], Albena Alexandrova[1,2], Evtim Lefterov[1]

[1]National Sports Academy "Vassil Levski", Sofia, Bulgaria
[2]Bulgarian Academy of Sciences, Sofia, Bulgaria

ABSTRACT

Contemporary sport induces a serious physical and mental stress in athletes. This could result in lowering of their sports performance. Thus, the evaluation of stress in athletes is an important milestone in their preparation. Establishing levels of stress would allow targeted work by coaches and sports specialists to increase the resistance of competitors to stress. In this regard, the use of non-invasive methods for stress testing is essential. Recently, the use of saliva as a biological research material becomes of increasing interest.

The aim of this study was to establish the potential of alpha amylase activity, protein and potassium concentrations in saliva to reflect adequately the degree of stress in athletes.

Eleven boxers, participants in the National Championship, took part in the study. Saliva was collected by salivetes three times: 1) one week before the competition, 2) before the draw of lot, and 3) before the first bout. The salivary alpha amylase activity (sAA), protein and potassium concentrations were detected with commercially available kits.

The sAA, protein and K+ concentrations rose significantly in stress conditions. The individual values of sAA showed large differences that could be explained by the poly-allelic expression of sAA whose activity depends on the number of alleles (2 to 14) with each individual.

All tested indices could serve as indicators for evaluation of stress level in athletes as a high correlation between the protein and K+ and sAA values was found.

In order to evaluate the changes in sAA and to compare the individual results between athletes we suggested the sAA to be presented in relative units. The activities, measured in calm conditions long time before a competition could be assumed as a baseline and the coefficient of increase in sAA in a stressful condition could be accepted as a "stress coefficient".

Keywords: *noninvasive methods, protein concentration, potassium concentration, saliva, salivary alfa amylase, sport, stress.*

INTRODUCTION

The competitive nature of sport requires athletes to show maximum capability under a great psychological pressure. The result of the competition is usually very important for a large rang of people such as athletes themselves, coaches and teammates, family, friends, fans and even for the state prestige. At the same time, nowadays, because of the nearly aligned forces of the elite sportsmen, the final result of the race is usually very unsure until the last moment. Thus each competition provokes variety of stressful situations and respectively a wide range of stress reactions in the athletes.

Currently, there are changes in the rules of many sports in order to increase their attractiveness. In general these changes are related to an increase in the number of bouts and reduction of the break between them (e.g. in boxing), which further increases the level of stress in athletes. Increased stress requires application of appropriate methods for its diagnosis. This would result in obtaining data about athletes' stress levels and a purposeful work aimed at increasing the sustainability to acute and chronic stress impact, to manage stress and to apply methods for relaxation and recovery.

There are different biochemical methods for the study and estimation of sports stress. Since the venous blood samples are unpleasant procedure for many athletes, in recent years there has been a growing interest in obtaining biological materials for research with non-invasive methods (Gröschl, 2008). Suitable alternative is a saliva sample, which has the following advantages: collected through non-invasive procedure that does not require special training, without higher risks of infections, easy store and processing (Kaufman and Lamster, 2002). One of the most informative methods to assess the stress levels is the evaluation of cortisol concentration in saliva, but it requires special equipment, supplies and prolonged laboratory processing. For the purposes of sports practice easily accessible and field applicable non-invasive methods are needed. Promising in this respect are salivary alpha-amylase activity (sAA), protein concentration, concentrations of K+ and Na+ in saliva (Richteret al., 1998; Minasian et al., 2004; Granger et al., 2007; Ullmann et al., 2010). In the scientific literature methods and apparatus for rapid determination of these parameters have already been described, but their interpretation for the purposes of sports practice is still a poorly developed area (Yamaguchi et al., 2004, Yamaguchi et al.,2006; Shimazaki et al., 2008).

This study aimed to test the capacity of the following indices: activity of alpha-amylase, protein concentration, and K+ concentration in saliva to be used as markers for early detection of sport stress. The accumulation of data and experience for correct interpretation of these indices will be a useful tool for evaluating the competitive stress in sport practice. This study is a continuation of our previous research in the area of stress in sport (Petrov et al., 2012, Petrov et al.,2014; Petrov et al.,, 2015).

MATERIALS AND METHODS

Participants

Eleven athletes from the boxing teams of National Sports Academy "Vasil Levski", Sofia, Bulgaria took part in the study; all of them participants in

the National Championship. The average age of the tested persons was 20.2 ± 1.17 years (from 19 to 23 years) and the average sports experience was 7.5 years. At the beginning of the study all respondents were informed about the aims, objectives and conduction of the study and signed a declaration of informed consent, according to the Helsinki Declaration on ethical treatment of humans (WMA, 2013) and Health Act of the Republic of Bulgaria.

DESIGN OF THE EXPERIMENTS

Saliva was collected from the athletes three times: 1) one week before the National Championship to obtain the baseline values of the tested indices, 2) before the draw of lot, and 3) before the first bout.

Salvia collection

Saliva was collected with cotton swab salivetes (Sarstedt AG & Co, Nuembrecht, Germany) without stimulation of salivary glands. All salivetes were previously marked with code numbers, time and date. In order to reduce the influence of other factors on the saliva composition the athletes were instructed one day before sample's collection to avoid alcohol consumption, cigarette's smoking, coffee drinking and heavy meal intake. Before the sample collection, the athletes rinsed their mouth with distilled water. Respondents themselves placed the swab under their tongue for 2 minutes, and then put the swab into the salivete. After sampling, the salivetes were transported to the laboratory as soon as possible in a cooler bag. Salivetes were centrifuged at 1000 g and the resulting saliva was stored at -20°C up to the biochemical analysis.

Protein concentration

The concentration of the protein was determined by the kit Total Protein liquicolor (REF 10570, HUMAN Gesellschaft fur Biochemica und DiagnosticaMbH, Wiesbaden, Germany). Each sample (50 µl) was added to the biuret reagent (1000 µl). The mixture was stirred vigorously and after 10 min incubation at 25°C the samples were subjected to photometry at a wavelength of 540 nm with the use of Biochemical Analyzer Human80. The amount of protein was estimated with the formula C = 80 x (ΔAsample/ ΔAstandard)[g/L].

Activity of alpha amylase

For determining the activity of alpha amylase a commercially available kit (Alfa-amylase Colorimetric test, REF E12 218A, EMAPOL, Gdansk, Poland) was used. The method is based on the capacity of α-amylase to catalyze the hydrolysis of the substrate 2-chloro-4nitrofenil-maltotriozid. The release rate of the product 2-chloro-4-nitrophenol is proportional to the increase in absorbance at a wavelength of 410 nm, and is a measure for determining the activity of α-amylase in the sample. The procedure was as follows: Each sample was previously diluted 1:100 and 10 µlof the latter was added to the reagent (1000 µl). The mixture was stirred vigorously and was incubated at 25°C for 1 min. After that the samples were read at 410 nm with the use of Biochemical Analyzer Human80. The absorption of each sample was detected again after 1, 2 and 3 minutes. The activity of α-amylase was calculated with the formula (ΔA sample/min) x 24820 (37°C) [U/L] and was presented as normal logarithm, ln (sAA).

K+ Concentration

For measurement of K+ concentration a turbidimetric test (Potassium liquirapid, REF 10118, HUMAN Gesellschaft fur Biochemica und DiagnosticaMbH, Wiesbaden, Germany) was used. The method is based on the ability of the potassium ions in a protein-free alkaline medium to react with sodium tetraphenyl borate to form a fine turbid suspension of potassium tetraphenyl borate. The resulting turbidity is proportional to the concentration of K+. Each sample (50 µl) was added to the precipitation solution (500 µl). The mixtures were thoroughly mixed and centrifuged at high speed for 10 min. In order to obtain a homogeneous turbidity 100 µl of the supernatant or 100 µl of the standard were pipetted into 1000 µl of the working reagent. The samples and standard were mixed well and after 5 min the absorption of the standard and the samples were read against working reagent within 5 to 30 min at a wavelength of 578 nm with a biochemical Analyser Human80. The amount of K+was estimated with the formula C = 5 x (ΔA sample / ΔA standard) [mmol/L].

Statistical analysis

The statistical analysis of the results was performed with the use of the Statistical Package SPSS 19. Variation and correlation analyses were applied and the nonparametric ANOVA for repeated measures (Friedman one-way analysis of variance by ranks) with post hoc analysis of Dunnswas used for verifying the statistical reliability of the resulting differences in the average values. Throughout the text, the dispersion of averages is presented with a standard deviation (± SD), and in the charts with their standard error (± SE).

RESULTS

The anthropometric data of the respondents and the result of their participation in the National Championship are presented in Table. 1. Seven of the athletes took prizes in the competition.

Table 1. *Anthropometric data of the boxers and their ranking in the national championship*

No	Age (years)	Height (cm)	Weight (kg)	Category (kg)	Ranking
1	20	169	62	60	2 place
2	21	175	59	56	2 place
3	20	176	76	75	3 place
4	19	165	54	49	3 place
5	21	184	72	75	
6	20	172	58	60	1 place
7	20	180	72.5	75	3 place
8	20	175	66.5	70	
9	19	173	60	56	
10	19	180	85	81	
11	23	174	65	64	1 place

The individual values of the sAA for each athlete are presented in Fig. 1. The activities were in the range of 3.56 to 6.86 ln (sAA) one week before the competition, from 4.87 to 7.85 ln (sAA) before the draw and from 5.18 to 8.24 ln (sAA) prior to the first bout. For most athletes the enzyme activity rose before drawing of lot and reached even higher values before the bout. However the sAA of athletes №4, №5 and №10 before the draw were greater than before the first bout.

Fig. 2 presents the average values of the sAA of the entire team in the three sampling points of the research. A statistically significant increase from the baseline before the draw the lot (6.83 ± 0.82 ln(sAA)vs 5.50 ± 0.84 ln(sAA)) and before the first bout (7.03 ± 0.89 ln(sAA) vs 5.50 ± 0.84 ln(sAA)) was observed. There was no statistically significant difference between the values before drawing of lot and before the first bout.

The individual values of the total protein concentration in saliva for each participant are presented in Fig. 3. The protein concentrations were within the range of 0.12 - 0.94 g/L before the competition, 0.30 - 2.78 g/L before the draw of lot and 0.48 - 3.66 g/L before the first bout. In general (except boxer №3) low values for protein concentration were observed a week before the competition when the athletes were in a relative physiological rest. In stress conditions such as drawing of lot and competition the values rose as for some boxers the highest value was registered before the draw and for others - before the first bout.

Fig. 4 presents the average values of the total protein concentration in saliva of all boxers during the study. The resulting pattern was similar to those of the dynamics of sAA: in comparison to the baseline values a significant increase in protein concentration before the draw of lot (1.59 ± 0.86 g/L vs 0.44 ± 0.29 g/L) and before the first bout (1.49 ± 1.02 g/L vs 0.44 ± 0.29 g/L) was observed. The difference in the average concentration of total protein before the draw the lot and before the first bout was not statistically significant.

The individual concentrations of K+ in the saliva of each participant showed a significant increase immediately before the draw of lot and before the bout. The values ranged within 13.7 - 26.4 mmol/L one week before the competition, before the draw of lot were min 20.0 and max 59.5 mmol/L, and before the first bout were from 27.6 up to 55.8 mmol/L (Fig 5). The tendency was the same as in the dynamics of protein concentration and sAA: in stress condition (before both the draw the lot and the bout) the values were higher than in a relative mental rest (one week before the competition), as for some boxers the highest values were registered before the draw of lot, while for others - before their first bout in the championship.

In Fig. 6 the average values of the saliva K+ concentration of all boxers are presented. A statistically significant increase from baseline was detected before the draw of lot (34.70 ± 10.54 mmol/L vs 20.43 ± 4.20 mmol/L). The average value before the first bout was also significantly higher (35.96 ± 8.45 mmol/L vs 20.43 ± 4.20 mmol/L) than those obtained one week before the competition. The difference in average before the draw the lot and before the first bout was statistically insignificant.

Table 2 presents the correlation matrix of the studied parameters. High, statistically significant correlations be-

tween the activity of sAA measured in the three stages of the study (0.812; 0.924 and 0.665) were identified. The protein concentrations measured before draw of lot and before bouts were also highly correlated (r = 0.752, p = 0.019). The individual values of the studied biochemical indicators were also strong mutually correlated. The concentration of total protein in the saliva before bouts strongly correlated with the sAA before the draw of lot and before fights (r = 0.738, p = 0.023; r = 0.736, p = 0.024). The K+ concentration one week before the competition and the sAA both before the competition and before bouts were also highly correlating (r = 0.792, p = 0.011; r = 0.738, p = 0.022).

Table 2. *Correlation matrix of the investigated biochemical parameters. The significant correlations are highlighted in gray*

		sAA one week before	sAA before draw of lot	sAA before the bout	Protein one week before	Protein before draw of lot	Protein before thebout	K+ one week before	K+ before draw of lot
sAA before draw of lot	**Correlation**	0.812	1						
	Significance	0.002							
sAA before the bout	**Correlation**	0.924	0.665	1					
	Significance	0.000	0.025						
Protein one week before	**Correlation**	-0.055	-0.035	0.064	1				
	Significance	0.879	0.924	0.860					
Protein before draw of lot	**Correlation**	0.385	0.460	0.388	0.494	1			
	Significance	0.243	0.155	0.238	0.147				
Protein before the bout	**Correlation**	0.613	0.738	0.736	0.655	0.752	1		
	Significance	0.079	0.023	0.024	0.056	0.019			
K+ one week before	**Correlation**	-0.173	-0.464	0.062	-0.094	-0.143	-0.231	1	
	Significance	0.611	0.151	0.857	0.797	0.674	0.549		
K+ before draw of lot	**Correlation**	0.436	0.231	0.461	0.213	0.639	0.514	0.366	1
	Significance	0.180	0.495	0.153	0.555	0.034	0.156	0.268	
K+ before the bout	**Correlation**	0.792	0.542	0.738	-0.018	0.435	0.512	-0.441	0.245
	Significance	0.011	0.132	0.022	0.963	0.242	0.158	0.234	0.525

DISCUSSION

The working hypothesis of this study was that some biochemical indices, obtained by noninvasive methods (saliva collection), could be used for adequate evaluation of the competitive stress features. This hypothesis was probated in real competitive conditions, the National Championship. The results showed a statistically significant increase in all tested biochemical indices (sAA, protein concentration and K+ concentration) measured either before the draw of lot or before the first bout in comparison to the same indices, measured one week before the competition. According to the coaches' opinion, pending the outcome of the draw of lot induces in boxers stress in the same range as before the bout. It is well known that high levels of stress influence negatively sports results (Bali, 2015). In this study we registered a notably very low activity of sAA (calculated as ln(sAA)) in the samples of the winner (athlete №6) in all three measurements.

In regards to raw sAA (before their logarithmic-transformation), we observed large variations in the values of the tested individuals. Kobayashi et al. (Kobayashi et al., 2012) also found a considerable inter-individual dispersion for raw sAA (coefficient of variation 67%). In order to improve the statistical distribution of salivary alpha-amylase numerical transformations, square root-, and natural logarithmic-transformation for sAA values were proposed. The numerical transformations square and root transformation were insufficient. The logarithmic transformation appears to improve the distribution of sAA better. However these mathematical transformations diminished inter-individual variation up to 20% - 30% without any physiological reasons.

The large differences in this indicator could be explained with the fact that the gene fors AA expression is poly-allelic and thus the enzyme activity depends on the number of alleles at each person (2 to 14) (Perry et al., 2007; Falchi et al., 2014). Elbers et al. (Elbers et al., 2011) reported a significant correlation between the number of genetic copies of sAA (AMY1) and its activity ($r = 0.45$; $p < 0.001$). Therefore, it can be assumed that both the basal activity of sAA at rest and its increased activity in stressful situations are proportional to the number of genetic copies of AMY1 in the tested individual. This assumption explains the high correlation coefficients in all three measurements. Therefore, based on the relative increase in sAA activity versus the sAA activity measured in physical and mental rest, a method for evaluation of the stress response magnitude could be proposed. In this study the activities measured one week before the competition could be assumed as a baseline and the coefficient of increase in sAA activity before the draw of lot or before the match, could be accepted as a "stress coefficient" (Fig. 7).

The substantial difference in the interpretation of the results represented directly and the use of the proposed "stress coefficient" may become clear when we compare Fig. 1 and Fig. 7. For example, in Fig. 1 the sAA of boxer №6 (gold medalist) showed the lowest values, whereas the second gold medalist (boxer №11) showed the highest values. However, when the results were recalculated with the use of the "stress coefficient", these athletes could be classified as competi-

tors with moderate level of stress reaction both before the draw of lot and before the first bout. Thus, this method of data presentation is in line with the concept that moderate stress leads to the highest level of performance (Bali, 2015).

The high correlation between the protein concentration and K+ concentration and sAA is interesting and could be used for developing of methods for express field assessment of pre-start stress with widespread use in sports practice. For example, K+ concentration may be determined without the use of any reagents, only with a portable analyzer with an ion-selective electrode.

CONCLUSIONS

The sAA and salivary protein concentration rose in conditions of pre-start and competitive stress. However, sAA showed large individual differences. In order to avoid this problem, we suggested the changes in sAA to be present in units relative to their values in physical and mental rest conditions. For this purpose, the samples should be collected by the competitors themselves at home with previously given salivetes. The basal values of the sAA should be measured in samples, collected several times during the day, in order to obtain a circadian rhythm, in a period long before major competitions.

The information received from the sAA overlaps to a large extent those, obtained by salivary protein concentration data. Thus, we believe that the measurement of the protein concentration has valuable advantages in practical terms that include lower cost, shorter time to determine and less fluctuation of results.

The measured concentrations of K+ in our experiments were within the range of the ion-selective electrode sensitivity. This observation allows an express measurement of this indicator in field conditions without the use of reagents. Therefore, the K+ concentration in saliva could serve as a perspective express indicator for evaluation of stress in sports practice.

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Correspondence:
Lubomir Petrov
National Sports Academy "Vassil Levski",
Sofia Bulgaria,
E-mail: dr.lubomir.petrov@gmail.com