

Influence of Intense Swimming Exercise on Selected Salivary Components

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Abstract

Original Research Article

Physical exercise may induce many changes in quantity and quality of the biochemical components of saliva. Therefore, a noninvasive sampling and testing of saliva can be used as a diagnostic method in monitoring of physical activity. Objectives of this study was comparison selected salivary components in swimmers before and after intense training. 19 subjects of both sexes, 18-24 years of age, were selected for the study, of which 10 were competitive swimmers and 9 were in the control group. Salivary samples were collected from all subjects in the morning and in the afternoon and from the swimmers before and after training. The parameters measured included: salivary flow rate, pH, total protein, alpha-amylase, peroxidase, cortisol, total antioxidant status (TAS), sialic acid (FSA, GSA, TSA), calcium and magnesium. A significant increase of salivary, alpha-amylase and GSA was noted in all swimmers after the morning workout, while after the afternoon swim training significant decrease in the salivary flow rate and cortisol, accompanied by increasing protein and alpha-amylase levels, were found. Regarding the output of salivary components, after the morning training session, a slight increase in the output was observed for all salivary parameters being investigated. However, it was statistically significant only for total protein, alpha-amylase, calcium, TSA and TAS. After the afternoon training a slight increase in the output was observed for all investigated parameters, except for cortisol which showed decreasing trend. A statistically significant increase in the output was recorded for total protein, alpha-amylase, calcium and TAS. The data suggest a significant increase in alpha-amylase levels and tendency for greater total protein concentration in saliva, following morning and afternoon swimming workout.

Keywords: Physical exercises, Swimmers, Saliva, Physiological, Biomarkers.

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INTRODUCTION

Physical activity induces changes in biochemical components of blood, which are reflected in saliva. Therefore, saliva constitutes an alternative diagnostic material, which can be used for monitoring of the body's response to physical activity and to psychological stress related to playing sports, especially that saliva can be collected in an easy, non-invasive manner. Both the salivary flow rate and its composition are dependent on the autonomic nervous system's activity and every change in this activity can cause changes in the composition of saliva. During physical activity a sympathetic system stimulation can occur and it is, strong enough to diminish or significantly reduce salivary secretion [1, 2]. When considering physical activity, its type, course, intensity, duration and the time of day need to be taken into account. However, studies have showed a differential impact of physical activity on salivary secretion, ranging from no influence [3] to

significant increase [4] or decrease [5, 6]. That can be caused, to some extent, by the time of day, because the sympathetic nervous system's activity is higher in the morning than at other times of the day [5]. Decrease in salivary secretion during intense exercises is explained by an increase in sympathetic nervous system's activity leading to the narrowing of blood vessels which deliver blood to salivary glands and consequently, to reduce salivary volume. The parasympathetic system's activity is known to promote its secretion [6]. Moreover, the physical activity-induced increase in vasopressin (antidiuretic hormone) levels can decrease saliva secretion. The influence of dehydration during exercise, evaporation of saliva due to breathing through the mouth as well as hyperventilation, cannot be ignored, either [7].

Physical exercises can also cause some changes in the concentration of organic and inorganic components of saliva. Salivary proteins are released

mainly after activation of the sympathetic nervous system. Moreover, the level of cortisol in the blood (glucocorticoid hormone produced by the adrenocortical glands, called the stress hormone) increases after physical exercise, which largely influences the body's metabolic rate. The unbound protein fraction of cortisol travels by passive diffusion to body fluids, including saliva. Since a correlation has been found between cortisol levels in blood serum and in saliva, salivary cortisol can be an indicator of the adrenocortical gland's secretions during physical activity [8]. Circadian rhythm of the blood cortisol was identified to reach its maximum level early in the morning, decreasing further into the day to reach the low level in the evening and the cortisol concentration was found to increase following physical exercise performed in the morning rather than in the evening [9]. Also, the concentration of alpha-amylase, a salivary enzyme, which constitutes half of total salivary protein, was shown to increase in response to psychological and physical stress [6]. Alpha-amylase, produced mainly in parotid and submandibular salivary glands, is a sensitive marker reflecting sympathetic nervous system activity and, constitutes an alternative to both catecholamines (adrenaline and noradrenaline) and cortisol assays [10].

Assessment of lactate concentration in blood serum can be also used for monitoring athletes' training. Lactate provides an anaerobic threshold based on the blood lactate levels measured at the lower limits of training intensity. In saliva, just like in the blood serum, a significant increase in the lactate concentration was observed and it correlated with its levels in the blood serum and saliva, after the swim training [11]. A study of Immunoglobulin A, the first line of defense against upper respiratory tracts' infections in response to physical activity, is not completely unambiguous. Since both the body's metabolic rate and oxygen consumption increase during physical activity (due to increased metabolic rate especially in skeletal muscles), the production of reactive oxygen species (ROS) increases, as well. Under the normal conditions, reactive oxygen species are eliminated by intracellular and extracellular defense mechanisms, including enzymatic and non-enzymatic antioxidants which delay or inhibit oxidation of substrates [12]. An increase in the antioxidant levels in the saliva was observed after physical activity [13]. Levels of trace elements and electrolytes have been assessed, with magnesium, sodium, and manganese contents increasing in saliva after physical activity [14].

The aim of the study was to evaluate selected salivary components and their circadian rhythm in swimmers before and after swim training and compare them with sedentary controls.

MATERIALS AND METHODS

Participants: 19 subjects aged 18-24 (mean 23) were included in the study. The Study group consisted of 10 competitive swimmers: 7 males, and 3 females. The Control group consisted of 9 subjects with sedentary lifestyles: 4 males and 5 females. Inclusion criteria were as follows: subjects at the age of 18 and over, generally healthy, not taking any medicine, who practice swimming for 20 hours a week, or who are currently not engaged in any physical activity.

All subjects were provided with a detailed description of the study protocol and then a written informed consent was obtained from them. The swimmers trained 21 hours per week in the morning (7.00-8.45 a.m.) and in the afternoon (4.00-5.45 p.m.). The swim training consisted of swimming the distance of 5000 meters.

Salivary sample collection and analysis

To ensure consistency and to account for diurnal fluctuation in the salivary parameters, all unstimulated mixed saliva samples were taken from swimmers in the morning and in the afternoon, before and after the swimming workout. In the study group, saliva samples were collected 4 times a day (i.e. in the morning and in the afternoon before and after swim training). The total number of saliva samples collected amounted to 40. In the control group, saliva was collected twice a day, in the morning and in the afternoon, at the time when the study group started the swim training. The total number of saliva samples collected from the control group amounted to 18.

Samples of saliva were collected after a thorough mouth rinse with distilled water. The subjects were sitting, head bent down and the mouth open. Saliva was taken with a plastic pipette and put into a graded test tube placed on crushed ice. Based on the measurement of the volume of the collected saliva sample, and the time needed to collect it, the salivary flow rate was calculated as ml/min (V). The samples were centrifuged for 10 minutes at a speed of 3500 rpm before biochemical assays. Sample collection was preceded by rinsing the mouth and clear supernatants were used for the assays to minimize the contribution of other sources of the tested salivary parameters, such as: oral bacteria, epithelial cells, leukocytes, and dietary residues. Total protein - P (by Lowry's *et al.*, method) [11], pH (by potentiometric method), alpha-amylase - Amy (by Caraway's colorimetric method-Alpha Diagnostic Kit), salivary peroxidase - SPO (using Nbs-SCN method) [15], salic acid total - TSA, bound - GSA and free - FSA (by periodate-resorcinol method) [16], cortisol - C (Elisa Kit from R&D Systems), total antioxidant status - TAS (TAS assay Randox), calcium - Ca (by method based on formation of chromogenic complex between calcium ions and o-cresolphthalein using Alpha Diagnostics kit), magnesium - Mg (by colorimetric method based on the reaction of

magnesium with Xylidyl Blue-I using Alpha Diagnostics kit) were assessed. Moreover, the data obtained from the measurements were expressed as an output, i.e. concentration of a specific component obtained within 1 minute.

This study protocol has been approved by The Bioethics Committee (No KB-327/2009) of The Medical University of Wrocław. All research was performed in accordance with relevant guidelines and regulations and a statement confirming that informed consent was obtained from all participants.

Statistical analysis: Depending on the type of variables distribution, Student's t-test or Mann-Whitney test was used for the independent variables analysis. For the dependent variables, Student's t-test or non-parametric Wilcoxon's test was conducted. The obtained data were analyzed statistically with the significance level set at $p < 0.05$ by STATISTICA 13.1 software package (StatSoft, Inc., Tulsa, OK, USA).

RESULTS AND DISCUSSION

Analysis of the Time of Day Variations

We found slight fluctuations in the average levels of salivary parameters related to the time of day. This was found in The samples collected both in swimmers before swim training and in the control group. Those levels were slightly lower in saliva collected in the morning hours than in the samples collected in the

afternoon for both groups, except for pH, cortisol, and GSA in the control group.

In the control group, the alpha-amylase level was significantly lower in the morning hours compared to the afternoon hours, but it increased in swimmers before the afternoon training session by 41% ($p > 0.05$) compared to the morning workout. However, cortisol level was significantly higher in swimmers before the morning training session compared to the afternoon session, with a similar tendency observed in the control group.

Levels of salivary parameters in swimmers before training compared with the control group

In the morning, significantly lower cortisol levels were found in swimmers' saliva compared to the control group. In the afternoon, lower salivary flow rate, higher total protein concentration, lower alpha-amylase activity and lower cortisol content were also noted.

Levels of salivary parameters before and after training:

After the morning training session, a significant increase in the levels of total protein, alpha-amylase and GSA was observed. Following an afternoon workout, a significant decrease in the salivary flow rate and cortisol concentration, accompanied by increased total protein and alpha-amylase, were observed (Table-1).

Table-1: Concentration of the test components in saliva

Salivary parameters	Day time	Non- swimmers (Control group)	Swimmers study group		Before training swimmers vs non-swimmers	Swimmers before vs after training
			before	After		
Salivary flow rate (V) ml/min	am	0.53±0.43	0.28±0.12	0.20±0.87	u=-1.837 p=0.066 ^b (↓47%) u=-2.245 p=0.025 ^b (↓38%)	t=3.221 p=0.089 ^a (↓29%) t=0.312 p=0.583 ^a (↓11%)
	pm	0.60±0.21	0.37±0.15	0.33±0.19		
	am vs pm	z=0.652 p=0.514 ^c (↑13%)	t=2.156 p=0.159 ^c (↑32%)	t=3.894 p=0.064 ^c (↑65%)		
pH	am	7.48±0.36	6.99±0.40	6.95±0.41	t=-2.756 p=0.013 ^a (↓6%) t=-1.229 p=0.235 ^a (↓4%)	t=0.034 p=0.855 ^a (↓0.57%) t=1.746 p=0.202 ^a (↑3.5%)
	pm	7.33±0.35	7.06±0.55	7.31±0.19		
	am vs pm	t=0.799 p=0.384 ^a (↓2%)	t=0.114 p=0.739 ^a (↑1%)	t=5.891 p=0.025 ^a (↑15%)		
Total protein (P) g/l	am	0.74±0.21	0.79±0.25	1.25±0.97	u=0.449 p=0.650 ^b (↑7%) u=1.959 p=0.050 ^b (↑59%)	z=2.803 p=0.005 ^c (↑58%) z=1.718 p=0.086 ^c (↑27.0%)
	pm	0.79±0.22	1.26±0.56	1.60±1.15		
	am vs pm	t=0.157 p=0.696 ^a (↑7%)	z=1.783 p=0.074 ^c (↑59%)	z=1.579 p=0.014 ^c (↑28%)		
α-amylase (Amy) j/ml	am	39.15±31.30	34.48±19.19	50.75±37.36	u=-0.040 p=0.967 ^b (↓12%) t=-2.877 p=0.005 ^b (↓95%)	z=2.803 p=0.0005 ^c (↑47%) z=1.885 p=0.059 ^c (↑28%)
	pm	143.94±86.55	48.50±33.98	62.02±35.99		
	am vs pm	t=11.665 p=0.003 ^a (↑268%)	z=0.764 p=0.444 ^c (↑41%)	z=0.561 p=0.575 ^c (↑22%)		

Salivary peroxidase (SPO) U/ml	am	1.35±0.87	1.24±0.65	2.05±2.94	u=-0.000 p=1 ^b (↓8%)	z=0.152 p=0.878 ^c (↑65%)
	pm	1.43±1.07	1.33±0.89	1.39±0.79	u=-0.122 p=0.902 ^b (↓7%)	z=0.152 p=0.878 ^c (↑4%)
	am vs pm	t=0.033 p=0.856 ^a (↑6%)	z=0.051 p=0.959 ^c (↑7%)	z=0.153 p=0.878 ^c (↓32%)		
Total Antioxidant Status (TAS) mmol/l	am	0.49±0.21	0.40±0.15	0.52±0.18	t=-0.979 p=0.341 ^a (↓4%)	t=2.323 p=0.144 ^a (↑30%)
	pm	0.51±0.30	0.39±0.17	0.47±0.17	t=-1.161 p=0.262 ^a (↓23%)	t=1.137 p=0.300 ^a (↑20%)
	am vs pm	t=0.049 p=0.826 ^a (↑4%)	t=0.061 p=0.806 ^a (↓2.5%)	t=0.398 p=0.535 ^a (↓10%)		
Total sialic acid (TSA) mg%	am	6.06±2.10	4.78±1.85	5.73±2.08	u=-1.102 p=0.270 ^b (↓21%)	t=1.152 p=0.297 ^a (↑20%)
	pm	6.25±4.51	7.19±3.68	3.81±4.45	u=0.571 p=0.567 ^b (↑15%)	t=0.112 p=0.741 ^a (↓47%)
	am vs pm	z=0.533 p=0.593 ^c (↑3%)	t=3.424 p=0.081 ^a (↑50%)	t=1.786 p=0.198 ^a (↓33%)		
Free sialic acid (FSA) mg%	am	2.23±1.52	2.05 ± 1.11	1.94±1.02	t=-0.283 p=0.781 ^a (↓8%)	t=0.043 p=0.836 ^a (↓5%)
	pm	2.76±1.93	3.32 ± 2.57	3.22±3.17	t=0.541 p=0.596 ^a (↑20%)	t=0.007 p=0.934 ^a (↓3%)
	am vs pm	t=0.146 p=0.528 ^a (↑24%)	t=1.885 p=0.187 ^a (↑62%)	t=1.316 p=0.267 ^a (↑66%)		
Glycosidically bound sialic acid (GSA) mg%	am	3.82±1.28	3.22±2.83	4.56±3.93	u=-1.102 p=0.270 ^b (↓16%)	z=2.701 p=0.007^c (↑42%)
	pm	3.50±2.88	3.87±1.54	4.59±2.30	u=1.306 p=0.191 ^b (↑11%)	z=1.274 p=0.202 ^c (↑17%)
	am vs pm	z=0.770 p=0.441 ^c (↓8%)	z=1.172 p=0.247 ^c (↑20%)	z=0.533 p=0.593 ^c (↑1%)		
Cortisol (C) µg/ml	am	18.43±17.16	4.01±2.74	3.48±2.96	u=-2.653 p=0.0007^b (↓78%)	t=0.171 p=0.684 ^a (↓13%)
	pm	7.28±8.22	2.00±1.21	1.24±0.61	u=-2.571 p=0.010^b (↓72%)	t=3.055 p=0.097 ^a (↓38%)
	am vs pm	z=1.836 p=0.066 ^c (↓60%)	t=4.523 p=0.047^a (↓50%)	t=5.447 p=0.031 ^a (↓64%)		
Calcium (Ca) mg%	am	4.00±1.47	3.92±1.55	5.56±2.04	t=-0.122 p=0.904 ^a (↓2%)	t=4.079 p=0.058 ^a (↑42%)
	pm	2.86±1.60	4.40±1.89	5.98±2.31	t=1.896 p=0.075 ^a (↑54%)	t=2.817 p=0.110 ^a (↑36%)
	am vs pm	t=2.488 p=0.134 ^a (↓28.5%)	t=0.379 p=0.545 ^a (↑12%)	t=0.186 p=0.671 ^a (↑7%)		
Magnesium (Mg) mg%	am	0.89±0.34	0.88±0.33	0.94±0.27	u=-0.204 p=0.838 ^b (↓1%)	z=0.866 p=0.386 ^c (↑7%)
	pm	0.65±0.23	0.71±0.27	0.71±0.28	u=0.367 p=0.713 ^b (↑9%)	z=0.051 p=0.956 ^c (const) 0%
	am vs pm	t=3.043 p=0.100 ^a (↓27%)	z=1.274 p=0.202 ^c (↓81%)	z=1.987 p=0.047^c (↓24%)		

a- t-student test; b- U-Mann-Whitney test; c- Wilcoxon test Significant differences at p<0.05

The effect of exercise on relative changes in salivary components concentrations, presented as 100% baseline value, is illustrated in Fig-1.

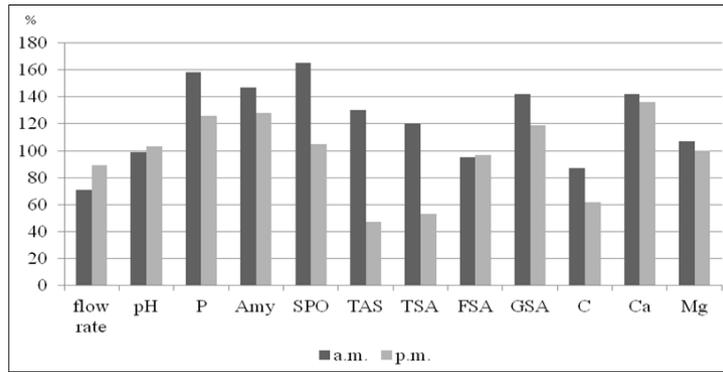


Fig-1: Effect of training on relative changes in salivary parameters, levels before exercise were set at 100%

Salivary components’ output in swimmers before training compared with the control group:

In the morning, slightly higher output was noted for all saliva components in swimmers. However, the output was significantly higher only for total

protein. The cortisol level output proved to be significantly lower. In the afternoon, slightly higher outputs were observed for all salivary components as well, but they were significantly higher only for total protein and calcium (Fig.2).

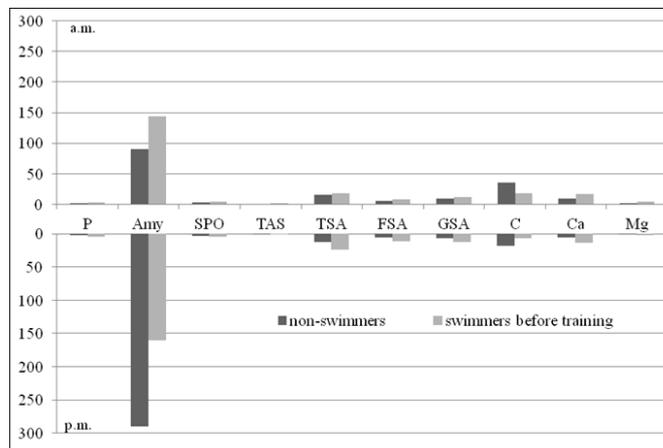


Fig-2: Output of salivary components (mg/µg/U per min.) of swimmers before training and non-swimmers in the morning and afternoon hours

Salivary Components’ Output Before and After Training in Swimmers

After the morning training session, a slight increase in the output was observed for all tested salivary components, but it was statistically significant only for total protein, alpha-amylase, calcium, TSA and TAS.

Following the afternoon training session, a slight increase in the output was observed for all salivary components, except for the cortisol for which a declining trend was observed. A significant increase in the output was found for total protein, alpha-amylase, calcium and TAS (Fig-3).

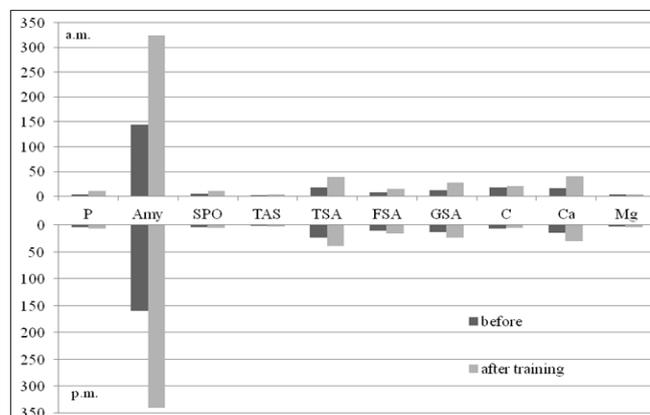


Fig-3: Output of salivary components (mg/µg/U per min.) of swimmers before and after training in the morning and afternoon hours.

Circadian rhythm patterns were demonstrated in the flow rate of saliva and in the levels of the salivary components [17]. This was taken into account while measuring salivary parameters twice a day, i.e. in the morning and in the afternoon. Our data confirms the presence of circadian rhythm patterns in alpha-amylase and cortisol levels contained in saliva. Just like in the Rohlender *et al.*, [18] study, lower alpha-amylase and higher cortisol levels were noted in the control group and in the swimmers' group before training in the morning hours as compared to the afternoon hours. Also, Raff and Trivedi [19] in their study on the circadian rhythm of salivary cortisol showed higher levels of cortisol in the morning hours when compared to the evening hours.

A slight decrease in the salivary flow rate, after swimming exercise observed in our study, is consistent with the previous results [5, 6, 20]. Decrease in the salivary flow rate can be caused by dehydration due to prolonged exercise, which was observed in athletes after a marathon run and other intensive exercise. In our study, swimmers were not exposed to dehydration, because they practiced in the water temperatures ranging from 26 to 28°C. Therefore excess heat produced by the athletes' own bodies during the exercise was released directly to the environment. Moreover, swimmers were allowed to drink energy drinks at will during the practice. However, Ligtenberg *et al.*, [21] demonstrated a significant increase in the unstimulated salivary flow rate following moderate and intense outdoor running at temperatures ranging between 7 and 15°C. Dimitriou *et al.*, [5] showed higher salivary flow rates in swimmers in the evening as opposed to the morning, just like in our study, which explains a higher activity of sympathetic nervous system in the morning hours versus at the other times of the day.

During exercises, total protein concentrations in saliva can increase, following an increase of β -sympathetic activity in salivary glands [3] which was confirmed by our data from both the morning and afternoon training sessions. Our data, just like that of the other studies, showed a significant increase in the total salivary protein levels after physical activity [1, 6, 21]. De Oliveira *et al.*, [22] suggested that an increase in the total salivary protein levels is caused exclusively by increased alpha-amylase secretion, therefore monitoring of the total protein concentration can serve as an alternative, non-invasive method for determining exercise intensity. Similarly, Diaz *et al.*, [23] considered the assessment of total protein in unstimulated saliva as an attractive marker due to fast testing ability as compared to traditional kinetic or immune assays.

Salivary alpha-amylase is believed to be a biomarker of stress and stimulates sympathetic nervous system activity. It has been successfully applied as an indicator of exercise intensity in well-trained subjects

[24]. The correlation between exercise and alpha-amylase activity in saliva has been extensively investigated. However, the obtained data were not consistent, as some studies showed a significant increase, but others showed no such change [25]. In our study a significant increase of salivary alpha-amylase activity after the exercise was observed, regardless of the time of the day. Diaz *et al.*, [23] found an increase of salivary alpha-amylase immediately prior to warming up and 5 minutes after swimming competition in male professional swimmers. Edmonson *et al.*, [26] noticed an increase of the enzyme activity in saliva in elite swimmers with a disability during regular training. Also Sinnott-O'Connor *et al.*, [27] found a significant increase of salivary alpha-amylase activity during intensive training and competition in Paralympic Swimmers.

Physical exercise is a powerful stimulant provoking the hypothalamic-pituitary axis and cortisol change. It has been assumed that cortisol levels in saliva reflected its concentration in plasma. Physical and psychological stress caused by exercise evokes changes in the cortisol secretion [5, 7, 13]. However, the changes depend on the size and duration of the stimulus. In the case of exercise or training, it is influenced by the type of physical exertion, the impact of training units and time of execution [28]. VanBruggen *et al.*, [29] showed that cortisol responses increased significantly for both serum and saliva only in response to high-intensity exercise on a cycle ergometer. Jacks *et al.* [30] investigated salivary cortisol levels in response to exercise at 3 different intensities and indicated that only exercise of high intensity and long duration resulted in significant increases in salivary cortisol. Tan and Long [31] noticed that the increase in post-training cortisol in saliva was correlated to the intensity in synchronized swimmers' pool training. O'Connor *et al.*, [32] found a relationship between salivary cortisol after swimming and depressed mood (measured by the Profile of Mood States questionnaire) as they noticed higher salivary cortisol in swimmers compared to the control group during baseline and overtraining, and also a positive correlation with depressed mood during overtraining. A similar result was obtained by Yazdanparastet *et al.*, [33]. Filaire *et al.*, [34] who noticed a significant increase in salivary cortisol concentrations after exercise in the handball players, which did not appear in swimmers. They stated that the type of sport played could influence the concentration of salivary cortisol. Our data did not reveal any significant differences in salivary cortisol levels before and after the swimming practice, either. However, we did observe lower salivary cortisol concentrations in the afternoon as compared to the morning measurements taken before and after exercise, which is in line with the circadian variation [19]. However, in contrast to Dimitrou *et al.*, [5] we did not find elevated levels of salivary cortisol to be caused by the exercise.

Physical activity leads to an elevated oxygen demand due to an increased rate of metabolism. This is known to lead to an increased production of reactive oxygen species, which are harmful. Therefore, a higher production of antioxidants is a type of biological response deactivation free radicals. The salivary antioxidant system consists of various molecules and enzymes such as peroxidase, superoxide dismutase as well as uric acid, ascorbic acid [35]. Sariri *et al.*, [36] and Darmichi *et al.*, [37, 38] found a significant increase in the salivary peroxidase activity, which returned close to the basal value one hour after an exercise, in athletes immediately after an intense run on the treadmill. Total antioxidant status (TAS) concentration was found to be higher in blood plasma of the rugby players after the dosed submaximal physical exercise [39] as well as in saliva of subjects who performed Tai Chi during a period of 6 months [40]. Our data revealed only slightly higher salivary peroxidase and TAS levels following a swimming practice.

Cavas *et al.*, [13] studied activity of selected antioxidative enzymes and free sialic acid concentrations in unstimulated mixed saliva in Judo athletes before and after a two-hour-long training session. His study revealed increased levels of antioxidative enzymes (catalase, superoxide dismutase, glutathione peroxidase) and free sialic acid in saliva after the training, but no changes in the total protein concentration. It was assumed that sialic acid consumes toxic hydrogen peroxide under physiological conditions. It is known that terminal N-acetylneuraminic acid (NANA) residues are removed by neuraminidase enzyme and released particles of sialic acid are recycled, or degraded by other enzymes. Therefore, released N-acetylneuraminic acid represents a form of defense against oxidative destruction, thus enabling us to determine that sialic acid as an alternative marker of oxidative stress. However, our data do not prove such a relationship because no significant increase in the levels of free sialic acid levels has been identified after workouts. Our data have shown a small increase in calcium concentration and a decrease in magnesium content after the swim training. These data are different from Chicharro *et al.*, [14] findings, which have shown a slight decrease of the mean calcium value and a significant increase in magnesium content in the mixed saliva, after a maximal exercise on cycloergometer.

CONCLUSION

The results of this study suggest a significant increase in the alpha-amylase levels and a tendency for greater total protein concentration in saliva, following an intense swimming exercise, performed in the morning as well as in the afternoon.

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