Might Salivary Lysozyme Be an Indicator of Prolonged Intense Training Load in Athletes? A Preliminary Study in Adolescent Male Gymnasts.

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Abstract

Lysozyme is one of the salivary antimicrobial proteins (AMP) which act as a defence at the mucosal surface. While in adult athletes, a decrease in salivary lysozyme (SLys) levels has been reported after prolonged intense training, to our knowledge, no studies have been conducted to study the relationship between SLys levels and long-term physical activity in children or teenagers.

The aim of this preliminary study was mainly to evaluate in a group of adolescent male gymnasts undergoing prolonged intense training load whether – in accordance with studies in adult athletes – there will also be a decrease in SLys and if so, whether this phenomenon will be so common that we detect it in a small group of study participants.

Twelve adolescent male gymnasts aged from 15.0 ± 1.6 years of national or international performance level were recruited to participate in this study. All participants of the study had their sample of saliva taken: I. Period) after the transitional period (rest), i.e. just before the beginning of the preparatory training period. II. Period) immediately after the end of the preparatory training period that was focused on maximal strength and power development. Preparatory training period lasting 6 weeks consisted of nine 2.5 hour training units (on average) over 6 days in every week. At the same time, three times per week (Mondays, Wednesdays and Fridays) two-a-day training sessions were incorporated. Intensity of the physical exercise was not determined.

We found a significant decrease in SLys levels after the preparatory training period (termed as II. period) compared to its level just before the start of the training (i.e. after the rest, termed as I. period).

The results of this preliminary study suggest that SLys measurements may be an indicator of prolonged training load in adolescent athletes. Although the intensity of the training load has not been determined, the national and international performance level of the gymnasts enrolled in the study allows at least a rough estimate of its level. However, larger studies on male and female adolescent athletes, applying relevant training load with monitoring of variables such as specific sports performance, physical fitness, nutrition, sleep quality, social and psychological factors, are desirable.

Key words: *lysozyme, saliva, athletes, gymnasts, training load, overtraining*

INTRODUCTION

Lysozyme or muramidase (EC 3.2.1.17), a low-weight enzyme with antibacterial activity named according to Greek word "lysis", is one of antimicrobial proteins of innate immune system. This enzyme glycoside hydrolase is able to hydrolyse of 1,4-β-D-glycosidic bonds between structural parts (N-acetylmuramic acid and N-acetyl-D-glucosamine residues) of peptidoglycan (murein),

the primary component of Gram-positive bacteria cell wall, resulting in disintegration of the cell wall (lysis of the bacterial cell) and rapid killing of Gram-positive organisms. The enzyme is abundant in secretions and body fluids such as saliva, tears, airway secretions, human milk, urine, blood plasma and can be also found in granules of polymorphonuclear or mononuclear leukocytes (Perera et al., 1998).

Salivary lysozyme cooperates in ensuring of oral tissue health with both, salivary antimicrobial proteins of immunoglobulin (sIgA, IgG, IgM) and nonimmunoglobulin nature (lactoferrin, lactoperoxidases, defensins etc.). Lysozyme and lactoferrin are the two most abundant antimicrobial proteins (AMP) and as parts of airway surface liquid are derived mainly from serous cells of submucosal glands but also from surface epithelium and leukocytes entering the oral cavity through gingival crevices (Dubin et al., 2004; Perera et al., 1998). Both, output and composition of saliva is under control of autonomic nervous system, thus, it means flow rate and composition of saliva are indirect indicators of autonomic nerve's activity (Chicharro et al., 1998). Whilst, increased rate of salivary fluid output occurs in response to parasympathetic stimuli (copious saliva with low levels of organic and inorganic compounds), sympathetic stimuli lead to low volume of saliva rich in proteins and K^+ (rev. by Chicharro et al., 1998).

It is well known that IgA as the most abundant antibody at the mucosal surface plays an important role in protection against infectious objects entering the respiratory tract. In spite of that fact, salivary IgA levels may not parallel incidence of respiratory tract infections (Peters et al., 2010). At least *in vitro,* bactericidal activity of secretory IgA, complement and lysozyme was demonstrated for complex of all mentioned antimicrobial proteins but not for any of them given individually (Hill et Porter, 1974). Therefore, it seems that might be useful to asses also other constituents of mucosal defence system (Gillum et al., 2013). In past, one of them – salivary lysozyme was examined in connections to both, psychological and physical stress conditions and appeared to be inversely correlated to subjectively perceived level of psychological stress (Perera et al., 1997; Perera et al., 1998). On the other hand, acute moderate or high intensity exercise has been shown to increase salivary lysozyme levels (Allgrove et al., 2008; Gillum et al., 2017a; Gillum et al., 2017b) in contrast to prolonged intense training causing its decrease in saliva. For instance, in work published by West and coworkers (West et al., 2010) elite rowers had approximately half the concentration of salivary lysozyme and lactoferrin than non-exercising control subjects over a 5-month training season (West et al., 2010). Cunniffe with colleagues (Cunniffe et al., 2011) reported declines in salivary lysozyme concentrations (paralleled by decrease in salivary IgA) in elite rugby players at certain periods throughout the season in a prospective longitudinal monitoring study during a period of 11 months. Moreover, drops in both markers seemed to occur during/after preceding periods of intense conditioning type work (Cunniffe et al., 2011).

To our knowledge, no studies have been conducted to study the relationship between SLys levels and long-term physical activity in children or teenagers. Therefore, we present this preliminary study comparing the amount of SLys in adolescent male gymnasts of national or international performance level at the end of transitional period (preseason's rest) with the SLys level immediately after finishing preparatory training period characterized by maximal strength and power development. Potential ability of SLys levels to reflect disproportion between high training load and insufficient recovery in children might be beneficial in diagnostics of states of pathological fatigue such as overtraining and overreaching, that may have a negative impact not only on their athletic performance but also on their overall development.

The aim of this study was to evaluate in a group of adolescent male gymnasts whether – in accordance with studies in adult athletes – there will also be a decrease in SLys and if so, whether this phenomenon will be so common that we detect it in a small group of study participants. This could help us to estimate the number of participants in the intended main trial. Moreover, we wanted to assess whether the sensitivity of the lysoplate method used for detection SLys in this

work (Tenovuo, 1989) is sufficient enough to reveal anticipated decline in SLys concentrations. Finally, we wished to check, whether the mode of specimen collection and handling is both, acceptable for participants and feasible.

MATERIAL AND METHODOLOGY

Participants

Twelve adolescent male gymnasts (aged from 12 to 17 years, with their other characteristics in Table 1) of national or international performance level were recruited to participate in this study.

Tab. 1: Subject characteristics, n = 12. Measurement of both VO2max (maximal oxygen uptake expressed per kilogram body mass) and Wmax (maximal power output) is described in "The bicycle ergometer test" section.

	Age [years]	**Body mass [kg]**	**Height [cm]**	**VO_2max (ml/min/kg)**	**Wmax (W/kg)**
Mean ± SD	15.0 ± 1.6	51.6 ± 8.6	160 ± 9.8	53.9 ± 4.0	4.3 ± 0.3

Experimental design

All participants enrolled in the study had their sample of saliva taken:

1) after transitional period (rest), just before the beginning of the preparatory training period *(termed as I. period*).
2) immediately after the end of the preparatory training period lasting 6 weeks that was focused on maximal strength and power development *(termed as II. period*) (table 2).

Characterization of the preparatory training period**:** Mondays, Wednesdays and Fridays were composed of a two-a-day training sessions (2 hours in the morning and 3 hours in the afternoon), whilst Tuesdays, Thursdays and Sundays of a one-a-day training session (lasting 2 hours, and 3 hours on Sunday). Saturdays were training sessions-free.

Tab. 2: *II. period characteristics* (II. period consisted of 9 training units during 6 days in every week lasting 6 weeks. At the same time, three times per week two-a-day training sessions were incorporated)

Training parameters	**Volume** (hours per: training unit / week)	**Frequency** (quantity of training units per: week / II. period)	**Intensity**
Average values	2.5 / 22.5	9 / 54	not determined

The subjects and their parents were provided both, written document describing the study and a verbal explanation. Consequently, all participants (including their guardians) provided written informed consent before volunteering for the study, and anybody who wished to withdraw could do so at any time. This study was approved by the Masaryk university's research ethics committee.

The bicycle ergometer test

To accurately quantify physical fitness exercise testing was used. All gymnasts participated in the bicycle ergometer test (Lode Excalibur Sport, BTL zdravotnická technika, a.s.) to obtain their VO_2max (maximal oxygen uptake expressed per kilogram body mass) and Wmax (maximal power output).

The test was performed at 75 rpm and began at an intensity of 1 W/kg W. The load was increased by adding a 0.5 W/kg weight to the basket every 1 min until exhaustion. In addition, VO_2max was measured during this test using Metalyzer 3B (Cortex). The test was considered as maximal if there was a plateau in VO_2 and/or a respiratory quotient higher than 1.10. The value of VO_2max corresponded to the highest 30 s value.

Saliva sampling and processing

To avoid variation of salivary lysozyme (SLys) levels during the day, whole unstimulated saliva samples were collected between 7 and 8 a.m. on the day of sampling after the subjects (with rinsed their mouth) had not been eating or drinking for 2 hours. Participants were in the seated position, leaning forward with their head tilted and were passively dribbling saliva into Eppendorf microtubes until microtubes became full. Finally, samples of saliva were centrifuged (1500 g for 10 min at room temperature) and supernatant stored at –20 °C until analysed for lysozyme.

SLys concentration was analysed as technical duplicates of the saliva samples (without any pretreatment) using the lysoplate method based on a diffusion of the sample into the agarose gel containing *Micrococcus luteus* (incubation in the wet-chamber at 4 °C). The diameter of the clearance zone measured after 24-hours is proportional to the logarithm of the concentration (Tenovuo, 1989). Lysozyme (Sigma-Aldrich, USA) was used for preparation of calibration solutions.

Statistical analysis

Obtained SLys concentration data (Table 2) were checked for normality using Shapiro-Wilk's test. As a normal distribution of data in both these groups (I. and II. training periods) was excluded, between training period-differences in salivary lysozyme levels were determining using non-parametric Wilcoxon signed-rank test. A 2-tailed level of 0.05 was considered to be statistically significant.

All calculations were done with help of statistical software STATISTICA 12.5.

RESULTS

In table 3 there are average salivary lysozyme concentrations in adolescent male gymnasts just before the beginning of the training period (after the preseason´s rest) after transitional period – "Period I" and immediately after the end of the pre-season training period – "Period II".

Tab. 3: Salivary lysozyme concentrations (SLys) in adolescent male gymnasts enrolled in the study determined after both, pre-season rest and 6-weeks lasting training load (n = 12)

	SLys (µg/ml)		Between period-differences
	Period I (after preseason rest)	Period II (after 6-weeks of training load)	
Mean	222.9	10.4	–212.5
SD	205.3	13.0	205.6

There was a significant difference (decrease, p = 0.0022) between SLys levels measured before (*I. period*) and after (*II. period*) 6-weeks lasting training load (preparatory training period).

DISCUSSION

Based on available data regarding adult athletes (West, 2010; Cunniffe, 2011) the aim of the present preliminary study was to evaluate whether the decreasing effect of prolonged physical training (6 weeks) on salivary lysozyme (SLys) concentrations may also occur in teenagers or, more precisely, if supposed decrease in SLys levels might be both, marked and frequent enough to be detectable in limited number of participants using less sensitive procedure (Tenovuo, 1989). With respect of the aim of the study identifying subject or training characteristics – that might influence the mucosal immune responses, including SLys levels – has not been done as thoroughly as been proposed by Gleeson and colleagues (Gleeson et al., 2004). In the present study, the training load was defined only by the overall duration, volume and frequency of training unit, without measuring the training intensity. The physical fitness of the participants has been desribed using VO_2max and Wmax parameters.

The advantage of the chosen sport branch (gymnastics) was the fact, that compared with other less strenuous branches of sport, gymnasts underwent relatively high training load in the preparatory training period (termed as "II. period" in this article). Thus, we could assume the potential physiologic response, including changes in SLys levels, might be expressed more clearly.

Indeed, we found a significant decrease in SLys levels after the preparatory training period (*II. period*) compared to its level just before the start of the training (*I. period*). This decrease, observed in this study in adolescent gymnasts, is consistent with previous results in adult athletes undergoing longer-term exercise (West et al., 2010; Cunniffe et al., 2011). However, in our study, there was a decline of SLys in the order of magnitude of weeks -and not months- of physical stress, as were in adults. In addition, a significant decrease in SLys concentration was observed after the training period, although no pretreatment of the saliva sample /the pretreatment significantly increasing the amount of detectable lysozyme in saliva (rev. by Tenovuo, 1989)/ was used prior to using the lysoplate method.

Achieved results and mentioned facts indicate that assessment of SLys in adolescent male athletes undertaking prolonged intense exercise might be sensitive sufficiently to detect changes in SLys levels, even if less sensitive procedure without pretreatment of the saliva samples was used. As states of pathological fatigue such as syndrome of overtraining arise mainly due to an imbalance in the training recovery ratio (with preponderance of training or competition activities) (Meussen et al., 2013) SLys might also be useful in diagnosing of these pathologies. Whether SLys determination might be useful even if the pathological fatigue condition is triggered by factors independent of the training patterns (e. g, eating patterns or poor sleep quality) (Cadegiani et Kater, 2018, Cadegiani et Kater 2019; Rietjens et al., 2005) remains – without precise knowledge of pathophysiology of overtraining and similar states – unclear at this time.

In relation to gender, we can speculate that the adolescent female athletes' SLys levels might differ from male ones as was reported for the acute exercise of adults in the study by Gillum and colleagues (Gillum et al., 2014) Men expressed higher SLys concentration than women in the mentioned study both, at pre-exercise and post-exercise time points.

In addition to gender, there are individual differences in physical capacity, training history and fitness besides others that are able to influence AMP levels, including SLys (West et al., 2006). Therefore, for basic orientation measurements of maximal oxygen uptake (VO_{2MAX}) and Wmax were performed in our study. Obtained values of VO_{2MAX} suggest its moderate level and besides other are comparable with those of professional rugby players (Cunniffe et al., 2011).

CONCLUSION

The results of this preliminary study suggest that SLys measurements may be an indicator of prolonged training load in adolescent athletes, even if the less sensitive lysoplate method is used. (Tenovuo et al., 1989). Although the intensity of the training load has not been determined, the national and international performance level of the gymnasts enrolled in the study allows at least a rough estimate of its level. In addition to this fact, we are aware not only of the general limitations of our study such as the limited number of the subjects, their non-random allocation and participation of male gymnasts only, but also those summarized by Gleeson and co-workers (Gleeson et al., 2004).

Regarding the method of sample collection and handling, it was acceptable to the participants and feasible.

Overall, larger studies on male and female adolescent athletes, applying relevant training load with monitoring of variables such as specific sports performance, physical fitness, nutrition, sleep quality, social and psychological factors, are desirable.

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