

Salivary Immuno Factors, Cortisol and Testosterone Responses in Athletes of a Competitive 5,000 m Race

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Abstract

The exercise-stress model can be a model of temporary immunosuppression that occurs after severe physical and psychological stress. It also allows for the study of interactions between the endocrine and the immune systems. This study examined changes in salivary hormonal and immune factors in athletes in response to physical and psychological stress in a 5,000 m running competition. Eighteen endurance-trained runners (9 males and 9 females) participated in this study. All participants completed a competitive 5,000 m race. Saliva samples were collected 10 min before (PRE) and 10 min after (POST) the competition. Saliva was analyzed for α -amylase activity, concentrations of salivary immunoglobulin A (SIgA), lactoferrin, cortisol, testosterone and total protein. Although the concentrations of salivary TP, SIgA, lactoferrin, cortisol and α -amylase activity were significantly increased immediately after a competitive 5,000 m race, the secretion rates of these factors were not significantly altered in both male and female groups. Additionally, basal levels of SIgA and α -amylase activity were significantly higher in female runners than in male runners. This gender difference still existed after the race. The secretion rates of testosterone decreased significantly after the race in the male, but not in the female group. Moreover, testosterone-to-cortisol (T/C) ratios were significantly lower post-competition compared to pre-competition in both male and female athletes. The T/C ratio had been used as a performance index for athletes. Whether there are correlations between these changes of their physiological characteristics and better running performance need further investigations.

Key Words: cortisol, gender differences, physical and psychological stress, salivary immune factors, testosterone

Introduction

Interactions between exercise stress and the immune system provide a unique opportunity to link basic and clinical physiology and to evaluate the role of underlying stress and immunophysiological mechanisms. Saliva contains numerous host defense factors,

such as immunoglobulin A (IgA), anti-microbial peptides and α -amylase, which play key roles in the mucosal immunity against microbial infection (39). Salivary IgA (SIgA) prevents the adherence of microbes to the mucosal surface and, thus, plays an important role in mucosal immunity (20). Alpha-amylase has been shown not only to be as an anti-

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bacterial protein by inhibiting bacterial growth and colonization in the oral cavity (24), but is also a biomarker of sympathetic nervous activity (34). Lactoferrin acts as an antibacterial protein by sequestering iron and directly interacting and damaging bacterial membrane (14). Salivary secretion of these host defense factors was shown to be affected by high-intensity exercise. In addition, some studies have indicated that SIgA concentrations and secretion rates are higher in athletic men compared to athletic women at rest (9), and also immediately prior to prolonged cycling exercise (1). Another study demonstrated that women had higher secretion rates of SIgA than men pre-exercise; lactoferrin values increased after 45 min at 75% VO_2 max running exercise in both sexes (8). It remains unclear whether gender would influence salivary hormonal and immunological factors.

Physical exercise has been shown to influence the secretion of steroid hormones, such as cortisol (C) and testosterone (T). Salivary cortisol represents the biologically active, free fraction of blood cortisol in response to exercise (26) and the concentration of this hormone in the saliva accounts for 70% of the non-bound blood cortisol (2). Cortisol is a catabolic hormone secreted from the adrenal cortex in response to physical and/or psychological stress, which stimulates muscle protein degradation (6). Significant increases in cortisol levels were observed in school children and different types of athletes during and after strenuous exercise (3). On the other hand, testosterone is an anabolic hormone which increases muscle mass and strength by enhancing muscle protein synthesis, and is an important stress hormone. Indeed, testosterone concentrations increased after an acute bout of exercise (12). Other studies noted that testosterone concentration decreased in response to prolonged endurance exercise (30). Moreover, some studies reported that testosterone levels had not changed after exercise (27, 28). The testosterone/cortisol (T/C) ratio is considered to reflect anabolic and catabolic balance. Therefore, depending on type, intensity, frequency and duration of a preceding exercise as well as an athlete's hydration state, levels of anabolic and/or catabolic hormones and the catabolic state of the body are changed (22). When the T/C ratio decreased by more than 30%, it has been proposed to indicate a state of overreaching (23). Investigators have also noted lower T/C ratios in elite performers than in medium performers (28). Therefore, the T/C ratio has also been used as a performance index for athletes.

There is a limited research on hormonal and immune responses of experienced (>4 years) endurance-trained runners. Vuorimaa *et al.* reported that both cortisol and testosterone concentrations of well-trained middle-distance runners were significantly elevated 20 min after continuous or intermittent ex-

ercise (36). A previous study has found gender differences in metabolic substrates and endocrine responses to stress during prolonged post-exercise and recovery period (34). A recent report also demonstrated that saliva flow rates and the secretion rates of lactoferrin, lysozyme and amylase, but not SIgA, are significantly higher in males than in females during a 16-week, 11 h per week moderate-vigorous physical activity in winter (11). In contrast, SIgA was significantly lower in women than men no matter at rest or after performing 2 h 65% VO_2 max cycling (1). However, the acute effects of 5,000 m competitive race on endurance-trained runners and gender differences have not been investigated. The aim of this study was to examine and compare the effects of 5,000 m running competition on salivary hormone and immune responses between male and female athletes.

Materials and Methods

Participants

Eighteen endurance-trained runners (nine males and nine females) from the college team of the National Taiwan University of Physical Education and Sport volunteered to participate in this study. The criteria for inclusion into this study included engagement in intense athletic conditioning for over four years, and healthy physical conditions without history of cardiovascular or endocrine/immune-related diseases. Participants who need to take any medication during the period of this study were excluded. All participants signed an informed consent form after having been fully informed of the risks and the purpose of the study. The study was reviewed and approved by the Human Ethics Committee of the National Taiwan University of Sport.

Determination of Physical Characteristics

The physical characteristics of endurance-trained runners were measured as in our previous study (33). An eight-electrode bioimpedance analyzer InBody 3.0 (Biospace, Seoul, Korea) was used to measure the body weight. Body height was measured using a stadiometer (Holtain, UK) to the nearest 0.1 cm in standing position and without shoes. Body mass index (BMI) was calculated as body weight (kg) divided by the square of height (m). Prior to entering the study, VO_2 max ($\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) of each subject had been measured with the Bruce Protocol that provides excellent accuracy and a standardized testing procedure for all participants on a treadmill (Medtrack ST65, Quinton, Seattle, WA, USA) (19). The breath-by-breath gas analysis was performed using a Vmax 29C gas analyzer (Sensormedics, Yorba Linda, CA, USA). VO_2 max was

confirmed when the following criteria were met: [1] a respiratory exchange ratio higher than 1.20; [2] a plateau in VO_2 max despite an increase in running speed; and [3] visible exhaustion.

Saliva Collection

Saliva samples were collected 10 min prior to (PRE) and 10 min after (POST) the 5,000 m race that was held at 15:00, and at least 2 h had passed after meal and brushing the teeth. In order to minimize possible contamination (e.g. by food), all participants were asked to thoroughly rinse their mouth with sterile distilled water and then spit out the water. Ten minutes later, they were seated and unstimulated whole-saliva specimens were collected for two (PRE) and five (POST) minutes. All salivary samples appeared clear with no visible color, although there was still possibilities of invisible contamination. Volume of the saliva collected was computed by weighting the tube immediately after collection, and the saliva density was assumed to be 1.00 g/ml. Salivary flow rate was calculated as volume (ml)/collection time (min). Saliva specimens were stored in sterile plastic containers at -80°C until use.

Assays

A Bio-RAD protein assay kit (Bio-RAD, Hercules, CA, USA) was used to measure the salivary TP concentrations. Concentrations of SIgA, lactoferrin, cortisol, testosterone and α -amylase activity were measured as described before (33). In brief, an enzyme-linked immunosorbent assay (ELISA) was used to determine the SIgA concentrations. Primary and secondary antibodies were anti-human IgA antibody (I-9889, Sigma, Poole, UK) and anti-human IgA conjugated with horseradish peroxidase (A3062, Sigma, Poole, UK), respectively. Lactoferrin levels were detected with the sandwich ELISA method. Primary antibody was sheep anti-human lactoferrin (ab36303, Abcam, Cambridge, UK). Secondary antibody and tertiary antibody were rabbit anti-human lactoferrin (ab15811, Abcam, Cambridge, UK) and alkaline phosphatase-conjugated goat anti-rabbit IgG (816122, ZYMED, South San Francisco, CA, USA), respectively. The α -amylase activity was determined using a kinetic reaction assay kit (Salimetrics LLC, State College, PA, USA) according to the manufacturer's instructions. Commercial ELISA kits (DRG Instruments, GmbH, Marburg, Germany) were used to measure cortisol and testosterone concentrations. All samples were measured in triplicates and data were expressed as absolute concentrations and protein was assayed relative to salivary flow rate (37). The inter-assay coefficient of variation was 2% for SIgA, 2.5% for lactoferrin and α -amylase, and 1.5%

Table 1. Anthropometrical and physical parameters of the athletes

Group	Male	Female
Number of athletes	9	9
Age (years)	19.3 ± 0.7	19.1 ± 0.5
Height (cm)	171.8 ± 1.6	$160.3 \pm 1.8^{\#}$
Body weight (kg)	57.8 ± 1.5	$49.9 \pm 1.7^{\#}$
BMI (kg/m^2)	19.6 ± 0.3	19.4 ± 0.3
VO_2 max ($\text{ml}/\text{kg}/\text{min}$)	59.9 ± 3.8	$47.5 \pm 3.9^{\#\#}$
5000 m running time (min)	17.1 ± 1.7	$20.4 \pm 1.9^{\#\#}$

Values are mean \pm SD. BMI, body mass index. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ significantly different from Male.

for cortisol and testosterone. The intra-assay coefficient of variation (CV) for the measurements of SIgA, lactoferrin, amylase activity, cortisol and testosterone was 3, 3, 4, 4 and 4%, respectively.

Statistical Analysis

All data are expressed as mean \pm SD. Statistical comparisons before and after the race were analyzed using paired *t*-test. Intergroup differences were treated with one-way analysis of variance. Significant difference was set at $P < 0.05$. A Tukey *post hoc* analysis was used when significant differences were found for the main effects.

Results

Anthropometrical and Physical Parameters

Nine males (age: 19.3 ± 0.7 years) and nine females (age: 19.1 ± 0.5 years) endurance-trained runners of national level participated in this study. Participant characteristics and their 5,000 m running time are summarized in Table 1. Although the male participants were taller and heavier ($P < 0.05$) than the female participants, their BMI values were not significantly different. Male athletes show higher VO_2 max values and better exercise performances ($P < 0.01$) than females.

Salivary Total Protein and Defense Factors

The concentrations of salivary TP, SIgA, lactoferrin as well as the α -amylase activity increased significantly ($P < 0.05$) after the 5,000 m race compared to the pre-race values in both male and female athletes (Table 2). In addition, the basal levels of α -amylase ($P < 0.05$) and SIgA ($P < 0.01$) were significantly higher in female runners than in male runners. This gender difference still existed after the race (Table 2, $P < 0.05$). After the race, the salivary flow

Table 2. Concentrations of salivary total protein, lactoferrin, SIgA and α -amylase activity

		TP ($\mu\text{g/ml}$)	α -Amylase (U/ml)	Lactoferrin (ng/ml)	SIgA ($\mu\text{g/ml}$)
Male (n = 9)					
	PRE	97 \pm 74	32.3 \pm 5.5	3,793 \pm 742	161 \pm 16
	POST	1,748 \pm 293*	59.2 \pm 9.5*	8,141 \pm 155*	293 \pm 19*
Female (n = 9)					
	PRE	997 \pm 180	66.2 \pm 16.3 [#]	4,988 \pm 709	249 \pm 27 ^{##}
	POST	1,644 \pm 135*	88.6 \pm 18.0 ^{*, #}	7,092 \pm 247*	300 \pm 25 ^{*, #}

Values are mean \pm SD. TP, total protein; SIgA, salivary immunoglobulin A; PRE & POST, before and after the 5,000 m race, respectively. * $P < 0.05$ significantly different from PRE. [#] $P < 0.05$; ^{##} $P < 0.01$ significantly different from male at that time point.

Table 3. Secretion rates of saliva, salivary total protein, lactoferrin, SIgA and α -amylase activity

		TP ($\mu\text{g/min}$)	α -Amylase (U/min)	Lactoferrin (ng/min)	SIgA ($\mu\text{g/min}$)	Saliva Flow Rate (ml/min)
Male (n = 9)						
	PRE	445.4 \pm 11.1	17.1 \pm 0.8	2010.3 \pm 111.3	85.3 \pm 2.4	0.53 \pm 0.15
	POST	479.4 \pm 52.7	16.6 \pm 1.7	2279.5 \pm 27.9	82.0 \pm 3.4	0.28 \pm 0.18*
Female (n = 9)						
	PRE	488.5 \pm 33.8	33.8 \pm 3.6 [#]	2543.9 \pm 155.9	126.9 \pm 5.9 [#]	0.51 \pm 0.22
	POST	526.1 \pm 25.7	30.5 \pm 3.8 [#]	2269.4 \pm 46.9	106.5 \pm 4.8 [#]	0.32 \pm 0.19*

Values are mean \pm SD. Abbreviations are as in footnote of Table 2. * $P < 0.05$ significantly different from PRE; [#] $P < 0.05$, ^{##} $P < 0.01$ significantly different from male at that time point.

Table 4. Concentrations and secretion rates of cortisol and testosterone in saliva

Group		Cortisol (nmol/l)	Cortisol (pmol/min)	Testosterone (nmol/l)	Testosterone (pmol/min)	T/C Ratio
Male (n = 9)						
	PRE	85.32 \pm 20.08	45.22 \pm 3.01	0.16 \pm 0.12	0.085 \pm 0.018	1.83 \pm 1.41
	POST	134.55 \pm 22.31***	37.67 \pm 4.01	0.09 \pm 0.08	0.025 \pm 0.014*	0.73 \pm 0.67*
Female (n = 9)						
	PRE	76.44 \pm 25.63	38.98 \pm 5.64	0.04 \pm 0.03 [#]	0.020 \pm 0.006 ^{##}	0.65 \pm 0.60 [#]
	POST	137.15 \pm 17.75***	43.88 \pm 3.37	0.03 \pm 0.03	0.009 \pm 0.006 [#]	0.28 \pm 0.21 ^{*, #}

Values are mean \pm SD. Abbreviations are as in footnote of Table 2. * $P < 0.05$, *** $P < 0.001$ significantly different from PRE; [#] $P < 0.05$, ^{##} $P < 0.01$ significantly different from male at that time point.

rates dramatically decreased in all athletes (Table 3). However, the secretion rates of total protein, α -amylase activity, lactoferrin and SIgA were not markedly different between before and after the race. Consistent with the basal levels of α -amylase and SIgA, the secretion rates of α -amylase activity and SIgA remained higher ($P < 0.05$) in female than in the male athletes, irrespective of pre-exercise or post-exercise levels.

Salivary Hormones

Cortisol concentrations of both the male and

female runners increased significantly ($P < 0.001$) after the 5000 m running (Table 4), but there were no significant changes in the secretion rates of cortisol. No significant gender differences in concentrations and secretion rates of cortisol were observed. However, the concentrations ($P < 0.05$), secretion rates ($P < 0.01$) of testosterone and the T/C ratio ($P < 0.05$) were much lower in females than males. Furthermore, the secretion rates of testosterone dropped dramatically after the race in male ($P < 0.05$), but not female athletes. The T/C ratio decreased markedly ($P < 0.05$) in both the male and female groups after the race.

Discussion

The aim of the current study was to examine the effects of the competitive 5,000 m race on the hormonal and immune responses of male and female endurance-trained athletes. The present study has revealed that: [1] the post-race concentrations of the salivary defense factors α -amylase, lactoferrin and SIgA were significantly increased; however, the secretion rates of these factors were not markedly different compared to the pre-race values; [2] the T/C ratios were significantly declined after race; and [3] gender differences in pre-race and post-race levels of α -amylase, SIgA, testosterone and T/C ratio were observed.

Strenuous exercise is known to decrease the flow rate of saliva (16). This is probably one explanation for the increased concentrations of total protein, lactoferrin, SIgA and α -amylase activities. Walsh *et al.* reported that salivary total protein concentration can be used to estimate the hydration status of athletes (38). Either in the condition of dehydration or drying oral surface caused by oral breathing during strenuous exercise (18), salivary total protein concentrations and osmolality were markedly elevated. In our studies, the salivary total protein concentrations were significantly elevated post-race. It is known that dehydration has an negative impact on saliva flow rate during exercise (38). In order to correct for this factor, secretion rates of the salivary defense factors were divided by salivary flow rates and the values were not markedly different compared to the pre-race values. Therefore, the levels of salivary defense factors before the 5,000 m race were regarded to be not significantly different from those of post-race. Similar findings have been reported after a competitive marathon race (25), a single bout of soccer-specific exercise (31) and repeated bouts of short-term, high-intensity cycling exercise (32) as well as high-intensity intermittent exercise, all of which did not affect the SIgA concentration (37). Eleven male runners ran for 2 h at 75% VO_2 max and the SIgA concentration decreased dramatically to below the pre-exercise concentrations (5). Thus, the changes of SIgA are dependent on the intensity, duration and mode of exercise. Additionally, other studies found that α -amylase increases significantly in response to physiological- and psychosocial-stress conditions, such as marathon (16), written examination (4) and the cold press test (34). However, there is no previous evidence of gender differences in salivary α -amylase in response to physical stressors. Here, we first report that female athletes show higher α -amylase levels than males before or after 5,000 m running. Because α -amylase is also a biomarker of sympathetic nervous activities, it might be implied that the female athletes in this study showed higher sympathetic responses to the competitive 5,000 m race.

In addition, a previous study showed that menstrual phase did not affect SIgA and lactoferrin secretion rates (8). Whether variations of ovarian hormones during the menstrual cycle affect α -amylase secretion required further investigations.

Studies have been performed to examine the impact of exercise, competition and examination on cortisol responses (3). In addition, cortisol responses to psychological stress in the absence of physical exercise are generally recognized (34). An earlier study reported that salivary cortisol concentrations of young male athletes were significantly increased after a 90-min training session (7). However, exercises of low intensity and short duration exert minimal effects on the cortisol response (17). Consistent with previous report that cortisol concentrations were higher when runners were in a state of dehydration than euhydration (21), our results showed that cortisol concentrations significantly increased after the 5,000 m race in both genders. Interestingly, we found that salivary cortisol concentrations before the race were much higher than the baseline level (below 60 nmole/L, data not shown) of these athletes. Cortisol is a hormone secreted in response to many factors such as physical (*e.g.* heat and cold), physiological (*e.g.* high-intensity exercise), and psychological (*e.g.* competition) stress (2, 6). Similar to previous findings (10, 13, 35), we found much higher post-exercise levels of cortisol, which may be due to these factors. Moreover, no gender difference of absolute concentrations and secretion rates of cortisol were found in this study.

A previous study found significant decreases of testosterone concentrations in male runners at 90 min after the start of the relay race, but the testosterone levels of female runners were not affected (15). In addition, *taekwondo* fighting simulation decreased the levels of testosterone in male fighters, but not in females (29). We had a similar finding in the gender difference of testosterone concentration. Our results also demonstrated that males have four times the salivary testosterone of female before the 5,000 m race, and three times the testosterone of females after the race. In sports science, T/C ratio has been shown to be an indicator of anabolic/catabolic balance, which is affected by intensity of the exercise (32). Previous studies showed that the T/C ratio following *taekwondo* fighting simulation decreased in both genders (29), and the T/C ratio was significantly lower after 10 min of running at 70% VO_2 max (21). Similarity of T/C ratio response to exercise was also observed in our results. Furthermore, the T/C ratio has also been used as a performance index for athletes (28). However, we further observed that an inverse correlation existed between the decreased percentage of T/C ratio and the 5,000 m running time in the competition for all athletes, but not in individual male nor female group.

This observation may be further supported by increasing the number of participants in each group in future studies. Whether changes of the T/C ratio are correlated to better running performance and the possible mechanisms needs further investigation.

In conclusion, the concentrations of salivary defense factors measured post-race were significantly increased. However, the secretion rates of these factors were not markedly different compared to the pre-race values. In addition, the T/C ratios significantly declined after the 5,000 m running race in both male and female athletes.

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