Original Article

Circadian effects on the acute responses of salivary cortisol and IgA in well trained swimmers

L Dimitriou, N C C Sharp, M Doherty

Objective: To examine whether time of day significantly affects salivary cortisol and IgA levels before and after submaximal swimming.

Methods: Fourteen male competitive swimmers (mean (SD) age 18 (3.2) years) volunteered to participate in the study. In a fully randomised, cross over design, each subject performed 5 × 400 m front crawl at 85 (1.2)% of their seasonal best time (277 (16) seconds), with one minute rest between each 400 m, at 0600 and 1800 hours on two separate days. Timed, unstimulated saliva samples were collected before and after exercise. Saliva samples were analysed for cortisol and IgA by radioimmunoassay and single radial immunodiffusion respectively.

Results: Significant time of day effects (am and pm respectively) were observed in IgA concentration (0.396 (0.179) vs 0.322 (0.105) mg/ml, p<0.05), IgA secretory rate (0.109 (0.081) vs 0.144 (0.083) mg/min, p<0.01), and saliva flow rate (0.31 (0.23) vs 0.46 (0.22) ml/min, p<0.001) before exercise (all values mean (SD)). Differences in cortisol levels before exercise (1.09 (0.56) vs 0.67 (0.94) µg/dl) approached significance (p = 0.059). The exercise protocol did not significantly affect IgA concentration and secretory rate (p>0.05) but, in comparison with values before exercise, caused significant alterations in cortisol (p<0.01) and saliva flow rate (p<0.01). There was no significant interaction effect of time of day by exercise on any salivary variables measured (p>0.05). However, most of the values of the salivary variables before exercise were significantly inversely related to their exercise induced response (p<0.05).

Conclusion: These results suggest a significant circadian variation in the variables measured before exercise, without showing a significant effect on their acute responses to exercise.

METHODS

Subjects

Fourteen healthy male competitive swimmers, who all habitually trained for 90–120 minutes in both the morning and evening, volunteered. They had a mean (SD) age of 18.81 (3.19) years, height of 1.79 (0.23) m, and weight of 70.78 (6.60) kg. They had been training for a mean (SD) of 8.68 (2.02) years, with a swimming distance of 21.34 (3.71) km/week and a seasonal best time (PB) of 277 (16) seconds. Two were club standard, one was county standard, two were regional standard, and nine were national standard. Assessment of their circadian chronotype characteristics showed that 11 were intermediate (neither morning nor evening type), two were moderate evening types, and one was a definite evening type.

A trawl of articles on the effects of circadian rhythms, on the acute responses of the mucosal immune system and salivary cortisol after swimming shows a dearth of information. Providing more information on this subject could help to identify the optimal time for training in terms of the least immunosuppressive effects and to emphasis the significance of obtaining experimental and control data at identical time points.

This study was conducted to determine the time of day effects, (morning (0600) v evening (1800)) before and after intermittent submaximal swimming, on salivary cortisol and IgA in competitive male swimmers. It was hypothesised that (a) the variables examined would show a circadian variation between morning and evening, and (b) their responses to exercise would be determined by their basal levels measured before exercise.
Circadian effects on salivary cortisol and IgA responses to training

Experimental design
In a fully randomised, cross over design, each subject performed two tests at 0600 and 1800 hours in the same indoor heated pool (water temperature 28.7 (0.5)°C, air temperature 26.6 (1.2)°C, and humidity levels 70.3 (5.8)%), with a minimum of 36 hours between the test sessions. The maximum time between the tests for any one subject was eight days. The time of day for testing was selected to correspond closely to the circadian peaks and troughs of most of the variables measured, and, coincidentally, are the most common times for swimming training.

This study was designed to eliminate many of the physiological variables known to cause alterations in salivary cortisol and IgA. Subjects were instructed to refrain from any intense physical activity, dietary supplements, and medicines for 24 hours before the test session. Furthermore, sex, tobacco, alcohol, and caffeine consumption were restricted for 12 hours, and eating for eight hours, before testing. Drinking water, chewing gum or mints, and teeth brushing were prohibited one hour before testing. Subjects were also instructed not to disrupt sleep patterns. On the night preceding each exercise test, each subject slept for a mean (SD) of 7.2 (1.5) hours. The last meal (eight hours before testing) was either plain pasta or rice only. On the first test day, the weight (Tefal scales; 0–120 kg, ± 500 g) and height (Harpenden stadiometer) of the subjects were measured. Mood was evaluated once before the swimming test and again about 25 minutes after, using the POMS-C questionnaire. A saliva sample was also collected at this time.

Warm up
As a warm up, a 600 m swim was completed at each subject’s preferred pace together with their chosen flexibility exercises. In addition, 2 × 100 m of front crawl was performed at the approximate split time of the 400 m target time to familiarise the subjects with target time and pace. The warm up was kept constant within subjects over the two testing sessions.

Swimming test
The swimming test consisted of 5 × 400 m front crawl repeats swum at 85 (1.2)% of their season’s best time (277 (16) seconds) with a one minute rest between each 400 m. Time was recorded (0.01 seconds) with a digital hand stopwatch. At the end of each 400 m, subjects were instructed to indicate their rating of perceived exertion (RPE, 0–10 Borg scale). At the end of the 5 × 400 m, swimmers exited the pool, dried themselves, and prepared for salivary sampling. The POMS-C and RPE tests were used to control psychological factors known to influence cortisol and IgA before, during, and after exercise.

Saliva sampling
Before saliva collection, subjects were required to rinse out their mouths for one minute with water to remove any substances such as chlorhexidine that may affect cortisol and/or IgA levels. Timed unstimulated saliva samples were collected in prechilled and preweighed plastic universal containers (20 ml) before the warm up of the swimming test and seven minutes after termination of the swimming test on both test occasions. Immediately after sampling, samples were frozen and stored at −20°C until assayed for IgA and cortisol concentrations.

Although blood has been traditionally used for cortisol assessment, saliva reflects the level of unbound cortisol more accurately than serum total cortisol. Unbound cortisol, as in saliva, gives a direct measurement of the biologically available molecule, whereas bound cortisol, as in serum, is physiologically inactive. In addition, measurement of serum cortisol requires venepuncture, which is associated with negative feelings, which could increase cortisol and decrease IgA.

Cortisol assay
After being thawed, saliva samples were analysed for cortisol using the radioimmunoassay “Magic cortisol Component Set” (Chiron Diagnostics Corporation, Norwood, Massachusetts, USA). A 90 µl sample of the standard was pipetted into tubes, followed by 100 µl antibody and 50 µl tracer. After incubation at room temperature for 3.5 hours, magnets were attached to the tubes for five minutes. The tubes were then decanted into a pan, and the radioactivity bound to them was counted in a gamma counter for two minutes.

Saliva flow rate (Salivafr)
The Salivafr was calculated by dividing the sample volume (ml) by the time (min) taken to produce it. For the estimation of Salivafr, it was assumed that saliva density was 1.00 g/ml.

IgA secretion rate (IgAfr)
IgAfr was calculated by multiplying the absolute IgA concentration (mg/ml) by the Salivafr, (ml/min).

IgA assay
Secretory IgA concentrations were determined by single radial immunodiffusion using a Bind A Rid kit (Binding Site Limited, Birmingham, UK). Calibrators and control and saliva samples (thawed) were mixed immediately before use, and 10 µl of each were pipetted into wells containing monospecific antibody in an agarose gel, and incubated at room temperature for four days. High IgA concentrations were diluted 1:2 with 1% sheep albumin. Precipitin ring diameters were measured with an eyepiece graticule. A calibration curve from the samples containing known amounts of antigen (mg/l) was constructed, and the antigen concentrations were determined by squaring the ring diameters and interpolating them from the standard curve. Note that a radial immunodiffusion reference table for IgA was also used.

Statistical analysis
All values reported are mean (SD). Data showing skewness and kurtosis were transformed to natural logarithms (ln). This logarithmic transformation of skewed and/or kurtotic data was undertaken to improve the statistical inference of the tests and further attenuate the impact of outliers. A two factor (time of day (0600 hours, 1800 hours) × (exercise condition (before and after exercise)) repeated measures of multivariate analysis of variance and analysis of variance was applied to the data of POMS-C, salivary cortisol, IgA, IgAfr, and Salivafr. This was to examine: (a) whether time of day significantly affects values before exercise, (b) the effects of exercise on the selected variables; (c) the interaction of time of day with their exercise induced responses. One way multivariate analysis of variance was used to determine whether time of day significantly affected RPE. Pearson product moment correlations were pursued between baseline values and exercise induced response of cortisol, IgAfr, and Salivafr, in the morning and evening.

The change in values for the variables caused by exercise was calculated by subtracting the value obtained before exercise from that obtained after exercise. p was set at <0.05.

RESULTS
Salivary cortisol
Cortisol levels measured before exercise did not show a significant time of day variation. However, after exercise there was a significant increase in cortisol levels. No significant interaction effect between time of day and exercise on cortisol was observed (table 1).
Table 1  Cortisol (µg/dl), IgA<sub>r</sub> (mg/min), IgA concentration (mg/ml) and Saliva<sub>r</sub> (ml/min) measured before and after exercise at 0600 (am) and 1800 (pm)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Before exercise</th>
<th>After exercise</th>
<th>Absolute change (SD)</th>
<th>Change (%)</th>
<th>Time effect</th>
<th>Exercise effect</th>
<th>Time × exercise interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td>am 1.09 (0.56)</td>
<td>1.32 (0.67)</td>
<td>0.23 (0.99)</td>
<td>21.1</td>
<td>F=4.277</td>
<td>F=5.963</td>
<td>F=0.288</td>
</tr>
<tr>
<td></td>
<td>pm 0.67 (0.94)</td>
<td>1.18 (0.73)</td>
<td>0.51 (1.26)</td>
<td>76.1</td>
<td>p=0.059</td>
<td>p=0.030*</td>
<td>p=0.600</td>
</tr>
<tr>
<td>IgA&lt;sub&gt;r&lt;/sub&gt;</td>
<td>am 0.109 (0.08)</td>
<td>0.076 (0.04)</td>
<td>0.032 (0.09)</td>
<td>9.4</td>
<td>F=14.665</td>
<td>F=6.358</td>
<td>F=0.025</td>
</tr>
<tr>
<td></td>
<td>pm 0.144 (0.08)</td>
<td>0.165 (0.06)</td>
<td>0.038 (0.10)</td>
<td>6.3</td>
<td>p=0.002**</td>
<td>p=0.062</td>
<td>p=0.877</td>
</tr>
<tr>
<td>IgA&lt;sub&gt;r&lt;/sub&gt;</td>
<td>am 0.396 (0.18)</td>
<td>0.443 (0.20)</td>
<td>0.047 (0.26)</td>
<td>11.9</td>
<td>F=7.77</td>
<td>F=0.70</td>
<td>F=0.104</td>
</tr>
<tr>
<td></td>
<td>pm 0.322 (0.10)</td>
<td>0.344 (0.14)</td>
<td>0.023 (0.15)</td>
<td>7.1</td>
<td>p=0.048*</td>
<td>p=0.418</td>
<td>p=0.752</td>
</tr>
<tr>
<td>Saliva&lt;sub&gt;r&lt;/sub&gt;</td>
<td>am 0.31 (0.23)</td>
<td>0.19 (0.10)</td>
<td>0.12 (0.23)</td>
<td>38.7</td>
<td>F=22.93</td>
<td>F=13.844</td>
<td>F=0.041</td>
</tr>
<tr>
<td></td>
<td>pm 0.46 (0.32)</td>
<td>0.33 (0.16)</td>
<td>0.14 (0.16)</td>
<td>30.4</td>
<td>p=0.000***</td>
<td>p=0.003**</td>
<td>p=0.843</td>
</tr>
</tbody>
</table>

Values are mean (SD) (n=14).
*Significantly different at p<0.05, **significantly different at p<0.01, ***significantly different at p<0.001.
Saliva<sub>r</sub>, Flow rate of saliva; IgA<sub>r</sub>, rate of IgA secretion; ↑, increase; ↓, decrease; Change, post-exercise value – pre-exercise value.

**DISCUSSION**

The results confirm the existence of circadian variation in the salivary variables, which supports the first hypothesis. However, swimming exercise intensity and subjects' mood state (before and after exercise) and perceived exertion during exercise did not show significant variations between the morning and evening tests (p>0.05, table 1). This is important to note because many studies have shown that factors such as exercise intensity, mood state, and perception do have a significant effect on cortisol levels, IgA concentration and IgA<sub>r</sub>. As most of the influential factors were either controlled or met, the data reported probably indicate interaction of circadian rhythms and exercise. When the same statistical analysis was performed on both raw and ln data (cortisol, IgA<sub>r</sub>, and POMS-C), similar results were found. However, analysis of cortisol levels before exercise showed significant differences between raw data (p = 0.059) and ln data (p = 0.010).

Cortisol

No significant circadian variation was observed in salivary cortisol levels before exercise (p>0.05, fig 1). However, in line with previous work, cortisol was higher in the morning than evening, possibly to promote gluconeogenesis and appetite. Exercise significantly increased cortisol levels (fig 1), possibly explained as the end product of hypothalamic-pituitary-adrenal axis activity stimulated in turn by the intensity and duration of the swimming test. Previous studies have shown that exercise at more than 60% of maximum oxygen intake increases cortisol levels. However, of the 28 post-exercise measures, a decrease in cortisol after exercise was observed, morning and evening as has been shown elsewhere. This decrease in cortisol after exercise can be explained by negative feedback regulation. However, the large intersubject variability in cortisol levels makes interpretation difficult. This suggests it is unsuitable as a monitoring variable for groups of athletes, although it may prove useful for monitoring individuals.

Despite the fact that the exercise induced cortisol response was found to be greater (76.1%) in the evening than the morning (21.1%), circadian rhythms did not have a significant effect on the magnitude of the cortisol response to submaximal swimming, which is in line with other studies. (table 1). This indicates that exercise induced cortisol response is independent of variations throughout the day. However, the significant inverse relations observed between the pre-exercise cortisol levels and the exercise induced cortisol response (cortisol level measured after exercise minus level before exercise, am and pm; r = −0.763, p = 0.002 and r = −0.817, p = 0.000 respectively) suggest that the exercise induced response of cortisol...
salivary cortisol is primarily dependent on the baseline levels measured before exercise, which in turn are mainly regulated by circadian rhythms. The fact that time of day was not found to significantly affect the exercise induced cortisol response may be attributable to the small number of subjects used together with the considerable intersubject variability noted before and after exercise.

IgA concentration, IgAsr, and Salivafr.

In this study, IgA concentration, IgAsr, and Salivafr, before exercise showed significant time of day variations, which supports the theory that the mucosal immune system is affected considerably by circadian rhythms. Despite the non-significant exercise induced changes observed in IgA concentration and secretory rate, Salivafr, which was significantly decreased (p<0.001) after exercise, may be the most influential factor in terms of protection against oral pathogens and infections. This can be supported by findings showing that people suffering from xerostomia (dry mouth syndrome) have a substantially increased incidence of oral infections and more pathogenic bacteria in the buccal cavity. These results appear to suggest that submaximal swimming may detrimentally affect the quantity of saliva produced, but not its quality, a finding supported by other studies.

Time of day did not have any significant effect on the magnitude of IgA concentration, IgAsr, and Salivafr, responses to submaximal swimming exercise. However, it has been reported that the magnitude of immune responses to exercise is independent of the circadian variation in lymphocyte subpopulations. The magnitude of the decrease, although non-significant between morning and evening for both IgA concentration and Salivafr, was greater in the morning—that is, when flow rate is low—than the evening—when flow rate is high. This emphasises that it may be more hazardous, in terms of susceptibility to oral infections, to exercise in the morning than the evening. In addition, correlation analysis revealed significant inverse relations (at am and pm) between the baseline values of IgA concentration (r = −0.523, p<0.001), IgAsr (r = −0.896, p<0.0001) and Salivafr, and IgAsr, p<0.000 1 as was also shown in this study. These results suggest that the responses of IgA concentration and secretory rate and an increase in cortisol, indicating that athletes should avoid early morning training. This will not be acceptable to elite competitors, whose regimens often require two or even three daily sessions. However, early morning sessions should perhaps be avoided by those returning to training after injury or illness, those close to periods of important competition (which are more associated with underperformance syndrome), and possibly those training at altitude, which itself imposes a degree of immunosuppression.

**Take home message**

Swimming did not induce any significant changes in morning and evening salivary IgA concentration and IgAsr, which supports previous results. In general, exercise increased IgA concentration and decreased IgAsr, although not significantly.

**Figure 1** Mean salivary cortisol and IgA levels measured before and after exercise at 0600 (am) and 1800 (pm). There was a significant difference in salivary cortisol concentration measured before and after exercise (p<0.05). There was a significant difference in salivary cortisol concentration measured before exercise between morning and evening (p<0.05).

**Figure 2** Mean salivary IgA secretory rates and saliva flow rates measured before and after exercise at 0600 (am) and 1800 (pm). There was a significant difference in salivary IgA secretory rate measured before exercise in the morning and evening (p<0.01). There was a significant difference in salivary IgA concentration measured before and after exercise (p<0.05).
swimmers and their coaches for their patience and participation in this study. Also we express our very grateful thanks to Professor Peter Radford for his strategic help, and to Professor Yannis Koutedakis, Dr Shantha Perrera, and Dr David M Aslin for their most useful advice on the saliva sampling procedures.

Authors' affiliations
L Dimitriou, N C O’Connor, Department of Sport Sciences, Brunel University, Osterley Campus, Borough Road Isleworth, Middlesex TW7 5DU, UK
M Doherty, Department of Sport and Exercise Science, University of Luton, Park Square, Luton, Beds LU1 3JU, UK

REFERENCES

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