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

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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) v 0.322 (0.105) mg/ml, p<0.05), IgA secretory rate (0.109 (0.081) v 0.144 (0.083) mg/min, p<0.01), and saliva flow rate (0.31 (0.23) v 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) v 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 (5.98) 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 (PBT) 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.

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.
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 x 100 m of front crawl was performed at the average 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 x 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 x 400 m, swimmers exited the pool, dried themselves, and prepared for salivary sampling. The POM S-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 chlorinated water to measure 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 “Mag ic 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 1.00g/ml.

IgA secretion rate (IgAsr)

IgA 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 was 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) x (exercise conditions (before and after exercise)) repeated measures of multivariate analysis of variance and analysis of variance was applied to the data of POM S-C, salivary cortisol, IgA, IgAsr, 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, IgA, IgAsr, 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).

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IgA<sub>r</sub> before exercise showed a significant time of day variation. Exercise did not significantly affect IgA<sub>r</sub>. No significant interaction effect was observed between time of day and exercise induced effects on IgA<sub>r</sub> (table 1).

**IgA concentration**

Before exercise, IgA concentration also showed a significant time of day variation. Exercise did not have a significant effect on IgA concentration. No significant interaction was observed between time of day and exercise induced effects on IgA concentration (table 1).

**Saliva,<sub>r</sub>**

A significant chronobiological oscillation of Saliva<sub>r</sub>, before exercise was found. Submaximal swimming significantly reduced the quantity of saliva produced at both times with a mean decrease of 0.13 ml/min. No significant interaction was observed between time of day and exercise induced effects on Saliva<sub>r</sub> (table 1).

**Swimming times and intensity**

One way multivariate analysis of variance with repeated measures indicated that no significant differences were observed between the two 5 x 400 m swims (326.4 (19.9) seconds in the morning v 326.5 (20.2) second in the evening; T=0.111, F<sub>1,13</sub> = 0.209, p = 0.95). Thus the two sessions were of similar intensity (84.8 (1.2)% of PBT in the morning v 84.8 (1.3)% of PBT in the evening; T=0.091, F<sub>1,13</sub> = 0.164, p = 0.97).

**RPE**

One way multivariate analysis of variance with repeated measures of RPE (0–10 Borg scale) indicated a non-significant effect of time of day (am v pm) on the perception of exertion (5.7 (2.0) v 5.5 (1.7); T=0.946, F<sub>1,13</sub> = 1.703, p = 0.230).

**Profile of mood states**

A two way multivariate analysis of variance with repeated measures on the POMS-C data showed that there was no significant circadian rhythm in perception of mood states between morning and evening under the instructions of “How do you feel right now?” (T=0.434, F<sub>1,13</sub> = 0.578, p = 0.740) before and after exercise. Collectively, global mood measured before exercise was better in the morning than in the evening. Exercise did not significantly affect global mood (T=1.326, F<sub>1,13</sub> = 1.768, p = 0.223). No significant interaction effect was found for exercise by time on the data of global mood (T=1.604, F<sub>1,13</sub> = 2.139, p = 0.223).

**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 many 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 between morning and evening 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 hypothalamo-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 measurements, 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

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**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 x exercise interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td>am 1.09 (0.56)</td>
<td>1.32 (0.67)</td>
<td>T0.23 (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>T0.51 (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>T0.032 (0.09)</td>
<td>9.4</td>
<td>F=14.665</td>
<td>F=6.158</td>
<td>F=0.025</td>
</tr>
<tr>
<td></td>
<td>pm 0.144 (0.08)</td>
<td>0.105 (0.06)</td>
<td>T0.038 (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</td>
<td>am 0.396 (0.18)</td>
<td>0.443 (0.20)</td>
<td>T0.047 (0.26)</td>
<td>11.9</td>
<td>F=4.77</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>T0.023 (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>Sal&lt;sub&gt;r&lt;/sub&gt;</td>
<td>am 0.31 (0.23)</td>
<td>0.19 (0.10)</td>
<td>T0.12 (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 (0.22)</td>
<td>0.33 (0.16)</td>
<td>T0.14 (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.

Sal<sub>r</sub>, Flow rate of saliva; IgA<sub>r</sub>, rate of IgA secretion; ↑, increase; ↓, decrease; Change, post-exercise value – pre-exercise value.

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Circadian effects on salivary cortisol and IgA responses to training

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 Saliva measured before exercise showed significant time of day variations, which supports the theory that the mucosal immune system is affected considerably by circadian rhythms. Swimming did not induce any significant changes in morning and evening salivary IgA concentration and IgAsr (figs 1 and 2), which supports previous results. In general, exercise increased IgA concentration and decreased IgAsr, although not significantly.

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. These results support the theory that, in future exercise and immunoendocrinological studies, data are collected at identical time points. The magnitudes of immune responses to exercise are independent of the circadian variation in lymphocyte subpopulations. The magnitude of the decrease, although non-significant between morning and evening for both IgAsr 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, (r = -0.891, p<0.000 and r = -0.840, p<0.0000 respectively) and Salivafr, (r = -0.906, p<0.000 and r = -0.665, p<0.001 respectively) and their exercise induced response. These results suggest that the responses of IgA and Salivafr, to exercise are affected by their baseline levels, which in turn are dependent on circadian rhythms.

A portion of the cortisol levels, IgA concentration, IgAsr, and Salivafr, measured before exercise could be attributed to anticipatory psychological stress. Psychological stress operates as a cofactor in the increase in cortisol level and the decrease in Salivafr, by sympathetic activation and IgAsr. Therefore, the baseline values of the variables cannot be considered true stress-free control conditions. This is a limitation of the study.

The results of this study suggest that the optimal time for training—that is, the time of day with the least immunosuppressive effect—is the evening, because basal and post-exercise cortisol levels are low, and the flow rate of saliva/mucosal barrier against oral pathogens is then at its peak (both before and after submaximal swimming). We suggest that, in future exercise and immunoendocrinological studies, data are collected at identical time points.

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REFERENCES

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