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CIRCADIAN RHYTHM OF WHITE BLOOD CELL AGGREGATION AND FREE RADICAL STATUS IN HEALTHY VOLUNTEERS

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Previous studies have demonstrated circadian rhythms in the onset of thrombotic events, which occur most commonly in the morning, and also in the fibrinolytic activity of the blood which has a peak in the evening and a trough in the morning. There has recently been increasing interest in the role of white blood cells (WBCs) and free radicals (FRs) in thrombosis. No one has yet investigated the potential circadian variation of WBC aggregation and FRs in humans. We studied the circadian rhythm of WBC aggregation and FR status in 10 healthy male volunteers. Six blood samples were collected at 4 hourly intervals from 12:00 (mid-day) until 08:00 the following morning. The volunteers carried out normal daily activities until 00:00 at which time they went to bed and they remained in bed until 08:00.

The following were measured on each sample: WBC aggregation; thiobarbituric acid reactive substances (TBARs), plasma thiols (PSH), red cell lysate thiols (LSH), glutathione (GSH) and superoxide dismutase (SOD) which are all altered in the presence of FR activity. The following parameters demonstrated significant circadian rhythms, WBC aggregation $p < 0.001$, TBARs $p < 0.015$, PSH $p < 0.001$, LSH $p < 0.002$. WBC aggregation was lowest at 09:00 and highest at 00:00–04:00. TBARs and PSH both had a peak at 16:00 and a trough at 04:00. LSH had a peak at 12:00 and a trough at 08:00. As the behaviour of WBCs and FR status influence the flow properties of blood, a circadian rhythm in WBC function and FR status may contribute to the time of onset of thrombotic diseases. Moreover, with many studies being currently undertaken in this area, our work indicates the need to standardize sample times.

KEY WORDS: circadian rhythm, white blood cells, free radicals

INTRODUCTION

The distribution of the time of onset of acute myocardial infarction (MI),[1,2] sudden cardiac death (SCD)[3,4] or an episode of myocardial ischaemia[5] is not in a random fashion over a 24 hour period. These events have a circadian rhythm with a marked morning increase in the frequency of their occurrence. It is of interest that similar circadian rhythms are found for a wide range of pathophysiological processes which may be involved in their pathogenesis.

Blood pressure[6] and heart rate increase in the morning which may precipitate atherosclerotic plaque rupture. Also in the morning the thrombotic tendency of the blood is increased due to increased platelet aggregability,[7] increased blood viscosity[8] and reduced fibrinolytic activity.[9] Animal studies have demonstrated a circadian

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rhythm in coronary artery tone,[10] which is also maximal in the morning. Furthermore, the endogenous circadian rhythm of cortisol and the rise in catecholamine levels with the assumption of upright posture mean that both of these hormones have high blood levels in the morning. Adrenaline and cortisol may both contribute to the increased vascular tone.[11]

All the above may contribute to the morning pathogenesis of MI, SCD and myocardial ischaemia. However, other factors may be involved. There is increasing interest in the role of the white blood cell (WBC) in the pathogenesis of coronary artery disease (CAD) and thrombosis. Epidemiological studies have demonstrated that a high WBC count in a patient is predictive of future MI[12] and thrombotic cerebrovascular accident (CVA).[13] Furthermore, the WBC count has been found to correlate strongly with the prevalence of CAD, non-fatal MI and SCD in the MRFIT study (Multiple Risk Factor Intervention Trial).[12] This study also reported that a fall in the WBC count of 1×10^9 WBC/l was associated with a 14% fall in the risk of SCD. In addition, the WBC count has been correlated with the severity of CAD found at coronary angiography.[14]

If the WBC count is an important risk factor for CAD then could its behaviour be linked to the circadian rhythm of CAD presentation? The WBC can adhere, aggregate and release, all features which can influence the blood flow in the microcirculation. The aim of this study was to determine whether a diurnal variation exists in various aspects of WBC behaviour which may contribute to thrombosis.

SUBJECTS AND METHODS

The study was performed on an inpatient basis in the Department of Medicine, Ninewells Hospital, Dundee. The local ethical committee gave approval for the study. Ten healthy male volunteers with a median age of 22 years (range 21–29 years) were enrolled, all were non-smokers who had taken no medication for at least 14 days and no alcohol for at least 3 days prior to the study. All gave informed consent.

During the study the subjects were ambulant between 08:10 and 00:10, they stayed within the hospital and refrained from vigorous exercise. All the subjects were either doctors, medical students or laboratory personnel and they carried out their normal daily activities during the study. On the day of the study the subjects consumed a normal self selected diet except they were asked to refrain from fatty foods. The meal times were breakfast 09:00, lunch 13:00, dinner 19:00 and supper 22:00. The subjects went to bed at 00:10 and remained in bed until 08:10.

BLOOD SAMPLE COLLECTION

All blood samples were drawn from different sites in the antecubital fossae using a 19-gauge butterfly needle. Light torniquet pressure was applied if required to assist venepuncture, the pressure was released for at least 10 s prior to the blood being drawn. The first sample from each subject was taken at 12:00, following this, samples were obtained at 16:00, 20:00, 00:00, 04:00, 08:00 and 09:00. Only WBC aggregation was measured for the 09:00 sample.

WHITE BLOOD CELL AGGREGATION (WBC AGGREGATION)

5 ml of blood were collected in heparinised tubes (10 units lithium heparin/ml of blood) and WBC aggregation was measured in whole blood within 30 min of venepuncture.[15] 2.5 ml of the heparinised blood was transferred to a plastic cuvette containing a 10 × 3 mm Teflon stir bar. The sample was stirred at 37°C at 120 rpm for 3 min, a 100 μl aliquot of blood was removed and dispersed in 20 ml diluent. (Isoton II to which formaldehyde had been added, 0.2% final concentration). 50 μl of 2 mM N-formyl-methionyl-leucyl-phenylalanine (FLMP), an aggregating agent, was added to the remaining blood in the cuvette, the sample was then stirred for a further 90 s. Another 100 μl aliquot of blood was removed and added to the diluent as before. 3–4 drops of the lysing agent, Ultra Lyse II, were added to the diluted blood samples. After fixation and erythrocyte lysis the relative numbers of single WBCs and aggregates were determined using a Coulter Counter. WBC aggregation was calculated by measuring the fall in the WBC count after the addition of the aggregating agent FLMP.

FREE RADICAL RELATED ASSAYS

Free radicals are short lived species and difficult to measure directly. The ability of the blood to scavenge FRs can be assayed by measuring red cell glutathione (GSH), plasma thiol (PSH), red cell lysate thiol (LSH) and superoxide dismutase (SOD).

For these assays 10 ml of blood was collected into a lithium heparin (10 U/ml) tube and centrifuged immediately at 3000 rpm for 10 min at 4°C. Plasma was separated and stored, the buffy coat was removed and the remaining red blood cells washed with normal saline and then centrifuged at 3000 rpm for 10 min at 4°C. The process of washing and centrifugation was then repeated. TBARs were measured by the thiobarbituric acid assay described by Aust.[16] GSH, PSH and LSH concentrations were measured by the method of Ellman.[17] SOD activity of red cell lysate was measured using the technique of Misra and Fridovich[18] on identical volumes of erythrocytes to standardize for any interference by haemoglobin.

CATECHOLAMINES

Plasma adrenaline and noradrenaline were assayed by a double isotope radio-enzymatic assay.[19] Two ml of blood were collected into a lithium heparin (10 U/ml) tube and centrifuged immediately at 3000 rpm for 10 min at 4°C. The plasma was separated and stored at −70°C until the assay was performed.

HAEMATOCRIT

Haematocrit was determined with an automated blood cell analyser (Coulter T540) within 15 min of blood sampling.

STATISTICAL ANALYSIS

Two way analysis of variance was performed on each index to evaluate significance

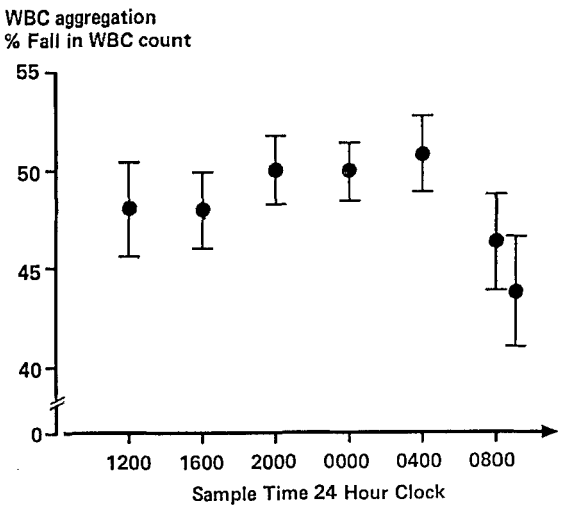

FIGURE 1 The circadian variation in WBC aggregation.

of circadian variation while allowing for between subject differences. The normal distribution of sample values was verified from a plot of the resulting residuals. The standard error of the difference of the means (SEDM) was also calculated; all analyses were performed using a statistical package (Statcalc Eds. A. Lee, P. McInernity, P. Mullins, University of Auckland, New Zealand).

RESULTS

Statistically significant circadian variations were demonstrated for WBC aggregation, TBARs, PSH and LSH (Figures 1–4). No statistically significant variations were demonstrated for erythrocyte GSH content or SOD activity (Figures 5, 6). Adrenaline, noradrenaline and haematocrit also had a significant circadian variation (Table I).

WBC aggregation had a significant circadian variation ($p < 0.001$) which was highest in the early morning 00:00–04:00 and lowest later in the morning at 09:00 (SEDM = 2.33). Both TBARs and PSH had peak levels at 16:00 and a trough at 04:00 (SEDM = 0.226 and SEDM = 12.02 respectively), LSH was maximal at 12:00 and lowest at 08:00 (SEDM = 23.79). These indices had circadian variations which were significant for TBARs $p < 0.015$, PSH $p < 0.001$ and LSH $p < 0.002$.

Adrenaline and noradrenaline both had significant circadian variations ($p < 0.001$) which followed the same pattern, both had high levels from 12:00–20:00 and a trough at 04:00. Haematocrit had a significant circadian variation ($p < 0.001$), the peak levels occurred at 12:00–16:00 and the trough levels at 04:00–08:00 (Table I).

DISCUSSION

The exact role of the WBC in vascular disease is not known but WBCs have physical and chemical properties which promote thrombosis and vascular damage.[20] The WBC

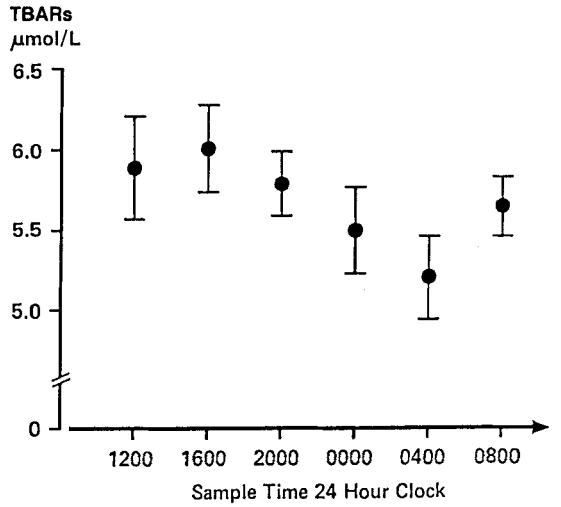

FIGURE 2 The circadian variation in plasma TBARS.

can adhere to other cells. If WBCs adhere to each other aggregates are formed which may occlude small blood vessels. The WBC can also adhere to endothelial cells which reduces the vessel diameter. Whole blood granulocyte aggregation measures the adhesion of neutrophils to each other in response to a chemotactic stimulus in an agitated suspension. Our study demonstrates a circadian variation in WBC aggregation with maximal aggregation occurring between 00:00 and 04:00 and minimal aggregation at 09:00. This fall at 09:00 occurred after the volunteers had been out of bed and ambulant for 1 h. Changes in posture are known to alter other cellular functions.[21] After a period of recumbency the assumption of an upright posture causes

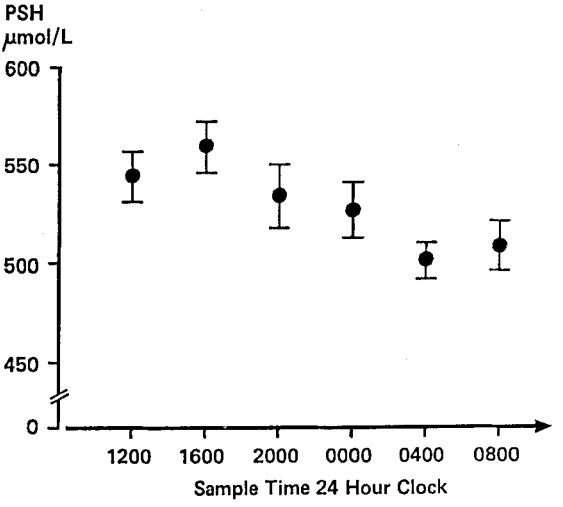

FIGURE 3 The circadian variation in PSH.

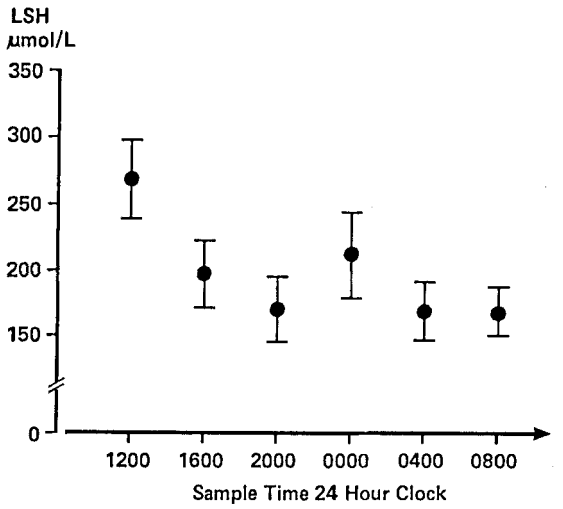

FIGURE 4 The circadian variation in LSH.

a compensatory increase in the heart rate, peripheral vascular resistance, plasma catecholamines, renin and angiotensin II. It is not known if the fall in WBC aggregation in this time period is due to an endogenous circadian rhythm or is related to the change in posture and physical activity. Circadian variations do exist for various WBC receptors[22] but this is not likely to be the explanation for our results as the number of FMLP receptors does not vary diurnally.[23]

Free radicals (FRs) are a highly reactive chemical species which have prothrombotic properties. FRs inhibit prostacyclin synthetase, inhibit antithrombin III and cause

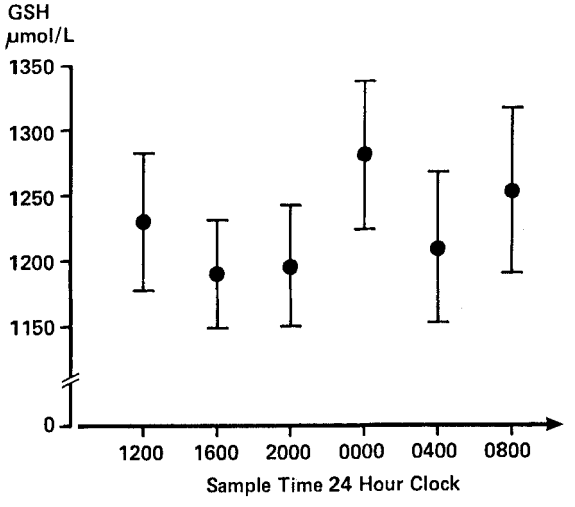

FIGURE 5 The circadian variation in GSH.

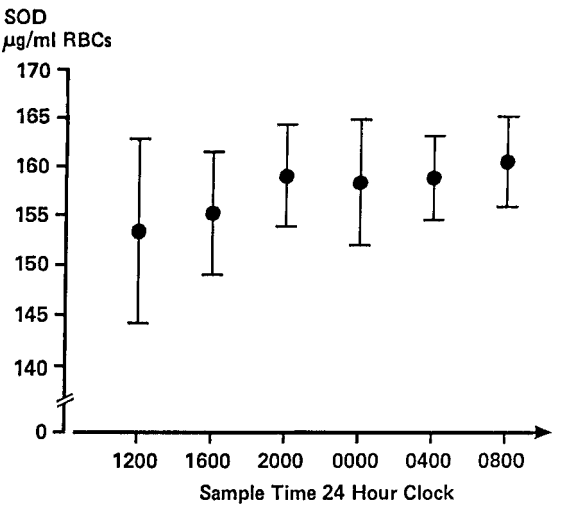

FIGURE 6 The circadian variation in SOD.

endothelial cell necrosis,[24] thus variation in FR activity influences the thrombotic tendency of blood.

FR activity can be measured in blood by the level of FR reaction products such as TBARs although this is only a very approximate index of peroxidation status when applied to plasma because it depends on alpha tocopherol, lipid level and the real level of lipid peroxides if any.[25] In addition, the level of defence mechanisms against FRs such as PSH, GSH, LSH and SOD can be measured. A significant circadian variation was detected in TBARs, PSH and LSH levels. SOD and GSH did not show a significant circadian variation. TBARs and PSH were the highest at 16:00 and lowest at 04:00. LSH had a peak at 12:00 and trough at 08:00.

Both TBARs and PSH levels are measured in plasma and both changed rapidly during the study period. We suggest that this variation may result from circadian variations in factors which generate FRs, such as sunlight exposure[26] and auto-oxidation of catecholamines[27] and also changes in plasma volume. The possible influence of light and hormones on FR indices has previously been investigated by Kolosova[28] who reported a biphasic circadian rhythm for liver lipid peroxidation products in rats. Peak levels were detected in the morning and early evening, this circadian rhythm was reversed by inversion of the light darkness cycle or of the

TABLE I
Mean values (± SD) of adrenaline, noradrenaline and haematocrit levels

Sample Time (24 h clock)	Adrenaline (ng/ml)	Noradrenaline (ng/ml)	Haematocrit
12:00	0.098 ± 0.032	0.67 ± 0.17	46.2 ± 2.5
16:00	0.092 ± 0.031	0.68 ± 0.13	46.1 ± 2.2
20:00	0.096 ± 0.062	0.68 ± 0.14	44.7 ± 1.8
00:00	0.063 ± 0.031	0.62 ± 0.13	45.1 ± 2.1
04:00	0.048 ± 0.033	0.42 ± 0.06	43.3 ± 2.4
08:00	0.074 ± 0.029	0.49 ± 0.09	43.2 ± 2.5

glucocorticoid circadian rhythm. The circadian variation in humans of one index of FR activity was investigated by Munian,[23] who measured the superoxide production of neutrophils. This study demonstrated a circadian variation, the lowest concentration of superoxide occurred at 14:00 however, only three time points were assessed over a 14 h period: 08:00, 14:00 and 22:00.

The plasma levels of TBARs and PSH may be affected by circadian fluctuations in plasma volume. We therefore measured haematocrit which should be similarly affected. TBARs, PSH and haematocrit all had high levels at 12:00–16:00 and low levels at 04:00–08:00. Plasma volume is known to vary with time, this may be a contributory factor to the circadian changes in TBARs and PSH which we detected.

The LSH curve although it shows a significant circadian variation does not seem to vary with TBARs and PSH levels. The lack of synchronisation of LSH levels with TBARs and PSH levels is probably due to the LSH being an intracellular/membrane FR scavenger. As both red cell GSH and SOD are true intracellular components it is not surprising that they do not show a circadian variation.

In conclusion this is the first study, to our knowledge, which reports a circadian variation of WBC aggregation and some putative indices of FR activity. It is important for two reasons. Firstly, knowledge of a circadian variation in factors affecting blood flow may provide further insight into the pathophysiology of the time of onset of thrombotic vascular events. Secondly, with many studies currently being undertaken in the area of FR and WBC pathology our work emphasizes the need for standardization of sampling time points. Accurate data regarding the circadian variation of these indices is important, as without it, comparisons between studies and individuals is difficult. The reasons for the variations detected are not fully understood and further work is required to investigate factors such as catecholamines, posture, plasma volume, exercise and light exposure.

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