Short Communication

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The diurnal pattern of salivary IL-1 β in healthy young adults

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Abstract:

Disruption in circadian rhythm affects the production of inflammatory cytokines. Understanding how it behaves in diseased conditions is essential. Despite the role of the interleukin-1 β (IL-1 β), a potent inflammatory cytokine, in human diseases, little is known about the steady-state circadian rhythm of IL-1 β in healthy individuals. This short study investigates the diurnal pattern of salivary IL-1 β throughout the day in healthy young adults. Twelve participants provided saliva samples at various times throughout the day. Salivary IL-1 β were assessed using enzyme-linked immunosorbent assay (ELISA) and Western blot analysis. Salivary IL-1 β levels were highest at 0430 h and lowest at 0000 h and shared a similar diurnal pattern to that of salivary IL-6. Western blot analysis showed that these levels correspond to the mature form of IL-1 β . Our findings are important as it established the diurnal pattern of salivary IL-1 β is fluctuating normally throughout the day. The findings also open an incredible opportunity for developing research conducted in the field with saliva as the diagnostic tool.

Keywords: healthy, interleukin-1 β , interleukin-6, saliva, young adult

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Introduction

Circadian rhythms are physical, mental and behavioural changes that follow a 24-h cycle, most prominently indicated by the sleep-wake cycle. They communicate tightly with the immune system in a bi-directional manner, and they are regulated by the main circadian pacemaker in the suprachiasmatic nucleus of the hypothalamus. Circadian rhythms are observed in most biochemical messengers, including hormones and cytokines. Growing evidence has shown that the disruption of the circadian control often accelerates the development of various chronic diseases [1]. The role of inflammatory cytokines in the progression of chronic diseases such as cancer, diabetes and obesity is crucial. Given that the disruption in the circadian system directly affects the production of inflammatory cytokines, understanding the diurnal patterns of cytokines, especially those involved in disease pathogenesis and progression, is important.

Interleukin-1 β (IL-1 β) is a pro-inflammatory cytokine, which is found to be elevated in various diseases, including cancer [2]. Despite the role of this cytokine in disease progression, little is known about the steady-state circadian rhythm of IL-1 β in healthy individuals. Compared to blood, saliva has proven to be a good alternative biological source for measuring cytokines and hormones in addition to its non-invasive and rapid collection process. Furthermore, saliva has an advantage over blood where it contains a low background of normal material and fewer complexes than blood. Circulating IL-1 β in the blood of healthy individuals is very low and often below the detection limit, whereas it is higher in saliva [3]. Although Reinhardt et al. [4] showed elevated salivary IL-1 β in the morning than in the evening in shift workers, their findings were restricted to males. Furthermore, the salivary IL-1 β diurnal pattern data may only be representative of individuals who engage in shift work lifestyles. Moreover, the participants' ages were not recorded thereby making it difficult to ascertain whether the data is representative of either younger or older adults or both. Accordingly, this brief study investigates the circadian rhythm of salivary IL-1 β throughout the day in healthy young male and female adults.

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Materials and methods

Ethical approval

The study was approved by the Brunei Darussalam Medical Health Research and Ethics Committee, and informed consent was obtained from all the participants.

Subjects

Twelve participants were recruited from the PAPRSB Institute of Health Sciences, Universiti Brunei Darussalam to participate in this study. There were seven males and five females (mean age: 25.9 ± 2.3 years). The mean body mass index (BMI) of the participants was 23.6 kg/m^2 . All the participants were non-smokers, and none were on medication or supplements that could affect immune activity during the study period. We instructed the participants to collect saliva samples at six time points [0430 hour (h), 0800 h, 1200 h, 1600 h, 2000 h, and 0000 h] throughout the day. Two days prior to the day of saliva collection, participants were instructed to wake up at 0700 h and go to bed at 0000 h to make the sleeping pattern consistent between participants. Participants were asked to refrain from eating, drinking, brushing their teeth 1 h prior to sample collection and any physical exercise 24 h prior to and during sample collection. Female participants were asked to provide the samples within the first 5 days of their menstrual cycle.

Saliva collection

Saliva was collected by trained research personnel using the passive drool technique as described in the guidelines for Salimetrics saliva collection methods (Salimetrics, LLC, State College, PA, USA). Participants were required to drool into a 15 mL sterile centrifuge tube to give a volume of approximately 1 mL. Saliva was centrifuged at 780 g for 15 min before storing at -80°C .

Salivary IL-1 β and IL-6 measurement

On the day of assay, the samples were completely thawed, vortexed and centrifuged at 780 g for 15 min. The concentration of salivary IL-1 β was quantified using an IL-1 β specific enzyme-linked immunosorbent assay (ELISA) kit (Salivary Interleukin-1 β ELISA Kit, Salimetrics LLC, State College, PA, USA). Salivary IL-6 was measured using an IL-6 specific ELISA kit (Human IL-6 ELISA Kit II, BD Biosciences, San Jose, CA, USA). The concentration of salivary total protein was measured using the bicinchoninic acid assay (BCA) Assay (Thermo Fisher Scientific Inc., Waltham, MA, USA). All assays were read on a microplate spectrophotometer (BioTek, Winooski, VT, USA) at 450 nm with a correction wavelength at 630 nm.

Western blot analysis of salivary IL-1 β

Frozen saliva samples were completely thawed. To 150 μL of saliva sample, 1200 μL (9 $\times$) of chilled 100% ethanol solution was added and stored at -20°C overnight to allow for the proteins in the saliva to precipitate. Samples were then centrifuged at 780 g for 20 min at 4°C . The supernatant was carefully aspirated from the sample without disturbing the pellet, and the pellet was left to air-dry for a few minutes. To the pellet, 40 μL of solubilisation buffer [50 mM Tris-HCl pH 7.5, 2 mM ethylenediaminetetraacetic acid (EDTA), 1% sodium dodecyl sulfate (SDS)] was added, and the samples were then boiled at 95°C until the pellet completely dissolved. Samples were diluted accordingly with the solubilisation buffer followed by the addition of NuPAGE[®] LDS Sample Buffer (4 $\times$) (Thermo Fisher Scientific Inc., Waltham, CA, USA). Samples were boiled at 95°C for 5 min and centrifuged for 30 s, just before loading. Proteins (15–30 μg) from saliva samples were resolved on 12.5% polyacrylamide gels, at 120 V for 1 h at room temperature, and then transferred to 0.45 μm polyvinylidene fluoride (PVDF) membranes using wet transfer system at 30 V for at least 2 h at 4°C . The blots were blocked for 1 h in 5% non-fat skimmed milk in Tris-buffered saline with 0.5% Tween 20 (TBST) at room temperature, followed by incubation with primary antibody (Abcam, Cambridge, UK). Primary antibody used was 1/1000 rabbit polyclonal anti-IL-1 β (Abcam, Cambridge, UK). Membranes were washed with TBST and incubated with secondary antibody [1/2000 anti-rabbit HRP (Cell Signalling Technology, Boston, MA, USA)]. After washing, the signal

was detected using Clarity- ECL Western Blotting Substrate (Bio-Rad, Hercules, CA, USA). Imaging of blots was performed with the VersaDoc MP 4000 system (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Salivary IL-1 β (Salivary Interleukin-1 β ELISA Kit, Salimetrics, State College, PA, USA) and IL-6 [5] levels are known to be affected by salivary flow rate and total protein concentration. However, in saliva there is no correlation between the levels of these biomarkers with the total protein. Hence, the results presented are non-adjusted salivary IL-1 β and IL-6 levels. Repeated measures analysis of variance (ANOVA) and post-hoc tests were carried out using IBM SPSS version 22 (IBM Corp., Armonk, NY, USA). A p-value of 0.05 was statistically significant.

Results

To investigate the diurnal cycle of salivary IL-1 β , saliva samples from healthy males and females collected from several time points throughout the day were analysed. There were no significant gender effects. ELISA of salivary IL-1 β revealed that IL-1 β was highest in the morning (i.e. 0430 h) than during bedtime at 0000 h (Figure 1A). The repeated measures ANOVA identified that there was a significant difference in the average salivary IL-1 β levels ($p = 0.004$). Post-hoc tests showed that the average salivary IL-1 β level at 0430 h was not significantly different to that at 0800 h ($p = 0.728$). However, the average salivary IL-1 β level at 0430 h was significantly greater than at later time points [1200 h ($p = 0.004$), 1600 h ($p = 0.006$), 2000 h ($p = 0.012$) and 0000 h ($p = 0.011$)]. IL-1 β is a pro-inflammatory cytokine released upon activation of a multi-protein innate immune pathogen-sensing complex called the inflammasome. The preformed IL-1 β [31 kilodalton (kDa)] has to be proteolytically processed by an important cysteine protease, caspase 1, upon inflammasome activation to result in the secretion of an active and mature form of IL-1 β (17 kDa). Western blot analysis of the participants' saliva was performed to determine whether salivary IL-1 β detected by ELISA corresponded to the active form of IL-1 β . The observed peak in salivary IL-1 β levels in the morning (Figure 1A) corresponded to the presence of the mature form of IL-1 β at those time points (Figure 1B) suggesting that the active form of IL-1 β , not the inactive form, is responsible for the diurnal pattern observed with salivary IL-1 β . We then wanted to see how the diurnal pattern observed with salivary IL-1 β compares to that of other inflammatory cytokines. The diurnal pattern of salivary IL-6, another pro-inflammatory cytokine, is established [5]. ELISA analysis showed that the diurnal pattern of salivary IL-6 closely mimics that of salivary IL-1 β (Figure 2), where salivary IL-6 levels were higher in the morning than in the evening. Although a repeated ANOVA test showed no significant difference between the average IL-6 levels at all time points ($p = 0.234$), the general diurnal pattern trend of salivary IL-1 β still closely resembles that of salivary IL-6.

Figure 1: Diurnal changes in the levels of salivary IL-1β.

(A) Salivary IL-1β levels throughout the day were measured by ELISA and (B) the presence of immature (31 kDa) and mature salivary IL-1β (17 kDa) were analysed by Western blot analysis. Western blot data is representative of one individual sample set for both males ($n = 7$) and females ($n = 5$). Data points on the graph (A) represent the mean $\pm$ standard error of mean (SEM).

Figure 2: The diurnal patterns of salivary IL-6 (pg/mL) and salivary IL-1 β (10 pg/mL).

Salivary IL-6 and salivary IL-1 β levels throughout the day were measured by ELISA. Data points on the graph represent the mean $\pm$ SEM.

Discussion

This study investigated the diurnal patterns of salivary IL-1 β and IL-6 in healthy young adults. The salivary IL-1 β levels peaked at 0430 h immediately after awakening, gradually decreasing and started to rise again at bedtime (0000 h), which is in agreement with previous findings [4]. ELISA measurements of salivary IL-1 β corresponded with the presence of the mature form of the cytokine (Figure 1). However, the presence of the mature form was only observable at 0430 h and 0800 h, and not at the other time points as the protein levels were below the detection threshold for Western blot analysis. Not surprisingly, our findings showed that salivary IL-6 shared a similar diurnal pattern to salivary IL-1 β as both cytokines are somnogenic and that the basal plasma of IL-6, at least, appear higher during the rest phase. Furthermore, systemic pro-inflammatory activity is elevated during the rest phase in healthy individuals. Izawa et al. [5] reported a similar diurnal pattern where salivary IL-6 is highest in the morning, lowest in the early afternoon and peaked again at bedtime. We recently proposed that salivary IL-1 β could be a potential salivary biomarker for cancer progression [2]. Although many studies have investigated different types of cytokines and chemokines as potential biomarkers for early diagnosis and progression of cancer, a limited amount of research has investigated the correlation between IL-1 β and cancer. Understanding the diurnal pattern of salivary IL-1 β in healthy individuals serves as the first step to establishing the salivary baseline level of this biomarker. Our current finding can further assist us to better understand the biological basis of salivary IL-1 β in human diseases such as cancer.

In most previous studies, immune diurnal variations were investigated using blood samples, but not saliva samples. The results of our study provide a new window for detecting an individual's immune diurnal pattern and circadian disruption by using saliva samples in the context of IL-1 β . In future research, salivary IL-1 β could be used to establish inflammatory circadian variations in the development of diseases such as cancer, for which the usefulness of salivary samples would permit larger scale studies. However, the findings of this short study are to be interpreted only within the context of certain limitations. Although the study was performed on healthy individuals, the participants were not from different age groups. Secondly, our sample size was relatively small. Although we found significant differences in levels of salivary IL-1 β at different time points, a larger sample size and additional samples collected over a 48-h period could enable us to investigate the correlational stability of salivary IL-1 β over 2 days. Although we did not observe any notable gender differences, a larger data set would allow us to clarify this as some salivary biomarkers, such as C-reactive protein, have been known to vary between males and females [5].

Knowledge of the diurnal pattern of salivary IL-1 β in the steady-state of healthy individuals is essential to understand the pathogenesis and progression of various diseases. Our findings are significant for the field of salivary biomarkers by which we can determine if a person's salivary IL-1 β is fluctuating normally throughout the day thereby by enabling the ability to identify deviation from the norm. The findings also open an incredible opportunity for developing research conducted in the field with saliva as the diagnostic tool.

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Conflict of interest: The authors declare that they have no conflict of interest.

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