Circadian rhythmicity of plasma lipid peroxidation and antioxidants in oral squamous cell carcinoma

S Manoharan, A A Baskar, T Manivasagam, P Subramanian

ABSTRACT

Introduction: Oral cancer is one of the leading cancers in India accounting for 30 to 40 percent of all cancers. Disturbances in lipid peroxidation and antioxidants status have been implicated in the pathogenesis of several cancers including oral cancer. However, circadian disturbances of oxidants and antioxidants in oral cancer patients were not reported.

Methods: The levels of plasma thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH) and activity of glutathione peroxidase (GPx) in ten oral cancer patients and an equal number of age-matched healthy subjects were assayed at every 6 hour intervals using colorimetric methods and their circadian characteristics were analysed using Cosinorwin computer software programme.

Results: Alterations in mesor, amplitude, acrophase and r value of the chosen parameters were noticed.

Discussion: The desynchronisation of plasma thiobarbituric acid reactive substances and the altered circadian characteristics of antioxidants observed in this study, may deserve further investigation for the early diagnosis, prognosis and for the efficacy of cancer chronotherapy.

Keywords: oral cancer, lipid peroxidation, antioxidants, circadian rhythms

INTRODUCTION

Cancer of the oral cavity presents challenging and unresolved problems for the human population and for a high risk region like India, it is of prime concern. Cancer of the oral cavity constitutes about 3-4% of all cancers in Western industrialised countries, mainly affects middle aged and elderly people, and is more common in men than in women. In India, where the habits of chewing tobacco with betel nut, reverse smoking and heavy alcohol usage are common, there is a striking incidence of oral cancer which accounts for as many as 30-40% of all cancers(1,2).

Free radicals induced oxidative stress has been implicated in the pathogenesis of several diseases including cancer(3). Free radicals can damage proteins, lipids, carbohydrates, and nucleic acids. The most important function of free radicals in vivo or in vitro is lipid peroxidation resulting in deleterious effects on membrane system and death of affected cells. However, the body has developed several endogenous antioxidants defence systems (non enzymatic and enzymatic) to deal with the production of reactive oxygen intermediates(4). The antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) and the small-molecule nonenzymatic antioxidants include vitamin E, C and reduced glutathione (GSH)(4).

Chronobiology is the study of temporal relationships of biological phenomena. Temporal coordination of biological processes with an approximately 24 hours cycle (circadian) is common throughout the animal and plant kingdom(5). Since overproduction of lipid peroxidation products and depleted antioxidants have an important role in the development of cancer, researchers have focused their interest on circadian fluctuations of the above-said biochemical variables. Lipid peroxidation and antioxidant defense mechanisms may relate to preventive and curative chronochemotherapeutic efficacy and management. The concentrations of thiobarbituric acid reactive substances and conjugated dienes in human plasma are often used as indices of lipid peroxidation. Circadian rhythms of antioxidants have been the subject of considerable interest in recent years(6) and significant 24-hour fluctuations of oxidants and antioxidants have been reported in healthy subjects(3,7,8) as well as in diseased patients(9). Previous reports from our laboratory demonstrated circadian fluctuation in lipid peroxidation and antioxidants in experimental animals(9,10).

A spectrum of biochemical alterations including plasma TBARS and antioxidants have been reported in patients with oral cancer(11,12). However, there has
been no report related to the circadian rhythmicity of plasma thiobarbituric acid reactive substances and antioxidants in patients with oral squamous cell carcinoma. Hence, the present study was undertaken to find out the circadian variations in lipid peroxidation and antioxidant status in plasma of oral cancer patients.

METHODS

Ten newly-diagnosed oral cancer patients, who had not undergone any previous treatment for their tumours, were chosen from Rajah Muthiah Dental College and Hospital, Annamalai University, India. An equal number of age and sex matched healthy subjects were also investigated. The subjects were males ranging in age from 45 - 60 years.

All the subjects were interviewed before being clinically examined in the outpatient department. The questionnaire contained data on demographical factors, types of habits, frequency and duration of habits. The clinical and pathological diagnosis were subsequently entered in the forms.

All the oral cancer patients in the present study were regular tobacco chewers, smokers and alcoholics. Other habits such as betel nut chewing, bidi and cigarette smoking were also common. The clinical status of all the oral cancer patients confirmed by histopathological examinations was found to be moderately differentiated squamous cell carcinoma. Healthy subjects were not habituated to tobacco chewing and smoking and were diagnosed as being free from cancer, infectious diseases and benign lesions.

Blood samples from both oral cancer patients and healthy subjects were collected at an interval of 6 hours (00:00, 06:00, 12:00, 18:00, 24:00) continuously throughout the 24-hour period. Minimum amount of blood from oral cancer patients and healthy subjects were collected into heparinised tubes. The blood samples from oral cancer patients were collected through intravenous catheter, inserted into the antecubital vein of the oral cancer patients and blood samples from healthy subjects were obtained by venous arm puncture into antecubital vein. The plasma was separated by centrifugation at 3000 rpm for 15 minutes.

The plasma thiobarbituric acid reactive substances were estimated by the method of Yagi(13). The deproteinised plasma was treated with thiobarbituric acid (TBA) at 90°C for an hour. After cooling, the pink colour extracted using butanol was read at 530 nm. Reduced glutathione was measured according to the method of Beutler and Kelley(14). The method was based on the development of yellow colour when 5, 5-dithio-bis-2-nitrobenzoic acid (DTNB) was added to the compound containing sulphydryl groups. The activity of glutathione peroxidase was determined by the method of Rotruck et al(15). The enzyme present in the plasma/erythrocyte lysate was allowed to react with H2O2 in the presence of reduced glutathione for a specified time period, and the remaining reduced glutathione content was estimated by the method of Beutler and Kelly(16).

The “Cosinorwin” computer software programme (www.septmr.com) was used to analyse the characteristics of biochemical rhythms such as acrophase, amplitude, mesor, r and p values. Acrophase represents the time at which the level of biochemical variable is highest over a 24-hour period. Amplitude

<table>
<thead>
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<th>Parameters</th>
<th>Circadian characteristics</th>
<th>Healthy subjects</th>
<th>Oral cancer patients</th>
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<tbody>
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<td>Thiobarbituric acid reactive substances</td>
<td>Acrophase (hours)</td>
<td>16:40</td>
<td>19:14</td>
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<td></td>
<td>Mesor</td>
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<td>Amplitude</td>
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<tr>
<td>Reduced glutathione</td>
<td>Acrophase (hours)</td>
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<td>02:00</td>
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<td>Mesor</td>
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<td>r value</td>
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<td>Glutathione peroxidase</td>
<td>Acrophase (hours)</td>
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<td>02:32</td>
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Table 1. Circadian characteristics of plasma TBARS, GSH and GPx activity in healthy subjects and oral cancer patients.
Fig. 1 Circadian patterns of plasma TBARS in (a) Healthy subjects; (b) Oral cancer patients. Dotted lines represent the raw data (with mean ± SD) and smooth lines represent the best fitting cosinor curve (obtained using “cosinorwin” computer software program).

Fig. 2 Circadian patterns of plasma glutathione in (a) Healthy subjects; (b) Oral cancer patients. Dotted lines represent the raw data (with mean ± SD) and smooth lines represent the best fitting cosinor curve (obtained using “cosinorwin” computer software program).

Fig. 3 Circadian patterns of plasma glutathione peroxidase in (a) Healthy subjects; (b) Oral cancer patients. U - µ moles of glutathione utilised/minute. Dotted lines represent the raw data (with mean ± SD) and smooth lines represent the best fitting cosinor curve (obtained using “cosinorwin” computer software program).
indicates half of the difference between maximum and minimum values of the biochemical variables (within the 24-hour period). Mesor denotes the mean value of the biochemical variables for equidistant data covering a 24-hour period. The range and the mesor are expressed with the same unit as the documented biochemical variables. The acrophase is expressed in hours. The values of the variables (mean ± SD) were plotted versus the time of blood collection\(^6,15\).

### RESULTS

Table I shows the circadian rhythmicity of plasma thiobarbituric acid reactive substances, GSH levels and G Px activity in healthy subjects and oral cancer patients. The acrophase of plasma TBARS and GSH were delayed approximately 2.5 hours and 2 hours, respectively, in oral cancer patients as compared to healthy subjects. The mesor values for TBARS were decreased in oral cancer patients as compared to healthy subjects. The acrophase of plasma TBARS and GSH were delayed approximately 2.5 hours and 2 hours, respectively, in oral cancer patients as compared to healthy subjects. Although the mesor values for glutathione were decreased in oral cancer patients, the amplitude was found to be zero in both groups. Delay in acrophase (approximately 3:30h) and decrease in mesor values were noticed for GPx activity in oral cancer patients as compared to healthy subjects. Altered \( r \) values in the rhythms of TBARS, GSH and GPx were noticed in oral cancer patients (Figs. 1-3).

### DISCUSSION

The circadian rhythms are daily oscillations in various biological processes that are regulated by an endogenous clock. Disruption of these rhythms has been associated with cancer in humans. One of the cellular processes that is regulated by circadian rhythm is cell proliferation, which often shows asynchrony between normal and malignant tissues. Investigation of the mechanisms by which the circadian clock controls cell proliferation and other cellular functions might lead to new therapeutic targets\(^7\).

Numerous studies have shown that circadian system alterations are not only a risk factor for tumour incidence, but also related to the progression of existing tumours. Circadian system alterations have been described in tumour bearing animals and in cancer patients\(^19\). Emerging data in the human and animal literature suggest that circadian regulation may be an important prerequisite for the maintenance of host defenses against cancer\(^19\). The circadian timing of surgery, anticancer drugs, radiation therapy and biologic agents can result in improved toxicity profiles, tumour control and host survival\(^20\). Several circadian rhythms relevant to the treatment of cancer have been identified in humans\(^21\). Rhythms are most significantly altered in patients with large tumour burden, poor performance status, or liver metastases. Endocrine rhythms may be more markedly disrupted in patients with hormone – sensitive tumours\(^22\).

In the present study, we have noticed a marked circadian fluctuation over a 24-hour period in plasma TBARS of oral cancer patients as compared to healthy subjects. The plasma thiobarbituric acid reactive substances peaks at 16:40h and 19:14h in healthy subjects and oral cancer patients, respectively. Luo et al\(^7\) have demonstrated a notable circadian rhythmicity for plasma malondialdehyde (MDA), peaking around 21:00h in healthy subjects, which was in keeping with our observation for TBARS. They suggested that the evaluation of circadian natures of superoxide dismutase and MDA may be important in the prevention, diagnosis and treatment of associated diseases. Singh et al\(^20\) have demonstrated a marked circadian variation in plasma lipid peroxide and antioxidants in patients with gynaecological malignancies as compared to healthy women. They also reported that MDA peaks during 18:00h and GPx in 18:00h, which corroborate with our results.

Elevated plasma lipid peroxidation has been reported in oral cancer patients\(^15\). Our results also revealed that the rhythms of TBARS and enzymatic antioxidants in oral cancer patients are not synchronised/exhibited a phasing with that of healthy subjects. Subramanian et al\(^10\) have reported that the temporal patterns of TBARS depends on the circadian fluctuations of serum antioxidants. The alteration in the acrophase of TBARS observed in oral cancer patients may therefore be related to alteration in the circadian rhythm of plasma GSH and GPx activity.

We also noticed significant and non-significant temporal variations in plasma GPx activity and glutathione level, respectively, in oral cancer patients as compared to healthy subjects. However, we have observed a phase delay of both GPx activity and GSH level in oral cancer patients as compared to healthy subjects. In the present study, we could not observe any detectable rhythmicity for plasma glutathione in both cancer patients as well as in healthy subjects. However, we observed a phase delay for glutathione levels in oral cancer patients as compared to healthy subjects. Valencia et al\(^9\) reported a non-significant intertime variability for glutathione in healthy subjects. They have reported that the circadian rhythmicity of glutathione could depend on various factors such as meal composition, protein dietary content, meal-related hormonal modifications and stress.

In the present study, the circadian rhythms of plasma glutathione peroxidase show peaks at about 22:55h in healthy individuals and at 02:32h in oral
cancer patients. The decrease in mesor value of glutathione peroxidase in oral cancer patients may be due to exhaustion of this enzyme in scavenging the excess lipid peroxides that are generated into the circulation.

The changes in plasma TBARS circadian pattern observed in oral cancer patients can therefore be related to possible circadian fluctuations in plasma reduced glutathione and glutathione peroxidase activity. The altered circadian patterns of variables studied, deserve further investigations for the early diagnosis and prognosis of effective chronotherapy of cancer.

REFERENCES