Circadian variation of the cell proliferation in the jejunal epithelium of rats at weaning phase

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Received 11 November 2004; revision accepted 4 May 2005

Abstract. Circadian variation in cell proliferation of the jejunal epithelium of 18-day-old rats was studied using the 2-h arrested metaphase score and crypt isolation method. A continuous decrease in the arrested metaphases occurred from 07.00 h to 13.00 h. From 17.00 h arrested metaphase values increased and were maintained at the higher level during the dark period as showed by Cosinor analyses ($P < 0.05$). These results indicate that in the young rat there is already a circadian variation in jejunal epithelial cell proliferation as early as 18 days. We can even suggest that the presence of a circadian rhythm at weaning contributes to the steady state of cell proliferation in the intestinal epithelium observed in adult life.

INTRODUCTION

The epithelium of the small intestine of adult rats is continuously renewed by cell proliferation in the proliferative compartment of the crypt, and that cells migrate up to replace those sloughed off at the villus tip (Leblond & Stevens 1958). In adult rats, the rate of cell proliferation in the crypt is in a steady state in relation to the lost cells (Quastler & Sherman 1959) and they follow a circadian rhythm (Pilgrim *et al.* 1963; Sigdestad & Leshner 1970; Tutton 1973; Al-Dewachi *et al.* 1976; Becciolini *et al.* 1983; Kennedy *et al.* 1983); this has also been shown in human rectal crypts (Marra *et al.* 1994). All these studies indicated that cell proliferation is higher in the dark period than in the light.

In contrast to the adult rats, in suckling and weaning phases, cell proliferation in crypts is not in a steady state (Wright *et al.* 1975; Yeh 1977; Trahair 1989). It has been suggested that the circadian rhythm of cell proliferation is not present and is not induced by experiencing light at birth (Scheving 2000), but that it develops shortly after weaning when nocturnal feeding replaces episodic maternal feeding (Scheving & Pauly 1973). Despite all these data, until the present, the circadian variation of cell proliferation has not been demonstrated in rat pups during suckling and/or weaning. Thus, studies concerning the influence of light and dark cycles on kinetic parameters such as cell cycle time, index of proliferation, rate of cell migration, cell

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differentiation, number of cells in the villus and number in the crypt have contributed to some extent the study of intestinal cell development during the young-to-adult transition.

Here, the main aim of the investigation is to verify whether there is circadian variation in cell proliferation in the jejunal epithelium of 18-day-old rats by counting arrested metaphases at each 2 h during 24 h, and calculating a metaphase arrest index. The hypothesis is to demonstrate that there is already a circadian variation in cell proliferation in the jejunal epithelium before complete weaning.

MATERIALS AND METHODS

Eighteen-day-old Wistar rats (which is in the weaning phase), both male and female, were maintained with their mothers until the experimental time. The rodents were maintained under conventional conditions with a 12 h light/dark cycle (06.30 h lights on, 18.30 h lights off) and ambient temperature between 23 °C and 25 °C. Food and water were provided *ad libitum*. Groups of three rats received intraperitoneal injections of vincristine sulphate (Oncovin, Lilly do Brazil Ltda, São Paulo) 0.5 mg/kg body weight at each of the following times of day: during the light period (07.00 h, 09.00 h, 11.00 h, 13.00 h, 15.00 h, 17.00 h) and during the dark period (19.00 h, 21.00 h, 23.00 h, 01.00 h, 03.00 h, 05.00 h). In each case, cohorts were killed 2 h after vincristine injection. Immediately after killing, the jejunum was removed from each rat, cut into 2-cm long pieces and were fixed, intact in Carnoy's fixative for 24 h. After fixation, the jejunum samples were hydrolysed in 1 N HCl at 60 °C for 6 min, stained with Schiff's solution for 1 h and maintained in 45% acetic acid (Wright *et al.* 1975) at 4 °C.

The most appropriate methods indicated for statistical analyses of biological circadian rhythms are the temporal series and the Cosinor method (Bennedito-Silva 2003). These methodologies have been used to analyse other biological rhythm studies (Halberg *et al.* 1972; Cipolla *et al.* 1987; Duarte *et al.* 2003; Da Rocha *et al.* 2004). In these investigations the COSANA 3.1 software was employed (Duarte *et al.* 2003; Da Rocha *et al.* 2004), modified by Bennedito-Silva (1988).

To obtain the arrested metaphase index, from tissue of each of the two hourly samples, 40 crypts were prepared by the squash method, and by microscopy, the number of arrested metaphase cells was counted. The average arrested metaphase counts were calculated by the ratio between the number of cells with arrested metaphases to the total number in the intestinal crypts – this was the arrested metaphase index. These arrested metaphase indices were used to construct two temporal series curves, one denominated as the real curve index and the other as the cosinor curve index. The last curve was obtained after the metaphase indices had been analysed from COSANA 3.1 software. These curves were used to perform the descriptive analyses.

The fitted period considered to use the COSANA software was set at 24 h and it allowed obtaining the following parameters: Mesor (midline-estimating statistic of rhythm), the amplitude, the acrophase and the *P*-value. Mesor is the mean value of the cosine function; Amplitude is one-half of the total cosine function and the acrophase is the slope of the phase from higher value of the cosine function.

RESULTS

The temporal series curve (real curve index and cosinor curve index) are shown in Fig. 1. Descriptive analyses from the real curve index showed that there was a decrease in cell proliferation from 07.00 h until 13.00 h. A peak in cell proliferation was observed at 15.00 h. At 17.00 h, cell

Figure 1. Temporal series of real curve index and cosinor curve index. Metaphases index and standard deviation calculated from 40 crypts in the jejunal epithelium of rats at the weaning phase. Each point represents the average of three rats.

Table 1. Statistical parameters obtained by COSANA software from averages of the arrested metaphase index values obtained from jejunal crypts of rats at the weaning phase

Period	Mesor	Amplitude inferior	Amplitude superior	SE amplitude	Acrophase inferior (h/min)	Acrophase superior (h/min)	% R	P-value	Variance
24 h	11.23	1.16	3.02	0.47	21.50	1.13	68.35	0.006	1.35

proliferation increased, once more reaching its highest value at around 21.00 h. The descriptive analyses could conclude that cell proliferation remained high during the dark period (Fig. 1).

When the cosinor curve index was analysed we were able to conclude that during the dark period cell proliferation was higher compared to during the light period. This observation was confirmed by the parameters produced by COSANA version 3.1 software (Table 1). The highest values remained occurring during darkness, with acrophase inferior at 21.5 h, acrophase superior at 01.13 hours and the *P*-value being statistically significant ($P = 0.006$).

In addition during lightness, especially in the morning, many crypts undergoing bifurcation were observed, in contrast to during darkness. However, quantification of this fission process of crypts was not performed here; however, investigation concerning crypt fission is necessary to determine whether this process exhibits circadian variation. Thus, these results allow us to conclude that during darkness cell proliferation is significantly higher ($P < 0.05$) than during the light period of the day in the jejunal epithelium of 18-day-old rats, demonstrating that there is already circadian variation of this process at the weaning stage.

DISCUSSION

In the literature, there are few descriptions concerning circadian variation of cell proliferation in the intestinal epithelium of adult rats. Al-Dewachi *et al.* (1976) showed that the zenith and

the nadir of labelling indices (LI) and mitotic indices (MI) in the small intestine of adult rats occurred at 02.00 h and 15.00 h, respectively. Tutton (1973) demonstrated a similar result with regard to the MI as a peak of this index was observed at 02.00 h and a drop at 14.00 h in the jejunum and ileum of adult rats. Other studies confirmed that the MI is higher during darkness than in the light in the intestinal epithelium of adult rodents (Pilgrim *et al.* 1963; Sigdestad & Leshner 1970; Kennedy *et al.* 1983). These investigations are important to establish mechanisms that regulate the steady state of cell proliferation in the intestinal epithelium of rats, which even now are poorly understood.

Although in the rat, considerable changes occur in cell proliferation, cell migration and expression of some digestive enzymes during the third week after birth until complete weaning, the pattern of cell proliferation in the small intestine during suckling and weaning phases is profoundly different from that of adult rats as the process is not yet in a steady state (Wright *et al.* 1975). Some studies have shown that kinetic processes (e.g. cell proliferation and migration) in the intestinal epithelium of rats during the suckling period are different from those observed in the adult because these processes become modified in the weaning phase (Yeh 1977; Trahair 1989; Gomes & Alvares 1998). Despite these data, there is no information concerning the presence of circadian variation in cell proliferation in this period of development.

Here we have observed by descriptive and Cosinor analyses, that mitotic activity in the jejunal epithelium of rats at the weaning phase is highest during regular darkness. Descriptive analyses of temporal series from the real curve index have shown two peaks of the arrested metaphases, at 15.00 h and 21.00 h, respectively, demonstrating that there is a proportion of cell populations cycling through the day with peaks at different times. The cosinor curve index analyses have shown that the highest arrested metaphase index occurs during the darkness (Fig. 1). The latter observation is confirmed by parameters obtained from COSANA software where the acrophase inferior and superior were found to be at 21.50 h and 01.13 h, respectively (Table 1). These results have allowed us to conclude that although there was a peak of cell proliferation in the evening, the highest mitotic activity in the jejunal epithelium occurred during darkness in these animals.

In an attempt to establish a relationship between cell proliferation and the cell cycle time (T_C), we performed experiments in which T_C was calculated in the jejunum and in the duodenum of these young rats by the stathmokinetic method, for lightness (13.00 h) and for dark (01.00 h). Previously, no significant difference had been found in T_C between light and dark (Wille *et al.* 2004). Comparing these data with the present results, we conclude that there is no correlation between the increase of arrested metaphase indices during darkness and cell cycle time in the small intestinal epithelium of these very young animals. However, the measured increases in arrested metaphase indices during darkness confirm the increase in the rate of entry cells into mitosis observed previously (Wille *et al.* 2004), and the present results confirm the data obtained beforehand in our laboratory that cell proliferation is indeed higher during darkness.

The presence of circadian variation in arrested metaphase indices during the weaning phase has indicated that although this phase is a period of transition between suckling and adult life (in which the small intestine will undergo considerable changes in terms of cell proliferation) the behaviour of the proliferative compartment, in terms of circadian variation, is similar to that of adults rats (Tutton 1973; Al-Dewachi *et al.* 1976; Becciolini *et al.* 1983; Kennedy *et al.* 1983) and in human rectal crypts (Marra *et al.* 1994); that is that the highest cell proliferation occurs during darkness and the lowest occurs in the light period of the day.

The observation of crypt bifurcation in the morning suggests that it might be occurring in response to the increase in the cell population numbers at night. No crypt fission was observed from night-time samples. To confirm this observation, further studies are necessary to quantify

crypt fission events and any increase in cell number in crypts during light and/or darkness. Yet, changes in kinetic processes in young and adult rats and the mechanism for its control are still not completely understood. Many factors as hormonal activity, effects of transcription factors, binding of growth factors and further exogenous factors such diet and the light and dark cycles are possible agents involved in control of cell proliferation during the intestinal growth in the rat (Klein & McKenzie 1983; Cummins & Thompson 2002). However, further studies are still required to determine the exact role and interactions of these sets of factors on control of cell proliferation and tissue development.

In conclusion we have shown that there is a circadian variation in the arrested metaphase indices in cells of the small intestine of rats in the weaning phase, and that light and dark cycles could be contributing to the steady state of cell proliferation, which ultimately affects adult life.

ACKNOWLEDGEMENTS

We thank Marinês Cordeiro for her dedicated technical assistance. We also thank Dr Luiz Silveira Menna-Barreto of the Physiology Department, Universidade Estadual de São Paulo, for conceding us the Cosinor software (COSANA 3.1 version). We are grateful to Professor Ana Maria Kaust of the Foreign Language Department, Universidade Estadual de Ponta Grossa, for her revision of the manuscript. Experiments described were accomplished according to current animal welfare laws of Brazil.

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