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COLCHICINE-INDUCED APOPTOSIS DAILY RHYTHMS IN MALE AND FEMALE SUCKLING MICE

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Abstract

Cell death in the intestinal crypts is increased in many conditions including exposure to several cytotoxic compounds, among them colchicine. In despite of *in vitro* studies´ abundance, *in vivo* investigations assessing the effect of this microtubule inhibitor on apoptosis are scarce and no data dealing specifically with colchicine-induced apoptosis in suckling mice are available. The aims were to determine the colchicine inductive effect on apoptosis in 14 d old mice intestinal crypts; to establish the existence of daily variations in the apoptotic indices and to examine whether there is sexual dimorphism in such variations. Experimental work was performed on C3H/S male and female 14 d old mice exposed to 12:12 light-dark conditions. Commencing at midnight and every 4 h thereafter animals were sacrificed having received an i.p. 1 µg colchicine dose per g body weight 4 h before. Samples of duodenum and colon were processed for hematoxylin-eosin staining and TUNEL technique. Twenty longitudinally sectioned crypts per organ and individual were monitored for apoptotic cells. The apoptotic index was expressed as the number of apoptotic cells per 1,000 nuclei for each animal. The arithmetic mean ± SE was calculated for each study time and gender. Differences between genders were determined by the Student's t test. Within group differences were tested by one-way analysis of variance (ANOVA). Results revealed that. 1) the apoptotic daily changes phasing in the small bowel between young males and females showed sexual dimorphism; 2) the absence of daily variations in the young female large bowel cell death indices; 3) a highly prominent induced apoptosis intensity in the small bowel; 4) the sensitivity and number of colchicine induced-apoptosis changes throughout the day are age-dependant. Colchicine is to be taken regularly as a treatment for various human diseases and poisoning results in a multi-organ dysfunction syndrome, particularly affecting tissues with rapid cell turnover among them the intestinal epithelium. The complex pattern of variables implied in the colchicine-induced intestinal apoptosis rhythms (gender, age, time of drug delivery) highlights the need of treatments design with colchicine dosing according with the patient condition.

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Introduction

In multicellular organisms homeostasis is maintained through a balance between cell death and cell proliferation (Danial and Korsmeyer, 2004; Fan and Bergamann, 2008). Cell death spontaneously occurs in the intestinal crypts; this process is increased in many conditions including exposure to several citotoxic compounds (Kasibhatla et al., 2004), radiation (Inagaki-Ohara et al., 2002), venous congestion (Wu et al., 2004), feeding on a combination of fish oil and pectin (Sanders et al., 2004) or by hypoxia/reoxygenation (Ozen et al., 2005).

Duncan and Heddle (1984) demonstrated that colchicine stimulates the apoptosis in large intestine crypts and more recently we reported the same effect on the small intestine crypts in adult mice (González et al., 2005). Colchicine is an agent capable of alterating the mitotic spindle formation by sequestering single tubulin molecules (Inoué, 1981; Salmon et al., 1984).

Several investigations have established the occurrence of daily rhythmicity in many aspects of the intestinal physiology including enzyme expression (Abolmaali et al., 2009), transcription of drug transporters (Stearns et al., 2008), DNA synthesis (Bjarnason and Jordan, 2002), mitosis (Barbeito et al., 2002) and enterocytes apoptosis at the cessation of feeding and commencement of fasting (Iwakiri et al., 2001). In a previous work we described the existence of temporal variations in the proliferation of C3H/S-mouse intestinal enterocytes at 7 and 14 days old (Barbeito et al., 2003) a much earlier developmental stage than those previously reported (Reyna et al., 1997; Barbeito et al., 2002). We also showed the existence of sex-differences in the temporal structure of mitotic index curves in these animals.

Spontaneous and radiation-induced cell death by apoptosis also displays clear circadian rhythms (Ijiri and Potten, 1986; Ijiri and Potten, 1988). We reported that colchicine-induced apoptosis in the adult male mice duodenal crypts showed daily variations (González et al., 2005). No data concerning possible differences for the circadian rhythmicity of apoptosis indices related to sex and gender in mice are available.

Due to the lack of works dealing specifically with colchicine-induced apoptosis in suckling mice, the following study was designed bearing in mind the above investigations. The aims were to determine the colchicine inductive effect on apoptosis in 14 day old mice intestinal crypts; to establish the existence of daily variations in the apoptotic indices and to examine whether there is sexual dimorphism in such variations.

Materials and Methods

Animals and Housing

C3H/S 14 days old male and female mice were used. These suckling animals were the progeny of breeder females that had been housed under standard conditions for circadian-

periodicity analysis in individual cages after conception. These conditions included: food and water available *ad libitum*, room temperature controlled at 22 ± 2 °C and light alternating in a 12 h light-dark cycle beginning at 06:00 h. Even illumination was provided by a 40W fluorescent light placed over the cages.

Experimental Design and Procedures

At 14 days of age, the mice were divided into control and experimental groups of males and females (n=4-8) for killing by decapitation and exsanguination at the following times of day: 00:00, 04:00, 08:00, 12:00, 16:00, and 20:00 h. Four h before sacrifice, each animal of the experimental lots was injected intraperitoneally with 1 µg of colchicine (Sygma Chemical Co., St. Louis, MO, USA) per g body weight. Samples of duodenum and colon were fixed in 10% buffered formalin and embedded in paraffin for subsequent sectioning at a thickness of 5 µm and staining with hematoxylin&eosin (Sygma Chemical Co., St. Louis, MO, USA).

A 10 % of the sections of the sample were nick end-labeled for DNA fragmentation using the TUNEL technique (Roche Diagnostics, Indianapolis, IN) according to manufacturer specifications.

Microscopic Observations

The preparations were keyed and examined by a single observer, under oil immersion objective (1,500 fold magnification) in order to score the number of apoptosis in 20 crypts sections. The definition of an apoptotic cell in the H-E stained samples attended to morphological criteria: cell shrinkage, intense eosinophilic cytoplasm, and nucleus showing typically condensed chromatin (pyknosis), as described by Kerr et al. (1972), Colombo et al. (2000), Coopersmith et al. (2002) and Vyas et al. (2007). Several apoptotic bodies clustered were given a single count. Any doubtful cells were disregarded. A parallel TUNEL assay was employed to corroborate the reliability of the apoptosis morphological assessment.

Apoptotic Quantification

The scanty depth and other morphological characteristics of the inmature crypts in suckling mice did not allow to divide them into three regions (tiers 1-4; 5-12 and 13-20, proceeding from bottom to top) (Barbeito et al., 2003) as in adult mice (Potten, 1991). Instead, the individual count was performed in longitudinally sectioned crypts showing a lumen that had no less than 14 cell tiers. The apoptotic index was expressed as the number of apoptotic cells per 1,000 nuclei for each animal.

Statistical Analysis

The arithmetic mean ± standard error (SE) was calculated for each study time and gender. Differences between sexes were determined by the Student's t test. Within group differences were tested by one-way analysis of variance (ANOVA).

Results

All results using H&E were in concert and confirmed as apoptotic cells by internucleosomal DNA fragmentation by TUNEL technique.

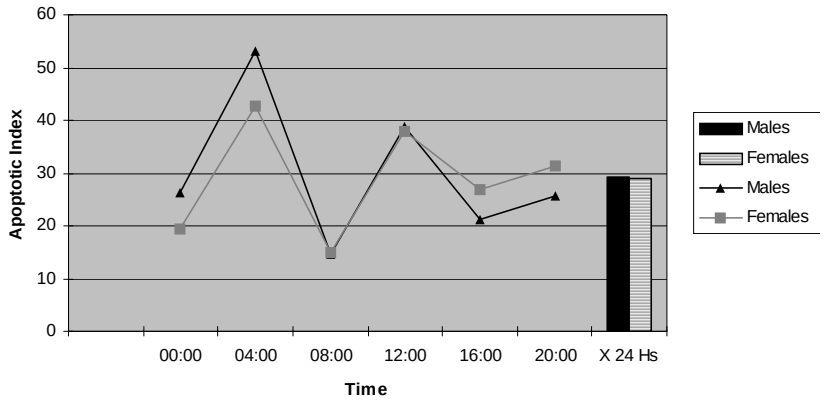

Figure 1. Apoptotic cells in duodenal crypts.

Duodenum

Males. The apoptotic indices varied significantly throughout the day (Table 1), exhibiting a peak at 04:00 h (53.06±6.45) and a trough at 08:00 h (14.5±4.75). The difference between these values was statistically significant ($p<0.001$).

Table 1. Apoptotic index every 1000 cells in duodenal crypts

	Males		**Females**	
Time	X±SE	n	X±SE	n
00.00	26.34±6.33	4	19.29±4.50	5
00.04	53.06±6.45	7	42.56±5.73	6
00.08	14.73±4.75	4	14.96±3.25	8
12.00	38.84±8.42	4	38.01±4.54	7
16.00	21.21±3.90	8	26.79±2.19	5
20.00	25.71±2.91	5	31.25±5.49	6
		32		37

X 24 hs 29.28 ± 5.63 X 24 hs 28.81 ± 4.34
p 04.00-08.00 < 0.001 p 04.00-08.00 < 0.001
p 12.00-00.00 < 0.001
p: statistical signification

Females. In these animals a bimodal apoptosis daily curve was registered with peaks at 04:00 and 12:00 h (42.56±5.73; 38.01±4.54 respectively, $p < 0.001$) and minimal indices at 00:00 and 08:00 (19.29±4.50; 19.29±4.50 respectively; $p < 0.001$).

The average apoptotic indices were similar in both sexes (males: 29.28±5.63, females: 28.81±4.34) (Table 1).

Colon

Males. In this group the peak was detected at 04:00 h (18.30±2.23). This value was higher than all the other values registered for male colonic apoptosis (Table 2).

Females. In contrast to the findings in the other sector and organs examined, female mice showed no circadian variation in the apoptotic intensity in the large intestine (Table 2).

The comparison of the results obtained from males and females showed similar average apoptotic indices in this organ (males: 7.96±2.35; females: 8.71±3.38) (Table 2).

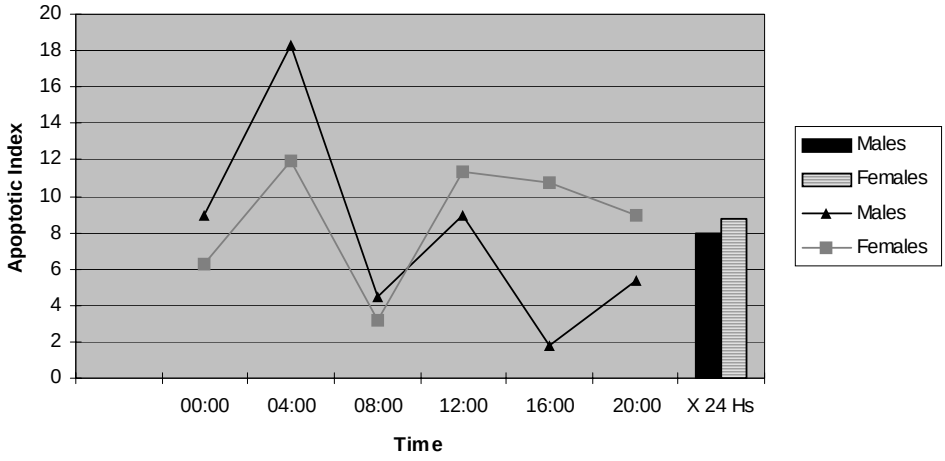

Figure 2. Apoptotic cellsin colonic crypts.

Table 2. Apoptotic indices every 1000 cells in colonic crypts

	Males		Females	
Time	X±SE	n	X±SE	n
00.00	8.93±1.78	5	6.25±2.68	4
00.04	18.30±2.23	4	11.91±2.34	6
00.08	4.46±1.71	4	3.22±1.18	5
12.00	8.93±2.39	5	11.31±2.90	6
16.00	1.79±0.98	5	10.71±3.17	4
20.00	5.35±1.00	4	8.93±5.49	5
		27		30

X 24 hs 7.96 ± 2.35 X 24 hs 8.71 ± 3.38

p 04.00-16.00 < 0.001 p=NS

p: statistical signification; NS: not significant

Discussion

It has been demonstrated that diverse cell types undergo apoptosis when they are treated with colchicine (Suzuki, 1998; Suzuki et al., 1998; Takeda et al., 2000; Cervinka et al., 2004; Kalvelyte et al., 2003; Jorda et al., 2005). In despite of *in vitro* studies´ abundance, *in vivo* investigations assessing the effect of this microtubule inhibitor on apoptosis are scarse. Our work demonstrated that colchicine induced apoptosis in the suckling mouse duodenal and colonic crypts enterocytes as it had been shown by Duncan and Heddle for adult mouse colonic crypts (1984) and in our laboratory for adult male mouse duodenal crypts (González et al., 2005).

The comparison of the apoptotic daily changes phasing in the small bowel between young males and females showed sexual dimorphism, a condition that has been reported in relation to mitotic activity in the same population (Reyna et al., 1997; Barbeito et al. 2003) or in others such as endocrine cells of the pars intermedia (Barbeito et al., 2000) in this mouse strain. The absence of daily variations in the young female large bowel cell death indices does not permit to consider a sexual dimorphism though it might be reflecting an uneven sensitivity to colchicine between genders.

In regard to the induced apoptosis intensity found for each intestinal sector, the levels achieved resulted highly prominent in the small bowel. This is in coincidence with the observations in adult mice of the same strain (Barbeito and González, unpublished data) in which the apoptotic indices values for the duodenal crypts had a many-times fold difference to rectal crypts. The dead cell index resulted higher in the small intestine; this agrees with the findings of Yamada et al. (2000) in guinea pig and 6 weeks old mouse after autumn crocus poisoning. Potten et al. (1992) reported an elevated apoptosis index for the small bowel of gamma-exposed mice and Gibson et al. (2005) found a 10-fold higher apoptosis index in large intestine than in jejunum. Differences between the induced apoptosis yield in the two intestinal sectors may be due to a differential gene expression related to cell death control like p53 and Bcl2 (Roberts et al., 1997; Gauthier et al., 2001).

It is known that apoptotic indices changes in accord to the time of day the cell insult is delivered, i.e. Ijiri and Potten (1990) found a circadian rhythm in the apoptotic cell yield indicating that the sensitivity and number of radiation-induced apoptosis varied along the day. Similar findings were reported by Duncan et al. (1983). Results from a previous study in adult male mice showed that the peak hour of day for inducing duodenal apoptosis was achieved when colchicine was injected at 04:00 (González et al., 2005). We also detected daily variations in the apoptotic indices for the duodenum from both sexes. However, the highest apoptotic index value was observed when animals received colchicine at midnight. The large bowel crypts only showed daily changes in the male individuals but in this case the maximal apoptotic index value was observed at 04:00, when the treatment was performed at 00:00 h. These results also differ from findings in adults which exhibited variations in both genders [these data have not been presented here]. The adult's male apoptotic highest values were obtained when the cells were challenged at 04:00 h, for the adult females the same result was registered when the colchicine administration was performed at 08:00 h. Thus, the sensitivity and number of

colchicine-induced apoptosis changes throughout the day are age-dependant. A similar age-dependant condition has been observed in assessments concerning the proliferation activity in diverse organs including the bowel (Barbeito et al., 2000, Barbeito et al., 2003).

Advances in colchicotheraphy have shown fascinating new fields for research (Fabre, 2005). Colchicine is used in the treatment of gout, inflammatory dermatoses such as scleroderma, Sweet´s syndrome and autoinmune bullous disease (Sullivan et al., 1998). It is also the most effective treatment for familial Mediterranean fever to be taken regularly on a life-long basis (Medlej-Hashim et al., 2004) and recently its potential therapeutic value in the prevention of p25 formation for the treatment of neurodegenerative disorders has been assessed (Jorda et al., 2005). The colchicine doses employed in these diseases' treatments are lower than those administered in antineoplastic chemotherapy. In spite of this, patients with hepatic alterations or ingesting barbiturates tend to exhibit a predisposition to colchicine intoxication (Arroyo et al., 2004). The drug´s effect on the intestinal epithelium is the cause of the initial signs in colchicine intoxication. As colchicine poisoning results in a multi-organ dysfunction syndrome, particularly affecting tissues with rapid cell turnover (Brvar et al., 2004), among them the intestinal epithelium, the complex pattern of variables implied in the colchicine-induced intestinal apoptosis rhythms showed here (gender, age, time of drug delivery, enterotoxicity species-differences) highlights the need of treatment design with colchicine dosing according to the patient condition.

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