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Abstract.

We studied the index of DNA synthesis (DNAs) of two cellular carcinomas: the hepatocellular ES12a and the mammary TN60 of mice, throughout one circadian cycle. In the results we observed that both tumors have circadian rhythms (CR), but the peaks of DNA synthesis (DNAs) vary. Besides, the mean of DNAs along 24 hours show significative differences, the TN60 have higher values than the ES12a. These observed CR in the DNAs index in both carcinomas mean that, at least in partly, the proliferation of cancer cells can be regulated by endocrine factor as it normally occurs in ordinary cells. The big problem we can find for the chronopharmacology is that it is impossible to know in advance the rate of proliferation of each tumor.

Key words: carcinoma, circadian rhythms, DNA synthesis, mice.

1. Introduction.

Cell Cycle lenght may vary, in numerous cell populations, in response to specific conditions associated with different stages of the organism`s life span. It is regulated by growth factors and other signaling molecules that stimulate or inhibit cellular proliferation (García et al. 2001). In mammals, circadian rhythms are generated by a central clock in the suprachiasmatic nucleous (SNC) located in the hypothalamus that constantly synchronize with environmental cues via circadian input pathways and controls the peripheral clocks through circadian output pathways (Reppert et al. 2002; Buijs et al. 2006).

The circadian system is perturbed by exposure to light at night with commensurate suppression of melatonin production and dysregulation of circadian genes that have been implicated in cancer development (Takahashi et al. 2008). Shift work that involves circadian disruption is deemed to be a probable carcinogen by the International Agency for Research on Cancer and epidemiologic studies. It has been demonstrated that circadian rhythm disruption increases the risk of breast, colon, prostate, lung, ovarian and hepatocellular carcinoma (Straif et al. 2007; Davis et al. 2001; Baan et al. 2009).

Tumor growth and evolution is a complex phenomenon controlled by an intríncate pattern of competing processes (Perez de Castro et al. 2007). Previous studies have demonstrated that many cellular neoplastic populations like some hepatomas and hepatocellular carcinomas show circadian variations in the synthesis of DNA (DNAs) (García et al. 2008; Nash and Echave Llanos 1971). In some cases, the observed rhythm was similar to that of the original cell population (Barbeito et al. 1995), but, some undifferentiated hepatocellular carcinomas did not show any mitotic circadian rhythm (Moreno et al. 1985). Moreover diurnal levels of pituitary gonadotrophins is of self-evident interest in the biology of endocrine related tumors such as breast and prostate cancer (Kelleher et al. 2014).

There are many techniques to analyze the different phases of the cell cycle. Estimation of S-phase index (SI) can be obtained by detection of labelled nuclei by immunohistochemical methods like bromodeoxiuridine (Brdu), a thymidine analogue, as an indicator of cellular proliferation (Thompson et al. 1999).

The following experiments were designed in order to study and compare the DNAs index of two different cellular carcinomas of adult male mice: the hepatocellular ES12a and the mammary TN60, throughout one complete circadian cycle.

2. Materials and methods.

2.1 Animals:

For these experiments, we used adult male C3H/S-strain mice. Conditions concerning animal management fully respected the policy and mandates of the Guide for the Care and Use of Laboratory Animal Research of the National Research Council. They were subjected to the following standardization conditions: water and food available *ad libitum,* ambient temperature maintained at 22 ± 2 °C, alternating light and dark periods restricted to 12 h each with illumination by fluorescent lamps beginning at 06:00 h.

2.2 Tumour-bearing animals:

After an appropriate period of synchronization (15 days), about 70 mg of the C3H/S-histocompatible mammary o hepatocellular carcinomas were grafted separately into the subcutaneous tissue of each animal's flank. These carcinomas were maintained by subcutaneous serial transplant in male mice. The graft-bearing animals, subsequently divided into lots of 5 to 8 mice each, were then housed for further two weeks under standardization conditions before the lots were separated into the following experimental-protocol groups.

2.3 Experimental groups:

The animals were divided in two experimental groups: Group I, mice bearing the ES12a hepatocellular carcinoma, and Group II, mice bearing the TN60 mammary carcinoma. When the tumors were about 2 ± 0.5 cm of diameters every group was divided into 6 lots of

4/8 animals each. Every lot were sacrificed every 4 h starting at 00:00 h throughout one complete circadian cycle. In the necropsy of the animals we extracted the solid tumor and these were processed as we mentioned before.

2.4 SI (S-phase index):

To determine the index of DNAs of tumor cells we used immunohistochemistry. One hour before being killed, all animals, received an intraperitoneal injection of 50mg/kg of 5-bromodeoxiuridine (Sigma, St. Louis, U.S.A.). Samples of apparently nonnecrotic tumor tissue were excised and fixed in 10 % buffered formalin for 24 h and embedded in paraffin. Sections (5 µm) were placed on silanized slides, dried overnight, deparaffinized in xylene, rehydrated through graded alcohols and washed in Tris-buffered saline (TBS) at pH 7.4. Endogenous peroxidase was blocked with 3 % H2O2 for 10 min. Antigen retrieval was achieved by washing the slides in TBS and irradiating them in citrate buffer, pH 6.0 at 750 W for two cycles of 5 min in a microwave oven. After microwaving, the slides were washed in TBS and incubated with primary antibodies (Bu 20a, 1/100, Dako, Carpinteria, CA, USA) for 1 h at room temperature. Envision was used as a detection system with 3'3-diaminobenzidine (Sigma, St. Louis, MO) as the chromogen. The sections were lightly counterstained with Mayer's hematoxylin (Martín and Badrán, 1998).

The specimens were then examined microscopically under an oil-immersion objective (at 1.500 X) in order to score the total number of labelled nuclei among a minimum of 3000 nuclei. From this data, the S-phase index (SI) was calculated and expressed as the number of labelled nuclei per 1000 nuclei.

2.5 Statistical analysis:

The results are expressed as means ± SE. For evaluating the statistical significance of differences among the means we used first the Anova and Student-Newmann-Keuls Multiple Comparisons Test.

3. Results.

As we can see on table 1 and figure 1 both tumors have circadian rhythms in their DNAs, and the minimum values were observed at the same time (between 08:00 and 12:00 h) but the peak of DNAs vary because in the ES12a is at 00:00 h (156.5 ± 28.3) and in the TN60 is at 20:00 h (190.4 ± 14.4).

Furthermore, the mean of DNAs along 24 hours have significative differences (p= 0.007) between ES12a and TN60. The last one, the mammary, has higher values than the hepatic carcinoma.

In figure 1 we could observe that in ES12a hepatocellular carcinoma the values between the maximum and minimum of their ADNs have much more significant differences (p 0.001) than also significant differences observed between the maximum and minimum values in TN60 mammary carcinoma (p 0.05).

4. Discussion.

The circadian variation in human normal tissues has been described since 1938 (Cooper 1938) but we could find very few reports on CR of DNAs in human tumor type, and the majority of these studies were made without check a full day. However, we can mention a report that analyze the cellular proliferation in human Breast cancers, in wich they found changes during each Estrous Cycle and each season throughout each year (Oh 2001).

In this study we observed CR in the DNAs index in both murine carcinomas: the hepatocellular ES12a and the mammary TN60, this means that, at least in a partly, the proliferation of cancer cells can be regulated by endocrine factor as it occurs in ordinary cells. As it can be observed in previous works and it has been demonstrated in this research, it is true that the mayority of solid tumors have rhythms in their proliferation activity. The differences in the average in the proliferation of tumors along a day are probably due a numerous factors: as the original tissue of the tumor, the degree of malignancy, degree of diferentiation of cell type, GF, and others mitotic controls. We can mentioned a lots of GF involves in control cellular proliferation, including HGF, EGF, TGFβ, IGF, etc (Akiel 2014). Probably one or more of these are responsible for regulating the CR of proliferation observed in this study.

The big problem that we can find is that it is impossible to know in advance the rate of proliferation of each tumor and obviously this study cannot be applied to human patient with cancer. We could try to know the rate of proliferation of specific neoplastic cell performing a cell culture of them, but we coudn`t be sure that the results found in culture are the real proliferation rate *in vivo;* as Loning and Kettner said: "The clock-controlled genes usually do not share overlapping expression patterns between tissues, suggesting a key role for the circadian clock in controlling tissue-specific function in vivo" (2013). All these preliminary considerations are nothing more than speculation since we are far from knowing at least the CR of proliferation of the most common malignancies in man. Perhaps if we could correlate our results in mice (with nocturnal habits) and we can transpose this to humans (with diurnal habits) we can suppose that the most important DNAs activity probably appears during diurnal moment for the implementation of anticancer drugs, to increase its positive effects. In addition, we must mention that "The

changes in lifestyle are coupled with a significant increased in the risk of deseases in all aspects of human health, including cáncer" (Loning and Kettner 2013).

If we knew the proliferative rate of each specific kind of tumor we could apply the principle of the chronopharmacology, which propose that the circadian rhythms can affect the effects of drugs, improving their benefits or increasing its adverse effects (Nakahata 2007). This would lead us to a significant improvent in the treatment and control of the cancer. Therefore, to increase the maximum efficiency of attack against tumor and protect normal host tissue are the biggest challenges for successful anticancer treatment (Loning and Kettner 2013).

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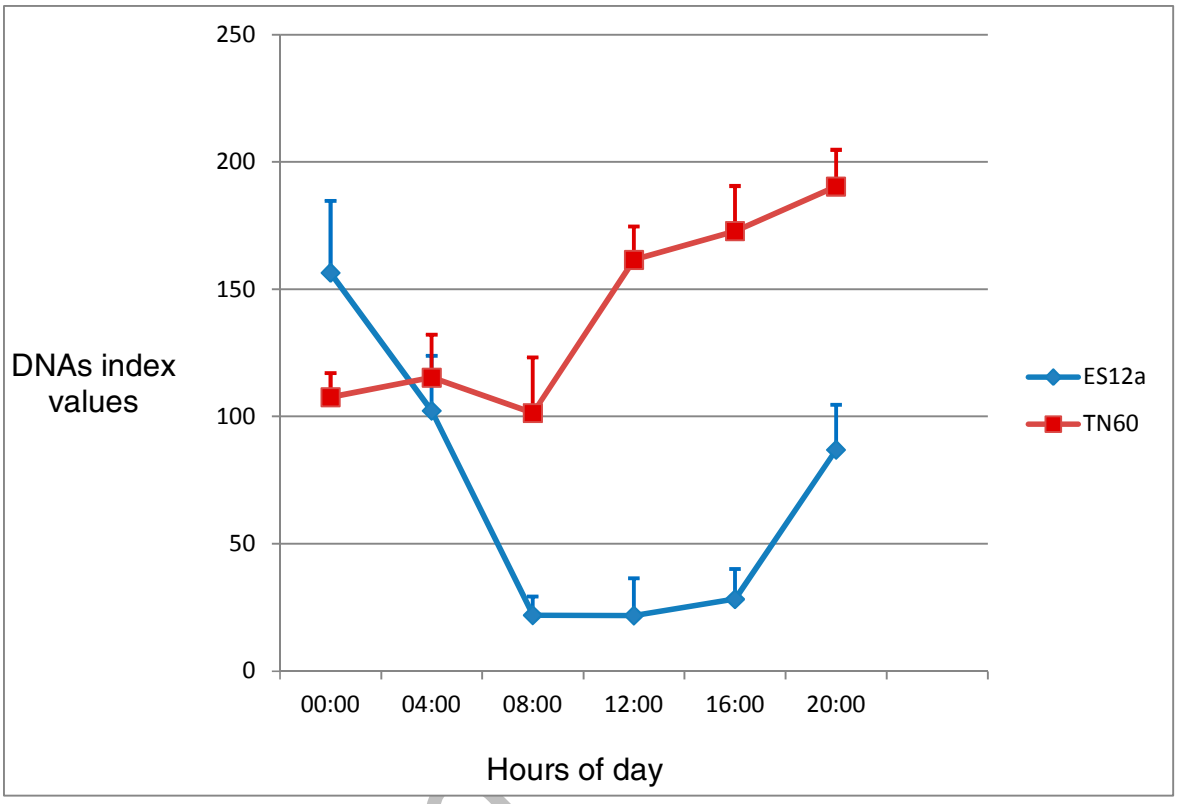

Fig. 1: Circadian curve of DNAs values of TN60 and ES12a in 24 hours.

Table 1: DNAs values along a circadian time span for ES12a and TN60 carcinoma.

DNAs index			
HD	**ES12a**	**TN60**	
	X ± ES (n)	**X ± ES (n)**	
00:00	1**56.5** ± 28.3 (8)	107.6 ± 9.5 (4)	
04:00	102.3 ± 21.6 (6)	115.3 ± 16.9 (6)	
08:00	22.0 ± 7.4 (5)	101.4 ± 21.9 (5)	
12:00	21.9 ± 14.6 (5)	161.6 ± 13.1 (6)	
16:00	28.3 ± 11.9 (6)	173.0 ± 17.7 (6)	
20:00	86.9 ± 17.7 (7)	**190.4** ± 14.4 (5)	
X	69.6 ± 20.7 (37)	144.8 ± 9.7 (32)	(p= 0.0073)

HD: hour of day
X: mean
ES: Standard error
n: number of animals
P: probability