

Nanodoses of melatonin induces apoptosis on human breast cancer cells co-cultured with colostrum cells

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ABSTRACT

The aim of this study was to investigate the effect of melatonin nanodoses adsorbed to PEG microspheres during different phases of the day on oxidative stress and apoptosis in human colostrum mononuclear (MN) cells co-cultured with breast cancer tumor cells (MCF-7). The MCF-7 cells were obtained from the American Type Culture Collection, and the MN cells were obtained from volunteer donors. The cells were preincubated for 24 h with or without 100 ng.mL⁻¹ melatonin (MLT), PEG microspheres (PEG) or 100 ng.mL⁻¹ MLT adsorbed to PEG microspheres (PEG-MLT). Superoxide, superoxide dismutase (SOD), intracellular calcium and apoptosis in the MN cells, MCF-7 cells and co-cultures were determined. The MN cells and co-cultured cells during the nocturnal period and the MCF-7 cells during the diurnal period exhibited increased superoxide release in the presence of PEG-MLT. MN cells treated with MLT during both periods of the day exhibited the highest SOD concentrations, whereas the MCF-7 cells had high SOD levels when incubated with PEG-MLT during the nocturnal period. The nocturnal period co-culture, independent of treatment, showed the highest levels of the enzyme. The highest amount of intracellular Ca²⁺ release was observed in MN cells and MCF-7 cells co-cultured with PEG-MLT during the nocturnal period. Irrespective of the phase of the day, the highest apoptosis index was observed in co-cultures of cells incubated with MLT-PEG. These data suggest that nanodoses of melatonin-modified release plus physiological melatonin produced in higher concentrations during the night can increase the effective activity of this hormone against tumors.

Keywords: Breast cancer; drug delivery; melatonin; MN cells; MCF-7.

1. INTRODUCTION

Clinical and experimental evidence has supported the hypothesis that colostrum is important in the protection against breast cancer [1] and can reduce the risk of disease in women who breastfeed [2,3]. Colostrum contains soluble and cellular components, such as lipids, carbohydrates, cytokines, viable leukocytes [particularly macrophages] [4], and hormones, which are important for immune defense [5] and exhibit a circadian rhythm [6].

Melatonin, one of the hormones present in milk that is produced by the pineal gland [7] exhibits a pronounced circadian rhythm, has an important role in the control of the sleep-wake cycle, and is directly connected to the regulation of several neuroendocrine axes [8].

Many of the benefits of melatonin and its metabolites are related to their antioxidant, anti-inflammatory [9,10] and pro-oxidative effects [11]. Oxidative stress, chronic inflammation and cancer are closely linked. Studies have shown the action of melatonin against free radicals [8]. It is known that oxidative stress can lead to chronic inflammation, which in turn can mediate most chronic diseases, among them cancer [12].

Melatonin has anti-tumor effects on human MCF-7 breast cancer cells, being able to increase apoptosis in these cells, likely because of modifications to intracellular Ca²⁺ release [13]. However, the oral bioavailability of this hormone is lower due to hepatic metabolism and variable rates of absorption [14-16]. Thus,

PEG-drug conjugates are being used as possible modified release systems for a variety of molecules and drugs [17-21].

The administration of drugs adsorbed to PEG microspheres has been an alternative treatment for a number of diseases [22], including breast cancer. Polymeric systems with melatonin can prevent the degradation hormone and increase of bioavailability within an organism, decrease degradation via metabolic enzymes and reduce or eliminate the immunogenicity of proteins [18,23]. The PEG microspheres when combined with melatonin, has shown to reduce cell viability and to induce apoptosis in breast cancer tumor cell lines [13].

Additionally, regarding disease infections, studies have reported different effects of melatonin depending on the phase of day [5,6] and on the actions associated with PEG microspheres in human colostrum phagocytes [24]; despite the influence of this hormone on anti-tumor mechanisms, the effects of interactions of colostrum MN cells and melatonin during different phases of the day on tumor cells have not yet been elucidated.

The action of melatonin on human cells suggests that this hormone exerts effects differently depending on time. Determining the best time for the actions of this hormone is important in order to allow adequate treatment, and this information can be the basis of possible therapeutic techniques. Additionally, the immunomodulatory potential of melatonin on colostrum cells in the initiation of tumor cell killing should be

considered in order to clarify its possible use as a treatment for breast cancer. The objective of this work was to analysis the effect of melatonin adsorbed to PEG microspheres on oxidative stress

2. MATERIALS AND METHODS

2.1. Subjects.

This cross-sectional study evaluated 60 mothers clinically healthy who had no diagnosed diseases, such as hypertension and diabetes, and who reported not consuming alcoholic beverages or tobacco at the Municipal Hospital of Barra do Garças, Mato Grosso, Brazil.

2.2. Polyethylene glycol [PEG] microsphere preparation.

Polyethylene glycol [PEG] 6000 was used for produce microsphere according to previously described protocol [19, 25]. 20 g of PEG 6000 was resuspended in 100 mL of a 2% sodium sulfate solution in phosphate-buffered saline [PBS] and incubated at 37°C for 45 min. After period, the PEG microspheres were diluted 3:1 in PBS and washed twice in PBS [500 x g, 5 min]. The PEG microspheres were resuspended in PBS. The formation of the microspheres was thermally induced by subjecting the solution to 95°C for 5 min. For adsorption, the suspensions of PEG microspheres in PBS were incubated with MLT [Sigma, St. Louis, USA; concentration 100 ng/mL] at 37°C for 30 minutes. After this period the PEG microspheres with adsorbed MLT was washed twice in PBS [500 x g, 5 min]. The loading efficient of adsorption the PEG microspheres with adsorbed melatonin [MLT] were then analyzed by fluorescence microscopy using a fluorescently labeled with a solution of Dylight-488 [Pierce Biotechnology, Rockford, USA; 10 µg/mL] [24].

2.3. Obtaining colostrum samples and colostrum cells separation.

Colostrum from each woman was collected in sterile plastic tubes. The collection of colostrum from the lactating participants was performed following the protocol developed by França et al. [6]. Two breast milk samples were collected [diurnal and nocturnal] from each mother at 3 days postpartum [colostrum] for a total of 120 samples.

The samples were centrifuged [160 x g, 4°C] for 10 min. Cells were separated by a Ficoll-Paque gradient [Pharmacia, Upsala, Sweden]. The cells were adjusted at a final concentration of 2×10^6 cells/ml.

2.4. Colostrum MN cells treatment.

Colostrum MN cells [diurnal and nocturnal collection] were pre-incubated for 24 h with or without 50 µL of melatonin [MLT -100 ng/mL final concentration] [5], 50 µL of polyethylene glycol [PEG] microspheres or 50 µL of melatonin adsorbed in PEG microspheres [PEG-MLT - 100 ng/mL final concentration]. Next, the cells were diluted in RPMI 1640 medium supplemented with 10% fetal bovine serum [FBS] [Sigma, St. Louis, MO, USA], penicillin [20 U/mL] and streptomycin [20 µg/mL] [Sigma, St. Louis, MO, USA] at 37°C for 24h in a humid atmosphere containing 5% CO₂.

2.5. MCF-7 cell culture.

Human breast cancer cells (MCF-7) were acquired from American Type Culture Collection [ATCC, USA]. The cells cultures were sustained in 75 cm² plastic culture flasks in RPMI 1640 medium supplemented with 10% fetal bovine serum [FBS]

and apoptosis in human colostrum MN cells co-cultured with breast cancer tumor cell lines [MCF-7].

[Sigma, St. Louis, MO, USA], penicillin [20 U/mL] and streptomycin [20 µg/mL] [Sigma, St. Louis, MO, USA] at 37°C in a humid atmosphere containing 5% CO₂. The cells were subcultured every 5±2 days.

2.6. MCF-7 Cell treatment.

Subconfluent [MCF-7 cells were treated with of trypsin [Sigma, St. Louis, USA] and adjusted for 5×10^5 cells/mL. Then, the trypsinized cells were pre-incubated for 24h with or without 50 µL of MLT [100ng/mL final concentration], 50 µL of PEG microspheres [PEG] or 50 µL of MLT adsorbed in PEG microspheres [PEG-MLT] [100 ng/mL final concentration]. After, the cells were diluted in RPMI 1640 medium supplemented with 10% fetal bovine serum [FBS] [Sigma, St. Louis, MO, USA], penicillin [20 U/mL] and streptomycin [20 µg/mL] [Sigma, St. Louis, MO, USA] at 37°C for 24 h in a humid atmosphere containing 5% CO₂. The cells were cultured in two periods [diurnal and nocturnal] according to colostrum collection.

2.7. Co-culture of MN cells and MCF-7 cells.

Cell co-culture systems incorporating MN and MCF-7 cells [26] were performed using the same protocol described above.

2.8. Release of superoxide anion.

Superoxide release was determined by cytochrome C [Sigma, ST Louis, USA] reduction [5,27]. Briefly, MN cells, MCF-7 cells and co-culture were treated according to the described above. The suspensions were then placed in PBS with 2.6 mM CaCl₂, 2 mM MgCl₂, and cytochrome C [Sigma, ST Louis, USA; 2 mg/mL]. The suspensions [100 µL] were incubated for 60 min at 37°C on culture plates and were subsequently read at 550 nm, and the results expressed as nmol/O₂⁻. All experiments were performed in duplicate.

2.9. CuZn-superoxide dismutase determination [CuZn-SOD – E.C.1.15.1.1].

Analysis of the CuZn-SOD enzyme in culture supernatant of MN, MCF-7 and co-culture was performed using the nitroblue tetrazolium [NBT] reduction method [Sigma]. The individual samples were placed in glass tubes, with another tube containing a standard solution. Each tube contained 0.5 mL of the sample, and the standard tube contained 0.5 mL of hydro-alcoholic solution. Next, 0.5 mL of chloroform-ethanol solution [1:1 ratio] and 0.5 mL of reactive mixture [NBT increased by EDTA] was added to the tubes. The experimental and standard solutions received 2.0 mL of buffer carbonate, and the pH was increased to 10.2 after the addition of hydroxylamine. The tubes remained still at room temperature for 15 min and were subsequently read at 560 nm [28].

2.10. Intracellular Ca²⁺ release determination.

The MN cells or/and MCF-7 cells were incubated with 5 µL of Fluo3-acetoxymethyl [Fluo3-AM; Sigma, St. Louis, USA - 1µg/mL] for 30 min at 37°C. After period, the cells were washed twice in PBS containing BSA [5mg/mL; 160 x g, 10 min, 4°C] and then analyzed by flow cytometry [FACSCalibur system; BD, San Jose, USA]. Fluo-3 was detected using a 530/30 nm filter for

intracellular Ca^{2+} . The rate of intracellular Ca^{2+} release was expressed as the geometric mean fluorescence intensity of Fluo-3.

2.11. Apoptosis assay.

The cells [MN cells or/and MCF-7 cells] were resuspended in 500 μ L of binding buffer with 5 μ L of annexin V-FITC] and 5 μ L of PI [Annexin V-FITC Apoptosis Detection Kit, AlexisTM, San Diego, USA and then incubated for 10 min at room temperature. As negative and positive controls untreated cells and cells treated with staurosporine [Sigma, St. Louis, USA] were used respectively. The fluorescence of the cells was analyzed by flow cytometry [FACS Calibur system; BD, San Jose, USA]. The

obtained data were analyzed using CellQuest software. The cells were classified as follows: viable cells [annexin⁻/PI⁻], early apoptotic cells [annexin⁺/PI⁻], late apoptotic cells [annexin⁺/PI⁺], and necrotic cells [annexin⁻/PI⁺].

2.12. Statistical analysis.

An analysis of variance [ANOVA] was used to evaluate superoxide release, culture supernatant SOD levels, intracellular calcium and apoptosis index of cells [MN, MCF-7 and MN/MCF-7] in the presence or absence of PEG microspheres with adsorbed melatonin. Statistical significance was considered when $P < 0.05$.

3. RESULTS

3.1. The effect of melatonin adsorbed to PEG microspheres on superoxide release.

Melatonin increased the release of superoxide from colostrum phagocytes compared to the spontaneous release. Additionally, independent of the phases of the day, the phagocytes exposed to melatonin adsorbed to PEG microspheres displayed increased superoxide release when compared to the phagocytes exposed to the PEG microspheres alone. Furthermore, the release of superoxide during the diurnal period decreased significantly in the presence of PEG-MLT (Figure 1A).

The MCF-7 cells showed higher superoxide release when treated with melatonin during both phases of day when compared to untreated cells. Superoxide release during the nocturnal period decreased significantly in the presence of melatonin adsorbed or not to PEG microspheres (Figure 1B).

nocturnal period displayed increased superoxide release (Figure 1C).

3.2. The effect of melatonin adsorbed to PEG microspheres on the superoxide dismutase [SOD] enzyme.

MN cells during both periods of the day exhibited the highest SOD concentrations in culture supernatant when the cells were treated with MLT. The PEG-MLT increased the SOD concentration in the culture supernatant of MN cells during the nocturnal period and decreased the concentration during the diurnal period [Table 1].

MCF-7 cells incubated with MLT and PEG-MLT during the nocturnal period had high SOD concentration, whereas during the diurnal period, the cells treated with PEG-MLT exhibited a decreased concentration. The enzyme concentration in the culture supernatant of the MCF-7 treated with MLT or MLT adsorbed to PEG microspheres was significantly higher during the nocturnal period than during the diurnal period [Table 1].

During the diurnal period, MN cells and MCF-7 cells in co-culture showed lower SOD levels when they were incubated with PEG microspheres. Higher SOD concentrations were observed when the co-culture was treated with MLT adsorbed or not to the PEG microspheres during the nocturnal period (Table 1). A comparison of the periods showed that the MN cells in co-culture with the MCF-7 cells, independent of treatment, showed the highest levels of SOD concentration during the nocturnal period compared to the diurnal period (Table 1).

Table 1. CuZn-superoxide dismutase concentrations in the culture supernatants from MN cells collected during both the diurnal and nocturnal periods, from MCF-7 incubated during the diurnal and nocturnal periods, and from co-cultures [MN cells and MCF-7 cells during both periods].

SOD [IU]	Phases	RPMI1640 Medium	PEG	MLT	PEG-MLT
MN cells	Diurnal	37.5 ± 5.7	24.5 ± 7.9*	41.3 ± 4.4*	21.0 ± 6.0*#
	Nocturnal	19.5 ± 2.1 [†]	19.9 ± 2.7	31.7 ± 3.8* [†]	26.3 ± 3.8*#
MCF-7 cells	Diurnal	35.7 ± 1.8	13.9 ± 3.8*	34.1 ± 3.5	24.5 ± 6.3*#
	Nocturnal	37.6 ± 4.3	38.8 ± 2.2 [†]	45.9 ± 1.5* [†]	49.0 ± 2.5* [†]
Co-culture	Diurnal	14.0 ± 4.1	5.1 ± 1.9*	9.6 ± 1.7	9.8 ± 2.4
	Nocturnal	18.4 ± 5.3	24.2 ± 2.4 [†]	28.1 ± 2.4* [†]	30.2 ± 2.5* [†]

Figure 1. Superoxide release from the colostrum MN cells, MCF-7 cells and co-cultures [colostrum MN and MCF-7 cells]. The results represent the mean ± SD of 10 experiments. RPMI1640 medium; polyethylene glycol [PEG] microspheres; melatonin [MLT]; melatonin adsorbed to the PEG microsphere [PEG-MLT]. * $p < 0.05$: comparing spontaneous release from non-treated cells to cells treated with MLT or PEG-MLT, considering the same collection period [diurnal or nocturnal]; # $p < 0.05$: comparing MLT and PEG-MLT, considering the same collection period [diurnal or nocturnal]; [†] $p < 0.05$ comparing superoxide release between the different collection periods [diurnal or nocturnal], considering the same treatment.

Melatonin increased the release of superoxide by cells in co-culture. The MN cells in co-culture with the MCF-7 cells exposed to melatonin adsorbed to PEG microspheres during the

International units [IU]; RPMI1640 medium; polyethylene glycol [PEG] microsphere; melatonin [MLT]; melatonin adsorbed to the PEG

microspheres [PEG-MLT]. The results represent the mean ± SD of 10 experiments. *p<0.05: comparing non-treated cells to cells treated with MLT or PEG-MLT, considering the same collection period [diurnal or nocturnal]; #p<0.05: comparing MLT and PEG-MLT, considering the same collection period [diurnal or nocturnal]; †p<0.05 comparing superoxide release between the different collection periods [diurnal or nocturnal], considering the same treatment.

3.3. The effect of melatonin adsorbed to PEG microspheres on intracellular Ca²⁺ release.

Table 2 shows the rate of intracellular Ca²⁺ release in MN cells, MCF-7 cells and co-culture of MN and MCF-7 cells. The PEG microspheres reduced the release of intracellular Ca²⁺ from the MN cells during the diurnal period. When these cells were incubated with MLT, independent of the phases of day, they showed higher intracellular Ca²⁺ release than the non-treated cells did. The highest intracellular Ca²⁺ release was observed in MN cells treated with MLT during the diurnal period. The MLT adsorbed to PEG microspheres increased intracellular Ca²⁺ release in the MN cells during the nocturnal period (Table 2).

MCF-7 cells exhibited low spontaneous intracellular Ca²⁺ release. When these cells were incubated with MLT or MLT adsorbed to PEG microspheres during the diurnal period, they exhibited increased intracellular Ca²⁺ levels (Table 2).

In co-culture, independent of the day period, the cells showed higher intracellular Ca²⁺ release when they were incubated with MLT adsorbed or not to PEG microspheres. The highest intracellular Ca²⁺ was observed during the nocturnal period when the cells were treated with MLT adsorbed or not to PEG microspheres (Table 2).

Table 2. Release of intracellular Ca²⁺ from the MN cells, MCF-7 cells and co-cultures [MN cells Intracellular Ca²⁺ release is represented by mean fluorescence intensity as determined by flow cytometry.

Intracellular Ca ²⁺	Phases	PBS	PEG	MLT	PEG-MLT
MN cells	Diurnal	12.5 ± 5.6	5.7 ± 2.2*	19.5 ± 1.0*	15.6 ± 1.5
	Nocturnal	4.8 ± 2.5 [†]	5.4 ± 2.6	10.9 ± 3.7* [†]	16.3 ± 3.1*
MCF-7 cells	Diurnal	5.6 ± 1.7	6.7 ± 1.5	21.5 ± 3.4*	27.1 ± 8.6*
	Nocturnal	5.4 ± 0.4	6.1 ± 0.4	6.0 ± 0.8 [†]	16.1 ± 3.1* [#]
Co-culture	Diurnal	5.3 ± 1.3	18.6 ± 1.9*	21.7 ± 4.7*	16.4 ± 4.5*
	Nocturnal	6.7 ± 1.3	6.1 ± 1.6 [†]	16.1 ± 1.4* [†]	25.9 ± 1.4* [†]

The results represent the mean ± SD of five experiments. Polyethylene glycol [PEG] microspheres; melatonin [MLT]; melatonin adsorbed to PEG microspheres [PEG-MLT]. Q1: Viable cells [annexin⁻/PI⁻]; Q2 [annexin⁺/PI⁻] and Q3 [annexin⁺/PI⁺]: total apoptotic cells; Q4: necrotic cells [annexin⁻/PI⁺]. *p<0.05: treated cells compared with non-treated cells; #p<0.05: comparing the different treatments [MLT and PEG-MLT]; †p<0.05 comparing different collection period [diurnal or nocturnal], considering the same treatment.

3.4. The effect of melatonin adsorbed to PEG microspheres on apoptosis rates.

To evaluate apoptosis induction in MN cells, MCF-7 cells and co-culture [MN and MCF-7 cells] cells were stained with annexin V and analyzed using flow cytometry [Table 3, Figure 2 and Figure 3].

The apoptosis index of the MN cells was similar when these cells were incubated with PEG but increased in terms of necrosis during the diurnal period. MLT and MLT-PEG, independent of the period of day, increased the apoptosis rates

(Table 3, Figure 2 and Figure 3). A comparison of the periods showed that nocturnal MN phagocytes treated with MLT or PEG-MLT exhibited decreased necrosis in the MN cells (Table 3).

The apoptosis rate was lower in the non-treated MCF-7 cells than in the treated MCF-7 cells. The PEG microspheres increased apoptosis in MCF-7 cells during the diurnal period and necrosis during both periods. The apoptosis rates, independent of the phase of day, increased in the cells that were treated with MLT, and necrosis increased during the diurnal period. MCF-7 treated with PEG-MLT exhibited increased apoptosis during both periods and increased necrosis during the diurnal period [Table 3]. A comparison of the periods in regard to the non-treated MCF-7 showed higher apoptosis rates during the nocturnal period than during the diurnal period [Table 3, Figure 2 and Figure 3]. When these cells were treated with MLT adsorbed or not, they showed decreased necrosis during the nocturnal period [Table 3].

Table 3. Apoptosis [%] and necrosis [%] of colostrum Mononuclear [MN] Cells, MCF-7 Cells and co-culture of colostrum MN Cells and MCF-7 Cells in the presence of MLT adsorbed to PEG microspheres.

Cells	Phases	Viables Q1	Apoptosis [Q2+Q3]	Necrosis Q4
MN	Diurnal	92.9 ± 3.6	6.7 ± 3.7	0.4 ± 0.2
	Nocturnal	85.1 ± 5.2	7.3 ± 1.5	0.8 ± 0.1
MN +PEG	Diurnal	80.7 ± 2.5	9.2 ± 2.9	10.2 ± 1.8*
	Nocturnal	86.7 ± 4.9	6.2 ± 3.8	0.9 ± 0.4 [†]
MN +MLT	Diurnal	84.2 ± 2.3	13.9 ± 2.1*	2.0 ± 0.3*
	Nocturnal	81.0 ± 4.7	16.1 ± 4.8*	0.6 ± 0.4 [†]
MN+PEG-MLT	Diurnal	83.1 ± 8.9	18.9 ± 7.7*	3.0 ± 0.9*
	Nocturnal	82.0 ± 3.8	16.5 ± 2.7*	0.3 ± 0.1 [†]
M CF-7	Diurnal	97.3 ± 0.8	0.4 ± 0.1	2.3 ± 0.4
	Nocturnal	63.5 ± 6.7 [†]	15.3 ± 0.7 [†]	2.4 ± 1.2
MCF-7+PEG	Diurnal	80.6 ± 0.9	4.9 ± 1.4*	14.5 ± 0.9*
	Nocturnal	57.9 ± 8.9 [†]	15.8 ± 3.2 [†]	6.2 ± 0.4* [†]
MCF-7+MLT	Diurnal	58.8 ± 3.1*	36.0 ± 2.8*	5.2 ± 3.2*
	Nocturnal	40.4 ± 8.5 [†]	35.1 ± 9.5*	1.0 ± 0.5 [†]
MCF-7+PEG-MLT	Diurnal	31.7 ± 3.2* [#]	57.2 ± 6.4* [#]	6.0 ± 1.1* [#]
	Nocturnal	51.1 ± 5.9* [#]	47.5 ± 4.3 [†]	0.7 ± 0.4* [†]
MN+ MCF-7	Diurnal	65.7 ± 7.9	24.4 ± 7.7	9.9 ± 1.6
	Nocturnal	56.7 ± 10.0	20.9 ± 4.1	4.5 ± 2.0 [†]
MN+ MCF-7+PEG	Diurnal	60.1 ± 2.7	30.7 ± 5.9	9.2 ± 2.8
	Nocturnal	52.9 ± 10.4	18.9 ± 4.0 [†]	8.2 ± 1.6
MN+ MCF-7+MLT	Diurnal	50.6 ± 6.7	40.6 ± 6.5*	8.9 ± 2.7
	Nocturnal	56.7 ± 6.2	48.3 ± 1.6*	4.9 ± 0.8 [†]
MN+MCF-7+PEG- MLT	Diurnal	33.6 ± 4.7* [#]	60.7 ± 3.6* [#]	5.8 ± 1.5
	Nocturnal	33.0 ± 20.9* [#]	62.8 ± 20.7* [#]	2.5 ± 0.4 [#]

The results represent the mean ± SD of five experiments. Polyethylene glycol [PEG] microspheres; melatonin [MLT]; melatonin adsorbed to PEG microspheres [PEG-MLT]. Q1: Viable cells [annexin⁻/PI⁻]; Q2 [annexin⁺/PI⁻] and Q3 [annexin⁺/PI⁺]: total apoptotic cells; Q4: necrotic cells [annexin⁻/PI⁺]. *p<0.05: treated cells compared with non-treated cells; #p<0.05: comparing the different treatments [MLT and PEG-MLT]; †p<0.05 comparing different collection period [diurnal or nocturnal], considering the same treatment.

In general, the apoptosis rate in the co-cultures of MN cells and MCF-7 cells was increased [Figure 2 and Figure 3]. During the diurnal period, the co-culture of cells with MLT and PEG-MLT increased the apoptosis rates during both of the periods that were evaluated. Irrespective of the phase of day, the highest apoptosis index was observed in the co-cultures of cells incubated with MLT-PEG microspheres. The PEG-MLT decreased the necrosis index in the co-culture of cells during the nocturnal period [Table 3]. Co-culture in the presence of MLT exhibited lower necrosis during the nocturnal period than during the diurnal period [Table 3].

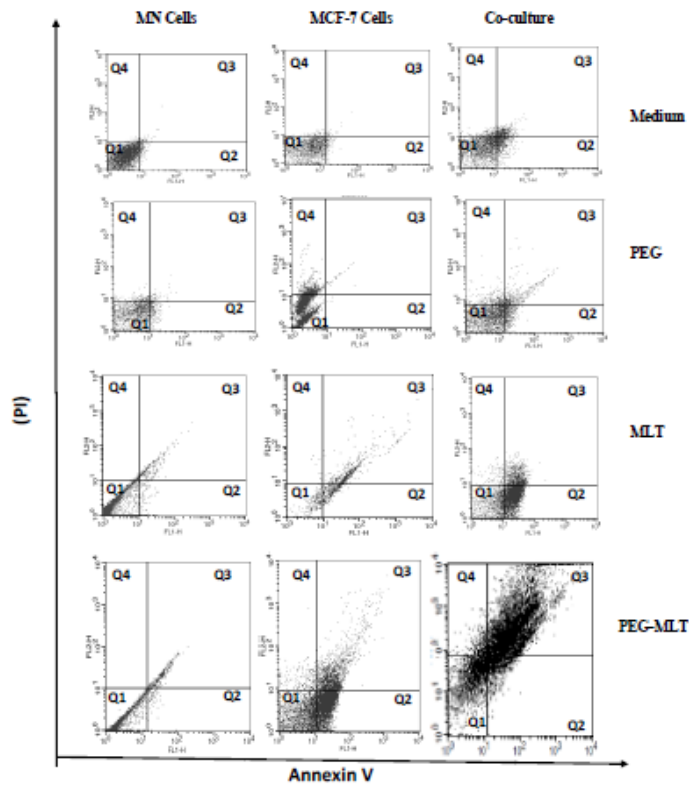


Figure 2. Apoptosis in MCF-7 human breast cancer cells in co-culture of diurnal colostrum MN cells in the presence of PEG microsphere adsorbed with melatonin. Modes of cell death were using flow cytometry with annexinV/PI staining. The summation of the upper-right [Q3] and lower-right [Q2] quadrants is presented as the percentage of total apoptosis. The upper-left [Q4] quadrant is the percentage of necrosis and lower-left [Q1] quadrant corresponds to viable cells. Data are representative of an experiment with the different treatments.

Discussion.

Breast milk itself undergoes chronobiological changes in cell immunity. The composition of breast milk contains several factors that interfere with human health and may alter disease progression as well as its longevity [29]. Nutrition and some nutrients can influence the immune system and regulate directly by signaling pathways, as well as regulating hormones that, in turn, modulate the immune system. [30]. In this study, melatonin adsorbed to PEG microspheres exhibited anti-tumor activity against MCF-7 human breast cancer cells, especially when in co-culture with colostrum MN cells, and this activity oscillated as a function of the time of day.

Polymeric matrices associated with hormones have increased the possibility of obtaining new drugs to activate cells

and act on tumor cells. The generation of free radicals is an important mechanism of protection for the organism [31,32]. In this work, we showed that both the release of superoxide from colostrum MN phagocytes and the SOD concentration increased during both phases of day in association with melatonin. The adsorption of melatonin into PEG increased superoxide and SOD, and this effect was more evident during the nocturnal period than during the diurnal period, suggesting a balance in the mechanisms of cellular oxidative stress. It was interesting that the MCF-7 cells treated with MLT or PEG-MLT exhibited an increase in superoxide release during the diurnal period, while higher concentrations of SOD were observed during the nocturnal period. Most likely, this imbalance in cellular oxidative metabolism may be associated with tumor cell survival. Similar results with other cell lines also suggested the involvement of an oxidative imbalance in target cells [33].

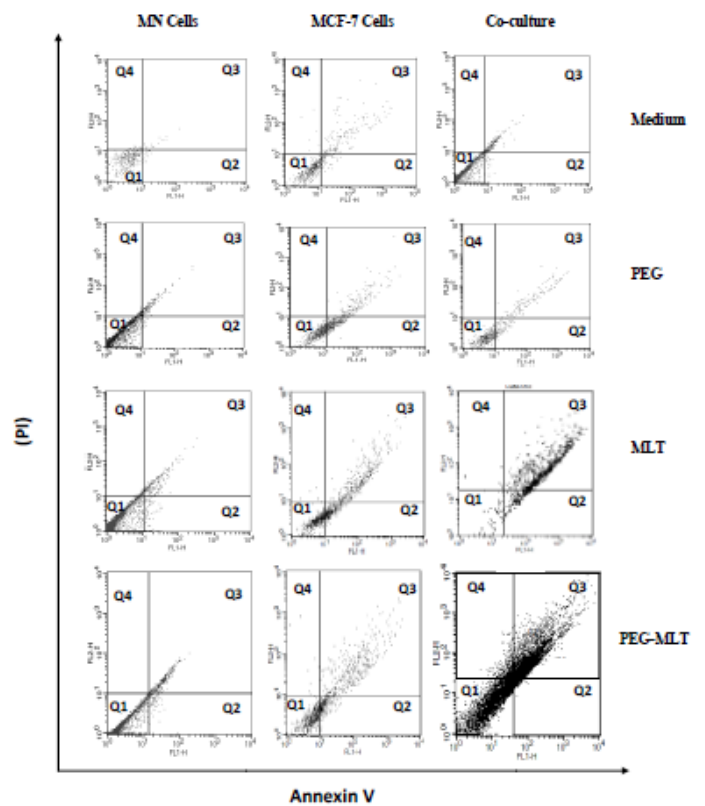


Figure 3. Apoptosis in MCF-7 human breast cancer cells in co-culture of nocturnal colostrum MN cells in the presence of PEG microsphere adsorbed with melatonin. Modes of cell death were using flow cytometry with annexinV/PI staining. The summation of the upper-right [Q3] and lower-right [Q2] quadrants is presented as the percentage of total apoptosis. The upper-left [Q4] quadrant is the percentage of necrosis and lower-left [Q1] quadrant corresponds to viable cells. Data are representative of an experiment with the different treatments.

In co-culture, the cells treated with PEG-MLT exhibited an increase in superoxide release and the highest levels of SOD concentration during the nocturnal period, suggesting that the antioxidant system presents variations depending on the phase of day and that variations in the oxidative mechanisms of colostrum MN cells remain, and most likely, the maintenance of this oxidative balance may be auxiliary in the control of tumor cells. Studies have reported that the effects of melatonin administration are different depending on whether the pineal gland is present and have suggested that the presence of a daily rhythm in terms of

melatonin may affect the sensitivity of specific target tissue to this hormone and may be most effective during the nocturnal period because tissues are most sensitive to the hormone at this time [34]. In this study, we confirmed that the effects of melatonin adsorbed to PEG microsphere on oxidative metabolism in MN cells were more evidence during the nocturnal period.

Previous studies showed that, in MCF-7 cell cultures, periodic exposure to melatonin was as effective as continuous exposure in terms of antiproliferative effects, although the amount of melatonin and the time of cell exposure to the hormone were obviously half when under pulsatile exposure [34]. Here, considering the phases of day, we observed that, in co-culture rather than in isolated cells, the treatment of melatonin adsorbed to PEG microspheres was capable of acting on the oxidative cellular mechanisms.

Some studies of free radical regulatory mechanisms have suggested that superoxide dismutase plays a protective role [20]. However, the fact that melatonin follows a day-night variation makes it an important immunomodulatory agent that can improve cell activation processes. Melatonin has an influence on the activity and the expression of superoxide dismutase, under both physiological and elevated oxidative stress [31], and acts on scavenged free radicals [35], suggesting that the actions of melatonin can be considered in support of its possible antitumoral effects.

An important mechanism for protecting the body in various processes has been attributed to the free radical generation [36,37] and physiological signaling pathways [38]. Excessively high levels of free radicals cause damage to cellular proteins, membrane lipids and nucleic acids [39] and increase DNA damage and ATP depletion, leading to the induction of apoptosis [40,41]. Cancer development and progression is characterized by dynamic changes in the expression and function of protein kinases [42] or their signaling pathways, which can lead to malignant transformation of breast cells [43]. Studies have reported that a modified release system with antibodies [44] or melatonin [13] are capable of activating the signaling pathways involving calcium intracellular mechanisms and of inducing apoptosis in breast cancer cells.

In this study, the intracellular calcium showed chronobiological variation in the different cells evaluated. In MN cells, melatonin increased the intracellular calcium during both periods. In the modified release system, this hormone was capable

of increasing the calcium during the nocturnal period. This variation was different in the MCF-7 cells since high levels of intracellular calcium were observed during the diurnal period, suggesting modified actions of melatonin on the MCF-7 cells. Melatonin introduced to the co-culture increased intracellular calcium release with a profile similar to that of the MN cells, which can be interpreted as immunostimulatory action towards immune cells in co-culture with malignant cells.

Changes in intracellular Ca²⁺ release by human cells may result in cell damage and activation of cell death pathways [45], and the release of large amounts of intracellular Ca²⁺ has been associated with induction of apoptosis in human cells. [13, 44, 46]. In this work, independent of the phases of day, the highest levels of apoptosis were observed in cells in the presence of melatonin adsorbed to the PEG microsphere, and the most expressive apoptosis was observed in the co-cultures. The MN cells, despite the increase in apoptosis in the presence of the treatment, maintained viability indices regardless of the time of day, specifically above 80%. Interestingly, during the nocturnal period, a lower index of necrosis was observed, which may be associated with the effects of endogenous melatonin. Further studies are needed to confirm the involvement of endogenous melatonin in the process of necrosis.

The role of melatonin as an antiproliferative agent in breast cancer has been extensively studied, showing that melatonin reduces the invasive and metastatic properties of MCF-7 cells [47,48]. Studies have shown that melatonin decreased cell proliferation and increased expression of p53 and p21 proteins in MCF-7 cells [49] which are proteins that are capable of inducing apoptosis [49].

The importance of the period of administration and of the use of the modified system of melatonin for the development of future anti-tumor therapies should be considered since the PEG microsphere system increases the bioavailability of hormones in terms of both concentration and time [13].

In the present study, the PEG microspheres were shown to be an important melatonin vehicle that was capable of improving immune responses through the activation of colostrum cells to act on breast tumor cells. This polymer has important properties that modulate and prolong the action of melatonin when this drug is administered orally, and it seems to be more effective during the nocturnal period, particularly under the activation of antioxidant systems.

4. CONCLUSIONS

In conclusion, these data suggested that a melatonin-modified release and, most likely, physiological melatonin produced in higher concentrations during the nocturnal period can potentialize the activity of this hormone in being more effective against tumors. In addition, an increase in apoptosis and the imbalance in oxidative status in the cancer cells were observed, as a result of the immunomodulatory effect of melatonin in co-

cultures of colostrum cells and human breast cancer cells. These results implied that chronobiological variations in soluble and cellular components present in the colostrum can act as anti-tumor agents during different phases of the day. However, clinical tests during the nocturnal period must be performed before application of melatonin as a supplementary agent for therapeutic use in order to confirm the efficacy of this treatment.

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