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Cell death induced by tamoxifen in human blood lymphocytes cultivated *in vitro*

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ABSTRACT. Many chemotherapeutic agents with a potential against solid tumors or leukemia can cause lymphopenia. Tamoxifen (TAM) is a synthetic non-steroidal anti-estrogen drug employed in female breast cancer treatment. The present study investigated the capacity of TAM to induce cell death in human lymphocytes cultivated *in vitro*. Lymphocytes were obtained from young (25-30 years; n = 3) and elderly women (58-77 years; n = 3) and cultivated for 24 or 48h, with or without TAM (20 µM). After the culture, cell viability, immunocytochemical response and ultrastructure were evaluated. TAM affected lymphocytes in a time- dependent manner, and cells obtained from elderly women were the most sensitive to TAM. Immunocytochemical analysis evidenced higher frequency of apoptosis in treated cells, and the ultrastructural study revealed autophagic vacuoles, differing from the controls. In summary, the treated lymphocytes were affected by TAM, leading to cell death by apoptosis and autophagy.

Key words: apoptosis, autophagy, chemotherapeutic, cytotoxicity, morphology, ultrastructure.

RESUMO. Morte celular induzida pelo tamoxifeno em linfócitos humanos cultivados *in vitro*. Muitos agentes quimioterápicos com potencial contra tumores sólidos ou leucemias podem causar linfopenia. O Tamoxifeno (TAM) é um agente antiestrogênio não-esteroidal empregado no tratamento de câncer de mama feminino. O presente trabalho investigou a capacidade do TAM em induzir morte celular em linfócitos humanos cultivados *in vitro*. Os linfócitos foram obtidos de mulheres jovens (25-30 anos; n = 3) e idosas (58-77 anos; n = 3) e cultivados por 24 ou 48h, com ou sem TAM (20 µM). Após a cultura, foram analisadas a viabilidade celular, a resposta imunocitoquímica e a ultraestrutura. Os resultados indicam que o Tamoxifeno induziu morte celular em linfócitos de ambos os grupos, entretanto, as células das mulheres idosas apresentaram-se mais sensíveis ao tratamento. A análise imunocitoquímica mostrou maior frequência de apoptose nas células tratadas e o estudo ultraestrutural revelou vacúolos autofágicos nos linfócitos expostos ao Tamoxifeno. Em conclusão, nosso estudo revelou que o TAM induziu morte celular por apoptose e autofagia.

Palavras-chave: apoptose, autofagia, quimioterápico, citotoxicidade, morfologia, ultraestrutura.

Introduction

Some drugs have a chemotherapeutic potential in relation to tumors or leukemia, due to reduced cell proliferation and a higher cell death rate. However many of these agents, despite their therapeutic potential, can also present severe cytotoxic effects in normal tissues, which lead to side effects observed during chemotherapy, such as mucositis, hair loss, myelosuppression. Moreover, chemotherapy can induce acute lymphopenia and chronic depletion of CD4 cells, resulting in a higher susceptibility to opportunistic infections (STAHNKE et al., 2001). In addition, these side effects seem to be more severe in the elderly patient population (BALDUCCI; CORCORAN, 2000; LICHTMAN; VILANI, 2000). Tamoxifen (TAM) ((Z)-1-[4-[2(dimetilaminoetoxi) fenil]-1,2-difenil-

1-butenol) is a synthetic non-steroidal anti-estrogenic drug widely used as a breast cancer chemotherapy drug for more than 30 years. Nevertheless, TAM is not only an anti-estrogenic agent, because it also has estrogenic properties, depending on the species, tissue and gene considered (OSBORNE et al., 2000). Recent studies indicated that the estrogenic action of TAM can cause endometrial cancer as a serious side effect in postmenopausal patients (AYCART et al., 2005; SHAHI et al., 2008). In pre-menopausal patients, some effects are similar to menopausal symptoms (CLEMONS et al., 2002; NYSTEDT et al., 2003). White (2003) stated that the low dose of tamoxifen used therapeutically in women, can be associated to a possible risk of liver cancers. Tamoxifen can also cause damage to the eyes (OMOTI; OMOTI, 2006; BAGET-BERNALDIZ et al., 2008).

This drug also has been indicated as an agent that induces chromosome aberrations in several cell types (SARGENT et al., 1996; STYLES et al., 1997; MIZUTANI et al., 2004), hepatotoxicity in animals (HARD et al., 1993; ALBUKHARI et al., 2009) and mutations in the lac I gene in transgenic rat livers (STYLES et al., 2001). Some studies also suggested that TAM can lead to DNA damage in many animal organs (DAVIS et al., 1998; CARTHEW et al., 2001) and in human leukocytes, both *in vivo* (HEMMINKI et al., 1997) and *in vitro* (HEMMINKI et al., 1995; WOZNIAK et al., 2007). In addition, *in vitro* studies indicated that tamoxifen enhances the apoptotic effect of cisplatin on primary endometrial cell culture (DRUCKER et al., 2003).

Tamoxifen in micromolar concentrations presents cytotoxic activity not mediated by estrogen receptors in some tumoral and non tumoral cell types, including blood cells, such as neutrophils (JAN et al., 2000). Studies using spleen cell culture pointed out that TAM caused the suppression of lymphocyte mitogenesis, indicating that TAM can be an immunosuppressive agent (BARAL et al., 2000).

Based on these facts, this study was undertaken to investigate the capacity of TAM to induce cell death in human lymphocytes cultivated *in vitro*, as well as the early ultrastructural modifications involved in this process.

Material and methods

Cells

Lymphocytes were obtained from samples of peripheral blood, obtained by venipuncture with an anticoagulant (EDTA) added. Three volunteers for each group of women (group A = 25-30 years old (n = 3) and group B = 58-77 years old (n = 3)) were used (project approved by Ethics in Research Committee of the Medical Sciences College /UNICAMP). Twenty milliliters of blood sample were centrifuged in a conical centrifuge tube for 15 minutes (1,300 g) to deposit erythrocytes. The interface between plasma and erythrocytes was carefully transferred using a pipette into another centrifuge tube with as few erythrocytes as possible, and centrifuged in a Percoll density gradient (Amersham Pharmacia Biotech) for 30 minutes (660 g), to separate blood cell types. The layer containing mononuclear cells (Percoll-50%-density: 1.06-1.08 g mL⁻¹) was washed twice in Hanks Solution to remove Percoll. These cells were resuspended in RPMI 1640 medium containing antibiotics (streptomycin 10 mg L⁻¹ and penicillin 1000U L⁻¹) and 10% fetal bovine serum (FBS) (Nutricell,

Campinas State São Paulo) and incubated at 37°C for 2 hours, for monocyte adhesion. Cells in the supernatant were diluted to 4.5 x 10⁵ lymphocytes mL⁻¹, and placed in 25 cm³ tissue culture flasks at 37°C.

Tamoxifen

TAM (Sigma) was dissolved in dimethylsulfoxide (DMSO) (Sigma), followed by an appropriate dilution in RPMI 1640, containing 10% (FBS) with a final concentration of 20 µM in the culture. Similar concentrations of DMSO diluted in RPMI were added to the control cultures. All cultures were maintained for 24 or 48 hours. The solvent concentration (DMSO) was less than 0.1%, which does not affect cell viability (BARAL et al., 2000). The concentration of TAM applied was based on previous studies, which revealed the cytotoxicity of this drug even in micro molar concentrations (JAN et al., 2000; MANDLEKAR; KONG, 2001; MAJUMDAR et al., 2001). The treatment period was limited to 24 and 48 hours because primary cell cultures tend to enter a process of cell death over a longer period, which could lead to not-specific phenotypic alterations.

Cell Viability

The viable cell count for all samples was obtained by the exclusion test of intact cells, by using 1% Trypan Blue and establishing the percentage of unstained alive cells for the total of resuspended cells. Cell viability was analyzed after 24 or 48 hours of culture, and counted in a hemocytometer chamber.

Apoptosis Detection

Following the manufacturer's protocol (Oncogene Research Products-Annexin V-Biotin Apoptosis Detection Kit), cells were incubated with annexin-biotin that presents a high affinity for phosphatidylserine, followed by incubation with FITC-conjugated anti-biotin. This procedure was employed to target apoptotic cells that express phosphatidylserine (PS) on the outer leaflet of the plasma membrane. The preparations were analyzed with a Zeiss Axioskop fluorescence microscope equipped with a set of filters for fluorescein. A total of 400 cells were counted for each sample, using low magnification to show a larger number of cells. The number of apoptotic cells from each blood sample was established based on the fluorescent tagging observed with the microscope.

Transmission electron microscopy

The cultured cells were deposited in a pellet by centrifugation, and washed in 0.1 M cacodylate buffer (pH 7.2), with 1.5% saccharose. Cells were fixed in 2.5% glutaraldehyde, 1.25% formaldehyde and 0.03% picric acid solution in 0.1 M cacodylate buffer (pH 7.2), with 1.5% saccharose for 1 hour at room temperature. The fixed cells were washed three times in the same buffer, post-fixed in 1% osmium tetroxide in the same buffer for 1 hour, and washed three times in distilled water. Then, the cells were included in 2% agar, dehydrated with a graded series of acetone solutions, and embedded in Epon. Thin sections of selected areas of the epoxy block were cut with an ultramicrotome using a diamond knife. Sections were mounted on copper grids, stained with alcoholic uranyl acetate and lead citrate, and examined with a Zeiss Leo 906 transmission electron microscope at an accelerating voltage of 60 KV (modified from MATOS et al., 1995).

Statistical Analysis

Statistical analysis was performed using One-Way ANOVA with Tukey's test, considering values of $p < 0.05$ as statistically significant. Data are presented in graphs as mean values \pm sd.

Results

Cell Viability

Statistic analysis indicated a significant difference in cell viability between treated cells and controls. A reduced viability was observed in treated cells from both young and elderly women (Figure 1). Meantime, this occurred after 48 hours contact for young women (group A), while in group B it was significant already after 24 hours. Also, the viability of group B cells was lower than group A in all studied conditions.

Apoptosis Detection

According to Figure 2, the percentage of apoptotic cells, identified by FITC-conjugated biotin, was higher in treated cultures comparing to respective controls, in both groups. We noticed that the group A presented a small increase of cells undergoing apoptosis, when control and treated cells were compared after 24 hours, but after 48 hours, this number was significantly higher. Moreover, group B presented a higher percentage of apoptotic cells, in both treated cultures and controls, when compared to group A,

but significant differences were not found for treated cells, when comparing the two culture periods.

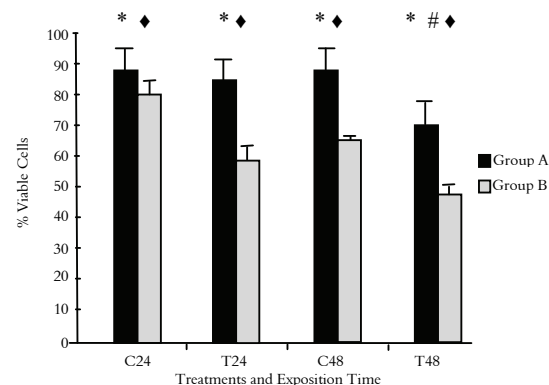


Figure 1. Cell viability of human lymphocytes cultivated *in vitro*, verified by Trypan Blue. Exclusion Test. C = control, T = treatment, A = young women (n = 3), B = elderly women (n = 3), 24 and 48 hours. The comparison between treatments and groups were made through One-Way ANOVA, followed by Tukey's test, with a significance level of 5%. Data are presented as mean values \pm sd. Significant differences are identified by * (comparisons between groups); # (comparisons between the treatments within group A) and ♦ (comparison between the treatments within group B).

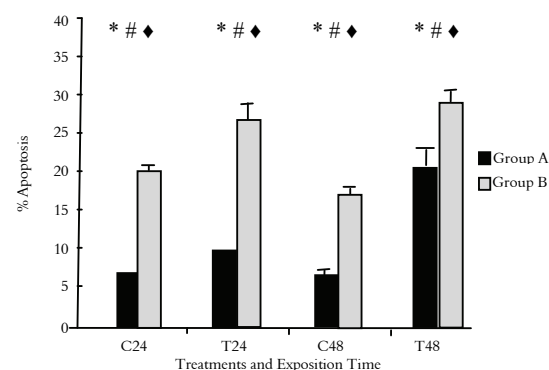


Figure 2. Frequency of apoptosis in human lymphocytes cultivated *in vitro*, verified by fluorescent marking of phosphatidylserine on the outer leaflet of the cell membrane. C = control, T = treatment, A = young women (n = 3), B = elderly women (n = 3), 24 and 48 hours. 400 cells were counted for each sample. The comparison between treatments and groups were made through One-Way ANOVA, followed by Tukey's test, with a significance level of 5%. Data are presented as mean values \pm sd. Significant differences are represented by * (comparison between groups); # (comparison between treatments within group A) and ♦ (comparison between treatments within group B).

Transmission Electron Microscopy

In thin sections of control lymphocytes (Figure 3A and C), spherical cells were observed containing a large nucleus with a pattern of condensed and loose chromatin, occupying most of the cell volume. The membranes are well preserved and, although the cells have only a thin

layer of cytoplasm, they also contain many mitochondria and ribosomes. The treatment with TAM revealed cells with a nucleus with the same condensed and loose chromatin pattern, decreased cell volume, but more organelles are present. Endoplasmic reticulum, a small Golgi complex and some large cytoplasmic vacuoles were found, which possibly represent autophagic vacuoles, as

suggested by the membranous whorls within the vacuoles (Figure 3B and D). Notice that, in these cells (Figure 3B and D), the membranes are well preserved but there are fewer microvilli. We can also observe that after 48 hours of treatment with TAM, there was a loss of the typical spherical cell shape as well as of microvilli, and the flattened nucleus has superficial depressions (Figure 3D).

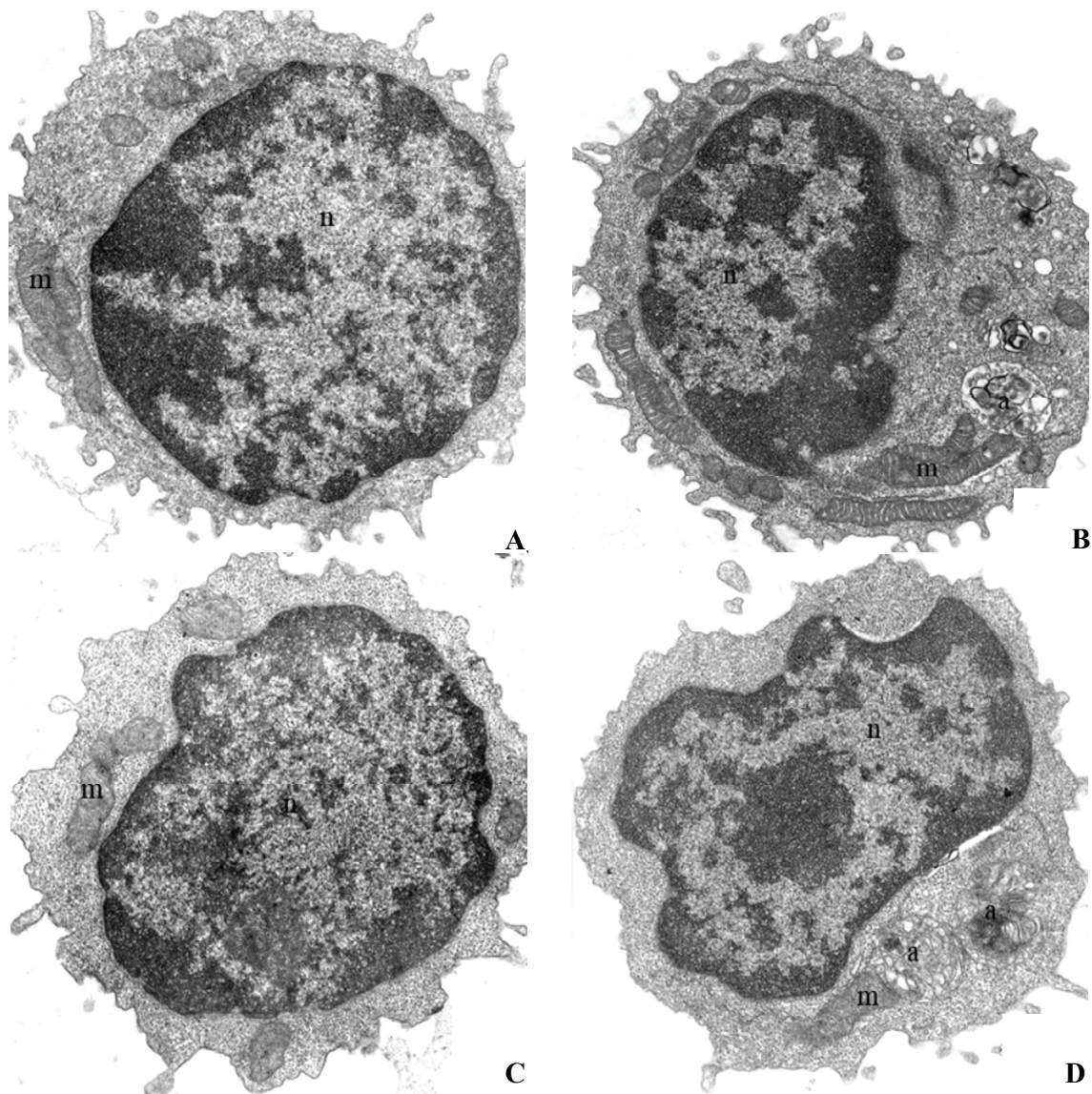


Figure 3. (A) Typical control lymphocyte, maintained for 24h in culture: spherical cells are found containing a large nucleus (n), with condensed and loose chromatin pattern, scant cytoplasm and mitochondria (m) (x 14,500); (B) Lymphocyte treated with 20 μ M of TAM for 24h: the typical cell is spherical, a typical nucleus (n), autophagic-like vacuoles (a) and mitochondria (m) (x 16,500); (C) Control lymphocyte maintained for 48h in culture: cell is spherical, with a typical nucleus, scant cytoplasm and mitochondria (m) (x 15,000); (D) Lymphocyte treated with 20 μ M of TAM for 48h, a group in which almost all cells have lost their spherical shape. The nucleus (n) is also not spherical, with superficial depressions, although the chromatin pattern still has condensed and loose areas; mitochondria (m) and autophagic-like vacuoles, one containing membrane whorls, which may be a degenerating mitochondrion (a) (x 16,500).

Discussion

Tamoxifen has been clinically used as a chemotherapeutic drug against breast cancer, and its potential to induce cell death in various cell types is still unclear. The present study is the first to show the morphological aspects of TAM-induced cell death in human lymphocytes cultivated *in vitro*.

The higher apoptosis rates observed in treated cultures are consistent with previous studies, which affirm that lymphoid cells can undergo apoptosis in response to a variety of stimuli, including chemotherapeutic drugs (FRIESEN et al., 1996). Moreover TAM is cytotoxic at micromolar concentrations (JAN et al., 2000; MANDLEKAR; KONG, 2001), also affecting non breast cancer cells (MAJUMDAR et al., 2001; PETINARI et al., 2004; KIM et al., 2007; GIL-SALÚ et al., 2008). The lower percentage of viable cells and the higher frequency of apoptosis in lymphocytes obtained from elderly women can be associated with the aging of the immune system, which is in functional decline (GRUBECK-LOEBENSTEIN et al., 1998), resulting in a dramatic reduction of responsiveness as well as functional deregulation. Some authors evidenced that lymphocytes of aged persons are more prone to undergo apoptosis, in comparison to lymphocytes of younger people (PAGLIARA et al., 2003). As previously documented, elderly patients are especially prone to increased toxicity due to morbidity and aged physiology. Chemotherapy may have such a strong effect on aged patients that some authors suggested the investigation of multiple organs should be carried out before treatment (SAWHNEY et al., 2005).

Although the data that refers to translocation of PS is consistent with apoptosis, our ultrastructural results are more compatible with autophagy. The large cytoplasmic vacuoles, which are probably autophagic vacuoles, are present only in the treated lymphocytes. This mode of cell death is characterized by massive degradation of cell contents, which includes essential organelles such as mitochondria, by means of complex intracellular membrane vesicle reorganization and lysosomal activity (WANG; KLIONSKY, 2003; GOZUACIK; KIMCHI, 2004). The diminished number of microvilli in treated cells suggests an effort to reduce the cell membrane area in contact with TAM. Previous studies have also shown that Tamoxifen induces signs of autophagy in breast cancer cells and non-breast cancer cells (KLIONSKY et al., 2003; QADIR et al., 2008). Autophagy has been observed

in cells treated with chemotherapeutic drugs other than TAM (KONDO; KONDO, 2006; MORETTI et al., 2007). Some authors showed that apoptosis and autophagy can occur in parallel, as seen in studies with malignant glioma cells, breast cancer cells and neurons (DAIDO et al., 2004; LAMPARSKA-PRZYBYSZ et al., 2005; MATYJA et al., 2005). Moreover, other studies indicate that accumulation of autophagic vacuoles can precede apoptotic cell death (CUI et al., 2007; WANG et al., 2007; OGATA et al., 2008). Recent studies have shown results similar to our observations. In those studies it was demonstrated that, in tumoral lineages with PS translocation, autophagosomes were identified. Previous studies already showed that PS translocation is not an exclusive characteristic of apoptotic cell death, and can be found in necrosis (LECOEUR et al., 2001; KRYSKO et al., 2004). Our data does not present specific ultrastructural aspects of apoptosis, perhaps because culture was restricted to 24–48 hours, and characteristics such as chromatin alterations require a longer period to become ultrastructurally identifiable. However, we observed cells that express phosphatidylserine (PS) on the outer leaflet of the plasma membrane, one of the most important early molecular modifications that can indicate apoptosis (AMARANTE-MENDES; GREEN, 1999).

Based on these results, we may conclude that lymphocytes treated with tamoxifen demonstrated lower cell viability as well as a higher cell death rate than their controls. Also, longer periods of treatment with TAM lead to more affected cells and more morphological changes. Besides that, lymphocytes obtained from elderly women are more likely to undergo apoptosis, in both control and treated cultures. Our data also suggests that apoptosis and autophagic cell death may be parallel events.

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