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I. B.

ASPECTOS MORFOLÓGICOS DA APOPTOSE INDUZIDA PELO TAMOXIFENO EM LINFÓCITOS HUMANOS CULTIVADOS *IN VITRO*

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) Naila Francis Paulo de Oliveira e aprovada pela Comissão Julgadora.

Tese apresentada ao Instituto de Biologia para obtenção do Título de Mestre em Biologia Celular e Estrutural na área de Biologia Celular.

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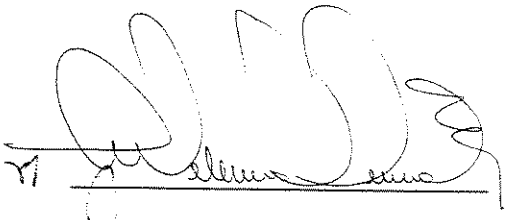
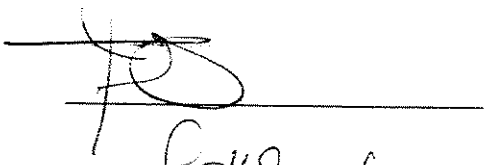
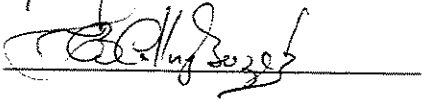
Profa. Dra. Mary Anne Heidi Dolder (Orientadora) 

Profa. Dra. Fátima Aparecida Böttcher Luiz 

Profa. Dra. Carla Beatriz Collares Buzato 

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Morphological Aspects of Tamoxifen-Induced Apoptosis in Human Lymphocytes Cultivated *in vitro*

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1-RESUMO

Alguns fármacos apresentam potencial quimioterápico contra neoplasias, por inibirem a proliferação celular e induzirem a morte celular. Muitos desses agentes podem apresentar efeitos citotóxicos em tecidos normais como linfopenia, devido à indução de apoptose com diminuição das células T CD4, podendo levar a implicações na resposta imune. O tamoxifeno (TAM) é um agente não esteróide antiestrógeno utilizado como quimioterápico coadjuvante no tratamento de câncer de mama. Trabalhos recentes têm demonstrado que o TAM pode causar câncer endometrial em pacientes pós-menopausa, como sério efeito colateral. O presente trabalho objetivou investigar a capacidade do TAM de induzir apoptose em linfócitos humanos cultivados *in vitro*. Para tanto, amostras de sangue de voluntárias jovens (grupo A= 25 a 30 anos;n=3) e idosas (grupo B= 58 a 77 anos;n=3), foram centrifugadas em gradiente de densidade de Percoll (Percoll-50%) para separação de linfócitos e monócitos. Os monócitos foram excluídos por cultura dependente de adesão por 2 horas. O sobrenadante contendo linfócitos controle foram cultivados em meio RPMI suplementado com 10% de soro fetal bovino por 24 e 48 horas, enquanto linfócitos, denominados tratados, receberam 20μM de TAM no meio de cultura. Após a cultura, as células foram: 1) contadas em hemocitômetro, sendo estipulado a porcentagem de células viáveis para o total de células analisadas, utilizando-se o método de exclusão pelo Azul Tripan; 2) incubadas com anexina-biotina, que possui alta afinidade pela fosfatidilserina, seguido de incubação com anti-biotina conjugada à fluoresceína, para marcação das células em apoptose e analisadas ao microscópio de fluorescência; 3) fixadas em formaldeído e coradas com Leishman para estudos em microscopia de luz (ML); 4) depositadas em lamínulas, fixadas em glutaraldeído seguido de pós-fixação com tetróxido de ósmio e

analisadas ao microscópio eletrônico de varredura (MEV); 5) fixadas em solução de glutaraldeído, ácido pícrico e formaldeído seguido de pós-fixação com tetróxido de ósmio e analisadas ao microscópio eletrônico de transmissão (MET); 6) a análise estatística foi realizada a partir de análise de variância one-way ANOVA com $p< 0,05$. As culturas tratadas demonstraram menor viabilidade celular e maior índice apoptótico que seus controles. Ainda, culturas controle e tratadas de linfócitos de mulheres idosas apresentaram maior porcentagem de células em apoptose quando comparadas com linfócitos de mulheres jovens. Os estudos em ML e MET revelaram maior condensação cromatínica e redução do volume celular, e ainda, ao MET foram observados vacúolos autofágicos no citoplasma. A MEV revelou células com perda de microvilosidades e perda da morfologia arredondada após 48 horas de tratamento. Conclui-se, então que os linfócitos foram afetados pelo tratamento com o TAM em ambos os grupos, embora o grupo de mulheres idosas se mostrou mais susceptível a apoptose.

2-ABSTRACT

Some drugs have a chemotherapic potential against neoplasms, through inhibition of proliferation and cell death induction. Many of these agents can display cytotoxic effects in normal tissues such as lymphopeny due apoptosis induction with a consequent decrease of T CD4 cells and its implications in the immune response. Tamoxifen is a synthetic non-steroidal antiestrogenic drug which is currently being widely employed in the treatment of female breast cancer. Recent studies have shown that TAM can cause endometrial cancer in postmenopausal patients, considered a serious side effect. The purpose of this work was to investigate the capacity of TAM to induce apoptosis in human lymphocytes cultivated *in vitro*. Samples of peripheral blood were obtained from young (group A= 25-30 years old;n=3) and old (group B= 58-77 years old;n=3) women and centrifuged in a Percoll density gradient, to separate lymphocytes and monocytes (Percoll-50%). Monocytes were excluded by culturing for 2 hours. The supernatant with control lymphocytes was cultivated in RPMI containing 10% fetal bovine serum, for 24 and 48 h as controls, whereas treated lymphocytes received TAM (20μM) added to the culture medium. After the culture, the cells were: 1) counted in a hemocytometer, and the viable cell number for each sample was obtained through an exclusion test of intact cells by using 1% Tripan Blue and establishing the percentage of unstained, alive cells for the total of ressuspended cells; 2) incubated with annexin-biotin, which has high affinity for phosphatidilserine (PS), followed by incubation with FITC conjugated anti-biotin, to target apoptotic cells and examined by fluorescence microscopy; 3) fixed in formaldehyde and stained with Leishman and examined by light microscopy (LM); 4) placed on coverslips, where the cells were fixed, post fixed in osmium tetroxide and examined with the scanning electron microscope (SEM); 5) fixed in in glutaraldehyde, formaldehyde and picric acid, post fixed in osmium tetroxide, and

examined in a transmission electron microscope (TEM); 6) statistical analysis was performed using one-way analysis of variance (ANOVA) with $p< 0.05$. The treated cultures showed less viable cells and more apoptotic cells than control cultures. Moreover, group B showed more apoptotic cells in comparison with group A, in both control and treated cultures. LM and TEM showed treated cells with more condensed chromatin and reduction of cell volume. In TEM some autophagic vacuoles in the cytoplasm were observed. SEM showed loss of microvilli and loss of spherical shape at 48 h, when compared with the controls. Thus, a response to TAM was observed in both groups, although group B was more susceptible to apoptosis.

3-INTRODUÇÃO

3.1 QUIMIOTERAPIA

A quimioterapia é o método que utiliza compostos químicos denominados quimioterápicos no tratamento de doenças causadas por agentes químicos ou biológicos. Quando aplicada ao câncer, a quimioterapia é chamada de quimioterapia antineoplásica. O primeiro quimioterápico antineoplásico foi desenvolvido a partir do gás mostarda, usado nas duas Guerras Mundiais como arma química. Após a exposição de soldados a este agente, observou-se que eles desenvolveram hipoplasia medular e linfóide, o que levou ao seu uso no tratamento dos linfomas malignos. A partir da publicação, em 1946, dos estudos clínicos feitos com o gás mostarda e a das observações sobre os efeitos de ácido fólico em crianças com leucemias, verificou-se avanço crescente da quimioterapia antineoplásica. Atualmente, quimioterápicos mais ativos e menos tóxicos encontram-se disponíveis para uso na prática clínica. Os avanços verificados nas últimas décadas, na área da quimioterapia antineoplásica, têm facilitado consideravelmente a aplicação de outros tipos de tratamento de câncer e permitido maior número de curas. Os efeitos terapêuticos dos quimioterápicos dependem do tempo de exposição e da concentração plasmática da droga (DeVITA *et al.*, 1993).

O DNA de todas as células age como modulador na produção de formas específicas de RNA transportador, ribossômico e mensageiro e deste modo determina qual proteína irá ser sintetizada pela célula. Entre as proteínas sintetizadas, as enzimas são responsáveis por inúmeras funções celulares, e a interferência nos processos controlados por elas, irá afetar a função e a proliferação das células, inclusive as neoplásicas. Os diferentes agentes antineoplásicos podem atuar sobre todas ou a maioria das fases do ciclo celular, ou ainda

em fases específicas do ciclo celular (FRANKS & TEICH, 1990). Outro mecanismo de ação muito importante dos quimioterápicos é via ativação de apoptose (LOWE & LIN, 2000; MAKIN & HICKMAN, 2000).

3.2 EFEITOS COLATERAIS DA QUIMIOTERAPIA

Os fármacos utilizados para o tratamento de neoplasias comprometem a proliferação e induzem a morte celular das células neoplásicas. Porém, muitos desses agentes além de seu potencial terapêutico, podem apresentar também severos efeitos citotóxicos em tecidos normais e consequentemente levar a efeitos colaterais frequentemente observados durante a quimioterapia, como infecções nas mucosas, perda capilar, mielosupressão, e distúrbios gastrintestinais (náuseas, vômitos e diarréia). STAHNKE *et al.*, (2001) mostraram que pacientes, sob tratamento com quimioterápicos, apresentam diminuição dos linfócitos T CD4, com implicações severas na resposta imune, levando ao aumento da susceptibilidade a doenças infecciosas oportunistas, devido à indução direta de morte celular por apoptose nas células linfóides diferenciadas. Estudos indicam que os efeitos colaterais da quimioterapia parecem ser mais severos em pacientes idosos (BALDUCCI & CORCORAN, 2000; LICHTMAN & VILLANI, 2000).

Assim como os efeitos terapêuticos, os efeitos tóxicos em tecidos normais também são dependentes do tempo de exposição e da concentração plasmática da droga. Ainda, a toxicidade é variável para os diversos tecidos e depende da droga utilizada (DeVITA *et al*, 1993).

3.3(z)-1-{4-[2-(dimetilamino)etoxi]fenil}-1,2-difenil-1-buteno: TAMOXIFENO (TAM)

O tamoxifeno (Fig. 1) é um agente sintético não esteróide anti-estrógeno aprovado pela FDA (Food and Drug Administration), utilizado desde a década de setenta como quimioterápico no tratamento de pacientes com câncer avançado de mama, e, anos mais tarde, no tratamento preventivo de pacientes que apresentam um risco potencial para o desenvolvimento do câncer de mama (KING, 1995; ALASTAIR & WOOD, 1998; MANDLEKAR & KONG, 2001; CLEMONS *et al.*, 2002; JORDAN *et al.*, 2003). Estudos de pacientes que receberam tal tratamento indicam uma redução da incidência de câncer mamário em torno de 2%, em mulheres que receberam administração do TAM durante um ano na fase pós-menopausa, e de até 60% de redução quando o tratamento foi feito por um período de cinco anos na pré-menopausa (HENDERSON *et al.*, 1992).

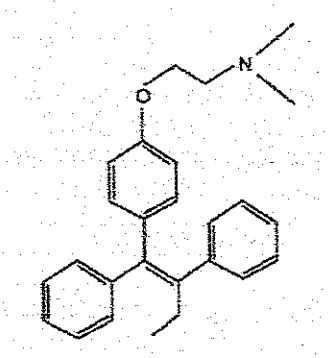

Fig. 1. tamoxifeno

O TAM inibe o estrógeno por competição pela ligação ao seu receptor (WAKELING *et al.*, 1984). O estrógeno (Fig.2) é um dos hormônios relacionados à proliferação celular e sua ação em tecidos como o mamário, por exemplo, é tida como possível promotora do câncer de mama. O complexo receptor de estrógeno-TAM, por sua

vez, inibe a ativação dos genes sensíveis ao estrógeno, impedindo a transcrição desses genes particularmente os que medeiam a proliferação celular (DICKSON & STANCEL, 1999; HOWELL *et al.*, 2000). Como resultado, o TAM bloqueia o ciclo celular na fase G1, atuando assim como um agente citostático (SUTHERLAND *et al.*, 1986; BUDTZ, 1999). Além do bloqueio do ciclo celular pelo mecanismo antiestrogênico, o tamoxifeno também pode agir através da indução de morte celular, atuando desta maneira como um agente citotóxico (PERRY *et al.*, 1995; BUDTZ, 1999).

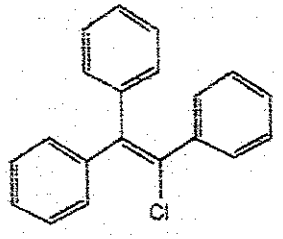

Fig. 2. estrógeno

Em estudos de pacientes com câncer de mama recebendo TAM diariamente por pelo menos 3 meses, foi mostrado, utilizando marcadores para apoptose e mitose, que a resposta clínica estava associada tanto com o decréscimo da proliferação quanto com o aumento de apoptose (KEEN *et al.*, 1997; CAMERON *et al.*, 2000).

O TAM é extensivamente metabolizado em humanos a metabólitos ativos (LIEN *et al.*, 1991; LIM *et al.*, 1994). Ambos os metabólitos, 4-hidroxytamoxifeno e N-desmetiltamoxifeno possuem afinidades maior ou igual ao receptor de estrógeno em relação ao TAM (FABIAN *et al.*, 1981).

Embora acredita-se que o mecanismo primário do tamoxifeno seja via receptor de estrógeno, pesquisas têm indicado um mecanismo adicional não mediado por receptor de

estrógeno, também capaz de induzir morte celular. Esse efeito já foi demonstrado em vários tipos celulares tumorais e não tumorais (JAN *et al.*, 2000). Acredita-se que o efeito antitumoral do TAM seja através de ambos os mecanismos: mediado e não mediado por receptor de estrógeno (MANDLEKAR & KONG, 2001). Entretanto o TAM não é somente um agente antiestrogênico, pois exibe também propriedades estrogênicas, dependendo da espécie, tecido e gene (OSBORNE, 1996). O efeito diferencial do tamoxifeno em vários tecidos pode ser devido à interação com o receptor α ou β, e enquanto o TAM é um agonista parcial do receptor α , é inteiramente antagonista de β (KATZENELLENBOGEN & KATZENELLENBOGEN, 2000; KATZENELLENBOGEN *et al.*, 2000). Recentemente MANDLEKAR & KONG (2001) mostraram que ambos os metabólitos do TAM são capazes de induzir apoptose em linhagens tumorais negativas para receptor de estrógeno. *In vivo*, a eficácia terapêutica do TAM em tumores que não apresentam receptor de estrógeno tem sido obtida em doses de 4 a 8 vezes mais altas que aquelas usadas para tumores positivos para receptor de estrógeno. Assim, o TAM também tem sido utilizado no tratamento de câncer pancreático, renal e ainda em gliomas e melanomas (MANDLEKAR & KONG, 2001).

Um dos efeitos colaterais mais sérios do TAM é o aumento da incidência de câncer endometrial em mulheres pós-menopausa, através de seu efeito estrogênico secundário (FORNANDER *et al.*, 1993; RUTQVIST *et al.*, 1995; FISHER, *et al*, 1996; 1998; MOURITS *et al.*, 2002). Em mulheres pré-menopausa, os efeitos são similares aos sintomas de menopausa como: fogachos, menstruação irregular, secura vaginal, prurido vulvar e edema (CLEMONS *et al.*, 2002; NYSTEDT *et al.*, 2003). Outros efeitos incluem

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anemia, leucopenia, trombocitopenia, elevação de níveis de triglicerídeos, diminuição de plaquetas, constipação, entre outros (Professional Information Brochure: Nolvadex; CLEMONS *et al.*, 2002). Raras vezes foram relatados eventos tromboembólicos e embolismo pulmonar. Ainda, estes eventos parecem ser mais freqüentes em pacientes com mais de 50 anos de idade (FISHER *et al.*, 1998).

Estudos com animais de experimentação têm indicado o TAM como agente indutor de aneuploidias (SARGENT *et al.*, 1996; STYLES *et al.*, 1997) e aberrações cromossômicas em fígado de ratos (STYLES *et al.*, 1997), além de causar câncer hepático (GREAVES *et al.*, 1993; HARD *et al.*, 1993) e induzir mutações no gene lac I em fígado de ratos transgênicos (DAVIES *et al.*, 1997). Outros estudos indicam que o TAM pode causar formação de adutos no DNA em vários órgãos de animais (RANDERATH *et al.*, 1994; OSBORNE, *et al.*, 1996; CARTHEW *et al.*, 2001), bem como no DNA de leucócitos humanos de pacientes tratados com TAM (HEMMINKI *et al.*, 1997).

O TAM pode também causar adutos em linfócitos humanos cultivados *in vitro* (HEMMINKI *et al.*, 1995; DAVIS *et al.*, 1998) e ainda induzir formação de micronúcleos em células humanas metabolicamente ativas (WHITE *et al.*, 1992; CROFTON-SLEIGH *et al.*, 1993; STYLES *et al.*, 1997). Além disso, outros autores mostraram que o TAM pode afetar o pH de organelas citoplasmáticas, inibindo a acidificação (ALTAN, *et al.*, 1999; CHEN *et al.*, 1999) e ainda diminuir a taxa de vesículas de secreção (ALTAN *et al.*, 1999). Alguns estudos *in vitro* têm indicado que o tamoxifeno quando administrado com estradiol, afeta a viabilidade de certos tipos celulares, como por exemplo as células endometriais, por desencadear o mecanismo de morte celular por apoptose (STACKIEVICZ *et al.*, 2001).

3.4 APOPTOSE

A morte celular por apoptose pode ser definida por uma série de modificações morfológicas e moleculares, desencadeadas pela ativação de uma série coordenada e programada de eventos executados por um conjunto específico de produtos gênicos, ou seja é um suicídio intencional baseado num mecanismo genético (MAJNO & JORIS, 1995; SASANO, 1995; KIMURA *et al.*, 1997; KIMURA *et al.*, 2000). Muitos estudos relatam que os genes supressores de tumor (p53, BRAC 1 e BRAC 2) e oncogenes (myc e família bcl-2) participam deste processo (SHEN *et al.*, 1998; SAKAHIRA *et al.*, 1999; LESLIE, 2001; WEI *et al.*, 2001). A apoptose encontra-se associada à processos normais como, por exemplo, o desenvolvimento embrionário, a manutenção da homeostase de populações celulares nos tecidos, aos mecanismos de defesa durante as reações imunes, ao envelhecimento celular, entre outros, ou pode ser desencadeada por tratamentos celulares com agentes nocivos (FALCIERI *et al.*, 1994).

Uma das modificações moleculares mais importantes ocorridas durante a apoptose é a exposição da fosfatidilserina na porção externa da membrana plasmática (FADOK *et al.*, 1992; KOOPMAN *et al.*, 1994; HOMBURG *et al.*, 1995; MARTIN *et al.*, 1995; SCHLEGEL *et al.*, 1995; VERMES *et al.*, 1995; VAN DER EIJNDE, 1998; VERHOVEN *et al.*, 1999). Têm sido sugerido que a translocação da fosfatidilserina pode ser atribuída a enzimas como as flipases, ou a translocase, as quais possuem alta afinidade pelos fosfolipídios e facilitam o movimento bidirecional na bicamada (VERHOVEN *et al.*, 1995). Frequentemente, a apoptose pode ser reconhecida em função de variadas modificações na morfologia celular, algumas delas mais facilmente observáveis em microscopia eletrônica como: retração celular, condensação da cromatina formando massas densas com tamanhos

e formas variadas, fragmentação do núcleo, formação de bolhas citoplasmáticas e corpúsculos apoptóticos, os quais podem conter fragmentos de núcleos e outras organelas citoplasmáticas. Estes eventos progridem rapidamente e não causam inflamação exudativa nos tecidos, ao contrário da necrose (KERR *et al.*, 1972; FALCIERI *et al.*, 1994; HÄCKER, 2000; KIMURA *et al.*, 2000). *In vivo*, as células apoptóticas normalmente são removidas por fagocitose efetuada pelas células sadias adjacentes, como macrófagos (MAJNO & JORIS, 1995), devido à exposição da fosfatidilserina que marca as células a serem fagocitadas (ELLIS *et al.*, 1991; SAVILL *et al.*, 1993; ROTELLO *et al.*, 1994; FRANC *et al.*, 1996; SAVILL, 1996; FADOK & HENSON, 1998).

3.5 APOPTOSE INDUZIDA PELO TAMOXIFENO

Em adição ao mecanismo antiestrogênico, mediado por receptor de estrógeno, o tamoxifeno em concentrações micromolares, possui atividade citotóxica não mediada por receptor de estrogênio em vários tipos celulares, tumorais (células de câncer de próstata, células de câncer de bexiga e gliomas), e não tumorais (neutrófilos e a linhagem MDCK) (JAN *et al.*, 2000). Pesquisas indicam que a morte celular não mediada pelo receptor de estrógeno induzida pelo tamoxifeno inclui modulação de proteínas sinalizadoras tais como a proteína quinase C (PKC), calmodulina e proto–oncogene c-myc, bem como alterações na fluidez da membrana plasmática (MANDLEKAR & KONG, 2001). O mesmo autor ainda acredita que a p38, c-Jun N-terminal quinase, o estresse oxidativo, a transição na permeabilidade mitocondrial e a geração de ceramida podem ter um papel importante na apoptose induzida pelo TAM. Recentes evidências sugerem que a mitocôndria possui um

papel central na apoptose como integrante da sinalização apoptótica e também na amplificação da resposta apoptótica (GREEN & REED, 1998). A disfunção mitocondrial é caracterizada pelo aumento na permeabilidade mitocondrial e perda do potencial de membrana, culminando na ativação da caspase-3. DIETZE, *et al.* (2001) demonstraram que em células HMEC-E6 (em suas primeiras passagens) tratadas com TAM houve diminuição do potencial de membrana mitocondrial, condensação mitocondrial e ativação das caspases-3 e 9.

4-OBJETIVOS

4.1 OBJETIVO GERAL

Tendo em vista os fatos acima descritos, o objetivo geral deste trabalho foi investigar a capacidade do tamoxifeno de induzir apoptose em linfócitos humanos cultivados *in vitro*, bem como as alterações envolvidas nesse processo.

4.2 OBJETIVOS ESPECÍFICOS

1) Detectar, através de teste de exclusão pelo Azul Tripan a capacidade do tamoxifeno de afetar a viabilidade celular, após 24 e 48 horas de tratamento.

2) Detectar, através de ensaio imunocitoquímico a capacidade do tamoxifeno de induzir apoptose em linfócitos humanos, após 24 e 48 horas de tratamento.

3) Observar as possíveis alterações morfológicas em microscopia de luz e em microscopia eletrônica de varredura e transmissão, ocorridas nos linfócitos submetidos ao tratamento com tamoxifeno em comparação com células controle não tratadas, após 24 e 48 horas de tratamento.

4) Analisar as possíveis diferenças na resposta ao TAM de linfócitos obtidos de mulheres jovens (25-30 anos) comparado àqueles obtidos de mulheres idosas (58-77 anos).

5-MATERIAL E MÉTODOS

5.1 Células

Os linfócitos foram obtidos a partir de coleta de sangue periférico por punção venosa, com seringa de 20 mL contendo EDTA como anticoagulante (aprovado pelo Comitê de Ética em Pesquisa da Faculdade de Ciências Médicas-UNICAMP/ n° 069/2003). Amostras de sangue de cada grupo de mulheres (grupo A= 25 a 30 anos;n=3 e grupo B= 58 a 77 anos;n=3) foram centrifugadas individualmente, por 15 minutos (1300g) para concentração dos leucócitos. Após a centrifugação, a interface branca (concentrado de leucócitos) entre o plasma e as hemáceas foi cuidadosamente coletada. A interface branca contendo menor quantidade possível de hemáceas foi centrifugada em gradiente de densidade de Percoll (Amersham Pharmacia Biotech), para separação dos tipos celulares sanguíneos (modificado de HJORTH, *et al.*, 1981). A solução de Percoll foi obtida pela adição de 1 mL de solucão salina 9% à 9 mL de Percoll. A partir desta solução, considerada como 100% foram distribuídas em tubo para centrífuga de 15 mL, três camadas de diferentes concentrações, obtidas pela diluição do Percoll 100% em solução salina fisiológica: primeira camada: Percoll 75%, segunda camada: Percoll 65% e terceira camada: Percoll 50%. Após centrifugação por 30 minutos (660g), em gradiente de densidade previamente preparado, as três camadas continham: hemáceas, leucócitos polimorfonucleares e mononucleares respectivamente. A camada contendo células mononucleares, correspondendo a densidade de: 1.06-1.08 g/mL, foi lavada duas vezes em Solução de Hanks para remoção do Percoll. Essas células foram ressuspendidas em meio RPMI 1640 suplementado com antibióticos (10 mg/L de estreptomicina e 1000U/L de penicilina) e 10% de soro fetal bovino (SFB) (Nutricell, Campinas-SP) e incubadas à 37°C por duas horas, para adesão dos monócitos. O sobrenadante contendo linfócitos em sua maioria, foi contado em hemocitômetro, ajustado

a uma concentração de 4,5 x 10^5 células/mL e transferido para frasco de cultura de 25cm^3 em meio RPMI com 10% de SFB.

Com base na literatura, a qual relata número variável de linfócitos periféricos entre os indivíduos, foi coletado amostras de sangue das voluntárias várias vezes, seguido de contagem em hemocitômetro, para padronização da concentração de células/mL que seriam lançadas para dar início ao experimento.

5.2 Tamoxifeno

As células foram então, imediatamente tratadas ou não com TAM e mantidas em cultura por 24 e 48 horas à 37°C. O TAM (Sigma) foi dissolvido em dimetilsulfóxido (DMSO) (Sigma) seguido de diluição apropriada em meio RPMI 1640 suplementado com 10% de SFB com concentração final na cultura de 20μM (JAN *et al.*, 2000; MANDLEKAR & KONG, 2001). A concentração do solvente (DMSO) foi menor que 0,1%, a qual não demonstra efeitos sobre a viabilidade celular (BARAL *et al.*, 1985; HEMMINKI *et al.*, 1995; BARAL *et al*, 2000). Concentração similar de DMSO diluído em RPMI foi adicionada às culturas controles não tratadas.

5.3 Viabilidade Celular

O número de células viáveis por amostras foi obtido a partir de testes de exclusão de células inviáveis utilizando-se corante vital Azul Tripan 1%. A viabilidade celular foi analisada após 24 e 48 horas de cultura, por contagem em hemocitômetro, determinando-se a porcentagem de células vivas não coradas para o total de células lançadas. As observações foram feitas em triplicata para cada voluntária.

5.4 Detecção de Apoptose

De acordo com indicações do fabricante (Oncogene Research Products-Annexin V-Biotin Apoptosis Detection Kit), as células foram incubadas com anexina-biotina, que possui alta afinidade pela fosfatidilserina, seguida de incubação com anti-biotina conjugada à fluoresceína e analisadas ao microscópio de fluorescência Zeiss Axioskop equipado com conjunto de filtros para fluoresceína. Este protocolo foi utilizado para marcação imunocitoquímica das células apoptóticas que expressam fosfatidilserina na porção externa da membrana plasmática. Células em necrose foram diferenciadas pela utilização de iodeto de propídeo. Foram contadas, ao total, 400 células de cada amostra e estabelecida a porcentagem de células em apoptose, de acordo com a marcação observada ao microscópio. As observações foram feitas em duplicata para cada voluntária.

5.5 Microscopia de Luz

Após a cultura, as células foram lavadas em PBS, fixadas em formaldeído 0,2% por 4 minutos, depositadas em lâminas, secas ao ar e coradas com Leishman 0,033% por 5 minutos (modificado de McCARTHY *et al.*, 1990).

Para a escolha do melhor fixador bem como do corante foram realizados vários testes anteriores à escolha do formaldeído e do Leishman como reagentes para microscopia de luz. Utilizou-se metanol, metanol ácido-acético (3:1, v/v) ou paraformaldeído 2% em PBS como fixadores, e corantes específicos para células sanguíneas: Wright, May-Grünwald-Giemsa ou Panótico. O metanol e o metanol-ácido-acético não conservaram a estrutura celular, e embora o paraformaldeído tenha conservado a célula, não permitiu bom contraste entre o núcleo e o citoplasma quando usado em conjunto com os corantes mencionados.

Além disso, os corantes foram diluídos e testados em diferentes tempos de coloração até chegar a um resultado satisfatório que permitisse o estudo morfológico.

5.6 Microscopia Eletrônica de Varredura

Após a cultura, as células foram coletadas por centrifugação e lavadas em tampão cacodilato 0,1 M (pH 7,2), acrescido de 1,5% de sacarose. As células foram, então, depositadas em lamínula, fixadas em glutaraldeído 2,5% em tampão cacodilato 0,1 M (pH 7,2), acrescido de 1,5% de sacarose por 1 hora, pós-fixadas em tetróxido de ósmio 1% no mesmo tampão por 1 hora, desidratadas em ordem crescente de acetona (70%, 80%, 90, 95% e 100%), secas ao ponto crítico, recobertas com ouro e analisadas ao microscópio eletrônico de varredura (JEOL S800 SV) operado a 15 KV (GENARI *et al.*, 1996).

5.7 Microscopia Eletrônica de Transmissão

As células foram coletadas por centrifugação e lavadas em tampão cacodilato 0,01 M (pH 7,2), acrescido de 1,5% de sacarose. As células foram, então, fixadas numa solução de glutaraldeído 2,5%, formaldeído 1,25% e ácido pícrico 0,03% em tampão cacodilato 0,05 M (pH 7,2), acrescido de 1,5% de sacarose por 1 hora. Em seguida, as células foram pós-fixadas em tetróxido de ósmio 1% por 1 hora no mesmo tampão, desidratadas em ordem crescente de acetona, envolvidas em ágar 2%, embebidas e incluídas em Epon, seccionadas, seguida de contrastação com acetato de uranila e citrato de chumbo e analisadas ao microscópio eletrônico de transmissão (Zeiss Leo 906) operado a 60 KV (modificado de HIRSCH & FEDORKO, 1968).

O protocolo acima foi precedido por outro que teve como base somente o glutaraldeído 2,5% como primeiro fixador diluído em tampão fosfato 0,1M (pH 7,4) acrescido de sacarose 1,5%, seguido da metodologia rotineira para microscopia eletrônica de transmissão. Este fixador não conservou as membranas celulares e, portanto, foi substituído pela solução já mencionada.

5.8 Análise Estatística

Os resultados foram submetidos à análise de variância One-way ANOVA, com intervalo de confiança de 95% e $p< 0,05$.

6-ARTIGO

MORPHOLOGICAL ASPECTS OF TAMOXIFEN-INDUCED APOPTOSIS IN HUMAN LYMPHOCYTES CULTIVATED *IN VITRO*

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ABSTRACT

Tamoxifen is a synthetic non-steroidal antiestrogen drug employed in the treatment of female breast cancer. Here, the capacity of TAM to induce apoptosis in human lymphocytes was investigated through quantitative (cell viability and immunocytochemical methods) and qualitative studies (light and electron microscopy). Statistical analysis was performed using one-way analysis of variance (ANOVA) with 95% confidence limits, with $p< 0.05$. Blood samples were obtained from young (group A;n=3) and old (group B;n=3) women and centrifuged in a Percoll density gradient, to separate mononuclear cells. Monocytes were excluded by adhesion to the substrate after culturing for 2 hours. The supernatant including control lymphocytes was cultivated in RPMI containing 10% fetal bovine serum, for 24 and 48 h, while the treated cells received TAM (20μM) in the same culture medium. Statistical analysis showed that the treated cultures demonstrated more apoptotic cells than found in control cultures. Moreover, group B showed more apoptotic cells in comparison with group A. Morphological studies showed treated cells with loss of microvilli, reduction of cell volume, nuclei with more condensed chromatin and some cytoplasmic autophagic vacuoles, differing from the controls. Thus, a response to TAM was observed in both groups, although group B was more susceptible to apoptosis.

INTRODUCTION

A chemotherapic potential in relation to neoplasms has been noted for some drugs due to a decrease in cell proliferation and a higher rate of cell death. However many of these agents, beyond their therapeutical potential, can also present severe cytotoxic effects in normal tissues, leading to side effects observed during chemotherapy, such as mucositis, hair loss, myelosuppression. Moreover, chemotherapy can induce acute lymphopeny and chronic depletion of CDT 4 cells, leading to increased susceptibility to opportunistic infections (Stahnke et al., 2001). In addition, these side effects seem to be more severe in the older patient population (Balducci & Corcoran, 2000; Lichtman & Villani, 2000). Tamoxifen (TAM) ((Z)-1-[4-[2(dimetilaminoetoxi]fenil]-1,2-difenil-1-buteno) is a synthetic non-esteroidal anti-estrogenic drug widely used as a breast cancer chemotherapic drug. TAM was approved by the Food and Drug Administration in 1977 for the treatment of women with advanced breast cancer and, several years later, also for adjuvant treatment of primary breast cancer (King, 1995; Alastair & Wood, 1998; Mandlekar & Kong, 2001; Clemons et al., 2002; Jordan et al., 2003). However, TAM is not only an antiestrogenic agent, because it also shows estrogenic properties depending on the species, tissue and gene considered (Osborne, 1996). Recent studies have shown that the estrogenic action of TAM can cause endometrial cancer (Fornander et al., 1993; Fisher et al, 1996; Rutqvist et al., 1995; Fisher et al, 1998; Mourits et al., 2002) as a serious side effect in postmenopausal patients. In premenopausal patients some effects are similar to menopausal symptoms (Clemons et al., 2002; Nystedt et al., 2003).

This drug also has been indicated as an agent that induces aneuploidy (Sargent et al., 1996; Styles et al., 1997), chromosomic aberrations in rat livers (Styles et al., 1997),

hepatic cancer in animals (Greaves et al., 1993; Hard et al., 1993) and mutations in the lac I gene in transgenic rat livers (Davies et al., 1997). Some studies have also indicated that TAM can cause the formation of DNA adducts in many animal organs (Carthew, et al., 2001), in human leukocytes from treated patients (Hemminki et al., 1997; Davis et al., 1998) and in lymphocytes cultivated in vitro (Hemminki et al., 1995), as well as inducing the formation of micronuclei in metabolically active human cells (White et al., 1992; Crofton-Sleigh et al., 1993; Styles et al., 1997). Moreover, other authors have shown that TAM can affect the pH of organelles in many different cell types, inhibiting acidification (Altan et al., 1999; Chen et al., 1999). In addition, TAM decreased the rate of vesicle secretion (Altan et al., 1999).

Recent *in vitro* studies have indicated that tamoxifen enhances the apoptotic effect of cisplatin on primary endometrial cell culture (Drucker et al., 2003) and moreover, endometrial cell culture receiving TAM and steroid hormones are likely to undergo apoptosis (Stackievicz et al., 2001). Also, 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). In adition, studies using spleen cell culture showed that TAM caused the suppression of lymphocyte mitogenesis, indicating that TAM can be an immunosuppressive agent (Baral et al., 2000).

Based on the above facts, the purpose of this study was to investigate the capacity of TAM to induces apoptosis in human lymphocytes cultivated *in vitro*, as well the structural modifications involved in this process.

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MATERIAL AND METHODS:

Cells

Lymphocytes were obtained from samples of peripheral blood, by venipuncture and an anticoagulant (EDTA) was added. Three volunteers for each group of women (group A= 25-30 years old and group B= 58-77 years old) were used (approved by Ethics in Research Committe of the Medical Sciences College /UNICAMP/ n° 069/2003). Twenthy milliliters of each blood sample was centrifuged in a conical centrifuge tube for 15 minutes (1300g) to deposit erythrocytes. The interface between plasma and erythrocytes was carefully pipetted into another centrifuge tube with a few erythrocytes as possible, and centrifuged in a Percoll density gradient (Amersham Pharmacia Biotech) for 30 minutes (660g), to separate blood cell types (modified from Hjorth et al., 1981). The layer containing mononuclear cells (Percoll-50%-density: 1.06-1.08 g/mL) was washed twice in Hanks Solution to remove Percoll. These cells were ressuspended in RPMI 1640 medium containing antibiotics (streptomicin 10 mg/L and penicilin 1000U/L) and 10% fetal bovine serum (FBS) (Nutricell, Campinas-SP) and incubated at 37°C for 2 hours, for adhesion of the monocytes. Cells in the supernadant were ajusted to 4,5 x 10^5 lymphocytes/mL and placed in 25 cm^3 tissue culture flasks at 37°C.

Tamoxifen

TAM (Sigma) was dissolved in dimetilsulfoxide (DMSO) (Sigma) followed by appropriate dilution in RPMI 1640 containing 10% (FBS) with a final concentration of 20μM (Jan et al., 2000; Mandlekar & Kong, 2001) in the culture.The treated cells were then cultivated for 24 or 48 hours. Similar concentrations of DMSO diluted in RPMI were added

to the control cultures, and they were also cultivated for 24 or 48 hours. The solvent concentration (DMSO) was less than 0.1%, which does not affect cell viability (Baral et al., 1985; Hemminki et al., 1995; Baral et al., 2000).

Cell Viability

The viable cell number for 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 ressuspended cells. Cell viability was analysed 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 which has high affinity for phosphatidilserine followed by incubation with FITC-conjugated anti-biotin, to target apoptotic cells that express phosphatidilserine on the outer leaflet of the plasma membrane and analysed by Zeiss Axioskop fluorescence microscope equipped with set of filters for fluorescein. Necrotic cells were stained with propidium iodide. 400 cells were counted for each sample and the number of apoptotic and necrotic cells from each blood sample was established based on a fluorescent tagging observed in the microscope.

Light Microscopy

After the culture, cells were collected by centrifugation and washed once in PBS. Then, they were fixed in 0.2% formaldehyde in PBS for 4 minutes, placed on slides, air dried and stained with 0.033% Leishman for 5 minutes (modified from McCarthy, 1990).

Scanning Electron Microscopy

After the culture, cells were collected by centrifugation and washed once in 0.1 M cacodylate buffer (pH 7.2), placed on coverslips, fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), with 1.5% saccharose. The fixed cells were washed three times in 0.1 M cacodylate buffer (pH 7.2) and postfixed in 1% osmium tetroxide in the same buffer for 1 hour. The cells were then washed three times in distilled water, and dehydrated in a graded series of acetone solutions (70%, 80%, 90%, 95% and 100%), critical point dried, sputtered with gold and examined with a scanning electron microscpe (JEOL S800 SV) at an accelerating voltage of 15 KV (Genari et al., 1996).

Transmission Electron Microscopy

After the culture, cells were pelleted 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% acid picric solution in 0.1 M cacodylate buffer (pH 7.2), with 1.5% saccharose for 1 hour. The fixed cells were washed three times in the same buffer, and postfixed in 1% osmium tetroxide in the same buffer for 1 hour, and washed three times in distilled water. The cells were then 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 Hirsch & Fedorko, 1968).

Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) with 95% confidence limits, with $p < 0.05$. Data are presented in text and figures as mean ± SD.

RESULTS

At 24 hours of treatment with TAM, the cells of the young women group (group A) showed a viability of 83±9.1%, similar to its control, that reached 87±8.0%. After 48 hours, the culture demonstrated diminished cell viability (66±8.1%) in contrast to its control that maintained 87±7.8% of cell viability (Fig. 1). The treated cells obtained from older women (group B) were clearly less viable (T24=62±2.1% and T48=45±2.9%) than their controls (C24=78±3.5% and C48=59±0.9%) for both culture periods. Moreover, this group demonstrated less cell viability in comparison with the first group that reached more than 80% of viable cells in most of the cases. According to Fig. 2, the percentage of apoptotic cells, identified by FITC-conjugated biotin, was higher in treated cultures (T24A=12±0.2%, T48A=25±2.6%, T24B=32±2.9% and T48B=35±2.6%) in comparison to their respective controls in both groups (C24A=8±0.2%, C48A=8±0.6%, C24B=20±0.8% and C48B=21±1.1%) Moreover, group B presented a higher percentage of apoptotic cells, in

both control and treated cultures when compared with group A. The group of older women reached around 20% of apoptosis for its controls and more than 30% in the treated cultures, while the group A did not reach 10% of apoptosis in the controls and varied between 12 and 25% for the treated cultures. We noticed that in group A there was a small increase of cells undergoing apoptosis when control and treated cells where compared after 24 hours, while, after 48 hours, the variation was higher (Fig. 2). A similar result can be observed in Fig. 1, in which group A demonstrates a diminishes viability after 48 hours of treatment while in group B, diminishes cell viability already after 24 hours.

Light microscopy showed treated and untreated cells (Fig. 3-6) with a spherical shape, the nucleus following the cell format, and a scant cytoplasm, with the chromatin well stained in contrast with the cytoplasm. Treated cells showed (Fig. 4 & 6) more condensed chromatin and reduction of cell volume. In addition to these characteristics observed, electron microscopy demonstrated other alterations in the treated cells. Control lymphocytes analysed with the scanning electron microscope presented a spherical shape and the cell surface totally covered by microvilli, as can be seen in Figs 7 & 9. The treated cells (Fig. 8 & 10) presented loss of microvilli, and after 48 hours the loss of microvilli was even greater (Fig. 10). This figure also shows the loss of the typical spherical shape after 48 hours of treatment with TAM. In thin sections of control lymphocytes (Fig. 11 & 13), spherical cells were observed containing a large nucleus with a condensed and loose chromatin pattern, occupying almost all the cell volume. The membranes are well preserved and, although the cells have scant cytoplasm, the cells have many mitochondria. The treatment with TAM showed cells containing a nucleus with more condensed chromatin, decreased cell volume and some cytoplasmic vacuoles, that are probable autophagic

vacuoles identified by their membranous content (Fig. 12 & 14). Notice that, in these cells (Fig. 12 & 14), the membranes are well preserved. As in scanning electron microscopy, we can also observe that after 48 hours of treatment with TAM, there was loss of the spherical shape and of microvilli.

DISCUSSION

Tamoxifen has been clinically used as a chemotherapic drug for breast cancer, however the potential it has to induce apoptosis in various cell types is still unknown. The present study is the first to show the morphological aspects of TAM-induced apoptosis in human lymphocytes.

The cell viability was affected by treatment with TAM, in both groups A and B, as shown by the Tripan Blue exclusion test. The two groups also present differences between themselves, in that a larger number of lymphocytes obtained from older women were affected by TAM. 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 chemotherapic drugs (Friesen et al., 1996), and that TAM is cytotoxic at micromolar concentrations (Jan et al., 2000; Mandlekar & Kong, 2001), and has an effect on nonbreast cancer cells (Perry et al., 1995; Majumdar et al., 2001). Since the expression of estrogen-receptor is either low or not measurable in lymphoid tissues (Weihua et al., 2003), it is possible that the TAM-induced apoptosis in lymphocytes is not mediated by estrogen-receptor.

The lower percentage of viable cells and more frequent occurrence of apoptosis in lymphocytes obtained from older women can be associated with the aging of the immune

system, which is in functional decline (Gravenstein et al., 1998; Grubeck-Loebenstein et al., 1998), and is associated with a dramatic reduction in responsiveness as well as functional disregulation (Effros, 2001; Pawelec et al., 2002). Pagliara et al. (2003) have shown that lymphocytes of aged persons are more prone to undergo apoptosis, in comparision to lymphocytes of younger people. In addition, others studies have shown that lymphocyte number is in decline in the elderly (Pawelec et al., 1998; Argentati et al., 2002). Although it is not yet well known, there appears to be a correlation between aging and the increase of less active enzymes, that are more susceptible to heat inactivation and proteolytic degradation (Effros, 2001; Linton & Thoman, 2001; Pawelec et al., 2002). Moreover, accumulation of oxidatively modified proteins, that increase during senescence, may reflect deficiencies in one or more parameters of a functional complex that maintains a delicate balance between the presence of antioxidants, repair, replacement or elimination of biologically damaged proteins and cells (Stadtman, 2001).

It is also important to keep in mind that cells that are still intact (viable–Trypan Blue) already present loss of the typical assymetry of the membrane bilayer and express phosphatidilserine (PS) on the outer leaflet of the plasma membrane (Fadok et al., 1992; Koopman et al., 1994; Martin et al., 1995; Verhoven et al., 1995). The exposure of PS is one of the most important molecular modifications during apoptosis (Fadok et al., 1992; Koopman et al., 1994; Homburg et al., 1995; Martin et al., 1995; Schlegel et al., 1995; Vermes et al., 1995; Van Der Eijnde, 1998; Verhoven et al., 1999). Castedo et al. (1996) have reported that phosphatidilserine translocation is an early event of the apoptotic process that occurs before loss of viability, and precedes nuclear fragmentation (Castedo et al., 1996; Chan et al., 1998). *In vivo,* these and others molecular changes are important for

recognition and elimination by adjacent cells, such as macrophages (Ellis et al., 1991; Savill et al., 1993, Rotello et al., 1994; Franc et al., 1996; Savill, 1996; Fadok & Henson, 1998).

In addition to PS translocation others alterations are consistent with the apoptotic process, such as, certain morphological alterations observed in this study. Loss of microvilli observed in treated cells (Fig. 8 & 10) showed by scanning electron microscopy was previously described as one of the first responses of the cells to an unfavourable environment, perhaps to reduce the exposed cell surface (Lin et al., 1973; Tilz & Albergger, 1974) and also can be observed during the apoptotic process (Fernadez-Segura et al., 1990; Nagata, 1996; Kondo et al., 1997; Häcker, 2000). Loss of the spherical shape occurred after 48 hours of treatment with TAM, may be associated with actin filament dysfunction (Karp, 1999). This phenomenon seems to occur concomitantly, downstream of caspase activation and the dephosphorylation of ezrin/radixin/moesin proteins, which normally stabilize microvilli on the actin cytoskeleton (Kondo et al., 1997).

Nuclear chromatin condensation and the reduction of cell volume, observed by light (Fig. 4 & 6) and electron microscopy (Fig 8 & 10; 12 & 14), are the most striking features of apoptosis (Kerr et al., 1972; Wyllie, 1980; Searle et al., 1982; Falcieri et al., 1994; Häcker, 2000; Kimura et al., 2000). Possibly, if the cultures had been kept more than 48 hours, we could have found cells with fragmented nuclei and apoptotic bodies, that are late events of the apoptotic process (Falcieri, 1994; Häcker, 2000; Kimura et al., 2000). The chromatin condensation was suggested to be a consequence of the activity of the nuclear protein called DFF (Liu et al., 1998), among others (Samejima et al., 1998). These nuclear proteins are cleaved by active caspases, probably caspases 3 and 6 (Lazebnick et al., 1994;

1995; Gohring et al., 1997; Liu et al., 1997; Hirata et al, 1998). Studies, using cell culture or cloned molecules, suggest that DFF is required only for chromatin condensation but not DNA fragmentation (Sahara et al., 1999; Sakahira et al., 1999). Meanwhile, cell volume decrease is associated with the loss of intracellular ions, mainly K^+, that has an essential role in caspase activation and nuclear activity during apoptosis (Gómez-Angelats et al., 2000).

The cytoplasmic vacuoles, which are probably autophagic vacuoles present in the treated lymphocytes, have also been observed in other studies during apoptosis in insects (Dai & Gilbert, 1999) and mammalian cells (Ohsawa et al., 1998). The cell death-associated autophagy was found in fungus (Cornillon et al, 1994), and in various physiological states of development (e.g: during insect metamorphosis, mammalian embryogenesis, as in regression of interdigital webs). It has also been encountered during adult phases (e.g: in intestine, postweaning mammary gland, ovarian atretic follicles) (Schweichel & Merker, 1973; Clarke, 1990; Shibahara et al., 1995; Zakeri et al., 1995; D'Herde et al., 1996; Beaulaton & Lockshin, 1997; Jochova et al., 1997). Autophagocytosis also appears to be associated with experimental and human neurodegenerative diseases (Alzheimer, Parkinson) (Cataldo et al., 1995; Anglade et al., 1997; Migheli et al., 1997).

However, some authors believe that the presence of autophagossomes is representative of another type of non-apoptotic cell death called autophagic cell death. Bursh et al. (1996) described the occurrence of autophagic cell death in human mammary carcinoma cells (MCF-7) treated with TAM. Later, these authors (2000) based on the studies of cytoskeleton, demonstrated that the same cell line used in the previous

experiment undergoing autophagic cell death presented a preserved cytoskeleton even during the period of nuclear destruction, while in classic apoptosis, demonstrated in human colon cancer cells (HT29/HI1) treated with tyrphostin, the cytoskeleton proteins were depolymerized or cleaved in early stages.

We suggest here that cells from our experiment underwent apoptosis, as indicated by the immunocytochemical study with fluorescence microscopy which is based on a specific test for apoptosis (Oncogene Research Products-Annexin V-Biotin Apoptosis Detection Kit), even though with transmission electron microscopy we found autophagossomes.

Thus we conclude that lymphocytes treated with tamoxifen demonstrated lower cell viability as well as a higher apoptotic rate than their controls. Also, longer periods of treatment with TAM result in more affected cells and more morphological changes. Moreover, lymphocytes obtained from older women are more susceptible to undergo apoptosis, in both control and treated cultures.

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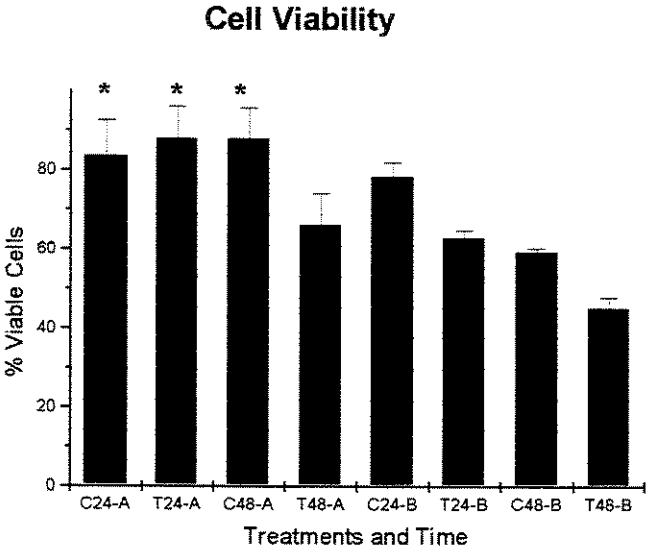
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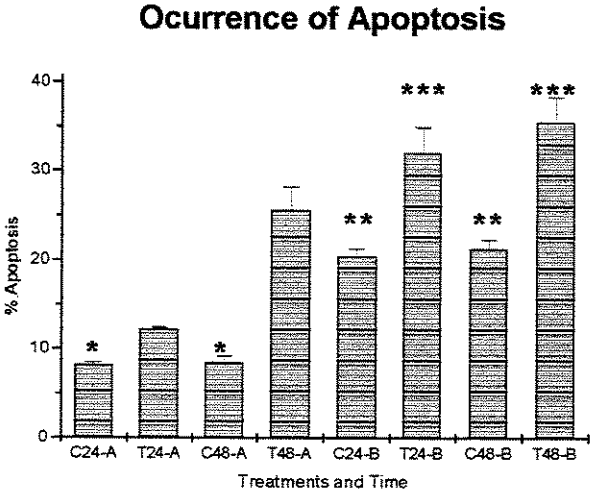
Treatments and Time

Fig. 1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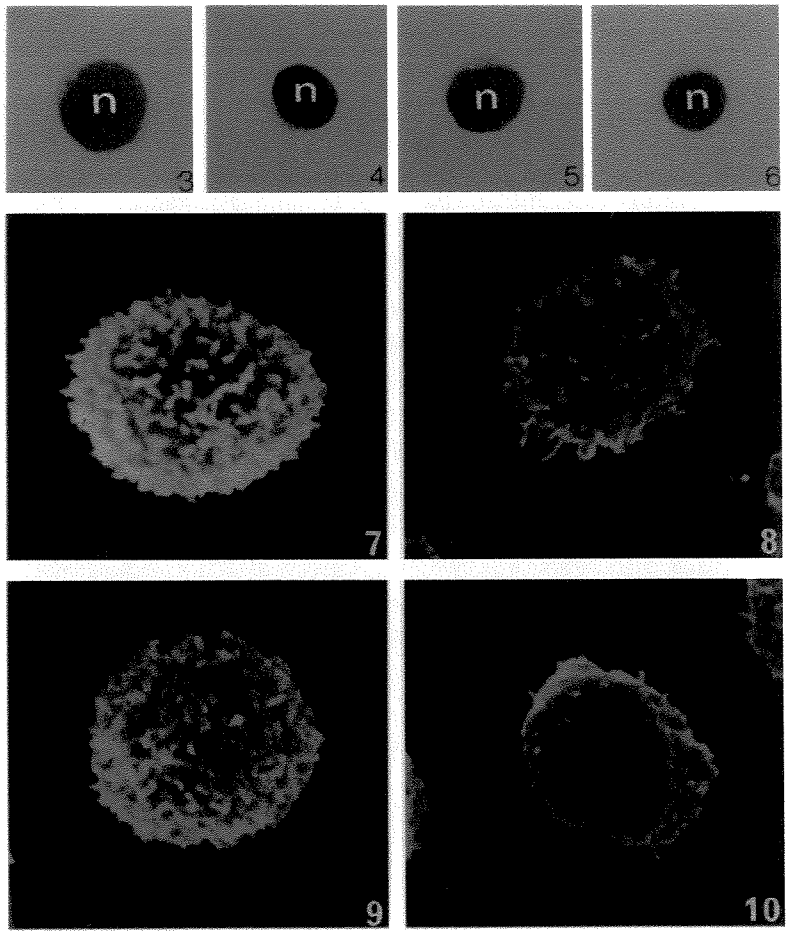
Treatments and Time

Fig. 2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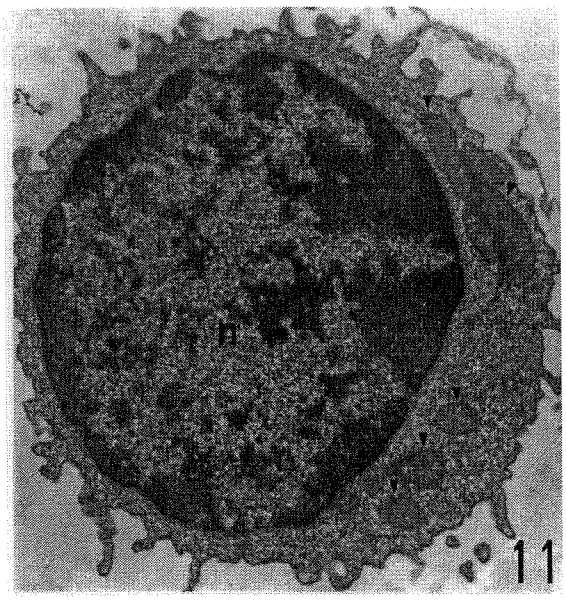
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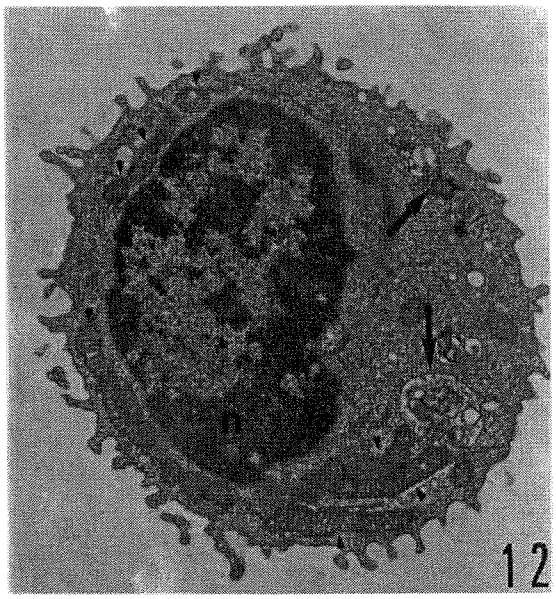
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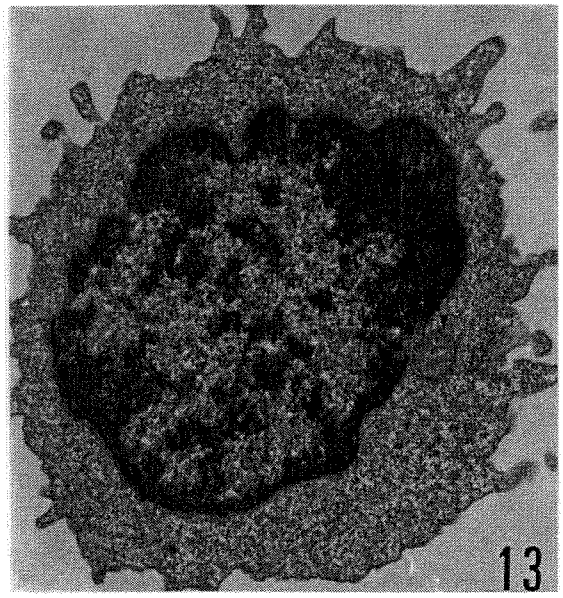
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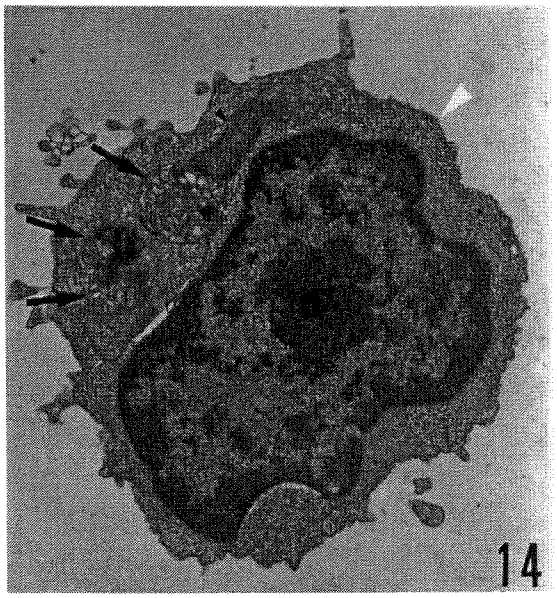
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LEGENDS

Fig.1 Cell viability of human lymphocytes after treatment with 20μM of TAM, verified by Trypan Blue exclusion test. C=control, T=treatment, A=young women, B=older women, 24 and 48 hours. Comparision of variables among treatments and groups were made by one-way analysis of variance (ANOVA) with 95% confidence limits. (*) indicate no statistically significance difference. (n=3 where n is the number of replicates).

Fig. 2 Apoptosis induction in human lymphocytes after treatment with 20μM of TAM, verified by fluorescent marking of phosphatidilserine on the outer leaflet of the cell membrane. C=control, T=treatment, A=young women, B=older women, 24 and 48 hours. 400 cells were counted for each sample. Comparision of variables among treatments and groups were made by one-way analysis of variance (ANOVA) with 95% confidence limits. (*) indicate no statistically significance difference. (n=2 where n is the number of replicates).

Fig. 3 Control lymphocyte maintained 24 h in culture: cell with a spherical shape, large nucleus (n), scant cytoplasm, and chromatin strongly stained in contrast with the cytoplasm (x 3.600).

Fig. 4 Lymphocyte treated with 20μM of TAM for 24 h: cell with a spherical shape, scant cytoplasm, large nucleus (n) with more condensed chromatin, strongly stained in contrast to the cytoplasm, and reduction of cell volume compared with the control (x 3.600).

Fig. 5 Control lymphocyte maintained 48 h in culture: cell with a spherical shape, large nucleus (n), scant cytoplasm, and chromatin strongly stained in contrast with the cytoplasm (x 3.600).

Fig. 6 Lymphocyte treated with 20μM of TAM for 48 h: cell with a spherical shape, scant cytoplasm, large nucleus (n) with more condensed chromatin, strongly stained in contrast to the cytoplasm, and reduction of cell volume compared with the control (x 3.600).

Fig. 7 Control lymphocyte after 24 h in culture: cell with spherical shape, covered by microvilli (x 9.000).

Fig. 8 Lymphocyte treated with 20μM of TAM for 24 h: cell with a spherical shape and some areas without microvilli (x 9.000).

Fig. 9 Control lymphocyte mantained 48 h in culture: cell with spherical shape, covered by microvilli (x 9.000).

Fig. 10 Lymphocyte treated with 20μM of TAM for 48 h: loss of spherical shape, large areas without microvilli (x 9.000).

Fig. 11 Control lymphocyte kept 24 h in culture: cell with a spherical shape, large nucleus (n) with condensed and loose chromatin pattern, scant cytoplasm, mitochondria (black head arrows) (x 15.000).

Fig. 12 Lymphocyte treated with 20μM of TAM for 24 h: cells with a spherical shape, nucleus (n) with more condensed chromatin compared with the control, autophagic vacuoles (arrows), mitochondria (black head arrows) (x 15.000).

Fig. 13 Control lymphocyte maintained 48 h in culture: cell with sperical shape, large nucleus with condensed and loose chromatin pattern, scant cytoplasm, mitochondria (black head arrows) (x 15.000).

Fig. 14 Lymphocyte treated with 20μM of TAM for 48 h: nucleus (n) with more condensed chromatin compared with the control, autophagic vacuoles (arrows), mitochondria (black head arrows), loss of spherical shape and of microvilli (white head arrow) (x 15.000).

Fig. 10 Lymphocyte treated with 20μM of TAM for 48 h: loss of spherical shape, large areas without microvilli (x 9.000).

Fig. 11 Control lymphocyte kept 24 h in culture: cell with a spherical shape, large nucleus (n) with condensed and loose chromatin pattern, scant cytoplasm, mitochondria (black head arrows) (x 15.000).

Fig. 12 Lymphocyte treated with 20μM of TAM for 24 h: cells with a spherical shape, nucleus (n) with more condensed chromatin compared with the control, autophagic vacuoles (arrows), mitochondria (black head arrows) (x 15.000).

Fig. 13 Control lymphocyte maintained 48 h in culture: cell with sperical shape, large nucleus with condensed and loose chromatin pattern, scant cytoplasm, mitochondria (black head arrows) (x 15.000).

Fig. 14 Lymphocyte treated with 20μM of TAM for 48 h: nucleus (n) with more condensed chromatin compared with the control, autophagic vacuoles (arrows), mitochondria (black head arrows), loss of spherical shape and of microvilli (white head arrow) (x 15.000).

7-CONCLUSÕES

O estudo do efeito do TAM sobre linfócitos humanos cultivados *in vitro* revelaram que:

1) A viabilidade celular foi reduzida pelo tratamento com o TAM em ambos os grupos.

2) O índice apoptótico foi maior nas células tratadas em relação aos controles em ambos os grupos.

3) Os estudos morfológicos demonstraram células tratadas com redução do volume celular, condensação cromatínica, presença de vacúolos autofágicos e perda de microvilosidades, características compatíveis com o processo apoptótico.

4) A maior parte das metodologias utilizadas (viabilidade celular, detecção de apoptose e microscopia eletrônica) demonstraram que maior tempo de exposição ao TAM traz maiores prejuízos às células.

5) As células obtidas de mulheres idosas se mostraram mais sensíveis à ação do tamoxifeno, possivelmente pela maior fragilidade dos linfócitos em função da idade.

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9-APÊNDICE

Tabela 1. Número de células/mL após o período de cultura, obtido por contagem em hemocitômetro a partir de teste de exclusão pelo Azul Tripan (viabilidade celular). Concentração inicial de 450.000 células/ mL. Foram feitas observações em triplicata para cada voluntária.

mulheres jovens	**Controle 24h**	**Tratado 24h**	**Controle 48h**	**Tratado 48h**
voluntária 1	407.250	419.850	418.500	324.000
	406.350	418.500	418.950	324.000
	409.500	418.950	417.150	318.600
voluntária 2	321.30	349.200	349.200	249.300
	320.850	342.900	346.500	249.750
	319.950	346.500	343.800	246.600
voluntária 3	400.500	419.400	419.850	323.100
	400.500	414.000	418.500	315.000
	401.400	415.350	417.150	315.900
mulheres idosas				
voluntária 1	340.650	292.500	261.000	224.100
	334.350	296.100	261.450	220.500
	337.500	289.350	262.350	217.350
voluntária 2	346.500	273.600	271.350	196.650
	348.300	272.250	271.350	193.500
	348.300	265.500	267.300	188.100
voluntária 3	373.500	286.650	270.000	195.750
	374.850	278.550	271.350	191.250
	376.200	283.500	273.150	197.100

Tabela 2. Número de células apoptóticas após o período de cultura obtido por contagem a partir de ensaio imunocitoquímico para fosfatidilserina (detecção de apoptose). Foram contadas ao total de 400 células por amostra e foram feitas observações em duplicata para cada voluntária.

mulheres jovens	**Controle 24h**	**Tratado 24h**	**Controle 48h**	**Tratado 48h**
voluntária 1	32	48	36	88
	32	48	34	96
voluntária 2	36	48	32	112
	32	50	34	108
voluntária 3	35	50	35	104
	36	49	35	108
mulheres idosas				
voluntária 1	85	136	88	148
	85	140	96	156
voluntária 2	76	116	80	128
	80	120	76	132

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