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DMSO MODIFIES STRUCTURAL AND FUNCTIONAL PROPERTIES OF RPMI-8402 CELLS BY PROMOTING PROGRAMMED CELL DEATH

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Apoptosis in lymphoid cells can be induced in different ways depending on cell type and acquired signal. Biochemical modifications occur at an early phase of cell death while at late times the typical morphological features of apoptosis can be visualized. The aim of this study is to verify by multiparametric analyses the plasma membrane fluidity, the intracellular Ca^{2+} concentration and the nitric oxide synthase (NOS) activity during cell death progression induced by DMSO treatment. The RPMI-8402 human pre-T lymphoblastoid cell line was induced to cell death by DMSO. Analyses rescued at early times of treatment prove a substantial modification of plasma membrane fluidity associated with an increase of intracellular Ca^{2+}. Moreover, these modifications are associated with an up regulation of NOS activity. Our results are consistent with the hypothesis that programmed cell death can be induced by up regulation of the intracellular Ca^{2+} associated with an increase of cell membrane fluidity. The apoptotic mechanisms seem to involve not only membrane damage and increased intracellular calcium levels but also production of nitric oxide.

Programmed cell death (PDC) or apoptosis represents the most common mechanism of eukariotic cell death consisting in an ordered sequence of cellular events that lead to the transcription of specific genomic sequences able to activate endogenous nucleases that cut the DNA in the linker region, producing nucleosomes or oligonucleosomes-DNA fragments. Cells that undergo apoptosis show morphological features due to chromatin condensation and decreased cell volume. Apoptosis in lymphoid cells can be induced in different ways depending on the cell system and acquired signals. Previously we found that dimethyl sulfoxide (DMSO) provokes cell death by PCD in human pre-T cell line RPMI – 8402 (1). This effect of DMSO may be caused by a perturbation of plasma membrane microviscosity, as previously suggested (2,3). It is well known that the biophysical properties of cell membranes have crucial impact on the activity of enzymes and membrane transport functions. In most cell lines, apoptosis is induced by an increase in intracellular Ca^{2+} associated with an enhancement of cell membrane permeability (4,5). Free intracellular Ca^{2+} is a pleiotropic regulatory substance in various types of cell; many of the effects of cytosolic Ca^{2+} involve the up-regulation of inflammatory genes, including those encoding nitric oxide synthase (6). Nitric oxide (NO) is a radical produced in mammalian cells from arginine in a reaction catalyzed by nitric oxide synthase (NOS) and it has been shown to induce programmed cell death in thymocytes (7). NO is synthesized by many cell types involved in immunity and inflammation. The principal enzyme involved is the inducible type-2 isoform of NOS (NOS-2), which produces

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high-level sustained NO synthesis. At high concentrations, as generated by NOS-2, NO is rapidly oxidised to reactive nitrogen species (RNOS) with a variety of biologic effects, including cell death (7-10).

The aim of the present study is to elucidate specific biochemical mechanism occurring at early stage of cell death progression. Untreated and DMSO-treated human RPMI-8402 cells were observed using both morphological and biochemical methods, including measurement of membrane fluidity, intracellular Ca^{2+} concentration $[Ca^{2+}]_i$, and nitric oxide synthase (NOS) activity.

MATERIALS AND METHODS

Cell cultures

Human RPMI-8402 cell line is a thymic lymphoma showing immunological features of pre-T cells as previously described (1,11). Cells were maintained in continuous suspension culture in RPMI-1640 medium supplemented with 10% FBS, 4 mM L-glutamine, 100 mM sodium pyruvate, and 25 mM Hepes. Cells were grown at 2.5 x 10^5/ml. with more than 98% viability as determined by trypan blue exclusion test. During the log - growth phase, cells were treated with 1.5% (v/v) DMSO (gas chromatography grade) up to 72 hrs. Samples were immediately processed for morphological and biochemical analyses.

Apoptosis estimation

Apoptosis was detected by morphological examinations, at the light and the electron microscope level, and by flow cytometry as previously described (1,12).

Gel electrophoresis DNA laddering assessment.

DNA was extracted with ethanol chloroform/isoamyl alcohol (24:1) and precipitated with 2.5 vol. of absolute ethanol. 10 mgr of DNA/sample were loaded into a 1.8% agarose gel, runnning at 20V, and observed by UV light.

Morphological analyses

Treated and untreated cells were fixed with 1.25 % glutaraldehyde in cacodylate buffer for one hour and post-fixed with 1 % OsO_4 in the same buffer for one hour at 4°C. Treated samples were stained over-night with saturated aqueous uranyl acetate solution, dehydrated and embedded in Spurr medium. Thin sections were counterstained with lead citrate. Ultrastructural observations were carried out using a Philips CM10 electron microscope operating at 60 kV.

Fluorescence measurements

To measure the fluidity of the hydrophilic region (outer membrane) of the cell membrane, the fluorescent probe 1- (4-trimethylaminophenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), a hydrophilic derivative of DPH, was used. Cell incubation with the probe was performed as described by Sheridan and Block (13). Briefly, 3 ml of TMA-DPH (10^{-3}M) was incubated for 5 min at room temperature (23 °C) with 2 ml of freshly prepared cells in 50 mM Tris-HCl buffer solution, pH 7.4. Fluorescence intensities (100 readings each) of the vertical and horizontal components of the emitted light were measured by a Perkin-Elmer LS 50 B spectrofluorometer equipped with two glass prism polarizers (excitation wavelength 365 nm, emission wavelength 430 nm). Membrane fluidity is expressed as the inverse of fluorescence polarization. Steady-state fluorescence polarization (p) of TMA-DPH was calculated by using the following equation:

$$p = (I_v - I_h)(G) / (I_v + I_h)(G)$$

where G is the correction given by the ratio of the vertical to the horizontal components when the excitation light is polarized in the horizontal direction (14) factor that corrects the r-value for an unequal detection of vertically (I_v) and horizontally (I_h) polarized light.

Intracellular Ca^{2+} concentration

Intracellular Ca^{2+} concentration was measured in intact cells using the fluorescent probe FURA 2-AM as previously described (15). Determinations were performed using a Perkin-Elmer LS 50 B spectrofluorometer at 37 °C according to the method of Rao (16). Fluorescence intensity was evaluated at a constant emission wavelength (490 nm) with changes in the excitation wavelength (340 and 380 nm). At the end of an experiment, maximum and minimum fluorescence values, at each excitation wavelength were obtained by first lysing the cells with 0.1% Triton X-100 (maximum) and then adding 10 mM EGTA (minimum). With the maximum and minimum values, the 340/380 nm fluorescence ratios were converted into free Ca^{2+} concentrations using a Fura –2 Ca^{2+} binding constant (135 nM) and the formula described by Grynkiewicz et al. (17).

NOS activity

NOS activity was quantified by measurement of the nitrite levels in supernates of sonicated cells as described by Chen and Mehta (18). The nitrite level was measured by the Griess reaction (19). In brief, cells were suspended in NO buffer (composition in mM: HEPES 25, NaCl 140, KCl 5.4, $CaCl_2$ 1, and $MgCl_2$ 1, pH 7.4) containing 1.44 mM NADPH and incubated with buffer plus L-arginine (1 mM) for 1 hour at 37 °C. The reaction was then stopped by freeze-thawing and, the samples were then sonicated. To reduces nitrate to nitrite each sample was incubated for 1 hour at 37 °C in presence of nitrate reductase (20 mU). After centrifugation at 10,000 rpm for 15 min, the supernate was allowed to react with the Griess reagent (19). The chromophore absorption was read at 543 nm. The nitrite concentration was determined with sodium nitrite in water as standard.

The assay was normalized to protein's content evaluated, in sonicated samples, by the method of Lowry et al. (20). NOS activity was expressed as mmoles of nitrite/mg protein/hour.

Statistical analysis

All experiments were performed four times. Analysis of variance was used to compare the results. When F-values were significant, the Bonferroni t-test was used. P values less than 0.05 were considered statistically significant.

Source of materials

DMSO and all cell lines culture material were from Sigma (St. Louis MO, USA). Reagents for electron microscopy were from Polyscience (Warrington, VA, USA), while products for electrophoresis were from Bio-Rad Laboratories (München, Germany).

RESULTS

DMSO induces in RPMI-8402 human cell line several biological modifications including cell growth arrest, phenotype changes and programmed cell death progression (21,22). Each of these events appears associated to the other in a treatment-time manner dependent.

Morphological analyses carried out at the level of the electron microscope (Fig. 1) prove that DMSO provokes several time-dependent cellular modifications. Untreated RPMI-8402 cells (section A) are characterized by a relatively high nucleus/cytoplasm ratio. The nucleus contains a

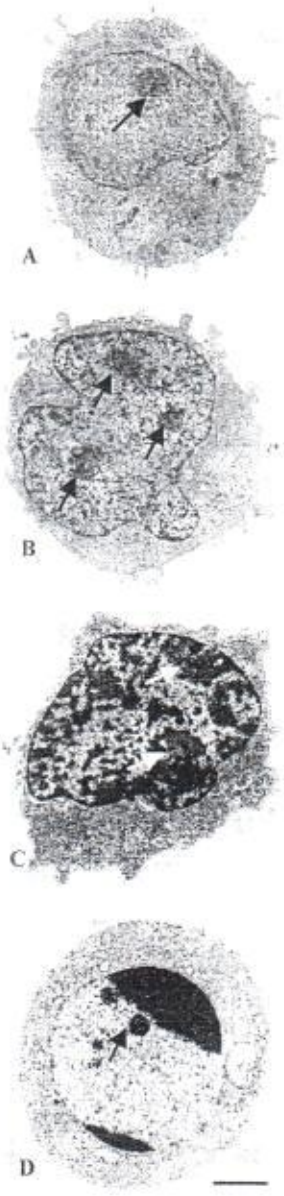

Fig. 1. *Morphological analysis of RPMI-8402 lymphoblastoid human pre-T cells. Section A displays the image of untreated cell; Sections B, C and D report respectively the cells after 8, 24 and 48 hours of DMSO treatment. The micrographs demonstrate the cell modification due to apoptotic program progression. At 8 hours, treated cells shows the decrease in number and size of cytoplasmic organelles. The cell surface initially is flat and at the nuclear level several chromatin aggregates are forming. Nucleoli are compacted. At 24 hours of treatment they can be visible the initial clumping of the chromatin, representative of nuclear condensation that increases throughout the treatment. These cells, after 48 hours show the typical apoptotic features with the presence of condensed chromatin at the nuclear periphery. The absence of cytoplasmic organelles and a flatting of the plasma membrane are also evident. Arrows: Nucleoli. Bar: 2 μm.*

Fig. 2. *Electrophoretic analysis of DNA, recovered at 0 (lane a), 8 hours (lane b), 12 hours (lane c), 24 hours (lane d), 48 hours (lane e), and 72 hours (lane f) of DMSO treatment. The ladder-like pattern of DNA banding, indicative of chromatin fragmentation, -begin to be evident after 48 hours of treatment.*

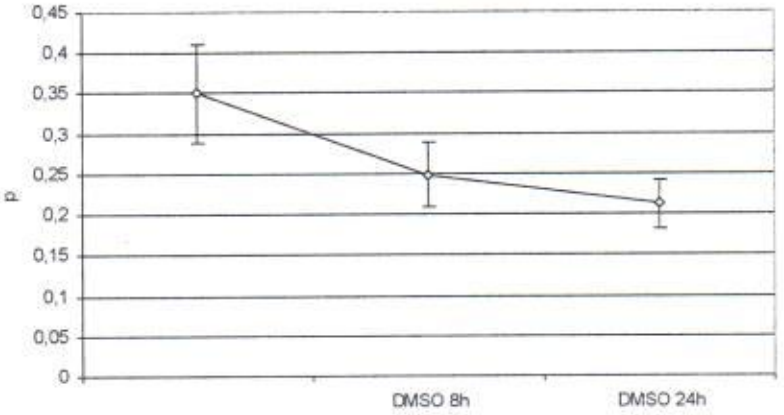

Fig. 3. *Plasma membrane steady-state fluorescence polarization (p) of TMA-DPH in RPMI-8402 cells in the basal state (0) and after 8 hours and 24 hours of incubation with DMSO. Means ± S.D. are shown.*

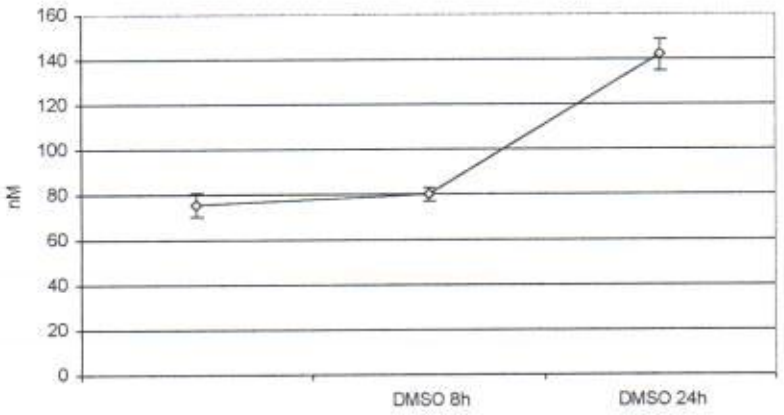

Fig. 4. *$[Ca^{2+}]_i$ in RPMI-8402 cells in the basal state (0) and after 8 hours and 24 hours of incubation with DMSO. Means ± S.D. are shown.*

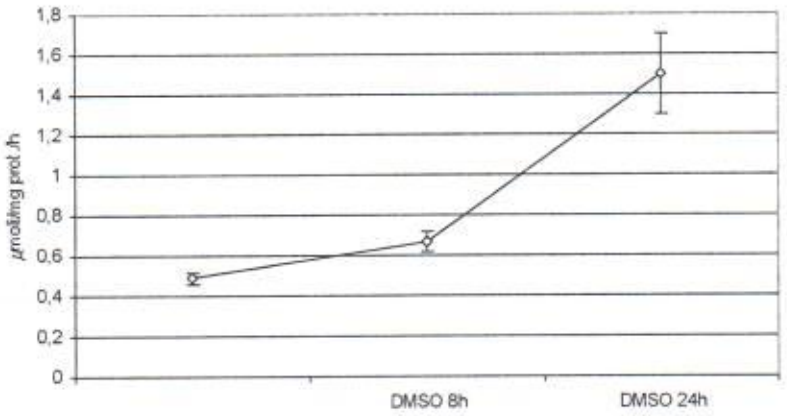

Fig. 5. *NO produced by RPMI-8402 cells before (0) and after 8 hours and 24 hours of incubation with DMSO. Means ± S.D. are shown.*

large amount of dispersed chromatin with some blocks of condensed chromatin confined to the nuclear periphery; one or more nucleoli are recognizable (arrows). The cytoplasm contains several mitochondria, RER profiles and Golgi apparati. The plasma membrane is irregular with the presence of microvilli or philipodia. DMSO treatment induces after 8 hours (section B) a rearrangement of the cytoplasmic compartment with a decrease in number and size of the organuli such as mithocondria or Golgi. The cytoplasmic membrane initiate the modification of and subsequent disappearance of the microvilli. At a nuclear level, more evident is the presence of the condensed chromatin which appears uniformly distributed. At 24 hours (section C) treated cells show an increase in density with intense modification both at the nuclear and cytoplasmic level. The nuclear chromatin is condensed in great compacted granular masses. These aggregates often are bound to nuclear membrane, probably constituting the initial step of micronuclei formation. The nucleoli are often disarranged are sometimes localized close to the nuclear membrane (arrows). At the cytoplasmic level it can be hard to distinguish structured organelles as mitochondria, RER or Golgi apparatus. Moreover, comparing these cells to others in the basal condition, a well defined remodelling of the cellular membrane arrangement to a flat, appearance and without the typical presence of microvilli can be demonstrated. After 48 hours of DMSO treatment (section D) several cells show typical apoptotic features. The nucleus is separated into mostly discrete fragments or roundish blocks linked to the nuclear membrane. Nucleoli are smaller, condensed and somewhat round. The cytoplasm is decondensed and several vacuoli are present. The plasma membrane is completely flat or, in dead cells, frequently destroyed.

DNA electrophoresis (Figure 2) shows the presence of ladder-type fragmentation, indicative of apoptotic cells, exclusively in the DMSO-treated samples, at 48 and 72 hours while at 0, 8, 12 and 24 hour no evidence of a DNA fragmentation is detectable.

Regarding biochemical data, DMSO treatment affects, as shown in figure 3, the plasma membrane fluidity of RPMI-8402 cells. In particular, a significant decrease of TMA-DPH p values (i.e. increased fluidity of the superficial part of the membrane) was observed both after 8 and 24 hours of incubation with DMSO (being time 0: 0.350±0.06; DMSO 8 hours: 0.248±0.04; DMSO 24 hours: 0.212±0.03; $p<0.05$).

As concerns the intracellular calcium concentration, it was not significantly changed after 8 hours of incubation with DMSO (being time 0 : 75.76±5.2 nM; DMSO 8 hours: 80±3 nM), while it was significantly increased after 24 hours (DMSO 24 hours: 142±7nM; $p<0.001$) (Figure 4). The increased intracellular calcium concentration can be due to an enhanced release from intracellular stores or to a higher influx through plasma membrane.

Finally, the incubation with DMSO caused a significant increase in the NOS activity of RPMI-8402 cells (Figure 5). In particular, the NO production was greatly enhanced after 24 hours (being time 0: 0.49±0.03 μmoles/mg prot./h; DMSO 8 hours: 0.665±0.05 μmoles/mg prot./h; DMSO 24 hours: 1.5±0.2 μmoles/mg prot./h; $p<0.05$).

DISCUSSION

It is accepted that apoptosis is a gene-controlled process of cellular self-destruction. It occurs during physiological regulation and in pathological situations in the cell life (23). In the immune system, several different intracellular and extracellular factors have been associated with the induction of apoptosis with the final responses dependant on cell system and the acquired signals (24,25). Previously we have demonstrated that DMSO induces apoptosis in human pre-T cell line RPMI – 8402 (1). Moreover it has been reported (26) that at early times of the apoptotic program the biochemical modifications are more evident than morphological changes. Typical morphological apoptotic features correspond to final step of cell death progression.

For this reason in this study, employing the RPMI-8402 cell line, we focused our attention to biochemical and morphological modifications, examining the role of plasma membrane fluidity, intracellular calcium concentration, and NOS activity during the first hours of DMSO treatment.

In this work, the incubation of RPMI-8402 cells with DMSO was associated with a significant increase in the fluidity of the more superficial part

of the plasma membrane, as demonstrated by the lower TMA-DPH p values. Our results strongly suggest that the membrane fluidity is an important physical property related to the induction of apoptosis.

These data are in agreement with previous results suggesting that apoptotic lymphocytes are characterised by a membrane fluidization that mainly occurs on the cell membrane surface (5). Functionally, the increased plasma membrane fluidity associated with apoptosis may represent a mechanism to translocate phosphatidylserine to the outer leaflet, mediating phagocytic recognition of apoptotic cells, or a consequence of this loss of lipid asymmetry.

Recently it was demonstrated that lipid asymmetry loss is promptly detectable after one hour of incubation time in plasma membranes of EL4 cells treated with apoptosis inducers (27), thus confirming that cell membrane perturbation is detectable at early stages of the apoptotic process.

It is well known that various cellular functions are modulated by the physical properties of the cell membrane (28). Secondary effects of plasma membrane modification might be changes in cytosolic Ca^{2+} concentration caused by altered transmembrane calcium flux. Our biochemical data show that DMSO treatment affects the intracellular calcium concentration in RPMI-8402 cells. The increase in $[Ca^{2+}]_i$ may activate calcium-dependent degradative enzymes, including Ca-dependent endonucleases that cause DNA fragmentation and death in apoptotic cells. In addition to calcium-activated nucleases other key molecules associated with the regulation of apoptosis may be activated by increased $[Ca^{2+}]_i$, such as cytokines and transcription factors (29,30). Recently it was demonstrated that inflammatory genes including NOS-2 may be regulated by oxidant-sensitive transcription factors (31). In agreement with the above-mentioned observations in this study we have found that the incubation with DMSO caused a significant increase in the NOS activity, probably through a calcium-mediated activation of transcription factors, such as NF-kB. Nitric oxide is important as a toxic defence molecule against infectious organisms. It also regulates the functional activity, growth and death of many immune and inflammatory cell types including T lymphocytes (8). Moreover, it is well known that peroxynitrite, a powerful oxidant produced in vivo by reaction of superoxide and NO, is able to mediate apoptosis (32).

In summary, RPMI-8402 cells treated with DMSO were found to exhibit both morphological and biochemical characteristics indicative of apoptosis. The apoptotic mechanisms involve not only membrane damage and an increase in intracellular calcium levels but also production of nitric oxide.

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REFERENCES

1. **Trubiani O., M. Ciancarelli, M. Rapino and R. Di Primio.** 1996. Dimethyl sulfoxide induces programmed cell death and reversible G1 arrest in the cell cycle of human lymphoid pre-T cell line. *Immunol. Lett. 50:51.*
2. **Hynie S. and V. Klenerova.** 1980. Effects of dimethyl sulfoxide and other dipolar aprotic solvents on rat hepatic adenylate cyclase. Potentiating effects on glucagons and guanylylimidodiphosphate stimulation. *Naunyn. Schmiedebergs Arch. Pharmacol. 310:231.*
3. **Nygren P., R. Larsson, J. Rastad, G. Akerstrom and E. Gylfe.** 1987. Dimethyl sulfoxide increases cytoplasmic Ca^{2+} concentration and inhibits parathyroid hormone release in normal bovine and pathological human parathyroid cells. *Biochim. Biophys. Acta. 928:194.*
4. **Pozzan T., R. Rizzuto, P. Volpe and J. Meldolesi.** 1994. Molecular and cellular physiology of intracellular calcium stores. *Physyol. Rev. 74:595.*
5. **Benderitter M., L. Vincent-Genod, A. Berroud and P. Voisin.** 2000. Simultaneous analysis of radio-induced membrane alteration and cell viability by flow cytometry. *Cytometry. 39:151.*
6. **Korhonen R., H. Kankaanranta, A. Lahti, M. Lähde, R.G. Knowles and E. Moilanen.** 2001. Bi-directional effects of the elevation of intracellular calcium on the expression of inducible nitric oxide synthase in J774 macrophages exposed to low and to high concentrations of endotoxin. *Biochem. J. 354:351.*
7. **Jun C.D., S.J. Park, B.M. Choi, H.J. Kwak, Y.C. Park, M.S. Kim, et al.** 1997. Potentiation of the activity of nitric oxide by the protein kinase C activator phorbol

ester in human myeloid leukemic HL-60 cells: association with enhanced fragmentation of mature genomic DNA. *Cell. Immunol. 176:41.*

8. **Coleman J.W.** 2001. Nitric oxide in immunity and inflammation. *Int. Immunopharmacol. 1:1397.*
9. **Agostini M., B. Di Marco, G. Nocentini and D.V. Delfino.** 2002. Oxidative stress and apoptosis in immune diseases. *Int. J. Immunopathol. Pharmacol. 15:157.*
10. **Acharya A.** 2002. H_2O_2 – induced apoptosis of thymocytes involves mobilization of divalent cations. *Int. J. Immunopathol. Pharmacol. 15:195.*
11. **Huang C.C., Y Hou, G.E. Moore and J. Minowada.** 1974. Cytogenetic study of human lymphoid T-cell lines derived from lymphocytic leukaemia. *J. Natl. Cancer Inst. 53:655.*
12. **Sawai M., K. Takase, H. Teraoka and K. Tsukada.** 1990. Reversible G1 arrest in the cell cycle of human lymphoid cell lines by dimethyl sulfoxide. *Exp. Cell Res. 187:4.*
13. **Sheridan N.P. and E.R. Block.** 1988. Plasma membrane fluidity measurements in intact endothelial cells: effect of hyperoxia on fluorescence anisotropies of 1-(4-trimethylaminophenyl)-6-phenylhexa-1,3,5-triene. *J. Cell. Physiol. 134:117.*
14. **Kuhry J.G., G. Duparatali, C. Bronner and G. Laustriat.** 1985. Plasma membrane fluidity measurements on whole living cells by fluorescence anisotropy of trimethylammoniumdiphenylhexatriene. *Biochim. Biophys. Acta. 845:60.*
15. **Mazzanti L., R.A. Rabini, E. Faloia, P. Fumelli, E. Bertoli and R. De Pirro.** 1990. Altered cellular calcium and sodium transport in diabetes mellitus. *Diabetes. 39:850.*
16. **Rao G.H.R.** 1988. Measurement of ionized calcium in normal human blood platelets. *Anal. Biochem. 169:400.*
17. **Grynkiewicz G., M. Poenie and R.Y. Tsien.** 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem. 260:3440.*
18. **Chen L.Y. and J.L. Mehta.** 1994. Inhibitory effect of high -density lipoprotein on platelet function is mediated by increase in nitric oxide synthase activity in platelets. *Life Sci. 55:1815.*
19. **Green L.C., D.A. Wagner, J. Glogowski, P.L. Skipper, J.S. Wishnok and S.R. Tannenbaum.** 1982. Analysis of nitrate, nitrite and [^{15}N] nitrate in biological fluids. *Ann. Biochem. 126:131.*
20. **Lowry O.H., M.Y. Rosenbrough, A.L. Farr and R.J. Randall.** 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem. 193:265.*
21. **Trubiani O., C. Pieri, M. Rapino and R. Di Primio.** 1999. The c-myc gene regulates the polyamine pathway in DMSO-induced apoptosis. *Cell. Prolif. 32:119.*
22. **Trubiani O., S. Guarneri, R. Paganelli and R. Di Primio.** 2002. Involvement of capsase-3 in the cleavage of terminal transferase. *Int. J. Immunopathol. Pharmacol. 15:201.*
23. **Ellis R.E., J. Yuan and H.R. Horvtitz.** 1991. Mechanism and functions of cell death. *Annu. Rev. Cell Biol. 7:663.*
24. **Ashwell J.D., N.A. Berger, J.A. Cidlowski, D.P. Lane and S.J. Korsmeyer.** 1994. Coming to terms with death: apoptosis in cancer and immune development. *Immunol. Today 15:147.*
25. **Linette G.P. and S. Korsmeyer.** 1994. Differentiation and cell death: lessons from the immune system. *Curr. Op. Cell Biol. 6:809.*
26. **Kroemer G., P. Petit, N. Zamzami, Y. Vayssière, and B. Mignotte.** 1995. The biochemistry of programmed cell death. *FASEB J. 9:1277.*
27. **Jessel R., S. Haertel, C. Socaciu, S. Tykhonova and H.A. Diehl.** 2002. Kinetics of apoptotic markers in exogenously induced apoptosis of EL4 cells. *J. Cell. Mol. Med. 6:82.*
28. **Kinnunen P.K.J.** 1991. Effect of phorbol ester on the release of atrial natriuretic peptide from the hypertrophied rat myocardium. *Chem. Phys. Lipids. 57:375.*
29. **Jayaraman T and A.R. Marks.** 1997. T cells deficient in inositol 1,4,5-triphosphate receptor are resistant to apoptosis. *Mol. Cell. Biol. 17:3005.*
30. **Gong G., G. Waris, R. Tanveer and A. Siddiqui.** 2001. Human hepatitis C virus NS5A protein alters intracellular calcium levels, induces oxidative stress, and activates STAT-3 and NF-kappa B. *Proc. Natl. Acad. Sci. USA. 98:9599.*
31. **Lim J.W., H. Kim and K.H. Kim.** 2001. NF-kappaB, inducible nitric oxide synthase and apoptosis by elicobacter pylori infection. *Free Radic. Biol. Med. 31:355.*
32. **Wei T., C. Chen, B. Zhao, W. Xin and A. Mori.** 1998. EPC-K1 attenuates peroxynitrite-induced apoptosis in cerebellar granule cells. *Biochem. Mol. Biol. Int. 46:89.*