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Biochemical Pharmacology 63 (2002) 437–453

Biochemical Pharmacology

In vitro induction of apoptosis vs. necrosis by widely used preservatives: 2-phenoxyethanol, a mixture of isothiazolinones, imidazolidinyl urea and 1,2-pentanediol☆

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Received 30 August 2001; accepted 13 November 2001

Abstract

Preservatives are added to many final products, such as detergents, cosmetics, pharmaceuticals and vaccines. We conducted an *in vitro* investigation of the apoptosis- and necrosis-inducing potential of brief applications (10 min) of four common preservatives: ethylene glycol monophenyl ether, 2-phenoxyethanol (EGPE), imidazolidinyl urea (IMU), a mixture of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one (CMI/MI), and 1,2-pentanediol, a ''preservative-non-preservative'' best known as pentylene glycol. Using HL60 cells, we monitored the kinetics of cell toxicity with the MTT test and analysed extranuclear end points of apoptosis, i.e. phosphatidylserine exposure and nuclear fragmentation. Preservative treatment resulted in a dose-dependent decrease of cell viability. The mode of cell death was dose-dependent: necrosis occurred at high concentrations while apoptosis, shown by DNA laddering, DNA subdiploid peak and caspase-3 activation, occurred at lower concentrations 0–24 hr after exposure to a single dose: CMI/MI induced apoptosis at low concentrations (0.001–0.01%) and necrosis at high concentrations (0.5–0.1%); IMU and EGPE required higher concentrations to induce apoptosis (IMU 0.01–0.1% and EGPE 0.01–0.5%) or necrosis (IMU 0.5–1% and EGPE only at 1%). PG induced apoptosis only at 5%. Externalization of PS, a hallmark of apoptosis, occurred early in HL60 treated with low concentrations of CMI/MI and EGPE and was concomitant with the subdiploid peak in HL60 treated with PG. However, it did not occur in HL60 treated with IMU. In conclusion, at appropriate concentrations, each of the four preservatives modulates the apoptotic machinery by a caspase-dependent mechanism. Thus, apoptosis could be a good parameter to evaluate the cytoxicity of these chemical compounds.

Keywords: Apoptosis; Caspase-3; Cytofluorimetric analysis; HL60; Necrosis; Preservatives

1. Introduction

Necrosis and apoptosis are two different forms of cell death, induced by different stimuli or also by the same stimulus: in fact the same stimulus induces apoptosis or necrosis in a dose-dependent-manner [1–5]. The morphological and biochemical features through which cell death is manifested are also different. Necrosis occurs when the stimuli are very strong and is characterized by swelling of the cytoplasm and cytoplasmic organelles, swelling of the nucleus and early rupture of the plasma membrane [1–3].

Apoptosis involves a biochemical and genetic regulatory network providing for cell suicide in response to non-lethal stimuli [6–8]. Apoptotic cells show characteristic markers, such as condensation of nuclear chromatin, loss of plasma membrane asymmetry with membrane scrambling of phosphatidylserine (PS) [9], activation of proteases (e.g. caspases) and endonucleases that cleave the DNA into

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Abbreviations: Ac-DEVD-*p*NA, acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline; AnnxV, annexin-V; EGPE, ethylene glycol monophenyl ether, 2-phenoxyethanol; FITC, fluorescein-labelled; CMI/MI, mixture of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one; HL60, human leukemia cells; MTT, 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; IMU, imidazolidinyl urea; PBS, phosphate buffered saline; PG, 1,2-pentanediol, pentylene glycol; PI, propidium iodide; PS, phosphatidylserine; RPMI 1640, Roswell Park Memorial Institute; Tris, tris(hydroxymethyl)aminomethane.

0006-2952/02/$ – see front matter
PII: S0006-2952(01)00910-8

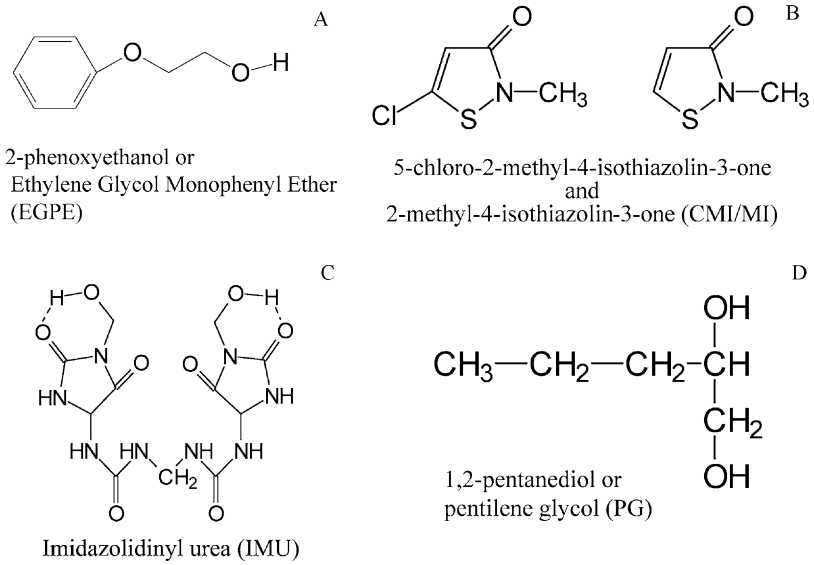


Fig. 1. Chemical structure of (A) EGPE, (B) CMI/MI, (C) IMU, and (D) PG.

multiples of 180 bp fragments observable as a classical ladder, segmentation of the cells into ''apoptotic bodies'', zeiosis and observable in cell culture, ''secondary necrosis'' [10–13].

Preservatives are an important class of chemicals used to inhibit the growth of microorganisms harmful to industrial products. The most frequently used biocides are EGPE [14], an aqueous solution of mixture of CMI/MI in an approximate ratio of 3:1 [15], IMU [16], and a ''preservative-non-preservative'' 1,2-pentanediol, pentylene glycol (PG) [17] (Fig. 1A–D). These chemicals are added to pharmaceuticals (i.e. in vaccine formulations, EGPE), cosmetic creams and lotions, and household detergents (IMU, PG and CMI/MI). *In vivo* they are toxic to microorganisms but also to humans. Other authors have investigated the potential toxicity of preservatives used in foods (butylated hydroxytoluene and butylated hydroxyanisole, sulphites and mercurial compounds) [18–23] and in topical ophthalmological preparations (benzalkonium chloride and its derivatives) [24,25]. To our knowledge, there are no reports on the effects of the preservatives tested in this study. Therefore, we investigated whether these compounds are able to induce apoptosis *in vitro*. Each of the compounds was tested at different concentrations and at different times after brief applications to the cells (10 min). We performed the experiments *in vitro* on HL60 tumour cells, which represent a good model to highlight the hallmarks of apoptosis. For each compound, the effect on cell viability was monitored by the 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) test and Trypan Blue dye exclusion assay while the induction of apoptosis was qualitatively monitored for DNA ladder formation by means of agarose gel electrophoresis and quantified by evaluating both subdiploid DNA content and annexin-V (AnnxV) binding by flow cytometry. The relationship between apoptosis induction and caspase-3 activation was also investigated.

2. Materials and methods

2.1. Cell line and reagents

The human promyelocytic leukemia cell line (HL60), originated from the American Type Culture Collection, was kindly provided by Marcella Cintorino (Istituto di Anatomia Patologica, Policlinico Le Scotte, Università degli Studi di Siena). Roswell Park Memorial Institute (RPMI 1640) and MTT were obtained from Sigma. Foetal calf serum was obtained from Gibco. AnnxV was obtained from Boehringer Ingelheim Bioproducts. EGPE was obtained from INDUCHEM. IMU was obtained from SEPPIC. CMI/MI was obtained, as dilute solution of 1.5% of the two isothiazolinones, from Rohm and Haas. PG was obtained from DRAGOCO.

2.2. Cell treatment

HL60 cells were grown in RPMI medium containing 10% foetal calf serum, 100 U/mL penicillin G and 100 μg/mL streptomycin, at 37° in a controlled humidified incubator in 5% CO_2. The cells were used between the 5th and 20th passage. Cells, plated at the concentration of 5×10^5/mL, were incubated at 37° in RPMI medium containing 10% foetal calf serum. After 1 hr of incubation, the cells were exposed to scalar concentrations of the different preservatives for 10 min, then collected by centrifugation, resuspended in fresh medium at 1×10^6/mL and incubated for different times. Cells treated with phosphate buffered saline (PBS) 1X served as controls.

The four preservatives were tested at the following concentrations: (1) EGPE: between 0.01 and 1%, its usual concentration as preservative being 0.5% and the maximum concentration accepted being 1%;[1] (2) CMI/MI: between 0.001 and 0.1%, its usual concentration as preservative being 0.05% and the maximum concentration accepted being 0.1% of diluted aqueous solution of 1.5% of the two isothiazolinones (being 0.0015% of non dilute solution of CMI/MI in a ratio 3:1) (see footnote 1); (3) IMU: between 0.01 and 1%, its usual concentration as preservative being 0.5% and the maximum concentration accepted being 0.6% (see footnote 1); (4) PG: between 0.01 and 5%, its usual concentration as preservative being 5%.

The duration of cell treatment with the preservatives was chosen on the basis of data available in the literature and our data from preliminary experiments.

2.3. Cytotoxicity and cell viability

In the preliminary study, the cytotoxic effect of different preservatives was estimated by measurement of the rate of mitochondrial metabolism of MTT [26]. Briefly, control and treated cells were seeded at 1×10^5 cells/well in 100 μL of medium in 96-well plates and 10 μL of a MTT (5 mg/mL in PBS) were added to each well. After 4 hr of incubation at 37°, 100 μL of a lysing buffer (10% sodium dodecyl sulphate; 45% dimethylformamide; adjusted to pH 4.5 with glacial acetic acid) were added to each well and the blue formazan crystals were dissolved by pipetting. The plates were read with a microplate reader (Bio-Rad) using a test wavelength of 595 nm and a reference wavelength of 655 nm. Viability was also analysed at different times by the Trypan Blue dye exclusion assay. All the cytotoxicity and cell viability assays were performed in triplicate.

2.4. DNA fragmentation assay

Immediately after exposure to preservatives, the cells were incubated at 37°. At different times of incubation, the cells were collected by centrifugation and the DNA was isolated from 2×10^6 cells for each treatment. After harvesting, the cell samples were washed with phosphate buffered saline and pelleted by centrifugation. The cell pellet was then treated with lysis buffer TTE (0.2% Triton X-100, in 10 mM Tris–HCl, 1 mM EDTA pH 7.4). To separate fragmented DNA from intact chromatin, the samples were centrifuged at 20,000 *g* for 10 min, the pellets were separated from the supernatant and both were treated with ice-cold 5 M NaCl to remove the histones from DNA. Genomic and apoptotic DNA was precipitated in ice-cold isopropanol overnight at −20°. After centrifugation, the pellet was rinsed by adding ice-cold 70% ethanol and the DNA was dissolved in TE buffer (Tris–HCl, 1 mM EDTA, pH 7.4) and incubated at 37° for 1–3 days. The DNA samples were heated in a 65° bath for 10 min and immediately loaded onto a 1% agarose gel containing ethidium bromide (0.5 μg/mL). Electrophoresis was carried out in TBE buffer (Tris–HCL/borate/EDTA electrophoresis buffer) until the marker dye had migrated 4–5 cm. Gels were examined and photographed under UV light.

2.5. Measurement of cellular DNA content

To quantify the percentage of apoptotic cells by analysis of DNA content, we used the simple flow cytometric method described by Nicoletti *et al.* [27]. At different times after exposure to preservatives, 1×10^6 cells for each sample were washed in PBS 1X and the pellet was fixed overnight in ice-cold ethanol 70% at −20°. Then, the cell suspension was centrifuged, washed twice with 1 mL of PBS and resuspended in 1 mL of a PBS solution containing RNAse (Type I-A, Sigma, 1 mg/mL final concentration) and propidium iodide (PI) (Sigma, 50 μg/mL final concentration). The tubes were placed on ice in the dark until the cellular red fluorescence of PI was collected on a linear scale using a FACScan flow cytometer (Becton Dickinson) equipped with an excitation laser line at 488 nm and a 575 ± 15 nm band pass filter. At least 20,000 events were collected for each sample using the Cell Quest software (Becton Dickinson) and the pulse processing module for doublet discrimination; debris was excluded from the analysis by an appropriate morphological gate of forward scatter (FSC) vs. side scatter (SSC).

2.6. Measurement of phosphatidylserine (PS) expression using fluorescein-labelled (FITC) Annexin V

AnnxV binding and PI uptake were assessed by flow cytometry using a commercial kit (Boehringer Ingelheim Bioproducts) according to the manufacturer's instructions. Briefly, at different times of incubation after exposure to preservatives, approximately 2.5×10^5 cells for each sample were washed twice in PBS and the pellet was resuspended in 200 μL of the binding buffer provided in the kit. A 5 μL of the fluorescein-labelled (FITC)-AnnxV kit stock solution were added to the cell suspension (1 μg/mL final concentration) and incubated for 10 min at room temperature in the dark. Then, the cells were washed in PBS and resuspended in 190 μL of binding buffer plus 10 μL of the PI stock solution (1 μg/mL final concentration). The cells were immediately analysed with a FACScan flow cytometer equipped with an excitation laser line at 488 nm and Cell Quest software. The FITC-AnnxV (green fluorescence) and the PI (red fluorescence) were collected on a log scale through a 530 ± 20 and 575 ± 15 nm band pass filter, respectively [28].

[1] Annex VI, Part 1, Dir. 76/768/EEC and subsequent adjournments.

2.7. Caspase-3-like activity assay

After treatment with the preservatives, the HL60 cells were washed in PBS and resuspended in ice-cold lysis buffer (50 mM HEPES, 1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol, 0.1% CHAPS, pH 7.4 supplemented with 5 mg/mL leupeptin). After centrifugation at 10,000 *g* at 4°, the supernatant was used for the assay of caspase-3 activity. The protein concentration in the lysate was determined by the Bradford assay (Bio-Rad). For the caspase-3 assay, 25 μg of cell lysate were incubated in 100 μL of assay buffer (50 mM HEPES buffer, 10 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol, 0.1% CHAPS, pH 7.4, 100 mM NaCl) containing 200 μM acetyl-Asp-Glu-Val-Asp-*p*-nitroaniline (Ac-DEVD-pNA) in presence or absence of the caspase-3 inhibitor 0.1 μM Ac-DEVD-CHO. The samples were incubated at 37° in a microtiter plate reader for 16 hr. The enzyme-catalysed release of *p*-nitroaniline was monitored at 405 nm. The conversion of the substrate was linear in time and amount of protein.

3. Results

3.1. Cytotoxic effect of the preservatives on HL60

To investigate the cytotoxic effect of the four preservatives, we added increasing concentrations of them to HL60 cells for 10 min. This cell line, established from acute promyelocytic leukemia, was selected as the model system since, it is commonly employed in studies of the antiproliferative and apoptotic effects of potentially active chemicals. After treatment, the cells were resuspended in fresh medium and cultured for up to 24 hr; aliquots were removed at 0, 3, 6 and 24 hr for monitoring of cell viability by the MTT test. Fig. 2A–D shows the viability of HL60 in the 24 hr period after treatment with different concentrations of EGPE (0.01–1%), CMI/MI (0.001–0.1%), IMU (0.01–1%) and PG (0.01–5%). At their lowest concentrations, EGPE, CMI/MI and IMU induced a moderate cytotoxic effect within 24 hr after treatment. When the concentration was increased to 0.1–0.5% for EGPE and IMU and to 0.01–0.05% for CMI/MI, the MTT test showed a pronounced decrease of cell viability at 3 hr, culminating at 24 hr. At their highest concentrations, EGPE (1%) and CMI/MI (0.1%) caused a decrease of cell viability at zero hour, suggesting sudden cell death. PG showed an evident cytotoxic effect only at the highest concentration; at lower concentrations, there was no appreciable time- or dose-dependent decrease of cell viability.

3.2. DNA content and DNA fragmentation in HL60 treated with the preservatives

To determine the mode of cell death, we analysed the dying cells for DNA subdiploid content by means of flow cytometry after staining the DNA with PI. At 3 and 6 hr after treatment, aliquots of HL60 treated with low concentrations of the preservatives were removed from the cultures for analysis of DNA content. As seen in Fig. 3A–D, only a small proportion of cells had subG1 DNA content, which is representative of cells with decreased PI staining and is an indicator of DNA fragmentation associated with apoptotic cell death. At 6 hr after treatment, the hypodiploid population increased for each one of the compounds and the reduction of the diploid peak was evident.

At higher concentrations, IMU (0.5–1%), CMI/MI (0.05–0.1%) and EGPE (1%), at 3 hr, there was no subdiploid peak, the diploid DNA peak was not modified and the reduction of cells in the G_2/M phase was barely evident. At 6 hr after treatment with EGPE 1%, there was a reduced cell number in the acquisition gate associated with a strong increase of cellular debris (excluded from the analysis, as previously indicated) suggesting that all cells were dead by necrosis.

Internucleosomal DNA fragmentation was confirmed by the pattern of DNA laddering into fragments of about 180 bp (and multiples) detected in agarose gel electrophoresis of extracts obtained at 6 hr from HL60 treated with preservatives (IMU 0.01–0.1%, EGPE 0.01–0.5%, CMI/MI 0.001–0.01% and PG 5%) (Fig. 4). The data for the percentage of hypodiploid cells are well correlated with DNA laddering.

3.3. Measurement of PS exposure in HL60 cells treated with the preservatives

To confirm that the cell death induced by low concentrations of these preservatives was due to apoptosis, we stained HL60 cells with AnnxV, which binds PS with high affinity. PS translocation from the inner to the external leaflet of the plasma membrane is considered one of the earliest events in apoptosis and can be analysed by staining with FITC-AnnxV. Cells were simultaneously stained with PI and analysed by flow cytometry at 3 hr after exposure to the different preservatives, when only few cells showed subdiploid DNA content.

Fig. 5A and B shows the results of the bivariate FITC-AnnxV-PI flow cytometry of HL60 treated with EGPE and CMI/MI. The lower left quadrant of the histograms shows the viable cells, which exclude PI and are negative for FITC-AnnxV binding. The upper left quadrant represents the early apoptotic cells, which are PI negative and AnnxV positive, indicating the translocation of PS to the external cell surface but integrity of the plasma membrane. The upper right quadrant represents the non-viable necrotic and late-stage apoptotic cells, which are positive for AnnxV binding and PI, while the lower right quadrant represents non-viable cells positive only for PI.

Treatment of HL60 with EGPE (0.01–0.1%) induced an increase in both AnnxV positive and AnnxV/PI positive

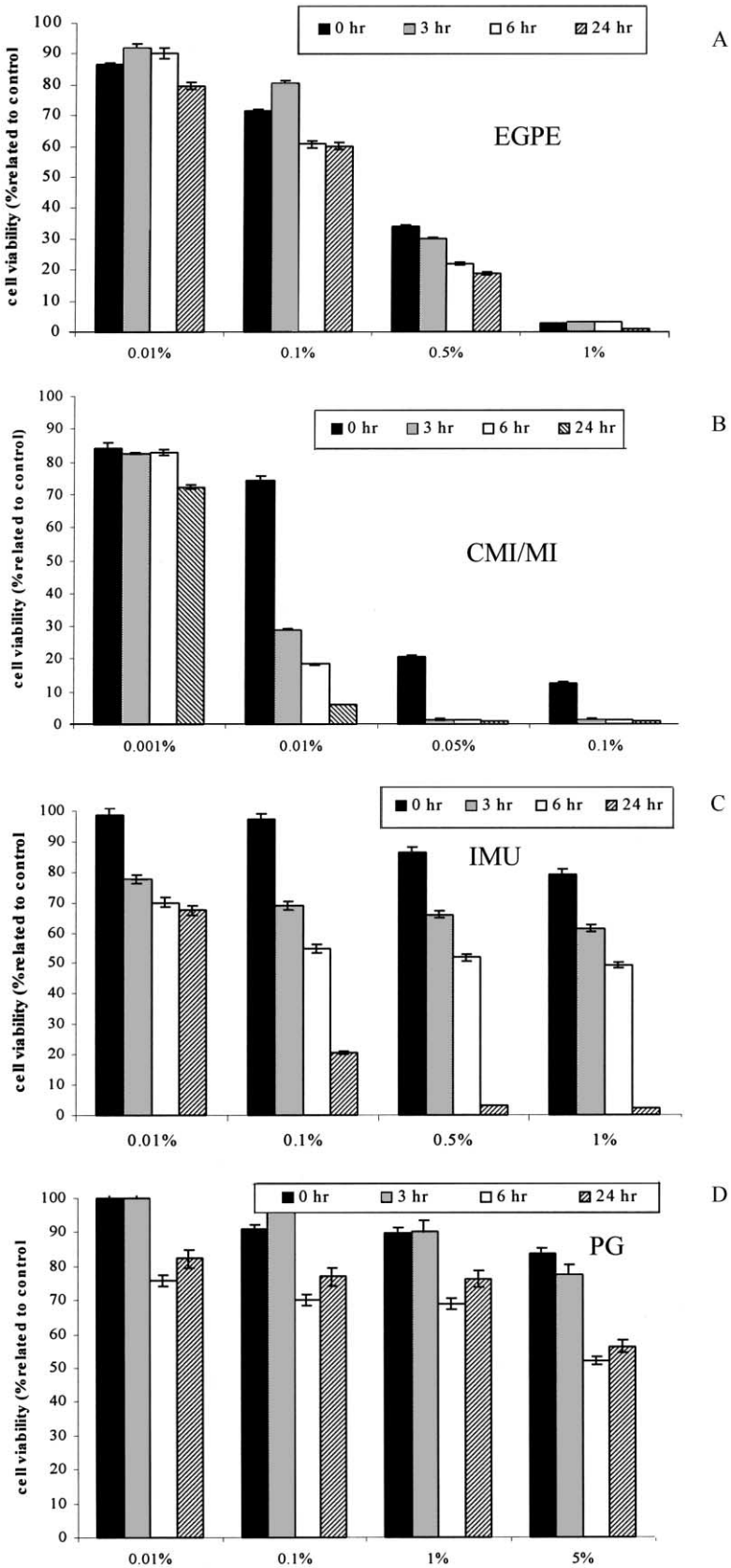


Fig. 2. Cell viability of HL60 cells 0–24 hr after exposure to different concentrations of four preservatives. (A) EGPE (0.01%–1%); (B) CMI/MI (0.001–0.1%); (C) IMU (0.01–1%); (D) PG (0.01–5%). Cells were incubated with each preservative for 10 min, resuspended in fresh medium and incubated at 37°. Cell viability was evaluated at 0, 3, 6, 24 hr by the MTT test. Values are expressed as percentage ± SEM (average of five separate experiments).

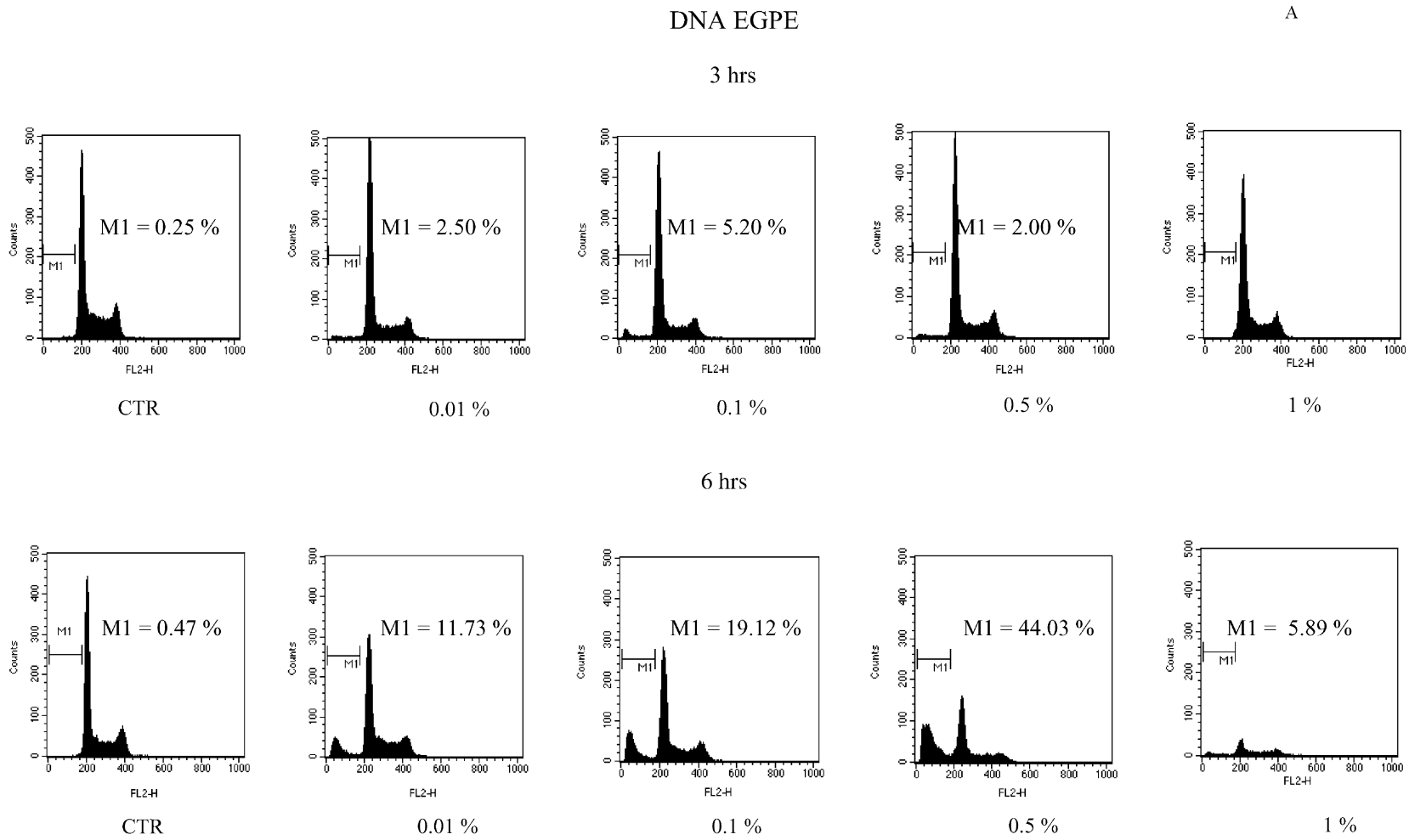
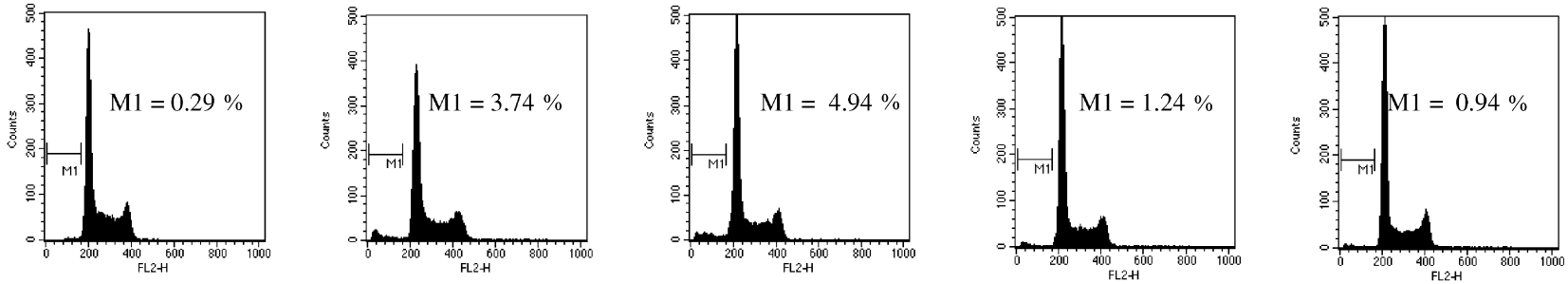


Fig. 3. Frequency distribution histograms of DNA content of PI-stained HL60 cells at different times after treatment with the four preservatives. The percentage of apoptotic cells was quantified as the accumulation of PI-stained cells in the sub-G_0/G_1 DNA histogram region. The cells were treated with different concentrations of the four preservatives. Samples were removed for assay at 3 and 6 hr after treatment with preservatives and the assay was stopped by fixation in ethanol. The percentage of cells containing sub-diploid amounts of DNA is indicated. Similar data were obtained in three independent experiments. (A) EGPE (0.01–1%); (B) CMI/MI (0.001–0.1%); (C) IMU (0.01–1%); (D) PG (0.01–5%).

DNA CMI/MI

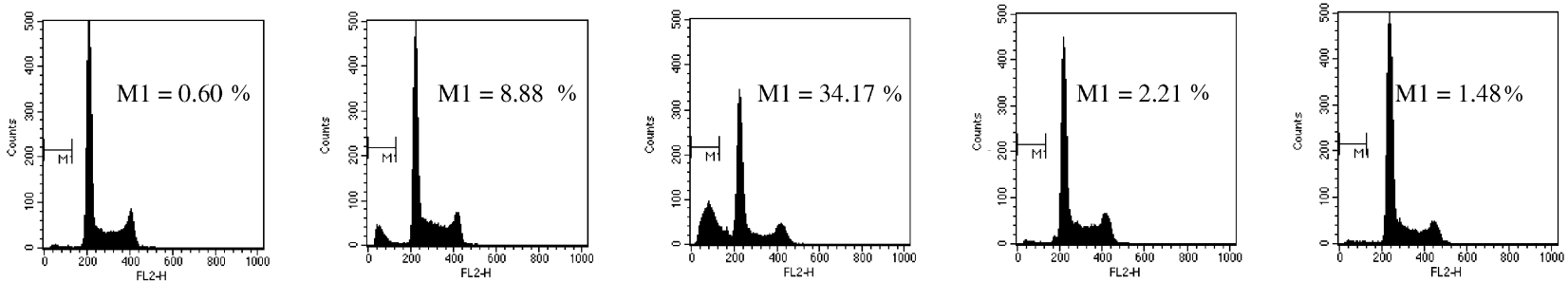
B

3 hrs



CTR 0.001 % 0.01 % 0.05 % 0.1 %

6 hrs

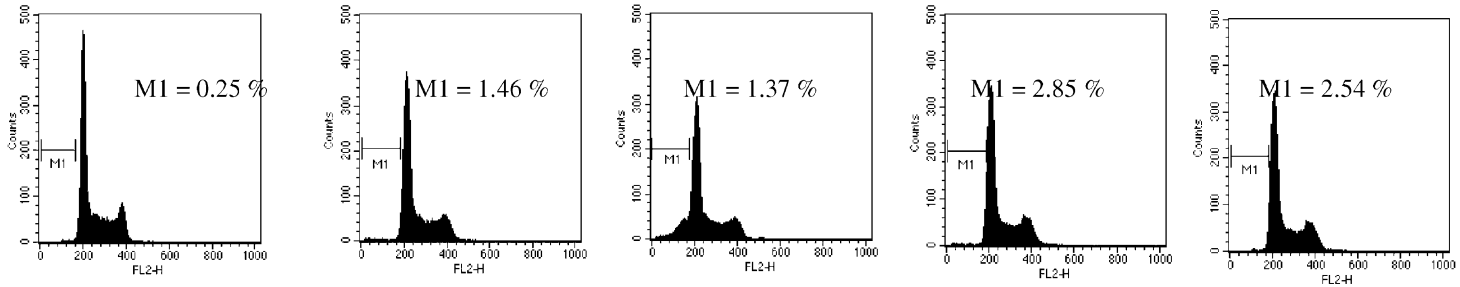


CTR 0.001 % 0.01 % 0.05 % 0.1 %

Fig. 3. (*continued*).

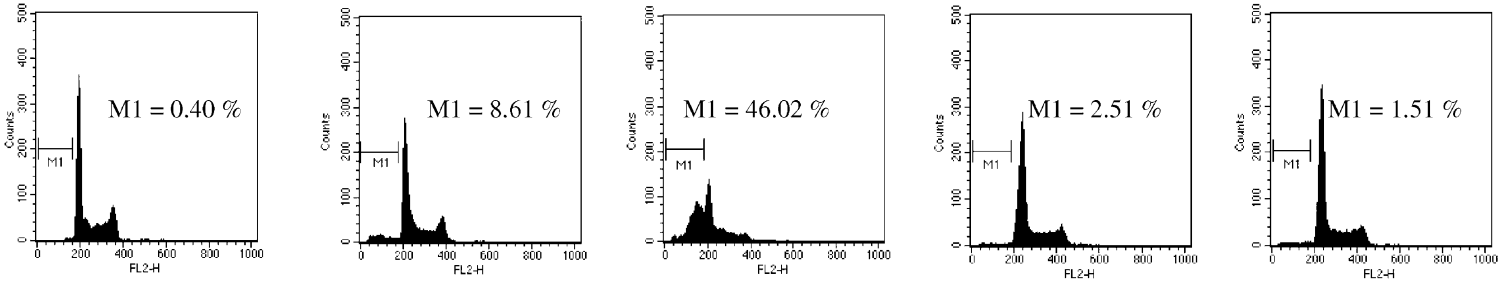
DNA IMU C

3 hrs



CTR 0.01 % 0.1 % 0.5 % 1 %

6 hrs



CTR 0.01 % 0.1 % 0.5 % 1%

Fig. 3. (*continued*).

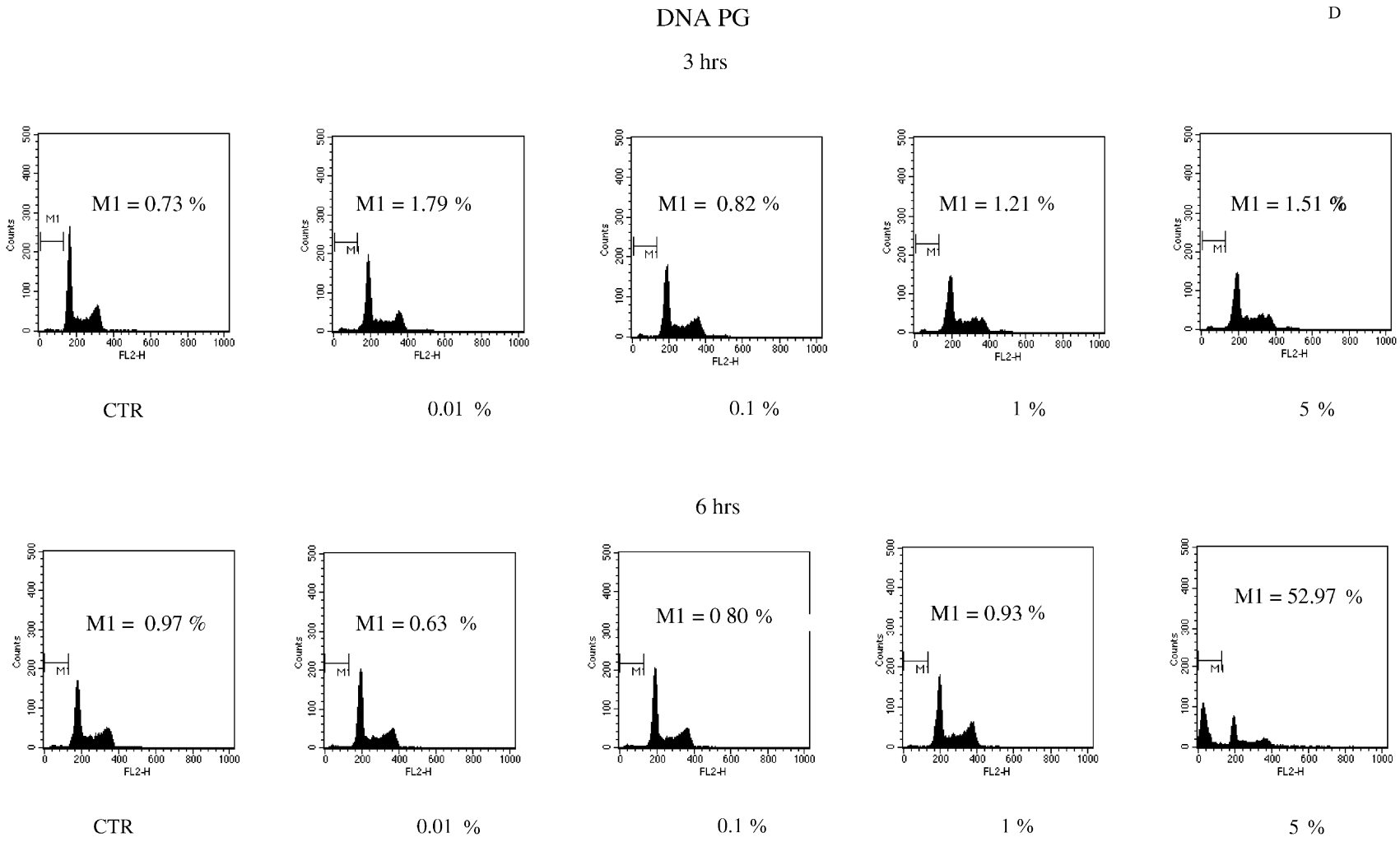


Fig. 3. (*continued*).

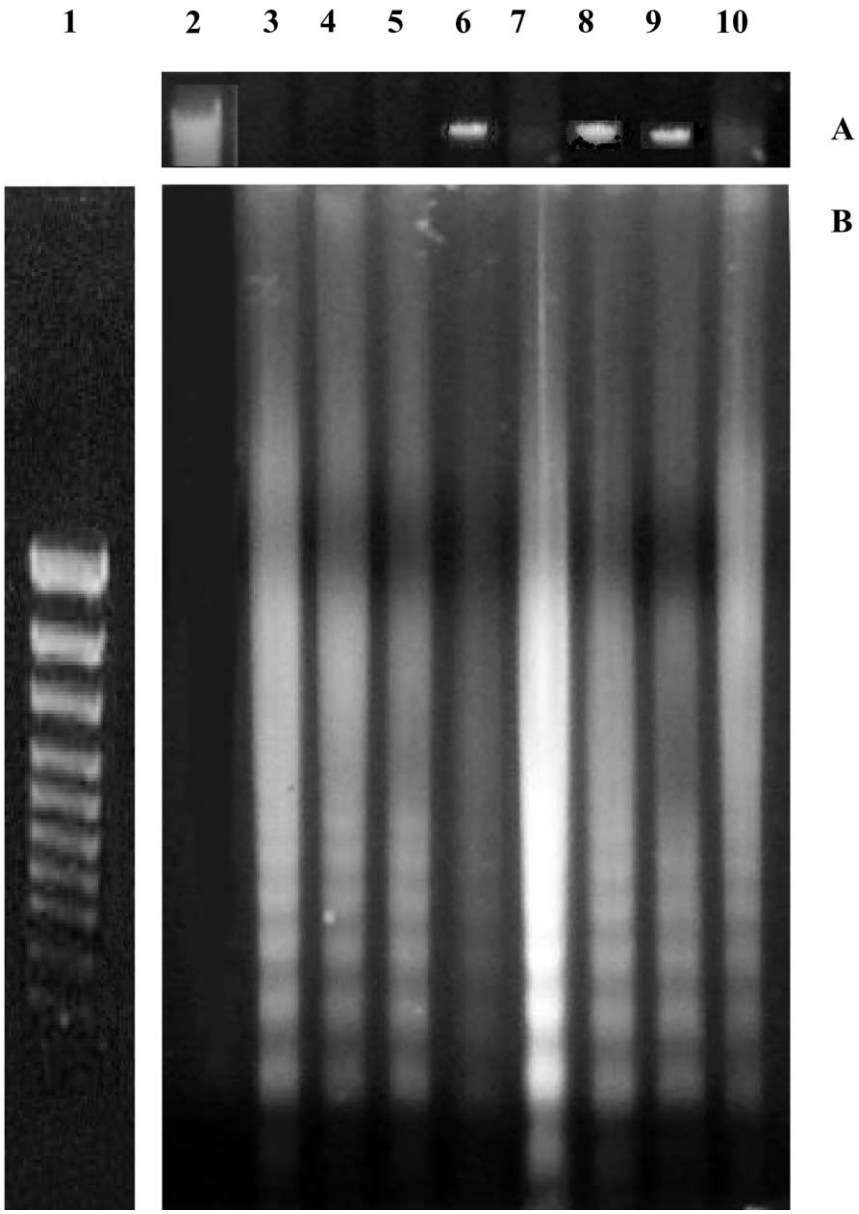


Fig. 4. DNA ladder formation following exposure of HL60 cells to the preservatives. Cells were incubated with each preservative for 10 min, resuspended in fresh medium and incubated at 37°. At 6 hr after treatment, cells were collected and DNA was extracted as reported previously. The results represent four independent experiments. (A) Intact DNA; (B) fragmented DNA; molecular-weight markers (lane 1), control cells (lane 2), cells treated with 0.01, 0.1, and 0.5% EGPE (lanes 3–5), cells treated with 0.001 and 0.01% CMI/MI (lanes 6, 7), cells treated with 0.01 and 0.1% IMU (lanes 8, 9), cells treated with 5% PG (lane 10).

cells after only 3 hr (in comparison to control) suggesting that EGPE initiated apoptosis in this cell population. At a higher concentration, EGPE 0.5%, most cells were positive for AnnxV and AnnxV/PI, confirming a late stage of apoptotic cell death, while at EGPE 1% the cells were only AnnxV/PI and PI positive, indicating death by late apoptosis or necrosis.

At different concentrations, the kinetics of PS exposure was similar in CMI/MI and EGPE at 3 hr after treatment: at the lowest concentration of CMI/MI (0.001%), we only detected cells positive for AnnxV, while at higher concentrations the cells were simultaneously positive for AnnxV and AnnxV/PI. With the AnnxV staining analysis, it is not possible to distinguish between primary necrosis and necrosis that is secondary to apoptosis; however, we inferred that the highest concentrations of CMI/MI (0.05–0.1%) and EGPE (1%) were necrotic, as confirmed by the lack of sub-diploid DNA peak, DNA laddering and Trypan Blue positivity.

For IMU and PG, we reported AnnxV-FITC binding as a frequency distribution histogram (Fig. 6A and B). In HL60 cells treated with IMU, PS exposure did not occur at 3 or 6 hr after treatment (Fig. 6A). PG treatment showed PS exposure only at 6 hr after treatment but not at 3 hr (Fig. 6B).

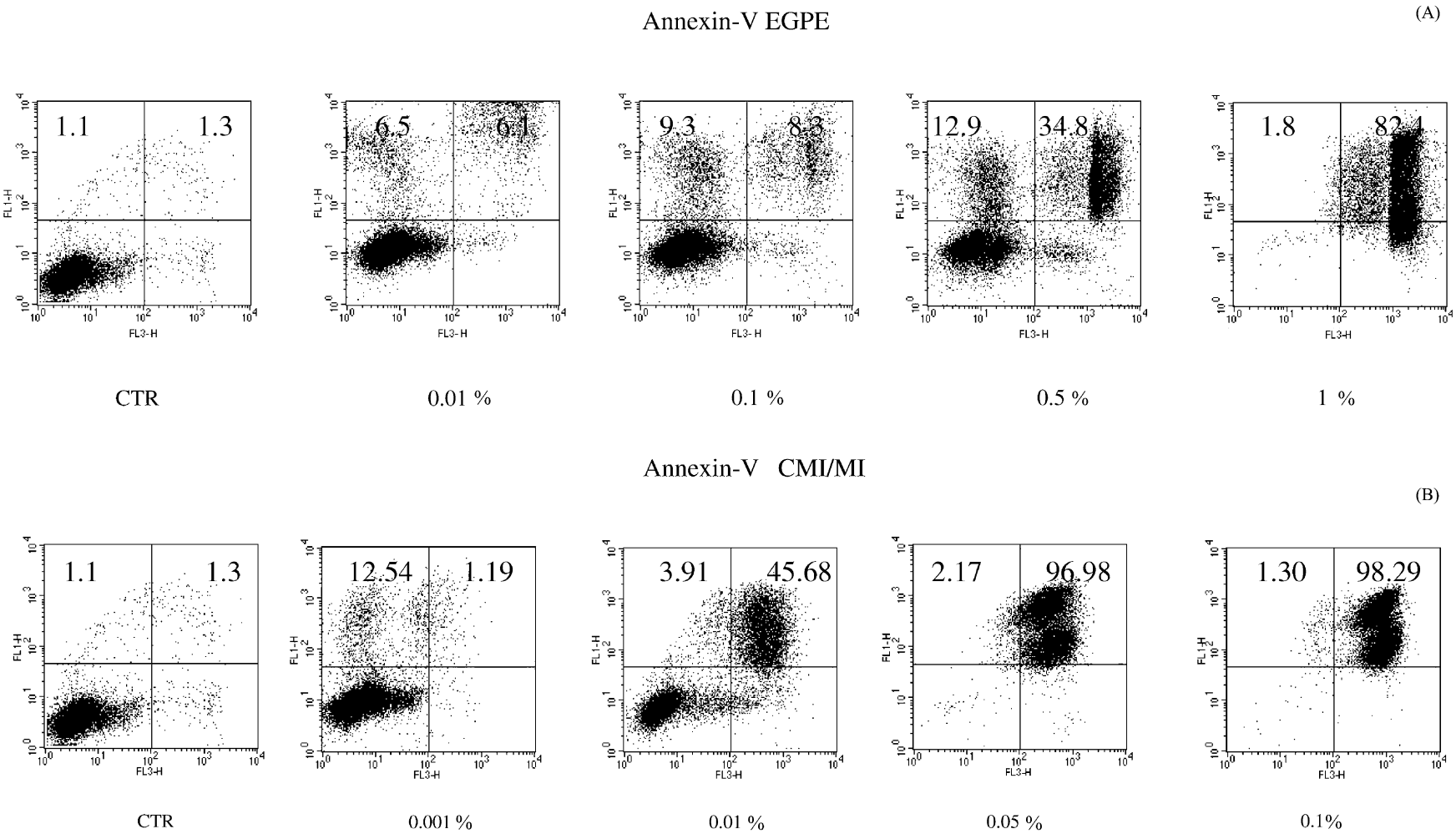


Fig. 5. Bivariate flow cytometry dot plots of FITC-AnnxV(FL1)-stained vs. PI(FL2)-stained HL60 cells treated with EGPE and CMI/MI. HL60 cells were stained at 3 hr after treatment with graded concentrations of (A) EGPE (0.01–1%) and (B) CMI/MI (0.001–0.1%) and compared with the untreated control. The cells were immediately analysed with a FACScan flow cytometer equipped with an excitation laser line at 488 nm and Cell Quest software. The FITC-AnnxV (green fluorescence) and the PI (red fluorescence) were collected on a log scale through a 530 ± 20 and 575 ± 15 nm band pass filter, respectively. The lower left quadrant (AnnxV−/PI−) represents viable cells, while the upper left (AnnxV+/PI−) and upper right (AnnxV+/PI+) quadrants show apoptotic and necrotic cells or late apoptotic cells, respectively. Similar data were obtained in three independent experiments.

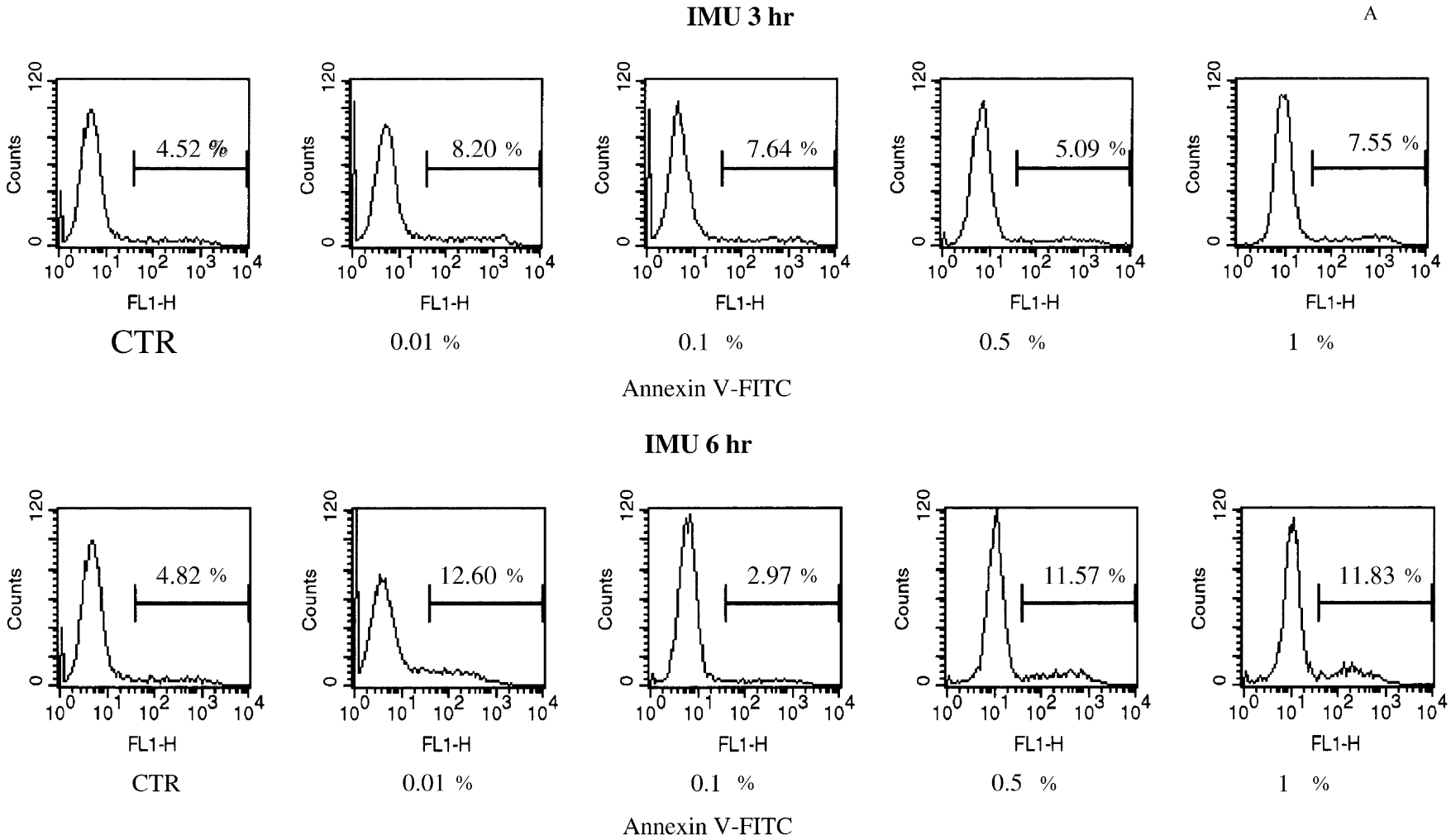


Fig. 6. Flow cytometric analysis of PS externalization in HL60 treated with IMU and PG. HL60 were stained at 3 and 6 hr after treatment with graded concentrations of (A) IMU (0.01–1%) and (B) PG (0.01–5%). The cells were immediately analysed with a FACScan flow cytometer equipped with an excitation laser line at 488 nm and Cell Quest software. The FITC-AnnxV (green fluorescence) and the PI (red fluorescence) were collected on a log scale through a 530 ± 20 and 575 ± 15 nm band pass filter, respectively.

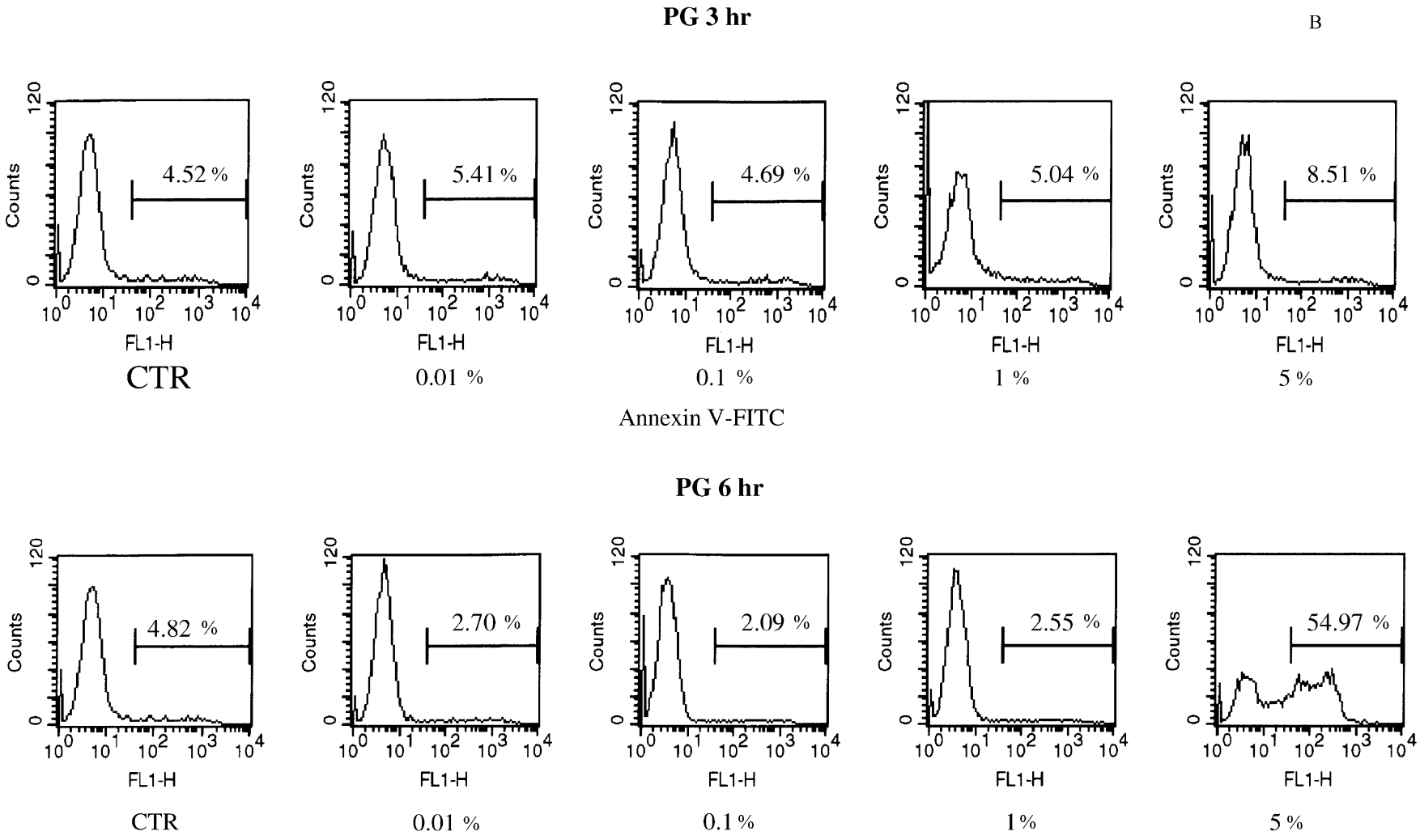


Fig. 6. (*continued*).

3.4. Caspase-3-like activity

To evaluate the role of caspase-3 in apoptosis induced by the four preservatives, we assayed caspase-like activity in lysates made from HL60 treated with graded concentrations of each preservative at 3 hr (this time was reported because it is the time in which caspase-3-like increase is more evident).

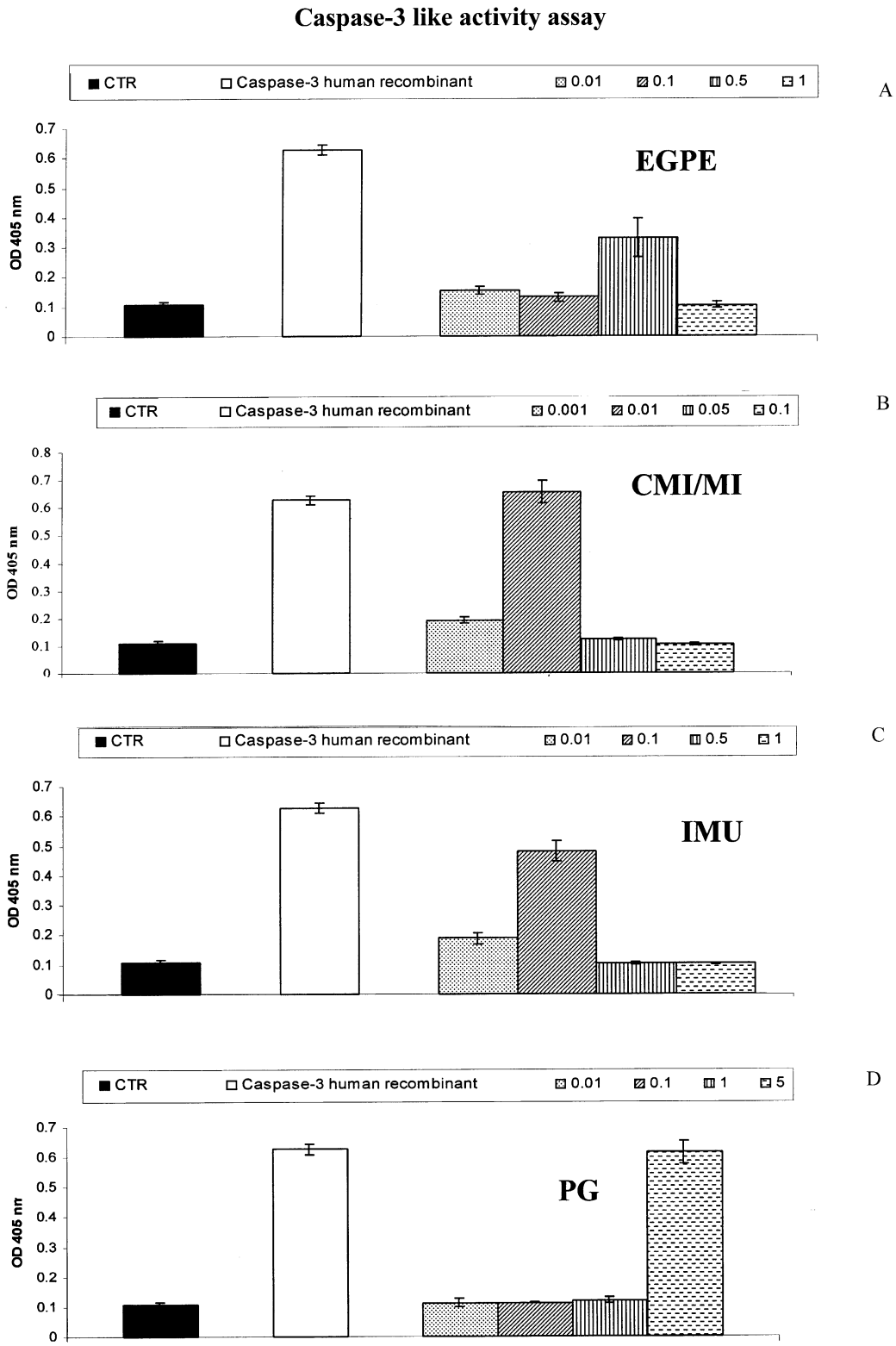


Fig. 7. Induction of caspase-3 activity in HL60 treated with the four preservatives. Lysates (25 μg), made from HL60 treated with different concentrations of the preservatives at 3 hr after treatment, were incubated in 100 μL of assay buffer containing the specific tetrapeptide substrate, Ac-DEVD-pNA (200 μM). Values are expressed as OD 405 nm value ± SEM. (A) EGPE (0.01–0.1%); (B) CMI/MI (0.001–0.1%); (C) IMU (0.01–1%); (D) PG (0.01–5%).

Fig. 7A–D shows that caspase-3 activation was dose-dependent at apoptotic concentrations while no activation of caspase-3 occurred at necrotic concentrations. The activation of caspase-3 at apoptotic concentrations was completely removed by addition to the mixture of the caspase-3 specific inhibitor Ac-DEVD-CHO during incubation (data not shown).

Note that the substrate for assaying caspase-3 activity (DEVD) can also be cleaved by caspase-7, so the activity displayed with this substrate is referred to as ''caspase-3-like'' [29].

4. Discussion

In this study, we compared the toxic effects of four preservatives belonging to different chemical classes and we investigated their potential to induce apoptosis in HL60 cells. Before discussing our results, we will consider several aspects of the chemistry and biological properties of the four preservatives. It has been reported that EGPE, an ethylene glycol phenyl ether [14,30], is able to lyse microbial cell membranes and red blood cells [31]. In addition, EGPE and other glycol ethers have been found to block junction-mediated intercellular communication, perhaps by means of membrane-mediated mechanisms [32,33].

IMU, a urea derivative, acts as a formaldehyde donor [16]. The formaldehyde reacts most rapidly with organic and inorganic anions, amino and sulphide groups and electron-rich groups to disrupt metabolic processes, eventually causing death of the organism [34]. There is growing evidence that several methylated compounds are potential formaldehyde generators in their biological reactions and can induce apoptosis [35].

CMI/MI is an aqueous solution containing a mixture of 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one [15]. While the chlorinated molecule is the more active of the two, both ingredients are considered biologically functional, although the mechanism of action is not well understood. Toxicity of CMI/MI in normal human fibroblast culture has been reported, as well as its allergenic and irritation potential in hen's egg chorioallantoic membrane [36–38].

PG, 1,2-pentanediol, acts by virtue of its humectant activity. Thus, it can be used contemporaneously as a humectant and antimicrobial agent in modern pharmaceutical and cosmetic ''self-preserving formulations'' [17,39].

There is very little information about the comparative effects of these four preservatives on cell cytotoxicity [36,40] and no data on the induction of apoptosis. Our results show that each of the tested preservatives is toxic to HL60 cells: PG is the least toxic compound while CMI/MI is the most toxic. These results are consistent with another report that CMI/MI is more toxic than IMU, as shown by the MTT test on single-layer human fibroblast cultures [36]. Amouroux *et al.* have reported acute cytotoxicity of IMU in sea urchin eggs, mainly due to inhibition of protein and DNA synthesis [40].

To elucidate the mode of cell death, we analysed parameters commonly used to establish death by apoptosis, such as specific internucleosomal DNA cleavage and the associated presence of a subdiploid DNA peak (markers of the later stage of apoptosis) and the exposure of PS (a marker of early apoptosis) [9,13,41]. Our results show that cells treated with low concentrations of CMI/MI (0.001–0.01%) and EGPE (0.01–0.5%) displayed all the classical alterations characteristic of apoptosis. For both preservatives, the exposure of PS preceded the nuclear signs of apoptosis, such as DNA fragmentation. In HL60 treated with PG, we observed the classical parameters of apoptosis only at the highest concentration (5%) while PS externalization occurred more slowly; in fact, it was evident only at 6 hr after treatment and was concomitant with the appearance of subdiploid DNA content. Reports of a time overlap among DNA laddering, subdiploid DNA content and PS exposure suggest that PS exposure is not always an indicator of early apoptosis [42,43]. HL60 cells treated with IMU at low concentrations exhibited the nuclear changes associated with apoptosis, i.e. DNA fragmentation and subdiploid DNA content, but did not show any externalization of PS on the outer leaflet of the plasma membrane. This is difficult to explain since it is different from the kinetics observed for CMI/MI and EGPE and several studies have indicated that plasma membrane scrambling during apoptosis is a general phenomenon associated with the intracellular events leading to DNA fragmentation, the ultimate stage of apoptosis [9,13].

This prompted us to assess another marker of apoptosis: caspase-3 activity [10–12]. Although, DNA fragmentation strongly suggests an apoptotic mechanism, many researchers consider caspase-3 activation to be an even more reliable hallmark of apoptosis than DNA laddering. The morphological and biochemical changes of apoptosis are orchestrated by a set of cysteine proteinases that became active during apoptosis, the caspases [10–12]. Caspase-3 is the major executioner caspase together with caspase-6 and caspase-7. The early transverse redistribution of PS, as well as other biochemical markers of apoptosis, depends on caspase-3 activity [44].

We assessed caspase-3 by colorimetric assay using a specific tetrapeptide substrate, Ac-DEVD-pNA [29]. We have shown that caspase-3 is increased in HL60 treated with low concentrations of each of the preservatives, including IMU. Thus, our data are in keeping with the general notion that caspase-3 is involved in most nuclear and cytoplasmic alterations associated with apoptosis. The lack of PS exposure of HL60 treated with IMU may not be correlated with caspase-3 activation or nuclear changes [42] or the PS exposure could be inhibited.

The molecular mechanisms responsible for PS translocation during apoptosis remain unclear [45] but presumably

involve both inhibition of a specific aminophospholipid translocase (APT) and activation of non-specific scramblase [44–47]. An intriguing hypothesis to explain our results is the potential interaction between IMU (or more likely the formaldehyde generated by IMU) and the membrane, which could block PS exposure during apoptosis triggered by IMU by interfering with APT or scramblase.

When HL60 cells were treated with higher concentrations of each preservative, the predominant form of cell death was non-apoptotic. We failed to detect DNA laddering or the appearance of a subG1 peak but all cells appeared AnnxV and PI positive, suggesting that the predominant form of cell death was by necrosis. The concept that low concentrations of a cytotoxic compound can induce apoptosis while high concentrations induce necrosis is not novel [19,29]. It has been reported that other preservatives, different from those tested in the present study, are able to induce necrosis or apoptosis in cell cultures *in vitro*. Oikawa *et al.* reported oxidative DNA damage and apoptosis by butylated hydroxytoluene, a synthetic phenolic antioxidant widely used as a food additive [19]. de Saint *et al.* [24] reported that benzalkonium chloride, the most commonly used preservative in many ophthalmic solutions, induces necrosis at high concentrations and apoptosis at lower concentrations.

Our results are an important contribution to the evaluation of cytotoxic effects of preservatives in a cell culture system. Moreover, we found that each of these chemically different compounds is able to modulate the apoptotic machinery at low concentrations but that different pathways seem to be involved. These preservatives are widely used in cosmetics and topical pharmaceutics. Moreover, CMI/MI is able to induce allergy and irritation *in vivo* [37,38,48]. Therefore, further studies will be designed to investigate their potential in inducing apoptosis and necrosis in more appropriate cell cultures, such as keratinocytes or human lymphocytes.

Acknowledgments

We thank Sandra Nuti, Simona Tavarini and Silverio Sbrana for assistance with the cytofluorimetric analysis, Lucia Bovalini for helpful comments and Peter Christie for the careful English revision. This research was supported by grant from Piano di Ateneo per la Ricerca.

References

[1] Majno G, Joris I. Apoptosis, oncosis and necrosis: an overview of cell death. Am J Pathol 1995;46(1):3–15.
[2] Denecker G, Vercammen D, Declercq W, Vandenabeele P. Apoptotic and necrotic cell death induced by death domain receptors. Cell Mol Life Sci 2001;58(3):356–70.
[3] Renehan AG, Booth C, Potten CS. What is apoptosis and why is it important? BMJ 2001;322(7301):1536–8.
[4] Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. Br J Cancer 1972;26(4):239–57.
[5] Ellis HM, Yuan J, Horvitz HR. Mechanism and functions of cell death. Ann Rev Cell Biol 1991;7:663–93.
[6] Wyllie AH. The genetic regulation of apoptosis. Curr Biol 1998; 5:97–104.
[7] Robertson JD, Orrenius S. Molecular mechanisms of apoptosis induced by cytotoxic chemicals. Crit Rev Toxicol 2000;30(5):609–27.
[8] Corcoran GB, Fix L, Jones DP, Moslen MT, Nicotera P, Oberhammer FA, Buttyan R. Apoptosis: molecular control point in toxicity. Toxicol Appl Pharmacol 1994;128(2):169–91.
[9] Bevers EM, Comfurius P, Dekkers DW, Zwaal RF. Lipid translocation across the plasma membrane of mammalian cells. Biochim Biophys Acta 1999;1439:317–30.
[10] Thornberry NA. Caspases: key mediators of apoptosis. Chem Biol 1998;5(5):R97–103.
[11] Adams JM, Cory S. Life-or-death decisions by the Bcl-2 protein family. Trends Biochem Sci 2001;26(1):61–6.
[12] Chang HY, Yang X. Proteases for cell suicide: functions and regulation of caspases. Microbiol Mol Biol 2000;Rev 64(4):821–46.
[13] Zhang JH, Xu M. DNA fragmentation in apoptosis. Cell Res 2000;10(3):205–11.
[14] Hall AL. Cosmetically acceptable phenoxyethanol. In: Kabara JJ, editor. Cosmetics and drug preservation, principles and practice. New York: Marcel Dekker, 1984. p. 79–108.
[15] Law AB, Moss JN, Lashen ES, Kathon CG. A new single-component, broad-spectrum preservative system for cosmetics and toiletries. In: Kabara JJ, editor. Cosmetics and drug preservation, principles and practice. New York: Marcel Dekker, 1984. p. 533–57.
[16] Rosen WE, Berke PA. GERMALL 115 a safe and effective preservative. In: Kabara JJ, editor. Cosmetics and drug preservation, principles and practice. New York: Marcel Dekker, 1984. p. 209–22.
[17] Enigl DC, Sorrells KM. Water activity and self-preserving formulas. In: Kabara JJ, Orth DS, editors. Cosmetics and drug preservation, principles and practice. New York: Marcel Dekker, 1997. p. 45–73.
[18] Yu R, Mandlekar S, Tony Kong A-N. Molecular mechanisms of butylated hydroxylanisole-induced toxicity: induction of apoptosis through direct release of cytochrome *c*. Mol Pharmacol 2000;58(2): 431–7.
[19] Oikawa S, Nishino K, Oikawa S, Inoue S, Mizutani T, Kawanishi S. Oxidative DNA damage and apoptosis induced by metabolites of butylated hydroxytoluene. Biochem Pharmacol 1998;56:361–70.
[20] Serrano A, Palacios C, Roy G, Cespón C, Villar ML, Nocito M, González-Porqué P. Derivatives of gallic acid induce apoptosis in tumoral cell lines and inhibit lymphocyte proliferation. Arch Biochem Biophys 1998;350(1):49–54.
[21] Stammati A, Zanetti C, Pizzoferrato L, Quattrucci E, Tranquilli GB. *In vitro* model for the evaluation of toxicity and antinutritional effects of sulphites. Food Addit Contam 1992;9(5):551–60.
[22] Takahashi N. Cytotoxicity of mercurial preservatives in cell culture. Ophthalmic Res 1982;14(1):63–9.
[23] Sugihara N, Shimomichi K, Furuno K. Cytotoxicity of food preservatives in cultured rat hepatocytes loaded with linolenic acid. Toxicology 1997;120(1):29–36.
[24] de Saint Jean M, Brignole F, Bringuier A-F, Bauchet A, Feldmann G, Baudouin C. Effects of benzalkonium chloride on growth and survival of Chang conjunctival cells. Invest Ophthalmol Visual Sci 1999;40(3):619–30.
[25] Debbasch C, Brignole F, Pisella PJ, Warnet JM, Rat P, Baudouin C. Quaternary ammonium and other preservatives' contribution in oxidative stress and apoptosis on Chang conjunctival cells. Invest Ophthalmol Visual Sci 2001;42(3):642–52.
[26] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.

[27] Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 1991;139:271–9.
[28] Van Engeland M, Nieland LJ, Ramaekers FC, Schutte B, Reutelingsperger CP. Annexin-V affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry 1998;31:1–9.
[29] Di Stefano A, Ettorre A, Sbrana S, Govani C, Neri P. Purpurin-18 in combination with light leads to apoptosis or necrosis in HL60 leukemia cells. Photochem Photobiol 2001;73:290–6.
[30] Lucchini JJ, Corre J, Cremieux A. Antibacterial activity of phenolic compounds and aromatic alcohols. Res Microbiol 1990;141(4):499–510.
[31] Breslin WJ, Phillips JE, Lomax LG, Bartels MJ, Dittenber DA, Calhoun LL, Miller RR. Hemolytic activity of ethylene glycol phenyl ether (EGPE) in rabbits. Fundam Appl Toxicol 1991;17(3):466–81.
[32] Musshoff U, Madeja M, Binding N, Witting U, Speckmann EJ. Effects of 2-phenoxyethanol on *N*-methyl-D-aspartate (NMDA) receptor-mediated ion currents. Arch Toxicol 1999;73(1):55–9.
[33] Loch-Caruso R, Trosko JE, Corcos IA. Interruption of cell-cell communication in Chinese hamster V79 cells by various alkyl glycol ethers: implications for teratogenicity. Environ Health Perspect 1984; 57:119–23.
[34] Bruch CW. Gaseous sterilization. Ann Rev Microbiol 1961;15:245–62.
[35] Szende B, Tyihak E, Trezl L, Szoke E, Laszlo I, Katay G, Kiraly-Veghely Z. Formaldehyde generators and capturers as influencing factors of mitotic and apoptotic processes. Acta Biol Hung 1998;49(2–4):323–9.
[36] Rivalland P, Vie K, Coiffard L, De Roeck-Holtzhauer Y. Cytotoxicity test of antibacterial agents on human fibroblasts cultures. Pharm Acta Helv 1994;69(3):159–62.
[37] Stejskal VD, Forsbeck M, Nilsson R. Lymphocyte transformation test for diagnosis of isothiazolinone allergy in man. J Invest Dermatol 1990;94(6):798–802.
[38] Luepke NP. Hen's egg chorioallantoic membrane test for irritation potential. Food Chem Toxicol 1985;23(2):287–91.
[39] Alany RG, Rades T, Agatonovic-Kustrin S, Davies NM, Tucker IG. Effects of alcohols and diols on the phase behaviour of quaternary systems. Int J Pharm 2000;196(2):141–5.
[40] Amouroux I, Pesando D, Noel H, Girard JP. Mechanisms of cytotoxicity by cosmetic ingredients in sea urchin eggs. Arch Environ Contam Toxicol 1999;36(1):28–37.
[41] Green DR. Apoptosis and sphingomyelin hydrolysis: the flip side. J Cell Biol 2000;150(1):F5–8.
[42] King MA, Radicchi-Mastroianni MA, Wells JV. There is substantial nuclear and cellular disintegration before detectable phosphatidylserine exposure during the camptothecin-induced apoptosis of HL60 cells. Cytometry 2000;40:10–8.
[43] Naito M, Nagashima K, Mashima T, Tsuro T. Phosphatidylserine externalization is a downstream event of Interleukin-1β-converting enzyme family protease activation during apoptosis. Blood 1997;89(6):2060–6.
[44] Vanags DM, Porn-Ares MI, Coppola S, Burgess DH, Orrenius S. Protease involvement in fodrin cleavage and phosphatidylserine exposure in apoptosis. J Biol Chem 1996;271:31075–85.
[45] Bratton DL, Fadok VA, Richter DA, Kailey JM, Guthrie LA, Henson PM. Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase. J Biol Chem 1997;272:26159–65.
[46] Fadok VA, Voelker DR, Campbell PA, Cohen JJ, Bratton DL, Henson PM. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol 1992;148:2201–16.
[47] Martin OC, Pagano RE. Transbilayer movement of fluorescent analogs of phosphatidylserine and phosphatidylethanolamine at plasma membrane of cultured cells: evidence for a protein-mediated and ATP-dependent process(es). J Biol Chem 1987;262:5890–8.
[48] de Groot AC, Weyland JW, Kathon CG. A review. J Am Acad Dermatol 1988;18(2 Pt 1):350–8.