The interference of rosmarinic acid in the DNA fragmentation induced by osmotic shock

Paola Sinibaldi Salimei [1], Gabriella Marfè [1], Livia Di Renzo [2], Carla Di Stefano [1], Maria Gabriella Giganti [1], Giuseppe Filomeni [3] and Maria Rosa Ciriolo [3]

[1] *Department of Experimental Medicine and Biochemical Sciences, University of Rome "Tor Vergata", Via Montpellier 1, 00133, Rome, Italy,* [2]*Department of Experimental Medicine and Pathology, University of Rome "La Sapienza", Viale Regina Elena, 324, 00161, Roma, Italy,*[3] *Department of Biology, University of Rome "Tor Vergata", Via della Ricerca Scientifica, 00133 Rome, Italy*

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1. ABSTRACT

The induction of cell death in human erythroleukemic cells (K562) by sorbitol shows the typical apoptotic changes in ultrastructural morphology, including blebbing, chromatin condensation and nuclear membrane breakdown.. Using a cytofluorimetric approach, we found that sorbitol induced production of reactive oxygen species (ROS) followed by DNA fragmentation in leukemic cells. In this study, we investigated effect of curcumin and rosmarinic acid on cell viability in three different cell lines: erythroleukemia K562 , papillary NPA, and anaplastic ARO thyroid cancers. Curcumin was able to induce apoptosis in a concentration- and time dependent manner in three cell lines , while rosmarinic acid was less effective on this process. To examine this possibility in cellular system, this study evaluated the capacities of both compounds acting as antioxidant   inhibiting sorbitol-induced apoptosis. K562, NPA and ARO cells were pre-incubated with 25 µM rosmarinic acid to allow the uptake and then the cell lines were treated with 1 M sorbitol. Afterwards, the cells were subjected to agarose gel electrophoresis to assess the DNA fragmentation.   In conclusion, the antioxidant activity of rosmarinic acid is able to inhibit sorbitol-induced apoptosis.

2. INTRODUCTION

Sorbitol is a hydrogenated form of carbohydrate obtained by the reduction of the carbonyl group of the glucose molecule to the hydroxyl group. It has been already demonstrated that sorbitol is able induce apoptosis to efficiently and rapidly when provided at high concentrations, as a part of the mechanisms related to hyperosmotic stress. In fact, hyperosmotic challenge has been shown to induce apoptosis in several cell lines such as human neuroblastoma (1), cardiac myocites (2), Hep-2 cells (3) and human gastric cells (4,5). Moreover, among the polyhydric alcohols, it has been demonstrated that xylitol (five hydroxyl groups) and erythritol (four hydroxyl groups) are also able to induce chromosomal DNA fragmentation but less efficiently than mannitol (six hydroxyl groups), which acts as sorbitol. In contrast, neither glycerol (three hydroxyl groups) nor ethylene glycol (two hydroxyl groups) are able to induce DNA fragmentation (6). These data indicate that polyhydric alcohols, with at least four hydroxyl groups in the molecule, increase their ability of inducing apoptosis.

Apoptosis is a morphologically and biochemically distinct form of cell death that occurs under
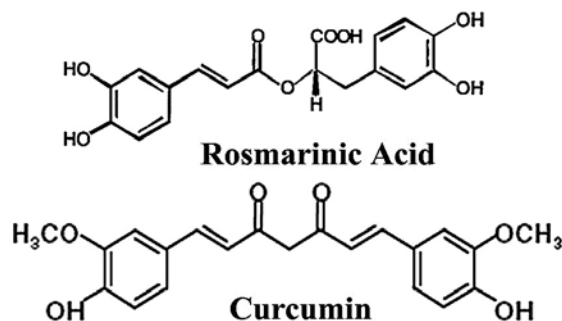
Figure 1 .Molecular structures of curcumin and rosmarinic acid.

a variety of physiological and pathological conditions (7,8). Defective apoptosis is recognized as the major criterion that contributes to cancer initiation and progression. Consequently, cancer cells can survive beyond their normally proposed life spans, expand clonal size, increase resistance to chemotherapy and facilitate metastatic activity (8,9).
Oxidative injury after differen stimuli including clinical and experimental ischemia/hypoxia, reperfusion and inflammation can induce cardiac and endothelial cell apoptosis (10,11,12,13), which is a fundamentally different way of cell death from necrosis. The severity of cellular damage by an oxidant injury determines which mechanism of cell death dominates (14). Accordingly, agents or antioxidants that can inhibit production of reactive oxygen species (ROS) can prevent apoptosis (10,15,16). However, the underlying molecular mechanisms by which antioxidative agents protect cells from stimulator-triggered apoptosis remain to be elucidated.

There is currently intense interest in polyphenolic phytochemicals such as flavonoids, proanthocyanidins and phenolic acids. Epidemiologic studies have shown that a high consumption of these polyphenolics is inversely related to the risk of cardiovascular diseases (17,18,19), and this phenomenon appears to be associated with their antioxidant capacity (20). Flavonoids constitute one of the antioxidant phytochemical groups and has found in a large number of fruits and vegetables.

There are several subclasses such as flavonols, flavones, isoflavones, flavonones, flavan-3-ols and anthocyanidins. These flavonoids are natural antioxidants that scavenge various types of radicals in aqueous and organic environments (21,22,23,24,25,26,27), as well as anti-inflammatory agents that inhibit adhesion molecules and matrix proteases (28,29,30). Whether these flavonoids act in vivo as antioxidants or anti-inflammatory agents appears to depend on their bioavailabilities.

According to the literature evidence that flavonoids are antioxidants and have the ability to scavenge free radicals, we examined the effects of two phenolis ptytochemical rosmarinic acid ((R)-a-((3-(3,4-dihydroxyphenyl)-1-oxo-2-propenyl)oxy)-3,4-dihydroxy)-

3,4 dihydroxy-benzene propanoic acid) and curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) (Figure 1). on apoptosis induced by sorbitol. From a molecular perspective, rosmarinic acid and curcumin have a similar chemical structures and the active centres of both are likely the phenolic hydroxyl groups.

We show that sorbitol is able to induce apoptosis efficiently in human K562 cells *via* the activation of the "mitochondrial pathway" and that the death process is strictly related to a massive production of reactive radical species. Furthemore, we have investigated the possibility that rosmarinic acid and curcumin could inhibit sorbitol-induced apoptosis
The final interesting result of the present work is the identification of rosmarinic acid as a potent antioxidant against ROS , and as an effective inhibitor of sorbitol-induced apoptosis.

3. MATERIAL AND METHODS

3.1. Cells lines Culture, Reagents
The NPA and ARO cells, a gift from Prof. Massimo Santoro, derive from human papillary and anaplastic thyroid carcinomas, respectively. The K562 cells, kindly supplied by Dr. Gianfranco Catalano, derived from patients in blast crisis stage. All cell lines were grown in RPMI 1640 medium (supplemented with 10% heated–inactivated fetal bovine serum, 100 U ml^{-1} of penicillin and 100 μg of streptomycin ml^{-1}). All cell lines were incubated with different concentrations of rosmarinic acid and curcumin (10-25-50-100 μM) and incubated for indicated time 24 h, 48 at 37°C. In a second experimental phase, all cell lines , pretreated with rosmarinic acid and curcumin at the concentration 25 μM for 1 h, were incubated with 1 M sorbitol for 60 min. The cells not treated were considered as controls. Curcumin and rosmarinic acid and sorbitol were purchased from Sigma-Aldrich.

3.2. DNA fragmentation assay
Briefly, cells were washed twice with phosphate-buffered saline (PBS) and lysed by addition of a hypotonic solution (1% NP-40 in 20 mM EDTA, 50 mM Tris-HCl pH 7.5). After centrifugation, the supernatant was collected and the extraction was repeated with the same lysis buffer. The supernatants was brought to 1% SDS and treated with RNase A (final concentration 5 mg/ml) for 2 h at 56°C followed by digestion with proteinase K (final concentration 2.5 mg/ml) at 45°C for at least 6 h. Before hydrolysis, a further cleaning of DNA was performed by phenol-chloroform extraction, followed by three successive ethanol precipitation in 2 M ammonium acetate. Pellets were dried for 30 min and resuspended in 200 ml Tris-EDTA pH 8.0. Aliquots of 20 μl containing 10 mg DNA were electrophoresed in 1.5% agarose gel. (31).

3.3. RNA isolation and cDNA preparation
The total RNA, extracted by guanidium thiocyanate method, was isolated from cells, both pretreated or no treated with the compounds . The total RNA pellet was dissolved in 100 ml of nuclease-free water. RNA was reverse transcribed by using a random

Table 1. Primers used in PCR analysis

Target gene	Forward primer 5'–>3'	Reverse primer 5'–>3'
Bax	CTTTTGCTTCAGGGTTTCAT	AAGTAAAAAGGGCCGCAAC
Caspase-3	TTTGTTTGTGTGCTTCTGAGCC	ATTCTGTTGCCACCTTTCGG
Bcl-2	GTCGCTACCGTCGTGACTT	CAGCCTCCGTTATCCTGGA
Bcl-xL	GCGGCTGGGATACTTTTGT	GCTATGCCCGTCAGGAAC
Beta-actina	GGCGGCACCACCATGTACCCT	AGGGGCCGGACTCGTCATATC

hexanucleotide primer with Superscript II Reverse Transcriptase from Invitrogen according to the manufacturer's instructions. Briefly, 1 mg of total RNA was transcribed with reverse transcriptase at 37 °C for 60 min with the final stage at 90 °C for 5 min.

3.4. Semi-quantitative RT-PCR analysis

Primer pairs sequences were designed on the basis of published gene sequences as reported in Table 1. One microliter of the RT reaction products was amplified by PCR in the presence of final concentration of 0.2 microM primers (sense and antisense mixture 1:1), 200 μM deoxynucleotide triphosphates (dNTPs) mixture and 0.5 U Taq DNA polymerase (Sigma). PCR reactions were conducted in a Stratagene RoboCycler Gradient 40 temperature cycler fitted with a Hot Top Assembly PCR .The program for amplification consisted of 1 cycle at 95°C for 1 min, followed by 1 min of denaturation at 94°C, 1 min of annealing different temperature and 2 min of extension at 72°C for 20 to 35 cycles in the linear range of the amplification cycle number, which was determined empirically in each case, and an additional incubation of 10 min at 72°C for final extension (Table 1). PCR products were analyzed by electrophoresis on 1.8% ethidium bromide-containing agarose gels and visualized and quantitated under UV transillumination of apparatus BioRad GelDoc 1000 (BioRad) with the program Quantity One.

3.5. Measurement of intracellular ROS

For detection of intracellular ROS, cells were incubated with both 1 M sorbitol and 50 mM DCF-DA (dissolved in dimethyl sulfoxide) at 37 °C. At each time cells were pelletted, washed and resuspended in ice-cold PBS. The fluorescence intensities of 2',7'-dichlorofluorescein, formed by the reaction of DCFH-DA with ROS, of more than 10,000 cells from each sample, were analyzed by recording FL-1 fluorescence by a flow cytometer. Prior to data collection, propidium iodide was added to the samples for gating out dead cells. Treatment with 100 mM tert-butylhydroperoxyde was used as a positive control. Experiments were repeated at least three times with similar results. The data are given as one representative histogram

3.6. Western blot analyses

Cell pellet was resuspended in lysis buffer containing 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 0.5% IGEPAL CA-630, and protease inhibitors. After 30 min incubation on ice, cells centrifuged at 14,000 $\times$ g for 15 min at 4°C, and supernatants were stored at –

80°C. 20 µg of proteins were loaded on 10% polyacrylamide gel and transferred onto a nitrocellulose membrane (Bio-Rad). Monoclonal anti-caspase-9 (1:1000) (Upstate) was used as primary antibody. The specific protein complex, formed upon anti-mouse secondary antibody treatment (1:5,000), was identified using Fluorchem Imaging system (AlphaInnotech – Analitica De Mori, Italy) after incubation with ChemiGlow chemiluminescence substrate (AlphaInnotech – Analitica De Mori, Italy).

4. RESULTS

4.1. Sorbitol induces apoptosis in K562, NPA and ARO cells

In preliminary studies, we established the effects of the dose and the time of incubation of sorbitol treatment in K562 cells on induction of apoptosis. In these experiments, the criterion for induction of apoptosis was the fragmentation of chromosomal DNA resulting from random cleavage at intranucleosomal intervals evaluated by 1.5% agarose gel electrophoresis. Titrations of sorbitol in the range from 0.1 to 1.0 M, in K562 cells incubated for 1 h at 37 °C, indicated that concentrations from 0.6 to 1.0 M was able to induce a fragmented chromosomal DNA (Figure 2A).

1n order to establish the type of death occurring (necrosis versus apoptosis), we performed cytofluorimetric analyses of K562 cells after double staining with annexin V/propidium iodide. Annexin V binds with high affinity to phosphatidyl-serine of early apoptotic cell membrane surface, while propidium iodide binds to DNA being a valid marker for late apoptotic or necrotic cells. Figure 2 B shows a time-dependent increase in annexin V positive cells upon treatment with 1 M sorbitol; the percentage of early apoptotic cells increased from 12.75 to 36.72 % after 10 and 30 min of treatment respectively, showing no significant rise in the values of necrotic cells.

4.2. Expression of apoptosis-related genes in all cell lines during the treatment with sorbitol

To identify the genes involved in sorbitol-induced apoptosis in three cell lines, we investigated the kinetics mRNA expression of some genes encoding apoptosis-promoting and inhibiting factors by semi-quantitative RT-PCR method. Subsequently, we examined the expression of apoptosis-related genes, in K562 NPA and ARO cells at the indicated time points treated with sorbitol. Expression of the bax and procaspase-3 genes increased, showing a peak after 60 min , and then
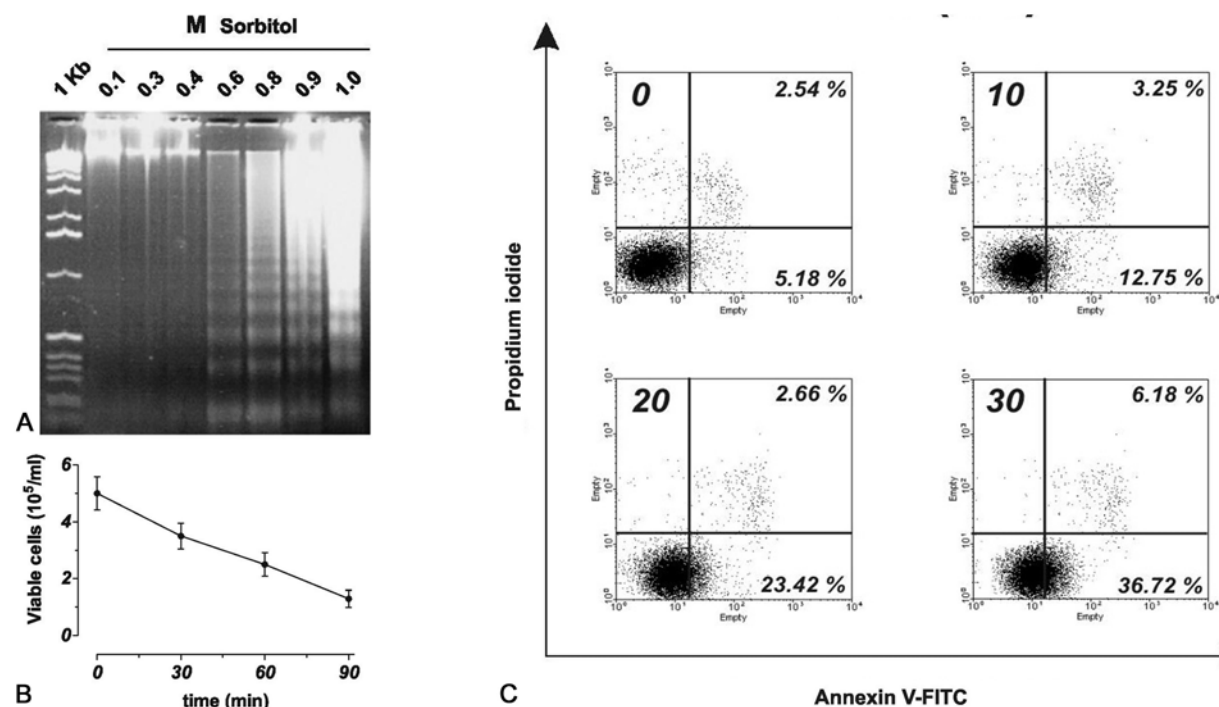
Figure 2. Sorbitol induces apoptosis in K562 cells. K562 cells, at a density of 2 x 10^5/ml, were incubated with increasing concentrations of sorbitol for 60 min. Cells were then harvested and DNA extracts were obtained as described in Materials and Methods and DNA ladder was quantified using Quantity One software. *Lane M*: molecular weight markers**.** K562 cells were incubated for 10, 20, and 30 min in media containing 1M sorbitol. At indicated time points, cells were harvested and stained with annexin V-FITC and propidium iodide as described in Materials and Methods. Data are from a typical experiment out of three giving comparable results. Fluorescence was analyzed by a FACS calibur instrument, and percentages of positive-stained cells were calculated using WinMDI version 2.8 software.

decreased up to 120 min. Expression of bcl-2 and bcl-x_L did not change remarkably comparing to control 60 min and 90 min, respectively, when they were barely detectable and their expression disapper completely at 120 min (Figure 3 A, B, C). Beta-actin DNA band was distribuited at similar levels in the samples.(Figure 3 A, B, C) These results suggest that two genes (bclxL and bcl-2) were not able to inihibit sorbitol-induced apoptosis and thus this process could be regulated by caspase-3 activation

4.3. Effect of curcumin on K562, ARO, NPA cells .

The induction of DNA fragmentation was demonstrated by incubating with different concentrations (10, 25 μM) of curcumin for 24-48 h. DNA fragmentation became visible at 25 μM curcumin treatment in K562 cells (Figure 4 A). In contrast, NPA and ARO cells did not showed DNA fragmentation at the same concentration (Figure 4 B). When cells were treated with 50 and 100 μM curcumin, DNA ladders were just visible after 24 h of treatment (Figure 4 C). The DNA ladder assay indicated that curcumin-induced cell death through no necrotic but apoptotic pathway.

### 4.4. Rosmarinic acid inhibits sorbitol-induced apoptosis

Hyperosmotic stress seemed to induce the apoptotic response in K562 cells via a massive production

of radical species. To verify whether oxidative stress was the mediator of sorbitol-induced hyperosmotic effects or the consequence of the observed mitochondrial impairment, K562 cells were pre-treated with different antioxidants before sorbitol addition. ROS production was determined by cytofluorimetric analyses upon DCFH-DA treatment.

Due to the evidence that ROS were concomitantly produced in the sorbitol-induced apoptosis, we focused our attention on diet-derived antioxidants, such as polyphenols. Among them we selected rosmarinic acid, which was found to be a potent inhibitor of both cellular ROS-producing enzymes (32), e.g. lipoxygenase (33) and cyclooxygenase (34,35), and NOS (36,37). K562 cells were pretreated with 25 μM rosmarinic acid for 1 h and incubated with 1 M sorbitol, in order to monitor cell response during the following hour. Figure 6 A shows that the cell viability rate in sorbitol-treated K562 cells, in the presence of rosmarinic acid, almost matched the control values, as well as the percentage of DCF positive cells (Figure 5 A), suggesting that this compound was able to counteract hyperosmotic-mediated ROS production and apoptosis completely. These data were also confirmed by the lack of activation of caspase-9, as demonstrated by Western blot analysis (Figure 5 B).
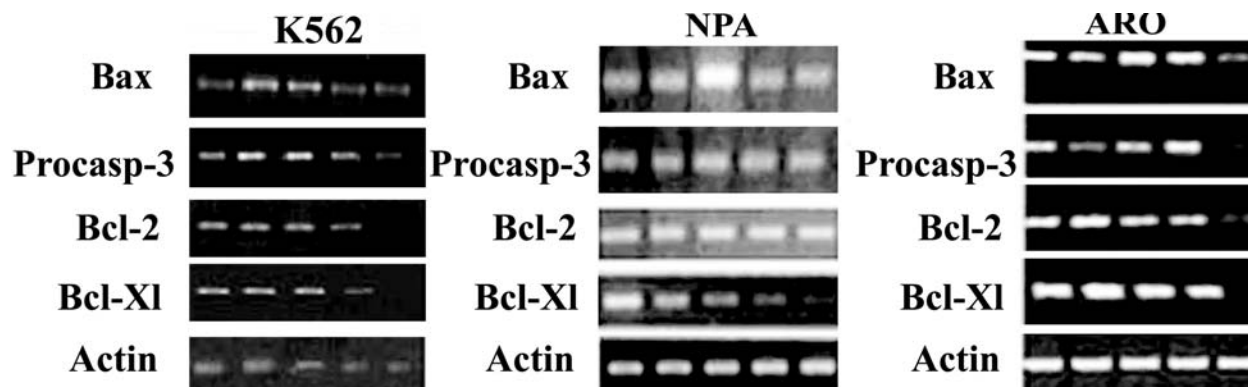
Figure 3..Expression of apoptosis-related genes during sorbitol treatment in the three cell lines.
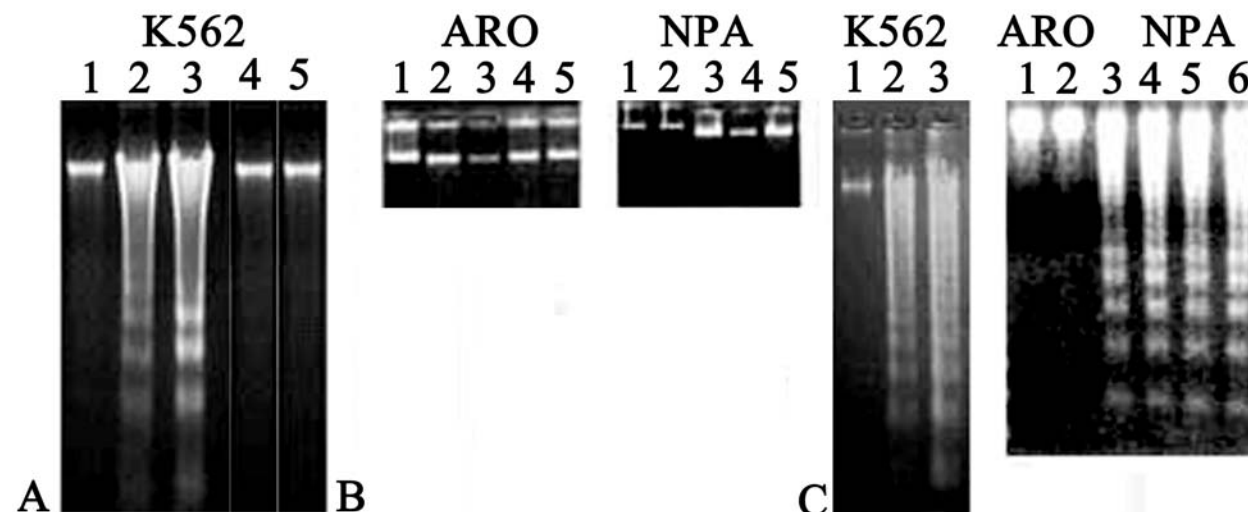
Figure 4. K562 cells, at a density of 2×10^5/ml, were incubated with curcumin at different concentration (10, 25 μM) for different times. Cells were then harvested and DNA extracts were obtained as described in Materials and Methods and DNA ladder was quantified using Quantity One soft NPA and ARO cells were incubated with curcumin at different concentration (10, 25 μM) for different times. DNA fragmentation is not evident. K562, NPA and ARO cells were incubated for 24 h with curcumin at the concentration 50, 100 μM.. At indicated time points, cells were harvested and DNA extracts were obtained as described in Materials and Methods and DNA ladder was quantified using Quantity One software.

Furthemorem, Figure 6 A shows that rosmarinic acid 25 μM is able to block sorbitol-induced DNA fragmentation in NPA and ARO cells, while curcumin at same concentration is not able to block the DNA fragmentation in all cell lines.(Figure 6 B, C).

5. DISCUSSION

Osmotic shock due to exposure of cells to high concentrations of sorbitol has been demonstrated to induce apoptosis by altering the plasma membrane structure so that membrane-embedded proteins cluster and activate their signalling pathways. It has already been reported that the exposure of cells to hyper-osmolar media causes a significant increase in complex protein kinase activity, the mechanism of which is still the aim of studing. However, it is known that the osmotic shock induced by sorbitol activates the stress-activated protein kinases c-Jun N-terminal kinases (JNKs) and p38 protein kinase as well as the extracellular signal-regulated kinases (ERKs) (38) furthermore, it stimulates the cytoplasmic cytochrome c accumulation (39) and the cleavage of procaspase-3 (40).

Such research attempts to extend our previous observation that sorbitol mediates the induction of apoptosis (1,2,3,4) by a mechanism independent of hypertonicity in different cell lines.

In the present study, K562, NPA and ARO cells were treated by sorbitol at concentrations in the range from 0.6 M to 1 M for 1 h and they underwent apoptosis. The characteristic fragmentation of chromosomal DNA into nucleosomal oligomers was detected within 60 min after treatment (41).
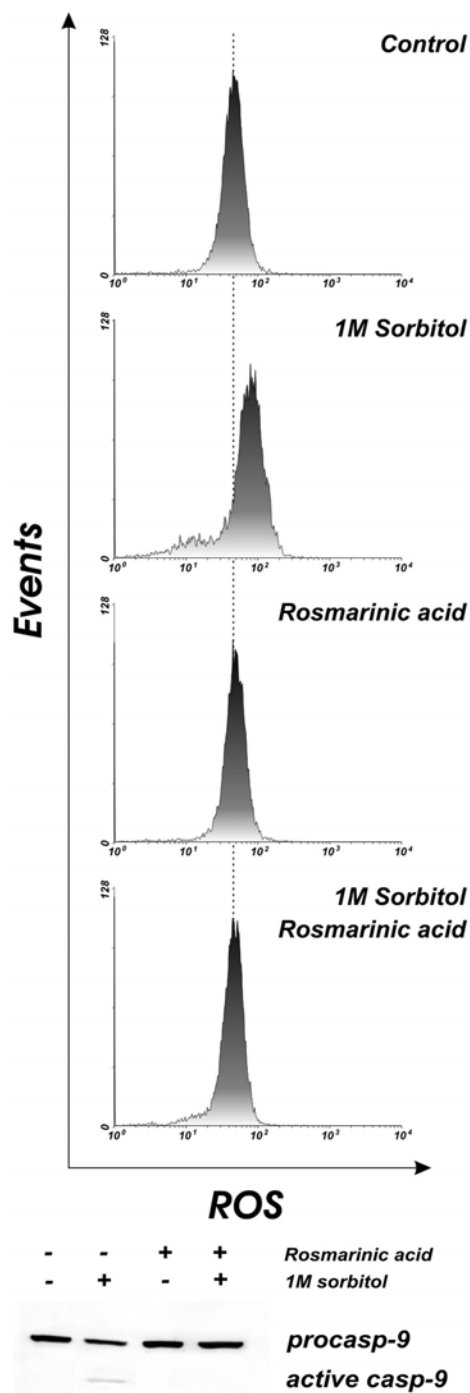
Figure 5. Rosmarinic acid counteracts sorbitol-mediated apoptotic events**.** K562 cells were incubated with 1M sorbitol for 60 min in the presence or absence of 25 μM rosmarinic acid as described in Materials and Methods. Cells were analyzed by a FACScalibur instrument for radical content upon DCFH-DA staining. Data are expressed as means ± S.D. (n = 5) and results obtained in the presence of Rosmarinic acid were considered significantly different from cells incubated with 1M sorbitol alone. K562 cells were incubated for 60 min in media containing 1M sorbitol in the presence or absence of 25 μM rosmarinic acid, harvested and lysed for Western blot analyses as described in Materials and Methods. 20 μg of total cell extracts was loaded for detection of pro-form (inactive) and cleaved (active) Caspase-9. Immunoblots are from one experiment representative of three that gave similar results.
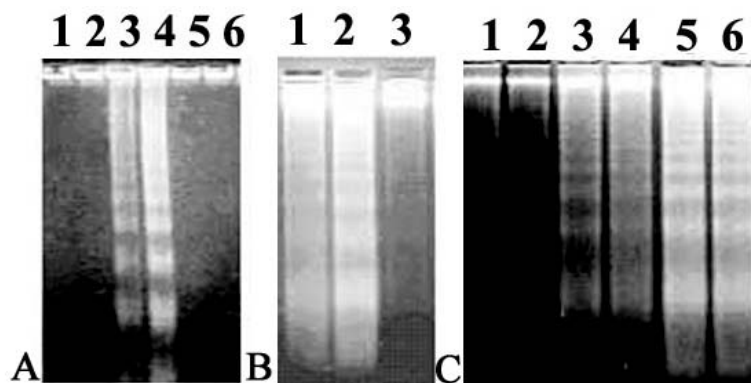
Figure 6. Curcumin induces apoptosis in K562 cells**.** NPA and ARO cells, at a density of 2 x 10^5/ml, were incubated with 1M sorbitol for 60 min in the presence or absence of 25 µM Rosmarinic acid as described in Materials and Methods.. Cells were then harvested and DNA extracts were obtained as described in Materials and Methods and DNA ladder was quantified using Quantity One software. K562 cells were incubated with 1M sorbitol for 60 min in the presence or absence of 25 µM curcumin as described in Materials and Methods.. Cells were then harvested and DNA extracts were obtained as described in Materials and Methods and DNA ladder was quantified using Quantity One software. NPA and ARO cells were incubated with 1M sorbitol for 60 min in the presence or absence of 25 µM curcumin as described in Materials and Methods . Cells were harvested and DNA extracts were obtained as described in Materials and Methods and DNA ladder was quantified using Quantity One software.

Furthemore, we identified ROS as the upstream mediators of the activation of apoptosis in the response to sorbitol treatment. In fact, we found that exposure of K562 cells to sorbitol induces a rapid production of radical species from different origins; their concentration could be partially decreased by pre-treatment with general antioxidant molecules. In particular, the rosmarinic acid, which was used as ROS-scavengers, rescued from sorbitol-induced apoptosis and buffered the increase in the percentage of DCF-positive cells. This evidence suggests that ROS are involved in the cell death and this scavenge is able to abolishe this process completely.

The curcumin chemiopreventive activity   in several animal tumor models has led investigators to examine its potential impact on apoptosis such as in human and rat T lymphocytes (42), whereas others have documented an induction of apoptosis in lines such as HL60 (43) , and in vivo in azoxymethane-induced colon tumors (44). In some cell lines, the results have even been conflicting, such as in HT-29 human colon cancer cells, that have been noted to be induced into apoptosis by some authors (45), whereas the others noted no effect of curcumin (46).

Curcumin, being a hydrophobic molecule (47) passes through the plasma membrane easily into cytosol. Recently, curcumin was shown to cause phosphatidylserine exposure, increase plasma membrane permeability and decrease mithocondrial membrane potential, cell shrinkage (48), and typical features of apoptotic cells (49). Curcumin is an interesting molecule because of the variety of biological effect it possesses in addition to its potent anticancer activity but, its exact mechanism of action is not very clear.

Results of the present study show that rosmarinic acid   but not curcumin   prevents sorbitol –induced apoptosis in different cell lines and suppression of caspase 9 activation as well as production of ROS were identified in its preventive mechanism.

Rosmarinic acid frequently found as a secondary metabolite in herbs and medicinal plants, has exhibited antimicrobial, antiviral, antioxidative, and anti-inflammatory activities (50,51,52).

The final interesting finding of the present work is the identification of rosmarinic acid as a potent antioxidant against ROS, and as an apoptosis effective inhibitor; in fact, pre-treatment with rosmarinic acid totally prevented i DCF-positive cell increse and sorbitol-mediated cell death. Particularly, all the mitochondrial-associated phenomena observed during hyperosmotic conditions did not take place: caspase-9 cleavage were completely unaffected.

Therefore, the biological effect of a flavonoid compound may depend upon its behavior as either an antioxidant or a prooxidant. However the differential biological effect of prooxidant and antioxidant activity of flavonoids is still undefined.

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Send correspondence to: Gabriella Marfè, University of Rome "Tor Vergata", Department Experimental Medicine and Biochemical Sciences, Via Montpellier 1, 00133, Rome, Italy, Tel: 390672596504 ; Fax: 390672596504, E-mail: gabriellamarfe@libero.it