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Cytotoxicity, DNA Fragmentation and Histological Analysis of MCF-7 Cells Treated with Acetylspermine

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ABSTRACT

Acetylspermine is polyamine analogue drug. It causes the generation of free radicals and induction of oxidative stress, associated with cellular injury. Proliferation cells assay with MTT of untreated MCF-7 cells was successively survived with maximum increment in its number after 48 h, whereas the survival cells of MCF-7 showed regular inhibition as it grown with different concentrations of acetylspermine. The maximum inhibitory effect of acetylspermine was shown at the dose 5.0 µM. The percent of inhibition reached 88% as compared with the corresponding control. The effect of acetylspermine on the antioxidant activities of MCF-7 demonstrated that the activity of both superoxide dismutase and peroxidase were increased as the concentration of acetylspermine increased up to 5.0 µM. The rate of increases was 5 and 3 folds more than their corresponding controls, whereas there was an obvious decline in the activities of polyphenol oxidase and catalase reaching the lowest values at the concentration 5.0 µM acetylspermine. Such previous controversial in the activities of the different enzymes manifested that acetylspermine application elevated the enzymes that induced the Reactive Oxygen Species (ROS). Moreover, our results proved that acetylspermine derivative treatment of MCF-7 causes morphological changes typical for apoptosis and plays an important role in destruction of cancer cells and the rate of destruction is dose dependent. The results clearly demonstrate that acetylspermine treatment augments the antioxidants defense mechanism, induced toxicity and provides evidence that it may have a therapeutic role in free radical mediated diseases.

Key words: Acetylspermine, antioxidant activity, cytotoxicity, apoptosis of acetylspermine

INTRODUCTION

The PAs biosynthetic pathway in plants has been thoroughly investigated and reviewed in detail (Evans and Malmberg, 1989; Tiburcio *et al*., 1990; Slocum, 1991; Martin-Tanguy, 2001). PAs are synthesized from arginine and ornithine by arginine decarboxylase (ADC) and ornithine decarboxylase (ODC). The intermediate agmatine, synthesized from arginine, is converted to Put, which is further transformed to Spd and Spm by successive transfers of aminopropyl groups from decarboxylated S-adenosylmethionine (dSAM) catalyzed by specific Spd and Spm synthases. The aminopropyl groups are derived from methionine, which is first converted to S-adenosylmethionine (SAM) and then decarboxylated in a reaction catalyzed by SAM decarboxylase (SAMDC). The resulting decarboxylated SAM is utilized as an aminopropyl donor. The SAM is a common precursor for both PAs and ethylene and SAMDC regulates both biosynthetic pathways. Polyamines, such as Spd and Spm and their obligate precursor Put, are polybasic amines that are implicated in many physiological processes in plants (Galston and Sawhney, 1990). Because of their polycationic

nature at a physiological pH, PAs occur in plant cells not only as free form but also as bound forms. The intracellular free PA pool depends on several processes including PA synthesis, PA degradation, PA conjugation, PA transport (Tiburcio *et al*., 1997) and interaction with other hormone like ABA (Lee *et al*., 1997). Polyamines being cationic in nature can associate with anionic components of the membrane such as phospholipids. PAs in chloroplast can covalently bind to chlorophyll-protein complex in the thylakoids (Del Duca *et al*., 1994). Catabolism of intracellular polyamine is a consequence of two enzymes, the spermidine/spermine N-1-acetyltransferse (SSAT) and peroxisomal N1-acetylpolyamine oxidase (Huang *et al*., 2005). The products of SSAT/PAO activities on spermidine and spermine are the reactive oxygen species, H_2O_2, spermidine, putrescine and 3-acetoamino-propanal, respectively (depending on the starting substrate). The activity of SSAT/PAO pathway has been linked previously with the cytotoxic response of several tumor types to specific polyamine analogue (Casero *et al*., 2003). However, recent studies have clearly demonstrated that an additional enzyme exists in the mammalian polyamine catabolic pathway, an inducible spermine oxidase (SMOP/PAOh1). This is a cytosolic protein that is selectively active on spermine producing H_2O_2, spermidine and 3-aminopropanal (Bellelli *et al*., 2004). The expression of this enzyme is induced by some of the same agents that induce SSAT, suggesting that induction of both of the polyamine catabolic pathways with the production of H_2O_2 (Devereux *et al.*, 2003). Intracellular polyamines do not exert toxic effects as long as physiological regulation by de novo synthesis, uptake, degradation via the interconversion pathway (Seiler, 1991), to maintain concentrations to certain limits. However, the prevention of Spm degradation by polyamine oxidase (PAO which is FAD dependent enzyme) can be lethal (Sarhan *et al*., 1991). If extracellular polyamines undergo oxidative deamination by diamines oxidase, an aldehyde and hydrogen peroxides accumulated. The accumulated products led to cell damage and cell death (Sharmin *et al*., 2001). Direct cytotoxicity actions of Spm were observed only at mM concentrations. The search for inhibitors of polyamine-related enzymes started with the aim to inhibit tumor growth. Selective inhibitors of the enzymes involved in polyamine biosynthesis did not result in practically useful anticancer drugs (Seiler, 2003).

This study aims to evaluate the metabolic efficiency of acetylspermine as anticancer against MCF-7 cell line of breast cancer.

Preparation of different concentrations of acetylspermine: Stock solution of acetylspermine was prepared in 1% phosphate saline buffers pH 7.2 and stored in refrigerator. The different concentrations of acetylspermine (1.25, 2.5 and 5.0 µM) were prepared through the dilution of stock by saline buffer.

Measurement of cytotoxicity to cells (MTT assay): This assay measures cell viability and proliferation according to Mosmann (1983).

Cell culture and acetylspermine treatment: The MCF-7 cells were routinely grown at 37°C in Dulbecco's modified Eagle's medium (DMED) containing 10% Fetal Bovine Serum (FBS) in humidified atmosphere containing 5% CO_2 and used for experiment when confluent. The cultured cells were sub-cultured twice each week. The MCF-7 were plated at 5×10^5 Cells cm^{-1} and placed in a humidified incubator containing 5% CO_2 and 95% air over night at 37°C. For application of acetylspermine, the medium was replaced with DMEM enriched with 10% FBS and acetylspermine was added at concentrations of (1.25, 2.5 and 5.0 µM) against control. Plates were incubated at 37°C for 48 h.

Estimation the activities of antioxidants enzymes, superoxide dismutase, peroxidase, polyphenol oxidase and catalase: Assay of Superoxide Dismutase (SOD) was carried out according to (Beauchamp and Fridovich 1971), peroxidase (PO), Polyphenol oxidase (PPO) and catalase (Cat), were carried out according the method adopted by Kar and Mishra (1976).

Analysis of DNA fragmentation: Extraction methods which allow the isolation of only fragmented DNA without contaminating genomic DNA according to (Herrmann *et al*., 1994) and the colorimetric estimation of DNA content was carried out according to (Perandones *et al*., 1993).

Histopathological examination: The HCl-denaturated-methyl green-pyronin (BDH) technique was based on method of (Iseki and Mori, 1986) and modified by Sen *et al*. (1999) was used.

RESULTS

Cytotoxicity of acetylspermine against MCF-7 cell line at 48 h was evaluated. The results of survival cells assay for MCF-7 by cells using MTT reduction method were shown Table 1 and Fig. 1. It revealed that Proliferation of untreated MCF-7 cells was successively survived with maximum increment in its number after 48 h. On the other hand, the survival cells of treated MCF-7 showed regular inhibition as it grown with different concentrations of acetylspermine. The maximum inhibitory effect of acetylspermine was shown at the dose 5.0 µM. The percent of inhibition reached 88% as compared with the corresponding control. In a similar manner the other treatments of acetylspermine gave a similar effect with ascending response as the incubation period reached to 48 h. The previous results evaluated that the percentage of cytotoxicity of spermine analogue was dose and time dependent.

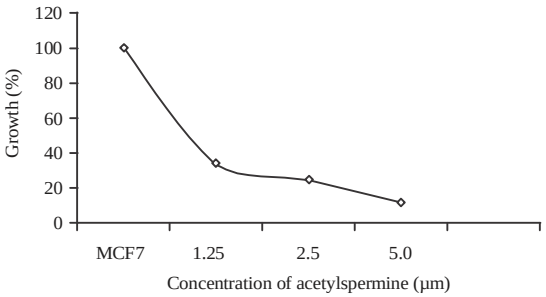

Fig. 1: Cytotoxicity of different concentrations of acetylspermine (µM) against MCF-7 cell line at 48 h

Table 1: Cytotoxicity of acetylspermine against MCF-7 cell line at 48 h

Treatments acetylspermine (µM)	Survival MCF-7 cells (%)	Mortality (%)
Control (MCF-7)	100	100
1.25	35	65
2.50	25	75
5.00	12	88

Table 2: Levels of antioxidants in MCF7 with different doses of acetylspermine at 48 h

	Treatment			
Concentration	SOD	PO	PPO	CAT
Control (MCF-7)	2.110±0.231	0.063±0.143	0.156±0.025	23.522±0.146
1.25	3.300±0.312**	0.092±0.015*	0.089±0.018*	12.426±0.163***
2.5	4.830±0.276***	0.163±0.027*	0.053±0.019*	8.230±0.271***
5	6.800±0.249***	0.320±0.025**	0.026±0.017**	8.200±0.233***

Units of enzymes activities are expressed as, SOD: Units min^{-1} mg $protein^{-1}$ (1 unit = the amount of enzyme that inhibits the auto-oxidation of pyrogallol by 50%), PO: Units min^{-1} mg $protein^{-1}$, PPO: Units min^{-1} mg $protein^{-1}$, CAT: µmol of hydrogen peroxides consumed min^{-1} mg $protein^{-1}$, *Significant, p>0.05, **Highly significant, p>0.01, ***Very highly significant, p>0.001, Student's t-test

The effect of acetylspermine on the antioxidant activities of MCF-7 were shown in Table 2 and Fig. 2. Results revealed that, the activity of all tested superoxide dismutase and peroxidase increased as the concentration of acetylspermine increased up to 5.0 µM. The rate of increases was 5 and 3 folds respectively more than control, whereas, the activities of polyphenol oxidase and catalase were declined to the lowest values at the concentration (5 µM) acetylspermine. The percentage of decreases was 83 and 65% irrespective to their corresponding control. The previous controversial in the activities of the different enzymes manifested that acetylspermine application enhance the elevation of enzymes that induce the Reactive Oxygen Species (ROS).

For histological analysis of MCF-7, an experiment was conducted in which the cell line was *in vitro* treated with acetylspermine for up to 48 h. After the elapse of incubation period, cells were stained with methyl green-pyronin as specific stain for the cytochemistry of DNA and RNA. Results in Fig. 3 revealed that control sample of MCF-7cells have a well developed bluish green color and RNA gain red color when stained with pyronine. The well developed colors of both DNA and RNA indicating the normal proliferation MCF-7 cells. On the other hand, when cells treated with

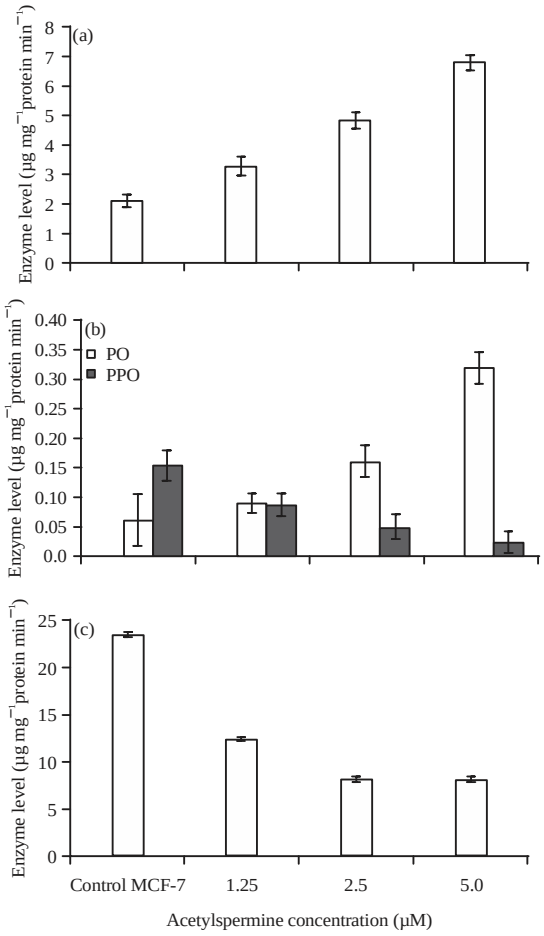

Fig. 2(a-c): Antioxidant enzymes activity (µg product/mg protein/time) in MCF-7 cells treated with different concentrations of acetylspermine for 48 h, (a) Superoxide dismutase (SOD), (b) Peroxidase (PO) and Polyphenol oxidase (PPO) and (c) Catalase (CAT)

acetylspermine (1.25-5.0 µM), the color of DNA and RNA was regularly diminished with increasing both the concentration of acetylspermine and time of the experiment. In this regards, the cells had reddish color as well as minute fragmentations were also appeared especially when treated cells incubated for 48 h. The previous histological bases in color of cells indicate that acetylspermine plays an important role in the destruction of cancer cells and the rate of destruction was dose and time dependent. In control group, the cells were filed with the nuclear chromatin substance and cytoplasm was strongly acidophilic, some few cells revealed the chromatin substance in the form of large a regular particle (X1000). While in MCF-7 treated with 1.25 µM product of acetylspermine, most of cells lost their nuclei and become appeared as ghost but very few still revealed remnants of the chromatin substance (X1000). Moreover, MCF-7 treated with 2.5 µM product of acetylspermine, the cells lost large quantity

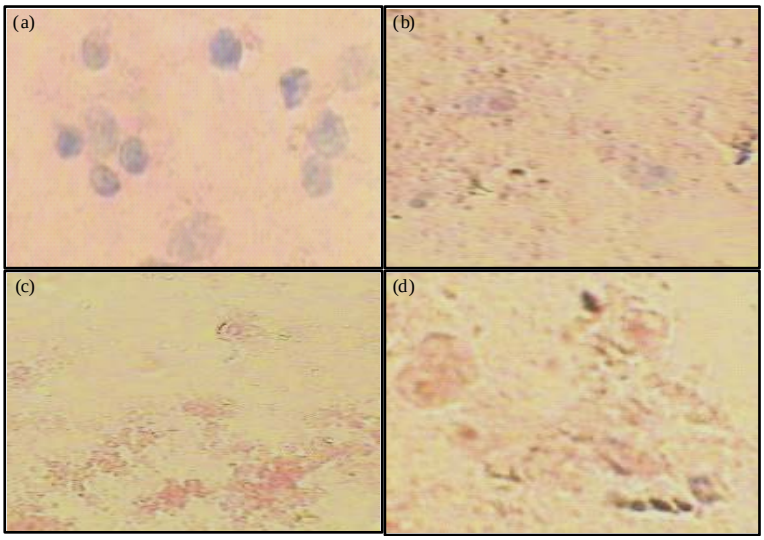

Fig. 3(a-d): HCl-denatured methyl green-pyronine staining in MCF-7 cells following 48 h, (a) Control cell lines (MCF-7) group, (b) MCF-7 treated with 1.25, (c) 2.5 and (d) 5.0 of acetylspermine: X 1000

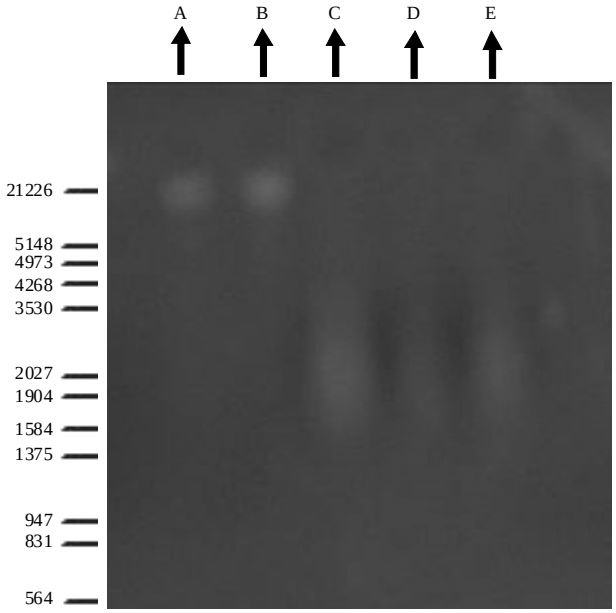

Fig. 4: Agarose gel electrophoresis of genomic DNA extracted from MCF-7 cell lines that treated with acetylspermine, A: Enzyme, B: Control, C: 1.25, D: 2.5 and E: 5.0 µg product of acetylspermine

of the chromatin substance or the remaining Chromatin become condensed and defeated to one side of the cell (X1000). Finally MCF-7 treated with 5 µM product of acetylspermine, all the cancer cells were completely destructed and appeared as remnants of cells (X 1000).

Studying the effect of different concentrations of acetylspermine on DNA fragmentation in MCF-7 cell lines, results in Fig. 4 revealed that acetylspermine induced a significant effect on the nucleoplasm of MCF-7 cell lines. In this regard, the reduction in the amount of the total DNA in control as well as treated cells was obviously in Agarose gel. Where in the treated cells there is an obvious seamier was developed.

DISCUSSION

The polyamines putrescine (put), spermidine (Spm) and spermine (SM) are naturally occurring polycationic amines

that are required for cell growth because of the critical role of polyamines in the regulation of cell growth. The polyamine metabolic pathway is an attractive target for antineoplastic strategies (Galston and Sawhney, 1990). The primary regulatory enzymes of polyamine biosynthesis include Ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase, Spermidene synnthase and spermine synthase. The enzyme spermidine/spermine N1-acetyltransferase (SSAT) and spermine oxidase play a significant limiting role in polyamine catabolism (Porter and Sufrin, 1986; Hyvonen *et al*., 1988; Milovica *et al*., 2001). Moreover, several polyamine analogues have efficacy against a variety of cancer cells including breast cancer. Recently, a novel class of polyamine analogues designated as oligoamines and acetyl amine have been developed. In this regards acetylspermine was applied to MCF-7 at the final concentration 1.25-5 μM Table 1 and Fig. 1. The results manifested that an obvious inhibition in human breast cell line to the percentage 88%. Moreover, an obvious increases in the activities of both superoxide dismutase (SOD) and peroxidase Table 2. The previous results are in concomitant with many trials for inhibiting cancer cell where, recently the terminally acetylated polyamine analogue is most extensively studied and the most promising compounds are currently under investigation in clinical trials as anticancer agents (Casero and Woster, 2001). The polyamine analogue is able to mimic the natural polyamines in their self-regulatory role but unable to act as substitutes for polyamines in their cell growth regulatory functions. It has recently been shown that the growth and the inhibitory activity of some of these compounds might be mediated by stimulation of polyamine catabolism and polyamine oxidase (Acetylspermine) activity in particular (Wang *et al*., 2001). The cytotoxicity of 3-acetamidopropanol and hydrogen peroxide are produced in the Acetylspermine catalyzed oxidative de-amination of acetylspermine. The induction of polyamine interconversion by some alkyl substituted polyamine analogue has been suggested to be involved in the growth inhibitory effects of those compounds in several cancer cell lines including breast cancer (Davidson *et al*., 1999). Moreover, H_2O_2 locally generated by the polyamine interconversion pathway in breast cancers has recently been suggested to be an effective death signaling pathway (Wallace *et al*., 2003). It is known that the fetal calf serum, a component of cell culture media contains an enzyme called Serum Amine Oxidase (SAO), capable of preferentially oxidizing spermine and spermidine at the primary amino groups, generating cytotoxic aldehydes, H_2O_2 and ammonia (Morgan, 1988).

Considering the possibility that spermine analogue derivative especially acetylspermine (that contain unprotected amino groups) might be oxidized by SAO, MCF-7 culture together with the spermine derivatives showed an obvious mortality in the cell line. As regard in the dose-response curve points following the co-treatment with spermine derivatives. The growth inhibitory properties were investigated after 48 h. The growth-inhibitory effects of acetyl derivative were accompanied by cell shrinkage observed by monitoring the cells by means of the phase- contrast microscope Fig. 3, since such change in the shape of treated cells Fig. 3a-c, might indicate apoptotic cell death, we further developed the investigation on cell morphology response to spermine analogue treatment in order to find out another evidence of apoptosis. Both control and treated cells were stained with Methyl green-pyronine MGP and visualized by means of a light microscope. Dense nuclei due to chromatin condensation and nuclear fragmentation (the brighter fluorescence) were observed in treated MCF-7 cells Fig. 3b-d but not in the control Fig. 3a. The pervious results of chromatin condensation and nuclear fragmentation are hallmarks of apoptosis (Kerr *et al*., 1994; Gooch and Yee, 1999); this observation strongly supported that the cytotoxicicty of the oxa-spermine derivatives take place via apoptosis. Also (McCloskey *et al*., 1996) claimed that the first evidence that polyamine analogue treatment can induce apoptosis has been demonstrated in MCF-7 and other human cell lines. The growth inhibitory and apoptotic effects of several terminally alkylated polyamine analogues have linked to their ability to stimulate the two- steps polyamine back conversion by a direct line analogue (Wang *et al*., 2001). It is known that apoptosis can be biochemically characterized by the oligonucleosomal DNA fragmentation (Compton, 1992). DNA isolated from control and treated cells was processed on Agarose gel electrophoresis in order to detect the typical DNA ladder, as a result of oligonucleosomal fragmentation. Before the beginning of DNA isolation procedure the cells were monitored by microscope for the presence of morphological changes. Although we have used different spermine analogue concentrations (in the range 1.25-5.0 μM), cell number and exposure time (48 h) in order to find the best condition for DNA fragmentation detection, we failed to obtain the typical DNA ladder in response to oxa-polyamine treatment Fig. 4. Finally, it could be stated that stimulation of polyamine interconvesion pathway and acetylspermine activity have been proposed to be involved in the mechanism of the cytotoxic and apoptotic effects of some polyamines is known that cancer cells show impaired apoptotic mechanisms, thus the induction of apoptosis is an important aim in anti-tumor strategies.

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