

Evaluation of Growth Inhibitory and Apoptosis Inducing Activity of Human Calprotectin on the Human Gastric Cell Line (AGS)

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ABSTRACT

Background: Calprotectin is cytotoxic agent that its anti-tumor effects are governed through suppression of topoisomerase II; a key enzyme in apoptosis. In previous studies, cytotoxicity and apoptotic effects of calprotectin are shown on different cancer cell lines, but not human gastric cancer cell lines. In the present study, cytotoxicity and apoptotic effects of calprotectin on human gastric adenocarcinoma cancer cell line (AGS) were evaluated. **Methods:** The AGS cells were exposed to the different concentrations of calprotectin for 24, 48 and 72 hours. Cell proliferation was assessed using dimethylthiazol diphenyl tetrazolium bromide assay and dye exclusion tests. For evaluation of cytotoxic mechanism in calprotectin on AGS cells, flow cytometric analysis was performed. **Results:** Our results revealed that calprotectin induces growth inhibition of AGS in a dose- and time-dependent manner. Results of this investigation showed that sensitivity of AGS cells to cytotoxic effect of human calprotectin was highly remarkable. In addition, growth inhibitory effect of this cytotoxic agent mostly was governed through induction of apoptosis in the AGS cells. **Conclusion:** These findings indicated that calprotectin induces growth inhibition and apoptosis in the AGS cells. *Iran. Biomed. J. 12 (1): 7-14, 2008*

Keywords: Human calprotectin, Human gastric adenocarcinoma (AGS), Growth inhibition, Apoptosis

INTRODUCTION

Gastric cancer is one of the most prevalent malignant tumors in the world. Although the incidence of gastric cancer is declining, it still remains a major health problem and a common cause of cancer mortality world wide [1, 2]. Gastric cancer carcinogenesis refers to accumulation of genetic alteration of multiple genes such as oncogenes, tumor suppressor and mismatch repair genes [3]. The dynamic balance between cell proliferation and apoptosis is very important to maintain the homeostasis in human body and gastric carcinogenesis is related to this imbalance [4]. Development of gastric cancer is believed to be a slow process with primary etiological determinants for gastric cancer being exposure to chemical

carcinogens and/or infection with *helicobacter pylori* [5, 6]. It has been reported that gastric cancer also expresses multidrug-resistance associated protein (MRP) and shows lower sensitivity to the several anti-cancer drugs [7].

The currently used chemotherapeutic agents for gastric cancer are not highly effective because detection and treatments of this cancer are performed in the advanced stages of the disease [1, 3]. Calprotectin is a heterodimeric protein complex with zinc and calcium binding capacity. This protein is predominantly found in cytosolic fraction of neutrophils [8, 9]. Calprotectin has zinc binding properties and reversible anti-microbial and anti-fungal activity [10] and exhibits growth inhibitory and apoptosis inducing activity against some normal and a broad spectrum of tumor cells with different

origins [8, 9, 11]. Although several reports suggest that cell death inducing activity of calprotectin is due to exclusion of zinc from target cells [10], there is still other possibility for cell death inducing mechanism of this protein. Previous studies demonstrate that calprotectin inhibits the activity of casein kinase II, which is involved in the phosphorylation of several enzymes including topoisomerase (topo) I and II [12]. Because the inhibitors of topo induce cell death through apoptosis in various cells death mechanisms [13], it is possible that the cell death induced by calprotectin is related to regulation of topo activity in target cells.

In this study, we evaluated the cytotoxic and apoptotic effects of human calprotectin in different concentrations and time intervals on human gastric cancer cells (AGS), in order to determine of human calprotectin effect in proliferation inhibition and apoptosis induction on AGS cells.

MATERIALS AND METHODS

Materials. Dithiothreitol and lymphoprep were obtained from Merck (Germany) and Amersham Company (USA) respectively. FCS was obtained from Gibco (Scotland) and Seromed (Germany). RPMI 1640 medium, penicillin, streptomycin, trypan blue (TB), MTT (dimethylthiazol diphenyl tetrazolium bromide) and etoposide all were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and were at least of analytical grade. *In situ* cell death detection kit (Annexin-V FITC) was purchased from IQ products (Netherlands). Flask, tubes and culture plates were obtained from Griner (Germany). Other chemicals used in this study were purchased from Sigma Chemical Co. All solutions were made in deionized double distilled water.

Cell line. The human gastric adenocarcinoma cell line (AGS, NCBI: C-131) was obtained from National Cell Bank of Iran, Pasteur Institute of Iran (Tehran). These cells were maintained in RPMI-1640 medium supplemented with 10% FCS in a humidified incubator (37°C and 5% CO₂).

Calprotectin purification. Human neutrophil was prepared from leukocyte-rich blood fractions (buffy coat) according to the method of Muller *et al.* [14] and Van Den Bos *et al.* [15]. Purification method of human calprotectin was described previously [16].

Incubation of calprotectin on AGS for cytotoxicity assay. AGS cells were cultured in

RPMI-1640 medium supplemented with 10% heat inactivated FCS, 2 mM glutamine, penicillin (100 IU/ml) and streptomycin (100 µg/ml) at 37°C in an incubator containing 5% CO₂. Harvested cells with trypsin (0.25%) were counted by Neubauer slide and TB and then were seeded into 96-well plates (1 × 10⁴ cell/well). The cells were incubated with different concentrations of calprotectin (0, 1.025, 2.05, 4.1, 8.2 and 16.4 µM) and etoposide (as positive control) (0, 1.025, 2.05, 4.1, 8.2, 16.4, 32.8 and 65.6 µM) for 24, 48 and 72 h. For each concentration of drugs, six wells of 96-well plates containing 1 × 10⁴ AGS cells were used. In each experiment, six AGS cultured wells with no drug were used as negative control. The cultured medium was controlled every day.

Viability test. Relative cell number was measured using TB test and MTT assay [17, 18]. The principle of the TB dye exclusion is that viable cells exclude the dye whereas dead cells take it up. Measurement of cytotoxicity is achieved by direct counting of viable and dead cells. The percentages of viability and cytotoxicity were calculated according to the following formulas:

$$\% \text{ Viability} = \frac{\text{number of viable cells}}{\text{total number of cells counted}} \times 100$$

$$\% \text{ Cytotoxicity} = 100 - \% \text{ Viability}$$

For MTT assay, the dye was dissolved in PBS at a concentration of 5 mg/ml and solution was filtered through a 0.45 µm filter to sterilize and remove small amount of insoluble residues present in some batches of MTT, then stored at 2-8°C for frequent use. Four hours before the end of incubations, 10 µl of MTT solution (5 mg/ml in PBS) was added to each well containing 100 µl cultured medium. Dissolved yellowish MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes at 4-h incubation. In contrast with dead cells, active mitochondrial dehydrogenases of living cells will cause this change. The cleavage and conversion of the soluble yellow dye to the insoluble purple formazan have been used to develop an assay system alternative to other assays for measurement of cell proliferation. The insoluble formazan produced was dissolved in solution containing 100 µl isopropanol and OD was read against blank reagent with multi-well scanning spectrophotometer (ELISA reader, Organon Teknika, The Netherlands) at a wavelength

of 570 nm. Six AGS cultured wells with 100 μ l deionized distilled water incubation at 10 min were used as positive control and six AGS cultured wells with no sample were used as negative controls. In addition, six wells containing only dye (100 μ l diluted MTT) were used as dye control. The percentage of cytotoxicity was calculated according to the following formulas:

$$\% \text{ Viability} = \left(1 - \frac{\text{mean absorbance of toxicant-treated cells}}{\text{mean absorbance of negative control}}\right) \times 100$$

$$\% \text{ Viability} = 100 - \% \text{ Cytotoxicity}$$

Flow cytometry analysis. For flow cytometry analysis, AGS cells were cultured into 6-well plates at a density of 5×10^5 cells with and without the cytotoxic agents at 18 and 36 h. All floated and adherent cells were harvested and centrifuged at $200 \times g$ for 10 min. Cell pellet was washed with 1X calcium binding buffer and centrifuged at $200 \times g$ for 10 min. Ten microliters of Annexin V/FITC was added to 100 μ l of cell suspension containing 10^6 cells, and incubated at 4°C for 20 minutes. Then, the cells were washed again with the calcium binding buffer and added 10 μ l of propidium iodide (PI) and incubated at 4°C for 10 min and analysis performed by a flow cytometer (Bio-Rad, USA). FL1 and FL2 channels were used for detection of Annexin/FITC and PI, respectively. Concentration of the calprotectin giving 50% cytotoxicity (LC_{50}) (70 $\mu\text{g/ml}$ or 2.87 μM) and LC_{50} of etoposide (30 $\mu\text{g/ml}$ or 52 μM) were selected in evaluation of apoptosis using flow cytometry. For each experiment of each drugs, 2 well of 6-well plates of 5×10^5 AGS cells were used and each experiment was repeated 2 times.

LC_{50} determination. LC_{50} was determined by probit analysis using the pharmacological PCS statistical package (Springer-Verlage, New York).

Statistical analysis. Results were expressed as mean \pm SD. Mean difference among groups was calculated by one- and two-way variance analysis (one- and two-way ANOVA). $P < 0.05$ was considered statistically significant.

RESULTS

Effect of human calprotectin on the AGS cell proliferation. The results of cytotoxicity effect of

calprotectin on AGS cells in different concentrations and time intervals are shown in Figures 1 (MTT assay) and 2 (TB dye exclusion method). Proliferation inhibition by human calprotectin was significant at all time intervals and both staining methods ($P < 0.001$ by one-way ANOVA). A significant positive correlation between the results obtained by MTT and TB assays in cytotoxicity evaluation of calprotectin on AGS cells are shown in Figures 1 and 2. The results obtained by MTT assay and TB method are positively correlated ($P < 0.005$, Fig. 3).

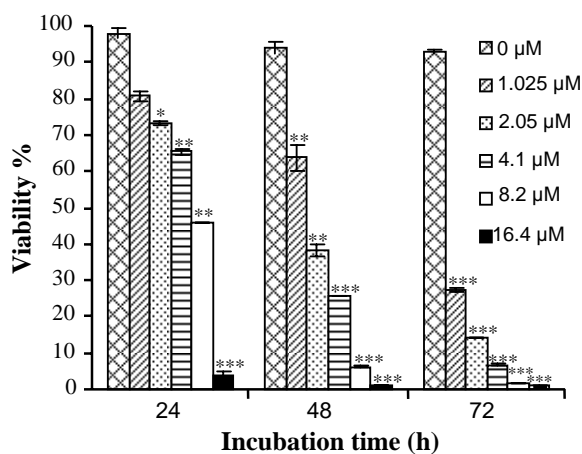


Fig. 1. Measuring the inhibitory effect of human calprotectin on cell proliferation by MTT assay. The AGS cells were treated with different concentrations of human calprotectin for 24, 48 and 72 h and viability was assessed using MTT assay as described in materials and methods. Results are expressed as percentage of viability compared to control and are presented as mean \pm SD. Significance levels are * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Inhibition of proliferation was found to be significant at all concentrations of human calprotectin ($P < 0.001$ by one-way ANOVA).

The results of Figures 1 and 2 showed that cytotoxicity effects of calprotectin on AGS cells are dose and incubation time dependent. The results obtained from the TB test and MTT assay revealed that the incubation of AGS cells with 16.4 μM of human calprotectin led to the complete inhibition of cell proliferation 48 h after incubation (Figs. 1 and 2). According to the Figures 3, there is significant correlation between both methods and they are linear and positive ($P < 0.01$). The 50% LC_{50} values of human calprotectin and etoposide on AGS cells at different time intervals obtained by TB test and MTT assay are shown in Figure 4. There are significant differences between LC_{50} obtained by TB test and MTT assay at different time intervals for both calprotectin and etoposide ($P < 0.05$).

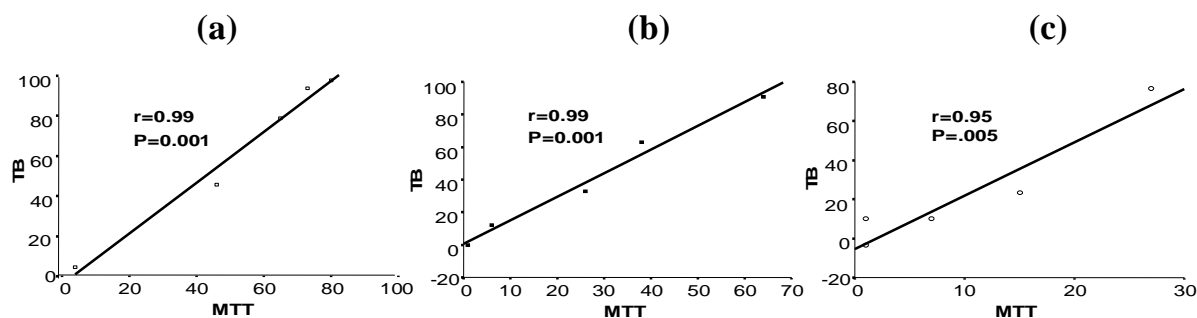


Fig. 3. Viability (%) of AGS cells incubated with different concentration of human calprotectin in TB and MTT assays after 24 (a), 48 (b) and 72 (c) h time intervals.

Measuring apoptotic cell induction. To quantify the frequency of apoptotic cell induced by human calprotectin, Annexin-V/PI double staining was performed. The cells were treated with calprotectin (2.87 μM) and etoposide (52 μM) (positive control) for 18 and 36 h. Cell surface expression of phosphatidylserine (PS) translocated from the inner cytoplasmic membrane is considered an early apoptotic event. By treating cells with either etoposide or calprotectin and then analyzing with flow cytometry, four populations are resolved. Alive cells are double negative and are seen in the lower left quadrant [A3]. Cells that are Annexin V-FITC (+)/PI (-) [A4] are apoptotic. The Annexin V-FITC (+)/PI (+) cell population [A2] has been described as necrotic or advanced apoptotic. The last quadrant [A1] Annexin V-FITC (-)/PI (+) may be bare nuclei, cells in late necrosis, or cellular debris.

Our data represent that the cytotoxicity of both calprotectin and etoposide on the AGS cells occurs through apoptosis. The apoptotic index values were determined using the flow cytometry results and have been shown in Table 1 and representative Figure 5. Treatment of the AGS cells with human calprotectin and etoposide for 18 h induced apoptosis in about 19.2% and 17.1 % of the treated cells, respectively. Human calprotectin and etoposide after 36-h of incubation induced apoptosis

in approximately 38% and 47.6% of the treated cells, respectively. These results demonstrated that there is no significant difference in apoptosis induction by etoposide and human calprotectin on human gastric cancer cell line.

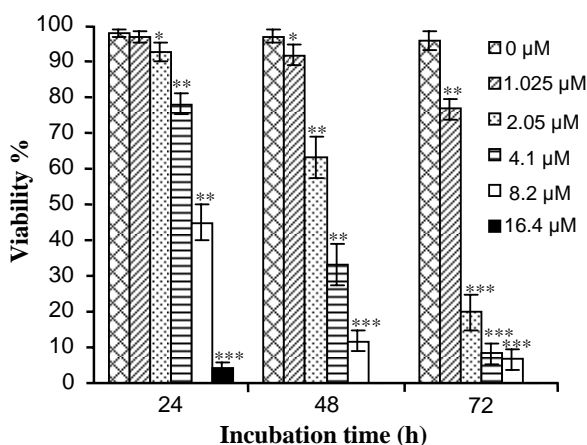


Fig. 2. Cytotoxicity measurement of Human calprotectin by TB dye exclusion assay. The cells were treated with various concentrations of human calprotectin for 24, 48 and 72 h. The anti-proliferative effect was measured using dye exclusion assay. Results were expressed as mean \pm SD. Significance levels are * P <0.05; ** P <0.01; *** P <0.001. Inhibition of proliferation was found to be significant at all concentrations of human calprotectin (P <0.001 by one-way ANOVA).

Table 1. Apoptotic index (n = 3) of the AGS cancer cells treated with human calprotectin (2.87 μM) and etoposide (52 μM) for different incubation times. Results were expressed as the means \pm SD.

Cells (%)	Agents		Agents	
	Calprotectin	Etoposide	Calprotectin	Etoposide
	18 (h)		36 (h)	
Alive	66.34 \pm 10.8	69.36 \pm 6.1	44.9 \pm 10.2	47.8 \pm 10.6
Early apoptotic	8.20 \pm 2.1	11.20 \pm 3.4	23.5 \pm 8.3	25.2 \pm 6.7
Late apoptotic	11.00 \pm 4.4	5.90 \pm 2.4	14.5 \pm 3.2	22.4 \pm 8.2
Necrotic	15.30 \pm 5.9	13.50 \pm 1.3	17.2 \pm 7.1	5.1 \pm 0.2

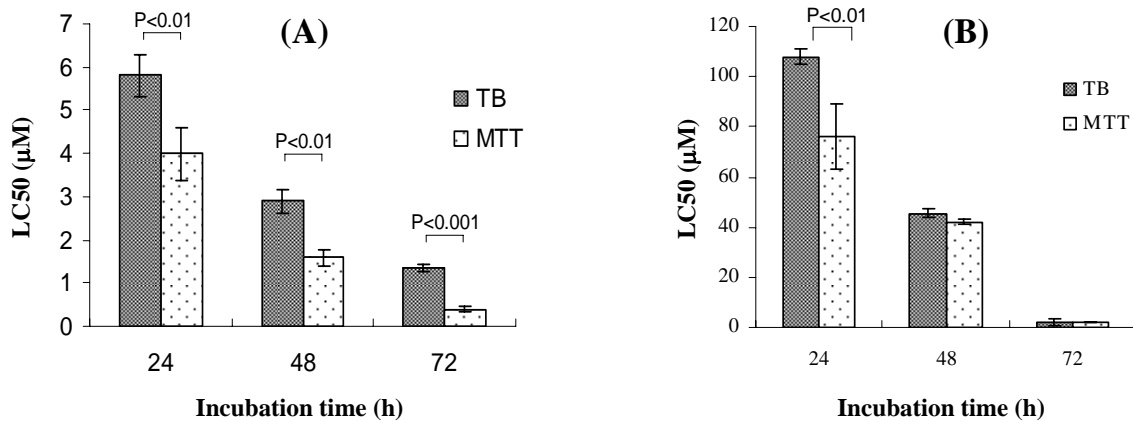


Fig. 4. Determination of LC₅₀ values for human calprotectin and etoposide affecting AGS cell line. The LC₅₀ values for (A) human calprotectin and (B) etoposide affecting AGS cells during 24, 48 and 72 h incubation. LC₅₀ values are assessed by TB and MTT assays. Results were expressed as mean \pm SD. There was significant difference between TB and MTT assay at all time intervals for both agents ($P < 0.05$). Inhibition of proliferation was found to be significant between LC₅₀ concentration of calprotectin and etoposide at both assays ($P < 0.05$). Comparison between proliferation inhibition of human calprotectin and etoposide by LC₅₀ concentration on AGS cell was found to be significant at all time ($P < 0.001$ by two-way ANOVA).

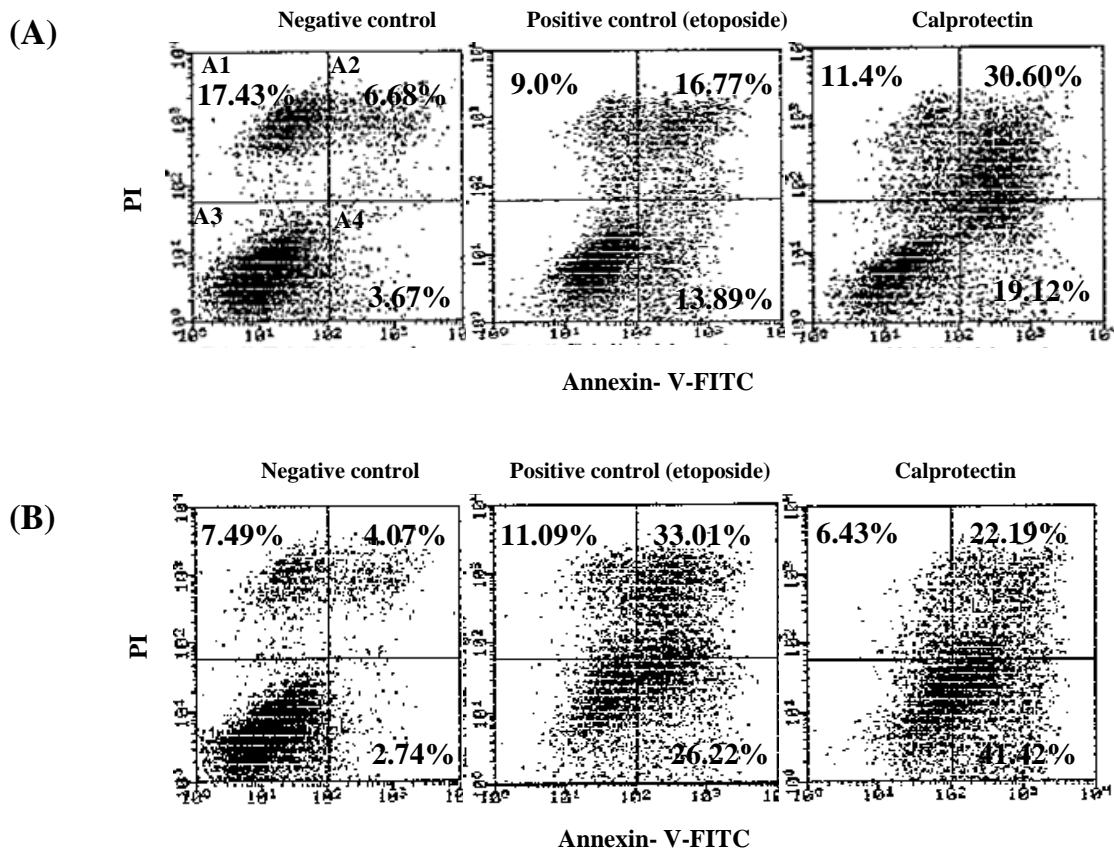


Fig. 5. Representative flow cytometry results in evaluation of the effect of human calprotectin on the induction apoptosis. After incubation for 18 and 36 h (A and B, respectively) with human calprotectin and etoposide (control positive), cancer cells were harvested, stained with Annexin V-FITC (FL-1) and propidium iodide (PI, FL-2) and analyzed by flow cytometry. Four populations are resolved. Viable cells or Annexin-FITC (-)/PI (-) [A3] are seen in the lower left quadrant. Cells that are Annexin V-FITC (+)/PI (-) [A4] are apoptotic (lower right). The cell population with Annexin V-FITC (+)/PI (+) [A2] has been described as necrotic or advanced apoptotic (upper right) and Annexin V-FITC (-)/PI (+) [A1] may be bare nuclei, cells in late necrosis, or cellular debris (upper left).

DISCUSSION

AGS cells are from a poorly differentiated gastric adenocarcinoma cell line [19] with wild type P53 [20]. In this work, these cells were incubated with human calprotectin. To evaluate their potency in the inhibition of cell proliferation and induction of apoptosis, MTT assay, TB test and flow cytometry were performed. According to the results of this study, it is possible to assume that human calprotectin can initiate signaling toward the AGS cell-death with the doses much lower than etoposide (as standard drug). Since the cells are more responsive to the inhibitory effect of human calprotectin compared to etoposide at various concentrations, the protein seems to be much more potent cytotoxic agent for AGS tumor cells in relative to the etoposide.

Previous investigations have shown that tumor cells may be drug-resistant due to the over-expression of some proteins (e.g. P-glycoprotein or MRP). These proteins confer resistance to the tumor by pumping the drug out of the cells [21]. It has been reported that also in gastric cancer, MRP are expressed [22]. Thus, higher resistance of AGS cells to etoposide in comparison with human calprotectin is probably due to the presence of such resistance system for etoposide in membrane of AGS cells.

The mechanism of cell apoptosis stimulated by calprotectin has not been fully elucidated. It has been reported that calprotectin may induce apoptosis of multiple tumor cells and consequent inhibition of carcinogenesis by inhibition of casein kinase I and II that inhibit phosphorylation of topo II [23] and activating caspases [24]. Previous studies demonstrated that calprotectin inhibits the activity of casein kinase II, which is involved in the phosphorylation of several enzymes including topo I and II [12]. Because the inhibitors of topo activity induce cell death through apoptosis in various cells [13], it is possible that cell death inducing activity of calprotectin is governed somehow by regulation of topo activity in target cells.

The ability to simultaneously measure multiple parameters on a cell by cell basis is probably the most powerful aspect of analytical flow cytometry. This allows flow cytometry to be used for a wide range of applications. Perhaps the most common use is the identification of the presence of antigens either on the surface of or within cells [25]. In normal cells, PS residues are found in the inner membrane of the cytoplasmic membrane. During apoptosis, the PS residues are translocated in the membrane and

are externalized. In general, though not always, this is an early event in apoptosis and is thought to be a signal to neighboring cells that a cell is ready to be phagocytosed. Annexin-V is a specific PS-binding protein that can be used to detect apoptotic cells [26]. Mikami *et al.* [27] by using flow cytometry showed that calprotectin, induces apoptotic cell death in various tumor cells, suggesting that calprotectin is an effector molecule against tumor cells in polymorphonuclear leukocytes. To explore the cell death-inducing mechanism of the factor, Mikami *et al.* [27] and Yui *et al.* [11] confirmed the involvement of target protein synthesis, generation of reactive oxygen species and loss of DNA content in the reaction. Ghavami *et al.* [24] by using annexin V-fluorescein isothiocyanate apoptosis detection kit showed the treatment of the colon carcinoma cells with different concentrations of human S100A8/A9 induces a significant increase in cell death. Annexin V/phosphatidylinositol and Hoechst 33258 staining revealed that the cell death was mainly of the apoptotic type [24].

The cytotoxic effect of calprotectin as LC₅₀ values, determined at all time intervals with the TB assay, was seen at higher concentrations compared to MTT assay. Similar results have been previously reported by other researchers [28, 29]. In another study, good correlation was observed between the MTT and the [³H]-TdR assay for drug sensitivity testing [30]. Heidari *et al.* [31] compared three methods (neutral red, [³H]-TdR assay and TB assay) for cytotoxicity effect of T-2 toxin. The results showed that all three methods are reliable and comparable for determining the LC₅₀ of cytotoxic drugs. These results indicated that the neutral red cytotoxicity assay may be preferable to other methods because of its simplicity, its use of nonradioactive materials and its objectivity [31].

In the present study, results showed that MTT assay in comparison to TB assay is a relevant method for determination of drug cytotoxicity effects. MTT assay is easy to handle, rapid, precise and a large number of probes can be assayed in a relatively short time and no radioactivity is necessary [32].

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