[Downloaded free from http://www.advbiores.net on Tuesday, March 28, 2023, IP: 178.173.134.149]

Original Article

Annexin V FITC conjugated as a radiation toxicity indicator in lymphocytes following radiation overexposure in radiotherapy programs

Mohammad Bagher Tavakoli, Majid Kheirollahi[1], Ali Kiani, Mohammad Kazemi[1], Shaghayegh Haghjooy Javanmard[2], Leili Mohebat[3]

Department of Medical Physics, School of Medicine, [1]Department of Genetics, Pediatric Inherited Diseases Research Center, Research Institute for Primordial Prevention of Non-communicable Disease, [2]Departments of Physiology and [3]Oncology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract

Background: Following human radiation exposure in hospital or accidents, dose assessments are of prime importance in radiation accidents. These issues are of continuing importance with respect to socioeconomic policy relating to the industrial and medical uses of ionizing radiation, and also for risk assessment among people who are occupationally exposed to low and/or high linear energy transfer (LET) radiation, such as astronauts, pilots, stewardesses, nuclear power plant workers, and victims of radiation accidents.

Materials and Methods: In this study, an assay for assessing radiation dose based on the induction of apoptosis in human T-lymphocytes was done to examine T-lymphocyte cells isolated from the fresh blood of 16volunteers, cultured and exposed to gamma rays. Radiation-induced apoptosis (RIA) was assessed by flow cytometric identification of cells displaying apoptosis-associated DNA condensation.

Results: Dose-response experiments showed that at 2Gy dose level of radiotherapy programs, the RIA frequency was significantly above control. Apoptotic levels significantly depend on the dose of radiation rather than the donor.

Conclusion: The results demonstrate the potential use of this assay as a biological indicator of radiation toxicity, optimizing patient dose in radiotherapy and biological dosimetry process.

Key Words: Annexin V, apoptosis, flow cytometry, phosphatidylserine, radiotherapy, toxicity

Address for correspondence:
Mr. Ali Kiani, Department of Medical Physics, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran. E-mail: kiani2231@gmail.com
Received: 28.04.2014, **Accepted:** 24.11.2014

INTRODUCTION

Apoptosis is a cell death process characterized by morphological and biochemical features occurring at different stages.[1-6] A critical stage of apoptosis involves the acquisition of surface changes by dying cells that eventually results in the recognition and uptake of these cells by phagocytes. Some changes occurred, such as the expression of thrombospondin binding sites, loss of sialic acid residues, and exposure of a phospholipid-like phosphatidylserine (PS) on the apoptotic cells' membranes. Phospholipids are asymmetrically distributed between inner and outer leaflets of the plasma membrane with phosphatidylcholine and sphingomyelin exposed on the external leaflet of the lipid bilayers, and PS is predominantly observed on

Access this article online	
Quick Response Code:	Website: www.advbiores.net
	DOI: 10.4103/2277-9175.158025

Copyright: © 2015 Tavakoli. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

How to cite this article: Tavakoli MB, Kheirollahi M, Kiani A, Kazemi M, Javanmard SH, Mohebat L. Annexin V FITC conjugated as a radiation toxicity indicator in lymphocytes following radiation overexposure in radiotherapy programs. Adv Biomed Res 2015;4:119.

[Downloaded free from http://www.advbiores.net on Tuesday, March 28, 2023, IP: 178.173.134.149]

the inner surface facing the cytosole.[3-5] Exposure of PS on the external surface of the cell membrane has been reported for activated platelets and senescent erythrocytes. This occurs in the early phases of apoptotic cell death, during which the cell membrane remains intact. This PS exposure may be an early sign of dying cells.[2,3,5] Annexin V, belonging to a family of proteins, with anticoagulant properties has proved to be a useful tool for detecting apoptotic cells, since it preferentially binds to negatively charged phospholipids like PS in the presence of Ca^{2+} and shows minimal binding to phosphatidylcholine and sphingomyeline. Changes in PS asymmetry, which are analyzed by measuring annexin V binding to the cell membrane, are detected before morphological changes associated with apoptosis have occurred and before membrane integrity has been lost. By conjugating fluorescein isothiocyanate (FITC) to annexin V, it is possible to identify and quantize the apoptotic cells, the discrimination of intact cells (FITC-PI-), early apoptotic cells (FITC+PI-), and late apoptotic or necrotic cells (FITC+PI+).[1]

The ideal of radiation therapy is to irradiate the tumor with a dose sufficient to kill all malignant cells while minimizing the damage to surrounding normal tissue. The dose is usually limited by the tolerance of the surrounding normal tissue.[1] To minimize normal tissue toxicity, tolerance dose limits have been established from clinical data and set using population averages.[2] Current radiotherapy guidelines typically limit doses such that the normal tissue late toxicity risk is less than 5% at 5 years post treatment (TD5/5). Although these guidelines limit the number of patients with late effects, these limits do not take into account patients who have normal, sensitive, or radio-resistant tissue. Even with rigid dose tolerance limits, patients respond with different levels of toxicity to a given treatment schedule.[3] The development of a predictive assay would allow patients who are prone to late toxicity to consider the risk-benefit ratio of radiation therapy and perhaps be considered for an alternative type of treatment, such as surgery.[4] Also, in a radiation accident, information on the absorbed dose and its distribution in the body is of great importance for an early assessment of irradiation's consequences for exposed individuals. Cytogenetic analysis of peripheral blood lymphocytes (PBLs) can provide a precise estimation of the radiation dose received.[6-9]

For the radiation doses used in radiotherapy (2-8 Gy) or received in a radiation accident (50-500 mGy), an assay by measuring radiation-induced apoptosis (RIA) has been developed to determine the dose absorbed by the individual tissue. The goal of the present study was the evaluation of this assay's usefulness in the biological dosimetry of radiation accident victims.[7,8] The most thoroughly developed biological indicator of exposure to ionizing radiation currently available is quantification of chromosomal aberrations in peripheral blood lymphocytes.[7,10] *In vivo* or *in vitro* irradiation of blood lymphocytes produces similar yields of chromosome damage per cGy, so that the observed levels of aberrations in exposed persons can be related to an *in vitro*-produced dose-response curve.[11,12] Because of the long life of some lymphocytes, chromosomal aberrations can be detected even years after an accident. Recently, an equally sensitive micronucleus (MN) assay has been suggested as an alternative method for determining radiation exposure.[7-14] This assay is easier and faster than scoring dicentrics and permits the screening of large numbers of cells. Together with the possibilities of computerized image analysis or flow cytometry, this makes the MN assay more attractive for routine procedures.[15,16] Theoretically, it enables resolution of doses smaller than the 50-100 mGy usually quoted as the lower limit for detection by scoring chromosome aberrations.[6] Recently, Boreham *et al*. have demonstrated that apoptosis-associated DNA unwinding in lymphocytes can be used as a potentially sensitive biological dosimeter at these doses.[13,17] We describe here an assay based on the induction of apoptosis in T-lymphocytes following exposure to ionizing radiation. By examining specific T-lymphocyte types, we avoid an error of variance in sensitivity of different cell lines of blood and have an independent measurement with which to confirm the dose.[18] It permits the detection of doses as low as 50mGy days after irradiation when the inter donor variation is greatly reduced. Results suggest that lymphocytes can be effectively used to predict the prognosis of charged particle therapy, too.[14-18]

MATERIALS AND METHODS

Blood sample collection

All experiments were performed on fresh heparinized human blood samples taken from 16 healthy adult donors. Following venipuncture, blood was diluted 1:1in RPMI 1640 medium (Life Technologies, Basel, Switzerland), and peripheral blood lymphocyte counts (PBLCs) were separated by Ficoll density gradient centrifugation using Ficoll Paque Plus (Sigma-Aldrich Gmbh Munich, Germany) [Figure 1]. The cells were incubated at 37°C in a 5% CO_2 atmosphere. The cell suspension was then divided into 10 portions, which were poured into six-well Spiel cell culture plates and irradiated in a tissue-equivalent phantom by a Cobalt-60 radiotherapy unit (Theratron Phoenix, Ottawa Canada) at a dose rate of 82.46cGy/min for four doses in Sayed Al-Shohada Hospitals Radiotherapy Department in Isfahan [Figure 2], and subsequently incubated under standard culture conditions again. Physical dosimetry was performed using a gas chamber

[Downloaded free from http://www.advbiores.net on Tuesday, March 28, 2023, IP: 178.173.134.149]

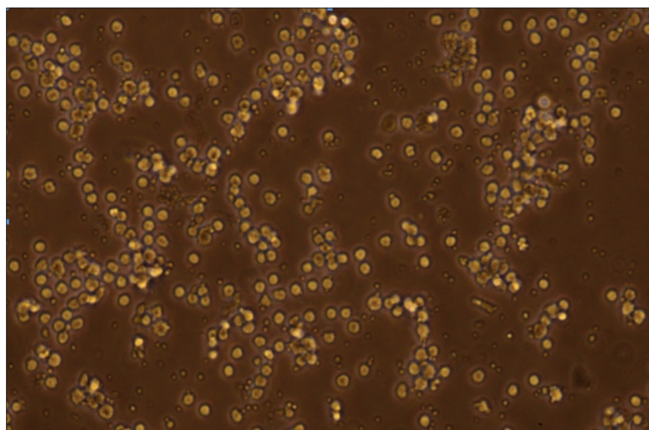

Figure 1: Extracted lymphocyte under dark field vision (×400)

Figure 2: Irradiation of samples in tissue equivalent phantom

dosimeter (Farmer 2570/1, Nuclear Enterprises, Zurich, Switzerland).

After approximately 12 h incubation, the cell suspensions were transferred into 5-ml test tubes and centrifuged at 1500 rpm (320 g) for 5 min at room temperature. The supernatant was aspirated and the cell pellet resuspended in approximately 300 µl of the remaining solution. FITC-conjugated annexin V (Becton-Dickinson, Basel, Switzerland) was added to these cell suspensions. After 15 min incubation at room temperature, 4 ml of BD FACS lysing solution (Becton-Dickinson) diluted 1:10 in double-distilled water was added to the suspension, and the specimens were left for 15 min at room temperature. The cells then centrifuged at 1500 rpm for 5 min, the supernatant was aspirated, and the cells were resuspended in 3 ml phosphate buffer solution (PBS) in order to wash them. Following another round of centrifugation (1500 rpm for 5 min) and aspiration, the cells were resuspended in 0.5 ml of FACS flow phosphate buffer (Becton-Dickinson), to which 5µl of propidium iodide (PI) stock (1 mg/ml in PBS) was added to stain the DNA. The cells were incubated at room temperature for 5 min. The cells were subsequently measured by flow cytometry. Forward and side light scattering and stain-induced fluorescence at two different wavelengths (530 nm, green and 640 nm, red) were simultaneously measured from each cell. The forward scatter (FSC) signal is proportional to the cell size or type, whereas the side scatter (SSC) signal is proportional to cellular granularity (DNA content). Using these two parameters, it was possible to discriminate the three major types of leucocytes (monocytes, granulocytes and lymphocytes) and gate the lymphocytes for acquisition [Figure 3a-d]. Data for 10000 cells were acquired in about 2-3 min. Apoptotic lymphocytes must be included in the cells selected during data acquisition. The apoptotic cells can be distinguished from the normal lymphocytes by their smaller size (FSC) and slightly larger granularity (SSC). However, nonappropriate lymphocytes and debris must first be excluded. Figure 3b shows how this population was identified. The population displaying high cellular DNA content and high FITC positivity contained the normal, FITC-positive cells; the population with high cellular DNA content but low annexin FITC positivity contains other lymphocytes. Below these two clusters, two further populations were observed, both with reduced DNA content: these are the apoptotic cells. The population with the lowest DNA values and low FITC positivity represents the debris. The two-dimensional FSC versus SSC dot plot showed the presence of these apoptotic cells (FITC-positive) within the selection gate [Figure 3a and 3c]. The threshold for data acquisition was set on the fluorescence signal of normal unstained cellular DNA content in order to reduce the contribution of spurious debris signals.

RESULTS

Sample were selected from people aged 24-37 years who were referred to the Iranian Blood Transfusion Organization in Isfahan to donate blood for humanistic proposes. They had no history of radiotherapy, smoking, malignant illness, and continued contact with physical or chemical agents. Their age distribution is listed in Table 1.

Samples were exposed to 0-, 2-, 4-, and 6-Gy doses of gamma rays. IA in T-lymphocytes was examined at next day after irradiation [Figure 2]. The RIA was consistently higher than apoptosis of the nonirradiated control values over the entire experiment. After irradiation of lymphocytes, three different populations of PBLs were observed [Figure 3c and d]: i) early apoptotic cells identified with annexin V, ii) late apoptotic cells stained with PI, and iii) non apoptotic cells [Figure 3b and b]. RIA may be defined as the

[Downloaded free from http://www.advbiores.net on Tuesday, March 28, 2023, IP: 178.173.134.149]

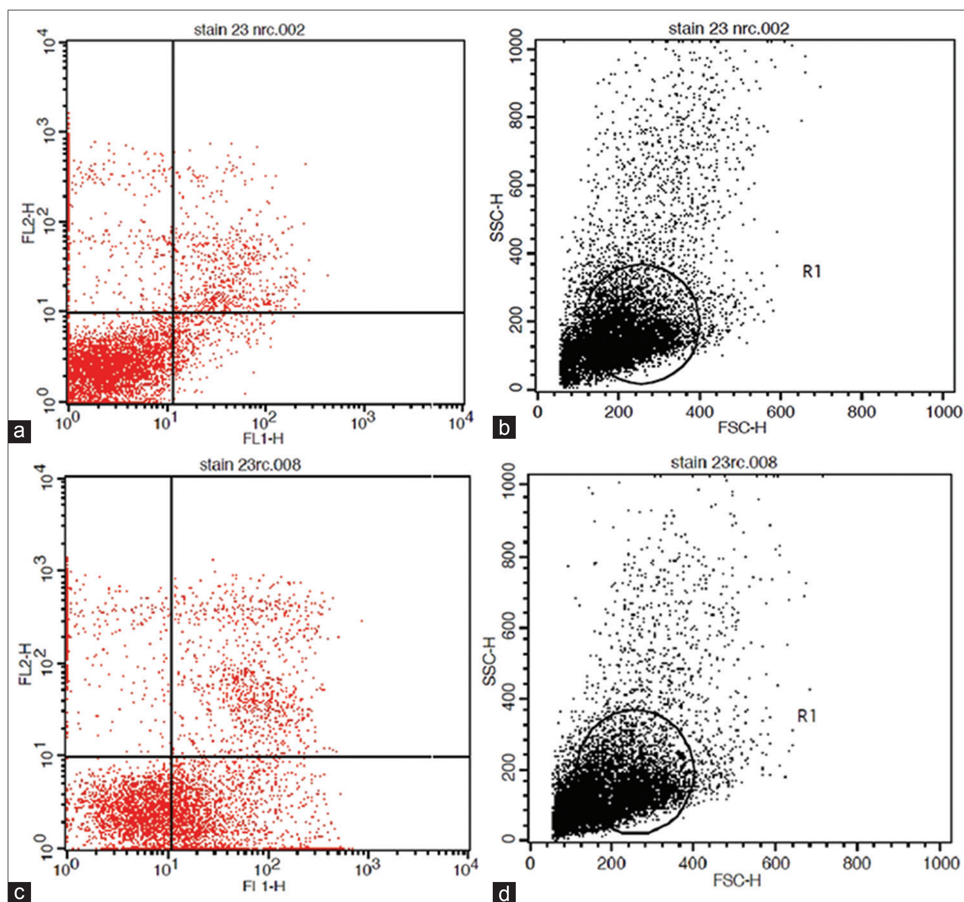

Figure 3: (a) Two parameter dot plot Pi/FITC fluorescent for non irradiated cells (b) Gated zone for appropriate non irradiated cells (c) Two parameter dot plot Pi/FITC fluorescent for irradiated cells (d) Gated zone for appropriate irradiated cells

Table 1: Age distribution of samples and related average and standard deviation

Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	AVA*	STDE**
Age	31	33	31	27	37	27	31	33	27	35	23	25	34	26	24	24	29.9	4.40

*Average, **Standard deviation. AVA: Average, STDE: Standard deviation

Table 2: Excreted dose and related average max and min of apoptosis

Dose (Gy)	0	2	4	6
Average-apoptose	4.81	17.44	26.31	35.31
MAX	8	28	34	47
MIN	0	9	17	20
STDE	1.91	6.78	4.57	7.46

STDE: Standard deviation

percentage of total PBL death induced by the radiation dose minus the spontaneous cell death in control (0 Gy). Table 2 shows the results of apoptosis assessment after the irradiation of cultured cells in the experiment.

To study the relation of apoptosis rate to the age of samples, their correlation for the exerted doses of gamma irradiations assessed. The correlation coefficient was <0.2 for all exerted doses. This suggests that apoptosis is not related to the ages of the samples and can be used as indicator of radiation dose. The result of measuring RIA is indicated graphically in Figure 4.

RIA values increased with radiation dose (0, 1, 2, and 6 Gy), and this can be fitted to a linear equation as follows: RIA = βD (Gy) + α. Alpha (α) is defined as background rate of apoptosis and Beta (β) as the apoptosis rate for unit dose of radiation ($\beta = \Delta RIA/\Delta D$ [Gy]). It seems that β represents an individual marker of radio sensitivity. In this manner $\beta = 5.02$ and $\alpha = 5.9$, and RIA = 5.02D + 5.9. RIA increased significantly with radiation dose and incubation time. The α value rose with the incubation time ($P < 0.001$) while the β value did not.

DISCUSSION

In the current study, we measured the stability of PBLs with regard to PS presentation on their

[Downloaded free from http://www.advbiores.net on Tuesday, March 28, 2023, IP: 178.173.134.149]

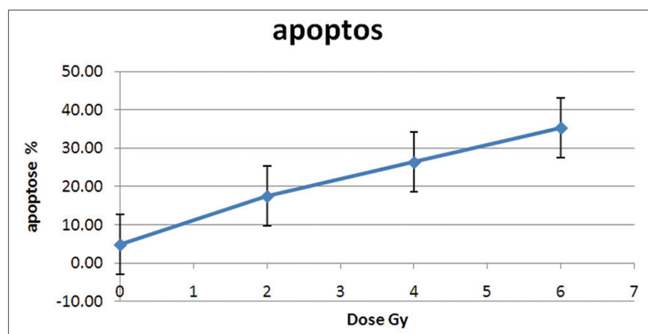

Figure 4: Exerted radiation dose and related apoptosis of PBLCs shown graphically

surface as measured by annexin-V binding FITC. It is a well-established marker for apoptosis and a potentially attractive biomarker for identifying radio toxicity patients in radiotherapy programs and radiation accidents.[1,3]

Annexin V binding to PBLs increases as samples stored before analysis so the apoptosis results increase[4]. It observed that time delay after irradiation and before flow cytometry positively affects the result of the annexin assay. It was noted by researchers that first-order RIA strongly changes with relay time of storage too[8]. Its protocol-dependent and relation of the relay time of cell storage in binding buffer and results makesthe reliability of annexin V-assay as a test for the measurement of radiation toxicitylate after an accident questionable. So this assay includes easily acquired clinical variables that may be of significant use in designing toxicity-based clinical trials in which time dependent phenomena may be affected by unpredictable patient time of arrival and variation in processing.

This work is important first, it quantitatively underscores fundamental differences between the annexin and other radiobiological tests such as dicentric and MN assay second to timing of patient enrollment. For research involving only clinical measurements or biochemical markers of illness that can be easily stored for later analysis, the variability in time of arrival is an inconvenience. However, when samples must be analyzed "emergently," the unpredictability of patient arrival can be expected to directly impact the observed utility of that measurement. In the case of annexin V as a marker of lymphocyte radiation induced apoptosis, sample degradation imposed by the demands of sample storage and the presumed limited availability of a flow cytometer would likely reduce the observed utility of a radio toxicity patients.

The negative predictive value of this approach appears high in specific subpopulations of lymphocytes (95-100%). Therefore, this tool may hold potential for physicians to identify suitable patients for dose escalation trials and to spare patients at risk for late radiation toxicity when a surgical option such as prostatectomy is available.[5]

CONCLUSION

Although the findings from this study hold promise, they require further study for several reasons. Validation of these results using a second, independent cohort is required to ensure reproducibility of these findings. This would also allow collection of potential confounders not included in the current study, such as dose variation of organs at risk. Ultimately such an assay could be tested for its utility by measuring lymphocyte sensitivity in patients before treatment decisions and by documenting whether the results had an impact on patient management and, more importantly, a reduction in long-term toxicity.

ACKNOWLEDGMENT

We thank the staff at the Central Research Lab of Isfahan University of Medical Sciences for assistance and good cooperation. The authors gratefully acknowledge the other contributor.

REFERENCES

1. vanEngeland 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.
2. Bordón E, Henríquez Hernández LA, Lara PC, Pinar B, Fontes F, Rodríguez Gallego C, *et al.* Prediction of clinical toxicity in localized cervical carcinoma by radio-induced apoptosis study in peripheral blood lymphocytes (PBLs). Radiat Oncol 2009;4:58.
3. Ilyenko I, Lyaskivska O, Bazyka D. Analysis of relative telomere length and apoptosis in humans exposed to ionising radiation. Exp Oncol 2011;33:235-8.
4. Hooker DJ, Mobarok M, Anderson JL, Rajasuriar R, Gray LR, Ellett AM, *et al.* A new way of measuring apoptosis by absolute quantitation of inter-nucleosomally fragmented genomic DNA. Nucleic Acids Res 2012;40:e113.
5. Bordón E, Henríquez-Hernández LA, Lara PC, Ruíz A, Pinar B, Rodríguez-Gallego C, *et al.* Prediction of clinical toxicity in locally advanced head and neck cancer patients by radio-induced apoptosis in peripheral blood lymphocytes (PBLs). Radiat Oncol 2010;5:4.
6. Kakizaki T, Hamada N, Sakashita T, Wada S, Hara T, Funayama T, *et al.* Vulnerability of feline T-lymphocytes to charged particles. J Vet Med Sci 2007;69:605-9.
7. International Atomic Energy Agency (IAEA). Handling of radiation accidents. In: Dolphin GW, editor. Biological Dosimetry with Particular References to Chromosome Aberration Analysis: A Review of Methods. Vienna, Austria:; 1969. p. 215-24.
8. Menz R, Andres R, Larsson B, Ozsahin M, Trott K, Crompton NE. Biological dosimetry: The potential use of radiation-induced apoptosis in human T-lymphocytes. Radiat Environ Biophys 1997;36:175-81.
9. Hosokawa Y, Sakakura Y, Tanaka L, Okumura K, Yajima T, Kaneko M. Radiation induced apoptosis is independent of caspase-8 but dependent on cytochrome C and the caspase-9cascade in human leukemia HL60 cells. J Radiat Res 2005;46:293-303.
10. Amaral A. Trends in biological dosimetry: An overview. Braz Arch Biol Technol 2002;45:119-24.
11. Pinar B, Henríquez-Hernández LA, Lara PC, Bordon E, Rodriguez-Gallego C,

[Downloaded free from http://www.advbiores.net on Tuesday, March 28, 2023, IP: 178.173.134.149]

Lloret M, *et al.* Radiation induced apoptosis and initial DNA damage are inversely related in locally advanced breast cancer patients. Radiat Oncol 2010;5:85.

12. Hodge S, Hodge G, Holmes M, Reynolds PN. Increased peripheral blood T-cell apoptosis and decreased Bcl-2 in chronic obstructive pulmonary disease. Immunol Cell Biol 2005;83:160-6.
13. Schnarr K, Boreham D, Sathya J, Julian J, Dayes IS. Radiation-induced lymphocyte apoptosis to predict radiation therapy late toxicity in prostate cancer patients. Int J Radiat Oncol Biol Phys 2009;74:1424-30.
14. Ishihara S, Iinuma H, Fukushima Y, Akahane T, Horiuchi A, Shimada R, *et al.* Radiation-induced apoptosis of peripheral blood lymphocytes is correlated with histological regression of rectal cancer in response to preoperative chemoradiotherapy. Ann Surg Oncol 2012;19:1192-8.
15. Prieto A, Díaz D, Barcenilla H, García-Suárez J, Reyes E, Monserrat J, *et al.* Apoptotic rate: A new indicator for the quantification of the incidence of apoptosis in cell cultures. Cytometry 2002;48:185-93.
16. Zhang HM, Nie JS, Li X, Niu Q. Characteristic analysis of peripheral blood mononuclear cell apoptosis in coke oven workers. J Occup Health 2012;54:44-50.
17. Zhang G, Gurtu V, Kain SR, Yan G. Early detection of apoptosis using a fluorescent conjugate of annexin V. Biotechniques 1997;23:525-31.
18. Hingorani R, Deng J, Elia J, McIntyre C, Mittar D. Detection of Apoptosis Using the BD Annexin V FITC Assay on the BD FACS Verse System: Flow cytometry base protocol. USA, San Diego, CA: BD Biosciences; 2011. p. 1-12.

Source of Support: This study is funded by Isfahan University of Medical Science, **Conflict of Interest:** None declared.