EFFECTS OF X AND GAMMA RAYS ON HUMAN LYMPHOCYTES

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Abstract. The purpose of this study is to investigate the effects of different doses and dose-rates of X- and gamma rays on human lymphocytes. After separating mononuclear cells with lymphocytes from human peripheral blood, they were irradiated, either with X-ray doses (0.25, 0.5, 1, 2 and 4 Gy) at a dose rate of 0.28 Gy/min or with doses (15.65, 31.25, 62.5, 125 and 250 mGy) at dose rate of 20 mGy/min or with ^{60}Co gamma ray doses (0.25, 0.5, 1, 2 and 4 Gy) at dose rate of 33 mGy/min. Using flow cytomtery the amount and percentage of apoptotic and lymphocytes were measured. In contrast to low X-ray doses with low dose rate, induction of apoptosis was significant at high X-ray doses with high dose rate. Moreover, apoptosis was significant at high gamma ray doses at low dose rate. We conclude that apoptosis is a good biological marker for radiation response, and the induction of apoptosis depends on dose, and not, on dose rate.

Keywords: X-ray, gamma ray, apoptosis, flow cytometry.

INTRODUCTION

When cells are exposed to ionizing radiation, they respond in a variety of ways that differ quantitatively and qualitatively according to the absorbed dose and the cell type. That generally reflects damage caused to well defined cellular components and molecular structures. Apoptosis represents highly complex response to ionizing radiation since it requires an active participation of the cell in its own death. Its occurrence depends on a series of parameters, including the intrinsic radiosensitivity of the cell, the extent of molecular damage and the capacity of the cells to repair it, and the microenvironment within the tissues or organs. Lymphocytes are particularly susceptible to DNA damage-induced apoptosis, a suicide response adapted to their high potential for mutation and clonal expansion [9].

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According to the absorbed dose and cell type, when cells are exposed to ionizing radiation, they respond in a variety of ways function of the radiation-induced cellular damages [9]. Indeed, after radiation-induced cellular damages, cells might be, either, completely repaired, or partially repaired and then might induce mutation, or, might come in the apoptosis processes. Apoptosis represents an extreme and highly complex response, in particular, involved in the response to ionizing radiation since it requires an active participation of the cell in its own death [9].

Apoptosis is the most common form of eukaryotic cell death [1]. It is essential in many physiological processes, including the embryonic development and the maturation of the immune system [21]. Apoptosis is morphologically characterized by increased cytoplasmic granularity, cell shrinkage, chromatin condensation, membrane blebbing, and the formation of distinctive nuclear bodies [1, 24], as shown in Fig. 1. Although apoptosis can occur spontaneously, it can be also induced by various physiological conditions and external stimuli such as ionizing radiation. It has been shown that tumor cells are susceptible to death by apoptosis in response to drugs and /or radiation treatment [1].

The interest for using apoptosis as a possible measure of radiosensitivity has increased substantially both with regard to the possibilities of using the extent of apoptosis as a biological dosimeter [3] and for estimating the radiosensitivity of cancer cells before radiotherapy [8, 10, 20]. The status and level of expression of proteins that regulate apoptosis have even been proposed to serve as radiation exposure indicators or sensors [1, 19, 23]. Two distinct but interconnected apoptotic pathways have been characterized, the cell surface death receptor pathway (extrinsic) and the mitochondria initated (intrinsic) pathway. Both pathways terminate in activation of effector caspases, which mediate the proteolytic events characterizing apoptosis [6, 26, 27].

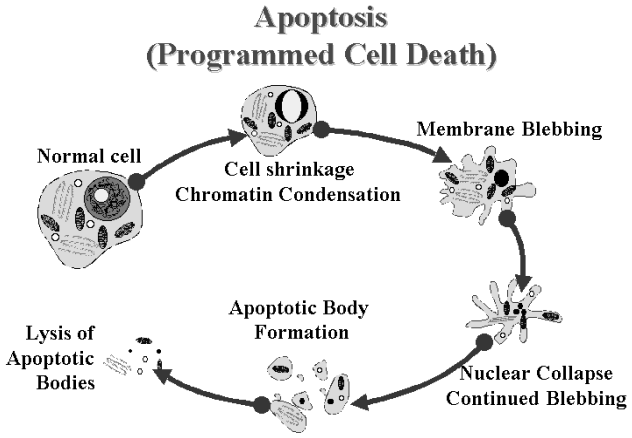

Fig. 1. Apoptosis (programmed cell death [5]).

The majority of hematopoietic cells die by apoptosis after exposure to ionizing radiation [13]. Numerous caspases are known to play a pivotal role in the execution of apoptosis, like caspase 3. The caspase 3 is generally accepted to be a key protease that is activated during the intr- and extr-insic pathways in apoptotic process [4], respectively, after activation of caspase 8 and caspase 9. In this work apoptotic cells were indicated by the detection of activated caspase 3 using CaspGLOWTM fluorescein active caspase-3 staining kit.

In this study we investigated the effects of different doses and dose-rates of X- and gamma rays on human lymphocytes by rapid detecting apoptosis in the cells using flow cytometry.

MATERIALS AND METHODS

SEPARATION OF MONONUCLEAR CELLS

Blood samples were collected from healthy volunteers in heparinized tubes. The PBMCs were isolated through the density gradient centrifugation. The blood was layered onto Histopaque 1077 (Sigma, Germany) and the PBMC monolayer was recovered [11, 25]. The cells were washed twice with 10 mL of PBS. The PBMCs were resuspended in RPMI 1640+Guloamax TMI medium, supplemented with FBS (20% v/v), penicillin (100 IU/mL) and streptomycin (100 µg/mL) and were distributed into 25 cm^3 culture flasks at a concentration of ($5–10 \times 10^6$) cells/mL.

X-IRRADIATION

X-ray irradiation of the cells was performed with a Pantak HF420 RX machine operating at 250 kV (the mean energy was 208 keV), 1 mm Cu filtering, 1 mA with a dose rate of 20 mGy/min, or 15 mA with a dose rate of 0.28 Gy/min. Dosimetry was performed on a regular basis with a 0.6 cm^3 ionizing chamber (NE 2571), which was connected to a dosimeter (Farmer dosimeter 2570). The chamber was placed in parallel to the irradiated cell flasks. Dose homogeneity was evaluated as being <1.5%.

Cells from each donor were irradiated with 0.25, 0.5, 1, 2, and 4 Gy with a dose rate of 0.28 Gy/min, or with 15.625, 31.25, 62.5, 125 and 250 mGy with a dose rate of 20 mGy/min then incubated for 24 hrs at 37 ºC in 5% CO_2.

^{60}Co GAMMA IRRADIATION

Gamma irradiation of the cells was performed using ^{60}Co of 1.25 MeV mean energy at a dose rate 33 mGy/min for doses (0.25, 0.5, 1, 2 and 4) Gy. Fig. 2 shows

the γ source used in the present work. Its active diameter is d = 15 mm, outer diameter is D = 18.2 mm and height is I = 36.8 mm.

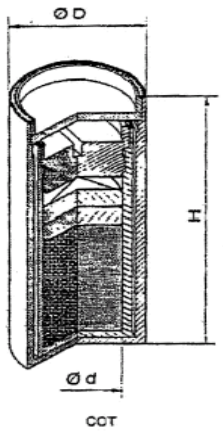

Fig. 2. ^{60}Co gamma ray source used in the present work.

STATISTICAL ANALYSIS

Results were always represented as mean±standard error of the mean (SEM) of four independent experiments with triplicate measurements. The t-test was performed to compare between the control and the irradiated samples. Values of $p < 0.05$ were considered significant, and those of $p < 0.001$ reflect high significance.

RESULTS

Caspase proteases which have essential role in apoptosis are: initiators (caspase – 2, 8, 9 and 10) and excutioners (caspase – 3, 6 and 7). All initiator and excutioner caspases have either a direct or an indirect role in the processing, propagation and amplification of apoptotic signals that results in the destruction of cellular structures [27]. In mammals, caspases, principally caspase 3, appear to be activated in a protease cascade that leads to inappropriate activation or rapid disablement of key structural protiens and important signaling, homeostatic and repair enzymes. Caspase 3 is a frequently activated apoptotic death protease. It has been revealed that caspase 3 plays a key role for some of the characteristic changes in cell morphology and certain biochemical events associated with the execution and completion of apoptosis [18]. Fluorescence intensity which is proportional to the amount of antibody bound per cell is proportional to the activation of caspase 3, as also recorded by Maher *et al.* [12]. The fluorescence intensity of activated caspase 3 indicates the amounts of apoptosis with dose. We observed a significant

increase at all doses of X-ray with high dose rate (0.28 Gy/min). Fig. 3 shows a dose related increase of fluorescence in apoptotic cells after normalization for 4 donors.

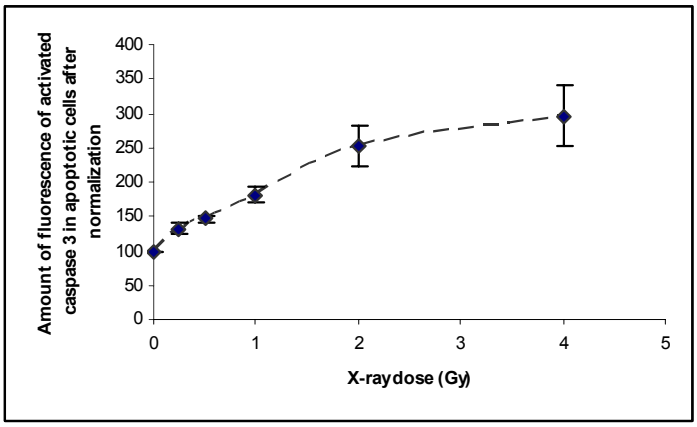

Fig. 3. Variation of amount of fluorescence of activated caspase 3 with X-ray dose in apoptotic cells after normalization for the mean of all donors. Dose rate = 0.28 Gy/min.

The effect of γ-ray doses 0, 0.25, 0.5, 1, 2, 4 Gy at dose rate 33 mGy/min on the amount of fluorescence of activated caspase 3 in apoptotic is shown in Fig. 4. The amount of fluorescence of activated caspase 3 in apoptotic cells is dose dependent. A significant difference is observed between control and all doses**.**

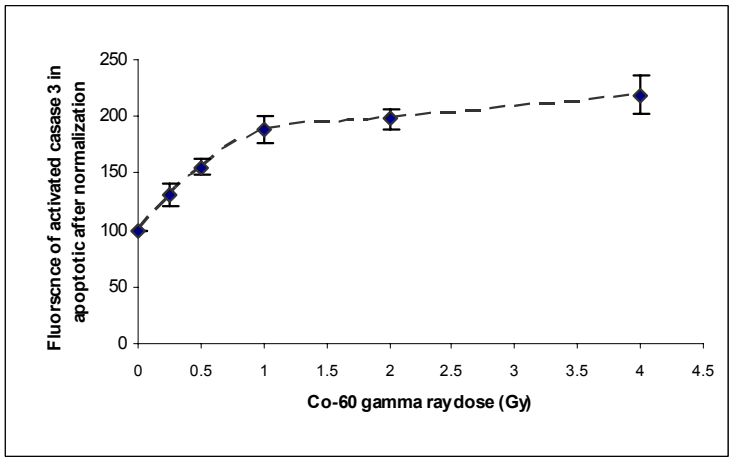

Fig. 4. Variation of ^{60}Co gamma ray dose with amount of fluorescence activated caspase 3 in apoptotic cells after normalization for the mean of all donors. Dose rate = 33 mGy/min.

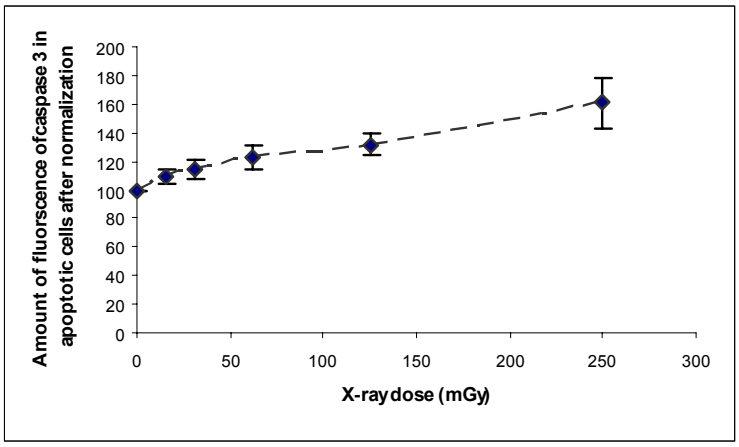

Fig. 5**.** Variation of amount of fluorescence of activated caspase 3 with X-ray dose in apoptotic cells after normalization for the mean of all donors. Dose rate = 20 mGy/min.

Effect of X-ray doses 0, 15.625, 31.25, 62.5, 125, 250 mGy at dose rate of 20 mGy/min on the amount of fluorescence of activated caspase 3 in apoptotic cells is shown in Fig. 5. The amount of fluorescence in apoptotic cells increases linearly with the dose**.**

For the donors at dose rate 0.28 Gy/min the mean of the percentage of apoptotic and lymphocytes cells with dose is sketched in Figs. 6 and 7, respectively.

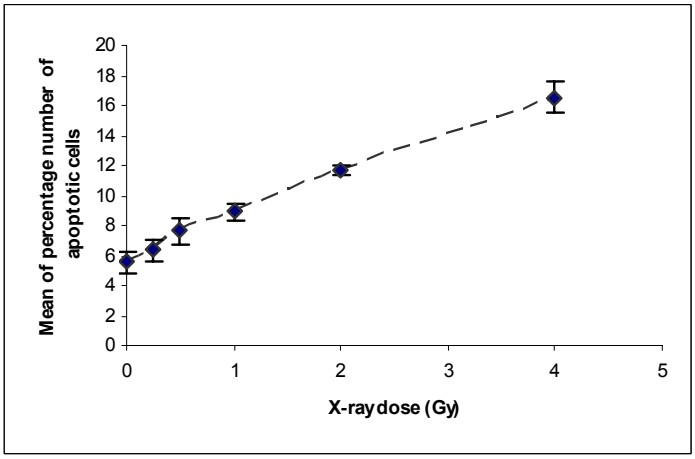

Fig. 6**.** Variation of mean of percentage of apoptotic cells with X-ray dose. The results represent mean±SEM for four independent experiments. Dose rate = 0.28 Gy/min.

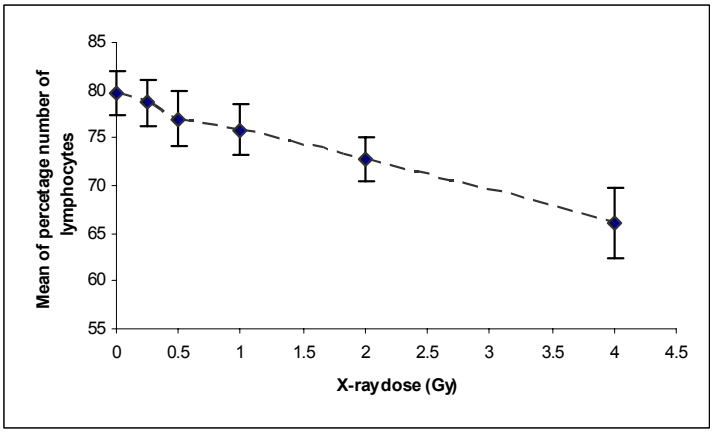

Fig. 7**.** Variation of mean of percentage of lymphocyte cells with X-ray dose The results represent mean±SEM for four independent experiments. Dose rate = 0.28 Gy/min.

At high doses of X-ray with high dose rate, an increase in the mean of the percentage of apoptotic cells was observed to be dose dependent and significant at 1, 2, 4 Gy. The present results agree well with others, like those of Payne *et al*. which observed that irradiation of human lymphocytes with X-ray doses (0–20 Gy) induced apoptosis [16]. A significant difference was observed at 2.5–5 Gy. Irradiation of CD4+ and CD8+ T-lymphocytes with X-ray doses (0–1.5 Gy) was done by (Wilkins *et al*.), the dose response curves were linear up to 0.5 Gy thereafter the response showed a plateau [23]. Also Holl *et al*. showed a dose dependent relation between doses and apoptotic signals in spleen cells after irradiation of mice with X-ray doses (0.2–4 Gy) [9]. A significant dose dependent induction of apoptosis was also observed by Boreham *et al*. [3] after irradiation of human lymphocytes irradiated with X-ray at doses 0, 0.2, 0.4, 0.6, 0.8, 1 Gy. Our results also showed a decrease of the percentage of lymphocytes with dose but they were significant only at 4 Gy. The results agree with that of Nomura *et al*. [15] as they showed that irradiation of X-ray doses (0–2.5 Gy) to different types of mice strains results in survival rate of white blood cells and splenic lymphocytes that decreased with increasing dose .

For low doses and low dose rate of X-ray the increase of percentage of apoptotic cells and the decrease of percentage lymphocytes were not always regular and insignificant, the results being shown in Figs. 8 and 9.

For low doses of X-ray with low dose rate the percentage of apoptotic cells was increased linearly but with no significance. This can be due to the repairing of most of the damage occurring at low doses. However, the effect of low dose rate on apoptosis was not clear as compared with the results of Meijer *et al*. [14].

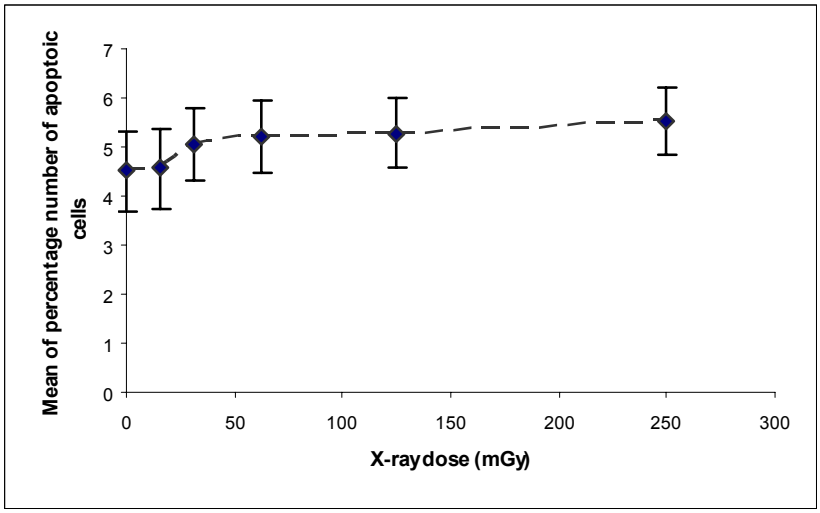

Fig. 8. Variation of mean of percentage of apoptotic cells with X-ray dose for all donors. Dose rate = 20 mGy/min.

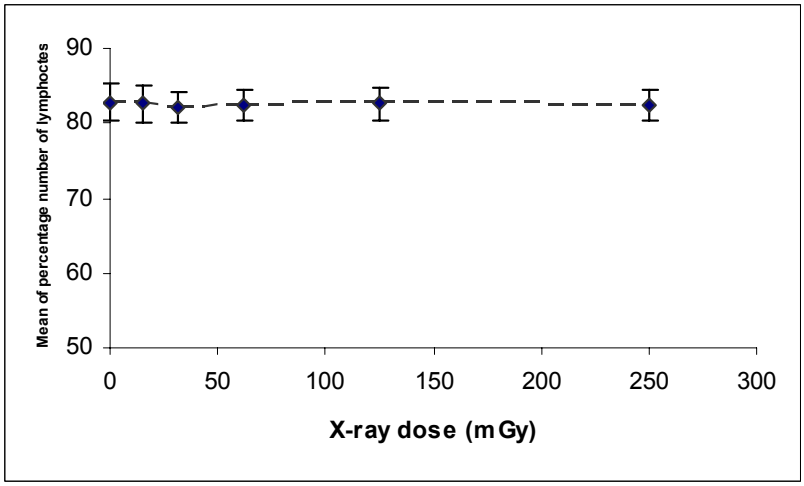

Fig. 9. Variation of mean of percentage of lymphocytes cells with X-ray dose for all donors. Dose rate = 20 mGy/min.

For ^{60}Co gamma ray doses the mean percentages of apoptotic cells were 4.97%, 5.76%, 6.71%, 8.16%, 10.95% and 14.16% for 0, 0.25, 0.5, 1, 2 and 4 Gy, respectively. The increase of apoptotic cells showed dose dependent and significant for all doses excepting 0.25 Gy. The variation of the mean of percentage of apoptotic and lymphocyte cells with the dose is shown in Figs. 10 and 11, respectively.

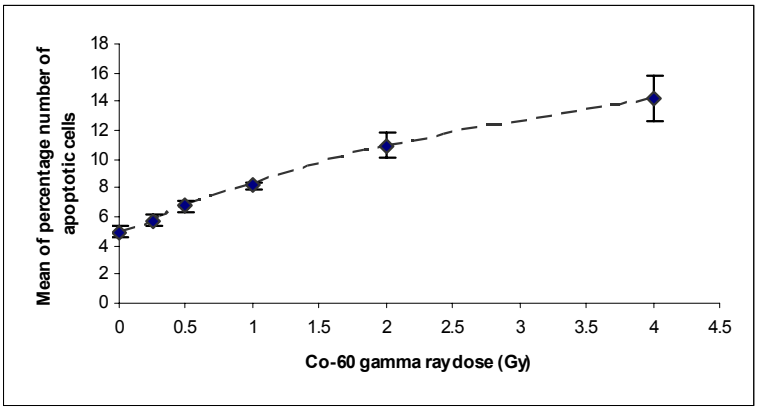

Fig. 10. Variation of mean of percentage apoptotic cells with ^{60}Co gamma ray dose. Dose rate = 33 mGy/min.

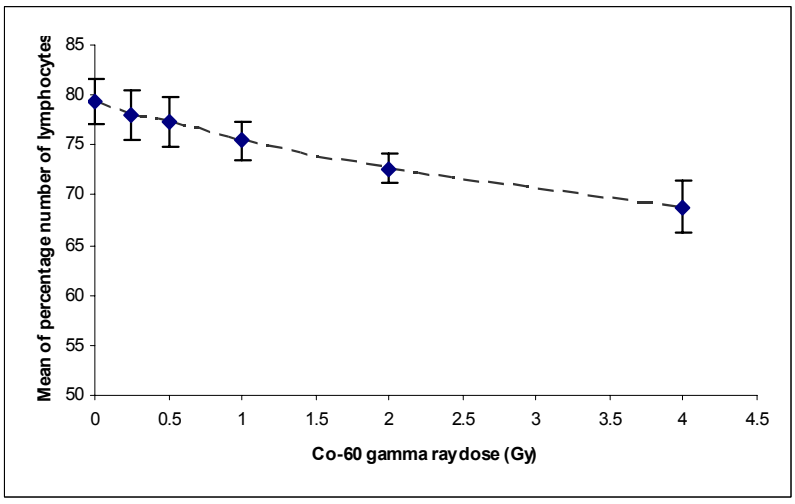

Fig. 11. Variation of mean of percentage lymphocyte cells with ^{60}Co gamma ray dose. Dose rate = 33 mGy/min.

With gamma ray doses at low dose rates, the increase of apoptotic cells was observed to be dose dependent and significant for all doses except for that of 0.25 Gy. This property of dose dependence was also observed by Belyaev *et al*. [2], Liegler *et al*. [11], Vral *et al*. [22], and Wilkins *et al*. [23]. A dose dependent decrease of percentage lymphocytes was also observed in our results. It is dose dependent. It is significant at 2 and 4 Gy, which agrees well with Liegler *et al*. [11]. Also they found that by increasing the radiation dose and length of the post irradiation period, the number of peripheral lymphocytes is decreased.

FITTING EQUATION

To describe the dependence of apoptosis induction on dose and dose rate we suggested a fitting equation of the form:

$$Y = a\,D^{b} + c \tag{1}$$

where: Y = the percentage of apoptotic cells; a = the amplitude parameter; D = the radiation dose; b = a shape parameter; c = a constant, which represents the background of the variable Y.

The fitting parameters a, b and c of our equation for percentage of apoptotic cells are given in Table 1. From the value of a, the percentage of apoptotic cells induced by high X-ray dose with high dose rate (0.28 Gy/min) was the same as that at high ^{60}Co gamma ray doses with dose rate of 33 mGy/min. At low X-ray doses with low dose rate of 20 mGy/min, the percentage of apoptosis was very low in comparison with the other two experiments. Figs. 12, 13, and 14 show the fitting curves for the three experiments.

Table 1

The fitting parameters in our model for the percentage number of apoptotic cells for three experiments

Type of irradiation	Parameter a ± std error	Parameter b ± std error	Parameter c ± std error	R^2
High X-ray dose with dose rate 0.28 Gy/min	3.44 ± 0.18	0.84 ± 0.03	5.52 ± 0.14	0.999
Low dose of X-ray with dose rate 20 mGy/min	0.11± 0.12	0.41± 0.18	4.47 ± 0.18	0.880
High dose gamma ray with dose rate 33 mGy/min	3.44 ± 0.40	0.74 ± 0.07	4.78 ± 0.31	0.994

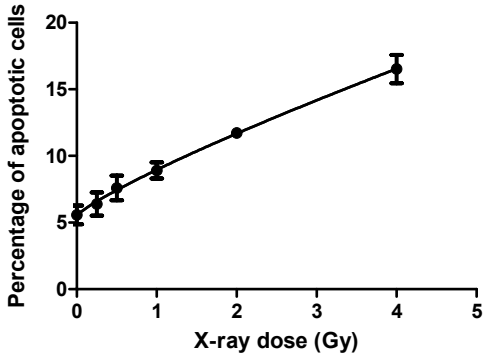

Fig. 12**.** Dose response curve for high doses of X-ray at 0.28 Gy/min dose rate with apoptotic cells.

From the value of *a* parameter, the percentage of apoptotic cells induced by high X-ray dose with high dose rate (0.28 Gy/min) was the same as that at high ^{60}Co gamma ray doses with dose rate of 33 mGy/min. At low X-ray doses with low dose rate of 20 mGy/min, the percentage of apoptosis was very low in comparison with the other two experiments; this might indicate at this range of low doses that irradiation may induces another type of lesions other than apoptosis, like DNA damage.

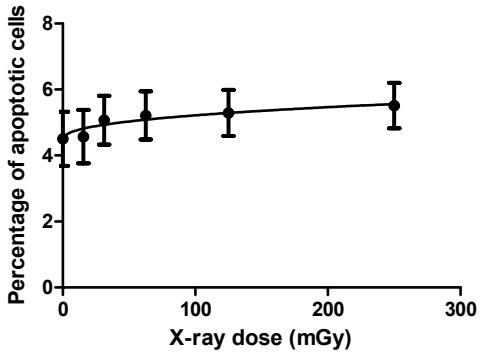

Fig. 13**.** Dose response curve for low doses of X-ray at 20 mGy/min dose rate with percentage of apoptotic cells.

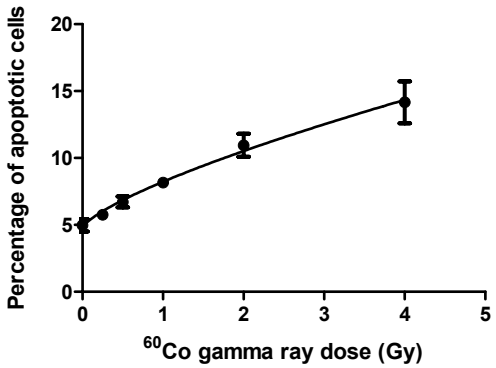

Fig. 14. Dose response curve for low doses of ^{60}Co gamma ray with dose rate of 33 mGy/min with percentage of apoptotic cells.

From the value of *a* which is shown in Table 1, it was noticed that the percentages of apoptotic cells induced by X-ray with high dose rate (0.28 Gy/min) and ^{60}Co gamma rays with low dose rate (33 mGy/min) are dose rate independent. This agrees with Fujikawa *et al*. [7] and Vral *et al*. [22] in which they concluded that the ability of lymphocytes to undergo apoptosis is independent of dose rate. Pecaut *et al*. [17] observed that the whole body irradiation of C57BL/6 mice with gamma radiation (0, 0.5, 1.5 and 3 Gy) at a dose rate 1 cGy/min and 80 cGy/min changed

the number of leukocytes and lymphocytes in lymphoid and organs and these were highly dependent on the dose, but not dose rate. A simple hypothesis concerning apoptosis dose-rate independence after irradiation is as follows: in lymphocytes, apoptosis occurs so rapidly after the production of DSBs by radiation that most cells with DSBs undergo apoptosis before the DSBs are repaired. The recombinational repair of DSBs in mammalian cells occurs only after cells with DSBs enter the S phase, because the Rad51 protein, indispensable for this repair, is synthesized in the S-G2 phases and degraded in the M phases [7].

From the present results, one can conclude that apoptosis is a good biological marker for radiation response, and induction of apoptosis depends on the dose, and not, on the dose rate.

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