ANALYSIS OF THE APOPTOTIC EFFECT OF ULTRAHIGH GAMMA DOSE RATES ON HUMAN PERIPHERAL BLOOD LYMPHOCYTES *IN VITRO*

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Relative biological effectiveness of ionizing radiation is determined by a number of factors, including a dose rate. Radiotherapy equipment employs low dose rates of up to a few Gy per minute. But very little is known about the biological effect of high and ultrahigh (≥ 10^8 Gy/min) dose rate radiation. Our study aimed to investigate the apoptotic effect of ultrahigh gamma dose rates on human peripheral blood lymphocytes. Blood samples were collected from seemingly healthy donors. Lymphocytes were isolated by density gradient separation. Lymphocyte suspensions were irradiated with low-rate doses on the Rokus-AM gamma-ray machine for clinical use (Russia) and with 10^8 Gy/s doses on the experimental pulse generators Angara-5-1 and Mir-M (Russia). Apoptosis was measured by flow cytometry using annexin V and propidium iodide double staining. We established that in comparison with low dose rates, ultrahigh gamma dose rates (with doses ranging from 1 to 6 Gy) induced significantly more pronounced apoptosis in peripheral blood lymphocytes ($p < 0.05$) with fewer necrotic cells. Total radiation-induced cell death did not differ significantly between the therapeutic gamma machine and the experimental pulse generators. Further research is needed to assess biological and medical significance of our findings.

Keywords: ultrahigh dose rate gamma radiation, ultrahigh intensity X-rays, dose rate, cell death, apoptosis, necrosis, lymphocytes

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АНАЛИЗ ИНДУКЦИИ АПОПТОЗА ЛИМФОЦИТОВ ПЕРИФЕРИЧЕСКОЙ КРОВИ ЧЕЛОВЕКА СВЕРХИНТЕНСИВНЫМ ГАММА-ИЗЛУЧЕНИЕМ *IN VITRO*

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Относительная биологическая эффективность ионизирующего излучения определяется рядом параметров, одним из которых является мощность дозы. В терапевтических лучевых установках используется облучение с мощностью дозы до нескольких Гр/мин. Эффект высоких и особенно сверхвысоких (10^8 Гр/мин и выше) мощностей дозы практически не изучен. Целью нашего исследования являлось определение влияния гамма-излучения, имеющего сверхвысокую мощность дозы, на индукцию апоптоза в лимфоцитах периферической крови человека. Лимфоциты получали из крови условно здоровых добровольцев выделением их на градиенте плотности. Образцы суспензии лимфоцитов при низкой мощности дозы облучали на установке «Рокус-АМ» (Россия), при мощности дозы около 10^8 Гр/с — на экспериментальных установках «Ангара-5-1» и «Мир-М» (Россия). Уровень апоптоза регистрировали методом проточной цитофлуориметрии с двойной окраской аннексином V и йодидом пропидия. Установили, что гамма-излучение со сверхвысокой мощностью дозы в диапазоне доз 1–6 Гр индуцирует апоптоз в лимфоцитах периферической крови достоверно выше, чем гамма-излучение с низкой мощностью дозы ($p < 0,05$), одновременно в меньшей степени индуцируя некроз. При этом общий уровень радиационной гибели лимфоцитов для терапетивческой и экспериментальных установок достоверно не различался. Дальнейшие исследования позволят уточнить биологическую и медицинскую значимость полученных результатов.

Ключевые слова: сверхинтенсивное гамма-излучение, сверхинтенсивное рентгеновское излучение, мощность дозы, клеточная гибель, апоптоз, некроз, лимфоциты

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Success of radiation therapy in patients with malignant tumors is determined by a number of biologic effects it induces, including cell death. Ionizing radiation causes molecular damage to cell organelles and compartments and has a particularly detrimental effect on the DNA structure. DNA damage either results in cell death or is repaired and cells subsequently recover their function. However, if mechanisms of DNA repair are affected by radiation, there is a risk of mutations and cancer formation [1].

There are a few types of cell death that can be induced by ionizing radiation [2, 3]. Of particular interest here is apoptosis, the programmed cell death that spares the surrounding healthy tissue, does not trigger inflammation and can be enhanced by radiation [4]. Certain cell types are resistant to radiation-induced apoptosis; others can modify their radiosensitivity depending on the stage of the cell cycle or microenvironment [5]. A lot of research is ongoing in the field aiming to find methods of control over apoptosis occurring in some cell types in response to radiation or chemotherapy in patients with malignant tumors [6, 7].

It has been proved that although many anticancer drugs promote apoptosis in tumors, they are not selective, which means they also attack healthy tissue [8]. Radiation therapy also has its downsides, including formation of necrotic lesions in the tumor and damage to the surrounding healthy cells. There is evidence that ionizing radiation stimulates apoptosis of lymphocytes, thymocytes and different precursor cells. Mature differentiated cells rarely become apoptotic in response to radiation but some authors report radiation-induced apoptosis in human breast cells, intestinal crypts and tonsils. In the experiment [9] apoptosis has been induced in mouse eggs and hepatocellular carcinomas [9].

High-current pulse electron accelerators capable of generating high-intensity bremsstrahlung radiation are employed by research studies aimed at developing new radiation technologies. It has been experimentally proved that these machines do have their drawbacks limiting their use in clinical practice, including unstable characteristics from pulse to pulse. At the same time, narrower electron beams are believed to be more sparing to healthy tissue, while high dose rates are expected to cause more profound damage to target areas [10]. Gamma machines used in clinical routine generate a therapeutic dose rate of 1.5–2 Gy/min allowing absorption of 100 quanta per second by a 10 μm cell at 100 keV energies. Intervals between bursts are as short as 1 to 10 ms, but cell repair mechanisms work faster. Increased radiation intensity can be achieved on high-current accelerators generating 1 to 100 GW relativistic electron beams, reducing intervals between bursts by up to 6 or 7 orders of magnitude and (hypothetically) modifying cellular response to irradiation. So far, experimental data on the impact of high dose rates on biologic objects remain controversial [11–15].

The aim of this study was to identify biologic effects induced by high dose rates generated by Angara-5-1 and Mir-M machines on human peripheral blood lymphocytes and to compare them to the effects induced by low dose rates generated by Rokus-AM gamma machine.

METHODS

The study included 3 stages:

1) preparation of mononuclear cell suspension;

2) irradiation of the obtained samples with gamma rays generated by Rokus AM machine and high-intensity bremsstrahlung radiation produced by Angara-5-1 and Mir-M;

3) determine the proportion of apoptotic cells in the irradiated samples using flow cytometry.

Preparing a suspension of human blood mononuclear cells

We used suspensions of healthy lymphocytes isolated from the blood of seemingly healthy donors. Blood samples (2.6 ml each) were collected into test tubes containing EDTA anticoagulant, diluted with phosphate buffered saline (PBS) in the ratio of 1 to 2, and then layered over the ficoll (ρ = 1.077; PanEco, Russia). The samples were centrifuged for 40 min at 400 g. The mononuclear layer containing 70 % to 90 % lymphocytes was collected from the interface, washed in PBS twice for 5 min at 200 g and transferred to RPMI 1640 medium with 10 % fetal bovine serum.

The obtained suspension of mononuclear cells was aliquoted in 12 portions (per sample): 2 controls were not irradiated; the remaining 10 were exposed to 5 different radiation doses in twos. The irradiated samples were incubated in the CO_2-incubator at 37 °C and 5 % CO_2. One sample from each pair was incubated for 24 h, another — for 48 h. Such time intervals were necessary to objectively estimate the level of radiation-induced apoptosis. All procedures from blood collection to incubation were performed at room temperature and took 3.5 to 4 h in total.

Irradiation of samples using Angara-5-1

Anagara-5-1 generates high-intensity bremsstrahlung radiation by means of 8 independent high-power sources [8]. They are activated simultaneously (the rms deviation is only 10 ns). The maximum output voltage at matched load is 1.5 MV. The voltage pulse is a half-sine in shape and has a duration of 40–60 ns at half of amplitude. The anode is 50 μm thick tantalum foil (Fig. 1).

Spectral analysis of bremsstrahlung radiation demonstrated that the majority of emitted quanta had energies ranging from 200 to 600 keV. The analysis was carried out on profiles of signals of AD3 diamond X-ray dosimeters (engineered at Dukhov Research Institute of Automatics, Russia) with various filters.

Dose measurements were taken using DPG-03 thermoluminescent dosimeters (TLDs) by Doza LLC, Russia, containing 3 polycrystalline magnesium borate detectors, and the KDT-02 TLD processing set (Electron Corporation, Ukraine). We experimentally evaluated dose dependence on the distance between the object and the diodes and estimated dose distribution depending on the position of the irradiated sample. According to our calculations, the generated dose was attenuated for every 1 cm of the sample's thickness by 10 % in the vertical direction and by 9 % — in the horizontal direction, relative to the center of the anterior surface of the object. We determined the desirable positions of the samples on the flange. The samples were placed in a duralumin cylinder with 7 mm thick bottom and walls. The bottom of the cylinder was positioned 57 mm away from the tantalum foil.

To adjust the doses, the samples were placed at different distances from the radiation source. The maximum dose varied from burst to burst, therefore, TLDs were installed in close proximity to the samples. Dose rates is also varied along with the dose, because pulse duration remained unchanged. For the maximum dose of 10 Gy its rate exceeds 100 MGy/s; for the minimum dose (less than 1 Gy) its rate was about 10 MGy/s.

Mononuclear cell suspensions were placed in plastic containers installed perpendicular to the electron beam

(Fig. 2, D). The liquid layer in the tubes was 4 mm thick. After irradiation the samples were transferred to culture dishes and loaded into the CO_2-incubator (see the details above).

Irradiation of samples using Mir-M

Mir-M is an experimental high-current nanosecond electron accelerator. Its peak output energy reaches 800 kV; half amplitude duration is 40 to 60 ns. The anode is made of 50–100 μm thick tantalum foil. Behind the foil an additional carbon composite Graflex filter is installed (0.5–1 mm thick) for capturing electrons that have passed through the foil. The flange of the exit window is made of 1 mm-thick aluminum. The studied dose rates were the same as for the experiment with Angara-5-1.

Two methods of dose measurements were used in the experiments.

Quick dose measurements were carried out using TLD dosimeters DTG-4 (Doza LLC) in the form of a monocrystalline tablet made of lithium fluoride activated with magnesium and titanium. The irradiated TLDs were read out using Doza-TLD measuring complex (Doza LLC) and DVG software of the same manufacturer. The margin of measurement error for the TLD/Doza-TLD complex was ± 30 %, with a confidence interval of 0.95. Therefore, for better accuracy the dosimeters were calibrated by irradiation using Rokus-AM machine (radiation source: cobalt 60, dose received: 7 Gy). Each irradiated TLD was read out and its individual characteristics were taken into account in the measurements.

For more accurate dosimetry we used Gafchromic EBT 2 films (Ashland, USA). The films were processed using DoseLab 6.5 (Moebius Medical Systems, USA). Because dose distribution along the beam axis was heterogeneous in the Mir-M experiment and the dose was attenuated 1.5–2 times for every 1 cm of the sample's thickness depending on the cathode, we tried to minimize the linear dimensions of the sample.

The samples were 0.6 ml stirred suspensions of mononuclear cells securely sealed in 2 mm deep wells of 16 mm in diameter. The suspension filled the well completely; thus the leukocytes were evenly distributed in the sample during irradiation.

The package for irradiation included: a 5 mm separator consisting of two 1 mm thick plastic sheets spaced at 3 mm intervals; a layer of 2 mm thick polyurethane foam with the sealed sample; 1 mm thick supporting plastic plate. Packages were installed along the same axis so that sample containers were precisely behind each other. The distance between the samples was 8 mm. The distance from the flange to the front-face of the first sample was about 1 cm. Dosimeters were placed behind the separator, adjacent to the anterior surface of the sealed sample (Fig. 1, A, C).

Irradiation of samples using Rokus-AM gamma machine

Rokus-AM is a 60Co-based therapeutic gamma machine. We used it to compare the effectiveness of low-intensity beams and high-intensity gamma radiation. The machine was operated at a dose rate of 0.9 Gy/min (15 mGy/s), which is 10^9–10^{10} times lower than that of nanosecond accelerators.

Cell suspensions were the same as in previous experiments; therefore, we did not have to account for individual characteristics of each sample.

Irradiation was performed using the same well as in the Mir-M experiment. The sample could be positioned horizontally, which allowed us to use an open 2 mm tall plastic container with thin walls, which fit in the well perfectly (Fig. 1, B). Since we had to ensure electron equilibrium, the sample was covered with a 5 mm thick plastic sheet and a 15 mm thick acrylic (plexiglass) sheet was put under the sample.

Irradiation parameters were as follows: distance from the source to the surface — 75 cm, field size — 10 × 10 cm. Irradiation was performed with a time shift of 1–1.5 h relative to the time of the irradiation on the high-intensity gamma sources.

Studying apoptosis in the samples

Following the incubation, we measured apoptosis in the samples of cell suspensions by flow cytometry. We applied two techniques: staining of unfixed samples using the

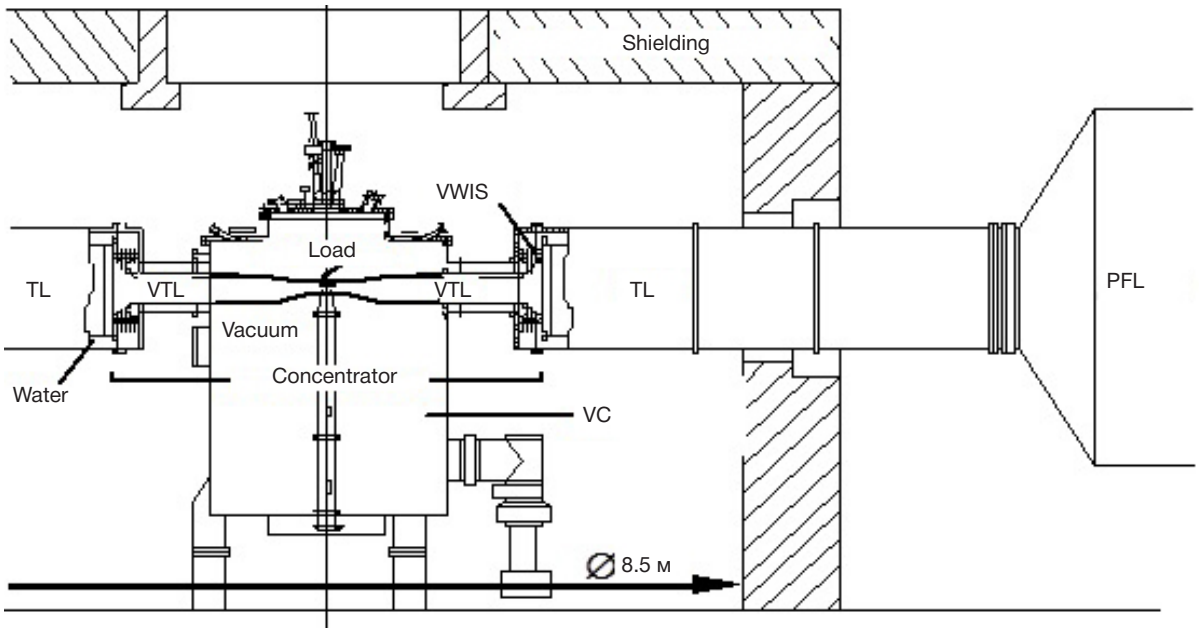

Fig. 1. Schematic of generation and delivery of the electromagnetic pulse by Angara-5-1 generator to the load in the vacuum chamber. PFL — double water pulse-forming line; TL — water transmission line; VWI — vacuum-water interface; VTL — magnetically insulated vacuum transmission line; VC — vacuum chamber with electromagnetic flux concentrator

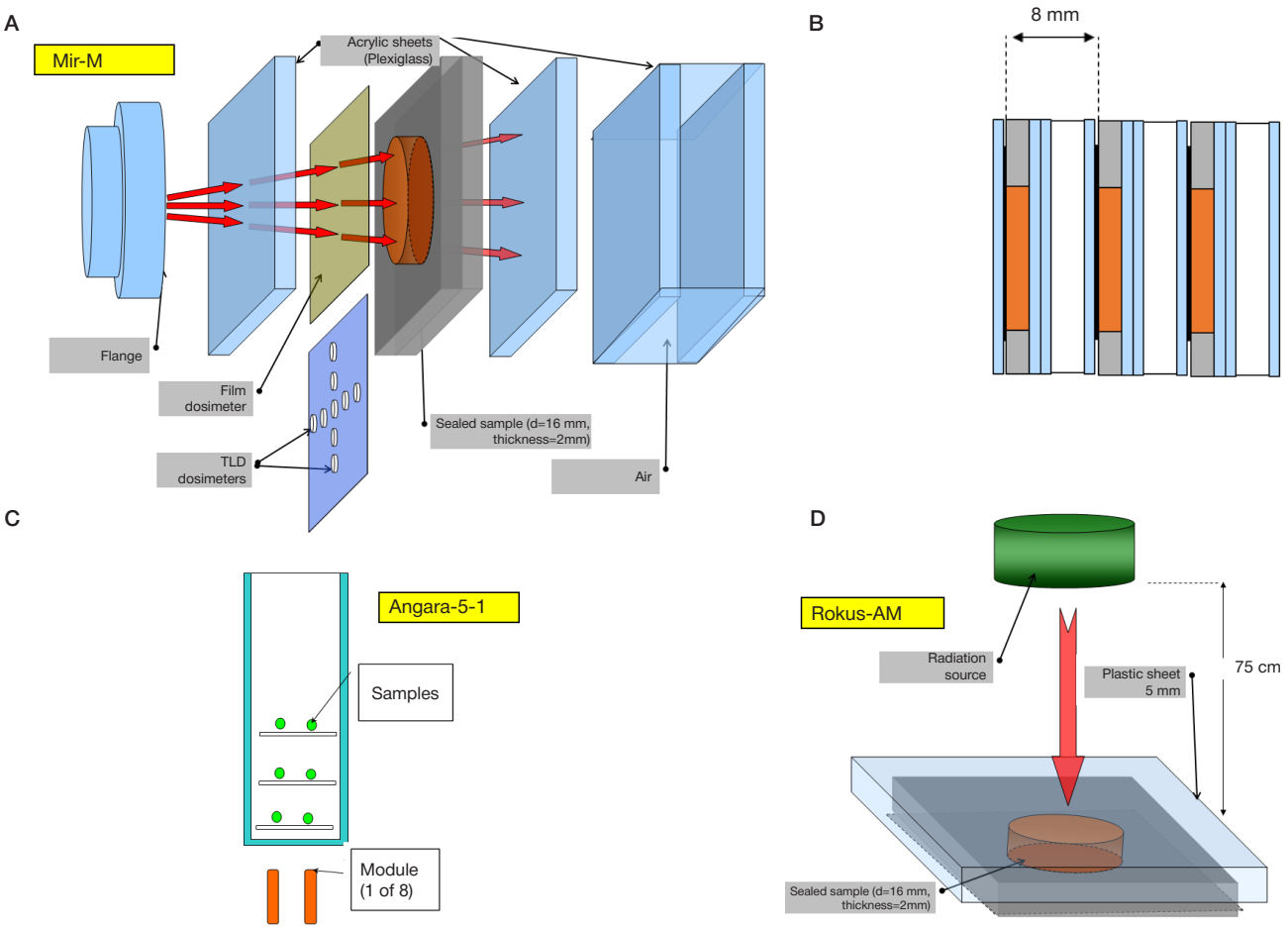

Fig 2. Position of the samples for irradiation by Mir-M **(A, B)**, Angara-5-1 **(C)** and Rokus-AM **(D)**

Annexin-V-FITC Kit (Beckman Coulter, USA) that contains annexin V and propidium iodide, and staining of fixed samples with propidium iodide.

For annexin V staining the irradiated and incubated samples were washed in PBS. A hundred μl of cell suspension containing 106 cells were stained using the Annexin-V-FITC kit according to the manufacturer's protocol. This staining technique helps to quantify the cells that have entered apoptosis (annexin V positive particles) and the cells that have died or are dying of necrosis (propidium iodide positive particles). It is also used to differentiate between the early apoptotic cells (annexin V positive) and the late apoptotic cells (annexin V positive, propidium iodide positive).

Apoptosis can also be detected by staining of fixed samples with propidium iodide: it appears as a subdiploid peak on the single-parameter histogram, which represents the amount of apoptotic bodies in the sample. The irradiated and incubated samples (incubation time was either 24 or 48 h) were left in 70 % ice cold ethanol for 72 h for fixation. Then the cells were washed twice in 1 ml PBS and resuspended in 100 μl PBS. To prevent propidium iodide from binding to RNA the suspension was incubated with 20 μl RNase (R4875, Sigma-Aldrich, USA) at 37 °C for 30 min. The incubated suspension was stained with 20 μl propidium iodide for 40 min at room temperature in the area protected from the light. Before flow cytometry was performed, the sample was replenished with 1 ml PBS. The final cell concentration was at least 10^6 cells.

Flow cytometry was performed on Cytomics FC 500 (Beckman Coulter). We measured the ratio of the subdiploid peak to the total number of cells.

RESULTS

We have established that apoptosis in the lymphocytes exposed to gamma rays generated by Rokus-AM linearly depends on the radiation dose (in the studied dose range). The longer the irradiated samples were incubated, the more apoptotic cells were there. The level of apoptosis increased by 8.0 ± 2.2 % (relative to the deemed 0 % in the non-irradiated samples) in the cells exposed to 5 Gy doses and subsequently incubated for 24 hours. But the samples incubated for 48 h showed a 10.0 ± 2.6 % increase in spontaneous apoptotic activity and a 27.0 ± 3.8 % increase in the proportion of apoptotic cells when exposed to the same 5 Gy radiation doses ($p = 0.004$) (Fig. 3).

The samples exposed to the beams generated by Angara-5-1 exhibited linear dependence of apoptotic activity on the dose; however, the line slope in this case was steeper in comparison with the line constructed for the Rokus-AM experiment (Fig. 3). Doses of 3 Gy generated by Rokus-AM induced apoptosis in 23.0 ± 3.1 % of cells in the samples incubated for 48 h, while high-intensity radiation (in doses comparable with 3 Gy) induced apoptosis in 31.0 ± 3.8 % of cells ($p = 0.050$) in the same sample. This means that high intensity bremsstrahlung radiation has a stronger proapoptotic effect than therapeutic doses of gamma rays.

Because differences between the two radiation types in terms of apoptosis induction in peripheral blood lymphocytes turned out to be significant, we went on to experiment with Mir-M (specially engineered for medical and biological research) in an attempt to better understand the apoptotic effect of high intensity radiation. We used markers of early and late apoptosis

and isolated a fraction of previously ignored necrotic cells (those cells appeared intact on the histogram).

Fig. 4. shows the changing levels of apoptosis (early apoptosis is shown in Fig. 4, A, late — in Fig. 4, B), necrosis (Fig. 4, C) and total cell death (Fig. 4, D) in the irradiated samples depending on the radiation dose and machine ability. We have compared the effect of "therapeutic" gamma radiation generated by Rokus- AM and high-intensity beams produced by Mir-M (only for the lymphocytes incubated for 48 h).

We have demonstrated that total cell death is the same for both machines, but the samples irradiated with 5 Gy doses generated by Rokus-AM tend to have higher apoptotic activity (Fig. 4, D). However, the analysis of cell death types reveals that the number of apoptotic cells (annexin V positive) is significantly higher ($p < 0.05$) in the samples irradiated with ≥ 4 Gy doses generated by Mir-M. The level of necrosis does not exceed 12.0 ± 2.2 % (6 Gy) for Mir-M and is 44.0 ± 8.1 % for Rokus-AM (6 Gy), at $p = 0.0029$.

DISCUSSION

Because Angara-5-1 and Mir-M are unique facilities (there are no other similar stations in the world), it is difficult to compare the results of our study with the findings of other researchers. A few authors studied the biologic effect of ultrahigh intensity photon beams [6, 7, 16], but they did not differentiate between various types of cell death induced by gamma rays and only estimated total cell death or compared anticancer effect of radiation in animals.

We have compared the apoptotic effect of high intensity bremsstrahlung radiation generated by Angara-5-1 and Mir-M and gamma rays produced by Rokus-AM on the peripheral blood lymphocytes of healthy donors and discovered that the former are more proapoptotic. Apoptosis increases linearly in the dose range below 6 Gy and shows dependence on post-irradiation incubation time (24 or 48 h). However, some authors report no differences in apoptosis levels stimulated by varying dose rates [16]. For example, Kotenko et al. have demonstrated that although higher dose rates cause more double-strand DNA breaks and affect mechanisms of DNA repair, the proportion of apoptotic cells remains stable. Similar results are reported by [17]. However, the authors studied therapeutic dose ranges, but never answered a question why disrupted mechanisms of DNA repair in combination with double-strand breaks do not enhance apoptosis. Probably the difference between those studies and our experiment lies in the specifics of the selected biologic models. We irradiated mononuclear cells isolated from peripheral blood, whereas in the studies [16, 17] human fibroblasts were used. Besides, Angara-5-1 and Mir-M generate extremely high dose rates of 100 MGy/s, which may have affected the outcome of our experiment. Perhaps, such dose rates "turn off" reserve DNA repair mechanisms that are still functional at 400 mGy/min (0.017–6.7 mGy/s) [17]. Maybe, double-strand breaks caused by such high dose rates have a specific spatial configuration that blocks repair mechanisms (the breaks are too "deep"). It should be noted that radiosensitivity of cells depends on the stage of the cell cycle and is the lowest for non-dividing cells, such as peripheral blood mononuclear cells (lymphocytes). Non-diving tumor cells constitute the largest population of radioresistant cells. In this light, our findings could be of some interest to the developers of radiation treatments based on the use of gamma rays with ultrahigh dose rates.

We have also discovered that necrosis induced by high intensity radiation is significantly less intense that necrosis induced by therapeutic gamma rays. In the dose range below 6 Gy, necrosis increases proportionally to the dose. The analysis of total cell death in peripheral blood lymphocytes shows that 2 different dose rates applied during one-time exposure to ≤ 6 Gy causes more or less the same number of cells to die, regardless of the type of particle accelerator. This is consistent

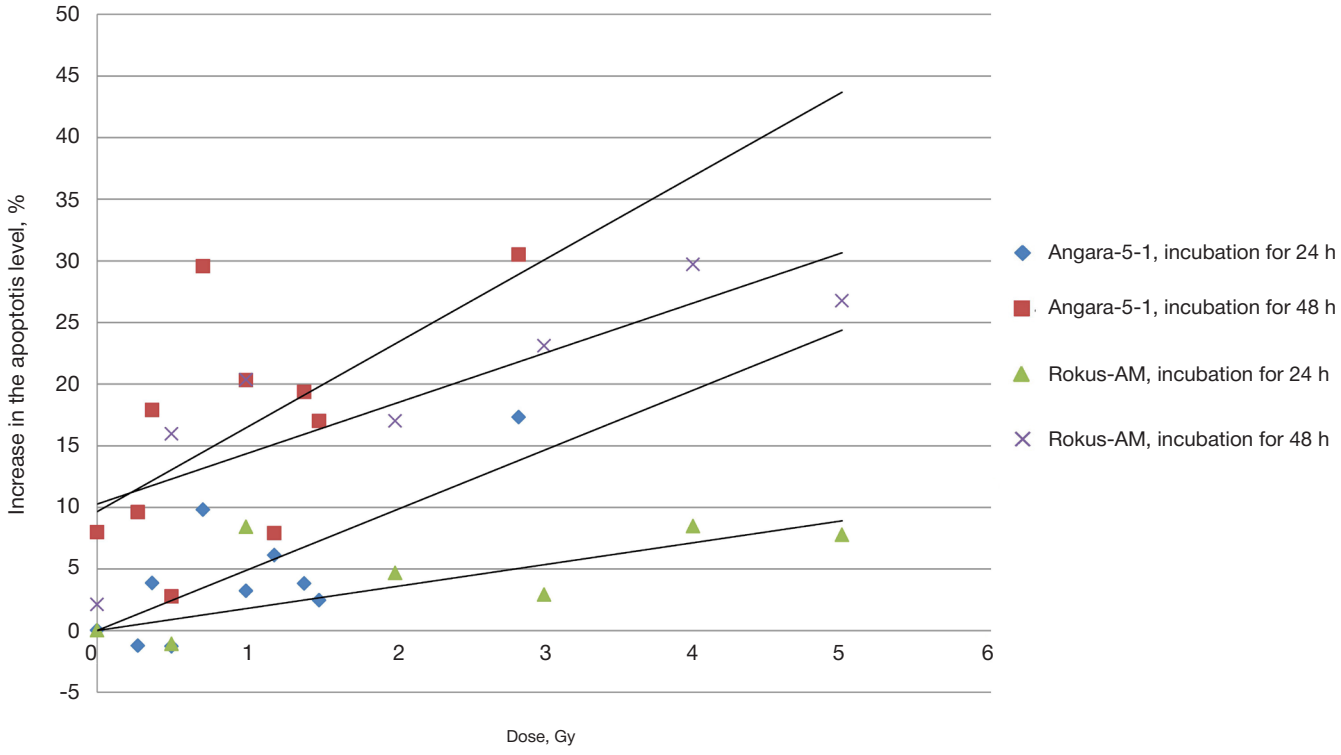

Fig. 3. Levels of apoptosis in the suspensions of peripheral blood lymphocytes irradiated by Rokus-AM and Angara-5-1 with doses from 0 to 5 Gy

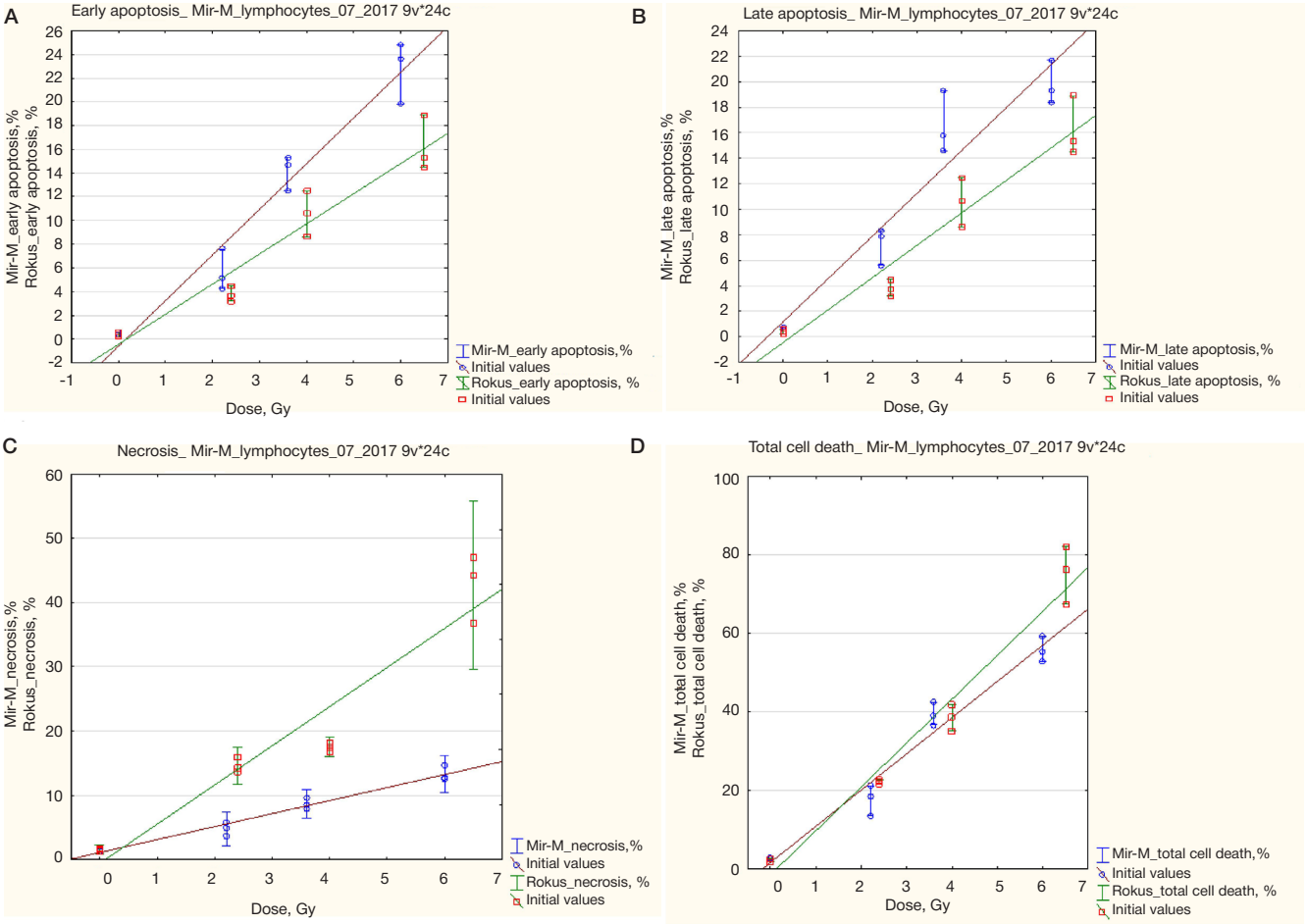

Fig. 4. Comparison of the effects induced by electron beams generated by high-current nanosecond electron accelerator Mir-M and gamma rays produced by Rokus-AM on human peripheral blood lymphocytes in the dose range from 0 to 6 Gy. **(A)** Early apoptosis (annexin V+/PI–); **(B)** late apoptosis (annexin V+/PI+); **(C)** necrosis (annexin V–/PI+); **(D)** total cell death. PI — propidium iodide. Positive and negative staining is represented by + and – respectively.

with the findings of other researchers. For example, in the work by Brüchne et al. [9] the depressing effects of therapeutic gamma rays and high intensity laser beams on KHT mouse fibrosarcoma were comparable.

CONCLUSIONS

In the dose range below 6 Gy, total cell death induced by high intensity gamma rays is comparable with that caused by therapeutic gamma machine. Higher dose rates induce apoptosis, while lower does rates induce necrosis.

Our findings suggest that higher dose rates could be more beneficial for patients than lower dose rates, because intense apoptosis is more “physiological” and will lead to fewer complications than massive necrotic cell death (tissue decay, intoxication, damage to healthy tissue, etc.). Further research is necessary to investigate mechanisms of apoptosis induction by ultra high dose rates in non-dividing (interphase) cells and to compare the effects of radiation at different stages of cell cycles.

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