IMPACT OF POST-CHERNOBYL RADIATION ON FLOW CYTOMETRY PARAMETERS OF HUMAN SPERM

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SUMMARY

Objective: The aim of this study was to analyse the long-term radiation effects on human sperm.

Methods: In total, 104 samples of male donors from 2 regions of Ukraine were tested. Group 1 consisted of 32 donors from the Ivano-Frankivsk region, group 2 included 72 volunteers from the Zhytomyr region. The average age of donors in both groups was 35 ± 6 years (range 24–49). To assess the level of apoptosis, membrane mitochondrial potential, concentration of reactive oxygen species, and ploidy of sperm, flow cytometry was performed.

Results: The individual equivalent dose of group 1 was < 0.4 mSv and of group 2 ≥ 0.4 mSv. Live spermatozoa with signs of apoptosis were significantly higher ($p < 0.05$) in group 2 in comparison to group 1 (15.6% and 11.2%, respectively). Spermatozoa without violating integrity were 73.2% in group 1 and approximately 16% higher than the indices of group 2. The percentage of dead necrotic spermatozoa was twice as high in men with a predicted equivalent dose of ≥ 0.4 mSv than in comparison group. A higher percentage of spermatozoa with low mitochondrial membrane potential, di- and tetraploid was found in group 2.

Conclusions: An equivalent individual dose of ≥ 0.4 mSv can cause a decrease in mitochondrial potential, an increase in the production of spermatozoa with pathological ploidy, as well as to provoke increasing apoptosis in cells.

Key words: male infertility, flow cytometry, long-term radiation, ploidy of spermatozoa, apoptosis, reactive oxygen species

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INTRODUCTION

Spermatogenesis is a story of the life of one cell's population, that is tightly connected with its surroundings as well as external influences (1, 2).

More than thirty years have passed since the Chernobyl disaster, but the medical consequences of this tragedy continue to confront us every day. Immediately after the accident, numerous studies were carried out highlighting the problem of acute radiation exposure on this generation and its possible harmful effects on the reproductive health of future generations (3, 4).

Aside from the distant consequences of the Chernobyl nuclear power plant explosion, there are many sources of gamma-radiation impacting the human body today that cannot be underestimated in the context of scientific and technological progress.

The impact of radiation on the gonads is dose- and time-dependent (5) and is described in many publications. For example, it was shown that long-term exposure to high levels of ionizing radiation causes testicular atrophy and lower doses result in pathology of motility of spermatozoa (6).

In 1970, the effect of roentgen rays on the testicles and spermatogenesis of prisoner-volunteers was studied by American scientists (7). It was concluded that the dose of 0.11 Gy caused decrease in the number of spermatozoa and increasing ionization to 3–5 Gy resulted in irreversible sterility. Other research has shown that 6 Gy causes permanent azoospermia (8).

It is known that every cell in the organism has redox homeostasis, which includes antioxidants (superoxide dismutase, catalase, glutathione peroxidase, peroxiredoxins, glutathione, lipoic acid, carotenoids, iron chelators). Antioxidants play a significant role in reducing reactive oxygen species (ROS) and, with that, preventing lipid peroxidation (9). In case when a cell is under the influence of stressors (environmental or xenobiotics) increase levels of ROS and the excess causes oxidative stress (10).

One of the effects of exposure to ionizing radiation on cells is based on the production of ROS. In this case, atomic structure is altered through direct interactions of radiation with target macromolecules or via products of water radiolysis (9).

Ahmad et al. (11) in their research studied the effect of ionizing radiation on occupationally exposed medical workers. This study shown that in the blood of individuals that worked with interventional radiography or computerized tomography, levels of superoxide, malondialdehyde (a product of lipid peroxidation and one of the main biomarkers of oxidative damage) and superoxide dismutase activity were significantly higher than in control individuals. The main sources of seminal ROS are leucocytes,

cytoplasmic retention and immature spermatozoa with abnormal head morphology (12).

MATERIALS AND METHODS

Ejaculate from male donors was obtained according to the conditions of medical ethics and subject to the signing of an agreement. Informed consent was obtained from all subjects involved in the study.

The study of ejaculate according to the main criteria recommended by the WHO was carried out immediately after the material was obtained. Sperm collection was carried out by masturbation in sterile plastic containers after preliminary abstinence from any sexual contact for 3–6 days. Before processing, the ejaculate was exposed for 20–60 minutes at 37 °C for complete liquefaction.

In total, 104 samples were received from male donors from 2 regions of Ukraine and were divided into two groups. Group 1 consisted of 32 donors from the Ivano-Frankivsk region with contamination ^{137}Cs 20–100 kBq/m^2, group 2 included 72 volunteers from the Zhytomyr region with radiation pollution of 185–550 kBq/m^2.

The average age of donors was 35 ± 6 years (range 24–49). All donors were free from any genital infection, and men with severe extragenital pathology were excluded from both groups. Also were excluded men who had treatment that could impair spermatogenesis or parameters of spermiogram as well as volunteers who abused alcohol and smoking.

Previously, patients were interviewed regarding the duration and locality of their stay before and after Chernobyl accident, their professional activities, living conditions, habits, nutrition, and for each of them, a dosimetric analysis was carried out and an equivalent dose was estimated based on the completed questionnaires.

The assessment of the individual equivalent full dose for the whole body was done according to the formula:

$$D_T(t) = \frac{A_o^{137}}{37} \cdot ND(t)$$

where Ao137 is radiation contamination of the populated area with cesium-137 at the end of April 1986 and ND (t) normalized equivalent dose received for the whole body over time t.

The determination of standardized equivalent doses was based on the use of a standardized exposure dose in atmospheric air at a height of 1 m above the earth's surface for a period of time t in the territory where cesium-137 pollution is equal to 37 kBq/m^2 at the end of April 1986. The data on the standardized exposure doses were obtained from the work of Likhtariov (13). The cesium-137 isotope was adopted as an indicator of radioactive contamination after the Chernobyl accident.

The normalized equivalent dose ND (t) is given by the equation (14):

$$ND(t) = M^{-1}C_1C_2F_1\left[F_2 + (1-F_2)F_3\right]P_\gamma(t)$$

where M is the percentage of external exposure in the total dose, C1 is the conversion coefficient for modifying the exposure dose to the absorbed tissue dose, C2 is the conversion coefficient for modifying the absorbed tissue dose into an equivalent dose, F1 is a correction factor that takes into account the effect of soil conditions on the exposure dose in air, F2 is the factor of staying outside (the proportion of time spent outside the building), and F3 is the screening factor of buildings.

The conversion factors C1 and C2 were taken from the report by Kenigsberg (15). The F1 factor is 0.368 for the period 1990–2004, while in the period 1986–1990 it linearly varied from 1 to 0.368. The value of the factor of staying outside – F2 was considered equal to 0.295 for the rural population and 0.2 for the urban population. Screening factors were taken as 0.212 and 0.1 for rural and urban residents, respectively, or taken from the report by Malko (14) in special cases. The factor characterizing the contribution of prolonged exposure to the accumulation of the total dose was 0.736 (15).

After performing the aforementioned calculations of individual equivalent dosage for both groups, we discovered that the dose for group 1 donors was less than 0.4 mSv, whereas the dose for group 2 donors was higher.

Flow Cytometry

To assess the level of apoptosis, the reagent kit Annexin V (An-V)-FITC Apoptosis Detection Kit I (BD Pharmingen, USA) was used. The washed cells were resuspended in 100 µl of 1x An-V binding buffer and 5 µl of An-V-fluorescence of isothiocyanate and propidium iodide (PI) were added, mixed well at vortex for 3 seconds at low speed, and then incubated for 15 minutes in the dark at room temperature (t=25 °C); 1 ml of buffer was added. This method allowed to detect the translocation of phosphatidylserine from the inner side of the cell membrane to the outer one, which reflects the early (reverse) stage of apoptosis (16, 17).

In the experiments, spermatozoa belonging to the An-V-negative/PI-negative type were assigned to the "normal" category; spermatozoa of the An-V-positive/PI-negative type were classified as the "early apoptotic category"; spermatozoa of the An-V-positive/PI-positive type were treated as "dead apoptotic", and spermatozoa of the An-V-negative/PI-positive type were assigned as "dead necrotic". The apoptosis index was the ratio of early apoptotic spermatozoa to the general group of living cells, which comprised two categories of PI-negative spermatozoa.

To determine the membrane mitochondrial potential of sperm, the washed cells were resuspended in 100 µl of isotonic sodium chloride solution; 10 µl of rhodamine (Rh) 123 was added and thoroughly mixed at vortex for 3 seconds at a low speed. Then the cells were incubated in the dark for 20 minutes at 37 °C; 5 µl of PI solution (concentration 400 µg/ml) and 1 ml of isotonic sodium chloride solution were added. Again, the suspension was mixed at vortex for 3 seconds at low speed (18, 19).

All the samples for assessment of the level of apoptosis and membrane mitochondrial potential were analysed using a particle analysing system (PAS) laser flow cytometer (Partec, Germany) with a wavelength of 488 nm. The signals were detected on the green and red filters, respectively. Signals were recorded and processed using FlowMax software (Partec, Germany).

The concentration of ROS was determined using dihydroethidium (DHE) from Sigma. This uncharged substance easily penetrates through membranes and, upon contact with O_2, oxidizes to ethidium bromide, which is accompanied by the emission of light quanta in the red range (20). ROS was recorded after DHE was added to a sperm suspension containing approximately 107 cells/

ml to a final concentration of 2 mM. At the same time, SYTOX™ Green, which is a welcome stain, was added to a final concentration of 0.05 mM. After that, the sperm suspension was kept for 15 minutes at a temperature of 37 °C, and then centrifuged for 5 minutes at 600 g. The suspension was resuspended in a Biggers-Whitten-Whittingham nutrient medium (BWW) consisting of 95 mM NaCl, 44 mM sodium lactate, 25 mM $NaCO_3$, 20 mM HEPES, 5.6 mmol D-glucose, 4.6 mM KCl, 1.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.27 mM sodium pyruvate, 0.3% weight per volume bovine serum albumin (BSA), 5 U/ml penicillin, and 5 mg/ml streptomycin (21).

The method of PI staining was used to study the ploidy of human sperm according to the protocol (22). In order to obtain correct results, two samples from each donor were analysed. PI was added (final concentration of 25 μg/ml) to a sperm suspension containing 10,000 cells/sample. The analysis of the stained samples was performed on a laser flow cytometer PAS (Partec, Germany) with a wavelength of 488 nm, detected signals on the red filter. Signal registration and processing were performed using FlowMax software (Partec, Germany). PI staining of DNA made it possible to distinguish three different cell populations by their relative ploidy: tetraploid (4N), diploid (2N), and haploid (1N).

Statistical Analysis

Results were expressed as arithmetic mean ± standard error of the mean. The statistical significance of differences between the two groups was determined using the Mann-Whitney U test, with $p<0.05$ being considered significant. Group 1 was used as a comparison group in the statistical analysis. Correlations between studied parameters of flow cytometry were tested by the Spearman's rank correlation coefficient and p-value was calculated for each coefficient, where $p<0.05$ was considered statistically significant.

RESULTS

According to the calculations performed, sperm donors of group 1 from the Ivano-Frankivsk region had range of individual equivalent dose < 0.4 mSv, whereas group 2 (Zhytomyr region with pollution of ^{137}Cs 185–550 kBq/m^2) had an equivalent dose ≥ 0.4 mSv (Table 1). There were no statistical differences in age of the participants in the two compared groups.

By comparing the spermiograms of both groups, we have found a significant difference in the indicators. However, according to the WHO criteria, the indicators still remained normal (Table 2). Thus, in group 2, pathological forms were observed twice as often,

Table 1. *Comparative analysis of individual characteristics of volunteers from two different regions of Ukraine*

Group	Number of volunteers	Median age (years)	Individual equivalent dose (mSv)
1	32	34.1 ± 5.6	< 0.4
2	72	35.7 ± 6.7	≥ 0.4

Table 2. *Volunteers' spermiogram parameters*

Parameter	Group 1	Group 2	p-value
Semen volume (ml)	4.1 ± 0.9	2.9 ± 0.1	0.001
Sperm concentration (million/ml)	43.3 ± 8.6	32.7 ± 14.6	< 0.001
Total motility (PR + NP, %)	78.8 ± 7.7	63.3 ± 11.4	< 0.001
Progressive motility (PR, %)	70.7 ± 9.8	51.7 ± 12.7	< 0.001
Sperm morphology (pathological forms, %)	14.2 ± 4.6	31 ± 11.9	< 0.001

NP – non-progressive motility

Table 3. *Comparison of parameters of flow cytometry in both groups*

Flow cytometry readings		Group 1	Group 2	p-value
Live spermatozoa	An-V-negative/PI-negative (%)	73.2 ± 7.1	57.1 ± 9.2	< 0.001
	An-V-positive/PI-negative (%)	11.2 ± 5.5	15.6 ± 5.7	< 0.001
Dead spermatozoa	An-V-positive/PI-positive (%)	9.9 ± 3.3	15.9 ± 5.1	< 0.001
	An-V-negative/PI-positive (%)	5.7 ± 2.2	11.4 ± 4.6	< 0.001
Mitochondrial membrane potential of spermatozoa	Low, Rh-negative/PI-negative (%)	20.9 ± 8.1	25.8 ± 6.9	< 0.01
	Normal, Rh-positive/PI-negative (%)	62.7 ± 8.7	46.5 ± 10.6	< 0.001
ROS-positive, % of total		32.0 ± 11.0	35.3 ± 8.4	0.05
Sperm ploidy	Haploid (%)	98.1 ± 0.9	96.7 ± 1.1	< 0.001
	Diploid (%)	1.5 ± 0.7	2.4 ± 0.95	< 0.001
	Tetraploid (%)	0.4 ± 0.3	0.9 ± 0.45	< 0.001

ROS – reactive oxygen species

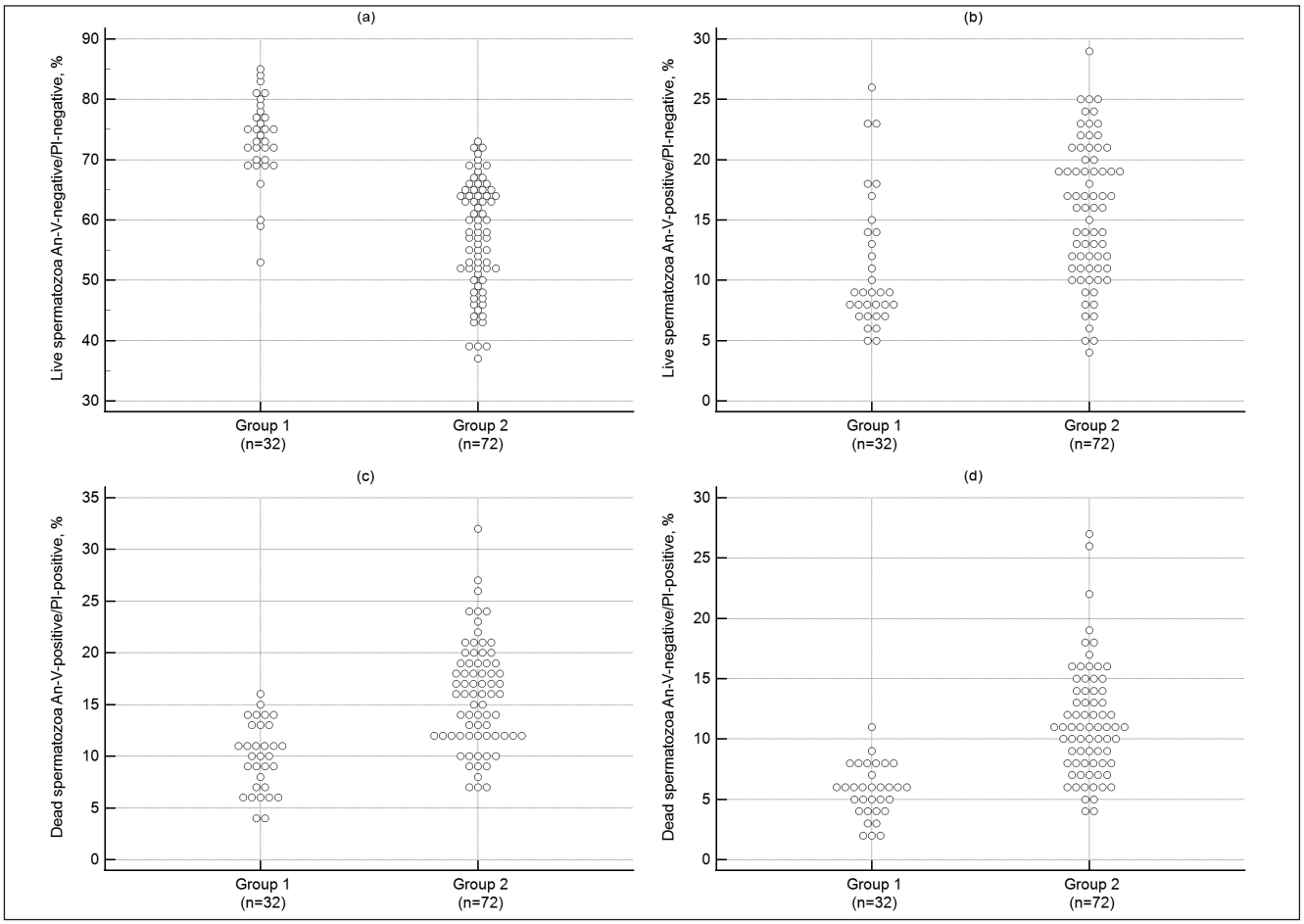

Fig. 1. *Data comparison of the level of apoptosis of spermatozoa between group 1 (Ivano-Frankivsk region) and group 2 (Zhytomyr region).*

and the sperm concentration indices were 24% lower compared to the volunteers in group 1.

A comparison of flow cytometry parameters, studied in the two groups (Table 3), revealed a reliable difference in all values ($p < 0.05$).

Live spermatozoa without signs of apoptosis, defined as An-V-negative/PI-negative, were 73.2% in group 1, an increase of 16% compared to group 2 (Fig.1a). Both early and late apoptosis rates were significantly higher in the group from the more contaminated area (Fig.1b, 1c).

The percentage of dead necrotic spermatozoa was twice as high in men with a predicted equivalent dose ≥ 0.4 mSv (Fig. 1d).

In group 1, only 62.7% of spermatozoa had normal mitochondrial membrane potential, but in group 2 it was even 15% lower. The latter group, however, showed a substantial percentage of diminished mitochondrial potential.

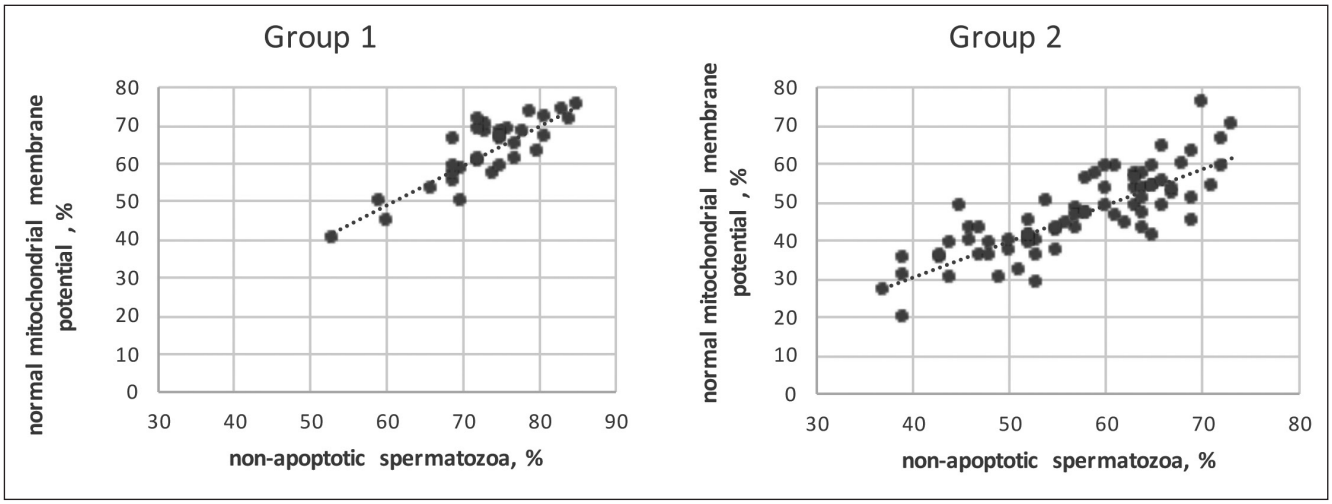

Fig. 2. *Correlation between normal mitochondrial potential of spermatozoa and non-apoptotic spermatozoa.*

Table 4. *Correlation analysis of flow cytometry parameters*

		Live spermatozoa		Dead spermatozoa	
		An-V-negative/ PI-negative (r, p-value)	An-V-positive/ PI-negative (r, p-value)	An-V-positive/ PI-positive (r, p-value)	An-V-negative/ PI-positive (r, p-value)
Mitochondrial membrane potential of spermatozoa	Low, Rh-negative/PI-negative, %	−0.59, < 0.001	0.57, < 0.001	0.18, 0.14	0.21, < 0.07
	Normal, Rh-positive/PI-negative, %	0.83, < 0.001	−0.54, < 0.001	−0.46, < 0.001	−0.46, < 0.001
ROS-positive		−0.44, < 0.001	0.49, < 0.001	0.09, 0.47	0.11, 0.36
Spermatozoa ploidy	Haploid	0.50, < 0.001	−0.33, 0.005	−0.23, 0.051	−0.38, 0.001
	Diploid	−0.52, < 0.001	0.37, 0.002	0.22, 0.06	0.40, < 0.001
	Tetraploid	−0.08, 0.51	−0.04, 0.96	0.02, 0.87	0.37, 0.11

r – Spearman's rank correlation coefficient; p-value < 0.05 was considered significant; An-V – Annexin V reagent kit; PI – propidium iodide; Rh – rhodamine; ROS – reactive oxygen species.

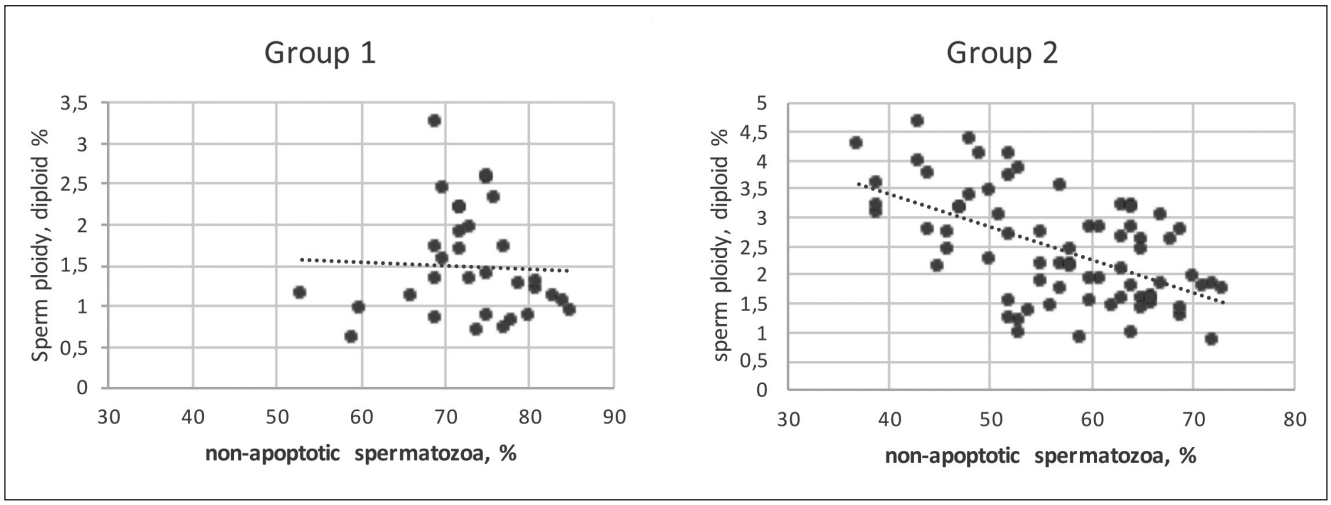

Fig. 3. *Correlation between diploid spermatozoa and non-apoptotic spermatozoa.*

The amount of di- and tetraploid spermatozoa was higher in group 2 when determining the ploidy of the sperm analysed, with the proportion being nearly twice as high as in group 1.

In the second group of volunteers, we carried out a correlation analysis of flow cytometry readings (Table 4).

According to the analysis, a moderate negative correlation was found between the percentage of apoptosis-free spermatozoa and the number of spermatozoa with low mitochondrial membrane potential. On the contrary, at normal potential, a strong positive correlation was observed in both groups (Fig. 2).

There was also a medium-negative correlation between the number of diploid spermatozoa and the level of cells without apoptosis in group 2 (Fig. 3).

In this study, a moderate positive correlation was found between the rate of early apoptotic spermatozoa and ROS. With an increase in the number of diploid spermatozoa in the ejaculate and in the presence of a weak positive correlation, the percentage of both apoptotic cells and dead necrotic cells decreased. No correlation was found between tetraploid spermatozoa and apoptosis rates.

DISCUSSION

Couples with a diagnosis of infertility are increasingly turning to assisted reproductive technology clinics. It is recognised that one third of such cases are associated with male factors. If the genetic material of the gametes has been damaged, fertilization itself does not guarantee a positive result. This is why a thorough approach to the diagnosis of infertility factors is the key to the effectiveness of the procedure. The routine spermiogram has been proven to be one of the main methods of diagnosing pathology. However, to clarify its causes, additional examination methods are required.

A study by Lazaros et al. (23) showed that there is a strong correlation between the detection of abnormal forms of spermatozoa in the ejaculate and the ploidy of these cells during flow cytometry examination.

In our study, we aimed to find out the correlation between ploidy and the apoptosis reaction of spermatozoa. It was found that the number of live non-apoptotic cells decreased with an increasing amount of diploid spermatozoa. We found a strong positive correlation between the indices of non-apoptotic spermatozoa, that is, cells that show progressive motility in the ejaculate, and the normal mitochondrial membrane potential. The dependence between sperm motility and normal membrane potential was described in the research work of Agnihotri et al. (24).

Mitochondria are not only a source of energy for cells, but also play an essential role in their apoptosis. Ionizing radiation causes damage to the membranes of mitochondria, thus disrupting the functioning of these organelles and, as a result, reducing cytochrome C release. The release of cytochrome C from cells

with mitochondrial stress triggers the apoptosis mechanism (25). This is also confirmed by our study, in group 2 a higher percentage of cells with early apoptosis and low mitochondrial membrane potential was observed (a medium correlation was found). When comparing the two groups in our experiment, a statistically significant difference in the figures of ROS-positive sperm was found. It is known that ionizing radiation provokes oxidative stress in the cell. The source of ROS in the sperm of volunteers in group 1 can be explained by the fact that ejaculate did contain a small percentage of morphologically defective spermatozoa, which is the source of seminal ROS. In turn, it was found that with the increase in the number of ROS-positive spermatozoa, the number of live apoptotic cells also increased ($r = 0.49$). With an increase in the equivalent individual radiation dose ≥ 0.4, which was observed in Zhytomyr region, the number of spermatozoa with defective genetic material increased significantly compared to the group of volunteers from western Ukraine.

CONCLUSION

Our study showed that an equivalent individual dose of ≥ 0.4 mSv can cause a decrease in mitochondrial potential, an increase in the production of di- and tetraploid spermatozoa, as well as a significant increase in the parameters of cells with apoptosis. Mitochondria are organelles sensitive to radiation, and when their potential is violated, an increase in apoptotic spermatozoa is observed. We recommend that in assisted reproductive technology, the ejaculate tests should be supplemented by flow cytometry in order to identify pathologies and prevent fertilisation with defective genetic material, which can lead to early pregnancy loss or genetic abnormalities in the offspring.

Conflicts of Interest

None declared

Adherence to Ethical Standards

The Commission of the Biological and Medical Ethics of Bukovinian State Medical University concluded (on 4 June 2021) that the research described in the paper was carried in accordance with the Declaration of Helsinki, Ethical Principles for Medical Research Involving Human Subjects (with amendments by the 64th WMA General Assembly, Fortaleza, Brazil, October 2013), and without violations of the Orders of Ministry of Public Health of Ukraine No. 690 (of 23 September 2009) and No. 944 (of 14 December 2009).

Data Availability Statement

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

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