

## REMOTE EFFECTS OF POST-CHORNOBYL IRRADIATION ON THE BIOCHEMICAL CHARACTERISTICS OF MALE SPERMATOZOA

**A.V. KLEPKO,**

*doctor of biological sciences, senior researcher  
National University of Life and Environmental Sciences of Ukraine,  
Kyiv, Ukraine*

*general\_ecology@ukr.net*

*ORCID 0000-0002-7061-453X*

**V.A. KRUPSKYI**

*National University of Life and Environmental Sciences of Ukraine,  
Kyiv, Ukraine*

**Abstracts.** *The present research aim was analysis of long-term radiation effects on biochemical characteristics of sperm collected from donors originating from radioactive-polluted territories of Ukraine, namely Zhytomyr oblast, Ivano-Frankivsk oblast, Kyiv oblast and Poltava oblast. Apart from this, the role of radiation component in sperm damaging was assessed.*

*It was found that in the most radiation polluted region - Zhytomyr the sperm samples were characterized by a large proportion of degenerative spermatozoa along with immobile sperms. Early signs of apoptosis development in spermatozoa were shown to progress gradually from Poltava to Zhytomyr. Thus, the apoptotic index for sperm samples was highest in Zhytomyr and lowest in Poltava. Also, raise in accumulated radiation dose was associated with elevation of ROS production in sperm samples.*

*It was shown that the ROS production for Zhytomyr donors was threefold more than for Poltava's donors and almost twofold more than for donors from Ivano-Frankivsk. Also we found differences in the mitochondrial potential ( $\Delta\psi$ ) of spermatozoa too. The total  $\Delta\psi$  tended to drop down depending on the mean value of radiation accumulated dose, for Kyiv and Zhytomyr the mean values being significantly less than for Poltava.*

*Thus, our investigations have shown that subjects living on radiation polluted territories may generate a great deal of damaged spermatozoa with the hidden molecular*

and cellular lesions. The latter would predispose inhabitants of radiation polluted regions to male-infertility.

**Keywords:** spermatozoa, ionizing radiation, apoptosis, reactive oxygen species, mitochondrial potential.

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### **Introduction.**

The territory of Ukraine is highly saturated with nuclear power plants (NPPs). The current exploitation of the NPPs is not so safe. Incidentally an unforeseen loss of radionuclides sometimes may occur resulting in wide scale pollution of territories by different radionuclides. Such events took place during Chornobyl disaster in April 1986. Recently, the accident on Fukushima Daiichi NPP that had been provoked by Great East Japan Earthquake on 11 March 2011 caused a discharge of a tremendous amount of radioactivity in the environment and the world ocean, in particular.

The short-lived radioisotopes, namely  $^{131}\text{I}$ ,  $^{132}\text{I}$ ,  $^{132}\text{Te}$ ,  $^{140}\text{Ba}$  and  $^{140}\text{La}$ , were only active for several months after Chornobyl catastrophe while the stable radioisotopes of  $^{90}\text{Sr}$ ,  $^{134}\text{Cs}$ ,  $^{137}\text{Cs}$  and  $^{238-242}\text{Pu}$  isotopes penetrated in soil, rivers, plants and animals and thereby spread far beyond the confined zone of the accident. However  $^{90}\text{Sr}$  and Pu-isotopes were not dispersed as far and therefore are believed to be of minor importance compared to the exposure by  $^{137}\text{Cs}$ . Yet,  $^{134}\text{Cs}$  significantly disappeared in a few years. In consequence to Chornobyl catastrophe about 85 PBq  $^{137}\text{Cs}$  and about 54 PBq  $^{134}\text{Cs}$  were emitted and dispersed over the northern hemisphere [1].

In total, 191,300 km<sup>2</sup> were contaminated with  $^{137}\text{Cs}$  in excess of 37 kBq•m<sup>-2</sup> of which 146,300 km<sup>2</sup> were in the Rus-

sian Federation, Belarus, and Ukraine.  $^{137}\text{Cs}$  contamination exceeding 185 kBq m<sup>-2</sup> was found almost exclusively in the Russian Federation, Belarus, and Ukraine on the area of 8,100 km<sup>2</sup>, 18,600 km<sup>2</sup>, and 4,700 km<sup>2</sup>, respectively [2, 3].

According to the radiobiologic law established by Bergonje and Tribando in the 20th century [4], ionizing radiation first of all damages physiologically active dividing cells which are present in abundance in highly proliferative male germinal epithelium. Apart from this, ionizing radiation produces highly reactive free radicals which cause lipid peroxidation and membrane damage. In contrast to other cells, spermatozoa do not possess the effective antioxidant system of lipid defense because of little catalase activity. This may cause severe damage to spermatozoid structure as it was shown by several authors [5, 6].

Furthermore, animal and human spermatozoa are very vulnerable to radiation impact because of unsaturated fatty acids presence in great abundance in their contents [7, 8].

The early phase of spermatozoid membrane integrity disruption is characterized by the loss of phospholipid asymmetry due to translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane. This translocation of phosphatidylserine is one of the earliest features of cells undergoing apoptosis. Spermatozoa showing evidence of phosphatidylserine exposure were found in human se-

men, their concentration being higher in infertile men than in normal sperm donors [9].

DNA-damage due to apoptosis has been found to occur in spermatozoa of poor quality. The presence of DNA fragmentation in ejaculated spermatozoa is more evident in atypical forms, confirming that morphology and sperm count correlate with testicular function.

Elevated percentages of apoptotic spermatozoa have also been found after infections of the reproductive tract, cancer and other pathologies [10]. However, scarce data have been published concerning the mechanisms involved in cell death of ejaculated sperm from normospermic donors. This is important to elucidate especially in those pathologies where normal spermatozoa are exposed to non-physiological damaging agents, for example elevated levels of reactive oxygen species (ROS) and radiation [6].

The presence of spermatozoa possessing a nuclear chromatin less tightly compacted than in normal mature spermatozoa has been reported in samples from infertile donors, in comparison with those from fertile men. Commonly, the percentage of immature cells is significantly higher in semen samples from infertile donor compared to control ones. The percentage of haploid immature spermatozoa is negatively correlated with cell concentration in semen, and this phenomenon is particularly evident in semen from oligozoospermic subjects [11, 12].

In order to investigate the fertilizing ability of DNA-damaged sperm, the intact murine sperm were exposed to gamma radiation prior to insemination. Fertilization rates of 64.3%, 59.9%, 58.5%, and 61.1% were achieved when sperm were subjected to 5, 10, 50, and 100 Gy, respectively, the control rate being 53.2%. The blastocyst develop-

ment was decreased from 49.8% in the control group to 20.3%, 7.8%, 3.4%, and 2.3% with sperm exposed to doses of 5, 10, 50, and 100 Gy, respectively. Of the transferred blastocyst in the control group, 69.8% were implanted and 33.9% developed into live fetuses. These rates were 57.1% and 21.4% for 5 Gy and 20 and 0% for 10 Gy showing a significant difference ( $p < 0.01$ ) with control. The present study clearly shows that DNA-damaged sperm (regardless of degree of damage) have the ability to fertilize the oocyte, but that embryonic development is very much related to the degree of DNA damage. However, the oocyte has the capacity to repair DNA damage of sperm when it is damaged less than 8%. Damage beyond this level will result in low rate of embryonic development and high early pregnancy loss [13].

Under ionizing irradiation different cell injuries may occur causing the loss of fertility potential and male infertility development. Regarding high radiation pollution of some territories in Ukraine, the pursuing of the research concerning the evaluation of male infertility risk in Ukrainian population will be of great concern and necessity.

The present research aims to analyze long-term radiation effects on biochemical characteristics of sperm collected from donors originating from radiation-polluted territories of Ukraine, namely Zhytomyr oblast, Ivano-Frankivsk oblast, Kyiv oblast, Poltava oblast. Apart from this, the role of radiation component in sperm damaging is to be assessed.

### ***Materials and methods.***

Semen samples were obtained from 479 men volunteers in the age interval 35±6 years who had given their written

consent for participation in research that was then approved by the commission on medical ethics of the National Research Center for Radiation Medicine which validated the complete accordance of the investigations to the ethical and juridical requirements of the Order No. 281 from 01.11.2000 of the Ministry of Public Health of Ukraine. All individuals were distributed in 4 groups with regard to the radiation pollution of territories in their settlements. Group 1 included inhabitants of area with  $^{137}\text{Cs}$  deposition density 185-550 kBq/m<sup>2</sup>, group 2 – 100-185 kBq/m<sup>2</sup>, group 3 – 20-100 kBq/m<sup>2</sup>, group 4 – 2-10 kBq/m<sup>2</sup>.

All subjects were thoroughly interviewed concerning their habits and aptitude for smoking, alcohol, anabolic and drug consumption. Moreover, detailed information about the places of residence before and after Chornobyl explosion as well as past and current diseases was inquired and recorded. Overall visual medical examination was also performed. Also all subjects were asymptomatic for genito-urinary infection.

Freshly ejaculated semen was obtained by masturbation into sterile container after 3-4 days of sexual abstinence. Ejaculates were allowed to liquefy at 37 °C for 30 min. Semen profile was assessed by light microscopy according to the procedure proposed by the World Health Organization [6]. All flow cytometry analyses were performed by flow cytometer PAS (Partec, Germany) equipped with a single 488-nm argon-ion laser. FL1 (FITC) signals were detected through a 530/30 nm band pass filter, FL2 (PI) signals were detected through a 585/42 nm band pass filter. 20 000 events were recorded and analyzed using “FlowMax” software (Partec, Germany). The sperm population was gated on the basis of the linear

forward (FSC) and side-scatter (SSC) properties.

Annexin V apoptosis detection kit I (BD Pharmingen, USA) was used to detect the translocation of PS from the inner to the outer leaflet of the spermatozoid plasma membrane [7]. An aliquot of semen specimen (50 µl of sample containing  $0.5 \times 10^6$ /ml sperm) was added to 145 µl of binding buffer prepared according to the manufacturer’s instruction, plus 5 µl of Annexin V-FITC, and incubated at room temperature for 10 min. Samples were then washed once with binding buffer, centrifuged at 600 g for 10 min, and, finally, the pellets were resuspended in 200 µl of binding buffer containing 1 µl/ml of propidium iodide (PI). The FITC-labelled spermatozoa are analyzed by flow cytometer PAS (Partec, Germany)

The different labelling patterns in the bivariate PI/Annexin V-FITC analysis identify the different cell populations. We qualified sperm as normal (negative Annexin V and PI), early apoptotic (positive Annexin V and negative PI), apoptotic (positive Annexin V and positive PI) and necrotic (negative Annexin V and positive PI). We defined as the apoptotic index the ratio between the positive Annexin V negative PI sperm and the total alive sperm (PI negative).

Intracellular generation of O<sup>2-</sup> was estimated using dihydroethidine (DHE) of “Sigma” production, a membrane permeating uncharged probe that reports overall cellular O<sup>2-</sup> production through oxidation to ethidium bromide and emitting red fluorescence [8]. ROS generation was induced by the addition of DHE to spermatozoa in suspension with concentration 10<sup>7</sup> cells per ml to give a final concentration of DHE equal 2 mM, SYTOX Green a cell viability stain with green fluorescence being also added to

the final concentration 0,05 mM. Then the spermatozoid suspension was incubated for 15 min at 37°C, centrifuged for 5 min at 600 g and resuspended in Biggers-Whitten-Whittingham (BWW) medium. The latter consisted of 95 mM NaCl, 44 mM sodium lactate, 25 mM NaHCO<sub>3</sub>, 20 mM HEPES, 5.6 mM D-glucose, 4.6 mM KCl, 1.7 mM CaCl<sub>2</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.2 mM MgSO<sub>4</sub>, 0.27 mM sodium pyruvate, 0.3% (w/v) BSA, 5 U/ml penicillin and 5 mg/ml streptomycin

Fluorometric assessment of mitochondrial membrane potentials was made on the basis of mitochondrial specific probe Rhodamine 123 (Sigma) (R123) [9].

Comparison of data for different donors' group was made using analysis of variances, Anova, and unpaired Student's t-test with amendment of Bonferroni. Confidence intervals for mean values were identified using t-statistic at P = 0.95 and standard errors. All statistical tests were two sided and P < 0.05 was considered statistically significant

Assessment of the whole body irradiation doses was carried out according to [10] on the basis of normalized doses of the whole body irradiation according to the following formulae:

$$ND(\Delta t) = E_r^{-1} C_1 C_2 P_i(\Delta t), \quad (1),$$

where ND(Δt) – normalized equivalent dose of whole body irradiation accumulated over the time Δt in the area with radioactive density of <sup>137</sup>Cs contamination equal to 37 kBq/m<sup>2</sup> at the end of April 1986; ER – the contribution factor of external radiation in the total dose; C1 – the conversion factor for converting exposition dose in tissue absorbed dose; C2 – the conversion factor for converting tissue absorbed dose

in equivalent dose; P<sub>γ</sub>(Δt) – the exposition dose in free air at the height 1 meter above the ground over the time Δt.

$$ID_{(T)} = \sum_{i=1}^n \frac{A_{oi} F_{li} [F_{2i} + (1 - F_{2i}) F_{3i}]}{37} \cdot ND(\Delta t_i) \quad (2),$$

$$T = \sum_{i=1}^n \Delta t_i, \quad (3),$$

where ID(T) – individual equivalent dose accumulated over time T; A<sub>oi</sub> – <sup>137</sup>Cs radioactive contamination density (kBq/m<sup>2</sup>) in i – place of residence; n – number of residence places changed by a subject in the period after Chernobyl catastrophe; F<sub>li</sub> – the correction coefficient taking into account a type of a soil in the i-place of residence; F<sub>2i</sub> – the outdoor occupancy factor in the i-place of residence; ND(Δt<sub>i</sub>) – normalized dose accumulated in the period of time Δt<sub>i</sub>; T – time span from Chernobyl catastrophe or later date (for those who were born after the accident) until the date of sperm examination; Δt<sub>i</sub> – period of time spent in i-place of residence.

Data about radioactive pollution of territories in Ukraine were retrieved from Internet resources [11] and special documents devoted to dosimetry of radiation polluted territories.

Comparison of data for different donors' group was made using analysis of variances “Anova” and unpaired Student's t-test with amendment of Bonferroni [11].

### **Research results and their discussion.**

In the research the inhabitants of Zhytomyr oblast, Kyiv and Kyiv oblast, Ivano-Frankivsk oblast, Poltava oblast and Kyiv city were enrolled. According to the data from radiodosimetric documents radiation contamination by <sup>137</sup>Cs of former two regions chang-

es in the range 20-1480 kBq/m<sup>2</sup>, in Ivano-Frankivsk and Poltava region it ranges 20-100 kBq/m<sup>2</sup> and 2-10 kBq/m<sup>2</sup>, respectively. Moreover, Zhytomyr and Kyiv regions in contrast to Ivano-Frankivsk and Poltava regions are polluted albeit partly by radioisotopes of plutonium (238-242Pu), americium (241Am), and strontium (90Sr). Taking these facts into account we made up four groups of men volunteers. Thus, group 1 comprised men from Poltava oblast, group 2 – from Ivano-Frankivsk oblast, group 3 – from Kyiv and Kyiv oblast and group 4 – from Zhytomyr oblast who had been living on urban or rural territories with relevant <sup>137</sup>Cs deposition densities. The data concerning group characteristics, individual equivalent dose means and age means are provided in Table 1.

The data on sperm quality of donors from different regions of Ukraine who

participated in randomized cross-sectional studies concerning fertility potential of spermatozoa are presented in Table 2.

It is seen that mean value of ejaculate volume gradually diminishes from 4.2 ml in group 1 to 2.2 ml in group 4. Meanwhile the mean value of sperm concentration in ejaculate was shown to be the highest in the samples from Poltava's inhabitants being equal to 95 million/ml. Interestingly, for Kyiv this index was more than for Ivano-Frankivsk, although the smallest magnitude was detected for Zhytomyr (43 million/ml). Similar mean values of progressive sperm motility were found in all 3 radiation polluted regions, while for Poltava's inhabitants the utmost meaning of the parameter was established to be equal 85%.

In the most radiation polluted region - Zhytomyr the sperm samples were

### 1. Comparative analysis of individual characteristics of men who participated in the epidemiological studies of sperm quality for human male population of Ukraine in the post-Chornobyl remote period

Group	Territorial radiation contamination by <sup>137</sup> Cs, kBq/m <sup>2</sup>	Individual age mean, years	Individual equivalent dose mean, mSv
I	2-10	32.3±0.4	0.28±0.04
II	20-100	32.9±0.5	3.78±0.12
III	100-185	31.7±0.8	7.98±0.24
IV	185-550	33.3±0.7	18.08±0.39

### 2. Sperm quality in different groups of donors

Group number	Volume of ejaculate (ml)	Concentration of spermatozoa (×10 <sup>6</sup> sperm / ml)	Sperm progressive motility, %	Sperm immotility, %	Degenerative sperm, %
I	4.2 ± 0.8	95 ± 17	85 ± 12	8 ± 2	7 ± 2
II	3.1 ± 1.0	60 ± 14*	61 ± 11	28 ± 4*	19 ± 9
III	3.2 ± 0.6	67 ± 22	69 ± 18	26 ± 5*	33 ± 8*
IV	2.2 ± 0,7*	43 ± 13*	45 ± 11*	33 ± 6*	53 ± 12*

\* – p<0.05, significant difference with control (group 1)

characterized by a large proportion of degenerative spermatozoa along with immobile sperms. In Ivano-Frankivsk and Kyiv these variables exceeded those for Poltava but were significantly less than for Zhytomyr. In the table 2 the data concerning sperm subpopulations for different donors' group are given.

Sperm viability ( $V^-/PI^- + V^+/PI^-$ ) was the highest in the samples from Poltava's donors, the mean value being 91%. For donors of Ivano-Frankivsk and Kyiv the differences in sperm viability were insignificant, while for Zhytomyr poor sperm viability was established. The quantity of necroses in spermatozoa tended to increase slightly with the rise of the mean value of accumulated dose for studied regions. Changes for non-viable apoptotic spermatozoa were more pronounced. Early signs of apoptosis development in spermatozoa were shown to progress gradually from Pol-

tava to Zhytomyr. Thus, the apoptotic index for sperm samples was highest in Zhytomyr and lowest in Poltava. Also, raise in accumulated radiation dose was associated with elevation of ROS production in sperm samples (table 3).

ROS production for Zhytomyr donors was threefold more than for Poltava's donors and almost twofold more than for donors from Ivano-Frankivsk (1 rem). We found differences in the mitochondrial potential ( $\Delta\psi$ ) of spermatozoa too. The total  $\Delta\psi$  tended to drop down depending on the mean value of radiation accumulated dose, for Kyiv and Zhytomyr the mean values being significantly less than for Poltava (table 4.).

There are two crucial targets for ionizing radiation detrimental effects in living cells. First of all, radiation affects DNA that results in damaging nucleotides and accumulation of single along with double strand breaks in DNA struc-

### 3. Determination of sperm subpopulations in different donors' groups using Annexin V and PI staining

Group number	Annexin V <sup>-</sup> /PI <sup>-</sup> , %	Annexin V <sup>+</sup> /PI <sup>-</sup> , %	Annexin V <sup>+</sup> /PI <sup>+</sup> , %	Annexin V <sup>-</sup> /PI <sup>+</sup> , %
I	91 ± 6	4 ± 2	2 ± 1	3 ± 3
II	81 ± 9	6 ± 2	11 ± 8	2 ± 1
III	70 ± 6*	11 ± 4*	17 ± 3*	2 ± 2
IV	58 ± 10*	19 ± 7*	12 ± 5*	12 ± 5*

\* – p<0.05, significant difference with control (group I)

### 4. Apoptosis index, ROS production and mitochondrial potential changes in sperm under different terrestrial radiation pollution

Group number	Apoptosis index, arbitrary units	ROS production DHE negative cells, %	Mitochondrial potential changes R123 positive cells, %
I	13 ± 2	28 ± 11	72 ± 22
II	14 ± 1	43 ± 9	55 ± 13
III	15 ± 3	51 ± 15	53 ± 15
IV	19 ± 2*	75 ± 12*	24 ± 7*

\* – p<0.05, significant difference with control (group I)

ture [4, 13]. However, in spermatozoon DNA is present in nucleoprotamine complex as inactive component due to highly condensed state of chromatin and the absence of whatever DNA transcriptional activity. Therefore DNA damaging just for spermatozoon functioning is of little importance. On the other hand, the spermatozoon is highly dependent on the integrity of its plasma and mitochondrial membranes due to the necessity to maintain ion-floods on due level. That is why membranes may represent the other essential target for radiation impact. It is well known sperm cell membranes are rich of unsaturated fatty acids [14, 15]. These compounds are very sensitive to irradiation, especially their double bonds which are key points in radiation assault through hydroperoxide or hydroxyl radicals. Consequently, lipid peroxidation chain reaction is launched resulting in further destruction of membranes. These conditions are very favourable for intercepting electrons from electron transporting chain by free oxygen molecules, the superoxide radicals being produced. This pool of superoxide radicals is associated exclusively with mitochondria. In addition, there is a second pathway for superoxide radicals generation that is accomplished through special enzyme-oxidase which is located in the heads of spermatozoa [8, 16, 17].

### ***Conclusion.***

Abatement of antioxidant enzymic activities in radiation damaged spermatozoa facilitate the propagation of free radicals, especially superoxide radicals, in mitochondria and spermatozoid heads. In turn, ROS penetrate into the nucleus and exert a direct effect on chromatin DNA causing overall disrup-

tion and formation of apoptotic bodies. In the last stage of apoptosis development cell death follows. Our results have established the gradual increase of apoptosis dependent cell deaths comparing to necrosis dependent cell death whose contribution to spermatozoid mortality significantly diminished for highly radiation polluted regions compared to low radiation polluted and non-polluted ones. This phenomenon is a hallmark of male-infertility progress. Thus, our investigations have shown that subjects living on radiation polluted territories may generate a great deal of damaged spermatozoa with the hidden molecular and cellular lesions. The latter would predispose inhabitants of radiation polluted regions to male-infertility. Therefore a special system of prophylactic arrangements needs to be elaborated to prevent further spreading of male infertility.

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**Клепко А.В., Крупський В.А. (2022)**

**ВІДДАЛЕНИЙ ВПЛИВ ІОНІЗУЮЧОГО ОПРОМІНЕННЯ ПІСЛЯ ЧОРНОБИЛЬСЬКОЇ АВАРІЇ НА БІОХІМІЧНІ ПОКАЗНИКИ СПЕРМАТОЗОЇДІВ ЧОЛОВІКІВ.**

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**Анотація.** Метою даного дослідження був аналіз тривалого впливу іонізуючої радіації на біохімічні характеристики сперми чоловіків-донорів, які проживають на радіоактивно забруднених територіях України, а саме Житомирської, Івано-Франківської, Київської та Полтавської областей. Крім того, оцінено роль радіаційної складової в пошкодженні сперматозоїдів чоловіків.

Встановлено, що в найбільш радіоактивно забрудненому регіоні – Житомирська обл., зразки сперми, поряд з нерухомими сперматозоїдами, характеризуються значною часткою пошкоджених сперматозоїдів. Встановлено, що ранні ознаки розвитку апоптозу сперматозоїдів поступово прогресують у спермі чоловіків з Полтавської області до Житомирської. Так, індекс апоптозу для зразків сперми був найвищим у чоловіків з Житомирського регіону, а найнижчим – з Полтавського. Крім того, підвищення накопиченої дози радіації було пов'язане з підвищенням виробництва активних форм кисню у зразках сперми.

Показано, що виробництво активних форм кисню у донорів з Житомирської області втричі більше, ніж у донорів з Полтавської, і майже вдвічі більше, ніж у донорів з Івано-Франківської області. Також ми виявили відмінності в мітохондріальному потенціалі сперматозоїдів. Сумарний мітохондріальний потенціал мав тенденцію до зниження залежно від середнього значення накопиченої дози радіації, причому для чоловіків з Київської та Житомирської областей середні значення значно менші, ніж для чоловіків Полтавської області.

Таким чином, наші дослідження показали, що чоловіки, які проживають на радіоактивно забруднених територіях, можуть генерувати велику кількість пошкоджених сперматозоїдів з прихованими молекулярно-клітинними ураженнями, що може призвести до розвитку чоловічого безпліддя.

**Ключові слова.** Сперматозоїди, іонізуюче випромінювання, апоптоз, активні форми кисню, мітохондріальний потенціал

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