

X-rays and metformin cause increased urinary excretion of cell-free nuclear and mitochondrial DNA in aged rats

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Abstract

Activation of cell death in mammals can be assessed by an increase of an amount of cell-free DNA (cf-DNA) in urine or plasma. We investigated the excretion of cf nuclear DNA (nDNA) and cf mitochondrial DNA (mtDNA) in the urine of rats 3 and 24 months in age after X-irradiation and metformin administration. Analyses showed that prior to treatment, the amount of cf-nDNA was 40% higher and cf-mtDNA was 50% higher in the urine of aged rats compared to that of young animals. At 12 h after irradiation, the content of cf-nDNA and cf-mtDNA in the urine of young rats was increased by 200% and 460%, respectively, relative to the control, whereas in the urine of aged rats, it was 250% and 720% higher. After 6 h following metformin administration, the amount of cf-nDNA and cf-mtDNA in the urine of young rats was elevated by 25% and 55% and by 50% and 160% in the urine of aged rats. Thus, these preliminary data suggest that X-rays and metformin cause a significant increase of cf-DNA in the urine of older rats caused by the active cell death in tissues. These results also suggest that metformin possibly initiates the death of the cells containing structural and functional abnormalities.

Keywords

Cell-free urine DNA, old and young rats, X-irradiation, metformin administration

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Introduction

The death of cells with structural and functional abnormalities in the tissues of organisms is an active process that supports the renewal of cell populations and the functional integrity and homeostasis of tissues.¹ The presence of circulating cell-free DNA (cf-DNA) in mammalian biological fluids, such as blood and urine, is due to continuous cell death in the tissues. According to the results of a number of studies, cell death accompanied by a release of cf-DNA fragments can occur not only through apoptosis and necrosis but also via mechanisms of autophagy. The process of autophagy is generally assumed as a mechanism of survival or a cytoprotective mechanism that removes damaged organelles, proteins, and other macromolecules.^{2,3} However, in the events of excessive increase in the amount of organelles and macromolecules with structurally functional disorders, the cell may be subjected to death by autophagic

degradation.^{1,3} An increase in the content of cf-DNA in plasma (serum), which is caused by an activation of cell death in tissues, occurs not only under external damaging influences^{4,5} but also with aging of the organism.^{6,7} The increase of cf-DNA in plasma with aging of the organism and in the absence of external influences may possibly happen largely due to the enhancement of programmed cell death.⁸ It should be noted that autophagic cell death in tissues, most likely, does not contribute significantly to the

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age-dependent increase in the content of cf-DNA in biological fluids, since the autophagic activity in tissues decreases along with the aging of the organism.⁹ Nevertheless, the activity of autophagic processes increases in response to such signals as starvation, hypoxia, oxidative stress, ionizing radiation (IR), infection, and other types, which are accompanied by a gain of the AMP/ATP ratio and an upregulation of 5'-adenosine monophosphate-activated protein kinase (AMPK) in cells.^{10,2} Autophagy in tissues can also be stimulated by the introduction of some pharmacological agents, as well as other compounds obtained from natural sources.¹¹ Metformin, which is widely used for the treatment of diabetes mellitus type 2, can be regarded as one of these drugs (activators of autophagy). Although the antidiabetic mechanisms of actions of metformin are not completely understood, its indirect participation in autophagy via activation of AMPK is generally accepted.¹² Perhaps the introduction of metformin in old animals, with suppressed autophagic activity and increased level of damage of cellular structures, will cause autophagy enhancement accompanied by the destruction of these dysfunctional cells. It is known that IR is also a potent activator of AMPK^{13,14} and an inducer of various cell death pathways, including apoptosis, necrosis, and autophagy.^{15,16} It can be presumed that activation of autophagy provides a significant contribution to the elimination of damaged cells from the tissues after exposure to IR.

We have previously demonstrated a significant increase in the excretion of fragments of cell-free nuclear DNA (cf-nDNA) and cell-free mitochondrial DNA (cf-mtDNA) in the urine of rats exposed to IR. Notably, the cf-mtDNA increase is much higher than that of cf-nDNA, which suggests the involvement of mitophagy (by autophagic mechanism) in postirradiation cell death.¹⁷ Metformin, as mentioned above, initiates the death of cells containing structural and functional abnormalities by the mechanism of autophagy. The intensification of cell death at the level of a whole mammalian organism can be estimated by a raise in the content of cf-DNA in urine or plasma. Since urinalysis is more preferable as a noninvasive approach, we conducted a comparative study for the excretion of cf-nDNA and cf-mtDNA in the urine of male rats (3 and 24 months age) after X-irradiation and administration of metformin.

Materials and methods

Animals and their treatment

The present study involved Fisher 344 male rats aged between 3 and 24 months, obtained from the nursery of the Branch of Institute of Bioorganic Chemistry RAS (Pushchino, Moscow region). During the experiment, the animals were kept under standard conditions in the vivarium of the Institute of Theoretical and Experimental Biophysics RAS. The experiments were conducted in accordance with all the

requirements established by the Institutional Committee for the Control of Animal Use in Biomedical Experiments. The animals were acclimatized for 1 week before the start of experiments. Rats were fed a special diet for mice and rats ad libitum, with free access to clean drinking water, and were housed three animals per cage under standard 12-h light/12-h dark cycle at a temperature of $22 \pm 2^\circ\text{C}$ and $45 \pm 5\%$ humidity. Irradiation of rats was performed using a commercially available X-ray apparatus RTU-12 (340 kVp, 20 mA; Medrent, Russia) with 1 mm aluminum and copper filters. An expert for radiation dosimetry controlled the absorbed dose using an ionization chamber and a clinical X-ray dosimeter (VacuDap, NTC-Amplitude, Moscow, Russia). Rats were irradiated by a sublethal dose of 5 Gy at a dose rate of 1 Gy/min. For the period of rat whole-body irradiation, the animal was placed in an individual plexiglass container to prevent from moving in the process of X-ray irradiation.

Metformin (1,1-dimethylbiguanide hydrochloride; Merck, Darmstadt, Germany) was introduced in rats at 300 mg/kg body weight perorally with drinking water twice during 1 day with an interval of 3 h. This dose does not exert genotoxic effect (in vivo)¹⁸ and even mice injected daily with 600 mg/kg body weight for 9 months did not exhibit significant kidney damage.¹⁹

Urine collection and DNA extraction

All chemicals were purchased from Sigma-Aldrich (St Louis, Missouri, USA) unless stated otherwise. For urine collection, rats were placed in individual metabolic cages (Hatteras Instruments, Cary, North Carolina, USA) equipped with glass containers. To the bottom of the container, 0.5 mL of 0.1 M ethylenediaminetetraacetic acid (EDTA; pH 8.0) solution was added and coated with a layer of paraffin oil. Such cages allow collection of urine separately from feces. In all cases, the urine of rats was collected (before and after irradiation or treatment with metformin) in the evening, starting at 6:00 p.m. For urine collection, rats were placed into metabolic cages before irradiation and before administration of metformin and at certain time points (6, 12, 24, 72 h) after these treatments. In all cases, the rats were housed in metabolic cages at $22 \pm 2^\circ\text{C}$ for 5.5–6.0 h until 5 mL of liquid (urine + EDTA) was collected in the container.

Urine samples were centrifuged (5000 r/min, 10 min), and supernatant was poured into another test tube and frozen at -20°C for subsequent DNA extraction. To isolate total urine DNA (mtDNA and nDNA), a frozen urine sample was thawed at room temperature, then placed on ice immediately prior to DNA isolation, and used for DNA extraction within 5 min. DNA was extracted using the Wizard Plus Minipreps DNA Purification System (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions and as described in the study by Su et al.²⁰ and Tsui et al.²¹ Three milliliters of urine sample

were mixed with 1.5 volumes of 6 M guanidine thiocyanate (Sigma-Aldrich) by inverting eight times. One milliliter of resin (Wizard DNA Isolation Kit, Promega) was added into the urine lysate and incubated for 3 h at room temperature under gentle stirring. The resin–DNA complex was centrifuged, transferred to a minicolumn (provided in the kit), and washed with a buffer provided by the manufacturer; then, the DNA was eluted with Tris-EDTA (TE) buffer. Each DNA sample was dissolved in 0.1 mL of Millipore Q water. DNA concentration was determined by reaction with PicoGreen, according to the manufacturer's protocol, after which fluorescence detection on a Tecan Infinite-unit 200 (Austria) followed. Samples of total DNA from rat urine were subjected to separation by electrophoresis on a 1% agarose gel stained with ethidium bromide to estimate the length of DNA fragments. Marker 100 base-pair DNA ladder (New England Biolabs, Canada) was used as a standard. Electrophoresis was carried out on equipment manufactured by Helicon (Moscow, Russia). For comparison, DNA samples isolated from the urine of rats collected prior to and 6 h after irradiation, as well as of aged rats 6 h after administration of metformin, were loaded on agarose gels for electrophoresis.

Quantitative analysis of nuclear and mtDNA by real-time polymerase chain reaction

The quantitative analyses of nDNA and mtDNA were carried out by real-time polymerase chain reaction (PCR; qPCR) using the TaqMan technology and the qPCR Thermal Cycler Prism 7500 (Applied Biosystems, Foster City, California, USA). The change in the relative mtDNA copy number was determined as the ratio between the copy number of the mitochondrial tRNA gene and that of the nuclear housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) which was amplified in the same tube.²⁰ The efficiency of the assay for amplifying both nDNA and mtDNA was measured using standard curves generated by a dilution series with 20, 10, 5, 2, 1, and 0.1 ng of total rat liver DNA per reaction. To determine the quantities of mtDNA and nDNA present in the tested samples, the average threshold cycle (C_t) values for nDNA and mtDNA were obtained from each reaction. The cycle number (C_t) at which the fluorescent signal of a given reaction crossed the threshold value was used as a basis for quantification of mtDNA and nDNA copy numbers.²² PCR assays were performed in duplicate or triplicate for each DNA sample. The following primers were used for tRNA gene amplification (73 bp) from mtDNA: forward—5'-AAT GGT TCG TTT GTT CAA CGA TT-3'; reverse—5'-AGA AAC CGA CCT GGA TTG CTC-3'; and a probe—R6GAAG TCC TAC GTG ATC TGA GTT-RHQ1. For amplification of the GAPDH gene (80 bp) from nDNA, the following primers were used: forward—5'-TGG CCT CCA AGG AGT AAG AAA C-3'; reverse—5'-GGC CTC TCT CTT GCT CTC AGT ATC-3'; and a probe—FAMCTG GAC CAC CCA

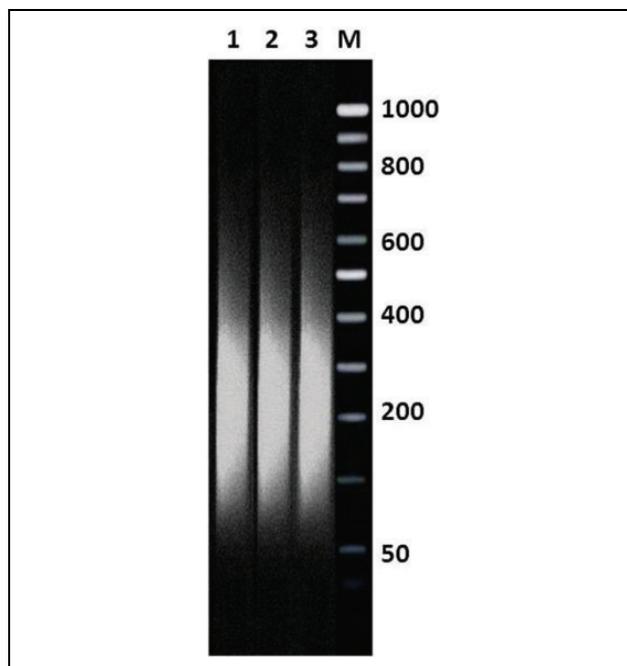


Figure 1. Electrophoresis of DNA extracted from rat urine, 1% agarose gel. (1) DNA from young rat urine collected before irradiation; (2) DNA from young rat urine collected 6 h after irradiation (5 Gy); (3) DNA from old rat urine collected 6 h after administration of metformin; M: 100 base-pair DNA ladder.

GCC CAG CAA-RTQ1. Primers and probes were chosen using Basic Local Alignment Search Tool database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), so that the amplicon from mtDNA had no significant homology with the nuclear genome.²³ Primers and probes for PCR were synthesized by Syntol, Inc. (Moscow, Russia). PCRs were performed in a 20 μ L volume containing 10 μ L of TaqMan Universal PCR Master Mix 29 Buffer (Applied Biosystems), 2 μ L of DNA solution, and 250 nM of each primer and probe. PCR cycles were as follows: 5 min at 95°C followed by 40 amplification cycles (95°C for 30 s, annealing and elongation at 60°C for 1 min). Fluorescence values were analyzed and calculated by the ABI PRISM 7500 software.

Statistical analysis

Statistical differences between the data obtained before and after treatment of rats were analyzed by the standard Student's *t*-test using GraphPad Prism software (version 5.0). The results are calculated and presented as a mean \pm SE of the mean (SEM, $n = 8$). A *p* value of <0.05 was considered to be statistically significant.

Results

The results of electrophoretic separation of total cf-DNA (nDNA and mtDNA) samples isolated from the urine of rats are presented in Figure 1. It demonstrates an electropherogram of urine cf-DNA collected from 3-month rats

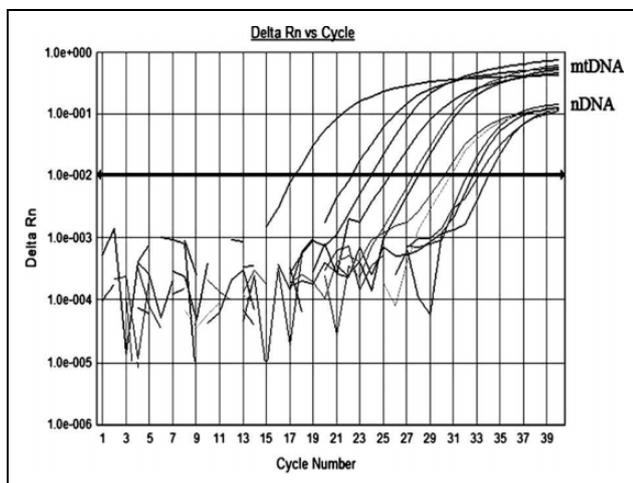


Figure 2. qPCR amplification curves for mtDNA and nDNA. Reactions were set up with DNA samples isolated from urine of different rats prior to irradiation. qPCR: quantitative polymerase chain reaction; mtDNA: mitochondrial DNA; nDNA: nuclear DNA.

prior to irradiation and administration of metformin and 6 h after irradiation as well as cf-DNA from the urine of 24-month rats collected after 6 h following metformin introduction. These results show that in all cases, the urine of rats contains degraded fragments of cf-DNA. The prevalent fraction of cf-DNA fragments is sized 300–600 bp. It is also seen that the fragments of cf-DNA do not differ significantly in size between rat urine samples collected before and after irradiation. These data also demonstrate that the size of transrenal cf-DNA fragments is not influenced by the age of rats and the introduction of metformin.

Figure 2 represents examples of kinetic curves of qPCR for cf-mtDNA and cf-nDNA from rat urine collected before irradiation. It is obvious that the exponential increase in the product amplification level of mtDNA occurs earlier than that of nDNA. The lower value of the threshold cycle indicates a greater copy number of mtDNA compared to nDNA. The reaction efficiency and the threshold fluorescence level were equal for all the reactions to be compared.

First of all, we carried out a comparative analysis for estimation of the contents of cf-mtDNA and cf-nDNA fragments in the urine of rats collected before X-ray irradiation and administration of metformin. The results testify that the quantity of cf-mtDNA in the urine of older rats is higher in comparison to that in the urine of 3-month rats. The copy numbers for fragments of both types of cf-DNA is much higher (cf-nDNA by 40% and cf-mtDNA by 50%) in the urine of 24-month rats, compared with the data obtained for their content in the urine of 3-month rats (Figure 3).

Summarized results of analysis of the content of cf-mtDNA and cf-nDNA in the urine of rats of different ages collected before and after exposure to IR at different times following irradiation (6, 12, 24, and 72 h) are presented in Figure 4. These results indicate that the quantitative content of cf-mtDNA and cf-nDNA in the urine of

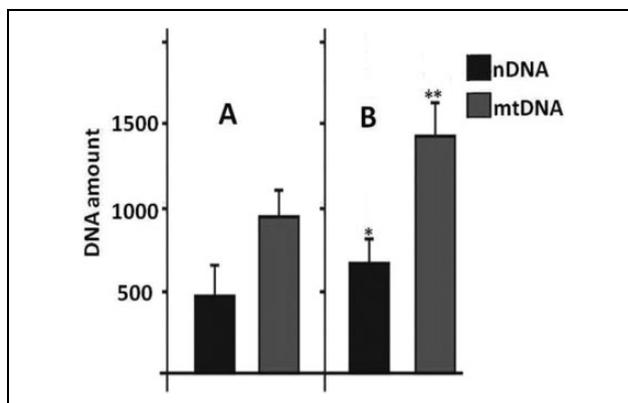


Figure 3. Contents of cf-DNA fragment copies in the urine of rats belonging to different age groups: (a) 3 months and (b) 24 months. Statistical significance was set at: * $p < 0.05$; ** $p < 0.01$. cf-DNA: cell-free DNA.

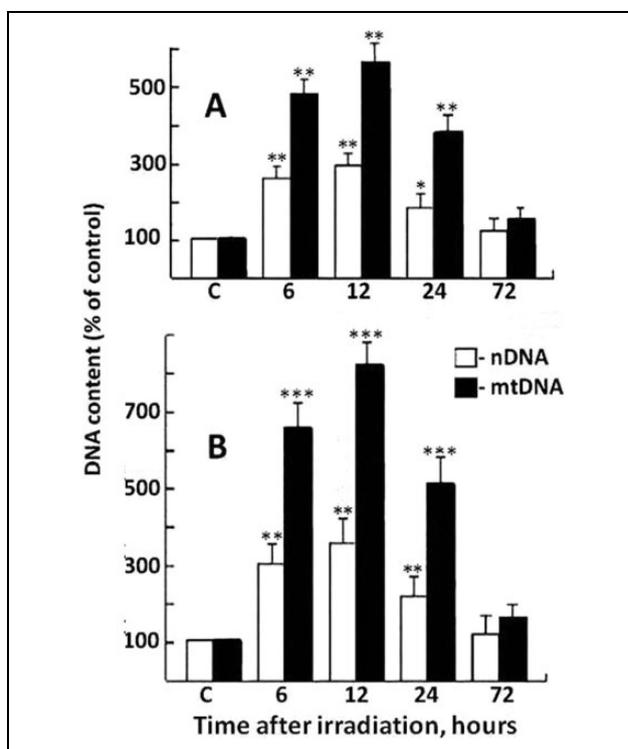


Figure 4. Percentage of cf-DNA from urine of rats belonging to different age groups, collected prior to irradiation (100%, C), and after 6, 12, 24, and 72 h following irradiation. (a) 3 months and (b) 24 months. Data are presented as mean \pm SEM ($n = 8$). Statistical significance was set at: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and $p < 0.05$ was considered statistically significant. cf-DNA: cell-free DNA; C: control.

3-month and aged rats change significantly depending on the duration of time between irradiation and urine collection.

Most notably, we observed a sharp rise in the levels of cf-mtDNA and cf-nDNA in urine in both the groups of rats after 6, 12, and 24 h following irradiation. A significant reduction of cf-DNA was registered in rat urine samples

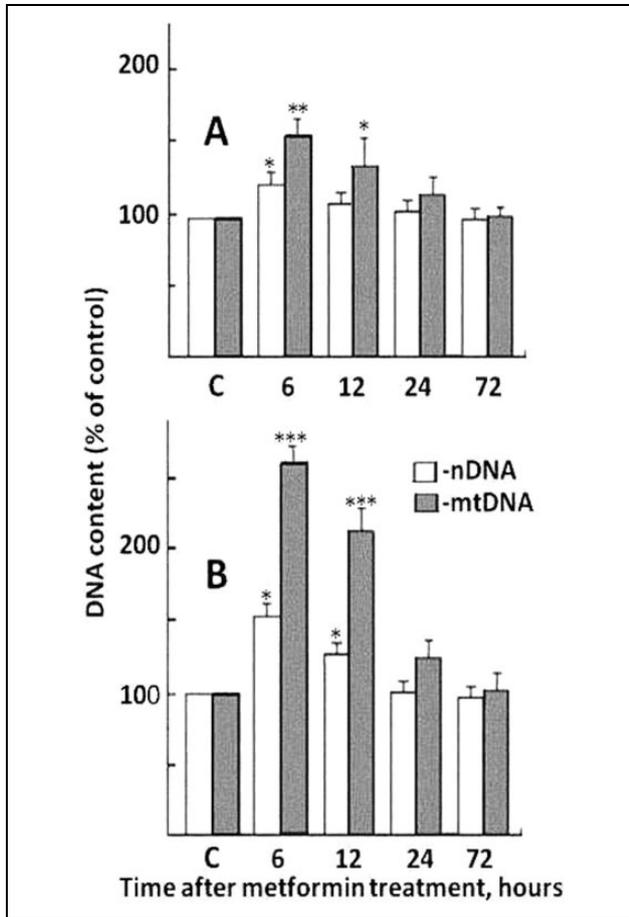


Figure 5. Percentage of cf-DNA from urine of rats belonging to different age groups, collected prior to administration of metformin (100%, C), and after 6, 12, 24, and 72 h following metformin treatment. (a) 3 months and (b) 24 months. Data are presented as mean \pm SEM ($n = 8$). Statistical significance was set at: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; and $p < 0.05$ was considered statistically significant. cf-DNA: cell-free DNA; C: control.

collected 72 h after irradiation. Comparison of analysis data obtained from 3- and 24-month-old rats shows that the percentage increase in the content of cf-DNA in urine is more pronounced in aged rats after irradiation. It can be seen that the urine of young rats following 6 and 12 h after irradiation contains 150–200% more cf-nDNA and 375–460% more cf-mtDNA (relative to the control data obtained from the same rats prior to irradiation). By the same time points following irradiation, we registered an increase in the urine of 24-month rats in the content of cf-nDNA and cf-mtDNA by 200–250% and 550–720%, respectively. Fragments of cf-mtDNA in the urine of aged rats exhibit the most prominent increase, as identified in these analyses.

Experimental data for determining cf-DNA in the urine of rats of different ages treated with metformin are presented in Figure 5. The obtained results show that metformin administration in rats also results in an increase of cf-DNA content in the urine of these animals. However,

the results of these analyses differ from the data obtained from irradiated rats, as may be expected. The increase in the content of cf-nDNA and cf-mtDNA fragments in the urine of these rats is registered only after 6 and 12 h following metformin administration. That is, the amount of cf-nDNA and cf-mtDNA fragments in the urine of 3-month-old rats after 6 and 12 h following metformin administration is increased by 25% and 55%, respectively. The urine collected after 12 h from the same rats exhibited elevated levels of cf-DNA only by the content of cf-mtDNA (by 30%). The percentage increase in the number of cf-nDNA and cf-mtDNA fragments in the urine of older rats after administration of metformin is significantly higher than that in 3-month-old rats (Figure 5). In particular, the levels of cf-nDNA and cf-mtDNA in the urine of aged rats are increased by 50% and 160%, respectively, after 6 h following administration of metformin. After 12 h following metformin administration, the levels of cf-nDNA and cf-mtDNA in the urine of the same animals exceed the control levels by 30% and 120%, respectively.

The demonstrated results show that regardless of the time point of cf-DNA analysis after irradiation or metformin administration, the quantitative content of cf-mtDNA fragments in the urine of young and aged rats is higher than that of cf-nDNA. These results preeminently indicate that mtDNA is represented by a larger number of copies containing PCR-amplified regions than in nDNA, in the composition of the total cf-DNA isolated from the urine of rats. We conducted a comparative analysis of changes of the content ratio cf-mtDNA/cf-nDNA in the urine of rats after X-irradiation and administration of metformin. The results are presented in Table 1, demonstrating that in most cases, the ratio cf-mtDNA/cf-nDNA has similar values (equal to 2.1–2.4; Table 1). However, the values for the ratio cf-mtDNA/cf-nDNA obtained from the analysis of cf-DNA contents in the urine of aged rats after 6 and 12 h following irradiation and treatment with metformin are significantly higher. In any case, the values of this ratio (cf-mtDNA/cf-nDNA) seem to be understated, given that mammalian somatic cells contain thousands of copies of mtDNA, even though they make up only about 1.0% of the total DNA content in the cell.

Thus, the results indicate that urine samples of rats collected after 6–12 h following IR irradiation and administration of metformin contain elevated levels of cf-nDNA and cf-mtDNA. Elevated levels of cf-nDNA and cf-mtDNA induced by IR and metformin in the urine of 24-month-old rats were significantly higher compared to those in the urine of 3-month-old rats.

Discussion

Excessive increase of cf-DNA contents in rat urine after IR irradiation indicates the induction of an active cell death process in the tissues of these animals. Irradiation of aged rats contributes to an even more significant increase in

Table 1. Ratio cf-mtDNA/cf-nDNA in the urine of rats of different age prior to irradiation and metformin administration and after treatment.

Treatment	Age, months	Control, prior to treatment	Time after irradiation and metformin administration (h)			
			6	12	24	72
X-irradiation	3	2.1 ± 0.1	2.3 ± 0.6	2.4 ± 0.3	2.2 ± 0.3	2.3 ± 0.2
	24	2.3 ± 0.2	2.7 ± 0.3*	2.8 ± 0.2*	2.3 ± 0.1	2.4 ± 0.4
Metformin administration	3	2.1 ± 0.1	2.4 ± 0.3	2.3 ± 0.1	2.4 ± 0.2	2.2 ± 0.3
	24	2.3 ± 0.2	3.8 ± 0.4*	3.3 ± 0.2*	2.3 ± 0.4	2.4 ± 0.2

cfDNA: cell-free DNA; mtDNA: mitochondrial DNA; nDNA: nuclear DNA.

*Values with significant difference from control for $p < 0.05$.

cf-nDNA and cf-mtDNA in their urine than the one observed in young rats. High levels of cf-DNA in the urine of aged rats subjected to IR is obviously associated with the presence of defective cells (damaged organelles and DNA) having low activity of reparation systems in the tissues of these rats prior to irradiation.²⁴ It should be noted that due to the reduction of activity in the functioning of protective and reparative systems, higher DNA damage and cell death rates can be observed in the kidneys, similarly to other tissues of aged animals, as compared to young animals.²⁵ It can also be assumed that elevation of cf-DNA level in the urine of irradiated rats is likely contributed by the development of radiation-induced nephropathy in these animals.²⁶

The process of autophagy can be stimulated under various cell disorders, upon oxidative stress, endoplasmic reticulum damage, imbalance of calcium homeostasis, and mitochondrial potential changes. Such and more severe disorders also occur in cells under the action of IR.^{16,27} Radiation-mediated induction of cell death via autophagy is less studied than programmed cell death. However, a number of studies indicate a possibility for radiation-induced cell death through the mechanism of autophagy. Radiation damage to mitochondria and post-irradiation energy expenditure lead to an increase in the ratio AMP/ATP. Perhaps it is the basic mechanism of AMPK activation in irradiated cells, which can stimulate autophagy.^{13,14}

As mentioned previously, metformin belongs to the group of compounds which can indirectly induce autophagy through AMPK activation.^{11,12}

Over 35 years ago it was established that antidiabetic biguanides are capable to reduce tumor induction and increase the life span in mice.²⁸ Currently, the research in this area gained a wide development, and antidiabetic drug metformin is regarded as an efficient tool for the prevention of carcinogenesis, aging, and combination therapy of tumors.^{29,30} It has been established that metformin exhibits the highest accumulation in mitochondria, inhibiting electron transport chain complex I and mitochondrial glycerophosphate dehydrogenase. This leads to the blockade of ATP synthesis and depletion of its intracellular pool, increasing the molecule ratio AMP/ATP.³¹ An elevated level results in activation of AMPK as a key regulator of

energy homeostasis.¹¹ The activation of AMPK stimulates catabolic pathways which lead to ATP generation and suppression of anabolic processes that consume ATP. AMPK activation also contributes to inhibition of mammalian target of rapamycin (mTOR) complex 1 (TORC1) and activation of uncoordinated-51-like kinase, which directly initiates autophagy.^{3,32} This is probably the most important mechanism of action of metformin. Nevertheless, its effects are not limited only to this mechanism. For instance, it has been shown recently that biguanides promote accumulation of AMP and related nucleotides in hepatocytes which inhibit glucagon signaling, suppress adenylate cyclase, and reduce cAMP level and activity of protein kinase A.³³ The results of our experiments demonstrated that in metformin-treated rats higher amounts of cf-nDNA and cf-mtDNA are excreted with urine. However, unlike the data obtained from irradiated rats, this increase is manifested not so dramatically, and is registered only after 6 and 12 h following metformin administration. Increased cf-DNA in the urine of X-irradiated rats may be due to cell death in tissues by various pathways as a result of cytotoxic and genotoxic effects of IR.^{15,16,27}

Since metformin does not exert genotoxic or cytotoxic effects and does not have nephropathic effects,^{18,19} on the contrary possessing a protective action, the observed increase of cf-DNA in urine might possibly be associated with cell death involving autophagy mechanisms. In older rats, metformin apparently activates the clearance of organs from higher amounts of damaged cells which can represent a potential risk for malignant transformation or development of a particular disease.²⁹

The analysis of the obtained data shows that in all experiments, the number of cf-mtDNA PCR copies, including the control data, is twice higher than the number of amplified cf-nDNA fragments (Table 1). However, the values of cf-mtDNA/cf-nDNA ratio obtained by analyzing the content of cf-DNA in the urine of aged rats after irradiation and administration of metformin (after 6 and 12 h) are significantly higher, compared with the data for urine samples from young rats. Since aging is accompanied by progressing induction and accumulation of damage in DNA, RNA, protein, and lipid macromolecules, additional damage caused by IR irradiation or autophagy activation in the

tissues of older animals results in enhancement of cell death and urinary excretion of cf-DNA. Upon additional damage of cells and DNA, autophagy may be involved in a comprehensive DNA damage response system.¹⁴ Therefore, it can be assumed that increased urinary excretion of cf-DNA in aged rats, following administration of metformin, is caused by the death of cells with structural and functional disorders. At least elevated excretion of cf-mtDNA with urine in aged rats after metformin administration results from selective removal of defective mitochondria (mitophagy) by autophagy mechanisms.^{2,3} Previously it has been demonstrated that activation of autophagy in aged rats caused by calorie restriction or administration of an antipolytic agent or everolimus (inhibitor of mTOR) results in a sharp increase in 8-oxy dG due to preferential degradation of damaged mtDNA copies.^{34,35} It is possible that other mechanisms also promote cell death under these conditions. It is known that although autophagy and programmed cell death occur in different ways, they are closely related, and their regulatory components may interact, contributing to the development of these processes and determining whether a cell lives or dies.³⁶ In conclusion, it can be mentioned that Menendez and coauthors have earlier expressed a hypothesis, according to which metformin can function in the tissues as a “sweeper” of dysfunctional precancerous cells preventing them from piling up and completely transforming into malignant cells.³⁷ The results of our experiments seem to demonstrate the real mode of action of metformin as a sweeper contributing to elimination of dysfunctional cells from the tissues in aged animals.

Declaration of conflicting interests

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