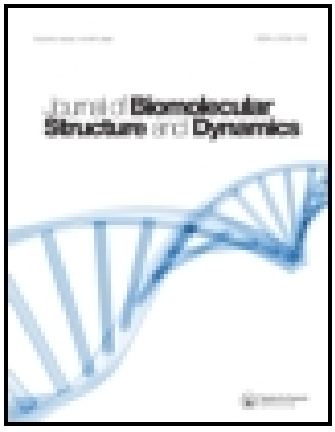

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Investigation of irradiated rats DNA in the presence of Cu(II) chelates of amino acids Schiff bases

N.H. Karapetyan[a], A.L. Torosyan[a], M. Malakyan[b], S.A. Bajinyan[b] & S.G. Haroutiunian[a]
[a] Department of Physics, Yerevan State University, Al. Manoogian 1, Yerevan 0025, Armenia
[b] Centre of Radiation Medicine and Burns, P.O. Box 25, Davidashen, Yerevan 0048, Armenia
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Investigation of irradiated rats DNA in the presence of Cu(II) chelates of amino acids Schiff bases

N.H. Karapetyan[a]*, A.L. Torosyan[a], M. Malakyan[b], S.A. Bajinyan[b] and S.G. Haroutiunian[a]

[a]*Department of Physics, Yerevan State University, Al. Manoogian 1, Yerevan 0025, Armenia;* [b]*Centre of Radiation Medicine and Burns, P.O. Box 25, Davidashen, Yerevan 0048, Armenia*

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The new synthesized Cu(II) chelates of amino acids Schiff bases were studied as a potential radioprotectors. Male albino rats of Wistar strain were exposed to X-ray whole-body irradiation at 4.8 Gy. This dose caused 30% mortality of the animals (LD_{30}). The survival of animals exposed to radiation after preliminary administration of 10 mg/kg Cu(II)(Nicotinyl-L-Tyrosinate)$_2$ or Cu(II)(Nicotinyl-L-Tryptophanate)$_2$ prior to irradiation was registered about 80 and 100% correspondingly. Using spectrophotometric melting and agarose gel electrophoresis methods, the differences between the DNA isolated from irradiated rats and rats pretreated with Cu(II) chelates were studied. The fragments of DNA with different breaks were revealed in DNA samples isolated from irradiated animals. While, the repair of the DNA structure was observed for animals pretreated with the Cu(II) chelates. The results suggested that pretreatment of the irradiated rats with Cu(II)(Nicotinyl-L-Tyrosinate)$_2$ and Cu(II)(Nicotinyl-L-Tryptophanate)$_2$ compounds improves the liver DNA characteristics.

Keywords: DNA; X-ray irradiation; melting; electrophoresis

Introduction

The study of biological effects of ionizing radiation is very important, since all living organisms are exposed to natural background radiation, which constitutes the cosmic rays and radiation from earth crust radioactive elements. At the same time, rapid development of nuclear power and widespread using of ionizing radiation research sources in various fields of science and technology created the potentially threatening radiation danger. Finally, the development of effective methods of radiation therapy of tumors and other pathological conditions is not possible without the direction changes in sensitivity of cells and tissues to radiation (Min, Lee, & Gu, 2003).

The most important task of modern radiobiology is the study of ionizing radiation lethal and mutagenic action mechanisms. Damages of the cells unique control systems play a crucial role in the development of radiobiological reactions leading to cell death, initiation of gene, and chromosome mutations. Ionizing radiation induces direct DNA damage and indirect damage through the radiolysis of water. Exposure to ionizing radiation inflicts single-strand breaks, double-strand breaks (DSB), base damages, and DNA-protein cross-links in the genomic DNA (Nikjoo, O'Neill, Terrissol, & Goodhead, 1994; Shikazono, Noguchi, Fujii, Urushibara, & Yokoya, 2009).

Possibility of protection from ionizing radiation, when an organism absorbs radiated energy, practically does not exist. In the previous investigations, no medicine has been invented that could successfully be applied in therapy of radiation diseases. Today, in the cases of accidental irradiation with high doses of radiation, it is possible to apply only conservative and symptomatic therapy with poor prospects that results of treatment will be positive. Therefore, the activity of numerous investigators is directed toward finding an effective radioprotective means that would successfully prevent development of radiation syndrome and protect cells and tissue from free radicals (Kljajic, Breyer, Horsic, & Milosevic, 1986; Weiss & Landauer, 2003).

Amino acid Schiff bases and their metal complexes are important class of compounds in medicinal and pharmaceutical field. They have antibacterial, antifungal, and antitumor activity (Malakyan, Bajinyan, Matosyan, Tonoyan, & Babayan, 2009).

Copper plays a key role as an integral component of many enzymes. While trace amounts of copper are required for normal metabolic processes, it can be extremely toxic in excess. It has the ability to generate free radicals and oxidize cellular components. Copper exists only in bound forms in the body both in metalloproteins and in low-molecular weight complexes to avoid its

*Corresponding author. Email: nelkh@ysu.am

inherent toxicity (Deschamps, Kulkarni, Gautam-Basak, & Sarkar, 2005).

The goal of this research was to identify radioprotective properties of new synthetic Cu(II) chelates of Schiff bases of Nicotinyl-L-Amino acids: Cu(II)(Nicotinyl-L-Tyrosinate)$_2$ and Cu(II)(Nicotinyl-L-Tryptophanate)$_2$. The differences in DNA structure of irradiated rats and the rats pretreated with Cu(II) complexes before irradiation were studied.

Investigations included survival studies as well as a DNA analyses. The defects in the structure of DNA were investigated using thermal denaturation and agarose gel electrophoresis methods.

Materials and methods

All using chemicals and reagents of analytical reagent grade were obtained from Sigma-Aldrich Stanford, California, USA.

The new Cu(II)(Nicotinyl-L-Tyrosinate)$_2$ and Cu(II)(Nicotinyl-L-Tryptophanate)$_2$ compounds (Figure 1) were synthesized at the Scientific Centre of Radiation Medicine and Burns (Yerevan, Armenia) (Malakyan et al., 2009), and kindly presented by Dr V. Matosyan and Dr V. Tonoyan for animals study.

The molecular weights of compounds are 620 D and 650 D, respectively. In experiments, *in vitro* Cu(II)(Nicotinyl-L-Tyrosinate)$_2$ and Cu(II)(Nicotinyl-L-Tryptophanate)$_2$ possess the expressed concentration-dependent SOD-mimetic activity (Malakyan et al., 2009).

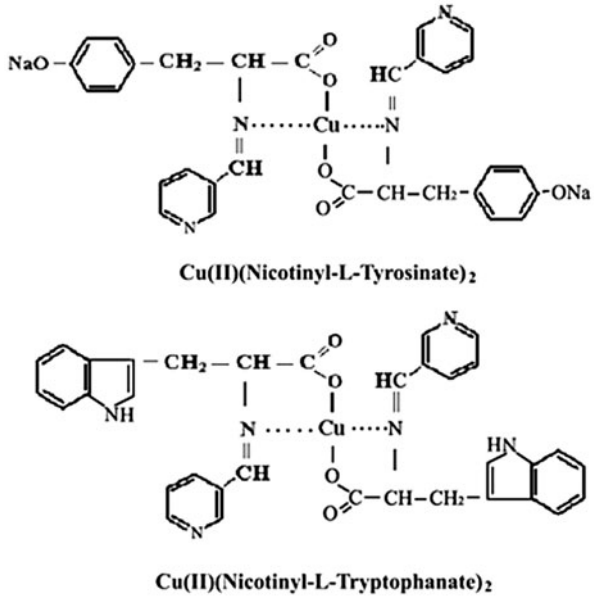

Figure 1. The chemical structure of Cu(II)(Nicotinyl-L-Tyrosinate)$_2$ and Cu(II)(Nicotinyl-L-Tryptophanate)$_2$.

Experimental animals and treatment

Male albino rats of Wistar strain (120–140 g, about 6–8 weeks old) were housed under standard conditions. The investigated rats were separated into five groups. Each group contained 10 animals. Healthy animals served as the control – group I. The animals of group II were exposed to X-ray irradiation at 480 R dose level (LD_{30}). The rats of the group III were pretreated subcutaneously with 10 mg/kg Cu(II)(Nicotinyl-L-Tyrosinate)$_2$ 6 h before exposure to X-ray irradiation at 480 R dose level. The rats of the group IV obtained 10 mg/kg Cu(II)(Nicotinyl-L-Tryptophanate)$_2$ subcutaneously 24 h prior to irradiation at 480 R. The rats of the group V obtained 10 mg/kg Cu(II)(Nicotinyl-L-Tyrosinate)$_2$ or Cu(II)(Nicotinyl-L-Tryptophanate)$_2$, but not irradiated. To be undergone to X-ray whole-body irradiation, rats were located in the distance 50 cm from the radiation source. 'RUM-17' roentgen facility was used for irradiation. The radiation dose rate was 1.78 Gy/min.

The animals survival rates were determined as the number of survived rats in percentage to total number of animals (10 rats per group) in the each of above mentioned groups after 30 days post irradiation. In order to perform analyses, rats were sacrificed under anesthesia on 7, 14, and 30 days after irradiation (per 3 rats at each point), and DNA were isolated from animals liver for analyses.

DNA isolation

DNA was isolated from rats livers using standard chloroform technique as described previously (Karapetyan, Ananyan, Torosyan, & Dalyan, 2007). The optical characteristics of DNA was determined by the ratio of $A_{260}/A_{280} = 1.8$, $A_{260}/A_{230} = 2$, which corresponded to the highly purified DNA (Sambrook, 1989). DNA concentrations calculated in base pairs, were determined spectrophotometrically with $\epsilon = 1.31 \times 10^4\ M^{-1}\ cm^{-1}$, at 260 nm (Wells, Larson, Grant, Shortle, & Cantor, 1970).

Spectroscopy

The absorption spectra and Ultraviolet melting curves of DNA samples were recorded on a Lambda 800 UV/VIS spectrometer (Perkin-Elmer). All the experiments were carried out in .1 SSC-buffered saline (1 SSC = .015 M sodium citrate, .15 M NaCl), pH 7.2.

Melting experiments were carried out at 25–95 °C temperatures interval, using 10 mm thermostatic quartz cuvettes. The heating rate was .5 °C/min, while absorbance at 260 nm was recorded. The degree of DNA denaturation $1-\theta$ vs. temperature was calculated using the formula:

$$1-\theta=\frac{A-A_{\min}}{A_{\max}-A_{\min}}$$

where θ is the degree of helicity, A, $A_{\min}$, and $A_{\max}$ are the absorbances of the experimental curve, the lower baseline (before melting), and the upper (after melting) baseline, respectively, at a given temperature T (Wartell & Benight, 1985). The melting temperature, T_m, is defined as the temperature at which half of the total base pairs are 'melted,' i.e. $1-\theta=.5$.

ΔT is the width of the melting interval equaled to the difference of temperatures, at which the tangent in the bend point crosses the levels $\theta=0$ and $\theta=1$, i.e.

$$\Delta T=\left(\frac{\partial\theta}{\partial T}\right)^{-1}_{T=T_0}$$

The hypochromic effect of DNA melting (Δh) was calculated by the formula:

$$\Delta h=\left(\frac{A_{95\,^\circ C}}{A_{25\,^\circ C}}-1\right)\times 100\%$$

where A_{25} °C and A_{95} °C are the absorbances corresponding to the entire helical and to the entire coiled conditions of DNA (Wartell & Benight, 1985).

Electrophoresis

The DNA samples containing 4 μg of DNA were separated in 1% agarose gel, using Tris-boric acid-EDTA buffer (.089 M Tris-borate, .002 M EDTA, pH 8.0). The separation was performed under condition of 5 V/cm. One kilobase DNA Ladder (Promega Product, USA) was used as a DNA fragments marker (250–10,000 base pairs). To detect DNA, after separation, gels were kept in a solution of ethidium bromide (.5 mg/ml). Photographs of gels were made in transmitted UV light, digital camera, and hp photosmart 735 brand (Sambrook & Russell, 2001).

Results and discussion

Pretreatment of animals with 10 mg/kg Cu(II)(Nicotinyl-L-Tyrosinate)$_2$ 24 h before irradiation or Cu(II)(Nicotinyl-L-Tryptophanate)$_2$ 6 h prior to radiation exposure to X-rays at 4.8 Gy dose level leads to radioprotection effect. According to the obtained results, in the group of animals pretreated with Cu(II)(Nicotinyl-L-Tryptophanate)$_2$, no mortality was observed, so 100% animal survival was registered. Relatively less expressed radioprotective effect was produced upon administration of Cu(II)(Nicotinyl-L-Tyrosinate)$_2$ to rats: survival in 30 days after exposure amounted 80%. In irradiated control group, survival of animals made only 50%.

The DNA samples were isolated from rats liver on 7, 14, and 30 days of irradiation and were analyzed using UV melting and electrophoresis methods.

DNA melting study

The DNA melting (the temperature-dependent dissociation of original double-stranded DNA) curves were observed using UV absorbance measurements. The heat denaturation process of normal DNA, the DNA from irradiated rats, and rats pretreated with Cu(II) complexes were analyzed. The melting temperature (T_m), the width of the melting interval (ΔT), and hypochromicity (Δh) give information about DNA stability and DNA secondary structure defects. Using this technique, it is possible to do conclusion about DNA intermolecular or intramolecular crosslinks (Cai, Cloutier, Sanche, & Hunting, 2005; Nadareishvili, Sanaya, Tevdoradze, & Mozdokeli, 1991; Privalov & Dragan, 2007). It is well known that ionizing radiation can modify DNA structure depended on the irradiation dose. For example, small doses of radiation cause inter- and intramolecular covalent crosslinks in DNA. High doses of radiation make the single-strand breaks and DSB (Cai et al., 2005; Georgakilas, Sakelliou, Sideris, Margaritis, & Sophianopoulou, 1998; Haveles, Georgakilas, Sideris, & Sophianopoulou, 2000; Lankinen, Vilpo, & Vilpo, 1996).

Figure 2 shows the normalized melting curves of DNA isolated from I, II, III, and IV groups of rats on 7,14, and 30 days after irradiation. The melting curves of DNA extracted from the rats liver of group V do not differ from melting curves of healthy rats DNA (these curves are not shown).

As can be seen in Figure 2, significant changes of the melting curves profile and shift of the curves to lower temperature range are observed for irradiated rats DNA (group II) in comparison to the healthy control. The same results related the melting parameters of the irradiated DNA are described in the literature (Iurgaĭtis, Lazurkin, & Bannikov, 1979). On the base of DNA melting curves, the differences in the melting temperature (T_m), interval of helix-coil transition (ΔT), and hypochromicity (Δh) were revealed for investigated groups. The DNA melting parameters calculated from obtained melting curves are summarized in Table 1.

The melting temperature T_m of irradiated rats DNA decreased to 68.8, 68.5, and 67.6 °C on 7, 14, and 30 days correspondingly, in comparison to 71.3 °C for normal DNA. Decreasing of T_m indicates the destabilization of DNA molecules. Probably, it can be explained by the DNA strand breaks and breaks of hydrogen bonds between the DNA strands during irradiation. These assumptions are in good agreement with published data (Cai et al., 2005; Nadareishvili et al., 1991).

The width of the melting interval ΔT of irradiated DNA increased to 8, 15, and 18.5 °C on 7, 14, and 30 days correspondingly, in comparison to 7.2 °C for normal DNA. This fact allowed assuming that irradiated DNA samples became more heterogenic. This

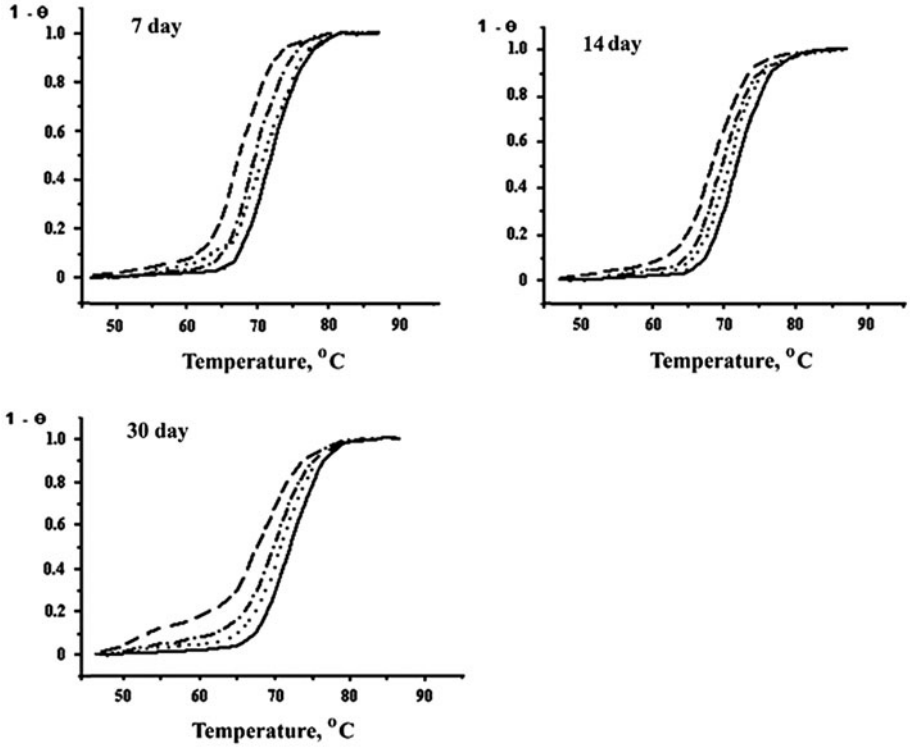

Figure 2. The melting curves of DNA isolated from I, II, III, and IV groups of rats on 7, 14, and 30 days after irradiation. (-): Norm control, (- -): irradiated control, (- • -): Cu(II)(Nicotinyl-L-Tyrosinate)$_2$ pretreated, and (• •): sCu(II)(Nicotinyl-L-Tryptophanate)$_2$ pretreated.

Table 1. The melting parameters of DNA isolated from I, II, III, and IV groups of rats on 7, 14, and 30 days after irradiation.

Study groups	T_m (°C)	ΔT (°C)	Δh (%)
Norm control	71.3 ± .2	7.2 ± .15	33 ± .15
7 Days			
Irradiated control, 480 R	68.8 ± .15	8 ± .25	19 ± .2
Cu(II)(Nicotinyl-L-Tyrosinate)$_2$ + 480 R	69.6 ± .1	7.25 ± .2	25.7 ± 15
Cu(II)(Nicotinyl-L-Tryptophanate)$_2$ + 480 R	70 ± .15	7.15 ± .15	24.4 ± .2
14 Days			
Irradiated control, 480 R	68.5 ± .15	15 ± .2	18.7 ± .25
Cu(II)(Nicotinyl-L-Tyrosinate)$_2$ + 480 R	70 ± .1	7.6 ± .15	24.7 ± .2
Cu(II)(Nicotinyl-L-Tryptophanate)$_2$ + 480 R	70.7 ± .15	7.9 ± 2	25 ± .15
30 Days			
Irradiated control, 480 R	67.6 ± .2	18.5 ± .15	18 ± .15
Cu(II)(Nicotinyl-L-Tyrosinate)$_2$ + 480 R	70.5 ± .1	9.3 ± .1	24.8 ± .2
Cu(II)(Nicotinyl-L-Tryptophanate)$_2$ + 480 R	71 ± .15	8.5 ± .2	27.6 ± .15

heterogeneity testifies the presence of the different defects in the secondary structure of irradiated DNA in comparison to the norm. It is shown that values of ΔT and T_m are determined by length of DNA helix sites. The most objective information on defects in secondary structure of DNA can be received from ΔT changes (Lyubchenko, Frank-Kamenetskii, Volgodskii, Lazurkin, & Gause, 1976).

The hypochromicity (Δh) is the most informative factor related to helicity of DNA samples. The defects of DNA secondary structure, such as single-strand breaks and DSB and partially untwisted regions, can decrease

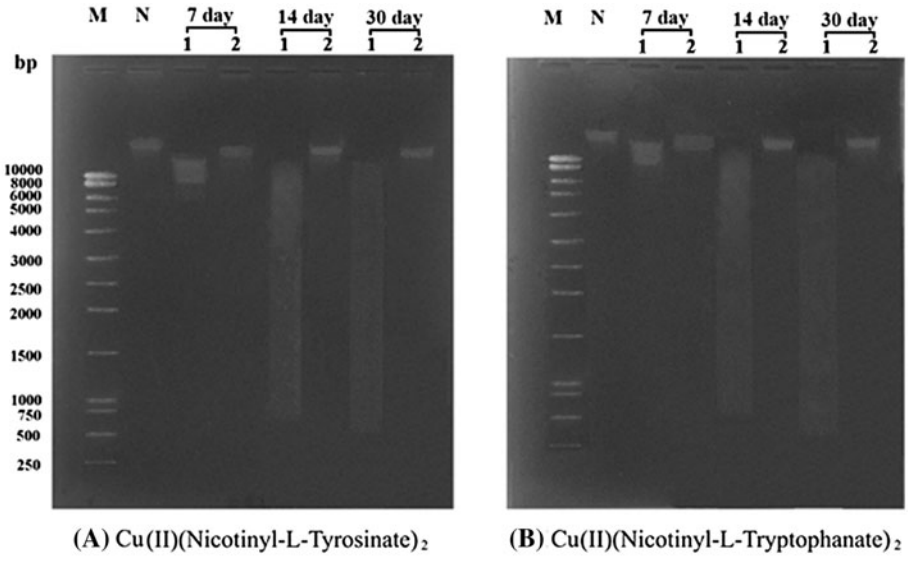

Figure 3. Electrophoresis of DNA isolated from I, II, III, and IV groups of rats on 7, 14, and 30 days after irradiation. M-marker and N-norm control. (A) 1-Irradiated control, 2-pretreated by Cu(II)(Nicotinyl-L-Tyrosinate)$_2$ on 7, 14, and 30 days. (B) 1-Irradiated control 2-pretreated by Cu(II)(Nicotinyl-L-Tryptophanate)$_2$ on 7, 14, and 30 days.

the hypochromicity of DNA. For native DNA, hypochromic effect is 30–37%. In this study, hypochromicity was equal to 33%. Irradiated rats DNA hypochromicity decreased to 19, 18.7, and 18% on 7, 14, and 30 days correspondingly. The decrease of this index indicates the presence of partially melted DNA regions.

As it is seen in Figure 2, pretreatment of rats with Cu(II)(Nicotinyl-L-Tyrosinate)$_2$ or Cu(II)(Nicotinyl-L-Tryptophanate)$_2$ (group III, IV) leads to shift the melting curves back to the norm. From the data presented in Table 1, may be seen, that the melting parameters of irradiated rats DNA closely approximated to the normal. Thus, for pretreated rats DNA, increasing of the T_m and Δh, decreasing of the ΔT in comparison to irradiated rats DNA were observed on 7, 14, and 30 days. This effect is more evident in case of Cu(II)(Nicotinyl-L-Tryptophanate)$_2$ pretreatment.

DNA electrophoresis

Agarose gel electrophoresis is an easy way to separate DNA fragments by their sizes and visualize them. Nucleic acids migration from negative to positive electrodes is due to the naturally occurring negative charge carried by their sugar–phosphate backbone. Double-stranded DNA fragments naturally behave as long rods, so their migration through the gel is relative to their size. Electrophoresis in native conditions reveals intermolecular crosslinks and DSB. Formation of DNA intermolecular crosslinks reveals the fraction with low mobility, that is a high-molecular weight. In the presence of DNA breaks, the fragments with high electrophoretic mobility that is a low-molecular weight detected on the agarose gel (Myllyperkiö, Koski, Vilpo, & Vilpo, 1999; Valenzuela, Núñez, Guerrero, Villalobos, & Ruiz de Almodóvar, 2000).

Using this technique it is possible to identify and estimate post-irradiation DNA damages. Figure 3 depicts the respective agarose gel electrophoretic patterns of DNA isolated from irradiated (group II) and Cu(II)(Nicotinyl-L-Tyrosinate)$_2$ or Cu(II)(Nicotinyl-L-Tryptophanate)$_2$ pretreated (groups III and IV) rats, compared with the norm (group I) and standard DNA marker (M). As can be seen, the norm rats DNA, group I (marked N) exhibited a homogeneous stain with clear edge, which corresponds to high-molecular DNA, with 15 kbp size. The data presented in Figure 3 clearly reveal absents of DNA with high-molecular weight on the electrophoresis treks of irradiated rats DNA on 7, 14, and 30 days (Figure 3(A) and (B), marked 1). Irradiated DNA patterns exhibit the high electrophoretic mobility on 7, 14, and 30 days. The presence of fractions with high electrophoretic mobility is a fact that demonstrates the fragmentation of DNA (Dahm-Daphi & Dikomey, 1995). The DNA fragments with different lengths are visualized in the irradiated fractions.

Thus, the fragments relevant to 8–14, .6–10, and .4–10 kbp were found after 4.8 Gy irradiation on 7, 14, and 30 days correspondingly. As can be seen, the DNA damages in irradiated patterns are increasing in time. This is probably due to the increasing of free radical processes after irradiation. At the same time there are published data, according to which activation of endogenous nucleases occurs during X-irradiation, which led to

accumulation of single-strand breaks and DSB in DNA (Bakayev, Yugai, & Luchnik, 1985). So, our data corresponds well with the literature, where revealed the different fragments with strand breaks in DNA samples obtained from irradiated animals (Torudd et al., 2005).

Figure 3 clearly shows that DNA samples isolated from rats pretreated with the Cu(II) chelates have better electrophoretic characteristics (Figure 3(A) and (B) marked 2), than irradiated rats DNA (Figure 3, marked 1). The DNA samples obtained from pretreated rats liver have the electrophoretic bands similar to healthy DNA. Thus, the fragments of DNA relevant to 14–15 kbp were detected on 7, 14, and 30 days. This fact indicates that the investigated compounds protect the DNA from damages. Oxidative stress is one of the major causes of DNA damage. The DNA oxidative damage occurs most readily at guanine residues due to the high oxidation potential of this base relative to cytosine, thymine, and adenine (Buechter, 1988). Ionizing radiation as oxidative stress inducer has sufficient energy to eject an electron from molecules, with the critical target being DNA to give DNA radical. When an electron is ejected, an unstable DNA radical is produced, which is exposed to rapid biochemical reactions, and results in the formation of potentially lethal single-strand breaks and DSB in the DNA (Ly, Bullick, Won, & Milligan, 2006).

On the basis of data obtained, radioprotective properties of Cu(II)(Nicotinyl-L-Tyrosinate)$_2$ and Cu(II)(Nicotinyl-L-Tryptophanate)$_2$ are possible to explain due to their SOD-mimetic activity.

Conclusion

According to the new data, the copper complexes exhibit antimicrobial, anti-inflammatory, antiulcer, anticancer, anticarcinogenic, analgesic, and radioprotectant activities. The newly prepared Cu(II) Schiff base compounds were screened for their radioprotective activity. The rats pretreated with Cu(II)(Nicotinyl-L-Tyrosinate)$_2$ and Cu(II)(Nicotinyl-L-Tryptophanate)$_2$ before irradiation had 80 and 100% survival at 30 days correspondingly. Examination of the DNA melting curves and DNA electrophoretic bands to help revealed an improvement of rat liver DNA characteristics in case of animal pretreatment with novel Cu(II) Schiff base complexes before ionizing radiation exposure. Based on obtained data, it is possible to conclude that Cu(II)(Nicotinyl-L-Tryptophanate)$_2$ protects the DNA from damages better than Cu(II)(Nicotinyl-L-Tyrosinate)$_2$.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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