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Protective effect of some amino acids synthesized derivatives and their chelates on *Escherichia coli* under X-ray irradiation

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The protective effects of novel synthesized derivatives of some amino acids — nicotinyl-L-tyrosinate and nicotinyl-L-tryptophanate schiff bases and their Cu(II) and Mn(II) chelates on growth, survival and membrane-associated ATPase activity of *E. coli* under X-ray irradiation were investigated. The specific growth rate and survival of *E. coli* were decreased at 10, 20 and 30 Gy doses. However, as 30 Gy was found to be the most effective irradiation dose, it was chosen for studying the radio-protective properties of different compounds. These compounds could increase the bacterial cell protection against X-ray irradiation in concentration-dependent manner. They had a role in stimulation of synthesis or regulation of activity of metal-dependent enzymes, required for reversing the X-ray irradiation damage. The study may prove useful for further estimation of the effectiveness of different compounds as radio-protectors on bacteria and other cells, especially mammalian cells under X-ray irradiation.

Keywords: X-ray irradiation, Radio-protective compounds, Bacterial growth and survival, ATPase activity, *Escherichia coli*

It is well-known that X-rays damage bacterial cells, but the exact mechanism of effects is still unknown. Damage by ionizing irradiation leads to cell death and occurs primarily through random deposition of energy in vital cellular macromolecules that are responsible for cell proliferation. DNA, the major and most important molecule in the cell is undoubtedly affected due to irradiation[1,2]. X-rays interfere also with many synthetic pathways in bacterial cells[3].

X-rays also cause critical lesions in cell membrane which is considered as one of its main target[4,5]. The alterations in membrane fluidity and permeability, reduction in electrophoretic mobility, loss of proteins and inactivation of membrane-associated proteins due to irradiation have been demonstrated[6]. These might affect transport properties and enzymatic activity in the cell membrane, particularly the primary transport systems and main enzymes, such as proton F_0F_1-ATPase in bacteria[7]. The changes in membrane properties affect *E. coli* cell growth and survival[8]. Penicillin and other membrane-associated antibiotics at non-lethal concentration have shown significant additional bactericidal effects on irradiated cells[5,9]. Similar effects have been noticed in case with extremely high frequency electromagnetic irradiation[8,10].

X-ray irradiation induce cell death probably via apoptosis[11,12]. Radioactive irradiation can stimulate intracellular production of specific compounds, such as hydroperoxides as a protective reaction to the stress in *E. coli*[13]. It causes peroxidation of lipids and alterations in membrane. It is demonstrated that interaction of oxygen with sites of energy deposition in DNA plays a smaller role in radiosensitization than on the membrane[5]. To overcome the harmful effects of irradiation, there is need for utilization of radio-protectors. The increased need for safe and effective sources of protection from the health hazards of unintended ionizing irradiation exposures is obvious[14-17]. Currently, the most commonly used radio-protectors that exhibit antioxidant reactivity are highly toxic in effective doses[12,18].

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Recently, the compounds of aminothiol family have shown potential as cyto-protectants for tissues treated with irradiation or radiomimetic chemicals[17,19]. The possible radio-protective effects of novel synthesized nicotinyl-L-tyrosinate (NLTyr) and nicotinyl-L-tryptophanate (NLTrp) schiff bases and their Cu(II) and Mn(II) chelates Cu(II)(nicotinyl-L-tyrosinate)$_2$ (CuNLTyr), Cu(II)(nicotinyl-L-tryptophanate)$_2$ (CuNLTrp), Mn(II)(nicotinyl-L-tyrosinate)$_2$ (MnNLTyr) and Mn(II)(nicotinyl-L-tryptophanate)$_2$ (MnNLTrp) on rats lethality, on their blood cells membrane properties[20] and DNA[21] have been demonstrated. Possible regulatory effect on the physicochemical characteristics of cell membrane is suggested to be due to the lipophilic properties and capability of these compounds to transfer across cell membrane[20-22]. The protective effects in rats might be due to antioxidant properties and binding of free radicals that damage cell and drive the apoptosis. Moreover, these compounds also probably inhibit radiation-induced DNA fragmentation and caspase activation[20,21].

In view of the versatile coordinating properties and the structural similarities of these compounds with natural biological substances, in this study, probable radio-protective effects of these compounds have been investigated on growth, survival and membrane-associated ATPase activity of *E. coli* under X-ray irradiation. *E. coli* is used for experiments, as it is a widespread gut commensal of vertebrates and a versatile pathogen organism[23,24], as well as is the best characterized simple organism of a special interest.

Materials and Methods

Reagents and others

NLTyr and NLTrp Schiff bases and their Cu(II) (CuNLTyr, CuNLTrp) and Mn(II) (MnNLTyr, MnNLTrp) chelates were from the Center of Radiation Medicine and Burns, Yerevan (Armenia)[21]. These compounds were derived from nicotine aldehyde and L-tyrosine or L-tryptophan, respectively. Peptone and DCCD from Carl Roth GmbH (Germany), glucose and agar from Sigma (USA) and other reagents of analytical grade were used in assays.

The data were processed for statistical averages at least from four replicates of independent measurements with determination of the standard errors (< 3%). The validity for difference between the data series was evaluated with the Student`s validity criteria (p) using SigmaPlot software[8,31]. When not mentioned, $p<0.001$ represented for the differences between the values without (control) and after X-ray irradiation.

Bacteria and preparation to assays

E. coli wild-type strain K-12(λ) was used in the study. Bacteria were grown in peptone medium (0.2% peptone, 0.5% NaCl and 0.2% K_2HPO_4, pH 7.5) under anaerobic conditions with glucose (0.2%) at 37°C as described elsewhere[8,10,25-28]. pH of growth and assays mediums was determined by a pH-potentiometer with selective electrode (HJ1131B, HANNA Instruments, Portugal).

Grown cells were harvested and concentrated by centrifugation (3600 *g*) at room temperature. The bacteria were washed and diluted into bi-distilled water and divided in different samples with the appropriate concentrations of synthesized compounds (NLTyr, NLTrp, CuNLTyr, CuNLTrp, MnNLTyr and MnNLTrp) and then subjected to X-ray irradiation.

X-ray irradiation technique

Bacteria were irradiated using X-ray machine of RUM-17 model (USSR). This device was operated at 180 V with a tube current of 15 mA without filters. The resulting dose-rate at the focus-to-surface distance of 15 cm was 19.6 Gy/min; calibration was done with a dosimeter (RadCal, USA). *E. coli* were irradiated with doses of 10, 20 and 30 Gy. After irradiation, bacteria were immediately transferred into the growth or assay mediums.

Bacterial growth calculation and survival determination

Bacterial growth was monitored with the Spectro UV–Vis Auto spectrophotometer (Labomed, USA) as described previously[10,28]. The specific growth rate was calculated over the interval, where the logarithm of absorbance of the culture at 600 nm increased linearly with the time and expressed as 0.693/doubling time.

Bacterial survival was determined by displacement of bacteria into the minimal salt medium (46 mM K_2HPO_4, 23 mM KH_2PO_4, 8 mM $(NH_4)_2SO_4$, 0.4 mM $FeSO_4$, 6 mM $MgSO_4$, pH 7.5) during 5 days[8,10,27]. The number of viable bacteria was calculated by counting colony-forming units grown on plates with solid nutrient medium.

ATPase activity assay

ATPase activity of membrane vesicles was measured spectrophotometrically by the amount of inorganic phosphate (P_i) liberated after adding 3 mM ATP as described[26,29,30]. The membrane vesicles from

lysozyme-treated spheroplasts were isolated by the method of osmotic lysis[8,30,31]. The protein was determined by the Lowry method using bovine serum albumin as a standard[26,29,30]. For ATPase assay, 50 mM Tris-Cl buffer with 0.4 mM $MgSO_4$ and 100 mM KCl (pH 7.5) was used. Corrections were made for blanks without ATP or membrane vesicles. Relative ATPase activity was expressed in μM P_i/mg protein/min^{-1}.

N,N'-dicyclohexycarbodiimide (DCCD), non-specific inhibitor of the F_0F_1-ATPase[8,29,31,32] at 0.2 mM was used in membrane vesicles treatment. The vesicles were treated with DCCD for 10 min prior to the assay. The DCCD-sensitive value was a difference between the values in the presence and absence of DCCD in parallel measurements[8,33].

Results

X-ray irradiation effects on *E. coli* growth and survival

The effects of 10, 20 and 30 Gy X-ray irradiation on *E. coli* revealed the loss of reproductive ability of bacteria. The depression of growth rate (Fig. 1a) and survival (Fig. 1b) compared with the non-irradiated (control) bacteria was dose-dependent. The growth rate of control was considered as 100%. The 10, 20 and 30 Gy irradiation depressed the bacterial growth up to 90%, 80% and 70%, respectively ($p<0.025$, $p<0.01$ and $p<0.02$, respectively) (Fig. 1a), as compared to the control. The survival of control bacteria within 5 days was decreased 9-fold, while the viability loss of X-ray irradiated bacteria with 10 and 20 Gy was ~10-fold ($p<0.02$) (Fig. 1b). In case with 30 Gy, the survival was depressed ~25-fold ($p<0.01$) (Fig. 1b). Thus, the most effective irradiation dose (30 Gy) was used for studying radio-protective properties of different compounds on *E. coli*.

Concentration-dependent effects of synthesized compounds on *E. coli* growth

The addition of any compound can negatively affect the growth characteristics of bacteria[5,24]. Therefore, the effects of novel synthesized compounds — NLTyr, NLTrp, CuNLTyr, CuNLTrp, MnNLTy and MnNLTrp at wide range of concentrations (physiologically permissible) on bacteria prior to irradiation were studied (not shown). The compounds were added into the growth medium directly before inoculation of bacteria. These compounds showed concentration-dependent effects on non-irradiated *E. coli* growth. The following concentrations of compounds were chosen for studying *E. coli* survival and ATPase activity under X-ray irradiation: 10^{-3} M for NLTyr and NLTrp, 10^{-7} M for CuNLTyr and CuNLTrp, and 10^{-5} M for MnNLTyr and MnNLTrp. These concentrations had little toxic effects on the bacteria.

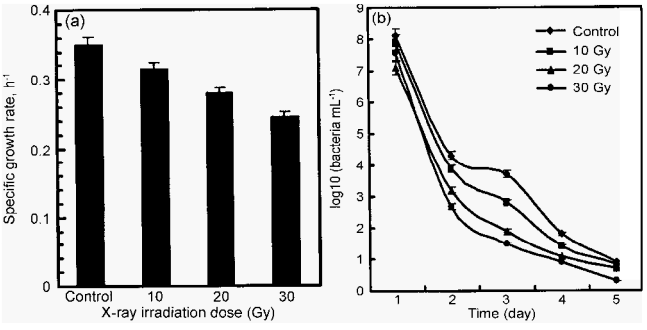

Fig. 1—*E. coli* specific growth rate (a) and survival (b) changes after X-ray irradiation of 10, 20 and 30 Gy [The control was non-irradiated cells. For details, see "Materials and Methods"]

Figure 2 shows the growth kinetics of *E. coli* till stationary growth phase in the presence of CuNLTyr and CuNLTrp at the above-mentioned concentrations (the other compounds had similar effects, data not shown). Compared with non-irradiated control, bacterial growth rate depressed 14% ($p<0.015$) and 20% ($p<0.03$) respectively with NLTyr and NLTrp, 11% with CuNLTyr and MnNLTyr ($p<0.025$ for two compounds) and 9% with CuNLTrp and MnNLTrp ($p<0.01$ and $p<0.05$) (Fig. 3).

Effects of synthesized compounds on growth and survival of *E. coli* after X-ray irradiation

X-ray irradiation depressed the growth and viability of *E. coli* cells, but the irradiation effects were

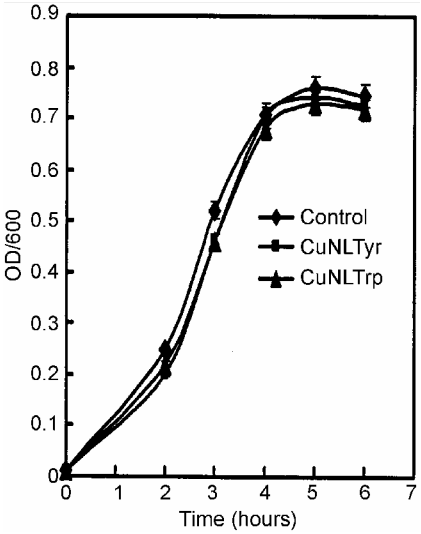

Fig. 2—*E. coli* growth kinetics in the absence and the presence of CuNLTyr and CuNLTrp [Bacteria were grown in peptone medium with 0.2% glucose at pH 7.5 under anaerobic conditions (see "Materials and Methods"). Substances (10^{-7} M) were added to the growth medium immediately before inoculation of bacteria. For the others, see legends to Fig. 1]

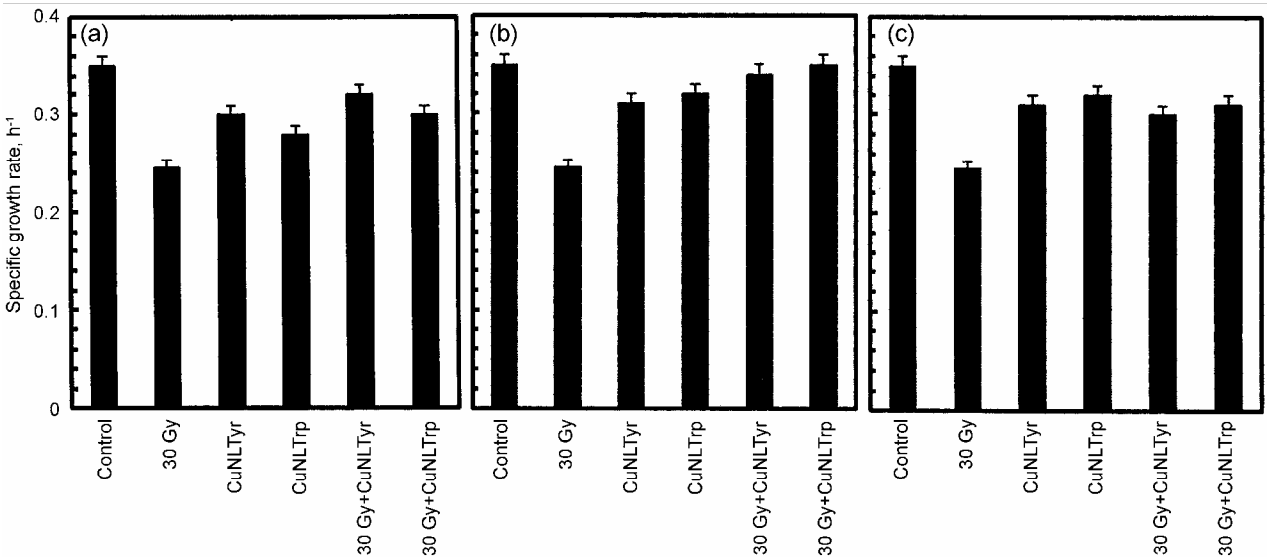

Fig. 3—*E. coli* growth rate change by the effect of of NLTyr (10^{-3} M), NLTrp (10^{-3} M) (a), and CuNLTyr (10^{-7} M), CuNLTrp (10^{-7} M) (b), and MnNLTyr (10^{-5} M), MnNLTrp (10^{-5} M) (c) under X-ray irradiation (30 Gy) [Control was without irradiation. Bacterial growth was determined as described in "Materials and Methods". For the others, see legends to Figs 1 and 2]

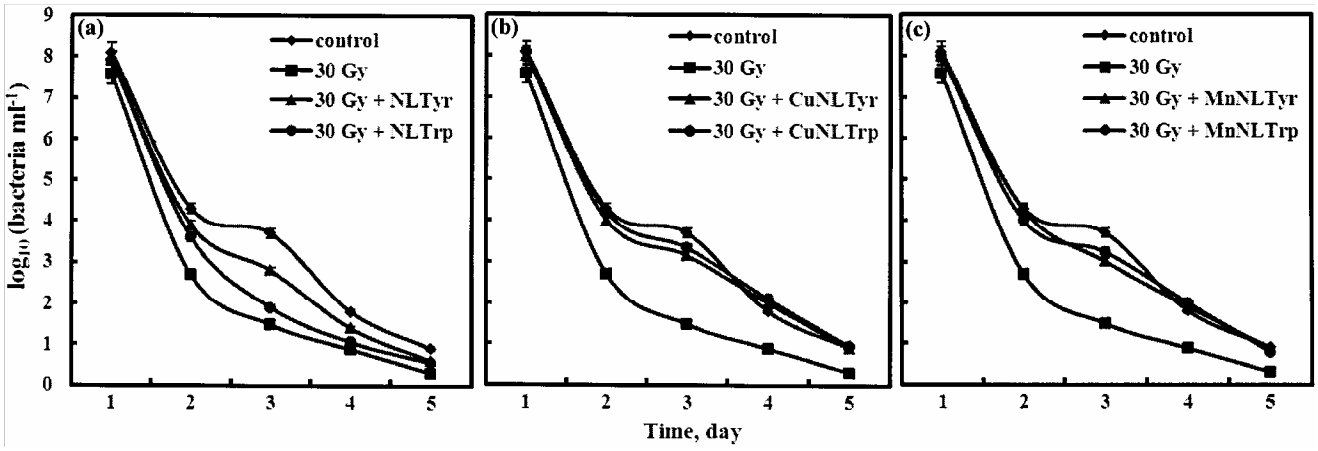

Fig. 4—*E. coli* survival change during 5 days by using of NLTyr (10^{-3} M), NLTrp (10^{-3} M), (a), CuNLTyr (10^{-7} M), CuNLTrp (10^{-7} M), (b), MnNLTyr (10^{-5} M) and MnNLTrp (10^{-5} M), (c) under X-ray irradiation (30 Gy) [Control was without irradiation. Bacterial survival was determined by counting colony-forming units as described in "Materials and Methods"]

reversed in the presence of NLTrp, NLTyr and CuNLTrp, CuNLTyr, MnNLTrp and MnNLTyr (Fig. 3). *E. coli* growth rate for all cases approximately approached to the control (non-irradiated) cells and was the same in case of CuNLTrp and CuNLTyr ($p = 0.356$; indicated about not significant differences compared with non-irradiated control) (Fig. 3b). The efficacy of NLTyr, NLTrp, MnNLTyr and MnNLTrp was less (Fig. 3a,c); the cell growth rate was 8.6% less with NLTyr ($p<0.02$), 14% less with NLTrp and MnNLTyr ($p<0.015$ and $p<0.025$, respectively) and 11.4% less ($p<0.03$) with MnNLTrp compared with non-irradiated control. Thus, the results indicated about the probable radio-protective effects of these compounds.

The radio-protective effects of these compounds were confirmed with bacterial survival changes (Fig. 4). In the mentioned concentrations, they had appreciable stabilizing effect on *E. coli*; the decline in the number of viable cells was less. The number of viable cells in X-ray-irradiated control decreased ~25-fold during 5 days observation ($p<0.05$). Nevertheless, the stabilizing effect of these compounds on *E. coli* cells viability was visible from the 2th day of observation. However, the effects of

NLTyr and NLTrp on survival were less than on the growth rate. Decrease in the number of viable cells during 5 days was ~13-fold and 14-fold, respectively ($p<0.02$), compared to 9-fold of non-irradiated control ($p<0.02$) (Fig. 4a). The survival of irradiated *E. coli* in the presence of NLTyr and NLTrp was ~44% and ~55% less, compared with non-irradiated control ($p<0.01$ for two compounds), but in the same time was ~44% and ~48% higher than with the irradiated sample ($p<0.025$ and $p<0.01$).

The protective effects were more visible with CuNLTyr and CuNLTrp (Fig. 4b). Decrease of the number of viable cells during 5 days was ~8.9-fold and ~8.6-fold, respectively ($p<0.05$ and $p<0.01$). These results were nearly similar with that of non-irradiated control ($p = 0.632$ and $p = 0.669$; non-significant differences in values of compared groups). Also, the cell viability with CuNLTyr and CuNLTrp was ~64.4% and ~66% higher, respectively ($p<0.015$ for two compounds), compared with the irradiated sample.

Two Mn-containing chelates of amino acid derivatives had the stabilizing effects on *E. coli*. The decline in the number of viable cells with MnNLTyr and MnNLTrp was ~9.1-fold and ~10-fold, respectively ($p<0.015$ for two compounds) (Fig. 4c). The effect of MnNLTyr was similar with the non-irradiated control ($p = 0.632$, indicated about non-significant differences in values of compared groups). But, the cell viability with MnNLTrp was 10% less ($p<0.025$), compared with non-irradiated control. However, the cell viability with MnNLTyr and MnNLTrp compared with the irradiated sample was ~64% and ~60% higher, respectively ($p<0.02$ and $p<0.01$).

Effect of synthesized compounds on membrane ATPase activity of X-ray irradiated *E. coli*

Exposure to ionizing irradiation of *E. coli* is known to damage cells and alter their normal biological functions. To survive, bacteria should have different systems like H^+-transporting F_0F_1-ATPase[8,31,33-35]. Probably, to recover the damage of systems, more energy is also needed and ATP-dependent processes are intensified. So, the ATPase activity might be changed, which may result the change in the. Indeed, after irradiation, the overall and DCCD-sensitive ATPase activity of *E. coli* membrane vesicles was increased ~2.5-fold and ~1.24-fold, respectively ($p<0.025$). This is suggested to be H^+-transporting F_0F_1-ATPase activity[8,35]. The influence of X-ray irradiation on the F_0F_1-ATPase could be, therefore, suggested by the increase in its activity[34].

Table 1—*E. coli* membrane vesicles ATPase activity under X-ray irradiation (30 Gy) and in the presence of novel synthesized derivatives of amino acids and their chelates

Assay conditions	ATPase activity (nmol P_i/min. μg protein)		
	- DCCD	+ DCCD*	DCCD-sensitive
Control non-irradiated (without compounds)	0.45 ± 0.03	0.16 ± 0.02	0.29 ± 0.03
Control X-ray irradiated (without compounds)	1.13 ± 0.05 (p = 0.102)	0.77 ± 0.05 (p = 0.002)	0.36 ± 0.03 (p = 0.003)
NLTyr (10^{-3} M)**	1.50 ± 0.04 (p = 0.018)	0.75 ± 0.04 (p = 0.102)	0.76 ± 0.02 (p = 0.05)
NLTrp (10^{-3} M)**	1.42 ± 0.02 (p = 0.011)	0.70 ± 0.03 (p = 0.034)	0.72 ± 0.05 (p = 0.068)
CuNLTyr (10^{-7} M)**	0.48 ± 0.02 (p = 0.088)	0.26 ± 0.02 (p = 0.042)	0.22 ± 0.03 (p = 0.011)
CuNLTrp (10^{-7} M)**	0.55 ± 0.03 (p = 0.05)	0.25 ± 0.01 (p = 0.074)	0.3 ± 0.02 (p = 0.013)
MnNLTyr (10^{-4} M)**	1.05 ± 0.05 (p = 0.05)	0.72 ± 0.04 (p = 0.004)	0.33 ± 0.03 (p = 0.042)
MnNLTrp (10^{-4} M)**	1.04 ± 0.05 (p = 0.074)	0.62 ± 0.03 (p = 0.011)	0.42 ± 0.05 (p = 0.034)

*DCCD in the concentration of 0.2 mM was used; p values were done for the comparison between compounds and non-irradiated control in each column

**in the brackets the concentration of the compound used was given

Interestingly, studied compounds had no effects on non-irradiated bacterial membrane ATPase activity, compared with control. But, X-ray irradiation in the presence of the studied compounds had different effects on *E. coli* (Table 1). Only in case of CuNLTyr and CuNLTrp, ATPase activity approached to its initial value (non-irradiated control). MnNLTyr and MnNLTrp had little recovering effect on irradiated cells ATPase activity. NLTyr and NLTrp showed higher effect on ATPase activity, compared with only irradiated control. Overall and DCCD-sensitive ATPase activities were increased 1.3-fold and 2-fold, respectively ($p<0.01$).

Discussion

Effects of 30 Gy X-ray irradiation on *E. coli* growth and survival and the probable protective effects of synthesized compounds on bacteria were investigated in the present study. The synthesized amino acid derivatives−NLTyr and NLTrp and their

Cu- and Mn-containing chelates under X-ray irradiation had different action. The protective effects on cell growth were more visible with CuNLTrp, CuNLTyr, followed by NLTyr, MnNLTyr, MnNLTrp and NLTrp. The CuNLTrp, CuNLTyr, MnNLTyr showed more protective effects on *E. coli* viability than MnNLTrp, followed by NLTyr and NLTrp.

The cell growth and viability are dependent, among other things, on the integrity of the cell membrane and initiation and regulation of various cell processes are associated with cell surface events[8,31,33]. Indeed, in this study, X-ray irradiation increased the membrane F_0F_1-ATPase activity. Probably, to recover the damage of cell, more energy is needed and thus the ATP-dependent processes are intensified[34]. It is known that proteins extract at its optimum concentration can promote the restoration of X-ray induced damage by involving in *E. coli* growth and division mechanisms[36]. So, amino acid residues and also their derivatives may play a significant role in repair of cellular structures damaged by the free radicals[37,38]. Because X-irradiation damages cellular membranes, it also probably increases the cellular uptake of these compounds[39].

A higher protective effect of bivalent metal chelates of amino acids is due to their lipophilic properties and easy penetration across the membrane[12,16,40]. Also, probably, they can stimulate *de novo* synthesis of metallo-element-dependent enzymes required for recovery of damaged cell constituents[40,41]. Also, Cu^{2+} and Mn^{2+} are indispensable catalytic and structural co-factors driving a wide array of important biochemical processes[40,41]. Moreover, the comparative differences of protective actions of Cu^{2+} and Mn^{2+} chelates might be due to their changed permeability by the cell membrane. Also, these ions are accumulated within bacterial cells by active transport mechanisms[40,43,44].

Interestingly, the studied compounds used alone had no effects on non-irradiated bacterial membrane ATPase activity, compared with control. But, under X-ray irradiation, only CuNLTyr and CuNLTrp had protective effect, with ATPase activity approaching to its initial value (non-irradiated control). This effect could be as a result of Cu^{2+} ion direct effect on the membrane, especially on the F_oF_1 ATPase activity, thus changing H^+-coupled transport by causing some conformational changes[42-44]. The study may prove useful for further estimation of the effectiveness of different compounds as radio-protectors on bacteria and other cells, especially mammalian cells under X-ray irradiation.

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