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Radioprotective Properties of Fullerenol: Cellular, Biochemical and Physicochemical Approaches

The search for optimal radioprotective methods and tools under low-dose radiation exposures represents a pressing issue in the field of modern radioecology. The objective of the study was to investigate the radioprotective properties of fullerenol $C_{60,70}O_y(OH)_x$, ($x+y$ = 24–28), a water-soluble polyhydroxylated fullerene derivative with an electron-deficient aromatic carbon structure. Tritium, a radionuclide of low decay energy, was selected to simulate an exposure to low-dose irradiation (< 0.05 Gy). We applied luminous marine bacteria *Photobacterium phosporeum* as a model cellular object to monitor radiation bioeffects; the bioluminescence intensity of the bacteria was used as a tested biological parameter. Tritium activated the bacterial luminescence; the addition of fullerenol (< $3 \cdot 10^{-3}$ g/L) "mitigated" the activation, thus revealing the radioprotective capacity of fullerenol for the marine microorganism. To evaluate the mechanisms of radioprotection of fullerenol in tritiated water, we investigated the effects of fullerenol on: (1) the content of reactive oxygen species and (2) the intensity of bioluminescence in the bacterial enzymatic reaction. Tritiated water produced moderate deviations from the control values, whereas the addition of fullerene brought these values closer to the 'control' ones. All observed effects were attributed to variations in the ionic balance of the aqueous medium, which resulted in the activation of bacterial functions through cell membranes.

Keywords: tritium, fullerenol, radioprotection, luminescence, bacteria, bioassay, enzymes, reactive oxygen species.

Introduction

Low-intensity radioactive contamination is causing increasingly serious environmental problems. These problems usually results from the intensive exploitation of natural resources and nuclear power plants operation. The sensitivity of organisms to low doses (<0.1Gy) is a subject of interest for scientists. Variations in sensitivity in different molecular environments are of particular interest; molecules of natural and artificial origin can serve as radiomodifying agents of varying efficacy.

We consider tritium (^{3}H) as a suitable object to examine low-dose bioeffects in water ecosystems. Tritium is a widespread radioisotope with a half-life of 12.4 years and a low energy of radioactive decay (5.7 keV) [1, 2]. Tritium occurs in nature mainly in the form of tritiated water (HTO) [3]. There are now three main sources of tritium: (1) natural formation in the upper layers of the atmosphere as a result of the splitting of nuclides by cosmic rays and reactions involving the capture of nitrogen and oxygen particles [4]; (2) residual activity from nuclear weapons tests [5]; and (3) decay products of the nuclear fuel cycle [3]. The monitoring of tritium concentrations in the vicinity of power plants is currently a subject of heightened interest. As tritium levels decline, there is a need to monitor lower levels of tritium activity in the environment [6–8].

The unique ability of ^{3}H to substitute protium (^{1}H) in biological macromolecules is a reason of tritium' chronic toxicity. Tritium decays to form primary products, namely ionized isotope of helium-3 (^{3}He$^+$), an electron, and antineutrino:

$$^3_1\mathrm{H} \xrightarrow{\beta^-} {}^3_2\mathrm{He}^+ + e^- + \tilde{\nu} \quad (1)$$

The bioeffects of tritium are caused by charged products of its radioactive decay (i.e. cation of helium $^{3}_{2}He^{+}$ and electron). The decay products initiate a charge transfer chain in external solutions and within the organisms themselves. It is important to note the extreme activity of the helium ion $^{3}_{2}He^{+}$, which rigidly accepts an electron from the aqueous environment or organic molecules, completing its outer shell to form a stable noble gas shell. The introduction of tritium into organisms results in the disruption of hydrogen bonds within living cells, thereby preventing the synthesis of organic structures [9, 10]. Furthermore, the electron-acceptor activity of $^{3}_{2}He^{+}$ leads to the disruption of the structure of crucial macromolecules responsible for the vital activity of living organisms, namely DNA, proteins, enzymes [11, 12].

Therefore, ionization of an aqueous medium is usually the main external factor under low-dose tritium exposures. Ionization affects the outer cell walls, resulting in membrane activation and related intracellular processes. This effect is known to promote the activation of bioluminescence of marine bacteria in tritiated water [12–17] and can be considered as a biophysical mechanism underlying the "hormesis" phenomenon [18–23].

The primary application of luminous bacteria is in environmental toxicity monitoring, as evidenced by numerous studies [12, 24–27]. Rapidness, accuracy, sensitivity and simplicity are the advantages of bacteria-based bioluminescent assays. The method is based on the changes in luminescence intensity under exposure to an analyzed sample. The bioassay detects ecosystem conditions upon exposure to toxic substances, in both acute and chronic forms of toxicity. Interference with bacterial metabolism at any level is indicated by a change in light emission. The effects of toxicants on the enzymatic chemiluminescent processes responsible for the luminescence of bacteria are of a particular interest. The system of two enzymatic reactions (2, 3) is commonly used as a model for such processes. This system is based on two bacterial enzymes, namely luciferase and NADH:FMN-oxidoreductase.

$$FMN + NADH \xrightarrow{\text{NADH:FMN-oxidoreductase}} FMN \cdot H^{-} + NAD^{+} \quad (2)$$

$$FMN \cdot H_2 + RCHO + O_2 \xrightarrow{\text{luciferase}} FMN + RCOOH + H_2O + h\upsilon \quad (3)$$

This coupled enzyme system has been employed as a luminescent enzymatic bioassay since 1990 [28]. A detailed description of this system has been presented in [29, 30]. The effects of various toxicant groups on the enzyme system have been reviewed in [31].

Bioluminescence systems of varying complexities (cell-based and enzyme-based) permit the elucidation of patterns of toxicant exposure at two distinct levels: cellular and biochemical.

Biological responses to low-dose radiation are usually explained by the involvement of reactive oxygen species (ROS) in metabolic processes [32, 33]. ROS comprise a group of mutually transforming and chemically active forms of oxygen-containing compounds [34] with lifetimes ranging from nanoseconds to hours [35]. ROS can occur in biochemical reactions as free radicals, ion-radicals (positively or negatively charged), and molecules. The group of ROS includes, but is not limited to, the following compounds: superoxide anion radicals ($O_2 \cdot -$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), singlet oxygen ($^1O_2$), hypochlorite (HOCl), hydroperoxyl radical ($HOO \cdot$), and others.

Traditionally, ROS are thought to cause oxidative stress and cell damage [33]. ROS are constantly produced by living organisms during respiration, as well as modified and consumed during metabolic activity. Currently, the beneficial functions of low and moderate doses of ROS are known and discussed; ROS are responsible for many vital physiological functions, namely proliferation, migration, differentiation and others [36–38]. Their roles vary considerably depending on the ROS types, the reactions in which they are involved and the target molecules with which they react. ROS are natural by-products of metabolic oxidative processes and are involved to cell signal transmission and homeostasis. The involvement of ROS in the bioeffects of nanostructures is currently under active investigation [39–41].

It is known that radioactive decay of radioisotopes in aerated aqueous solutions leads to the ROS formation [14, 42, 43], which can affect inhabitants of aquatic environments. The ROS involvement in the bioeffects of radionuclides and gamma-radiation has been studied in [12, 15, 42, 43]; luminescent bacteria and their enzymatic reactions have been used as model biological objects in these studies. Biological responses to radiation can vary depending on the molecular composition of aqueous solutions. The presence of organic molecules in water solutions can alter the ionic and radical states of radionuclides, affecting, thereby, the environment of aquatic inhabitants. For example, humic substances, products of natural oxidative decomposition of organic matter in sediments, are known to be native attenuators of radiotoxicity in aqueous solutions. Direct and indirect mechanisms of radioprotective activity of humic substances have been reviewed [15, 44].

Fullerenols are water-soluble derivatives of fullerenes with considerable potential as artificial radioprotectors. They represent a promising class of compounds with applications in physics, chemistry, nanobiotechnology, pharmacology, and biomedicine. The hypothetical structure of the fullerenol is illustrated in Figure 1B.

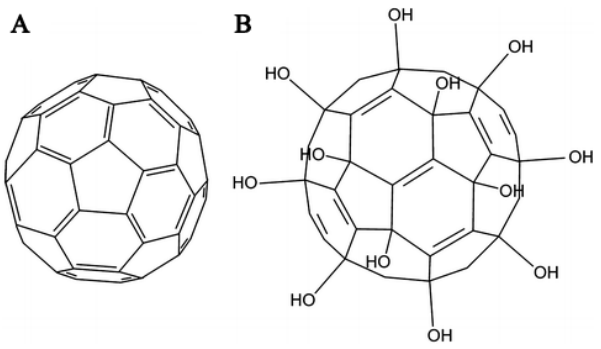

Figure 1. Hypothetical structure of fullerene C_{60} (*A*); fullerenol $C_{60}(OH)_{24}$ (*B*) [45]

Considerable attention has been devoted to the physicochemical properties of fullerenes and fullerenols, with a particular focus on their capacity to generate and capture ROS and reactive nitrogen forms [46, 47]. Additionally, fullerenol has been demonstrated to influence the formation of water radiolysis products, including H_2O_2 and hydroxyl radicals. Furthermore, it has been shown to prevent DNA and protein damage.

The idea of using fullerene derivatives to protect cells is related to their chemical and biological properties [49, 50]. Fullerenes and their derivatives are known to be effective radical traps and antiradical agents due to their highly conjugated π-system and low-energy vacant molecular orbitals [51]. The radioprotective activity of fullerenols has been reported earlier in [52, 53]. However, these studies focused on higher organisms; marine microorganisms and their enzymes have not yet been used.

The objective of the study was to investigate the radioprotective activity of fullerenol $C_{60,70}O_y(OH)_x$, (x = 22–24, y = 2–4) in tritiated water (HTO) under exposure to low-dose radiation (< 0.05 Gy). Marine luminescent bacteria were selected as the model biological object for this study. The impact of fullerenol on the luminescence intensity of the bacteria, the rate of their enzymatic reactions, and the ROS content in the bacterial suspensions were investigated.

Experimental

Preparations and Reagents

The bacterial samples were prepared from lyophilized bacteria *Photobaterium phosphoreum* according to the standard technique [15, 54].

The enzymatic kit was previously described in reference [55]; it was produced at the Institute of Biophysics, SB RAS, Krasnoyarsk, Russia. The chemicals required for the enzymatic assay and assay procedure were provided as follows [55].

Tritiated water, HTO, JSC Isotope, Russia, was used as a source of tritium. The preparation of HTO was described in [15]. The final specific radioactivities in the aqueous media were 0.03 and 500 MBq/L.

Fullerenol $C_{60,70}O_y(OH)_x$, where (x+y = 24–28), was synthesized and characterized as described in [55]. The preparation was produced by fullerene hydroxylation in nitric acid followed by the hydrolysis of the polynitrofullerenes [56]. The fullerenol preparation was characterized by infrared spectroscopy in the KBr matrix using a Fourier spectrometer VERTEX 70 (Bruker Optik GmbH, Ettlingen, Germany). The number of -OH groups was estimated by X-ray photoelectron spectroscopy using a UNI-SPECS spectrometer (SPECS Gmbh, Berlin, Germany) [57, 58] Chromatographic analysis showed that the fullerenol preparation involved 60 % of $C_{60}O_y(OH)_x$ and 40 % of $C_{70}O_y(OH)_x$.

Fullerenol solutions were prepared in distilled water. The bioluminescence intensity was preliminary measured in a wide range of fullerenol concentrations (10^{-16}–10^{-1} g/L) in order to select concentrations, which did not change the bioluminescence intensity. The following fullerenol concentrations were selected for further experiments: 10^{-15}–$3{\cdot}10^{-3}$ g/L. The results obtained were in agreement with the data presented in [55, 59, 60].

The chemiluminescence reagents were described in [60].

Luminescent Assay System Composition

Bacterial Assay

Bacterial bioluminescence kinetics was studied in non-radioactive samples (controls), as well as in radioactive samples in the absence and presence of fullerenol.

Enzymatic Assay

The solutions of chemicals were prepared as described in [60].

The enzyme solutions were non-radioactive (control) samples, radioactive samples and radioactive samples with fullerenol.

Bioluminescence Registration

A standard procedure for the bioluminescence measurements was described in detail in [12, 54]. Bioluminescence intensities of the control and radioactive samples were measured and compared in the presence and absence of fullerenol similar to [15].

The bioluminescence intensity was registered as described in [61, 62].

Chemiluminescence Measurements

The luminol chemiluminescent method [63–65] was used.

Statistical processing

All measurements were carried out in 4-6 replicates. Statistical evaluations were carried out employing the Student's t-test. The experimental error of the measurements did not exceed 12 %.

Results and Discussion

We studied the effects of HTO on bacterial luminescence in the absence and presence of fullerenol. The results are shown in Figure 2.

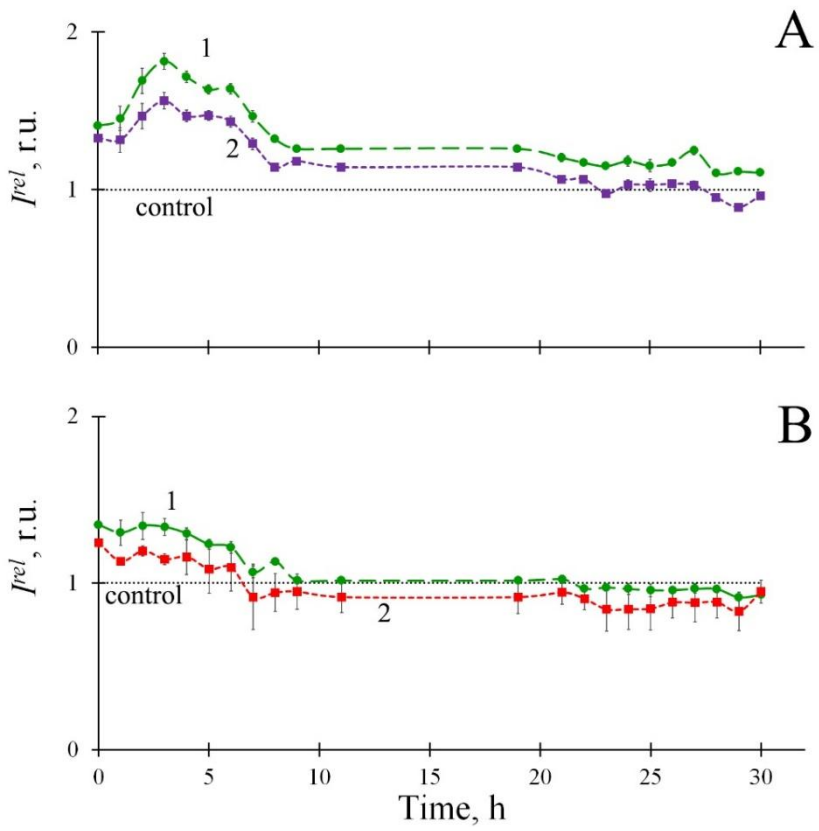

Figure 2. Kinetics of bacterial bioluminescence, I^{rel}, in HTO in the absence (1) and presence (2) of fullerenol. Specific activity of HTO: (A) 500 MBq/L; (B) 0.03 MBq/L. Fullerenol concentration: (A) 10^{-11} g/L; (B) 10^{-9} g/L

Two concentrations of HTO were chosen as examples, namely 500 and 0.03 MBq/L. The independence of bacterial luminescence response on concentration/radioactivity of HTO was found and discussed previously [13, 26] in a wide range of HTO concentrations (10^{-3} – $2 \cdot 10^{2}$ MBq/L).

Activation of bioluminescence ($I^{rel} > 1$) can be seen in the absence of fullerenol (Fig. 2A, B, curves 1) from the start of the chronic exposure. Over time, activation decreased to the control values.

Tritium activation was observed in previous works [12, 16, 26]; it was associated with the "hormesis" model [18–23], which always includes an activation stage. The activation was explained by the ionization of the aqueous medium with subsequent stimulation of cellular processes, membrane and enzymatic, as well as accumulation of ROS.

In the presence of fullerenol (Fig. 2A, B, curves 2) a shift of the kinetic curves closer to the control was observed, revealing a 'mitigation' of the tritium effects. This mitigation can be explained by the ability of fullerenol to reversibly accept/donate electron density, thereby changing a radical state of aqueous media [41]. Therefore, Figures 2A, B demonstrate the radioprotective ability of fullerenol in HTO. The ability is quantitatively comparable to that of humic substances — natural detoxifying and radioprotective agents [15].

To elucidate the mechanism of radioprotection, the effect of fullerenol (at 10^{-11} and 10^{-9} g/L) on the (i) content of ROS (ROS^{rel}) in bacterial suspension and (ii) the intensity of bioluminescence of the enzymatic system (I^{rel}) — were studied in HTO (500 and 0.03 MBq/L).

As an example, Figure 3 combines three types of the experimental measurements in HTO in the absence and presence of fullerenol: (A) bacterial luminescence intensity, I^{rel}, (B) ROS content in the bacterial suspension, ROS^{rel}, and (C) luminescence intensity of the enzyme system, I^{rel}. The exposure time of 4 h was chosen for the presentation. Bacterial luminescence intensity, I^{rel}, is presented in Figure 3A (green and red columns) according to the data in Figure 2A.

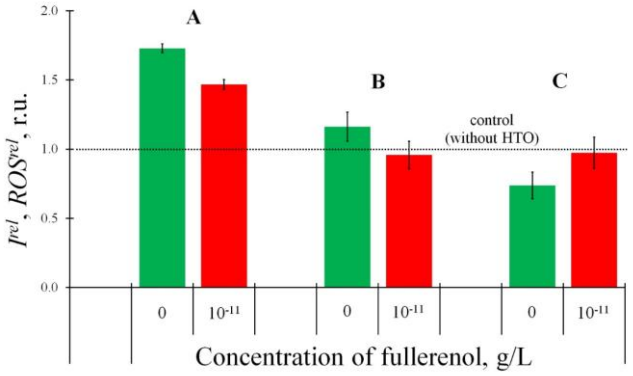

Figure 3. Relative luminescence intensity, I^{rel}, and ROS content, ROS^{rel}, in the absence and presence of fullerenol, 10^{-11} g/L. Time of exposure was 4 h; HTO specific radioactivity was 500 MBq/L. (*A*) — bacterial luminescence, (*B*) — ROS content, (*C*) — luminescence of the enzyme system

We registered an insignificant but reliable increase of ROS content in the bacterial suspension, ROS^{rel}, exposed to HTO — up to *ca.* 1.2 (Fig. 3B, green column). A similar increase in ROS content under similar conditions was previously observed in [12, 15]; it was attributed to the intensification of bacterial metabolic processes, resulting in additional ROS production. It is noteworthy that the luminol chemiluminescence method did not register an increase in the content of ROS in HTO media without bacteria [12–14], but the addition of bacteria resulted in a significant increase in the content of ROS [12]. It was concluded that the higher level of ROS in bacterial suspensions exposed to HTO is the result of a multi-step process involving: ionization of the aqueous media due to radioactive decay of tritium, stimulation of bacterial membrane receptors, intensification of intracellular processes including oxidative ones, and extra ROS production into the external media.

The addition of fullerenol (10^{-11} g/L) brought ROS^{rel} closer to the control (red column, Fig. 3B), probably, due to reversible electron-donor activity of fullerenol and related decrease of ion-radical oxygen particles (components of ROS group).

Suppression of the bioluminescence intensity of the enzyme system, I^{rel}, down to *ca.* 0.75 (green column, Fig. 3C) could be explained in a similar way: it is probably caused by additional ionization of the media in the presence of HTO. Obviously, the suppression of the intensity of the bioluminescent enzyme system can not be responsible for the activation of bacterial bioluminescence by HTO (Fig. 2A, B). This result corresponds to the conclusion [12–17] on the determining role of the bacterial cell membrane in the activation of bacterial bioluminescence under the exposure to tritium. An approach of I^{rel} to the control (Fig. 3C, red column) could be similarly explained by deionization of water media induced by fullerenol.

Therefore, we can explain the radioprotective ability of fullerenols in bacteria media by (1) the tendency to reversible electron donation/reception and (2) the predominant role of the cell membrane of bacteria exposed to tritium. The same ability of fullerenol in HTO is probably responsible for moderate effects on ROS levels and luminescence of the enzymatic system. However, the latter two factors are not decisive for the radioprotective ability of fullerenol towards bacterial cells.

Conclusions

The aim of this work was to evaluate the radioprotective potential of fullerenol, a water-soluble polyhydroxylated fullerene derivative with an electron-deficient aromatic carbon structure, and an effective catalyst due to its ability to reversible electron acceptance. A radionuclide with a low decay energy, tritium, was chosen to simulate an exposure to low-dose irradiation (< 0.05 Gy). The luminous marine bacterium was used as a model cellular object to monitor the effect of radiation on its luminescence as a physiological function of the organism. Tritium activated the bacterial luminescence; the addition of fullerenol ($< 3 \cdot 10^{-3}$ g/L) "mitigated" the activation effect, bringing the kinetic curve closer to the "control" (bacteria without tritium). Thus, the radioprotective ability of fullerenol for a marine microorganism was demonstrated.

Biochemical and physicochemical mechanisms of radioprotection were of particular interest. We studied the ROS content in bacterial suspensions and the luminescence intensity of the bacterial enzymatic reaction; two concentrations of tritiated water were used (0.03 and 500 MBq/L), concentrations of fullerenol varied at $< 3 \cdot 10^{-3}$ g/L. Tritium produced moderate deviations from control values (positive ones for ROS content and negative ones for luminescence of the enzyme system). The addition of fullerene brought these values closer to the 'control' ones. All effects of tritium and fullerenol were attributed to changes in the ionic balance in aqueous media, resulting in activation of bacterial functions via the cell membrane. A direct influence of tritium and fullerenol on intracellular enzyme processes was not confirmed. The variation of ROS content in bacteria suspensions is probably a secondary result related to bacterial metabolism and its adaptation to external media.

Further studies should be aimed to explore bacterial membrane functions under various conditions of low-dose radioactive exposures. Additionally, variations of potential radioprotectors of different chemical structure are of practical interest.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. CRediT: **Olga Vladislavovna Kolesnik** data curation, formal analysis, investigation, methodology, visualization, writing — original draft, writing — review & editing; **Aleksey Sergeevich Grabovoy** formal analysis, investigation, visualization, writing — original draft; **Gennadii Alexandrovich Badun** — conceptualization, methodology, resources; **Grigory Nikolaevich Churilov** – methodology, resources; **Nadezhda Stepanovna Kudryasheva** conceptualization, data curation, methodology, project administration, validation, supervision, validation, visualization, writing — original draft, writing — review & editing.

Conflicts of Interest

The authors declare no conflict of interest.

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