DOI 10.1007/s10517-018-4309-1

Comparative Analysis of the Formation of γH2AX Foci in Human Mesenchymal Stem Cells Exposed to ^{3}H-Thymidine, Tritium Oxide, and X-Rays Irradiation

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Translated from *Kletochnye Tekhnologii v Biologii i Meditsine*, No. 3, pp. 205-208, September, 2018
Original article submitted February 19, 2018

We performed a comparative study of the formation of γH2AX foci (a marker of DNA double-strand breaks) in human bone marrow mesenchymal stem cells after 24-h incubation with ^{3}H-thimidin and tritium oxide with low specific activities (50-800 MBq/liter). The dependence of the number of γH2AX foci on specific activity of ^{3}H-thymidine was described by a linear equation y=2.21+43.45x (R^2=0.96), where y is the number of γH2AX foci per nucleus and x is specific activity in 1000 MBq/liter. For tritium oxide, the relationship was described by a linear equation y=2.52+6.70x (R^2=0.97). Thus, the yield of DNA double-strand breaks after exposure to ^{3}H-thymidine was 6.5-fold higher than after exposure to tritium oxide. Comparison of the effects of tritium oxide and X-ray radiation on the yield of DNA double-strand breaks showed that the relative biological efficiency of tritium oxide in a dose range of 3.78-60.26 mGy was 1.6-fold higher than that of X-ray radiation. Improvement of the methods of analysis of DNA double-strand breaks repair foci is highly promising in the context of creation of highly sensitive biodosimetry technologies for tritium compounds in humans.

Key Words: *DNA double-strand breaks; ^{3}H-thymidine; tritium oxide; mesenchymal stem cells*

Growing world production of nuclear energy and the development of thermonuclear technologies raised concern about the potential consequences of tritium ingestion for human health. The situation is complicated by the fact that the biological effects of tritium, and in particular, its organic compounds have been studied insufficiently. International community still does not have a unanimous opinion on rationing of tritium compounds intake for humans. According to European standards, the concentration of tritium compounds in drinking water should not exceed 100 Bq/liter, while in Australia, the norm is 75,000 Bq/liter [3].

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The mean free path of the β-particle emitted by tritium is 0.4-0.6 μ, which is much less than the diameter of the nucleus in a somatic cell [1]. Thus, tritium can be dangerous to human health only after intake into the body. Being of a hydrogen isotope, tritium can be a part of water molecules (tritium oxide, HTO) and inorganic or organic molecules (organically bound tritium, OBT). From the viewpoint of classical biochemistry, HTO behaves as an ordinary water in living cells and body [2]. A part of HTO exchanges with hydrogen atoms and can be incorporated in various organic molecules (nitrogenous bases, amino acids, lipids, sugars, *etc*.) [6]. In contrast to HTO, OBT is unequally distributed in cells and tissues, which can lead to high microlocal doses and cause serious damage to proteins, lipids, and nucleic acids [5].

Among various DNA lesions, DNA double-strand breaks (DNA DSBs) are the most critical for cell fate

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[11]. Reparation of these DNA lesions is a slow process; at the same time, misrepaired and unrepaired DSBs can lead to cytogenetic disorders, cell death, inactivation of tumor suppressor genes, or activation of oncogenes [4].

Previous studies on DNA DSBs induction and repair after exposure to tritium compounds were made using high specific activities and radiation doses. At the same time, even professional workers are exposed to significantly lower doses of tritium (<100 mGy) [7]. Until present, no systematic studies of the induction and repair of DNA DSBs in mammalian cells after exposure to tritium compounds with low specific activity have been carried out. This is due to the fact that classical methods of DSBs analysis based on changes in DNA fragmentation (electrophoretic mobility, viscosity, sedimentation, *etc*.) did not allow estimating changes in the amount of DSBs at doses less than one or even tens of Gy [14]. In recent years, highly sensitive methods of indirect quantitative evaluation DSB is cells based on immunocytochemical analysis of proteins involved in DSB repair are intensively developed. Complex dynamic microstructures generated during DNA DSB repair and consisting of thousands of copies of proteins can be visualized by immunocytochemical staining as bright spots called DNA repair foci. It is believed that one focus corresponds to a single DNA DSB repair site [13]. Immunocytochemical analysis of phosphorylated core histone H2AX (γH2AX) is most popular. Phosphorylation of H2AX is mediated by ATM, ATR and DNA-PK kinases in response to the formation of DSBs and indicates its recognition [12]. It is shown that, after low linear energy transfer radiation exposure, one γH2AX foci corresponds to one DNA DSB [8].

Our aim was to study quantitative changes in the number of γH2AX foci (marker of DNA DSBs) in human cultured mesenchymal stem cells (MSC) after exposure to low doses of ^{3}H-thymidine, tritium oxide, and X-rays. The choice of MSC as an object of research was due to their high proliferation capacity and potential transmission of the accumulated DNA damage and mutations to the differentiated progeny of exposed cells.

MATERIALS AND METHODS

Primary human MSC culture (BioloT; passage 5-6) was used in the experiments. The cells were cultured in low-glucose DMEM (1 g/liter glucose) (Thermo Fisher Scientific) containing 10% fetal calf serum (Thermo Fisher Scientific) under the standard conditions in a CO_2 incubator (37°C, 5% CO_2) over two passages (the medium was changed every 3 days).

A sterile solution of ^{3}H-thymidine or tritium oxide with different concentration (final activity 50-800 MBq/liter) was added to the culture medium and incubated under standard conditions of CO_2 incubator for 24 h.

The cells were exposed to 100 kV X-rays at a dose rate of 40 mGy/min (0.8 mA, 1.5 mm Al filter) using RUB RUST-M1 X-irradiator. After irradiation, the cells were incubated for 0.5 h under standard conditions of CO_2 incubator.

Cells on coverslips were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 15 min at room temperature, washed twice with PBS (pH 7.4), and permeabilized in 0.3% Triton-X100 in PBS (pH 7.4) containing 2% BSA to block non-specific binding. The slides were incubated for 1 h at room temperature with primary rabbit monoclonal antibodies against γH2AX protein (clone EP854 (2) Y, Merck-Millipore) diluted 1:200 in PBS (pH 7.4) containing 1% BSA, washed with PBS (pH 7.4), and incubated for 1 h at room temperature with secondary antibodies IgG (H+L) conjugated with rhodamine fluorochrome (Merck-Millipore), diluted 1:400 in PBS (pH 7.4) containing 1% BSA. ProLong Gold medium with DAPI (Life Technologies) was used for DNA counterstaining and prevention of photo-fading.

Cells were viewed and imaged using a Nikon Eclipse Ni-U (Nikon) fluorescent microscope equipped with a ProgRes MFcool high-resolution video camera (Jenoptik AG) using UV-2E/ filter sets (λ_{ex}=340-380 nm and λ_{em}=435-485 nm) and Y-2E/C (λ_{ex}=540-580 nm and λ_{em}=600-660 nm). At least 200 cells per point were analyzed. The foci in the cells nuclei were counted manually.

Statistical and mathematical analysis of the obtained data was carried out using statistical software package Statistica 8.0 (StatSoft, Inc.). The results are presented as the means of three independent experiments±standard error.

RESULTS

Comparative study of DNA DSB formation in human MSC after 24-h exposure to different concentration of ^{3}H-thymidine and tritium oxide showed that the dependence of the number γH2AX foci on activity of ^{3}H-thymidine can be described by a linear equation $y=2.21+43.45x$ (R^2=0.96), where y is the number of γH2AX foci per cell nucleus and x is activity in 1000 MBq/liter. For tritium oxide and X-rays, the dependence can be described by a linear equation $y=2.52+6.70x$ (R^2=0.97), where y is the number γH2AX foci per cell nucleus and x is activity in 1000 MBq/liter (Fig. 1).

When describing the dose dependences by linear equations ($y=a+bx$), the slope b reflects the increment

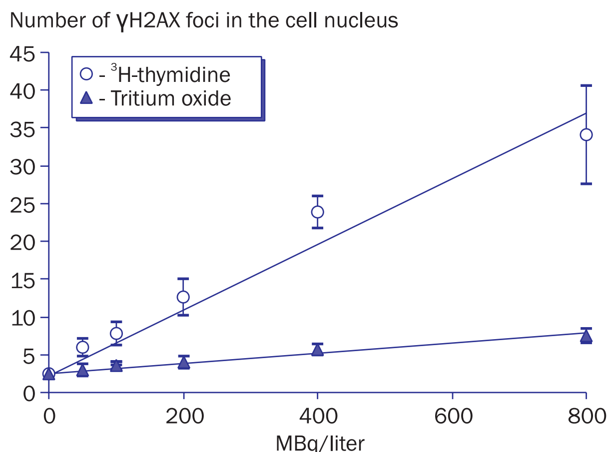

Fig. 1. Dependence of γH2AX foci formation in human MSC on specific activity of ^{3}H-thymidine and tritium oxide.

of the effect per dose unit. Comparison of coefficients *b* of the two dose dependencies obtained for different types of influences showed how much one of them is more pronounced than the other.

Our findings indicate that the quantitative yield of DNA DSBs after exposure to ^{3}H-thymidine in activity range of 50-800 MBq/liter is 6.5-fold higher than after exposure to tritium oxide, which suggested that the greatest biological danger is not the tritium itself, but the possibility of tritium being incorporated into organic compounds, especially nitrogen bases, which can cause the main genotoxic effects.

It was interesting to calculate the dose loads per cell nucleus after exposure to tritium compounds. To this end, we compared the yields of DNA DSBs in human MSC produced after tritium β-decay with those induced after X-rays exposure.

When calculating the doses received by cells, the distribution of tritium oxide in the cell nucleus was assumed homogeneous. In case of homogeneously distributed β-radiation, using the average energy of the spectrum, the dose rate of β-radiation in a layer of soft biological tissue of an infinite thickness and length $\dot{D}\infty$ can be represented as follows:

$$\dot{D}\infty=5.7\times10^{-7}q\langle E\rangle Gy/h, \quad \mathbf{(1.1)}$$

where q is the specific activity (Bq/ml) and <E> is average energy of β-particles (MeV).

The average energy of tritium β-particles is equal to <E>=5.68 keV.

The dose rate is:

$$\dot{D}=D\infty\times t, \quad \mathbf{(1.2)}$$

where t is irradiation time (h).

The results of comparative evaluation of the yield of DNA DSBs after exposure to tritium oxide and X-ray in a dose range of 3.78-60.26 mGy are presented in Figure 2.

It was found that in the studied dose range, the dependence of the number of γH2AX foci on the dose of tritium β-radiation can be described by a linear equation $y=2.52+0.09x$ ($R^2=0.96$), where γ is the number of γH2AX foci per cell nucleus, and x is the dose in mGy (Fig. 2). For X-rays, the dependence is described by a linear equation $y=2.64+0.06x$ ($R^2=0.99$), where y is the number γH2AX foci per cell nucleus and x is activity in mGy. Calculations showed that in the studied dose range, the relative biological efficiency of tritium oxide is 1.6-fold higher than that of X-ray exposure. These findings agree with published data [9,10].

In case of ^{3}H-thymidine, the assumption of its homogeneous distribution in the volume of the cell nucleus is incorrect and the doses should be calculated from the position of microdosimetry and heterogeneous ^{3}H-thymidine distribution in the cell nucleus. However, in a very rough approximation, which is often done when calculating the doses of ^{3}H-thymidine in a living body, we can obtain relative biological efficiency close to ~10: 1.6 (relative biological efficiency of tritium oxide)×6.5 (effectiveness of ^{3}H-thymidine in comparison with tritium oxide). To clarify the quantitative yield of DNA DSBs depending on the dose of ^{3}H-thymidine β-radiation, it is necessary to make a correct calculation of the dose received by cells taking into account micro distribution of ^{3}H-thymidine in the cell volume and its accumulation in DNA of living cells.

The obtained results will be used in further studies aimed at substantiation of radiation protection regulations of exposure to tritium compounds.

Improvement of the methods of analysis of DNA DSB repair foci is highly promising in the context of creation of highly sensitive biodosimetry technologies for tritium compounds in humans.

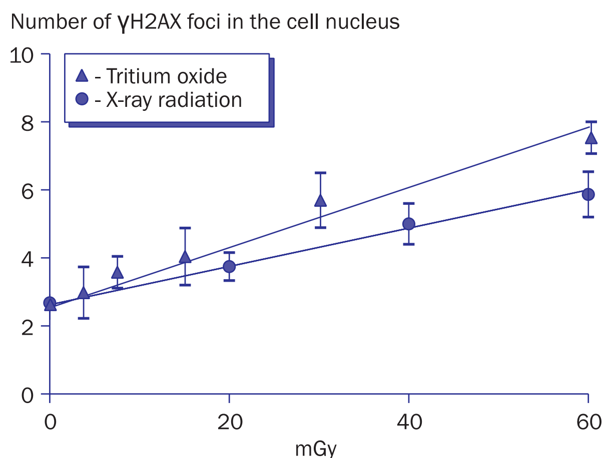

Fig. 2. Dose dependences of γH2AX foci formation in human MSC upon exposure to tritium oxide and X-ray radiation.

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