

The Role of miR-34a in Tritiated Water Toxicity in Human Umbilical Vein Endothelial Cells

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Abstract

In this work, we investigated the toxic effects of tritiated water (HTO) on the cardiovascular system. We examined the role of microRNA-34a (miR-34a) in DNA damage and repair in human umbilical vein endothelial cells (HUVECs) exposed to HTO. Cell proliferation capacity was evaluated by cell counting, and miR-34a expression was detected using quantitative PCR (QT-PCR). The Comet assay and γ -H2AX immunostaining were used to measure DNA double-strand breaks (DSBs). Reverse transcription polymerase chain reaction was used to measure the expression level of c-myc messenger RNA (mRNA). The cells exposed to HTO showed significantly lower proliferation than the control cells over 3 days. The DNA damage in the HTO group was more severe than that in the control group, at each time point examined. The expression of miR-34a mimics caused increased DNA DSBs whereas that of the miR-34a inhibitor caused decreased DNA DSBs. The proliferation viability was the opposite for the miR-34a mimics and inhibitor groups. The expression levels of c-myc mRNA in cells transfected with miR-34a mimics were lower than that in cells transfected with the miR-34a-5p inhibitor, at 0.5 hours and 2 hours after transfection. In summary, miR-34a mediates HTO toxicity in HUVECs by downregulating the expression of c-myc.

Keywords

MiR-34a, tritiated water, DNA damage, c-myc

Introduction

Cardiovascular disease (CVD) shows a high incidence rate and imposes heavy burdens on individuals, families, and communities. Although it is known that exposure to high doses of ionizing radiation increases the risk of CVD owing to the damage it causes to blood vessels and cardiac structures, it is still unclear whether there is an association between radiation exposure and CVD.¹⁻³ Due to the increased environmental contamination by radionuclides, the impact of radiation exposure on CVDs is being considered significant. Among the many environmental radionuclides, tritiated water (HTO), a by-product of nuclear power production, is an important one.

MicroRNA (miRNA) is defined as a class of evolutionarily conserved small noncoding RNAs approximately 22 nucleotides in length. The function of miRNAs is to suppress gene expression; therefore, they take part in many cellular processes, including DNA damage response and repair.⁴ MiR-34a is one of the direct target genes of p53, and the expression of ectopic miR-34a can induce apoptosis, cell cycle arrest, differentiation,

or restraint of migration and invasion.⁵⁻⁸ In our previous study, we showed that miR-34a is downregulated after HTO exposure and further studied the functions of miR-34a to elucidate its

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role in mediating cell responses to HTO exposure.⁹ To investigate the correlation of radiation with CVDs, we examined the role of miR-34a in repairing DNA damage in HUVECs exposed to HTO. MiR-34a is known to have many target genes, of which c-myc is an important one as it plays a role during DNA damage and repair, after radiation. For example, one study showed that c-Myc suppression of DSB repair and V(D)J recombination occur through inhibition of the nonhomologous end-joining pathway.¹⁰ Hence, in this study, we determined the changes in c-myc expression after HTO exposure, in response to both miR-34a mimics and an inhibitor

Materials and Methods

Cell Culture

The HUVECs, which were originated from the vascular endothelium of humans, were cultured in Dulbecco modified Eagle medium (Gibco, Grand Island, New York) and supplemented with 10% fetal bovine serum (Gibco). The cells were incubated at 37°C in a humid environment with 5% CO₂.

Transfection With miRNAs

The product descriptions of the miR-34a-5p mimics and inhibitor (GenePharma, Shanghai, China) are available on the Web site of GenePharma (http://www.genepharma.com/En/product_slist.asp?Parentid=15&sortname=miRNA). Per their Web site, “genePharma miRNA inhibitors are chemically modified and optimized nucleic acids, designed to specifically target microRNA molecules in cells. GenePharma miRNA mimics are small, single-stranded, chemically synthesized and optimized nucleic acids, designed to mimic endogenous mature microRNA molecules in cells.” The miR-34a-5p mimics and inhibitor were transfected into HUVEC cells at a final concentration of 12.5 nmol/L using Lipofectamine 2000 (Invitrogen, Carlsbad, California) in 24-well plates, according to the manufacturer’s instructions.

RNA Isolation and Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA was obtained using TRIzol reagent (Invitrogen), according to the manufacturer’s instructions. An miRNA extraction kit was used for extraction and a TaqMan MicroRNA Reverse Transcription Kit was used for augmentation. Real-time polymerase chain reaction (PCR) was performed using miR-34a-5p and U6 TaqMan probes (purchased from Applied Biosystems, Carlsbad, California). U6 was used as the endogenous control to normalize the cycle threshold values for miR-34a-5p.

Comet Assay

The alkaline comet assay was completed using OxiSelect Comet Assay Kit (Cell Biolabs Inc, San Diego, California), according to the manufacturer’s instructions. The centrifuge

tube contained 1.0×10^3 cells, which were resuspended in 10 μ L ice-cold phosphate-buffered saline (PBS; without Mg²⁺ and Ca²⁺). The Comet Agarose was placed in a water bath at 90°C to 95°C for 20 minutes and cooled to 37°C over 20 minutes. The cell samples were then mixed with Comet Agarose at a ratio of 1:10 (v/v) and immediately pipetted (100 μ L/well) onto an OxiSelect Comet Slide. The mixture was tiled on the slides, which were incubated in a prechilled lysis solution (2.5 mol/L NaCl, 20% EDTA, 10% dimethyl sulfoxide, 10% lauryl sarcosinate; pH 10) at 4°C in the dark for 1 hour and incubated in a prechilled alkaline solution (0.3 mol/L NaOH, 0.2% EDTA; pH 13) at 4°C in the dark for 30 minutes, before performing electrophoresis at 25 V/300 mA for 30 minutes. After electrophoresis, the slides were washed using prechilled DI H₂O 3 times and placed in 70% ethanol for 5 minutes, after which the slides were removed from ethanol and air-dried. After air-drying the agarose and the slides, 100 μ L diluted Vista Green DNA Dye was added to the slides, and they were left for 15 minutes at room temperature. The diluted Vista Green DNA Dye was made from 1 \times Vista Green DNA Dye, which was diluted 1:10 000 using Tris-EDTA buffer (10 mmol/L Tris, pH 7.5 and 1 mmol/L EDTA). A total of 100 cells were analyzed per slide. The percentage of tail DNA is defined as the fluorescence intensity in the tail divided by the fluorescence intensity in the cell, multiplied by 100. All experiments were performed in triplicate and repeated at least twice.

Immunofluorescence Staining

The cells were treated as described in the figure legends. The cells were fixed with prechilled (–20°C) 4% paraformaldehyde for 15 minutes. Then, the slides were washed thrice with PBS, before being immersed in cold methanol at –20°C for 10 minutes. The slides were then blocked with 1% bovine serum albumin for 1 hour at room temperature, and the primary antibody, anti- γ -H2AX (Millipore, Darmstadt, Germany), was added to the slides, which were then kept at 4°C overnight. The slides were washed thrice before adding the secondary antibody, DyLight 649-conjugated goat antimouse IgG (H+L; 1:10,000; Multisciences, Hangzhou, China), and incubated for 1 hour in the dark at room temperature. The slides were then covered with DAPI solution for 15 minutes in the dark at room temperature and added to antiquencher. Images were captured using a laser scanning confocal microscope (Leica Microsystems, San Francisco).

Evaluation of Cell Proliferation Capacity by Cell Counting Kit 8

Cell proliferation capacity was assessed using the Cell Counting Kit 8 (CCK-8; Dojindo Laboratories, Shanghai, China) assay. The cells were divided into 6 groups; the first 2 groups were the control and HTO-exposed groups, and the remaining 4 groups were transfected with miR-34a-5p mimics, control for the miR-34a-5p mimics, the miR-34a-5p inhibitor, and control for the miR-34a-5p inhibitor, respectively. The last 4 groups of

transfected cells were also exposed to HTO. Then, all the cells were cultured for 3 days; 50 μ L CCK-8 stock solution was added daily to the 24-well plates of all 6 groups while keeping the plates at 37°C for 3 hours. Absorbance at 450 nm was measured once daily using a microplate reader (Promega, Beijing, China).

RNA Extraction and Reverse Transcription PCR

Total RNA was extracted from the HUVEC lines using TRIzol (Invitrogen), according to the manufacturer's instructions. To determine the complementary DNA (cDNA), 1 μ g total RNA was used for cDNA synthesis using the RT Kit (reverse transcriptase PCR [RT-PCR] Kit; TaKaRa BIOTECHNOLOGY, Dalian, China) and oligo (dT) in a 10- μ L reaction. The PCR primers for the 2 genes were designed using the Gene Runner and Prime 5 software programs. The following primers were used for amplification: for c-myc-F, 5'-ACAGGAACTATGACCTCGACT-3' and for c-myc-R, 5'-CCGAAGGGA-GAAGGGTGT-3'. The PCR reaction mixture contained 0.25 μ L cDNA sample, 0.125 μ L DNA polymerase (2.5 U/ μ L), 1 μ L dNTP (2.5 mmol/L), 5 μ L 5 \times PrimeSTAR buffer (Mg²⁺ plus), and 0.5 μ L of each primer (20 pmol/L); dH₂O was used to make the total volume to 25 μ L. 18S rRNA was used as the reference gene for RT-PCR. The PCR cycling conditions for the c-myc gene were: 3 minutes at 98°C; followed by 10 seconds at 98°C, 15 seconds at 40°C, 50 seconds at 72°C for 40 cycles; and finally, 10 minutes at 72°C. The cycling conditions for 18S rRNA were 10 seconds at 98°C, 15 seconds at 55°C, and 50 seconds at 72°C for 40 cycles. The PCR products were separated in a 1% agarose gel, stained with GelRed (Biotium, San Francisco, California, USA), and then photographed using a multicolor fluorescent and chemiluminescent imaging system (Alpha, Shanghai, China).

Statistical Analyses

The mean \pm standard deviation (SD) was used for assessment, when appropriate. Incidences of statistical significance were defined as $P < .05$.

Results

Exposure to HTO Causes Changes in Cell Growth, DNA DSBs, and miR-34a Expression in HUVECs

The results of the cell counting experiments showed that HUVEC growth was significantly slower with HTO exposure than that without it, over a period of 3 days (Figure 1A). The induction and repair of DNA DSBs were detected in HUVECs exposed to HTO. The expression of γ H2AX, a sensitive DNA DSB biomarker, increased rapidly (Figure 1B), and immunofluorescent foci were formed at 0.5 hours after HTO exposure, peaked at 2 hours, and then reached a plateau. The comet assay also indicated increased DNA damage, as demonstrated by the higher residual level of the tail moments (Figure 1C) in HUVECs at 0.5, 2, and 4 hours after HTO exposure. Taken

together, these results show that DNA damage in the HTO-exposed HUVECs was severe, resulting in slower cell growth.

Accordingly, the expression of miR-34a in HUVECs changed after HTO exposure; miR-34a expression at 0.5 and 4 hours were lower than that at the other time points examined, with the highest expression at 2 hours. Quantitatively, the highest miR-34a expression was 11.6-fold higher than the lowest expression (Figure 1D).

Transfection of miR-34a into HUVECs

The level of miR-34a was altered by transfecting either miR-34a mimics or the miR-34a inhibitor into HUVECs. When the concentration of the miR-34a mimics was 12.5 nmol/L, the level of miR-34a in the cells was 5037.58-fold higher than that in the control (Figure 2A). However, when treated with the same concentration of miR-34a inhibitor, the level of miR-34a lowered to 66.99% of the level in the control (Figure 2B).

MiR-34a-Regulated Cell Growth and DNA Damage and Repair

MiR-34a was overexpressed or suppressed by transfecting mimics or the inhibitor into the cells, as described earlier. The cell counting experiments showed that cell proliferation in the miR-34a mimics group was slower than that in the miR-34a inhibitor group (Figure 2C).

Comet assay and γ -H2AX immunostaining showed that the cells of the miR-34a mimics group had more severe DNA damage than the cells of the control group (Figure 2D and E). Unlike the control group, the DNA damage caused by HTO exposure is exacerbated in the miR-34a mimics group; however, this increased damage could be attenuated by the miR-34a inhibitor. Taken together, these results demonstrate that miR-34a regulates DNA damage and repair after HTO exposure.

Exposure to HTO Alters the c-Myc Expression Levels in HUVECs

The expression of c-myc is shown in Figure 3. The c-myc expression at 0.5 and 2 hours, in the HTO group, was higher than that in the control group; its expression at 0.5 and 2 hours in the miR-34a mimics group was lower than that in the respective control group, and the opposite effect was seen in the miR-34a inhibitor group at the same time points.

Discussion

Since the mid-1960s, it has been widely recognized that high doses of radiation administered to patients as part of the treatment for cancers, such as Hodgkin disease and carcinoma of the breast, can cause damage to the heart and pericardium. Development of cardiac disease in patients treated with mediastinal radiotherapy for Hodgkin disease reportedly accounts for 25% of all deaths that were not associated with Hodgkin disease

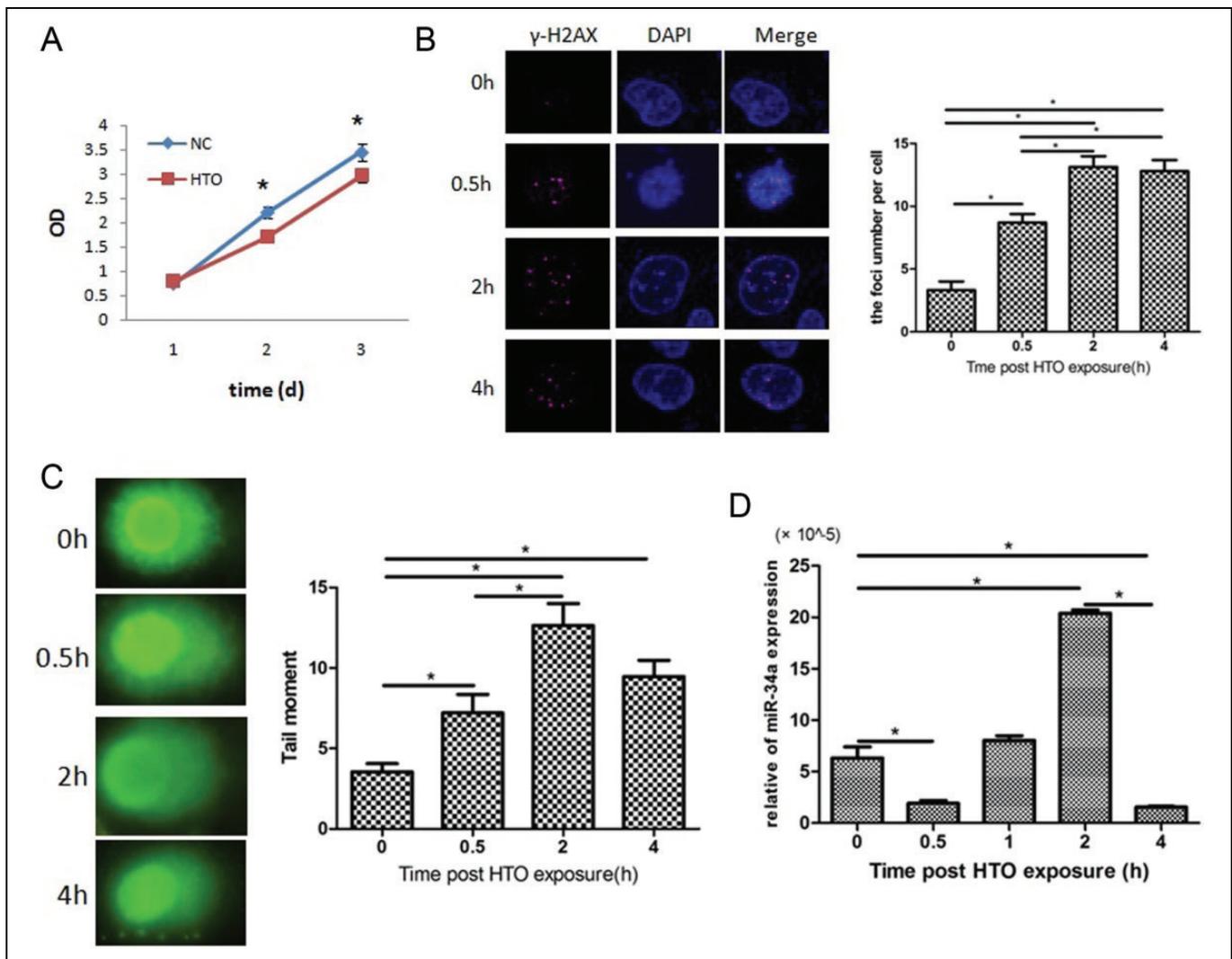


Figure 1. Cell proliferation, DNA double-strand breaks (DSBs), and the changes in microRNA-34a (miR-34a) expression in human umbilical vein endothelial cells (HUVECs), after exposure to tritiated water (HTO). A, Cell proliferation as assayed by cell counting. B, γ -H2AX expression, as seen by immunofluorescence staining. C, Tail moment in the comet assay. D, MiR-34a expression after HTO exposure, at different time points. The reported values are the means \pm standard deviation (SD; n = 3); *P < .05, in comparison to the control group.

itself. Generally, estimates of relative risk are in the range 2.2 to 3.1, although 1 study estimated it to be as high as 7.2. In 2011, Baker et al wrote a review that examined the sources of radiation and the risk of developing CVD.¹¹ The evidences presented in the review suggest that exposure to low-to-moderate radiation doses also correlates with CVD development, apart from the well-known correlation between exposure to high radiation doses and CVD development. The HTO, discarded from nuclear plants, is an oxidized form of tritium that can produce β -particles in living bodies. In previous studies, β -rays have been found to cause chromosome aberrations in human lymphocytes, bone marrow cells, spermatozoa, and fertilized mouse eggs.¹²⁻¹⁵ It also led to scission of puC18 plasmid DNA.¹⁶ Additionally, HTO can cause hematopoietic impairment and thymic lymphoma, which induce life-shortening effects in female B6C3F1 mice.^{17,18} When infant rats were

exposed to an HTO-contaminated environment, their blood pressures were found to increase significantly.¹⁹

Although HTO-produced β -rays have a low linear energy transfer,²⁰ the life-threatening effects of long-term exposure to β -rays were more marked than those of long-term exposure to X-rays or γ -rays.¹⁸ A dose-dependent increase in DNA single-strand breaks in response to HTO has been found in the marine mussel, *Mytilus edulis*.²¹ It has been reported that the values of RBE, which HTO induces as a result of X-rays on chromosome aberrations in human spermatozoa, ranged from 1.89 to 3.00.¹³ Irradiation of mice can damage vascular endothelial cells and lead to cardiac toxicity.²² However, little is known about the molecular mechanism by which HTO induces vascular endothelial damage. Here, we first establish a model of HTO exposure in HUVECs to explore the mechanism by which HTO affects HUVECs.

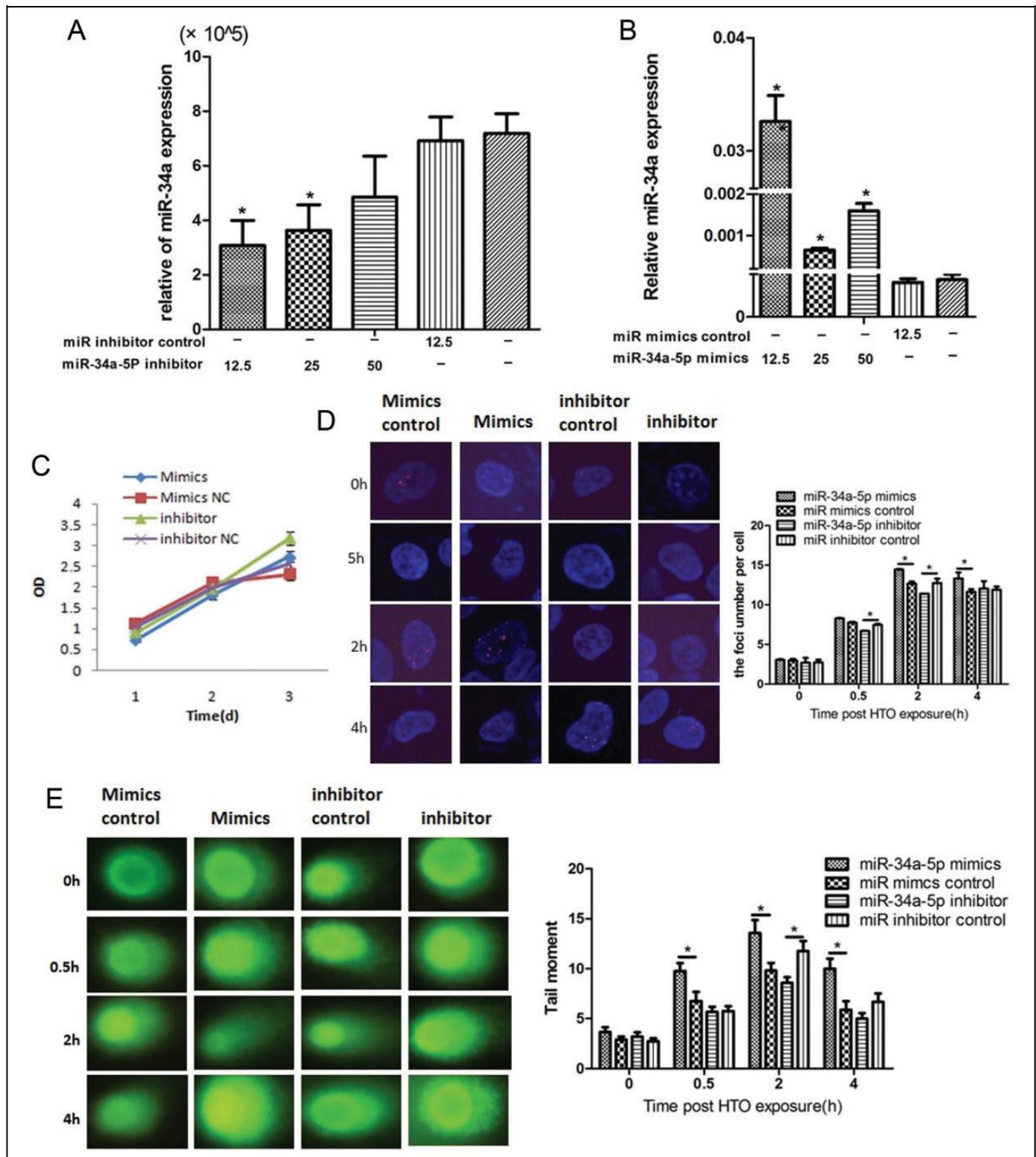


Figure 2. Optimal transfection conditions and cell proliferation and DNA double-strand breaks (DSBs) in human umbilical vein endothelial cells (HUVECs), after tritiated water (HTO) exposure. A, Transfection conditions for the microRNA-34a (miR-34a) inhibitor. B, Transfection conditions for the miR-34a mimics. The reported values are means \pm standard deviation (SD; $n = 3$); $*P < .05$, in comparison to the control group. C Cell proliferation, as assayed by cell counting. D, Confocal images of immunofluorescent foci in cells transfected with miR-34a mimics and inhibitor immunostained using anti- γ -H2AX at the indicated time points, after HTO exposure. E, Quantitative analysis of Olive tail moment in neutral comet assay of DNA DSBs in cells transfected with miR-34a mimics and inhibitor at the indicated time points, after HTO exposure. The reported values are the means \pm SD ($n = 3$); $*P < .05$, in comparison to the control group.

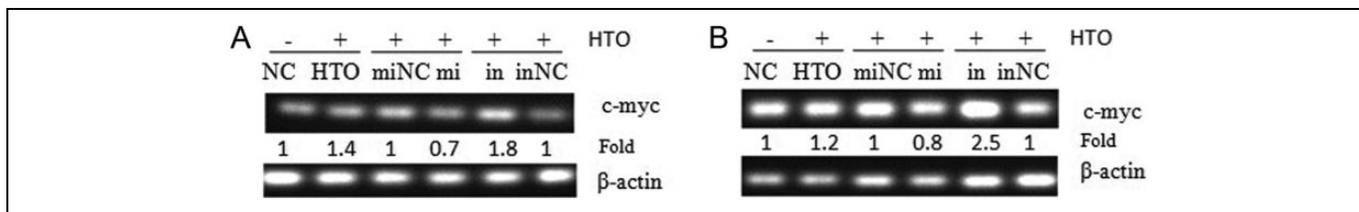


Figure 3. Expression of c-myc in human umbilical vein endothelial cells (HUVECs) at 0.5 hours (A) and 2 hours (B), after tritiated water (HTO) exposure.

MicroRNA is a family of small noncoding RNAs (~20 nt) in organisms ranging from nematodes to humans.²³ Recently, miRNAs have received much attention owing to their ability to increase or decrease the expression of target genes by binding to the 3'-untranslated regions (UTRs) or other regions (eg, the 5'-UTR coding sequences) and regulating protein expression.²⁴ Therefore, miRNAs play an important role in diseases or injuries and might be a new potential therapeutic target.²⁵ The miRNAs have already been shown to play critical roles in proliferation, apoptosis, differentiation, migration, cancer, and stress responses.^{26,27} The miRNAs regulate the biological pathways for stress response or induce apoptosis in response to irradiation, both in vivo and in vitro. Reports have already shown that miRNA regulates the expression of target genes, including apoptotic genes and other specific genes.²⁸

Previous studies have shown that miR-34a has an antiproliferative role and acts as a tumor suppressor in cancers. Additionally, miR-34a can lead to cell apoptosis or cell cycle arrest.^{29,30} Low-energy X-ray irradiation reportedly upregulates miR-34a expression in normal and breast cancer cell lines.³¹ Downregulation of miR-34a by an miR-34a inhibitor reduced cellular apoptosis induced by DNA damage.³⁰ In our study, after exposing HUVECs to HTO, at different time points, we observed the changes in miR-34a expression, which may mediate DNA damage. MiR-34a overexpression exacerbated DNA damage; our results show that suppressing miR-34a expression may protect cells from HTO-induced DNA damage.

It is well known that miR-34a has many target genes, including c-myc and STAG2.³² c-Myc plays a role in DNA damage-induced apoptosis by reducing the apoptotic response during DNA repair.³³ Li et al reported in their study that "expression of c-Myc reduces both signal and coding joins with decreased fidelity during V(D)J recombination. Mechanistically, c-Myc directly interacts with Ku70 protein through its Myc box II (MBII) domain. Interestingly, c-Myc directly disrupts the Ku/DNA-PKcs complex in vitro and in vivo."¹⁰ Our previous study also confirmed that c-Myc participates in the repair of ionizing radiation-induced DSBs, by regulating ataxia telangiectasia-mutated gene (ATM) phosphorylation and DNA-PK kinase activity.³⁴ In this study, we found that miR-34a inhibition could enhance c-myc gene expression at different time points, thus attenuating DNA damage, in HUVECs exposed to HTO. This finding shows that regulating c-myc expression is one of the mechanisms by which miR-34a participates in HTO-induced DNA damage and repair.

In summary, HTO can induce DNA damage in HUVECs; miR-34a may mediate DNA DSB repair by regulating the c-myc gene. Hence, this study provides new insights into the mechanism of HTO toxicity and the association of radionuclides with CVDs.

Author's Note

Feng Mei Cui and Liang Liu contributed equally to this work. Conceived and designed the experiment: Fengmei Cui and Yongming He. Performed the experiments: Qiu Chen, Liang Liu, Lulin Zheng, Wei Zhu and Fengmei Cui. Contributed the HTO: Guangliang Bao. Analyzed the data: Yu Tu, Jianping Cao and Liang Sun. Wrote the paper: Liang Liu, Fengmei Cui and Yongming He.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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