Tritiated Water Induces Toxicity in Human Umbilical Vein Vascular Endothelial Cells via IL8

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Abstract
We aimed to determine the toxic effects of tritiated water (HTO) on 12 generations (T1-T12) of human umbilical vein vascular endothelial cells (HUVECs) and elucidate the underlying mechanisms. We evaluated cellular senescence, interleukin (IL) 8 concentrations, and angiogenesis using β-galactosidase staining, enzyme-linked immunosorbent assay, and in vitro assays, respectively. The adhesion properties of contaminated cells and differentially expressed genes were assessed using the xCELLigence RTCA SP system and gene chip analysis, respectively. We found that long-term exposure to low levels of HTO can reduce the adhesion of HUVECs to the cellular matrix as well as their angiogenic capacity, while increasing their permeability, senescence, and adhesion to monocytes. Interleukin 8 activated the p38 and Epidermal Growth Factor Receptor (EGFR) pathways in HTO-treated cells and hence was identified as a key candidate of biomarker. The present study clarified the toxicity of HTO in vascular endothelial cells and identified IL8 as a novel protective target with important theoretical and practical values.

Keywords
tritiated water, human umbilical vein vascular endothelial cells, interleukin 8

Introduction
The extensive application of ionizing radiation in industry, medicine, scientific research, and various other fields can exert negative effects on human health as well as on massive economic and social benefits. In recent years, the public has become increasingly concerned about health issues resulting from environmental exposure to ionizing radiation, and tritium exposure accounts for the highest proportion of environmental contamination. Tritium is a major environmental contaminant produced by nuclear power plants that can each emit up to tens of thousands of TBq of tritium annually. With the continuous development of nuclear energy and an increasing number of nuclear reactors becoming operational, tritium emissions will continue to increase along with the increased concern regarding its effects on human health.

Tritium can directly or indirectly induce various biological effects including DNA strand breaks, micronucleus formation, cell necrosis or apoptosis, chromosomal aberration, and various other phenomena, thus negatively affecting human health.1-4 The half-life of tritium is 12.33 years, and the average

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penetration distance of its electrons in water is 0.56 µm, which is considerably less than the average diameter of a human cell.\(^{5,6}\) Therefore, it does not cause damage due to external irradiation but rather long-term internal radiation that results from the inhalation, oral intake, or absorbance of radioactive particles through the skin. After entering an organism, tritium can replace hydrogen atoms in biological macromolecules or bind to cellular components to form organically bound tritium (OBT)\(^7\). Exposure increases along with increased tritium levels in the environment,\(^8,9\) which might ultimately result in toxicity and genotoxicity to mammalian cells.\(^{10,11}\)

Humans should be most concerned about the effects of long-term exposure to low levels of tritium, especially on the cardiovascular system. Having a low proliferation rate, cardiomyocytes have historically been considered as one of the most radioresistant cell types. However, risk of cardiovascular diseases, including myocardial fibrosis, decreased ventricular wall motion function, coronary heart disease, and myocardial infarction, is significantly increased when tumors are treated by radiation compared with that by other modalities.\(^{1,2,13}\) Hauptmann et al.\(^14\) compared mortality from circulatory system diseases in 90, 284 radiological technologists in the USA during 1997 with their employment history and procedures. During an accrued total of 1, 107, 100 person-years, 1070 technologists died from diseases of the circulatory system. In subsets of deaths from cerebrovascular diseases (\(n = 174\)) during the decades before 1940, 1940 to 1949, and 1950 to 1959, compared with 1960 and later, the respective relative risks (with 95\% CI) were 0.90 (0.45-1.78), 1.54 (0.74-3.23), and 2.40 (1.09-5.31; \(P\) trend = 0.004). The respective relative risks (with 95\% CI) of death from ischemic heart disease (\(n = 633\)) were 0.98 (0.71-1.35), 1.00 (0.71-1.42), and 1.22 (0.81-1.82; \(P\) for trend = 0.026). Although the link between the incidence of cardiovascular diseases and mortality caused by ionizing radiation has reached general consensus, the specific molecular mechanism underlying this link remains unclear.

Here, we used gene chip technology to screen differentially expressed genes (DEGs) in human umbilical vein vascular endothelial cells (HUVECs) exposed to tritiated water (HTO). We then assessed changes in HUVEC morphology and function to understand the mechanisms underlying tritium-mediated vascular injury.

**Materials and Methods**

**Materials and Reagents**

Human umbilical vein vascular endothelial cells and monocyte/macrophage type 1 (THP-1) cells were acquired from the Chinese Academy of Sciences Cell Bank. High-glucose Dulbecco’s modified Eagle’s medium (DMEM) and phosphate-buffered saline (PBS) were sourced from Wisent Inc. Gibco fetal bovine serum (FBS), trypsin, and attachment factor were sourced from Thermo Fisher Scientific Inc. Cell senescence β-galactosidase staining kits were purchased from Beyotime Biotechnology. Evans blue (EB) and collagen were sourced from Sigma-Aldrich Corp. In vitro angiogenesis assay kits, Millicell hanging cell culture inserts, and 24- and 96-well plates were purchased from Millipore Sigma Co Ltd., and HTO (5 mCi) was obtained from PerkinElmer Life and Analytical Sciences Inc. E-Plate 16× was sourced from ACEA Bioscience Inc. Dimethyl sulfoxide was obtained from Beijing Solarbio Science and Technology Co., Ltd., and anti-Interleukin (IL)8, goat anti-rabbit, and goat anti-mouse antibodies were all from Abcam Plc.

**Culture and Passage of HUVECs**

We cultured HUVEC in DMEM (high glucose) supplemented with 10% FBS at 37 °C under a humid 5% CO\(_2\) atmosphere. When the cells reached confluence, the medium was removed and the cells were dissociated using trypsin in PBS. After sedimenting the cells by centrifugation at 1000 rpm for 5 minutes, the cells were suspended in high-glucose DMEM and seeded into 3 or 4 new dishes (depending on the density) at a ratio of 1:3 or 1:4 cells:medium.

**HUVEC Exposure and IL8 Neutralization**

Cells were prepared as described above, and the experiment was started by exchanging the growth medium in the dishes. The HUVECs were incubated with or without (T0) 3.7 × 10\(^3\) Bq/mL of tritium in high-glucose DMEM. Thereafter, each generation from 1 to 12 (T1-12) was exposed to tritium and washed 3 times in tritium-free medium to minimize tritium artifacts.

Each generation (T1-12) of cells was divided into groups (\(n = 4\) each), and IL8 concentrations were determined by enzyme-linked immunosorbent assay (ELISA). Cells in group 1 were incubated with an anti-IL8 antibody. The concentration of IL8 in the medium of group 2 was determined by ELISA, then a sufficient amount of anti-IL8 antibody was added to neutralize half the initial IL8 amount. Group 3 was incubated with the same amount of universal IgG antibodies as group 1. Group 4 was incubated for 12 hours with the same amount of universal IgG antibodies as in group 2. We also added 1.46, 3.15, and 4.22 ng/mL of IL8 to T5, T8, and T10, respectively.

**Cell Senescence Assays**

Cellular senescence was determined using senescence β-galactosidase staining kits with X-Gal as the substrate. The medium was removed, then the cells were washed once with PBS and fixed for 15 minutes at room temperature using the fixative provided in the kit. The fixative was removed, and the cells were rinsed in PBS 4 times. Thereafter, we added 1 mL of the β-galactosidase staining solution, incubated the cells in a CO\(_2\)-free atmosphere at 37 °C overnight. The staining solution was discarded on the following day, and PBS (2 mL) was added. Stained cells were then assessed by standard light microscopy and counted using ImageJ software.
Angiogenesis Test

Angiogenesis was detected according to the manufacturer’s instructions. Briefly, 900 μL of ECMatrix and 100 μL of 10× dilution buffer was slowly mixed in sterile centrifuge tubes. Thereafter, 50 μL of the mixture was transferred to each of 96 wells in cooled plates and incubated at 37 °C for at least 1 hour. Subsequently, cell suspensions (5 × 10^4/mL; 150 μL) were added, and plates were incubated at 37 °C for 12 to 18 hours. The cells were then photographed using a microscope, and the length of blood vessels was measured.

Cell Adhesion Test

The adhesion properties of the contaminated cells were tested using the xCELLigence RTCA SP system. Briefly, cells were suspended as described above, sedimented by centrifugation, and the supernatant was discarded. The cells were then resuspended at a final concentration of 1.6 × 10^6/mL, then 50 μL of the medium was added to each of the well in the E-Plate 16, which was placed on the RTCA Station for contact tests. A further 100 μL of the cell suspension was added to each well and replaced on the RTCA Station for data capture at 5-minute intervals. The first reading was taken as test point 1.

In the HUVEC-THP-1 adhesion test, each generation of HUVECs was seeded in 6-well plates and cultured until they reached 85% confluence. THP-1 cell suspension (700 μL) was mixed with of Snarf solution (0.7 μL) and incubated at 37 °C for 30 minutes. Portions (50 μL) of the THP-1 cell suspension were transferred to each well and incubated at 37 °C for 1 hour. The cells were washed 3 times in PBS to remove non-adherent monocytes and then were visualized by fluorescence microscopy. Ratios of THP-1 cells to HUVECs were calculated.

Cell Permeability Test

The level to which EB penetrated the cytoskeleton was detected using a microplate reader to determine the permeability of cell monolayers. Cytoskeleton mixed with 1% collagen was added to 24-well plates and incubated at 37 °C for 10 hours. Excess liquid was discarded and the plates were dried at 37 °C for 12 hours. Adhesion factors (60 μL) were added to each well, then the plates were incubated at 37 °C for 30 minutes. Excess adherence factors were discarded and the cell suspensions were diluted to 2.7 × 10^5/mL, and 250 μL of growth medium was added to cover the bottom of each well. The plates were covered and incubated at room temperature for 15 minutes. Growth medium (200 μL) was replaced with 200 μL of cell suspension plus 500 μL of medium, then the plates were incubated at 37 °C under a 5% CO_2 atmosphere until the cells formed monolayers. The solutions in each well were diluted 1:100, transferred to new plates, and mixed for 3 minutes. Cell permeability was then evaluated at a wavelength of 620 nm using a microplate reader.

Western Blotting

Cell suspensions produced as described above were separated by centrifugation at 1000 rpm for 5 minutes, then the supernatants were discarded and cell pellets were weighed. The cells were mixed with sodium dodecyl sulphate loading buffer (1 mg/10 μL) and lysed on ice for 20 minutes. The mixtures were boiled at 100 °C for 10 minutes, then 15-μL portions were resolved by electrophoresis on 10% polyacrylamide gels, then transferred to polyvinylidene fluoride membranes. Nonspecific binding on the membranes was blocked with skim milk for 1 hour. The membranes were incubated overnight with p-EGFR, p-p38, and anti-histone H4 antibody, washed 3 times, and then incubated with anti-rabbit antibody for 1 hour.

Enzyme-Linked Immunosorbent Assay

After equilibrating the plates to room temperature, blanks, standards, and samples were added to the wells and incubated at 37 °C for 90 minutes. After 5 washes, biotinylated antibody working solution (100 μL/well) was added. The plates were covered and incubated in darkness at 37 °C for 60 minutes, washed 5 times, and then enzyme conjugate diluent, and working solutions were added to the wells (100 μL/well each). The plates were covered and incubated at 37 °C for 30 minutes, washed another 5 times, and then mixed with the chromogenic substrate 3,3',5,5'-tetramethylbenzidine (100 μL/well) and incubated in darkness at 37 °C for 15 minutes. Stop solution (100 μL/well) was immediately added and the optical density at 450 nm (within 3 minutes) was measured using the microplate reader.

Chip Analysis

This analysis was completed at Shanghai Kangcheng Biotechnology Co Ltd. Samples of RNA prepared from homogenized cells using 2-phase separation and precipitation were stored at −80 °C. Total RNA was purified and reverse transcribed to complementary DNA (cDNA). The cDNA was then hybridized, purified, and scanned. Data were extracted and analyzed using Agilent Extraction Software.

Statistical Analysis

Data were statistically analyzed using SPSS 17.0 (SPSS Inc), and Student t tests were adopted for intergroup comparisons. Values with P < 0.05 were considered statistically significant.

Results

Tritiated Water Induced Morphological Changes and Senescence in HUVECs

Figure 1 shows that HTO significantly and dose dependently altered cell morphology, including irregular appearance, increased number of particulates in the cytoplasm, increased number of dead cells, blurred borders, and roughed cell
outlines. The T9 and T10 HUVEC generations were significantly larger in size than the controls, indicating that long-term exposure to HTO induces morphological changes in these cells. Figure 2 shows that cellular senescence significantly increased after exposure to tritium, starting from the T6 generation.

Changes in the Adhesion and Permeability of HUVECs After Exposure to HTO

Cellular adhesion, including cell-matrix and cell-cell adhesion, is considered a prerequisite for angiogenesis. Cell-to-cell adhesion, such as monocyte adherence to vascular endothelial cells, is considered an early indication of atherosclerosis. Membrane permeability started to increase from the T3 generation (Figure 3A). Figure 3B and C shows that HUVEC adhesion to cell-matrix decreased, whereas that to THP-1 cells remarkably increased, from the T3 generation.

Effects of HTO on Angiogenesis in HUVECs

Angiogenesis is an important physiological process implicated in both wound healing and development. The proliferation of endothelial cells and the formation of vascular loops through ducted branching are important events in angiogenesis and can serve as a measure of the angiogenic capacity of vascular endothelial cells. Figure 4 shows that the angiogenic capacity of HUVECs began to decline from the T3 generation as evidenced by decreased branching and cellular proliferation in the angiogenesis assays.

Screening DEGs

Figure 5 shows DEGs in the irradiated (3.7 × 10^3 Bq/mL) group compared with those in the control. Each data point in Figure 5A represents the hybridization intensity of each gene signal. Green and red points represent significantly downregulated and upregulated genes in the irradiated group compared with those in the control group. The heat map of the messenger
RNA cluster findings in Figure 5B shows that 4562 and 1433 genes were significantly upregulated and downregulated, respectively, in the irradiated group (fold change > 2, $P < 0.05$) compared with those in the control.

We found that among the DEGs, IL8, C-X-C motif chemokine ligand 8, laminin subunit α5, protein kinase, X-linked (PRKX), r-prox-melocytic leukemia, and sphingosine-1-phosphate receptor 1 genes were involved in cell adhesion and angiogenesis regulatory pathways. The results of further analysis (Figure 5C) showed that IL8, PRKX, and ROCK2 levels increased with the exposure to both low and high doses of HTO. Interleukin 8 was identified as the most reliable indicator of downstream effects because both ROCK2 and PRKX are non-end point proteins.

**Tritiated Water Induced Toxicity in HUVECs via IL8**

We further investigated the role of IL8 in tritium-mediated toxicity by measuring IL8 expression in HUVECs exposed to HTO using an ELISA. Figure 6A shows significantly elevated IL8 secretion in the T8 and T10 generations. The addition of an IL8 neutralizing antibody significantly increased the angiogenic capacity of tritium-treated HUVECs evidenced by an increase in the number of Nb nodes in the T5, Nb segments in T5 to T10, and the area of Tot meshes in the T8 generations (Figure 6B and C). In addition, p38 and EGFR were activated in cells incubated with HTO. Both p-p38 and p-EGFR increased after the addition of IL8 neutralizing antibodies (Figure 6D), indicating that recovery in these cells is linked to p38 and EGFR signaling.

**Discussion**

The potential health hazards of tritium cannot be ignored due to its mobility, cyclicity, and theoretical ability to exchange with hydrogen atoms in any macromolecules and compounds. Johnson et al. reported that the relative biological effects (RBEs) of tritium β-rays range from 1.0 ± 0.5 to 1.3 ± 0.3, which is comparable to the estimated value of 1.2 ± 0.3 for chronic X-rays. However, consensus on the RBE of tritium has not yet been reached. The UK Advisory Group on Ionizing Radiation released a report suggesting that given the large number of radiobiology studies that focus on tritium, its RBE should be classified as a minimum of 2 and that 3 should be considered for safety.

Although the biological effects of high doses of tritium have been defined and genetic and specific tumor effects have been identified in animal models after long-term exposure, epidemiological investigations into the health risks associated with exposure to tritium are not yet publicly available. A follow-up survey of 1562 workers with possible internal exposure to tritium radiation (Beral et al. found that the death rate due to prostate and kidney cancer in this group after 10 years was over twice of that in a group that had not been exposed to internal radiation. Despite this finding, the authors postulated that the increase in prostate cancer mortality could not be attributed solely to tritium because it does not accumulate in the prostate. A nested case–control study published by Rooney et al. showed that an increased risk of cancer is associated with exposure not only to tritium but also to $^{51}$Cr, $^{59}$Fe, $^{60}$Co, or $^{65}$Zn. Therefore, which radionuclides are responsible for increased risk of cancer remain unclear. According to a survey of staff at a nuclear power plant in Canada between 1957 and
Figure 3. Changes in HUVEC adhesion and permeability after exposure to HTO. A, Permeability of HUVECs after exposure to HTO. B, HUVEC adhesion to matrix. C, HUVEC adhesion to THP-1 cells. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\). HTO indicates tritiated water; HUVECs, human umbilical vein vascular endothelial cells; THP-1, monocyte/macrophage-1.

Figure 4. Effects of HTO on angiogenesis in HUVECs. A, Angiogenesis of different cell generations. B, Length. C, Branch point. D, Score. *\(p < 0.05\), **\(p < 0.01\). HTO indicates tritiated water; HUVECs, human umbilical vein vascular endothelial cells.
A cumulative absorbed dose increase of 1 Sv throughout the body could result in increased morbidity due to leukemia (52.5%), excluding chronic lymphocytic leukemia, compared with that in patients who were not exposed to radiation. Such conflicting data can explain why the health risks of tritium remain obscure, even though it is one of the main radionuclides associated with environmental exposure. Thus, tritium remains a current focus of investigators interested in the effects of environmental radiation exposure and health professionals alike, and this is likely to continue until its risks are fully understood.

One of the most significant biological effects of long-term exposure to low-level tritium is cardiovascular toxicity. Radiation can induce cardiovascular diseases, including myocardial fibrosis, ventricular wall motor dysfunction, and coronary heart disease, all of which are the consequences of significant changes to the vascular system. Vascular homeostasis is an important sign of health, and the integrity of vascular endothelial cells is an important factor in its maintenance. Pathological thrombosis might be induced after the vascular endothelium becomes damaged, and this will lead to atherosclerosis, which ultimately affect all organs in the body.
including the heart, kidney, brain, and liver. We previously confirmed that HTO can induce DNA damage in HUVECs. The present study further showed that long-term exposure to low-level HTO reduces HUVEC adhesion to cell matrices and its angiogenic capacity, while increasing their permeability, senescence, and adhesion to monocytes. This in turn leads to disrupted functions, potentially reducing their ability to maintain vascular homeostasis.

After confirming that long-term, low-level HTO exposure is toxic to HUVECs, we investigated the underlying mechanism(s). We identified IL8 as a candidate and evaluated its expression and function in cells incubated with HTO. This cytokine is primarily produced by monocyte-macrophages, and to a lesser extent, by fibroblasts, epithelial cells, and endothelial cells under appropriate stimulation. Its predominant function is to attract and activate neutrophils that cause local inflammatory reactions that mitigate cellular damage. It also promotes angiogenesis. We found that IL8 secretion increased, whereas angiogenesis decreased in HUVECs exposed to HTO, indicating that the IL8 secreted by HUVECs functions in activating the inflammatory response, as well as the p38 and EGFR pathways. These results are consistent with those of previous studies, including an investigation by Schröder et al., who exposed EC to 0.01 to 2 Gy of X-rays. They found that the amount of IL8, granulocyte macrophage colony-stimulating factor, and platelet-derived growth factor-BB increased in cell supernatants at different time points, with a nonlinear, dose-dependent relationship to the radiation. Shao et al. also found that ferulic acid can significantly improve the outcomes of HUVECs exposed to radiation by promoting cell viability and angiogenesis and arresting the G2/M cell cycle, which might be associated with decreased levels of HMGB1, IL-6, and IL8. Besides, PRKX expression was also dependent on the amount of exposure, indicating a possible biological dosimeter.

Recently, few papers revealed the effect of HTO on Zebrafish. Arcanjo et al. managed to develop protocols to perform total tritium and total OBT activity concentrations measurements in zebrafish eggs and larvae. Gagnaire et al. reported that HTO exposure induced DNA damage, reactive oxygen species production, and modulated the expression of genes involved in detoxification processes. It can be seen that in-depth research on the environmental toxicology of HTO is needed.

In summary, long-term exposure to HTO might cause the senescence of vascular endothelial cells, decrease their permeability, alter their adhesion properties, and reduce their capacity to maintain vascular homeostasis, and might be mediated by IL8. We clarified that HTO is toxic to vascular endothelial cells and showed that IL8 could have important theoretical and applications as a novel protective target.

**Authors’ Note**
Hong-Bin Yan, Yi-Tong Liu, Zhen-Yan Li, and Zhuo-Jun Wu are have contributed equally to this work as the co-first authors.
Hong-Bin Yan, Yi-Tong Liu, Zhen-Yan Li, Zhuo-Jun Wu, Feng-Mei Cui, and Qiu Chen conceived and designed the study. Meng Zhang and Pei-Jun Xue conducted the experiments. Yu-Long Liu, Kong-Zhao Wang, Yong-Ming He, and Yu Tu contributed in data analysis. Hong-Bin Yan, Yi-Tong Liu, Zhen-Yan Li, and Zhuo-Jun Wu wrote the manuscript.

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

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