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Source: Radiation Research, 195(3) : 265-274

Published By: Radiation Research Society

URL: https://doi.org/10.1667/RADE-20-00208.1

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RADIATION RESEARCH **195**, 265–274 (2021)
0033-7587/21 $15.00
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DOI: 10.1667/RADE-20-00208.1

A 3D *In Vitro* Model of the Human Airway Epithelium Exposed to Tritiated Water: Dosimetric Estimate and Cytotoxic Effects

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Baiocco, G., George, I., Garcia-Argote, S., Guardamagna, I., Lonati, L., Lamartinière, Y., Orsière, T., Rousseau, B., Ottolenghi A., Jha, A., Lebaron-Jacobs, L., Grisolia, C. and Malard, V. A 3D *In Vitro* Model of the Human Airway Epithelium Exposed to Tritiated Water: Dosimetric Estimate and Cytotoxic Effects. *Radiat. Res*. 195, 265–274 (2021).

Tritium has been receiving worldwide attention, particularly because of its production and use in existing fission reactors and future nuclear fusion technologies, leading to an increased risk of release in the environment. Linking human health effects to low-dose tritium exposures presents a challenge for many reasons. Among these: biological effects strongly depend on the speciation of tritiated products and exposure pathway; large dosimetric uncertainties may exist; measurements using *in vitro* cell cultures generally lack a description of effects at the tissue level, while large-scale animal studies might be ethically questionable and too highly demanding in terms of resources. In this context, three-dimensional models of the human airway epithelium are a powerful tool to investigate potential toxicity induced upon inhalation of radioactive products in controlled physiological conditions. In this study we exposed such a model to tritiated water (HTO) for 24 h, with a range of activity levels (up to ~33 kBq μl^{-1} cm^{-2}). After the exposures, we measured cell viability, integrity of epithelial layer and pro-inflammatory response at different post-exposure time-points. We also quantified tritium absorption and performed dosimetric estimates considering HTO passage through the epithelial layer, leading to reconstructed upper limits for the dose to the tissue of less than 50 cGy cumulative dose for the highest activity. Upon exposure to the highest activity, cell viability was not decreased; however, we observed a small effect on epithelial integrity and an inflammatory response persisting after seven days. These results represent a reference condition and will guide future experiments using human airway epithelium to investigate the effects of other peculiar tritiated products. © 2021 by Radiation Research Society

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INTRODUCTION

Efforts are ongoing to improve knowledge on tritium management in fission and fusion facilities (*1*). These address tritium release mitigation strategies, waste management improvement, tritium monitoring, dosimetry and epidemiology, as well as the refinement of knowledge in the fields of radiotoxicity and radiobiology (see e.g., TRANSAT, http://transat-h2020.eu/). Concerning these last topics, it is acknowledged that the radiotoxicological and radiobiological consequences of tritium contamination strongly depend on the speciation of tritiated products (*2*). Tritium commonly occurs as tritiated water (HTO) or organically-bound tritium (OBT) (*3*), but can also exist in many other forms, depending on nuclear-related construction materials (*4*). For example, tritiated tungsten nanoparticles are of interest (*5*), as they will be produced by plasma interactions with the wall components of the International Thermonuclear Experimental fusion Reactor (ITER). These could be potentially released into the environment after a loss-of-vacuum-accident (*6*). In another example, during the decommissioning of nuclear facilities, operations may be performed to eliminate tritiated materials, possibly generating airborne dust in the form of, e.g., tritiated steel or cement dust particles of micrometric size (*1*).

Furthermore, different exposure pathways are possible under accidental exposure scenarios (i.e., inhalation, skin absorption and/or ingestion). Overall, the chemical speciation determines, for a given exposure pathway, both the kinetics associated with the distribution of tritiated products at the organ/tissue level (including clearance) and the associated distribution at the cellular and subcellular levels. Considering the average range of beta (β) electrons emitted

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by tritium in biological tissue (~0.5 μm, corresponding to an average decay energy of 5.7 keV), it is clear that both the distribution of energy deposition events at the subcellular level and the cumulative dose to target cells will vary substantially when different tritiated products are considered. In turn, it will result in different biological effects.

In vitro models are a powerful tool to investigate the effects of tritiated products in controlled experimental conditions. *In vitro* three-dimensional (3D) systems cultured at an air-liquid interface have been established, mimicking the functioning of the human airway epithelium. Such models have the potential to recapitulate toxicity responses through the respiratory function, when exposed to a variety of pathogens or chemicals (*7*). We recently used such a model to assess the toxicity of tritiated tungsten particles (*8*). To our knowledge, these models have never been adopted to investigate the effects of tritiated products. When investigating tritiated products with a peculiar chemical speciation, as nano- or micrometric metal or cement particles, it is expected that this will pose challenges in terms of dosimetry. A comparison using controls exposed to similar nontritiated particles would disentangle the effects of radiation from the possible toxicity related to foreign non-biological materials. In all cases, it is essential to have a well-characterized control condition based on exposure to ''tritium only''. It is generally accepted that this condition can be established administering HTO to the 3D tissue model. Indeed, exposure to HTO is by far the most important type of tritium exposure and when it enters the body by inhalation, it adds to the surface liquid layer on the respiratory epithelium (*9*).

Given the above information, we used the human airway epithelium model MucilAir™ in this study to quantify cytotoxicity and tritium absorption after administration of HTO to the tissue for 24 h, at different activity levels (up to ~33 kBq μl^{-1} cm^{-2}). A simple dosimetric model was developed to estimate the upper limits of the dose to cells, starting from the administered activity and considering water transport through the tissue. At the end of the 24-h exposure, we measured cell viability (resazurin assay), epithelial integrity [through the transepithelial electrical resistance (TEER)] and the secretion of interleukin 8 (IL-8), chosen as one of the most abundant pro-inflammatory mediators released by airway epithelial cells, at different time points up to seven days. No major radiation-induced effects were observed. This is logical given the low-dose levels (less than 50 cGy cumulative dose for the highest tested activity) that we reconstructed with the dosimetric model.

Results from this study can therefore guide future experiment building using similar models, providing indications that higher activity levels are needed to establish a reference positive control condition based on HTO exposure. This will be particularly important when investigating the effects of the administration of other peculiar tritiated products.

MATERIALS AND METHODS

Cell Culture and Cellular Morphology

The fully differentiated primary human bronchial epithelial MucilAir model was purchased from Epithelix Sarl (Geneva, Switzerland). The MucilAir cultures used in this study originated from primary human cells isolated from the human nasal cavity of a pool of donors (human non-smokers) without respiratory pathologies. Signed informed consent and ethical approval were obtained by the supplier. All batches of MucilAir were tested negative by the supplier for mycoplasma, human immunodeficiency virus 1 (HIV-1), human immunodeficiency virus 2 (HIV-2), as well as hepatitis B and C. MucilAir models are characterized by a pseudostratified columnar epithelium presenting beating cilia and mucus production. The MucilAir model mimics the upper respiratory tract structure of the human lung, including basal, goblet and ciliated cells. Representative TEM pictures of the tissue have been provided elsewhere, in our previously published work (*8*).

Cell culturing was performed according to the supplier's instructions. Briefly, cells were kept at the air-liquid interface (ALI) in 24-well transwell inserts of 6.5-mm diameter and 0.4-μm pore size (Corning, St. Quentin Fallavier, France). The epithelium was cultivated with MucilAir serum-free culture media (Epithelix Sarl; Geneva, Switzerland) on the basal side. Cells were maintained at 37°C and 5% CO_2 for up to five weeks (one week before the exposure and four weeks after exposure). The basolateral cell culture media was changed twice per week, while the apical side was washed once a week with sterile saline solution (NaCl 0.9%, $CaCl_2$ 1.25 m*M*, HEPES 10 m*M*). Renewing the basolateral media preserves the tissue homeostasis, and washing the apical compartment removes mucus and surface dead cells. Cellular morphology was assessed using optical microscopy twice a week.

Exposure Protocol

The MucilAir tissues were cultured for one week before use. After that, they were exposed to HTO for 24 h. HTO stock solution (Amersham Biosciences, Little Chalfont, U.K.) was diluted in saline solution and 30 μl was applied on the apical compartment. Tested activity levels were: 0.4, 2.3, 6.7, 33, 66 and 330 kBq/well. Basolateral media was collected and replaced the first time at the end of the 24-h exposure period, then twice a week in the post treatment phase. At the end of the 24-h treatment period the apical side was washed and the mucus collected, then the procedure was repeated twice a week after treatment. Negative control cells were exposed to saline solution only.

Dosimetric Model

A simplified analytical approach was adopted to estimate upper limits for the dose to tissue cells. According to the information provided by the supplier, water absorption by the tissue is such that 20 μl of water can be transferred from the apical to the basolateral compartment in 24 h. The following thickness values are also provided as estimates: ~10 μm for the mucus layer and cilia; ~30 μm for the cell layer; and ~10 μm for the porous membrane. The surface of the insert available for cell culture is 0.33 cm^2. The tissue is considered to be water-equivalent in terms of density. Tritium β decay electrons are assumed to release all their energy locally at the emission point, which is justified based on their short range compared to tissue dimensions. Also, only the average energy of 5.7 keV (average range in water ~0.5 μm) is considered instead of the full energy spectrum. Based on these data, a tritium decay electron emitted in the cell layer and therein stopped causes an energy release of 9.3E–16 j in a mass of 9.90E–04 g, which leads to an S value (dose to target cells per decay of the source) of 9.22E–10 Gy/decay. Initial additional assumptions for the dosimetric estimate are: 1. HTO administered in the apical compartment is diffusing

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through mucus, cells and the porous membrane at a constant volumetric flow rate, and then reaches the basolateral compartment; 2. During diffusion in the tissue layer, HTO is replacing the water content in cells, and the full tissue volume is considered to be available for HTO; 3. The cross-sectional area available for water flow through all layers remains the same, therefore, a constant flow rate also means a constant flow velocity. These assumptions suggest that dosimetric estimates are to be considered at upper limits, higher than real dose values. Their validity is later addressed critically in the Discussion. A schematic representation of the experimental model MucilAir and HTO flow is shown in Fig. 1A.

To verify the calculated S value, we also implemented a software replica of the experimental tissue model using the radiation transport code PHITS version 3.02 (*10*). In this case, the full energy spectrum of tritium β decay electrons can be used for the source particles. The distribution of tritium sources can be varied in time, mimicking the flow of HTO through the tissue according to the above-mentioned assumptions. Two snapshots of the simulation reporting electron flux in color scale are given in Fig. 1B and C, with HTO respectively at administration or when the basal side of the tissue is reached (HTO at the level of the plastic membrane).

Quantification of Tritium Absorption

The cellular uptake of tritium and the transfer through the lung epithelium after treatment were quantified for a test exposure condition corresponding to the highest activity level. Apical and basolateral media were removed and stored at –20°C. Cells were also trypsinized and collected.

Apical media, basal media and cells were mineralized to insure a good tritium recovery by dissolution using 4–10 volumes of 30% H_2O_2 and incubation for 12 h at room temperature. Quantification was performed by liquid scintillation counting after addition of 4 ml of liquid scintillation cocktail (UltimaGold; Perkin Elmer Inc., Cambridge, UK).

Epithelial Integrity (TEER Measurement)

Transepithelial electric resistance (TEER) measurement is a method used to study epithelium permeability and tight junction integrity (*11*). After HTO exposure and at day 4 and day 7, TEER was measured using a STX2 electrode (World Precision Instruments, Hertfordshire, UK) and the electronic circuit of the EVOM Epithelial Volt/Ohm Meter (World Precision Instruments). Saline solution, 200 µl (0.9% NaCl, 1.25 m*M* $CaCl_2$ and 10 m*M* HEPES), was added onto the apical surface and removed immediately after measurement. To calculate the actual TEER value of each sample, the mean resistance of a cell-free transwell filter was subtracted from the resistance measured across each MucilAir epithelium.

Metabolic Activity

To measure cellular metabolism we performed the resazurin assay (Sigma-Aldrich; Saint-Quentin Fallavier, France). This test is based on the measurement of the activity of mitochondrial reductase that catalyzes the reduction of the non-fluorescent substrate resazurin into the fluorescent resorufin. The MucilAir inserts were transferred to a new 24-well plate containing 6 µ*M* Resazurin in saline solution. Resazurin solution (200 µl) was also applied on the apical surface and the plate was incubated for 1 h at 37°C and 5% CO_2. Then, 100 µl of the apical solution was transferred to a 96-well plate and the fluorescence of the transformed product was measured (excitation filter = 544 nm; emission filter = 590 nm). The MucilAir inserts were then re-transferred to a new 24-well plate containing fresh MucilAir culture media (700 µl per well). The remaining apical solution was removed, avoiding unsettling of the epithelium, and the inserts were put back into the incubator.

TABLE 1
Results of the Dosimetric Estimates in Terms of Cumulative Dose (24 h Exposures) Starting from the Administered Activity

Activity (kBq/well)	Cumulative dose (cGy)
0.4	0.05
2.3	0.31
6.7	0.89
33	4.38
66	8.77
330	43.84

Pro-Inflammatory Response

Interleukin-8 (IL-8) is one of the most abundant pro-inflammatory mediators released by airway epithelial cells and its elevated levels indicate an inflammatory process (*12*). We have quantified the levels of IL-8 induced by exposure to HTO in two test conditions, namely 23 and 330 kBq/well. Briefly, basolateral media was removed and centrifuged (10,000*g*, 10 min at 4°C) to eliminate cellular debris, then stored at –20°C. IL-8 was quantified using an ELISA kit (R&D Systems™, Minneapolis, MN). The optical density was measured at 450 nm with a microplate photometer Elx800 (Biotek, Colmar, France).

Statistical Analysis for Experimental Data

Results are expressed as mean ± SD. For the statistical evaluation, one-way ANOVA followed by Dunnett's multiple comparisons test were performed using GraphPad Prism version 6 (GraphPad Software, La Jolla, CA).

RESULTS

Dosimetric Estimate

The information on water transport through the tissue can be used to estimate a water flow of 0.83 µl/h. The activity level does not influence water transport through the layers. The cumulative number of decays occurring in the three layers is then given by the administered activity levels (in kBq/µl) multiplied by the flow rate and the exposure length. If the full tissue volume and cross-sectional area are considered available for flow, the number of decays occurring in tissue cells is obtained by multiplying this total number of decays by a factor of 3/5 (volumetric ratio of tissue layer to the sum of the three layers). The cumulative dose is obtained by further multiplying the number of decays in the tissue by the S value of 9.22E–10 Gy/decay. Results are given in Table 1.

The S value for a decay occurring in the tissue was further verified using full Monte Carlo simulations with the code PHITS (*10*). In particular, we used PHITS to verify that considering only the average energy instead of the full energy spectrum for tritium β decay electrons does not lead to a significant difference in the energy deposition. If electrons emitted in the cell layer are allowed to exit it, the average energy deposited per decay is lower only by a few percentages with respect to the expected 5.7 keV value. In reality, this loss is compensated

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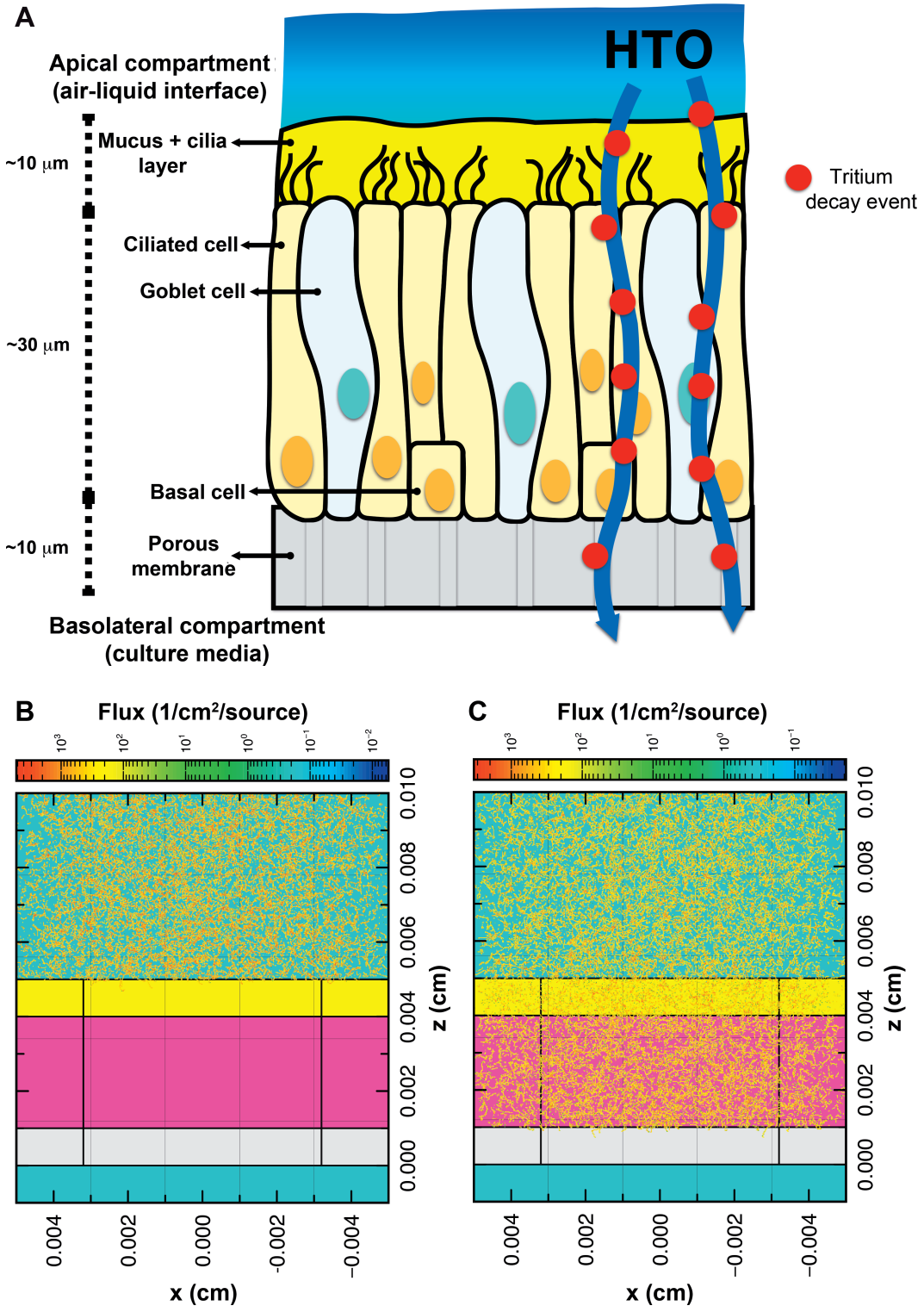


FIG. 1. Panel A: Schematic representation of the MucilAir tissue with thickness values used for the dosimetric estimate. HTO flow is indicated by the arrows from the apical to the basolateral compartment. Tritium decay events during the flow (red dots) are accompanied by a local release of the β electron energy. Cumulative dose to tissue cells only is considered in the estimate. The MucilAir tissue representation is adapted from (*31*). Panels B and C: Snapshots of PHITS simulations. Electrons emitted from the decay of tritium sources (electron flux scale, in color) are superimposed to a software replica of the experimental model (only a portion of the whole), when HTO is administered (panel B) or when, after flow through the tissue, it reaches the level of the plastic membrane (panel C).

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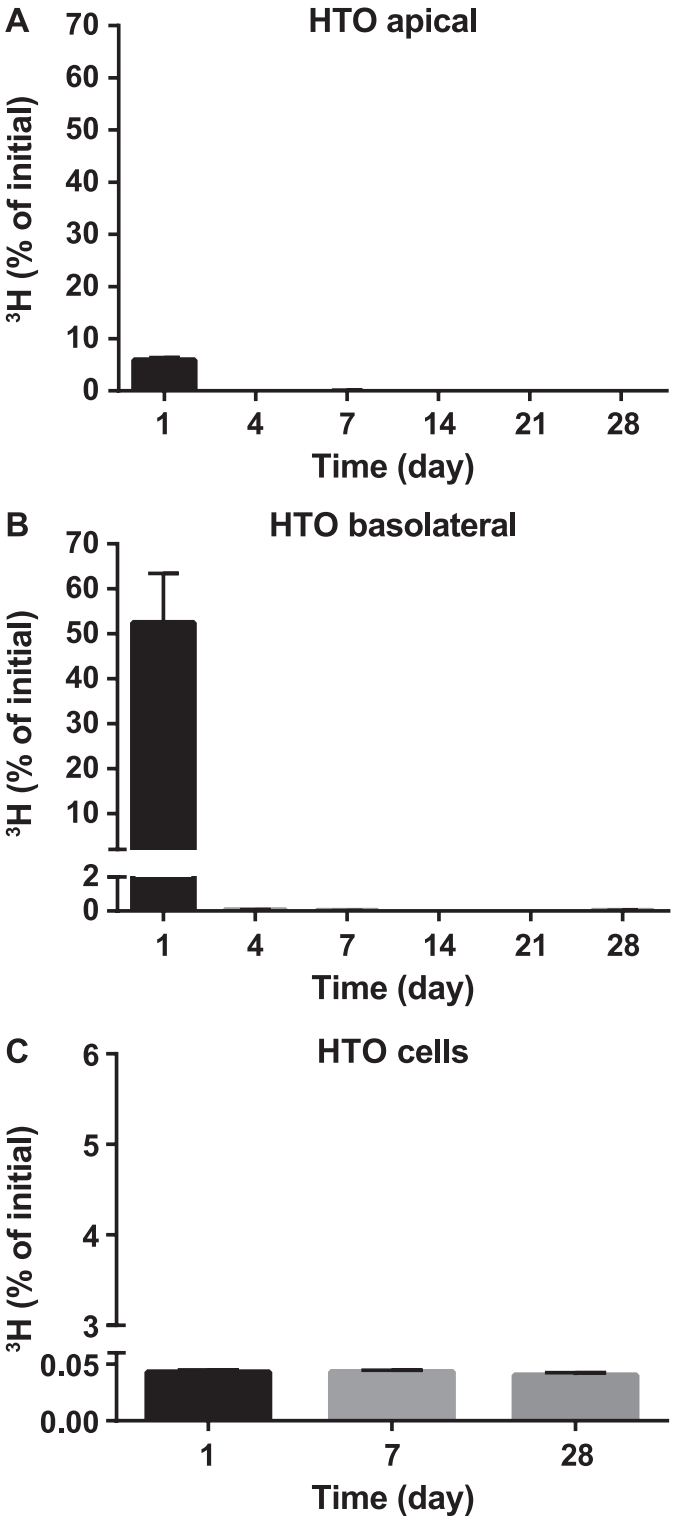


FIG. 2. Transepithelial passage of tritium: extracellular and intracellular quantification. Tritium content was assessed in both apical and basolateral media of MucilAir (panels A and B, respectively), as well as in cells (panel C), using liquid scintillation counting after mineralization of the samples. The results are expressed as mean tritium percentage in the exposure solution ± SD at the end of the 24 h treatment (day 1), and at day 4, 7, 14, 21 and 28 post-exposure (n = 6–18).

by energy depositions from electrons reaching the tissue that are emitted outside of it (in the mucus layer or when HTO sources are going through the porous membrane). The tissue layer is therefore in a condition of electronic equilibrium: the fluxes of tritium decay electrons generated outside cells and depositing energy in cells and that of electrons generated in cells and depositing energy outside cells can be assumed to be equal. This is not true only for the transient condition from administration to the moment HTO reaches the membrane, which is much shorter than the total exposure time (approximately, considering the thickness of the different layers and HTO flow rate, this time can be estimated as 1 out of the 24 h of the exposure). This transient condition and the use of the full energy spectrum can be disregarded, and the use of the analytical approach is fully justified.

Transepithelial Passage of HTO and its Intracellular Accumulation

To evaluate the absorption of HTO by the epithelial pulmonary MucilAir tissue, the extracellular and intracellular amount of tritium were quantified from day 1 to day 28 after exposure using liquid scintillation counting, upon exposure of the tissue to the highest activity level (330 kBq/well). Data are shown in Fig. 2. The extracellular quantification (mean percentage tritium ± SD) was performed on mineralized apical and basolateral cell culture media. At the end of exposure to HTO, a low fraction of tritium (6% at day 1), was present in the apical media, while a high amount (52% at day 1) was able to translocate in the basolateral compartment. From day 1 to day 28 after exposure to HTO, the quantity of tritium still present in the apical compartment severely and rapidly decreased up to less than 0.1% compared to day 1. A similar behavior was observed in the basolateral samples. Finally, to quantify the intracellular level of tritium upon MucilAir exposure to HTO, tritium quantification was performed on mineralized cells that were previously trypsinized. Tissue cells appear to retain a negligible amount of the total activity, as less than 0.05% of tritium was found in cells regardless of the time point. Also, no further release of such internalized activity appears to occur from cells to culture media.

Epithelial Integrity and Cell Viability after HTO Exposure

TEER and viability were tested upon exposure to HTO at increasing concentrations, from 0.4 up to 330 kBq/well at day 1 (end of exposure), day 4 (TEER only) and day 7 after treatment. Results are shown in Fig. 3. TEER was not decreased at any tested activity at day 1 and day 4. At day 7 a small decrease in TEER was observed for the highest activity level ($P < 0.05$). Viability was not decreased by the exposure to HTO at any activity level, neither at the end of the 24-h exposure, nor 7 days afterwards. Overall viability

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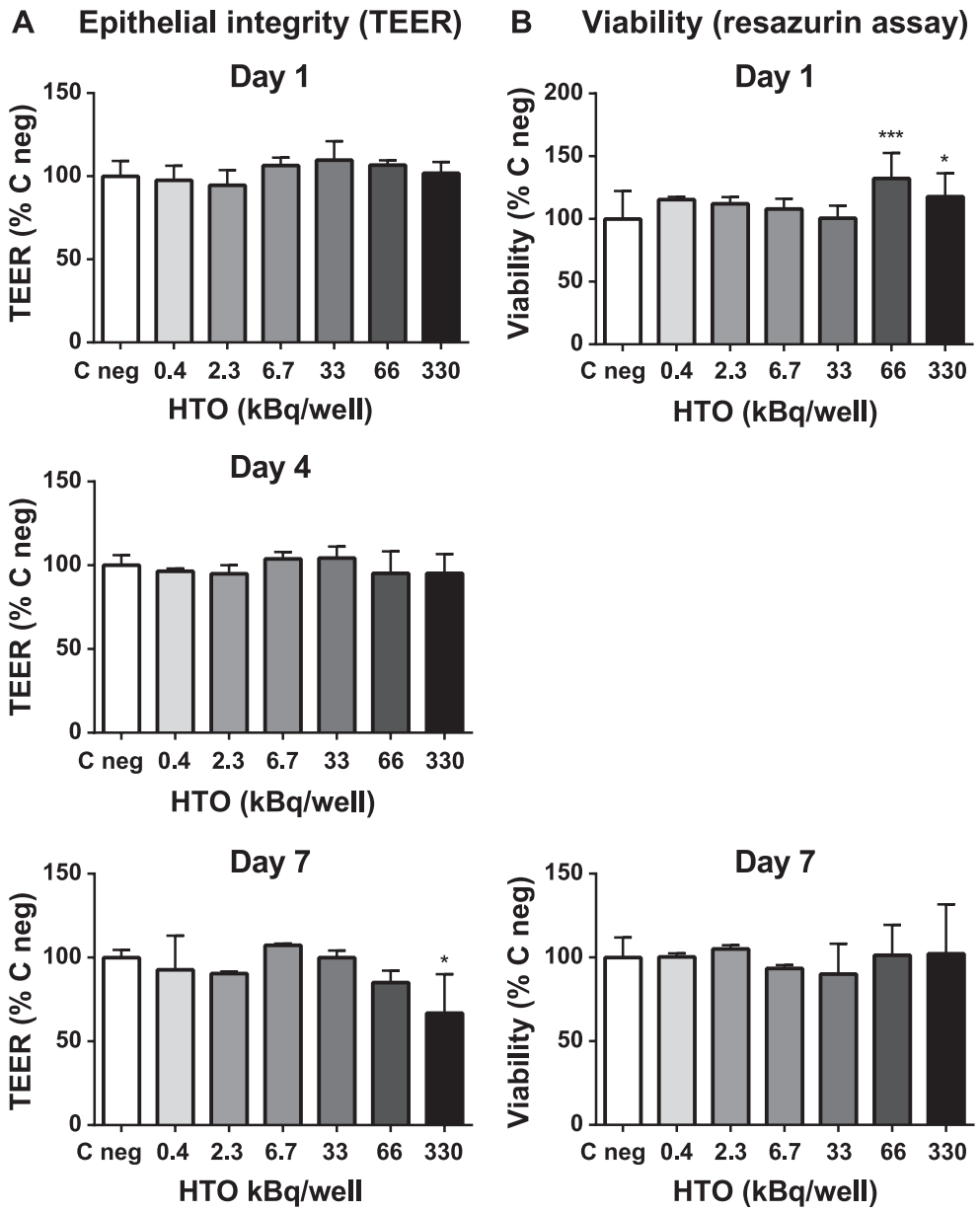


FIG. 3. Evaluation of epithelial integrity (TEER) (panel A) and viability (resazurin assay) (panel B) for tissue cells in the MucilAi model, as a function of HTO activity levels. Measurements are carried out at the end of the 24-h exposure (day 1), after four days (day 4) and seven days (day 7). Data (mean ± SD) are shown normalized to control conditions (C neg = no administration of HTO) at the same time point. Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple comparisons test: $*P < 0.05$ and $***P < 0.001$ (n = 2–20).

data confirms that culture conditions remained good over the entire investigated time frame.

IL-8 Release

The pro-inflammatory cytokine IL-8 was quantified by ELISA from samples of cell culture media from the basolateral compartment, upon MucilAir exposure for two chosen activity levels, 23 and 330 kBq/well, both at the end of the exposure and after 7 days. Data are presented in Table 2. IL-8 secretion was enhanced by HTO at the highest activity only, both at day 1 and day 7.

DISCUSSION

In the frame of refining, reducing and replacing animal experimentation, alternative *in vitro* methods have been developed over the last few years. Fully differentiated respiratory epithelia represent functional models displaying metabolic activity, mucus production and cilia beating, and they allow air-liquid exposure that more closely resembles *in vivo* conditions (*7, 13*). They are thus powerful tools to investigate respiratory tract toxicity in reconstructed physiological conditions (*14*). This applies also to radiotoxicity, potentially induced in the case of contamination by

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TABLE 2
Quantification of the Release of the Pro-Inflammatory Cytokine IL-8 in MucilAir Basolateral Compartments

	Day 1	Day 7
C. neg	100 ± 23.8	100 ± 17.3
HTO 23 kBq	114.3 ± 24.6	112.7 ± 23.9
HTO 330 kBq	137.5 ± 36.5[a]	131.3 ± 35.4[b]

Notes. The amount of IL-8 released by MucilAir was quantified using ELISA from samples of cell culture media from the basolateral compartment, collected at the end of the 24 h exposure (day 1) and 7 days after exposure. Results are compared to the negative control (C neg, corresponding to 4,290 pg/µl at day 1 and to 6,700 pg/µl at day 7) and expressed as mean percentage ± SD. Statistical significance was determined using one-way ANOVA followed by Dunnett's multiple comparisons test: [a]$P < 0.001$ and [b]$P < 0.05$ (n = 8 to 24).

inhalation of radioactive products. The toxicity scale is commonly expressed in terms of varying concentrations of pollutants. When dealing with radiotoxicity, an estimate of the physical dose to cells (Gy) would be of importance, but this is far from easy to achieve. This is even more true when dealing with radioactive sources decaying via the emission of low-energy/short-range particles, e.g., tritiated products; in such case, the temporal kinetics and the spatial distribution of sources at the sub-cellular level (thus on the micrometric scale) play a major role in determining the dose to cells and their sensitive targets. Both these aspects strongly depend on the chemical speciation of the tritiated product under consideration and are not easy to measure experimentally.

From the perspective of adopting the MucilAir model to investigate radiotoxicity effects induced by tritiated particles of different chemical speciation and sizes relevant to nuclear industry, in this study our goal was to set a reference condition for its exposure to tritium in the form of tritiated water. In the case of HTO exposure, one could reasonably assume a homogenous distribution of sources in the tissue cells, as well as no perturbation of standard exchange and transport mechanisms with respect to nontritiated water.

We therefore propose a simplified calculation scheme for dosimetric estimates, based on nominal water transport through the tissue, when water (30 µl in our experimental protocol) is administered to the apical compartment of the MucilAir model. Based on a constant volumetric flow rate of 0.83 µl/h and considering the thickness of the different layers (mucus layer and cilia, cell layer and porous membrane), we obtained a conversion of tested activity levels (in the range 0.013–11 kBq/µl) to cumulative dose to tissue cells for an exposure length of 24 h (correspondingly, 0.05–43.85 cGy). It is important to address here how the different assumptions made in the calculations affect such conversion. In particular, reported dose values are derived assuming a cross-sectional area available for water flow that stays the same for all layers, and is equal to the whole tissue surface. This means that the flow velocity stays the same and that the full tissue volume is considered to be available for HTO distribution. In reality, only a portion of the tissue is available for water flow, and this also suggests an increase in flow velocity. If this would be taken into account, fewer decays would be considered as occurring in the tissue during the exposure period, leading to a lower cumulative dose. It should be noted that the nominal volumetric flow rate through the tissue is the result of both passive (pressure gradient) and active ionic channels available in the tissue layer (ENaC, CFTR and aquaporins) (*15, 16*) that facilitate water transport. Aquaporins are membrane-bound water channel proteins with distinctive expression pattern in the nasal epithelium. They ensure rapid water transport across cell membranes in response to osmotic gradients (*17*). The expression of aquaporin 3 is dominant in the basal layer of the MucilAir tissue (*18*). The flow via active channels also occurs through a cross-sectional area that is smaller than the whole tissue surface and at a different flow velocity. Concerning the passage through the porous membrane, if the permeability of the membrane (in terms of overall surface available for the flow) is much smaller than that of the tissue layer, this abrupt change could lead to some degree of accumulation of HTO in the basal side of the tissue. This, on the contrary, would lead to a higher number of decays occurring in the tissue (higher dose than in our estimates). It is reasonable to hypothesize that this latter effect is less important, and to overall consider our estimates as upper dose limits.

We also performed liquid scintillation counting measurements to quantify the transepithelial passage of HTO and intracellular accumulation of tritium activity. At the end of the exposure for one day, we detected 52% of the total administered activity in the basolateral compartment. This is reasonably close to the expectations based on the nominal water flow (20 out of 30 µl passage through the tissue in 24 h). Intracellular activity accumulation was measured to be very low: less than 0.05% of the total activity was found in cells at the end of the exposure and at any later time point. This absence of accumulation in the epithelial tissue is in accordance with biokinetics of HTO after inhalation, as inhaled HTO is translocated to blood almost completely and instantaneously, and then distributes uniformly throughout the body (*4*). For HTO, it is therefore assumed that all activity deposited in the respiratory tract is absorbed instantaneously. Such a low retention level is also expected because of the very small volume tissue cells occupy with respect to the volume of the HTO solution (namely less than 5%). This volumetric ratio is further reduced, if we consider that only a portion of the tissue is available for water distribution, as mentioned above. The possibility of HTO loss in the protocol for cell preparation (including trypsinization and washing) could also contribute to the final result in terms of internalized activity. It is also important to consider that the presence of HTO in cells leads to organically bound tritium (OBT) production. In our case we cannot exclude that the small residual activity measured in cells is indeed due to their OBT content. The

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two contributions are both included when measuring the total residual activity, but they cannot be distinguished. Overall, given all these considerations and the fact that the exposure is a result of transient phenomenon (HTO flow) it seems however reasonable that only a very small fraction of the total activity is retained in cells and measured. A much lower level of activity with respect to expectations is measured instead in the apical compartment at day 1. This could be due to evaporation that inevitably occurs during the incubation of culture plates (24 h at 37°C) and tritium exchange with the incubator atmosphere that is close to 100% humidity.

Experimental results presented in this work show no significant decrease on cell viability (resazurin assay), but a small but significant decrease in TEER at day 7, upon exposure of the MucilAir model to HTO with activity levels up to 11 kBq/µl. A pro-inflammatory response is observed at day 1 and day 7 after exposure to the highest activity level, as indicated by the increase of IL-8 secretion. It is important to note that IL-8, though certainly not exhaustive of the inflammatory response pathway, was chosen as one of the most abundant pro-inflammatory mediators released by airway epithelial cells, shown to be upregulated by, e.g., nanoparticles of different kinds (*19*) including tungsten (*8*). This also makes it an ideal candidate for future adoption of this model for tritiated particles, as discussed later. The persistent inflammatory response measured after exposure of mammary epithelial cells to low-dose tritium was hypothesized to be related to accumulation of tritium in the form of OBT in cellular DNA or lipids (*20*), though intracellular activity is measured to be very low in our case. Also, it was recently shown that, on HUVEC cells exposed to HTO (3.7 and 3,700 kBq/ml), the increase of IL-8 secretion could play a pivotal role in cell defense (*21*). The slight decrease of TEER values in the same condition could be associated with this inflammatory state. For example, virus infection of airway epithelial cells has been shown to both decrease TEER of epithelial monolayers and increase IL-8 and IL-6 levels compared to controls (*22, 23*). Another hypothesis for the decrease of the TEER, in the absence of cytotoxicity, could be the activation of ionic channels (*24*).

More generally, radiotoxicological consequences of a contamination by tritiated water in cells (or animal models) have been mainly identified during experiments at high tritium concentrations (corresponding to high dose rates) and short-term exposures (*1*). Furthermore, in our study, cell exposure to tritium is a result of HTO passaging through an epithelial layer, which, combined with the relatively low initial activity level, leads to reconstructed dose rates (to be considered as upper limits) that are generally lower than those investigated *in vitro* (~44 cGy/day for the highest tested activity). Previously published work by Nias and Lajtha (*25*) (already pointing out the need for investigations at low-dose rates) showed that the growth rate of HeLa cells continuously exposed to ~30–50 cGy/day HTO is modified with respect to nonirradiated cells only from day 9–8 onwards, respectively. Siragusa *et al*. (*26*) administered HTO to V79 cells in suspension or adherent cultures. Starting from an activity level 1,000 times higher than the maximal tested in this work, their dosimetric model (*27*) led to dose rates in the range 2.2–2.4 Gy/h depending on culture conditions. Basically, no effect on clonogenic survival was measured for the shortest exposure length (~10 min), corresponding to a cumulative dose close to our maximal value. Despite the very different dose rate and exposure length in this latter study, the dedicated dosimetric model is of interest for our application. Their model exploits full Monte Carlo simulation of electron tracks and takes into account the specific cell geometry, but the authors stated that, in case of a uniform distribution of HTO, this was not necessary, and dosimetric results were the same if only the average energy of tritium electrons is considered and regardless of cell nuclei dimensions. This supports the adoption of a simplified analytical approach in our case, as is also justified in this work with a reference calculation using the code PHITS.

Little and Lambert (*28*) performed a systematic review of experimental studies on the relative biological effectiveness (RBE) of tritium including data from *in vitro* and *in vivo* measurements. A discussion of tritium RBE (and the many factors it depends on) is beyond the scope of this work, but in some of the considered *in vitro* studies the effects of dose rates as low as ours were investigated. Nevertheless, it is of note that all studies report end points considered to be most relevant to carcinogenesis (cell mutations and chromosome aberrations), and do not assess tissue-level coordination of cell function and inflammation, which, if chronic, might also be indicative of a pre-cancerous stage. As for *in vivo* experiments, dosimetric estimates are generally performed using biokinetic models, and final reconstructed dose rates can be quite low (*29*). Organismal response and biological changes induced in different tissues can be assessed. However, most of the *in vivo* studies dealing with tritium toxicity are done using ingestion, and direct effects to the respiratory system are generally not considered with such exposure pathway. Animal contamination with tritium in drinking water, as in (*29*), appears to have an effect on inflammation in the vascular system, even at a very low dose rate (~30 µGy/h for month-long exposures), but both detriment and adaptation are possible. Intraperitoneal injection of HTO is also used to mimic internal contamination by tritium, though in a single exposure. Nowosielska *et al*. (*30*) demonstrated that doses between 0.01 and 0.1 Gy do not lead to immunosuppression (evaluating the possible enhancement of artificial lung tumor metastases) and do not affect the serum levels of pro- and anti-inflammatory cytokines, but may enhance some components of anticancer immunity.

Overall, linking health effects to low-dose tritium exposure remains challenging. Information on cellular end points relevant to late health effects can be obtained *in vitro*, but often without taking into account homeostatic phenom-

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ena at the tissue-level. On the other hand, it is necessary for *in vivo* experiments that provide data on organismal response to be large in scale, and they are highly demanding in terms of resources. In all cases, dosimetric models are needed to assess tritium doses and associated uncertainties. Epidemiological studies on exposed cohorts particularly suffer from large uncertainties in tritium dosimetry, and also from suboptimal sizes.

Studying the response of MucilAir to HTO administration in this work, we set a reference condition for the investigation of effects induced by other peculiar tritiated products as tritiated particles. We demonstrate that this kind of model is suitable for the assessment of epithelial integrity and pro-inflammatory response, but tritium concentration higher than the maximal here tested (11 kBq/µl, corresponding to a localized tissue exposure lower than ~44 cGy/day according to our calculations) are needed to measure significant radiation-induced cytotoxic effects. In the general context of research on biological effects after inhalation of radioactive contaminants including tritiated products, and for the consequent improvement of risk estimates and radiation protection standards, a great deal of information can be obtained from exposure of 3D *in vitro* tissue models of the human airway epithelium.

ACKNOWLEDGMENTS

This project has received funding from the A*MIDEX project (ANR-11-IDEX-0001-02), funded by the Investissements d'Avenir French Government program, managed by the French National Research Agency (ANR), and from the Euratom Research and Innovation Programme 2014–2018 under grant agreement no. 754586. The views and opinions expressed herein do not necessarily reflect those of the European Commission.

Received: August 28, 2020; accepted: November 23, 2020; published online: December 30, 2020

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