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PASSAGE OF URANIUM THROUGH HUMAN CEREBRAL MICROVASCULAR ENDOTHELIAL CELLS: INFLUENCE OF TIME EXPOSURE IN MONO- AND CO-CULTURE *IN VITRO* MODELS.

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PASSAGE OF URANIUM THROUGH HUMAN CEREBRAL MICROVASCULAR ENDOTHELIAL CELLS: INFLUENCE OF TIME EXPOSURE IN MONO- AND CO-CULTURE MODELS.

ABSTRACT:

Purpose: Depleted uranium (DU) has several civilian and military applications. The effects of this emerging environmental pollutant on human health raise some concerns. Previous experimental studies have shown that uranium (U) exposure can disturb the central nervous system. A small quantity of U reaches the brain *via* the blood, but the effects on the blood-brain barrier (BBB) remain unclear.

Materials and Methods: In the present work, two cell culture models were exposed to DU for different times to study its cytotoxicity, paracellular permeability and extracellular concentration of U. The well-known immortalized human cerebral microvascular endothelial cells, hCMEC/D3, were cultured on the filter in the first model. In the second model, human primary cells of pericytes were cultured under the filter to understand the influence of cell environment after U exposure.

Results: The results show that U is not cytotoxic to hCMEC/D3 cells or pericytes until 500 µM (1.6Bq.L^{-1}). In addition, acute or chronic low-dose exposure of U did not disturb permeability and was conserved in both cell culture models. However, U is able to reach the brain compartment. During the first hours of exposure, the passage of U to the abluminal compartment was significantly reduced in the presence of pericytes. Electronic microscopy studies evidenced the formation of needle-like structures, like urchin-shaped precipitates, from 1 h of exposure. Analytical microscopy confirmed the U composition of these precipitates. Interestingly, precipitated U was detected only in endothelial cells and not in pericytes. U was localized in multilamellar or multivesicular bodies along the endo-

lysosomal pathway, suggesting the involvement of these traffic vesicles in U sequestration and/or elimination.

Conclusions: We show for the first time the *in vitro* passage of U across a human cerebral microvascular endothelial cells, and the intracellular localization of U precipitates without any cytotoxicity or modification of paracellular permeability. The difference between the results obtained with monolayers and co-culture models with pericytes illustrates the need to use complex *in vitro* models in order to mimic the neurovascular unit. Further *in vivo* studies should be performed to better understand the passage of U across the blood-brain barrier potentially involved in behavioral consequences.

Keywords: pericytes; human endothelial cell; uranium; permeability.

INTRODUCTION:

Uranium (U) is a heavy metal and radionuclide that is found naturally in the Earth's crust, and so is also present in underground water, air, plants and animals. In addition, certain human activities, industrial, nuclear or military, lead to the production of U waste, which increases the risk of human low-dose exposure. This waste is mainly composed of depleted uranium (DU) and is now considered an emerging environmental pollutant (Faa et al. 2018). DU contains a low level of ^{235}U and is around 40% less radioactive than naturally occurring U. Health risk concerns have been raised for populations exposed to U, especially regarding toxicity in the kidney and central nervous system (CNS). Previous studies have shown that U ingestion affects animal behavior: impairment of locomotor activity and learning processes and increased anxiety (Briner and Murray 2005; Lestaevel et al. 2015). In fact, after ingestion of DU-contaminated drinking water (40 $mg.L^{-1}$), there were even traces of U (quantity < 3 ng/L) in the brain of exposed animals and U was detected in specific brain areas - the striatum, the cortex and the hippocampus (Houpert et al. 2007). This localization of U is heterogeneous and, dose and exposition mode-dependent (Houpert et al. 2005; Tournier et al. 2009).

These animal data strongly suggest that radionuclide circulating in the blood could reach the brain after U exposure. Nevertheless, the brain is protected from harmful circulating compounds and xenobiotics by a unique specialized barrier, the blood-brain barrier (BBB). This barrier constitutes a specialized physical, metabolic and immunological barrier that represents the largest surface area of exchange between the blood and the brain. The BBB is localized at the level of the cerebral microvessels and is dependent on the expression of tight junctions (TJ) by cerebral endothelial cells. The permeability of these cells is highly controlled (Weiss et al. 2009; Obermeier et al. 2013). The cerebral endothelial cells lying on the basal lamina interact closely with other cell types such pericytes, glial cells, astrocytes, and neurons. All these cells together constitute the functional and organic unit that protects the brain and ensures its best functioning: the neurovascular unit (NVU) (Liebner et al. 2011) . Therefore, in order to study the permeability of any agent in the CNS, it is more reliable to use

not only brain endothelial cells as the simplest model of the BBB, but a more complex model with other cells. The role of the BBB after U exposure remains unexplored. There is only one study showing a possible vascular transfer of U after rat brain perfusion, but without impairment of BBB integrity (Lemercier et al. 2003). In fact, one *in vitro* study on rat brain endothelial cells (RBE4 cells) evidenced no toxicity of U, despite the intracellular presence of U (Dobson A et al. 2006). Human studies on BBB transfer of U are lacking and *in vitro* models represent a great opportunity to fill the gap.

The aim of this work was to create a human *in vitro* model to study the effects of U exposure. The first step was to use only endothelial cells to form the BBB model. Human cerebral microvessel endothelial cells (hCMEC/D3), an immortalized line derived from human temporal lobe microvessels, was selected since in order to study the BBB *in vitro* it is more reliable model, widely used and well characterized in the literature (Weksler BB et al. 2005; Weksler B et al. 2013). The culture was static, using a Boyden-like chamber and composed of two compartments. The luminal compartment represented the blood side and the abluminal the cerebral side of the BBB thus mimicking the physiological situation. A second model was developed with human pericytes cultivated under the filter in order to reproduce the cell environment of the BBB. The objectives were to determine if U was cytotoxic for human cerebral endothelial cells and pericytes, and to study the influence of time of exposure in mono- and co-culture models on BBB permeability, the extracellular concentration of U and the intracellular localization of U.

MATERIAL & METHODS:

1. Cell culture

i. hCMEC/D3 cell line

The human cerebral microvascular endothelial cell (hCMEC/D3) line was obtained from the Institut Cochin after a Mutual Transfer Agreement (LS12102) with the IRSN. They were stored at -150 °C in freezing medium (95% serum and 5% dimethylsulfoxide). In the present study, the hCMEC/D3 cells were used between passages 27 and 35. After thawing, the cells were seeded in T-75 cm^2 flasks, previously coated with collagen-I (Trevigen, Inc., Gaithersburg, MD, USA) from rat tail tendons, dissolved in distilled water (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) at 150 $\mu g.mL^{-1}$. Cells in T-flasks were kept in an incubator with a humidified atmosphere with 5% CO_2 at 37 °C, for 3-4 days.

ii. Human Brain Vascular Pericytes

The human brain vascular pericytes (HBVP) were from ScienCell Research Laboratories (ScienCell, San Diego, CA, USA). One vial containing $5x10^5$ cells in 1 mL was seeded in one poly-D-lysine coated T-75 cm^2 flask, with the same medium previously used for hCMED/D3 cells.

When the human endothelial cells and pericytes reached confluence, the cells were counted after trypsinization using a hemocytometer (Malassez, Preciss France) and seeded at the necessary concentration on other collagen-I coated supports.

iii. Model setup

Two *in vitro* models of culture were established. The first was comprised only the hCMEC/D3 cell line (model A) and the second was a co-culture of hCMEC/D3 cells with HBVP (model B). Transwells of 6- or 12-well format (24 mm or 12 mm diameter, respectively), polyester membrane, 0.4 µm of pore diameter (Corning, NY, USA), were coated with collagen-I from rat tail tendons (Trevigen, Inc., Gaithersburg, MD, USA) at 150 $\mu g.mL^{-1}$. The hCMEC/D3 cells were then seeded at day (D) 0 on the insert at a 50000 cells/cm^2 concentration and kept in the incubator. The medium was changed at D3, in both the luminal and abluminal compartments, and cultures were used for experiments at D6. EndoGRO™ Basal Medium (EMD Millipore Corp.) supplemented with EndoGRO™-MV Supplement Kit, Penicillin-Streptomycin at 10.000 $U.mL^{-1}$ (Life Technologies,

Thermo Fisher Scientific, Waltham, MA, USA) and HEPES 1M (Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) was used. All cultures were done with the same media.

To create a "close-contact" co-culture system between the hCMEC/D3 cells and HBVP (model B), pericytes were added on the day before the seeding (D-1) of the hCMEC/D3 cells on the apical side of the filter precoated with collagen-I (150 µg.mL^{-1}). The pericytes were seeded at a 50000 cells/cm^2 concentration on the bottom side of the filter and the inserts were kept inverted inside a Petri dish, in the incubator, until the next day. At D0, the inserts were returned into the right position inside the well. The cell culture medium used (i.e. EngoGro Basal Medium supplemented) was the same each side of the filter.

2. Uranium exposure

Whatever the speciation, after exposure, U is the uranyl ion in the formal oxidation state +VI [$U(VI)O_2^{2+}$] in aqueous media. In the body, U forms complexes with citrate, bicarbonate, or proteins in the plasma (Keith et al. 2013). In order to mimic that, the U utilized for *in vitro* experiments was in the form of uranyl nitrate hexahydrate, after the reaction with bicarbonate solution. A range of experimental concentrations (50 pM to 500 µM) was used to test every possible effect of U in the BBB, even if the highest concentrations (above 100 µM) were unlikely to be attained *in vivo* (Jiang et al. 2007). These values could be compared to the concentration of uranium in biofluids after humaun exposure. In the blood, uranium concentrations are in the order to 0.02 nM up to 0.4 nM (Dang and Pullat 1993). These concentrations measured in human blood are several orders of magnitude below the first concentrations used in the present study

A 10 mM stock solution was prepared by dissolving the DU powder (ORANO, France) in 100 mM $NaHCO_3$ solution. DU has a physical half-life of 4.5 billion years and the radioactive specific activity of DU was 1.4 x10^4 Bq.g^{-1}, and its isotopic composition was ^{238}U = 99.74%, ^{235}U = 0.255%, and ^{234}U = 0.0055% (pH = 7.4). DU solutions used for experiments were extemporaneously prepared by dilution of the stock solution in cell culture medium.

Different U exposure durations were performed to mimic both acute and chronic exposures when the human endothelial cells and pericytes reached confluence. As the status of the cells at the time of exposure can influence the cell responses, all exposures were realized on confluent cells to obtain the monolayer of endothelial cells. The acute exposure was reproduced with 24, 4 and 1 hour of exposure before the experiment. As for the chronic exposure, it was performed in the last 3 days of the 6 days of experiment (D3D6). All these conditions are summarized in **Figure 1**.

3. *U cytotoxicity assays*

A cell proliferation Kit I (MTT) from Roche (Cat. No. 11 465 007 001) was used to assay the cell's viability and proliferation. Cells were seeded on 96-well plates coated with collagen-I at 50000 cells/cm² concentration. U was added to the medium at different concentrations, from 50 pM to 500 µM, and kept in the incubator. On the day after the seeding, the yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) salt was added to each well and, 4 hours later, the solubilization agent was also added to each well. Each condition was done in sextuplicate. The reaction occured overnight in the incubator, allowing the evaluation of the plate on the next day with a microplate reader at 570 nm. In order to analyze the results, the mean absorbance value measured for only the medium, without cells, was subtracted from all the conditions, to remove the background noise. Afterwards, the absorbance of control cell samples was considered as 100% and all the test conditions were represented as percentages of the control.

4. *Paracellular permeability assay*

Paracellular permeability through the hCMEC/D3 monolayer was measured at D6 in 6- or 12-well plates as already described (Weksler BB et al. 2005). At the time of analysis, the luminal content of the inserts was removed; they were transferred into new 6- or 12-well plates containing transport buffer (HBSS with 10 mM of HEPES and 1 mM of sodium pyruvate, Invitrogen) in the lower

chamber. In the upper chamber, 50 µM Lucifer Yellow dilithium salt (LY) in transport buffer was added and incubated at 37°C 5% CO_2. For the positive control, cells were subjected to 1.4 M of mannitol 30 minutes before the experiment. For the negative control, cells were unexposed to U and only 100 mM $NaHCO_3$ solution was added. To quantify the LY passage, the abluminal compartment was analyzed at each time point (10, 25, 45 min) using a microplate reader, with an excitation filter of 485 nm and an emission filter of 535 nm. Paracellular permeability was calculated using the clearance principle, as described by Siflinger-Birnboim and colleagues (Siflinger-Birnboim et al. 1987). The clearance was calculated for the coated insert without cells, called PS_f, and for each coated insert with cells, called PS_t, each with $mL.min^{-1}$ dimensions. By using these values it was possible to obtain one other parameter, PSe, by applying the formula:

$$\frac{1}{PS_e} = \frac{1}{PS_t} - \frac{1}{PS_f}$$

Finally, PS_e was divided by the surface area of the filter to give the endothelial permeability coefficient (Pe) in 10^{-3} cm/min or as the percentage of permeability normalized to the permeability coefficient for the control conditions of untreated cells. Each condition was tested in triplicate in order to evaluate statistical significance of these data.

5. *Transmission Electronic Microscopy (TEM)*

The cells were fixed at 2.5% glutaraldehyde in PBS [-][-], for 1 h at room temperature (RT.) Then, the cells were postosmicated with osmium tetroxide and carefully washed in buffer before inclusion in EPON resin. At this point, the samples were cut to the desired thickness (100nm) using an ultramicrotome. Uranyl acetate and lead citrate were not used to contrast grids in order to avoid false positives with U. Ultrathin sections were examined in a JEOL 1011 electron microscope equipped with Orius 1000 camera.

6. *Analytical microscopy*

For chemical analysis, the ultrathin sections were examined with a Scanning Transmission Electron Microscope (TEM/STEM Tecnai12 G2 Biotwin Electron Microscope, FEI company of ThermoFisher scientific group) using an accelerating voltage of 100 kV and equipped with a CCD camera Megaview III (Olympus Soft imaging Solutions GmbH). Several subcellular structures were analyzed with the Energy Dispersive X-ray analyzer (EDX) equipped with a Super Ultrathin Window (SUTW) model sapphire (EDAX). Accumulation of U was detected by focusing the electron beam (20nm diameter) on specific precipitates and spectra were collected for 100 seconds.

7. *ICP-MS evaluation of cellular and extracellular luminal and abluminal U concentrations*

First, to identify the ability of U to enter each type of cell and to quantify the intracellular U content, a monoculture of hCMEC/D3 cells or pericytes was performed in the bottom of 6-well plates coated with collagen-I at 25000 cells/cm^2. After seeding, cells were exposed to U during 24 h. The tested U concentrations were 1 µM, 10 µM, 50 µM, 100 µM and 250 µM. After exposure, the cells were washed repeatedly with PBS [-] [-] supplemented with Bovine Serum Albumin 0.2% and then collected after trypsinization with 200 µL of 69% nitric acid (Aristar quality grade, VWR Prolabo).

Second, to identify U passage across the BBB (*i.e.* hCMEC/D3 cell line) U content in the extracellular compartments (*i.e.* luminal and abluminal) was quantified. After the U exposure in mono- and co-culture models (in 6-well formats – see section 2. Uranium exposure), the culture medium was removed and stored at 4°C.

The duplicated or triplicated samples were diluted with 2% nitric acid. As previously described (Gueguen et al. 2015), U was quantified by Inductively-Coupled Plasma Mass Spectrometry (ICP-MS) (ICP-MS, X series II, Thermo Electron, France) using a 7700X series (Agilent Technologies, Les Ulis, France), calibrated with a SPEX CertiPrep U standard solution (Jobin Yvon, Longjumeau, France). The results of the analysis were expressed as parts per trillion (1 ppt = 1 ng.L^{-1}).

8. Statistical Analysis

All results were expressed as mean ± standard deviation (SD) from at least three independent experiments, each one performed in triplicate for each tested condition, unless stated otherwise. The significance of variability between the results from various groups was determined by one-way ANOVA when the normality assumption was met, and the Kruskal–Wallis test if not. Post-test analysis was verified by Dunn's test. This analysis was performed using the SigmaPlot 11.0 software. The results were considered statistically different when $p<0.05$. The association between LY permeability and U concentration was analyzed by a generalized estimating equation (GEE) regression (Zeger and Liang 1986) and conducted using the Zelig Package of R software (R Software 2016). The associated symbols are * for $p< 0.05$, ** for $p<0.005$ and *** for $p<0.001$.

RESULTS:

1. U Cytotoxicity

Cytotoxicity tests showed no noteworthy difference between the viability of the control and of the cells subjected to concentrations up to 500 µM of U (**Figure 2Error! Reference source not found.**). Even if a tendency to decreased cell viability was notable for the 250 µM conditions, there was a statistically significant decrease on the percentage of living cells (of about 50% for each type of cell) only for 500 µM ($p < 0{,}001$), *i.e.* hCMEC/D3 cell line or pericytes.

2. U effect on *in vitro* paracellular BBB permeability

BBB properties were evaluated after exposure of the *in vitro* model composed of hCMEC/D3 monolayer to different concentrations of U. Paracellular permeability (Pe) to LY was studied by adding U at concentrations from 1 to 500 µM during the permeability test (**Figure 3**). Mannitol was used as a positive control to induce osmotic shock, and therefore the disarrangement of the cells and the disruption of the TJ, which interferes with the barrier properties of the monolayer. In comparison with the negative control, LY permeability increased by a factor of three ($1.31 \pm 0.19 \times 10^{-3}$ cm.min^{-1} and $3.71 \pm 1.25 \times 10^{-3}$ cm.min^{-1}, respectively). During U exposure, paracellular permeability of the hCMEC/D3 monolayer was not affected, since the permeability remained unchanged in every condition when compared to the negative control.

In order to determine if the duration of U exposure had an effect on the paracellular permeability of the BBB, the hCMEC/D3 monolayer alone (model A) and co-cultured with pericytes (model B) were subjected to a non-cytotoxic concentration of U (50 µM) for different times. Several time windows of exposure of the model were defined in order to mimic acute (1, 4 and 24 h) and chronic exposures (D3D6) as described in **Figure 1**. After 6 days, LY permeability was evaluated to verify possible

modifications of BBB properties. The comparison between models A and B determined whether the presence of pericytes modified the response obtained with the hCMEC/D3 monolayer alone.

Control permeability value for models A and B was 0.96 x10^{-3} cm.min^{-1} and 1.37 x10^{-3} cm.min^{-1}, respectively (**Figure 4**). This difference was statistically significant ($p = 0.009$) suggesting that the co-culture with pericytes affected the BBB permeability of the hCMEC/D3 monolayer when exposed to U. Comparing each time of U exposure to its control in model A or B, the results showed no detectable statistical difference between the groups (**Figure 5**). This indicates that neither acute nor chronic exposure to U seems to affect the permeability of the monolayer in each model. Interestingly, when all parameters were taken into consideration (models A and B, passage of cells and format of wells), the results of the comparison were altered. Model A, passage 34 for the hCMEC/D3 cells (*i.e.* the most used passage), 6-w format, was considered and showed a statistical difference between the data obtained with the 6- and 12-well formats ($p = 1.5 \times 10^{-5}$) by the GEE analysis. This occurred because the Pe was reduced to 50% in the 12-well format in comparison to the 6-well format.

4. Extracellular luminal and abluminal U concentrations

Despite there being no modification of the BBB permeability, the extracellular concentration results showed that U was capable of accessing the abluminal side from 1 h of exposure in both types of culture (models A & B). Only three conditions were analyzed for the quantification of extracellular U between both compartments (abluminal and luminal): 1 h, 4 h and D3D6. Mass balance (MB) was checked for every experiment and validated when it was between 65 and 135%. After 24-h exposure, the MB was not respected (around 21.54 ± 11.05% for model A) while the non-absorbance of U in the filter and plastic and the quantification of U in cells represent less than 1% of total U added at the beginning of the exposure (*i.e.* 50 µM).

In model A, the percentage of total U in the abluminal compartment increased progressively from 54.26 ± 17.41% (1 h) to 61.80 ± 4.18% (D3D6) and was significantly different ($p<0.05$) in D3D6 conditions in comparison with culture exposed for 1 h (**Figure 5**). In model B, the same tendency was

observed and the luminal and abluminal compartments in D3D6 conditions were statistically different ($p<0.001$) from 1-h exposure. Nevertheless, there were some differences between models A and B regarding the percentage of total U detected at the end of the experiment. After 1-h exposure, there was a statistically higher quantity in the luminal compartment in model B (x 1.6 times, 86.14 ± 12.82%) than in model A ($p<0.001$). Inversely, there was statistically less in the abluminal compartment in model B (16.96 ± 12.24%) than in model A (x 2.7 times, 45.74 ± 17.41%) ($p<0.001$). After 4- and 72-h exposure, in model B, the presence of pericytes had not significantly modified U passage through the cells.

5. Intracellular localization of U by microscopical analyses

Using ICP MS analysis, U was quantified inside each type of cell and represented only 1% of added mass at the beginning of the experiment (data not shown). In order to identify U localization, transmission electronic microscopy (TEM) analyses were performed in control conditions and after different times of U exposure. In controls, one or two layers of hCMEC/D3 cells were detected with no signs of damage: TJ, endosomal activity and vesicles with multilamellar/multivesicular/cottony-like bodies were detected. After U exposure, TJ were always detected as correlated with the LY permeability results (**Figure 4**). To distinguish the different types of cells in model B, we characterized the presence of pericytes in one side of the filter by extensive granular endoplasmic reticulum. Interestingly, electron-dense needle-like structures were detected from 1 h of exposure, but were not observed in both cell types. After 1 h of exposure, these urchin-shaped precipitates were detected only in hCMEC/D3 cells in models A and B (**Error! Reference source not found.**C & D). They were localized between the cells and the filter, between the cells and, more rarely, on the luminal side of these cells (**Figure 6E & F, Figure 7D & E**). The structures were localized also inside the cells, mainly in the multilamellar/multivesicular vesicles and sometimes in structures without lipid membrane (**Figure 7A & B**). Unexpectedly, these structures were not found in pericytes, and after other times of exposure. Analytical microscopy by TEM/STEM-EDX was performed on several

electron-dense needle-like structures detected inside or outside the cells to confirm the presence of U. U formed fine plate-like structures that were isolated or grouped in urchin-shaped precipitates reaching 150 nm in diameter (**Figure 7B & C, E & F**).

DISCUSSION:

U is an emerging environmental pollutant and its neurological effects were described after exposure in human and rodent models (Dinocourt et al. 2015). The quantity of U detected in the brain is low and heterogeneous (Houpert et al. 2005; Houpert et al. 2007) and the mechanism of U transfer into the brain remains unclear. The BBB regulates the access of endogenous and exogenous molecules (Weiss et al. 2009) and its impairment could be involved in behavioral effects (Obermeier et al. 2013). Thanks to human mono- and co-culture *in vitro* models, we have shed light on the effects of U after different time exposures on paracellular permeability, extracellular concentration of U and its intracellular localization.

The cytotoxicity of U was studied in hCMEC/D3 cell lines and human primary pericytes (HBBP). No toxic effect was observed in either type of cell until 500 µM after 24 h of exposure. There are studies with U in rat lung epithelial cells that indicate a dose-dependent cytotoxicity, with a significant decrease in cell viability after 72 h of exposure to 500 µM (Periyakaruppan et al. 2007). In renal epithelial cells, viability was reduced by 20% at 500 µM and there was minimal alteration at 250 µM after 24-h exposure (Carrière 2015). Recently, Pierrefite-Carle et al. reported 50% loss of viability around 390 µM in an osteosarcoma cell line (Pierrefite-Carle et al. 2017). There is only one report on rat brain endothelial cells that found no toxic effects at 10 and 50 µM both for 3 and 6 h of exposure (Dobson AW et al. 2006). Furthermore, despite the fact that cytotoxic tests of U have never been performed in human endothelium and pericytes, all of these studies support the fact that short-term exposure does not cause any visible effects on the cell's viability until relatively high concentrations, namely 500 µM.

Pericytes are contractile smooth muscle-like cells that cover the abluminal surface of microvessels. They make contact with endothelial cells by holes in the basal lamina (BL) and by extensions that cover 20 to 30% of the vascular circumference. In the present study, primary cells isolated from human brain were used, which are easily available and have been previously used *in vitro* (Stratman et al. 2009; Hatherell et al. 2011). Pericytes were cultivated under the filter to mimic the NVU organization. There are several reports of co-culture models of endothelial cells (ECs) and pericytes, but the influence of pericytes depends on the *in vitro* models. In rat and mouse models, transendothelial electrical resistance (TEER) increased in brain endothelial cells (Nakagawa et al. 2007; Daneman et al. 2010), whereas, in pig models, it was reduced in co-culture with pericytes due to an induction of MMPs (Zozulya et al. 2008). In our study, the paracellular Pe was increased in co-culture with pericytes in comparison to monoculture. This was not in agreement with the increase of TEER observed by Hatherell and coworkers in co-culture of hCMEC/D3 cells and HBPV (Hatherell et al. 2011). On the one hand, *in vitro*, the differentiation state of pericytes could influence the TEER (Thanabalasundaram et al. 2011). On the other hand, *in vivo*, pericytes do not have an effect on TJ protein expression, but decrease expression of the genes involved by favoring vascular permeability (Daneman et al. 2010). Regardless of the permeability marker, the pericytes' effects remain unclear and it is difficult to state whether there is a beneficial effect of co-culture with HBPV in this model.

No effect of the different times of exposure to U was observed on the paracellular Pe in these two models. The present study shows, for the first time, that the permeability of the monoculture of human cerebral microvascular endothelial cells is not disturbed by U after acute or chronic exposure. The extracellular concentration of U was assayed and our results show that U is able to reach the abluminal compartment, here mimicking the cerebral area in both mono- and co-culture models. U passage was detected from 1 h of exposure and the rate of passage was progressive until 72 h of exposure, when equilibrium was reached. Interestingly, there were significant differences between the two models after 1 h of exposure. The U content in the luminal compartment was higher in co-culture models than in monoculture models. Despite the increased permeability of the monoculture of hCMEC/D3 cells in the co-culture model, the extracellular concentration of U evidenced differences that were indirectly

correlated. The paracellular permeability was higher, but the U passage was reduced when pericytes were cultured under the filter. The passage was significantly slower only after 1 h of exposure, but no difference was detected for other times of exposure. This modification could be linked to the physical presence of pericytes under the filter and/or to the induction of signal pathways between ECs and pericytes.

Therefore, it was necessary to understand how U can cross the monoculture of human cerebral microvascular endothelial cells. Transcellular passage was suggested, but paracellular passage was unfortunately not excluded because the high paracellular Pe of hCMEC/D3 cell lines can facilitate the passage of small molecules, like U, between the cells. This indicates that, only when acutely exposed, the co-culture model responds differently than the mono-culture. Therefore, pericytes seem to influence ECs rapidly since they respond differently to U exposure after 1h of exposure, but these interactions do not influence the extracellular concentration of U for longer times of exposure. The difference could also be linked to the intracellular content of each type of cell. Nevertheless, the U content represented only 1% of mass added at the beginning of the experiment in each type of cell after 1 h of exposure (data not shown). One paper states that U enters rat brain endothelial cells (RBE4 cells) after only 15 minutes in the presence of 10 and 50 µM (Dobson AW et al. 2006). The RBE4 cells were cultivated in flasks, thus facilitating accumulation of U inside the cells. The use of filters prevents this artefact and permits the observation of U passage across the model. TEM and EDX analyses were performed and electron-dense needle-like structures were observed in hCMEC/D3 cell lines in mono- and co-culture after 1 h of exposure. EDX analyses confirmed the U composition of these urchin-shaped precipitates. These structures were localized in intracellular vesicles, including multilamellar and multivesicular bodies, along the endo-lysosomal pathway, among others observed in the cytoplasm. In addition, in some cases the precipitates were observed closer to the basolateral and apical side of the hCMEC/D3 cells, or outside the cells (between the cell layer and the filter). This study evidenced for the first time U precipitates in human endothelial cells. The same structures have been previously detected in renal epithelial cells and in osteoblastic cell lines (Mirto et al. 1999; Pierrefite-Carle et al. 2017). It is also well established that speciation of U influences the formation of

precipitates of uranyl phosphate (Mirto et al. 1999; Carriere et al. 2004). As observed in osteoblastic cell lines (Pierrefite-Carle et al. 2017), TEM analysis showed U precipitates in multivesicular bodies in endothelial cells, but not in lysosome-like vesicles, or in autophagic vesicles. Recently, Carmona et al. detected uranium in defined perinuclear regions of the cytoplasm and suggested its accumulation in organelles after exposure to SH-SY5 dopaminergic cells (Carmona et al. 2018). In the same cells, Vidaud et al. demonstrated also that urano-proteome was mainly localized in cytoplasm (Vidaud et al. 2019).

Multi-transport could be involved in U entrance to cells. Muller and coworkers suggested that U passage is mediated mainly by absorptive endocytosis in renal cell lines, namely through sodium-dependent phosphate co-transporters (NaPi-IIas) (Muller et al. 2006). Nevertheless, these co-transporters are not expressed in brain (Hilfiker et al. 1998), but subtype III is localized in neurons, astrocytes and endothelial cells (Inden et al. 2013). The question of the entry mechanism is far from resolved, and there are many possible lines of investigation.

The absence of the urchin-shaped precipitates in pericytes suggests that at the beginning of the exposure, ECs stock the U as much as possible to avoid it reaching the brain. The formation of the precipitates could participate in cell detoxification process in order to protect brain parenchyma. The presence of ectopic precipitates without cytotoxicity could be explained by a very limited uranium incorporation depending on efflux and / or uptake mechanisms and intercellular variability. This observation was also suggested in neuronal cells exposed to low concentrations (Carmona et al. 2018). However, beyond their capacity to accumulate U, the cells are obliged to release it, to both the luminal and abluminal sides, allowing it to reach the brain. Furthermore, it is possible that pericytes also release U in a soluble form to the abluminal compartment, since they are also capable of uptake. The presented results support the notion that the interaction between the cell types influences the cell response when they are treated by U. Nevertheless, the observed effects of DU could be further explained by its chemical rather than radiological properties, which could influence these observed cell responses. This shows the importance of the development of new *in vitro* neurovascular unit models close to the *in vivo* structure, with addition of neuronal cells, for example.

U is known to be implicated in some neurological disorders (Dinocourt et al. 2015). Lemercier et al. have already demonstrated that, after U perfusion, significant U content was detected in different rat brain areas, but without BBB disruption (Lemercier et al. 2003). In previous *in vivo* model of rats chronically exposed for different times to U-contaminated drinking water, U was also detected in different brain areas (Paquet et al. 2006). To detect the impairment of BBB, S100β protein was analyzed in peripheral blood by immunoassay (Feriel et al. 2015). No sign of BBB disruption was observed (cf. Suppl. data). Nevertheless, interestingly, age dependence of S100β protein blood concentration was observed, as the highest level of S100B protein on post-natal day 21 (PND21) was seen in aged control rats (4.32 ± 1.04 µg.L^{-1} for PND21 rats *versus* 0.24 ± 0.07 µg.L^{-1} for 6-month-old rats *versus* 0.09 ± 0.02 µg.L^{-1} for 9-month-old rats) (data not shown). These results are in accordance with the *in vitro* findings showing no disruption of the monoculture of hCMEC/D3 cells after U exposure.

CONCLUSION:

In conclusion, these results show for the first time the passage of U across the monoculture of human cerebral microvascular endothelial cells, and the intracellular localization of U precipitates without any toxicity of the barrier. Future *in vivo* studies must be conducted to elucidate translocation and retention of uranium particles into the brain and cellular and cognitive consequences in experimental animals and humans.

CONFLICT OF INTEREST

None declared.

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BIBLIOGRAPHY:

Briner W, Murray J. 2005. Effects of short-term and long-term depleted uranium exposure on open-field behavior and brain lipid oxidation in rats. Neurotoxicology and teratology. 27(1):135-144.

Carmona A, Malard V, Avazeri E, Roudeau S, Porcaro F, Paredes E, Vidaud C, Bresson C, Ortega R. 2018. Uranium exposure of human dopaminergic cells results in low cytotoxicity, accumulation within sub-cytoplasmic regions, and down regulation of MAO-B [Research Support, Non-U.S. Gov't]. Neurotoxicology. 68:177-188. eng.

Carriere M, Avoscan L, Collins R, Carrot F, Khodja H, Ansoborlo E, Gouget B. 2004. Influence of uranium speciation on normal rat kidney (NRK-52E) proximal cell cytotoxicity [Comparative Study Research Support, Non-U.S. Gov't]. Chem Res Toxicol. 17(3):446-452. eng.

Carrière M, Gougeta, B., Galliena, J., Avoscana, L., Gobinb, R., Verbavatzb, J., Khodjaa, H. 2015. Cellular distribution of uranium after acute exposure of renal epithelial cells: SEM, TEM and nuclear microscopy analysis. Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms. 231(1-4):268–273.

Daneman R, Zhou L, Kebede AA, Barres BA. 2010. Pericytes are required for blood-brain barrier integrity during embryogenesis. Nature. 468(7323):562-566. eng.

Dang HS, Pullat VR. 1993. Normal concentration and excretion ratio of uranium in serum of normal individuals in India. Health physics. 65(3):303-305. eng.

Dinocourt C, Legrand M, Dublineau I, Lestaevel P. 2015. The neurotoxicology of uranium [Review]. Toxicology. 337:58-71. eng.

Dobson A, Lack A, Erikson K, Aschner M. 2006. Depleted uranium is not toxic to rat brain endothelial (RBE4) cells. Biol Trace Elem Res. 110(1):61-72. English.

Dobson AW, Lack AK, Erikson KM, Aschner M. 2006. Depleted uranium is not toxic to rat brain endothelial (RBE4) cells [Research Support, U.S. Gov't, Non-P.H.S.]. Biological trace element research. 110(1):61-72. eng.

Faa A, Gerosa C, Fanni D, Floris G, Eyken PV, Lachowicz JI, Nurchi VM. 2018. Depleted Uranium and Human Health [Review]. Current medicinal chemistry. 25(1):49-64. eng.

Feriel J, Adamo F, Monneret D, Trehel-Tursis V, Favard S, Tse C, Puybasset L, Bonnefont-Rousselot D, Imbert-Bismut F. 2015. S100B protein concentration measurement according to two different immunoassays [Letter]. Clinical chemistry and laboratory medicine : CCLM / FESCC. 53(7):e169-171. eng.

Gueguen Y, Suhard D, Poisson C, Manens L, Elie C, Landon G, Bouvier-Capely C, Rouas C, Benderitter M, Tessier C. 2015. Low-concentration uranium enters the HepG2 cell nucleus rapidly and induces cell stress response. Toxicol In Vitro. Eng.

Hatherell K, Couraud PO, Romero IA, Weksler B, Pilkington GJ. 2011. Development of a three-dimensional, all-human in vitro model of the blood-brain barrier using mono-, co-, and tri-cultivation Transwell models [Research Support, Non-U.S. Gov't]. J Neurosci Methods. 199(2):223-229. eng.

Hilfiker H, Kvietikova I, Hartmann CM, Stange G, Murer H. 1998. Characterization of the human type II Na/Pi-cotransporter promoter [Research Support, Non-U.S. Gov't]. Pflugers Archiv : European journal of physiology. 436(4):591-598. eng.

Houpert P, Frelon S, Monleau M, Bussy C, Chazel V, Paquet F. 2007. Heterogeneous accumulation of uranium in the brain of rats. Radiat Prot Dosimetry. 127(1-4):86-89. eng.

Houpert P, Lestaevel P, Bussy C, Paquet F, Gourmelon P. 2005. Enriched but not depleted uranium affects central nervous system in long-term exposed rat. Neurotoxicology. 26(6):1015-1020. eng.

Inden M, Iriyama M, Takagi M, Kaneko M, Hozumi I. 2013. Localization of type-III sodium-dependent phosphate transporter 2 in the mouse brain [Research Support, Non-U.S. Gov't]. Brain Res. 1531:75-83. eng.

Jiang GC, Tidwell K, McLaughlin BA, Cai J, Gupta RC, Milatovic D, Nass R, Aschner M. 2007. Neurotoxic potential of depleted uranium effects in primary cortical neuron cultures and in Caenorhabditis elegans [Research Support, U.S. Gov't, Non-P.H.S.]. Toxicol Sci. 99(2):553-565. eng.

Keith DA, Rodriguez JP, Rodriguez-Clark KM, Nicholson E, Aapala K, Alonso A, Asmussen M, Bachman S, Basset A, Barrow EG et al. 2013. Scientific Foundations for an IUCN Red List of Ecosystems. PloS one. 8(5):e62111. eng.

Lemercier V, Millot X, Ansoborlo E, Menetrier F, Flury-Herard A, Rousselle C, Scherrmann JM. 2003. Study of uranium transfer across the blood-brain barrier. Radiat Prot Dosimetry. 105(1-4):243-245. eng.

Lestaevel P, Dhieux B, Delissen O, Benderitter M, Aigueperse J. 2015. Uranium modifies or not behavior and antioxidant status in the hippocampus of rats exposed since birth [Research Support, Non-U.S. Gov't]. The Journal of toxicological sciences. 40(1):99-107. eng.

Liebner S, Czupalla CJ, Wolburg H. 2011. Current concepts of blood-brain barrier development [Research Support, Non-U.S. Gov't

Review]. The International journal of developmental biology. 55(4-5):467-476. eng.

Mirto H, Barrouillet MP, Henge-Napoli MH, Ansoborlo E, Fournier M, Cambar J. 1999. Influence of uranium(VI) speciation for the evaluation of in vitro uranium cytotoxicity on LLC-PK1 cells. Human & experimental toxicology. 18(3):180-187. Eng.

Muller D, Houpert P, Cambar J, Henge-Napoli MH. 2006. Role of the sodium-dependent phosphate co-transporters and of the phosphate complexes of uranyl in the cytotoxicity of uranium in LLC-PK1 cells [Research Support, Non-U.S. Gov't]. Toxicology and applied pharmacology. 214(2):166-177. eng.

Nakagawa S, Deli MA, Nakao S, Honda M, Hayashi K, Nakaoke R, Kataoka Y, Niwa M. 2007. Pericytes from brain microvessels strengthen the barrier integrity in primary cultures of rat brain endothelial cells. Cell Mol Neurobiol. 27(6):687-694. eng.

Obermeier B, Daneman R, Ransohoff RM. 2013. Development, maintenance and disruption of the blood-brain barrier [Research Support, N.I.H., Extramural

Research Support, Non-U.S. Gov't

Review]. Nature medicine. 19(12):1584-1596. eng.

Paquet F, Houpert P, Blanchardon E, Delissen O, Maubert C, Dhieux B, Moreels AM, Frelon S, Gourmelon P. 2006. Accumulation and distribution of uranium in rats after chronic exposure by ingestion. Health Phys. 90(2):139-147. eng.

Periyakaruppan A, Kumar F, Sarkar S, Sharma CS, Ramesh GT. 2007. Uranium induces oxidative stress in lung epithelial cells [Research Support, N.I.H., Extramural

Research Support, U.S. Gov't, Non-P.H.S.]. Archives of toxicology. 81(6):389-395. eng.

Pierrefite-Carle V, Santucci-Darmanin S, Breuil V, Gritsaenko T, Vidaud C, Creff G, Solari PL, Pagnotta S, Al-Sahlanee R, Auwer CD et al. 2017. Effect of natural uranium on the UMR-106 osteoblastic cell line: impairment of the autophagic process as an underlying mechanism of uranium toxicity. Arch Toxicol. 91(4):1903-1914. eng.

R Software. 2016. R: A Language and Environment for Statistical Computing Version Version 1.0.136. Vienna, Austria: R Foundation for Statistical Computing.

Siflinger-Birnboim A, Del Vecchio PJ, Cooper JA, Blumenstock FA, Shepard JM, Malik AB. 1987. Molecular sieving characteristics of the cultured endothelial monolayer. Journal of cellular physiology. 132(1):111-117. eng.

Stratman AN, Malotte KM, Mahan RD, Davis MJ, Davis GE. 2009. Pericyte recruitment during vasculogenic tube assembly stimulates endothelial basement membrane matrix formation. Blood. 114(24):5091-5101. eng.

Thanabalasundaram G, Schneidewind J, Pieper C, Galla HJ. 2011. The impact of pericytes on the blood-brain barrier integrity depends critically on the pericyte differentiation stage. The international journal of biochemistry & cell biology. 43(9):1284-1293. eng.

Tournier BB, Frelon S, Tourlonias E, Agez L, Delissen O, Dublineau I, Paquet F, Petitot F. 2009. Role of the olfactory receptor neurons in the direct transport of inhaled uranium to the rat brain [Research Support, Non-U.S. Gov't]. Toxicol Lett. 190(1):66-73. eng.

Vidaud C, Robert M, Paredes E, Ortega R, Avazeri E, Jing L, Guigonis JM, Bresson C, Malard V. 2019. Deciphering the uranium target proteins in human dopaminergic SH-SY5Y cells. Archives of toxicology. 93(8):2141-2154. eng.

Weiss N, Miller F, Cazaubon S, Couraud PO. 2009. The blood-brain barrier in brain homeostasis and neurological diseases [Review]. Biochimica et biophysica acta. 1788(4):842-857. eng.

Weksler B, Romero IA, Couraud PO. 2013. The hCMEC/D3 cell line as a model of the human blood brain barrier. Fluids and barriers of the CNS. 10(1):16. eng.

Weksler BB, Subileau EA, Perriere N, Charneau P, Holloway K, Leveque M, Tricoire-Leignel H, Nicotra A, Bourdoulous S, Turowski P et al. 2005. Blood-brain barrier-specific properties of a human adult brain endothelial cell line [Research Support, N.I.H., Extramural

Research Support, Non-U.S. Gov't

Research Support, U.S. Gov't, P.H.S.]. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. 19(13):1872-1874. eng.

Zeger SL, Liang KY. 1986. Longitudinal data analysis for discrete and continuous outcomes. Biometrics. 42(1):121-130. eng.

Zozulya A, Weidenfeller C, Galla HJ. 2008. Pericyte-endothelial cell interaction increases MMP-9 secretion at the blood-brain barrier in vitro. Brain Res. 1189:1-11. eng.

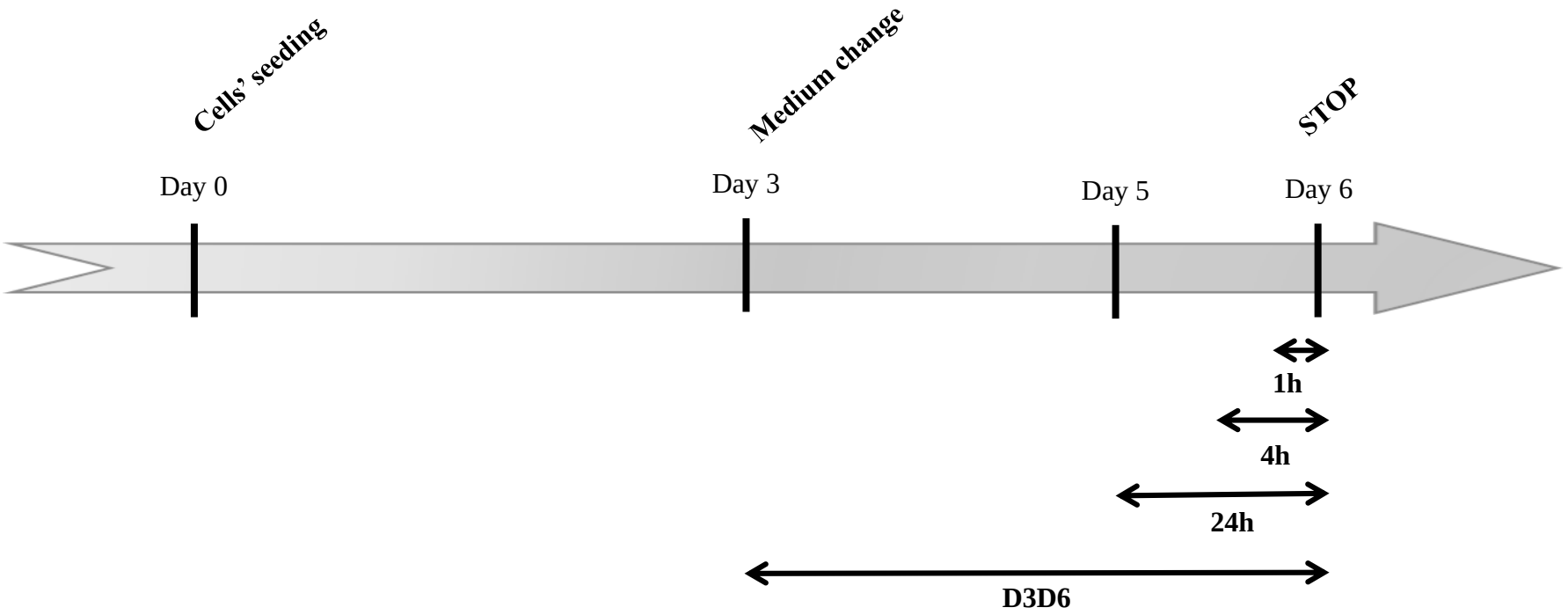


Figure 1: Representation of the timeline used to mimic acute and chronic exposures.

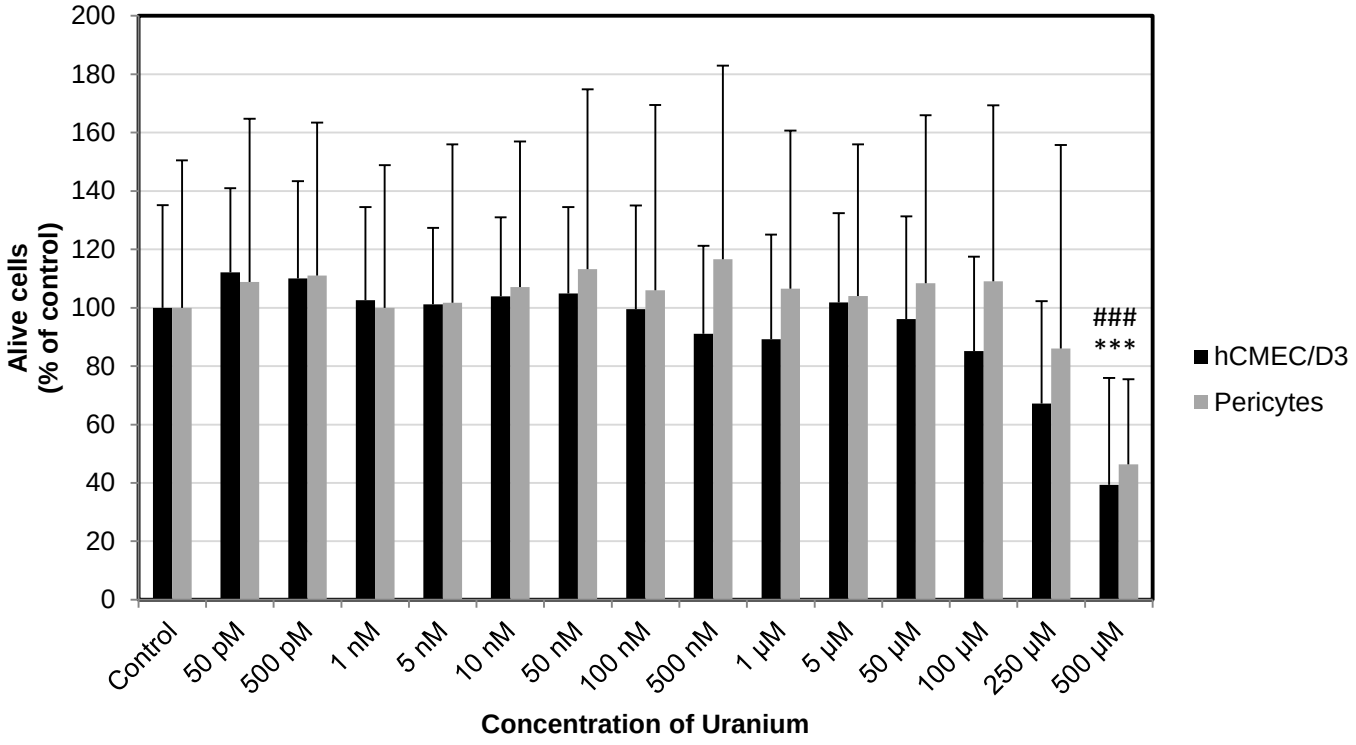


Figure 2: Evaluation of the cytotoxicity of 24-h exposure to U in the hCMEC/D3 cell line and in human brain pericytes by MTT assay. Results expressed as the viability of the cells normalized to the control (non-exposed cells). Mean±SD. hCMEC/D3:***$p<0.001$, Pericytes:$^{###}p<0.001$. N=3 in sextuplicate.

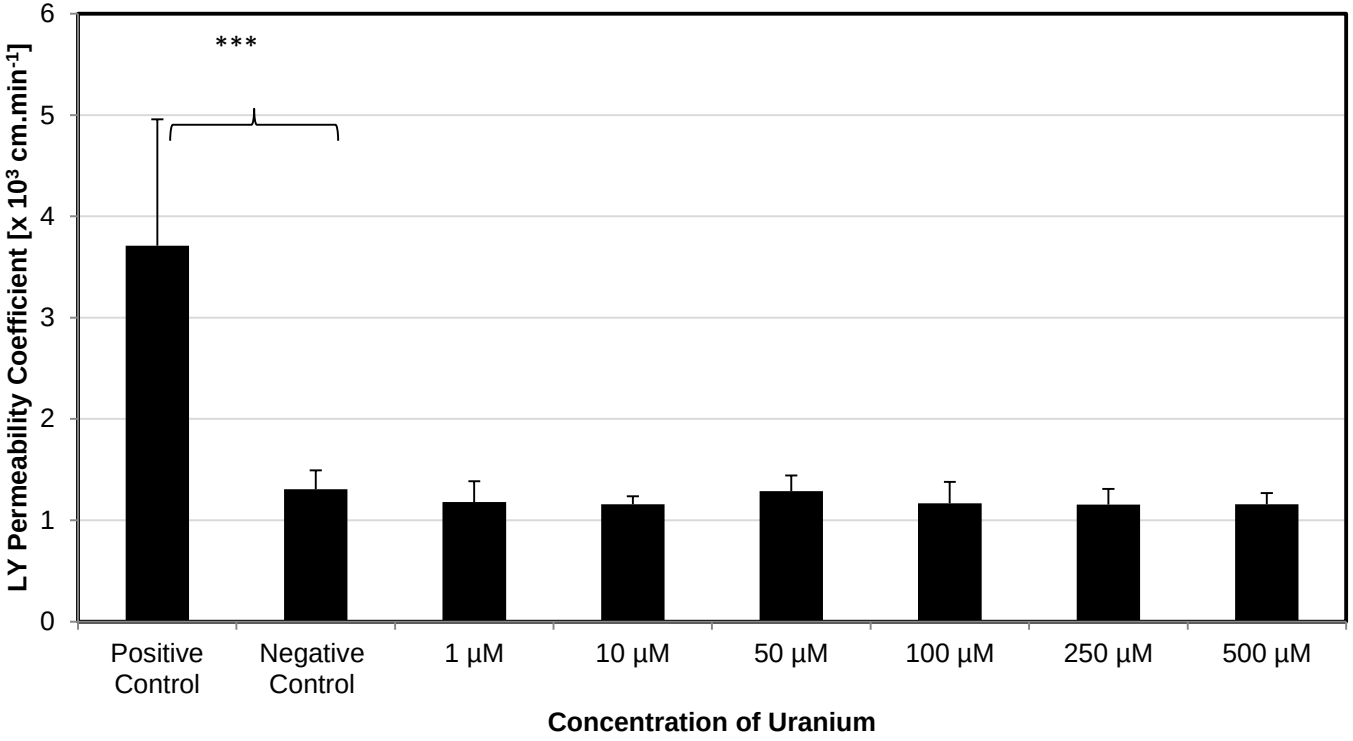


Figure 3: LY Permeability of hCMEC/D3 monolayer subjected to 1 to 500 µM of U at the time of the test. Results expressed as Mean±SD. ***p<0.001. N=3 in triplicate.

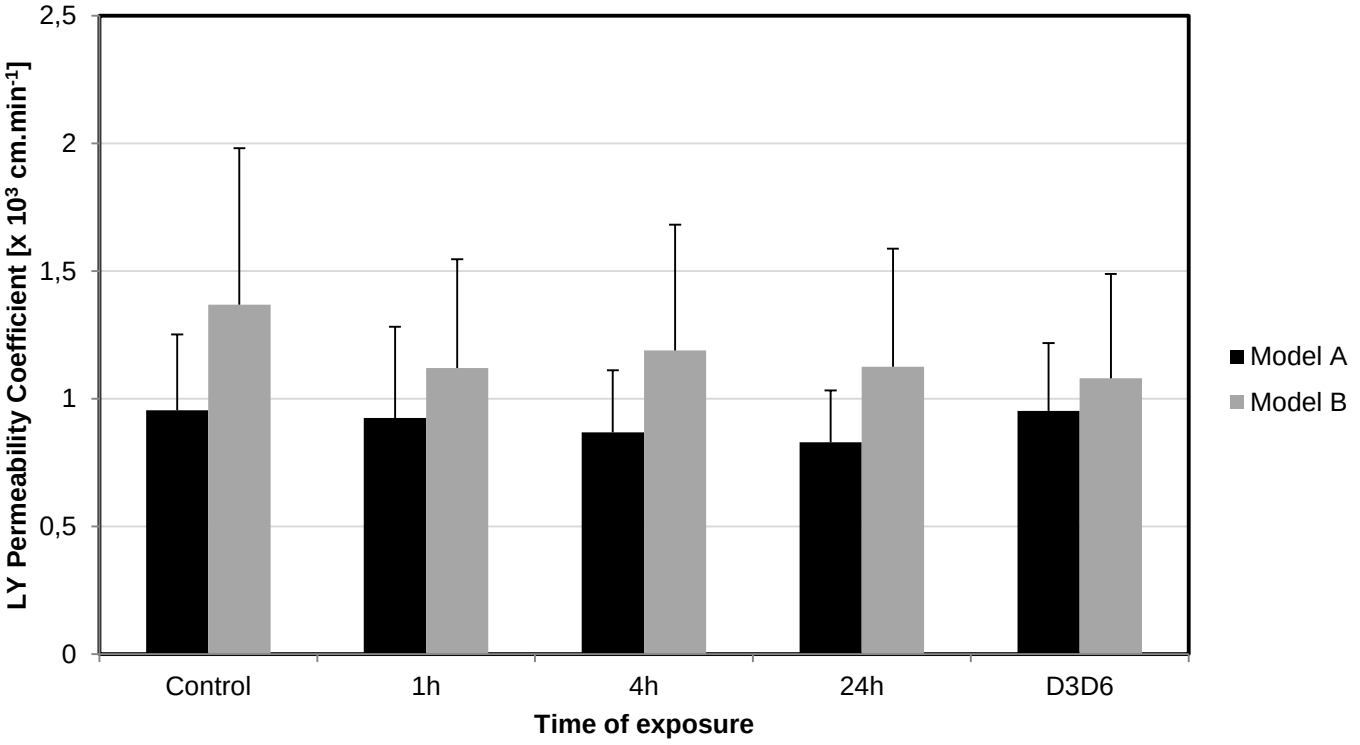
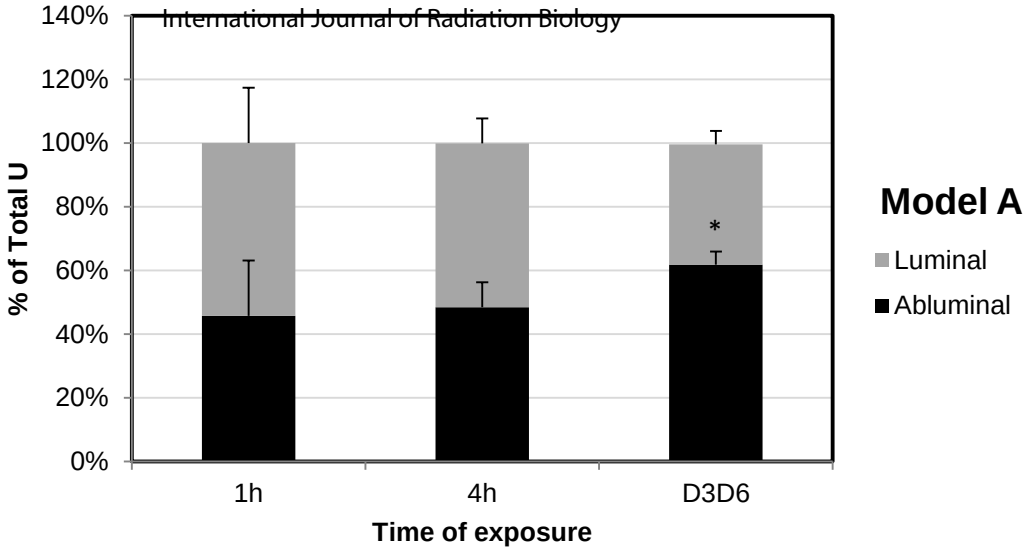


Figure 4: LY permeability test of model A (hCMEC/D3 cells alone) and model B (hCMEC/D3 cells and pericytes) exposed to 50 µM U for different times. Results expressed as Mean±SD. N=3 - 8 in triplicate.



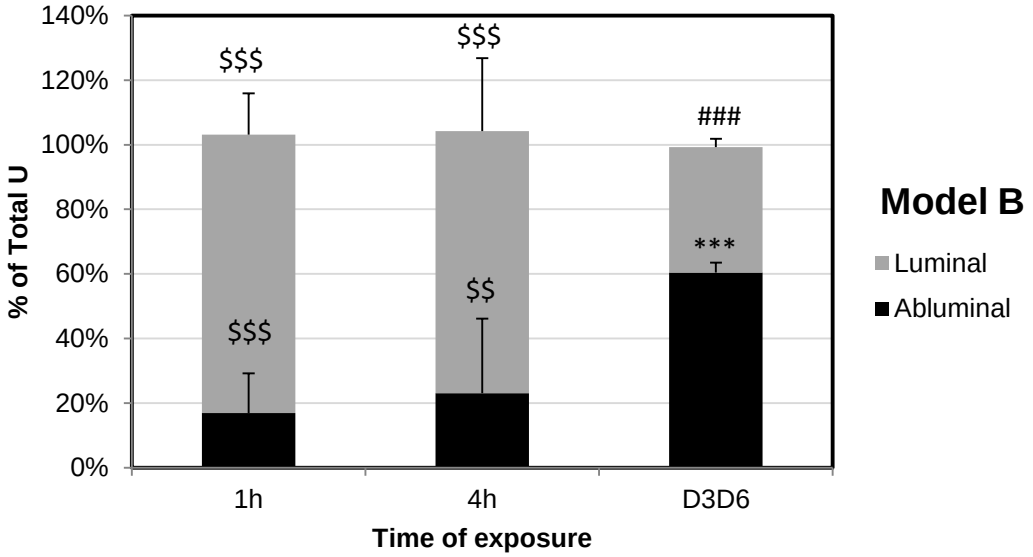


Figure 5: Percentage of Total U in extracellular compartments (luminal and abluminal) in model A and B exposed to 50 µM of U for different times. Results expressed as Mean±SD. N=3 in sextuplicate experiments. In each model, the percentage of Total U for 4 h and D3D6 conditions was compared to 1-h exposure for the luminal (* p<0.05, ***p<0.001) and abluminal compartments (###p<0.001). For each time of exposure, the percentage of Total U in each compartment was compared between model A and model B ($$ p<0.005, $$$p<0.001).

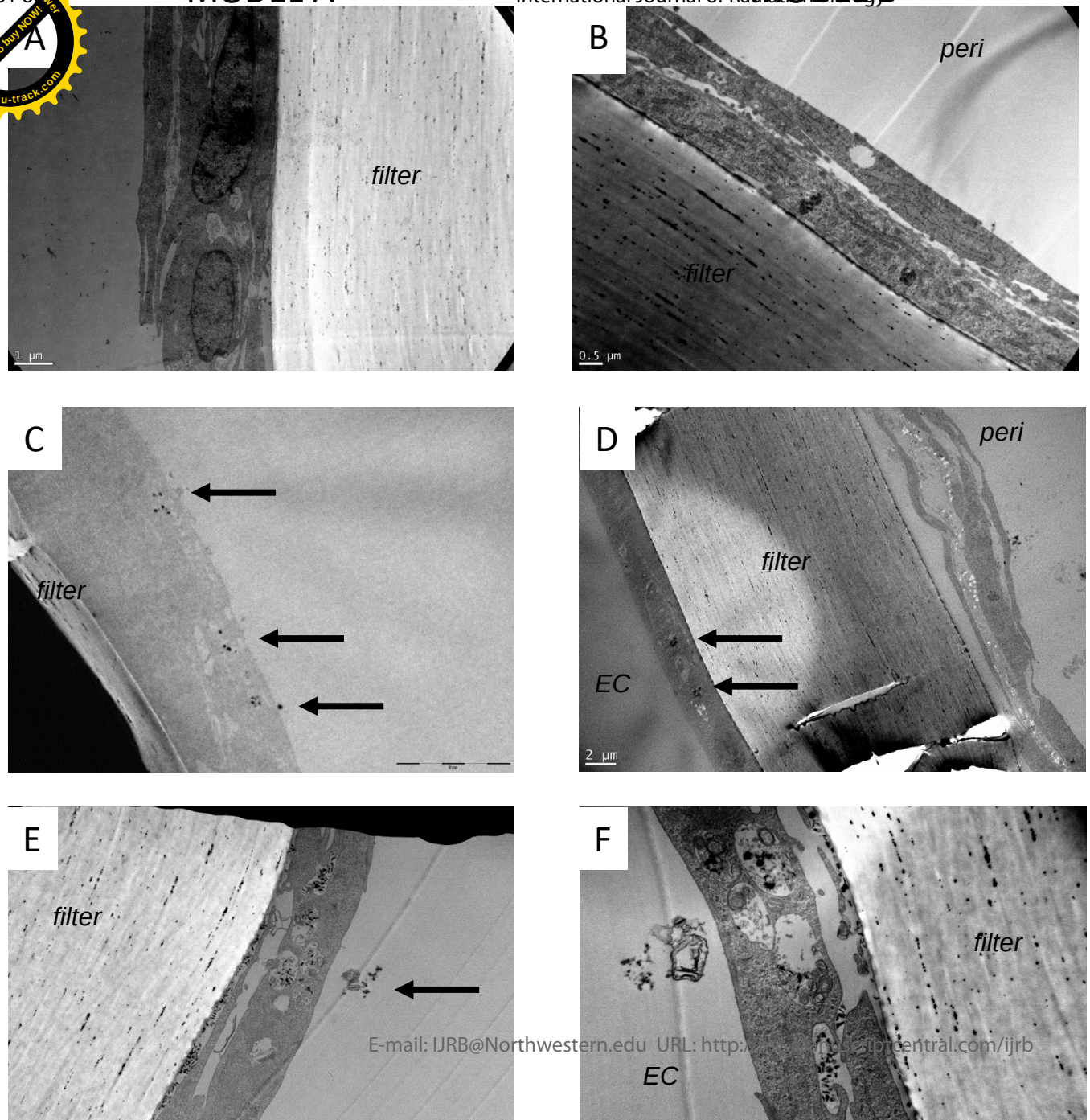


Figure 6: TEM ultramorphology of models A (A, C, E) and B (B, D, F): (A-B) Control, (C-F) – After 1 h of U exposure. Scales are indicated in the figure. EC: endothelial cell, Peri: pericytes. Electron-dense needle-like structures are indicated by black arrows.

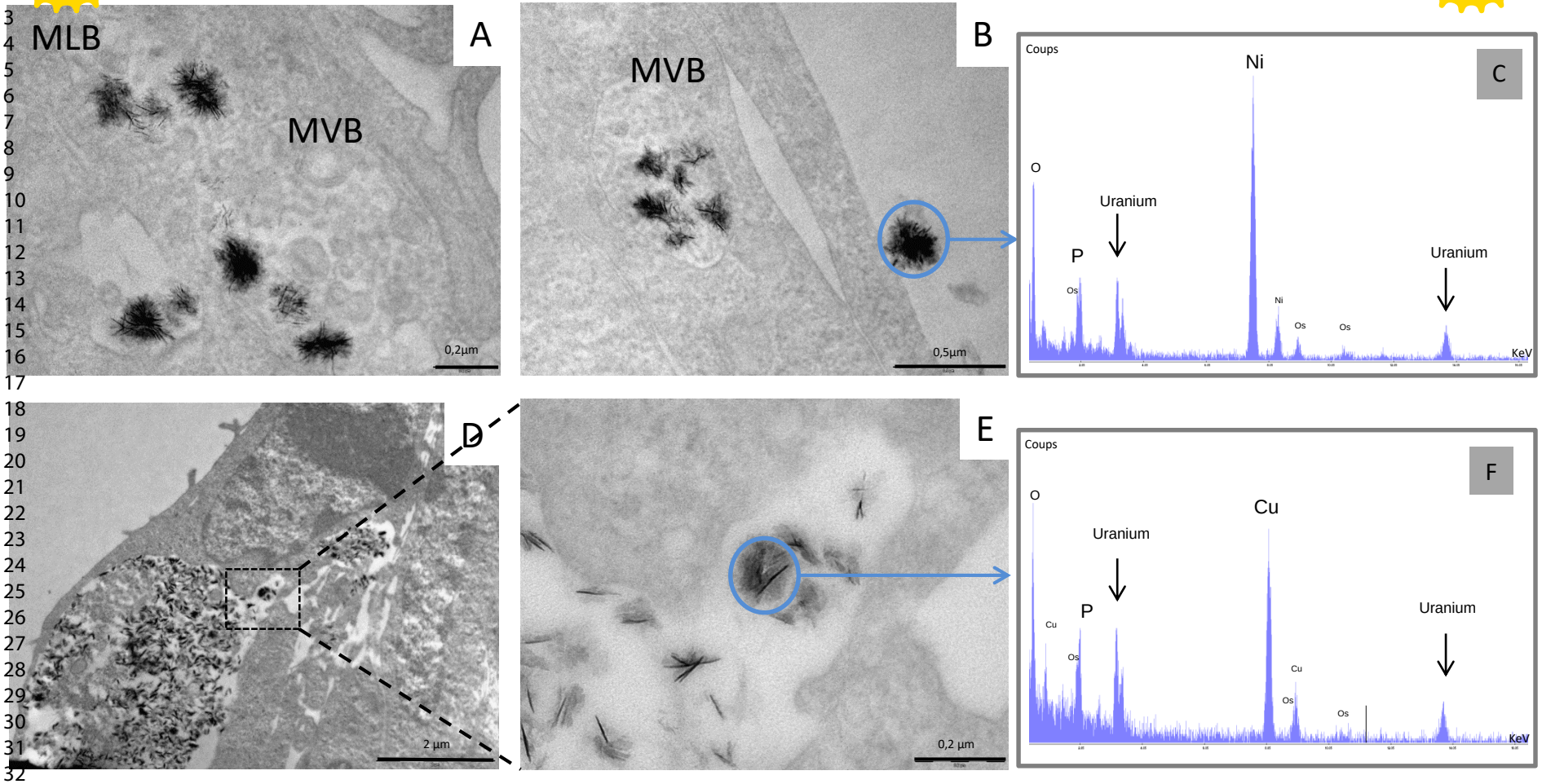


Figure 7: TEM/STEM-EDX observation of hCMEC/D3 cells in models A (A-B) and B (D-E) after 1 h of U exposure. MLB: multilamellar bodies, MVB: multivesicular bodies. Energy dispersive X-ray spectroscopy microanalysis of uranium-exposed models (C-F). The two highest peaks (Ni and Cu) correspond to the elements of the grids.