Characterization of the human neuro-vascular unit in order to perform in vitro exposure to low doses of uranium

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Abstract

Contamination by uranium is a relevant concern due to its natural presence and to the quantities generated by man-made activities. After in vivo studies, evidences indicated Central Nervous System impairment and small traces of depleted uranium (DU) were found in the brain of the animal models, despite that there is no evidence of a Blood-Brain Barrier (BBB) breakdown. It was necessary to construct in vitro models to understand the mechanisms of DU interaction with cerebral endothelium, starting by a BBB model and evolving to Neurovascular Unit (NVU) models. The two here used models include (1) human cerebral microvessel endothelial cells (hCMEC/D3) (2) co-cultured with human pericytes. These models showed that DU is not cytotoxic and does not disturb the tight junctions in concentrations below 500 µM. More, it is at least capable of entering into endothelial cells and pericytes in a concentration dependent manner. Therafter, using a concentration of 50 µM all the models indicate that DU does not affect paracellular permeability despite of the exposure duration. However, they also show that hCMEC/D3 possess, at a small level, a detoxification system of DU after the uptake but also that it is capable of passing through the cells to the lower compartment. This response is slightly modulated by the presence of pericytes, stating the importance of complexifying the model in order to mimic the in vivo case.

Keywords: Depleted Uranium; Blood-Brain Barrier; Neuro-vascular Unit; *in vitro* modelling

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Introduction

Uranium (U) is naturally present in the Earth's crust [1] and therefore within certain foods and drinking water. The production of enriched U for weapons and nuclear reactors results in a by-product called depleted U (DU), which has considerably less radioactivity [1] than other types. This material has several applications that could cause its dispersion in the environment and provoke an exposure [2]. The toxicity of U is both chemical and radiological. However, for low dose exposures to natural or DU the greatest effects are caused by chemical toxicity.

U is fixed either in the kidneys or in the skeleton. The kidneys are the main target of U toxic effects, being responsible for excreting 80 to 90% of it [3], and they are known to suffer modifications in the urinary excretion. Soft tissues, as the liver, lungs and testis, are also able to fix U in a much smaller quantity [4]. The whole brain accumulates trace amounts of U, regardless of the time of exposition, in a heterogeneous and dose-dependent form. Using mostly rat models, several reports indicate behavior alterations ensuing DU exposure. The toxicity threshold and the effects depend on the concentration, the route and time of exposure which may be due to different U oxidation forms present in different routes of exposition and U affinity.

The mechanisms by which U is able to move from the blood into the brain are still unknown. There is a study in rat model reporting that the Blood Brain Barrier (BBB) is intact after U contamination [5]. Beside the fact that there are other structures that act as interfaces between the blood and the brain, the BBB represents the largest contact surface, justifying the investigation in this one.

The BBB is a highly selective physical, metabolic and transport barrier [6]. In order to protect the Central Nervous System

(CNS), the BBB prevents the contact between the brain parenchyma and compounds circulating in the blood both by the presence of tight continuous endothelium permitting the isolation from fluctuations in nutrients, hormones and metabolites [7]. The BBB is composed by endothelial cells (EC), different from the ones existing at the periphery regarding structure and metabolism. They are held closely together by inter-endothelial junctions, namely Tight Junctions (TJ). They actively transport hydrophilic molecules in a strictly controlled way through polarized expressions of membrane receptors and of influx/efflux transporters on both the luminal and the abluminal surface.

The function of the brain EC requires a close relation with the surrounding cells, since it is this microenvironment that induces their special characteristics and barrier properties. EC together with neighbouring pericytes, glial cells, neurons and basal lamina form the Neurovascular Unit (NVU). This is the organic and functional unit resulting from the interaction of neural and non-neural cells that protects the brain and ensures its best functioning [8, 9].

Besides the fact that in vivo models are non-human and much more expensive, have reduced throughput and carry extra difficulties, there is an increasing number of directives to limit their use. In order to understand the many mechanisms that are involved in the permeability of the BBB, it is possible to use in vitro models. The present work utilizes two iterations of a static model in a Boyden-like chamber with two compartments: luminal, mimicking the cerebral one, and abluminal, mimicking the blood. The first model uses an immortalized human cerebral microvessel endothelial cell (hCMEC/D3) line and the second one combines hCMEC/D3 with human primary pericytes. Tests performed on the two cell types demonstrated that DU is not cytotoxic until it reaches very high concentrations (500 μM) to both cell types when exposed during 24h (data not shown). Furthermore, it was seen that DU does not disturb the permeability of EC, and therefore the TJ, in both the first and the second model (data not shown). With this data it was possible to establish a working concentration of 50 μM and test the effects of different time exposures, mimicking acute and chronic exposures.

Materials and Methods

Cell Culture hCMEC/D3 were obtained from a MTA with Institut Cochin (Paris, France), pericytes were purchased from ScienCell (San Diego, CA, USA). hCMEC/D3 and pericytes were maintained in EndoGROTM Basal Medium, supplemented with EndoGROTM-MV Supplement Kit, Penicillin-Streptomycin at 10,000 U/mL and HEPES 1M at 37ºC in the presence of 5% CO2.

Models Arrangement In the first model, hCMEC/D3 are seeded (50000 cells/cm_2) on the luminal side of Transwells polyester inserts, either in 6 or 12-well format, with 0.4 μm pore size pre-coated with collagen-I (150 μg/mL). In the second model, pericytes are seeded (50000 cells/cm_2) on the abluminal side of Transwells polyester inserts, either in 6 or 12-well format, with 0.4 μm pore size pre-coated with collagen-I (150 μg/mL). The following day the hCMEC/D3 are seeded (50000 cells/cm_2) on the luminal side of the pre-coated with collagen-I Transwells. The medium was changed in all models at Day (D) 3 and the experiments carried at D6.

Permeability studies In the present work Lucifer Yellow (LY) test was applied. After the 6-day culture, the luminal content of the inserts is removed; they are transferred into 6- or 12-well plates containing 2.6 or 1.5 mL, respectively, of transport buffer (HBSS with 10mM of HEPES and 1mM of sodium pyruvate) and 1.5 or 0.5 mL of 50 μM LY solution is added to each chamber, depending on the size of the chamber. At 10 and 25 minutes, the inserts are changed into fresh transport buffer containing wells. The plates are kept on the incubator. After 45 minutes, the transport buffer of the abluminal compartment for 10, 25 and 45 minutes and the one of the luminal compartment after the 45 minutes are collected. 200 μL of buffer from the abluminal compartment for each time point and 20 μL of the luminal compartment, diluted at 1:10 in fresh transport buffer to a final volume of 200 μL, is collected for each condition and added to black 96-well plates (Corning, NY, USA). Together with the samples, solutions of known crescent

concentration between 0 and 20 µM are added to create a standard. The fluorescence of the plate is read using a microplate reader. The data is analyzed using the clearance principle as described by [10].

Uptake Extracellular The cells are seeded at a 50000 cells/cm² concentration on 24 mm or 12 mm Transwells. 1 mL of the luminal and of the abluminal medium is collected at D3, for each insert. The medium in the inserts is changed and U is added to the medium at different time points, according with the different contaminations in study. At D6 the medium is again collected. The samples of each condition are diluted with 2% nitric acid, to be in the range of the standard of 1 to 1000 parts per trillion. U is quantified by Inductively-Coupled Plasma Mass Spectrometry (ICP-MS) (ICP-MS, PQ, Excell, Thermo Electron, France) using a 7700X series (Agilent Technologies, Les Ulis, France), calibrated with a SPEX CertiPrep uranium standard solution (Jobin Yvon, Longjumeau, France).

Uptake Intracellular The cells are seeded on the bottom of 6- or 12-well plates coated with collagen-I at 25000 cells/cm2. For EC, U is added after 24h of seeding and the samples collected after 48h of seeding. For the pericytes, U is added after 48h of seeding and the samples are collected after 96h of seeding. The cells are washed repeatedly with PBS [-] [-] more or less supplemented with Bovine Serum Albumin and then collected with 200 µL of 69% nitric acid (Aristar quality grade, VWR Prolabo). Each concentration is tested in duplicate. The samples of each condition are diluted with 2% nitric acid, to be in the range of the standard of 1 to 1000 parts per trillion. U is quantified by Inductively-Coupled Plasma Mass Spectrometry (ICP-MS) (ICP-MS, PQ, Excell, Thermo Electron, France) using a 7700X series (Agilent Technologies, Les Ulis, France), calibrated with a SPEX CertiPrep uranium standard solution (Jobin Yvon, Longjumeau, France).

Statistical Analysis All results are expressed as mean ± Standard Deviation (SD) from at least three independent experiments, each one with triplicates for each condition tested, unless stated otherwise. The significance of variability between results from various groups was determined by One-way ANOVA if normality was verified and Kruskal–Wallis 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 statically different when $p<0.05$. The associated subtitle are * for $p< 0.05$, ** for $p<0.005$ and *** for $p<0.001$.

Results

Effects of different exposition times Several time windows of contamination were defined in order to mimic both acute and chronic exposures. The acute exposure was reproduced by 24, 4 and 1h of exposure before the permeability experiment. A long chronic exposure was modelled with the presence of DU in the medium all culture long, D0D6. Two smaller 3-day exposures were done by adding DU to the medium in the moment of seeding and keeping it until D3, called D0D3, and also by only adding DU in the last 3 days of culture, D3D6. After the 6 days of culture, a LY permeability test is performed, whose results are depicted in Figure 1.

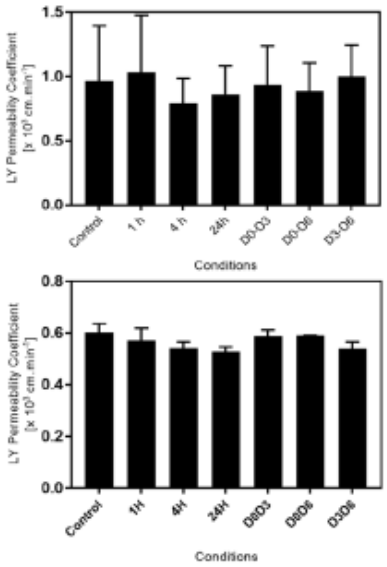

Figure 1 - LY permeability test of (top) mono-culture and (bottom) co-culture subjected to 50 µM of *DU* DU during different time lengths. Results expressed as Mean ± SD. N=3 in triplicate.

All the conditions present values of permeability coefficient within the normal values, similar to the non-contaminated

control, in both models. In addition, there is no detectable difference between the groups or between the models. Previous tests indicated that the viability is not affected by DU until 500 µM (data not shown) and these different exposures at 50 µM show that DU does not affect the cell's permeability, which is also in agreement with the literature. However, animal models showed that U is able to reach the brain. Therefore, U must pass through the BBB by another route besides paracellular transport, for instance by transcellular transport. For that to be possible, it is necessary that DU enters the cell, which was verified next.

Intracellular U Uptake In order to test the incorporation of U, the cells are seeded on the bottom of a 6-well plate, the U is added after 24h at concentrations from 1 to 250 µM and 24h after that the cells are photographed using a phase contrast microscope, thoroughly washed and the intracellular concentration of U is measured using ICP-MS. The results of the DU found inside the EC and pericytes are shown in Figure 2.

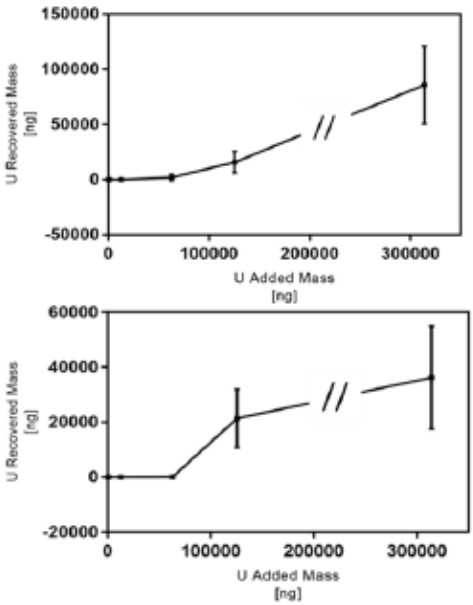

Figure 2 - Intracellular U mass in (top) hCMEC/D3 and (bottom) pericytes subjected to 1 to 250 µM of U. Results expressed as Mean ± SD. N=3 in duplicate.

It is visible that the cells are capable of incorporating U and the quantity found inside the cells rises with the quantity added to the medium, starting at very small values but increasing considerably in the last 2 points, at 100 and 250 µM, which is in agreement with previous studies. In addition, by comparing the results of both cell types it is clear that EC uptake much more DU than pericytes, which is not surprising taking in account their barrier properties. This data may suggest a detoxifying system of EC that saturates at higher concentrations: it is able to efflux DU when there is only a small amount but loses its capacity in the presence of more abundant quantities.
Since it was shown that U enters the cells, it leaves to know how it reaches the brain. For that, it is necessary to know if the cells release the incorporated U, to which side and at what kinetics.

Extracellular U uptake In order to understand what happens to DU once it enters the cells, both models were subjected to the same acute and chronic contamination profiles as done before. Following to the different exposures, the quantity of U in each compartment of the model was measured at D3 and D6. The results of the acute contaminations in both models are depicted in Figure 3.

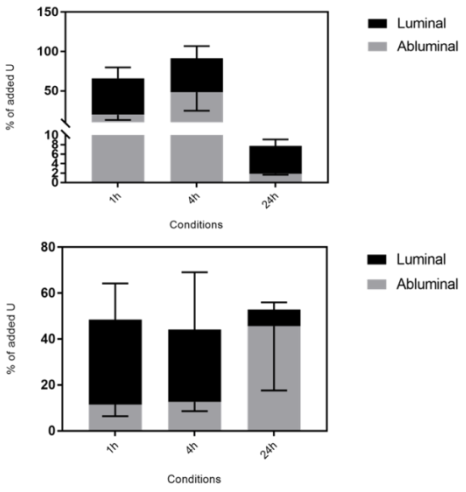

Figure 3 - Extracellular concentrations of DU at D6 in luminal and abluminal compartments following acute exposure of the (top) mono-culture and the (bottom) co-culture models. N=3 in triplicate.

For 1h and 4h exposures, in the first model, the majority of U stays in the luminal compartment (46 and 43%, respectively) but the abluminal quantity is bigger in the 4h than in the 1h case (48% and 20%, respectively). By doing the mass balance,

there is a considerable quantity of DU (between 10 and 35%) not found neither in the luminal nor the abluminal side, possibly resting inside the cells. From 1h to 4h, the quantity in the luminal side decreases, the quantity in the abluminal increases and the quantity presumably inside the cells decreases, suggesting a passage by a transcellular route.

In the co-culture model, there are 37% and 32% for the luminal, 12% and 13% for the abluminal, respectively. In this model 4h is not a long enough contamination to have a substantial quantity in the abluminal side, since the two profiles are quite similar. In addition, in the first model there are differences between the 1h and 4h exposures but in the co-culture there is none. This indicates that, when acutely exposed, the co-culture model responds differently than the mono-culture.

Analyzing the 24h for the first model, the quantity of DU in both compartments is considerably smaller (6% in the luminal and 2% in the abluminal) and, when to the 4h results, the quantity of DU diminishes in both sides. This suggests that as time passes, the cells incorporate U from both compartments, stocking it. It is also visible that the quantity of DU that the cells are capable of withholding is about 90 % of the total amount added. In the second model, there is 46% that reaches the abluminal side and only 7% that remains on the luminal one. The quantity of U in the lower compartment is considerably higher than in the 4h condition showing that the majority of U passes from the luminal side to the cells and to the abluminal side. Comparing the models, it also has a reduced quantity of U in the luminal but a considerably larger quantity in the abluminal side (7% and 46%, respectively) that was considered as statistically different ($p<0.05$). This requires confirmation, since the experiment for the 24h condition in the co-culture model was only repeated twice, lacking one repetition.

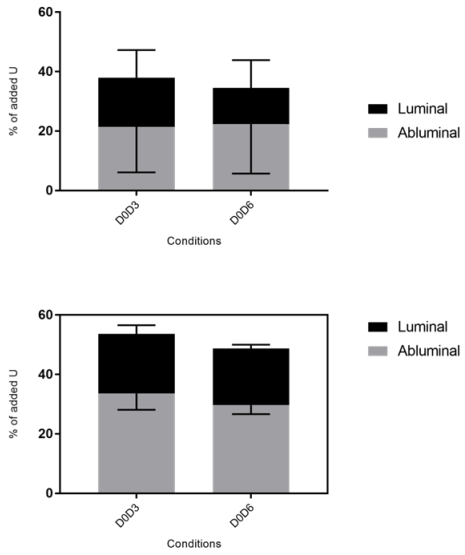

Figure 4 - Extracellular concentrations of DU at D3 in luminal and abluminal compartments following chronic exposure of the (top) hCMEC/D3 and (bottom) hCMEC/D3 + pericytes models. N=3 in triplicate.

The chronic contaminations results at D3 are depicted in Figure 4. In the first model, it is visible that D0D6 and D0D3 present very similar values for both compartments (12% and 17% for the luminal, 22% and 21% for the abluminal, respectively). This shows reproducibility of the method, since until D3 both were subjected to the same conditions (3 days of contamination). Even more, U found in the abluminal compartment is also due to its free passage during cell growth, since U is added during cell seeding at D0 when the cells are not yet confluent. It is visible that a long exposure causes the release of DU to both compartments, since the quantity found at D3 for D0D3 and D0D6 was significantly higher than at 24h.

In the second model, it is evident that D0D6 and D0D3 have the same response (19% and 20% for the luminal, 30% and 34% for the abluminal, respectively). This continues to suggest reproducibility of the method. By comparing these conditions with the 24h obtained before, there is a change in the pattern. The quantity of U decreases in the abluminal side and increases in the luminal one in a mild form. This suggests that the cells were capable of incorporating the U from the abluminal side and releasing it to the luminal one.

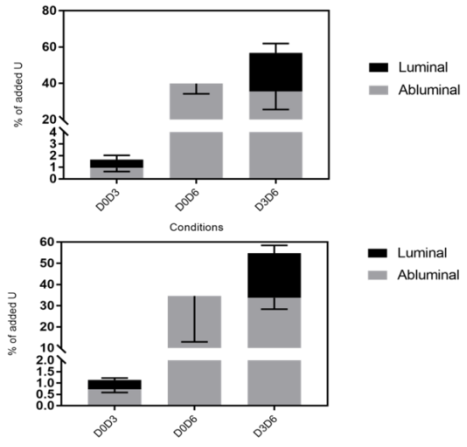

Figure 5 - Extracellular concentrations of DU at D6 in luminal and abluminal compartments following chronic exposure of the (top) hCMEC/D3 and (bottom) hCMEC/D3+pericytes models. N=3 in triplicate.

Lastly, Figure 5 represents the results of chronic contaminations at D6. In the first model, the D0D3 condition presented values of 0.7% for the luminal and 1% for the abluminal side, which means that there is U in both compartments even though the contamination was stopped at D3. Again, this data suggests that cells uptake the U, stock it and then have a detoxifying system that releases U.

The D0D6 condition presents a slight increase in the abluminal quantity (from 22% to 40%) from D3 to D6, showing that a long chronic exposition has different effects. This condition does not have a value for luminal compartment at D6 because since the cells are contaminated twice, at D3 and D6, it wouldn't be possible to analyse the results and know from which contamination the U came from.

Lastly, the D3D6 condition presents values (21% in the luminal and 36% in the abluminal) slightly different of the ones obtained with D0D3 at D3. These conditions were expected to be quite similar since they are both 3-day long contaminations. The small difference may be explained by the fact that cells achieve confluence around D3, meaning that as the time passes the number of cells increases in the monolayer. This means that by starting the contamination at D3, there are more cells that allow the passage of U to the abluminal compartment, justifying the increase from D0D3 to D3D6 (21% to 36%).

Discussion

The fact that the U was capable of entering the cells was already shown in the previous experiment and corroborated by published works [11, 12]. With this experiment there is the certainty that DU is not only capable of entering the cells but also that it can exit, as supposed before. In addition, it also indicates that the DU can be expelled to the abluminal side. For this to happen it is necessary that DU passes through the cells, since they are alive and the permeability is not altered, as seen before. It was shown for the first time in an all-human in vitro model that U is able to reach the abluminal, here mimicking the cerebral, compartment just as it was seen in the animal models.

The fact that a great quantity of U seems to be stocked inside the cells in every condition is not surprising. In vivo, these cells are responsible for protecting the brain from any harmful substance present in the blood current. Therefore, it may be hypothesized that EC stock the U as much as possible to avoid that it reaches the brain. However, beyond its capacity to stock the U, the cells are obliged to release it, doing it to both the luminal and the abluminal side, allowing it to reach the brain. In order to confirm it, further uptake tests should be performed by measuring the quantity of U found in each compartment and also inside the cells to allow a mass balance.

All together these results suggest that the time of exposure influences the response of the system, as was already discussed for in vivo models. Furthermore, since the presence of DU in the abluminal side is undeniable it also shows that EC allow the passage of DU, by some mechanism, as it happens in the animal models, when trace amounts of DU are found after chronical ingestion.

The mechanism through which the U is capable of accessing the cells is still an open question. One work [13] performed in LLC-PK1 cells, a kidney epithelial cell line, states several different conclusions. First, the uptake was significantly reduced when performed at 4°C, which implicates a transport system and not a diffusion

process, and then the group demonstrates that U uptake is concentration-dependent. Furthermore, it was shown that using several inhibitors of absorptive endocytosis, the uptake of U was compromised and it had a significant linear correlation with FITC-albumin. However, it was also seen that cytotoxicity was not reduced when endocytosis was inhibited, leading to the theory that U is also taken up by a transport system at a lesser degree. This system is probably the type IIa sodium-dependent phosphate transporter (NaPi-IIa). Despite the fact that this work already suggests possible mechanisms, EC are a particular type of cells with a high control of what compounds can enter and exit. Furthermore, it is known that, specifically, type II sodium-dependent phosphate transporters are not expressed in brain EC [14]. Thus, it is necessary to verify the actual mechanisms in EC in the future.

Many metals are essential and required for optimal CNS function, as calcium, copper, magnesium, manganese, zinc, etc. In order for them to reach the brain in a controlled fashion, the EC have active or receptor-mediated transport systems. Other metals, considered non-essential, like mercury, cadmium and lead, can also access the brain [15]. There are already some investigations about the entry of these metals through the BBB that could provide some indications of what could happen with U. There is even a paper stating the existence of a synergistic passage of different metals to the BBB [15]. In this work, it was found that some metals, or mixture of metals, could induce the passage of other endogenous metals to the brain. For example, rats with embedded DU fragments have an increase uptake of cobalt in advanced age. This collaborative action between metals could indicate a similar transport mechanism and/or a common one, which could saturate.

Regarding the stocking of metals, EC seem to accumulate iron in an extracellular concentration-dependent manner and, when the storage capacity is exceeded, iron is transported across the BBB [129]. For iron transporters, such as holotransferrin, lactoferrin, and melanotransferrin, they are described in vitro to be transported across the brain endothelial cell monolayer, without any degradation in the lysosomal compartment. These molecules are thus transcytosed through BBB by a receptor-mediated transport [16-18]. Drawing the parallelism, it seems to be the approximately the same effect found with U: the uptake is dependent of the extracellular concentration and after the capacity of stocking is overwhelmed, EC expel U to both compartments. In fact, there are also other reports that indicate that after the entrance of lead to the EC, the metal is expelled by a transport protein both present in the luminal and abluminal membranes [19]. It is possible that the same happens with U, leading to its release towards both compartments, as seen in this work. As for exactly what transporter could be responsible for this passage, it is still unknown. Some suggest the divalent metal transporter 1 (DMT-1) as a possible answer [20], since this transporter is known to have a wide range of substrates, mediating the passage of numerous metals to the BBB, both essential (as iron, cooper and cobalt) and non-essential (as cadmium and nickel). However, it is still possible that U is capable of entering through more than one mechanism, as was found in LLC-PK1 cells and as it happens in EC for mercury and iron [21].

One other possible insight could be the work developed with bacteria strains from the Chernobyl zone [22]. This strain has a high resistance to U thanks to multiple detoxifying mechanisms. One of the found mechanisms involves the release of inorganic phosphate that forms a uranyl-phosphate complex with U which is then effluxed to the extracellular domain. Even more, these inorganic phosphates can also be released to complex with U extracellularly and avoid its entrance. This inorganic polyphosphates are also expressed in the mammalian brain [23].

It is visible that the question of the entry mechanism is far away from answered, leading to many possible lines of investigation. Beyond the internalization mechanism, the cellular processes that occur after the uptake of U by the EC are still unknown. In order to provide some clues regarding this question, results of Transmission Electron Microscopy of EC subjected to the different contaminations lengths are still awaited in the scope of this project to allow the visualization of the cell's ultrastructure**.**

Conclusion

The presence of natural U in the Earth's crust, together with man-made activities, civil, military or nuclear, that create large amounts of DU increase the risk of human contamination. There is data that suggests behavioural changes in exposed workers [24] and cognitive impairment in exposed Gulf War veterans [25-27]. These symptoms lead to the investigation of the possible routes and consequences for this contamination in numerous animal models. Results show that DU affects certain physiological systems, as the CNS. Rat models subjected to chronic exposures to low doses of U showed signs of behavioral modifications, as transient memory changes and locomotion alterations, and variations in the metabolism of certain neurotransmitters. In fact, there were even traces of U in certain areas of the brain of contaminated animals [28-30]. However, results indicate that the BBB integrity seems to be intact after DU perfusion [5], meaning that DU can reach the brain without disturbing intercellular junctions. This creates questions of how does the DU reach the brain, how the DU interacts with the BBB, what are the effects of DU in the BBB, and what is the influence of the other cell types in the microenvironment of the BBB, namely the human NVU. *In vivo* models provide with a more complete response but they are non-human, much more expensive, have reduced throughput, in addition to the increasing number of directives to limit their use. Therefore, in order to investigate this subject, it was necessary to develop *in vitro* models to mimic the *in vivo* situation, which is nowadays a big challenge. Even if further characterization of the already existing models are necessary regarding function of BBB and NVU phenotypes, the results obtained about TJ characteristics (paracellular permeability and presence of TJ proteins between cells) are very promising and allow to investigate DU interaction with cerebral endothelium.

In this work two different human models with increasing complexity were used using a time window of 6 days of culture. The first model to be developed was the simplest, mimicking only the BBB by culturing a human EC line, hCMEC/D3, in a Boyden-like chamber over a semi-permeable porous membrane. The second model includes also human primary pericytes on the other side of the filter.

It was demonstrated that DU is not cytotoxic until very high concentrations (500 µM) both to hCMEC/D3 and pericytes when exposed during 24h. Furthermore, it was also shown that DU does not disturb the permeability of EC, and therefore the TJ, in both the first and the second model. With this data it was possible to establish a working concentration of 50 µM and test the effects of different time exposures, mimicking acute and chronic contaminations. The results indicated that the permeability of the BBB also does not change when the time of exposure varies. At this point, it is difficult to exactly mimic the *in vivo* situation since these models are static whereas *in vivo* DU exposure comes from blood flow, so in the future laminar flow should be added to improve these models. Even so, a permeability approach allows working in a concentration independent way. This working concentration of DU is fixed in order to be easily detectable by the measurement devices in the following tests.

The data obtained lead to think that U could surpass the BBB by some alternative route besides paracellular transport. The possibility of transport by transcellular pathways induced the investigation of whether U was capable of entering the cells. Uptake tests show that DU is capable of entering in a concentration-dependent manner in both cell types but mostly in EC.

Afterwards, it was investigated how the quantity of DU changed in both compartments of the model throughout different exposures, acute and chronic. It was shown for the first time, in our human models, that DU is capable of accessing the abluminal compartment, here mimicking the cerebral. In addition, it was shown that the response to acute and to chronic exposures is different. Furthermore, it indicates that EC incorporate the DU, stock it and then release it by a detoxifying system. This system liberates the DU back to the extracellular domain to both compartments, allowing it to reach the abluminal side. Finally, the data seems to show an influence of the communication between EC and pericytes due to the slight differences in the quantity of DU found in the abluminal side between the mono-culture and the co-culture.

In the end, all the tests show that the mono-culture model is already robust, providing

with a lot of information regarding the reaction of the BBB upon DU contamination. Nonetheless, the addition of another cell type in order to complexify the model into the NVU seems to be relevant, justifying the last step of the present work. This data highlights the fact that it is necessary and more efficient to work on a group of different cell types than on an isolated one.

Future Perspectives

All the here presented results are comforting regarding exposed populations since the BBB helped by its vicinity plays its role of protecting the brain by avoiding, at a certain level, U to reach easily the cerebral parenchyma. Now, it is necessary to continue the investigation. Great insight will be provided by the data from TEM approach combined with DU permeability (on-going investigation).

Concerning the model developing, each cell type should be characterized in terms of gene expression when in mono and co-culture, to confirm there is no alteration regarding the phenotype when in contact with other cell types.

It would be interesting to develop models even more complex, for instance, by instead of using a NT2-N cell line (on-going investigation), using a NT2-NA which besides neuron-like cells also contains astrocytes. Even more, it could also be used a microglia cell line. In theory, since it is more closely related to the *in vivo* structures, the response should be more reliable.

Furthermore, in each model, it should be investigated what are effects of DU in the different cell types of the NVU regarding gene regulation and protein expression.

These new inquiries will be essential to speculate on what cellular and molecular mechanisms are involved in brain DU uptake in order to find the best target to care the disorders observed after U exposure. In a more general future perspective, the data collected with this model could one day be useful to understand which are the signalling pathways of each NVU component that are involved in behaviour disorders observed in human and in rodent models after DU exposure.

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