Pump-Priming Grant

Report

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Title: Investigating the effects of brain death on kidney mitochondrial function

Objective

The aim of this study was to investigate how brain death affects mitochondrial health in kidneys, using clinical plasma samples from brain dead donors (DBD).

Method

Study cohort A total of n=21 plasma samples were requested from the Quality in Organ Donation Biobank (QUOD) and the Oxford Transplant Biobank (OTB). Seven plasma samples were collected from healthy living kidney donors (OTB biobank). The remaining n=14 plasma samples were collected from deceased DBD donors at time of organ donation. Seven samples were from DBD donors that donated kidneys which resulted in immediate renal function (IF) and the other seven samples were from DBD donors who donated kidneys that developed delayed graft function (DGF) after transplant. We selected donors whose donated kidneys had matching outcomes for both kidneys: either immediate function and high eGFR (estimated glomerular filtration rate) (>60 mL/min) 12 months after transplant or delayed graft function (DGF) and low eGFR (<45 mL/min) 12 months after transplant (Table 1).

Cell model Human Embryonic Kidney cells (HEK293) were used in this study as a stable in vitro model for kidney cells, while Human Umbilical Vein Endothelial Cells (HUVECs) were used as a model of endothelial cells. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 25mM glucose, sodium pyruvate and sodium bicarbonate (Sigma® D6546-500ML), 15% foetal calf serum, penicillin-streptomycin (50 µg/mL), and L-glutamine (4mM) and incubated at 37°C with 5% CO_2. The culture medium was changed every day until the cells reached desired confluency (80%). Cells were then isolated and seeded on 96-well black wall plates with transparent bottoms for follow-up assays at a density of 50,000 cells/well.

Assays The DHE, JC-1 and MitoXpress Xtra assays were used for monitoring ROS production, mitochondrial membrane potential and oxygen consumption rate, respectively. Ten µL of brain dead donor and living donor plasma were added in each cell-containing well prior to the assays to assess how plasma from different donor types affects the mitochondrial parameters mentioned above. The same assays were repeated in the presence of a mitochondrial protective compound (CC4066) and heat-inactivated plasma.

Cell viability was assessed using Propidium Iodide (PI) staining.

Mitochondrial morphology assessment by Mitotracker staining 300nM of Mitotracker deep red FM in media was added to the HEK293 and HUVEC cells (± pooled DBD/LD plasma) and incubated for 30min at 37°C. After incubation cells were washed in respective media and were imaged on an Olympus SpinSR SoRA spinning disc confocal microscope. Images were captured every 3 minutes per

cycle with a total of 90 cycles for HEK293 (4:30 hours) and 80 cycles for HUVEC (4:00 hours) cells. Excitation wavelength was 635nm, at 3% of strength, and emission detection at 700nm with magnification 40X, imaging an area of 243.75 X 243.75 µm. Images were analysed using Fiji (Schindelin et al, 2012) and the MiNA, mitochondrial network analysis plugin, averaging 3 cells per timepoint/position in the well (total 4 positions imaged/well).

Results

Following incubation of HEK293 cells with plasma from Living donors (LD), DBD donors donating kidneys with immediate kidney function (IKF) or delayed graft function (DGF) we found no significant difference in cellular oxygen consumption rate, measured in the MitoXpress Xtra assay (Figure 1 A,B), with only a small trend towards higher oxygen consumption rates when cells where incubated with plasma from the IKF group compared to cells alone (no plasma) (p=0.06).

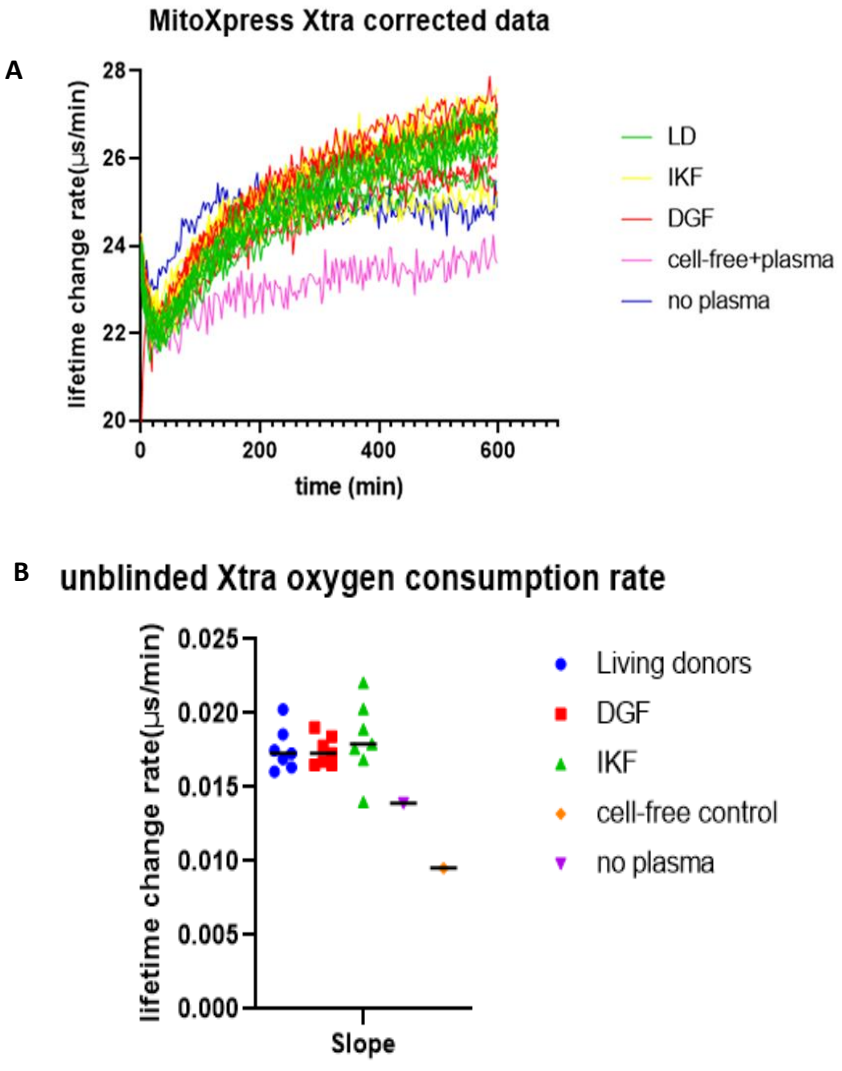

Figure 1. MitoXpress Xtra assay, measuring cellular oxygen consumption rate following incubation of DBD plasma with HEK 293 cells. (A) Fluorescence timecourse of all samples analysed. Each line represents a different sample. Lines are colour-coded according to the biological group analysed. (B) Rate of change in lifetime of the oxygen probe as a surrogate marker for oxygen consumption rate. The slope of each individual curve in A was

measured. Each point represents an individual donor. There was no significant difference between LD, IKF and DGF groups. LD-living donor, IKF-immediate kidney function, DGF-delayed graft function. Cell-free control was plasma added to a well with no cells, to assess the effect of plasma on the oxygen probe. No plasma served as negative control (HEK 293 cells incubated with no donor plasma).

The JC1 assay was used to measure changes in the mitochondrial membrane potential over time and following incubation of HEK293 cells with different types of plasma (higher red/green ratios represent higher mitochondrial membrane potential). No significant differences were observed between groups (Figure 2 A,B).

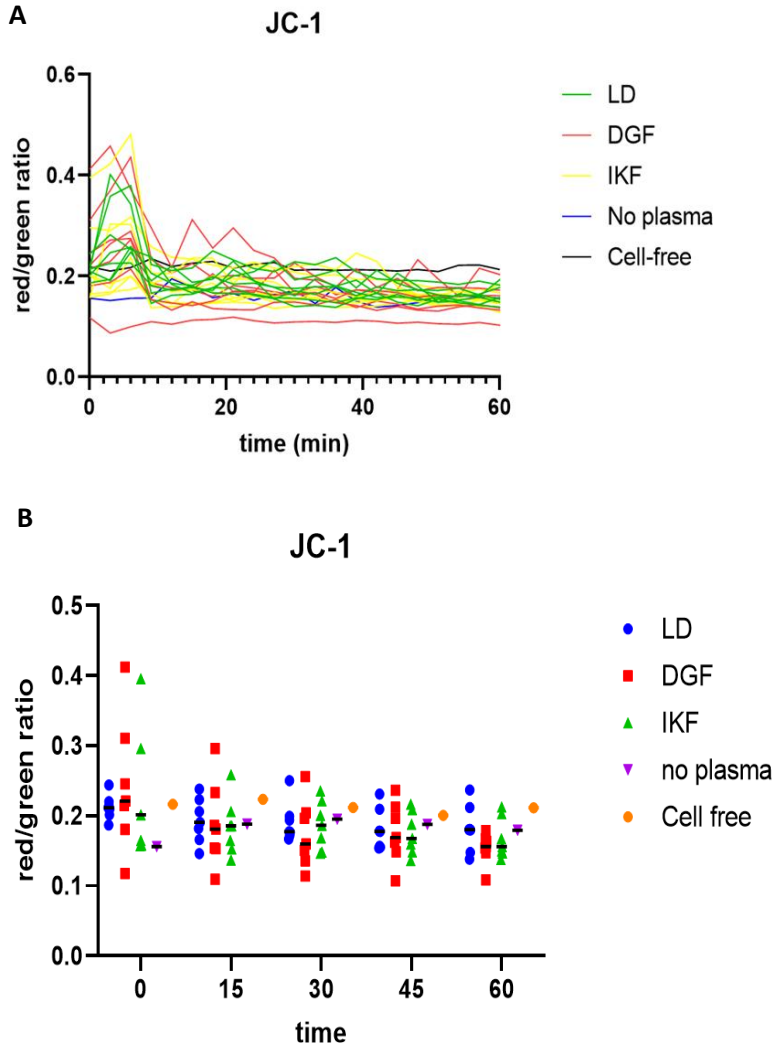

Figure 2. JC-1 results of the effect of DBD plasma on HEK 293 cells, measuring the ratio of red (595 nm) to green (535 nm) fluorescence. (A) Continuous trend of change in red/green fluorescence ratio over 1 hour. (B) Red to green fluorescence ratio at different timepoints. Each point represents an individual donor. No significance of difference was found between LD, IKF and DGF however a larger variance was observed in DGF and IKF. LD-living donor, IKF-immediate kidney function, DGF-delayed graft function. Cell free control was adding plasma to a well with no cell. No plasma served as negative control (HEK293 cells alone with no plasma).

Despite no gross changes to oxygen consumption rates and mitochondrial membrane potential, we found that incubation of HEK293 cells with plasma from donors in the DGF group caused increased production of Reactive oxygen species (ROS), as indicated by significantly higher DHE fluorescent intensity (Figure 3)

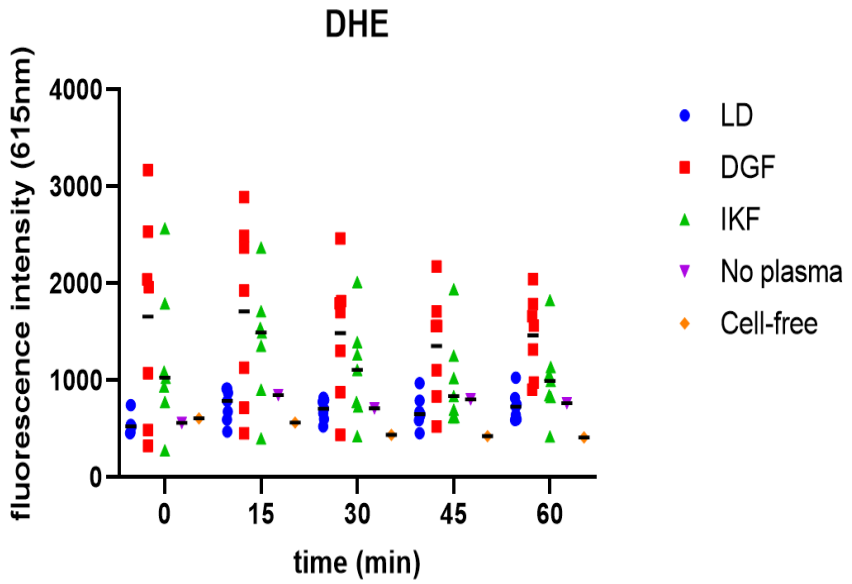

Figure 3. The effect of DBD plasma incubated with HEK 293 cells on ROS production (DHE fluorescence). The assay measured the intensity of fluorescence at 615 nm and the figure shows DHE fluorescence at different timepoints. In the cell free control, plasma was added to a well with no cells. No plasma served as negative control (HEK 293 cells only). Each point represents an individual donor. The DHE fluorescence in the DGF group was significantly higher than LD (Welch's t test, p=0.0010) and IKF (Welch's t test, p=0.0142). DHE fluorescence in the IKF group was higher than LD (Welch's t test, p=0.0168). LD-living donor, IKF-immediate kidney function, DGF-delayed graft function.

Heat-inactivation of the donor plasma at 60°C for 30 minutes, before incubation with HEK293 cells appeared to significantly reduce the amount of ROS produced (decreased DHE fluorescence) in the DGF-plasma group, but not in the other groups (Figure 4).

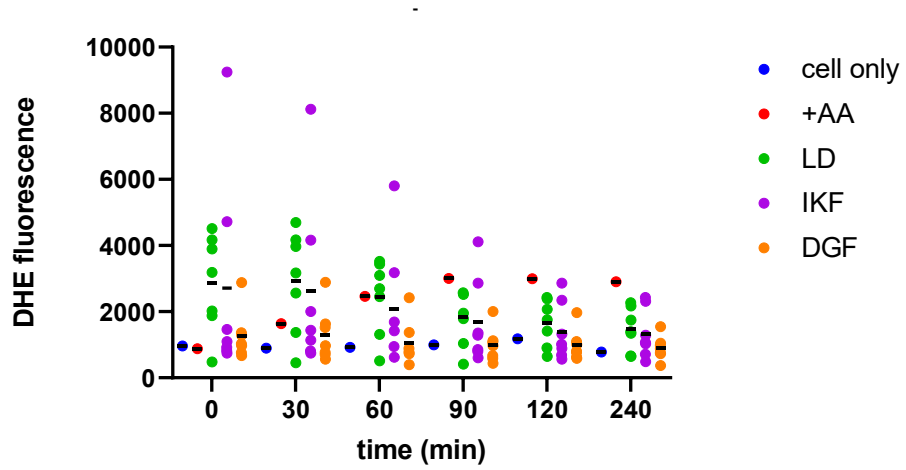

Figure 4. The effect of heat-inactivated DBD plasma incubated with HEK 293 cells on ROS production (DHE fluorescence). The assay measured the intensity of fluorescence at 615 nm and the figure shows DHE fluorescence at different timepoints. "Cell only" served as negative control (HEK 293 cells only). Each point

represents an individual donor. LD-living donor, IKF-immediate kidney function, DGF-delayed graft function. AA= antimycin A, positive control.

The experiments (as reported above) were repeated on HUVEC cells, as a model of endothelial cells, but overall incubation with plasma from deceased and living donors did not cause significant changes to the parameters measured (data not shown).

Mitochondrial morphology analysis from the Mitotracker and confocal imaging experiments is currently underway. Figure 5 below reports representative images of HEK293 cells incubated with plasma and stained with Mitotracker.

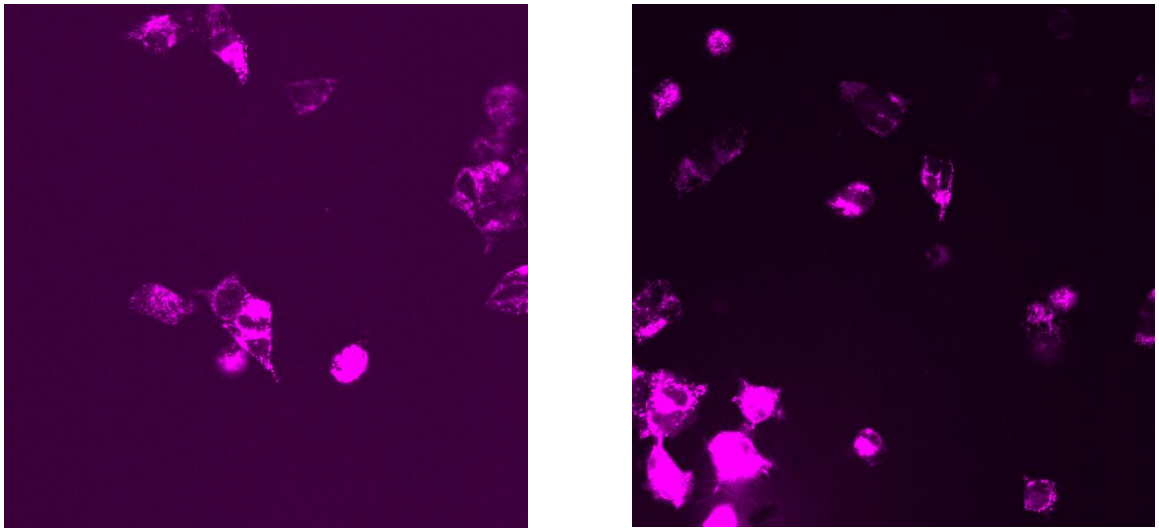

Figure 5. Representative images of HEK293 cells stained with Mitotracker and imaged by confocal microscopy. Left, HEK293 cells with no plasma added, right HEK293 cells incubated with a pooled sample of plasma from donors in the DGF group.

The mitochondrial protective compound CC4066 did not appear to have an effect on DHE fluorescence from HEK293 cells incubated with different plasma samples from kidney donors, in this setting (data not shown).

Outputs (publications/presentations)

A publication stemming from this work is currently under preparation for submission towards the end of the year. Additionally, data from this work have been used as pilot/background data in a fellowship grant application submitted to Kidney Research UK (Intermediate fellowship) (unsuccessful) and an MRC New Investigator grant (outcomes awaited Oct/Nov 2025).

Next Steps (what is it leading to)

Following on from this work, we have performed a metabolomics analysis of the plasma samples used in the study, to investigate whether the plasma samples presented specific metabolite signatures associated with mitochondrial injury and poorer kidney outcomes following transplant, as these signatures could provide clues for pathways to target therapeutically in the future. We have been able to identify specific metabolite and pathways of interest, which we intend to further validate at the tissue and plasma level in an independent cohort of clinical samples from the QUOD biobank.