

Review of the effectiveness and safety of methods to avoid mitochondrial disease

Minutes of morning presentations and discussion (non-confidential evidence)

10.30am Friday 25 March 2011

HFEA, 21 Bloomsbury Street, London, WC1B 3HF

Present – Panel

Dr Robin Lovell-Badge (Panel co-chair), MRC National Institute for Medical Research
Professor Peter Braude, Kings College London
Professor Keith Campbell, University of Nottingham
Professor Anneke Lucassen, Human Genetics Commission

Present - Researchers submitting evidence

Professor Joanna Poulton (JP), Nuffield Dept Obstetrics & Gynaecology
John Radcliffe Hospital, Oxford
Professor Hubert Smeets (HS), Department of Clinical Genetics, Maastricht UMC
Dr Shamima Rahman (SR), National Commissioning Group for Rare Mitochondrial Disease of Adults and Children, National Hospital for Neurology, UCLH
Professor Douglas Turnbull (DT) & Dr Mary Herbert (MH), Newcastle University
Dr Shoukhrat Mitalipov (SM), Oregon Health and Science University
Dr Rhiannon Lloyd (RL), Institute of Zoology, Zoological Society of London

Present – HFEA staff

Peter Tompson (PT) (Meeting Chair)
Hannah Darby
Chris O'Toole
Helen Richens
Juliet Tizzard

Apologies

Professor Neva Haites (Panel co-chair), University of Aberdeen
Professor Sir Richard Gardner (Panel member)

1. Introduction and welcome

- 1.1. Peter Thompson (PT), chair of the meeting, introduced the session and thanked participants for attending. He explained that the Secretary of State had asked the HFEA to carry out a scientific review of treatments to avoid mitochondrial disease. The HFEA established a panel to collate and summarise the current state of expert understanding on the safety and efficacy of methods to avoid mitochondrial disease through assisted conception. PT clarified that ethical issues are not within the scope of the review. PT outlined the public call for evidence and that this workshop would allow the panel to consider the evidence submitted. The panel will prepare a report for the HFEA to submit to the Department of Health in mid-April. The Secretary of Health will use the scientific review to inform his decision as to whether to hold a public consultation on introducing regulations in the Act.
- 1.2. PT made apologies for two members of the panel – Professor Sir Richard Gardner and Professor Neva Haites. All participants briefly introduced themselves. The Chair explained the format of the day.
- 1.3. One participant asked whether the researchers who submitted evidence would get to see the report before it goes to the Department of Health. The Chair explained that participants

would see draft minutes of the session. They will not see the report before it is submitted to the Secretary of State.

- 1.4. Helen Richens gave an overview of the research that had been submitted. She outlined that they had received statements, references of published studies and unpublished manuscripts and confidential data. The research is largely split between the three techniques of preimplantation genetic diagnosis (PGD), spindle transfer and pronuclear transfer.

2. Presentation: Professor Joanna Poulton

- 2.1. Professor Joanna Poulton (JP) gave a short presentation on behalf of herself and Dr Dagan Wells at Nuffield Department of Obstetrics and Gynaecology, University of Oxford. She made the following key points:
 - Supports further research on viable human oocytes but feels it is too soon to use the techniques in assisted conception
 - Need to exploit alternative less invasive methods, such as PGD
 - Need to research biological basis of mitochondrial transmission and segregation
 - Need to research whether the cell's own mitochondrial quality control can be manipulated
 - Need to increase the supply of donated oocytes
- 2.2. JP gave an overview of mitochondrial DNA (mtDNA) biology, including the concept of heteroplasmy, where there is a variation in load of mutant mtDNA, and how mutant mtDNA has to exceed a certain threshold in order to exhibit mitochondrial disease. The distribution of mutant mtDNA can be different in different tissues and may also change with time. It is possible to get a complete loss of mitochondrial DNA mutants from blood, which means a blood sample from a patient may not allow you to assess the mutant level of mtDNA in the rest of the body.
- 2.3. JP explained the concept of a mitochondrial bottleneck whereby only a small number of mtDNA passes from the mother to the offspring, which means that you can get wide variation of the level of mutant mtDNA between mother and offspring. JP stated that this makes it difficult to counsel patients. The literature indicates that a major component of the bottle neck occurs by the time the oocytes are mature. However JP stated that there is no doubt that there is segregation of mtDNA post-natally. JP told the panel that there is insufficient evidence about the biological basis of the mitochondrial bottle neck and on the effects of segregation at a later stage.
- 2.4. JP presented the different possible methods of avoiding transmission of mutant mtDNA, including oocyte donation, PGD and pronuclear or spindle transfer. She explained that oocyte donation resets the mutant mtDNA with wild type. JP stated that PGD is a possibility. However its use depends on the patient having enough oocytes so that some have a level of mutant mtDNA below the threshold. It is not known how applicable this is to the average woman with a history of mitochondrial disease.
- 2.5. JP stated that pronuclear transfer and spindle transfer clearly addresses the problem of re-setting the mutant mtDNA. However it is not clear whether doing these manipulations affects the segregation post-natally. This needs to be researched.
- 2.6. JP presented data from a Poulton et al *Diabetologia* 38, 868 (1995) showing that patients have varied levels of heteroplasmy in different tissues. She referred to a recent French study (Monnot S et al *Hum Mutat.* 2011, 32:116-25), in which the authors looked at mtDNA

mutations at two separate positions (m.3243A>G and m.8993T>C/G) and concluded that there is a systematic difference between the way that these two types of mutation segregate during embryogenesis. One mutation (m.8993T>C/G) resulted in oocytes with a wide range of mutant level, extending from nearly 100% mutant mtDNA to 100% wild type. Whereas the other mutation (m.3243A>G) resulted in mutant mtDNA around the middle distribution range. JP stated that she did not know whether this was a true difference related to the mutation or whether it was caused by the way the samples were collected. She stated that there still needs to be research carried out to understand the basic processes involved. It is not known whether manipulation of an early embryo affects post-natal segregation. JP said that further research to investigate cells from manipulated human and unmanipulated heteroplasmic embryos needs to be considered to see whether that affects segregation or differentiation into tissues.

- 2.7. JP stated that further research needed to be carried out into the cell's own way of controlling mitochondrial quality. She explained that there are processes during development that may affect the mutant mode, such as disruption of mutants by autophagy and whether or not mtDNA actually replicates during early embryogenesis.
- 2.8. JP also pointed out that it is now known that mitochondrial membrane potential in differentiated tissues is very different from embryonic cells. Embryonic cells tend to have a lower mitochondrial membrane potential and it may be that mitochondrial membranes actually drive differentiation. JP stated that it is known that transcription rates are determined by intracellular adenosine triphosphate (ATP) levels. However how these might affect developing tissues is still open to question.
- 2.9. JP pointed out that the demand for human oocyte donation has increased. She referred to a study in the mouse (Sato, AT 2005 *PNAS* 102:16765) where mtDNA disease was successfully treated by nuclear transfer. She thought that the success rate of reaching blastocysts with spindle transfer in the macaque monkey (61%) and pronuclear transfer in humans (8.3%) was good. She stated that the latter rate was understandably less with embryos derived from abnormally fertilised oocytes than normal human zygotes. JP queried what the actual proportion of healthy babies born per donated oocyte would be from the techniques relative to straight IVF using donor oocytes.
- 2.10. JP concluded that the Secretary of State needed to consider this a priority area of research, not just around nuclear transfer but of the basic biology of mitochondrial transmission. She stated that the techniques are not ready for assisted conception treatment yet. There also needs to be research into what motivates women to donate oocytes, to help increase the supply.
- 2.11. PT took questions from the panel. JP informed the panel that they did not know when mtDNA replication began because this is not an MRC priority area for research. There is information in the mouse but not the human.
- 2.12. One panel member asked whether, if you started with a patient who has a relatively low level of abnormal mitochondria, you could still end up with either very high or low levels of mutant mtDNA in the oocytes. JP referenced a case where a patient had 50% mutant mtDNA. The patient produced about seven oocytes that were almost 100% mutant and one that was 100% wild type. JP pointed out there is little research done in this area so it is not known whether the oocytes collected are representative of those ovulated naturally.

- 2.13. One panel member asked whether it was possible that the threshold effect was a nuclear interaction. JP thought that is likely that there are big nuclear factors and therefore having nuclear determinants from another nucleus may affect this.
- 2.14. Doug Turnbull (DT) clarified that the 8.3% success rate quoted for nuclear transfer was for abnormally fertilised embryos and that this figure in itself is not meaningful. DT quoted a figure of around 50%.
- 2.15. JP left the meeting.

3. Presentation: Professor Hubert Smeets

- 3.1. Professor Hubert Smeets (HS) presented evidence to the panel on PGD for mtDNA disorders. He presented data from an unpublished meta-analysis of all pathogenic mtDNA mutations in published literature. He also presented his team's practical experience with PGD at Maastricht University.
- 3.2. HS explained that the threshold is critical in mtDNA. Prenatal diagnosis can be hampered by it being difficult to correlate the mutation load with disease severity, the distribution being unclear and not knowing how the mutation will behave in time. This information is not known for most mtDNA mutations.
- 3.3. HS's team researched whether PGD could be a better alternative for healthy offspring and an option for all mtDNA carriers. They hypothesised that all mtDNA mutations will have a different pathogenic threshold but there may be a bottom-line below which no clinical manifestation occurs, irrespective of the mutation. This is based on the rationale that there might be a minimal percentage of wild type mtDNA that is sufficient to prevent symptoms. They aimed to characterise a general minimal threshold of mutant mtDNA below which the chance of an embryo being affected is acceptably low, independent of the exact mtDNA mutation. This would provide a generally 'safe' and 'common' cut-off point for PGD.
- 3.4. HS explained his team systematically reviewed 159 different heteroplasmic mtDNA mutations derived from 327 pedigrees. They used data on muscle only because the mutation should be mostly constant from the affected individuals and unaffected maternal relatives. His team excluded mutations that were not truly pathogenic, three mutations that were overrepresented, mutations where there was no data on mutant percentage, cancer related variants and homoplasmic mutations. They had data on muscle mutant percentages of 385 affected individuals and 19 unaffected individuals. HS acknowledged that this is a small number because muscle tissue is not readily available.
- 3.5. HS showed the panel that the affected individuals had a higher mutant load than the unaffected individuals. 55% of the unaffected carriers had a mutation level of less than 40%. Only 5% of the affected individuals have mutation level of less than 40%. HS explained they combined data on tRNA and protein-coding mutations, and just included data from familial mutations. They then compared the muscle mutant level in affected all cases (proband and affected maternal relatives) with unaffected carriers. HS explained because of the low number of muscle samples from unaffected carriers, they had to make assumptions on the general proportion of affected individuals in these families to give an a priori chance of being affected. They based this on the average proportion of affected siblings of the probands with a familial mtDNA mutation found in the literature, which was about 0.477.
- 3.6. HS concluded that their study found that a mutant level of less than 18% will give more than a 95% chance of being unaffected. HS clarified that the 18% applied to all the mutations

that his team had looked at. He proposed that this could be a 'safe' cut-off at which the risk of being affected is acceptably low and embryos would be eligible for transfer in PGD. This would provide mtDNA mutation carriers a fair chance of having a healthy offspring, irrespective of the exact mtDNA mutation. HS explained that if you had specific data on a particular mutation then you would not need to rely on this general cut-off point. The exact cut-off point for a specific mutation would need to be determined case-by-case, looking at disease severity, family circumstances and risk perceptions.

- 3.7. HS presented his team's practical experience with PGD for common mtDNA mutations: the MELAS mutation and the 3243 and 8993 mutations. They have had four couples and carried out a number of cycles, though they have not had any pregnancies. HS explained they usually take two blastomeres from each embryo, and presented data on the mtDNA mutation load in the biopsied blastomeres. Different mutations had different mutation loads. The embryos that were not transferred were dissected and HS explained there was generally a comparable mutation load between the blastomeres, though there were occasional outliers. HS stated the specific cut-off points they use for the different mutations.
- 3.8. PT invited questions from the panel. One panel member asked what proportion of patients had some embryos below the 18% threshold. HS stated that in their experience their patients generally always had embryos under this threshold but that their experience is not extensive. Another panel member asked whether they would always biopsy two cells. HS confirmed they did, as they occasionally have outliers. One participant clarified that homoplasmic patients would not benefit from PGD. One panel member raised the issue of segregation at blastocyst between the trophoctoderm and the inner cell mass and whether there would be a differential load which then goes through development.

4. Presentation: Dr Shamima Rahman

- 4.1. Dr Shamima Rahman (SR) presented on behalf of Professor Michael Hanna of the National Commissioning Group funded service for Diagnosis and Management of Rare Mitochondrial Diseases in Adults and Children, University College London Hospital and Professors Peter Clayton and Francesco Muntoni from the Great Ormond Street Hospital and the University College London Institute of Child Health.
- 4.2. SR gave an overview of mitochondrial disease epidemiology and genetics. She stated that mitochondrial diseases are disorders of the mitochondrial respiratory chain. They can be inherited through the nuclear genome or the mitochondrial genome. Studies suggest that 1 in 5000 births have a mtDNA mutation and of those, 1 in 500 has a homoplasmic mtDNA mutation and another 1 in 500 has the 3242 mutation. That makes a combined prevalence of 1 in 250 of the mtDNA mutations.
- 4.3. SR pointed out that mitochondria are not autonomous organelles but are much under the influence of the nucleus. 1500 nuclear genes are needed for mitochondrial function. SR stated that, in her clinical experience as a paediatrician, only a minority of patients have mtDNA mutations. She estimated that about 20% of childhood mitochondrial disease is thought to be caused by mtDNA mutations and about 50% of adults with mitochondrial disease. The rest are assumed to have nuclear gene mutations, though it is not possible to identify the gene responsible in the majority of cases.
- 4.4. SR presented a series of case studies to the panel. The first was of prenatal diagnosis of the 8993T>G mutation. A couple already had a severely affected son with Leigh syndrome. The mother had no detectable mutation in her blood. She had a prenatal test when pregnant with her second pregnancy as there is evidence that there would be a clear

answer for someone with a low mutation load. However the woman miscarried before the prenatal test. SR stated that prenatal testing usually gives clear results from mothers with a low mutation load for this particular mutation. For women with a more intermediate mutation load, SR stated that PGD would be helpful for this mutation.

- 4.5. SR presented a case study of a novel mtDNA mutation in a girl aged three. The mother was also found to have the same mutation. The mother had another baby who is well so far. SR pointed out that very little is known about genotype phenotype correlation, critical threshold and recurrent risks for most mtDNA mutations.
- 4.6. SR presented a case study of a family with the 3243A>G mutation. SR stated that this is the most common mtDNA mutation and is present in 1 in 500 of the general population. It has extremely heterogeneous clinical phenotypes and the precise genotype-phenotype correlation has not yet been established. In the case study, a young woman died at the age of 18 after a very severe course of MELAS syndrome. Her mother did not want to be tested. One sister has a 50% mutation load and suffers from migraines; she has a ten year old child who she does not wish to be tested. The other sister has a 50% mutation load and suffers from fatigue and deafness. She does not want to take any risks in her reproductive options. PGD is unlikely to be suitable as it is unlikely she will have oocytes with a low enough mutation load. The newer techniques being developed may present an option.
- 4.7. SR presented a case study on a recessive disorder where the mother had a number of pregnancies. Her second child suffered from Leigh syndrome, caused by SURF1 mutation, and died. The mother had chorionic villus sampling (CVS) on her next pregnancy. The baby was not affected but had a cardiac malformation and terminated at 20 weeks. SR stated that the guilt the mother felt around this meant she did not seek prenatal testing on her next two pregnancies. One was a healthy child, the next again suffered from Leigh syndrome and died. For her final pregnancy she had CVS and has now had a healthy unaffected child. SR stated that this shows how women's reproductive decisions can be dynamic.
- 4.8. SR presented a final case study on another recessive disorder. A mother had a child who died at five months from heart failure caused by complex 1 deficiency. She had normal mtDNA. The mother opted to have donor egg IVF for her next pregnancy.
- 4.9. SR concluded that currently prenatal diagnostic techniques are effective for only a minority of patients with mtDNA disease (8993T>G mutation). Nuclear transfer techniques are a potentially exciting option for some patients with mtDNA disease. However most childhood-onset mitochondrial disease is caused by recessive nuclear gene mutations and can be prevented by conventional prenatal diagnosis techniques, providing the causative gene is identified. SR thought that this is the most effective way of preventing mitochondrial disease and resources should be put there. She concluded that more research is needed.
- 4.10. PT invited questions from the panel. One panel member clarified that PGD for mitochondrial disease caused by nuclear mutations is effectively the same as any other PGD. The panel member also raised the point that PGD for high mutant loads may not eliminate the disease but it would lower the risk. SR pointed out that the mothers of her patients want a zero risk after their experience of having a severely affected child, though she acknowledged every family is different.
- 4.11. PT told participants that in carrying out this review, it is fully recognised that the conditions being considered are very serious.

5. Presentation: Professor Doug Turnbull & Dr Mary Herbert

- 5.1. Doug Turnbull (DT) was presenting on behalf of Lyndsey Craven, Laura Irving, Alison Murdoch and Mary Herbert from Newcastle University. DT gave an overview of mitochondria biology and stated that all mitochondrial genes are involved in the synthesis of the mitochondrial respiratory chain. Mitochondria produce more than 90% of cellular energy by oxidative phosphorylation, they contain their own genome and their metabolism is regulated by nuclear and mitochondrial genes.
- 5.2. DT informed the panel that there is a misconception around heteroplasmy, with the assumption that everyone is homoplasmic, and heteroplasmy is rare. DT clarified that heteroplasmy is the norm. Most people are heteroplasmic for benign polymorphisms; some people are heteroplasmic for pathogenic mutations. DT mentioned that Bert Vogelstein had carried out work that showed a lot of variation and that it was different between different tissues.
- 5.3. DT presented his experience as part of the MRC mitochondrial disease cohort to the panel. DT informed the panel that they had 381 patients with mitochondrial disease in Newcastle, 300 of who have mtDNA mutations. He acknowledged there is a bias as they see more adults than children, unlike SR, and that they are known to be a centre which specialises in these types of mutations.
- 5.4. DT stated that there is a misconception about nuclear-mitochondrial interaction. He pointed out that 50% of a person's nuclear genes are alien to their mitochondrial genes because they are paternal genes. DT stated that the biggest difference between mitochondrial and nuclear genes is between the African genotype and the European genotype. He stated that one in ten in people in the UK is born to a mixed race family; therefore the nuclear-mitochondrial interaction is happening all the time. He stated that he has no evidence in his patients that mixed-race patients are more commonly affected and that none of his patients are mixed-race.
- 5.5. DT discussed the options for reducing the risk of transmitting mtDNA disease through PGD and through nuclear transplantation. He stated that PGD involves selecting embryos with the lowest ratio of mutated to wild type mtDNA. In his experience they have found that the level of mtDNA mutation is broadly even between blastomeres, with the same level in blastomeres as in the trophectoderm. DT stated that PGD is important but, whilst effective for some mutations, it is not effective for the patients who are homoplasmic. DT mentioned that virtually none of the patients with 3243 mutation that HS presented were below the 18% lower threshold limit.
- 5.6. DT presented the option of transplantation of the nuclear genome into a cytoplasm containing healthy mitochondria, either by pronuclear transfer or transfer of the meiotic spindle between Metaphase II eggs. DT confirmed to the panel that his team has done some of this research and they had also worked quite closely with Dr Shoukhrat Mitalipov (SM). DT informed the panel that there is also evidence that the technique has prevented the transmission of mitochondrial disease in a rodent model (Sato *et al*, PNAS, 2005).
- 5.7. DT explained that the two challenges with transplanting the nuclear genome are minimising the carryover of mtDNA and minimising the impact of the manipulations on subsequent embryonic development. DT informed the panel that they are getting less than 2% carry over in their experiments (mtDNA carry over not detectable in 4/9 cases, about 2% detected in 5/9 cases). In unpublished data concerning monkey offspring, mtDNA carry over was not

detectable in 2/3 cases and was <1% in 1/3 cases. DT pointed out that the levels in the muscle samples presented by HS were at least 40%, often 70 to 80% and some samples were up to 90%. DT concluded that transplanting the nuclear genome provides a highly effective means of minimising the risk of transmission of mtDNA disease. However he emphasised that there is a learning curve with using the technique.

- 5.8. DT presented evidence on the impacts of nuclear transplantation on subsequent embryonic development. DT pointed out that while there is concern around epigenetic impacts, there is very little evidence that there is any growth defect in either rodents or primates. DT referenced the paper that first described the technique in mice (Science, 1983). He pointed out that it had been used for numerous generations in mice and is not associated with any increase in abnormalities. He referenced one study which described gene expression defect and reduced birth weight (Development, 1993) and a more recent study that revealed no change in gene expression and no apparent growth deficiency (Biology of Reproduction, 2009). He also referenced evidence from studies in non-human primates by SM (Tachibana *et al*, Nature 2009 and ongoing studies). DT concluded that, based on animal studies, nuclear transplantation is not associated with an increased incidence of abnormalities.
- 5.9. DT presented evidence from his own team's work on human embryos. He clarified that this was using abnormally fertilised human embryos, which is not ideal for some studies. He stated that they are reaching blastocyst stage in both their published proof of concept work using nuclear transfer and their unpublished work using spindle transfer. DT accepts that there is currently limited evidence in humans. However, what there is, is compatible with development to the blastocyst stage.
- 5.10. Mary Herbert (MH) presented their research team's next steps to the panel. She explained that they want to determine whether embryos created by nuclear transplantation have higher rates of abnormalities compared with embryos that have not been manipulated. She explained that they will be looking at the proportion of embryos that develop to the blastocyst stage. MH pointed out that the work they have done so far found that this drops by 50%, but that is using abnormally fertilised zygotes. She explained that normally fertilised zygotes are technically much easier to work with and is confident that they would get respectable development to blastocyst stage with these.
- 5.11. MH outlined that the team will look at blastocyst cell number in early expanded blastocysts, hatching blastocysts and hatched out blastocysts. They will also look at the number of cells in the inner cell mass. MH explained that they will then look at epiblast specification and carry out epigenetic analysis of blastocysts. She explained that they are interested in developing higher resolution criteria for looking at human blastocysts. They have carried out work that they hope to publish that looks at Nanog expression (the pluripotency gene). MH presented images showing epiblast specification, epigenetic modification and Nanog expression in cells of the inner cell mass. MH explained that they will be looking to see if the normal progression of Nanog expression is seen in the manipulated embryos. MH referenced work they had published (Santos *et al* (2010) Vol 25 Hum Rep) and stated that DNA methylation was confined to Nanog expressing cells in the inner cell mass of human blastocysts.
- 5.12. MH outlined the genetic and biochemical analyses that her team are planning on blastocysts from reconstituted embryos. They will be looking at the nuclear genome, doing karyotypes and array-based comparative genomic hybridization (CGH). In the mitochondrial genome they will look at the mtDNA copy number and mtDNA mutations. In

the biochemical analysis they will look at mitochondrial function, specifically at membrane potential and reactive oxygen species (ROS) production.

- 5.13. MH raised the issue of co-ordinating the logistics of egg donors and recipients. She explained that because this is difficult to do, it will be important to be able to vitrify zygotes and then manipulate them. MH said their evidence so far is encouraging. She presented data showing embryos, which they vitrified and then manipulated, developed to the same stage as embryos vitrified and cultured with no manipulation. MH clarified that the data showed development to the 8-cell stage and that these are just preliminary results. MH emphasised that they were encouraged by these results and that there is no evidence that the membrane becomes leaky after vitrification.
- 5.14. PT invited questions from the panel. MH clarified that the genetic analysis will be carried out on blastocysts. Panel members and MH discussed the merit of deriving embryonic stem cells from the manipulated blastocysts. MH stated that if they derived stem cells and they found abnormalities in the stem cells, they would not know whether those abnormalities came from the stem cells or not. She thought that it was more important to focus on analysing the blastocysts in high resolution to see if they are normal. She also pointed out it would use a lot of material to generate the stem cell lines.
- 5.15. One panel member asked whether their blastocyst development rate of manipulated embryos was similar to their routine blastocyst development rate. MH informed the panel that they do not culture blastocysts as part of their routine programme. They only cultured blastocysts in research experiments and these had a 30 – 40% development rate. The same panel member clarified that these embryos, being used as the centres standard of blastocyst development, will be of low quality. MH stated that they get some frozen embryos donated to research that are of better quality.
- 5.16. One panel member asked whether they were looking at other markers particularly for the trophectoderm, for example CDX2 and GATA 6. MH stated that they can screen successfully for GATA6 and Nanog together, though she did not show the images today.
- 5.17. MH clarified that when they referred to manipulated embryos, they had transferred pronuclei between different embryos. One panel member suggested that another possible experiment would be to take out the pronuclei and replace it into the same embryo in order to better identify the impact of the manipulation technique. This will be useful if the group extended their experiments into areas where they may have mitochondrial mutations.
- 5.18. One participant mentioned a meta-analysis of animal studies by Justin St John that showed that mtDNA replication does start during the blastocyst stage and this may influence when a good time to sample is.
- 5.19. DT informed the panel that there has been an analysis of a human foetus that was heteroplasmic, which found that there was an even distribution of the level of mutation 3243 throughout all tissues. DT stated that the massive spread between tissues is a misconception that does not hold true in the only analysis that has been done in humans. A panel member asked how this sits with the data JP presented earlier showing post mortem variation. DT stated that he thought that JP was showing a particular patient with a particular sort of mutation, which is not necessarily even. DT emphasised that though there will be some change, it will not be the case where one tissue has a zero mutant load and another has 100%.

6. Presentation: Dr Shoukhrat Mitalipov

- 6.1. Dr Shoukhrat Mitalipov (SM) presented his published work on non-human primates to the panel. His team are aiming to develop non-human primate model for germline therapy, looking specifically at mitochondrial gene replacement. They transplanted nuclear material from one monkey oocyte into a cytoplasm of another monkey's oocyte at the metaphase II (MII) stage. They used Indian-origin and Chinese-origin rhesus macaque monkeys (two sub-species) to provide two different mtDNA haplogroups. SM explained that it was easier to work with MII oocytes than germinal vesicle oocytes and that there were fewer ethical issues than working with zygotes. He explained that the mitochondria distribution is very even in the rhesus monkey and the spindle area of the M II oocyte contains no mitochondria.
- 6.2. SM described how they used a spindle imaging system to remove the spindle and that this removal was straightforward. His team remove the spindle into a small karyoplast (cytoplasm within a membrane) and used this to safely transplant the spindle back into another oocyte. He stated that only about 1% of mtDNA gets transplanted within the karyoplast.
- 6.3. SM explained they initially used electrofusion with the technique. However most of the embryos arrested at early cleavage stage and never developed to blastocyst. After fusing there would be premature resumption of meiosis. SM explained that they then tried spindle-chromosome transfer by fusion using Sendai virus extract. This gave efficient fusion within 20 – 30 minutes, intact MII spindle and normal fertilisation and embryo development to blastocyst.
- 6.4. SM stated they had about 60% development rate to blastocyst with the spindle transfer technique, which was the same as in the control embryos. *In vitro* studies showed the manipulated embryos had the same cell numbers and Nanog expression as the control embryos. SM stated that they developed three embryonic stem cell lines with a 33% isolation efficiency, comparable with the control embryos. They had a 33% pregnancy rate, which SM confirmed is usual in monkeys, and generated three pregnancies and four healthy infants. The offspring and cell lines carry mtDNA from the cytoplasm donors and nuclear DNA from the spindle donors. SM stated that the monkeys had no abnormalities at two years of age. There is no detectable mtDNA heteroplasmy in any tissues (mostly blood, placental tissue and skin) collected.
- 6.5. SM concluded that his spindle transfer technique presents a novel assisted reproduction approach that allows isolation and transfer of chromosomes in mature oocytes. It yields developmentally competent oocytes suitable for fertilisation and producing embryonic stem cells or healthy offspring. SM stated that the replacement of mtDNA in oocytes appears safe and efficient and that the procedure can be applied to human oocytes. He stated that the potential clinical applications include mitochondrial gene replacement therapy to prevent transmission of mtDNA mutations and the treatment of infertility caused by cytoplasmic defects in oocytes.
- 6.6. PT invited questions from the panel. SM confirmed that all four offspring were male, possibly because male blastocysts develop faster and these were selected for implantation. However SM informed the panel that they have now produced four more infants and three of these were female. One panel member asked about the sensitivity of the spindle to temperature. SM stated that it is crucial to maintain the oocytes at 37°C, however other than that it is very stable as long as it is not activated with electricity.

6.7. One panel member asked how SM analysed the tissues of the monkeys. SM explained they produced an additional four infants that were sacrificed before term so they could collect tissues including oocytes to assess whether there are higher levels of heteroplasmy. SM stated that so far they had not found this.

7. Presentation: Dr Rhiannon Lloyd

- 7.1. Dr Rhiannon Lloyd (RL) stated to the panel that she had two main concerns around the proposed mtDNA replacement therapies. Firstly, the potential of pathogenic mtDNA carry over from the original zygote/oocyte to the recipient egg. Secondly, the potential disruptions in nuclear-mtDNA dialogue caused by placing the nucleus from one mtDNA background into that of another.
- 7.2. RL stated that she was drawing on evidence from published animal somatic cell nuclear transfer studies, published animal cybrid studies and an unpublished and unvalidated human mtDNA haplotypes analysis of her own. RL pointed out that though mtDNA carry over was not reported in the spindle transfer experiments in monkeys or the pronuclear transfer in human embryos, mtDNA carry-over is a common phenomenon following somatic cell nuclear transfer and embryonic cell nuclear transfer. RL stated that it was reported in 129/235 cases, although levels were usually below 15%. She pointed out that there are instances where mtDNA carried over increased in abundance during development to high levels (59% in one bovine study). She acknowledged that the researchers in these cases would not have given due care to controlling mtDNA carry over and that many of these cases were whole cell nuclear transfer, not karyoplast transfer.
- 7.3. RL outlined the implications for mitochondrial replacement therapies. She explained that mtDNA carry over though common, was at a level generally well below that which caused mitochondrial disease, which means there is little risk to the offspring in its lifetime. However RL continued that the mtDNA carried over is sometimes preferentially amplified to levels that could cause mitochondrial disease and if this happened there would be a potential risk to the offspring in its lifetime. Furthermore RL explained that the mtDNA carried over could enter the germline, which could lead result in a potential risk to subsequent generations especially if the mtDNA bottleneck causes dramatic shifts in mitochondrial genotype in a single generation.
- 7.4. RL discussed disruptions in the nuclear-mtDNA dialogue. She gave an overview of the role mitochondria play in numerous cell processes, including producing cellular energy via the electron transport chain (ETC). RL explained the ETC is formed of protein subunits encoded by both the nuclear and mitochondrial genomes. She stated that these sub-units must be physically and chemically compatible to interact and form a functional ETC. RL gave the example of numerous transcription/replication factors from the nucleus must recognise and bind mtDNA so it can provide proteins for the ETC. She emphasised that nuclear-mitochondrial interactions are a result of thousands of years of evolution.
- 7.5. RL presented the panel with her hypothesis that nuclear-mitochondrial interactions are disrupted following nuclear transfer, leading to 'unhealthy' mitochondria and compromised cell function. RL presented data from cross-species cybrids, which showed that the higher the level of evolutionary divergence between the nucleus and mitochondria, the higher the level of mitochondrial dysfunction. She stated that this was illustrated by elevated lactate, reduced oxygen consumption and reduced ETC-complex activity. RL acknowledged that cross-species cybrids were an extreme example of evolutionary divergence.

- 7.6. RL discussed the implications for mitochondrial replacement therapies. She proposed that the risk to offspring or subsequent generations depends on whether the nucleus from the original cell is evolutionary diverged from the mitochondria present in the recipient cell. RL then outlined the evidence that human mtDNA is evolutionary diverged. She explained that it is well known that there are distinct human mtDNA haplogroups. Haplogroups are groups of related mtDNAs, which share the same specific identifier/single nucleotide polymorphisms. In Europe, 95% of the population belong to one of ten haplogroups and particular groups are often associated with distinct phenotypic traits.
- 7.7. RL presented a preliminary and unvalidated study she had carried out to estimate if different haplogroups encode ETC protein subunits that are likely to be physically/chemically compatible with one another. RL explained that compatibility can be estimated by quantifying the number of different amino acids observed between haplogroups with distinct physical/chemical properties, which have been shown by other studies to influence protein-protein interactions. RL used her output model to demonstrate that mitochondria dysfunction is not expected from a transfer between haplogroups that had mtDNA sub-units with identical physical/chemical properties. However she explained that it may be expected between haplogroups that had mtDNA sub-units with distinct physical/chemical properties.
- 7.8. RL explained to the panel that the implications for mitochondrial replacement therapies may be of little risk if the mtDNA haplogroups were the same. However there may be a potential risk if the mtDNA haplogroups are different.
- 7.9. RL acknowledged Professor Bill Holt, Zoological Society of London, Professor Matthew Guille, University of Portsmouth and Anne Gouraud, Zoological Society of London.
- 7.10. PT invited questions to the panel. One panel member asked whether the different haplotypes may also affect replication and distribution. A participant stated that there is minimal evidence that mtDNA haplotypes have an effect on disease, the only good evidence is around haplotype J and Leber hereditary optic neuropathy. He pointed out that they were looking at serious disease versus minimal effects of haplotypes. He also stated that normal people are heteroplasmic for haplogroups. RL acknowledged that the consequences could be quite subtle, particularly during early development. However she emphasised that it is the functional differences that are being picked out. One participant stated Martin Brand at The Buck Institute had looked at it and not found any difference between haplogroups.
- 7.11. A panel member raised the issue of mixed-race children and whether any studies had been done to see if there has been any drift in the mitochondrial sequence to match the nuclear genome, in subsequent generations. RL stated that there were natural hybrid studies in lower organisms which shows that the F1 progeny had no effect on their fitness because they have half of the compatible genome. However when they repeatedly back-crossed subsequent generations, they became less fit and showed signs of mitochondrial dysfunction. A participant stated that it is difficult to compare human and animal models, especially mice because they are highly inbred. The participant stated that the issue of mixed race couples has never been raised as an issue or as diagnostic criteria at any mitochondrial meeting. Another participant stated that she knew of two cases of Leigh syndrome in mixed-race couples and in both cases the mother did not carry the mutation.
- 7.12. One participant clarified that there are two separate issues being discussed: one about mitochondrial disease and the other about mitochondrial-nuclear function. He stated that it is known that the physiology of animals can be changed by having different

mitochondrial and nuclear backgrounds, however whether this impacts on disease is a different matter.

8. Open discussion

- 8.1. PT introduced the open discussion. One panel member put the discussion into context by giving an overview of developments in assisted reproduction from the first IVF case, to ICSI, PGD and cytoplasmic transfer. He described that the techniques they have been discussing today involve, for the first time, germ line modification. He explained that it will be the child who takes on the risk of the technique. He suggested to participants that the key test was whether they feel confident that a child born from this technique, and their children, will be fine.
- 8.2. One participant emphasised that there is a risk, but that the mothers who have mtDNA mutations are putting themselves at high risk anyway by trying to conceive naturally. He stated that there are groups of families, who have a high mutant level of mtDNA, for whom PGD is not an option.
- 8.3. Another participant made the point that ICSI for severe male factor infertility could be seen as a germ line experiment where the child is potentially put at risk, so the discussion needs to be put into context. Another participant emphasised that it was important to clearly distinguish the techniques they were discussing from cloning, as these technique do not alter the nuclear DNA.
- 8.4. The group discussed the use of PGD for avoiding mtDNA disorders. One participant said that the use of PGD depends on having embryos which have a mutant mtDNA level below the threshold. His team had been quite successful in getting them. A panel member pointed out that in translocation PGD only about 60-65% of cases have a transferable result and that the concept of poor outcomes in PGD is not a new one. He stated that there is an issue of risk reduction and whether PGD is worse than transferring nuclei.
- 8.5. A participant clarified what was meant by the term homoplasmy and that these mutations can cause a variety of phenotypes. He pointed out that there is a difference in the segregation of certain mutations, with mutation 8993 being the classic example of one that can be very high or very low. He explained that there is a need in homoplasmic or high heteroplasmic mutations for some sort of nuclear transplantation technique.
- 8.6. One panel member stated that the pronuclear and spindle transfer data presented shows high viability but that no experiment has been done using abnormal mitochondria. He pointed out that as other evidence suggests that there is a mitochondrial bottleneck and that abnormal mitochondria can be preferentially replicated, experiments involving abnormal mitochondria in primate and human models could be done. Another participant pointed out that there have only been two studies showing an increase in mutation load in tissues. These are specific tRNA mutations, which are tissue specific segregating mutations. He explained that there is evidence of some spread of mutation between different tissues but no good evidence of high levels of segregation. There is also evidence from RS that it is possible to lose mutations from replicating tissue. He stated that concerns over segregation had been exaggerated, especially as with only a 2% carry over of mtDNA the chance of this segregating to higher levels in any tissue is so remote that it is not a risk. He also pointed out that mtDNA mutations in any other species will be different to humans.
- 8.7. One panel member questioned the correlation between the mtDNA mutation load in a biopsied embryo during PGD and in the level in the resulting child. Another participant

stated that David Thorburn may have submitted evidence on measuring the mutation load during PGD and then in pregnancy. Another participant pointed out that a blood sample at the very early stage does not reflect what is actually happening. The participant stated that they have had good correlation between CVS and PGD. The group discussed losing mutations in blood for different mutations.

- 8.8. MH clarified to the panel that once they optimised the procedure for nuclear transfer, the level of carry over was non-detectable for almost half of the embryos. She emphasised that there is a learning curve in reducing the levels of carry over.
- 8.9. One panel member summarised his understanding of the concept of the bottleneck and selection. This was that there is little mtDNA replication until the early post-implantation embryo stages so there is a very low level of mitochondria in each cell at a time when tissues are allocated between gastrulation. As each founder population for each tissue is very small, there may end up with different numbers of abnormal mitochondria. The germ line in particular is developed from a very low number of founding cells, which may be why the germ line can be different from other tissues. He stated that whether or not selection occurs had not been clear from today's discussion, nor had whether it is possible for a low level of heteroplasmy to lead to a high mutant load in certain tissues.
- 8.10. Another participant stated that for the mutations that had been discussed, there is no evidence of selection for different tissues. He agreed that there is a kind of segregation between tissues but that the only evidence available shows there is not a meaningful difference in several tissues from the same foetus.
- 8.11. One participant raised the issue of missing a generation and the segregation occurring in the germ line, so a further generation may end up with mutant mitochondria. The same participant also raised the issue of what epigenetic modifications may arise from transferring nuclear material.
- 8.12. The meeting discussed the proportion of patients that are heteroplasmic and treatable with PGD, versus those who are homoplasmic. DT thought that about 20% of their patients at Newcastle would not be suitable for PGD and the remaining 80% could be suitable for PGD. He stated that PGD could never be better than spindle or pronuclear transfer because PGD transfers embryos with higher levels of heteroplasmy. This means the bottle neck or distribution in tissues means the child may still develop the disorder. DT explained that the transfer techniques had less than a 1% carry over of mtDNA.
- 8.13. One participant thought that PGD is of value for mutations where there is a high level of segregation and it is possible to get no mutation load in offspring. However for other mutations there is less segregation between oocytes and much higher levels of heteroplasmy.
- 8.14. One panel member asked about the long term health implications of using agents, such as cytochalasin B and the Sendai virus, in the transfer techniques. SM stated that they have produced over 150 monkey using various techniques. He informed the panel that cytochalasin B is reversible so there is usually a normal cytoskeleton. The Sendai virus does not contain any viral RNA. He stated that all the monkey infants produced from the spindle transfer technique have been healthy. They have also produced many cell lines. SH pointed out that it is very expensive to fund the experiments and this limits the number of monkeys it is feasible to produce. SH clarified that you would need to use the Sendai virus in humans.

- 8.15. One panel member suggested that there are two separate issues here: the issue of the mitochondria and the risk of carry-over; and the issue of manipulation involving agents that are not commonly used and transferring the spindle. Another participant pointed out that some of the risks are indefinable and this is the case for any technique that is being done for the first time in humans.
- 8.16. The group discussed the issue of producing embryonic stem cell lines. One panel member thought it would be useful and reassuring to produce stem cell lines after carrying out pronuclear or spindle transfer in embryos with abnormal mitochondria, to see the proportion of abnormal mitochondria in various cell types and whether the mitochondria function is normal. A participant pointed out that it is difficult to look at the mitochondrial function in cultured cells as the techniques used are not sensitive enough. The cultured cells would also not represent matured tissues. He was not convinced of the scientific value of carrying out these tests. Another panel member said that he was planning experiments to produce stem cell lines in the next 1 – 2 years, however it is hard to determine how useful these will be. The group debated whether embryonic stem cells would give more information than looking at Nanog expression in the blastocyst and whether it is important to test cells after they have been through the mitochondrial bottleneck and any selection or preferential replication mechanism.
- 8.17. The group discussed whether 2% mtDNA carry over will make any difference. One participant pointed out no patient has been shown to have mtDNA disease at that level. He also pointed out that there is no evidence of abnormal segregation. Another panel member emphasised that the difference is the technique being used and that no patient has undergone nuclear transfer.
- 8.18. A panel member queried whether SH had done any experiments increasing the size of the karyoplast to see if the mtDNA that is transferred will be preferentially selected against. SH informed the panel that they had done some unpublished experiments with 50-50 heteroplasmy that he can discuss with the panel in the confidential session. The group also discussed the reasons why polar bodies are not suitable to use.
- 8.19. One panel member queried how clinicians would monitor patients if they carried out the nuclear transfer techniques for real. The group discussed that carrying out CVS would be reasonable. Another participant emphasised it was important, as a next step, to carry out experiments in normal human embryos and in embryos from altruistic donors who suffer from mitochondrial disease.
- 8.20. PT brought the meeting to a close and thanked the participants for their contributions.