1 Risks inherent to mitochondrial replacement

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11 The UK Government has recently been debating whether or not to legislate to
12 allow *mitochondrial replacement* (MR) to be used in the clinic. However, we are
13 concerned that some of the science of MR has been misunderstood, or otherwise
14 given only fleeting consideration. We set out our arguments below and offer a
15 way forward to ensure that MR can safely deliver the health benefits it promises
16 for those suffering from mitochondrial-related diseases

17

18 Recent innovations that enable mitochondrial DNA (mtDNA) mutations to be
19 eliminated from the germline, by replacing mutated mitochondria within an
20 oocyte with mitochondria from a healthy donor female[1-3], offer hope for the
21 eradication of several debilitating and lethal mitochondrial diseases. The
22 potential for clinical application of MR has received widespread support[4,5], but
23 has also provoked safety and ethical concerns from the public and biomedical
24 practitioners[4,6]. In addition to currently addressed safety concerns related to
25 technical details of the procedures[7], a further safety concern exists that cannot
26 be easily addressed by methodological refinements. Embryos produced by all
27 variants of MR (pronuclear transfer; maternal spindle transfer; polar body
28 transfer) will acquire genetic material from three different individuals (nuclear
29 DNA from the prospective parents, and mtDNA from a donor female), and some
30 of these novel combinations of genetic material may not be fully compatible with
31 one another (i.e. may be mismatched). For example, various combinations of
32 donor mtDNA and recipient nuclear genomes have experimentally been shown
33 to negatively affect offspring health and fitness in vertebrate and invertebrate

34   models, even though the donated mitochondria were putatively healthy[8]. This

35   evidence has, however, been suggested to have low relevance to humans[3,7,9,10] for

36   three proposed reasons (Box 1). Here we address each of those reasons, and

37   explain why none of them refute compellingly the potential for mitochondrial-

38   nuclear (mito-nuclear) mismatches to affect the outcomes of MR in humans.

39

40   **Box 1.** ***Three proposed reasons why MR should not result in alterations of***

41   ***human phenotypes***

42

43   **Reason 1. MR, like sexual reproduction, randomly shuffles mitochondrial**

44   **and nuclear genomes each generation.**

45   This is based on the argument that sexual reproduction results in the random

46   mixing of two parental genomes. Thus, under sexual reproduction, the father's

47   haploid genome is as evolutionarily 'foreign' to the mother's mtDNA, as will the

48   mother's nuclear genome be to a donor's mtDNA under MR[9]. Under the

49   additional assumption that the mito-nuclear combinations found in the offspring

50   are a random subset of those determined at fertilization (i.e. the absence of

51   selection is assumed), there will be little scope for high performing mito-nuclear

52   allelic combinations to be preserved across generations. MR has therefore been

53   described as being equivalent to sexual reproduction, in terms of generating

54   healthy offspring containing novel combinations of mitochondrial and nuclear

55   alleles. In section 1, we explain why the process of co-transmission of mtDNA

56   and maternal nuclear DNA, coupled with selection, renders this proposed reason

57   unconvincing.

58

59   **Reason 2. Genetic diversity in humans is too low to cause incompatibilities.**

60   It has been suggested that mito-nuclear mismatches are unlikely to occur in

61   humans because the genetic diversity within the human population is so small

62   that any disruptions will be negligible[7]. It has been argued that mito-nuclear

63   compatibility should be widespread, given that humans are "a freely

64   interbreeding species"[10]. In section 2, we outline why the potential for mito-

65   nuclear incompatibilities in humans remains a credible possibility.

66

Reason 3. Incompatibilities do not occur in non-human primates.
Empirical data in a primate model[3] has been used as evidence that mito-nuclear mismatching will not occur, or will not be important, in humans. This reasoning is based on the production of four healthy male macaques born to three mothers, following MR-assisted IVF attempts on twelve mothers[3]. The individuals were apparently derived from two distinct, although unspecified[1], sub-species of *Macaca mulatta*[9]. In section 3, we outline why the macaque studies, to date, do not provide a strong base on which to dispel concerns regarding mito-nuclear incompatibilities manifesting in humans.

1. MR is more likely than sexual reproduction to disrupt coevolved mito-nuclear genetic combinations

1.1 Co-transmission. During sexual reproduction, but not during MR, offspring invariably receive an entire haploid copy of the nuclear genome from their mother, alongside their maternally-inherited mtDNA. In other words, mitochondrial alleles co-transmit with 50% of the autosomal nuclear alleles in 100% of the cases (and with two-thirds of the X-chromosome linked alleles, since females carry two copies of the X-chromosome and males carry only one). In contrast to sexual reproduction, MR can create entirely novel allelic combinations of mito-nuclear genotypes, because the mtDNA has been donated from a third-party (the donor female) – thus the co-transmission rate between the patient's nuclear DNA and the donated mtDNA is 0%.

1.2 Selection. The co-transmission of mtDNA and nuclear alleles facilitates the preservation of high performing (coevolved) combinations, across generations. The greater the percentage of co-transmission between mtDNA and nuclear DNA, the higher the potential for mito-nuclear co-adaptation. In natural conceptions, embryos carrying better-performing mito-nuclear allelic combinations may be more likely to survive through development, to reach reproductive age, and ultimately to successfully reproduce. Because the best-performing combinations may be more likely to be passed on, coadapted mito-nuclear allelic pairings are likely to be preserved across generations within any particular maternal lineage. Similarly, germline selection against incompatible mito-nuclear combinations

100   might occur at the oocyte stage, with poorly-performing oocytes potentially re-

101   adsorbed. By contrast, MR-assisted IVF creates combinations of mito-nuclear

102   alleles that are potentially novel (i.e. never before placed together), and not

103   previously screened by natural selection (or previously screened and selected

104   against). This lack of prior screening means that the sample of oocytes and

105   embryos created under MR will contain individuals that may be inherently more

106   likely to exhibit incompatibilities between the mitochondrial and nuclear

107   genomes[8]. The mito-nuclear allelic combinations carried by the offspring will be

108   under selection across life-stages, from before fertilization through to the

109   sexually mature adult (with this selection manifested as differential patterns of

110   survival or fertility among offspring carrying different mito-nuclear allelic

111   combinations). Reduced fertility, especially of males, as a result of epistatic

112   interactions during hybridization between alleles at different loci, including

113   those spanning different genomes, is expected theoretically[11] and supported

114   empirically, including for mito-nuclear complexes in *Drosophila melanogaster*

115   [12,13].

116

117   2. Genetic diversity in humans

118   The human population is generally thought to show lower mean levels of genetic

119   divergence at nuclear loci than other species[e.g. 14]. While the probability of MR

120   resulting in mito-nuclear incompatibilities would presumably be low if there was

121   complete genetic admixture within the nuclear genome, it is clear that genetic

122   population stratification does exist[15-17]. This stratification has its origins in

123   historical and demographic patterns of selection and migration[18], and positive

124   assortative mating between individuals of similar phenotypes may contribute to

125   its maintenance[19,20].

126

127   However, the level of divergence across mtDNA sequences is also relevant when

128   it comes to the question of whether or not MR may result in mismatched mito-

129   nuclear genotypes. In humans, the percentage divergence in mtDNA between

130   major human haplogroups is around 0.5% (Fig 1, Table S1), essentially

131   equivalent to the divergence exhibited across mtDNA haplotypes within the fruit

132   fly, *D. melanogaster* (0.4%; Fig 1, Table S2), which exhibit clear signatures of

133   mito-nuclear incompatibilities, particularly in males[12,13]. Haplogroup matching,

134   proposed as a way of circumventing this issue[21,22], might not always be

135   successful in preventing mito-nuclear incompatibilities. By definition, when

136   probing variation within human macro-haplogroups, divergence across mtDNA

137   haplotypes will persist (~0.1%, see Table S3 for estimates within haplogroup H,

138   the most common European macro-haplogroup, or 0.2% if the non-coding region

139   is included in the analysis [Fig 1]; similar patterns are found within H1 [Table S4,

140   Fig 1]). The mechanisms of the incompatibilities are largely unknown and the

141   identity of the causative interacting loci is undetermined[23]. However, it seems

142   that several loci of small effect are involved in *Drosophila*[24]. While this suggests

143   that less distantly-related genomes may result in smaller incompatibility

144   effects[24], it will be difficult to make predictions about the likelihood of

145   incompatibilities based on the specific alleles that delineate haplotypes, given

146   that it has been previously shown that single nucleotide differences in the

147   mtDNA can cause male sterility when interacting with particular nuclear

148   genotypes[13,25]. Further research into the degree of mismatch manifested with

149   increasing mitochondrial genetic divergence between putative donor and

150   patients should be a priority.

151

152   **3. Proof-of-principle studies do not allow epidemiological predictions of**

153   **incompatibilities**

154   Several studies demonstrated the technical feasibility of surgical MR[1–3,26], using

155   macaques, human cell lines and mice. The number of mitochondrial × nuclear

156   genotype combinations covered by all these studies together appears to be 15, or

157   less. In addition, with the exception of two studies[3,27], no maternal replicates

158   were used per mito-nuclear combination, preventing an examination of whether

159   any effect is due to a particular maternal effect associated with the study subject

160   or inherent to a particular mitochondrial × nuclear genotype combination. In

161   other words, these studies[1–3,26,27] were not designed to test for mito-nuclear

162   incompatibilities, and cannot be used to predict the population-wide likelihood

163   of mito-nuclear incompatibilities manifesting post-MR. Doing so will likely lead

164   to a high rate of Type II errors – the failure to detect effects that are present.

165

166 There are also additional issues with some of these studies. For example,
167 Tachibana et al.[3] used 98 human oocytes (from 7 donors) for MR, and concluded,
168 there was no difference in zygote survival to normal IVF controls. However, this
169 conclusion might warrant reappraisal. In their study, the authors derived six
170 embryonic stem cells (ESCs), from 19 blastocysts, from a starting stock of 64
171 oocytes that underwent MR treatment (ESC success rate: 6/64 = 9%, blastocyst
172 rate: 19/64 = 30%). This compares to nine ESCs, from 16 blastocysts, from a
173 starting stock of 33 oocytes in the control group (ESC rate: 9/33 = 27%,
174 blastocyst rate: 16/33 = 48%). The differences in ESC isolation rate between
175 treatment and control groups are in fact statistically significant (ESC: Fisher's
176 exact test, 1df, two-tailed, p = 0.035; Blastocysts: p = 0.078). This suggests
177 further evaluation of developmental success post-MR should be a priority.
178
179 Paull et al.[26] obtained 7 blastocysts out of 18 MR oocytes. The cell lines derived
180 from these blastocysts showed lower activity in all four respiratory chain
181 enzyme complexes than control cells. While differences were not statistically
182 significant, they represent reductions of between 2 to 19 % (average across four
183 enzymes: 11%) compared to parthogenetically-induced controls, and given the
184 low sample sizes involved, again suggest that further scrutiny into possible
185 effects of MR is warranted.
186
187 Finally, Craven et al[2] report that development to blastocyst stage was
188 approximately 50% lower for zygotes receiving MR treatment (18/80 = 22.5%)
189 than for controls; a difference that is likely to be statistically significant, although
190 the controls were unmanipulated and therefore do not represent a true control
191 for the manipulation.
192
193 In the light of these three examples, it is noteworthy, that MR affected
194 development and respiration in many other studies on non-primate vertebrates
195 and invertebrates[8].
196
197 **Conclusions**

198   MR-assisted IVF could place novel allelic combinations of interacting mtDNA and
199   nuclear genes alongside each other in the offspring, and these combinations
200   might not have been previously screened by selection (or in the worst case may
201   have already been removed from the population by selection). Therefore, mito-
202   nuclear allelic combinations created following MR (which are characterized by
203   0% co-transmission from parents to offspring) are theoretically not equivalent to
204   those found in individuals produced under sexual reproduction. This insight is of
205   fundamental importance, but apparently underappreciated in the literature
206   pertaining to MR. Given that mito-nuclear allelic combinations contribute to
207   encoding life's critical function of energy conversion, natural selection must be
208   assumed to be particularly intense on these combinations. We suggest that it is a
209   real possibility that novel combinations created under MR could result in mito-
210   nuclear mismatches. This possibility has also been predicted by evolutionary
211   theory[28] and experimentally supported in several taxa[8,29], including several with
212   comparable   levels   of   mitochondrial   genetic   diversity   to   the   human
213   population[12,13].

214

215   Lack of evidence from small-scale proof-of-principle experiments for MR effects
216   should not be used to conclude mito-nuclear incompatibilities are unlikely to
217   manifest   post-MR,   because   these   experiments   cover   few   mito-nuclear
218   combinations and their statistical inferences, in some cases, appear open to
219   question. In fact, there is actually an extensive, but largely overlooked, body of
220   experimental evidence that indicates mito-nuclear interactions are important in
221   determining health outcomes in humans[30–42], as well evidence for mito-nuclear
222   incompatibilities following the similar procedure of somatic cell nuclear transfer
223   in cattle[43,44]. Furthermore, the only previous attempt of using pronuclear
224   transfer in humans was not successful[45]. Future work should, therefore, address
225   to what extent the risk of mismatching can be reduced by matching the donor
226   and maternal mitochondrial haplotypes, since genetic variation across many
227   interacting loci are likely to be involved[24], and given the genetic variation
228   between and within human mtDNA haplogroups that we have outlined here. As a
229   suggested design, two oocytes should be used for every donor; each enucleated.
230   One of these is assigned to a control, and re-populated with the donor's own

7

231   nuclear genetic material, and the other to the MR treatment. By then comparing

232   the success of MR-treated to control eggs, and provided sufficient replication

233   across donors, this design would provide an explicit test for mito-nuclear

234   incompatibilities post-MR.

235

236   Acknowledgements

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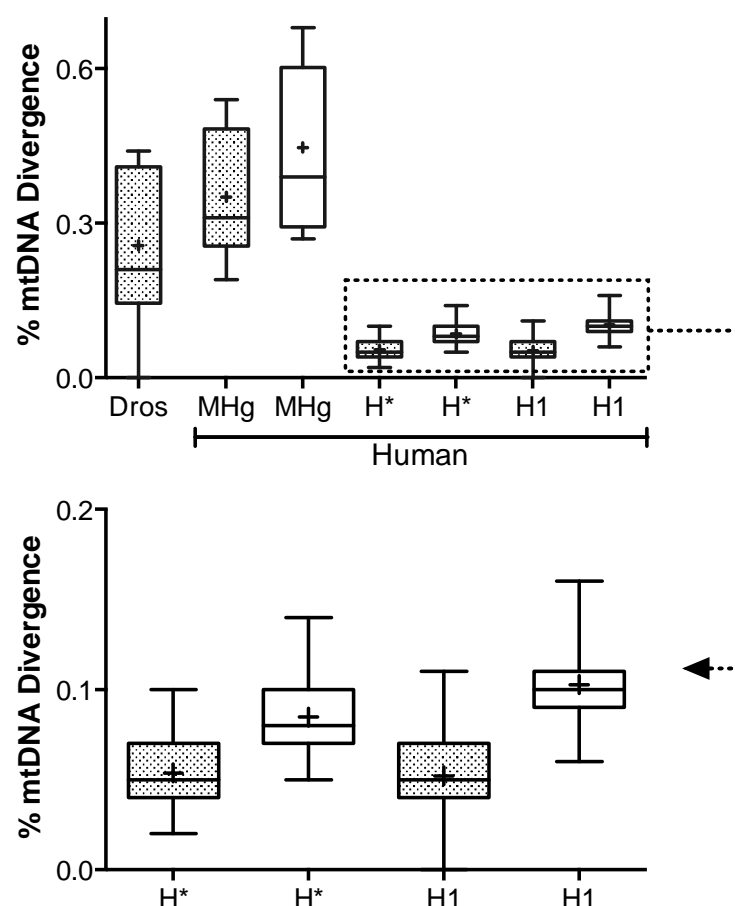364

365   **Figure 1:**

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368   **Figure 1**: Boxplots depicting variation in mtDNA divergence (%) across naturally-
369   occurring human mtDNA sequences, with comparison to mtDNA divergence across
370   global fruit fly (*Drosophila melanogaster*) populations. The *Drosophila* (Dros) plot is
371   based on protein coding regions of 13 *Drosophila melanogaster* populations that were
372   previously used in published studies showing effects of mitochondrial
373   replacement[12,13,24]. Human data are first presented using sequence polymorphisms
374   found only in the protein coding region (denoted by hashed boxes; to enable direct
375   comparison to the Drosophila plots, in which non-protein coding sequences were
376   unavailable), and secondly using the full sequence data (protein and non-coding
377   regions; denoted by open boxes). Human data are presented at three scales; first at the
378   scale of human mitochondrial macro haplogroups M, N, R, L0, and L3 (MHg), second
379   at the scale of human mitochondrial haplogroup H (sub-clades H1 to H10 [H*]) – the
380   most common haplogroup among Europeans, and third at the scale of haplotype,
381   specifically 20 mitochondrial haplotypes sampled from haplogroup H1 (H1). Box
382   plots show median values (line within box), 2nd, and 3rd quartile (box outline),
383   maximum data range (whiskers), and mean (+). At each scale, plots are generated
384   using pairwise divergence estimates for all combinations of mtDNA sequence.
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