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Report on the safety of 'mitochondrial replacement' techniques: epigenetic issues

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Abstract

This report examines safety issues raised by proposed 'mitochondrial replacement' techniques for preventing the transmission of mitochondrial genetic diseases. It focuses on the question of epigenetic problems in embryos created by these techniques, and concludes that there are major safety concerns that the HFEA has not properly addressed

The major defect of the HFEA's 2011 report is that it virtually ignores the issue of epigenetics, although it does suggest detailed analysis of epigenetic modifications and gene expression under the category of 'additional research' that 'will not necessarily inform the decision as to whether it is safe to proceed to clinical application of MST and PNT'. In our view, data on metabolism and gene expression in blastocysts is not something that would be 'nice to have' in assessing the impact of the techniques on the health of offspring: it is core and essential. It is imperative that the HFEA insists upon epigenetic and transcriptomic data before approving MST/PNT. There are two separate sets of issues: (i) epigenetic effects caused by the manipulation of embryos, and (ii) epigenetic effects of abnormal mitochondria on the nucleus of oocytes.

There is abundant evidence that assisted reproductive technologies can cause epigenetic perturbations in embryos and offspring produced by them. Enucleation of eggs is traumatic, and has been compared to major transplant surgery; damage to the developmental potential of eggs from these procedures was observed in both recent papers on MST. There is no body of data that would validate use of these techniques in a clinical setting. A recent study on ICSI showed that there may be genetic variation in the degree to which individuals are vulnerable to these manipulations.

An issue which is not addressed in the 2011 report, but which may pose significant hazards to offspring produced using MST or PNT, is aberrant epigenetic marking of both maternal and paternal nuclei in oocytes and embryos containing mutated mitochondrial DNA. We summarise evidence showing that mitochondria affect nuclear epigenetic markings and cell regulation in general. These changes can persist over long periods of time and appear to be central to diseases such as diabetes and cancer. Scientists from the Newcastle centre have recently proposed that these effects may explain aspects of the pathologies involved in mitochondrial diseases. The nuclei of oocytes from mothers carrying mitochondrial mutations will have been exposed to such mitochondria during the period of egg maturation, which is a period of intense epigenetic activity. Thus these nuclei cannot be considered as uncontaminated by the mitochondrial genetic defect. However, both PNT and MST are based exactly on that assumption. It is thus very possible that offspring produced using PNT or MST may suffer from some of the symptoms of the mitochondrial conditions that PNT and MST are designed to avoid. Until such a possibility is ruled out for each

condition, a much safer and precautionary approach to preventing transmission of mitochondrial diseases would be to avoid the use of the maternal nucleus and to rely on conventional egg donation.

The two recent papers from US groups on MST provide little reassurance as to the safety of the techniques.

We argue that the HFEA should take a precautionary approach to the safety of MST/PNT, since the HFE Act insists that the welfare of the child is the most important consideration. The existence of a safe alternative technique, ie egg donation means that it would be unacceptable not to take such an approach to the safety of MST/PNT. We list research that should be undertaken before approving MST/PNT, notably studies on epigenetic changes in embryos produced by them.

1. Introduction

The major defect of the HFEA's 2011 report is that it virtually ignores the issue of epigenetics, although it does suggest detailed analysis of epigenetic modifications and gene expression under the category of 'additional research' that 'will not necessarily inform the decision as to whether it is safe to proceed to clinical application of MST and PNT'. We find this omission difficult to understand, especially given the importance currently being given to epigenetics.

Instead, the report and the research of the Newcastle and the two US groups relies upon the criteria of survival to the blastocyst stage, and morphology of the blastocyst. However, only the most crude effects upon embryo viability are reflected in embryo death, and embryos can survive to the blastocyst stage with metabolic and gene expression abnormalities that will have profound effects later in prenatal development or postnatal life. As McEvoy et al note[1]:

> When such disruption coincides with the commencement of embryonic genome activation (from the two-cell stage onwards, depending on species) errors may increase even though, in contrast to some physical manipulations, genetic codes are conserved. Ironically, the subacute nature of at least some of the aberrant changes induced by in vitro production of embryos allows the changes to remain undetected in the short term. Blastocyst production, a hallmark for the efficiency of in vitro embryo culture systems, can often be achieved despite detrimental environmental effects. Indeed, Walker et al. (1992) reported that more blastocysts were produced from ovine zygotes in vitro than from equivalent zygotes in vivo. This finding should cause us to question the normality of blastocysts produced in artificial environments where subnormal embryos are perhaps less stringently de-selected than in dynamic conditions in vivo.

Data on metabolism and gene expression in blastocyst is not something that would be 'nice to have' in assessing the impact of the techniques on the health of offspring: it is core and essential. It is imperative that the HFEA insists upon epigenetic and transcriptomic data before approving MST/PNT.

There are two separate sets of issues: (i) epigenetic effects caused by the manipulation of embryos, and (ii) epigenetic effects of abnormal mitochondria on the nucleus of oocytes.

2. Epigenetic effects of manipulations

The first of these issues is well known, and we do not understand why the 2011 report fails to discuss them. There is abundant evidence that assisted reproductive technologies can cause epigenetic perturbations in embryos and offspring produced by them. The most well-known of these is the 'large offspring syndrome' and many other congenital malformations, and perinatal death in cloned animals. These effects are by no means completely accountable for by errors in reprogramming the gene expression of somatic cell nuclei. Similar phenomena had been observed in animal IVF before somatic cell cloning became possible, and some have now been seen in human IVF. Research has shown that they can be traced to the effects of in-vitro culture[2,3] and superovulation[4]. There is also evidence that they can be seen in ICSI[5] and, of course, most strikingly in cloned animals. A major element of these latter procedures is the use of micro-pipettes to inject sperm and to remove nuclei. These manipulations are bound to be damaging to oocytes, and this is experienced in reduced developmental potential, even at early stages of these embryos. This was observed in both recent papers on MST[6,7]. A general problem with most of the discussion about MST/PNT is that the genetic reductionist view of cells and organisms dominates, and no attention is given to cellular structure and the danger of disrupting it. Instead, it is generally assumed that, in order to create a healthy embryo, all that is necessary is to ensure that it has the right DNA. We are concerned that the effects of disrupting the oocyte cytoskeleton with nocodazole and cytochalasin will be felt in the embryo long after these chemicals have been washed out. As McEvoy et al note (ref 1):

> Nuclear transfer is not a robust technology in either murine or domestic animal studies and most reconstituted eggs never generate viable offspring. Initial enucleation of the oocyte, via a skilled yet crude excision process, and subsequent introduction of a donor nucleus is the equivalent of major transplant surgery and undoubtedly traumatizes both the cytoplasm and nucleus.

We would argue that the effects of the procedures are potentially more severe than transplant surgery, since they will potentially be felt in every cell of the child produced by these techniques

Indeed, one aspect of the present proposal is that it constitutes the first use of nuclear transfer manipulations in a clinical setting. We are not aware of any body of data that validates their use outside of the research setting, and we would have thought that this was essential for the most limited clinical use.

It is to be expected that these manipulations will lead to nuclear epigenetic changes, and the burden of proof should remain upon those who would expect that they do not. We note that, again, the HFEA recommends 'additional' research on this issue, without discussing the basis of the concerns that would lead to the recommendation. Since these manipulations are in essence the same as those involved in cloning, where the most extreme epigenetic problems have been observed, it would seem vital to ensure that such effects are not found in embryos derived from MST and PNT. Failure to do so would risk liability as a result of damage to the health of offspring produced through these techniques. A recent study (ref 5) showed that the vulnerability of mouse embryos to these effects in ICSI was dependent upon the genetic background (strain). This implies that, in humans some individuals may be more vulnerable to them than others, which introduces a further level of uncertainty into efforts to ensure the safety of MST/PNT in humans.

2. Mitochondrial regulation of nuclear epigenetic markings

An issue which is not addressed in the 2011 report, but which may pose significant hazards to offspring produced using MST or PNT, is aberrant epigenetic marking of both

maternal and paternal nuclei in oocytes and embryos containing mutated mitochondrial DNA. Although these conditions are designated 'mitochondrial diseases' there is now abundant evidence that altered mitochondrial states can affect nuclear epigenetic markings which can in turn affect mitochondrial function, resulting in some cases in vicious cycles, so the molecular pathologies in these conditions are better thought of as involving aberrant nucleo-mitochondrial states. Scientists from the Newcastle centre have recently proposed that these effects may explain aspects of the pathologies involved in mitochondrial diseases that cannot be accounted for purely by reference to the mitochondrial DNA mutation and its direct effects on oxidative phosphorylation[8]. They state that:

> 'The altered DNA methylation of the nuclear genome could therefore mediate the downstream consequences of pathogenic mtDNA mutations…. the tissue-specific expression of nuclear genes could contribute to the tissue selectivity in mtDNA diseases, and a variable epigenetic signature could explain the phenotypic variability of mitochnodrial disorders.'

Hiendleder et al[9] go so far as to suggest that mitochondrial pathologies are 'strikingly similar' to those seen in animals produced by nuclear transfer, although they do not expand upon the point. It seems likely that future research will show that many of the pathologies seen in mitochondrial disease are due to epigenetic defects in nuclear DNA, produced as a result of the primary mutation.

The nuclei of oocytes from mothers carrying mitochondrial mutations will have been exposed to such mitochondria during the period of egg maturation, which is a period of intense epigenetic activity. In PNT the maternal and paternal pro-nuclei will also undergo the post-fertilisation phase of epigenetic modification under the influence of defective mitochondria. It is thus reasonable to expect that these nuclei will contain epigenetic marks (including possibly of imprinted genes) which may later contribute to pathology. There is now abundant evidence that such epigenetic states can be long-lived and can even be inherited across multiple generations. **Thus these nuclei cannot be considered as uncontaminated by the mitochondrial genetic defect. However, both PNT and MST are based exactly on that assumption. It is thus very possible that offspring produced using PNT or MST may suffer from some of the symptoms of the mitochondrial conditions that PNT and MST are designed to avoid. Until such a possibility is ruled out for each condition, a much safer and precautionary approach to preventing transmission of mitochondrial diseases would be to avoid the use of the maternal nucleus and to rely on conventional egg donation.**

2.1 Evidence of nuclear epigenetic modifications as a result of altered mitochondrial states

There is now clear evidence that the nuclear-mitochondrial molecular 'cross-talk' is both a normal feature of cellular physiology, that it can be perturbed in disease states including diabetes and cancer, and that such perturbations are a central part of the pathology of these states.

That nuclear-mitochondrial molecular crosstalk occurs, in order to regulate cellular functioning is, a priori, to be expected, given the central role of mitochondria in many aspects of cellular function, in addition, of course, to oxidative phosphorylation (OXPHOS). Wallace has argued that this interaction is vital to cell sensing of fluctuations of the availability of calories for growth and division, on a short-term basis[10]. He points out that mitochondria produce the co-factors for modification of nuclear DNA histones and transcription factors – ATP, acetyl-CoA and S-adenosyl methionine. These co-factors are, of course, also central to regulating to many cellular signalling pathways. When

mitochondria produce less ATP and acetyl-CoA Phosphorylation and acetylation of histones is reduced, chromatin condenses and becomes less transciptionally active. Mitochondrial activity also regulates the overall redox balance of the cell and the level of reactive oxygen species (ROS), and these can also affect nuclear gene expression (see below).

It is also increasingly accepted that mitochondrial haplogroups are not selectively neutral, but represent regional adaptations, and can conversely lead to susceptibility to a variety of diseases. Bellizzi et al[11] recently demonstrated that the J haplogroup confers an increased overall level of nuclear methylation, apparently via transcriptional regulation of the MATA1 gene which encodes a DNA methyl transferase. The authors suggest that the effect on MATA1 transcription is mediated by cellular ATP levels. The link between mitochondria and nuclear methylation is also shown by the results of Smiraglia et al[12] who constructed cybrids lacking mitochondrial DNA. This resulted in altered nuclear methylation, which was reversed when mitochondrial DNA was re-introduced.

A further example of mitochondrial epigenetic control of nuclear genes is the stabilisation of the transcription factor HIF1α by ROS. This transcription factor, which is thought to help cells respond to hypoxia, and is elevated in pre-implantation embryos and cancer cells is thought to control the expression of as many as 5% of all nuclear genes[13]. Thus, altered mitochondrial function arising as a result of mtDNA mutations can have a profound and broad ranging effect on transcription in the nucleus, and cellular functioning as a whole. ROS are also known to activate MAPK signalling kinases, which encourage mitogenesis[14].

Takasugi et al[15] showed that there is a tissue dependent pattern of methylation of transcription control regions of nuclear genes encoding mitochondrial proteins, and suggested that this underlies the differing protein composition of mitochondria in different tissues. A recent review by the Newcastle group[16] stated that in cells with mitochondrial deletion there is altered regulation of transcription of genes involved in the TCA cycle and amino acid metabolism and that in cells with disturbed mitochondrial function there is altered transcription of nuclear genes that attempts to compensate.

In summary, there is clear evidence that control of nuclear gene expression through epigenetic changes involving methylation of nuclear DNA, methylation, phosphorylation and acetylation of histones and transcription factors and a variety of signalling effects mediated by ROS and redox balance, is a normal part of cellular regulation.

As might be expected from this, perturbed epigenetic states have been shown to be part of diseases of energy metabolism such as diabetes. It has now been shown that episodes of poor glycemic control can lead to later long term diabetic complications[17]. This effect was shown to be due to changes in gene expression due to histone methylation, mediated by ROS, such as superoxide, produced by mitochondria. A recent paper[18] showed the vicious cycle of signalling involving Protein KinaseCβII, a mitochondrial protein p66^{shc}, ROS generation, and acetylation/demethylation of histone H3 leads to a memory effect in vascular cells even after glucose levels have been returned to normal. The significance of this data in respect to oocytes is that it demonstrates that mitochondrially-mediated epigenetic changes in nuclear DNA can be long lasting.

Finally, it is also becoming clear that mitochondria play a key role in oncogenesis. This was first proposed by Warburg in 1956, and it is known that there are reduced levels of mitochondrial DNA in tumours, which appears to be regulated by p53, and this leads to resistance in these cells to apoptosis. It is also known that some mitochondrial

haplogroups confer increased sensitivity to cancer. Persistent OXPHOS defects can also lead to perturbation of nucleotide pools leading to nuclear genome mutagenesis and genome instability. Singh's group[19] has proposed a model to explain these observations, in which OXPHOS damage first leads to nuclear epigenetic changes, which attempt to repair the damage; but that persistent damage to mitochondria leads to mutagenesis in the nucleus, resistance to apotosis and cancer. The invasive phenotype of cells depleted of mtDNA was reversed by the reintroduction of wild type mitochondria[20] and it has also been shown that cybrid cancer cell lines with additional mtDNA mutations formed tumours in nude mice 7 times as large as those with functional mtDNA[21]. Many other findings have shown the role of (ROS) oxidative stress in cancer and Xie et al[22] added to this picture by showing that in cells lacking mtDNA, methylases are activated which silence the transcription of a number of genes known to be silent in prostate cancer.

These findings underscore the significance of mitochondria to nuclear epigenetic interactions. This is a rapidly evolving field in which much is still unclear. However, what is abundantly clear is that an assumption that the nuclei of cells containing defective mitochondria are epigenetically unaffected cannot be sustained. These cells are, of course, undergoing extensive epigenetic modification of nuclear DNA under the control of ooplasmic factors which are likely to include mitochondrially-derived molecules.

There is little evidence to date concerning the relevance of the above findings to the situation in oocytes and in newly fertilised embryos. There is one piece of evidence that suggests at least that ooplasmic factors, possibly mitochondria, can affect the epigenetic reprogramming of the paternal pronucleus that occurs after fertilisation. Cheng-Guang et al[23] used the observed intrastrain differences between C57BL/6 and DBA/2 mouse strains to investigate effects of ooplasm on pronuclei. Using androgenones (zygotes with two paternal pronuclei) in order to avoid masking effects on embryo viability of the maternal pronucleus. They found a suppression of embryo viability when D2 ooplasm was injected into oocytes containing B6 cytoplasm, but no reciprocal effect. The authors stated that the simplest explanations of their findings is that inter-strain genetic differences in male pronucleus paternal modification are mediated by ooplasmic factors and that these affect the expression of imprinted genes required for blastocyst formation. While this result cannot be taken as conclusive, since it was obtained using androgenones, it suggests that there may be risks to the male pronucleus in PNT, since it is first exposed to the maternal cytoplasm containing defective mitochondria.

3. Recent papers on MST/PNT

The two recent papers from US groups on MST provide little reassurance as to the safety of the techniques. The Tachibana et al study is, to date, the only study using normal human eggs, since Pauli et al used parthenogenetic embryos. In both studies the transfer procedure for MST led to premature activation of the eggs, resulting in chromosomal abnormalities and reduced development to the blastocyst stage. These easily observable effects of manipulation suggest that more subtle epigenetic effects may also be present. Pauli et al claim to have subjected their cell lines to epigenetic analysis, but do not present their data; in any case, the relevance of such data in homozygous cell lines derived from parthenotes is questionable. Such embryos have aberrant imprinting by definition, and the markings observed in ES cell lines passaged in culture may have little relation to those in an embryo developing in utero.

4. Summary of scientific evidence and its implications for safety of MST/PNT

The evidence presented above shows that, as might be expected from a basic understanding of cell biology, there is a continual and critically necessary molecular dialogue between mitochondria and the nucleus, and that incorrect input from mitochondria can result in damage to nuclear epigenetic marks and to the rest of the cell. Although there appears to be little published data on these interactions in maturing oocytes and single cell embryos, there is certainly no reason to think that they would not be occurring. In fact, these periods are exactly when the most intense epigenetic modifications, including imprinting, occurring. **Therefore, it cannot be safely concluded that maternal nuclei from women at risk of passing on mitochondrial disease are healthy, and it is to be expected that they will carry aberrant epigenetic markings.** The only real question is how serious those defects are likely to be, but that can only be determined by research: it would be highly dangerous to make 'informed guesses' in an area about which so little is understood.

To this epigenetic damage, MST and PNT add the likely further complications involved in the nuclear transfer manipulations. These must be taken very seriously, and evidence from other species provides little reassurance as to safety. It has been relatively easy to clone mice, and so it is not surprising that it has been a comparably easy to perform PNT/MST in that species. It has been less easy, but possible to clone monkeys, and so it has likewise been possible to produce offspring using MST. However, human embryos seem particularly sensitive to the manipulations of nuclear transfer, which may be one reason that, despite the efforts of the Newcastle laboratory amongst others, no significant success has been achieved in 'therapeutic cloning'. In fact, the scientific community, faced with the extreme difficulties of doing this attempted to resort to the highly scientifically dubious recourse of using animal oocytes, in order to try to crack the problem using a brute force of numbers approach. This too has now been abandoned in favour of IPS cells. It seems likely that a significant part of the difficulty of nuclear transfer in humans is due to the vulnerability of human embryos to damage, both physical and epigenetic, arising from nuclear transfer manipulations.

Thus, in summary, there is considerable evidence that there may be safety problems associated with MST/PNT as a result of epigenetic aberrations. We are currently in a situation in which there is much too little data on these points and much more research is needed before MST/PNT can be judged as even probably safe.

5. Conclusion: the need for a precautionary approach

In our view, the HFEA should take a precautionary approach to the issue of the safety of MST/PNT, although this is not evident in the 2011 report. There are two main reasons for this.

Firstly, the very nature of the development of new reproductive technologies demands a precautionary approach, if for no other reason than to counterbalance the positively anti-precautionary approach seen in the past, for example in the development of ICSI and ooplasm transfer. More importantly, we are dealing here with the creation of a child that might be congenitally impaired as a result of these technologies. The HFE Act insists that the welfare of the child be put above all other considerations, and this must include the child's physical welfare. This situation, in which there is reason to believe that there may be severe risks, but no conclusive evidence is available, is exactly the situation in which the Precautionary Principle should apply: it does not demand conclusive proof of safety,

only a higher standard of proof. Statements that, 'The evidence currently available does not suggest that the techniques are unsafe[24],' will not do. Decision making based on such statements risks the sort of collapse in public confidence in regulation that has occurred repeatedly over the past 20 years in the history of British scientific regulatory committees

The case of MST/PNT adds a second element that reinforces the need for a precautionary approach: here, there is a perfectly safe alternative (egg donation), which avoids all the risks detailed above. Thus, the risks to be imposed upon the child by a non-precautionary approach are justified only by the wish of the mother to be genetically related to the child. As we emphasised in a response to the main consultation, this wish is perfectly understandable, but the benefits of acceding to it are not medical but social, and compared to the risk to the welfare of the child seem somewhat minor, no matter how strongly parents may feel. In our view, this consideration, on its own, even in the absence of the massive social implications of altering a child's germ line for the first time, makes the use of MST/PNT ethically wrong. **But even if the HFEA wishes to take a different ethical position on that issue, the fact of availability of egg donation must dictate a precautionary approach to the question of risks from MST/PNT.** We would imagine that any other approach would expose both the clinicians and the HFEA to the risk of legal action, should children be born with complications as a result of the techniques.

We suggest that, before MST/PNT of approved, at a minimum, robust published data, for more than one laboratory should address, in addition to the core experiments demanded in the 2011 report, the following:

1. Effects of nuclear transfer procedures on embryo viability.
2. Epigenetic and transcriptomic profiles of eggs for mothers at risk of transmitting mitochondrial diseases.
3. Epigenetic and transcriptomic profiles of embryos produced by MST and PNT.
4. Studies on the genetic variation underlying the degree of vulnerability of different individuals to epigenetic damage as a result of these techniques.
5. Basic research on nuclear/mitochondrial interactions in maturing oocytes.

The other experiments defined as 'additional' by the committee in 2011 are also clearly essential, especially 'karyotype analysis and comparative genomic hybridisation/copy number variation arrays of embryos derived from MST or PNT'.

Evidence prepared by Dr David King.

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