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Mitochondrial replacement therapy—a new remedy for defects in reproduction

ARUNA PAL[1] and SAMIDDHA BANERJEE[2]

West Bengal University of Animal and Fishery Sciences, Kolkata, West Bengal 700 037 India

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ABSTRACT

Mitochondria is an important subcellular organelle with the prime function being energy metabolism and supply of energy to the body cells for carrying out the vital functions. Energy is the primary requisite for the reproductive organs of both male and female for carrying out the normal functions. In the present article, we have described how mutation in mitochondrial DNA lead to defects in male and female reproduction. Mitochondria is an integral part of the mid-piece of sperm and also has role in other parts of male reproductive system. Similarly, mitochondrial DNA has role in female reproductive system including ovulation, zygote activation, fertilization, oocyte maturation and embryo development. Mitochondrial defect are collectively named as "mystondria" (mysterious diseases of mitochondria) and may be corrected through mitochondrial replacement therapy, popularly known as *three parent baby concept,* since there are no other scope for cure or treatment. Two approaches for mitochondrial replacement therapy are pronuclear transfer and spindle transfer. The first three parent baby was developed in April 2016 through mitochondrial replacement therapy. The present review is aimed at functional relevance of three-parent baby concept in animal reproduction.

Key words: Mitochondrial DNA, Ovulation, Oxidative phosphorylation, Reproduction, Sperm, Three parent baby

Reproduction is the basic and primary function of any living organism and is the basis for life. In the current era of modern civilization, due to the fast and busy life pattern, reproductive disorders are commonly encountered. Infertility is the commonest problem encountered in the current days. These pose an urgent need to study the factors which effects the reproductive disorders, particularly at molecular level.

Currently due to excessive environmental pollution, there is an increasing tendency for mutations. Moreover, population control has always been a greatest challenge in developing country like India. A lot of studies have been conducted so far for understanding the reproductive functions at molecular level with a series of genes as growth hormone gene (Pal *et al.* 2014), FSH gene, IGF1 gene, thyroid hormone, LH, prolactin and others (Lal *et al.* 2016, Pal *et al.* 2006, 2004). Genome wide association studies for a series of genes were also conducted by a group of researchers on reproductive characteristics with pig as a model (Onteru *et al.* 2011).

Apart from genes studied from genomic DNA, current focus is also on mitochondrial DNA. Since mitochondria is responsible for providing energy to the cells through oxidative phosphorylation including reproductive cells at any age, any defect in mitochondrial function lead to disorders in reproductive process. Heteroplasmy is a common phenomenon in mitochondrial mutation, with levels close to or above threshold (>60%) are at a very high risk of transmission. Due to unpredictable epigenetic and genetic, and genotypic and phenotypic mito-nuclear relationship, diseases arising due to mitochondrial defect are collectively named as "mystondria" (mysterious diseases of mitochondria). Mitochondrial diseases are severely debilitating, often fatal and characteristically complex in nature. Since, there is no scope for cure or treatment, the only remedy is through mitochondrial replacement therapy (Fogleman *et al.* 2016). Hence the current topic is described on the effect of mitochondrial DNA on reproduction, how defects in mitochondrial DNA causes adverse phenotypic effect and its possible mode to overcome the problem. Mitochondrial replacement therapy is one of the most advanced topic discussed in relevance to animal science.

Basics of mitochondria

Mitochondrial DNA or mt DNA constitutes the genetic material (Fig. 1) and is located in the mitochondria of the eukaryotic cell and popularly known as 'power house'. In humans, there are 16,569 base pair of mitochondrial DNA which encode for only 37 genes. In human genome, mitochondrial DNA is the first significant part to be sequenced. 13 genes are involved in the process of oxidative phosphorylation and rest of the genes are responsible for the creation of transfer RNA (tRNA) and ribosomal RNA

Present address: [1]Assistant Professor (arunachatterjee @gmail.com), [2]PhD Scholar (banerjee.samiddha90@gmail.com).

Table 1. Incidences or types of reproductive abnormalities affected by mitochondrial gene

Reproductive process	Reference
Sperm motility and abnormality	Patel *et al.* (2016)
Asthenozoospermia and Oligozoospermia	Sampson *et al.* (2001)
Low level of mt DNA in semen	Kumar and Sangeeth (2009)
Defective sperm motility	Kumar and Sangeetha (2009)
Ovulation, zygote activation fertilization	Babayev and Seli (2015)
Oocyte maturation, embryo development	Babayev and Seli (2015)
Mitochondrial copy number in oocytes, ovarian ageing	May *et al.* (2016)
Litter size, percentage of abortion, age at first maturation, age at parturition, conception rate associated with Cytochrome B gene of mitochondria in female sheep and pig as model	Pradhan *et al.* (2017), Pal *et al.* (2016)
Libido, reaction time and seminal characteristics as semen concentration, semen volume, semen motility, sperm abnormality, live and dead count, acrosome integrity test and its association with Cytochrome B gene in male sheep	Pal *et al.* (2016)
Whole mitochondrial genome sequencing and its association with female reproductive traits of sheep	Pal (2017), Hyslop *et al.* (2016)

(rRNA) that coverts amino acids into proteins. Similar trend is also observed for livestock species.

Mitochondrial DNA having the separate evolutionary origin from the nuclear DNA also evolves faster than the nuclear genetic markers (Alexandre *et al.* 2013). Mitochondrial DNA controls the energy metabolism of the cell by the process of oxidative phosphorylation, hence it is susceptible to oxidative attack due to its location at the electron transport chain site where reactive oxygen species are generated making it far more mutation prone than the nuclear DNA. Due to this mutation prone nature of the mitochondrial DNA, it is variedly associated with several genetic disorders in the mammalian body (Frank and Hurst 1996).

Mitochondrial DNA are transmitted matrilineally (Fig. 2) in most organisms (Schwartz and Vissing 2002) although paternal inheritances of mitochondrial DNA can also be seen in some cases (Schwartz and Vissing 2002). Mammals inherit their mitochondrial DNA from the oocyte population in the mother present during fertilization (Chappel 2013). The natural selection of mitochondria only occurs in the females, highlighting the fact of 'Mother's Curse' which is an evolutionary effect that males inherit deleterious

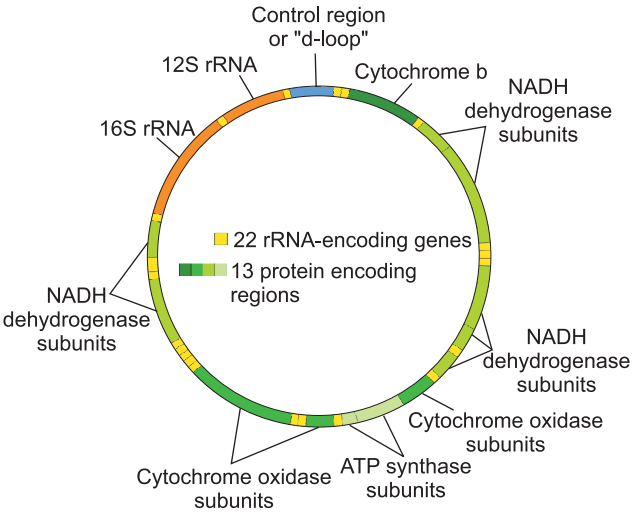

Fig. 1. Mitochondrial DNA, genes for mitochondria.

mitochondrial genome mutations from mother while those mutations are beneficial or neutral or less deleterious to female.

Impact of mitochondrial DNA on male reproduction and fertility

There are several evidences which show that mitochondrial DNA mutations in male causes deleterious effects. For instance, mitochondrial DNA haplogroup T in humans is associated with reduced sperm motility (Patel *et al.* 2016) . In many taxas, mitochondrial abonormalities are important contributers to shorter life span. Male causing mitochondrial disfunctionalities are predicted by 'mother's curse' hypothesis. Laber's Hereditary Optic Neuropathy (LHON) caused by base pair substitution in germline mitochondrial DNA affect majority of the male individuals. Recent evidences have also indicated the paternal inheritance of mitochondrial DNA (Schwartz and Vissing 2002).

Male infertility is generally associated with asthenozoospermia or oligo asthenozoospermia. This is generally reported in patients who suffer from mitochondrial diseases and it is observed that these diseases are caused due to point mutations or multiple deletions of mitochondrial DNA (Sampson *et al.* 2001). Sperms are shown to be more susceptible towards deletion of mitochondrial DNA which may affect sperm motility and fertility. A link has also been observed between the semen quality and the respiratory chain functions in the sperm mitochondria, however semen quality also depends upon point mutations, mitochondrial DNA single nucleotide polymorphisms (SNPs) and also mitochondrial DNA haplogroups (Kumar and Sangeetha 2009). In the semen of infertile men, a very low level of somatic mitochondrial DNA deletions have been observed (Kumar and Sangeetha 2009). This factor causes infertility through the effect of sperm motility.

Limited studies have been undertaken in livestock species till date. Mutations in Cytochrome B was found to

be associated with libido and seminal characteristics of male Garole sheep (Pal 2017). Later on, with NGS studies, whole mitochondrial genome sequencing, mutations in other mitochondrial genes were observed to be correlated to the male reproductive function in Garole sheep male as animal model (Pal 2017).

Mechanism of action-how mutation in mitochondrial DNA effects reproduction

Mitochondria are the major sources of ATP in the body and they play very important role in the process of spermatogenesis, differentiation and proper functioning of the germ line (Venkatesh *et al.* 1997). However genetic alterations or the abnormalities can lead to serious consequences in spermatogenesis and fertilization process. Mitochondrial DNA dysfunctions can lead to complete arrest of spermatogenesis and thus leading to male infertility (Frank and Hurst 1996).

The vital factors in male infertility to be observed are the energy and vigour of the sperms. Sperms require a huge amount of energy for its optimal functioning and its survival. Hence the location of sperm mitochondria is in its mid piece (Connel *et al.* 2002), so that it can provide large amount of energy as quickly as required (Shamsi *et al.* 2008).

Sperm motility and propelling depends on the densely packed group of mitochondrial DNA at the flagellum (Cardullo and Baltz 1991). This pack of energy is used to propel and drive the sperm which a very important factor in male fertility. If sperm motility is reduced, it causes infertility of males hence affecting the process of reproduction. Motility of the sperm heavily depends on the ATP generated by oxidative phosphorylation in the mitochondrial sheath (Kumar and Sangeetha 2009). Therefore these are the important traits to be observed in male fertility.

Mitochondrial DNA being associated with the inner mitochondrial membrane are exposed to highly mutagenic O_2 radicals since oxidative phosphorylation that takes place in the inner mitochondrial membrane generate super oxide radicals as the by product in the respiratory chain. The leakage of these super oxide radicals makes the mitochondria a major intracellular source of reactive oxygen species (Kumar *et al.* 2007). Hence the mitochondrial DNA becomes mutation prone in nature. In case of male reproduction, the reactive oxygen species (ROS) plays a vital role in the system (Venkatesh *et al.* 2009). To perform the normal physiological process, ROS are generated at low level by spermatozoa. But in case of defective mitochondrial DNA, defective protein is formed and defect in electron transport chain leading to increased ROS to pathological level, causing defect in spermatogenesis process and male infertility. The presence of free oxygen radicals was observed long before and was reported in 1943 (MacLeod 1943). The role of these radicals was reported later in 1989. Optimal or lower levels of reactive oxygen species are important for the proper functioning of the spermatozoa like hyperactivation, acrosome reaction, oocyte fusion,

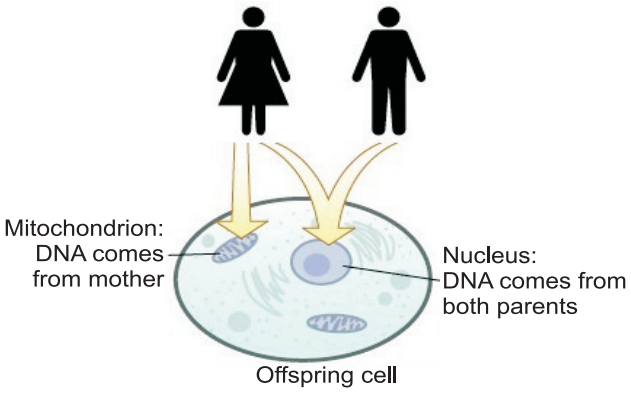

Fig. 2. Mode of transmission of mitochondrial DNA .

capacitation, motility and fertilization (Grivean and Le 1997, Agarwal *et al.* 2004). Normal spermatozoa functions get stimulated when they are incubated with low concentration of hydrogen peroxide radicals. Other superoxide radicals such as nitric oxide and superoxide anion are also observed to have promoted sperm capacitating and acrosome reactions (Zini *et al.* 1996). It is well reported that generation of reactive oxygen species by the spermatozoa is to maintain its own normal function. However excess generation can lead to abnormality in sperm function under some pathological conditions (Aitken and Clarkson 1988).

Impact of mitochondrial DNA on female reproduction and fertility

Mitochondria being the most prominent organelle in the embryo play a vital role in the reproductive system of the female also. Each and every physiological changes going on in the reproductive system depends on the energy provided by the mitochondria and the mitochondrial DNA.

Ovulation in animals, zygote activation, fertilization etc. depends upon the mitochondrial DNA and the energy supplied by the mitochondria (Babayev and Seli 2015). Dysfunctionality in the mitochondrial DNA can cause in the abnormality in the embryo development and fertilization. The mitochondria present in the oocyte are major energy suppliers of the developing embryo, in fact oocytes largely depends on the ATP produced by oxidative phosphorylation. With oocyte maturation, ATP consumption rate also increases. In mice, mitochondrial DNA is responsible for oocyte maturation and embryo development (Van *et al.* 1995). In pigs, immature oocytes can be a cause of depleted mitochondrial membrane potential (Lee *et al.* 2014).

Mitochondrial DNA copy number present in an oocyte is responsible for the determination of the quality of the oocytes and also several ovarian disorders. It is reported that an individual with low quality oocyte and several ovarian disorders contained a lesser number of molecules of mt DNA compared to an healthy individual containing 256 000 number of copies of mt DNA (May *et al.* 2016). Mitoguardin-1/2 had been identified as an important factor for ovarian endocrine functions and ovulation in a study

conducted in fruit flies (Xiao *et al.* 2017).

Even mammalian eggs and early embryo has to be dependent on oxidative phosphorylation because the glycolytic pathway is abstracted due to the suppression of the glycolytic enzyme phosphofructokinase (Barbehenn *et al.* 1974). Mitochondria also helps in the regulation of oscillations of calcium waves (ubiquitous signals) during fertilization since it is an efficient way of transmission of intracellular biological information (Ajduk *et al.* 2011). Oocyte ageing is associated with altered metabolic stress response and lower mitochondrial DNA copy number that correlate with intracellular NADH and FAD measured by Fluorescence Lifetime Imaging Microscopy, FLIM (Babayev *et al.* 2016). Mitochondrial cAMP signaling is an essential part of the cytoplasm-mitochondrion crosstalk. It maintains mitochondrial homeostasis, regulates mitochondrial dynamics, and modulates cellular stress responses and other signaling pathways (Fan *et al.* 2016).

Studies had revealed that mutations in cytochrome B is associated with female reproductive functions in Garole sheep. Similarly, association of cytochrome B with female reproductive function was detected in Ghungroo pig as model (Pradhan *et al.* 2017). Later on whole mitochondrial genome was sequenced and association was observed for reproductive functions for different genes with sheep as model (Kumar and Sangeetha 2009).

Mitochondrial DNA plays an essential role in the life cycle by controlling the energy production processes of the body. It is also vital in the aspect of animal and human reproduction process. Improvement in mitochondrial processes and in the mitochondrial DNA quality improvement leads to better reproductive processes and healthier individuals.

The study of mitochondrial DNA is helpful for early detection of genetic abnormality, three parent baby (zygote has cytoplasm from healthy female, nucleus from biological mother and sperm from father) in human and marker assisted selection in animals.

Mitochondrial replacement therapy

Mitochondrial replacement therapy is a special form of *in vitro* fertilization process in which the future baby's mitochondria comes from another individual (Pal *et al.* 2016). This process is carried out when the mother carries defective or dysfunctional mitochondria. Several vital mitochondrial diseases are caused due to dysfunctional or mutated mitochondria in the offspring since it is maternally inherited. Mitochondrial dysfunctions can lead to several multi organ defects and failures. Here, in this paper we have tried to highlight several facts where we have focused on the effect of reproduction in animals due to this dysfunctional mitochondrial DNA. There has been limited study based on this problem and scarcity of information regarding this where animal reproduction or reproductive organs have been affected because of any defects in the mitochondria. It has been conducted successfully by some workers in primates (Barritt *et al.* 2001). Based on our practical experience while working with animals in farm, we have seen that 30% of the total population of the animals suffer from debility, which further leads to reproductive failure and gradually multi organ failure, ultimately mortality in adverse cases (Pal 2017, Saey 2016).

Three parent baby concept

The mitochondrial replacement therapy was first carried out in 2016 when a baby was born via this technique to prevent mitochondrial diseases from the mother to the child. He was referred to as the first 'three parent baby' since the nuclear DNA was inherited from the parents and the mitochondrial DNA was inherited from an unrelated woman (Sanders 2009, Saey 2016). Earlier the babies born from this particular parent did not survive since the mother carried a defective mitochondria and since the mitochondria is inherited from the mother's cytoplasm (Saey 2016, Hamers 2016), the dysfunctional mitochondria were carried by the earlier offsprings which lead to their death. The current baby born from the three parent concept became free from all the inherited genetic disorders (Saey 2016). The technique was carried out via *in vitro* fertilization .

Mitochondrial replacement therapy--Pronuclear transfer

The first attempt taken by the scientists to keep away faulty mitochondria from being passed to the offspring from the mother is the pronuclear transfer (McDermott 2016). The first 'mitochondrial replacement' technique developed to stop mitochondrial diseases is called pronuclear transfer. It was first done in mouse embryos in 1983. Pronuclei are nuclei from the egg and sperm that are in the fertilized egg, called a zygote, but have not yet fused into a single nucleus (Fig. 3).

In this technique, the egg from mother with the abnormal mitochondria and the egg from donor with the normal mitochondria was fertilized at the same time. The fertilised pronuclei from the donor's egg was sucked out and discarded, and then the pronuclei from the mother's fertilized egg was transferred to the empty donor's egg with the normal mitochondria and finally the embryo with normal mitochondria and maternal and paternal genome was transferred to the uterus (Tibbitts 2014, Zhang *et al.* 2016).

This technique is considered to be unethical since it is destroying two embryos (Lee 2009). Secondly, from a more technical point of view, the abnormal mitochondria can also gloom into the nuclei during the transfer process, there unacceptably higher number of mitochondria from the mother's egg may find their way to the donor's egg including the disease carrying ones (Lee 2009). Then, scientists reported that refinements in the technique produced embryos in which less than 2% of the mitochondria were carried from the mother's egg into the donor egg (Hyslop *et al.* 2016). But an earlier study suggested that even 1% carryover could be dangerous because mutant mitochondria may replicate, eventually taking over the cell and crippling its energy production (Saey 2016).

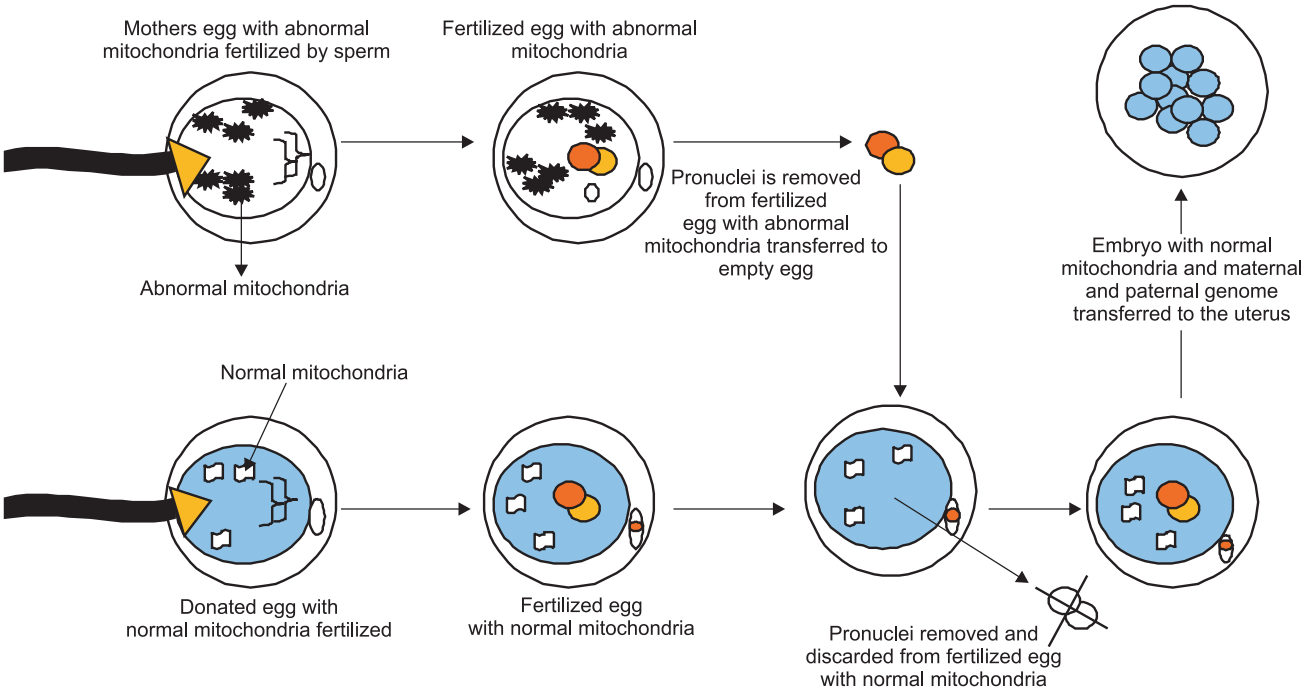

Fig. 3. Pronuclear transfer of mitochondrial replacement therapy.

Rather than considering this technique as a curative treatment for babies who are to be born with defective mitochondria, it is considered to be as a type of infertility treatment since these babies can have health issues related to dysfunctional mitochondria later on.

Mitochondrial replacement therapy—Spindle transfer

DNA resides in body's 46 chromosomes. Cell division leads to the splitting of 46 chromosomes to two sets of 23 each. The chromosomes attach themselves to several protein fibre called spindles. In this technique, two unfertilized eggs, one from the mother and another from the donor is taken. The nuclear membrane surrounding the nucleas is broken. By that time the spindle do not separate itself into the chromosomes. The spindle and its attached chromosomes are removed from the donor's egg and discarded. The same thing is done with the mother's egg except the spindle attached to the chromosomes are kept. This spindle attached to the chromosome are injected into the donor's empty egg and the father's sperm is taken and the egg is fertilized (Fig. 4). Then finally the embryo with normal paternal and maternal genome with normal mitochondria is transferred to the mother's uterus (Saey 2016).

The first three parent baby boy was born in 2016 by this method i.e. the spindle transfer method. The pioneer of spindle transfer is Mitalipov in Portland (Lee 2009). In the year of 2009 he showed that healthy baby monkeys can be born by this technique.

Spindle fibre transfer technique also has some downside. During the transfer of the spindle attached with chromosome, the chromosomes may fall off which may result into transfer of only a fewer number of chromosome in the embryo or too many if some are left in the egg of the donor. In both the cases this may lead to abnormality.

Zhang *et al.* (2016) performed spindle fibre in five embryos of which one was successful by which the baby boy was born last year. It was found out that he had only 1% of his mother's mt DNA. At the age of three months he appeared to be healthy. But it is still a matter of concern that whether his health will be affected or not in the long run. His health may also be affected due to the mismatch between the parent's nuclear DNA with the donor's mitochondrial DNA. Besides the risk of even trace levels of mitochondria ballooning, another study suggests that mismatches between the parents' nuclear DNA and the donor mitochondrial DNA could affect aging in a study conducted in mice (Mc Dermott 2016).

However several controversies exists regarding the techniques as well as the term. According to Cohen, the term 'three parent baby' is wrong since the offspring do not carry any trait of the donor because mitochondria does not contribute in transferring any basic trait to the offspring from the parents and therefore the donor can hardly be called as a parent. Andrew R. La Barbera, also agrees to this point since mitochondria is not a genetic material and a baby conceived using this techniques just have two parent.

A panel of experts also commented that it is ethical to make baby boys through this technique but not girls since mitochondrial DNA is passed through the mother not the father. So the baby boys born through this technique would never pass the donor's mitochondrial DNA.

Relevance of three parent offspring in Animal Science

It was observed from the present study that mitochondria

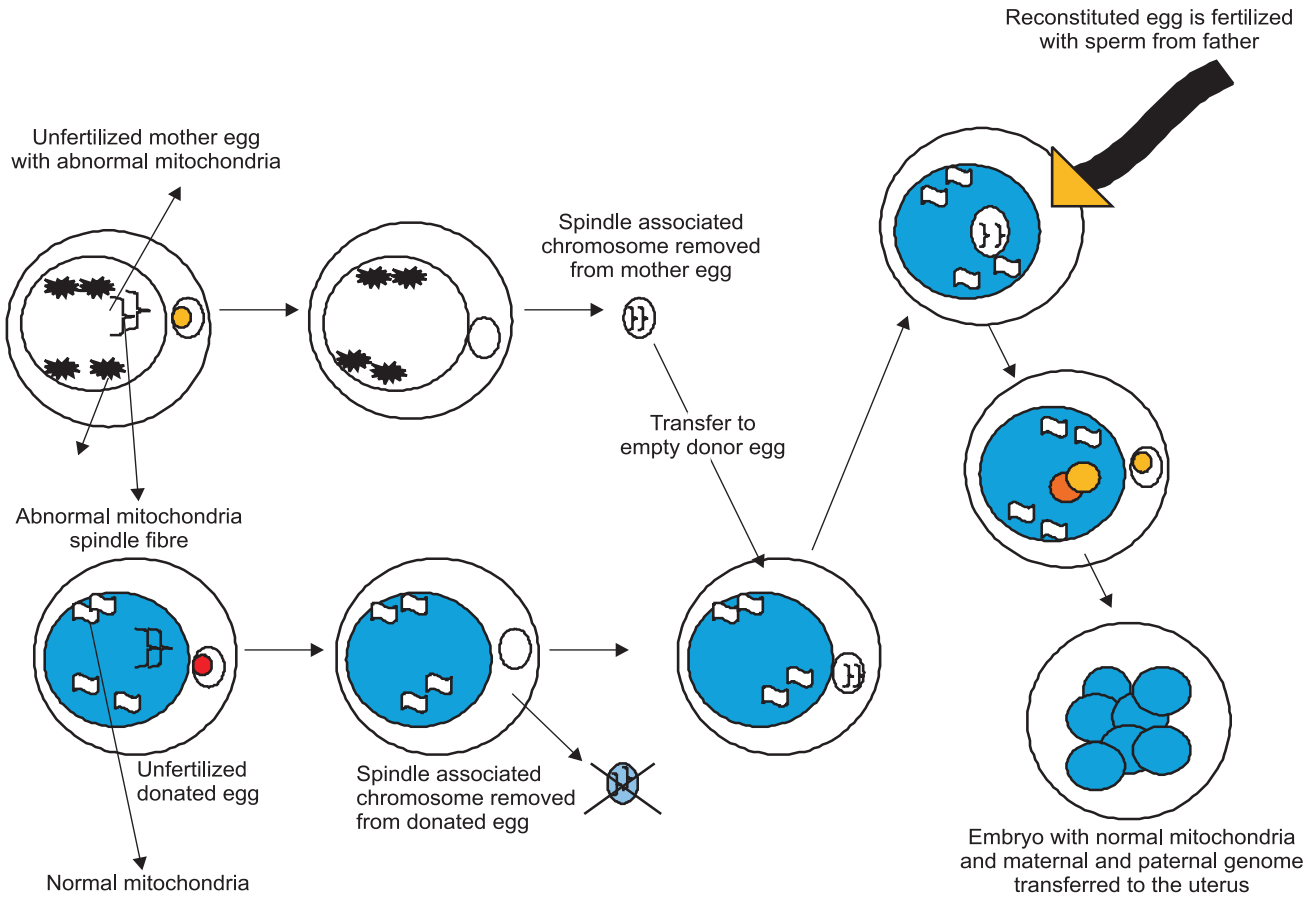

Fig. 4. Spindle transfer for mitochondrial replacement therapy.

is one of the most important cell organelle and has various important function in supplying energy. Any defect in mitochondrial DNA leads to diseases and effects reproduction. A way in the correction of the problem in Animal Science is two fold.

Marker assisted selection: First is detection of the animals carrying defective mitochondria, cull them and select only the animals carrying healthy mitochondria. One important issue is the heteroplasmy of defective mitochondria. When the percentage of defective mitochondria (with mutant DNA) is more than 60%, it is considered as defective or deleterious. During the selection process, the individuals having more than 60% defective mitochondria will be identified and culled. When the selection will be based on all 37 mitochondrial genes, the process may be regarded as genomic selection. This method is applicable for livestock reared in farms. Thus the healthy stock of livestock will be created.

Mitochondrial replacement therapy or three parent baby concept: It is considered as a costly technique although if it is massively applied it can be a cost effective one. In the field of animal science, high producing elite animals with mitochondrial defect need not be culled, but can be easily treated using this technique. Donor mitochondrial DNA may be collected from a female with healthy mitochondria. Thus the unique traits of high production of the animal can be conserved easily. Disease resistant animals can also be produced via this technique.

Endangered animals in the wildlife can also be produced by this method. There is no scope to cull an endangered animal. But when defective mitochondrial mutation is present in endangered animal, it can be treated with mitochondrial replacement therapy. Mitochondrial defects cause extreme debility, exercise intolerance and ultimately death due to mitochondrial replacement therapy.

Till date there is lacunae of study on defects in mitochondrial gene in animals and its effect on debility and diseases. In our lab, we are working on mutations in mitochondrial gene causing debility, exercise intolerance and having deleterious effect on the vital functions of the body organs, such as liver, kidney and heart in sheep and pig maintained in our farm (Pradhan *et al.* 2017, Pal *et al.* 2016). Initially we started with Cytochrome B gene, now working on all 37 mitochondrial genes (whole mitochondrial genome sequencing) through next generation sequencing approach (Pal *et al.* 2017). Still there are lot of scope for working out on mitochondrial defects as it causes substantial mortality and morbidity in animals. Care should be taken to include the parameter on marker assisted selection.

CONCLUSION

Very limited research and studies have been undertaken in the field of mitochondrial genes and its effect on reproduction and diseases in animal science. Although most of the cases remain undetected in a farm, to our practical experience, we could observe that about 30% of the animals suffer from mitochondrial defect, symptomatically revealed as extreme debility with death after multi-organ failure. Three parent baby is one of the most recent concept and is highly effective in correction of mitochondrial defects leading to reproductive disorders and other diseases without loosing our indigenous germplasm. Lack of curative treatments and prenatal diagnosis had led to this alarming situation which can be prevented through the mitochondrial replacement therapy.

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