OPINION

Alternative sources of gametes: reality or science fiction?

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Although great progress has been made in both the investigation and treatment of infertility, a considerable number of patients still fail to conceive. Spermatogenic failure and/or oocyte ageing appear to be responsible for a large proportion of cases. The use of donor gametes may bring legal, ethical and even social problems of acceptance that can discourage infertile couples from the donor route. Fortunately, emerging reproductive technologies and preliminary results from animal experiments provide some hope for alternative sources of gametes through which these infertile patients can finally conceive their own genetic child. In conjunction with intracytoplasmic sperm injection (ICSI), fertilization of human oocytes with immature sperm precursors, e.g. spermatids and even secondary spermatocytes, has resulted in healthy babies. Pregnancies have also resulted from the use of spermatids derived from in-vitro spermatogenesis. In the mouse, even primary spermatocytes appear able to participate in normal embryogenesis. In view of the possibility for transplantation and even xenotransplantation of spermatogonia to a host testis in animals, a similar use of human male stem cells might provide an attractive source for the treatment of males with arrested spermatogenesis, as well as male cancer patients. Transplantation of somatic cell nuclei and their haploidization within oocytes may prove to be a practical way of eradicating age-related aneuploidy and so constitute an innovative source of healthy oocytes. Most importantly, however, the safety of the procedures described here needs to be proven before their application to the human arena. Finally, we discuss the implications of cytoplasmic quality and of genetic imprinting in the context of these manipulations.

Key words: alternative sources/assisted reproduction/gametes/oocytes/spermatozoa

Introduction

Until ~20 years ago, almost the only way to conceive was by natural intercourse. As a treatment option, artificial insemination was quite successful when donor spermatozoa were used, but this had limited results with homologous spermatozoa. Although the situation has changed radically since the first human IVF pregnancy in 1978, nonetheless ~40% of couples can still fail to achieve fertilization because of poor sperm quality (Van Uem et al., 1985). The establishment of intracytoplasmic sperm injection (ICSI) (Palermo et al., 1992, 1995a,b, 1996) has greatly enhanced the chance of fertilization in couples with previous fertilization failure after standard IVF, adding another conspicuous portion of the infertile to the reproductive population. The application of ICSI became even wider when it was observed that this made pregnancies possible for azoospermic patients. The rapid acceptance of ICSI in clinical practice has been furthered by its ethical acceptability and by the fact that it generally avoids any need to use donor spermatozoa.

The use of IVF and ICSI has not been problem-free. The confidence of baby-boomers that they can reproduce at ‘any age’ has often postponed the wish to achieve pregnancy, and this is particularly evident in women aged ≥40 years. While infertility due to relatively advanced female age can now be successfully treated with oocyte donation, most couples are more interested in generating their own child, and the astounding accomplishments of reproductive medicine in recent years have raised their expectations.

The decreased fertility of older women stems in large part from a decline in the frequency of intercourse, in the number of primordial follicles and, in particular, from a higher incidence of oocyte aneuploidy (Tietze, 1957; Dailey et al., 1996). In the metaphase II (MII) oocyte, the frequency of aneuploidy, primarily the result of a non-disjunction of bivalent chromosomes at meiosis I, is estimated to be 4.9, 11.5 and 29.8% in the 25–34, 35–39, and 40–45 year old age groups respectively (Dailey et al., 1996). That oocyte aneuploidy is one major reason for the low ongoing pregnancy rates in older women, is suggested by the higher pregnancy rate in this group where young donor oocytes are used. Of course, age is also a predisposing factor for autosomal trisomy in the offspring (Hassold and Jacobs, 1984).

An interest in finding a treatment for azoospermia and for oocyte aneuploidy has stimulated many investigators to seek alternative sources of spermatozoa and oocytes for the infertile couples who specifically desire to conceive their own genetic
child. In recent years, several leads commonly used in cloning science have opened routes, real or theoretical, for safe ways to ‘manufacture’ gametes that will make that goal possible.

Here we discuss the prospects for techniques that may lead to the production of viable gametes with the same parental genome. We consider those that relate to male germ cell isolation and transplantation, the procedures of nuclear transplantation, somatic cell haploidization and finally issues involved in cytoplasmic–haploidization and finally issues involved in cytoplasmic–heteroplasmic transfer.

Sources of sperm precursors

Spermatids

The effectiveness of ICSI with freshly ejaculated spermatozoa has been extended to spermatozoa collected directly from the obstructed epididymis. However, for patients whose azoospermia is not obstructive in origin, the only option is retrieval directly from the testis. These cases, identified as non-obstructive, generally present considerable maturation arrest of the spermatogenic line (Martin-Du Pan and Campana, 1993). Fortunately, this maturation failure often occurs only in some of the tubules, so allowing the collection of spermatozoa from others (Silber et al., 1997). However, in some cases (e.g. Sertoli cell-only syndrome), a complete failure of sperm development extends to all tubules.

The apparent absence of even testicular spermatozoa in many azoospermic men has stimulated an interest in utilizing more immature stages—an approach first suggested by the success of Ogura and Yanagimachi in mice (1993). However, a necessary feature of spermatogenesis, and of a viable germ cell, is the process of haploidization, the youngest haploid cell being the round spermatid. Round spermatids from hamsters and mice are able to replicate their DNA, participate in syngamy and support complete development when incorporated into oocytes either microsurgically (Ogura and Yanagimachi, 1993) or by electrofusion (Ogura et al., 1993, 1994). Studies performed in the rabbit have given similar results (Sofikitis et al., 1994).

The success achieved with this approach in animals has stimulated an interest in the injection into human oocytes of immature spermatogenic cells retrieved from the testis. Fertilization and early cleavage have been observed following the use of human round spermatids in this way (Vanderzwalmen et al., 1995), and a healthy girl was born after ICSI with elongated spermatids (Fishel et al., 1995, 1997). In the same year, two normal infants were born after ICSI with round spermatids (ROSI) obtained from ejaculates of azoospermic men (Tesarik et al., 1995; Tesarik, 1996). However, the rates of fertilization and pregnancy with round spermatids have been disappointing (Tesarik and Mendoza, 1996; Amer et al., 1997; Antinori et al., 1997; Vanderzwalmen et al., 1997; Yamanaka et al., 1997; Palermo et al., 1999).

While the poor results with spermatids in these years have tended to discredit ROSI, there may be various reasons for this. It is still unclear whether elongating spermatids carry a much greater chance for success than round spermatids. One further problem in using round spermatids is a difficulty in distinguishing them from other round cells such as spermatocytes, monocytes and polymorphonuclear leukocytes. Furthermore, the acrosomal granule can sometimes be misidentified, even under the inverted microscope with Hoffman modulation contrast optics, the system most commonly used for identification and aspiration in wet preparations (Tesarik and Mendoza, 1996; Antinori et al., 1997; Vanderzwalmen et al., 1997; Silber and Johnson, 1998; Veheyen et al., 1998). Recently, a more reliable method has been described using a phase-contrast objective adapted to an inverted microscope together with a Petri dish with an especially thin glass bottom (Verheyen et al., 1998).

Another problem concerns oocyte activation. Calcium is the universal intracellular signal for triggering oocyte activation (Vitullo and Ozil, 1992; Homa and Swann, 1994; Tesarik et al., 1994; Palermo et al., 1997; Fissore et al., 1999), and at sperm–oocyte fusion a factor present in the spermatozoon initiates repetitive transient calcium fluxes in the oocyte (Vitullo and Ozil, 1992; Ozil and Swann, 1995). An absence of activation has been found to be the most common cause of fertilization failure after ICSI (Moomjy et al., 1998), and suboptimal activation after sperm penetration can arrest the fertilization process (Fishel et al., 1996). An insufficiency of the activation factor in round spermatids retrieved from men with complete failure of spermatogenesis appears to be at least partly responsible for the poor results of ROSI (Tesarik et al., 1998a). This is in contrast to round spermatids from men with continuing spermiogenesis, which seem able to induce a calcium signalling similar to that brought by mature spermatozoa (Sousa et al., 1996). It is possible that abnormal calcium signalling after ICSI might also result in chromosomally abnormal embryos (In’t Veld et al., 1995; Tesarik et al., 1998b). The high frequency of apoptotic germ cells in patients with maturation arrest (Tesarik et al., 1998a) may compromise ROSI by several mechanisms. In fact, caspase-activated hydrolases may cause damage both to spermatid DNA and to developmentally relevant cytoplasmic components, e.g. oocyte- activating factors, which may be an additional factor for the lower success with round spermatids.

Unfertilized human oocytes injected with mature spermatozoa apparently show a relatively high incidence of premature chromosome condensation (PCC) (Schmiady et al., 1996), which may occur even when spermatids are used for ICSI (Tesarik et al., 1998a,b). Unlike mature spermatozoa, whose condensed –S–S– stabilized protamines prevent sperm nuclei being driven to metaphase prematurely by oocyte metaphase promoting factor (MPF), round spermatids feature a dispersed chromatin in which the histone/protamine substitution has hardly begun. From this viewpoint, nuclear maturity appears to be important and could explain the slightly higher fertilization rate using elongated rather than round spermatids (Antinori et al., 1997). Interestingly, PCC may not always lead to developmental failure, as recently demonstrated by the birth of normal offspring after injection of mouse oocytes with round spermatids followed by delayed oocyte activation (Ogura et al., 1999). Moreover, it is possible that PCC may be helpful in nuclear reprogramming of immature germ cells, similar to...
the observed behaviour of somatic cell nuclei (Cibelli et al., 1998; Wells et al., 1999).

While healthy babies have been delivered worldwide, some authors have questioned the utility of ROSI as the treatment for azoospermic patients. Silber and Johnson (1998) compared 143 consecutive cases involving testis biopsies of men with non-obstructive azoospermia due to spermatogenic failure, and 62 controls with obstructive azoospermia and normal spermatogenesis. In no case were round spermatids seen in the absence of elongated spermatid and maturation arrest was found always to be a failure of progression beyond meiosis, not merely from the round to the elongated spermatid. This suggests that one should search exhaustively for mature spermatid or elongated stages before resorting to the injection of spermatids. On the other hand, ROSI might serve as a strategy to avoid the harmful effect of extensive biopsy in searching for spermatozoa. Ischaemia of the testis can be produced by testicular sperm extraction (Schlegel and Su, 1997) and the risk is increased when the intervention involves multiple incisions of the tunica albuginea (Jarow, 1991). Moreover, complete maturation arrest at the spermatid stage has been reported (Re et al., 1979; Aumuller et al., 1987), and round spermatids can be identified in many patients even when late spermatids or spermatidia were absent (Amer et al., 1997; Tesarik, 1998). Since the clinical value of ROSI needs to be evaluated further, patients should be informed about the uncertain safety and lower reproductive outcomes when round spermatids are to be used for conception.

**Spermatocytes**

The nuclei of secondary spermatocytes have been shown to complete meiosis when injected into mouse oocytes (Kimura and Yanagimachi, 1995). By 2 h after injection of secondary spermatocytes into mature oocytes, some spermatocyte nuclei exhibit premature chromosome condensation and microtubule attachment. Subsequent to electrical activation of oocytes containing a spermatocyte, however, both oocyte and spermatocyte chromosomes resumed their meiosis to form two pronuclei and two polar bodies (one of paternal and one of maternal origin). Of 2-cell embryos transferred to foster mothers, 24% reached full term. In the one example where human secondary spermatocyte nuclei were injected into electro-activated mature oocytes, this was followed by extrusion of a female second polar body and a male pseudo polar body, by formation of two pronuclei and by embryo development, with the reported delivery of a healthy child (Sofikitis et al., 1998).

Mouse primary spermatocytes also can complete meiosis within maturing or mature oocytes and can participate in embryogenesis although with lower efficiency than can secondary spermatocytes (Kimura et al., 1998; Sasagawa et al., 1998). Primary spermatocyte nuclei injected into electro-activated mature oocytes transformed to MII and the extrusion of two polar bodies was observed (PbII of oocyte origin and PbI of spermatocyte origin). When a polar body of spermatocyte origin is transferred into another mature oocyte, a male pronucleus may form, and the ‘haploidized’ primary spermatocyte can support embryo development. Similarly, when primary spermatocyte nuclei were injected into MII oocytes, the high level of MPF induced chromosome condensation and spindle formation (Sasagawa et al., 1998). Such oocytes first extruded two polar bodies, one of oocyte origin, and another of spermatocyte origin, and upon activation by an electric pulse they resumed meiosis to form two pronuclei, and two additional polar bodies were extruded. However, only two out of 258 such embryos transferred at the 2-cell stage developed to term. The reason for this poor success rate remains to be determined.

Mechanical manipulation and suboptimal media, as well as incomplete imprinting and DNA repair might account for this low success and poor embryonic development. The high incidence of chromosomal abnormality in primary spermatocyte-injected oocytes (Kimura et al., 1998) might be another plausible cause for this. Slightly better results have been reported using immature, instead of MII mouse oocytes, for the first step of nuclear haploidization (Ogura et al., 1998).

Though still imperfect, the use of spermatocytes might one day offer a promising treatment for those patients with spermatogenic arrest, most frequently seen at the end of prophase (the primary spermatocyte level) (Remy and Martin-Du Pan, 1993). While 50% of infertile males might present anomalies of either synopsis (chromosome pairing) at zygotene or desynapsis (precocious separation of paired homologues) at late pachytene, or anomalies of the synaptonemal complex (Lange et al., 1997), nevertheless, spermatocytes may provide a future alternative through which to treat spermatogenic arrest, particularly if healthy cells can be selected. In fact, in-vitro culture can simultaneously allow the selection of healthy cells and transmeiotic differentiation (Tesarik et al., 1999b), and represents an interesting alternative to direct injection of immature germ cells or their nuclei into oocytes. After the application of this technique in cases of maturation arrest at the primary spermatocyte stage (Tesarik et al., 1999a), the first two babies born showed no numerical or structural chromosomal anomalies (Tesarik et al., 1999b).

**Genomic imprinting**

In diploid cells, allelic exclusion results in one of two alleles of some genes being inactivated. This inactivation of either paternal or maternal alleles, termed ‘imprinting’, may be exerted through an epigenetic modification of their DNA by methylation of the 5′ position of selected cytosine residues (Bergman and Mostoslavsky, 1998); and it is reversible through demethylation, when the silenced gene is reactivated. Tissue-specific genes are methylated in most of the tissues in which they are not expressed, and are unmodified in their tissue of expression (Yeivin and Razin, 1993). In contrast, the housekeeping genes, which harbour a 5′ CpG island, are unmethylated in all tissues (Bird, 1986). Methylation is also involved in the maintenance of gene repression on the inactive X chromosome in female somatic cells (McCarrey and Dilworth, 1992).

Genomic imprinting is critical for normal development, and its disruption during gametogenesis or in early development underlies certain genetic diseases (e.g. Prader–Willi and Angelman syndromes), and can promote the development of malignant childhood tumours (Tycko et al., 1997). The importance of genomic imprinting in mammalian development...
was first recognized in 1977 (Lyon and Glenister, 1977). Gene inactivation experiments have since confirmed that imprinted genes in the female and male regulate embryonic and placental growth respectively (Barlow, 1995). Dramatic methylation changes have been observed during the early steps of embryo development. Most of the DNA in the early blastomeres is unmethylated, and is related to their totipotency; however, an extensive wave of de-novo methylation following implantation modifies most of the genome except the housekeeping genes (Bergman and Mostoslavsky, 1998). Aberrant methylation may be detrimental to embryo development, and murine embryos that express low values of the maintenance methyltransferase do not develop to term (Razin and Shemer, 1995). On the other hand, over-expression of the H19 gene in transgenic mice leads to late gestational death (Brunkow and Tilghman, 1991), and maternal duplication in the region of the Snrpn gene (paternal imprinted allele) causes postnatal lethality in mice (Cattanach et al., 1992). In addition, methylation of the promoter of the tumour suppressor gene can contribute to tumour formation.

While the exact timing of imprinting events in human gametogenesis is still unclear, there are some speculations based on indirect evidence (Tycko et al., 1997), and the use of immature germ cells in conjunction with ICSI may help to elucidate at which stages imprinting is incomplete. A pilot study did not reveal any differences from controls in the expression of several paternally and maternally imprinted genes in mouse embryos derived from ROSI (Shamanski et al., 1999). Differential methylation is not seen in germ cells of either sex at an early stage of their development. In spermatogenesis, the erasure of previous imprintings occurs prior to meiosis probably in primordial germ cells or replicating gonocytes. According to DNA methyltransferase activity, re-establishment of the imprinting appears to occur to a large extent in the preleptotene, leptotene and zygotene stages (Shamanski et al., 1999). However, the finding of residual methyltransferase activity in round spermatids has provoked the suggestion that imprinting is completed only after meiosis. Some minor elements of this might be incomplete even after spermiation, since methylation of genes, e.g. Pgk-2, ApoA1 and Oct-3/4 appeared to occur as sperm transit the epididymis (Ariel et al., 1994).

Transplantation of spermatogonia

In 1994, Brinster and Zimmermann reported that donor male stem cells could partially repopulate sterile mouse testes when injected into seminiferous tubules, and this produced some fertile spermatooza (Brinster and Zimmermann, 1994). A remarkable aspect of the colonization was the faithful reconstruction of the complex cellular associations of normal spermatogenesis (Dym and Clermont, 1970; Ewing et al., 1980; Russell et al., 1990). Subsequently, whether stem cells of the rat could colonize mouse seminiferous tubules was investigated (Clouthier et al., 1996). The rat cells were transplanted to the testes of immunodeficient mice, and when epididymides of eight mice were examined, three belonging to mice with the longest transplants (>110 days) contained rat spermatooza of normal morphology (Clouthier et al., 1996). The occurrence
be a necessary first step for successful transplantation of human spermatogonia.

Sources of oocytes

The feasibility of cloning in animals has raised concerns about the indiscriminate application of this to man, and as consequence several countries have imposed a ban on human cloning by legislation or regulation. Nevertheless, techniques such as nuclear transfer developed from cloning research might be a solution for some of the problems we face today in reproductive medicine.

A key step in the cloning procedure is the isolation of a karyoplast and its transfer into an enucleated ooplasm. Oocytes can be enucleated by micromanipulation with a glass micropipette, or by chemical treatment. Then, after insertion of the karyoplast, electrical stimulation or viral agents can promote its fusion with the enucleated recipient ooplasm.

Nuclear transplantation studies using embryonic and somatic cells have shown that successful embryo reconstitution requires nuclear reprogramming determined by maturation promoting factor (MPF) (Czolowska et al., 1984; Willadsen et al., 1986; Stice and Robl, 1988; Campbell et al., 1993). MPF is one of the major cytoplasmic controls of the cell cycle. MPF consists of a cyclin B, a regulatory component, and p34\(^{cd2}\), a catalytic subunit. During the cell cycle, the concentration of p34\(^{cd2}\) remains unchanged, whereas the concentration of cyclins varies. MPF activity begins to increase in the oocyte cytoplasm just before germinal vesicle breakdown and is sustained at a high level throughout metaphase I (MI). MPF values decrease at anaphase and telophase I, but increase again as the cell enters MII only to decline rapidly upon fertilization or oocyte activation. During transplantation, all nuclei transferred into a cytoplast with high MPF activity undergo nuclear envelope breakdown and chromosome condensation. The cell cycle is restarted by a suitable artificial stimulus such as electric pulses or exposure to a medium containing ethanol or strontium (Whittingham, 1980). In addition, the stage in the cycle of the donor nucleus and recipient cytoplasm are important factors for the normality of the ‘ploidy’ in the reconstituted cells (Kono, 1997). Altering the timing of oocyte activation with respect to the fusion of the donor nucleus provides a number of possible approaches to synchronizing the respective cell cycles.

Maternal age is viewed now as the underlying cause of the chromosomal aneuploidy seen in at least 5% of human conceptions. That age-related aneuploidy is the main reason for the poor embryo implantation observed in older women, is indicated by the high pregnancy rate obtained in aged infertile women when young donor oocytes are used. Ageing of the ooplasm has been considered to be responsible for producing an abnormal meiotic spindle (Battaglia et al., 1996) with spindle abnormalities as the source of incorrect segregation of chromosomes/chromatids at MI (Dailey et al., 1996). One approach to correction of these oocyte defects is transplantation of the nucleus of immature oocytes into the cytoplast of a younger woman (Zhang et al., 1999).

In a preliminary study in the mouse, germinal vesicle (GV) karyoplasts were isolated and transferred into immature recipient cytoplasts (Takeuchi et al., 1999a) (Figures 1a,b, and 2a,b). Not only was oocyte integrity restored in >90% of cases, but 90% of these reconstituted oocytes then extruded a polar body, and when fixed for cytogenetic analysis they displayed a normal chromosomal constitution. This preliminary study suggests that nuclear transplantation in the mouse can be highly efficient and does not cause genetic damage (Takeuchi et al., 1999a). In attempting the same procedure on human oocytes, the restoration rate at the GV stage was >80%, but the maturation rate was only 60%, an outcome that may be explained by the utilization of a less-than-optimal culture medium for in-vitro maturation (Takeuchi et al., 1998, 1999b). While further cytogenetic information is needed about oocytes produced by grafting an old nucleus with a younger cytoplasm, this approach appears to be the only treatment option for age-related aneuploidy. Although it needs to be refined, the technique should stimulate and inspire further research aimed at the treatment of oocyte ageing.

Recently, cytoplasmic transfer was suggested as an approach through which to restore normal growth in developmentally compromised embryos where some ooplasmic deficiency might exist. The importance of ooplasmic factors is postulated for
Alternative sources of spermatozoa and oocytes

the continued development of the zygote, particularly during early cleavage, when transcription of the embryonic genome is minimal (Van Blerkom, 1995; Liu et al., 1997). In an attempt to restore a compromised embryo, healthy ooplasm aspirated from donor MII oocytes was injected into oocytes of a patient who had experienced multiple assisted reproductive cycles with implantation failure attributable to poor embryo development, and normal babies resulted (Cohen et al., 1998). However, since this procedure was performed on MII oocytes, it cannot correct any chromosomal imbalance that may appear earlier in meiosis.

Both of the above approaches, nuclear transfer and ooplasmic transfer, have one main problem: older women (aged >40 years) produce too few oocytes. However, a sufficient number of oocytes might be created by a form of cloning—nuclear transplantation of an older patient's somatic cell into an enucleated ooplasm obtained from a younger donor (Figure 3a). The construction of viable gametes from somatic cells would benefit older women, women with premature ovarian failure, or those considered as poor responders, as well as constitute a landmark in the history of reproductive medicine.

It has been shown that ooplasm from a GV-stage oocyte is able to initiate a meiosis-like reduction division in mitotic nuclei originating either from germ cell lines or from more differentiated somatic cells (Kubelka and Moor, 1997). Although this approach to haploidization of somatic cell nuclei may appear similar to cloning, the resulting haploid oocytes obviously still need the contribution of the paternal gamete to produce biparental progeny. In preliminary experiments, we have been able to haploidize somatic cells transplanted into immature oocytes (Takeuchi et al., 1999c) (Figure 3b,c).
However, further cytogenetic information as well as the effect of the centrosome present in the fresh ‘manufactured oocyte’ on the fertilization process and later embryo cleavage must be better understood. It is also unknown whether imprinting of the reconstructed gamete derived from a cultured somatic cell is compatible with a sperm- or oocyte-derived nucleus. And since shorter mean telomere length has been reported in nuclear-transfer sheep derived from somatic cells (Schiels et al., 1999), it needs to be established whether somatic cell-derived gametes would bear any impact on senescence and the life span of the resultant offspring.

It is likely that the high rate of embryonic and fetal death arising from epigenetic and centriolar errors in animal cloning (Edwards and Beard, 1998) would also apply in cases where somatic cell haploidization is used to produce a ‘new’ egg for fertilization. Nevertheless, sperm entry and sperm function might modify this outcome, since this establishes a completely new system not found in a simple cloning situation. Recently, mouse clones were produced using metaphase nuclei derived from 4-cell-stage mouse embryos (Kwon and Kono et al., 1996), and more efficiently than with somatic cell nuclei (83% of reconstituted embryos developed into blastocysts, of which 57% resulted in live young). This suggests that the ability of cytoplasm to support embryonic development and correct genomic imprinting differs markedly between fertilized and parthenogenetic 1-cell embryos. However, for the moment, any application to man using adult nuclei should be precluded until the reason for the fetal losses are fully understood, or until their use is proven to be safe in animal experiments.

**Cytoplasmic implications**

Each mitochondrion contains 2–10 copies of mitochondrial DNA (mtDNA) in all human tissues, except platelets and oocytes which contain only one copy per mitochondrion. mtDNA is 20 times more susceptible to mutation than nuclear DNA (Kagawa and Hayashi, 1997), due to the location of the mtDNA close to the site of reactive oxidative species formation, and a lack of protective histones in mtDNA (Tritschler and Medon, 1992). Contrary to widely held notions, involving base excision and nucleotide excision repair pathways, mitochondria can efficiently repair oxidative damage to their DNA (Bohr and Dianov, 1999). Moreover, the mutation rate is highly variable across the genome. Some regions show nucleotide substitution rates similar to those of nuclear DNA, whereas synonymous sites and small tRNAs mutate ~20 times more rapidly, and tRNAs ~100 times more rapidly than their nuclear counterparts (Pesole et al., 1999). In most related diseases, patients’ cells carry a mixture of both mutant and wild-type (normal) mtDNA. This heterogeneous state of cells is called heteroplasmasy, while the homogeneous state of cells containing pure mutant or normal mtDNA is termed homoplasmasy (Kagawa and Hayashi, 1997). One factor that appears to be important for the phenotypic expression of mitochondrial diseases is the relative proportion of wild-type and mutant mtDNA (Newman et al., 1991; Boulet et al., 1992), and the ‘dose’ of mutant mtDNA also has an influence on the severity of the phenotype (Marchington et al., 1998). The level of mutant mtDNA varies in different tissues and changes with time (Poulton, 1996), probably related to the oxidative metabolism inherent in each organ.

In contrast to nuclear DNA, mtDNA is always maternally inherited. In recent years, mtDNA has been the subject of increasing attention due both to the subtle role it may have in early development (Van Blerkom, 1989), and its place in maternal age-related reduction of embryonic competence (Gaulden, 1992). Mitochondria are also a source of a variety of hereditary disorders. However, the mechanisms controlling the segregation and inheritance of mtDNA in mammals are controversial and poorly understood.

The transmission of mitochondrial disorders is not always uniform, even though the mitochondrial genome is maternally inherited. Mutant mtDNA can arise de novo through the large-scale rearrangement of mtDNA without any familial history, or be maternally inherited as in the cases of mtDNA point mutations. Mitochondrial diseases can also follow an autosomal dominant pattern of inheritance that causes variable deletion of mtDNA or the expression of an autosomal recessive leading to profound cytochrome oxidase deficiency. Finally, X-linked transmission is also seen as a possible inheritance mode (Poulton, 1996).

The proportion of mutant mtDNA transmitted from mother to offspring is variable because of a genetic bottleneck. For example, during germ-line development in early bovine embryogenesis, the number of mitochondria increases 100-fold, from ~1000 per oogonium to ~100 000 per oocyte, while the number of mtDNA increases only ~10-fold, from 10 000 to ~100 000 (Chen et al., 1995). As result, each oocyte harbours ~1 mtDNA molecule, instead of the usual 5–10 (Chen et al., 1995; Robin and Wong, 1998). Only a small number of mtDNA molecules replicate and give rise to the entire cytoplasmic genotype (~100 000 mtDNA molecules) during the late stage of oogenesis (Hauswirth and Laips, 1985). This single restriction/amplification event or bottleneck may be a necessary solution to the accumulation of mtDNA during the ageing process (Cortopassi et al., 1992), an accumulation that also occurs in oocytes (Chen et al., 1995). During and after the mitotic division of cleavage, both mutant and normal mtDNA are distributed unevenly into the daughter cells that give origin to the fetus and its germ cells, thus enabling a complete switch within one or two generations (Meirelles and Smith, 1998).

As of now, there is no effective treatment for mitochondrial diseases. Following transfer by the cytoplasm fusion method of normal mtDNA into a cell containing mutated mtDNA (Kagawa and Hayashi, 1997), dilution of abnormal mtDNA to below the pathogenic level proved to be a potentially effective way of restoring both biochemical and morphological phenotypes of defective mitochondria. Pursuing the same logic, nuclear transplantation in human germ line cells may offer an attractive therapeutic alternative in providing normal mitochondria at least, in patients compromised by a point mutation disorder. Theoretically, reconstitution of oocytes with healthy donor cytoplasm could diminish the transmission of defective mtDNA. However, since the transplanted nuclei always carry a thin surrounding cytoplasm (Takeuchi et al., 1999a), a variable number of mitochondria may be transferred as well.
However, the first polar body might be a promising source of karyoplasts in which the mitochondria content is negligible (Tsai et al., 1999) and therefore be a preferable route to a minimization of mtDNA transmission. After injection of the first polar body, a mature enucleated mouse oocyte was later brought to a state of syngamy by ICSI and resulted in normal offspring (Wakayama and Yanagimachi, 1998).

Nearly all the known activities required for mtDNA replication and expression are nuclear-encoded gene products, necessitating communication between these two intracellular compartments. Several reports indicated that mitochondria located in the perinuclear vicinity are preferentially replicated or initiate replication at an earlier stage than those further away from the nuclei, and give rise to a higher ratio of karyoplast-derived mitochondrial genotypes in daughter cells (Davis and Clayton, 1996). The same observation was supported by an experiment using mouse pronuclear transplantation and later karyoplast-derived mtDNA assessment (Meirelles and Smith, 1998). Moreover, perinuclear mitochondria from avian and amphibian oocytes replicated more actively and appeared to segregate to the somatic cells of the fetus, while another subcortical group appeared to become localized in the primordial germ cells (D’Herde et al., 1995).

Despite evidence for preferential replication of karyoplast-derived mitochondria in animals, the question of interspecies differences must be resolved before extrapolating this to man. A recent report has suggested that the genotype of mtDNA from recipient cytoplasm may become the dominant category of mtDNA in calves resulting from nuclear transfer (Takeda et al., 1999). Out of 21 calves, 20 showed a genotype identical to that of the recipient cytoplasm mtDNA. What will be the exact contribution of the karyoplast-derived mtDNA in offspring coming from nuclear transplantation? Can nuclear transplantation using a GV karyoplast actually minimize the transmission of maternal mutant mtDNA? As yet, the unpredictability of mtDNA segregation and a complex threshold variety involved in the phenotype expression do not enable us to reach a firm conclusion.

If the transfer of cytoplasm and nuclei to oocytes becomes safe and effective, this would offer women facing IVF failure a new option through which to conceive their genetically related offspring. As a treatment to improve the developmental potential of the embryo, one could assume that cytoplasm transfer might have negligible impact on the genetic constitution of the offspring, since this involves so little mtDNA. In the case of nuclear transplantation, the biparental character of the reconstituted oocytes may have significant social, psychological and legal implications. Certainly, the significance of mitochondria transferred with a karyoplast, and the putative preferential replication of the perinuclear mtDNA, both need to be better understood to ascertain the exact contribution of the donor and recipient mitochondria in the children that result.

Conclusions
Alternative sources of gametes are not merely science fiction, but already are a concrete fact. As of now, it appears that most of the manipulations are unlikely to have any immediate major impact on assisted reproduction in man. However, while it is important to stress that the genetic normality of the offspring and the safety of the procedures tested in animal experiments must first be firmly established, the results in the latter are encouraging enough to justify further research.

First, the utilization of immature germ cells may represent the only possibility for treatment of many azoospermic men. Although the success rate of this approach is still low, reports of healthy deliveries using round spermatid injection (ROSI) provide clear evidence, in man as well as in mouse, that the morphological steps of sperm formation are not a necessary corollary for participation in syngamy. Furthermore, healthy deliveries in mice using primary and secondary spermatocytes, and in a report in man using secondary spermatocytes, indicate that even spermatogenic cell precursors can sometimes support normal development. On the other hand, the use of immature germ cells has raised concerns about the implications of incomplete genetic imprinting, and the unexplained short life span and retarded growth observed among some offspring when extremely immature germ cells (e.g. primary spermatocytes) were used in mice.

Xenogeneic testicular transplantation of human spermato- gonia may one day provide a valuable, if indirect option for treatment of patients with spermatogenetic arrest. Our failure in initial attempts to repopulate mouse seminiferous tubules with human spermatozonia may point to a need for a more suitable animal model. However, autologous spermatogonial transplantation has led to reinitiation of spermatogenesis within the seminiferous tubules in the cynomolgus monkeys (Schlatt et al., 1999). Therefore, autologous transplantation of spermatozonia may offer to oncological patients the possibility to reinitiate spermatogenesis with their own cryopreserved stem cell after completion of chemotherapy or radiotherapy.

Nuclear transplantation has proven to be a highly efficient procedure in mice, in that >90% of reconstituted oocytes were able to extrude a polar body and displayed a normal chromosomal constitution. With human oocytes, however, lower maturation rates have been the rule, probably due to the suboptimal procedures currently available for human oocyte in-vitro maturation. Nonetheless, nuclear transplantation might ultimately provide an attractive treatment option for the age-related aneuploidy seen especially in poor responders and in older patients.

Even more audacious is the attempt to tailor gametes by haploidization of nuclei from somatic cell sources, theoretically, at any time and at any age. Despite its possible promise, centrosome evaluation as well as genetic assessment of the ‘manufactured oocytes’ need to be further pursued to ascertain the potential clinical value of somatic cell haploidization.

Finally, since the role played by karyoplast-derived mtDNA in the mitochondrial phenotype of the offspring is still uncertain, the ethical and social implications of karyoplast transfer have to be taken into consideration before this can become even a relatively routine practice.

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References


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