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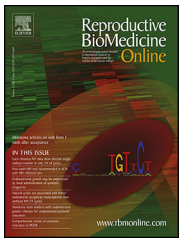

EDITORIAL

Can oocyte quality be augmented?

One of the great challenges in reproductive medicine today is the management of cases with poor-quality oocytes, a problem compounded by the falling ovarian reserve towards extinction as the menopause approaches. Donor eggs and embryos can bypass the problem, but not everyone accepts third-party genetic involvement. Preimplantation genetic screening to avoid aneuploid embryos is another option, and some young women now pin hopes on oocyte cryopreservation for preserving fertility to advanced reproductive ages, but neither procedure guarantees a good outcome. An ideal solution would be to repair or "rejuvenate" patients' own oocytes, but success with this approach has been elusive because the factors responsible for cellular health and competence are poorly understood.

Almost two decades ago, investigators tested whether transfer of cytoplasm from donor oocytes or zygotes can improve human oocyte quality (Barritt et al., 2001), an approach tried following evidence of reversal of the 2-cell block by a similar means during mouse embryo culture (Pratt and Muggleton-Harris, 1988). There were no confirmed candidates for the cytosolic factors or organelles supposed to be deficient, although mitochondria were chief suspects (Jansen, 2000) and it was hoped that a successful programme would lead to more specific treatment. At least 30 babies were born after IVF with cytoplasmic transfer by the time the procedure was suspended after coming under the purview of the US Food and Drug Agency (FDA) which said "any further ooplasm transfer protocol should be done under Investigational New Drug (IND) exemptions and an IND submission to the agency would be required to treat additional patients" amid concerns about biological safety and the ethics of creating children who inherit DNA from three parents, albeit only the tiny fraction of donor mitochondrial DNA (mtDNA) (http://www.fda.gov/ohrms/dockets/ac/02/briefing/3855b1_01.pdf). Two unexpected 45,XO conceptuses occurred in this small series, and unanticipated abnormalities emerged from animal studies of mitochondrial heteroplasmy (Lane, 2013). The developers of cytoplasmic transfer were rightfully cautious, acknowledging its experimental character and discouraging widespread application until more data were available, saying that "at present, there is insufficient evidence to demonstrate that any of these techniques is effective by itself" (Barritt et al., 2001).

A new technology called AUGMENT℠ that owes some of its rationale to cytoplasmic transfer has recently been launched by OvaScience, a company based in Boston (http://www.ovascience.com), which is also developing other fertility-enhancing technologies. Their product offers to "augment" oocytes with mitochondria transferred from putative ovarian stem cells (OSC; also called oocyte precursor cells) obtained from biopsies of the patient's ovarian cortex. After cryopreservation, thawing and enzymatic disaggregation, the mitochondria are isolated from OSC for injection into oocytes by an ICSI-like technique. According to the rationale, this infusion may boost ATP or reduce harmful reactive oxygen species (ROS) because the donor organelles are presumed to be better-preserved during their long dormancy.

OSC were discovered by a company founder and were announced triumphantly as ushering in a "paradigm shift in reproductive biology" (Woods and Tilly, 2013). They represent the kernel of the patent-protected technology, their use happily avoiding the potential hazard of heteroplasmy encountered previously in cytoplasmic transfers in animals. Nevertheless, the FDA reacted similarly to augmented oocytes, making the technology unavailable at this time in the USA and thereby unintentionally encouraging reproductive tourism to countries where it is licensed, including Canada where the first AUGMENT℠ baby was born recently (http://www.firststepsfertility.ca/services/augment/). Currently, an application to the Human Fertilisation and Embryology Authority (HFEA; http://www.hfea.gov.uk) seeks permission to open a clinical trial in Britain.

The commercial launch of AUGMENT℠ mostly received positive reviews in the media, has been welcomed by some physicians and undoubtedly raises the hopes of patients waiting for news of a breakthrough. On the other hand, voices in the reproductive science community have urged caution and expressed anxiety when the brand-new fertility treatment leapt suddenly from the laboratory to the clinic. The technology has two distinct parts, each of which we consider in turn.

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What evidence supports the existence of OSC and their physiological role?

Since publication of a classic paper by Solly Zuckerman in 1951 (Zuckerman, 1951), it was almost universally agreed that mammalian oogenesis is completed before or shortly after birth, depending on the species. Over the following five decades many studies confirmed the theory. It was therefore a shock when Johnson et al. (2004) at the Tilly Lab in Boston claimed that, rather than becoming extinct, oogonia (or OSC) persist in adult mouse ovaries and can restore follicles lost by atresia and ovulation. Nevertheless, these stem cells must have only limited durability because there was never any doubt that the follicle stock declines with age. In another astounding claim the following year, the same group presented evidence that the OSC are derived from bone marrow and circulate in the bloodstream from where they "seed" the ovaries (Johnson et al., 2005). Since these claims struck at the root of ovarian biology and had clinical implications, independent investigators designed rigorous studies involving transplantation, molecular phenotyping, genetic modification, oocyte tracing and mathematical models to verify or falsify Zuckerman's theory. Most studies found no signs of follicular renewal or derivation of germ cells from the circulation (Begum et al., 2008; Bristol-Gould et al., 2006; Eggan et al., 2006; Faddy and Gosden, 2007; John et al., 2007; Zhang et al., 2014), although it was hard to dismiss the possibility that a residue of stem cells hangs on through the lifetime of the ovary. There was, however, one Chinese study that appeared to have evidence for the existence and role of OSC after birth. Thus, using OSC isolated from postnatal mice and multiplied *in vitro*, Zou et al. (2009) claimed to have produced baby mice, but unanswered technical questions remain, the study has not been replicated independently and final proof of their physiological role is awaited (Grieve et al., 2015).

Bukovsky et al. (2005) proposed that new oocytes are formed in adult human ovaries based on observations in culture using germ-cell markers. But it was another paper from the Boston group that provided experimental evidence for the existence of OSC in postnatal human ovaries (White et al., 2012). They used anti-DDX4 (VASA) antibodies, to extract the alleged stem cells from the ovarian cortex, which were closely matched to the molecular phenotype of oogonia in fetal ovaries. Since the cells were rare they had to be multiplied in culture before xenografting to human ovarian tissue where they formed structures resembling primordial follicles. Since genuine follicles require granulosa cells, these results implied, again contrary to conventional scientific wisdom, that both oocyte and granulosa cell precursors survive to adult ages.

What conclusion can be drawn from these dramatic and sometimes conflicting studies? The balance of evidence strongly denies that new follicles are formed continuously after birth, but the survival of a population of stem cells of uncertain potency cannot be summarily dismissed. Indeed, their existence is not particularly surprising since stem cells are now known to be almost ubiquitous and provide many contemporary puzzles in biology. We need to know more about the lineage and potency of OSC.

OvaScience places OSC at the centre of their technology for improving oocyte health without needing a presumption of germline competence. However, and unfortunately, many technological details are proprietary information, and we have to deduce protocols from White et al. (2012). The cells were isolated from disaggregated tissue using antibodies to DDX4, a protein that is not required for oocyte development but specifically marks the germline as well as embryo stem cells, although the inability to form teratomas tends to confirm a germline rather than pluripotent character (White et al., 2012). Among many questions about the protocol, we wonder if enzymatic treatment of cells altered surface epitopes that would not otherwise recognize the anti-DDX4 antibody, and how the procedure isolates DDX4+ cells when the protein was thought to be intracellular (Albertini and Gleicher, 2015).

We urge the company to release details to fill knowledge gaps. We need to know if OSC are a residue of the canonical germline that differentiated in the fetal ovary or if they have an independent lineage (Baker, 1963). Could OSC be a sub-population of germ cells that failed to make the grade? And if a scattered distribution has prevented them from making syncytial relationships as in the founder population, does it matter? Finally, we ask whether OSC mitochondria really are more vigorous than in oocytes. The hypothesis is grounded on presumptive developmental quiescence, something that is not strictly equated with biochemical quiescence that is more likely to be protective. It is possible that the theoretical advantages of using undifferentiated, non-growing cells are lost when specimens are passed through a series of treatments for preparing the intact tissue and subsequently as the isolated cells adapt to culture conditions and multiply. Under such circumstances, might the phenotype change?

Can an infusion of mitochondria from OSC reverse poor oocyte quality?

While biological ageing evidently has pleiotropic causes, mitochondrial dysfunction is one of the strong candidates because the organelles are responsible for efficient generation of energy by oxidative phosphorylation (OXPHOS) and are exposed in the process to potentially genotoxic ROS. Their genes have, moreover, a much higher risk of mutation than the nucleus, the DNA of which is better protected by repair mechanisms and is cloaked in histone molecules (Wallace, 2010). Besides a key role in aerobic respiration, mitochondria are involved in other cellular processes, including calcium homeostasis and apoptosis.

There is experimental support for a mitochondrial hypothesis and therapy as a prescription for improving oocyte health and survival (Van Blerkom, 2011). For example lower levels of ATP are associated with changes in mitochondrial morphology, distribution and polarity in oocytes from older ovaries (Ben-Meir et al., 2015; Simsek-Duran et al., 2013; Van Blerkom et al., 1995). Since spindle assembly and function are energy-demanding processes, a deficiency of ATP might affect chromosome behavior leading to aneuploidy during meiosis and chaotic karyotypes during mitotic cleavage, perhaps tipping the important balance between ROS and oxidative defence in the cell (Eichenlaub-Ritter et al., 2011). The redox state of cells, as signaled by *Sirt1* expression, affects vulnerable mitochondrial targets for oxidative damage, and resveratrol

supplementation is correspondingly beneficial for oocyte health (Takeo et al., 2014). Through another mechanism, coenzyme Q10 (CoQ10) boosts respiration to preserve oocyte quality and quantity (Ben-Meir et al., 2015), and disruption of the nuclear gene *Pdss2* needed for CoQ10 production creates phenotypes that resemble aged oocytes, in which expression of the gene was reduced. Mitochondrial therapy cannot, of course, have a lasting impact when their dysfunction originates in mutations among the relatively larger number of nuclear genes required for organelle function.

When fully mature, the mouse oocyte possesses ~10^5 mitochondria, but this vast population probably has little redundancy and is not a particularly high density when cell volume is the denominator. In most species, this is a fixed endowment for preimplantation development because little or no net mitogenesis occurs before the blastocyst stage (Aiken et al., 2008). Afterwards, the organelles are distributed among the primary germ layers and primordial germ cells (PGC) during gastrulation at which stage a deficiency in their number or activity could be harmful, and perhaps an argument for an early infusion of fresh mitochondria.

The average human oocyte probably has more mitochondria than in mice, but they have a wide-ranging number of genomes per cell of up to an order of magnitude within a cohort from the same patient (Barritt et al., 2002). Perhaps eggs at the low end of the distribution are inadequately endowed, but this assumes an equivalence between number of genomes and mitochondria, which is unsafe until the number of mitochondrial chromosomes per organelle is known. Even so, it is not inevitable that a cell with fewer mitochondria will be impaired because the organelles are not equally active, depending on their niche and the stage of the cell cycle. Since the quality of oocytes in a cohort is heterogeneous it is desirable to identify cells with a moribund metabolism or overactive from stress for elimination or potential treatment, and this can already be done non-invasively by measuring pyruvate uptake and oxygen consumption (Harris et al., 2009).

There is far less information about mitochondria in OSC than in oocytes, but if they are homologous with PGC/oogonia we might expect their stock of organelles to be smaller than in most somatic cells, perhaps ranging from 10^1 up to 10^3 (Shoubridge and Wai, 2007). Regrettably few details are available for the AUGMENT^{SM} protocol, but it seems to be an immense challenge to harvest enough for treatment and would daunt experienced biochemists. Estimates of the number of mitochondria injected per cell and their purity are not public knowledge, although it is likely they are far fewer than in the 5–15% of donor egg cytoplasm used in the earlier cytoplasmic transfer experiments (Barritt et al., 2001), and only a tiny fraction compared to when pronuclei or spindles are transferred to enucleated eggs for patients with severe mitochondrial diseases where the goal is to replace nearly all the organelles (Gorman et al., 2015).

The main justification for augmenting oocytes has been to compensate for mitochondrial dysfunction. First we must consider, if only for dismissal, the question of whether female germ cells carrying harmful mtDNA are selectively eliminated in early development, for that would deny a general problem. Since this possibility is denied by the tragic evidence of inter-generational transmission of mitochondrial diseases to children (Shoubridge and Wai, 2007; Wallace, 2010) and in animal models where a majority of mitochondria are defective (Inoue et al., 2000), it appears that prenatal development is not absolutely dependent on OXPHOS. Of course, only maternal mitochondria are inherited. There is one (albeit chancy) compensation for an open pathway to pathogenesis, which is the rapid segregation of pathogenic mtDNA when mitochondria enter the "bottleneck" PGC stage resulting in highly variable heteroplasmy in oocytes and, hence, babies. Apart from these rare diseases (<1:5,000), what happens in normal ageing? According to data in mice, possession of only one or two chromosomes per mitochondrion would make the expression of recessive mutations more likely, but how common is pathogenic mtDNA, and what are the chances of an adverse phenotype, when it takes a critical threshold of 60% to 90% heteroplasmy to express serious harm in postnatal somatic cells (Shoubridge and Wai, 2007)? There is already good evidence of mutated mtDNA in human oocytes (Jacobs et al., 2007), but the extent and character should be investigated now for all stages of the germline, which is feasible by next-generation deep probing even in small single cells (Yao et al., 2015). Maybe these data will help to explain why the genetic load is not larger, and why damage accumulating in oocytes of older mothers is not passed down via PGC to put their grandchildren at greater risk (Elson et al., 2010). The evidence from mitochondrial diseases suggests there is no efficient filter for removing defective mitochondria, and yet an animal model suggests there can be selective elimination of harmful mutations (Fan et al., 2008). Thus, our understanding of how female germ cell health is safeguarded is still superficial.

A knockout mouse model for Leigh syndrome throws further light on how far development is independent of OXPHOS. When the nuclear gene *Surf1* was deleted to abolish cytochrome *c* oxidase activity, oogenesis and embryogenesis were hardly affected, but prenatal losses were heavy after gastrulation when there is greater demand for energy (Agostino et al., 2003). Oocyte mitochondria have few cristae (Sathananthan and Trounson, 2000), and, although they are not necessarily shut down under normal conditions, the cell may be able to shift to alternative pathways which may help explain some of the pathological data (Scantland et al., 2014). Taken together, these findings suggest that benefits accruing to augmented oocytes, if any, are unlikely to be observed in the embryology lab, and the main gains from treatment should be looked for after implantation, and from a spared biochemical pregnancy rate.

In reviewing a technology intended to augment oocytes it may seem perverse to consider whether it might actually cause harm, but we cannot anticipate all the implications of an invasive treatment of a complex cell. Mitochondria perform multiple roles, never act alone and are functionally compartmentalized: it would be naïve to compare a mitochondrial infusion with the administration of hormones or vitamins to relieve a known physiological deficiency. In oocytes they are differentiated compared to oogonia and most somatic cells, so we need to understand how OSC mitochondria behave in recipient cells and ask whether those from somatic cells would serve equally well. Perhaps transferred organelles respond to the ooplasm by shutting down OXPHOS to protect the cell from excess ROS, or maybe they continue functioning and raise ATP levels above an optimum predicted by the "quiet hypothesis" (Leese, 2002). Augmentation technology raises many questions for which there are, as yet, few answers.

Summing up

Among the most important questions we need to ask are: what do OSC represent; how many of them are recovered; how does their amplification affect them; how many mitochondria does each one contain; are their mitochondria healthy? And what is the evidence for a mitochondrial deficiency in the treated eggs; how is this diagnosed; how many mitochondria are being transferred; does transfer result in a significant boost to ATP; and how does the AUGMENT℠ protocol restore competence if aneuploidy is pre-existing at metaphase II in stimulated cycles? We need this information because mitochondrial metabolism is controlled at various levels in oocytes and may be unrelated to the number of actual mitochondria, for example being controlled by substrate availability, oxygen diffusion coefficients, ATP turnover, ATP synthase regulation, inner transmembrane potential and redox, to name but a few of the variables.

We are airing serious doubts about the AUGMENT℠ technology for improving oocyte quality based on the insecurities of our current knowledge about the character of the putative OSC and benefits claimed for mitochondrial transfer. If OvaScience has proprietary data to inform the controversy they should release them along with protocols for harvesting the cells and their mitochondria for independent testing and verification.

Those who worry that the technology has prematurely entered the clinic acknowledge that patients are already receiving treatment. The problem of poor-quality oocytes has prevented such patients from fulfilling their hopes of parenthood since the first days of IVF technology, and women at advanced reproductive ages may understandably regard the OvaScience technology as "a last chance", just as people have always reached for unsubstantiated therapies when orthodox medical care is helpless for intractable diseases, from Alzheimer disease to Zellweger syndrome. It would be arrogant to ridicule them for turning scientific opinion aside, and we ought to salute them with a respectful silence. If there is a silver lining to the product rolled out by OvaScience, it is that the company has drawn attention to a problem that needs much greater scientific effort and imagination.

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