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DNA damage in preimplantation embryos and gametes: specification, clinical relevance and repair strategies

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BACKGROUND: DNA damage is a hazard that affects all cells of the body. DNA-damage repair (DDR) mechanisms are in place to repair damage and restore cellular function, as are other damage-induced processes such as apoptosis, autophagy and senescence. The resilience of germ cells and embryos in response to DNA damage is less well studied compared with other cell types. Given that recent studies have described links between embryonic handling techniques and an increased likelihood of disease in post-natal life, an update is needed to summarize the sources of DNA damage in embryos and their capacity to repair it. In addition, numerous recent publications have detailed novel techniques for detecting and repairing DNA damage in embryos. This information is of interest to medical or scientific personnel who wish to obtain undamaged embryos for use in offspring generation by ART.

OBJECTIVE AND RATIONALE: This review aims to thoroughly discuss sources of DNA damage in male and female gametes and preimplantation embryos. Special consideration is given to current knowledge and limits in DNA damage detection and screening strategies. Finally, obstacles and future perspectives in clinical diagnosis and treatment (repair) of DNA damaged embryos are discussed.

SEARCH METHODS: Using PubMed and Google Scholar until May 2021, a comprehensive search for peer-reviewed original English-language articles was carried out using keywords relevant to the topic with no limits placed on time. Keywords included 'DNA damage repair', 'gametes', 'sperm', 'oocyte', 'zygote', 'blastocyst' and 'embryo'. References from retrieved articles were also used to obtain additional articles. Literature on the sources and consequences of DNA damage on germ cells and embryos was also searched. Additional papers cited by primary references were included. Results from our own studies were included where relevant.

OUTCOMES: DNA damage in gametes and embryos can differ greatly based on the source and severity. This damage affects the development of the embryo and can lead to long-term health effects on offspring. DDR mechanisms can repair damage to a certain extent, but the factors that play a role in this process are numerous and altogether not well characterized.
In this review, we describe the multifactorial origin of DNA damage in male and female gametes and in the embryo, and suggest screening strategies for the selection of healthy gametes and embryos. Furthermore, possible therapeutic solutions to decrease the frequency of DNA damaged gametes and embryos and eventually to repair DNA and increase mitochondrial quality in embryos before their implantation is discussed.

WIDER IMPLICATIONS: Understanding DNA damage in gametes and embryos is essential for the improvement of techniques that could enhance embryo implantation and pregnancy success. While our knowledge about DNA damage factors and regulatory mechanisms in cells has advanced greatly, the number of feasible practical techniques to avoid or repair damaged embryos remains scarce. Our intention is therefore to focus on strategies to obtain embryos with as little DNA damage as possible, which will impact reproductive biology research with particular significance for reproductive clinicians and embryologists.

Key words: DNA damage repair / gametes / sperm / oocyte / zygote / blastocyst / mitochondria / preimplantation / embryo / ART

Introduction

All biological cells are continually exposed to physiological and non-physiological stressors that cause damage to DNA. To compensate, robust DNA-damage repair (DDR) mechanisms are in place to detect damage, and repair it if possible or direct the cell to an activity profile that minimizes harmful effects of this damage. The activity of DDR mechanisms in many different types of somatic cells is well characterized. Less is known about DDR in reproductive and early embryonic cells, especially in preimplantation embryos, which are capable of developing independently from their external environment, utilizing stored maternal mRNA transcripts synthesized during oogenesis (Wrenzycki *et al.*, 2007).

DDR in the blastocyst is of paramount importance to prevent abnormal development in the embryo and foetus (Derijck *et al.*, 2008). It is believed that all major DNA repair pathways found in somatic cells are also active in embryos (Zeng *et al.*, 2004; Jaroudi *et al.*, 2009). Damage to DNA in embryos may result in an extended cell-cycle delay, with damage sensing being an important aspect of cell-cycle checkpoint control (Mu *et al.*, 2011; Pacchierotti *et al.*, 2011). Furthermore, the programmed cell death mechanisms, apoptosis and autophagy, are activated in response to received damage, as well as being crucial for normal embryonic development (Agnello *et al.*, 2015).

The increasing use of ART has recently raised concerns about unknown parental genome modifications affecting the development of the embryo and the future diseases in adulthood stemming from these gamete/embryo origins (Bonduelle *et al.*, 2005). In this context, an understanding of the blastocyst's capacity to repair damage would advise assisted reproduction practitioners and may help to produce healthy, viable embryos.

This review aims to summarize the sources of damage an embryo might be exposed to, the effects this damage has on development and viability and the DDR mechanisms active throughout the different stages of gamete and embryo development. Novel diagnostic and therapeutic measures allowing the detection and (whenever possible) repair of compromised embryos are suggested.

Methods

Using PubMed, a broad online bibliographic search for peer-reviewed original articles was carried out using keywords relevant to the topic with no limits placed on time. Keywords include 'DNA damage repair', 'gametes', 'sperm', 'oocyte', 'zygote', 'blastocyst' and 'embryo'. References from retrieved articles were also used to obtain additional articles. Literature on the sources and consequences of DNA damage on germ cells and embryos was also searched. Additional papers cited by primary references were included.

DNA damage in embryos and gametes

The origin of DNA damage in sperm

DNA damage can arise from many different origins, both from exogenous sources (e.g. ultraviolet and ionizing radiation, genotoxic substances) and endogenous sources (e.g. reactive oxygen species (ROS), DNA polymerase errors; Hakem, 2008; Tubbs and Nussenzweig, 2017). These insults can result in a wide variety of different types of damage, with specific agents tending to cause specific DNA damage. For example, DNA polymerase errors result in DNA base mismatches and single-base indels, whereas UV-C radiation tends to cause covalent linkages between adjacent pyrimidines (Chatterjee and Walker, 2017).

Like all cells of the body, gametes, zygotes and embryos are continually exposed to sources of DNA damage, although the sources of this damage and its severity are unique to these cells. The male gamete is more vulnerable to damage than the female gamete (Derijck *et al.*, 2008; Ménézo *et al.*, 2010). Damage in spermatozoa originates primarily from three processes (García-Rodríguez *et al.*, 2019): defective chromatin condensation during spermiogenesis (Marcon and Boissonneault, 2004); abortive apoptotic processes (Sakkas *et al.*, 2002); and oxidative stress (OS; Agarwal *et al.*, 2003). Chromatin condensation defects (i.e. inappropriate protamination) arise from chromatin remodelling during spermiogenesis, the purpose of which is to package the DNA into the smallest shape possible, altering sperm head size into an optimal configuration for motility (Miller *et al.*, 2010).

Due to the mitochondria and nucleus being in different cellular compartments, and the compacted nature of sperm chromatin, endonucleases (i.e. caspases) cannot reach the nucleus to initiate DNA fragmentation (Aitken and Koppers, 2011). Apoptosis in sperm is therefore mainly induced by ROS released from the mitochondria (De Iuliis *et al.*, 2009). Spermatozoa undergoing apoptosis have reduced motility and a decreased but not entirely absent ability to fertilize an oocyte (Aitken *et al.*, 1998). This phenomenon therefore allows DNA damage in the sperm to be passed into the developing embryo, often leading to impaired embryonic development and miscarriage (Aitken *et al.*, 2009).

Exogenous lifestyle factors such as diet, exercise and smoking can moderate levels of ROS, leading to effects on male fertility (Vujkovic *et al.*, 2009; Wright *et al.*, 2014; Jurewicz *et al.*, 2018). Ageing can also affect sperm quality: DNA fragmentation is elevated in sperm from older men compared with younger men, with an associated decrease in motility (Colasante *et al.*, 2019). Ionizing radiation is a known inducer of DNA damage in cells across the body. Sperm cells in men exposed to radiation exhibit higher fragmentation, lower motility and abnormal morphology compared to non-exposed men (Zhou *et al.*, 2016). As well as accidental or occupational exposure, sperm can accrue damage from chemotherapy. Despite the quick turnover rate of spermatozoa, damaged sperm can be found 2–5 years after cancer therapy has ended. This is due to damage induced in spermatogonia (sperm stem cells), which can be damaged by radiation and cytotoxic agents (Paoli *et al.*, 2018). However, spermatogonial stem cells seem to be highly resistant to DNA damage, possessing a lower average mutation rate than somatic cells (Murphey *et al.*, 2013). In a 2018 experiment, mice were treated with a chemotherapeutic regime that led to total azoospermia. In the ensuing 20 weeks after treatment ceased, spermatogenesis gradually recovered and high numbers of motile sperm could be observed, although their levels remained significantly lower than in the controls (Xavier *et al.*, 2018). In a nationwide register study conducted in Sweden between 1994 and 2014, it was observed that radio- or chemo-therapy did not increase the risk of congenital malformations in the offspring of men receiving treatment for testicular germ cell cancer (Al-Jebari *et al.*, 2019).

Whereas oogenesis is completed at birth in a female, in males, spermatogenesis is carried out throughout the male lifespan. It has been estimated that the average man produces over 525 billion sperm in his lifetime (García-Rodríguez *et al.*, 2019). Individual spermatogonia may undergo hundreds of rounds of replication, resulting in errors caused by DNA polymerase. These errors can accumulate over time in the spermatogonial genome, eventually passing into offspring (Gao *et al.*, 2016).

The origin of DNA damage in oocytes

Gamete production in females is drastically different to that in males, so oocytes face different risks of DNA damage. During embryonic development in females, primordial germ cells proliferate to form a resident population of primary oocytes (Spiller *et al.*, 2017; García-Rodríguez *et al.*, 2019). These then enter meiotic arrest until the female begins puberty, during which time they are metabolically inactive and less likely to acquire mutations through replication. When they begin dividing again during the pre-ovulatory stage of the menstrual cycle, they are once more vulnerable to damage (Zenzes, 2000).

Like sperm, oocytes are also exposed to potentially dangerous levels of ROS. In fact, oocytes are major producers of ROS due to their relatively high metabolism (Lopes *et al.*, 2010; Rocha-Frigoni *et al.*, 2016). Furthermore, controlled ROS levels are essential for normal reproductive functions such as oocyte maturation, folliculogenesis and luteolysis (Agarwal *et al.*, 2005). However, oocytes generally seem to be quite resistant to OS, probably due to the high antioxidant levels in follicular fluid (Carbone *et al.*, 2003).

Long-lived, non-replicating or slowly dividing cells are at higher risk of accruing non-repairable DNA damage over time. Due to being present in the body for around 50 years until menopause, oocytes are uniquely affected by ageing processes (Huber and Fieder, 2018). Oocyte quality is negatively correlated with ageing, which is characterized by morphological and cellular alterations (Sun *et al.*, 2018), and an increased vulnerability to OS and DNA damage (Mihalas *et al.*, 2017b). Aged oocytes can sustain high levels of damage from ROS produced by dysfunctional mitochondria (Bentov *et al.*, 2011; Li *et al.*, 2016; Min *et al.*, 2021). In normal mitochondrial function, a small amount of ROS is released due to electron leakage from the electron transport chain during the ATP production process (Goud *et al.*, 2008). This ROS eventually damages mitochondrial DNA (mtDNA) and leads to the presence of dysfunctional mitochondria in aged oocytes (Zhang *et al.*, 2017), resulting in ATP deficiency and elevated ROS production (Bentov *et al.*, 2011). ATP deficiency in oocytes influences processes such as spindle assembly checkpoint, chromatid separation and spindle detachment (Zeng *et al.*, 2007; Howe and FitzHarris, 2013). Impairments in these mechanisms contribute to aneuploidy and chromosomal misalignment (Liu and Keefe, 2002; Capalbo *et al.*, 2013). Oocytes from aged females facemuch greater risks of chromosome segregation errors, such as trisomy 21 (Wang *et al.*, 2011). The exact mechanism behind why this specific aneuploidy, which causes Down syndrome in affected offspring, is more prevalent in aged mothers has not been fully clarified. One theory is that trisomy 21 oocytes lag behind disomic oocytes in development and are more likely to escape timed apoptosis, and therefore constitute a greater proportion of the ovarian reserve in an aged woman (Hultén *et al.*, 2010). Other studies have pointed to specific polymorphisms that influence the risk of offspring being affected (Zampieri *et al.*, 2012).

The origin of DNA damage in embryos

Once fertilization successfully takes place between male and female gametes, a zygote is formed. The mitosis of the zygote results in the formation of the embryo as the first step of embryonic development. Both zygotes and embryos are vulnerable to damage by ROS, although they are naturally protected by the low oxygen concentration of the

oviduct and uterus (Burton *et al.*, 2003) and high antioxidant content of the follicular and tubal fluids (Burton *et al.*, 2003; Gupta *et al.*, 2006; Luddi *et al.*, 2016). Dietary intake of antioxidants such as zinc, selenium, vitamin C and vitamin E have been associated with positive effects on embryonic and foetal development (Tara *et al.*, 2010; Hovdenak and Haram, 2012). Supplementation of B vitamins such as cobalamin (Vitamin B12) to *in-vitro* culture media improves oocyte quality and increases expression of methyltransferases (Zacchini *et al.*, 2017).

One major hurdle in laboratory embryo culturing was eventually overcome after it was understood that ROS was the cause of the problem. Preimplantation embryos of many different species were arresting their development at a certain stage during *in-vitro* culture, giving rise to the so-called '2-cell block' (Zanoni *et al.*, 2009). A breakthrough study by Nasr-Esfahani *et al.* (1990) showed that a rise in ROS such as H_2O_2 at the maternal-zygotic transition, in combination with iron in the culture media, was causing membrane damage leading to developmental arrest and cell death. Solutions to circumvent the two-cell block include transfer of embryos to an oviduct (Whittingham and Biggers, 1967), cytoplasm transfer from a non-blocking strain (Muggleton-Harris *et al.*, 1982), the addition of iron chelators-like EDTA (Abramczuk *et al.*, 1977) and transferrin (Nasr-Esfahani *et al.*, 1990), and careful regulation of embryonic media components (Lawitts and Biggers, 1991; Gardner and Lane, 1996).

Gametes, zygotes and early embryos can also sustain damage arising from *in-vitro* manipulation, for example during ART. The removal of these cells from their protective habitat is the first hazard they face. For example, sperm is often centrifuged and removed from the antioxidant-rich seminal plasma. What is likely the most significant factor, however, is the non-physiological levels of O_2 present during most ART procedures (Agarwal *et al.*, 2006). Several studies have found associations between air quality parameters such as nitrogen oxide, carbon monoxide, sulphur dioxide, ozone and particulate matter and IVF success rates (Legro *et al.*, 2010; Choe *et al.*, 2018; Li *et al.*, 2020). One large-scale cohort study performed on women seeking ART therapies found associations between residential air pollution exposure and metabolites related to OS and inflammation, such as tryptophan and vitamin B3 (Gaskins *et al.*, 2021). UV and visible light may also induce OS damage on unsaturated phospholipids and cholesterol within cell membranes (Girotti, 2001). Several commonly used ART sperm treatment techniques such as incubation and cryopreservation have been shown to increase levels of DNA fragmentation (Dalzell *et al.*, 2004). It is thought that cryopreservation and vitrification of sperm and oocytes can lead to mitochondrial dysfunction and an increase in ROS generation (Dai *et al.*, 2015; Darr *et al.*, 2016).

Damage carried in the spermatozoa and oocytes can persist throughout fertilization and can make its way into the developing embryo. The nature and extent of this damage depend on its severity and the capability of each cell type to repair this damage. This will be discussed in the section 'DDR (DNA-damage repair) in cells and embryos'.

The impact of DNA damage on embryonic development

Outside of the necessary amount of genetic rearrangement and mutation required for evolution by natural selection to take place (Tubbs and Nussenzweig, 2017), DNA damage to spermatozoa or oocytes is universally negative. Levels of damage correlate with the likelihood of unsuccessful fertilization, miscarriage or impaired embryo quality (Ribas-Maynou and Benet, 2019; Rémillard-Labrosse *et al.*, 2020).

There is a strong relationship between DNA damage in sperm and infertility, although the exact nature of this relationship is complex. Unequivocally, spermatozoa DNA damage is detrimental for male fertility (Castilla *et al.*, 2010; Gunes *et al.*, 2015; Ioannou *et al.*, 2016; Leaver, 2016). Somewhat surprisingly, multiple studies have shown that damaged sperm are still able to fertilize oocytes, although the resultant zygotes have drastically reduced blastulation and pregnancy rates (Ahmadi and Ng, 1999; Fatehi *et al.*, 2006; Sedó *et al.*, 2017). This phenomenon is probably due to the fact that spermatozoa are (comparatively) not transcriptionally active, as their DNA is tightly packaged and generally inaccessible (Ren *et al.*, 2017), and any damage sustained will not impair their motility or oocyte activation ability, which is mainly driven by stored enzymes and high energy metabolism (Miki, 2007; Anifandis *et al.*, 2016). However, when exposed to very high levels of genotoxic insults, sperm cells eventually lose their ability to fertilize oocytes due to collateral peroxidative damage to the sperm plasma membrane (Aitken *et al.*, 1998).

The resilience of human oocytes is less well documented than spermatozoa, most likely because of the difficulty of obtaining oocytes for research purposes (García-Rodríguez *et al.*, 2019). However, it is widely accepted that the extended prophase meiotic arrest during the female's life until puberty constitutes a serious vulnerability to accumulating DNA damage (Mihalas *et al.*, 2017b). This arrest may also be a causal factor in menopause by limiting the overall 'shelf-life' of oocytes (Huber and Fieder, 2018). Animal studies have demonstrated that oocytes near ovulation are the most susceptible to radiation induction of mutational events (Jacquet *et al.*, 2005; Adriaens *et al.*, 2009).

Pre-pubertal oocytes exist in an immature state where fertilization and pregnancy can be achieved following *in-vitro* maturation, although the developmental competence is lower than post-pubertal oocytes (Revel *et al.*, 1995; Ptak *et al.*, 1999). Nuclear transfer from pre-pubertal lamb oocytes to adult sheep oocytes results in developmental arrest and failure due to incomplete DNA methylation profiling (Ptak *et al.*, 2006). Proper DNA methylation imprinting should therefore be investigated as a possible qualitative biomarker for oocytes retrieved from fertility preservation techniques such as ovarian tissue cryopreservation used on pre-pubescent girls (Martinez *et al.*, 2017; Yasmin *et al.*, 2018).

To prevent a developing embryo from inheriting damaged DNA, oocytes possess robust apoptosis pathways, controlled by transcription factors such as Trp63. In Trp63-deficient mice, the pro-apoptotic factors *Puma* and *Noxa* are not induced, even after γ-irradiation (Kerr *et al.*, 2012). Apoptosis also regulates the programmed degeneration, 'follicular atresia', of the ovarian follicles throughout a woman's life. The apoptosis regulator BAX (aka Bcl-2-like protein 4), which is expressed in both granulosa cells and oocytes, plays a central role in this process. Homozygous *Bax* knockout mice retain large numbers of primordial follicles throughout life and even into old age (Perez *et al.*, 1999).

The avoidance of DNA damage, especially in ART techniques, is important to prevent developmental abnormalities leading to birth defects. DNA damage in sperm has been shown to alter the metabolic profiles of preimplantation embryos used in ICSI (Uppangala *et al.*,

2016). Moreover, spermatozoa carrying extensive oxidative damage are still able to achieve fertilization when ICSI is used, raising concerns about the safety of the procedure (Twigg et al., 1998). If this oxidative damage is not repaired, it may give rise to abnormalities in the developing embryo; oxidative damage is suspected to play a causal role in the aetiologies of both foetal alcohol syndrome and thalidomide teratogenicity (Wani et al., 2017; Bhatia et al., 2019). Downregulation or knockout of DDR genes leads to increased teratogenicity of toxic compounds such as phenytoin and methamphetamine (Wells et al., 2010).

DNA-damage repair (DDR) in cells and embryos

DDR mechanisms

In order for cells to survive and propagate, they must be able to repair damage sustained to their DNA. DDR is therefore a crucial process for organisms to restore loss of function and avoid premature death. All three domains of life, *Bacteria*, *Archaea* and *Eukaryota*, have DDR mechanisms with some shared and some unique features (Weller et al., 2002; Bauer et al., 2015; White and Allers, 2018).

DNA damage can negatively affect cellular function. In the body, there are several mechanisms to minimize the effect of dysfunctional cells and prevent them giving rise to other disorders such as cancer (Table I). To accomplish this, it may not always be possible or necessary to fully repair the damaged DNA: simply preventing DNA replication in the cell may be sufficient. For this reason, some DDR genes, such as *p53*, also promote senescence and apoptosis in addition to initiating repair processes (Halazonetis et al., 2008; Lorda-Diez et al., 2019).

Although the same DDR mechanisms are present in all cell types within an organism, tissue-specific properties may be observed (Sun et al., 2019). This is because different cells will be exposed to different types and severities of damage based on their location and function in the body, and also because certain tissues may have differing levels of tolerance to DNA damage. For example, the rapidly proliferating cells of the small intestine are more prone to DNA replication mismatches, and so display high mismatch-repair (MMR) activity (Chao and Lipkin, 2006). Conversely, skin cells are frequently exposed to UV radiation and develop UV photoproducts, so have more active mechanisms for repairing them, such as nucleotide excision repair (NER; Melis et al., 2013).

DNA damage and ageing are linked together in an intricate relationship. It is thought that the ageing phenotype is caused by the accumulation of DNA damage over time (Kirkwood, 2005; López-Otín et al., 2013; Pan et al., 2016). The speed of the ageing process may be determined by this rate of accumulation and may be increased by defective DDR mechanisms or by more exposure to genotoxic insults (Nasto et al., 2013). *Caenorhabditis elegans* mutants with boosted DNA repair capacity exhibit resistance to oxidizing agents and UV radiation, and a longer lifespan than the wild type (Hyun et al., 2008).

Cellular senescence also plays an important role in the relationship between DNA damage and ageing. Senescence is a stable form of cell-cycle arrest that helps cells prevent genomic instability and the accumulation of damage (Calcinotto et al., 2019). Outside of its role in mammalian embryonic development (Muñoz-Espín et al., 2013), senescence is primarily a cancer prevention mechanism (Van Deursen, 2014). Senescence can be induced by a variety of factors: genotoxic insults, cancer, disease and DNA damage itself (Collado et al., 2007; Sturmlechner et al., 2017). It is thought that telomere shortening from the ageing process is also a primary trigger for senescence (Herbig et al., 2004). After successive cell replications, the protective t-loop structure at the ends of telomeres is lost, and the exposed chromosome end is recognized as a double-strand break (DSB; Wang et al., 2004). Once this occurs, senescence is activated through DDR pathways such as ATM, p53 and Mre11 (Takai et al., 2003). There is evidence that senescence may be a causal factor of ageing: aged tissues have a greater proportion of senescent cells than young tissues (Wang et al., 2009); and removal of p16(Ink4a)-positive senescent cells delays ageing symptoms in a progeroid mouse (Baker et al., 2011). However, some authors argue that senescence only contributes to ageing because old organisms lack a sufficient cell-replacement mechanism to compensate for cell functionality lost via senescence (López-Otín et al., 2013; Karin et al., 2019).

In addition to ageing and senescence, another main detrimental effect DNA damage has on an organism's health is induction of cancer. The link between exposure to toxic chemicals and incidence of cancer has been known for hundreds of years, at least since the work of 18th-century doctors such as Percival Pott and John Hill (Brown and Thornton, 1957; Lawley, 1994). Mutagenic agents that bind to cellular macromolecules and create DNA adducts give rise to tumours, although different chemicals are more likely to induce tumour formation than others (Otteneder and Lutz, 1999). The link between genetic alterations and cancer incidence is one of the major foundations of cancer research, especially since the discovery of oncogenes and tumour-suppressor genes (Vogelstein et al., 1988; Halazonetis et al., 2008; Basu, 2018). Many DDR-dysfunction diseases such as Xeroderma pigmentosum and Nijmegen breakage syndrome are characterized by high susceptibility to cancer (Seemanová and Jarolím, 1999; Black, 2016).

DDR in gametes

The repair of DNA damage is of critical importance to maintain fertility and embryonic viability, and for healthy development. To accomplish this, numerous DDR mechanisms are active in the germ cells, often at levels many times higher than background levels (Ménézo et al., 2010).

For a long time, it was thought that the extremely tight packaging of sperm DNA rendered them inaccessible to DDR enzymes (Ward and Coffey, 1991). We now know that NER, base excision repair (BER), MMR, DSB repair and post-replication repair mechanisms are all active in the male germ line (Gunes et al., 2015), but spermatozoa themselves have a very limited DNA repair repertoire. Some mechanisms are only partially present in sperm. For example, in BER, DNA glycosylases cleave aberrant base lesions, leaving behind an abasic site, which is then recognized by an apurinic/apyrimidinic endonuclease (Wallace, 2014). Spermatozoa do not contain any apyrimidinic endonuclease 1, so the abasic sites can only be fully repaired after fertilization when the spermatozoon reaches the oocyte (Smith et al., 2013; Aitken et al., 2014).

For oocytes, the extended length of time that they remain in a growth-arrested phase presents them with increased risk of DNA damage but more opportunities to repair it (Kerr et al., 2012).

Table I General features of DNA-damage repair mechanisms and recent evidence of their activity in reproductive cells/embryos.

DNA-damage repair mechanism	Type of damage	Source of damage	Main gene/protein pathways involved (in humans)	Evidence of activity in reproductive cells/embryos
Mismatch repair (MMR)	Non-complementary base pairs; base pair anomalies e.g. 8-oxo-guanine, UV photoproducts	Replication errors e.g. strand slippage; chemical damage of nucleotides	Mut proteins: MutS, MutH, MutL	High activity of MMR genes in murine embryonic stem cells (Tichy *et al.*, 2011) Transient expression of several key MMR genes in rhesus monkey oocytes and embryos (Zheng *et al.*, 2005)
Base excision repair (BER)	Non-bulky lesions: base adducts, abasic sites, chemically damaged bases: oxidized, alkylated, deaminated bases; uracil	Reactive oxygen species (ROS), spontaneous decay of DNA, environmental chemicals, radiation	DNA glycosylase, AP-endonuclease APE1, DNA polymerase β, *PARP1*, *XRCC1*, *OGG1*	Human sperm expresses OGG1 protein (Smith *et al.*, 2013) Several BER proteins are expressed in zebrafish embryos (Fortier *et al.*, 2009) Human and rat male germ cells can perform efficient BER (Olsen *et al.*, 2001)
Single-strand break (SSB) repair	Single-stranded breaks: loss of nucleotide, damaged 5' or 3' termini	Oxidative stress, formation of 8-OHdG, abasic sites, mis-repair of DSBs, failed antioxidant defence, abortive topoisomerase	*ATR*, *RAD17*, *TREX1*, *RPA2*, *BRCA1*, *APTX*, *XRCC1*	Several SSB repair mRNAs detected in rhesus monkey oocytes; *BRCA1* expressed throughout embryonic development (Zheng *et al.*, 2005) High expression of SSB repair mRNAs such as APTX in human GV stage oocytes (Menezo *et al.*, 2007)
Double-strand break (DSB) repair	DSBs, intra- and inter-strand crosslinks (ICLs)	Chemicals, genotoxins, lipid peroxidation, ionizing radiation, ROS, failed DNA replication	Non-homologous end joining (NHEJ): MRN complex (Mre11, Rad50, Nbs1), *XRCC4*, DNA Ligase IV, DNA-PKcs, Ku70, Ku80 Homologous recombination (HR): MRN complex (Mre11, Rad50, Nbs1), *BRCA2*, *ATR*	DSB repair mRNA templates for both NHEJ and HR detected in human MII oocytes and blastocysts (Jaroudi *et al.*, 2009) HR is utilized (more than NHEJ) in early swine embryos (Bohrer *et al.*, 2018) HR and NHEJ are both active in mouse zygotes (Derijck *et al.*, 2008)
Nucleotide excision repair (NER)	Bulky lesions e.g. pyrimidine dimers, thymine dimers, 6,4-photoproducts, intra-strand crosslinks	UV radiation, ROS, cancer therapeutics	*RPA1*, *RPA2*, *XPA*, XPB, XPC, XPD, Centrin-2, *DDB1*, *DDB2*, *ERCC1*, DNA Ligase I	NER proteins such as Rad23b are expressed in MII mouse oocytes (Wang *et al.*, 2010) Many NER mRNA transcripts are detected in human MII oocytes and blastocysts; higher expression in oocytes than blastocysts (Jaroudi *et al.*, 2009)

GV, germinal vesicle.

However, the number of studies investigating DDR mechanisms during human oogenesis is limited, and many questions have so far only been answered with regards to studies in animal models. Nevertheless, it is known that there are many DDR mechanisms active in the human female germline at every stage of development: from primordial follicles to post-ovulatory metaphase II-arrested oocytes and beyond into zygotes and embryos (Martin *et al.*, 2019). Enzymes for BER, NER, single-strand break repair, DSB repair and homologous recombination (HR) can be detected throughout germinal vesicle (GV) and M2 stage oocytes and in zygotes (Wang *et al.*, 2010; Martin *et al.*, 2019).

Due to ethical restrictions, studies involving the role of DDR in prepubertal cell populations are mainly restricted to animal models or data inferred from follow-up studies such as the Childhood Cancer Survivor Study (CCSS; Green et al., 2009). During the time that primary follicles are in a growing pool in preparation for maturation and ovulation, expression of *TP63* is high. This gene is responsible for sensing damage to the follicles and directing them towards apoptosis in response (Suh et al., 2006). Entry into the follicular growth phase coincides with a decrease in *TP63* expression levels and a marked increase in DDR genes (Martin et al., 2019). Exposure of rat ovaries to the follicle-damaging agent Bisphenol A has been shown to rapidly induce DSB repair genes such as *PRKDC*, *XRCC6*, *BRCA1*, *MRE11A*, *RAD50* and *SMC1A* (Ganesan and Keating, 2016).

Once oocytes reach the GV stage, they are arrested at the diplotene stage of prophase I until, following puberty, some follicles are selected for maturation and ovulation. During this time, the oocyte undergoes a drastic change in chromatin distribution resulting in transcriptional silencing. This event is critical for development: interruption of this process causes infertility in mice (Dumdie et al., 2018). It is thought that oocytes can carry out DNA repair at this stage despite no transcription taking place; this is because they possess a store of mRNA transcripts assembled during follicular growth (Jaroudi et al., 2009). These stored transcripts play important roles in maintaining the viability of embryos. DDR efficiency is lowered in aged females due to a reduction in the mRNA levels of stored DDR genes (Hamatani et al., 2004).

Nevertheless, DNA repair activity is decreased during this arrested stage: expression levels of ATM kinase in mature GV stage oocytes are considerably lower than at other stages of oocyte/blastocyst development (Marangos and Carroll, 2012). As a result, oocytes may enter into M-phase carrying with them damage that increases the risk of lowering embryo quality (Yuen et al., 2012; Ma et al., 2013). However, this increased tolerance of DNA damage may allow the oocyte to expend less energy on DDR and preserve a healthier metabolism (Martin et al., 2019). DNA-repair capacity may also be affected by species differences: rodents have a higher DSB repair capacity than primates (Wang et al., 2017).

Immediately after completing meiosis I, the oocyte initiates meiosis II and ovulation can occur. However, this process is halted at the metaphase II stage (M2 oocyte) and will not proceed unless the oocyte is fertilized. M2 oocytes are highly capable of performing DDR: using transcriptional profiling, high expression of genes involved in BER, MMR, NER, HR and non-homologous end joining (NHEJ) has been found in mouse, monkey and human M2 oocytes (Zeng et al., 2004; Zheng et al., 2005; Menezo et al., 2007; Jaroudi et al., 2009; Stringer et al., 2018). The DDR transcripts and proteins that are active in the early and arrested oocyte are also used in the zygote until the embryo genome is activated and it can begin transcribing its own DDR genes (Jaroudi et al., 2009; García-Rodríguez et al., 2019).

As already mentioned, sperm carrying DNA damage may still be able to successfully fertilize the oocyte. However, natural compatibility checks that act as barriers are in place to prevent heavily damaged or abnormal sperm from forming embryos (Springate and Frasier, 2017). In recent years, concern has been raised over the possibility that ART techniques such as ICSI bypass these natural compatibility checks and could increase the likelihood of offspring with developmental disorders (Georgiou et al., 2006; Schmid et al., 2007; Esteves et al., 2018). Even spermatozoa frozen for 15 years with severe cryodamage can still fertilize via ICSI; although in this case, the authors used mice to observe the normal offspring development (Ogonuki et al., 2006).

DDR in embryos

After fertilization has occurred, DDR activity in the zygote is increased, characterized by an up-regulation of the BER pathway from both sperm and oocyte BER enzymes (Lord and Aitken, 2015). This allows the zygote an opportunity to repair oxidative damage before the initiation of S-phase. Damage induced to embryos close to the start of S-phase, when DNA is replicated, is accompanied by a lengthier cell-cycle delay and higher number of mis-repaired DSBs, as measured by γH2AX foci (Pacchierotti et al., 2011).

Owing to the fact that DDR mechanisms are less robust in the spermatozoon than they are in the oocyte, DDR in the zygote is considered to be a maternal trait (Derijck et al., 2008). Damage carried in the sperm DNA is repaired using enzymes stored in the oocyte (Khokhlova et al., 2020). For example, DSBs in the male pronucleus are repaired mainly by HR using the female allele as a template (Ma et al., 2017). In fact, HR seems to be more commonly utilized than NHEJ in the early embryo; this is probably because NHEJ has a high risk of introducing deleterious mutations to the embryo (Bohrer et al., 2018). The importance of maternal DDR mechanisms can be clearly demonstrated with the use of maternal DNA repair mutant strains. Female mice with deficiencies in HR, NHEJ or MMR components produce embryos with more frequent chromosome/chromatid aberrations and microsatellite instabilities (Gurtu et al., 2002; Marchetti et al., 2007).

After fertilization, a significant genetic shift occurs in the zygote. It is generally understood that transcription is silenced in the oocyte until after the early divisions in the embryo, at which point transcription is reactivated in a minor and major wave characterizing the maternal to zygotic transition (MZT; Baroux et al., 2008). The timing of the MZT and the degree of transcriptional silencing in the oocyte may exhibit significant species-specific differences (Wang et al., 2014). In humans, MZT occurs at the 4–8 cell stage and progresses in a gradual manner: as the stored maternal mRNAs are depleted, zygotic transcription slowly compensates for their loss (Li et al., 2013). After activation of zygotic transcription, the embryo is once again sensitive to DNA damage and may utilize checkpoint kinase 1 (Chk1) to control the DDR response and regulate cell cycle arrest (Zhang et al., 2014). The DNA-repair gene *RAD18* is involved in suppressing DNA damage checkpoints and has a critical role in regulating the MZT at the mid-blastula transition (Kermi et al., 2015).

In humans, blastocyst implantation occurs roughly 9 days after ovulation. Communication between the blastocyst and the maternal endometrium via miRNAs and extra-cellular vesicles is important to mediate this process (Liang et al., 2017; Kurian and Modi, 2019). Once implanted, the embryo has access to the maternal nutrient and oxygen supply and a radical shift in metabolism is observed (Leese, 2012).

For DNA damage in a zygote's genetic material, there are three outcomes: it may be repaired; it may be passed into the offspring; or it may be so severe that the embryo fails to implant (Khokhlova et al., 2020). The type of damage and its severity affects which outcome will occur. It is thought that a certain level of damage in spermatozoa can

be repaired by the oocyte after fertilization. This capability is present even when oocytes are fertilized by sperm from a different species: when the DDR-inhibiting agent caffeine is added to hamster oocytes fertilized with human sperm, the frequency of chromosome aberrations increases (Genescà *et al.*, 1992; García-Rodríguez *et al.*, 2019). However, if the fertilizing spermatozoon is carrying more severe DNA damage, the chances of impaired blastocyst development and embryonic loss are drastically increased (Shoukir *et al.*, 1998; Dumoulin *et al.*, 2000; Seli *et al.*, 2004). Cell death may be the safest outcome for a zygote carrying severe DNA damage, but this should be a rare event (Jaroudi and SenGupta, 2007); apoptosis-induced cell death in a light-to-moderately damaged zygote would be unwarranted when DDR mechanisms exist. In fact, anti-apoptotic proteins of the BCL-2 family are highly expressed in zygotes and early blastocysts; their ablation may contribute to implantation failure (Opferman and Kothari, 2018). Instead, apoptosis is more frequently observed in late cleavage or blastocyst stages to remove damaged cells and protect the composition of the inner cell mass (Brison, 2000).

The phenomenon that damage-responsive apoptosis processes are more frequently observed in later stage blastocysts than earlier stages points towards temporal regulation of DNA damage response elements. Emerging evidence also indicates that different DDR mechanisms are active at different time points of preimplantation embryo development. Expression of three key MMR genes (*MSH2*, *MSH3* and *PMS1*) are higher in M2 oocytes than blastocysts, indicating a greater role of MMR at this stage of development (Jaroudi *et al.*, 2009). Cell cycle regulators p53 and p21 delay development of embryos fertilized by irradiated sperm at the zygote and morula/blastocyst stages, respectively (Adiga *et al.*, 2007).

While apoptosis inevitably leads to cell loss, autophagy may save the cell from this fate. Autophagy literally 'self-eating' allows the cell to remove damaged organelles and is a crucial aspect of cellular repair mechanisms. Autophagy and DNA repair are closely linked: DNA damage can induce autophagy; autophagy can stimulate DDR activation (Nazio *et al.*, 2018; Guo and Zhao, 2020). In embryonic studies, the strongest links to autophagy have been observed in mitochondria. Dysfunctional or damaged mitochondria can lead to accumulation of DNA damage; autophagy of mitochondria (mitophagy) is therefore important for preventing DNA damage and also for regulating the number of mitochondria required for healthy cellular function (Rambold and Lippincott-Schwartz, 2011). Blastocysts exposed to environmental toxicants such as polychlorinated biphenyls display diffuse mitochondrial damage and a subsequent up-regulation of autophagy markers (Ptak *et al.*, 2012). Also, induction of autophagy via rapamycin and pp242 enhances the development of embryos following somatic cell nuclear transfer, thereby improving cloning efficiency (Shen *et al.*, 2015). Our studies have indicated that placental autophagy in growth restricted androgenetic embryos (having only paternal genes) is regulated by the paternal genome. Conversely, autophagy is practically absent in respective placentae of normal size parthenogenetic embryos (having only maternal genes); instead, parthenogenetic embryos display heightened apoptosis (Ptak *et al.*, 2014). We believe this increased autophagy and apoptosis was caused by up-regulation of aplasia Ras homologue member I (*ARHI*) and pleckstrin homology-like domain family A member 2 (*PHLDA2*), respectively. High autophagic activity in early placentae can be a successful temporary counterbalance to the reduction of foetal growth and retarded vasculogenesis observed in pregnancies after transfer of *in-vitro* cultured embryos (Toschi *et al.*, 2016).

All-in-all, embryos and gametes have many different mechanisms and pathways to prevent damage and promote healthy development, but even isolated dysfunctions can severely impact viability (Table I). Dysregulation of DDR genes and mechanisms in somatic and germ cells not only predisposes to cancer and rapid-ageing diseases but also affects fertility. *BRCA1* in particular seems to be an important regulator of ovarian ageing and a defender of oocyte survival (Titus *et al.*, 2013). Genetic variation in DDR mechanisms has also been implicated with the timing of menopause onset (Day *et al.*, 2015; Gajbhiye *et al.*, 2018). The MMR gene *MLH1* is critical for female fertility in both mice and *Drosophila melanogaster* (Baker *et al.*, 1996; Vimal *et al.*, 2018). Several studies have investigated the importance of DDR mechanisms by down-regulating their activity and observing the effects on oocyte fertility and embryo development. Unsurprisingly, this leads to impaired blastocyst formation and quality (Bohrer *et al.*, 2018). Knockdown of the DDR regulator cyclin-dependent kinase 12 (*CDK12*) leads to embryonic lethality caused by a collapse of the replication fork and an overabundance of unrepairable DSBs (Juan *et al.*, 2016).

Diagnostic and therapeutic strategies of embryonic DNA damage

Diagnostic strategies of embryonic DNA damage

The accumulation of DNA damage in developing zygotes is widely known to decrease their viability. In fertility clinics across the world, evaluation of oocyte/embryo viability is a critical step of ART methodology. There is no established global consensus on how to assess embryo viability, and different clinics around the world utilize different methods, although some are subject to governmental, national or international supervision (Balaban *et al.*, 2011). However, assays of DNA damage are rarely used (Lundin and Ahlström, 2015). Instead, most assessments of embryo viability focus on morphological parameters, such as cell number and size, cytoplasmic granularity, shape anomalies, etc. Where DNA damage is concerned, it is primarily encompassed in the category of 'sperm quality' and not investigated in the oocyte or embryo (Gardner and Sakkas, 2003; Lundin and Ahlström, 2015). Having said that, some assessment criteria include screening for chromosome abnormalities (Gardner *et al.*, 2015).

In 2002, the 'Quiet Embryo Hypothesis' was put forward by Leese, who argued that on the basis of nutritional markers (e.g. amino acid turnover) of normal and impaired development, embryos with high levels of metabolism exhibited lower viability (Leese, 2002). He based this theory partly on evidence suggesting that caloric restriction down-regulates metabolic rate and slows the generation of ROS (Masoro, 2000; Raha and Robinson, 2000). Leese's hypothesis was later linked to DNA damage by Baumann *et al.* (2007) who speculated that embryos receiving more severe damage must devote more resources to repair and maintenance processes, and would therefore have high metabolic activity. A further experiment found a correlation between

DNA damage in human embryos and amino acid metabolism (Sturmey et al., 2009). Moreover, this damage was not accompanied by morphological defects, so would not be recognized by traditional embryo grading techniques. Amino acid profiling (AAP) is a promising embryo grading technique in that it is non-invasive and has a low chance of causing iatrogenic damage. Several studies have shown that this technique can predict implantation potential and developmental competence (Brison et al., 2004; Hemmings et al., 2012; Huo et al., 2020). However, AAP still faces some difficulties before it can be widely used in clinical settings: it relies heavily on high-performance liquid chromatography, which necessitates long assay times and uses inorganic solvents unsuitable for IVF clinics (Leese et al., 2021).

One aspect of DNA damage that is almost always overlooked when considering embryo viability is mitochondrial health status. The diagnosis of mitochondrial mutations is based mainly on patient and family history despite the fact that mitochondrial diseases have very different phenotypic effects and many mutations are unique to one patient (Gorman et al., 2015). Additionally, the diagnosis of maternal mitochondrial abnormalities is complicated due to the fact that different tissues can contain different proportions of mutated mtDNA, thereby affecting the result depending on which tissue was analysed (Naue et al., 2015). Women with no obvious symptoms of disease and an average content of mutated mtDNA may produce gametes with high levels of mutations thereby making the problem more complicated (Larsson et al., 1995; Cree et al., 2009).

Embryo and oocyte quality assessment remains a great challenge of ARTs exacerbated by the lack of appropriate diagnostic techniques. It is known that ARTs themselves can also cause additional DNA damage that compromises oocyte and embryo quality (Ramos-Ibeas et al., 2019). The currently used embryo quality assessment techniques such as blastomere biopsy are controversial and pose additional health risks to offspring (Zacchini et al., 2019). Thus, new imaging techniques based on non-invasive and non-destructive spectroscopy are needed to allow for a direct evaluation of both oocyte and embryo genome quality in preimplantation diagnosis.

As already mentioned, aged oocytes are at a heightened risk of oxidative damage, in particular due to dysfunctional mitochondria. Elevated ROS production catalyses the peroxidation of stored lipids, which produces electrophilic aldehydes (4-hydroxynonenal, malondialdehyde). Exposure of oocytes to lipid peroxidation products causes a cascade of events involving mitochondrial ROS production and lipid peroxidation, leaving the oocytes in a strong OS condition which will irreversibly deteriorate their quality (Lord et al., 2015; Mihalas et al., 2017a). Aged oocytes are negatively affected by shortened telomere lengths resulting from the accumulation of OS and DNA replication during foetal life (Keefe et al., 2007). Oocytes from aged mice exhibit high numbers of DSBs, chromosome abnormalities (Li et al., 2008) and increased OS-damage sensitivity due to down-regulation of DDR mechanisms (Titus et al., 2013).

In aged oocytes, the problem of lipid peroxidation is exacerbated by a high lipid content. Our unpublished data (Fig. 1) have demonstrated that maternally aged mouse oocytes tend to have higher amount of lipids than oocytes from young mice, and moreover, they possess more numerous lipid droplets of the biggest size (10–100 μm^2) in comparison to young oocytes. As in other cellular systems, these large droplet clusters represent a deteriorated lipid fraction, indicating a compromised developmental potential.

Therapeutic strategies of embryonic DNA damage

In recent years, novel techniques have been proposed to minimize or reverse the level of embryonic DNA damage with the aim of improving embryonic developmental potential. Work in our laboratory has

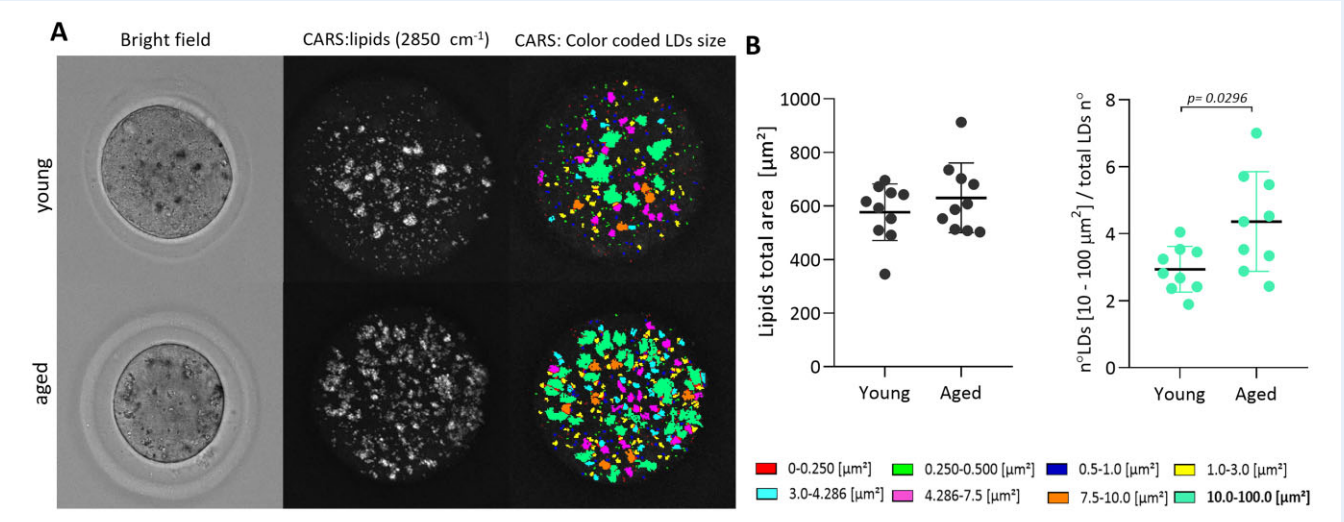

Figure 1. Lipid content increase in aged oocytes. (**A**) Higher amount of lipids in aged oocytes demonstrated by anti-Stokes Raman spectroscopy (CARS) microscopy. Legend indicates the colour associated with each of the eight lipid droplets (LDs) size ranges. (**B**) Histograms show that the LDs total area tends to increase in aged oocytes and a significant increase in 10–100 μm^2 lipid droplets is seen in maternally aged oocytes. Values represent mean ± SD; Student's *t*-test performed.

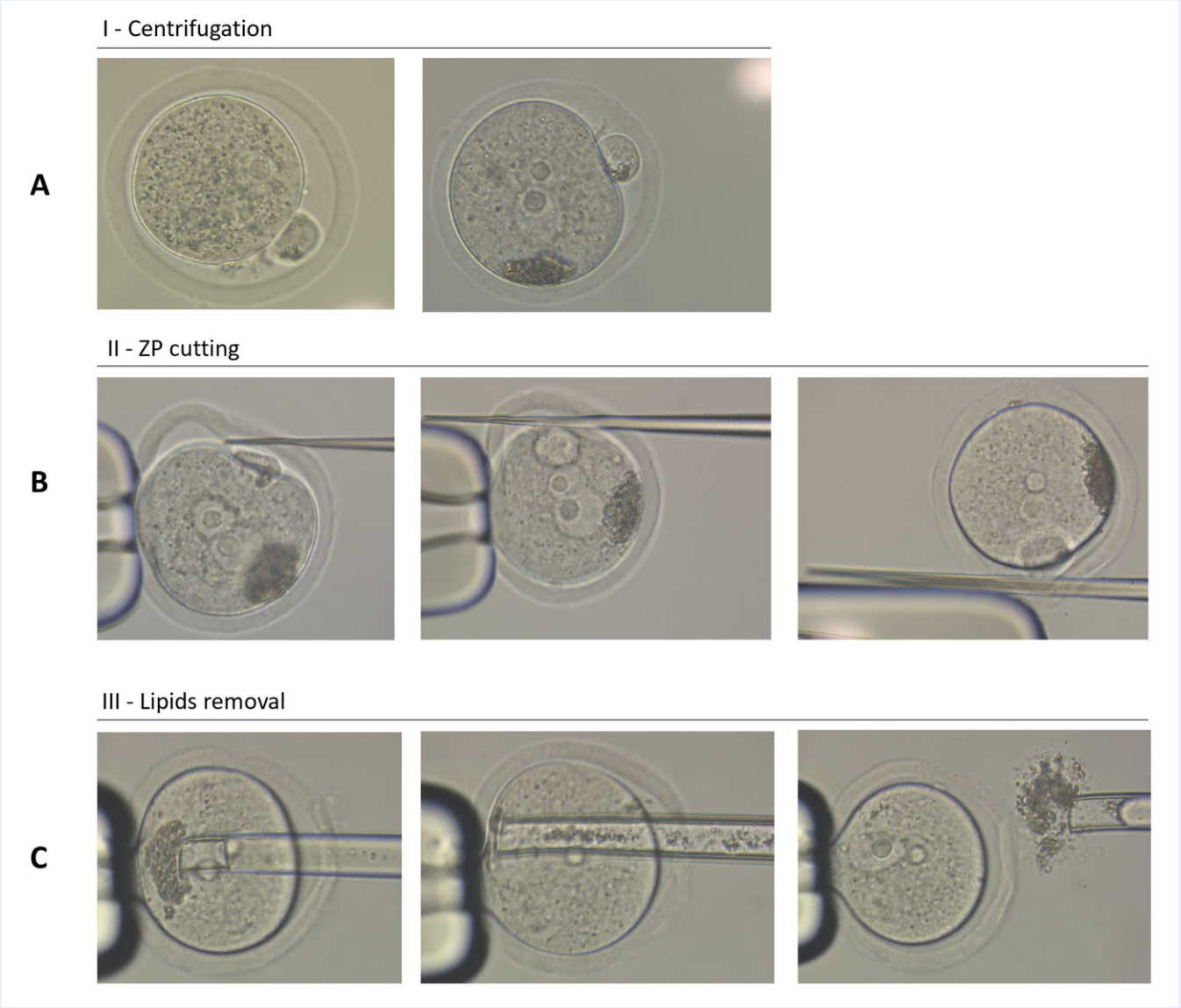

Figure 2. Lipid removal method. (**A**) Polarization of lipids by centrifugation. (**B**) Zona Pellucida (ZP) cutting mechanically executed to permit removal of cytoplasmic lipids. (**C**) Removal of the lipids by aspiration via micromanipulation.

led us to believe that the removal of lipids from aged oocytes/zygotes can be used as a method to reverse the ageing process and restore developmental competence to the same level of young oocytes/zygotes (Fig. 2). In this way, it is possible to block the peroxidation which has been associated with low embryo development outcome rates (Reis *et al.*, 2003). Mechanical de-lipidation of the oocyte allows the removal of big clustered droplets (around 60% of total lipids; Bisogno *et al.*, 2020). This technique has been confirmed to be a safe method without negative effects on embryo and offspring development in oocytes from pigs (high lipid content) (Nagashima *et al.*, 1995) and mice (low lipid content; Arena *et al.*, 2021). As oxidizable substrates such as pyruvate and other amino acids are the main energy substrate used by the preimplantation embryo (Leese and Barton, 1984; Thompson *et al.*, 1996), and lipids are essentially needed only in conditions of delayed implantation (Arena *et al.*, 2021), the removal of deteriorated lipids constitutes an advantageous strategy for improving the quality of compromised oocytes.

Other cellular components are also targets for therapeutic strategies to lessen DNA damage. Dysfunctional and damaged mitochondria, especially in aged oocytes, are a major source of DNA damage inherited by blastocysts and embryos. Therefore, genome-editing tools that can be used to correct mtDNA abnormalities may represent a valuable resource for improving embryonic potential in an ART setting. Mitochondria do not have very robust DDR mechanisms, and a common outcome following the generation of DSBs is the elimination of mtDNA particles from the cell by mitochondrial exonucleases (Minczuk *et al.*, 2008; Moretton *et al.*, 2017; Peeva *et al.*, 2018). This phenomenon has been used to design proteins that specifically target and bind mutated mtDNA, to introduce DSBs and facilitate their degradation (Fig. 3).

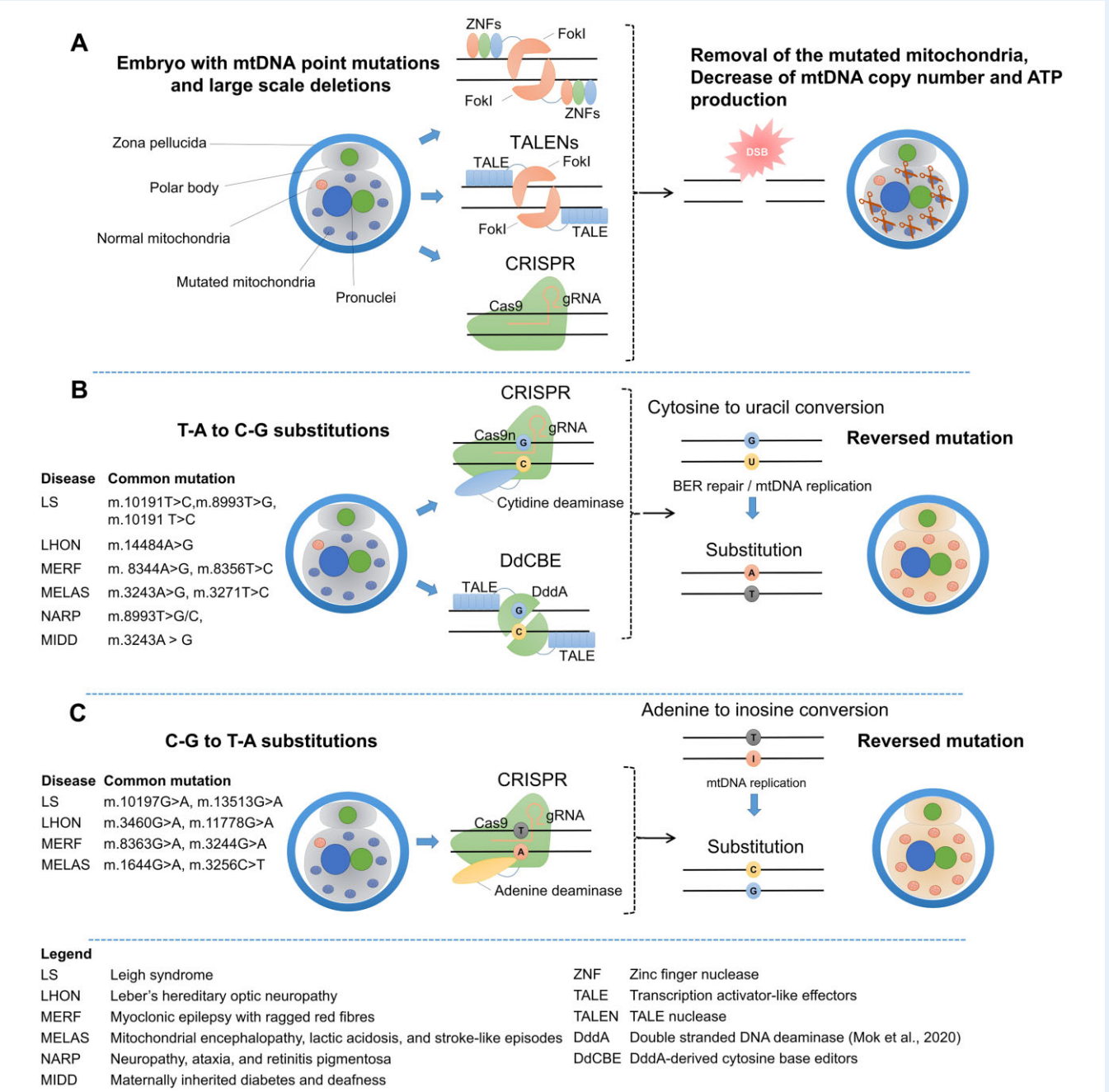

Figure 3. Gene editing methods for the removal of the mitochondrial (mt) DNA mutations. (**A**) Removal of mutated mtDNA via double-strand break (DSB) introduction with the use of ZNFs, TALENs or CRISPR. (**B**) Reversal of mtDNA mutations with the use of CRISPR and TALENs cytosine base editors (CBE) in base excision repair (BER). (**C**) CRISPR-based adenine base editor (ABE). The diagram shows examples of mtDNA mutations associated with human mitochondrial diseases Leigh syndrome (LS), Leber hereditary optic neuropathy (LHON), myoclonic epilepsy with ragged red fibres (MERRF), and mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS), neurogenic muscle weakness, ataxia and retinitis pigmentosa (NARP) and maternally inherited diabetes and deafness (MIDD), as reviewed in Mustafa *et al.* (2020). mtDNA, mitochondrial DNA; TALENs, transcription activator-like effector nucleases.

The first of these approaches combined highly specific zinc-finger proteins with non-specific restriction nucleases. The resulting fusion protein, known as zinc-finger nuclease (ZFN), can be designed to introduce a DSB into a desired target locus of the mutated mtDNA and allow subsequent elimination of the linearized mtDNA by the cellular mechanisms (Moretton *et al.*, 2017; Peeva *et al.*, 2018). This strategy was used for the first time in 2008 to selectively degrade a point mutation responsible for the development of mtDNA diseases, Leigh and

neurogenic muscle weakness, ataxia, and retinitis pigmentosa (NARP) syndrome (Minczuk *et al.*, 2008).

The next generation of mtDNA editors was based on transcription activator-like effectors (TALEs), a group of proteins found in bacteria *Xanthomonas* to aid the infection of plant species. Compared to ZNFs, TALEs possess a simpler, more customizable structure of the DNA-binding domain, which makes them easier to design for specific mutation sites (Miller *et al.*, 2011). The subunits of TALEs can be combined with Fok nuclease to create the fusion protein known as TALE nuclease (TALEN). TALENs are an ideal tool to introduce DSBs into mutated mtDNA (Cermak *et al.*, 2011; Hockemeyer *et al.*, 2011). In a landmark 2013 study, TALENs were used to bind and cleave the breakpoint region of an mtDNA with a large 5 kb deletion in human cells. The attempt was successful and led to a drastic decrease in the mutated mtDNA level (Bacman *et al.*, 2013).

Two years later, TALENs were successfully used to degrade specific mtDNA sequences in a heteroplasmic mouse oocyte model (Reddy *et al.*, 2015). Oocytes containing two mitochondrial haplotypes derived from BALB and NZB mice were injected with mRNA encoding mitochondria-targeting TALENs. This procedure resulted in the depletion of the selected mitochondrial haplotype, thus preventing haplotype transmission and offspring heteroplasmy. The same team fused mouse oocytes with human fibroblast cells carrying one of two mitochondrial disorders, Leber's Hereditary Optic Neuropathy or NARP, and demonstrated that TALENs could even be used to selectively degrade human mtDNA sequences inside mouse oocytes. In this case, the mutant mtDNA gene was not completely eliminated, but the mutation load, 'heteroplasmy shift', in these hybrid cells was drastically reduced (Reddy *et al.*, 2015).

In recent years, CRISPR/Cas9 has shown some promise in several mitochondrial genome-editing experiments. However, despite being a robust nuclear genome-editing tool, CRISPR methodology had found difficulty in the delivery of guide RNA (gRNA) to the inside of the mitochondrion (Loutre *et al.*, 2018; Jeandard *et al.*, 2019). Fortunately, the problem was solved by Hussain and colleagues by combining an appropriate mitochondrial localization sequence with gRNA. This system was tested on a model *MT-ND4* mtDNA gene mutation present in mouse embryonic fibroblasts, leading to a reduction in heteroplasmy (Hussain *et al.*, 2020).

Removal of mutant mtDNA molecules via the introduction of DSBs seems to be a promising approach for mtDNA-editing approaches. However, this methodology relies on the assumption that after mutated mtDNA removal, the number of remaining mitochondria will be sufficient for proper cell functioning, or that the remaining molecules will serve as the template for the creation of new 'healthy' mitochondria that can replace the mutated ones (Moretton *et al.*, 2017; Peeva *et al.*, 2018). While this may be the case in somatic cells, this assumption could be problematic in germ cells. It is believed that mitochondrial replication occurs only during the oocyte growth phase, so the ovulated oocyte cannot replicate its mitochondrial genome (St. John *et al.*, 2010). Furthermore, the mutation load in some oocytes may be high enough that the removal of mutated mtDNA would leave the cell with a dangerously low mtDNA copy number (Blok *et al.*, 1997; Brown *et al.*, 2001). It is known that low oocyte mtDNA copy number can affect many factors including oocyte meiotic division, IVF outcome, embryo development and embryo implantation (May-Panloup *et al.*, 2005; Santos *et al.*, 2006; Zeng *et al.*, 2007; Fragouli *et al.*, 2015). A critical threshold of oocyte mtDNA content (~50 000 copies) is thought to be required for fertilization and embryonic development in mammals (Wai *et al.*, 2010).

Thus, new techniques are currently being sought to allow editing without resulting in DSBs and mitochondrial genome degradation. Recently, two types of base editors have been described: cytosine base editors (CBE) and adenine base editors (ABE; Fig. 3). Both of them together can be used to engineer the reversal of all possible point mutations in the genome without introducing DSBs (Komor *et al.*, 2018). CBE fusion proteins are a combination of Cas9 endonuclease with cytosine deaminase from the APOBEC1 family (Lee *et al.*, 2021). CBE mediates deamination of cytosine which leads to the conversion of cytosine to uracil. The uracil is subsequently recognized by DNA replication machinery as thymine, resulting in a C-G to T-A transition (Komor *et al.*, 2018). In complementary fashion, ABE can convert an A-T base pair back into a G-C base pair.

In two early experiments, CBE and ABE induced point mutations into mouse zygotes which resulted in the creation of Duchenne muscular dystrophy and Alzheimer's disease mouse models (Kim *et al.*, 2017; Sasaguri *et al.*, 2018). Later, ABE was used to correct a nonsense mutation in the Duchenne muscular dystrophy gene in mouse embryos and adult mouse tissues (Ryu *et al.*, 2018). This shows that the newly developed technology is precise enough not only to introduce mutations but also to reverse them in the nuclear genome. As with CRISPR, CBE and ABE have faced difficulties in passing Cas9 gRNA through the mitochondrial membrane. A solution was found last year using CBE in combination with an interbacterial toxin. In their ground-breaking study, Mok *et al.* (2020) combined a newly discovered deaminase toxin together with DNA-binding TALE proteins and a mitochondrial localization peptide to create a fusion protein called 'DddA-derived cytosinebase editor' (DdCBE). With the use of DdCBE, the team was able to correct up to 27% of mutant mtDNA carrying *MT-ND6* gene mutation in human cells without changing the mtDNA copy number. Although the authors reported low off-target activity, both within mtDNA (up to 0.13%) and in the nuclear genome, additional research and testing are required to ensure that DdCBE can be used safely as a therapeutic tool.

Although it seems promising, this technology is far from being adopted in ART. Unfortunately, there is evidence that inactivated Cas9 protein can disrupt DNA replication, causing DNA structural abnormalities including mutations. In addition, there are reports of off-target activity in CRISPR-derived technologies leading to the generation of insertions and deletions in random positions of the genome (Doi *et al.*, 2021; Lee *et al.*, 2021). Reliable mutation editing could one day be a possibility in ART, and practitioners may be routinely considering mtDNA factors such as heteroplasmy level in their patients' germ cells, but for the time being, this methodology ultimately needs to be improved and standardized to improve reliability and reduce discrepancies between experimental results (Kantor *et al.*, 2020).

Non-invasive methods for DNA damage screening

Spent media evaluation

Many of the most well-established and commonly used cellular DNA-damage detection techniques are unsuitable for determining embryonic

potential. For example, immunofluorescent labelling of phosphorylated histone H2AX at serine 139 (γH2AX), as well as analysis of DDR proteins such as ATM, ATR, DNA-PKcs, Chk1, Chk2 or Rad51 (Rinaldi *et al.*, 2017; Stringer *et al.*, 2020), requires cellular fixation and thus cannot be used for embryo grading. The comet assay is another popular analytical technique that is also is unsuitable, given that it requires cell lysis prior to gel electrophoresis (Cortés-Gutiérrez *et al.*, 2017).

DDR machinery requires the uptake of specific nutrients from the environment (Sturmey *et al.*, 2009). Thus, disturbances in the uptake and release of specific metabolites in embryonic cell media can provide information about DNA damage. The profiling of spent media by nuclear magnetic resonance spectroscopy revealed that embryos stressed with the genotoxic agent cisplatin undergo extensive uptake of pyruvate, lactate, glucose and amino acids proline, lysine, alanine, valine, isoleucine and thymine (D'Souza *et al.*, 2016, 2018). The same technique showed that embryos derived from spermatozoa carrying DNA lesions exhibit increased pyruvate uptake from the spent media and release less alanine compared to control embryos (D'Souza *et al.*, 2019).

Besides nutrient uptake analysis there have been promising studies based on the measurement of embryo respiration, which may directly reflect the mitochondrial condition of the embryo (Hiramoto *et al.*, 2017). As mitochondria are the main intracellular oxygen consumers, the rate of oxygen uptake may reflect mitochondrial activity, condition and ATP production (Brand and Nicholls, 2011). Oocyte and embryo respiration can be analysed using techniques such as scanning electrochemical microscopy (Shiku *et al.*, 2001; Goto *et al.*, 2018), Seahorse device (Muller *et al.*, 2019) and electrochemical chip devices configured for the measurement of single embryo respiration (Hiramoto *et al.*, 2017). A series of recent studies has indicated a correlation between human oocyte respiration rates and embryo viability (Tejera *et al.*, 2011, 2012; Goto *et al.*, 2018). Still, those studies have almost exclusively focused on general gamete and embryo quality, leaving further questions unanswered about whether cellular oxygen consumption can reflect DNA damage or mitochondrial mutation status.

Another way to non-invasively assess embryo quality is the measurement of nucleic acids in the culture medium. Genomic and mitochondrial circulating free DNA (cfDNA) can be detected in the secretome of human cleavage-stage embryos (Stigliani *et al.*, 2013). So far, the effectiveness of this methodology is limited, due to the very small amounts of nuclear DNA and mtDNA in the media and interference with DNA from other sources, such as from granulosa cells (Hammond *et al.*, 2017; Zhang *et al.*, 2019). However, this technique does allow genotyping via gene variants, and the amount of mtDNA in the culture medium may be representative of the mtDNA content in the embryo itself (Zhang *et al.*, 2019). Thus, it is possible to screen the spent media for nuclear (n) DNA and mtDNA and thereby evaluate DNA mutations; however, no study to date has examined the correlation between mtDNA in spent media and embryo heteroplasmy. Nevertheless, nuclear cell-free DNA has been found to facilitate the diagnosis of X-link, thalassemia and cystic fibrosis mutations (Assou *et al.*, 2014; Liu *et al.*, 2017; Kuznyetsov *et al.*, 2020).

The main source of DNA in the medium is most likely apoptotic cells, thus there is a positive correlation between embryo fragmentation and DNA content in spent media (Stigliani *et al.*, 2013). Therefore, embryos that lose their genome integrity through damage or the removal process of low-quality cells by embryo self-correction mechanisms will likely expel more DNA to the culture medium (Orvieto *et al.*, 2020). Still, while some studies show a positive correlation between embryo condition and cfDNA amount in the spent media, others show opposite results (Rule *et al.*, 2018). Thus, the effectiveness of cfDNA analysis for the embryo evaluation awaits further long-term evaluation.

Spent media analysis has also included some attempts to analyse the micro (mi) RNAs released by damaged gametes or embryos. The idea is based on the assumption that miRNAs secreted by the trophectoderm to the external environment can be used as biomarkers (Capalbo *et al.*, 2016). Recent evidence links the appearance of miRNA miR-294 in the spent medium with blastocyst DNA damage and apoptosis following embryo UV irradiation (Makri *et al.*, 2020). It has also been shown that miRNA is involved in DDR processes in the gametes and embryos (Tulay *et al.*, 2015). For the time being though, this approach remains largely unexplored.

Raman spectroscopy

In recent years, the emergence of Raman spectroscopy (RS) as a valuable analytical tool has generated much hope and excitement in the field of embryology. RS works on the principle that as light is shone through a sample, it can scatter in an 'inelastic' manner, giving a broad range of information about the molecular constituents involved. This allows the elucidation of protein secondary structure, amino acid residues, RNA, DNA, sugars, lipids and more (Ellis *et al.*, 2013). What makes RS so conducive for embryological studies is its non-reliance on labels such as fluorescent dyes. This allows for the direct observation of embryos with minimal impact on their developmental potential. Another advantage is that the spectral signature produced by RS provides detailed multicomponent information in a single measurement, reducing the sample acquisition time (Scott *et al.*, 2008). Moreover, RS can be performed on wet samples at ambient temperatures.

Several studies show that the vibrational spectra of RS directly correspond to oocyte and embryo quality. For instance, oocytes subjected to OS exhibit a significantly altered vibrational spectrum, especially in the bands related to lipids and proteins (Bogliolo *et al.*, 2013). Differences in lipids and proteins can also be observed between fertilized zygotes and unfertilized oocytes, and between control and UV irradiated embryos (Perevedentseva *et al.*, 2019).

The utility of RS as an embryo grading technique was first demonstrated by Seli and colleagues in 2007 when they analysed the spent IVF culture media profiles of Day 3 human embryos (Seli *et al.*, 2007). By retrospectively comparing the spectra from embryos that implanted and those that did not, they noticed several distinct differences, in particular an increase in the relative contribution of -SH and a decrease in -CH and -NH signals. The authors raised the possibility that OS may be responsible for some of these differences. This same group went on to develop a viability index that could be used to predict embryo reproductive potential, and in a later study confirmed the model's sensitivity, specificity and accuracy (Scott *et al.*, 2008).

Later studies using RS on embryo spent media have further expanded our understanding of compositional changes that may be related to embryo viability. In 2013, Zhao *et al.* (2013) recorded higher phenylalanine levels in the Day 3 spent media of successful pregnancy embryos compared with unsuccessful ones. They also noticed significant differences in sodium pyruvate concentrations, a favoured energy

source for cleavage-stage embryos (Brinster, 1965). In a similar study in 2016, the team of Parlatan *et al.* (2016) specifically examined the amino acid composition of spent media and observed the most pronounced differences in the band ratios correlating to glycine, glutamine and proline. The authors also claimed that this RS-based analysis gave better pregnancy prediction rates than traditional morphological scoring. In the same year, Fabian *et al.* (2016) investigated differences in embryo spent media between obese maternal mice and normal mice. Although some differences between these experimental groups, relating to amide I region (1620–1690 cm^{-1}), were observed, the team was not able to definitively identify the exact molecular changes.

However, there are several significant hurdles currently keeping RS from being more widely used for analysing spent media and imaging embryos directly. For one thing, the radiation generated by spontaneous Raman scattering is dwarfed by the much more abundant Rayleigh scattering. Consequently, the weak signal must be compensated for by long integration times (Ellis *et al.*, 2013). Additionally, experiments must be carefully designed so that the high powered lasers used in the technique pose no risk of causing damage to embryos. A recent study by Perevedentseva *et al.* (2019) investigated the optimal parameters for laser image acquisition without affecting the embryo's developmental capability. This team recommended the use of longer wavelengths where possible, with a total exposure time of around 40–55 s using a 532 nm wavelength up to 3 mW power. Moreover, they demonstrated the use of RS to observe changes in spectra caused by UV radiation exposure, detecting marked changes in bands corresponding to cytochrome C (cyt c), a mitochondrial hemeprotein involved in apoptosis (Abu-Yousif *et al.*, 2008).

Direct non-invasive embryo evaluation

To gain a better picture of the biochemical changes occurring during early embryonic development, it may be necessary to directly observe oocytes and blastocysts themselves. Thankfully, RS is a powerful tool for this task. The first group to do this was Wood and colleagues in 2008 who utilized a combination of Fourier transform-infrared and RS to analyse intact whole mouse oocytes at different stages of maturation (Wood *et al.*, 2008). The most striking observation was a marked change in lipid deposit distribution: in GV stage oocytes lipids are located in two locations, whereas M2 oocytes possess only a large central lipid deposit. However, this methodology was performed on fixed oocytes and would not be transferable to a clinical ART setting.

In fact, RS can even be used to highlight the drastic effects of fixation on cells, as was demonstrated by Heraud and team in 2017 who used RS on fixed and unfixed oocytes (Heraud *et al.*, 2017). They observed significant alterations related to the backbone geometry of nucleotides and phosphate ion structure. Additionally, elevated S–S vibration was observed in fixed oocytes, which suggests that formalin fixation may cause disulphide bridge formation to increase. In recent years, the number of embryological studies taking advantage of RS' non-destructive nature has increased: it has even been used to characterize the biochemical changes occurring during the maternal-zygotic transition (Ishigaki *et al.*, 2017). In particular, a decrease in maternally derived β-sheet protein secondary structures and a concomitant increase in zygotic α-helix structures was observed. Another study used RS to look at the biochemical changes induced by oocyte vitrification, a technique to rapidly freeze oocytes that has a higher pregnancy success rate than slow-freezing methods (Rusciano *et al.*, 2017).

Modern methods like RS have helped to highlight the inadequacy of traditional morphological scoring. Morphologically low-scoring embryos have high concentrations of lipids and hydroxyapatite as demonstrated by RS (Ishigaki *et al.*, 2017). Rangan *et al.* (2018) used RS to distinguish between healthy and apoptotic cells in hamster ovaries, based on the analysis of specific nucleic acid, protein and lipid-associated marker bands within the 650–850 cm^{-1} spectral region. Research on somatic cells has shown that DNA conformational changes associated with DNA damage can be detected using RS. In this case, DNA breaks or base modifications could be visualized by several bands characteristic for the DNA backbone (1250–970 cm^{-1}), and DNA base pairs (1730–1250 and 970–600 cm^{-1}; Sofińska *et al.*, 2020). Due to the novelty of RS, the only available studies demonstrating its use for the direct detection of DNA damage in gamete/embryos are limited to studies on single sperm cells (Da Costa *et al.*, 2018; Huang *et al.*, 2020). However, several studies have reported that RS can be used to evaluate mitochondrial function (Brazhe *et al.*, 2013, 2015; Morimoto *et al.*, 2019; Wu *et al.*, 2021). This is based on the degree of cyt c oxidation, given by characteristic bands of the vibrational spectrum of both 750 and 600 cm^{-1} (Morimoto *et al.*, 2019). Because cyt c is an essential component of mitochondrial electron transport, the cytochrome oxidation status corresponds with mitochondrial membrane potential and ATP production (Hüttemann *et al.*, 2008); the analysis of specific cyt c bands may reflect the mitochondrial status of the oocytes and embryos. Recently, it has been shown that RS possesses similar or even greater sensitivity compared to the fluorophore-based tests for ATP, ROS production and mitochondrial membrane potential (Morimoto *et al.*, 2019).

Results from our own experimentations (unpublished data from our group) show an increase in intensity of the 750 cm^{-1} band of RS spectra obtained in the progression of delayed embryonic implantation, also known as diapause. This band corresponds to cyt c reduction and indicates a lowered mitochondrial activity; this is a characteristic physiological state of diapausing embryos (Hand *et al.*, 2018; Hussein *et al.*, 2020; Arena *et al.*, 2021). Our results illustrate the feasibility of RS in the analysis of embryonic mitochondrial status and could potentially find applications not only for DNA damage assessment but also in oocyte and embryo screening for mitochondrial abnormalities.

A new variant of RS, called coherent anti-Stokes RS (CARS), has been employed in several recent publications demonstrating its suitability to study aspects of early embryonic development. Notably, this technique has been used to shed light on the distribution and metabolism of lipids in oocytes and blastocysts. CARS has a major advantage over traditional RS in that the image acquisition time is much quicker, decreasing the chance of damage from laser irradiation (Jasensky *et al.*, 2016; Perevedentseva *et al.*, 2019). Additionally, although CARS spectroscopy is sensitive to the same vibrational signatures of molecules as seen in RS, it can obtain a much higher signal intensity, which is beneficial in the analysis of individual oocytes and embryos (Petrov *et al.*, 2007; Jaeger *et al.*, 2016). However, CARS has limitations regarding its low spectral resolution, and subsequently cannot be used to identify specific chemical species (Sinjab *et al.*, 2020). Nevertheless, these studies have illuminated differences in lipid droplet composition and distribution in oocytes and early embryos in humans and many other

mammalian species (Borri et al., 2019; Arena et al., 2021). To our knowledge, no study has yet been published with CARS microscopy being used to detect levels of DNA damage in cells. However, CARS shows promise in this field: it has been used to directly visualize DNA structure in chromatin and analyse thymine molecule arrangement in a microcrystalline sample (Guerenne-Del Ben et al., 2019; Dementjev et al., 2020).

Conclusion

In conclusion, DDR mechanisms are active throughout many stages of embryonic development, from the earliest stages of life at conception and from even earlier in the cellular processes of gametogenesis. The eventful lifecycle of germ cells and the lengthy journey blastocysts face before implantation renders them uniquely vulnerable to DNA damage from a multitude of different sources. Different mechanisms of DDR and damage control are activated at different stages of the preimplantation embryo's journey. Zygotes and early blastocysts are especially reticent to undergo apoptosis: every attempt is made at producing a viable embryo, so comprehensive DDR mechanisms must be in place to repair this damage as much as possible. Modern manipulation techniques used in ART may themselves induce significant DNA damage to sperm, oocytes and zygotes. Concerns about the risks of genetic or epigenetic disorders being inherited by offspring conceived by ART have not been fully explored. For a full evaluation of these risks, a precise knowledge of the capabilities of DDR in preimplantation embryos is required, and more studies should be performed in this field. Improvements in direct or indirect DNA-damage detection methods would allow ART practitioners to identify and select optimal embryos with the best viability, improving ART success rates.

Data availability

The data underlying this article are available in the article.

Authors' roles

The main body of work and general content editing was completed by RM; the subsections regarding mitochondrial genome editing and non-invasive methods for DNA damage screening, as well as Fig. 3 were provided by Ł.G.; the lipid peroxidation subsection and Figs 1 and 2 were provided by S.B.; the final draft revision and feedback were performed by G.E.P.

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Conflict of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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