The effect of cryopreservation on the genome of gametes and embryos: principles of cryobiology and critical appraisal of the evidence

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BACKGROUND: Cryopreservation has been extensively used in assisted reproductive technology, agriculture and conservation programmes for endangered species. The literature reports largely positive results regarding the survival of frozen–thawed cells and clinical outcomes. Nonetheless, it is unclear whether or not cryopreservation of sperm, oocytes and embryos causes any disruption in their genetic integrity. Drawing on
the available published evidence, this review paper describes in detail the physical and biochemical factors of cryopreservation that could potentially affect genomic integrity.

**METHODS:** A critical review of the published literature using PubMed with particular emphasis on studies which include assessment of genetic stability after cryopreservation of oocyte, sperm and embryos. The search was performed in 2014 and covered the period from the beginning of electronic records until July 2014. No language restrictions were applied.

**RESULTS:** Cryopreservation is associated with extensive damage to cell membranes, and results in alteration of the functional and metabolic status of the cells and mitochondria. Some evidence suggests an increase in DNA single-strand breaks, and degree of DNA condensation or fragmentation in sperm after cryopreservation. The extent of these changes may vary between different individuals and different techniques. The addition of antioxidants to the cryopreservation media and the use of well-controlled cooling regimes could potentially improve such outcomes. Limited numbers of studies on oocytes provide controversial results regarding the effect on DNA fragmentation, sister chromatid exchange (SCE) and aneuploidy. The only study on human embryos suggested that vitrification affects DNA integrity to a much lesser extent than slow freezing. Animal studies show increases in mitochondrial DNA mutations in embryos after cryopreservation. The limited numbers of long-term follow-up studies in humans provide reassurance that derives mostly from retrospective studies with some methodological weaknesses.

**CONCLUSIONS:** This review provides an overview of studies performed to date on the effect of cryopreservation on the oocyte, sperm and embryos. Controversy of the reported data has highlighted the gaps in our knowledge not only for clinical studies, but also for basic research in human embryos. New perspectives for future research are proposed.

**Key words:** cryopreservation / genome / oocytes / sperm / embryos

### Introduction

Over the last 60 years, the field of cryobiology has undergone a tremendous advancement. This trend continues with new techniques being developed at a rapid pace that cumulatively provide critical new opportunities in both human and animal reproduction. In the context of the cryobiology of reproductive cells (sperm and oocytes), embryos, blastomeres and reproductive tissues (ovarian and testicular tissue), sperm cryopreservation has the longest history and is the most widely used in human reproductive medicine, due to their higher cryoresistance, large numbers and straightforward protocols. One intriguing advantage of reproductive cryotechnologies is that they allow a unique suspension of time. Traditional obstacles to reproduction such as age, death, extinction, non-synchronous maturation and the availability of male and female reproductive cells become less important. With recent efforts to reduce multiple birth rates following assisted human reproduction (Tiitinen, 2012), cryopreservation has become more important practically. In countries with strong elective single-embryo transfer policies, the proportion of frozen cycles being undertaken is reported to be as high as 42.8% (Tomás et al., 2012).

Unfortunately, concerns about cryopreservation have tended to focus simply on the survival and viability of cells following the cooling and thawing processes, the assumption being that having survived the process and resulted in a live birth, the cryopreserved sample or tissue was in essence completely identical to its ‘fresh’ state. However, little is known about the genomic integrity of such cells and tissues. Contrary to the belief that cryostorage can effectively ‘stop time’, there is some evidence (Honda et al., 2001) to suggest that cells may still ‘gain’ age throughout the freezing–thawing cycle.

The aim of this review is to summarize the evidence from literature regarding potential effects of cryopreservation on the structural and functional integrity of the genome of reproductive cells and embryos with a particular emphasis on the possible mechanisms involved and to suggest areas for future research.

### Methods

We performed an electronic literature search of PubMed, Embase and Cochrane Systematic review, CENTRAL databases. The following combinations of key words were used:

- Sperm/Oocyte/Embryo cryopreservation/vitrification/slow freezing AND DNA integrity/fragmentation;
- Sperm/Oocyte/Embryo cryopreservation/vitrification/slow freezing a AND embryo;
- Sperm/Oocyte/Embryo cryopreservation/vitrification/slow freezing AND acridine orange;
- Sperm/Oocyte/Embryo cryopreservation/vitrification/slow freezing AND comet;
- Sperm/Oocyte/Embryo cryopreservation/vitrification/slow freezing AND DNA condensation;
- Sperm/Oocyte/Embryo cryopreservation/vitrification/slow freezing AND miscarriage;
- Sperm/Oocyte/Embryo cryopreservation/vitrification/slow freezing AND follow up;
- Sperm/Oocyte/Embryo cryopreservation/vitrification/slow freezing AND TUNEL;
- Sperm/Oocyte/Embryo cryopreservation/vitrification/slow freezing AND spindle;
- Sperm/Oocyte/Embryo cryopreservation/vitrification/slow freezing AND Sister chromatid exchange;
- Sperm/Oocyte/Embryo cryopreservation/vitrification/slow freezing AND aneuploidy;
- Sperm/Oocyte/Embryo cryopreservation/vitrification/slow freezing AND imprinting;
- Sperm/Oocyte/Embryo cryopreservation/vitrification/slow freezing AND mtDNA;
- Sperm/Oocyte/Embryo cryopreservation/vitrification/slow freezing AND methylation;
- Sperm/Oocyte/Embryo cryopreservation/vitrification/slow freezing AND gene expression.

Cryoprotectant/DMSO/Ethylene glycol/PROH/Glycerol AND genome/

No language restrictions were applied. The search was performed in 2014 and covered the period from the beginning of electronic records until July 2014. We
also reviewed the reference lists of all retrieved studies and reviews to find additional references. We searched for mostly experimental, cohort, case controlled and randomized controlled trials (RCTs) performed on oocytes, sperm or embryos of vertebrate animals only and published in peer-reviewed journals. The intervention in focus was freezing to \(-196^\circ\text{C}\), i.e. cryopreservation or vitrification with the use of different cryoprotectant media. Owing to the dearth of these types of studies, we included experimental as well as cohort studies. Publications on immature oocytes (GV, MI), sperm retrieved from testicular biopsy, ovarian or testicular tissue preservation were excluded from the review.

Studies were selected in two steps. Initially, titles and abstracts were scrutinized. The full manuscripts of the citations likely to meet selection criteria were then retrieved and studied.

The first author (J.K.) conducted a review of abstracts generated by the search. The relevant full-text papers were scrutinized by J.K. and any uncertainty was discussed with A.T. or Y.K. The PRISMA checklist and PRISMA flowchart (Fig. 1) were used.

**Extreme factors of cryopreservation**

Significant milestones in human reproduction were reached with freezing and the subsequent thawing and use of human sperm (Bunge and Sherman, 1953), embryos (Bunge and Sherman, 1953) and then with only limited success for frozen oocytes (Chen, 1986). Many studies have been devoted to the mechanisms of cryodamage. Several theories have been developed where ‘solution effect’, intracellular ice formation, minimal cell volume and phase transition of membranes were identified as the main elements responsible for cryoinjury (Lovelock, 1953; Mazur, 1963; Farrant, 1977; Meryman *et al.*, 1977; Belous and Bondarenko, 1982).

Although gametes provide special problems for the cryobiologist compared with somatic cells—limited numbers, ultimate use, generational impact and significant differences in cryosensitivity among the gametes of different species—the basic principles of cryopreservation are applicable to all cells.

One of the major causes of cell destruction during freezing and thawing is the formation of intracellular ice crystals; thus, a major aim is to reduce the amount of ice crystal formation during cryopreservation which can be achieved by increasing the total solute concentration with different cryoprotectants, increasing the degree of cellular dehydration, and changing the speed of cooling and/or warming. Treatment of the cells with cryoprotectants forces the movement of the water by osmosis and leads to dehydration of the cell. While shifting water out of a cell may help to...
reduce the amount of intracellular ice crystal formation, this may inflict a new and different problem for a cell in the form of osmotic shock (Love-lock, 1953; Mazur, 1963) and also a detrimental change in volume (Meryman et al., 1977).

Mazur (1963) showed that the rate of cooling substantially affects the probability of intracellular crystallization, dictated by the rate that water is transported across the membrane. Thus, the rate of cooling controls the rate at which water is converted to ice and, as a result, influences the rate at which the concentration of the extracellular solutions increase, and thus the rate at which water leaves the cell. If large cells with relatively low permeability are cooled slowly, it is possible to increase the probability that these cells will undergo dehydration, which will prevent early intracellular ice crystal formation. In contrast, during vitrification, ice crystal nucleation and growth is prevented by the use of very rapid cooling in aqueous solutions of highly concentrated cryoprotective agents (Rall and Fahy, 1985). At sufficiently low temperatures, these solutions become so viscous that they solidify without the formation of ice essentially forming a glass.

Warming is a ‘reversal’ of the cooling process, the basic aims of which are 2-fold: to rehydrate the cell and to remove cryoprotectant that has permeated the cell. In order to avoid osmotic shock, the gradual stepwise removal of cryoprotectant is important (Leibo et al., 1974). It is now well established that changing the cooling/thawing rate and use of different freezing/thawing media components can minimize unwanted effects of cryopreservation on biological material.

Despite advances in cryopreservation techniques and improved measurable outcomes, it is clear that cryopreservation still can cause extensive damage to membranes, resulting in, for instance, decreased metabolism of cells, and can disturb the bioenergetics processes of cells by damaging the mitochondria. In addition, little attention has been paid to the functional integrity of genetic material in reproductive cells after cryopreservation (Albertini and Olsen, 2013). The combined elements of freezing and thawing represent a dynamic process during which a number of physical and chemical factors, such as osmotic and hydrostatic pressure, ionic intracellular content, pH and temperature, fluctuate over a wide non-physiological range which may impact on genomic integrity (Table I). The capacity for reproductive cells to retain motility, fertilization and capacity for further cell division after cryopreservation may be of little value under conditions in which DNA, nucleoproteins (mainly histones and protamines) and mitochondrial DNA (mtDNA) can be affected adversely.

### Impact of extreme factors of cryopreservation on DNA integrity

DNA structure is very well known and described in detail elsewhere. Once it was believed that DNA is an extraordinarily stable macromolecule. However, the primary structure of this molecule was found to be quite dynamic and subject to constant change (Friedberg, 1995) and limited chemical stability (Lindahl, 1993). The phenomenon of gene transposition or alteration of nucleotide sequences is well described (Lindahl and Karlström, 1973; Finnegan, 1990). Many such changes arise as a consequence of errors introduced during replication, recombination and repair itself. Other base alterations arise from the inherent instability of specific chemical bonds that constitute the normal chemistry of nucleotides under physiological conditions of temperature and pH. Finally, the DNA of living cells reacts very easily with a variety of chemical compounds and a smaller number of physical agents, many of which are present in the environment.

Several physical and chemical factors that fluctuate during the process of cryopreservation are known to have a potential effect on DNA structure (Table II). For example, cryopreservation involves changes in osmotic pressure which itself causes chromosome aberrations or elevations in sister chromatid exchange (SCE) frequencies in hamster ovary cells and lymphocytes (Galloway et al., 1987; Kalweit et al., 1990). Thus, hyperosmotic stress may cause DNA damage or inhibit DNA repair leading to chromosomal aberrations (Kultz and Chakravarty, 2001). In principle, three mechanisms by which hyperosmotic stress could lead to an increased frequency of DNA double-strand breaks (dsb) have been suggested (Kultz and Chakravarty, 2001): first, cell shrinkage resulting in increased ionic strength, macromolecular crowding and physical distortion of the nuclear matrix may cause changes in DNA stiffness and bending (curvature stress). This could translate into mechanical strain on the DNA molecule in areas where chromatin packaging of DNA is too rigid and enhance the likelihood of breakage in these regions. Second, hyperosmotic stress could cause DNA dsb via formation of free radicals (McCarthy et al., 2010). This notion is supported by the fact that several free radical scavenging enzymes, including catalase, superoxide dismutase and glutathione peroxidase, are activated during hyperosmotic stress in mammalian cells (Kalweit et al., 1990). Third, DNA dsb could result from changes of chromatin compactness and DNA accessibility during hyperosmotic stress. Such changes may disturb the equilibrium between constitutive DNA repair and DNA damage or allow enhanced access of nucleases or free radicals to certain regions of DNA.

In eukaryotes, DNA is linked with both histone and non-histone chromosomal proteins to form chromatin. The presence of the chromatin structure is an important mechanism in securing DNA stability and function (Ljungman and Hanawalt, 1992). It is reasonable to suggest that histones and protamines can also undergo structural and functional configuration changes during freezing and thawing, as in any other proteins. For example, low temperature as well as ionic imbalance and pH fluctuation are all known to alter protein structure (Privatov, 1990).

In addition to the nuclear genome, cells possess an extra-nuclear genome represented by mtDNA. Comparisons of DNA sequences in different organisms reveal that the rate of nucleotide substitution

<table>
<thead>
<tr>
<th>Table I</th>
<th>Physical and chemical factors of cryopreservation with potential consequences for biological structures.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Physical factors</strong></td>
<td><strong>Chemical factors</strong></td>
</tr>
<tr>
<td>Fluctuation in osmotic pressure</td>
<td>Redistribution of the ionic components</td>
</tr>
<tr>
<td>Mechanical forces of ice crystals</td>
<td>Changes in pH</td>
</tr>
<tr>
<td>Electrical tension between crystals</td>
<td>Phase transition in biopolymers</td>
</tr>
<tr>
<td>Increased hydrostatic pressure</td>
<td>Redox transformation</td>
</tr>
<tr>
<td>Electrical tension between crystals</td>
<td>Specific (toxic) and non-specific effects of cryoprotectants</td>
</tr>
<tr>
<td>Redistribution of the ionic components</td>
<td>Free radicals</td>
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</tbody>
</table>
The egg is its coat or shell, which is a specialized extracellular matrix consisting largely of glycoprotein molecules, some secreted by the egg and others by surrounding cells. The volume of the highly hydrated nucleus is also much bigger in oocytes than in somatic cells. These peculiarities of oocytes make them different from other cells and have created problems in applied cryobiology. Until relatively recently, the efficiency of oocyte cryopreservation remained low across most species; the pregnancy rate per thawed oocyte after slow freezing in humans published until 2005 was 2.3% (Cutting et al., 2009). The introduction of vitrification has given more promising results with a 96.9% survival rate and 65.2% pregnancy rate per embryo transfer reported (Cobo et al., 2008a) and more reassuring benchmarks and key performance indicators quoted generally (Alpha Consensus, 2012). The advances in cryobiology became so pronounced that in some centres, authors claim that vitrified oocytes are as good as fresh. The controlled randomized clinical trial in a single centre failed to demonstrate the superiority of using fresh oocytes compared with vitrified oocytes in terms of ongoing pregnancy rate in donor cycles (Cobo et al., 2010). To date, live offspring have been obtained from frozen–thawed mouse (Whittingham, 1977), rabbit (Al-Hasani et al., 1989), cow (Fuku et al., 1992) and human (Chen, 1986) oocytes. The number of births derived from vitrified human oocytes was quoted as 800 in 2009 (Cutting et al., 2009) since the first report. This number is certainly likely to be much higher in 2014, with a single centre reporting the birth of at least 1027 babies (Cobo et al., 2014); however, no papers could be found reporting the worldwide estimate of children born to date. In spite of encouraging results for oocyte survival and clinical pregnancy rate, information on the effect on the genetic structure of oocytes remains scarce.

### Oocyte cryopreservation

Oocytes not only contribute genetic information for the developing embryos, but also provide them with energy, nutrients and a mitochondrial genome. Altered expression of the genetic information could be caused by disturbances in DNA, protein-histones, cytoskeleton system, DNA repair system and systems that regulate gene expression (imprinting, transcription apparatus, etc.). The eggs of most species are giant cells, containing sets of all the materials needed for initial development of the embryo until they reach the stage of implantation in mammals or self-feeding in others. Eggs are typically spherical or ovoid, with a diameter of ~100 μm in humans and sea urchins, 1–2 mm in frogs and fishes and many centimetres in birds and reptiles. A typical somatic cell, in contrast, has a diameter of only ~10 or 20 μm. The egg typically contains nutritional reserves in the form of yolk, which is rich in lipids, proteins, polysaccharides, cortical granules, mitochondria, spindles and macrotubules and microfilaments. The higher content of lipids is relevant for cryopreservation, since it could affect the efficiency of this process (Zhou and Li, 2013). Another distinctive characteristic of the egg is its coat or shell, which is a specialized extracellular matrix

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**Table II** Effect of different extreme factors on the DNA.

<table>
<thead>
<tr>
<th>Physical or chemical factors (in non-physiological range)</th>
<th>Consequence for the DNA structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ructuation of pH</td>
<td>Deamination, depurination, depyrimidation</td>
</tr>
<tr>
<td></td>
<td>The extremes of high or low pH destabilize DNA helix and change the melting point (Williams et al., 2001)</td>
</tr>
<tr>
<td></td>
<td>Neutral or acid pH causes more chromosomal aberration during freezing—drying of mouse spermatozoa than alkaline (Kaneko et al., 2003)</td>
</tr>
<tr>
<td>Temperature</td>
<td>Cold denaturation (Privalov, 1990; Marenduzzo et al., 2001)</td>
</tr>
<tr>
<td>Reactive oxygen species</td>
<td>Fragmentation, base loss, single-strand breaks and double-strand breaks, production of 8-hydroxyguanine DNA–protein cross-links (Dizdaroglu and Jaruga, 2012; Jena, 2012)</td>
</tr>
<tr>
<td>Ethylene glycol</td>
<td>Increases hyperchromicity, ellipticity and premelt slope of chromatin. Destabilizes high melting region of polypeptide-bound DNA and the extent of higher ordered structure in model complexes and chromatin (Schwartz and Fasman, 1979). Effects melting temperature and causes the conversion from the B to the C form of chromatin (Nelson and Johnson, 1970)</td>
</tr>
<tr>
<td>Dimethyl sulphoxide</td>
<td>Causes DNA methylation, conformational changes in the DNA and chromatin (Nelson and Johnson, 1970)</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>Increases DNA methylation (Hu et al., 2012)</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Changes the conformation of DNA, has a destabilizing effect and decrease T melting (Nakanishi et al., 1974)</td>
</tr>
<tr>
<td>Hyperosmotic stress</td>
<td>Results in chromosomal aberrations and DNA double-strand breaks; modulates DNA–protein binding; alters chromatin compactness (Kultz and Chakravarty, 2001); causes misfolding of proteins (Onganesy et al., 2007); induces production of reactive oxygen species (McCarthy et al., 2010)</td>
</tr>
<tr>
<td>Reactive oxygen species</td>
<td>Destabilizes mitochondria and nuclear envelope (specifically effects DNA–protein cross-links, mitochondrial genome to the electron transport chain, and its lack of protective histones)</td>
</tr>
<tr>
<td>Reactive oxygen species</td>
<td>Modulates DNA–protein binding; increases DNA methylation (Hu et al., 2012)</td>
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</tr>
</tbody>
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During evolution has been 10 times greater in mitochondrial genomes than in nuclear genomes, which presumably is due to a reduced fidelity of mtDNA replication, DNA repair or both. The proximity of the mitochondrial genome to the electron transport chain, and its lack of protective histones, apparently makes mtDNA more susceptible than nuclear DNA to various kinds of damage (Yakes and Van Houten, 1997). It is well established that cryopreservation causes an excessive production of reactive oxygen species (ROS) (Mazzilli et al., 1995; Tselkas et al., 2000; McCarthy et al., 2010), a well-recognized factor known to damage mtDNA (Wei, 1998).
(ii) aneuploidy rate,
(iii) SCE rate,
(iv) DNA fragmentation,
(v) methylation and gene expression.

**Effect on spindles**

Initial work on cryopreservation of oocytes strongly suggested the increased sensitivity of meiotic spindle tubulins not only to cryopreservation (Bromfield et al., 2009; Coticchio et al., 2009), but even to simple cooling (Pickering et al., 1990; Wang et al., 2001a; Zenzes et al., 2001; Gomes et al., 2012), exposure to osmotic stress (Mullen et al., 2004, 2007) or cryoprotectants (Van der Elst et al., 1988; Huang et al., 2006). Further studies, with some exceptions (Cobo et al., 2008b; Noyes et al., 2010), repeatedly demonstrated that vitrified oocytes were more likely to maintain a normal meiotic spindle than slow-frozen oocytes (Huang et al., 2006; Larman et al., 2007a; Cao et al., 2009; Martinez-Burgos et al., 2011). It also emerged that the meiotic spindle is capable of regeneration following cryopreservation (Chen and Yang, 2009; Tamura et al., 2013). However, the window of opportunity for spindle recovery was not unlimited. The optimal time for post-freezing recovery seems to vary depending on the method of cryopreservation, initial oocyte quality, patient age, method of spindle assessment and species (Bromfield et al., 2009; Chen and Yang, 2009; Rienzi et al., 2004; Ciotti et al., 2009; Asgari et al., 2011). Leaving oocytes for longer than ‘optimal time’ will result in progressive loss of bipolar spindle structure coincident with chromosome displacement.

**Effect on aneuploidy genesis**

The main concern regarding possible detrimental effects on the oocyte’s meiotic spindle is the potential for disturbance in alignment of chromosomes, i.e. non-disjunction or abnormal chromosome segregation and a concomitant increase in aneuploidy rates (Maro et al., 1986; Wang et al., 2001b; Bromfield et al., 2009; Sharma et al., 2013).

Some authors believe that polyplody may also occur secondary to polyspermic fertilization following oocyte freezing and thawing (Bos-Mikich and Whittingham, 1995). However, this mechanism has no relevance in clinical practice, since, at the time of writing, ICSI is always used following cryopreservation of human oocytes. The evidence regarding effects of oocyte cryopreservation on subsequent aneuploidy is scarce with a reported increase in chromosomal abnormalities mostly in mouse studies (Kola et al., 1988; Bouquet et al., 1992, 1993, 1995; Sterzik et al., 1992). However, more recent reports, from the same investigators, showed no increase in aneuploidy when either slow freezing or vitrification was applied, in spite of an increase in spindle abnormalities (Huang et al., 2007, 2008).

Limited numbers of human studies have failed to show increases in aneuploidy rate following oocyte cryopreservation. Cobo et al. (2001) showed no increase in aneuploidy when chromosomes 13, 18, 21 and X were assessed by fluorescence in situ hybridization (FISH) in 21 human blastocysts derived from cryopreserved oocytes in comparison with control. These authors concluded that the slow freezing method was safe, despite their assessment being limited to only four chromosomes and using only a relatively small number of embryos. Recent studies suggest that even though all 24 types of chromosome contributed to cleavage stage aneuploidy, chromosomes 22, 16, 19, 21 and 13 were the most frequently observed in the order of prevalence (together accounting for 59% of all abnormalities in embryos (Fragouli et al., 2013). It is also suggested that FISH would under detect at least 20–40% of chromosomal abnormalities carried by embryos (Wells et al., 2008) and is a poor method of choice for prediction of aneuploidy in blastocysts (Treff and Scott, 2012).

Another paired randomized controlled study (also found no increase in aneuploidy rate in human embryos derived from vitrified oocytes, using a comprehensive chromosome screening method to simultaneously detect all 24 chromosomes (Forman et al., 2012). While this technique overcomes many of the limitations of FISH, it may not be capable of detecting de novo duplications and deletions (Treff and Scott, 2012). Furthermore, this study excluded almost 22% of oocytes that apparently did not fertilize.

Fortunately, most gross chromosomal abnormalities are generally lethal and thus are not transmitted to future offspring. Thus, it becomes more important to understand the effects of sublethal damage to DNA caused by cryopreservation, which could be passed on to offspring and possibly future generations.

**Effect on SCE**

SCE is known to be a sensitive indicator of mutagenesis (Carrano et al., 1978; Latt and Schreck, 1980). SCE is a well-established reproducible and robust marker used to assess alterations in cellular homologous recombination (Stults et al., 2014) and has also been used to assess the influence of cryopreservation on reproductive cells. A significant increase in the average incidence of SCE was reported in mouse embryos derived from frozen–thawed (Bouquet et al., 1993) or vitrified oocytes (Ishida et al., 1997). Similarly, equilibration with dimethylsulphoxide (DMSO, 1.5 M) only at room temperature has also been shown to result in a significant increase in SCE frequency (Bouquet et al., 1993). The common feature of agents that elevate SCE frequencies is an action on DNA, either directly or indirectly, via possible alterations in DNA replication or, chromatin structure (Latt and Schreck, 1980); cryopreservation and/or pre-freezing manipulations might cause an alteration either in DNA structure or oocyte components implicated in repair mechanisms (Bouquet et al., 1993).

No other recent studies were found in the literature and none in human oocytes.

**Effect on DNA fragmentation**

Many studies have assessed DNA fragmentation in sperm following cryopreservation; however, this method has only rarely been applied for assessment of oocyte DNA after cryopreservation and it is difficult to draw clear conclusions from the current information available.

Human oocytes showed no increase in the number of DNA fragmented oocytes assessed with the terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling (TUNEL) assay after slow freezing and vitrification (Martinez-Burgos et al., 2011). However, not a single oocyte in this study was reported to have evidence of DNA fragmentation. It would be essential to have a negative control as a proof of method validity. This study was also limited to only a small number of donors (n = 10) of unreported background. Another small (n = 15) study also reported no increase in DNA fragmentation assessed by TUNEL following slow freezing of human oocytes and no signs of apoptosis as assessed by caspase activation (Gualtieri et al., 2009). In contrast, significant increases in DNA fragmentation were observed following vitrification.
assessed by the single cell gel electrophoresis assay (COMET assay), from 6.1% to 53.6% of mouse oocytes showing comet tails (Huang et al., 2009). It is unclear whether this difference could be attributed to different species, cryopreservation protocols or methods of DNA assessment. No human studies were found where COMET was used for assessment of DNA integrity after oocyte cryopreservation.

Some authors not only assessed DNA fragmentation levels following oocyte cryopreservation, but also suggested how to diminish the unwanted effect. For example, the presence of COMET tails in bovine oocytes became virtually undetectable when a droplet system was used for vitrification instead of traditional straws (Stachowiak et al., 2009). In addition, improved genomic integrity was achieved when ‘proper’ handling (avoiding loading oocytes with too much media on cryoloop and leaving oocytes in vitrification media for more than 60 s) during vitrification of mice oocytes was applied (Wang et al., 2009).

Other simple measures, such as changing the container, protocol or cryoprotectant type, also were shown to reduce DNA damage (as measured by COMET assay in bovine oocytes) (Sharma et al., 2010)—see Table III. Despite these studies being confined to animal species, important lesson can be learned. For example, most published studies on oocyte cryopreservation, but also suggested how to diminish the unwanted effect. For example, the presence of COMET tails in bovine oocytes became virtually undetectable when a droplet system was used for vitrification instead of traditional straws (Stachowiak et al., 2009). In addition, improved genomic integrity was achieved when ‘proper’ handling (avoiding loading oocytes with too much media on cryoloop and leaving oocytes in vitrification media for more than 60 s) during vitrification of mice oocytes was applied (Wang et al., 2009).

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### Effect of different cryoprotectant media

The duration of equilibration in cryoprotectant media may be relevant, since simple equilibration without further freezing appears to influence genetic integrity assessed by COMET (Aye et al., 2010). The cryoprotectant propylene glycol (PrOH) appears to be genotoxic for a Chinese hamster ovarian cell line (Aye et al., 2010) and mouse oocytes (Berthelot-Ricou et al., 2011). When bovine oocytes were exposed to DMSO or PrOH, the proportion of normal chromosomes or spindles

### Table III Summary of studies showing the possibility of how to reduce a negative effect of cryopreservation on the genome of reproductive cells and embryos.

<table>
<thead>
<tr>
<th>Parameter assessed, type of cells studied, species</th>
<th>Reported findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acridine orange staining Semen, human</td>
<td>Chromatin integrity was preserved better when computerized freezer was used in comparison with vapour freezing</td>
<td>Hammadeh et al., (2001)</td>
</tr>
<tr>
<td>COMET assay, semen, human</td>
<td>Addition of antioxidants into cryoprotectant media such as ascorbic acid and resveratrol improved the DNA integrity index after slow cooling cryopreservation</td>
<td>Branco et al. (2010)</td>
</tr>
<tr>
<td>DNA fragmentation TUNEL assay</td>
<td>Repeated freezing and thawing increases the percentage of DNA fragmentation. However, when the sperm sample was not washed from cryoprotectant, the extent of damage was less</td>
<td>Thomson et al. (2010)</td>
</tr>
<tr>
<td>TUNEL assay</td>
<td>Usage of density gradient centrifugation before freezing improves the rate of normal DNA synthesis</td>
<td>Brugnon (2013)</td>
</tr>
<tr>
<td>COMET assay, oocytes Bovine</td>
<td>Droplet system and open pull straws—no detectable DNA cryoinjuries in comparison with a traditional straw</td>
<td>Stachowiak et al. (2009)</td>
</tr>
<tr>
<td>COMET assay Oocytes, buffalo</td>
<td>Open pulled straw with ethylene glycol and propylene glycol better than French mini-straw with the same cryoprotectants</td>
<td>Sharma et al. (2010)</td>
</tr>
<tr>
<td>TUNEL assay</td>
<td>Dimethylsulphoxide is not genotoxic in comparison with ethylene glycol and propylene glycol</td>
<td>Aye et al. (2010)</td>
</tr>
<tr>
<td>Immunostain, polscope</td>
<td>There is an optimal time for ICSI following cryopreservation of oocytes that varies between 1 and 3 h, depending on several variables</td>
<td>Chen and Yang (2009)</td>
</tr>
<tr>
<td>Meiotic spindle</td>
<td>Culture media supplementation with antioxidants (β-mercaptoethanol) reduced % of DNA fragmentation after vitrification</td>
<td>Hosseini et al. (2009)</td>
</tr>
<tr>
<td>Oocyte</td>
<td>Assisted hatching, blastocoele aspiration can significantly minimize the DNA damage</td>
<td>Kader et al. (2010a, b)</td>
</tr>
<tr>
<td>Human, mouse, bovine</td>
<td>Slow freezing with glycerol was significantly better in comparison with vitrification and slow freezing with propanediol</td>
<td>Kader et al. (2009)</td>
</tr>
</tbody>
</table>

*TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling; COMET, the single-cell gel electrophoresis assay.*
was also significantly decreased and the degree of DNA methylation was changed with a more pronounced effect after PrOH exposure (Hu et al., 2012). Some of the detrimental effects of PrOH were explained by its capability to cause protracted increases in calcium levels (Larman et al., 2007b; Berthelot-Ricou et al., 2011). No studies investigating a genotoxic effect of cryoprotectants in human oocytes were found.

**Imprinting and gene expression**

The DNA methylation rate was also affected in mice oocytes and embryos derived from these oocytes following vitrification or even just exposure to vitrification solution with DMSO and ethylene glycol (Liang et al., 2014). The little evidence that exists suggests that both slow freezing and vitrification modify the gene expression profile of human oocytes in comparison with fresh controls (Monzo et al., 2012). While the oocytes in these studies were selected from the pool of those that failed to fertilize following ICSI, both vitrification and slow freezing caused an alteration of gene expression but in different ways. The mouse study also demonstrated differences in expression of histone deacetyltransferase1 (HDAC1) in embryos derived from the vitrified oocytes (Li et al., 2011). The crucial question remains; whether these changes have any impact on the function or long-term fate of the cryopreserved oocytes and subsequent offspring.

**Long-term follow-up studies**

To date, there are no long-term follow-up studies for children born from cryopreserved oocytes and most data reported consists of case reports and retrospective studies. However, preliminary outcome data offer ‘reassurance’ (Chian et al., 2008) with the 149 children born after slow freezing of oocytes and the 221 children born after vitrification largely anecdotally reported as being ‘healthy’ (Wenerholm et al., 2009). Another review reported only 1.3% of congenital anomalies for 900 babies born following cryopreservation of oocytes (Noyes et al., 2009). However, this review did not provide any information about how congenital anomalies were assessed and reported. The quality of studies varied from conference abstracts to case reports and case series. Different methods of freezing were pooled together with no details on the cryoprotectant media, cooling rate etc.

It is encouraging to see that some attempts have been made to start a prospective registry for children born from cryopreserved oocytes (HOPE registry, Ezcura et al., 2008). However, the primary outcome in this study, the results of which are still pending, remains to be live birth rate as a measure of success.

In summary, very limited information is available currently on the impact of cryopreservation on the genome of the oocyte and its possible effect on the offspring. But owing to the nature of the structures present within and the role played by the oocyte during early development, this is a critical issue to address. Further experiments and long-term clinical studies should be carried out following the use of cryopreservation techniques. More studies are urgently needed to investigate the effect of cryoprotectants as well as different cryopreservation regimes (slow and vitrification) on the integrity and methylation of DNA and gene expression. The very limited evidence from animal studies suggests that simple steps, such as modification of the freezing container, can completely diminish the negative impact of cryopreservation. Similar studies are urgently needed in human supported by further investigations in animal models. There should also be an international centralized prospectively collected registry database for all children derived from frozen oocytes.

**Sperm cryopreservation**

Since one of the primary functions of spermatocytes is to deliver an undamaged haploid paternal genome to the egg, they possess tightly packed genetic material which is distinctively different from somatic cells. From the earliest work on sperm cryopreservation, the traditional measures of success after cryopreservation were motility and fertilization ability. It is generally agreed that cryopreservation results in a decrease in both parameters; however, its effect on the integrity of DNA remains a controversial subject (Di Santo et al., 2012; Paoli et al., 2014).

**Effect on DNA integrity in sperm**

Some studies report alteration in the DNA integrity of human sperm following cryopreservation (Kalthur et al., 2008; Thomson et al., 2009a; Zribi et al., 2010; Meamar et al., 2012), whereas others have found no effect (Duru et al., 2001; Issachenko et al., 2004; Kadirvel et al., 2009; Vutuyavich et al., 2010).

The discrepancy in the results might be explained by the difference in individual cryoresistance, methods of cryopreservation or methods of DNA assessment used. A variety of methods are used to assess DNA integrity in sperm after cryopreservation including the TUNEL, Oxy DNA, COMET and acridine orange (AO) assays and chromatin dispersion (SCD) test or SCSA (sperm chromatin structure assay). Some of these tests measure DNA damage directly (TUNEL): others measure damage after denaturation (SCD or COMET) at acid or alkaline pH (Avendaño and Oehninger, 2011). Some methods are based on the fact that the emission spectra of dyes are sensitive to the status of DNA-associated proteins. For example, AO binds to native (double-stranded) DNA as a monomer and emits green fluorescence, whereas denatured DNA emits red fluorescence. Some data suggest that TUNEL assay might be more sensitive in detecting DNA damage than AO staining since the latter has some methodological weaknesses such as subjectivity in interpreting the results, rapid fluorescence fading and heterogeneous slide staining (Martins et al., 2007). However, even TUNEL’s higher sensitivity could be affected by different pretreatment procedures and the presence of DNA–protein cross-linkages that would limit the accessibility of the enzymes to nuclear DNA (Negoeescu et al., 1996).

AO staining of human sperm (Royere et al., 1988, 1991; Chohan et al., 2004; Ngamwutiwong and Kunathikom, 2007) showed that the level of native DNA significantly decreased after cryopreservation. However, even before treatment, the percentage of native DNA varied significantly between individuals (Sukcharoen, 1995; Hoshi et al., 1996). It is generally agreed that success of cryopreservation depends on the initial quality of cells (Hammadeh et al., 1999) and that low-quality spermatocytes are more likely to be less cryoresistant (Kalthur et al., 2008; Kopeika and Kopeika, 2008). The percentage of condensed chromatin (assessed by aniline blue) or presence of single-strand breaks (assessed by alkaline COMET assay) were significantly higher after cryopreservation in samples from infertile men when compared with those of fertile donors (Hammadeh et al., 2012).
1999; Donnelly et al., 2001). This may indicate that chromatin in the sperm from fertile men withstands cryodamage better than sperm from the infertile.

**Effect of different cryoprotectant media**

The impact of different components in the cryoprotectant media has been also studied in relation to DNA integrity (Yildiz et al., 2007). The presence of low-density lipoproteins in the media reduces the degree of DNA damage after cryopreservation in boars (Hu et al., 2008). In humans, however, the currently widely used cryoprotectant media failed to show any protection of the DNA integrity after cryopreservation (Thomson et al., 2009b). It is notable that all commercial media have glycerol as the main cryoprotectant, and further that the effect on the DNA integrity of equilibration in cryoprotectant without freezing was not assessed. This is significant, as even simple equilibration with specific cryoprotectants (without freezing) has been shown to reduce survival of embryos derived from cryoprotectant-treated sperm of zebrafish (Danio rerio) (Kopeika et al., 2003a, b, 2004). Some authors suggest that glycerol may have a toxic effect on mitochondria and this would subsequently cause activation of caspases (Wündrich et al., 2006).

While the debate regarding the presence or absence of an effect of cryopreservation on the DNA in human sperm continues, mostly animal studies have tried to elucidate the mechanisms involved in the DNA changes caused by cryopreservation.

**Mechanisms of cryodamage**

**Reactive oxygen species**

The ability of semen from fertile men to resist freezing damage may be due to some protective constituents in seminal plasma. Seminal plasma contains an abundance of antioxidant enzymes including superoxide dismutase and catalase, both of which remove ROS. The total antioxidant capacity of semen from fertile men has been found to be significantly greater than that for samples from infertile men (Lewis et al., 1995) and may confer protection against the trauma of cryopreservation.

Therefore, if natural abundance of antioxidants in seminal plasma improves chromatin cryoresistance, it is reasonable to expect that artificial addition of ROS scavengers might enhance chromatin cryoprotection during freezing–thawing cycles. As expected, the introduction of antioxidants such as melatonin, vitamin E and C, taurine, ascorbic acid or Opuntia ficus-indica into cryoprotectant media are shown to improve the integrity of DNA after cryopreservation (Branco et al., 2010; Kalthur et al., 2011; Cabrita et al., 2011; Succu et al., 2011; Meamar et al., 2012). However, it is not clear whether or not this effect is just a chance association or whether it reflects a direct involvement of the antioxidants in DNA protection from the cryodamage.

A recent study has found no correlation between the surplus of ROS following cryopreservation and the degree of DNA damage when good and poor freezability boar ejaculates were compared (Yeste et al., 2013). In contrast, a study on buffalo sperm showed a strong correlation between the surplus of ROS and DNA fragmentation level for samples stored at 4°C and no increase in ROS and/or DNA fragmentation when samples were frozen—thawed (Kadirvel et al., 2009). While the mechanism of ROS involvement in cryodamage of the DNA still remains to be unveiled, new theories are emerging to explain the DNA changes following cryopreservation.

**Change in osmotic pressure, degree of chromatin condensation, disulphide cross-linkage**

A recent study on marsupial sperm has elegantly demonstrated the importance of the osmotic changes in provoking chromatin relaxation and/or DNA fragmentation (Johnston et al., 2012). These authors showed that the water flux into the nucleus and/or general ice crystal damage to the sperm cell could result in a subsequent physical “tearing” of the tertiary structure of the DNA–protamine complex, leading to an increase in the incidence of relaxed chromatin. The detrimental effect of hypo- or hyperosmotic solutions on DNA integrity was also demonstrated in mouse (Yildiz et al., 2010).

The cryopreservation is also shown to increase the degree of chromatin condensation in sperm of men (Ruyere et al., 1988, 1991). These authors hypothesized that this abnormal ‘overcondensation’ may impaire subsequent conception rate via causing impairment in the process of decondensation. Although decondensation processes even in normal conditions are not fully understood (Paurset et al., 1991), disruption of disulphide cross-linkages established between cysteine molecules of protamines seems to be critical (Perreau et al., 1984). In vitro experiments using the detergent sodium dodecyl sulphate (SDS) demonstrated that sperm were less likely to decondense after cryopreservation indicating that the processes of freezing and thawing of sperm induces changes in disulphide bonds and/or causes denaturation preventing the biochemical action of SDS on the proteins.

Thiol status of DNA as a possible indicator of the DNA denaturation was investigated during cryopreservation of boar sperm (Courtens et al., 1989) and an increase in thiol (SH) groups after cryopreservation was established. The presence of disulphide bonds is essential for maintaining protamine–DNA chromatin structural organization. The alteration in composition and structural organization of chromatin may not only affect fertilization, but also early embryo development (Balhorn, 2007). It has been postulated that poor packaging may contribute to the failure of sperm decondensation and, consequently, result in fertilization failure (Sakkas et al., 1996). Moreover, some authors suggest that the destabilizing effect of cryopreservation on nucleoprotein union, i.e. disruption of disulphide bonds, might result in an increased subsequent DNA fragmentation (Yeste et al., 2013). If there is an underlying problem with the sperm chromatin compaction, that can also exacerbate the negative effect of cryopreservation on the DNA integrity (Yamauchi et al., 2010).

Addition of zinc to freezing media seems to alleviate the degree of DNA damage following cryopreservation (Kotdawala et al., 2012). Whether this positive effect of zinc is realized through its possible role in stabilizing nucleo-protamine complexes (Balhorn, 2007) remains unclear.

**Apoptosis**

More recent studies (Thomson et al., 2010; Zrbi et al., 2010) demonstrated increased DNA fragmentation in association with apoptosis, after cryopreservation. It is important to distinguish whether established changes in DNA integrity are a consequence of processes in viable cells rather than activation of apoptosis (Thomson et al., 2009b). The limitation of a number of the studies cited above is that they assessed DNA
damage in the total sperm population that included both morphologically and functionally normal and abnormal cells. It has been proposed that assessment of the DNA integrity in a subpopulation of morphologically and functionally normal sperm only would have more biological relevance, since only these cells will have potential to fertilize or be selected for ICSI (Avendaño and Oehninger, 2011). Assuming that any increased DNA fragmentation established in the neat, unprocessed sperm sample after cryopreservation is a reflection of changes secondary to apoptotic or necrotic processes only, then separation of functionally viable populations of cells should result in a relative improvement of the DNA integrity measures in comparison with the neat sample. However, sperm processing (selection) techniques, such as discontinuous density gradients, aimed at isolating the highest quality sperm population, did not result in improved readings of the DNA fragmentation index in thawed ram sperm (García-Alvarez et al., 2010). Furthermore, the detrimental effect of cryopreservation on the DNA integrity of human sperm was observed even in a subpopulation of sperm selected with density gradient centrifugation (Thomson et al., 2009b; Moubasher et al., 2013). Therefore, increased DNA fragmentation is present even in potentially functional sperm cells when non-motile sperm and other debris are removed.

**Activation of endonuclease**

Another hypothesis regarding the mechanism of DNA fragmentation deserves attention. Spontaneous DNA degradation within sperm nuclei can take place as a result of endonuclease activity released from sperm with membrane damage (Ward and Ward, 2004). The addition of supernatant obtained from a sperm sample subjected to an ‘extreme freeze’ (plunging samples into liquid nitrogen without cryoprotectants) not only increased the fragmentation rate in DNA of mouse sperm but also led to a drop of subsequent implantation rate (Pérez-Crespo et al., 2008).

**Effect on function and subsequent embryo development**

Irrespective of the mechanisms, the question remains as to the biological impact of sperm DNA fragmentation in terms of fertilizing ability, subsequent embryo development and resultant offspring. Unfortunately, there is no direct correlation between certain sperm functions (e.g. motility) and genomic integrity, especially following iatrogenic changes. Indeed, sperm with severe DNA damage due to γ-irradiation retained good motility (Bordignon and Smith, 1999) and fertilizing ability (Ahmadi and Ng, 1999). However, abnormal sperm chromatin structure is associated with an increase in miscarriage rate (Robinson et al., 2012) without affecting fertilization and pregnancy rate (Lin et al., 2008). It has been hypothesized that if cryopreservation of sperm causes damage to the DNA, then it could be repaired by the oocyte’s intrinsic repair system following fertilization. To test this hypothesis, different inhibitors and stimulators of the DNA repair system were added to the freshly fertilized embryos of the loach (Misgurnus fossilis) derived using cryopreserved sperm. Embryo survival dropped significantly when an inhibitor of the DNA repair system (3-aminobenzamide) was added (Kopeika et al., 2004), but the negative effect of sperm cryopreservation on embryo survival was completely reversed when embryos were exposed to caffeine (Kopeika et al., 2003a). The original study was subsequently repeated in trout species using the same inhibitor (3-aminobenzamide) of the repair system and it was suggested that at least 10% of sperm DNA damage could be repaired by the DNA repair system of the oocyte after fertilization (Pérez-Cerezas et al., 2010). A mammalian study also demonstrated the persistence of DNA aberration in murine embryos derived from sperm subjected to freezing–thawing (Yamauchi et al., 2012). Animal studies also showed that cryopreserved sperm not only contributed aberrant DNA into oocytes during fertilization, but also altered gene expression in surviving embryos and increased telomere length (Pérez-Cerezas et al., 2011). Moreover, both slow freezing and vitrification of human sperm cause DNA changes in key paternal genes involved in early embryo development (Valcarce et al., 2013). Therefore, to assess the effects of cryopreservation on sperm, it is essential to perform long-term follow-up studies on resultant children.

**Long-term follow-up studies**

Initial attempts to follow-up outcomes from treatments involving cryopreserved sperm focused on a cohort of 3381 pregnancies resulting from IVF with frozen donor sperm (Thépot et al., 1996; Lansac et al., 1997; Lansac and Royere, 2001). No evidence was found either of an increase in birth defects or chromosomal abnormalities compared with the general population. As with many follow-up studies, no details on cryopreservation protocol were provided, making it difficult to identify key causative aspects of the process. Also the focus of these follow-up studies was mainly limited to pregnancy and time of birth. Very limited information from animal studies suggests that fertilization of eggs with DNA-damaged sperm may ‘generate effects that only emerge during later life’ (Fernández-Gonzalez et al., 2008). These authors found aberrant growth, premature ageing and mesenchymal tumours in mice produced by ICSI from suboptimally frozen–thawed sperm. We found no similar studies for humans.

In summary, despite the growing evidence that cryopreservation affects the genetic integrity of sperm, there are still insufficient data available on the potential impact of these changes on future offspring. A number of factors are reported (including different cooling and warming regimes, antioxidants and stimulants of the DNA repair system) which might either reduce or reverse the side effects of sperm cryopreservation to improve outcomes (Table III). Detailed suggestions for future research and clinical practice are discussed in the end of this review.

**Embryo cryopreservation**

The first child after embryo cryopreservation was born in 1984 (Zeilmaker et al., 1984) and the procedure is now considered a routine and important component of IVF treatments. In contrast to gametes, the fact that embryos are diploid may be considered advantageous, as stable ploidy tends to increase the resistance of cells to both mutagenic and non-specific stress factors (Cherfas and Zøy, 1984). However, on the other hand, embryos have actively dividing cells. This might make their genetic apparatus more vulnerable to the insult of extreme factors when compared with highly packed and inactive chromosomes of sperm.

With the exception of one study (Li et al., 2012), no work has been done studying the effects of cryopreservation on the DNA integrity of human embryos.
Effect on DNA integrity

Animal evidence suggests that slow freezing as well as vitrification of blastocysts affects the DNA integrity, as assessed by TUNEL (Kader et al., 2009). Cryoprotectants such as ethylene glycol, 1,2-propanediol or glycerol all are shown to increase DNA fragmentation in porcine blastocysts (Rajaei et al., 2005) even without a cycle of freezing and thawing. Application of assisted hatching, blastocoele aspiration (Kader et al., 2010a), changing cooling rate or cryoprotectants (Kader et al., 2009) have all been shown to improve genetic integrity in mouse embryos (Table III). SCE levels were decreased in mouse embryos by reducing the equilibration time from 40 to 5 min when vitrifying with ethylene glycol, Ficoll and trehalose (Ishida et al., 1997). The only study examining DNA integrity in human embryos showed in a small number of embryos that slow cooling caused a significant decrease (76.5 ± 3.1%) in the DNA integrity index in comparison with fresh controls (92.4 ± 7.4%), whereas vitrification caused no changes (93.8 ± 1.3%) (Li et al., 2012). It is not possible to establish if these changes in DNA integrity were caused directly by the freezing procedure or were secondary to apoptosis.

The incidence of aneuploidy and polyploidy was not influenced regardless of the method used (slow cooling with 1,2 propanediol, ultrarapid cooling with DMSO, ultrarapid cooling with 1,2 propanediol) to freeze 2-cell mouse embryos. The level of mitotic crossing-over was increased substantially in embryos frozen—thawed by the ultrarapid cooling with DMSO (Bongso et al., 1988). However, the level of polyploidy was shown to increase in human embryos. This was attributed to a phenomenon of blastomere fusion triggered by cryopreservation at the 2- to 10-cell stage (Balasik et al., 2000).

As with other types of cells, a surplus of free radicals is suspected to be involved in the increase in DNA fragmentation in bovine embryos after cryopreservation (Hosseini et al., 2009). However, supplementation of the culture media with known antioxidants did not provide a complete rescue for the level of DNA fragmentation, suggesting that more than one mechanism for DNA fragmentation might exist during cryopreservation or the antioxidant media were used in suboptimal quantities to achieve the desired effect.

Methylation, gene expression and mtDNA

Whereas the effect of cryopreservation on the nuclear genome has been the focus of at least some investigations, to date there is only one study investigating the effect of cryopreservation on mitochondrial DNA in blastomeres. Zebrafish, as a model species, combined with mtDNA assessment was chosen since mtDNA is known to be more prone to mutation under stress conditions. A significant increase in the level of single-nucleotide variations of mtDNA following cryopreservation of zebrafish blastomeres was observed (Kopeika et al., 2005). The majority of established mutations in this study were thymine—cytosine substitutions that were thought to occur during the single-stranded phase of mitochondrial DNA replication. No studies were reported on any other type of cells or in other species.

It would be important to assess the effect of cryopreservation on mtDNA in embryos and oocytes of mammals including humans. It is also important to establish if cryopreservation of cells that undergo active DNA replication could have a potentially different impact on the genetic integrity.

Gene expression was shown to be different in frozen—thawed embryos, when compared with fresh ones (Tachataki et al., 2003; Mamo et al., 2006; Park, 2006; Dhand et al., 2007; Larman et al., 2011; Aksu et al., 2012; Saenz-De-Juano et al., 2012; Shaw et al., 2012) in different species such as humans, rabbit, mouse and bovine.

Even minimal embryo manipulation involving blastocyst collection and embryo transfer using blastocysts generated in vivo and cultured in vitro for <1.5 h has been associated with loss of maternal allele methylation causing later changes of gene expression in mouse extraembryonic tissue (Rivera et al., 2008). When the methylation status of H19/IGF2 differentially methylated domain (i) in mouse embryos was analysed, it was found that not only loss of methylation, but also H19 expression was much more pronounced in embryos subjected to vitrification (Wang et al., 2010). These studies demonstrate that even under ‘optimal’ or standard culture conditions, the methylation status of embryonic DNA is labile and thus cryopreservation, which involves a range of extreme factors, is likely to have consequences for methylation and subsequent gene expression.

Long-term follow-up studies

Altered methylation could potentially give rise to imprinting disorders. An unexpectedly high incidence of imprinting disorders including Beckwith–Wiedemann syndrome (BWS) and Angelman syndrome in children conceived with assisted reproductive technologies (ART) has been reported (Gosden et al., 2003). Culture media are known to be capable of altering gene expression and imprinting (Manipalviratn et al., 2009; De Waal et al., 2013). The National Hospital Discharge Registry of Denmark was used to see if there was an increase in reported imprinting-related diseases, mental retardation, cerebral palsy and malignancy with the follow-up period varying between 12 months and 13 years. No significant differences were observed between the cryopreservation and non-ART groups. Even though this is one of the largest studies so far, because of the rarity of imprinting disorders (BWS 1 in 13 7000 and Angelman syndrome 1 in 300 000) (Manipalviratn et al., 2009) even larger studies will be needed to form a robust conclusion.

A variety of imprinted genes are involved in regulating embryo development and cell proliferation; therefore, imprinting disorders are believed to be associated with intrauterine growth restriction, low (Nelissen et al., 2011) and high birthweight (Wang et al., 2010). As a consequence, several studies investigated the change in the distribution of birthweight among babies born from ART as indirect evidence of imprinting disorders. The data are so far controversial, showing both presence (Dumoulin et al., 2010; Nelissen et al., 2012) and absence (Vergouw et al., 2012) of an effect of culture media or cryopreservation (Källén et al., 2005; Shih et al., 2008; Pinborg et al., 2010) on newborn weight. Recent evidence suggests that babies derived from vitrified embryos have higher birthweight in comparison with fresh or slow-freezing cycles (Levi Setti et al., 2013; Liu et al., 2013).

There are several potential difficulties in interpretation of these studies. From the biological perspective, birthweight depends on an extensive list of different factors including, but not limited to, maternal age, parity, bodyweight, ethnicity, social status, smoking, underlying medical conditions (e.g. diabetes), length of subfertility, pre-eclampsia, medications in pregnancy, congenital infection in pregnancy, congenital malformation and gestational age. Each of the multiple and variable steps of ART need also to be taken into consideration when birthweight is assessed, including details of ovarian stimulation (dose and type of drugs used), estradiol level during the stimulation, type and dose of trigger used, culture
media, methods of fertilization, methods and frequency of embryo development assessment and handling, time of embryo transfer, quality and number of embryos transferred and methods of cooling and thawing used. It is clear even from the non-exhaustive list above that no retrospective studies would be able to distinguish which variable contributed primarily to the observed changes in birthweight.

A recent systematic review suggests that ART is associated with a 30–40% increase in birth defect risk (Hansen et al., 2013). Both the patient’s characteristics and some aspects of the ART process itself seem to contribute to this increased risk. However, it is still not clear precisely which type of ART exposure contributes to long-term effects for the offspring. There is much less clarity on the impact of embryo cryopreservation. A number of studies on children born from cryopreserved embryos have been published (Heijnsbroek et al., 1995; Sutcliffe et al., 1995; Wennerholm et al., 1998; Aytoz et al., 1999). These authors all concluded that cryopreservation induced no major pathological features. The systematic review of pregnancy outcomes, such as preterm birth, weight, perinatal mortality, birth defects and childhood morbidity, of children born after cryopreservation of embryos is also reassuring (Wennerholm et al., 2009). However, the majority of data come from retrospective studies many of which do not include information regarding the cryopreservation protocol used, and the stage at which embryos were frozen. Furthermore, the outcome measures are not defined according to the same criteria and also the methods of outcome assessment are also not always strict. Large-scale properly controlled multicentre studies are needed concerning the health of the progeny born using cryopreserved embryos.

In summary, children born following embryo cryopreservation appear to be unaffected by this procedure. Indeed, as far as embryo cryopreservation is concerned, some authors suggest that this procedure may result in higher pregnancy rates (Roque et al., 2013) ‘healthier’ babies or more favourable perinatal outcome (Maheshwari et al., 2012). However, this reassurance derives from studies with a high coefficient of heterogeneity, measuring safety outcome via miscarriage rate and clinical pregnancy rates and giving no details about cryopreservation protocols. The reason for apparently better outcome in frozen cycles is also not entirely clear. Some authors speculate that the endometrial and hormonal environment for embryos might be less hostile in a frozen cycle (Shih et al., 2008; Halliday et al., 2010; Evans et al., 2014). This is supported by a lower rate of blastogenesis disorders in frozen cycles in comparisons with fresh: the latter are considered to be of possible environmental disturbance, rather genetic, origin (Halliday et al., 2010). However, the question remains whether or not cryopreservation has any impact on the embryo genome directly or indirectly (in terms of reprogramming). In contrast to human sperm, where there exist a number of studies looking at DNA integrity after cryopreservation, with the exception of one, when DNA integrity index was assessed as a marker of apoptosis (Li et al., 2012), there are no relevant studies on human embryos. Animal studies show increased DNA single-strand breaks in embryos following cryopreservation, but more studies are needed to assess the molecular effects of exposure to cryoprotectants, particularly whether cryopreservation causes any structural and functional conformation or ‘reprogramming’ in the embryonic genome. Clinical outcomes following cryopreservation are largely reassuring with no significant increase in congenital malformation rate, but many studies have methodological weaknesses. Well-designed prospective multinational long-term studies are needed. The effect of cryopreservation on mtDNA also needs to be investigated in different species, including humans.

Conclusions
This review was devised to provide an overview of the literature assessing possible effects of cryopreservation on the structural and functional integrity of the genome of reproductive cells and embryos. This effect can be assessed in at least two possible ways: directly, by looking at the structures of concern at the molecular level and indirectly, by assessing gene expression afterwards or looking at phenotypic characteristics in the offspring. Both approaches have advantages and disadvantages as well as clinical, methodological and ethical challenges. The biological plausibility of the interaction between the variety of the extreme factors of cryopreservation and structural and functional integrity of the genome is outlined above. The mechanisms by which the process of cryopreservation might influence the integrity of the genome are outlined in Fig. 2.

Sperm
To date, direct studies have demonstrated that cryopreservation most likely affects DNA integrity in sperm; however, this effect might vary dramatically and largely depends on the initial quality of the semen sample. A better understanding of the reason for this variability in sperm cryo-resistance might better help to reduce or eliminate unwanted effects. A very limited number of animal studies suggest that the effect of cryopreservation on sperm DNA may have implications for embryo gene expression and for the later life of offspring. However, it remains unclear if the changes in DNA integrity caused by freezing have any implications for future offspring in humans. Clearly, a more evidence-based approach is needed for implementing standards for sperm cryopreservation protocols and RCTs are urgently needed to establish the superiority of freezing regimes (vapour versus computerized) and cryoprotectant usage. More clarity is also required in establishing if routine addition of zinc and/or antioxidants, such as ascorbic acid, resveratrol or rifafine, to cryoprotectant media should be recommended. DNA integrity should be considered as one of the primary outcomes in future investigations. Furthermore, different approaches need to be developed for the assessment of DNA damage in non-toroidal S/MAR linker regions, since the currently used methods, such as TUNEL, comet or SCSA, are mainly focused on nuclear toroids DNA breakage, that have low predictive value for reproductive outcome (Noblanc et al., 2013). Furthermore, the currently used sperm DNA damage tests may not be entirely fit for purpose since they were originally developed for somatic cells with completely different chromatin structure (Björndahl and Kvist, 2014). Highly variable chromatin stability in sperm (superstabilized versus decondensed) could cause potentially high false-negative or false-positive rate when these tests are applied to sperm.

More animal and human studies are needed to establish the involvement of the oocyte repair system in restoring sperm contributed ‘imperfections’. This might provide the possibility to manipulate the repair mechanism and might even help not only to repair iatrogenic-caused lesions but also influence intrinsic problems in cases of recurrent miscarriages or age-related decline in oocyte quality.

Oocytes
There is big gap in our understanding of the effect of cryopreservation on oocytes. The oocyte has been the most challenging biological system to freeze for a number of reasons including relatively large cell size and low surface area to volume ratio, poor membrane permeability and relatively
high content of water and lipids. Although significant improvement has been achieved in survival and fertilization rate of oocytes with vitrification, the effect of high concentrations of cryoprotectants on eggs remains understudied. From the very scant follow-up data that are available, it is premature to conclude that there is no increase in birth abnormalities following oocyte cryopreservation. Regulatory and professional bodies worldwide should develop a prospective registry for all births derived from cryopreserved oocytes enabling greater access for epidemiological studies. It is also time to urge all to expand the assessment of success beyond clinical pregnancy rate or live birth rate. Meanwhile, it is crucial to continue direct studies in human and animal cells to assess DNA integrity, methylation, DNA repair systems and consequent gene expression after different cryopreservation methods.

Embryos
The effect of cryopreservation on the embryo genome has also been grossly under-examined. In spite of the routine and widespread use of embryo cryopreservation in clinical practice, there are fewer studies focusing directly on DNA or chromosomal integrity following freezing and thawing of embryos than in oocytes, possibly as a result of embryo-specific ethical restrictions and clinical need. However, the animal studies suggest that there are certain steps that could be undertaken to minimize the negative effects of freezing on genetic integrity.

Optimization of freezing protocols may allow us not only to minimize unwanted effects, but also develop additional methods towards improving the health and well-being of future generations by selecting the ‘fittest’ embryo that could result in a birth of one healthy baby.

Suggestions for the future
From a practical perspective, we recommend the followings steps should be undertaken by the international community in an attempt to clarify if methods of cryopreservation could have any implication for genetic integrity and function in sperm, oocytes and embryos.

Avoiding using global terms such as cryopreservation in the context of assessing the effects (or be more precise regarding the exact nature of the cryopreservation?)

As the current review highlights, there is a vast number of variables that could potentially influence the outcome of cryopreservation including but not limited to: initial quality of material, the conditions under which this material was obtained, cryoprotectant media, length of exposure, freezing vial or vessel, rate of cooling and thawing. Animal studies, quite convincingly, demonstrate that fluctuation in these variables (Table III) could potentially cause a significant effect on the genetic integrity. For example, the slow freezing method using glycerol and antioxidants for storage of a good quality sperm sample might give a
Expanding the current methods of assessment of the genome

There has been tremendous progress in the methods used for DNA assessment in the last decade. Whole-genome sequencing is a valuable and powerful technology, the cost of which reduces dramatically year on year but is thus far under-utilized in the context of the scientific questions raised in the current review. The search should expand beyond the techniques that focus on DNA fragmentation only, since other types of DNA changes, such as nucleotide substitutions, deletions and insertions, can also take place and will be un-noticed with TUNEL or COMET assays.

Having a more targeted search and multi-assay approach

The current review summarized possible mechanisms that could be involved in affecting DNA during cryopreservation (Fig. 2).

Methods to assess levels of 8-oxoguanine and 8-hydroxy-2-deoxyguanosine, known by-products of oxidation, might be of more value than a simple FISH test, since a surplus of free radicals is expected to play a role during cryopreservation.

We also believe that the study of regions of more rigid DNA with higher compactness also deserves more attention, since this is more likely to be affected by osmotic pressure. Overall, each assay is designed to detect a certain type of chromatin DNA damage; therefore, if overall chromatin structure needs to be ascertained, a multi-assay approach would be required.

Targeting the ‘weakest link’ of cells

Any biological system has some cellular structures that are more prone to mutations under stress conditions than others. Examples of these are mtDNA or DNA macro satellites and therefore it would be reasonable to investigate the structure and function of these after cryopreservation.

Targeting in more detail the systems that respond to the presence of DNA damage

This might be especially relevant for sperm. There are multiple studies demonstrating DNA fragmentation in normal ejaculate as elucidated above. However, there is little or no practical value in demonstrating an increase in DNA fragmentation after cryopreservation, since it does not prove that the sperm cells with fragmented DNA actually fertilize the eggs following ICSI. Therefore, studies assessing response of the DNA repair system in the embryo derived from cryopreserved sperm would be of more practical value. These may open up areas for the potential improvement of the outcome by means of stimulating the DNA repair system.

Introducing standards of cryopreservation for clinical practice

As more evidence becomes available, national and international organizations should aim to provide guidance to the clinics on optimal cryopreservation protocols and media for different types of cells. Different protocols might be needed for high and poor quality cells. There is a large variety of commercially designed cryoprotectant media currently available, with some of them not disclosing exact content. The value of adding different antioxidants, zinc and free radical chelators need to be assessed systematically. There must be some regulation on what can and cannot be included in the media used for clinical use. It is also important to elucidate how some of the unintended deviation in the protocol might affect the quality of cryopreserved–thawed material.

Promoting basic research

It appears that before conducting RCTs, we still have a gap in fundamental knowledge that needs to be addressed urgently. More basic research is needed. As demonstrated above, in spite of the routine use of embryo cryopreservation, very few studies have investigated the cellular consequences of this process on subsequent development.

It is difficult to understand how it could be biologically plausible that reproductive cells or embryos subjected to the completely un-natural process of cryopreservation would have absolutely no structural or functional response to it. Therefore, what we need to learn is the mechanism of cellular response to the insults of cryopreservation in an attempt to preserve the genome. We also need to understand thoroughly how to minimize and optimize every possible step that could potentially cause a harmful perturbation in the reproductive cells. Eventually, we may discover some of the cellular defence mechanisms that make cryopreserved cells/embryos more able to survive.

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J.K. conceived the idea, proposed the scope, identified the articles and wrote the manuscript. A.T. identified and analysed the articles and co-wrote the manuscript. Y.K. identified and analysed the articles and reviewed the final version of manuscript.

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