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Clinician-induced (iatrogenic) damage incurred during human infertility treatment: Detrimental effects of sperm selection methods and cryopreservation upon the viability, DNA integrity, and function of human sperm

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ABSTRACT

Human infertility is becoming increasingly prevalent and is now estimated to affect 10% of couples worldwide. Although phenomenal development in assisted reproductive technology (ART) has yielded a powerful and sophisticated discipline with which to combat many types of human infertility, success rates vary markedly and rarely exceed 40%. Mounting evidence suggests that laboratory techniques used routinely in ART may inadvertently impart detrimental effects upon gamete and embryo viability and competence. In this mini-review, we discuss how routine cryopreservation methods, commonly used in ART, may exert iatrogenic (clinician-induced) damage upon sperm structure, DNA integrity, and function. It is recommended that future research programmes aim to refine or replace current cryobiology protocols in order that the efficacy of ART can be optimised accordingly.

1. Assisted reproductive technology (ART)

Infertility affects approximately 10% of couples globally and has led to phenomenal growth in a series of clinical laboratory techniques designed to combat such conditions, collectively termed assisted reproductive technology (ART)[1,2], resulting in the birth of more than 4.6 million babies worldwide. Infertility may arise as a result of male or female factors, a combination of both factors, or may arise for unknown reasons (idiopathic infertility[2]. Female infertility often occurs as a result of mechanical factors such as endometriosis, uterine lining defects, anovulation, or cervical polyps and cysts[3], while male factor infertility is commonly associated with significant reductions in seminal sperm (azoospermia), reductions in sperm concentration (oligozoospermia), poor sperm motility (asthenozoospermia), or abnormal sperm morphology (teratozoospermia). Other major causative male factors include varicocele (anomalous enlargement of veins in the scrotum resulting in compromised blood drainage in the testicles), cryptorchidism (absence of one or both testes from the scrotum), or idiopathic factors[4].

While the clinical and laboratory aspects of ART have evolved enormously since their first successful implementation in 1978, global pregnancy and live birth rates remain relatively low and rarely exceed 40%[5]. Pregnancy and delivery via routine conventional ART procedures such as *in vitro* fertilisation (IVF; whereby sperm and oocytes are co-incubated in culture media) or intracytoplasmic sperm injection (ICSI; whereby a single

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sperm is microinjected into the ooplasm) remain as low as 22.4% and 23.3% respectively[5]. As the number of couples seeking infertility treatment increases, it is essential to further develop ART success rates to improve chances of successful conception[6].

Over recent years, the unpredictable success rate of ART has drawn much attention, particularly in terms of the potential for ART to promote clinician-induced (iatrogenic) damage to gametes and embryos. Characterising the extent of such damage and identifying potential sources would provide an opportunity to upgrade, modify, develop and improve routine protocols[7]. ART procedures such as IVF and ICSI are performed on pre-treated/manipulated gametes outside of their natural environment, mainly to enhance and facilitate the fertilisation process. For example, oocytes are normally exposed to the enzyme hyaluronidase prior to ICSI to remove cumulus cells, followed by mechanical corona removal to expose the oocyte for maturity identification and injection. Semen is also extensively processed in order to pre-select good quality sperm exhibiting high motility, normal morphology and concentration. It is a distinct possibility that such manipulative techniques may inadvertently compromise important features relating to gamete integrity, structure, and function.

2. Oxidative stress and DNA fragmentation

Reactive oxygen species (ROS) like hydrogen peroxide are oxygen-derived molecules that have the capacity to act as powerful oxidants and are normally formed in low concentration in the both the male and female genital tracts. Oxidative stress, an elevation in the steady-state levels of ROS that exceeds the body's antioxidant defences[8,9], has been implicated in a number of different reproductive scenarios ranging from endometriosis to oocyte maturation, as well as being indicated in the etiology of defective embryo development, and a number of sperm conditions such as asthenozoospermia, and sperm DNA damage (Figure 1)[9-11]. While it is generally accepted that it is difficult for an *in vitro* system to mimic the exact physiological conditions of an *in vivo* system, multiple factors inherent in an ART setting may lead to elevated levels of oxidative stress and a suboptimal ART outcome[9,12].

Oxidative stress can induce damage to sperm membranes and DNA, ultimately leading to non-viable sperm. The use of damaged sperm in ART has been implicated in alterations in oocyte and/or embryo development. Critically, however, sperm selected for use in ART most likely originate from an environment experiencing oxidative stress. It follows, therefore, that DNA damage may have already occurred in a large percentage of such sperm prior to semen processing[9,14]. Similarly, oocytes and embryos may contribute to increased levels of ROS due to inadequate protective antioxidant mechanisms present *in vivo*. Indeed, cells cultured *in vitro* may be exposed to a relative "hyperoxic" environment compared to conditions *in vivo*[9,15].

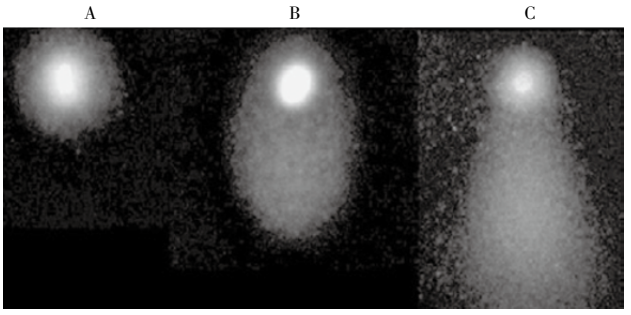

Figure 1. Representative micrographs of human sperm following an electrophoresis comet assay for DNA fragmentation.
The majority of DNA from normal sperm migrates as a single cohesive mass, while in sperm with DNA fragmentation, a comet-like structure is created with the fragmented DNA forming the "tail". The further the migration, the more profound the damage produced. A, B and C represent undamaged DNA, intermediate DNA damage, and extensive DNA damage in human sperm, repectively. Figure was reproduced from Enciso *et al.*[13] with permission.

3. Effects of ART treatment upon sperm

The perceived role of sperm has tended to be generalised to the mere transportation of paternal DNA into the oocyte. However, sperm play a crucial role which extends beyond the early stages of fertilisation, including embryogenesis, and the events leading to implantation[16]. In an ART treatment, semen samples are subjected to a variety of processing methods and chemicals (*e.g.*, sperm washing, retardation of motility by polyvinylpyrrolidone, cryopreservation), in order to enhance sperm parameters prior to use, to facilitate the fertilisation procedure, and to maximise the chances of successful fertilisation. However, it follows that each of these treatments may impart detrimental effect upon the sperm being processed.

3.1. Sperm washing as a means of selecting sperm for ART

Sperm preparation techniques such as density gradient wash (DGW) and swim-up (SUP) are routinely used in ART to produce a processed sample that is enriched with mature, motile, and morphologically normal sperm[17]. SUP depends on a migratory approach following the formation of a sperm pellet by brief centrifugation, whereby sperm with the greatest motility "swim-up" into culture medium, while DGW depends on sperm density and sedimentation[18]. DGW and SUP sperm isolation techniques may play a role in selecting sperm without DNA damage, and they are able to select non-fragmented sperm following cryopreservation[18,19]. Sperm quality has been shown to be superior following density gradient compared to SUP processing[20,21].

However, DGW and SUP require centrifugation, which may increase sperm ROS production, thereby damaging not only the sperm membrane which affects motility and fertilising capability but also sperm DNA[22]. Furthermore, time-dependant incubation of prepared sperm at RT or 37

°C reduces sperm motility and also significantly increases sperm DNA fragmentation and chromatin decondensation[23].

3.2. Alternative methods for sperm selection

Several newer selection methods have been developed with the objective of improving sperm preparation protocols for ART, aiming to isolate mature, structurally intact, and non-apoptotic spermatozoa with high DNA integrity[17]. One group of new selection techniques separate sperm by size and electronegative charge[17,24], do not involve centrifugation and may avoid the generation of ROS[17,25]. Another sperm preparation technique, based on the externalisation of phosphatidylserine to the outer surface of the sperm membrane, a feature of early apoptosis, has been proposed as a basis for selection of non-apoptotic spermatozoa[17]. While not as efficient as established techniques such as DGW or SUP in selecting a large proportion of viable sperm, a combination of DGW and phosphatidylserine techniques resulted in the selection of sperm with a 50% reduction in DNA fragmentation rates[26], and significantly higher rates of survival following cryopreservation-thawing compared to samples prepared by DGW only[27].

Morphological selection techniques have also been proposed based largely upon sperm ultra-morphology. Assessing the sperm acrosome, post-acrosomal lamina, neck, tail, mitochondria, nuclear shape, and chromatin content, motile sperm organelle morphology evaluation has been utilised prior to a modified version of ICSI, termed intracytoplasmic morphologically-selected sperm injection[28]. While such an approach particularly benefits situations where identification of specific sperm organelles is required, *e.g.*, the acrosomal component in cases of globozoospermia[29,30], there is a significant debate over the clinical viability of motile sperm organelle morphology evaluation followed by intracytoplasmic morphologically-selected sperm injection, considering that it is an elaborate and highly skilled procedure that involves prolonged sperm manipulation, adding significantly to routine ICSI processing times.

3.3. Cryopreservation

Cryopreservation is an essential tool for infertility treatment, and it is used not only in sperm donation programmes or in cases of poor semen quality but also following cases involving surgical sperm retrieval, or fertility preservation for cancer patients prior to treatment. Cryopreservation involves cooling of the sperm to sub-zero temperatures (−196 °C) by means of controlled-rate slow freezing or vitrification ('glass-like' solidification of cells), resulting in the temporary suspension of all biological activity (Figure 2). Samples treated in such a manner can be stored for long periods of time[31]. A combination of cryoprotectant solutions (glucose, sucrose, ethylene glycol, propylene glycol, dimethyl sulfoxide, and 2-methyl-2,4-pentanediol) is added to sperm samples in order to avoid damage as a result of cryopreservation or thawing procedures. However, high cryoprotectant concentrations may result in cellular damage, cause biochemical changes, and induce lethal osmotic injury[32,33].

Cryopreservation techniques are under constant scrutiny to reduce potential risks to gametes and embryos. Newer cryoprotectants can replace water in cells during freezing[34], while vitrification has been utilised to replace slow-freezing technologies[35], allowing higher cryoprotectant concentrations within cells and faster freezing to preserve cells in a 'glass-like' state, which avoids the formation of damaging ice crystals[35,36]. Simplified or adapted vitrification protocols have reported even better survival and fertilisation rates compared to original protocols, and there is thus a significant hope for considerable further improvements in the near future[34,37-39].

Figure 2. Students learning the intricacies of cryopreservation (A) and vitrification (B) as part of the MSc in Clinical Embryology at the University of Oxford, U.K.
Comprehensive training, coupled with the continued modification of such techniques as a result of investigative research, is highly likely to lead to improved success rates. For more information on the Oxford MSc in Clinical Embryology, see www.obs-gyn.ox.ac.uk/MSc.

4. Vulnerability of sperm DNA integrity to freeze-thaw cycles

Sperm cryopreservation can detrimentally affect sperm integrity and the dynamics of DNA fragmentation[40,41]. Increased levels of DNA fragmentation and DNA oxidative damage are often observed in thawed sperm[42]. Further incubation of post-thaw semen also results in a significant increase in DNA fragmentation[19]. The act of cooling, freezing, and thawing also damages sperm membrane structures, leading to a significant reduction in sperm viability and motility. Cryopreserved sperm are prone to oxidative stress damage, leading to an increase in the number of ROS present, but with a coincident reduction in the level of important antioxidants such as glutathione[43]. Cellular lipids and proteins can be damaged by changes in the concentration of oxidants/antioxidants[43]. Furthermore,

oxidative stress is known to reduce conception rate, compromise embryonic development, and correlate to an increased risk of miscarriage and childhood mortality[44-47].

However, while several studies indicate that sperm chromosomes and sperm viability were not affected by cryopreservation[48], other studies suggest that DNA fragmentation measurements are often under-estimated due to skewing of the TUNEL assay by DNA compaction and cell vitality[49]. Furthermore, fertilisation rates following ICSI were higher for fresh sperm (73.8%) compared with frozen sperm (68.7%) in a study carried out by Borges *et al*[50]. Cryopreserved asthenozoospermic (sperm with reduced motility) and oligoasthenozoospermic (low sperm count with reduced motility) were prone to further severe damage and consequently reduced fertilisation rates. Studies suggest that DNA fragmentation in sperm is mostly induced during sperm transport through the seminiferous tubules and the epididymis[9,51,52], mediated by ROS production by immature sperm and by the nitric oxide-producing epithelial cells that line the epididymis, where sperm are highly packed, facilitating transfer of free radicals from immature to mature sperm. This is particularly a reason for concern regarding ART protocols, where a similar mechanism may occur within the pellet of centrifuged semen where sperm would also be highly packed[9].

Interestingly, the eggs of marine invertebrates are known to undergo a 'respiratory burst' at fertilisation, involving an increase in levels of ROS within the zygote, suggesting that mitochondrial activity is stimulated by calcium release in the ooplasm following gamete fusion[53-55]. Intriguingly, Lopes *et al*. demonstrated that levels of oxygen consumption and ROS production were higher during fertilisation and cell cleavage stages within bovine zygotes[55], while Vandaele *et al*. indicated that the short term exposure of bovine cumulus-oocyte-complexes to levels of hydrogen peroxide led to improved embryo development[56]. While these studies indicate a possible role for ROS within embryo development, it is also possible that an abundance of ROS, introduced either within the oocyte or sperm as a result of ART, may account for deficient/abnormal embryogenesis. Indeed, cumulus cells are routinely removed for clinical procedures like ICSI, which may result in a deficiency of ROS within the zygote when it is required. It is therefore important that routine ART techniques associated with the potential induction of ROS production, such as overnight incubation and SUP, are investigated further in order to improve success rates.

5. Sperm function may be compromised by the use of cryopreservation

Besides DNA damage, cryopreservation may exert detrimental effects upon key sperm proteins associated with fertility[57]. However, specific effects of iatrogenic damage incurred by ART upon critical sperm proteins have not yet been extensively determined, mostly pertaining to difficulties in determining specific functions for the numerous proteins involved in sperm viability. The human sperm proteome, characterised in great detail by Johnston *et al*.[58], is thought to contain more than 1 760 proteins, 1 350 being identified in the soluble fraction, 719 in the insoluble fraction, and 309 identified in both fractions.

A number of studies have begun to explore the human sperm proteome in order to identify immune-dominant sperm surface antigens[59], and to identify new biomarkers for male infertility[60]. Furthermore, Pixton *et al*. mapped the sperm proteome of a human male who had experienced failed IVF and discovered at least 20 proteins with abnormal expression patterns compared to fertile controls[61]. Indeed, knock-out mouse models have shed light on numerous sperm proteins which may play crucial roles in spermatogenesis or sperm function during fertilisation (Table 1)[62]. More recently, the human sperm proteome has been used to compile a protein profile of capacitated versus freshly ejaculated human sperm[63]. However, while the effects of cryopreservation upon sperm DNA integrity has been exhaustively investigated, the effects of cryopreservation upon the vast array of sperm proteins thought to be involved in determining fertility remain largely un-investigated. As such, it is crucial that new studies aim to investigate related changes in vital sperm proteins. One key point to consider is how clinical activity/processing may exert influence over the quality of sperm DNA and protein, and ultimately, the 'fitness' of sperm to carry out their required reproductive function.

Kashir *et al*. reported that cryopreservation of human sperm significantly reduced levels of the sperm-specific oocyte activation factor, phospholipase C (PLC) zeta compared to fresh human sperm (by 20%-56%), possibly leading to a compromised fertilisation capacity of such sperm[6]. Indeed, specific levels of PLC zeta correspond to successful fertilisation and embryonic development, with an absence or reduction of this protein associated with male factor infertility[4,64]. Cao *et al*. further observed that levels of heat shock protein 90 were significantly reduced in thawed human sperm following cryopreservation[65]. Similarly, sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis on freeze-thawed boar sperm further revealed significantly reduced levels of heat shock protein 90[66]. Surface proteins that act as mediators in the fertilisation pathway[67], such as P25b, are reduced in bull sperm following cryopreservation with consequential reduction in fertilisation rates[68]. Moreover, 2-dimensional poly acrylamide gel electrophoresis (2D-PAGE) analysis of cryopreserved sea-bass sperm revealed degradation in a total of 21 proteins[69].

Prior to fertilisation, sperm endure several membrane and biochemical processes in the female tract, collectively known as capacitation, in order to facilitate penetration into the oocyte[70]. Among the many intracellular modifications occurring during sperm capacitation, are elevations in protein tryrosine phosphorylation (P-Tyr)[71] and cAMP[72]. In a recent study, cryopreservation was not found to affect the regulatory pathways of capacitation and the acrosome

Table 1
Key genes involved in fertilisation (modified from Matsuk & Lamb[62]).

Gene	Effect of mutation upon functional outcome in knock-out mice	Fertility status
Ace	Compromised ability of sperm to fertilise ova	Sub-fertile
Acr	Delayed fertilisation *in vitro*	Fertile
*Adam*2	Altered sperm protein expression and adhesion defects during fertilisation	Infertile
*Adam*3	Altered sperm protein expression and adhesion defects during fertilisation	Infertile
*B*4*galt*1	Defects in sperm-egg interaction	Infertile
*Cadm*1	Oligoasthenoteratozoospermia, block at elongating spermatid stage	Infertile
*Camk*4	Impaired chromatin packaging during spermiogenesis	Infertile
*Cplx*1	Zona pellucida penetration defect *in vivo* secondary to abnormal acrosomal exocytosis	Sub-fertile
Crem	Defective spermiogenesis with aberrant post-meiotic gene expression	Infertile
Crisp	Decreased sperm-egg fusion *in vitro*	Fertile
*Fndc*3*a*	Spermatid defects with multinucleated syncytia resulting in azoospermia	Infertile
HexbB	Reduced *in vitro* fertilisation rates at older ages	Progressive infertility
*Mfge*8	Defective sperm-egg binding	Sub-fertile
*Mmel*1	Decreased fertilisation and early embryo loss	Sub-fertile
*Pgap*1	Sperm-egg interaction defects, defective sperm entry into oviduct	Infertile
*Piwil*1	Block in spermatogenesis beginning at the round spermatid stage	Infertile
*Plcb*1	Apparent decrease in fertilisation rates	Sub-fertile
*Plcd*4	Defective zona-pellucida induced acrosome reaction	Infertile
Prnd	Dysmorphic spermatids with loss of motility of spermatozoa, defective acrosome reaction	Infertile
*Pvrl*2	Sperm morphological defects, impaired egg binding and penetration	Infertile
*Rasip*1	Defective fusion of sperm with zona-free egg	Infertile
*Rbmxl*2	Abnormal sperm incapable of fertilisation	Infertile
*Spam*1	Sperm defects in hyaluronic-acid binding	Sub-fertile
*Tyst*2	Defective sperm-egg binding and motility in viscous media	Infertile
*Wipf*3	Sperm head defects and decreased fertilisation	Infertile
Zpbp	Abnormal acrosome and globozoospermia	Infertile

reaction; however, levels of P-Tyr and cAMP were significantly reduced in cryopreserved sperm[73].

6. Conclusions

Sperm selection and cryopreservation are commonly-used techniques in ART. However, current selection protocols may underlie an increased incidence of DNA fragmentation in sperm, thus compromising viability. Growing evidence suggests that cryopreservation may also exert a number of detrimental effects upon key sperm proteins involved in capacitation, acrosome reaction and fertilisation. However, our understanding of such effects is hindered by our limited understanding of the exact functional role of the multitude of proteins making up the sperm proteome.

The extensive development of ART has not only revolutionised clinical treatment of infertility but also facilitated significant advances in our understanding of human reproduction. However, pregnancy and birth rates resulting from such technology remain frustratingly low. Modifications of current ART protocols and the development of novel techniques associated with advances in science and technology are essential in order to provide improved clinical and diagnostic tools for patients. Recognising the potential for iatrogenic damage upon gametes and embryos during ART will facilitate the improvement, development, and introduction of modified ART protocols which are highly likely to improve success.

Conflict of interest statement

We declare that we have no conflict of interest.

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