Implication of apoptosis in sperm cryoinjury

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Abstract  Apoptosis is an ongoing physiological phenomenon that has been documented to play a role in male infertility, if deregulated. Caspase activation, externalization of phosphatidylserine, alteration of mitochondrial membrane potential and DNA fragmentation are markers of apoptosis found in ejaculated human spermatozoa. These markers appear in excess in subfertile men and functionally incompetent spermatozoa. Sperm cryopreservation is a widely used procedure in the context of assisted reproductive techniques. Cryopreservation and thawing is a procedure that inflicts irreversible injury on human spermatozoa. The damage is manifested by a decrease in recovery of viable spermatozoa with optimum fertilization potential. This review describes the implication of apoptosis as one of the possible mechanisms involved in sperm cryoinjury. Evidence shows significant increase in some apoptosis markers following cryopreservation and thawing. On the other hand, the increase in sperm DNA fragmentation following cryopreservation and thawing requires further investigation. Specific technical measures should be applied to minimize the induction of apoptosis in human spermatozoa during cryopreservation and thawing. These include standardization of freezing protocols and cryoprotectant use. Selection of non-apoptotic spermatozoa may also prove to be of benefit.

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Apoptosis in ejaculated human spermatozoa

Apoptosis: general concepts

Apoptosis, a programmed cell death mechanism, is vital to many eukaryotes as it regulates cell count and removes unnecessary cells that compromise survival. It involves a series of biochemical events that trigger cellular morphological alterations eventually leading to cellular termination. Apoptotic changes are typified by nuclear fragmentation, chromatin condensation, mitochondrial enlargement and irregular changes to the plasma membrane (Kerr et al., 1972).

The removal of dead cells by neighbouring phagocytic cells has been termed efferocytosis. Dying cells that undergo the final stages of apoptosis display phagocytotic molecules, such as phosphatidylserine, on their cell surface.
Phosphatidylserine is normally found on the cytosolic surface of the plasma membrane, but is redistributed during apoptosis to the extracellular surface by a hypothetical protein known as scramblase. These molecules mark the cell for phagocytosis by cells possessing the appropriate receptors, such as macrophages. Upon recognition, the phagocyte reorganizes its cytoskeleton for engulfment of the cell. The removal of dying cells by phagocytes occurs in an orderly manner without eliciting an inflammatory response (Li et al., 2003; Martin et al., 2004; Vandivier et al., 2006).

Apoptosis is largely regulated by the activation of cysteine proteases called caspases. These are cysteinyl-aspartate-specific proteases that are produced as inactive zymogens, consisting of an NH2-terminal domain, a small 10 kDa subunit and a large 20 kDa subunit. Apoptosis is achieved by activation of caspases which cleave with high specificity at the aspartate residue (Fuentes-Prior and Salvesen, 2004). Their stimulation leads to the morphological changes and characteristics of apoptotic cells. Apoptotic initiator caspases include caspases-2, 8, 9 and 10, which activate effector caspases (3, 6 and 7) leading to cleavage of various substrates and completion of the apoptosis process. Caspase-3 activation decides the fate of the cell in the apoptotic cascade, where the cell cannot be reverted back to normal state, thus caspase-3 is considered the most important effector caspase, and its activation marks the ‘point of no return’ in apoptosis (Earnshaw et al., 1999).

Different pathways are involved in apoptosis activation. The Fas receptor plays an important role in activating effector caspases (Hetz et al., 2005; Medema et al., 1998; Thornberry and Lazebnik, 1998). The presence of the Fas receptor occurs in <10% of ejaculated healthy spermatozoa and >50% of ejaculated semen with oligozoospermia (Sakkas et al., 1999), suggesting that these apoptotic pathways occur in spermatozoa and that a possible link between sperm quality and Fas receptor presence exists.

Apoptosis manifestations in human spermatozoa

Apoptosis in human spermatozoa has not been fully understood. The presence of caspases in spermatozoa is one of the best markers for cellular apoptosis. Another marker of apoptosis is the externalization of phosphatidylserine on the sperm membrane. This is a relatively early apoptotic marker (Martin et al., 1995). The exposed phosphatidylserine is recognized by various receptors on phagocytes for destruction. DNA fragmentation is a marker for late-stage apoptosis in spermatozoa. DNA fragmentation can be caused partially by activation of caspase-3 (Grunewald et al., 2009), which inactivates poly(ADP-ribose) polymerase (PARP), a DNA repair enzyme, subsequently inhibiting reparation of the damaged DNA. A recent study showed a positive correlation between PARP protein presence and sperm maturity. PARP homologues were present in ejaculated spermatozoa as PARP-1 (75 kDa), PARP-9 (63 kDa) and PARP-2 (60 kDa). Upon staurosporine-induced apoptosis, immature spermatozoa showed moderately reduced concentrations of PARP-1 and undetectable concentrations of PARP-2, suggesting the involvement of PARP homologues in the response to pro-apoptotic stimuli (Jha et al., 2009). Another study showed an increase in the number of early apoptotic spermatozoa upon exposure to oxidative stress and PARP inhibition (Mahfouz et al., 2009). The release of apoptosis-inducing factor from the mitochondria also promotes DNA fragmentation in the nucleus (Martin et al., 2007).

Apoptosis induction in human spermatozoa

There are several factors that may lead to induction of apoptosis in human spermatozoa. High concentrations of reactive oxygen species (ROS) is one of the factors that have been positively correlated with induction of apoptosis (Wang et al., 2003). When concentrations of antioxidant enzymes fall or the ROS concentration rises, the ROS effect overwhelms the cell leading to apoptosis. In these situations, apoptosis is mediated by activation of the BCL-2 family of proteins which sense the apoptotic stimuli and trigger the permeabilization of the outer mitochondrial membrane through the BAX and BAK proteins and the release of cytochrome c (McCintock et al., 2002). In turn, caspase-9 is activated along with APAF-1 to form an apoptosisosome, resultanty initiating the apoptosis cascade (Arnoult et al., 2003).

Toxins originating from salmonella and Chlamydia trachomatis have been shown to increase apoptosis and DNA fragmentation in human spermatozoa (Eley et al., 2005; Gorga et al., 2001). The negative effects of chlamydia and mycoplasma infections on the sperm DNA could be alleviated using proper treatment. DNA fragmentation levels decreased in men infected with chlamydia and mycoplasma following antibiotic therapy (37.7% versus 24.2%; Gallegos et al., 2008). The findings associated with chlamydia toxins may be of special interest as the organism remains responsible for a significant percentage of sexually transmitted diseases in North America. Apoptosis in human spermatozoa was also found to be higher in patients with Hodgkin disease and patients with testicular cancer. Thus, the induction of apoptosis can be a basic response to neoplastic disease (Gandini et al., 2000). Environmental toxins have also been documented as a cause of infertility in men. Exposure to pesticide reduces the sperm quality and pregnancy rates (Roeleveld and Bretveld, 2008). This may be due to compounds such as dibromochloropropane, which exerts a direct damaging effect on the sperm DNA (Bretveld et al., 2007).

Apoptosis and male fertility

Numerous studies have shown a relationship between apoptosis markers (caspase activation, phosphatidylserine externalization and DNA damage) and male infertility (Brugnon et al., 2006; Moustafa et al., 2004; Said et al., 2004; Taylor et al., 2004; Zedan et al., 2009). Phosphatidylserine externalization and DNA fragmentation were shown to occur more frequently in spermatozoa that were positive for active caspase-3 (Weng et al., 2002). Apoptosis markers appeared significantly higher in low-motility spermatozoa as compared with high-motility spermatozoa (Weng et al., 2002). Similarly, percentage of apoptosis has been shown to be negatively correlated with motility and normal morphology and positively associated with sperm tail defects (Chen et al., 2006). These findings suggest a possible relationship between increased caspase activity, DNA damage and/or phosphatidylserine externalization and sperm quality in terms of routine parameters.
Elevated apoptosis markers have also been found to negatively correlate with oocyte penetration capacity. Semen samples with lowered penetrative ability contained spermatozoa with higher concentrations of apoptotic markers (phosphatidylserine externalization, disrupted mitochondrial membrane potential, activated caspase-3) when compared with semen samples with normal penetrative ability (Grunewald et al., 2008).

Sperm cryopreservation

The processes of freezing human semen and achieving successful fertilization via intrauterine insemination were established many decades ago (Bunge and Sherman, 1953). Sperm cryopreservation provides a useful and effective method in infertility management for many men. Cancer patients can use cryopreservation to preserve their fertility prior to treatments such as radiation and chemotherapy. Also, men undergoing vasectomy procedures will use this method to maintain fertility. Subsequent uses of the preserved semen include intrauterine insemination, IVF and intracytoplasmic sperm injection. However, freezing and thawing methods expose spermatozoa to much physical and chemical damage, and thus several improvements have been made to the process of cryopreservation and thawing (Nawroth et al., 2005).

Cryoprotectants are low-molecular-weight and highly permeable chemicals that serve to protect spermatozoa from freeze damage by ice crystallization. There are four main known cryoprotectants: glycerol, ethylene glycol, dimethyl sulphoxide and 1,2-propanediol. Cryoprotectants act by decreasing the freezing point of a substance, reducing the amount of salts and solutes present in the liquid phase of the sample and by decreasing ice formation within the spermatozoa (Royere et al., 1996).

There are two main freezing techniques used in sperm cryopreservation — slow freezing and rapid freezing (vitrification). The slow freezing method may be manual or automated involving a semi-programmable freezer. It is performed by simultaneously decreasing the temperature of the semen while adding cryoprotectant in a stepwise manner and eventually plunging the samples into liquid nitrogen. This method can cause ice crystallization to form if the cooling rate is too fast or too slow. The rapid freezing method, vitrification, is a process by which the samples are exposed to nitrogen vapour, equilibrated for some time and are then directly plunged into liquid nitrogen. This method does not allow ice crystallization to occur (Nawroth et al., 2005). Cryoinjury is not limited to the freezing process but may also occur during thawing. Ice crystals melt during thawing and could result in damage of the sperm organelles. Therefore, the thawing phase and cryoprotectant removal must be conducted in a stepwise manner, similar to freezing (Verheyen et al., 1993). Normozoospermic semen samples appear to be more tolerant to damage induced by freezing and thawing compared with oligozoospermic samples. It was reported that motile spermatozoa could be recovered after five refreeze—thaw cycles in normozoospermic samples and after two refreeze—thaw cycles in oligozoospermic specimens samples (Verza et al., 2009).

Mechanisms and manifestations of cryoinjury in spermatozoa

There are many risks associated with cryopreservation. Freezing to subzero temperatures causes irreversible injury in spermatozoa, which gets aggravated during the thawing procedure, thereby reducing the sperm cryosurvival rate. Cryoprotectants themselves can be toxic if used in high concentrations since spermatozoa are vulnerable to osmotic changes induced by cryoprotectants (Gao et al., 1993). Spermatozoa can also undergo intracellular and extracellular ice formation, excessive dehydration (due to increased solute concentration) and denaturation of proteins due to shifts in pH as well as membrane damage caused by close proximity freezing of cells (Figure 1).

The cooling rate plays an important role in determining the extent of cryoinjury to the spermatozoa. During freezing, ice nucleates in the extracellular matrix, eliciting an osmotic gradient. During freezing, water will move across the membrane from the intracellular to the extracellular space and intracellular ice formation will occur with rapid cooling rates. If the cooling rate is too slow, water can join the ice phase of the extracellular space and the cells become osmotically inactive due to intracellular ice formation and loss of cell membrane integrity (Frim and Mazur, 1983). Thus, the cooling rate is very critical — too rapid will cause severe intracellular ice formation but too slow can expose the cells to toxicity damage by high solute concentrations. Sperm motility, plasma membrane integrity and mitochondrial function are inversely correlated with cooling rates, which indicates that too fast or too slow cooling rates can cause these parameters to be compromised (Henry et al., 1993).

Osmotic changes during the cryopreservation process expose spermatozoa to changes in osmotic conditions...
resulting in cellular damage. In relation to cryopreservation, survival of human spermatozoa was decreased at hyposmotic conditions due to cellular swelling leading to lysis. Spermatozoa were initially resistant to hyperosmotic conditions; however, significant cell damage occurred when returned to isosmotic conditions (Curry and Watson, 1994; Meyers, 2005).

The membrane integrity is an important factor for the sperm motility and viability. Membrane fluidity has been positively correlated with the recovery of motile, viable spermatozoa from a cryopreserved sample (Giraud et al., 2000). The integrity of the sperm membrane is affected during cryopreservation and thawing processes. Alterations in sperm membrane integrity have been well observed in ultrastructural studies (Barthelemy et al., 1990). The stability of the membrane is affected by changes in temperature, volume changes associated with the movement of water, cryoprotectants and osmotic stress due to increased salt concentration. During cryopreservation, the initial cooling process causes phase transitions of the membrane lipids and impairs the function of membrane proteins, which are responsible for ion transport and metabolism (Oehninger et al., 2000). These changes compromise the membrane integrity and causes loss of function. Glycerol has been found to have a direct effect on the membrane by altering its fluidity via increasing the order of the fatty acids (Hammerstedt et al., 1990). Cryopreservation of human spermatozoa is also known to have negative effects on sperm motility and velocity due to membrane swelling and acrosomal leakage and degeneration. It has been reported that irregular interaction between DNA and nuclear proteins can lead to impaired motion parameters in spermatozoa (Royere et al., 1996).

**Implication of apoptosis in sperm cryoinjury**

**Increased apoptosis in cryopreserved spermatozoa**

Studies have revealed that apoptosis markers tend to increase in spermatozoa following cryopreservation and thawing. In an animal model, cryopreservation increased apoptosis manifestations including mitochondrial membrane potential, caspase activation, membrane permeability and phosphatidylserine externalization (Martin et al., 2004). One study has shown that cryopreservation was significantly associated with activation of caspases-3, 8 and 9, as well as disruption of the mitochondrial membrane potential. However, no significant changes were observed in DNA fragmentation (Paasch et al., 2004). Similarly, cryopreservation led to an increase in the percentage of spermatozoa showing membrane translocation of phosphatidylserine in samples collected from both patients and healthy donors, while this increase was not associated with any significant changes in DNA integrity (Duru et al., 2001).

In general, the occurrence of sperm DNA fragmentation during cryopreservation remains to be elucidated. The current body of evidence suggests that sperm DNA fragmentation is associated with an increase in oxidative stress during cryopreservation, rather than the activation of caspases and apoptosis (Thomson et al., 2009). The alteration in the mitochondrial membrane fluidity that occurs during cryopreservation will lead to a rise in mitochondrial membrane potential and the release of ROS. Subsequently, released ROS cause DNA damage in spermatozoa that present with high frequencies of single- and double-strand DNA breaks. The process of ROS generation during cryopreservation and thawing of spermatozoa has been well documented. It has been reported that ROS production by both human spermatozoa and seminal leukocytes increases on cooling to 4°C (Wang et al., 1997). Thus, cryopreserved semen samples containing leukocytes may be more prone to DNA fragmentation. In addition, the cryopreservation process has been shown to diminish the antioxidant activity of the spermatozoa making them more susceptible to ROS-induced damage (Lasso et al., 1994). In a recent study, the addition of an antioxidant to cryopreservation medium was tested to see if it could improve the post-thaw integrity of cryopreserved human spermatozoa; specifically from men with abnormal semen parameters. It was found that vitamin E was significantly associated with post-thaw motility, but neither sperm viability nor DNA fragmentation were affected (Taylor et al., 2009). Similarly, increased ROS concentrations in post-thaw spermatozoa were significantly reduced by both ascorbate and catalase (Li et al., 2009).

The occurrence of the sperm DNA fragmentation may not be the result of cryopreservation but may be more associated with the thawing. A rapid increase in sperm DNA fragmentation over time was shown with the highest rate of fragmentation occurring during the first 4 h after thawing. Therefore, thawed sperm samples should be used in the clinical setting as quickly as possible (Gosalvez et al., 2009). The DNA integrity during cryopreservation also appears to be independent of the method used (slow versus vitrification) or presence of cryoprotectants (Isachenko et al., 2004).

Comparison of fresh versus cryopreserved spermatozoa has shown that cryopreservation significantly increases the percentage of spermatozoa with activated pan-caspases from 21% to 47%. Interestingly, the concentration of activated caspase positively correlated with increasing glycerol concentration from 7% to 14% (Grunewald et al., 2005). In support, a significant increase of activated caspase-1 in healthy donors, caspase-8 in unselected infertility patients and caspase-9 in both patient and donor groups were reported after cryopreservation (Wundrich et al., 2006).

The extent of apoptosis in spermatozoa appears to be related to the type of cryoprotectant used and the protocols for cryopreservation. Annexin V binding was used to evaluate phosphatidylserine externalization after different cryopreservation protocols. The percentage of annexin V-negative spermatozoa was highest in spermatozoa cryopreserved by TEST-yolk buffer, followed by those cryopreserved with sperm maintenance media. Spermatozoa subjected to cryo-shock (no cryoprotectant) had the least annexin V-negative presentation (Glander and Schaller, 1999). Similarly, the application of 14% glycerol resulted in higher amounts of activated caspase than 7% glycerol. Therefore it was postulated that glycerol may also contribute to activation of caspases directly via toxic effects to mitochondria during sperm cryopreservation (Wundrich et al., 2006).
Apopotosis in cryopreserved subfertile semen samples

Spermatozoa from infertile men seem to be more prone to cryoinjury. It was noted that the severity of cryoinjury was greater in subfertile males and the extent of damage was correlated to the degree of oligo-asthenoteratozoospermia (Oehninger et al., 2000). Poor quality semen may be more prone to DNA damage and cell death after cryopreservation than normal semen samples. Increased concentrations of ROS cause peroxidative damage to the sperm plasma membrane, leading to reduced membrane integrity. This can deleteriously affect sperm motility by damaging the axonemal structure of the spermatozoa (Saleh and Agarwal, 2002). Annexin V, the early marker of apoptosis, was detected at higher concentrations in spermatozoa with low motility compared with spermatozoa with high motility (Weng et al., 2002). Most importantly, more recent findings documented that spermatozoa with lower motility are more susceptible to cryodamage and, consequently, have lower fertilizing capacity (Borges et al., 2007).

Sperm DNA in infertile men was found to be less resistant to damage during cryopreservation compared with spermatozoa from fertile men (Donnelly et al., 2001). Cryopreserved semen samples from cancer patients were also found to have higher DNA fragmentation compared with healthy donors (Said et al., 2009). It has been reported that men with oligozoospermia present with higher rates of sperm DNA fragmentation both pre- and post-cryopreservation compared with fertile men (de Paula et al., 2006).

Methods to decrease apoptosis during sperm cryopreservation

Optimization of the sperm preparation techniques, concentrations of cryoprotectant and freezing and thawing protocols should minimize the induction of apoptosis during sperm cryopreservation, which in turn should translate into higher success rates during assisted reproductive techniques (Figure 1). Before freezing, semen samples should be processed in a way to enhance the post-freeze sperm quality. The swim-up method to separate motile spermatozoa and the two density gradient centrifugation techniques are both used to ensure the isolation of functionally and morphologically normal spermatozoa (Sakkas et al., 2000). In a comparative study, it was found that semen samples from infertile patients prepared by a double density-gradient technique yielded higher recovery of motile spermatozoa following cryopreservation—thaw compared with those samples prepared by swim-up (Allamaneni et al., 2005). On the other hand, both density-gradient centrifugation and swim-up techniques were demonstrated to significantly reduce the extent of apoptotic spermatozoa compared with raw semen (Ricci et al., 2009).

The exclusion of apoptotic spermatozoa before cryopreservation may lead to recovery of higher quality spermatozoa that are functionally normal following thawing. Magnetic cell sorting (MACS) using annexin V-conjugated microbeads is a technique that has the ability to separate non-apoptotic from apoptotic spermatozoa based on phosphatidylserine externalization. It has been well documented that the separation of a distinctive population of non-apoptotic spermatozoa with intact membranes using annexin V-MACS may optimize cryopreservation—thaw outcome. The technique if performed prior to the freezing, was found to enhance the percentage of spermatozoa with higher motility, intact membrane mitochondrial potential and survival rates following cryopreservation (Grunewald et al., 2006; Said et al., 2005). The use of MACS can also improve motility and enhance oocyte penetration potential (Said et al., 2006a, b, 2008).

It is also important to consider the protective capacity of the seminal plasma when performing cryopreservation. The seminal plasma contains protective agents that can increase the resistance to freezing damage; however, it remains a controversy whether freezing raw or prepared samples yield better results. Sperm preparation methods are commonly used to select mature and functional spermatozoa but these procedures eliminate the presence of the antioxidant supply from the seminal plasma. Supplements can always be included *in vitro* to compensate for this deficiency. Multiple studies show the advantage of using TEST-yolk buffer as a cryoprotectant with glycerol to prevent damage in spermatozoa (Hallak et al., 2000; Naillie et al., 2004). Using TEST-yolk buffer in combination with glycerol is preferred compared with using glycerol alone. This combined approach was shown to preserve higher motility, morphology and sperm membrane integrity (Hallak et al., 2000). It also decreases the number of spermatozoa with phosphatidylserine externalization (Duru et al., 2001) and prevents excess chromatin structure damage and morphology changes (Hammadeh et al., 2001).

Conclusions

Cryopreservation is an integral component of fertility management and much of its successful application will affect success rates of assisted reproduction treatments. Whether sperm cryopreservation is applied as a fertility preservation measure or for a backup for assisted reproduction, the recovery rate of functionally competent spermatozoa is critical. Apoptosis has been correlated with decreased fertilizing capacity of spermatozoa and male infertility. Increased apoptosis markers have been documented in response to cryopreservation and thawing in human spermatozoa. This poses a serious threat to success rates following the use of cryopreserved spermatozoa for assisted reproduction. Thus, different aspects associated with apoptosis deserve attention during sperm cryopreservation in order to conserve vital sperm functions after thawing. Technical measures should be applied to provide maximal protection to the spermatozoa during cryopreservation and thawing to prevent induction of apoptosis. Appropriate use of cryoprotectants and sperm selection technologies appears to have the most impact on preventing apoptosis and thereby improving sperm cryosurvival rates.

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