The effect of cryopreservation on DNA damage, gene expression and protein abundance in vertebrate species

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Abstract

Cryopreservation techniques allow the long-term storage of a wide variety of biological materials without significant deterioration in quality. Immediate post-thaw survival is most often used to assess the effect of the freeze-thaw process on cells. However, this parameter provides no information on possible subtle effects of cryopreservation, including DNA damage, and alteration of mRNA levels and protein function that may not be evident immediately post-thaw. These potential adverse effects do not necessarily result in cell death. While there are many comprehensive reviews of gamete and embryo cryopreservation in vertebrate species, we review here the publications relating to the impact of cryopreservation on the genome of sperm, embryos and oocytes.

Introduction

Correct DNA replication and gene expression are crucial for embryo development. Any abnormal genomic process may lead to irreversible damage. Cryopreservation is a complicated procedure involving physical and chemical factors that expose cells to extreme conditions. Therefore, it is important to determine whether cryopreservation protocols could adversely affect the genome.

Sperm cryopreservation has been successful in vertebrate species (Stewart, 1951; Hammerstedt et al., 1990). Although cryopreservation of mammalian oocytes (Nobuya et al., 2005; Vincent et al., 1989), embryos (Edgar et al., 2000) and ovarian tissue (Gosden et al., 1994; Donnez et al., 2004) have been successful, fish oocytes and embryos have still not been successfully cryopreserved, with the main problems being identified as their large size, high yolk content and complex membrane systems (Zhang and Rawson, 1996).

Whilst some studies on the effect of cryopreservation at a molecular level, such as DNA damage and gene expression, reported instances in which no adverse effect of cryopreservation was observed in the porcine, human (Hamamah et al., 1990) and bovine species (Stachowiak et al., 2009), a second group found there were indications of negative effects in the ovine (Succu et al., 2008) and human (Tachataki et al., 2003) species. It has been suggested that these negative effects might subsequently be corrected or repaired during further growth and development (Succu et al., 2007). Any loss of DNA integrity and changes in gene expression as a result of cryopreservation may explain some of the differences in viability between fresh and post-thaw cells. Moreover, it has been shown that alteration of gene expression may also lead to major defects in the brain, ear, eye and kidneys (Favor et al., 1996). The objective of this review is to discuss the effect of cryopreservation on the genome in spermatozoa, oocytes and embryos of vertebrate species.

The effect of cryopreservation on spermatozoa

During fertilization, the spermatozoon penetrates the oocyte, donating the paternal genome and activating the oocyte to trigger embryo development. The egg cell destroys mitochondria of sperm and, as a result, only maternal mitochondrial DNA remains (Aurelio et al., 2004). The structure of spermatozoa has been reported for about 300 fish species from more than 100 families (Matos et al., 2002) and sperm DNA is at least 6-fold more condensed than that of somatic cells (Ward and Coffey, 1991). From the earliest work on sperm cryopreservation, the standard test for sperm viability is motility and ability to fertilize the mature egg (Stewart, 1951; Hammerstedt et al., 1990; Rana and McAndrew, 1989). However, if cryopreservation is to be used for routine storage of genetic material from different species, studies on the effect of cryopreservation at the molecular level must be carried out to make sure that the process does not cause detrimental changes.

There is evidence that cryopreservation can cause DNA damage of spermatozoa in humans (Donnelly et al., 2001a,b; Hammadah et al., 2001), monkeys (Li et al., 2007), rams (Peris et al., 2004) and fish species (Labbe et al., 2001; Zilli et al., 2003). The comet assay is a popular method for detecting DNA damage as it is rapid, less labor intensive and less expensive than other methods. The resulting stained electrophoresis image shows the damaged DNA separated from the intact DNA, resembling a comet with a head and tail. Labbe et al. (2001) demonstrated the effect of cryopreservation on DNA stability of rainbow trout sperm by using this method. The results showed that freezing only slightly affected sperm DNA stability but significantly increased the percentage of damaged nuclei. This result differed from that of a later report on a different species by Zilli et al. (2003) who demonstrated significant damage at the DNA level (%DNA and MT, P<0.01) in sea bass sperm with the same assay. Similar results were obtained by Donnelly et al. (2001b), who found sperm DNA of infertile men to be affected by cryopreservation. Interestingly, this was not seen in fertile men. It is possible that the semen from fertile men has better resistance to cryopreservation due to the presence of antioxidants protecting against cryodamage (Lewis et al., 1995). However, there is evidence that even the DNA integrity of sperm from fertile men can be affected by the freezing procedure (Donnelly et al., 2001a).

The process of cryopreservation can destabi-
lize the chromatin and further increase DNA susceptibility to denaturation. Indeed, this has already been shown in human (Spanò et al., 1999) and ram (Peris et al., 2004) sperm. The sperm chromatin structure assay (SCSA), which is a flow cytometric technique, was used in these studies. SCSA uses the metachromatic properties of acridine orange to stain sperm. When acridine orange binds with intact DNA (double-stranded), a green fluorescence is seen. Red fluorescence emits when binding is to denatured DNA (single-stranded) (Love, 2005). Peris et al. (2004) showed that sperm quality degenerates after the freeze-thaw protocol due to DNA damage after three hours of incubation. A similar result was also obtained on isolated erythrocytes and leukocytes of largemouth bass (Fisher et al., 1994). In addition, human and porcine spermatozoa have also been compared using acridine orange staining after cryopreservation. Hamamah et al. (1990) demonstrated that the cryopreservation procedure affected the percentage of DNA in human sperm but did not appear in boar according to acridine orange staining and by analyzing chromatin structure by a quantitative microspectrophotometric study of Feulgen-DNA complexes. It is possible that the boar DNA-protamine complex is more resistant to freezing or this could be related to the poor quality of human sperm (Kopeika et al., 2007).

Few reports are available on the effect of the freezing protocol on cellular proteins. In human (Cao et al., 2003) and boar (Huang et al., 1999), levels of heat-shock protein 90 (HSP 90) have been studied. The results demonstrated that HSP 90 decrease substantially in boar sperm after cryopreservation when analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blot (Huang et al., 1999). Cao et al. (2003) found significantly decreased levels of HSP 90 after cryopreservation of human sperm. These results might be associated with a reduction in frozen-thawed sperm motility. Surface proteins are involved in the steps leading to fertilization (McLesley et al., 1998) and Lessard et al. (2000) concluded that PSZb surface protein may be degraded during cryopreservation resulting in a reduction in fertility in bull sperm. Protein analysis of fish sperm (Zilli et al., 2005) demonstrated degradation of 21 protein after cryopreservation found in sea bass sperm using two-dimensional polyacrylamide gel electrophoresis. This may partially explain the lower sperm motility and hatching rate of embryos derived from cryopreserved sperm.

The effect of cryopreservation on oocytes

The oocyte contributes not only the maternal nuclear genome, but also the mitochondrial genome and nutrients to the zygote and embryo. Cryopreservation of mammalian oocytes by controlled slow freezing and vitrification has been successful, producing live offspring (Otoi et al., 1996; Nobuya et al., 2005; Vincent et al., 1989). However, as yet there has been no successful cryopreservation of fish oocytes (Tsai et al., 2009).

Although cryopreservation of mammalian oocytes has been successful, the effectiveness of oocyte cryopreservation in some species, including bovine, is still low because of complex structure, high chilling sensitivity and low membrane permeability (Masiop, 2003; Prentice and Anzar, 2011). Recently, DNA damage in the oocyte has been found after cryopreservation and this could be one of the reasons for reduced developmental ability of cryopreserved oocytes (Stachowiak et al., 2009; Men et al., 2003a,b). Stachowiak et al. (2009) compared three different vitrification methods (in open pulled straw, in droplet system and in 0.25 mL straws) on the levels of DNA integrity using the comet assay. They found the open pulled straw method resulted in a significant level of DNA damage. However, the two minimum sample size vitrification methods were safe for cryopreserving bovine oocytes (Stachowiak et al., 2009). These results differ from the results presented by Men et al. (2003b), who demonstrated that oocyte DNA exhibited different degrees of damage following similar freezing methods. The different results between these two papers might be due to some differences in vitrification procedures. Also Stachowiak et al. (2009) removed granulosa cells from oocytes prior to cryopreservation but Men et al. (2003a) left granulosa cells on the surface of oocytes. However, removal of granulosa cells does aid cryoprotectant penetration of the oocytes, a necessary and important factor in successful vitrification (Papis, 1996).

Some studies have been undertaken on mRNA and protein levels affected by cryopreservation. Succu et al. (2007) analyzed the effect of vitrification on maturation promoting factor (MPF) using three different cryodevices: open pulled straws, cryoloops and cryotops. Cryoinjuries in ovine oocytes are capable of reversal after a period of time of thawing (Succu et al., 2007). Although MPF was affected by the different cryodevices, it was restored after two hours of culture in the open pulled straw and cryoloop groups but not in the cryotop group. In the following year, Succu et al. (2008) reported a quantitative assay of developmental related genes (beta actin, H2A.Z histone, Poli A Polymerase, Heat shock protein 90β, P34cdc2, Cyclin b, Na/K-ATPase and Type I cadherin) in frozen-thawed ovine oocytes to determine the potential influences of vitrification method on the oocyte mRNA abundance. The results show that freezing procedures lower the ovine oocyte mRNA levels as measured by quantitative real-time PCR. Similarly, a decrease in CD9 molecule expression was reported in frozen-thawed mouse oocytes (Wen et al., 2007). As CD9 regulate signal transduction events that are critical to growth regulation (Li et al., 2004), data from the study by Wen et al. provide an indication of cryo-impact that could be linked to the poor developmental capacity of oocytes after cryopreservation.

The effect of cryopreservation on embryos

Most sperm and oocyte studies have investigated the effects of cryopreservation on DNA damage. There is evidence that cryopreservation could also lead to DNA damage (Park et al., 2006) and embryonic cells (Kopeika et al., 2005) due to addition or removal of cryoprotectants, chilling injury, lethal intracellular ice formation during the cooling or the thawing procedure. As a result, the process of cryopreservation can cause embryo damage and result in DNA fragmentation and reduced developmental ability (Park et al., 2006; Kopeika et al., 2005).

As maternal mRNA and proteins from the oocyte support the earlier cleavages of the embryo, studies on specific gene expression during embryonic development are very important. Successful cryopreservation of human embryos has been reported, and the thawed embryos showed the same implantation ability as fresh embryos at the same stage (Edgar et al., 2000). Although there are a limited number of human embryos for research, studies on gene expression can offer valuable information. Tachataki et al. (2003) used real time RT-PCR to analyze the expression pattern of the tuberous sclerosis TSC2 gene during preimplantation development of human embryos. The results of gene expression obtained in this study are similar to the results reported for the mouse housekeeping gene (β-actin), heat shock protein gene (Hsp 70), genes related to
oxidative stress (MnSOD and CuSOD), cold stress (CIRPβ, Rho3), cell-cycle arrest (Trp53) (Boonkuso1 et al., 2006), apoptosis (Bax, Bcl2 and p3) (Dhali et al., 2007) and glucose transporter (GLUT1) (Uechi et al., 1997). These studies concluded that cryopreservation affects the normal pattern of gene expression during embryonic development. Nevertheless, cryopreservation has a differential effect on these genes as seen by upregulation of stress-related and GLUT1 genes (Uechi et al., 1997) and the downregulation of apoptosis and TSC2 gene (Tachataki et al., 2003). Investigations of the activity of cytoplasmic enzymes (lactate dehydrogenase and glucose-6-phosphate dehydrogenase) have been conducted on zebrafish and turbot embryos even with zero survival rates after cryopreservation by Robles et al. (2005). Their results showed significantly lower enzymatic activity linked to cell rupture due to ice crystal formation during vitrification. Some researchers even tried to find certain genes that have the potential to improve the successful cryopreservation of fish embryos, such as antifreeze protein (Young and Fletcher, 2008) and the Aquaporin-3 gene (Hagedorn et al., 2002). Antifreeze protein can protect cell membranes from cryo-damage while the Aquaporin-3 gene increased membrane permeability to water and cryoprotectant.

The efficacy of translation is often affected by transcriptional modulation of regulatory genes. Moreover, it has been discovered that small non-protein-coding RNAs (small nucleolar RNAs, microRNAs, short interfering RNAs and small double-stranded RNAs) also regulate gene expression, including translation in developmental processes (Mattaick and Makunin, 2006). As proteins are important in regulation of developmental processes including differentiation, growth, survival and morphogenesis (Chi and Epstein, 2002), studies of proteins under the chilling and freezing conditions should be explored.

Cryopreservation of ovarian tissue is a viable alternative to cryopreservation of oocytes or embryos in humans and animals. Grafts of cryopreserved ovarian tissue have resulted in live-born mice (Carroll and Gosden, 1993), sheep (Gosden et al., 1994) and humans (Donnez et al., 2004). However, the expression of heat shock proteins, DNA-damage-inducible protein 45 and death-related apoptosis genes were significantly increased as measured by DNA microarray techniques in mouse ovarian tissue after cryopreservation (Liu et al., 2003). This work suggested that although competent follicles and mature oocytes were obtained after freezing, any change in the expression level should be considered important and should be avoided.

Conclusions

Whilst cryopreservation has been proven to be a successful approach for the long-term storage of biological material, there is growing evidence of potentially damaging effects at the molecular level. There is a need for long-term studies to determine the possible impact of subtle genomic and proteomic changes resulting from cryopreservation. Dulioust et al. (1995) demonstrated significant differences in body weight, pre-weaning development and learning capacity in mice derived from cryopreserved embryos. It has been reported that children born from cryopreserved embryos presented no major pathological features (Wennerholm et al., 1997). Studies on the long-term effects can be quite sensitive to detect some genetic related effects, although such effects are more difficult to explain. More molecular biology and long-term studies are necessary to assess the consequences of cryopreservation in depth.

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