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SPERM CRYOPRESERVATION: IS THERE A PLACE FOR FREEZE-DRYING IN SUB SAHARAN AFRICA

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ABSTRACT: *The need to improve sperm cryopreservation has generated much interest in Freeze-drying(FD). Haven has been demonstrated in animals as a good alternative for sperm preservation and maintenance of genetic integrity, its place for human sperm is currently being proposed. With FD, sperm can be stored at room temperature or 4^0C unlike the conventional use of Nitrogen liquid. Thus, resulting in substantial reduction in the cost of storage and shipping. The concern for FD on the sperm motility, viability and to some extent the DNA fragmentation is species specific and can be circumvented by the use of intracytoplasmic sperm injection (ICSI), resulting in the good embryonic development and successful pregnancy. The review provides an overview of the conventional Nitrogen liquid storage and FD. Embracing the concept of FD could reduce the overhead cost of sperm cryopreservation especially in our setting with scarce resources.*

KEYWORDS: Freeze-Drying, Nitrogen Liquid Storage, Sperm Preservation.

INTRODUCTION

The limited life span of sperm requires significant coordination to retrieve both sperm and oocytes at the same time (Moce *et al*., 2016) which may be inconvenient or impossible in some cases. In light of this, research involved with extending the lifespan of sperm by cooling the cells has been rigorously pursued (Snijder *et al*., 2012). In recent years, interest has risen in new preservation technique that facilitates sperm storage and distribution with Freeze-drying (FD) proposed as a useful alternative method for sperm preservation and maintenance of genetic resources in different animal species (Vayena *et al*., 2009). Freeze-drying or Lyophilization is an innovative technology for preserving sperm. It mainly involves the removal of all moisture or water molecules in a semen sample, often done in the preservation of food and pharmaceuticals (Sharma *et al*., 2014). It involves cooling the samples below the triple point of water and then reducing pressure. The temperature at which the different state of a substance coexists. Below this point, a substance changes directly from a solid to a gaseous state at a reduced pressure. Thus, cooling below the triple point at a reduced pressure causes solid, water, ice to sublimate directly into a vapour state and later reconstituted by addition of water. In the process, spermatozoa can be stored for a prolonged period in a refrigerator (4 degree Celsius) or even at ambient temperature and as well as easily and safely transported (Kaneko 2016).

Historical Background

The concept of Lyophilization first applied in the food industry in 1958 (Kaneko 2016) and about cells, Loi et al. 2013 demonstrated that haven stored in a dehydrated state at room

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temperature, for three years, somatic cells could direct embryonic development of enucleated oocyte up to the blastocyst stage. Thus, heralded the focus research attention on the adaptation of FD technology in the preservation of mammalian sperm to sustain long-term storage at room temperature. The first attempts at FD sperm performed in 1949 by Polge et al. with the mixing of fowl semen with ringer's solution containing glycerol in the process removed 90% of water. The following storage at room temperature for two hours and rehydrated, 50% of the spermatozoa regained motility. Though, fertility was not determined. Following this, there have been unsuccessful attempts at FD of human (Sherman 1954), and bull (Bialy and Smith 1957) spermatozoa. However, Wakayama and Yanagimachi in 1998, reported the offspring obtained from FD mouse sperm. The authors concluded that although FD can damage the sperm structures, DNA, and intracellular factors needed to activate oocyte preserved, and viable offspring produced by ICSI with the use of FD spermatozoa (Wakayama and Yanagimachi 1998, Manjunath *et al*., 2012, Gil *et al*., 2014). To date, sperm preservation by FD has only been studied and applied in the maintenance of laboratory, domestic and wild animal strains.

Freeze-Drying Protocol

FD imposes stress on the biomaterials and may cause irreversible structural changes in the biomolecules occasioned by ice crystals formation, osmotic dehydration, and mechanical forces. While the drying component leads to the removal of bound water surrounding biomolecules. The outcome of the spermatozoa is affected by the various factors that affect the stages in the process (Hochi *et al*., 2011). However, the DNA fragmentation of the spermatozoa is one of the leading causes of failed embryonic development. In light of this, researchers have directed emphasis towards measures to prevent sperm DNA damage during FD process (Walters *et al*., 2009). In FD, the nature of the solvent and other components of the sample determine the temperature needed to achieve complete freezing of the sample. During primary drying, the pressure in the freezer is reduced, and heat is applied to initiate sublimation of the ice crystals. While the secondary drying involves the final desorption of the remaining water and this is achieved by raising the temperature of the product and reducing the partial pressure of the water vapour in the container (Hochi *et al*., 2011, Benson *et al*., 2012).

Sample Preparation for Freeze-drying technique

Sample preparation involves the mixture of the balanced salt solution with suitable osmotic pressure and PH that is easily dried and can provide a useful matrix to support the survival of spermatozoa (Gil et al. 2014). Studies have shown that chromosome integrity and fertilization ability of spermatozoa can is maintained by adding a reduced amount of Ethylene diamine tetra acetic acid (EDTA) to the solution during FD and subsequent preservation (Hochi *et al*., 2011, Fernandez-Gonzalez *et al*., 2015). Nakai *et al*. (2007) reported that EDTA and EGTA (Ethylene Glycol-bis (2-amino-ethylether)-N, N, N, N,-tetraacetic acid) are effective at inhibiting endonuclease activity. Thus, preventing DNA fragmentation. Slightly alkaline (PH 8) solution maintain chromosome integrity and developmental ability of mouse spermatozoa (Kaneko 2016). Furthermore, the addition of Trehalose, a non- reducing disaccharide, characterized by its capacity to stabilize and protect membranes under extreme environmental conditions thereby allowing the anhydrobiotic organism to survive dehydration- rehydration cycle can improve sperm DNA integrity after FD procedure (Men at al 2013). Though, no improvement in the fertilization and subsequent development to the blastocyst stage. As a result, other sugars such as Lactose and sucrose combined with EDTA have been utilized in a study with FD boar sperm. Thus, Garcia Campos et al. (2014) reported that FD medium supplemented with lactose

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and sorbitol (50mM, NaCl, 10mM TRIS, 10mM EDTA, 0.117M sorbitol, 0.15M lactose) resulted in 98% and 100% DNA integrity at a storage temperature of 19 and 25 degree Celsius. Currently, FD solution with 10mM TRIS and 1mM EDTA and the PH of 8.0 is the most simple and stable approach to sustaining sperm fertility after FD (Kaneko 2015).The application of this solution resulted in the preservation of mouse and rat spermatozoa for 3-5years at 4 degree Celsius after FD and the offspring derived, healthy with normal life spans and reproductive potential (Kaneko and Sarikawa 2012).

Semen samples for FD can be fresh, frozen, as well as ejaculated or epididymal sperm. Recent studies used spermatozoa that were ejaculated or collected from the caudal epididymis for FD most of which were completely mature (Sharma *et al*., 2014). Thus, confers some degree of resistant to damage caused by physical stress due to their condensed DNA (Kaneko, 2014). However, the DNA of testicular spermatozoa are still immature with uncondensed DNA. Thus, predispose them to damage during FD (Kaneko 2016). However, the application of diamine treatment has been shown to enhance the resistance of testicular spermatozoa (Zhan *et al*., 2010). Irrespective of the sample, the FD protocol as well as the kinetic, the degree of drying is regulated by the vacuum pressure, temperature, and the drying period, FD solution and storage period (Hara *et al*., 2014) and maintenance of cellular function via the drying condition (Hara et al. 2014). In a study of mouse spermatozoa, it was noted that the proportion of development to the blastocyst stage at a pressure of 0.37mbr was significantly higher than at 0.04 or 1.03mbr (Kawase *et al*., 2009). Kwon *et al*., (2004) reported that a prolongation of the FD period for boar spermatozoa from 4-24 hours compromise the ability of the FD spermatozoa in vitro participation in embryonic development. However, the different value for the pressure and the FD period depend on the FD equipment, the sample and the content of the FD medium used (Loi *et al*., 2013, Hara 2014). Following the FD, the spermatozoa lose their motility and membrane integrity while some maintain their genetic integrity. FD sperm can produce calcium oscillations when introduced into the oocyte by ICSI. Thus, make successful fertilization possible. ICSI, therefore, becomes the only tool that allows live offspring obtained from FD sperm (Choi *et al*., 2011).

Conventional Method Protocol with LN2

The conventional method of sperm cryopreservation involves physical sperm storage in liquid Nitrogen (LN2), and this can be done using fast freezing or slow freezing technique or with a programmable freezer. In these methods, a low molecular weight cryoprotectant is added to the processed semen sample to prevent ice formation that can compromise the viability of the cell (Di Santo *et al*., 2012). The commonly used cryoprotectant is the glycerol which protects sperm cell membranes and prevents osmotic stress within the cell (Sharma *et al*., 2014). However, glycerol has been shown to alter the acrosomal membranes and change the mitochondrial environment (Di Santo *et al*., 2012). Manual slow freezing often takes 2-4 hours to complete. The sample is gradually cooled from room temperature to 5 degree Celsius at a rate of 0.5-1degree Celsius/min until the sample becomes frozen and the temperature is further lowered from 5degree Celsius to -80degree Celsius at a rate of 1-10degree Celsius/min. Finally, the sample is plunged into LN2 at -196degree Celsius (Kusakabe and Tateno, 2011). The drawback of this method is that ice crystals can form within the cells if cooling becomes too fast. Also, the cells can shrink if the cooling becomes too slow due to osmotic shock. Hence, the need for controlled cooling rate (Lee et al. 2013). In the rapid freezing method, the sample comes into direct contact with Nitrogen vapour at -80degree Celsius following the addition of cryoprotectant to the sample (Sharma *et al*., 2014). The straw with the sample is stored 15-

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20cm away from the LN2 (vapour phase) for 15mins and then plunged into the LN2 at -196degree Celsius. The challenge with controlling cooling rate in this protocol might be responsible for the variation in the post-thaw sperm quality (Sharma *et al*., 2014).

An attempt at overcoming the lack of control over cooling rate lead to the introduction of the automated programmable freezer. Here the freezing program is chosen haven loaded the sample onto the plate in it, and the temperature is automatically adjusted. Again, this is only beneficial when handling a large number of samples (Walters *et al*., 2009) and also associated with delay cooling rate which may compromise the viability of the sperm cells (Kaneko, 2016). Conventional sperm preservation using LN2 requires a continuous supply of LN2 and maintenance of equipment for a long term preservation. Unfortunately, significant sperm samples stored may be lost if the supply of LN2 is compromised especially during a natural disaster such as earthquakes (Vayena *et al*., 2009). Also, the presence of a pathogenic microorganism in the storage tanks is a potential risk of transmission of disease via stored semen sample. Though the cryovials for sperm storage are a closed system, the possibility of LN2 seeping into the cryovials cannot be ruled out completely (Jensen *et al*., 2011). Also persistent storage at -196 dgree Celsius in LNS might pose a significant challenge during audit and transportation of samples.

In the search for improvement in sperm preservation, attention has been on the result in the animal field where the FD technique documented as an alternative for storing sperm (Gianaroli *et al*., 2012). Unfortunately, FD is highly detrimental to sperm membranes making the outcome an immotile sperm and unviable (Unger *et al*., 2009). However, the overall integrity of the genetic material is preserved with less DNA damage when compared with cryopreservation in LN2 (Montag *et al*., 2011). The animal data reinforce suitability of this technique. FD spermatozoa allow viable offspring by ICSI in mice (Li *et al*., 2009), rabbit (Montag et al. 2011), rat (Kaneko 2014). Study of the subsequent generation rule out genomic instability (Gianaroli et al., 2012) suggesting that the integrity of the genetic material is sustained with no remarkable deterioration for an extended period when sperm preserved by FD technique (Zhang et al. 2010). Currently, it is restricted due to the absence of human studies.

In Sub-Saharan Africa, infertility due to abnormal sperm account for about 50% of cases (Eva *et al*., 2016). Thus, necessitates the use of artificial insemination (AI) as a treatment option. In a bid to prolong the life span of sperm for future use, sperm cryopreservation with liquid nitrogen has been the practice. However, the use of liquid nitrogen is expensive and requires adequate space. Furthermore, there is a high tendency of viral cross-contamination between samples (Hamoun *et al*., 2017). Hence, FD with the advantages of being cheap, no nitrogen liquid, and limited space for storage (Mohamed *et al*., 2016) may be a suitable alternative for the Sub-Sahara Africa with scarce resources.

CONCLUSION

Freeze-drying and LN2 freezing have a negative impact on sperm structures but at different levels as expressed by the diverse incidence of the evaluated parameters following the application of each technique. More importantly, is the damage to cell membranes which is higher after FD and thus loss of motility which is an essential requirement to complete organic fertilization. As a result, make the relatively difficult micro-insemination the only tool to achieve fertilization (Hochi *et al*., 2011). DNA fragmentation tends to be more prevalent with

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LN2 freezing (Gianaroli *et al*., 2012). Cryopreservation in LN2 is recognized, and successful technique but the possibility of a simplified storing system would present positive aspects such as the exclusion of LN2 or dry ice and the potential to ship samples at room temperature. Though FD spermatozoa could maintain their function and abilities to interact with oocyte cytoplasm after prolonged storage at low temperature (Hochi *et al*., 2011), the acceptable outcome of transferable blastocysts and production of live offspring derived from FD sperm samples are still subject to the contention that requires more research in large domestic species. However, Lyophilization could be useful for special cases such as in handling samples of patients with viral infection as FD has been reported to induce inactivation of viruses (Unger *et al.*, 2009). It is currently still considered as an experimental technique due to lack of information on its fertilizing ability in human.

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