

Methods of Cryopreserving Sperm in Infertile Men with Oligospermia and Cryptospermia Patterns

M. Gholamitabar Tabari (MSc)¹, S.G.A. Jorsaraei (PhD)*¹, Y.R. Yousefnia Pasha (MD)¹

1. Fatemeh-Zahra Infertility & Reproductive Health Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, I.R. Iran

J Babol Univ Med Sci; 18(11); Nov 2016; PP: 35-43

Received: May 23th 2016, Revised: Jul 27th 2016, Accepted: Sep 28th 2016.

ABSTRACT

BACKGROUND AND OBJECTIVE: Cryopreservation of sperm currently plays a significant role in maintaining the fertility of couples who undergo infertility treatment, particularly in patients with oligospermia and cryptospermia. To avoid recurrent biopsies, we need to dedicate a lot of time to sperm collection so that the patient is placed in infertility treatment cycles when the necessary conditions are provided. This study is dedicated to Cryopreservation of sperm in oligospermia and cryptospermia samples.

METHODS: For this study, we referred to NCBI database and selected articles related to various methods of cryopreserving sperm using certain keywords such as "oligospermia", "cryptospermia" and "cryopreservation of sperm" and articles that surveyed cryopreservation in terms of medical and functional views.

FINDINGS: To cryopreserve sperm, three methods are used; slow, fast and glass. Using several preservatives in culture medium is necessary for survival of sperm. Since cryopreservation may weaken sperm mobility in patients with severe spermatogenesis disorder, researchers seek to devise new methods to reduce these effects. Using zona pellucid, volvox globator algae, alginate-agarose, cryoloop, straw and microinjection needle are among the methods advised for preserving sperms and increasing their survival rate.

CONCLUSION: According to the results of this study, using different tools to achieve best results depends on the type of samples obtained from these patients.

KEY WORDS: *Oligospermia, Cryptospermia, Infertile.*

Please cite this article as follows:

Gholamitabar Tabari M, Jorsaraei SGA, Yousefnia Pasha YR. Methods of Cryopreserving Sperm in Infertile Men with Oligospermia and Cryptospermia Patterns. J Babol Univ Med Sci. 2016;18(11):35-43.

*Corresponding author: S.G.A. Jorsaraei (PhD)

Address: Fatemeh Zahra Infertility and Reproductive Health Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, I.R. Iran

Tel: +98 11 32274881

E-mail: alijorsara@yahoo.com

Introduction

About 15% of couples suffer from infertility. There are factors and problems that induce infertility in men, affect 1 in every 20 men and include half of the cases in need of assisted reproductive therapy as a participating factor. Several disorders and diseases may cause infertility in men. Moreover, there are also unknown causes for men's infertility that are inexplicable (1). Men's infertility may have different reasons. Varicocele or varicose veins in testicles is the most common disease after puberty and is one of the effective factors in men's infertility. Although varicocele is one of the most common causes of damaged sperm parameters, we need to consider that it may not cause a serious damage in all individuals. Genetic and chromosomal abnormalities are among the known factors that may cause infertility in men. There are several other factors that induce infertility: changes in environmental conditions, which lead to increased number of pollutants (2), disorders occurred in the hormonal system (3), changes in the immune system (4) as well as lack of elements and antioxidants (5, 6), environmental toxins such as cadmium, mercury, bisphenol and dioxins (7), agricultural pesticides such as diazinon and hinosan (8) may have negative effects on the process of spermatogenesis and cause infertility by reducing sperm parameters.

However, since there are several assisted reproductive techniques such as intracytoplasmic sperm injection or microinjection provide the possibility to have children for men with very low sperm count and poor parameters. Therefore, patients with oligospermia and cryptospermia with low sperm count can successfully benefit from this technique (9, 10). Oligospermia is a condition, in which the sperm count in the semen is below 20 million per ml and may be below 5 million in severe conditions. Cryptospermia refers to men with less than 100000 sperm per ml in their semen.

As of now, there is no reliable and definitive medication for this such conditions. Therefore, a natural pregnancy is an issue for these people, yet not impossible. In such cases, only very accurate techniques in repeated semen samples may achieve sperm (11-13). Considering that achieving the required sperm for microinjection may not be possible for samples with very low sperm count and weak parameters during puncture and egg collection, all necessary measures need to be considered before any action. The best technique for such cases is collecting and cryopreserving sperm, in order to prevent the stoppage of puncture. Several fertility centers use testicular biopsy method coincided with the day of egg extraction to prevent the stoppage of microinjection or

intracytoplasmic sperm injection (ICSI). Some others have suggested egg cryopreservation.

However, all these interventions only delay the problem of sperm shortage. Therefore, sperm cryopreservation in these patients before the day of egg extraction will be very helpful and it must be done before ovarian stimulation (14). To avoid recurrent biopsies from patients with severe oligospermia or cryptospermia, some fertility centers dedicate a great deal of time to sperm collection and after cryopreservation and preparation of the necessary conditions, start the cycle of infertility therapy (15). This study aims to investigate cryopreservation of sperm in oligospermia and cryptospermia samples.

Methods

For this study, we referred to NCBI database and selected articles related to various methods of cryopreserving sperm using certain keywords such as "oligospermia", "cryptospermia" and "cryopreservation of sperm" and articles that surveyed cryopreservation in terms of medical and functional views.

Results

Sperm cryopreservation techniques: The mechanism of cryopreservation is based on the fact that when intracellular water turns into ice in the right temperature, in a way that the cell is not damaged, the molecular movements are effectively stopped and the biochemical processes of cell are delayed or stopped completely. In this condition, cell viability will increase. Sperm cryopreservation is categorized into three methods: slow freezing, ultra-rapid freezing and vitrification.

Slow freezing is done through several steps with cell temperature decreasing gradually. However, in ultra-rapid method, sperms are exposed to nitrogen vapor at a distance of 1.5-3 cm from the surface of nitrogen for a certain period of time. In this condition, the temperature of sperm declined up to -60°C. Then, sperm are transferred into liquid nitrogen container. We need to consider that both of these methods do not show very ideal results due to formation of ice crystals. Therefore, several studies have been carried out to reduce time of the process and removal of ice crystals and expensive equipment have been used solve these issues. Vitrification is one way of avoid cell damage due to formation of ice crystals. In this method, samples are directly and quickly immersed in liquid nitrogen so that the cryopreservation process is done extremely fast. In this method, samples are chilled at a very high speed

(72000 k/min) and are cryopreserved in a very short period (5-8 seconds) (16).

In 2003, a group of researchers presented a new technique for vitrification. Based on this new technique, researchers try not to use cryopreservation culture medium, since it has toxic and lethal effects on sperm. By dragging sperm into CryoLoop, samples are directly immersed in liquid nitrogen. Using this method significantly improved the viability and motility of sperm compared with conventional methods (17). Some researchers believe that the process of melting-freezing reduces motility, changes penetration pattern in cervical mucus, changes acrosome structure and plasma membrane and slows the proteolytic activity of acrosin (18).

Nowadays, despite the use of modern protocols for cryopreservation, the qualitative and quantitative parameters of living sperm after melting are unsatisfactory compared with the sample before being frozen and the fertility rate obtained from frozen sperm is lower than fresh sample (19). Melting and vapor phases of cryopreservation not only kill the sperm, but also damage the acrosome. The effect of cryopreservation on sperm during melting and vapor phases is identical for fertile people and people with oligospermia. Therefore, the quality of sperm in people with oligospermia after freezing and melting is not considerably different (20).

Results of a study show that the osmotic pressure caused by protective materials in cryopreservation medium damages plasma membrane of sperm. Moreover, severe temperature reduction in sperm during cryopreservation causes considerable damage to the anterior portion of sperm head and the lower acrosome membrane. The induced shock may lead to rupture and reduction in the acrosome matrix. Acrosome enzymes are necessary for fertilization ability and they help sperm reach oocyte plasma membrane and thus damage to these enzymes is accompanied by reduced fertilization. Therefore, the percentage of components in cryopreservation medium must be modified, so that the sperm is faced with minimum damage. This is especially important in patients with oligospermia and cryptospermia who have limited number of living and moving sperm (21). The main principle in cryopreservation process is the reduction of damage caused by formation of ice crystals and toxic salts inside cells. The cooling process of cell must be done slowly and the intracellular water must be removed properly.

One must note that before or during the cooling process, a suitable substitute needs to be considered for the water extracted from cell (22). Considering that the freezing process decreases the fertility capacity of

sperm by causing damage to the cell membrane, damage to sperm motility, change in morphology and acrosome damage, damage to DNA structure and sperm function, more attention has been paid in recent years to assessment of different methods of cryopreservation to investigate their effect of sperm motility. Different preservative medium also protect sperms against negative effects of freezing process. All these factors help us chose the best and most effective method with minimum damage to sperm. Three most common medium used for sperm cryopreservation include TYBG, HSPM and GEYC (23).

The only compound in TYBG medium that maintains the extracellular osmotic pressure is dextrose. Glucose, fructose and glycine are used in GEYC medium and glucose, sucrose and glycine are present in HSPM medium. It seems that GEYC and HSPM benefit from better maintainers to maintain extracellular osmotic pressure compared with TYBG medium (24). Moreover, one of the studies indicated that there is not a significant difference between GEYC and HSPM medium regarding fertility of the frozen sperm. However, the percentage of cycles resulting in pregnancy for frozen samples in HSPM medium was more than GEYC medium (25).

Various maintainers are used along with sperm cryopreservation culture medium. Some of these maintainers are egg yolk, milk, dual ion buffers (with negative and positive ions), citrate, fructose, tris buffer, glucose, dextrose and glycine (26). Therefore, considering the effect of various maintainers on cryopreservation culture medium, HSPM is suggested to be used instead of GEYC as a better diluent. The advantage of HSPM medium over TYBG and GEYC is that it is a sterile medium and is biochemically defined. Since this medium lacks egg yolk or any type of non-human protein, the possible presence of unwanted antibodies in response to intrauterine insemination is prevented. Some studies show that the best efficiency in motility and viability rate of sperm belongs to the slow freezing method. Moreover, regarding different freezing mediums, viability rate of sperm after melting in HSPM medium is significantly higher than TYBG and GEYC mediums. Therefore, due to lack of access to freezing devices in all fertility centers, using HSPM cryopreservation medium in vapor phase is advised for sperm samples with natural parameters (27).

One of the issues in the process of sperm cryopreservation is the fact that there is still no medium to prevent sperm damage during the process of freezing completely. Damage to sperm can be detected immediately when their motility has slowed down after melting. Thus, adding preservatives before

freezing is essential to better viability of sperm. However, there is another theory, according to which slower motility of sperm during freezing and melting may be due to having contact with medium preservatives (28). In 2005, researchers concluded that in preserving the structure of sperm, DMSO acts better than glycerol or 1,2-propanediol during the freezing process (29).

Investigating the effect of selenium on natural sperm, another group of researchers in 2014 found that 5 µg/L selenium increases the motility, morphology and viability rate of sperm after melting process (30). The effect of antioxidants on the quality of melted sperm in ultra-rapid method indicates that vitamin E at 1 and 2 mmol concentration increase the motility rate, the progressive motility and viability rate of natural sperm, which was significant in progressive motility and viability rate compared with control group. Although this increase is observed in oligospermia samples, the difference was not statistically significant compared with control group. Ascorbic acid (vitamin C) is another case that has no special effect on any of sperm parameters (31).

Sperm of people with oligoasthenoteratospermia is more prone to damage after freezing compared with normal people. Centrifugation before freezing helps choosing sperm resistant to freezing. In this regard, hypotaurine is one of the antioxidants with protective effects of sperm function (32). Some researchers reported that freezing with machines maintains the quality of sperm better than freezing using vapor phase (33). The progressive motility of sperm may slow down significantly after vitrification. In addition, vitrification causes significant decrease in viability and morphology of sperm and causes significant increase in apoptosis rate without changing the sperm count. In other words, vitrification has negative effects on vital parameter and apoptosis rate of sperm in infertile men, though less than other methods (34).

Assessment of motility, viability and acrosome reaction in sperm, before and after freezing, indicates that in the absence of cryopreservation medium and only in the presence of seminal plasma, vitrification technique decreases motility rate, viability and acrosome reaction in human sperm less, compared with HSPM medium (35). Although cryopreservation weakens the motility of sperm in patients with severe spermatogenesis disorder but does not endanger fertilization and pregnancy (36).

Comparison between fresh and frozen sperm in patients with oligospermia demonstrates that parameters of sperm, fertilization and pregnancy were similar in both groups and sperm cryopreservation can be helpful in patients with oligospermia (14). For

reaching a better conclusion regarding parameters of sperm after melting, researchers use different methods and tools to freeze sperm in patients with oligospermia including:

1. Using Zona pellucida: although the idea of sperm cryopreservation for patients with special characteristics or patients with very low sperm count goes back to more than one decade ago, there are various biological issues to overcome it. Early attempts to freeze spermatozoa and place them in empty animal or human zona pellucida, experimentally filled with preservatives (37-40), led to the conclusion that viability and fertilization of sperm with human zona is less than animal zona such as hamster. The researchers did not reject the possibility that when human zona is used, the presence of ZP3-binding protein may cause induction in acrosome reaction. Although this method requires special skills, equipment and perseverance, live birth by human or hamster zona has been reported by some of the relevant experts.

One of the advantages of this method is that it somehow prevents wasting the time for tasks like screening and isolation of motile sperm which is necessary after melting in conventional methods. Moreover, due to less contact of freezing substance and prevention of possible disadvantages due to risk of biological contamination, this method is mentioned as an appropriate method. However, its utilization difficulties could not attract the attention of experts (41-44).

2. Using Volvox globator: in this method, spermatozoa is injected into the spherical area of algae and it is then frozen. Placement of sperm and its recycling after melting is done using special needle of microinjection (45). Using spherical algae volvox globator is a promising, inexpensive and easy method to maintain motile sperm and has high performance. Although this method sounds attractive and perfect for sperm cryopreservation, it is accompanied by certain limitations. The contrast between human gametes and genetic material of algae and constant level of algae are among the limitations of this method.

3. Using alginate-agarose: the suggestion of using non-biological carriers such as polysaccharide alginate-agarose is in fact the substitution of a non-toxic material for cryopreservation of low count sperm. In this method, a small amount of sperm is placed in alginic acid drops and they can be melted and transformed into liquid again to recycle sperm. One of the advantages of this method is the ineffectiveness of alginic acid, which almost causes no change. Moreover, in this method, alginic acid prevents sperm from sticking to the wall of the pipette or contamination with foreign biological material. At the

same time, for patients with low sperm count, the samples can be divided into some parts to use them in several stages. Reduced sperm motility after melting is one of the disadvantages of this method (46, 47). In this method, some materials can be used as preservatives.

First, sperm is combined with preservatives and is added to alginate before gelatinization. Then, samples are frozen in small, round sizes. We need to consider that since samples need to be dissolved in acid citrate in slow freezing method and a small amount of alginic acid is left in the membrane of the sperm in this condition, dividing the samples into small pieces of agarose and placing them in 0.25 cc straws and going through vitrification can preserve the parameters of sperm to a large extent (47).

4. Using CryoLoop: in this method, sperm samples were divided into small volumes of 15 to 20 μ l and were placed on special cryos for embryo freezing (48-50); Or, sperm samples were placed on a roll of freezing material covered with Nylon Ring and directly immersed in nitrogen liquid. This method is easily accessible and does not require additional arrangements or supplies. The risk of contamination due to openness of the system is one of the disadvantages of this method (51, 52).

5. Using straw: using pipette is another conventional method. Stretched pipettes act like straw and can be obtained in different sizes. Sperm should be prepared in 1 to 5 μ l suspension and be placed in pipettes through vitrification technique (53). In this condition, the combination of sperm and freezing substance was placed inside the small pipette and was then immersed in liquid nitrogen. One of the advantages of this sterile method is its simplicity and easiness. However, it is not that appropriate for samples that are severely damaged and do not benefit from suitable parameters, because the sperm may stick to the wall of pipette and the samples may be lost (14, 54).

6. Using microinjection needle (ICSI pipette): for patients with severe oligospermia or cryptospermia, special microinjection needles are advised. Both methods of slow freezing and vitrification can be used and the sperm can be kept inside it (50, 55). Sterility, simplicity and easiness are the advantages of this method. However, this method cannot be used for a long period and there is the possibility of contamination. We should also consider that microinjection needles are extremely fragile (56).

7. Micro droplet: sperm cryopreservation is done using small 1 to 40 μ l drops. These drops contain sperm and the freezing substance and are placed on a cold surface or directly immersed in liquid nitrogen (53, 57). The small drops are then placed in special

culture containers and covered with mineral oil (58-60). In this method, there is little chance that sperm stick to the surface of the freezing device. The disadvantages of this method include the risk of contamination as well as the shape and the size of culture containers, which may cause problems for using or storing sperm in liquid nitrogen.

8. Microspheres agarose: in this method, sperm is kept on very delicate filaments of agarose and one of the advantages is that it is non-biological. However, it is not clinically evaluated yet (47).

Discussion

Some researchers concluded that if we use straw in sperm cryopreservation, particularly in samples with oligospermia or cryptospermia, the motility rate, viability rate and morphology of sperm in processed and washed samples are better than samples that did not go through the washing process after melting. This signifies the importance of processing and washing the sperm in oligospermia and cryptospermia samples (61). Some researchers refrain from using some preservatives for sperm cryopreservation due to their toxicity.

A study shows that elimination of preservatives necessitates the use of a special technique for sperm cryopreservation. Optimization of sperm cryopreservation techniques can be applicable for natural samples as well as samples such as oligospermia and cryptospermia. The capillarity in pipettes helps us place sperm inside the special pipettes (straws) and then immerse it in liquid nitrogen without using preservatives. In addition to the previous method, use of special biopsy pipette is also advised. Evidence indicate that although both methods benefit from high reliability, the chance of preserving natural sperm samples or oligospermia and cryptospermia samples is higher in biopsy pipettes (62).

Another issue that is highly important in sperm cryopreservation is the risk of contamination. FDA regulatory agency tends to support the methods that do not cause any kind of contamination during the process of freezing or while storing the sperm in liquid nitrogen. Therefore, it has been observed that placing sperm on CryoTubes or Petri dishes without hermetic packaging or without being sealed increased the risk of contamination. For this reason, recycling rate, viability rate and motility rate of frozen sperm may differ by 59-100%, 8-85% and 0-100%, respectively.

Although the extent of this difference is related to the population of patients, initial quality of the sample, the frozen sperm count, the freezing device, type of substance added for freezing and the protocol of

freezing and melting, the risk of contamination during the freezing or melting process should not go unnoticed as well (63). Now, a question is proposed here: can any sample go through the cryopreservation process? Needless to say, finding alive and motile sperm after melting, particularly in cases of severe oligospermia and cryptospermia, is a very difficult task. Of tens of samples with this problem, maybe we can only preserve the motility and viability of sperm in only a few samples after melting. Therefore, a limited percentage of such samples can be used for assisted reproductive techniques. We have to consider that the primary parameters of sperm are the major determining factors of freezing and melting processes. In support of this hypothesis, we must note that the basic parameters of sperm, except for morphology, are closely related to sperm motility.

According to the World Health Organization definition, sperm samples with count, motility and viability rate less than the 5th percentile have the least level of motility and viability. Therefore, World Health Organization emphasizes that for sperm samples with parameters (or one of the parameters) less than the 5th percentile, the patient needs consultation and his fertility should be measured more accurately. Using any kind of method in such cases may not have favorable consequences.

Therefore, the sperm should be prepared for cryopreservation with more attention using an appropriate method and we need to design an accurate plan for an optimized recycling during the melting process. In this regard, samples such as oligospermia, severe oligospermia, cryptospermia, testicular cancer and parameters less than the 5th percentile require more accurate assessment (64). In cases where the sperm count is less than 1 million sperm per milliliter of semen, like the case of patients with severe oligospermia or cryptospermia, we may not find any difference between the outcome of egg fertilization and the use of fresh or frozen sperm. The desired result strengthens the theory that we should not start the

process of sperm cryopreservation without accurate assessment of the criteria and effectiveness of sperm (65).

According to some experts, we may be able to achieve higher fertilization rates and better quality embryo using frozen sperm from patients with azoospermia or cryptospermia (obtained through biopsy method). However, if we use fresh sperm in these patients, we experience better implantation. This result attracted the attention of so many experts and obliged them to consider sperm cryopreservation as an alternative and inevitable solution (66).

It seems that semen samples in people with normal level of sperm are more resistant to the damage due to freezing and melting, compared with semen samples in people with oligospermia, cryptospermia and asthenospermia. Although obtaining sperm from testis and epididymis by biopsy method was encouraging for patients and it is an effective component in the treatment of male infertility, we still need to do a lot of work in regard with freezing low count sperm biologically and technically and we need to do so much investigation to introduce appropriate techniques for its clinical use. Human sperm can be successfully frozen and recycled for later use. Sperm cryopreservation currently plays a key role in preserving the fertility of couples undergoing infertility treatment, patients with cancer undergoing chemical therapy or radiotherapy with the possibility of damage to the gonads, pelvic or testicular surgery, patients with degenerative diseases such as diabetes, multiple sclerosis or spinal cord injury, men undergoing surgical sterilization such as vasectomy, screening and quarantining of sample donor, oligospermia and cryptospermia.

Acknowledgments

Hereby, we express our deepest sense of gratitude and indebtedness to our colleagues at Health Research Institute.

References

1. Mclachlan RI. Approach to the patient with oligozoospermia. *J Clin Endocrinol Metab*. 2013;98(3):873-80.
2. Jorsaraei SGA, Firoozjaee A, Yousofnia Pasha Y, Tahmasbpour Marzony E, Sarabi E. The effects of single dose treatment of diazinon on testes structure and spermatogenesis in rat. *Yakhteh Med J*. 2010;12(1):39-42.
3. Jorsaraei SGA, Shibahara H, Ayustawati, Hirano Y, Suzuki T, Tahmasbpour Marzony E, Suzuki M. The leptin concentrations in seminal plasma of normozoospermic, asthenozoospermic and oligoasthenozoospermic men and its relationship to semen parameters. *Iran J Reproduct Med*. 2010;8(3):95-100.
4. Hosseinzadeh Colagar A, Tahmasbpour Marzony A, Chaichi MJ. Zinc levels in seminal plasma are associated with sperm quality in fertile and infertile men. *Nut Res*. 2009;29(2):82-8.
5. Hosseinzadeh Colagar A, Tahmasbpour Marzony E. Ascorbic acid levels in seminal plasma of fertile and idiopathic infertile men: determination and its relationship to sperm quality. *J Clin Biochem Nutr*. 2009; 45:144-9.
6. Hosseinzadeh Colagar A, Pouamir M, Tahmasbpour Marzony E, Jorsaraei GA. Relationship between seminal malondialdehyde and sperm parameters quality in the fertile and infertile men. *Brazil Arch Biol Technol*. 2009;52(6):1387-92.
7. Wong EW, Cheng CY. Impacts of environmental toxicants on male reproductive dysfunction. *Trend Pharmacol Sci*. 2011;32(5):290-9.
8. Jorsaraei Sg B A, Yosefnia Pasha Yr, Alizadeh Navaei R. The in vitro effects of Hinosan and Diazinon on human sperm parameters. *J Babol Univ Med Sci*. 2005;7(2):30-4.
9. Bendikson KA, Neri QV, Takeuchi T, Toschi M, Schlegel PN, Rosenwaks Z, et al. The outcome of intracytoplasmic sperm injection using occasional spermatozoa in the ejaculate of men with spermatogenic failure. *J Urol*. 2008;180(3):1060-4.
10. Corea M, Campagnone J, Sigman M. The diagnosis of azoospermia depends on the force of centrifugation. *Fertil Steril*. 2005;83(4):920-2.
11. Tournaye H, Camus M, Goossens A, Liu J, Nagy P, Silber S, et al. Recent concepts in the management of infertility because of non-obstructive azoospermia. *Hum Reprod*. 1995;10(1):115-9.
12. Ron-El R, Strassburger D, Friedler S, Komarovski D, Bern O, Soffer Y, et al. Extended sperm preparation: an alternative to testicular sperm extraction in non-obstructive azoospermia. *Hum Reprod*. 1997;12(6):1222-6.
13. Jaffe TM, Kim ED, Hoekstra TH, Lipshultz LI. Sperm pellet analysis: a technique to detect the presence of sperm in men considered to have azoospermia by routine semen analysis. *J Urol*. 1998;159(5):1548-50.
14. Koscinski I, Wittemer C, Lefebvre-Khalil V, Marcelli F, Defossez A, Rigot J M. Optimal management of extreme oligozoospermia by an appropriate cryopreservation programme. *Hum Reprod*. 2007;22(10):2679-84.
15. Abdelhafez F, Bedaiwy M, El-Nashar S A, Sabanegh E, Desai N. Techniques for cryopreservation of individual or small numbers of human spermatozoa: a systematic review. *Hum Reprod Update*. 2009;15(2):153-64.
16. Yavin S, Arav A. Measurement of essential physical properties of vitrification solutions. *Theriogenol*. 2007;67(1):81-9.
17. Isachenko E, Isachenko V, Katkov Ii, Dessole S, Nawroth F. Vitrification of mammalian spermatozoa in the absence of cryoprotectants: from past practical difficulties to present success. *Reprod Biomed Online*. 2003;6(2):191-200.
18. Donnelly ET, Steele EK, McClure N, Lewis SE. Assessment of DNA integrity and morphology of ejaculated spermatozoa from fertile and infertile men before and after cryopreservation. *Hum Reprod*. 2001;16(11):1191-9.
19. Vayena ERP, Griffin P. Current practices and controversies in Assisted Reproduction. WHO, Geneva. 2001:152-65.
20. Cross NL, Hanks SE. Effects of cryopreservation on human sperm acrosomes. *Hum Reprod*. 1991;6(9):1279-83.
21. Peiravi T, Mafat Gh, Soleimani Rad J, Farzadi L. Effect of vapor phase cryopreservation on human sperm acrosomes in fertile and subfertile men. *Journal of reproduction and infertility*. 2004;5(3):208-16.
22. Alan O, Gardner DK. Handbook of In Vitro Fertilization. 2nd ed. Chapter 16: Cryopreservation of oocytes and embryos. Shaw JM, Oranrathachai A, Trounson AO. Editors. CRC Press Inc. 2000.
23. Prins GS, Weidel L. A comparative study of buffer systems as cryoprotectants for human spermatozoa. *Fertil Steril*. 1986;46(1):147-9.
24. Nallella KP, Sharma RK, Allamaneni SS, Aziz N, Agarwal A. Cryopreservation of human spermatozoa: comparison of two cryopreservation methods and three cryoprotectants. *Fertil Steril*. 2004;82(4):913-8.

25. Mahadevan M, Trounson AO. Effect of cryoprotective media and dilution methods on the preservation of human spermatozoa. *Andrologia*. 1983;15(4):355-66.
26. Jezek D, Schulze W, Kalanj-Bognar S, Vukelic Z, Milavec-Puretic V, Krhen I. Effects of various cryopreservation media and freezing-thawing on the morphology of rat testicular biopsies. *Andrologia*. 2001;33(6):368-78.
27. Talebian A, Sadeghi M R, Akhondi MM, Ardakani H, Bolorzadeh M. The assessment and comparison of different media and methods of sperm cryopreservation (Sperm banking). *J Reproduct Infertil*. 2005;6(2):140 -52. [In Persian]
28. Critser JK, Huse-Benda AR, Aaker DV, Arneson BW, Ball GD. Cryopreservation of human spermatozoa. III. The effect of cryoprotectants on motility. *Fertil Steril*. 1988;50(2):314-20.
29. Keros V, Rosenlund B, Hultenby K, Aghajanova L, Levkov L, Hovatta O. Optimizing cryopreservation of human testicular tissue: comparison of protocols with glycerol, propanediol and dimethylsulphoxide as cryoprotectants. *Hum Reprod*. 2005;20(6):1676-87.
30. Nasri S, Amidi F, Rezaeian Z. Effect of Selenium on the motility, morphology and viability of sperm cells after freezing and thawing procedure. *J Qazvin Univ Med Sci*. 2014;18(1):11-7. [In Persian]
31. Nazm Bojnordi M, Movahedin M, Amanpour S, Ghasemi Hamidabadi H. Effect of antioxidants supplementation on human sperm parameters after freezing. *J Mazandaran Univ Med Sci*. 2008;18(63):20-27. [In Persian]
32. Brugnion F, Ouchchane L, Pons-Rejraji H, Artonne C, Farigoule M, Janny L. Density gradient centrifugation prior to cryopreservation and hypotaurine supplementation improve post-thaw quality of sperm from infertile men with oligoasthenoteratozoospermia. *Hum Reprod*. 2013;28(8):2045-57.
33. McLaughlin EA, Ford WC, Hull MG. A comparison of the freezing of human semen in the uncirculated vapour above liquid nitrogen and in a commercial semi-programmable freezer. *Hum Reprod*. 1990;5(6):724-8.
34. Ramzani M K M A, Adib M. Effect of vitrification on apoptosis and some of parameter of sperm in infertile men. *Feiz*. 2010;14(1):18-25.
35. Darvishnia H, Shams Lahijani M, Akhoundi MM, Mobaraki M, Sadeghi MR. The Effects of cryopreservation by vitrification on motility, viability and spontaneous acrosome reaction of human spermatozoa. *Iran J Biol*. 2008;21(4):198.
36. Hauser R, Yogev L, Amit A, Yavetz H, Botchan A, Azem F, Lessing J B, Ben-Yosef D. Severe hypospermatogenesis in cases of nonobstructive azoospermia: should we use fresh or frozen testicular spermatozoa?. *J Androl*. 2005;26(6):772-8.
37. Walmsley R, Cohen J, Ferrara-Congedo T, Reing A, Garrisi J. The first births and ongoing pregnancies associated with sperm cryopreservation within evacuated egg zonae. *Hum Reprod*. 1998;13(4):61-70.
38. Cohen J, Garrisi G J, Congedo-Ferrara TA, Kieck KA, Schimmel TW, Scott RT. Cryopreservation of single human spermatozoa. *Hum Reprod*. 1997;12(5):994-1001.
39. Montag M, Rink K, Dieckmann U, Delacretaz G, Van Der Ven H. Laser-assisted cryopreservation of single human spermatozoa in cell-free zona pellucida. *Andrologia*. 1999;31(1):49-53.
40. Liu J, Zheng X Z, Baramki T A, Compton G, Yazigi R A, Katz E. Cryopreservation of a small number of fresh human testicular spermatozoa and testicular spermatozoa cultured in vitro for 3 days in an empty zona pellucida. *J Androl*. 2000;21(3):409-13.
41. Borini A, Sereni E, Bonu, Flamigni C. Freezing a few testicular spermatozoa retrieved by TESA. *Mol Cell Endocrinol*. 2000;169(1-2):27-32.
42. Hsieh Y, Tsai H, Chang C, Lo H. Cryopreservation of human spermatozoa within human or mouse empty zona pellucidae. *Fertil Steril*. 2000;73(4):694-8.
43. Levi-Setti PE, Albani E, Negri L, Cesana A, Novara P, Bianchi S. Cryopreservation of a small number of spermatozoa in yolk-filled human zonae pellucidae. *Arch Ital Urol Androl*. 2003;75(4):195-8.
44. Hassa H G F, Yildirim a, Can C, Sahinturk V, Tekin B. A new protection solution for freezing small numbers of sperm inside empty zona pellucida :Osmangazi-Turk solution. *Cell Preservat Technol*. 2006;4(3):199-208.
45. Just A, Gruber I, Wober M, Lahodny J, Obruca A, Strohmer H. Novel method for the cryopreservation of testicular sperm and ejaculated spermatozoa from patients with severe oligospermia: a pilot study. *Fertil Steril*. 2004;82(2):445-7.
46. Herrler A, Eisner S, Bach V, Weissenborn U, Beier HM. Cryopreservation of spermatozoa in alginic acid capsules. *Fertil Steril*. 2006;85(1):208-13.

47. Isaev DA, Zaletov SY, Zaeva VV, Zakharova EE, Shafei RA, Krivokharchenko IS. Artificial microcontainers for cryopreservation of solitary spermatozoa. *Hum Reprod.* 2007;22(Suppl 1): i154.
48. Desai NN, Blackmon H, Goldfarb J. Single sperm cryopreservation on cryoloops: an alternative to hamster zona for freezing individual spermatozoa. *Reprod Biomed Online.* 2004;9(1):47-53.
49. Nawroth F, Isachenko V, Dessole S, Rahimi G, Farina M, Vargiu N, et al. Vitrification of human spermatozoa without cryoprotectants. *Cryo Letters.* 2002;23(2):93-102.
50. Schuster TG, Keller LM, Dunn RL, Ohl DA, Smith GD. Ultra-rapid freezing of very low numbers of sperm using cryoloops. *Hum Reprod.* 2003;18(4): 788-95.
51. Desai N, Culler C, Goldfarb J. Cryopreservation of single sperm from epididymal and testicular samples on cryoloops: preliminary case report. *Fertil Steril.* 2004;82(2):264.
52. Isachenko E, Isachenko V, Katkov, Ii, Rahimi G, Schondorf T, Mallmann P, et al. DNA integrity and motility of human spermatozoa after standard slow freezing versus cryoprotectant-free vitrification. *Hum Reprod.* 2004;19(4):932-9.
53. Isachenko V, Isachenko E, Montag M, Zaeva V, Krivokharchenko I, Nawroth F, Dessole S, Katkov, Ii, Van Der Ven H. Clean technique for cryoprotectant-free vitrification of human spermatozoa. *Reprod Biomed Online.* 2005;10(3):350-4.
54. Desain G, Goldfarb J. A convenient technique for cryopreservation of micro quantities of sperm,. conference location, 1998. Conference
55. JO Sohn, SH Jun, LS. Park, EK Kim, TG. Chung, R. Lee. Comparison of recovery and viability of sperm in ICSI pipette after ultra rapid freezing or slow freezing,. *Fertil Steril.* 2003;80(3):127.
56. Gvakharia M, Adamson GD. A method of successful cryopreservation of small numbers of human spermatozoa. *Fertility and Sterility.* 2001;76(3):101.
57. Gil-Salom M, Romero J, Rubio C, Ruiz A, Remohi J, Pellicer A. Intracytoplasmic sperm injection with cryopreserved testicular spermatozoa. *Mol Cell Endocrinol.* 2000;169(1-2):15-9.
58. Quintans CJ, Donaldson MJ, Asprea I, Geller M, Rocha M, Pasqualini RS. Development of a novel approach for cryopreservation of very small numbers of spermatozoa. *Hum Reprod.* 2000; 15: 99.
59. Sereni E, Bonu MA, Fava L, Sciajno R, Serrao L, Preti S ,Distratis V, et al. Freezing spermatozoa obtained by testicular fine needle aspiration: a new technique. *Reprod Biomed Online.* 2008;16(1):89-95.
60. Bouamama N, Briot P, Testart J. Comparison of two methods of cryoconservation of sperm when in very small numbers. *Gynecol Obstet Fertil.* 2003;31(2):132-5.
61. Ghasemian FFR, Mohammadi Sardoo M, Bahadori Mh. Evaluation of serial thawing-refreezing on human spermatozoa resistance using cryovials and straws. *Int J Fertil Steril.* 2012 6(3):157-64.
62. Kuznyetsov V, Moskovtsev SI, Crowe M, Lulat A G, Librach CL. Vitrification of a small number of spermatozoa in normozoospermic and severely oligozoospermic samples. *Syst Biol Reprod Med.* 2015;61(1):13-7.
63. Moskovtsev SI, Lulat AGM, Librach L. Cryopreservation of human spermatozoa by vitrification vs. slow freezing: canadian experience. Canada: Intech Open Access Pub; 2012.
64. Degl'innocenti S, Filimberti E, Magini A, Krausz C, Lombardi G, Fino MG, et al. Semen cryopreservation for men banking for oligospermia, cancers, and other pathologies: prediction of post-thaw outcome using basal semen quality. *Fertil Steril.* 2013;100(6):1555-63.
65. Bessonnat J, Brouillet S, Sintzel S, Gillois P, Bergues U, Boutte-Busquet C, et al. In cryptozoospermia or severe oligozoospermia is sperm freezing useful?. *Basic Clin Androl.* 2014;24:15.
66. Hauser R, Bibi G, Yogev L, Carmon A, Azem F, Botchan A, et al. Virtual azoospermia and cryptozoospermia--fresh/frozen testicular or ejaculate sperm for better IVF outcome? *J Androl.* 2011;32(5):484-90.