Impact of Cryopreservation Process on Viability, Nitric Oxide and DNA Apoptosis in Fertile Human Spermatozoa

Sara Saeednia[1], Hossein Bahadoran[1]*, Fardin Amidi[2], Mohammad Hossein Asadi[1]

1. Dept, Anatomy, Baqiyatallah University of Medical Sciences, Tehran, Iran.
2. Dept, Anatomy, Tehran University of Medical Sciences, Tehran, Iran.

Dr. Hossein Bahadoran is an assistant professor of anatomy department in Baqiyatallah University of Medical Sciences. He teaches anatomical sciences, including gross anatomy and histology. His research fields are anatomical variations and stem cells.

Article info:
Received: 13 Mar 2013
Accepted: 7 Aug 2013

Key Words:
Viability,
Nitric Oxide,
DNA Apoptosis,
Cryopreservation,
Spermatozoa.

ABSTRACT

Introduction: Infertility is a common phenomenon in modern societies. Today, use of assisted reproductive technologies (ARTs) for treatment of infertility is common, and cryopreservation is one of these technologies. Cryopreservation has impact on the function and percentage of fertility of human sperm. In this study, motility, viability, nitric oxide, and DNA apoptosis were assessed before and after cryopreservation process of human semen samples in normozoospermic men.

Methods: We divided 20 semen samples of normozoospermic men into 2 groups: fresh group as a control and frozen–thawed group. Each semen sample has been aliquoted to 4 parts in cryotube for assessment of viability by eosin and negrosin staining, motility by invert microscope, nitric oxide and DNA apoptosis content by flow cytometry.

Results: Normozoospermic men frozen–thawed semen samples showed significant ($p<0.05$) difference in viability, motility, nitric oxide and DNA apoptosis compared with fresh semen samples.

Conclusion: cryopreservation process has impact on viability, motility, intracellular nitric oxide and DNA apoptosis content in fertile human semen samples.

1. Introduction

Cryopreservation has been recently used as assisted reproductive technologies (ARTs) . For example before chemotherapy, radiotherapy, some of surgical treatments and vasectomy semen sample has been saved as fertility insurance. Also, freezing of semen has been done for azoospermic men and patients who have been cheeked up for HIV and hepatitis in laboratory. Frozen semen will insure later fertility for patients [1, 2, 4]. Today, despite of various protocols using for cryopreservation, the quality and fertility of frozen sperm has been decreased [5].

*** Corresponding Author:**
H. Bahadoran
Address: Department of Anatomy, Baqiyatallah University of Medical Sciences, Tehran, Iran.
Tel: +98 (21) 22289941
Fax: +98 (21) 26127336
E-mail: bahadoran_h@yahoo.com

In cryopreservation, semen is encountered to cold shock and atmospheric oxygen. Samples keep in -196°C in liquid nitrogen. This temperature is enough for stopping the biological function of cell. Both of Freezing and thawing create over modification in water content of cell.

On the other hands, sperm looses more cytoplasm at the end of differentiation, that possess antioxidants for counteract with reactive oxygen species (ROS) and lipid peroxidation (LPO) . For this reason, spermatozoon is susceptible to LPO as a result of cryopreservation and produces mechanical stress to cell membrane [3]. LPOs generated in enzymatic reaction of cell and human spermatozoa are able to make this [6]. Because of high affinity of ROS to react by other molecules, ROS lead to structural and functional modification of cells [3]. ROS production is a normal physiological process but changes in balance between ROS production and seminal antioxidants scavenger, lead to cell damage and male infertility. ROS may play a beneficial role in normal physiological function [1] such as maturation, capacitation and acrosome reaction [3]. Reactive oxygen species (ROS) that are generated by sperm metabolism, above physiological defense have bad effect on plasma membrane of sperm, DNA integrity, physiological function and sperm quality. Frozen sperm creates many modifications in sperm parameters. Studies show that after cryopreservation, 25-75 percentage of sperms lose their motility and decrease their viability because of generation of intracellular ice crystal and osmotic stress [7]. Over production of ROS, cryopreservation declined ATP concentration by suppression of oxidative phosphorelation or glycolsis and lead to decrease of sperm motility. Freezing of sperm significantly increased ROS production. Generation of ROS such as superoxide onion and hydrogen peroxide led to generation of cytochrome C from mitochondria and production of Apaf complex in cytoplasm and Caspase activity was happened. Caspase activate endonoclases and lead to DNA fragmentation and apoptosis [8, 9]. Nitric oxide (NO) , a highly reactive gas with a short half-life, is synthesized from the enzymatic conversion of L-arginine to L-citrulline by NADPH-dependent NO synthases (NOSs) . The NO-generating system has been demonstrated in the human reproductive tract, where NO plays a role in a variety of reproductive functions. In vitro studies, it has been shown that low concentrations of NO enhance the motility of mouse, hamster, and human spermatozoa, the acrosome reaction (AR) of mouse and bull spermatozoa, and the zona pellucida-binding ability of human spermatozoa.

On the other hand, higher NO concentrations seem to exert opposite effects on the motility, viability, and metabolism of human spermatozoa in vitro [10].

In this study, viability, motility, intracellular nitric oxide, and DNA apoptosis in normozoospermic men before and after cryopreservation were assessed.

2. Materials and Methods

Sampling Collection and Preparation

Semen samples from fertile men (>25% rapid motility or >50% progression in a semen sample, and fresh sperm concentration >20×106/ml) were obtained by masturbation and collected into sterile containers, following 3–5 days' abstinence from sexual activity. After liquefaction at 37 °C with 5% CO2 in air, the semen samples were examined for sperm concentration according to the World Health Organization (WHO) guidelines [11].

The approval of the Research Ethical Committee of Baqiyatallah University of Medical Sciences was obtained prior to the study, and all subjects were informed about this study.

We divided 20 semen samples of normozoospermic men into 2 groups: fresh group as a control and frozen–thawed group. Each semen sample has been aliquoted to 4 parts in cryotube for assessment of viability, motility, DNA apoptosis and nitric oxide, and equal volume of sperm freezing solution (Vitrolife, Sweden) were added to each cryotube.

Cryopreservation of Semen Samples

After addition of equal volume of sperm freezing solution (Vitrolife, Sweden) to each cryotube, they were inserted on the liquid nitrogen vapor at _180°C (15 – 30 centimeter up the liquid nitrogen) for 20 – 30 minute and transferred to liquid nitrogen at _196 °C and stored for two weeks.

Thawing Process

After two weeks, cryotubes containing semen samples were thawed at room temperature for 5 min, and incubated at 37 °C for 20 min. The freezing medium was removed by centrifugation (1000 rpm for 5 min) .

Assessment of Viability

The eosin–nigrosin dye exclusion staining was used to assess the sperm viability. The smears were assessed by

Table 1. Motility percentage of spermatozoa in normozoospermic men.

	Group	Motility Total Motility%	Immotility%
Normozoospermic Men	Fresh	100%	0%
	Frozen – thawed	88%	12%

ANATOMICAL SCIENCES

oil immersion light microscopy at ×1000 magnification (Fig1) . Live spermatozoa were appeared white where as dead spermatozoa with disrupted membranes appeared red. Vitality was quantified by counting a minimum of 200 spermatozoa on each slide and the proportion of live spermatozoa was expressed as a percentage.

Assessment of Motility

For assessment of motility, 10 µl of each seamen sample was put on glass lam and covered with lamella. Assessment was done with invert microscope by 40X magnification in multiple views according to the fifth edition (2010) of World Health Organization (WHO) guidelines [11].

Measurement of Nitric Oxide in Spermatozoa

Briefly for NO measurements, each sample was loaded with DAF-2/DA and incubated in the dark

(120 min, 37 °C) before analyzing by fluorescence-activated cell sorter (FACS) . Excitation wavelength 488 nm and emission wavelength 530 nm was used. The mean fluorescence intensity of the analyzed sperm cells was determined after gating the cell population by forward and side scatter light signals.

The final gated populations are usually consisted of 8000–12000 sperm cells. Fluorescence in these cells was recorded on a frequency histogram by logarithmic amplifiers. Acquired dot plot representing total events and final gated population of spermatozoa, and histogram of unstaning spermatozoa have been shown in Fig 4: A and B.

Measurement of DNA Apoptosis in Spermatozoa

We used in situ cell death detection kit, fluorescein (Roche, 11684795910, Germany) for detection and quantification of apoptosis (programmed cell death) at single cell level, based on DNA strand breaks (TUNEL technology) . Acquired dot plot representing total events and final gated population of spermatozoa, and histogram of unstaining spermatozoa have been shown in Fig 6: A and B.

Statistical Analysis

Data are expressed as the mean ± SEM. Data of viability, nitric oxide and DNA apoptosis content was analyzed by independent t-test. Differences were regarded statistically significant if $p < 0.05$. Data of motility was analyzed by Chi-Square Test.

3.Results

Viability in Spermatozoa

Viability of frozen–thawed group had significant reduction compared with fresh group ($p<0.01$) (Fig.2, Table 2) .

Table 2. Viability, nitric oxide and DNA apoptosis content in normozoospermic men.

	Motility Fresh	Frozen – thawed
Viability	59.54 ± 4.07	26.50 ± 2.11 *
Nitric Oxide	96 ± .12	9.41 ± .83 *
DNA Apoptosis	7.37 ± .48	34.97 ± 1.27 *

Results are expressed as mean ± SEM. P-values <0.05 were considered significant.

ANATOMICAL SCIENCES

*: Significant difference compared with fresh group ($p < 0.05$) .

Motility in Spermatozoa

Total motility percentage of sperm in fresh group was 100% (consider each group from 100%) compared with frozen–thawed group that was 88% of samples. Immotile percentage of sperm in fresh group was 0% whereas in frozen–thawed group was 12% (Table 1) .

Nitric oxide content in spermatozoa

Nitric oxide content of frozen–thawed group had significant elevation compared to the fresh group ($p<0.01$) (Fig. 3, Table 2) .

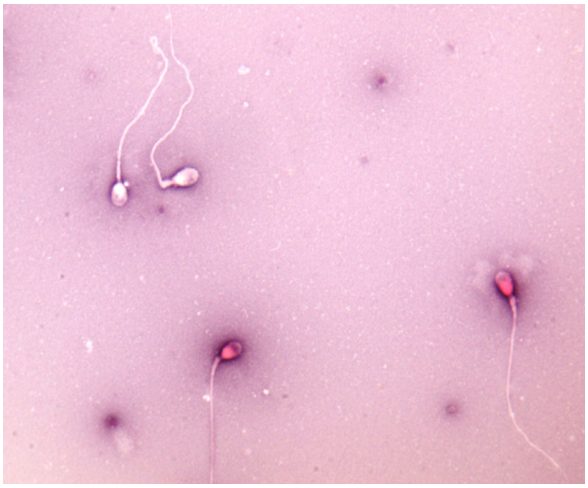

Figure 1. Human spermatozoa staining with eosin–nigrosin dye which assessed by oil immersion light microscopy at ×1000 magnification. Live spermatozoa Live spermatozoa were appeared white whilst dead spermatozoa with disrupted membranes have taken up the eosin stain and appeared red.

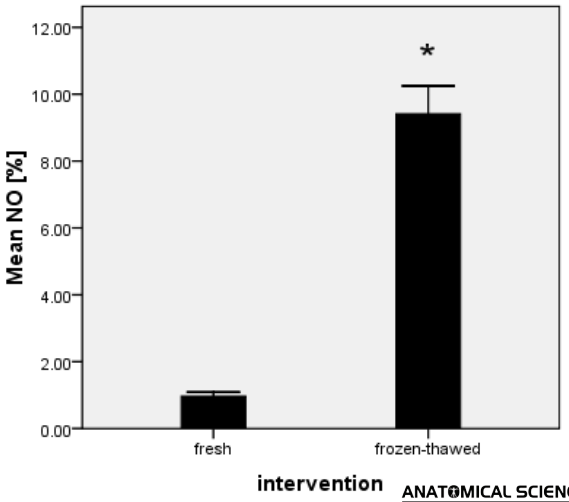

Figure 3. Nitric oxide content in normozoospermic men. P-values <0.05 were considered significant.* : Significant difference vs.compared with fresh group ($p<0.05$) .. Error bars: +/- 1SE.

4. Discussion

The aim of the present study was to determine that cryopreservation of normal human spermatozoa could influence on viability, motility, intracellular nitric oxide and DNA apoptosis compared to fresh semen samples. It was found that cryopreservation process decreased viability and motility and increased nitric oxide and DNA apoptosis in spermatozoa of normozoospermic men.

Mammalian spermatozoa were among the first cells to be successfully cryopreserved. However, both freezing and thawing expose the cells to severe stresses [12]. In

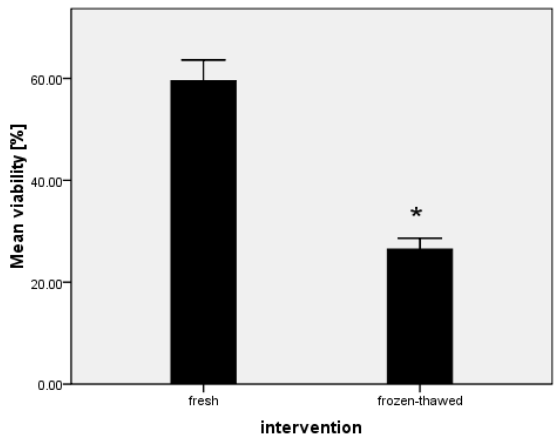

Figure 2. Viability in normozoospermic men. P-values <0.05 were considered significant.* : Significant difference vs.compared with fresh group ($p<0.05$) . Error bars: +/- 1SE.

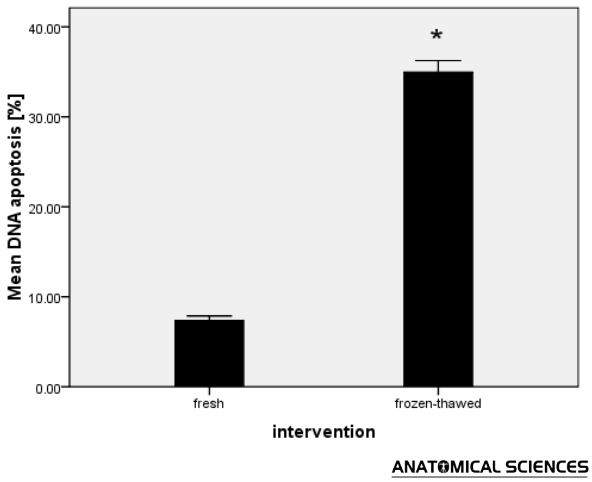

Figure 5. DNA apoptosis in normozoospermic men. P-values <0.05 were considered significant.* : Significant difference vs.compared with fresh group ($p<0.05$) . Error bars: +/- 1SE

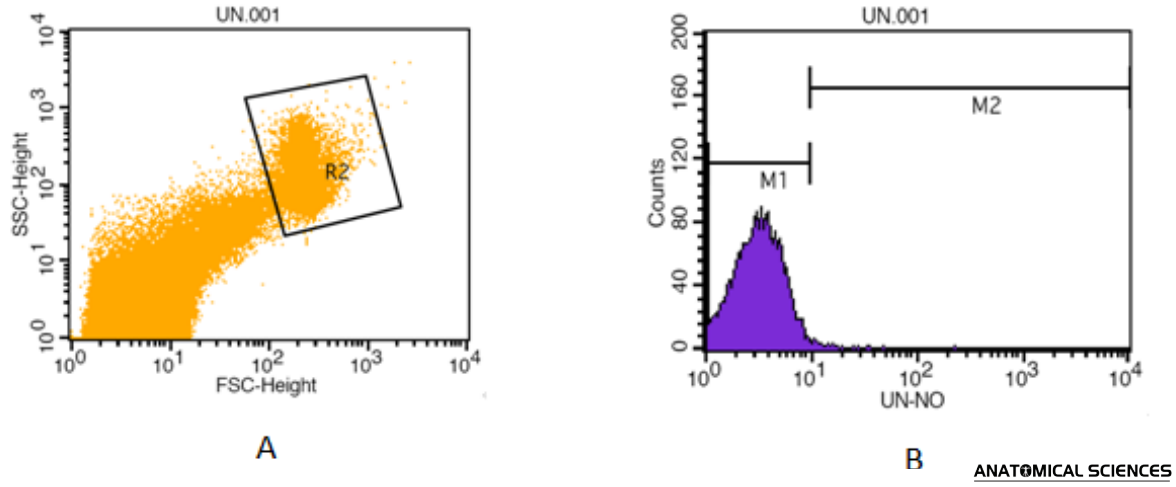

Figure 4. (A) Dot plot representing total events acquired and final gated population of spermatozoa. (B) Histogram of unstaining spermatozoa.

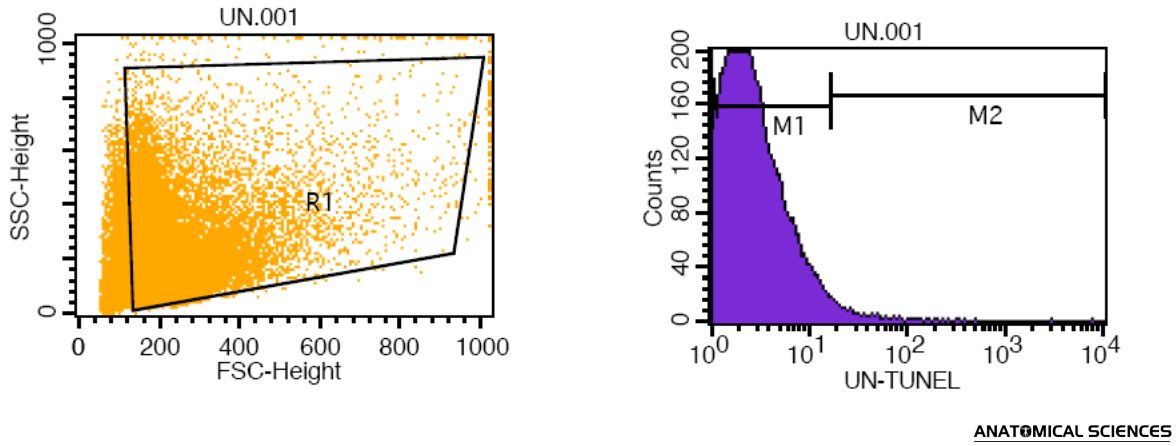

Figure 6. (A) Dot plot representing total events acquired and final gated population of spermatozoa. (B) Histogram of unstaining spermatozoa.

cryopreservation, semen has been encountered to cold shock and atmospheric oxygen. Samples were kept in -196°C in liquid nitrogen. This temperature is enough for stopping the biological function of cell. Both of freezing and thawing create over modification in water content of cell. On the other hand, sperm looses more cytoplasm at the end of differentiation, that possess antioxidants for counteract with reactive oxygen species (ROS) and lipid peroxidation (LPO) . For this reason, spermatozoon is susceptible to LPO as a result of cryopreservation and produces mechanical stress to cell membrane [3]. Freezing induced LPO in cell membrane of sperm and led to over production of free radicals concentration in the samples.

Exposing to high concentration of ROS, modifies sperm motility, viability, DNA integrity, penetration into cervical mucus, structure of acrosome and activity of acrosome protease [12, 13, 14, 15, 16].

Compared to the raw fresh semen, frozen spermatozoa show a shorter lifespan and a lower fertility. This was due to the great difference between fresh and frozen sperm in the generation rate of O2- and H2O2 or in the intracellular concentration of free calcium ions (Ca2+) [19].

Also after cryopreservation, sperm viability was decreased because of generation of intracellular ice crystal and osmotic stresses [7].

This research show that viability of frozen–thawed group have significant reduction compared with fresh group ($p<0.01$) .

Motility is one of the most important features that play a role in fertilizing ability of spermatozoa [18].

Baumber J et al, reported that exposure to high concentration of reactive oxygen species (ROS) can lead to disruption of mitochondrial and plasma membranes that results in chromosomal and DNA fragmentation and a reduction in sperm motility [20].

Studies show that after cryopreservation, 25-75 percentages of sperms lose their motility [7]. In this study, total motility percentage of sperm in fresh group was 100% compared with frozen–thawed group that was 88% of samples. Up to now, two ways for apoptosis have been known: mitochondrial (intrinsic) and cell surface receptor (extrinsic) . Apoptosis with intrinsic origin (mitochondrial) has been induced in response to different situation such as activation of P53 protein, distraction of DNA, chemotherapy and cytoplasmic stress [8].

Cryopreservation of sperm may lead to changes in the permeability, integrity, and symmetry of the plasma membrane and induced apoptosis. This condition is related to the creation of oxidative stress and reactive oxygen spices (ROS) [21].

Generation of ROS such as superoxide onion and hydrogen peroxide lead to generation of cytochrome C from mitochondria and production of Apaf complex in cytoplasm and activating Caspase. Caspase activate endonoclases and lead to DNA fragmentation and apoptosis. In cryopreservation process, DNA fragmentation has been increased [8, 9]. Several previous studies show that during freezing and thawing of sperm in buffalo, human, bull and stallion, DNA integrity was unchanged [21].

But many other researches indicated that generation of excessive oxidative stress (OS) during cryopreservation lead to DNA fragmentation [16]. As a result, due to adverse effects of cryopreservation on sperm chromatin, assessment of the DNA integrity of frozen/ thawed spermatozoa is important [21]. Our results show that DNA apoptosis of frozen–thawed group have significant elevation vs. fresh group ($p<0.01$) .

In vitro studies, it was shown that low concentrations of NO enhance the motility, the acrosome reaction (AR) , and the zona pellucida-binding ability in human spermatozoa. On the other hand, higher NO concentrations seem to have adverse effects on the motility, viability, and metabolism of human spermatozoa in vitro [10]. Our results is the same and show that in normozoospermic men when nitric oxide content in fresh group have significant reduction compared with frozen–thawed group ($p<0.01$) , viability and percentage of total motility have significant elevation compared to frozen–thawed group.

On the other hand, when nitric oxide content in frozen–thawed group has significant elevation compared with fresh group, viability and percentage of total motility have significant reduction compared to the fresh group. Results of the present study cleared that cryopreservation process decreased viability and motility and increased nitric oxide and DNA apoptosis in spermatozoa of normozoospermic men.

Acknowledgement

The authors of this study wish to thank Research and Technology Foundation (Iran National Science foundation (INSF)) for their financial support. The authors are also grateful to all participants, without whom this work would have been impossible.

Conflict of Interest:

None of the authors declared probable conflicts of interest.

References

1. Crosier, A. E., B. S. Pukazhenthi, et al. Cryopreservation of spermatozoa from wild-born Namibian cheetahs (Acinonyx jubatus) and influence of glycerol on cryosurvival. Cryobiology. 2006; 52 (2) : 169-81.

2. O'Connell M, McClure N, Lewis SE. The effects of cryopreservation on sperm morphology, motility and mitochondrial function. Hum Reprod. 2002; 17 (3) : 704-9.

3. Bansal AK, Bilaspuri GS. Impacts of Oxidative Stress and Antioxidants on Semen Functions. Vet Med Int. 2010; 7: 686137.

4. Baumber J., B. A. Ball, et al. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. J Androl. 2000; 21 (6) : 895-902.

5. Yildiz, C., C. Fleming, et al. Fresh and frozen-thawed sperm quality, nuclear DNA integrity, in vitro fertility, embryo development, and live-born offspring of N-ethyl-N-nitrosourea (ENU) mice. Cryobiology. 2008; 57 (2) : 156-62.

6. Koppers AJ, De Iuliis GN, Finnie JM, McLaughlin EA, Aitken RJ. Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. J Clin Endocrinol Metab. 2008; 93 (8) : 3199-207.

7. Marcus-Braun, N., G. Braun, et al. Effect of cryopreservation on quality and fertilization capacity of human sperm. Eur J Obstet Gynecol Reprod Biol. 2004; 116: 63-6.

8. Garcia-Macias, V., P. de Paz, et al. DNA fragmentation assessment by flow cytometry and Sperm-Bos-Halomax (bright-field microscopy and fluorescence microscopy) in bull sperm. Int J Androl. 2007; 30: 88-98.

9. Dominguez-Fandos, D., M. I. Camejo, et al. Human sperm DNA fragmentation: correlation of TUNEL results as assessed by flow cytometry and optical microscopy. Cytometry A. 2007; 71: 1011-8.

10. Alberto Revelli, Costanzo Costamagna, Federica Moffa, Elisabetta Aldieri, Simona Ochetti, Amalia Bosia, et al. Signaling Pathway of Nitric Oxide-Induced Acrosome Reaction in Human Spermatozoa. Biology of Reproduction. 2001; 64: 1708–1712.

11. Department of Reproductive Health and Research. WHO laboratory manual for the examination and processing of human semen. 5th ed. World Health Organization, Switzerland. 2010: 36-41.

12. Tahereh Talaei, Tahereh Esmaeelpour, Fatemeh Aekiyash, Soghra Bahmanpour. Effects of cryopreservation on plasma membrane glycoconjugates of human spermatozoa. Iranian Journal of Reproductive Medicine. 2010; 8: 119-124.

13. Katrina Taylor, Peter Roberts, Kathy Sanders, Peter Burton. Effect of antioxidant supplementation of cryopreservation medium on post-thaw integrity of human spermatozoa. Reproductive Bio Medicine. 2009; 8: 184-189.

14. Critser JK, Arneson BW, Aaker DV, Huse-Benda AR, Ball GD. Cryopreservation of human spermatozoa. II. Post-thaw chronology of motility and zona-free hamster ova penetration. Fertil Steril. 1987; 47: 980-4.

15. McLaughlin EA, Ford WC, Hull MG. Motility characteristics and membrane integrity of cryopreserved human spermatozoa. J Reprod Fertil. 1992; 95: 527–534.

16. Alvarez JG, Storey BT. Evidence that membrane stress contributes more than lipid peroxidation to sublethal cryodamage in cryopreserved human sperm: glycerols and other polyols as sole cryoprotectant. J Androl. 1993; 14: 199–208.

17. J. Gadea, M. Molla, E. Selles, M.A. Marco, F.A. Garcia-Vazquez, J.C. Gardon. Reduced glutathione content in human sperm is decreased after cryopreservation: Effect of the addition of reduced glutathione to the freezing and thawing extenders. Cryobiology. 2011; 62: 40–46.

18. Cátia S. Branco, Márcia E. Garcez, Fábio F. Pasqualotto, Bernardo Erdtman, Mirian Salvador. Resveratrol and ascorbic acid prevent DNA damage induced by cryopreservation in human semen. Cryobiology. 2010; 60: 235–237.

19. Alemayehu Lemma. Effect of Cryopreservation on Sperm Quality and Fertility, Artificial Insemination in Farm Animals. 2011; 12: 191–216.

20. Baumber J, Ball BA, Linfor JJ, Meyers SA. Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. Journal of Andrology 2003; 24: 621–628.

21. Mack SR, Zaneveld LJ. Acrosomal enzymes and ultrastructure of unfrozen and cryotreated human spermatozoa. Gamete Res. 1987; 18: 375–383.

22. Herrero MB, Cebral E, Boquet M, Viggiano JM, Vitullo A, Gimeno MA. Effect of nitric oxide on mouse sperm hyperactivation. Acta Physiol Pharmacol Ther Latinoam. 1994; 44: 65–69.

23. Yeoman RR, Jones WD, Rizk BM. Evidence for nitric oxide regulation of hamster sperm hyperactivation. J Androl. 1998; 19: 58–64.

24. Hellstrom WJG, Bell M, Wang R, Sikka SC. Effects of sodium nitroprusside on sperm motility, viability, and lipid peroxidation. Fertil Steril. 1994; 61: 1117–1122.

25. Zhang H, Zheng RL. Possible role of nitric oxide on fertile and asthenozoospermic infertile human sperm functions. Free Radical Res. 1996; 25: 347–354.

26. Herrero MB, Viggiano JM, Perez-Martinez S, deGimeno MF. Evidence that nitric oxide synthase is involved in progesterone-induced acrosomal exocytosis in mouse spermatozoa. Reprod Fertil Dev. 1997; 9: 433–439.

27. Zamir N, Barkan D, Keynan N, Naor Z, Breitbart H. Atrial natriuretic peptide induces acrosomal exocytosis in bovine spermatozoa. Am J Physiol. 1995; 269: E216–E221.

28. Sengoku K, Tamate K, Yoshida T, Takaoka Y, Miyamoto T, Ishikawa M. Effects of low concentrations of nitric oxide on the zona pellucid binding ability of human spermatozoa. Fertil Steril. 1998; 69: 522–527.

29. Nobunaga T, Tokugawa Y, Hashimoto K, Kubota Y, Sawai K, Kimura T, et al. Elevated nitric oxide concentration in the seminal plasma of infertile males: nitric oxide inhibits sperm motility. Am J Reprod Immunol. 1996; 36: 193–197.

30. Weinberg JB, Doty M, Bonaventura J, Haney AF. Nitric oxide inhibition of human sperm motility. Fertil Steril. 1995; 64: 408–413.

31. S. S. du Plessis, D. A. McAllister, A. Luu, J. Savia, A. Agarwal, F. Lampiao. Effects of H2O2 exposure on human sperm motility parameters, reactive oxygen species levels and nitric oxide levels. Andrologia. 2010; 42: 206–210.

32. Alberto Revelli, Costanzo Costamagna, Federica Moffa, Elisabetta Aldieri, Simona Ochetti, Amalia Bosia, et al. Signaling Pathway of Nitric Oxide-Induced Acrosome Reaction in Human Spermatozoa. Biology of Reproduction. 2001; 64: 1708–1712.

33. Armand Zini, Eve DE Lamirande, Claude Gagnon. Low Levels of Nitric Oxide Promote Human Sperm Capacitation In Vitro. Journal of Andrology. 1995; 16 (5) : 424-31.

34. C. Roessner, U. Paasch, H.-J. Glander, S. Grunewald. Activity of nitric oxide synthase in mature and immature human spermatozoa. Andrologia. 2010; 42: 132–137.