

Impact of sperm cryopreservation on child sex after intracytoplasmic sperm injection

Ahmed M. Omar^a, Mahmoud F. Abdel Hamid^a, Amr H. Abbassy^b

Departments of ^aDermatology and Venerology,
^bObstetrics and Gynecology, National Research
Centre, Cairo, Egypt

Correspondence to Ahmed M. Omar, MD,
Department of Dermatology and Venerology,
National Research Centre, Dokki,
PO Box 12622, Cairo, Egypt
Tel: +20 111 251 1369;
e-mail: dr.ahmedomar75@gmail.com

Received 27 November 2014

Accepted 28 January 2015

**Journal of the Arab Society for Medical
Research** 2015, 10:41–46

Background/aim

Sperm cryopreservation causes extensive damage to sperm membranes and its ultrastructural morphology, affecting the fertilization ability by decreasing the percentage of normal intact acrosomes and consequently the acrosome activity. This retrospective study aims at detecting the effect of sperm cryopreservation on the baby's sex after intracytoplasmic sperm injection (ICSI) in terms of the susceptibility of X versus Y chromosome bearing spermatozoa to cryopreservation.

Patients and methods

This retrospective study included 87 ICSI cycles performed with post-thawed spermatozoa. The patients were classified into two groups (I and II) according to the total sperm count before freezing.

Results

This study included 87 ICSI cycles performed with post-thawed spermatozoa. Patients were classified into two groups (I and II) according to the total sperm count before freezing. Group I included 43 patients with a sperm count less than 0.1×10^6 /sample (countable samples). Group II included 44 patients with a sperm count more than 0.1×10^6 /sample (uncountable samples). The numbers of fertilized M II, good embryos, clinical pregnancy, and male babies were significantly higher in group I compared with group II.

Conclusion

ICSI using post-thawed spermatozoa of countable samples yielded a higher male sex ratio (80.8%) compared with uncountable samples (28.6%). Thus, spermatozoa that successfully survived the freeze–thaw procedure exhibited an improved chromatin structure and nuclear maturity. These data suggest that sperm cryopreservation may improve the fertilization rate, enhance early embryo development parameters, as well as pregnancy outcome after ICSI.

Keywords:

baby sex, intracytoplasmic sperm injection, sperm cryopreservation

J Arab Soc Med Res 10:41–46
© 2015 The Arab Society for Medical Research
1687-4293

Introduction

Cryopreservation of human spermatozoa is routinely used in many assisted insemination and fertilization programmes [1]. Cryopreservation causes extensive damage to the sperm membranes and decreases the percentage of motile spermatozoa and the velocity of their movement [2–4], decreasing the fertilization ability by decreasing the percentage of normal intact acrosomes and consequently the acrosome activity [5–7].

As early as 1971, Pedersen and Lebech [8] described severe impairment of sperms in terms of ultrastructural morphology. Several investigators have confirmed this damage at the level of the membranes and acrosomes after freezing [9–12], which appear swollen and ruptured [13]; however, a comparison of the ultrastructural morphology of fresh and frozen–thawed testicular retrieved spermatozoa showed intactness of the nuclear membranes and chromatin in frozen–thawed samples.

Cryopreservation increases sperm DNA fragmentation, as concluded by Li *et al.* [14] and

Pérez-Cerezalesin *et al.* [15]. Further, Steele *et al.* [16] using the comet assay showed that control ejaculated sperm DNA was significantly more damaged than testicular sperm DNA from control men; they also showed that the percentage of undamaged DNA in testicular sperm from fertile men was significantly greater than the percentage of undamaged DNA in ejaculated sperm from the same men; this may explain the higher percentage of recovered spermatozoa in testicular samples compared with ejaculated samples and may also explain the higher pregnancy rates that were observed in frozen–thawed surgically retrieved spermatozoa used for intracytoplasmic sperm injection (ICSI) [17].

Despite the fact that sperm cryopreservation reduces sperm quality, samples from fertile men are significantly better than those from infertile individuals [18,19]. An explanation for this discrepancy may be the higher rate of reactive oxygen species production in low-quality samples than in normal-quality samples [20–24].

As ejaculate contains several cellular components other than spermatozoa, their sublethal damage results from the combined effects of cell dehydration/rehydration, membrane lipid phase transition, alteration in the enzyme activity or energy metabolism, and activation of lipid peroxidation cascade with generation of reactive oxygen species [23,25,26]. This may explain the lower results of ejaculated spermatozoa compared with other sperm sources. Several authors reported that the clinical pregnancy rate is similar when frozen-thawed testicular spermatozoa or fresh gametes are used for ICSI [27–32]. Severe impairment of sperm motility depends largely on the initial quality of the sample, the cryoprotectant, or the method of freezing [10,23,33–36].

The present study aims at detecting the effect of sperm cryopreservation on the baby's sex after ICSI in terms of the susceptibility of X versus Y chromosome bearing spermatozoa to cryopreservation.

Patients and methods

This is a retrospective study of patients who underwent cryopreservation of motile ejaculated (26 cases) and testicular (61 cases) spermatozoa using sperm freeze (FertiPro NV, Beernem, Belgium) as a cryoprotectant, programmed freezing protocol, and sperm storage in liquid nitrogen as the refrigerant (-196°C). One of the authors was the working embryologist.

Clinical history (full history and genital examination), laboratory data (hormonal profile if needed, including follicular stimulating hormone, leutenizing hormone, testosterone, and prolactin), prethawing and post-thawing sperm count, motility, and vitality were determined.

Sample analysis was performed according to the guidelines of the WHO [37,38] under a light microscope (Olympus CH 30 RF 200; Olympus Company Limited, Tokyo, Japan).

Testicular tissue samples were collected in a HEPES buffered Earle's balanced salt solution; the biopsy samples were shredded with two sterile microscopic glass slides. Fine pincers were used for further mincing [39], and the sample was examined under an inverted microscope (Olympus 1 × 70 S8F2; Olympus Company Limited). If no spermatozoa were detected the tissue fluid was treated with erythrocyte lysing buffer and the sample was centrifuged [40]. A dish with microdrops was then prepared (2 mm each drop) from the pellet, covered with sigma oil, and examined under an inverted microscope [41,42].

The sample was diluted 1 : 1 (v/v) with the sperm freeze medium, which was added dropwise from the side of a 15-ml falcon tube (Falcon 2095; Becton Dickinson, New Jersey, USA) over 10 min to minimize hyperosmotic stress, while continuously shaking the tube. The diluted semen was loaded into straws using an automatic pipette. The straws were sealed at one end with a cotton plug. After aspiration each straw was sealed on the other side. The straws were labeled individually with the name of the patient, file number, date of cryopreservation, and nature of the sample (Brady, New Jersey, USA), and placed in the chamber of a computer-controlled biological freezer (Nicoool LM 10; Air Liquide, Paris, France) and cooled with the freezing program described by Yavetz *et al.* [43]. The rate of freezing was as follows: from room temperature to 10°C at a rate of $-1.6^{\circ}\text{C}/\text{min}$ for 6 min (program 5), and from 10 to -120°C at a rate of $-5.5^{\circ}\text{C}/\text{min}$ for 20 min (program 10). The sample was then removed from the controlled-rate freezer and plunged directly into the liquid nitrogen storage tank at -196°C [43].

When a straw had to be thawed, it was removed from the liquid nitrogen tank according to the patient identification number, file number, registration data, and cryopreservation tank map and thawed at 37°C for 10 min. One end of the straw was cut and the straw was placed near the tip of a conical falcon tube and the other end was cut to let the sample fall into the tube. The cryoprotectant was removed by placing the sample in a tube to which 10 ml of Earle's balanced salt solution containing 0.5% human serum albumin was added slowly dropwise. Equilibration at 37°C for 10 min and then centrifugation at 1500 rpm for 10 min were carried out. The supernatant was removed and the pellet was resuspended in a fixed volume of 200 ml. The sample was then evaluated using the guidelines of WHO [37,38] and the results were recorded.

The ICSI procedures involved sperm and oocyte preparation [30,44]. The ICSI procedure was performed as described by Van Steirteghem *et al.* [45], Al-Hassani *et al.* [46], Merchant *et al.* [35]. A duration of 16–18 h after injection, the oocytes were examined for the presence of pronuclei and polar bodies [47,48], and at 25 h after injection the oocytes were monitored for early cleavage [34]. The embryos were monitored at exactly 48 h for four-cell stage (day 2 transfer), or at exactly 72 h for 7–9-cell stage (day 3 transfer). The embryos were then transferred to the uterine cavity according to the protocol followed by Merchant *et al.* [35], Racowsky *et al.* [49], Alikani *et al.* [50,51], Ziebe *et al.* [52]. Luteal support was provided with either

human chorionic gonadotropins (hCG) or natural micronized progesterone (600 mg/day).

Results

This study included 87 ICSI cycles performed with post-thawed spermatozoa. The patients were classified into two groups (I and II) according to the total sperm count before freezing. Group I included 43 patients with a sperm count less than 0.1×10^6 /sample (countable samples). Group II included 44 patients with a sperm count more than 0.1×10^6 /sample (uncountable samples).

There was no statistically significant difference in male clinical parameters, sperm freezing and thawing data, and female clinical parameters between pregnant and nonpregnant ladies in groups I and II, as shown in Tables 1–3, respectively.

There was a statistically significant difference in the numbers of fertilized M II and good-quality embryos between pregnant and nonpregnant ladies in groups I and II, whereas other ICSI parameters were statistically not significant, as shown in Table 4.

All patients underwent a pregnancy test 2 weeks after embryo transfer; 28 of 87 (32.2%) cases showed a positive pregnancy test as detected by the b-hCG in their serum. Gestational sac and presence of pulsating fetal heartbeat were detected by means of transabdominal ultrasound in 27 of 87 (31%) cases 2 weeks after the pregnancy test. Only one of the ejaculated samples resulted in a positive pregnancy (1/26, 3.8%), compared with 26 of 40 (65%) from retrieved spermatozoa in groups I and II, as shown in Table 5. Twelve of the 27 positive pregnancies resulted in successful deliveries and the sex of the babies is shown in Table 6; seven of 27 cases are still pregnant and eight of 27 aborted. This study showed that the take baby home was 11 males and seven females in group I and II as shown in Table 6.

The percentage of good embryos to fertilized M II and the number of clinical pregnancies were significantly higher in group I compared with group II. In contrast, no significant difference between groups I and II was detected in other parameters, as shown in Table 7.

To detect a possible explanation to these findings, we compared the female clinical parameters and ICSI data in groups I and II, as shown in Table 8. The results showed that the number of good and fair embryos and the number of embryos transferred was significantly

Table 1 The difference in male clinical and endocrinal parameters between pregnant and nonpregnant women

Male clinical and hormonal parameters	Pregnancy test positive		Pregnancy test negative	
	N	Mean $\pm$ SD	N	Mean $\pm$ SD
Age (years)	22	38.2 $\pm$ 7	52	39.8 $\pm$ 6.6
Infertility duration (years)	22	10.3 $\pm$ 10.9	50	10.9 $\pm$ 6.53
Testicular size (ml)	21	12.4 $\pm$ 3.1	48	12.3 $\pm$ 2.9
FSH (mIU/ml)	11	12.8 $\pm$ 9.2	24	10.8 $\pm$ 7.4
LH (mIU/ml)	8	12 $\pm$ 8.7	16	12.9 $\pm$ 11.9
Testosterone (ng/ml)	4	3.14 $\pm$ 2.7	18	4.9 $\pm$ 2.5
Prolactin (ng/ml)	5	21.3 $\pm$ 18.2	16	12.9 $\pm$ 14

Missing values were either repeated cases or missed data; All data are insignificantly changed; FSH, follicular stimulating hormone; LH, leutenizing hormone.

Table 2 The difference in prefreezing and post-thawing data between pregnant and nonpregnant women

Prefreezing and post-thawing data	Mean $\pm$ SD	
	Pregnancy test positive (N = 27)	Pregnancy test negative (N = 60)
PF total count $\times 10^6$	3.6 $\pm$ 12.4	1.14 $\pm$ 2.25
PF motility%	28.3 $\pm$ 17.2	33.4 $\pm$ 23.7
PF total motile count $\times 10^6$	0.5 $\pm$ 1.65	0.44 $\pm$ 1.4
Freezing duration (months)	3.9 $\pm$ 3.77	4.1 $\pm$ 4.24
PT total count $\times 10^6$	3.2 $\pm$ 11.7	0.87 $\pm$ 1.8
PT motility%	21.8 $\pm$ 14	22.1 $\pm$ 18.7
PT total motile count $\times 10^6$	0.23 $\pm$ 0.76	0.24 $\pm$ 0.94

All data are insignificantly changed; PF, prefreezing; PT, post-thawing.

Table 3 The difference in female clinical, hormonal, and biological parameters between pregnant and nonpregnant women

Female clinical, hormonal and biological parameters	Pregnancy test positive		Pregnancy test negative	
	N	Mean $\pm$ SD	N	Mean $\pm$ SD
Female age (years)	26	30.3 $\pm$ 6.15	53	32 $\pm$ 6.8
Female FSH (mIU/ml)	5	12.2 $\pm$ 2.2	16	10.8 $\pm$ 6.1
Number of cumulus masses	27	13.8 $\pm$ 7.36	60	13.2 $\pm$ 9.9
Number of mature oocytes (M II)	27	11.7 $\pm$ 7	60	11 $\pm$ 8.5

Missing values were either repeated cases or missed data; All data are insignificantly changed; FSH, follicular stimulating hormone.

Table 4 The difference in intracytoplasmic sperm injection parameters between pregnant and nonpregnant women

ICSI parameters	Mean $\pm$ SD	
	Pregnancy test positive (N = 27)	Pregnancy test negative (N = 60)
Number of injected M II	11.7 $\pm$ 7	10.9 $\pm$ 8.2
Number of fertilized M II	7.8 $\pm$ 4.8	5.35 $\pm$ 4.4*
Number of embryos	7 $\pm$ 3.8	5.27 $\pm$ 4.34
Number of good embryos	2.8 $\pm$ 2.1	1.8 $\pm$ 2.2*
Number of fair embryos	2 $\pm$ 2.3	1.33 $\pm$ 1.5
Number of bad embryos	2.15 $\pm$ 3	2.15 $\pm$ 3
Number of embryos transferred	4.22 $\pm$ 1.5	3.45 $\pm$ 1.91

ICSI, intracytoplasmic sperm injection; *Significant difference using t-test at $P < 0.05$.

higher in group I, which may explain the higher pregnancy rate (Table 7).

A comparison between the two groups regarding the sex of delivered babies showed that the number of male babies was significantly higher in group I when compared with group II, as described in Table 9.

Discussion

Cryopreservation is associated with extensive damage to cell membranes, and results in alteration of the functional and metabolic status of the cells and mitochondria. Evidence suggests an increase in DNA single-strand breaks, and degree of DNA condensation or fragmentation in sperm after cryopreservation [7,24]. Pregnancy rate was significantly higher in group I compared with group II [18/43 (41.9%) vs. 9/44 (20.4%)]. The difference is attributed to the high percentage of good embryos/fertilized M II (115/275, 41.8%) in group I versus group II (67/257, 26%) ($P < 0.05$) and to the mean numbers of embryos transferred, which was statistically higher in group I (4.16 ± 1.65)

Table 5 The percentage of frozen ejaculated sperm samples and testicular retrieved spermatozoa among pregnancy test results in groups I and II

Sperm sources	n (%)		Total
	Pregnancy test positive	Pregnancy test negative	
Ejaculated samples	1 (3.8)	25 (96.2)	26
Testicular samples	26 (42.6)	35 (57.4)	61
Total	27 (31)	60 (69)	87
P value	0.000		

Table 6 The number and percentage of delivered cases and their baby's sex in groups I and II

Outcomes	Baby sex			Total babies
	Male	Female	Male and female	
Single	5 (71.5)	2 (28.5)	–	7
Twins	–	1 (25)	3 (75)	8
Triplets	1 (100)	–	–	3
Total babies	8 (44.5)	4 (22.2)	6 (33.3)	18

Table 7 A comparison between groups I and II regarding embryological records

Embryological data	n (%)	
	Group I (N = 43)	Group II (N = 44)
Fertilization rate	275/496 (55.4)	257/474 (54.2)
Good embryos/fertilized M II	115/275 (41.8)	67/257 (26)*
Chemical pregnancy	18/43 (41.9)	10/44 (22.7)
Clinical pregnancy	18/43 (41.9)	9/44 (20.4)*
Implantation rate	28/179 (15.6)	13/142 (9.2)

*Significant difference using *t*-test at $P < 0.05$.

compared with group II (3.23 ± 1.87) ($P < 0.05$). This difference could not be explained by female factors as the differences in mean age, serum follicular stimulating hormone level, number of cumulus masses, mature oocytes, number of injected oocytes, fertilized oocytes, and embryos were statistically insignificant between groups I and II. This may point to the effect of sperm factor on the ICSI outcome as some changes caused by cryopreservation resemble the changes occurring during normal capacitation that contribute to more efficient oocyte activation and/or pronuclear formation after ICSI [53,54].

The mean number of fertilized M II and the number of embryos were significantly higher in pregnant ladies. No significant differences between pregnant and nonpregnant ladies were detected in other parameters.

ICSI using frozen–thawed samples in group I yielded a higher male sex ratio (80.8%) compared with countable samples (28.6%). This study is contradictory to a previous work that showed no significant difference in sex ratio when frozen–thawed spermatozoa were used for artificial insemination [55], as artificial insemination donor [56] or as IVF donor [57]. In contrast, Sidhu *et al.* [58] had reported that more male children (101 male versus 83 female children) were born after IUI using cryopreserved thawed spermatozoa [53]. However, to our knowledge, no studies have reported

Table 8 The differences in female clinical parameters and intracytoplasmic sperm injection data between groups I and II

Female clinical and ICSI parameters	Group (I) (N = 43)	Group (II) (N = 44)
Female age (years)	31 ± 6	31.6 ± 7.2
Female FSH (mIU/ml)	9.5 ± 3.8	13.24 ± 6.78
Number of cumulus mass	12 ± 7.6	13.27 ± 9.43
Number of Mature oocytes (M II)	11.5 ± 7.2	10.8 ± 8.37
Number of injected M II	11.5 ± 7.2	10.77 ± 8.4
Number of fertilized M II	6.4 ± 4	5.84 ± 5.3
Number of embryos	6.1 ± 3.6	5.48 ± 4.76
Number of good embryos	2.67 ± 2.51	$1.52 \pm 1.68^*$
Number of fair embryos	1.09 ± 1.6	$1.98 \pm 1.91^*$
Number of bad embryos	2.35 ± 2.8	1.95 ± 3.13
Number of embryos transferred	4.16 ± 1.65	$3.23 \pm 1.87^*$
Number of sacs	1.56 ± 0.7	1.44 ± 0.73

FSH, follicular stimulating hormone; ICSI, intracytoplasmic sperm injection; *Significant difference using *t*-test at $P < 0.05$.

Table 9 The distribution of baby sex between groups I and II

Baby sex	n (%)		
	Male	Female	Total
Group I	9 (80.8)	2 (18.2)	11
Group II	2 (28.6)	5 (71.4)	7
Total	11 (61.1)	7 (38.9)	18
P value	0.049		

*Significant difference using *t*-test at $P < 0.05$.

differences in sex ratio when ICSI was performed using cryopreserved thawed spermatozoa.

During spermatogenesis the chromatin becomes highly condensed within a protamine matrix [59]. The DNA is organized into loops, attached at their bases to the nuclear matrix, anchored to the base of the sperm tail by the nuclear annulus, and stabilized by disulfide bonds [60,61]. This tight packing of the DNA reduces exposure to free radical attack. It has been proven that hidden anomaly such as higher levels of loosely packaged chromatin and damaged DNA can be present in sperm nuclei from men with deficient spermatozoa. The most frequent visible changes are related to the protamine-deficient, nicked, and partially denatured DNA [62,63].

A properly performed cryopreservation may selectively affect defective rather than normal spermatozoa [64]. Furthermore, spermatozoa that successfully survived the freeze–thaw procedure also exhibited an improved chromatin structure and nuclear maturity. These data suggest that sperm cryopreservation when performed correctly may not only improve the fertilization rate but may also enhance early embryo development parameters as well as pregnancy outcome after ICSI [65].

As most of the cases had few uncountable sperms, limited number of spermatozoa have been retrieved and used for oocyte injection. This finding may be explained by the fact that cryopreservation acts as an artificial selecting process with minimal effect on the Y chromosome (60 megabase) due to its smaller size and lower molecular weight compared with the larger X chromosome (160 megabase) [53]. We think that the flow cytometric sorting used to separate spermatozoa bearing Y chromosome based on sex chromosome content might be of added value for use as a preconception method of influencing baby sex [66] to avoid having children affected by sex-linked disease. There are over 1100 X-linked diseases and ~60 Y-linked diseases. The embryonic sex data (as determined by PGD) show that the proportions of XX embryos after X-sort and XY embryos after Y-sort were consistent with the post-sort FISH results.

If sperm cryopreservation sorting did adversely affect the sperm function, one would expect lower rates of fertilization, cleavage, and pregnancy, which was not the case in this study.

In our study seven cases are still pregnant and the baby's sex has not been determined yet; the sex of these unborn children may or may not confirm the results of this study.

In conclusion, ICSI using post-thawed spermatozoa of countable samples yielded a higher male sex ratio

(80.8%) compared with uncountable samples (28.6%). Thus, it is suggested that sperm cryopreservation might affect the pregnancy outcome after ICSI, which needs further controlled studies on a larger scale to be validated.

Acknowledgements

Conflicts of interest

None declared.

References

- Sherman JK. Cryobanking of semen in AIH. In: Emperaire JC, Audebert A Hafez SE, editors. *Homologous artificial Insemination*. Plenum Press, New York, 1980. p. 66.
- Critser JK, Arneson BW, Aaker DV, Huse-Benda AR, Ball GD. Cryopreservation of human spermatozoa. I. Postthaw chronology of motility and of zona-free hamster ova penetration. *Fertil Steril* 1987; 47:980–984.
- Critser JK, Arneson BW, Aaker DV, Huse-Benda AR, Ball GD. Cryopreservation of human spermatozoa. II. Postthaw chronology of motility and of zona-free hamster ova penetration. *Fertil Steril* 1987; 9:1214–1219.
- Hammerstedt RH, Graham JK, Nolan JP. Cryopreservation of mammalian sperm: what we ask them to survive. *J Androl* 1990; 11:73–88.
- Mack SR, Zaneveld LJ. Acrosomal enzymes and ultrastructure of unfrozen and cryotreated human spermatozoa. *Gamete Res* 1987; 18:375–383.
- Cross NL, Hanks SE. Effects of cryopreservation on human sperm acrosomes. *Hum Reprod* 1991; 6:1279–1283.
- Jia G, Fu X, Cheng K, Yue M, Jia B, Hou Y, Zhu S. Spermatozoa cryopreservation alters pronuclear formation and zygotic DNA demethylation in mice. *Theriogenology* 2014; pii:S0093–S0691X00672–4.
- Pedersen H, Lebech PE. Ultrastructural changes in the human spermatozoon after freezing for artificial insemination. *Fertil Steril* 1971; 22:125–133.
- Mahadevan MM, Trounson AO. Relationship of fine structure of sperm head to fertility of frozen human semen. *Fertil Steril* 1984; 41:287–293.
- 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; 50:314–320.
- Check ML, Check JH, Long R. Detrimental effects of cryopreservation on the structural and functional integrity of the sperm membrane. *Arch Androl* 1991; 27:155–160.
- Henry MA, Noiles EE, Gao D, Mazur P, Critse JK. Cryopreservation of human spermatozoa. IV. The effects of cooling rate and warming rate on the maintenance of motility, plasma membrane integrity, and mitochondrial function. *Fertil Steril* 1993; 60:911–918.
- Nogueira D, Bourgain C, Verheyen G, Van Steirteghem AC. Light and electron microscopic analysis of human testicular spermatozoa and spermatids from frozen and thawed testicular biopsies. *Hum Reprod* 1999; 14:2041–2049.
- Li MW, Meyers S, Tollner TL, Overstreet JW. Damage to chromosomes and DNA of rhesus monkey sperm following cryopreservation. *J Androl* 2007; 28:493–501.
- Pérez-Cereales S, Martínez-Páramo S, Cabrita E, Martínez-Pastor F, de Paz P, Herráez MP. Evaluation of oxidative DNA damage promoted by storage in sperm from sex-reversed rainbow trout. *Theriogenology* 2009; 71:605–613.
- Steele EK, McClure N, Lewis SE. Comparison of the effects of two methods of cryopreservation on testicular sperm DNA. *Fertil Steril* 2000; 74:450–453.
- Ben Rhouma K, Marrakchi H, Khouja H, Attalah K, Ben Miled E, Sakly M. Outcome of intracytoplasmic injection of fresh and frozen-thawed testicular spermatozoa. A comparative study. *J Reprod Med* 2003; 48:349–354.
- Mangoli V, Dandekar S, Desai S, Mangoli R. The outcome of ART in males with impaired spermatogenesis. *J Hum Reprod Sci* 2008; 1:73–76.
- Muratori M, Marchiani S, Maggi M, Forti G, Baldi E. Origin and biological significance of DNA fragmentation in human spermatozoa. *Front Biosci* 2006; 11:1491–1499.

- 20 Zalata A, Hafez T, Comhaire F. Evaluation of the role of reactive oxygen species in male infertility. *Hum Reprod* 1995; 10:1444–1451.
- 21 Donnelly ET, Steele EK, McClure N, Lewis SEM. Assessment of DNA integrity and morphology of ejaculated spermatozoa from fertile and infertile men before and after cryopreservation. *Hum Reprod* 2001; 16:1191–1199.
- 22 Donnelly ET, McClure N, Lewis SE. Cryopreservation of human semen and prepared sperm: effects on motility parameters and DNA integrity. *Fertil Steril* 2001; 76:892–900.
- 23 Muratori M, Maggi M, Spinelli S, Filimberti E, Forti G, Baldi E. Spontaneous DNA fragmentation in swim-up selected human spermatozoa during long term incubation. *J Androl* 2003; 24:253–262.
- 24 Kopeika J, Thornhill A, Khalaf Y. The effect of cryopreservation on the genome of gametes and embryos: principles of cryobiology and critical appraisal of the evidence. *Hum Reprod Update* 2015; 21:209–227.
- 25 Barthelemy C, Royere D, Hammah S, Lebos C, Tharanne MJ, Lansac J. Ultrastructural changes in membranes and acrosome of human sperm during cryopreservation. *Arch Androl* 1990; 25:29–40.
- 26 McLaughlin EA, Ford WC, Hull MG. Motility characteristics and membrane integrity of cryopreserved human spermatozoa. *J Reprod Fertil* 1992; 95:527–534.
- 27 Gil-Salom M, Romero J, Minguez Y, Rubio C, De los Santos MJ, Remohí J, Pellicer A. Pregnancies after intracytoplasmic sperm injection with cryopreserved testicular spermatozoa. *Hum Reprod* 1996; 11:1309–1313.
- 28 Friedler S, Raziel A, Soffer Y, Strassburger D, Komarovskiy D, Ron-EL R. Intracytoplasmic sperm injection of fresh and cryopreserved testicular spermatozoa in patients with non-obstructive azoospermia – a comparative study. *Fertil Steril* 1997; 68:892–897.
- 29 Friedler S, Raziel A, Soffer Y, Strassburger D, Komarovskiy D, Ron-EL R. The outcome of intracytoplasmic injection of fresh and cryopreserved testicular spermatozoa in patients with obstructive azoospermia – a comparative study. *Hum Reprod* 1997; 3:1872–1877.
- 30 K pker W, Schlegel PN, A-Hassani S, Fornara P, Johannisson R, Sandman J, *et al.* Use of frozen thawed testicular sperm for Intracytoplasmic sperm injection. *Fertil Steril* 2000; 73:453–458.
- 31 Kalsi J, Thum MY, Muneer A, Pryor J, Abdullah H, Minhas S. Analysis of the outcome of intracytoplasmic sperm injection using fresh or frozen sperm. *BJU Int* 2011; 107:1124–1128.
- 32 Ishikawa T, Shiotani M, Izumi Y, Hashimoto H, Kokeguchi S, Goto S, Fujisawa M. Fertilization and pregnancy using cryopreserved testicular sperm for intracytoplasmic sperm injection with azoospermia. *Fertil Steril* 2009; 92:174–179.
- 33 Englert Y, Delvigne A, Vekemans M, Lejeune B, Henlisz A, de Maertelaer G, *et al.* Is fresh or frozen semen to be used in in vitro fertilization with donor sperm? *Fertil Steril* 1989; 51:661–664.
- 34 Yoshida H, Hoshiai H, Fukaya T, Ohi T, Kakuta C, Tozawa H, *et al.* Fertilizability of fresh and frozen human spermatozoa. *Assist Reprod Technol Androl* 1990; 1:164–172.
- 35 Merchant R, Gandhi G, Allahbadia GN. In vitro fertilization/intracytoplasmic sperm injection for male infertility. *Indian J Urol* 2011; 27:121–132.
- 36 Xu XR, Tan FQ, Zhu JQ, Ye T, Wang CL, Zhu YF, *et al.* Detection of DNA damage caused by cryopreservation using a modified SCGE in large yellow croaker, *Pseudosciaena crocea*. *Acta Biol Hung* 2014; 65:405–413.
- 37 World Health Organization. *Laboratory manual for the examination of human semen and sperm-cervical mucus interaction*. 4th ed. Cambridge, UK: Cambridge University Press; 1999.
- 38 Cooper TG, Noonan E, von Eckardstein S, Auger J, Baker HW, Behre HM, *et al.* World Health Organization reference values for human semen characteristics. *Hum Reprod Update* 2010; 16:231–245.
- 39 Verheyen G, De Croo I, Tournaye H, Pletincx I, Devroey P, van Steirteghem AC. Comparison of four mechanical methods to retrieve spermatozoa from testicular tissue. *Hum Reprod* 1995; 10:2956–2959.
- 40 Nagy Z, Devroy P, Verheyen G, Tournay H, Van Steirteghem AC. An improved treatment procedure for testicular biopsy specimens offers more efficient sperm recovery: case series. *Fertil Steril* 1997; 68:376–379.
- 41 Henkel RR, Schill WB. Sperm preparation for ART. *Reprod Biol Endocrinol* 2003; 1:108.
- 42 Van Peperstraten A, Proctor ML, Johnson NP, Philipson G. Techniques for surgical retrieval of sperm prior to intra-cytoplasmic sperm injection (ICSI) for azoospermia. *Cochrane Database Syst Rev* 2008; 2:CD002807.
- 43 Yavetz H, Lessing JB, Niv Y, Amit A, Barak Y, Yovel I, *et al.* The efficiency of cryopreserved semen versus fresh semen for in vitro fertilization/embryo transfer. *J In Vitro Fert Embryo Transf* 1991; 8:145–148.
- 44 Ola B, Afnan M, Sharif K, Papaioannou S, Hammadih N, Barratt CLR. Should ICSI be the treatment of choice for all cases of in-vitro conception?. *Hum Reprod* 2001; 16:2485–2491.
- 45 Van Steirteghem A, Liu J, Joris H, *et al.* High success rates by intracytoplasmic sperm injection than by subzonal insemination. Report of a second series of 300 consecutive treatment cycles. *Hum Reprod* 1993; 8:1055–1060.
- 46 Al-Hassani S, K pker W, Baschat AA, Sturm R, Bauer O, Diedrich C, *et al.* Mini-swim up: a new technique of sperm preparation for intra cytoplasmic sperm injection. *J Assist Reprod Genet* 1995; 2:428–433.
- 47 Nagy Z, Liu J, Joris H, Devroey P, Van Steirteghem A. Time course of oocyte activation, pronucleus formation and cleavage in human oocytes fertilized by intracytoplasmic sperm injection. *Hum Reprod* 1994; 9:1743–1748.
- 48 Tesarik J, Greco E. The probability of abnormal preimplantation development can be predicted by a single static observation on pronuclear stage morphology. *Hum Reprod* 1999; 14:1318–1323.
- 49 Racowsky C, Stern JE, Gibbons WE, Behr B, Pomeroy KO, Biggers JD. National collection of embryo morphology data into Society for Assisted Reproductive Technology Clinic Outcomes Reporting System: associations among day 3 cell number, fragmentation and blastomere asymmetry, and live birth rate. *Fertil Steril* 2011; 95:1985–1989.
- 50 Alikani M, Cohen J, Tomkin G, Garrisi GJ, Mack C, Scott RT. Human embryo fragmentation in vitro and its implications for pregnancy and implantation. *Fertil Steril* 1999; 71:836–842.
- 51 Alikani M, Calderon G, Tomkin G, Garrisi J, Kokot M, Cohen J. Cleavage anomalies in early human embryos and survival after prolonged culture in-vitro. *Hum Reprod* 2000; 15:2634–2643.
- 52 Ziebe S, Petersen K, Lindenberg S, Andersen AG, Gabrielsen A, Andersen AN, *et al.* Embryo morphology or cleavage stage: how to select the best embryos for transfer after in-vitro fertilization. *Hum Reprod* 1997; 12:1545–1549.
- 53 M n zo YJ, Veiga A, Pouly JL. Assisted reproductive technology (ART) in humans: facts and uncertainties. *Theriogenology* 2000; 53:599–610.
- 54 Palermo GD, Neri QV, Schlegel PN, Rosenwaks Z. Intracytoplasmic sperm injection (ICSI) in extreme cases of male infertility. *PLoS One* 2014; 9:e113671.
- 55 Grefenstette I, Royere D, Barth l my C, Tharanne MJ, Lansac J. Outcome of 470 pregnancies after artificial insemination with frozen sperm. *J Gynecol Obstet Biol Reprod (Paris)* 1990; 19:737–744.
- 56 Lansac J, Thepot F, Mayaux MJ, Czyglick F, Wack T, Selva J, Jalbert P. Pregnancy outcome after artificial insemination or IVF with frozen semen donor: a collaborative study of the French CECOS Federation on 21,597 pregnancies. *Eur J Obstet Gynecol Reprod Biol* 1997; 74:223–228.
- 57 Lansac J, Royere D. Follow-up studies of children born after frozen sperm donation. *Hum Reprod Update* 2001; 7:33–37.
- 58 Sidhu RS, Sharma RK, Agarwal A. Effects of cryopreserved semen quality and timed intrauterine insemination on pregnancy rate and gender of offspring in a donor insemination program. *J Assist Reprod Genet* 1997; 14:531–537.
- 59 Sidney R, Grimes JR. Nuclear proteins in spermatogenesis. *Comp Biochem Physiol* 1986; 83:495–500.
- 60 Ward WS. Deoxyribonucleic acid loop domain tertiary structure in mammalian spermatozoa. *Biol Reprod* 1993; 48:1193–1201.
- 61 Barone JG, DeLara J, Cummings KB, Ward WS. DNA organization in human spermatozoa. *J Androl* 1994; 15:139–144.
- 62 Evenson DP, Darzynkiewicz Z, Melamed MR. Comparison of human and mouse sperm chromatin structure by flow cytometry. *Chromosoma* 1980; 78:225–238.
- 63 Foresta C, Zorzi M, Rossato M, Varotto A. Sperm nuclear instability and staining with aniline blue: abnormal persistence of histones in spermatozoa in infertile men. *Int J Androl* 1992; 15:330–337.
- 64 Spano M, Cordelli E, Leter G, Lombardo F, Lenzi A, Gandini L. Nuclear chromatin variations in human spermatozoa undergoing swimup and cryopreservation evaluated by the flow cytometric sperm chromatin structure assay. *Mol Hum Reprod* 1999; 5:29–37.
- 65 Kuczyrski W, Dhont M, Grygoruk C, Grochowski D, Wolczyrski S, Szamatowicz M. The outcome of intracytoplasmic injection of fresh and cryopreserved ejaculated spermatozoa – a prospective randomized study. *Hum Reprod* 2001; 16:2109–2113.
- 66 Karabinus DS, Marazzo DP, Stern HJ, Potter DA, Opanga CI, Cole ML, *et al.* The effectiveness of flow cytometric sorting of human sperm (MicroSort ) for influencing a child's sex. *Reprod Biol Endocrinol* 2014; 12:106.