



Assessment of sperm DNA integrity and ICSI outcome after sperm cryopreservation

Page 1 a 14

[Avaliação da integridade do DNA do esperma e resultado da ICSI após a criopreservação do esperma]

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ABSTRACT

The association between suboptimal sperm parameters and DNA damage in mature spermatozoa indicates that some individuals may have intrinsic issues with spermatogenesis. The objective of this study is to evaluate the impact of cryopreservation and reactive oxygen species (ROS) on sperm DNA stability, as assessed by Sperm Chromatin Structure Assay (SCSA) motility and viability, and ICSI Result. Participants contacted the Fertility Clinic and provided written informed consent. All data were subsequently handled and processed in a confidential and anonymous manner. There was a source of 300 participants (600 semen samples) in this study, divided into 3 groups. All female partners underwent ICSI, (some of the oocytes injected by fresh sperms and others injected by cryopreserved sperms for same patient (liquid nitrogen at -196°C, in accordance with conventional cryopreservation protocols). Cryopreservation of spermatozoa from fertile men does not seem to adversely affect sperm DNA integrity in either semen or prepared samples. However, the morphology of spermatozoa in both semen and prepared samples is significantly compromised by the cryopreservation process. No evidence that cryopreserving spermatozoa from healthy males would compromise the genetic material of the sperm either in the semen or in the laboratory-prepared samples.

Keywords: male infertility, DNA fragmentation, oxidative stress

RESUMO

A associação entre parâmetros espermáticos abaixo do ideal e danos ao DNA em espermatozoides maduros indica que alguns indivíduos podem ter problemas intrínsecos com a espermatogênese. O objetivo deste estudo foi avaliar o impacto da criopreservação e das espécies reativas de oxigênio (ROS) na estabilidade do DNA dos espermatozoides, conforme avaliado pelo Sperm Chromatin Structure Assay (SCSA), motilidade e viabilidade e resultado da ICSI. Os participantes entraram em contato com a Clínica de Fertilidade e forneceram consentimento informado por escrito. Todos os dados foram posteriormente tratados e processados de forma confidencial e anônima. O estudo contou com 300 participantes (600 amostras de sêmen), divididos em 3 grupos. Todas as parceiras foram submetidas à ICSI, (alguns dos oócitos injetados por espermatozoides frescos e outros injetados por espermatozoides criopreservados para a mesma paciente (nitrogênio líquido a 196°C, de acordo com os protocolos convencionais de criopreservação). A criopreservação de espermatozoides de homens férteis não parece afetar negativamente a integridade do DNA do esperma em amostras de sêmen ou preparadas. No entanto, a morfologia dos espermatozoides, tanto no sêmen quanto nas amostras preparadas, é significativamente comprometida pelo processo de criopreservação. Não há evidências de que a criopreservação de espermatozoides de homens saudáveis comprometa o material genético do esperma no sêmen ou nas amostras preparadas em laboratório.

Palavras-chave: infertilidade masculina, fragmentação do DNA, estresse oxidativo

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INTRODUCTION

The special interactions between sperm DNA and nuclear proteins result in highly compressed sperm chromatin (predominantly protamine). The replacement of histone by transition proteins and subsequently by protamine occurs during the latter stages of spermatogenesis, when the spermatid nucleus is reshaped and compacted (Soto-Heras *et al.*, 2023; Yap, 2023). While protamine does a good job of enclosing the DNA in sperm chromatin, histones continue to wrap up to 15% of the DNA at particular DNA sequences, indicating a nonrandom link between histones and DNA. This looser packing of histone-bound DNA sequences suggests Cryopreservation of spermatozoa from fertile men does not appear to negatively impact sperm DNA integrity in either semen or prepared samples. However, the morphology of spermatozoa in both semen and prepared samples is significantly impaired by the cryopreservation process (Ribas-Maynou *et al.*, 2022).

The ratio of histones to polyamines in sperm is increased in infertile males compared to fertile controls. It is possible that an excessive amount of nuclear histone leads to less efficient chromatin compaction and more DNA damage (Jut *et al.*, 2024).

Sperm damage throughout the course of spermatogenesis, DNA or its chromatin structure might get damaged at any time. The correlation between low sperm parameters and DNA damage in mature spermatozoa suggests innate spermatogenesis issues in some individuals (Babaei *et al.*, 2024). The correct transfer of genetic information relies on sperm DNA being intact. This evaluation is clinically relevant because of its correlation with birth rates overall, not only those resulting from spontaneous conception.

The Sperm Chromatin Structure Assay (SCSA) has established that a DNA Fragmentation Index (DFI) greater than 30% is associated with unfavorable pregnancy outcomes following Assisted Reproductive Technology (ART), demonstrating not only the efficacy of ART but also the effectiveness of this method. Intracytoplasmic sperm injection (ICSI) can result in live births, even when the sperm have

extensive chromatin damage (Robertson *et al.*, 2024). Although cryopreservation reduces sperm fertility in most animals, assisted reproduction utilizing frozen-thawed semen has practical advantages.

Cryopreservation of sperm is a hotly debated topic in the study of infertility in males since the freezing process can impair several sperm activities, some of which are inaccessible through the conventional semen quality analysis (Wang *et al.*, 2024).

Cryopreservation has recently been linked to research on reactive oxygen species (ROS) generation & DNA damage in sperm. The freezing-thawing process considerably affects intracellular H₂O₂ level & DNA integrity in semen, which may account for the lower fertilization rate and IVF/ICSI outcome observed with frozen-thawed spermatozoa. However, data on the effects of cryopreservation on these factors is limited (Hughes and Silva, 2023, Makris *et al.*, 2023). These measures are thus suggested for use as a supplement in the evaluation of sperm quality following freeze-thaw in semen. This research sought to determine whether sperm DNA stability (as measured by Sperm Chromatin Structure Assay (SCSA)), motility, and viability, and ICSI success were affected by cryopreservation and reactive oxygen species.

MATERIALS AND METHODS

This study involved 300 patients, conducted under the Quality Education Assurance Unit at the Faculty of Medicine, Al-Azhar University, Cairo, Egypt. It aimed to evaluate the effects of cryopreservation and reactive oxygen species (ROS) on sperm DNA stability (using SCSA), motility, viability, and ICSI outcomes. All couples were referred to the Assisted Reproductive Unit and underwent the ICSI cycle between February 2022 and February 2023. Based on sperm parameters, 300 male volunteers were divided into three groups, each with 100 participants. Each group was then separated into two subgroups: fresh and frozen samples.

Group 1 consisted of normal female partners and male partners with oligozoospermia (fresh and frozen).

Group 2: Normal female partners with male partners who have asthenozoospermia (fresh and frozen).

Group 3: Normal female partners with male partners who have teratozoospermia (fresh and frozen).

The study included both fresh semen and cryopreserved samples from the same participants.

Semen analysis is conducted according to WHO guidelines (2021). After 2 to 5 days of sexual abstinence, semen samples are collected through masturbation. The sample is collected in a sterile, clean, and wide-mouthed container to minimize collection errors, and the container must be from a batch proven to be safe for spermatozoa. The semen specimen should be maintained at body or room temperature and analyzed within an hour of collection. The physical and microscopic examination includes assessing the appearance of the ejaculate, liquefaction, viscosity, volume, odor, and pH, as well as recording concentration, motility, and the presence of abnormal forms.

Principle of the Method: The Halo Sperm G2 test is based on the SCD (sperm chromatin dispersion) assay. Intact, unfixed spermatozoa are embedded in an inert agarose microgel on a prepared slide. For sperm cells with fragmented DNA, an initial acid treatment denatures the DNA. Following this, a lysing solution removes most nuclear proteins, resulting in nucleoids with extensive halos of spread DNA loops emerging from a central core in sperm with minimal DNA damage. In contrast, sperm with fragmented DNA will exhibit either no halo or a barely discernible halo.

A minimum of 300 spermatozoa per sample are scored according to the following criteria:

Narrow Halo: The halo width is equal to or less than one-third of the minor core diameter.

Halo-Free Sperm: Absence of a halo.

Degraded Sperm: Sperm lacking a halo, with an irregular or faintly pigmented center.

Large Halo: Sperm exhibiting a halo with a width equal to or greater than the minor core diameter.

Medium-Sized Halo: Sperm with a halo that is intermediate in size, falling between large and very small halos.

Cryopreservation of spermatozoa: Dropwise addition of Sperm freezes™ cryoprotectant (FertiPro NV, Sint-Martens Latem, Belgium) was made to cryo-vials (Nalge Company, Rochester, NY, USA) containing spermatozoa, with gentle spinning to distribute the cryoprotectant evenly. Before usage, sperm freeze™ was brought to room temperature from its storage temperature of 4 degrees Celsius. Following a 10-minute chilling period at ambient temperature, the mixture was frozen using Stationary phase vapor-based cooling. Vapor from Cryogenic nitrogen (-80°C) was used to suspend samples for storage according to Tamburrino *et al.*, 2023 method as about ten centimeters must increase the liquid nitrogen level for 15 minutes. Afterwards, liquid nitrogen was used to freeze the samples (around -196°C) for later use (Tamburrino *et al.*, 2023).

To avoid the spermatozoa in the cryovials from bursting, the lids were unfastened, and the liquid nitrogen was drained from the containers. For around 15 to 20 minutes, the samples were permitted to defrost at ambient temperature, and are typically thawed at a 37°C water bath. This temperature matches body temperature and helps restore the sperm's motility without causing thermal shock. When the samples thawed completely, a comparable amount of each cryovial received BWB (Biggers-Whitten-Whittingham) buffer, followed by centrifugation of the cells. at two hundred g for six minutes to get rid of any remaining residues of the Sperm freeze™ cryoprotectant. The pellet was centrifuged and then re-suspended in BWB (~400µl) at a concentration proportional to the number of spermatozoa recovered (Zwamel, 2023; Aksu *et al.*, 2024).

Handling of sperm specimens were treated for ICSI by adding one ml of sperm gradient medium to a new sample, applying a centrifugation speed of 1800rpm for ten minutes discarding the supernatant, followed by adding two milliliters in the sperm washing process to the resultant pellet & centrifuging again. This resulted in the required quantity of motile & anatomically functional sperm cells for assisted reproductive techniques (WHO, 2023).

All female partner undergoes ICSI, (some of the oocytes injected by fresh sperms and others injected by cryopreserved sperms (were preserved in liquid nitrogen at -196°C using conventional cryopreservation methods).

Oocytes were retrieved using transvaginal ultrasonography (US) with general anesthesia administered 34-36 hrs. After HCG hormone (human chorionic gonadotropin injection, using the Labotect aspiration pump from Germany, a single-lumen, 17-gauge oocyte pick-up needle was employed to Collect follicular fluid 115–120 mm Hg of negative pressure (Reproline Medical, Germany). Suction of 14 cc of follicular fluid Transferred into sterile tubes (Falcon, Healthcare Co., China), with isolated oocyte-cumulus cell complexes being treated with Gamete Buffer media (Cook, Limerick, Ireland), put in a set of four plates with fertilization medium (Cook, Limerick, Ireland), and then incubated for thirty minutes at 37°C in 6% carbon dioxide while being viewed through a dissecting microscope (Zeiss Stemi 2000-C Stereo Microscope, C60 Labotect, Germany).

Using a dissecting microscope (Zeiss Stemi 2000-C Stereo Microscope), oocyte-cumulus cell complexes were extracted, washed in Gamete Buffer media (Cook, Limerick, Ireland), placed in four dishes holding fertilization medium (Cook, Limerick, Ireland) and maintained at 37°C during incubation in six percent carbon dioxide for approximately half an hour (C60, Labotect, Germany). Oocytes were denuded by soaking them for 30–45 seconds in a solution containing 80 IU/ml of hyaluronidase enzyme (Life Global, Europe), then placing them in a solution of Gamete Buffer medium and removing the corona cells using a sterile drawn pipette.

After denudation, the oocyte was inserted ten microliter aliquots of fertilization medium (Cook, Limerick, Ireland) in injection plates, covered with 3ml of sterile, equilibrated mineral oil. Quick evaluations of oocyte maturity and quality were made utilizing an enverted microscope incorporating Hoffman optics (Olympus 1x71, Japan) a hot stage, & automated manipulators for oocyte grading (Narishige, Japan). Prophase I, Metaphase I (MI), Metaphase II (MII), & Post-Maturation were identified as the maturation phases. Before the ICSI process, the oocytes were incubated in a culture medium

at 37 degrees Celsius with six percent carbon dioxide (WHO, 2023; Govahi *et al.*, 2024).

The samples were processed then maintained in an incubated state until the injection subsequent to evaluation and preparation of the sperm from the semen in the manner as detailed earlier. A single sperm cell with normal morphology was injected into each oocyte while immobilized in polyvinylpyrrolidone (PVP) (Irvine, USA). Sperm samples collected during ICSI procedures were analyzed individually. Aseptic technique necessitated the use of a holding pipette and an injection needle, both of which were used to administer the injection into the sterilized dish. According to Van Steirteghem method, sperm was injected into the intracytoplasmic ally. After injection, oocytes were washed and introduced into Global Total Medium (Life Global, Europe), where they remained until fertilization at 37 degrees Celsius, 6% carbon dioxide, and 90-95% humidity (Thompson *et al.*, 2024 and Ozturk *et al.*, 2020).

Evaluation occurred $17\pm$ hours after the microinjection procedure. Oocytes that had been injected were checked for pronuclei and other signs of injury. Oocytes were considered fertilized if they had 2 pronuclei (2PN) and the 2nd polar body had been extruded (Suzuki *et al.*, 2024). A total of 72 hours' post-injection, each embryo was graded and transferred based on its cell quantity and shape, specifically its blastomeric size and fraction of nucleate fragments. Following oocyte retrieval, the best embryos from day 3 were transferred to the uterus 48-72 hours later in $30\mu\text{l}$ of Global medium (Life Global, Europe), which includes ten percent human serum albumin (HSA), was applied for embryo transfer (ET) catheter (Labotect, Germany) according to Li and Gao 2024. If embryo transfer was delayed until day 5, embryo quality and grade are checked prior to E.T (Bartolacci *et al.*, 2024). Transfers of embryos on days 3 and 5 were performed using protocols established by the American Society for Reproduction. Cryopreserving the surplus of healthy embryos was a need. After 6-7 weeks of amenorrhea, A transvaginal ultrasound scan was conducted to evaluate whether a clinical pregnancy had been developed, and a serum - HCG determination was performed as a chemical pregnancy test (deemed positive at 20 IU/L), with the presence of an intrauterine gestational sac. (Munoz *et al.*, 2024).

Statistical analysis software was employed to analyze the data. To compare the analyzed parameters before and after therapy in each study group, the paired t-test and McNemar's test were applied (Borges *et al.*, 2024).

RESULTS

No substantial difference among the groups for male age, male body mass index (BMI), female age, female BMI, or duration of infertility, Table 1.

Table 1. General characteristics of male studied patients (n=300)

Parameters	Oligospermia (N=100)	Asthenospermia (N=100)	Teratospermia (N=100)	P value
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD	
Male age /Year	32.10 $\pm$ 2.3	31.10 $\pm$ 2.1	33.10 $\pm$ 2.0	> 0.05
BMI	24.76 $\pm$ 2.0	23.76 $\pm$ 3.0	24.90 $\pm$ 2.0	> 0.05
Female age /Year	29.90 $\pm$ 2.0	30.10 $\pm$ 2.1	30.00 $\pm$ 1.9	> 0.05
BMI (Kg/m ²)	22.96 $\pm$ 2.8	23.76 $\pm$ 3.0	23.90 $\pm$ 2.6	> 0.05
Infertility years	4.06 $\pm$ 1.6	4.16 $\pm$ 1.6	4.96 $\pm$ 1.5	> 0.05

Values are presented as Mean $\pm$ SD and T-test and McNemar's test were applied

(***) $P \leq 0.001$ indicates high significance (**); $P \leq 0.01$ denotes very significant (*); $P \leq 0.05$ is considered significant (*); $P > 0.05$ is not significant.

There were significant differences between the Freeze-thawed semen group compared to the Fresh semen group regarding Sperm count ($P \leq 0.001$), sperm motility, sperm Progressive motility ($P = 0.001$) & DNA fragmentation index ($P \leq 0.001$), however, no significant differences amongst the Freeze-thawed semen group compared to the Fresh semen group regarding Abnormal forms ($P = 0.0052$), Head defects ($P > 1.0000$), sperm Midpiece defects ($P = 0.0109$) & sperm tail defects ($P = 0.7908$), Shown as Table 2, Figure 1 and 2

There were significant differences between the Freeze-thawed semen group compared to the Fresh semen group regarding Blastocyst formation rate on Day, however, no significant differences between the Freeze-thawed semen group compared to the Fresh semen group regarding Total Collected oocytes number ($P = 0.9305$), Mature oocytes ($P < 0.0001$), Fertilization rate on day 1 ($P = 0.0839$), Cleavage rate on Day 3 ($P = 0.0083$), Grade A embryos ($P = 0.1661$), Grade B embryos ($P = 0.6959$) and Grade C embryos ($P = 0.5365$). Table 3

Table 2. Semen parameters of Oligozoospermia Studied patients (n=100)

Parameters	Fresh semen	Freeze-thawed semen	P value
	Mean $\pm$ SD	Mean $\pm$ SD	
Sperm count ($\times 10^6$ /ml)	8.60 $\pm$ 2.1	7.30 $\pm$ 1.9	$\leq 0.001^*$
Sperm motility/ml	51.30 $\pm$ 2.1	44.20 $\pm$ 2.0	$< 0.001^*$
Progressive motility/ml	23.60 $\pm$ 1.5	20.60 $\pm$ 1.2	$< 0.001^*$
Abnormal forms	93.40 $\pm$ 1.2	93.90 $\pm$ 1.3	0.0052
Head defects	80.40 $\pm$ 3.8	80.40 $\pm$ 4.2	1.0000
Midpiece defects	40.30 $\pm$ 2.8	41.30 $\pm$ 2.7	0.0109
Tail defects	20.60 $\pm$ 2.4	20.70 $\pm$ 2.9	0.7908
DNA fragmentation	25.30 $\pm$ 3.0	27.20 $\pm$ 2.0	$< 0.001^*$

Values are presented as Mean $\pm$ SD and t-test and McNemar's test were applied

(***) highly significant $P \leq 0.001$, (**) very significant $P \leq 0.01$, (*) significant $P \leq 0.05$ (*), not significant $P > 0.05$

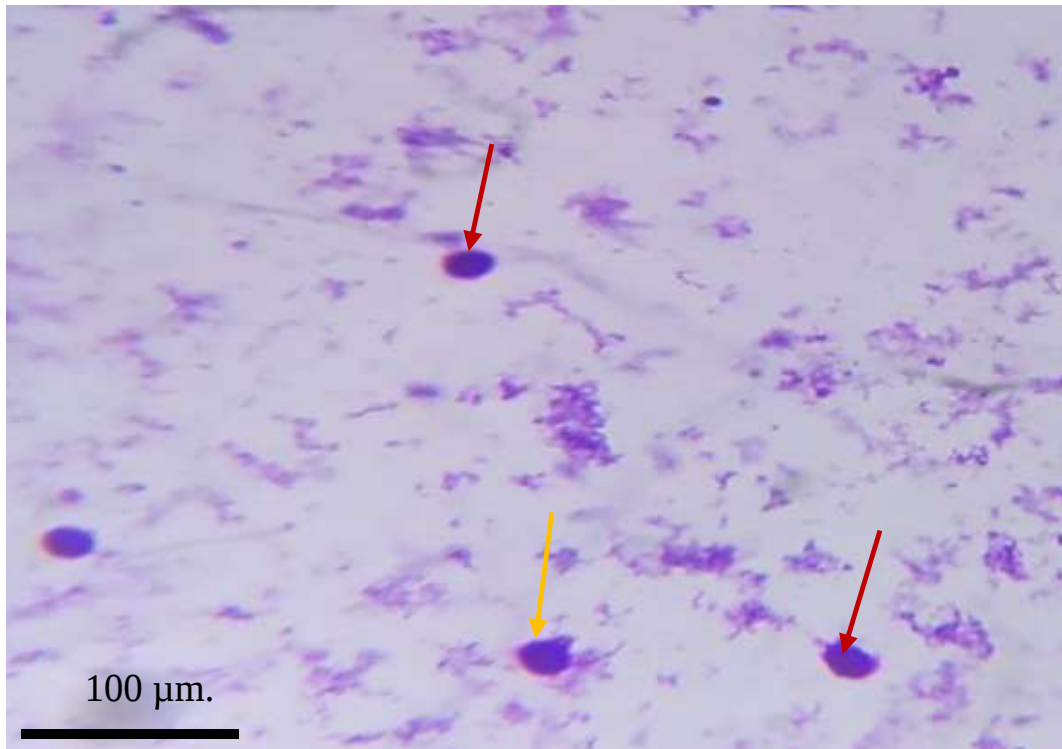


Figure 1. The light photomicrograph for fresh semen sample in Oligozoospermia group shows sperm with fragmented DNA (red arrow), sperm without fragmented DNA (yellow arrow), (Magnification x 40) stained with halo sperm G2 kit (Halotech DNA).

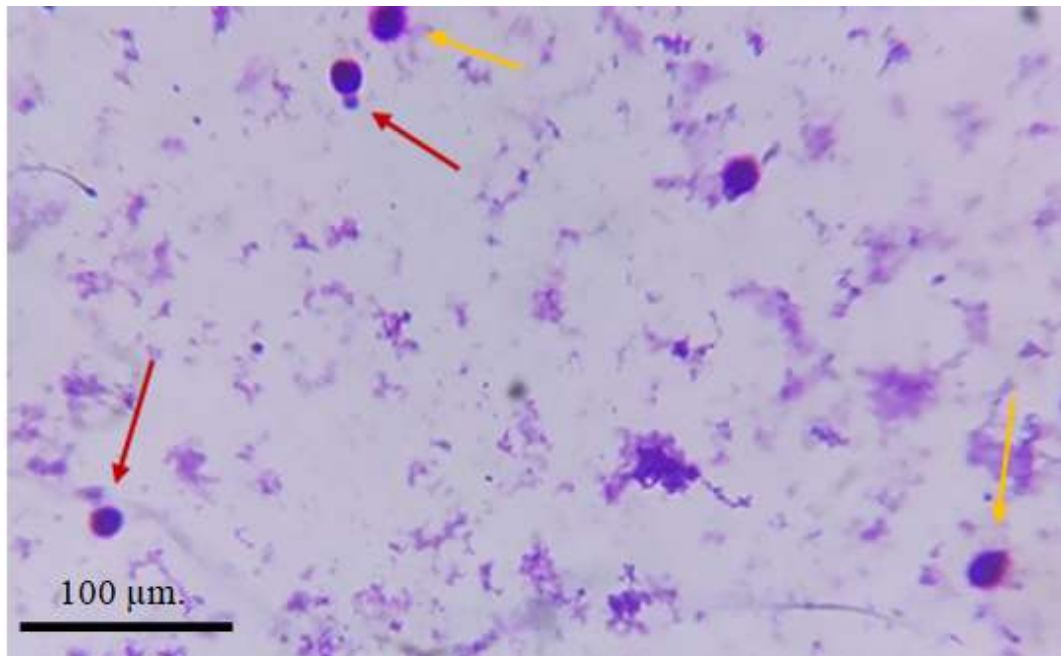


Figure 2. The light photomicrograph for cryopreserved semen sample in Oligozoospermia group shows sperm with fragmented DNA (red arrow), sperm without fragmented DNA (yellow arrow), (Magnification x 40) stained with halo sperm G2 kit (Halotech DNA).

Table 3. ICSI outcome among Oligozoospermia Studied patients (n=100)

Parameters	Fresh semen	Freeze-thawed semen	P value
	%	%	
Total Collected number (n=1318)	660.0 (50.07%)	658.0 (49.9%)	0.9305
Mature oocytes (n=1100)	551.0 (83.4%)	549 (90.2%)	< 0.0001
Fertilized oocytes (n=880)	450 (81.6%)	430 (78.3%)	0.0839
Fertilization rate on Day 1 (n=880)	450 (81.6%)	430 (78.3%)	0.0839
Cleavage rate on Day 3 (n=816)	425 (94.4%)	391 (91.0%)	0.0083
Blastocyst rate on Day 5 (n=640)	358 (84.3%)	282 (72.1%)	≤ 0.001*
Grade A embryos (N =363)	211 (59.0%)	152 (53.9%)	0.1661
Grade B embryos (N =207)	113 (31.5%)	94 (33.3%)	0.6959
Grade C embryos (N =70)	34 (9.5%)	36 (12.8%)	0.5365

Values are presented as Mean±SD and t-test and McNemar's test were applied

(***) highly significant $P \leq 0.001$, (**) very significant $P \leq 0.01$, (*) significant $P \leq 0.05$ (*), not significant $P > 0.05$

There were significant differences between the Freeze-thawed semen group compared to the Fresh semen group regarding Sperm count ($P \leq 0.0001$), sperm motility ($P \leq 0.0001$), & sperm Progressive motility ($P \leq 0.0001$), however, no significant differences between the Freeze-thawed semen group compared to the Fresh semen group regarding Abnormal forms ($P=0.1178$), Head defects ($P=0.0575$), sperm Midpiece defects ($P=0.0061$) sperm tail defects ($P=0.7427$) & DNA fragmentation ($P=0.8209$, shown Table 4, Fig. 3.

There were significant differences between the Freeze-thawed semen group compared to the Fresh semen group regarding Cleavage rate on Day 3 & Grade A embryos. However, no significant differences between the Freeze-thawed semen group compared to the Fresh

semen group regarding Total Collected oocytes number ($P=1.000$), Mature oocytes ($P=0.0766$), Fertilization rate on day 1 ($P=0.7378$), Blastocyst formation rate on Day 5 ($P=0.0271$), Grade B embryos ($P=0.2260$) & Grade C embryos ($P=0.0756$), Table 5.

There were significant differences between the Freeze-thawed semen group compared to the Fresh semen group regarding sperm motility, sperm Progressive motility, Abnormal forms ($P < 0.0001$), and DNA fragmentation index ($P \leq 0.0001$), however, no significant differences between the Freeze-thawed semen group compared to the Fresh semen group regarding Sperm count ($P=0.8078$), sperm Head defects ($P=0.9023$), sperm Midpiece defects ($P=0.3032$) & sperm tail defects ($P=0.7639$) shown in Table 6, Figures 4, 5.

Table 4. Semen parameters of Asthenozoospermia Studied patients (n=100)

Parameters	Fresh semen	Freeze-thawed semen	P value
	Mean ± SD	Mean ± SD	
Sperm count ($\times 10^6$ /ml)	17.60 ± 3.1	12.60 ± 2.8	< 0.0001*
Sperm motility/ml	21.20 ± 1.1	18.20 ± 1.0	< 0.0001*
Progressive motility/ml	11.60 ± 1.3	8.60 ± 1.2	< 0.0001*
Abnormal forms	94.40 ± 2.2	94.90 ± 2.3	0.1178
Head defects	78.40 ± 3.8	79.40 ± 3.6	0.0575
Midpiece defects	30.30 ± 2.5	31.30 ± 2.6	0.0061
Tail defects	18.70 ± 2.1	18.80 ± 2.2	0.7427
DNA fragmentation	21.30 ± 3.0	21.21 ± 2.6	0.8209

Values are presented as Mean±SD and t-test and McNemar's test were applied

(***) highly significant $P \leq 0.001$, (**) very significant $P \leq 0.01$, (*) significant $P \leq 0.05$ (*), not significant $P > 0.05$

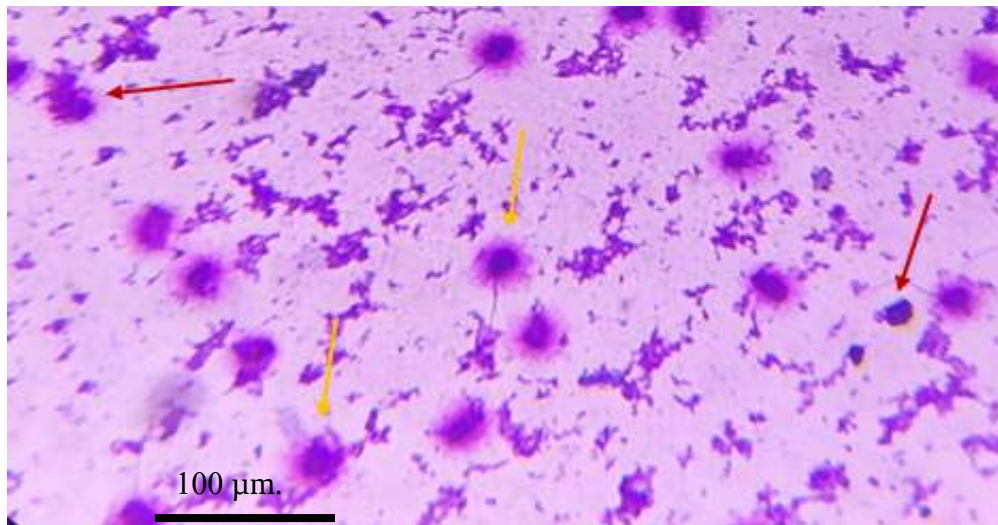


Figure 3. The light photomicrograph for cryopreserved semen sample in Asthenozoospermia group shows sperm with fragmented DNA (red arrow), sperm without fragmented DNA (yellow arrow), (Magnification x 40) stained with halo sperm G2 kit (Halotech DNA).

Table 5. ICSI outcome among Asthenozoospermia Studied patients (n=100)

Parameters	Fresh semen	Freeze-thawed semen	P value
	%	%	
Total Collected number (n=1270)	635.0 (50.0%)	635.0 (50.0%)	1.000
Mature oocytes (n=1239)	623 (98.1%)	616 (97.0%)	0.0766
Fertilized oocytes (n=993)	501 (80.4%)	492 (79.8%)	0.7378
Fertilization rate on Day 1 (n=933)	501 (80.4%)	492 (79.8%)	0.7378
Cleavage rate on Day 3 (n=899)	465 (92.8%)	434 (88.2%)	0.001*
Blastocyst rate on Day 5 (n=820)	431 (92.7%)	389 (89.6%)	0.0271
Grade A embryos (N =507)	294 (68.2%)	213 (54.7%)	< 0.001*
Grade B embryos (N =227)	109 (25.3%)	108 (30.4%)	0.2260
Grade C embryos (N =86)	28 (6.5%)	58 (14.9 %)	0.0756

Values are presented as Mean±SD and t-test and McNemar's test were applied

(***) highly significant $P \leq 0.001$, (**) very significant $P \leq 0.01$, (*) significant $P \leq 0.05$ (*), not significant $P > 0.05$

Table 6. Semen parameters of Teratozoospermia Studied patients (n=100):

Parameters	Fresh semen	Freeze-thawed semen	P value
	Mean ± SD	Mean ± SD	
Sperm count ($\times 10^6$ /ml)	18.12 ± 4.1	17.92 ± 4.1	0.8078
Sperm motility/ml	53.31 ± 5.8	40.20 ± 5.4	< 0.0001*
Progressive motility/ml	21.18 ± 1.3	15.30 ± 1.1	< 0.0001*
Abnormal forms	99.60 ± 0.4	99.80 ± 0.2	< 0.0001*
Head defects	70.40 ± 5.8	70.50 ± 5.7	0.9023
Midpiece defects	15.30 ± 6.8	16.30 ± 6.9	0.3032
Tail defects	10.70 ± 2.3	10.80 ± 2.4	0.7639
DNA fragmentation	45.30 ± 3.0	50.20 ± 3.3	< 0.0001*

Values are presented as Mean±SD and t-test and McNemar's test were applied

(***) highly significant $P \leq 0.001$, (**) very significant $P \leq 0.01$, (*) significant $P \leq 0.05$ (*), not significant $P > 0.05$

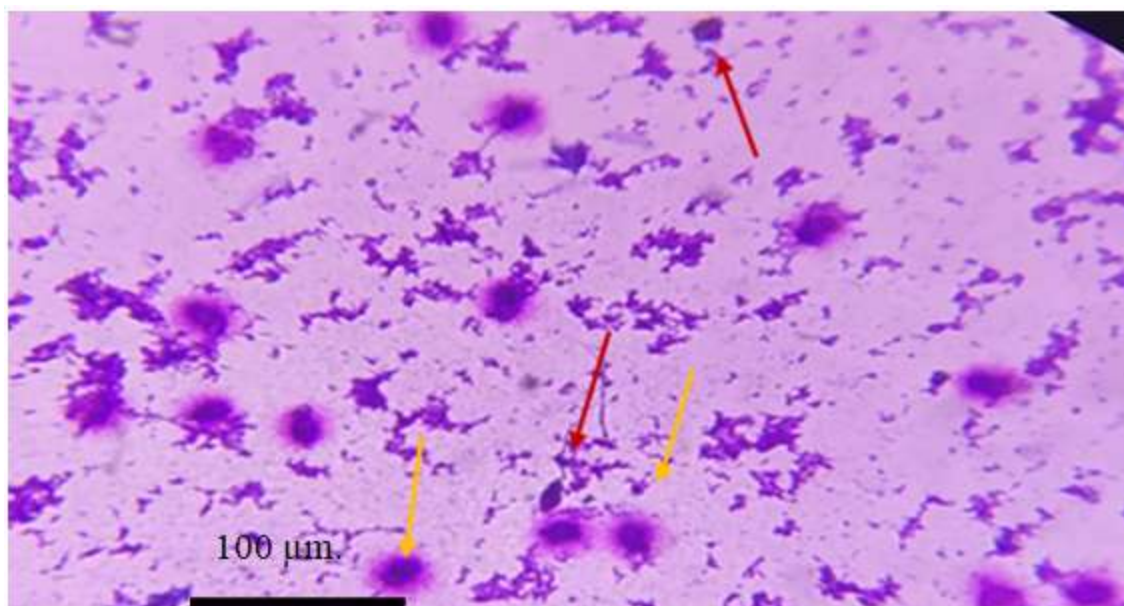


Figure 4. The light photomicrograph for fresh semen sample in teratozoospermia group shows sperm with fragmented DNA (red arrow), sperm without fragmented DNA (yellow arrow), (Magnification x 40) stained with halo sperm G2 kit (Halotech DNA).

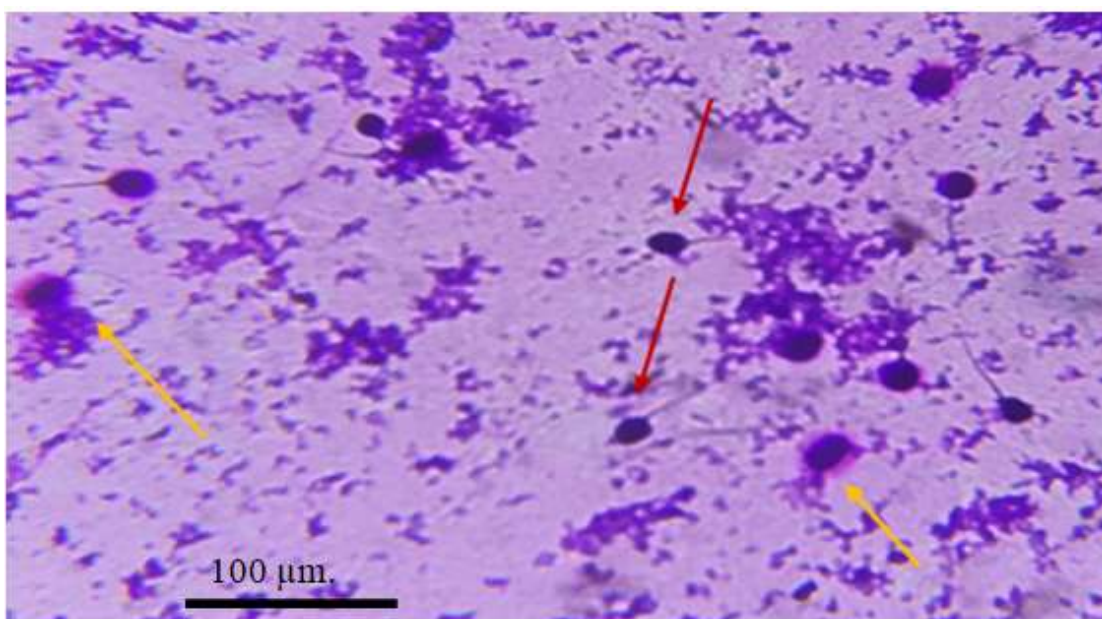


Figure 5. The light photomicrograph for cryopreserved semen sample in teratozoospermia group shows sperm with fragmented DNA (red arrow), sperm without fragmented DNA (yellow arrow), (Magnification x 40) stained with halo sperm G2 kit (Halotech DNA).

There were significant differences between the Freeze-thawed semen group compared to the Fresh semen group regarding Grade A embryos and Grade B embryos ($P < 0.001$), however, no significant differences amongst the Freeze-thawed semen group compared to the Total

Collected oocytes number ($P = 0.3515$), Mature oocytes ($P = 0.1971$), Fertilization rate on day 1 ($P = 0.0037$), Cleavage rate on Day 3 ($P = 0.0063$), Blastocyst rate on Day 5 ($P = 0.7852$), and Grade C embryos ($P = 0.0570$), Table 7.

Table 7. Oocytes outcome among the Teratozoospermia group

Parameters	Fresh semen	Freeze-thawed semen	P value
	%	%	
Total Collected number (n=1502)	759.0 (50.5%)	743.0 (48.8%)	0.3515
Mature oocytes (n=1307)	654 (86.1%)	653 (87.8%)	0.1971
Fertilized oocytes (n=989)	513 (78.5 %)	476 (72.9%)	0.0037
Fertilization rate on Day 1 (n=989)	513 (78.5 %)	476 (72.9%)	0.0037
Cleavage rate on Day 3 (n=913)	480 (93.6%)	433 (90.1%)	0.0063
Blastocyst rate on Day 5 (n=711)	375 (78.2%)	336 (77.6%)	0.7852
Grade A embryos (N =295)	196 (66.4%)	99 (33.6%)	< 0.001 *
Grade B embryos (N =219)	90 (41.1%)	129 (58.9%)	< 0.001 *
Grade C embryos (N =197)	89 (45.2%)	108 (54.8%)	0.0570

(***) highly significant $P \leq 0.001$, (**) very significant $P \leq 0.01$, (*) significant $P \leq 0.05$ (*), not significant $P > 0.05$

DISCUSSION

The ability of human spermatozoa to bind to an extract of chicken perivitelline membrane coated on micro-well plates decreases following cryopreservation (Nazari *et al.*, 2022). Moreover, the average speed of increasingly motile spermatozoa decreases by about 30% after cryopreservation in both fertile and infertile patients, with a more significant reduction observed in infertile males compared to fertile donors (Mangoli *et al.*, 2024).

Cryopreservation damage to OATS-containing semen may negatively impact fertilization and embryo quality, but pregnancy rates are likely to stay consistent. Cellular damage during freezing is often attributed to membrane rupture from intracellular ice crystals during rapid cooling, osmotic effects, or mechanical force from extracellular ice during gradual cooling (Ekpo *et al.*, 2022 and Nel-Themaat, 2024). After seven days of storage, specimens held at -70°C experienced a greater decrease in motility compared to those stored at -196°C , with the difference becoming more pronounced after three months. However, no differences in sperm morphology were noted between the two groups. (Dzickońska *et al.*, 2022; Bondarenko *et al.*, 2024).

Spermatozoa from infertile males are more susceptible to damage from cold temperatures compared to those from fertile men. Freeze-thawing has been shown to significantly impair sperm chromatin, morphology, and membrane

integrity in both fertile and infertile males. (Anagnostopoulou *et al.*, 2024).

The infertile group exhibited significantly greater chromatin condensation compared to the fertile group (Hekim *et al.*, 2024).

Leukemia patients, both motile sperm count and curvilinear velocity are significantly lower before and after freezing and thawing compared to healthy donors (Todorovic *et al.*, 2022 and Zhang *et al.*, 2024). Another study found that viable spermatozoa have much lower chromatin resistance to heat denaturation, and a higher incidence of abnormal chromatin structure compared to infertile spermatozoa (Hallam *et al.*, 2024).

This suggests a connection between variations in chromatin condensation and childbirth. Increased DNA instability and susceptibility to denaturing stress have been associated with chromatin structure defects in infertile males (Balder *et al.*, 2024). Other studies have shown that the compactness of mammalian spermatozoa nuclei changes after freezing and thawing (Baity *et al.*, 2024).

This could explain why insemination with frozen-thawed semen often results in lower pregnancy rates or why sperm motility may improve after thawing, but pregnancy rates remain low. It also seems that DNA in sperm from men with reproductive issues is more vulnerable to damage from cold temperatures, with a higher proportion of DNA-fragmented

spermatozoa observed in the semen of infertile men compared to fertile men (Badr 2024, Pezo *et al.*, 2024).

Additionally, there is an inverse relationship between the proportion of fragmented DNA in spermatozoa and fertilization rates in both in vitro fertilization and in vivo chorionic syncytial embryo transfer. (Liu *et al.*, 2023, Marinaro and Schlegel (2023). It is also recognized that a considerable percentage of men with a sperm evaluation that might be deemed typical by WHO standards, however who have been diagnosed with unexplained infertility, have DNA disruptions in the strands (Kandil *et al.*, 2024).

Mutagenic events are closely linked with sperm DNA damage. Nevertheless, spermatozoa with compromised genetic material are still able to fertilize, and mutations and abnormalities may not show until the embryo has split or the fetus has developed (Cheung *et al.*, 2023).

DNA strand breaks result in chromosomal damage, and most sperm-derived genetic mutations are caused by chromosomal breakage as opposed to chromosomal rearrangement as in the oocyte (Akhavizadegan *et al.*, 2025). Also, research on genetic disorders caused by germline mutations has revealed a preponderance toward male rather than maternal inheritance (Xu *et al.*, 2023).

The morphology of the sperm, as measured by the Tygerberg criteria, is widely recognized as a crucial element in assessing the success of an in vitro fertilization (IVF) cycle (Jia *et al.*, 2024).

IVF fertilization rates are known to be lower in cases when sperm tail abnormalities are present. To maximize success rates with ICSI, only spermatozoa with normal-shaped, completely compacted nuclei should be utilized (Robinson, *et al.*, 2023).

Fertilization rates and the likelihood of a healthy pregnancy are both lower in sperm with significant sperm head abnormalities (Zenoaga and Martinez, 2024). A possible explanation for why infertile men's semen may withstand freezing damage is that it contains defensive elements found in seminal plasma. Superoxide dismutase (SOD) and catalase, found in

abundance in seminal plasma, detoxify the body by neutralizing harmful reactive oxygen species such as hydroxyl radicals and

CONCLUSIONS

DNA damage does not appear to occur during the cryopreservation of spermatozoa from fertile males, whether in fresh or cryopreserved samples. However, cryopreservation significantly alters the morphology of spermatozoa in both cases. Preserving DNA integrity is essential for applications such as using frozen-thawed donor sperm for IVF or ICSI, as well as for long-term storage prior to treatments like chemotherapy or radiation therapy.

In contrast, spermatozoa from infertile men experience severe DNA integrity compromise and significant morphological deterioration in both semen and prepared samples following freezing. Further research is necessary to identify optimal methods for preserving DNA integrity in sperm samples from infertile men before their use in IVF or ICSI.

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