Toxicology Reports 6 (2019) 889–896

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Improvements in morphology and membrane stability obtained from *TPP-TAB*, a cryopreservation medium treated infertile smoker sperm cells – An *in vitro* study

Parameswari Ranganathan (M.Sc PhD), T.B. Sridharan*

Department of Industrial Biotechnology, School of Biosciences and Technology, VIT University, Vellore, India

ARTICLE INFO

Keywords:
Sperm stability
E4 medium
ROS
Cryo injury
Tea poly phenol
Terminaliaarjuna
Glycerol

ABSTRACT

Background: Plants are the major sources of antioxidants, which maintains oxidant: antioxidant state or is to protect from excessive reactive oxygen species in case of cryo medium selection though maintain sperm stability.
Bottleneck: Cryo preservation of infertile smokers is challengeable with the available medium due to over production of ROS hitches, were cause loss of sperm physiology. Hence, a novel medium is needed to store/protect sperm cells of infertile smokers where they attending/ongoing IVF or oncogenic surgical treatment.
Aim: The aim of this study, is to check the stability of sperm cells by TPP-*T.arjuna* bark (*TAB*) (E4) cryo medium preserved infertile smoker's against ROS/cryo injury- as a continuous study.
Materials and methods: 42 infertile smoker's subjects with 28 control subjects were selected. Surface morphology (acrosome) of sperm by scanning electronic microscope, sperm membrane proteins by colorimetric method, sperm head and tail defects by CASA method and finally sperm cell stability is checking its zeta electric potential charges, were all done with E4 cryo medium treated frozen/thawed selected study subjects.
Results: Sperm morphology and zeta potential shows there is no damage along the stability of cells maintained during E4 medium cryopreservation in infertile subjects.
Summary: This is the first study is too established for infertile smokers sperm stability was checked for six months with E4 cryo medium.

1. Introduction

Cryo preservation, a form of storage procedure which help to preserves cell (sperm cell, culture cell and stem cell) for future determinations and cryo medium is type of safeguarding medium which helps to preserve as well as protect the physiology and biology of cells from environmental and heat shock substances under sub-zero condition (-196 °C) [1]. In the socioeconomic world, male infertility a noticeable health disorder. In addition their lifestyle (includes sedentary life style, junk food habits, smoking, alcoholic, drugs etc.,) occupation exposure to electromagnetic waves/rays, environment factors, (exposure to relatively more temperature, pollution) and genetic factors. As per our earlier report lifestyle and occupation factors are giving more chances to be infertile when compare to genetic factors [2]. Smoking, one of leading life style habit, and infertile smoker's sperm cell preservation is challengeable so far [3]. Glycerol, is one of the medium were used for all type species sperm cell storage. Also, researches have indicated that, diverse nature of ingredients (which includes DMSO, ethylene glycol, vit- E, taurine, zinc, aspartic acids, glutamic acid, EDTA, fructose, trehalose and antibiotics like penicillin) with different set of experiments (either *in vivo* or *in vitro*) were conducted and compared with or without glycerol medium [4]. In exhaustive, the compounds with different time and different selected dosage of combinations were designed as a preservatives and reported in various *in vitro* studies [5], still glycerol only opted in sperm cryopreservation as per expertized reports; glycerol maintains temperature limits by adjusting its electrolyte during freezing. In case of, higher pH in glycerol medium, it allows for risk of microbial contamination and it was overcome by employing light acidic pH (6.8 or 6.9) which in turn to natural vaginal pH [6]. Acidic nature of female tract protects the sperm from infection [7]. But, our previous report stated that, during cryo preservation glycerol exchanges lipid bilayer due to continuous heat shock substance and creates insecurity between sperm cell as well as its membrane which results in sperm cell death also it was compared as well as confirmed with experiment listed cryo mediums. [8]. Another, medium called Trehalose, one of the non-porous disaccharide compound preserves sperm cells through its high

* Corresponding author.
E-mail addresses: eashvit@gmail.com (R. Parameswari), tbsridharan@vit.ac.in (T.B. Sridharan).

https://doi.org/10.1016/j.toxrep.2019.08.015
Received 18 January 2018; Received in revised form 21 August 2019; Accepted 23 August 2019
Available online 24 August 2019

viscous nature and inhibit ice crystal growth formation by its kinetic nature [9]. Freeze/thaw process of sperm will reduce the live cells proportion due to lipid peroxidation (LPO), resulting in loss of sperm cell. Generally freeze/thaw process reduces the motility, count and integrity of selected species sperm biology [10]. As an alternative of cryo medium, *vitrification,* one of the recent advanced technique which is reported for defending embryos; meantime it cannot be opted for sperm cell storage due to temperature maintenance failure [11]. Temperature, is one of major determining factor which plays a significant role in sustaining the structure of the sperm cells throughout cryopreservation process [12]. Recent reports demonstrated that with antioxidants (taurine, glutathione, vitamin C&E, catalase) and amino acids (valine, histidine, proline and glycine) supplementation, can overcome damages of sperm in human, bull and different fish species [5,10–12]. Many protocols with different medium composition are available with few restrictions and drawbacks like cryo injury, cell damage and temperature maintenance. During freeze/thaw process, reactive oxygen species (ROS) will be produced more and further its function has to reduce antioxidants, finally unevenness between free radicals and antioxidants status in sperm cells, results in sperm membrane damage or sperm DNA damage. However, endogenous antioxidants are not enough to protect homeostasis in case of oxidative stress; in this manner supplementation of antioxidant is needed to protect sperm cells.

In order to overcome those practical difficulties, antioxidants from natural sources like medicinal plants can be used as a cryo guard [13]. Recent novel studies have stated that therapeutic plants are chief sources of antioxidants, with various physiological activities, that are non-toxic, eco-friendly and low cost.

In India, more than 45, 000 medicinal plants are available. But, 3000 plants only were officially documented as medicinal plants in treatments. At the same time more than 6000 plants were used by traditional practitioners without documentation as per *AYUSH*, government of India (GOI) reports. Therapeutic plants are natural scavengers and comprise of flavonoids, carotenoids, vitamin C (spermatogenesis) and E (steroidogenesis), poly phenols, follow components like Zn (sperm motility and morphology) and act as hunters against ROS which is produced from free/thaw process. In this aspect, a plant species named, *Terminalia arjuna (T.arjuna)* belonging to *Combretaceae family* and reported as natural antioxidant properties with sperm DNA damage inhibition activity present in *T.arjuna* bark which is evidenced by our recent Parameswari et al., (2017) also our study reported, *Terminalia arjuna* bark (TAB) *is* reported as metal (Zn, Se and Ca) rich nutrient which is also helpful in scavenging function, as an additional source where infertile patients from smoking habit (smoke majorly affects zinc production from prostate secretion) [14]. Instead of chemical substance, above said properties are the basis for our earlier study [8] and the cryo medium was prepared from naturally available plants bark (T.*arjuna* bark) and leaf (tea poly phenol), meanwhile it was also compared with commercial (glycerol) extender. The present study aims to continue as well as to check the stability of TPP-*T. arjuna* bark (TAB) extender (E4 medium) on sperm morphology, motility and sperm membrane stability against ROS/cell damage as a continuous study in the end of 6th month.

2. Materials and methods

2.1. Ethics and study subjects

This study is part of major research work and it was approved by institutional review board of VIT University, with Ref. No.VIT/UHEC-3/NO.11; based on which informed consent form was obtained and maintained confidentially.

2.2. Subject selection

Male infertile partners (Aged 27–-39 years) with fertile female partners were only targeted for the study. In the current study, subjects who have had a history of smoking for at least 8 -11years (before enrolment in the study) were categorized based on smoking index.

Smoking Index (SI): The number of cigarettes smoked/ day × Number of smoking years [15]

Also subjects who have habits like drug abuse, regular junk food eaters are all included in the modernized life style factors for negative control. Also, idiopathic reasoned infertile subjects are also selected for negative control group.

2.3. Initial semen analysis

Semen samples were collected from International Institute for Training and Research in Reproductive Health - Bangalore Assisted Conception Centre (IIRRH-BACC), Bangalore, Karnataka, India. After 3–5 days of abstinence the semen samples were collected through masturbation using a clean sterile, wide-mouthed container and confirmed as non-toxic. After collection the samples were labelled and allowed to liquefaction for 30 min. at ambient temperature. Once liquefied, the samples were analyzed for semen quality (sperm concentration, morphology, total and progressive motility) by Computer Assisted Semen Analysis (CASA), according to WHO 2010 guidelines [16].

2.4. Sperm preservation analysis

Our previous study [8] addresses the composition and types of extender are listed in Table 1. Briefly E1- normal glycerol extender, E2- is citrate egg yolk extender. E3 (*T. arjuna* bark; purified 1% *T. arjuna bark extract* in 20 ml glycerol) and E4 (purified 1% *T. arjuna* bark (TAB)-Tea poly phenol (TPP) in 20 ml glycerol) were developed in our laboratory. In addition, antibiotics (penicillin-streptomycin, 1%); amino glycosides (6%), were added to improve the freeze/thaw quality. Penicillin-streptomycin is a broad spectrum antibiotic which serves as an inhibitor of microbial contamination at the time of preservation. Amino glycosides was added to inhibit microbe's protein synthesis. Trehalose was used to keep glassy state of cryo medium and was also helpful to maintain temperature at given cooling frequency.

2.5. Freeze/Thaw analysis

This study was initially started with 4 different kinds of extender (listed in Table 1) and outcome with natural antioxidant rich *TPP-T. Arjuna bark* (E4 medium) medium has proved for sperm DNA damage inhibition and for maintaining sperm parameters in the selected infertile smokers subjects (n = 42) when compared to other medium. So, the same E4 medium is continued in the straws to check sperm stability for prolonged period with these same subjects and the results were documented in the end of 6th month. Simultaneously sperm motility and morphology were taken as a serious concern in case of our medium selected and outer surface of morphology was monitored by Scanning electronic microscope (*SEM- oxford instrument's ZEISS 18. EVO model)*.

Table 1
List of four extender with its composition.

Recipe	E1	E2	E3	E4
Glycerol (%)	3	–	–	–
pH	7.2	7.2	6.9	6.9
Citric acid (g)	–	1.8	–	–
Egg-yolk (ml)	–	20	–	–
Penicillin-Streptomycin (IU)	–	–	2500	2500
Amino glycosides (IU)	–	–	2000	2000
Terminalia Arjuna Bark (ml)	–	–	20	20
Tea poly phenol (ml)	–	–	–	20
Trehalose (g)	–	0.4	0.4	0.4

Sperm head and tail defects and motility (whether the sperm cells are still in motile) were noted directly by CASA according to World Health Organization (WHO), 2010 guidelines [16].

2.5.1. Post thaw sperm markers quality analysis

Thawed sperm cells were lysed with 100 µl of sperm lysis buffer (0.1% DTT, 0.1% SDS and 0.1% urea) and incubated for 10 min. After this, the cells were washed with PBS buffer (pH 7.4) and centrifuged in 10,000 rpm for 30 min. The collected pellets were taken for post-thaw sperm markers analysis.

2.5.2. Zinc estimation (sperm membrane)

The level of zinc in the sperm membrane was estimated by our laboratory standardized procedure and modified [17]. In detail, the collected pellets from after thawing, the samples allowed for digestion with light acidic nature with pH 6.7 and absorbance was measured at 283.2 nm using flame atomic spectrometer against zinc chloride ($ZnCl_2$) as a standard. The total zinc concentration was expressed in mg/mL.

2.5.3. 8-OHdG analysis (8-Hydroxy-2′-deoxyguanosine) (sperm DNA damage marker)

After thawing, spermatozoa samples were treated with sodium chloride salting wash using 6 M NaCl (300 µl), which helps to precipitate the proteins. This whole mixture was allowed to centrifuge for 20 min. at 16,000 rpm, 4 °C, for 10 min. The collected supernatant was transferred to a new sterile Eppendorf tube with 500 µL of cold isopropanol. The mixture was inverted several times slowly till the DNA precipitate appears. After precipitation, again the mixture was centrifuged at 20,000 rpm, 4 °C, for 10 min. to get the pellet. The supernatant was discarded and then 500 µL of 70% ethanol was added in the pellet and then mixed slowly to remove any excess salts. Finally, the tubes were allowed to centrifuge for 15 min. at 14,000 rpm, 4 °C. The pellet obtained was the final sperm genomic DNA. The supernatant was discarded carefully, and the DNA pellet was air dried. After thorough drying, 50–100 µL of TE buffer was added to dissolve the sperm DNA. Sperm DNA samples dissolved in the TE buffer were loaded in 0.8% of agarose gel and ran at 35–50 V to 30 min. with reference to 1-Kb DNA ladder, to obtain an isolated genomic DNA. The remaining TE buffer-dissolved isolated samples underwent for enzymatic DNA digestion with DNase I, nuclease PI and alkaline phosphatase for 8 − OH dg measurement through the HPLC system containing a Younglin make ACME 9000 gradient pump, the C_{18} column with 250 mm × 4.6 mm of E95520, Kromasil syringe sample injector. The same Younglin packed 1050 UV detector (258 nm) is connected with the electrode to monitor 8 − OHdg in the meantime. Composition of mobile phase is 5% ethanol, 10-mM KCL, 10-mM NH_4 H_2 PO_4 and 1-mM EDTA with the pH 4.8–5.0 (flow rate 1 mL/min). With the standard dG and 8 − OHdg, the unknown samples dG and 8 − OHdg were calibrated and expressed in ng/ml [18]

2.5.4. Zeta potential (sperm membrane stability)

According to Ishijima et al. [19], zeta potential was done to check sperm membrane changes while selecting sperm cells in the AI, ICSI or IVF processes. The sperm cells were washed twice with HEPES (serum-free) + HTF (human tubinal fluid) buffer with pH 7.45 adjusted with a negative charge. For this analysis, the performance was done with a new glass tube selected with a positive charge (+1 to +5 kV) after rinsed and confirmed whether it is free of contaminants. The 0.1 mL of washed sample was carefully transferred to the tube and allowed to RT for 1 min to adjust the charge between the sample and the test tube. After 1 min, the samples were placed in centrifuge 3500 rpm for 5 min. The supernatant was discarded and the samples are dried (without changing the electric charge, the centrifugation was conducted and always note that centrifugation will not affect the net charge of the sample). Then, 200 µl of the serum-accompanied HEPES + HTF buffer was pipetted out and allowed to each dried sample and nullify the charge of each tube. This was repeated twice and the sample collected from the bottom of the tube was transferred to the polyethylene glycol (PEG)-coated tube and processed for measuring the sperm membrane charge to zeta potential instrument. The zeta potential of sperm cells was expressed in millivolts. The normal charge of the live sperm cell should be —12 to +20 mV, the mature sperm cell charge is between —20 and —16 mV and the abnormal sperm cell will reflect +60 mV.

2.5.5. Reactive oxygen species (ROS) effects by NBT (nitro blue tetrazolium) staining

ROS NBT staining was developed in our Gene Cloning and Technology lab authorized. Briefly, the sperm cells were incubated with 0.1% of a nitro blue tetrazolium salt (NBT) (10 mg of NBT in 10 ml of PBS/DMSO with pH 7.4) and allowed for 90 min. in dark condition (covered with an aluminum foil) at cold temperature (—18 °C). After incubation, a smear of NBT stained cells was made on a glass slide and the cells were viewed under an inverted microscope. The NBT-stained ROS effects were viewed at diverse magnifications and the results were expressed in scores [20].

2.5.6. Total sperm membrane protein analysis

The total sperm membrane protein level was checked with few modifications; sperm membrane protein estimation was done according to Lowry's method (1951), [21]. Briefly, 0.1 mL of the lysed sample was made up to 1 mL with distilled water. To this, 4.5 mL of alkaline copper reagent was added, mixed and allowed to stand at RT for 10 min. Later, 0.5 mL of Folin's phenol reagent was added and shaken well. The blank and standards were treated in a similar manner. The blue complex formed was measured at 620 nm after 20 min. against the blank using the ELISA microtiter plate reader (BIO-RAD, Model 680, Microtiter plate Reader). The concentration of the solution is calculated to be equal to 1 mL solution and the final total protein concentration was expressed in mg/mL.

2.6. Control samples

Meantime the listed control samples are stored (−80 °C deep freezer) for without any cryo ingredient/treatment and it has to been compared with fresh untreated infertile as well as freeze/thawed infertile semen samples. In addition, for comparison E1, E2 and E3 medium were also continued for comparison with E4 medium to identify a novel cryo medium for infertile smoker's sperm cells.

2.7. Development of TPP- TAB cryo preservation medium (E4) kit by lyophilization process

Lyophilization or freeze drying process involves three cycles, namely, freezing, primary drying and secondary drying [22].

Steps involved in Lyophilization

Freezing: Aqueous tea poly phenol *Terminalia arjuna* medium filled in amber glass vials are loaded in the lyophilize chambers. The samples will be freeze at —40 °C for minimum of 4 h. The vacuum decreased inside the chamber to 380000 milli Torr (normal atmospheric vacuum pressure is 760000 milli Torr).

Primary drying: After freezing, the samples' vacuum will be reduced to 300 milli Torr and heat will applied in the loaded chambers and the temperature will move from -40 °C to 0 °C in minimum 16 *h.* (every 5° drop will take a time of 2 h.).

Secondary drying:Under proper vacuum condition, the cryo medium will be dried from 0 °C to 35 °C in minimum 14 h. In final stage of secondary drying, vacuum will reach 0 milli Torr (free from air). After the temperature reaches the set point, the cryo medium will be removed from the chambers and sealing to be done. Before sealing, homogeneity and moisture checks will be carried out as per the National Accreditation Board for Laboratories (NABL) requirement.

2.8. Statistics

All the results were represented as mean ± standard error of mean (SEM). Significance differences with subjects are expressed with $P < 0.001$ and $P < 0.05$ All the statistical analysis were done with Graph Pad prism version 6.0.

3. Results

Previous part of trail was done with comparison to fresh and one month preserved with E1 to E4 cryo medium. Also results proved that E4 cryo medium is better when compared to other mediums [8]. So the E4 (tea poly phenol-*Terminalia arjuna*) medium was decided to be continued for 6 month in same frozen condition. The 2nd trial was checking sperm stability in the end of 3rd and 6th month by checking its morphology, motility, maturity by zeta potential charges and final product of E4 medium by lyophilized method. From the results, we observed that the percentage of rapid motility and sperm count in the 3rd and 6 month of continuous study was maintained with minimum loss when compare to 1st and fresh infertile results.

Mean time the both preserved and fresh infertile results was compared with unpreserved control samples which was listed in Table 2. The control semen sample was stored without any preservative medium under −80 °C deep freezer. From the control sample results, it sperm motility and morphology reduction was noticed. However, this reduction has not affected the sperm cell quality which was listed in Table 2. Like freeze/thaw samples, motility, zeta potential and sperm membrane stability also calculated and compared with infertile sperm cells. Also Table 3 explains about E1, E2 and E3 medium preserved infertile sperm cell and its values in 3 and 6th month. When compare to E4 medium all the other three medium was less in protecting sperm cells biology and integrity by its ROS and 8OHdG values.

Also the level of membrane proteins denotes that the sperm cell membrane is maintained in the E4 medium. Sperm membrane protein, an important dominant factor in semen which helpful to maintain seminal fluidity and helps to sperm cells mobility

Like this quote, a considerable thing in the concentration of sperm membrane protein or total seminal protein will always rich, even in the infertile subjects. Hence, from the present study there is no changes in the level of protein and also freeze/thaw method does not affect the sperm membrane and it helps to maintain sperm motility, if even infertile.

E4 medium preserved infertile sperm cells and its morphology (both before and after trials) are shown in Fig. 1; according to sperm morphology, acrosome part is major concern for capacitation process when sperm-egg fusion happens.

At the end of 3rd and 6th month, a neat acrosome part with undamaged sperm cells were maintained in infertile smokers and it was shown in Fig. 1 of A, B and C. A is untreated fertile sperm cell (control). B is 1st month frozen/thawed by E4 cryo medium infertile smoker sperm cell. C is 6th month frozen/thawed by E4 cryo medium infertile smoker sperm cell which noted neat acrosome in the image under scanning electronic microscope. F is untreated infertile cigarette smoker sperm cell (negative control) with its free radical/leukocytes.

Fig. 2 demonstrated the sperm motility in the E4 medium treated infertile smokers and its comparison is done with fertile subjects. In detailed, A is untreated fertile non-smokers of control subject's sperm motility. While C and D is 1st and 6th month of E4 cryo medium treated infertile smoker's sperm cell motility movement. B is untreated fresh infertile smoker sperm cell motility. Results showed that the motility was maintained.

Generally sperm membrane is composed of protein and lipid molecules. In case of cryo injury, free or oxidative radicals (ROS) will degrade sperm membrane which results in slow progressive motility and abnormal morphology

When sperm cells undergo cryogenic process, it causes generation of ROS, creating an imbalance between oxidant: antioxidant status leading to sperm physiology and biology losses. Present study shows, infertile smoker's samples were maintained from severe damages when preserved with E4 medium and at the same time the level of ROS is carried to maintain oxidant: antioxidant status and the sustained sperm biology by its motility which was given in Fig. 3 as well as in Table 2. The same it has been compared with 1st month and fresh infertile samples results and respective significance were obtained ($p < 0.05$).

E4 medium treated infertile sperm cells stability are shown in Fig. 4. According to electric charge stability for sperm cells was identified by its electrophoretic mobility charges in the range between -40 to +40 mV. After 6th −40 to +40 mV. After 6th month also the cryo

Table 2
3nd and 6th month trail comparison with 1st month trail of E4 medium of TPP-TAB treated sperm cell values.

Parameters	Control (n = 28), fresh	Control (n = 28), Untreated Frozen/ thawed 6th month	IF-S (n = 42) (fresh)	IF-S (n = 42) Untreated Frozen/ thawed 6th month	E4 medium frozen /Thawed trails in month/ 2nd trail in 3rd and 6th month (n = 42)		
					1st	3rd	6th
Sperm count (million/ml)	59.46 ± 0.74	55.32 ± 0.09	9.20 ± 3.26	7.91 ± 0.38	8.12 ± 0.83	7.89 ± 0.11	7.12 ± 0.92
Total motility (%)	49.75 ± 0.6	32.17 ± 0.004	12.50 ± 2.53	10.29 ± 0.94	8.84 ± 1.21	8.32 ± 1.05	7.86 ± 0.21
Rapid motility (%)	43.21 ± 0.9	29.76 ± 0.004	2.31 ± 0.75**a	1.95 ± 0.05*b	2.01 ± 0.89***c	1.93 ± 0.16**a	1.69 ± 0.34**a
Normal morphology (%)	17.71 ± 0.96	14.32 ± 0.35	3.33 ± 0.36**a	2.98 ± 0.09**a	3.03 ± 0.47***c	2.97 ± 0.14**a	2.90 ± 0.22***c
Head/tail defects (%)	47.71 ± 0.96	35.59 ± 0.26	11.19 ± 2.34	13.29 ± 3.84	13.25 ± 3.21**a	13.98 ± 4.05**a	14.11 ± 4.49**a
Zinc (mg/ml) (sperm membrane)	28.79 ± 1.02	19.38 ± 0.42	2.45 ± 0.02	2.33 ± 0.005	2.38 ± 0.91***c	2.29 ± 0.54*b	2.25 ± 0.43*b
ROS (scores)	1.85 ± 0.73	2.04 ± 0.19	17.40 ± 1.71**a	21.32 ± 2.58***c	3.12 ± 0.09	3.02 ± 0.19	3.00 ± 0.10* b
8-OH-2-guanosine (%)	0.87 ± 0.003	0.95 ± 0.24	23.45 ± 2.98**a	25.82 ± 1.64*b	1.09 ± 0.21	1.09 ± 0.21	0.99 ± 0.32**a
Sperm membrane proteins (mg/ml)	7.94 ± 0.98	7.04 ± 1.95	5.37 ± 1.39	5.31 ± 0.34	5.22 ± 1.17	5.14 ± 1.25	4.85 ± 1.39*b

The list of semen parameters, biochemical, oxidative damage marker along sperm head and tail defects with membrane proteins level in smoking infertile subjects of frozen/thaw with E4 extender and compared with fresh untreated control subjects. Values are represented here mean ± standard error of mean (SEM), **a: negative correlation with $p < 0.01$ significant, *b: positive correlation with $p < 0.05$ significant, ***c: positive correlation with $p < 0.001$ significant.

Table 3
3nd and 6th month trail comparison with 1st month trail of E1, E2 and E3 cryo medium treated sperm cell values.

Parameters	1st month			3rd month			6th month		
	E1	E2	E3	E1	E2	E3	E1	E2	E3
Sperm count (million/ml)	2. 21 ± 0.34	4.45 ± 0.45	6.89 ± 0.26	1.69 ± 0.55	3.84 ± 1.32	5.25 ± 0.59	0.95 ± 0.35	0.56 ± 0. 39	3.19 ± 1.74
Total motility (%)	1.23 ± 0.34	2.86 ± 0.08	3.45 ± 0.96	1.02 ± 0.78	2.4 ± 0.21	3.02 ± 0.19	0.86 ± 0.90	1.75 ± 0.43	1.98 ± 0.95
Rapid motility (%)	0.26 ± 0.08	1.05 ± 0.06	1.37 ± 0.97	0.19 ± 0.11	0.56 ± 0.21	0.85 ± 0.24	0.12 ± 0.08	0.37 ± 0.09	0.65 ± 0.78
Normal morphology (%)	0.85 ± 0.09	1.23 ± 0.04	2.28 ± 0.32	0.41 ± 0.21	0.92 ± 0.24	2.02 ± 0.51	0.23 ± 0.18	0.65 ± 0.38	1.42 ± 0.85
Head/tail defects (%)	0.34 ± 0.002	1.4 ± 0.18	2.5 ± 0.78	0.55 ± 0.12	2.55 ± 0.72	3.19 ± 0.94	0.68 ± 0.21	4.11 ± 1.28	5.32 ± 0.99
Zinc (mg/ml) (sperm membrane)	1.32 ± 0.28	1.43 ± 0.48	2.28 ± 0.62	0.93 ± 0.38	1.21 ± 0.85	2.1 ± 0.93	0.48 ± 0.43	0.84 ± 0.32	1.45 ± 0.75
ROS (scores)	16.12 ± 0.83	16.02 ± 0.79	10.23 ± 0.44	24.09 ± 2.34	22.43 ± 1.54	14 ± 1.42	29.48 ± 4.32	35.65 ± 6.82	18.43 ± 3.84
8-OH-2-guanosine (%)	21.67 ± 2.86	20.32 ± 2.45	2.45 ± 0.82	25.94 ± 3.58	28.47 ± 5.76	3.64 ± 1.02	31.20 ± 2.84	38.54 ± 5.92	7.93 ± 1.07
Sperm membrane proteins (mg/ml)	2. 21 ± 0.34	4.45 ± 0.45	6.89 ± 0.26	2.04 ± 0.19	4.09 ± 0.21	6.21 ± 0.43	1.57 ± 0.08	3.40 ± 0.03	5.43 ± 0.85

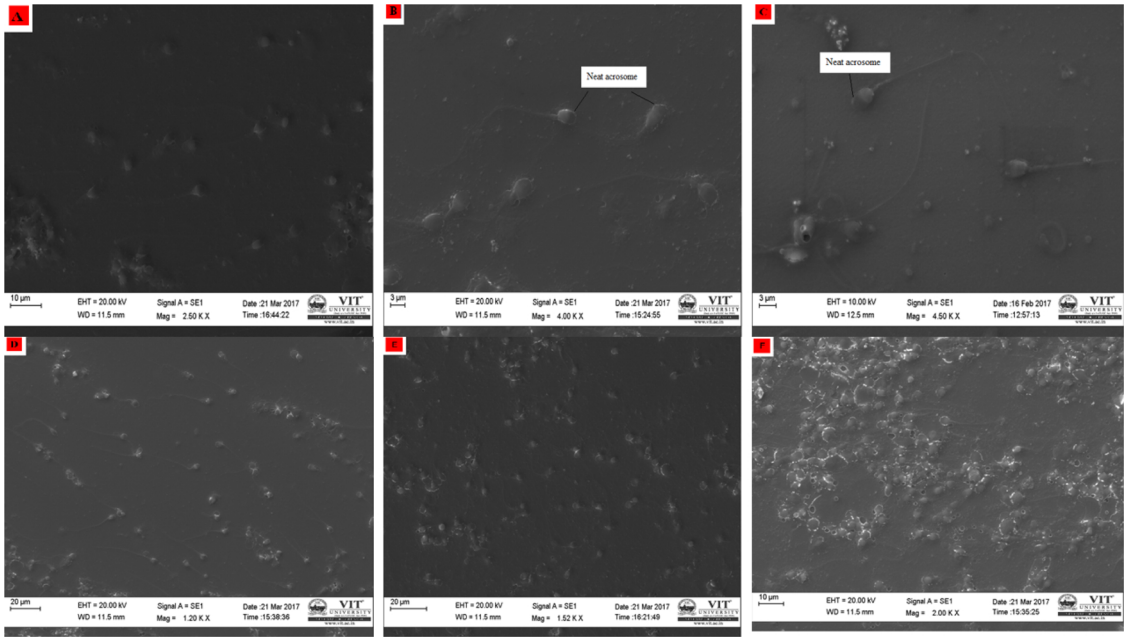

Fig. 1. Before and after E4 cryo medium Freeze/thawed sperm cell morphology and its comparison under scanning electronic microscope.
A is fresh fertile sperm cell (control) under scanning electronic microscope. B is 1st month frozen/thawed by E4 cryo-medium infertile smoker sperm cell which noted neat acrosome in the image under scanning electronic microscope. C and D is 6th month frozen/thawed by E4 cryo-medium infertile smoker sperm cell which noted neat acrosome in the image under scanning electronic microscope. E and F are untreated infertile cigarette smoker sperm cell with its free radical/leukocytes deposition was visualized under scanning electronic microscope

preserved sperm cells with E4 medium was maintained in its maturity (−7.9 mV) and it was compared with control (−38.6 mV) sperm cell maturity. Meanwhile it was compared with fresh (−13.4 mV) and 1st (−11.1 mV) month preserved sperm cells. According to Ishijima et al [19] the stability of sperm cells was identified by its electrophoretic mobility charges in the range between -16 to −20 mV. Our obtained results agreed with it. Finally the product of Tea poly phenol and *Terminalia arjuna* bark compositional, E4 cryo preservation medium was given in Fig. 5.

4. Discussion

Smoking is one of the major mutating factor and damages spermatogenic stem cells resulting in spermatogenesis error like Down syndrome, Kline filter syndrome or XY micro deletion diseases. Infertile smoker sperm preservation is essential when they go for ART, IVF or therapeutic medications. There are many reports published and are only to preserve healthy sperm cells in humans as well as animals [4,5,23,24] and few are for those who underwent oncology treatment. During cryopreservation, sperm undergoes oxidative stress. In case of fresh/fertile semen, it will be resolved by its own antioxidants which are present in seminal plasma or with the help of cryo protectants [10,11]. As per clinician as well as andrologists research report, infertile smokers are recommended to take 2 to 3 times more amount of ascorbate and it is essential to neutralize oxidants against *de nova* mutation when trying to conceive. In infertile smoker, the level of ROS is high and so far there are no reports for preserving those sperm cells. The reason is, that infertile subjects cannot produce enough amounts of

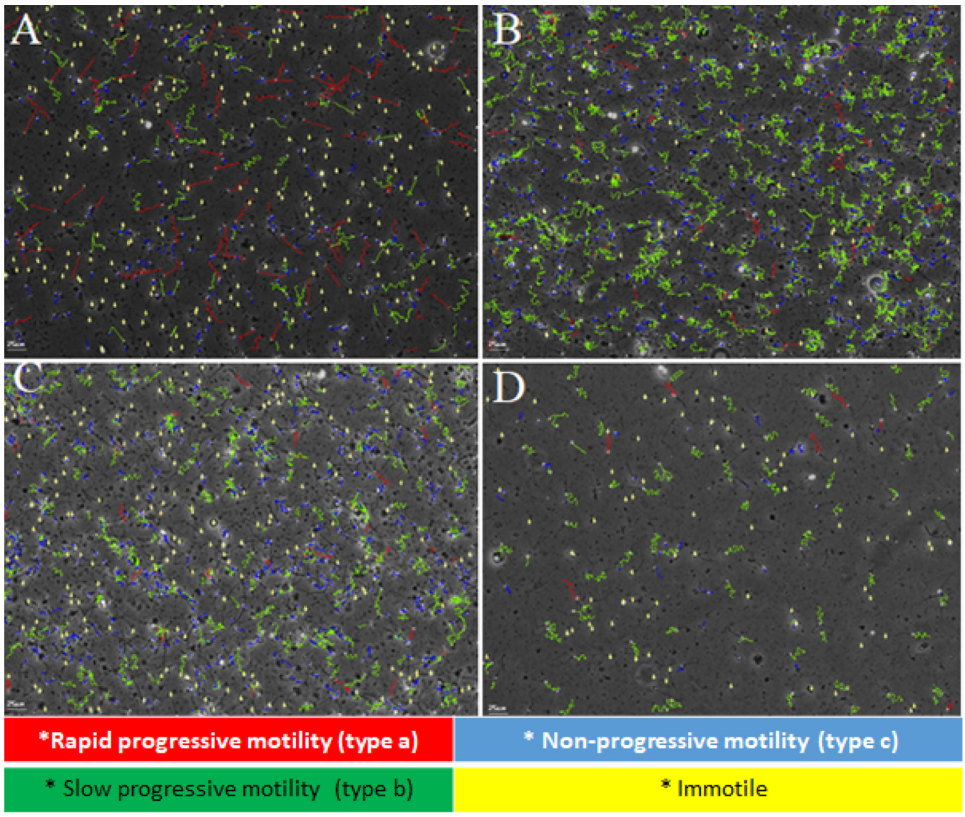

Fig. 2. Infertile Sperm motility by CASA analysis with E4 cryo medium frozen/thawed and its comparison.
A is untreated fertile non-smokers of control subjects sperm motility. While C and D is 1st and 6th month of E4 cryo medium treated infertile smoker's sperm cell motility movement. B is untreated fresh infertile smoker sperm cell motility

antioxidants due to over production or unbalanced state of Oxidative species: reactive oxygen species (OS: ROS). Many researches have reported that the sperm is dependent on seminal plasma for extracellular antioxidants to defeat oxidative species [9,23]. However, seminal plasma of semen is separated and discarded during cryo preservation processing to increase the stability against ROS [11]. Hence, the researchers were appealed to provide antioxidants sources which protects spermatozoa during processing [13]. Recently researchers revealed that natural herb/medicinal plants act as antioxidant scavengers and are used as cryo medium while it improves sperm quality [25]. However, to the best of our knowledge there are no reports about E4 medium of TPP – TAB composited antioxidants effects on sperm parameters; meanwhile first study in the comparing with untreated control was equally stored and reported. Remarkably, from the findings of present experimental design of 1st, 3rd and 6th month comparison, TPP – TAB medium plays a major role in reduction of oxidative species and also on intensity of damage marker (8OHdG) reduction. Also there is no report about reduction of DNA damage marker (8 – OHdG) while using cryo medium. But here, we examined both untreated control was stored without any cryo preservation was reported with DNA damage marker (8 – OHdG), sperm cell stability and sperm membrane protein levels. Meantime the other medium from the present study such as E1, E2 and E3 was not at the level as equal to E4 medium in the management of ROS and 8OHdG markers.

Zeweil, et al [26] reported that, use of pomegranate juice in cryo preservation helps to improve sperm motility and also acts as antioxidant booster. On the other side, alcoholic Sargassum extract made cryo medium inhibit free radicals with significant changes in rapid motility, moreover these works are limited in dosage (250 to 500 µg/mL) and also it was tried only with the control group [27]. In case of our study, we have worked with infertile subjects and significant positive effects in reducing free radicals were noticed. Reports from 2003, 2006, 2011,2012, 2015, 2017 and 2018 of earlier studies, reveals that different type of antioxidants (Tris, taurine, curcumin, inositol and

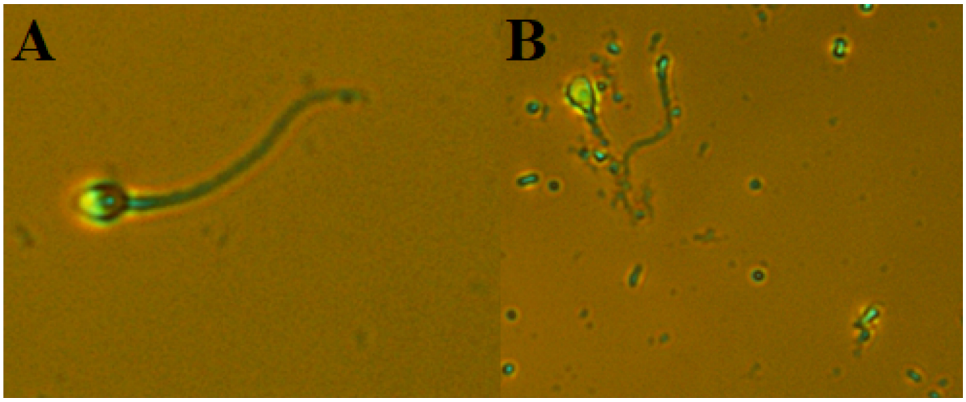

Fig. 3. ROS effects on E4 cryo preserved sperm cells by NBT staining.
A shows the focused sperm cells without damage in the end of 6th month. B shows the initial fresh infertile sperm cells (400x magnification)

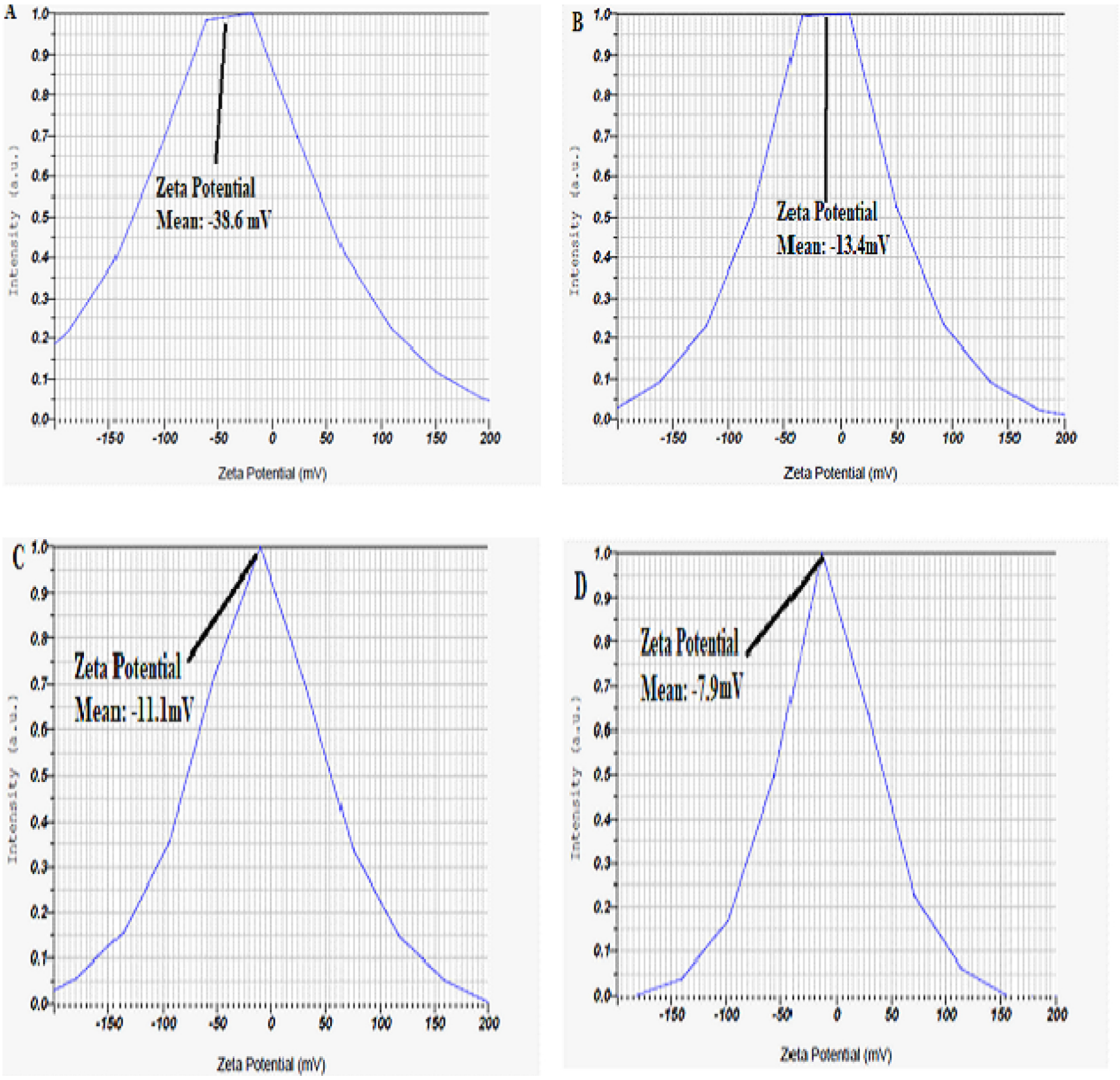

Fig. 4. E4 cryo preserved infertile sperm cell membrane stability by zeta electric potential charges and its comparison in 1st and 6th month trail.
A denotes the control fertile sperm cell membrane stability with its zeta potential charge is -38.6 mV. B denote the fresh untreated infertile smoker sperm cell stability under zeta electrical charges with -13.4 mV. C is first month freeze/thawed infertile smokers sperm cell stability with its zeta electrical charge is -11.1. D is 6th month freeze/thawed infertile smokers sperm cell stability with its zeta electrical charge is -7.9. X-axis denote the zeta electric charges and Y axis denote its intensity in aspiratory unit (a.u). -40 to +40 mV are identified as stable when selecting sperm cells for AI, ICSI and IVF procedures.

Fig. 5. E4 cryo medium (Tea poly phenol and *Terminalia arjuna* bark is the major composition in the E4 cryo medium product).

saffron) were used as cryo medium and trial was done up to 3 months only [3,5,6–8,10,11,22,24]. To sort out this problem, our study basically aims to identify a cryo protective extender for infertile smokers using natural antioxidants of TPP – TAB compounds with trehalose, as a cryo survival compound (E4 medium). Hence in the future we have decided to continue for checking mitochondrial and acrosome stability.

When freezing with E4 medium (up to 6th month), the control and infertile sperm cells were stored without preservation was carried out for comparison with positive and negative control effects up to duration (6th month). The outcome of TPP-*TAB* medium destructs the free radicals/ROS and also it reduces sperm membrane protein damages as well as it maintains the sperm motility, sperm stability and sperm morphology during preservation and it was compared with unpreserved control and infertile sperm cells.

Declaration of Competing Interest

None to declare.

Acknowledgements

The authors are thankful to Management of VIT and DST-FIST sponsored SEM facility VIT University and Dr. Kamini A Rao, Medical Director, BACC, India. Also thankful to Department of Clinical Biochemistry, CMCH, Vellore.

References

[1] C.U. Mussagy, J. Winterburn, V.C. Santos-Ebinuma, J.F. Pereira, Production and extraction of carotenoids produced by microorganisms, Appl. Microbiol. Biotechnol. 103 (2019) 1095–1114.
[2] R. Parameswari, T.B. Sridharan, Cigarette smoking and its toxicological overview on human male fertility—a prospective review, Toxin Rev. (2019), https://doi.org/10.1080/15569543.2019.1579229.
[3] P. Ranganathan, K.A. Rao, J.J. Sudan, S. Balasundaram, Cadmium effects on sperm morphology and semenogelin with relates to increased ROS in infertile smokers: an in vitro and in silico approach, Reprod. Biol. 18 (2018) 189–197.
[4] A.K. Anil Joshi, S.M. Mathur, K. Naqvi, J.P. Mittal, Influence of osmolality of complete semen extender on motion characteristics of frozen-thawed ram spermatozoa, Asian-Aust. J. Anim. Sci 19 (2006) 1716–1721.
[5] M. Bottini-Luzardo, F. Centurion-Castro, M. Alfaro-Gamboa, R. Ake Lopez, J. Herrera-Camacho, Effect of addition of coconut water (Cocos nucifera) to the freezing media on post-thaw viability of boar sperm, Trop. Anim. Health Prod. 45 (2012) 101–106.
[6] M.N. Bucak, P.B. Tuncer, S. Sarıözkan, P. Alkım Ulutas, K. Çoyan, N. Başpınar, B. Özkalp, Effects of various antioxidants on cryopreserved bull sperm quality, Res. Vet. Sci. 87 (2009) 468–472.
[7] G. Guha, V. Rajkumar, R.A. Kumar, L. Mathew, The antioxidant and DNA protection potential of Indian tribal medicinal plants, Turk. J. Biol. 35 (2011) 233–242.
[8] R. Parameswari, R. Kamini, P. Manigandan, A.S. Vickram, K. Archana, T.B. Sridharan, TB, tea Polyphenol-T. Arjuna Bark as sperm antioxidant extender in infertile smokers, Cryo Lett. 38 (2017) 95–99.
[9] M. Hezavehei, M. Sharafi, H.M. Kouchesfahani, R. Henkel, A. Agarwal, V. Esmaeili, A. Shahverdi, Sperm cryopreservation: a review on current molecular cryobiology and advanced approaches, Reprod. Bio Med. Online 37 (2018) 327–339.
[10] E. Isachenko, V. Isachenko, I.I. Katkov, et al., Vitrification of mammalian spermatozoa in the absence of cryo protectants: from past practical difficulties to present success, Reprod. Bio Med. Online 6 (2003) 191–200.
[11] H. Cai, J.D. Niringiyumukiza, Y. Li, Q. Lai, Y. Jia, P. Su, W. Xiang, Open versus closed vitrification system of human oocytes and embryos: a systematic review and meta-analysis of embryologic and clinical outcomes, Reprod. Biol. Endocrinol. 16 (2018) 123.
[12] A. Varghesea, J. Savai, N. Pandita, R. Gaud, In vitro modulatory effects of Terminalia arjuna, arjunic acid, arjunetin and arjungenin on CYP3A4, CYP2D6 and CYP2C9 enzyme activity in human liver microsomes, Toxicol. Rep. 2 (2015) 806–816.
[13] D. Mortimer, Current and future concepts and practices in human sperm cryo banking, Reprod. Bio Med. Online 9 (2004) 134–151.
[14] R. Parameswari, R. Kamini, K. Mano, M. Aruna, A.S. Vickram, M. Rameshpathy, T.B. Sridharan, Human sperm DNA damage inhibition and antioxidant activity of T. Arjuna bark: an in vitro study, 3 Biotech 7 (2017) 188.
[15] W. Franklin, F.C. Lowell, Unrecognized airway obstruction associated with smoking: a probable forerunner of obstructive pulmonary emphysema, Ann. Intern. Med. 54 (1961) 379–386.
[16] WHO, Laboratory Manual for the Examination of Human Semen and Semen-Cervical Mucus Interaction, 5th edn., Cambridge University Press, Cambridge, 2010.
[17] D.S. Chu, Zinc: a small molecule with a big impact on sperm function, PLoS Biol. 16 (2018) e2006204.
[18] C. Cao, T. Lai, M. Li, H. Zhou, D. Lv, Z. Deng, S. Ying, Z. Chen, W. Li, H. Shen, Smoking-promoted oxidative DNA damage response is highly correlated to lung carcinogenesis, Oncotarget 7 (2016) 18919.
[19] S.A. Ishijima, M. Okuno, H. Mohri, Zeta potential of human X- and Y-bearing sperm, Int. J. Androl. 14 (1991) 340–347.
[20] R. Parameswari, K.A. Rao, T.B. Sridharan, Deterioration of semen quality and sperm DNA integrity as influenced by cigarette smoking in fertile and infertile human male smokers- A prospective study, J. Cell. Biochem. 120 (2019) 11784–11793.
[21] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[22] M.V. Ramirez-Mares, H. Kobayashi, E.G. de Mejia, Inhibitory effect of Camellia sinensis, Ilex paraguariensis and Ardisia compressa tea extracts on the proliferation of human head and neck squamous carcinoma cells, Toxicol. Rep. 3 (2016) 269–278.
[23] H.J. Kim, J.H. Lee, Y.B. Hur, C.W. Lee, et al., Marine antifreeze proteins: structure, function, and application to cryopreservation as a potential cryoprotectants, Mar. Drugs 15 (2017) 27.
[24] G. Raad, L. Lteif, R. Lahoud, J. Azoury, J. Azoury, J. Tanios, M. Hazzouri, J. Azoury, Cryopreservation media differentially affect sperm motility, morphology and DNA integrity, Andrology 6 (2018) 836–845.
[25] M.M. Zanchi, V. Manfredini, D.S. Brum, L.M. Vargas, et al., Green tea infusion improves cyclophosphamide-induced damage on male mice reproductive system, Toxicol. Rep. 2 (2015) 252–260.
[26] H.S. Zeweil, S. Elnagar, S.M. Zahran, et al., Pomegranate peel as a natural anti-oxidant boosts bucks' fertility under egyptian summer conditions, World Rabbit Sci. 21 (2013) 33–39.
[27] A. Sobhani, T.E. Eftekhaari, M.E. Shahrzad, M. Natami, S. Fallahi, Antioxidant effects of brown algae Sargassum on sperm parameters: CONSORT-compliant article, Medicine 94 (2015) 1–6.