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Effects of Crocin on Human Sperm Viability, Motility, Morphology, DNA Fragmentation and Reactive Oxygen Species Levels after Freezing and Thawing

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ABSTRACT

Objectives: To investigate the effects of crocin supplementation during solid surface vitrification (SSV) and liquid nitrogen vapor freezing (LNF) of human spermatozoa on post-thaw sperm parameters.

Materials and Methods: Thirty-six normozoospermic semen samples were used in the study. Post prepared semen samples were divided into five aliquots: one served as non-cryopreserved control; two were vitrified, with or without crocin supplementation (SSV ± 10 µg/ml crocin); and the last two aliquots were frozen in liquid nitrogen vapor, with or without crocin supplementation (LNF ± 10 µg/ml crocin).

Results: After cryopreservation, sperm motility (93.54 ± 3.55%, 70.10 ± 10.94% and 53.58 ± 12.06% in controls, SSV and LNF groups, respectively, $p < 0.001$) and sperm viability (79.82 ± 18.86%, 57.74 ± 21.99% and 49.69 ± 22.25%, $p < 0.001$) decreased significantly in both methods. However, the SSV groups had a significantly higher sperm motility (70.10 ± 10.94% and 53.58 ± 12.06%, $p < 0.001$) and viability (57.74 ± 21.99% and 49.69 ± 22.25%, $p < 0.001$) than the LNF groups. Supplementation with crocin 10 µg/ml in cryoprotective agent did not improve sperm motility and viability in both cryopreservation methods. Also, no effect was noted on sperm morphology, sperm deoxyribonucleic acid (DNA) integrity, and both the intracellular and extracellular reactive oxygen species (ROS) levels.

Conclusion: Crocin supplementation during vitrification and liquid nitrogen vapor freezing did not improve the outcome of post-thaw sperm.

Keywords: crocin supplementation, sperm cryopreservation, vitrification, vapor freezing .

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ผลของสารโครซินต่ออัตราการรอดชีวิตของตัวอสุจิมนุษย์, การเคลื่อนไหว, ลักษณะรูปร่าง, การแตกหักของดีเอ็นเอและระดับสารอนุมูลอิสระ หลังขบวนการแช่แข็งและการละลาย

วรัญญา เรืองชัยนิคม, อุบล แสงอนันต์, ธีระพร วุฒยวนิช, วราภรณ์ ภิรมย์เลิศอมร, อุษณีย์ แสนหมี่

บทคัดย่อ

วัตถุประสงค์: *เพื่อศึกษาผลของการเติมสารโครซินในขบวนการแช่แข็งและละลายอสุจิมนุษย์ ทั้งวิธีการแช่แข็งแบบเนื้อแก้วและการแช่แข็งในไอของไนโตรเจนเหลว โดยดูอัตราการรอดชีวิต, การเคลื่อนไหว, ลักษณะรูปร่าง, การแตกหักของดีเอ็นเอและระดับสารอนุมูลอิสระของตัวอสุจิ*

วัสดุและวิธีการ: *การศึกษานี้ใช้น้ำอสุจิที่ผ่านเกณฑ์มาตรฐาน จำนวน 36 ตัวอย่าง นำไปผ่านกระบวนการปั่นแยกตัวอสุจิตามวิธีมาตรฐาน แล้วนำอสุจิที่ได้แบ่งเป็น 5 ส่วน ส่วนที่ 1 (กลุ่มควบคุม) ไม่ผ่านขบวนการแช่แข็ง อีก 2 ส่วนทำการแช่แข็งแบบเนื้อแก้ว (SSV) โดยเติม/ไม่เติมสารโครซิน (10 ไมโครกรัม/มิลลิลิตร) และ 2 ส่วนสุดท้ายทำการแช่แข็งในไอไนโตรเจนเหลว (LNF) โดยเติม/ไม่เติมสารโครซิน (10 ไมโครกรัม/มิลลิลิตร)*

ผลการศึกษา: *อสุจิหลังผ่านขบวนการแช่แข็ง พบว่าอัตราการเคลื่อนไหว (ร้อยละ 93.54 ± 3.55, 70.10 ± 10.94 และ 53.58 ± 12.06 ในกลุ่มควบคุม, SSV และ LNF ตามลำดับ, $p < 0.001$) และอัตราการรอดชีวิต (ร้อยละ 79.82 ± 18.86, 57.74 ± 21.99 และ 49.69 ± 22.25, $p < 0.001$) ลดลงในทั้งสองวิธีอย่างมีนัยสำคัญทางสถิติ แต่อย่างไรก็ตามการแช่แข็งแบบเนื้อแก้วมีอัตราการเคลื่อนไหว (ร้อยละ 70.10 ± 10.94 และ 53.58 ± 12.06, $p < 0.001$) และอัตราการรอดชีวิต (ร้อยละ 57.74 ± 21.99 และ 49.69 ± 22.25, $p < 0.001$) มากกว่าวิธีการแช่แข็งในไอไนโตรเจนเหลวอย่างมีนัยสำคัญทางสถิติ การเติมสารโครซินในขบวนการแช่แข็งไม่ได้เพิ่มอัตราการเคลื่อนไหวและการรอดชีวิตของตัวอสุจิ ในการแช่แข็งทั้งสองวิธี และไม่มีผลต่อลักษณะรูปร่าง, การแตกหักของดีเอ็นเอและระดับสารอนุมูลอิสระทั้งในและนอกตัวอสุจิ*

สรุป: *การเติมสารโครซินในขบวนการแช่แข็งและละลาย ไม่ว่าจะด้วยวิธีแช่แข็งแบบเนื้อแก้วหรือการแช่แข็งในไอไนโตรเจนเหลวไม่ได้ช่วยเพิ่มผลลัพธ์ที่ดีต่อตัวอสุจิ*

คำสำคัญ: *การเติมสารโครซิน, การแช่แข็งอสุจิ, การแช่แข็งแบบเนื้อแก้ว, การแช่แข็งในไอของไนโตรเจนเหลว*

Introduction

The biotechnological applications of cryopreservation are evolving and advancing at a rapid pace. Sperm cryopreservation has now become an important part of assisted reproductive technology, and it is routinely practiced worldwide. Male fertility preservation is performed for various reasons.

Recent researches on sperm physiology and cryobiology have contributed to the improvement in sperm cryopreservation techniques. Cryopreservation can irreversibly damage the sperm structure and affect deoxyribonucleic acid (DNA) integrity through dehydration, osmotic shock, formation of intra- and extracellular ice crystals, and the generation of reactive oxygen species (ROS)[1]. Mature spermatozoa are very susceptible to damage from ROS because their plasma and mitochondrial membranes contain polyunsaturated fatty acids, and they possess no antioxidant defense mechanism in their cytoplasm[2]. Male patients, with high levels of ROS in seminal fluid, are known to have abnormal sperm morphology, decreased sperm viability, and lower fertilizing potential[3]. During cryopreservation, an excessive amount of ROS associated with increased production and reduced antioxidant activity causes oxidative stress and apoptosis that affect sperm motility and DNA integrity resulting in decreased fertility potential of the spermatozoa[4-7]. The separation of motile spermatozoa from the seminal plasma, during the preparation step before cryopreservation is possibly another contributory factor, as it removes the anti-oxidant defense system in the seminal plasma[8]. Although cryoprotective agents may help prevent ice crystallization, they have intrinsic toxicity and may cause osmotic stress, resulting in sperm membrane destabilization and protein denaturation[9].

Our previous study showed that rapid freezing of spermatozoa gave significantly better outcomes than the standard slow programmable freezing, even after repeated freezing and thawing[10]. Despite this dramatic success, we seek to further improve the method such that cryopreserved sperm would have a survival rate approaching 90%. Our literature search indicated that supplementation with antioxidants, such as vitamin C[11], vitamin E[12], glutathione, steroids, leptin[13], and crocin[14] might improve the outcome of sperm cryopreservation.

Crocin is a carotenoid antioxidant that helps eliminate ROS, especially superoxide anion. Crocin has been used as an antioxidant in medical treatment, as well as in assisted reproductive technology (ART). As cryopreservation generates free radicals that decrease sperm quality, researchers are interested in whether crocin would improve the outcomes of sperm freezing. Sapanidou et al[14] added crocin to the bovine sperm cryopreservation medium. They found a significant reduction in ROS formation and lipid peroxidation. The sperm motility, sperm viability, and acrosomal integrity increased significantly. The fertilization rate and the number of embryos were also increased. Crocin, exert an antioxidant effect on apoptosis signaling pathways and prevent DNA fragmentation and morphological changes of apoptosis that are induced by tumor necrosis factor-α and serum-glucose deprivation[15]. A study in red deer sperm by Dominguez-Robolledo et al[16] found that the addition of crocin 1 mM to sperm cryopreservation media significantly reduced ROS and increased sperm motility. However, a dose greater than 2 mM had a negative effect and significantly increased lipid peroxidation. Unfortunately, previous studies in experimental animals were conflicting, and there has been no study in humans. In this study, we investigated the effects of crocin supplementation during solid surface vitrification (SSV) and liquid nitrogen vapor freezing (LNF) of human spermatozoa on post-thaw sperm parameters.

Materials and Methods

Participant selection criteria

Semen samples were collected from male partners of infertile couples, who visited our infertility clinic at Maharaj Nakorn Chiang Mai University Hospital. They collected sperm samples into sterile containers by masturbation after abstinence of two to seven days. Only semen with normal parameters, according to the World Health Organization reference values (WHO

2010) were included in the study. All participants gave their written informed consents for the use of their semen for research. The exclusion criteria were: 1) medical diseases or used medication that could affect spermatogenesis, 2) positive serology for human immune-deficiency viruses (HIV),3) a history of exposure to radiotherapy/chemotherapy, 4) varicocele, genital infection or leukocytospermia, and 5) did not understand Thai language or did not want to participate in the study.

Semen preparation

Semen samples were allowed to liquify at 37°C for 30-60 minutes. Semen samples were layered on top of 80% and 40% discontinuous Sil-Select Plus gradients (Fertipro NV, Beernem, Belgium), then centrifuged at 350 g for ten minutes. The sperm pellet was washed twice with 4 ml of Earle's Balanced Salt Solution (EBSS; Biological Industries, Kibbutz Beit Haemek, Israel), supplemented with 0.3% human serum albumin (HSA; Life Global, Guilford, CT, 0.03M sodium pyruvate (Cat. No. H0887; Sigma) and centrifuged at 200 g for five minutes. The final pellet was suspended in 500 µl of the same medium and divided into five aliquots. The first aliquot (100 µl) served as a non-frozen control and was immediately assessed for sperm motility, sperm morphology, kinematics of sperm movement, sperm viability, DNA integrity, extracellular and intracellular ROS. The flow chart of the study is shown in Fig. 1.

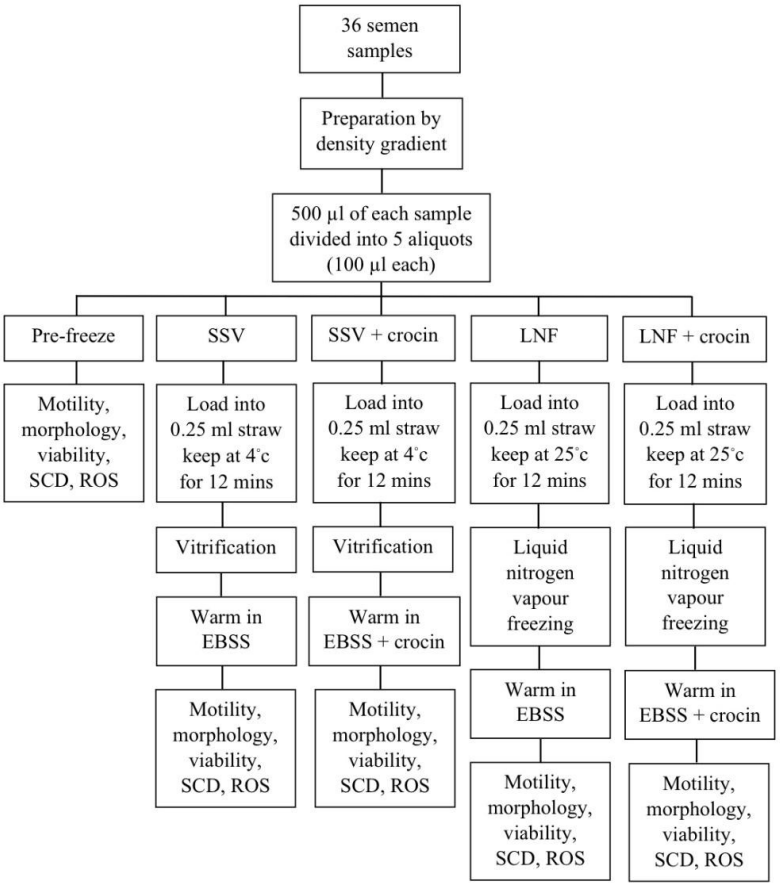

Fig. 1. Flow diagram of the study.

SSV: solid surface vitrification, LNF: liquid nitrogen vapor freezing, SCD: sperm chromatin dispersion, ROS: reactive oxygen species, EBSS: Earle's balanced salt solution

Cryopreservation methods

Vitrification was performed on the second and third aliquots. Each aliquot containing 100 µl of prepared sperm was mixed dropwise with an equal volume of the cryoprotective medium. The second aliquot was mixed with plain cryoprotectant, while the third aliquot was mixed with the same cryoprotectant supplemented with crocin (Sigma Chemical, St. Louis, MO, USA) 10 µg/ml. The in-house medium contained 10% glycerol, 10% human serum albumin (HAS), 133 mM glycine, 5.5 mM glucose, 100 mM Trehalose, 12.2 mM sodium pyruvate, and 20 mM hydroxyethyl piperazineethanesulfonic acid (HEPES). The mixtures were loaded into 0.25 ml straws and left to incubate at 4˚C for 12 minutes, then inserted into a pre-cooled in-house aluminium block, previously submerged in liquid nitrogen.

The fourth and fifth aliquots were cryopreserved by the liquid nitrogen vapor technique. The aliquots were mixed dropwise with an equal volume of warm (37°C) cryoprotective media (SpermFreeze, Lifeglobal, USA), the fourth aliquot without crocin supplementation, and fifth aliquot with crocin 10 µg/ml. The mixtures were loaded into straws and incubated at room temperature for 12 minutes, then placed in a horizontal position ten centimeters above liquid nitrogen for 15 minutes, before plunging into liquid nitrogen.

After at least one week of storage in liquid nitrogen, the straws were warmed in water at room temperature (25-28 °C). Warmed samples were washed in Earle's Balanced Salt Solution (EBSS) (second, fourth aliquots without crocin supplementation and third, fifth aliquots with crocin 10 µg/ml) and centrifuged at 200 g for five minutes to remove the cryoprotectants. Post-warmed samples were immediately assessed for sperm motility and kinematics, sperm morphology, viability, extracellular and intracellular ROS level and DNA integrity.

Sperm assessment

Sperm motility and kinetics were assessed using an HTM IVOS II computer-assisted semen analyzer (CASA; Hamilton Thorne Biosciences, Beverly, MA), equipped with a Clinical Human Motility II software. The kinematic parameters measured included: the velocity of smooth average cell path (VAP), mean curvilinear velocity (VCL), mean straight-line velocity (VSL), the amplitude of lateral head displacement (ALH), percent linearity (LIN = VSL/VCL x100) and percent straightness (STR = VSL/VAPx100).

The washed samples were smeared on glass slides and labeled accordingly. They were stained with Diff-Quick, and then assessed with HTM IVOS II computer-assisted semen analyzer (CASA; Hamilton Thorne Biosciences, Beverly, MA). For every slide, at least 200 spermatozoa were read in duplicates.

Sperm viability assessment, 10 µl from each aliquot was mixed with 10 µl of 0.5% Eosin-Y (Sigma Chemical) on a glass microscopic slide. Viable sperm appeared unstained, whereas the stained ones (red) were dead. At least 200 spermatozoa were counted in duplicates.

The level of extracellular ROS was assessed by a chemiluminescence technique, using a Glomax 20/20 luminometer (Turner Biosystems Inc., Sunnyvale, CA, USA). This method was used because it is currently the most sensitive and most used method to measure ROS levels. In principle, ROS and specific reagents will react and emit photons that pass through the photomultiplier tubes (PMT) of the luminometer. The results are measured as relative light units (RLU) of counted photons per minute (CPM) or as millivolts per second (mV/s). An earlier study determined a cut-off value of ROS for normal semen as < 20 RLU/sec/10^6. In our study, the intra-assay coefficient of variation (CV) of this method was 9.7% and the inter-assay CV was 9.8%.

The level of intracellular ROS (hydrogen peroxide radicals) was assessed by an imaging flow cytometry technique, using Amnis® FlowSight (Merck, Darmstadt, Germany)[17]. The machine combines the speed, sensitivity, and phenotyping abilities of flow cytometry with the detailed imagery (x20 magnification) and functional insight of microscopy. At least 5,000 live spermatozoa were assessed per sample.

We employed a sperm chromatin dispersion (SCD) test to assess sperm DNA fragmentation. The

principle of the technique involves sperm embedded in an agarose matrix and lysed to deproteinize the nuclei. Spermatozoa with intact DNA will show extended halos of DNA dispersion. The halos represent relaxed DNA loops, while non-dispersed chromatin displays DNA fragmentation.

Statistical analysis

The sample size was calculated based on a post-cryopreservation sperm motility. We regarded 10% absolute increase in sperm motility to be of clinical significance if the crocin supplementation was effective, with 90% power with an alpha error of 0.05, 25 subjects were required.

Data expressed as mean were compared by repeated measure analysis of variance (ANOVA) if they were normally distributed. Otherwise, they were transformed into normal distribution before ANOVA tests. If there were significant differences, Tukey HSD post-hoc test was done. A p value < 0.05 was statistically significant.

Results

Thirty-six normozoospermic semen samples were included in this study. Participants' age and pre-processing sperm parameters are shown in Table 1.

Table 1. Mean age (± SD) and sperm parameters (± SD) of participants.

Parameters	Mean ± SD
Age (years)	34.8 ± 5.0
Volume (ml)	3.3 ± 1.6
Sperm concentration (millions/ml)	62.7 ± 41.5
Total motility (%)	71.1 ± 16.0
Progressive motility (%)	65.6 ± 16.3
Normal morphology (%)	10.8 ± 4.6

SD: standard deviation

Sperm motility (93.54 ± 3.55%, 70.10 ± 10.94% and 53.58 ± 12.06% in controls, SSV and LNF groups, respectively, p < 0.001) and sperm viability (79.82 ± 18.86%, 57.74 ± 21.99% and 49.69 ± 22.25%, p < 0.001) decreased significantly after cryopreservation in both methods (Table 2). The solid surface vitrification (SSV) group had a higher post-thawed sperm motility (70.10 ± 10.94% vs 53.58 ± 12.06%, p < 0.001) and viability (57.74 ± 21.99% vs 49.69 ± 22.25%, p < 0.001) than LNF groups (Table 2). Supplementation with crocin 10 µg/ml in the cryoprotective agents did not improve sperm motility and sperm viability in both methods.

Table 2. Sperm motility, viability, morphology, ROS levels, and DNA integrity in controls and post-cryopreserved samples, with or without crocin supplementation in the cryoprotective media.

Parameters (n = 36)	Control	Vitrification		Liquid nitrogen vapor	
		SSV	SSV + crocin	LNF	LNF + crocin
Total motility (%)	93.54 ± 3.55	70.10 ± 10.94 [a,b,c]	71.96 ± 12.23 [a,b,c]	53.58 ± 12.06 [a]	55.16 ± 17.79 [a]
Progression (%)	89.34 ± 5.24	63.61 ± 11.52 [a,b,c]	65.33 ± 12.20 [a,b,c]	47.20 ± 12.57 [a]	48.90 ± 17.00 [a]
Viability (%)	79.82 ± 18.86	57.74 ± 21.99 [a,b,c]	59.92 ± 22.91 [a,b,c]	49.69 ± 22.25 [a]	49.90 ± 21.34 [a]
Morphology (%)	16.22 ± 7.98	14.47 ± 9.67	14.15 ± 7.18	14.04 ± 8.89	14.46 ± 9.00
ROS (RLU/sec/106)					
Extracellular	1.01 ± 1.33	3.22 ± 7.10	2.66 ± 6.54	4.66 ± 9.48	3.69 ± 8.96
Intracellular	315.83 ± 250.61	275.07 ± 229.61	334.25 ± 313.91	282.13 ± 258.69	347.7 ± 420.11
DNA fragmentation (%)	44.69 ± 10.16	39.64 ± 12.19	40.53 ± 14.13	41.67 ± 9.57	43.97 ± 12.36

ROS: reactive oxygen species, DNA: deoxyribonucleic acid, SSV: solid surface vitrification, LNF: liquid nitrogen vapor freezing

Repeated measure ANOVA, [a] p < 0.001 significant differences vs. control, [b] p < 0.001 significant differences vs. LNF, [c] p < 0.001 significant differences vs. LNF + crocin

VAP, VCL, and ALH significantly decreased after cryopreservation by both methods when compared to controls. STR significantly increased in the SSV group (Table 3). Sperm DNA fragmentation and sperm morphology were not different between controls and cryopreserved groups (Table 2). Crocin supplementation did not show any change in the percentage of sperm DNA fragmentation and sperm morphology in both cryopreservation groups. Both cryopreservation methods increased extracellular ROS when compared with controls, but the increase was not significantly different (Table 2, $p > 0.05$). Supplementation with crocin in cryoprotective agents decreased ROS level, but this decrease did not reach statistical significance in both groups ($p > 0.05$). Both cryopreservation methods non-significantly decreased intracellular ROS when compared with non-cryopreserved controls. Crocin supplementation slightly increased intracellular ROS levels in both methods, but this increase did not reach statistical significance in both groups ($p > 0.05$).

Table 3. Sperm kinematics in controls and post-cryopreserved samples, with or without crocin supplementation in the cryoprotective media.

Parameters (n = 36)	Control	Vitrification		Liquid nitrogen vapor	
		SSV	SSV + crocin	LNF	LNF + crocin
VAP	64.61 ± 12.05	46.91 ± 10.82 [a]	51.34 ± 11.34 [a]	50.19 ± 12.02 [a]	49.83 ± 10.93 [a]
VSL	43.81 ± 12.71	35.67 ± 10.64 [b]	39.98 ± 11.21	36.29 ± 11.21 [c]	37.65 ± 10.62
VCL	127.84 ± 25.28	95.20 ± 17.72 [a]	101.88 ± 20.23 [a]	105.98 ± 24.04 [a]	105.19 ± 20.62 [a]
ALH	7.12 ± 1.37	5.30 ± 0.88 [a]	5.41 ± 1.07 [a]	5.80 ± 1.24 [a]	5.70 ± 1.03 [a]
STR	67.72 ± 9.93	73.45 ± 8.27 [d]	74.90 ± 7.75 [e]	70.33 ± 8.73	70.66 ± 8.84
LIN	36.92 ± 9.06	38.61 ± 7.48	40.25 ± 7.60	35.79 ± 7.47	35.95 ± 7.30

SSV: solid surface vitrification, LNF: liquid nitrogen vapor freezing

Repeated measure ANOVA [a] $p < 0.001$ significant differences vs. control, [b] $p = 0.022$ significant differences vs. control, [c] $p = 0.042$ significant differences vs. control, [d] $p = 0.046$ significant differences vs. control, [e] $p = 0.005$ significant differences vs. control

Discussion

Crocin was chosen as a supplement in this study because previous in vitro experimental and clinical studies have shown its beneficial effects on sperm parameters and embryo quality[14, 15], and there has been no study on its use in human sperm cryopreservation. Sperm viability, DNA fragmentation, and morphology did not change significantly after cryopreservation, which was in agreement with our previous studies[10]. Post-cryopreserved sperm had a significant decrease in total motility, progressive motility, and many sperm kinematic parameters. However, the importance of CASA sperm kinematics is not completely understood and still under debate. Current data indicates that VSL and VCL are important kinetic parameters that are related to the speed of sperm motion. They are better correlated with fertility in humans than the percentage of motile sperm[18]. A study by Donnelly et al[19] found that the percentage of morphologically normal sperm correlated most strongly with IVF fertilization rate, and VAP correlated most significantly with pregnancy rate. In our study, it was reassuring that cryopreservation did not result in a significant change in sperm morphology, but it decreased total and progressive motility, VAP, VSL, VCL, and ALH. The decrease in these sperm kinematics could be due to cryoinjury itself or because of ROS, or both. LIN (= VSL/VCL x 100) did not change significantly in both cryopreservation groups, because there was a proportionate reduction in both VSL and VCL. On the other hand, the percent straightness (STR = VSL/VAP x 100) significantly increased in the vitrification group, compared with a non-significant increase in the liquid nitrogen vapor group. Clinically, post-cryopreserved sperm in the vitrification group would cover more distance in a shorter period of time than those in the liquid nitrogen vapor group, implying a better sperm kinematic. A previous study found that samples from which IVF pregnancy resulted had significantly higher LIN and STR than those from which pregnancy was not achieved[19].

We evaluated both the intra- and extracellular ROS levels in the non-frozen controls and both cryopreserved groups. We hypothesized that cryoinjury would increase the levels of ROS both inside and outside the cells. The findings that extracellular ROS levels were increased after both cryopreservation methods, and decreased, although not significantly, by the addition of the antioxidant crocin, was compatible with our assumption. However, the non-significant decrease in intracellular ROS after both cryopreservation methods was unexpected. Our results were in agreement with similar studies that demonstrated a non-significant decrease in intracellular ROS after programmable freezing of boar sperm[20], and liquid nitrogen vapor freezing of fish sperm[21]. In another study on human sperm cryopreservation, using the liquid nitrogen vapor techniques, there was a significant reduction in the percentage of ROS-positive sperm by transferase-mediated dUTP nick end labeling (TUNEL) assay after freezing compared to that before freezing[22]. The reasons behind the reduction in the amount of intracellular ROS were not known, but these authors postulated that it was probably due to cryoinjury to mitochondria, which resulted in both the reduction in intracellular ROS production and the energy required for sperm motility. Unfortunately, they did not measure extracellular ROS to support their assumption. The increased levels of extracellular ROS in our study suggested that ROS production was increased and then transported to the outside. This increase should come from the spermatozoa rather than exogenous sources, as leukocytospermia was one of our exclusion criteria, and we processed the sperm through density gradient centrifugation to clean up dead spermatozoa and white cells before cryopreservation. The measured levels of intracellular ROS might have been falsely low, probably due to the inactivation of esterase enzymes. This process could occur through protein oxidation by ROS itself or through cryoinjury. With a decrease in the activity of esterase enzymes, less amount of dichlorodihydrofluorescein diacetate (DCFH-DA) would be converted to dichlorodihydrofluorescein (DCFH), resulting in a lower level of the fluorescent signals from 2' , 7' - dichlorofluorescein (DCF). We postulated that crocin supplementation counteracted the detrimental effect of ROS on the esterase enzyme, thereby; increase the amount of the fluorescent signals. We observed from the imaging flow cytometer that the presence of intracellular ROS localized in the sperm head and proximal sperm tail. This was compatible with current knowledge that intracellular ROS were mainly produced from two sources: 1) the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system at the plasma membrane of the sperm head, and 2) the NADH-dependent oxidoreductase system in the mitochondria[6]. The intensity of fluorescent signals from the sperm head was observed to be more intense and more obvious than those from the sperm tail. We had no information on the amount of ROS that was contributed by each compartment. However, the lower intensity of intracellular ROS signals in the tail compartment might have been due to its smaller size, rather than a lower production rate. ROS are known to diffuse readily through specialized aquaporin pores in the cell membranes. ROS produced from mitochondria could, therefore, exit the tail compartment more readily than those from the head compartment due to its higher surface to volume ratio. Our finding that crocin supplementation in the cryopreservation media did not significantly reduce the amount of extra-cellular ROS could be due to many factors. Pharmacokinetic studies indicate that crocin ($C_{44}H_{64}O_{24}$; MW 976.972 Daltons) is a very polar substance and is not absorbed into the blood circulation as an intact molecule after oral ingestion. There is no information on its diffusion across the cell membrane[23]. In general, a polar molecule with > 5 hydrogen bond donors, > 10 hydrogen bond acceptors, and a molecular size > 500 Daltons are usually not membrane permeable[24]. We, therefore, assumed that crocin might remain largely outside the cells to combat ROS that were released from the spermatozoa. In this regard, crocin might act passively to scavenge ROS after they had exerted their detrimental effects inside the spermatozoa. The other reason for its ineffectiveness was that the amount of crocin required for optimal antioxidant activity could vary

among different individuals, making it difficult to see a beneficial effect when the same dose was used in all subjects. The third possible reason was that crocin was added to the cryoprotective media and sperm washing solution, but it was absent in sperm preparation. As ROS were known to be released after osmotic change and centrifugation[25], this could limit the beneficial effect of crocin in our study. Despite an adequate sample size in this study, we encountered many limitations that should be addressed in future studies. First, we should incorporate both intra- and extracellular antioxidants to see if the combination could significantly improve post-cryopreserved sperm survival. The inclusion of an agent that directly inhibited ROS production might also be beneficial. This cocktail of antioxidants and ROS inhibitors should be present both in the cryopreservation and washing media throughout the entire process of sperm processing, freezing, and washing. It was also advisable to use a direct method of intracellular ROS measurement, instead of an indirect one that was employed in this study.

Conclusion

In conclusion, the supplementation of crocin during vitrification and liquid nitrogen vapor freezing did not improve the outcome of post-thaw sperm.

Potential conflicts of interest

The authors declare no conflicts of interest.

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