Review article

Antioxidants Effects on Human Sperm Cryopreservation: A Scoping Review

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ABSTRACT

Background

Sperm Cryopreservation has become a routinely used procedure in assisted reproductive technology. This procedure keeps sperm preserved for later use for patients who will undergo chemotherapy, have ejaculation problems, and many other medical indications. This procedure allows the patients to preserve their fertility potential for having their offspring in the future by storing the sperm in a very low temperature of liquid nitrogen. However, this technique raises some concerns regarding the reduction in post-thawed quality. This adverse effect is caused by thermal shock, osmotic shock, and oxidative stress during the freezing and thawing process. Among these, oxidative stress is considered the primary factor contributing to the significant decline in post-thawing sperm parameters. The administration of antioxidants during cryopreservation is promised to have beneficial impacts on cryopreserved semen.

Objective

This review focused on discussing the beneficial impacts of antioxidant supplementation in human sperm cryopreservation.

Methods

We conducted a scoping review by the recommendations of the Joanna Briggs Institute and the Systematic Reviews and Meta-Analyses Extension for Scoping Reviews (PRISMA-ScR). This review focused on discussing the beneficial impacts of antioxidant supplementation in human sperm cryopreservation.

Results

From 444 records, 17 articles have been selected and extracted from four databases: Wiley, Ebsco, PubMed, and ScienceDirect.

Conclusion

The results indicated that the use of antioxidants, including both natural and synthetic ones, provides beneficial effects and better reproductive results for cryopreserved human sperm.

Keywords

sperm cryopreservation; oxidative stress; antioxidant, fertility; scoping review

INTRODUCTION

Cryopreservation is a procedure that preserves cells or tissues by cooling the samples to a very low temperature using cryogenic agents[1]. This technique is widely used in the reproductive field, aiming to keep the gametes, embryos, or reproductive tissues and maintain their reproductive potential for future use. Reproductive issues related to male factors, such as cancer treatment that involves chemotherapy and radiotherapy, erection or ejaculation problems, hormonal problems due to obesity, congenital abnormality and systemic disease that affect sperm quality are some indications for the sperm cryopreservation procedure[2-6].By freezing the sperm, a man can maintain their reproductive potential and keep the opportunity of having his offspring through assisted reproductive technology[4].

However, instead of the advantage of sperm cryopreservation, this method has some issues regarding the post-thawing sperm quality. The

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cryopreservation process causes the change in sperm structure and function due to thermal shock, osmotic shock, and mainly oxidative stress during the freezing and thawing process[4,7]increase in ROS level during cryopreservation becomes the primary source of DNA alteration that induces apoptosis[8]. The high level of ROS will alter DNA integration, affecting abasic sites, cross-linking, nitrogen base modification, and DNA strand damage[9]. Oxidative stress due to ROS imbalance and sperm structural damage will significantly affect the number of sperm oocyte-activating factors, directly causing a reduction in the fertilization rate. Furthermore, the decrease in sperm structural and functional damage was hypothesized to be the root of the low fertilization rate, implantation rate, and miscarriage of IVF patients[9-11] The administration of antioxidants during the cryopreservation process is believed to help reduce the oxidative stress level, thus can maintain the sperm parameters and increase the fertilization rate[10-12]. Many studies have been conducted on the use of antioxidants in sperm cryopreservation, utilizing various types of antioxidants, doses, procedures, and measured outcomes to address this issue. However, there are still gaps in knowledge regarding the optimal and standardized results that can provide the best option for patients. Based on this, we need to conduct a scoping review to compile information on the effect of antioxidant administration during cryopreservation on sperm quality.

METHODS

This scoping review followed the recommendations of the Joanna Briggs Institute (JBI) and The Systematic Review and Meta-Analysis Extension for Scoping Reviews (PRISMA-ScoR), except for calculating the risk of bias assessment, as this is not indicated for scoping reviews. The databases used in this study are International Journal Websites, PubMed, Wiley, Ebsco, and ScienceDirect. The keywords applied for the title and abstract are ("sperm cryopreservation" OR "semen cryopreservation" OR "sperm freezing") AND ("antioxidant" OR "antioxidant" OR "antioxidants" OR "antioxidant"). The article selection was also based on inclusion and exclusion criteria, including year of publication, language, type of literature, and open-access accessibility. The title and abstract of the articles also screened article themes. Databases were last accessed on August 9, 2024. All screened papers were collected and uploaded into Mendeley for the duplication removal process. Abstract and title screening was performed by MM, followed by full-text Screening by MM, MP, and DR. No disagreement arose between the reviewers.

For data extraction, we use the PCC framework (Population, Concept, Context) to resolve the research question. The population (P) consisted of men with normal sperm parameters. At the same time, the concept (C) was the adverse impact of oxidative stress on cryopreserved sperm, and the context was to explain the effect of antioxidant administration on cryopreserved sperm.

Following the JBI guidance, we use descriptive statistics to collate the data of selected papers. Relevant information related to the research question was manually extracted and inputted into an Excel spreadsheet. Descriptive data of the articles, including title, first author, time of the study, freezing method, and objective of the study, were described in Table 1. Characteristics of the subjects, including the number of participants, age, and country, are presented in Table 2. The effects of antioxidants on cryopreserved sperm, including the type of antioxidants used, dosage, intervention, parameters measured, and results, are reported in Table 3.

The papers extracted were selected based on inclusion and exclusion criteria as follows:

Inclusion Criteria

- The theme of the study was sperm cryopreservation
- The Independent variable was the antioxidant
- The year of publication was 2014-2024
- Written in English
- Full text available
- Open access articles
- Original research articles
- Human as the subject of study

Exclusion Criteria

- Freezing method other than the standard method
- Abnormal sperm parameter of the subject

RESULTS

We used the keywords "sperm cryopreservation" OR "semen cryopreservation" OR "sperm freezing" and "antioxidant" OR "antioxidant" OR "antioxidants" OR "antioxidant" for the article selection process.

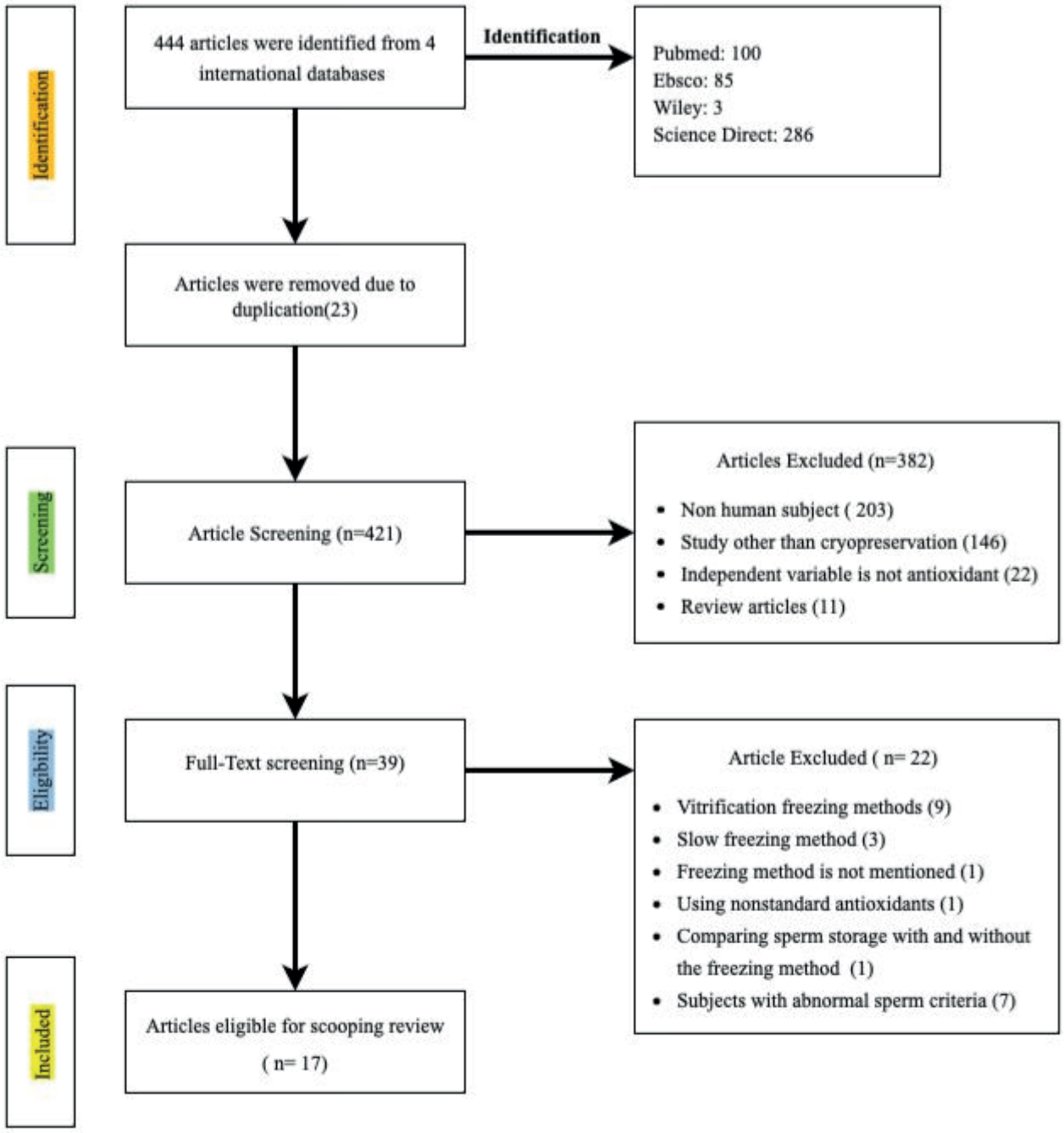

Figure 1. PRISMA Diagram

Along with the searching process using the mentioned keyword, we screened for publication years from 2014 to 2014, full-text and open-access articles, an English language preference, and original research articles, resulting in 444 articles being included in the review process.

The identified 444 articles were then subjected to a duplication check, during which 23 articles were removed. We continued the process with abstract screening, where we excluded 382 articles because they used non-human subjects, the study focused on cryopreservation, the independent variables were not

antioxidants, and they were review articles. The abstract screening was then followed by a full-text screening, during which we omitted 22 articles due to the use of a nonstandard freezing method or the absence of method details, including the use of nonstandard antioxidants. This treatment process compared the freezing and non-freezing groups and used abnormal sperm patients, resulting in 17 articles that were eligible for a scoping review.

We extracted the data from these articles, including the title, author, country of study, methods, and results. The study overview is presented in Table 1.

Table 1. Overview of Selected Studies

No	Title	Authors, Year	Objective
1	Canthaxanthin Protects Human Sperm Parameters during Cryopreservation	Najafi et al, 2019	to examine the effects of canthaxanthin on human sperm motility, viability, morphology, acrosome reaction, chromatin packaging, and DNA integrity after the freeze-thaw process with the rapid freezing method.
2	Carboxylated Poly-L-lysine Potentially Reduces Human Sperm DNA Fragmentation after Freeze-Thawing, and Low-Dose Resveratrol Enhances Its Function	Tachibana et al, 2023	to determine that resveratrol (RES) synergizes with CPLL and enhances the SDF-reducing effects that occur during the freeze-thawing of human sperm.
3	Protective Effects of Curcumin on the Outcome of Cryopreservation in Human Sperm	Santonastaso, 2021	to assess the effects of curcumin supplementation in a freezing medium on preventing cryo-damage in human semen
4	Green Tea Extract as a Cryoprotectant Additive to Preserve the Motility and DNA Integrity of Human Spermatozoa	Alqawasmeh et al, 2021	to evaluate the GTE as a potential additive in cryopreservation media of human spermatozoa
5	L- proline as a Novel Additive to Cryopreservation Media improved post-thaw quality of human spermatozoon via reducing oxidative stress	Moradi et al, 2022	to evaluate the effects of L- L-proline supplementation in cryopreservation medium on normozoospermic semen samples
6	Leptin Improves Sperm Cryopreservation via Antioxidant Defense	Fantoura, 2016	Demonstrating the effect of cryopreservation on sperm DNA fragmentation (DNAf) and investigating the possible effects of sperm capacitation techniques and Leptin in vitro incubation
7	Novel Additive for Sperm Cryopreservation Media: Holotheria parva Coelomic Cavity Extract Protects Human Spermatozoa Against Oxidative Stress—A Pilot Study	Khasavi et al., 2019	tested the effects of the extract of the coelomic cavity of five Holotheria parva, a marine organism rich in antioxidants, for its ROS-scavenging activity and cryoprotective effects on oxidative stress
8	The Protective Effects of Melatonin against Cryopreservasi-Induced Oxidative Stress in Human Sperm	Karimfar et al., 2018	to evaluate changes in post-thaw motility, viability, and intracellular ROS and malondialdehyde (MDA) in response to the addition of Melatonin to human sperm freezing extender
9	Melatonin Affects Membrane Integrity, Intracellular Reactive Oxygen Species, Caspase3 Activity, and AKT Phosphorylation in Frozen-Thawed Human Sperm	Najafi, 2018	evaluated the effects of various concentrations of Melatonin (0–15 mM) on human sperm parameters and levels of intracellular reactive oxygen species during cryopreservation
10	Oxidative Stress Measurement in Frozen/Thawed Human Sperm: The Protective Role of an In Vitro Treatment with Myo-Inositol	Ponchia, 2021	to determine the in vitro impact of myoinositol in ameliorating sperm oxidative status during sperm cryopreservation
11	Phosphatidylcholine and L-Acetyl-Carnitine-Based Freezing Medium Can Replace Egg Yolk and Preserves Human Sperm Function	Sicchieri et al, 2020	Comparing the efficacy of a synthetic cryoprotectant supplemented with L-α-phosphatidylcholine (PC) and L-acetyl-carnitine (ANTIOX-PC) and the standard egg-based TEST-yolk buffer (TYB) in sperm cryopreservation
12	Supplementation of Cryoprotectant with Pinus massoniana Bark Extract Improves Human Sperm Vitality and Fertility Potential	Li et al., 2020	to determine whether adding PMBE to cryoprotectant could improve human sperm quality after cryo-resuscitation and to investigate the potential regulatory mechanisms.
13	Cryoprotective Effect of Sericin Supplementation in Freezing and Thawing Media on the Outcome of Cryopreservation in Human Sperm	Aghaz et al., 2018	to investigate the effects of sericin supplementation in freezing and thawing media on frozen-thawed human sperm quality

No	Title	Authors, Year	Objective
14	Supplementation of cryopreservation medium with TAT-Peroxiredoxin 2 fusion protein improves human sperm quality and function.	Liu et al., 2018	To investigate the potential effects of TAT-PRDX2 protein supplementation to the cryopreservation medium on post-thaw sperm
15	The effect of Vitamin B12 Supplementation on Post-Thaw Motility, viability, and DNA damage of human sperm	Hosseinabadi, 2020	to investigate the effects of vitamin B12 supplementation on human sperm parameters during the cryopreservation process.
16	Addition of Tempol in Semen Cryopreservation Medium Improves the Post-Thaw Sperm Function	Bateni, 2014	to evaluate the role of Tempol in the cryopreservation of human semen using a commercially available cryopreservation medium.
17	Effect of Selenium on Human Sperm Parameters after Freezing and Thawing Procedures	Rezaeian al, 2016	To evaluate the effects of pre-freezing treatment of human semen samples with Selenium on semen parameters after the thawing procedure.

The seventeen articles above describe the antioxidants used in the study and its objectives. A total of 16 types of antioxidants were used in human sperm cryopreservation, with various parameters evaluated.

Table 2. Subject Characteristic

No	Number of participants	Age	Country
1	25	not mentioned	Iran
2	24	38±6.5 y.o	Japan
3	60	36.5 ± 6.2 y.o	Italy
4	45	37±2.7 y.o	China
5	30	not mentioned	Iran
6	45	35.3±4.8 y.o	Brazil
7	50	30 (median age)	Iran
8	43	32.51 ± 4.13 y.o	Iran
9	44	not mentioned	Iran
10	25	not mentioned	Italy
11	63	not mentioned	Brazil
12	not mentioned	not mentioned	China
13	51	32.51 ± 4.13	Iran
14	50	26-35 y.o	China
15	30	not mentioned	Iran
16	23	not mentioned	Iran
17	42	32.1 ± 3.9 y.o	Iran

The average age of the participants was 33.8 ± 4.1 years; however, some studies did not provide data on the participants' ages. Iran has become the country with the most studies conducted on the use of antioxidants in sperm cryopreservation. The next was China, followed by Brazil, Italy and Japan. The number of participants involved in the study ranged from 25 to 63. However, one study conducted in China did not specify the number of participants; instead, it mentioned the criteria for the sample, which were men with normozoospermia. Most of the patients were men who were coming to the fertility center and were admitted for some infertility issues due to female factors or others. (Table 2)

Various antioxidants were used in studies of human sperm cryopreservation. The antioxidant administrated can also be grouped as natural antioxidants such as curcumin, greentea extract, Holoteria parva, canthaxantin, and pinus manosiana[15,16,17,18,19] or chemical ones, for example L-proline, carboxylated poli L lysine, Melatonin, myoinositol, tempol and Selenium[20,21,22,12]. Both regimens were thought to have beneficial effects on human sperm cryopreservation. The study employed various dosage types. Some studies used only one dose[23], attempting to compare the antioxidant and non-antioxidant groups. Others used various doses of antioxidants and attempted to compare among intervention groups, aiming to evaluate the best results given[21,24,22]. One study using phosphatidylcholine and L acetylcysteine and was trying to create an antioxidant combination supplementation thought that it would enhance the result on cryopreserved sperm[25] (Table 3)

Table 3. The Effect of Antioxidants on Cryopreserved Sperm

No	Antioxidant	Dosage	Intervention	Parameters	Results
1	Canthaxanthin	0; 0,1;1;10;25 µM	canthaxanthin with different concentrations was added to a freezing medium	sperm viability, morphology, acrosome reaction, DNA denaturation, chromatin packaging, DNA fragmentation	10 and 25 µM canthaxanthin improved chromatin packaging, acrosome integrity, DNA denaturation, and fragmentation, total motility, viability, normal morphology, while one µM canthaxanthin only improves the chromatin packaging, acrosome integrity, and DNA denaturation
2	Carboxylated poly L lysine (CPLL)	0,1mM	comparing the freezing media supplemented with and without CPLL	SDF, ROS levels in mitochondria and living sperm and lipid peroxidation (LPO), MMP (mitochondrial membrane potential)	CPLL can reduce SDF via inhibition of intracytoplasmic ROS and Lipid Peroxidation
3	Curcumin	2,5; 5; 10 and 20 µM	comparing different concentrations of curcumin supplementation in freezing medium	sperm parameters, DNA fragmentation, intracellular ROS, and glutathione peroxidase 4 (GPX4)	Supplementation with 20 µM curcumin in a freezing medium caused increases in progressive and nonprogressive motility, reduced ROS level and DNA fragmentation, and upregulating GPX4
4	Green tea Extract	1 ng/mL	the semen was cryopreserved with and without 1.0 ng/mL GTE.	motility, ROS level, and DNA fragmentation	the addition of 1.0 ng/mL GTE to cryopreservation media significantly increased sperm motility and DNA integrity
5	L proline	0, 1, 2 and 4 mmol/L	Cryopreservation media were supplemented with different concentrations of L- proline (0, 1, 2 and 4 mmol/L).	sperm parameters, SDF. Sperm dispersion chromatin, chromomycin, ROS, MDA, TAC	4mmol L proline increased motility and viability, TAC, decreased ROS and MDA, and improved chromatin damage.
6	Leptin	1 ng	comparing the capacitated sperm supplemented with and without Leptin prior to freezing	oxidative measurement and SDF	the addition of Leptin before freezing to capacitated sperm reduced DNA fragmentation and enhanced SOD and glutathione peroxidase
7	Holoteria parva	25, 50, 100, 250, 250, 500 and 1000 µg/ml	comparing different concentrations of Holoteria parva supplementation in sperm freezing medium	motility, morphology, vitality, DNA fragmentation	the addition of 250 µg/ml Holoteria parva had a significant effect on sperm motility, while ROS levels decreased on 250 and 500 µg/ml dosages
8	Melatonin	0.001, 0.005, 0.01, 0.05, 0.1, and 1 mM.	comparing the different concentrations of Melatonin supplementation in sperm-freezing medium	motility, viability, intracellular ROS, and MDA	All doses increased motility and viability and reduced intracellular ROS and MDA, except at 0.001 mM. The most effective dose is 0.01mM
9	Melatonin	3mM	comparing two groups: supplemented with Melatonin and without Melatonin	motility, viability, membrane integrity, intracellular ROS (H2O2 and O2- level), caspase three activity, and AKT phosphorylation.	Melatonin significantly decreased intracellular ROS, caspase 3 activity, the number of dead and apoptotic sperm, and increased viability, motility, and AKT phosphorylation.

No	Antioxidant	Dosage	Intervention	Parameters	Results
10	Myoinositol	20 mg/mL	comparing the group without myoinositol, the group supplemented with myoinositol pre-freezing and post-freezing	vitality, motility, oxygen consumption rate, proteomic analysis (CO group)	myoinositol increased sperm vitality and motility, oxygen consumption rate, and level of carbonylated protein) compared to non-treated samples.
11	Phosphatidylcholine dan L acetylcysteine (ANTIOX-PC)	3% w/v Phosphatidylcholine and 6 % w/v acetylcysteine	comparing 1 group supplemented with phosphatidylcholine dan l acetylcysteine (ANTIOX-PC) and the other group with standard egg-based test yolk	motility and chromatin quality (DFI)	ANTIOX PC retained higher nonprogressive motility and lower immotile sperm compared to TEST Yolk Buffer (TYB).
12	Pinus massoniana	50 µg/mL	6 groups: fresh, CPA, CPA+PMBE (50 µg/mL PMBE/ Pinus Montana Bark Extract), CPA+ procyanidins(23.8 microgram/mL, procyanidin B2) CPA+DMSO (5%DMSO)	oxidative stress (dichlorofluorescein diacetate staining and MDA content) and antioxidant capacity (glutathione peroxidase activity and reduced glutathione content)	PMBE promotes post-thaw vitality and acrosome reaction, enhances mRNA and protein expression of SOD2, reduces intracellular ROS, and preserves antioxidant capacity and mitochondrial function
13	Sericin	1: 0; 0,5; 1; 2,5; and 5%	comparing freezing media supplemented with sericin and post-thawing media supplemented with sericin	morphology, motility, viability, and DNA fragmentation	exp 1: 2,5 and 5% concentration of sericin in the freezing medium increased viability and total motility and also decreased DNA fragmentation significantly/ exp 2: The addition of 5% sericin in the thawing medium increased total motility, viability, and decreased DNA fragmentation compared to those without supplementation
14	TAT peroxiredoxin	150 µg/mL	each patient type was divided into three groups: fresh, frozen without TAT peroxiredoxin, freezed+TAT peroxiredoxin	motility, viability, mitochondrial potential, DNA damage, ROS level and lipid peroxidation	The addition of 150 µg/ml TAT-PRD reduced intracellular ROS and MDA and enhanced post-thaw sperm motility and viability compared to the control but had no significant effect on OAT. TAT-PRDX reduced spontaneous acrosome reaction and increased Ca ionophore-induced acrosome reaction.
15	Vitamin B12	0, 0.5, 1, 2, 2.5 mg/ml	comparing five different concentrations of vitamin B12 added to freezing media	motility, viability, DNA fragmentation,	2 mg/ml was considered the optimal concentration of vitamin B12 for evaluating sperm DNA fragmentation. The results showed that 1 and 2 mg/ml of vitamin B12 significantly increased post-thawing motility and viability compared with the 0 mg/ml vitamin B12 ($p < .05$). Also, by supplementing with 2 mg/ml of vitamin B12, DNA fragmentation decreased when compared to the control
16	Tempol	5 µM	comparing the freezing media supplemented with and without Tempol	DNA fragmentation, motility, viability, ROS	tempol improved motility and viability post thawing and reduced DNA fragmented sperm and percentage of ROS-positive sperm

No	Antioxidant	Dosage	Intervention	Parameters	Results
17	Selenium	5µg/ ml	comparing washed and unwashed sperm groups, each group was further divided into two subgroups, where one subgroup supplemented and the other not supplemented with five µg/mL selenium	morphology, viability, DNA damage, motility	motility in unwashed Selenium-treated samples was higher compared to washed untreated samples. In washed sperm samples, DNA damage decreased in treated samples compared to untreated ones. The morphology of washed treated samples was higher than that of unwashed untreated samples. Morphology in unwashed treated samples is higher than in both unwashed samples. Washed-treated samples had higher normal morphology compared to washed-untreated samples

The parameter assessed in these review studies includes: a) sperm parameters (motility, viability, and morphology), b) DNA damage markers (DNA fragmentation index, DNA denaturation, and chromatin packaging index), c) oxidative markers (total antioxidant capacity, ROS level, internal antioxidant level, for example, Superoxide dismutase (SOD), Glutathion peroxidase, malondialdehyde (MDA) and level of carbonylated protein) d) sperm function (acrosome reaction, oxygen consumption level, caspase 3 activity, and number of apoptosis sperm), e) sperm structural damage (mitochondrial function, acrosome integrity and lipid peroxidation level).

The results of the studies explained the impact of antioxidant agents on sperm cryopreservation. From the variables assessed during the studies, here is a brief description of the result

- Motility

  Most antioxidants administered during sperm cryopreservation yield positive results, showing a significant increase in motility, except for Leptin, Pinus manosiana, and Selenium. The increase in motility was calculated for the percentage of progressive and total motility for each sample.

- Viability

  More than 10 studies out of 17 presented a significant increase in post-thawed sperm viability compared to the control group. A study using various doses of Melatonin showed significant increases in all treatment groups (0.005 mM, 0.01 mM, 0.05 mM, 0.1 mM, and 1 mM). However, we did not find any significant differences in studies using carboxylated polylisin, green tea extract, Leptin, Holoteria parva, phosphatidylcholine, L-acetyl cysteine, and Selenium.

- Morphology

  A study using canthaxanthin and Selenium showed a significant increase in the morphology of cryopreserved sperm supplemented with antioxidants.

- GPX4mRNA expression

  GPX4 mRNA expression showed a significant increase only in studies using curcumin and green tea extract.

- Oxidative markers level (SOD, glutathione peroxidase and TAC)

Some antioxidants were confirmed to reduce Intracellular ROS levels; these included carboxylated polylysine, curcumin, L-proline, Holoteria parva (at all treatment doses), Melatonin (at all treatment doses), Pinus masonii, TAT peroxiredoxin, and Tempol. However, only carboxylated polylysine showed a reduction of extracellular ROS levels. Regarding the antioxidant level, SOD and glutathione peroxidase tended to increase significantly in a study using leptin supplementation. On the other hand, only Leptin had successfully increased the TAC level, while only myoinositol was proven to increase the oxygen consumption rate.

- Acrosome reaction and integrity

  Functional tests of sperm, assessing the acrosome reaction, revealed a significant increase in the study using Pinus mannosiana and TAT peroxiredoxin.

In contrast, the evaluation of acrosome structure, measuring acrosome integrity, presented a higher level in the canthaxanthin study.

- Mitochondrial Membrane Potential (MMP)

  Mitochondrial function was assessed by measuring the mitochondrial membrane potential level, which was successfully increased by carboxylated polylysine and TAT peroxiredoxin.

- Sperm DNA Fragmentation (SDF) and Chromatin Packaging

  One of the important parameters of sperm function was sperm DNA fragmentation. From the 16 types of antioxidants discussed in this review, only eight antioxidants demonstrated a reduction in SDF on cryopreserved sperm, including canthaxanthin, curcumin, green tea, Leptin, sericin, vitamin B, Tempol, and Selenium. On the other hand, a study using canthaxanthin and L-proline demonstrated a reduction in chromatin packaging.

- Malondialdehyde (MDA) and Lipid Peroxidation (LPO)

  MDA was the important compound produced as a side product of lipid peroxidation, and it was found to decrease in a study using L-proline, Melatonin (at all doses), Pinus mannosiana, and TAT peroxiredoxin. At the same time, carboxylated polylysine was reported to have a significant reduction in lipid peroxidation.

- Proteomic Analysis

  Proteomic analysis has been performed in a few studies, and myoinositol has been shown to decrease the proteomic analysis results.

- Apoptotic Sperm Cell and Caspase 3 Activity

  Some studies have attempted to evaluate the reduction of apoptotic sperm cells and also caspase-3 activity as a marker. Melatonin demonstrated the positive result at a dose of 3 mM.

DISCUSSION

To date, sperm cryopreservation is commonly practiced in the reproductive technology field, with average post-thawing results still becoming an issue due to its significant reduction in sperm quantity and quality(4). The natural characteristics of sperm demonstrate their fragility in facing thermal shock, osmotic shock, and oxidative stress during and after the process[26]. The minimum cytoplasmic content in sperm, the loss of endogenous antioxidant enzymes in seminal plasma during the sperm preparation session, and the triggering of ROS production and antioxidant imbalance during steps in cryopreservation resulted in a lower number and quality of sperm for the in vitro fertilization procedure[27]. The potential effect of antioxidants in neutralizing and minimizing oxidative stress in various cell types throughout the body, both in vivo and in vitro, has drawn the attention of many scientists to focus on the study of the beneficial impacts of antioxidant supplementation during sperm cryopreservation[4].

To date, numerous studies have focused on sperm cryopreservation. Some of the studies used animals as the research objects, usually in the husbandry field, with the objection trying to obtain the best post-thawing sperm for animal breeding or as part of biotechnology research[28-29]. In this review, we aim to focus on human sperm cryopreservation to achieve more applicable results. From 17 studies, various antioxidants were used to supplement the freezing media. Natural antioxidants, including Curcumin, Greentea, Pinus manosiana, and Holoteria parva, were claimed to give promising results with minimum side effect[15-17,19]. On the other hand, more studies were likely to use the synthetic ones based on the prominent and standardized effects[31,20,32,33,21,21,25]. However, both types of antioxidants have their study backgrounds and standard procedures to follow.

The freezing process, where sperm is mixed with freezing media and then exposed directly to liquid nitrogen at an extremely low temperature (-196 °C), is believed to be the primary source of oxidative stress [34]. Thus, the attempt to minimize the adverse impact of oxidative stress focused on this step. Only one study using Leptin was designed to give antioxidants prior to freezing by incubating the sperm sample with Leptin overnight before freezing. Prior to freezing, some sperm preparation procedures aimed to select good immotile quality sperm. There were various types of sperm preparation, including simple washing, swim-up, density gradient centrifugation (DGC), and microfluidic[9,27]. In this review, we selected studies using the DGC method, as it is the most widely applied method for sperm preparation in fertility clinics[35]. It was said to have a higher risk of ROS production during the process[36]. Despite the objective of selecting the best sperm to be preserved, sperm preparation is known to have adverse consequences due to its higher rate of

oxidative stress resulting from the loss of antioxidant enzymes, as well as the removal of seminal plasma and repetitive centrifugation [27].

Regarding the results, each study assessed basic parameters, including sperm motility, viability, and morphology. Satisfactory results were obtained in the motility and viability assessments. Ninety percent of the study reported an increase in motility in post-thawing sperm following antioxidant supplementation, and about 70% reported viability improvement. However, Morphology increased only in 2 studies using Canthaxantin and Selenium [18,12]. Sperm motility reduction was the most commonly documented effect of sperm cryopreservation, with a decrease ranging from 25% to 75% in post-thawed sperm, and specifically 50% to 75% for progressive motility reduction. Motility is highly sensitive to oxidative stress due to its strong correlation with mitochondrial and plasm membrane function that are susceptible to high ROS levels, and antioxidants were proven to protect these two main aspects during cryopreservation [15,31]. On the other hand, viability was highly dependent on membrane integrity. Sperm membrane composition of PUFA is known to be highly sensitive to ROS. Antioxidants act as ROS scavengers and induce antioxidant enzyme production, thus preventing the defect of the membrane [19,23]. On the other hand, the morphology condition came from the spermatogenesis process, which was quite problematic to improve with antioxidants. (reference) Moreover, structural damage to sperm is highly affected by many factors, rather than oxidative stress, such as mechanical damage caused by crystal ice formation and osmotic shock, which ascribe to damage to the lipid and protein in the plasma membrane, causing defects in the sperm's head, midpiece, tail, and acrosome. However, this kind of damage was known to be irreversible. Thus, the administration of antioxidants produced the least effect on morphology improvement [37]. The structural defect of sperm was also assessed by measuring acrosome integrity, where only canthaxanthin showed a positive result regarding this parameter[18]. Sperm DNA fragmentation and chromatin packaging measurement were the following parameters that should be taken into account. The increase in SDF during cryopreservation is primarily induced by rapid osmotic changes that occur during freezing, which are believed to trigger the production of ROS from mitochondria, increasing [38].

Furthermore, excessive ROS was thought to increase lipid peroxidation, leading to the formation of MDA as the end product. Some antioxidants, with their specific mechanisms, such as electron donation, can terminate the ROS chain reaction, reduce mitochondrial ROS production, maintain membrane fluidity, support endogenous antioxidant production, and prevent apoptotic signaling[9]. These antioxidants have been proven to have a significant effect on lipid peroxidation, MDA levels, the level of apoptotic sperm cells, and caspase 3 activity.[32,21,19,39,20,31.]

The sperm function evaluation was also assessed by measuring the MMP and acrosome reaction levels, showing that TAT peroxiredoxin yielded positive results for both parameters[39]. Additionally, other antioxidants that were reported to increase the MMP were Pinus mannosiana and carboxylated poly L-lysine to increase the acrosome reaction [19,20]. Furthermore, the evaluation of oxidative markers reported promising results in reducing intracellular ROS levels by most of the antioxidants in the studies [20,22,19,17]. Another study using Leptin also confirmed the increase in TAC and extracellular antioxidants; however, we also obtained favorable results showing an increase in oxygen consumption rate. Moreover, Leptin has also been proven to increase endogenous antioxidants, such as SOD and glutathione peroxidase[33].

Furthermore, the sperm function evaluations were also determined by the proteomic analysis and apoptosis markers. The evaluation of these markers was performed by studies using Melatonin and Myoinositol, reporting a significant decrease in both parameters[31,23]. Tracing back the antioxidants characteristic of this review study reveals that they share some standard mechanisms for preventing or neutralizing oxidative stress conditions. Some of the potential benefits include the capability of directly scavenging ROS by binding to the free radical compound through their chemical structures or an indirect mechanism by inducing the production of endogenous antioxidants. Both are known to be effective methods in reducing and balancing ROS intra- and extracellularly. Some antioxidants have been reported to possess specific characteristics that mitigate rapid osmotic changes, such as osmoprotectors, genomic stability agents, immune response barriers associated with oxidative stress, stabilizers of plasma membranes, and even the recovery of the seminiferous epithelium. [32,16,21,31,20,24.]

From 17 studies, the basic parameter is to measure sperm quality (motility, viability, and morphology). Furthermore, some studies tried to evaluate the molecular aspects of cryopreservation damage and oxidative markers [24,15,12]. However, various molecular parameters across the studies made it impossible for us to compare the results thoroughly. On the other hand, variations also arose from the detailed procedure of DGC preparation, such as gradient concentration, duration of centrifugation, centrifugation speed, and temperature[27]. Regarding the cryopreservation steps, we can find that there was variation in cryoprotectant media, exposure to LN2 vapor prior to the immersion in LN 2, duration of storage, and centrifugation speed post-thawing[33,15,40.] All of the factors mentioned above are claimed to have the potential to affect the cryopreserved sperm. Variation also comes from the tools or evaluation tools to assess the parameters of studies [32,24,24]; this could lead to different interpretations of the results and somehow cannot be fully compared among studies.

Summary

Overall, antioxidants tended to give functional improvement rather than structural one. Many more studies with comprehensive parameters and standardized freezing protocols are needed to determine the most optimal antioxidant supplementation regimen. However, limitations in human resources, such as collecting and preserving samples, have led to many aspects of the effect of antioxidants on maintaining sperm parameters after freezing remaining vague and, to some extent, unknown. Thus, further and deeper studies regarding antioxidants and sperm cryopreservation are required.

Contribution Summary Statement

Meidona Nurul Milla (MNM) performed the screening, wrote the manuscript, developed the main conceptual idea, and designed the review under the supervision of Dicky Mohammad Rizal (DMR), Mulyoto Pangestu (MP), and Dewajani Purnamasari (DP). Dewajani Purnamasari performed a complete abstract assessment during the screening process, while Meidona Nurul Milla and Mulyoto Pangestu conducted the full-text screening.

Conflict of Interest

There is no conflict of interest regarding this review paper.

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Authors' Contribution

Data gathering and idea owner of this study: Meidona Nurul Milla, Dicky Mohammad Rizal

Study design: Meidona Nurul Milla, Dewajani Purnamasari, Mulyoto Pangestu

Data gathering: Meidona Nurul Milla, Mulyoto Pangestu

Writing and submitting manuscript: Meidona Nurul Milla

Editing and approval of final draft: Meidona Nurul Milla, Dicky Mohammad Rizal, Mulyoto Pangestu

Appendix 1

Manual Data Extraction Instrument adapted from Pollock et al [42]

Domain	Element	Detail
Publication	Publication Type	Published articles
	Titel	As listed
	Year	Publication date
	Author/s	Name/s
	Country	Where Data Gathered From
	Research design (category)	Quantitative
Design	Methodology	Laboratory in vitro experimental
	Aims/Concept/Objectives	As stated in the publication
	Participant Age at the time of study	The age range in years
Participants	Participant sex/gender	male
	Preservation Intervention Type/s Included	Standard sperm freezing method with antioxidant supplementation in freezing media
Findings	Quantitative data of sperm parameters, oxidative parameters, DNA damage parameters	Results stated in the articles
	Conclusions	As reported in the paper

Appendix 2: Search Strategy

1. sperm cryopreservation OR semen cryopreservation OR sperm freezing: 15412
2. antioxidant OR anti oxidant OR antioxidants OR antioxidant: 823786
3. 1 &2: 3059
4. Filtering full-text dan open access articles: 586
5. Filtering English language articles: 561
6. Filtering research articles: 444

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