Received: 30 September 2019 | Revised: 27 November 2019 | Accepted: 13 December 2019

DOI: 10.1111/and.13514

INVITED REVIEW

First International Journal of Andrology ANDROLOGIA WILEY

The efficacy of antioxidants in sperm parameters and production of reactive oxygen species levels during the freeze-thaw process: A systematic review and meta-analysis

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Abstract

To investigate the impact of antioxidants in sperm parameters and reduction in reactive oxygen species production during the freeze-thaw process. PubMed, Scopus, Web of Science, Embase and Cochrane central library were systematically searched. Of the 1583 articles, 23 studies were selected for data extraction. Our results show that antioxidants improved sperm progressive motility (standardised mean difference (SMD) = 1; 95% CI: 0.62, 1.38; $p < .001$) and viability (SMD = 1.20; 95% CI: 0.50, 1.91; $p = .001$) and reduced sperm DNA fragmentation (SDF) and hydrogen peroxide (H_2O_2) production, but there was no significant improvement in total sperm motility after thawing. Acetyl-l-carnitine/l-carnitine, melatonin and catalase had a significant positive impact on progressive motility. The role of tempol and melatonin in improving viability was significant compared to other antioxidants. Moreover, a significant reduction in SDF was observed after addition of butylated hydroxytoluene, tempol and vitamin E. However, the prevention of H_2O_2 production was significant only after the addition of tempol. Our overall results displayed the positive impact of antioxidants on progressive sperm motility, viability and reduction in SDF and H_2O_2 production, but no significant impact of antioxidants on total sperm motility was seen during the freeze-thaw process.

KEYWORDS

antioxidant, DNA fragmentation, freeze-thaw, reactive oxygen species, sperm parameter

1 | INTRODUCTION

Since the 1960s, sperm cryopreservation has been introduced as a routine practice in infertility clinics (Najafi et al., 2018). This technique is being utilised for several indications, including donor insemination programmes, before treatments which my hurt fertility potential such as chemotherapy, radiotherapy, orchiectomy and vasectomy, testicular sperm retrieval in azoospermic cases and epididymal sperm aspiration (obstructive azoospermia), gender reassignment, spinal cord injury and hypogonadotropic hypogonadism (Banihani & Alawneh, 2019; Pabon & Meseguer, 2019; Wierckx et al., 2012; Yang, Xu, Cui, Wu, & Liao, 2019). Freezing spermatozoon is performed before intracytoplasmic sperm injection (ICSI) to rule out cycle cancellation for failure to collect semen specimen on the day of the in vitro fertilisation/ICSI attempt (Montagut et al., 2015). It also can be a helpful technique for fertility preservation in men serving in the military and travelling husbands (Dean, Caplan, & Parent, 2016).

Inherent reactive oxygen species (ROS) production during the freeze-thaw process causes oxidative stress (OS) and sperm cryo-injury (Condorelli et al., 2017). In fact, a drastic change in temperature

from 37 to −196°C during cryopreservation leads to OS after production of ROS (Hatef, Taromchi, Nejatbakhsh, Farrokhi, & Shokri, 2017). Excessive generation of ROS causes physical and chemical changes in the sperm membrane, decreases sperm motility, vitality, DNA integrity and fertilising potential due to peroxidation of polyunsaturated fatty acid (PUFA) of sperm membrane. Combined with limited availability of free radical scavenging system, it makes the spermatozoa more vulnerable to free radicals induced sperm damage (Karimfar et al., 2015; Malo et al., 2010). Recent research has shown that the freeze-thaw process may decrease 25–75 percent of sperm motility subsequent to mitochondrial damage in the mid-piece of spermatozoa. In fact, ROS have been cited to be responsible for sperm DNA damage during the freeze-thaw process (Najafi, Halvaei, & Movahedin, 2019).

ROS are unstable and highly reactive lacking an electron in their outer shell and include a) superoxide anion (O_2^-), hydroxyl radical and hydroperoxyl radical, b) nonradical species, such as hypochlorous acid and hydrogen peroxide (H_2O_2), and c) reactive nitrogen species and free nitrogen radicals, such as nitroxyl ion, nitrous oxide and peroxynitrite (Condorelli et al., 2017).

Different antioxidants (enzymatic and nonenzymatic) have been investigated to neutralise OS (Zhang et al., 2016). Recently, Amidi et al. reviewed the effectiveness of vitamin E, glutathione, superoxide dismutase (SOD), catalase (CAT), vitamin C, L-cysteine, ergothioneine, melatonin, selenium and natural herbs such as genistein, resveratrol, quercetin, Rhodiola sacra aqueous extracts and rosemary on the quality of human and animal spermatozoa during cryopreservation. They reported the protective effect of antioxidants against ROS production on human, goat, ram, bull, canine and boar sperm quality. However, they concluded that more research is warranted to a draw conclusive evidence regarding the use of antioxidants in a clinical setting (Amidi, Pazhohan, Nashtaei, Khodarahmian, & Nekoonam, 2016).

More recently, Hezavehei et al. discussed the different conventional freezing techniques, advanced methods of cryopreservation and novel strategies (such as addition of cryoprotectants, antioxidants, fatty acids, antifreeze proteins, nanoparticles, animal serum or plant essential oils), which have been shown to protect the human and animal spermatozoa against cryo-injury. These authors examined different studies which investigated the effect of addition of various cryoprotectants (permeable and nonpermeable) and antioxidants on sperm freezing media and their ability to reduce cell damage. They indicated that supplementing the freezing media by various antioxidants (vitamin E analogous, glutathione, l-cysteine, melatonin, l-carnitine, taurine, hypotaurine, CAT, SOD, cholesterol-loaded cyclodextrins) improves sperm motility, viability, DNA integrity, acrosomal reaction and membrane integrity and reduces lipid peroxidation and ROS production. They concluded that additional studies are needed to assess the clinical use of different antioxidants (alone or in combination with each other) on different sperm parameters to identify the best conditions for sperm freezing (Hezavehei et al., 2018).

To the best of our knowledge, this is the first systematic review and meta-analysis aiming to evaluate the effect of antioxidants on reducing the detrimental effects of ROS and improving sperm quality during the freeze-thaw process.

1.1 | METHOD

This study was done by following the checklist provided in the Preferred Reporting Items for Systematic Review and Meta-Analyses (PRISMA). Comprehensive research was performed in PubMed, Embase, Scopus, Web of Science and Cochrane electronic databases from February 1988 until April 2018. The following terms were considered in the titles and abstracts: ("spermatozoa" OR "spermatozoon" OR "sperm") AND ("cryopreservation" OR "cryofixation" OR "cryonic suspension" OR "freezing" OR "freeze-thaw process" OR "post-thaw" OR "frozen-thawed" OR "rapid freezing" OR "slow freezing" OR "vitrification" OR "chilled storage") AND ("antioxidants" OR "antioxidant effect" OR "antioxidant effects"). All references of the final articles selected for the study were investigated via a manual search in order to identify more relevant and eligible articles.

1.2 | Selection criteria

The inclusion criteria were a) interventional study, b) reports on the impact of antioxidants on sperm parameters and functions after thawing and comparison with the control group (group without addition of antioxidants), c) addition of antioxidants to cryomedia before freezing, d) assessment of the effect of antioxidants after thawing by an examiner in a blind fashion, e) studies involving semen samples from adult human men (over 18 years old), f) semen samples received after an abstinence of 3–5 days, g) sperm freezing following a minimum of 30 min of liquefaction, h) freezing each sample for at least 24 hr, i) normal semen sample was selected according to WHO guideline 2010 of sperm volume ≥ 1.5 (ml); sperm concentration ≥15 × 10^6/ml, total motility ≥ 40%, progressive motility > 32%, viability > 58% and normal morphology > 4%, j) asthenozoospermic sample was selected if total motility < 40% or progressive motility < 32%, k) oligoozoospermic sample selected if sperm concentration <15 × 10^6/ml and L) men who had fathered a child in the past year were identified as fertile men. The exclusion criteria were animal studies, narrative reviews, conference abstracts (not containing enough data) and editorials. All articles were screened by two authors (R.B and I.H) according to the title, abstract and full-text. Conflicts about studies were resolved by a third author.

1.3 | Data extraction

Data were extracted from the selected articles by two authors (R.B and I.H) and discrepancies were resolved via consultation. In addition, the following information was acquired from each article:

author's name, year of publication, geographic location of participants, and concentration of antioxidant, sample size, sperm motility (total and progressive), sperm vitality, ROS level and SDF.

1.4 | Statistical analysis

Standardised mean differences (SMD) with 95% confidence interval (95%CI) for total sperm motility, progressive sperm motility, viability, ROS level and SDF between intervention and control groups were calculated for both antioxidant and control groups. Also, Plot digitizer software was applied to digitalise and extract the data when presented in the graphic form only. The concentration of all antioxidants was presented in mM/ml and μM/ml. The random-effect model (hedges) was applied to all analyses.

p values < .1 resulting from Cochran's *Q* test and I^2 values of >50% were considered to exhibit significant heterogeneity among the studies. Subgroup analysis was conducted according to the antioxidant type since the impact of antioxidants on post-thaw sperm quality varied from antioxidant to antioxidant and also depending on the selected concentration. Sensitivity analysis was performed using a removal method in which low-quality studies were removed and analysis was repeated. Additionally, the potential publication bias was evaluated by visual examination of Beggs' funnel plots and Egger's regression test. All tests were carried out using STATA software (StataCorp, Texas, USA). Statistically significant differences were defined when *p* values were less than .05.

2 | RESULTS

2.1 | Study selection process

Study selection methods are summarised in Figure 1. During the initial search of five databases according to the subject and search strategy, 1583 articles were identified. After the first and second screenings according to the titles and abstracts respectively and excluding animal and in vivo studies, 137 articles were available for further evaluation; 68 duplicate articles were removed, and 69 abstracts were available for examining and reviewing the full-text article. During reviewing of the full-texts, 22 conference abstracts were found without full-texts, 4 articles were written in other languages than English, one of them was a duplicate report, two of them were not interventional studies (one of them was a clinical approach, one article was association study) and one abstract was referred to a fake journal and no full-text could be found. Thirty-nine full-text articles were selected for third screening. During manual search and cross-verification of references, 15

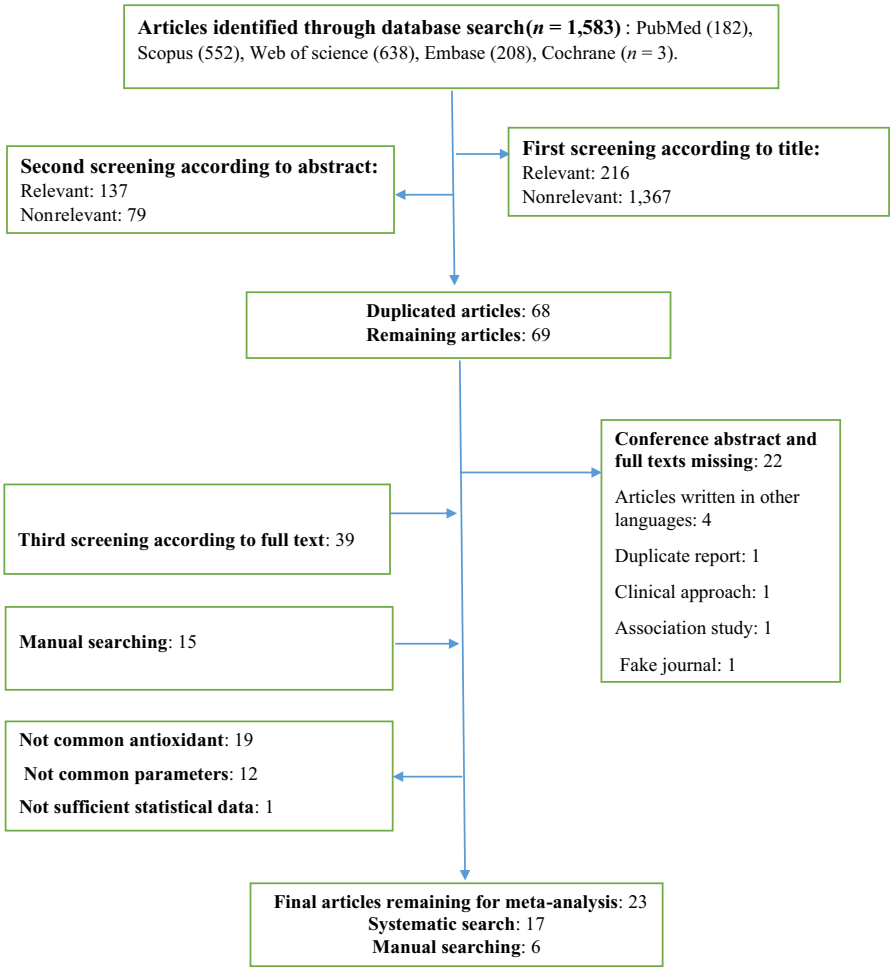

FIGURE 1 Systematic review and meta-analysis flow chart showing the database searches, the number of abstracts screened, the full texts retrieved and the articles analysed

relevant articles were found and included in the earlier list. Finally, 54 articles were selected for assessment. During full-text screening, no common antioxidants were identified in nineteen articles (Asadmobini, Bakhtiari, Khaleghi, Esmaeili, & Mostafaei, 2017; Brugnon et al., 2013; Fontoura et al., 2017; Isachenko et al., 2008; Kotdawala et al., 2012; Lu et al., 2018; Meamar et al., 2012; Naeini, Bafrani, & Nikzad, 2014; Najafi et al., 2016; Oehninger, Duru, Srisombut, & Morshedi, 2000; Parameswari et al., 2017; Pariz & Hallak, 2016; Saeednia et al., 2015; Sbracia, Grasso, Sayme, Stronk, & Huszar, 1997; Sobhani, Eftekhaari, Shahrzad, Natami, & Fallahi, 2015; Taylor, Roberts, Sanders, & Burton, 2009; Vickram et al., 2015; Werner et al., 2017), twelve articles lacked common sperm parameters (Banihani, Agarwal, Sharma, & Bayachou, 2014; Branco, Garcez, Pasqualotto, Erdtman, & Salvador, 2010; Donnelly, McClure, & Lewis, 1999; Esteves, Spaine, & Cedenho, 2007; Gadea et al., 2011; Garcez et al., 2011; Garcez, Branco, Lara, Pasqualotto, & Salvador, 2010; Jenkins, Aston, & Carrell, 2011; Li, Lin, Liu, Xiao, & Liu, 2010; Minaei et al., 2012; Nabi et al., 2017; Shabani Nashtaei et al., 2017) and one article lacked sufficient data (Karimfar et al., 2015). Finally, 23 articles (Aliabadi, Jahanshahi, Talaei-Khozani, & Banaei, 2018; Askari, Check, Peymer, & Bollendorf, 1994; Azadi, Tavalaee, Deemeh, Arbabian, & Nasr-Esfahani, 2017; Bateni et al., 2014; Deng et al., 2017; Duru, Morshedi, Schuffner, & Oehninger, 2000; Esteves, Sharma, Thomas, & Agarwal, 1998; Ghorbani et al., 2015; Kalthur et al., 2011; Keshtgar, Iravanpour, Gharesi-Fard, & Kazerooni, 2016; Martinez-Soto, Dioshourcade, Gutiérrez-Adán, Landeras, & Gadea, 2010; Merino et al., 2015; Moubasher, Din, Ali, El-sherif, & Gaber, 2013; Najafi et al., 2018; Nekoonam, Nashtaei, Naji, Zangi, & Amidi, 2016; Rossi, Mazzilli, Delfino, & Dondero, 2001; Taylor et al., 2009; Thomson et al., 2009; Varghese et al., 2005; Wang, Sikka, Veeraragavan, Bell, & Hellstrom, 1993; Zhang et al., 2016; Zou et al., 2017; Zribi et al., 2012) were entered into the meta-analysis for data extraction. From each article, the optimal dose was selected. Of the various doses examined, if no significant difference in the antioxidant effect on the sperm parameters was observed, the maximum dose used was selected.

2.2 | Study characteristics

The eligible studies and their results are summarised in Tables 1 and 2. All eligible studies selected for meta-analysis are summarised in Table 1. All these interventional studies were published between 1993 and 2017. In addition, the studies were conducted in European countries (n = 3), United States of America (n = 6), Asia (n = 10), Australia and Africa (n = 4). Moreover, nineteen articles investigated nornozoospermic samples, two articles examined both normozoospermic and asthenozoospermic semen samples and one examined asthenozoospermic sample, one normozoospermic and oligozoospermic semen sample. The number of subjects in each study ranged from 8 to 59. The present study contained 522 normal semen samples and 72 abnormal (asthenozoospermic) samples (Table 1).

3 | META-ANALYSIS RESULTS

3.1 | The impact of antioxidants on total sperm motility

Twelve trials were utilised to investigate the effectiveness of antioxidants on this parameter in fertile men. The efficacy of addition of quercetin, tempol, vitamin E, acetyl-l-carnitine/l-carnitine and pentoxifylline to the cryomedium of normal semen sample prior to cryopreservation, on total sperm motility was evaluated and compared to the controls (group without the addition of antioxidant). When subgroup meta-analysis was conducted according to antioxidant type, significant improvement was found in the post-thaw total sperm motility when quercetin (SMD = 1.97; 95%CI: 0.04, 3.90; p = .046, n = 2), tempol (SMD = 1.33; 95%CI: 0.64, 2.01; p < .001, n = 2) and vitamin E (SMD = 0.77; 95%CI:0.31, 1.22; p = .001, n = 4) were added to cryomedium; however, no significant improvement was observed subsequent to addition of pentoxifylline (SMD = −1.20; 95% CI: −3.66, 1.25; p < .001, n = 2) and acetyl-l-carnitine/l-carnitine (SMD = −0.44; 95% CI: −2.31, 1.43; p = .644, n = 2) (Table 2; Figure 2).

3.2 | The impact of antioxidants on progressive sperm motility

Data from nine articles were used to assess the efficiency of acetyl-l-carnitine/l-carnitine, melatonin, vitamin E and CAT on progressive sperm motility of fertile men after thawing and compared to the control groups. Data from two other available articles showed no significant improvement in sperm progressive motility of asthenozoospermic patients subsequent to the addition of acetyl-l-carnitine/l-carnitine (SMD = 1.84; 95%CI: −1.38, 5.05; p = .263, n = 2) (Table 2). In the subgroup meta-analysis, progressive sperm motility increased after adding acetyl-l-carnitine/l-carnitine (SMD = 1.20; 95%CI: −0.02, 2.42; p = .005, n = 3), melatonin (SMD = 1.17; 95%CI: 0.75, 1.58; p < .001, n = 2) and CAT (SMD = 0.65; 95%CI: 0.32, 0.98; p < .001, n = 2). The effect of vitamin E (SMD = 0.80; 95%CI: - 0.21, 1.81; p = .119, n = 2) on progressive sperm motility was not significant (Table 2; Figure 3).

3.3 | The impact of antioxidants on sperm viability

Ten articles were examined to evaluate the efficacy of quercetin, tempol, melatonin, acetyl-l-carnitine/l-carnitine, vitamin E on sperm viability during the freeze-thaw process. According to two other available articles, no significant improvement was found on sperm viability subsequent to adding acetyl-l-carnitine/l-carnitine to cryomedium in asthenozoospermic men (SMD = 2.48; 95%CI: −1.36, 6.32; p = .205, n = 2). Subgroup meta-analysis of normal semen samples showed the significant positive effect of tempol (SMD = 1.39; 95%CI: 91.0, 1.86; p < .001, n = 2) and melatonin (SMD = 1.09; 95% CI: 0.68, 1.50; p < .001, n = 2) on sperm viability after thawing. However, these improvements were not significant after the addition of quercetin

TABLE 1 Characteristics of the randomised clinical trials included in the meta-analysis

Antioxidant	Concentration	Sample. size	Duration of freezing	Parameters	Reference
Melatonin	0, 3 mM	33	14 days	Progressive motility, viability, ROS (H_2O_2)	Najafi et al., 2018
	0, 0.1 mM	20	14 days	Progressive motility, viability, ROS (H_2O_2)	Deng et al., 2017
Quercetin	0, 10 µm	24	.....	Total motility, viability, DNA fragmentation	Azadi et al., 2017
	0, 50 µm	9	7 days	Total motility, viability, DNA fragmentation	Zribi et al., 2012
Genistein	0, 10 µm	20	Long-term storage	DNA fragmentation	Martinez-Soto et al., 2010
	0, 100 µm	20	.....	DNA fragmentation	Thomoson et al., 2009
Catalase	0, 200 U	50	At least 1 day	Progressive motility	Moubasher et al., 2013
	0, 200 U	25	7 days	Progressive motility	Rossi et al., 2001
Vitamin E (Normozoospermia)	0, 80 µm	20	14 days	DNA damage	Nekoonam et al., 2016
	0, 200 µm	12	At least 1 month	Total and progressive motility, viability	Keshtgar et al., 2016
	0, 5,000 µm	38	14 days	Total and progressive motility DNA fragmentation	Kalthur et al., 2011
	0, 200 µm	22	14 days	Total motility	Taylor et al., 2009
	0, 10,000 µm	25	14 days	Total motility, viability	Askari et al., 1994
Acetyl-l-carnitine/L-carnitine (Normozoospermia)	0, 6 mM	33	14 days	Total and progressive motility, viability	Zhang et al., 2016
	0, 4.3 mM	30	2 days	Progressive motility	Aliabadi et al., 2018
	0, 5, 20 mM	10	1 day	Total motility, viability	Duru et al., 2000
	0, 0.176 mM	25	...	Progressive motility	Varghese et al., 2005
Acetyl-l-carnitine/L-carnitine (Asthenozoospermia)	0, 15 mM	35	14 days	Progressive motility, viability	Zou et al., 2017
	0, 6 mM	37	14 days	Progressive motility, viability	Zhang et al., 2016
Butylated hydroxytoluene	0, 0.5 mM	20	....	ROS (O^-_2), DNA fragmentation	Ghorbani et al., 2015
	0, 1 mM	8	....	ROS (O^-_2), DNA fragmentation	Merino et al., 2015
Pentoxifylline (Normal samples)	0, 5 mM	15	2 days	Total motility	Esteves et al., 1998
	0, 3 mM	20	14 days	Total motility	Wang et al., 1993
Tempol	0, 5 µm	24	....	Total motility, viability, DNA fragmentation, ROS (H_2O_2)	Azadi et al., 2017
	0, 0.5, 5, 50 µm	19	....	Total motility, viability, DNA fragmentation, ROS (H_2O_2)	Bateni et al., 2014

(SMD = 3.78; 95%CI: −1.18, 8.73 p = .135, n = 2), vitamin E (SMD = 0.08; 95%CI:-0.38, 0.53; p = .737, n = 2) and acetyl-l-carnitine/l-carnitine (SMD = 0.31; 95% CI:-1.83, 2.45; p = .776, n = 2) (Table 2; Figure 4).

3.4 | The impact of antioxidants on sperm DNA fragmentation

Ten articles were investigated to evaluate the impact of butylated hydroxytoluene (BHT), quercetin, vitamin E, tempol, genistein on reduction in SDF of normozoospermic specimens during the freeze-thaw process (Figure 5). According to subgroup meta-analysis, this reduction was significant after addition of BHT (SMD = −2.50; 95%CI:-3.71, −1.29; p < .001, n = 2), vitamin E (SMD = −1.51; 95%CI: −2.15, −0.87; p < .001, n = 2) and tempol (SMD = −0.82; 95%CI:-1.26, −0.38; p < .001, n = 2), but no significant reduction was detected after the addition of quercetin (SMD = −2.19; 95%CI: −5.34, 0.96; p = .174, n = 2) and genistein (SMD = −0.22; 95%CI: −0.66, 0.22; p = .338, n = 2) to the cryomedium (Table 2).

3.5 | The impact of antioxidants on reduction in ROS level

Four trials were reviewed to evaluate the efficacy of melatonin and tempol on inhibition of H_2O_2 production in normal semen samples

TABLE 2 Outcome of meta-analysis

Sperm outcome	No. of trials	SMD	Pooled %95 CI	Heterogeneity *p*-value for effect	I^2 (%)
Total motility					
Total	12				
Fertile men					
Quercetin	2	1.98	0.04, 3.90	.003	88.7
Tempol	2	1.33	0.64, 2.01	.150	51.7
Vitamin E	4	0.77	0.31, 1.22	.076	56.3
Acetyl-l-carnitine/L-carnitine	2	−0.44	−2.31, 1.43	.001	91
Pentoxifylline	2	−1.20	−3.60, 1.25	<.001	94.4
Progressive motility					
Total	11				
Fertile men	9				
Acetyl-l-carnitine/L-carnitine	3	1.20	−0.02, 2.42	<.001	92.5
Melatonin	2	1.17	0.75, 1.58	.412	0
Vitamin E	2	0.80	−0.21, 1.81	.032	78.3
Catalase	2	0.65	0.32, 0.98	.732	0
Infertile men					
Acetyl-l-carnitine/L-carnitine	2	1.84	−1.38, 5.05	<.001	98.1
Viability					
Total	12				
Fertile men	10				
Quercetin	2	3.78	−1.18, 8.73	<.001	96.8
Tempol	2	1.39	0.91, 1.86	.394	0
Melatonin	2	1.09	0.68, 1.50	.870	0
Acetyl-l-carnitine/L-carnitine	2	0.31	−1.83, 2.45	<.001	93.8
Vitamin E	2	0.08	−0.38, 0.53	.816	0
Infertile men	2				
Acetyl-l-carnitine/L-carnitine	2	2.48	−1.36, 6.32	<.001	98.3
DNA fragmentation					
Total	10				
Fertile men	10				
Butylated hydroxytoluene	2	−2.50	−3.77, −1.29	.115	59.7
Quercetin	2	−2.19	−5.34, 0.96	<.001	95.3
Vitamin E	2	−1.51	−2.15, −0.87	.148	52.3
Tempol	2	−0.80	−1.26, −0.38	.442	0
Genistein	2	−0.22	−0.66, 0.22	0.645	0
ROS level (H_2O_2)					
Total	4				
Fertile men	4				
Melatonin	2	−2.76	−6.45, 0.94	.006	86.9
Tempol	2	−0.43	−0.85, 0	.883	0
ROS level (O_2^-)					
Total	2				
Fertile men					
Butylated hydroxytoluene	2	−0.95	−2.82, −0.92	.003	89

during the freeze-thaw process (Figure 6). According to subgroup meta-analysis, tempol (SMD = −0.43; 95%CI:-0.85, 0; p = .051, n = 2) had sufficient potential for reducing the H_2O_2 level significantly. However, this reduction was not significant following the addition of melatonin (SMD = −2.76; 95%CI: −6.45, 0.94; p = .143, n = 2) (Table 2). Only two articles were available to assess the efficacy of

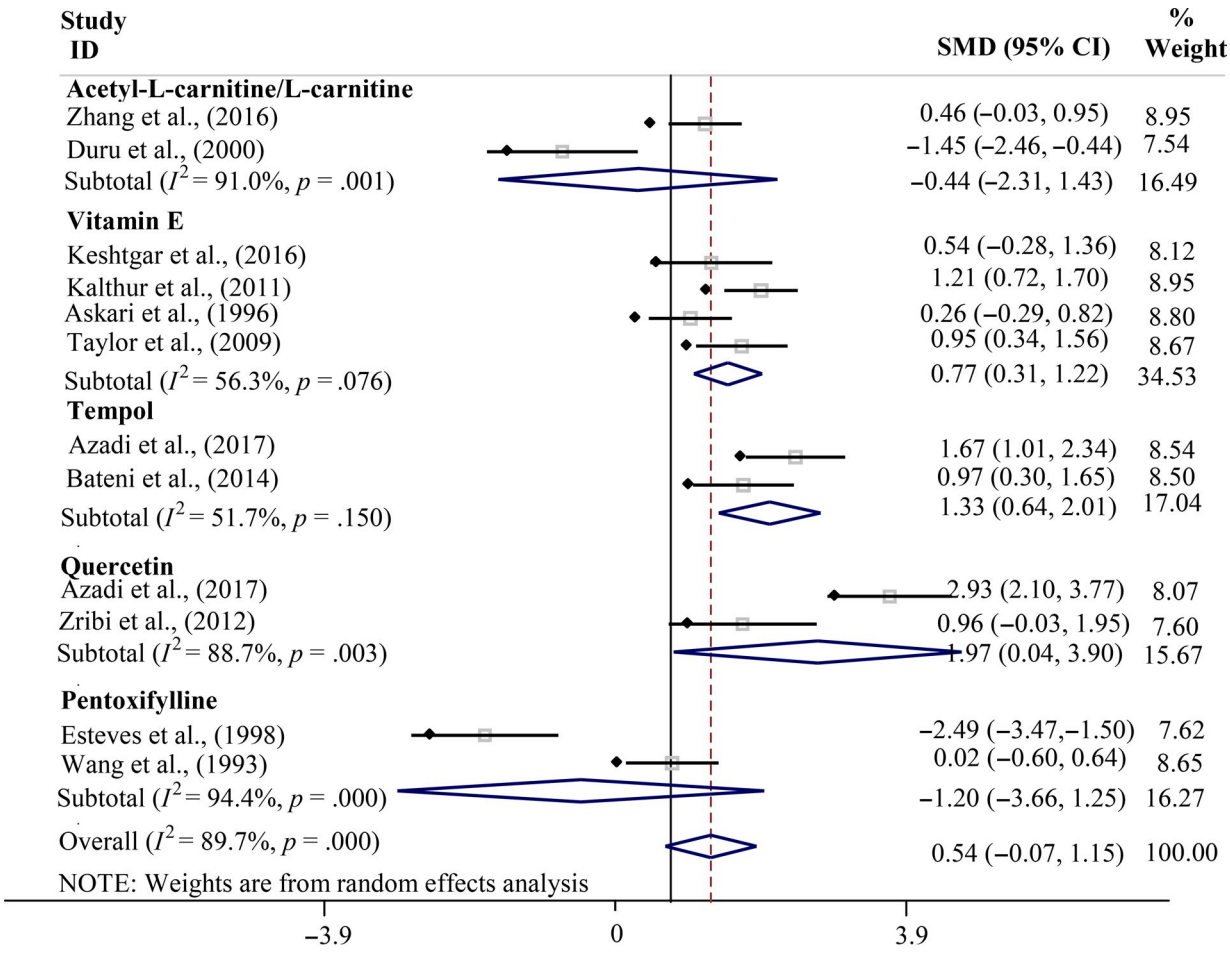

FIGURE 2 Subgroup meta-analysis of the effect of antioxidants on sperm total motility. SMD; standard mean difference; CI: confidence interval

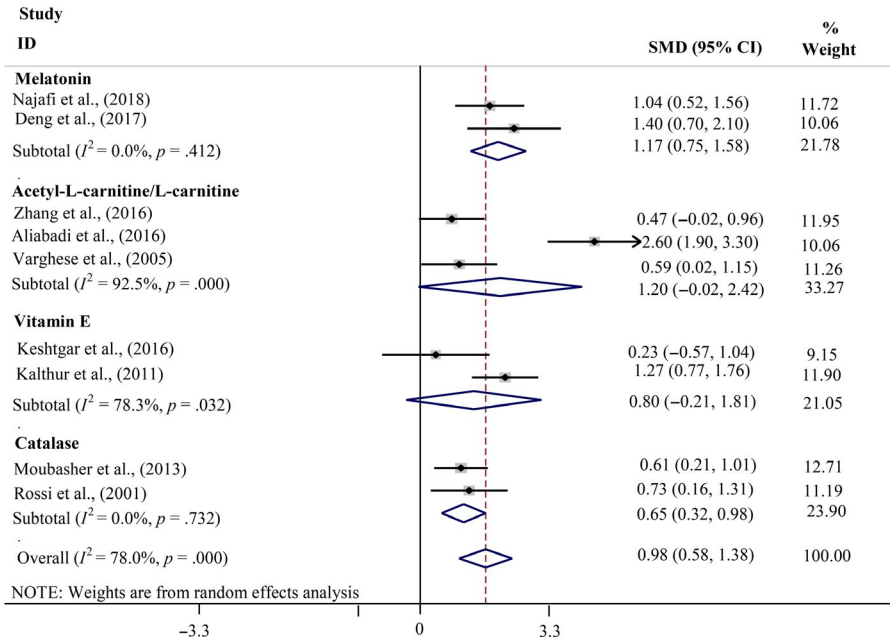

FIGURE 3 Subgroup meta-analysis of the effect of antioxidants on sperm progressive motility. SMD: standard mean difference. CI, confidence interval

antioxidant on reducing O^{-}_{2} production. The results indicated no significant reduction in post-thaw O^{-}_{2} subsequent to addition of BHT to cryomedium before freezing (SMD = −0.95; 95%CI: −2.82, −0.92; p = .320, n = 2) (Table 2).

4 | DISCUSSION

A substantial amount of studies has been performed to investigate the efficacy of antioxidants on sperm parameters during the freeze-thaw process. However, the results of these studies have been inconsistent and controversial. To the best of our knowledge, this is the first meta-analysis that evaluates the efficacy of antioxidants in improving sperm quality after thawing. Sperm viability and motility are the most vulnerable parameters during the freeze-thaw process which endanger the male fertility potential. Although many factors are involved in creating this phenomenon, ROS have been elucidated as the significant contributing factors since sperm cells have inadequate cytoplasm, low antioxidant capacity and considerable amounts of PUFA in their plasma membranes (Azadi et al., 2017; Li et al., 2010). ROS may also induce DNA fragmentation and finally impair the development of foetus and offspring (Shabani Nashtaei et al., 2017). Actually, the presence of a significant amount of l-carnitine and acetyl-l-carnitine in the epididymis suggests the crucial effect of l-carnitine on sperm metabolism and spermatogenesis. In vitro l-carnitine has been utilised as a principal sperm motility booster because fatty acid translocation in the mitochondria which is necessary for the production of ATP may be affected by l-carnitine (Zhang et al., 2016). Recent research has indicated that melatonin is a time and dose-dependent antioxidant, which improves sperm motility by altering mitochondrial respiratory complexes I and IV, improving the potential of the mitochondria transport chain, and scavenging ROS (Deng et al., 2017). Excessive production of ROS during the freeze-thaw process impairs the ability of scavenging system of spermatozoa which leads to peroxidation of PUFA of sperm membrane and a considerable decrease in sperm progressive motility, and integrity of membrane. Rossi et al. suggested that SOD and CAT via prohibition of production of ROS and lipid peroxidation result in good recovery of sperm parameters after the freeze-thaw process (Rossi et al., 2001). However, sperm progressive motility after the supplementation of l-carnitine to cryomedium in asthenozoospermic samples was the same as control group. Zou et al. (2017) showed 7.5 mM of acetyl-l-carnitine as the optimal dose to improve post-thaw sperm motility in asthenozoospermic samples. This result could be attributed to the saturation of l-carnitine transferase I on the outer mitochondrial membrane (Zou et al., 2017). The difference observed between the current meta-analysis and the study performed by Zou et al. (2017) may originate from the fact that asthenozoospermic samples in the latter investigation showed increased ROS level compared to normal samples. Abnormal samples require a higher level of antioxidants to neutralise ROS and protect sperm quality in the thawing phase. The current meta-analysis indicates a significant role of antioxidants on sperm viability in normal semen samples. Since sperm cryopreservation impairs sperm survival rate, using antioxidants improves sperm viability after thawing.

Tempol has been considered to be a cell-penetrating antioxidant, which scavenges superoxide anions and acts as an SOD mimetic agent. It promotes the transformation of O^{-}_{2} to H_2O_2 and decreases the generation of hydroxyl radicals, which have been identified as the

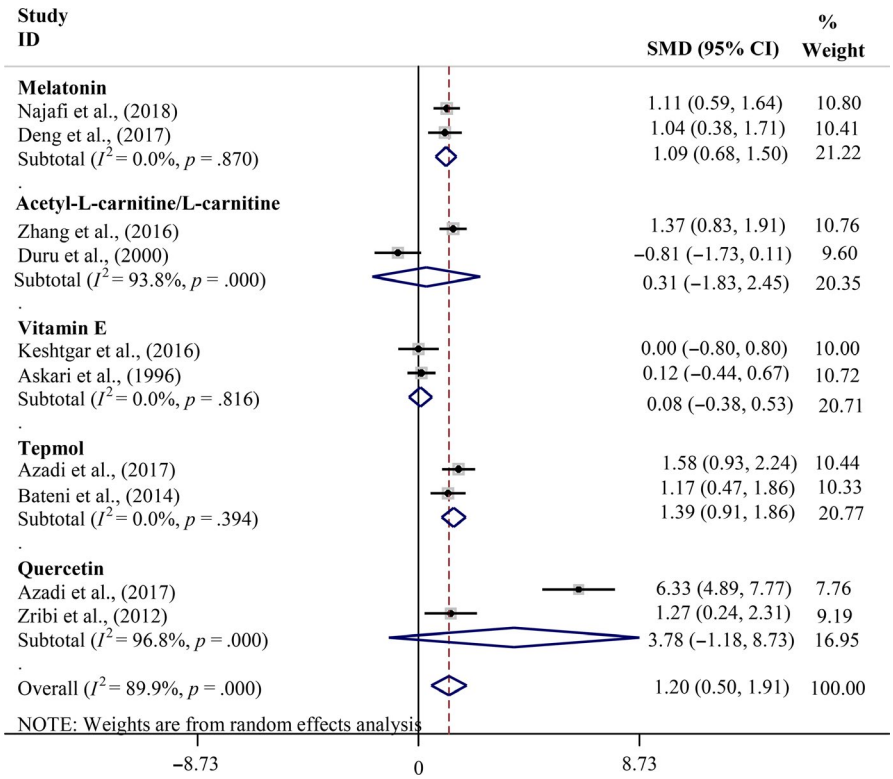

FIGURE 4 Subgroup meta-analysis of the effect of antioxidants on sperm viability. SMD: standard mean difference: CI: confidence interval

most toxic agents via Fenton or Haber Weiss reactions at intra- and extracellular levels (Bateni et al., 2014). It is believed that this nonenzymatic antioxidant improves sperm viability by reducing superoxide anions and H_2O_2 levels (Azadi et al., 2017). Santiani and coworkers studied the effect of tempol on ram sperm cryopreservation and concluded that tempol as a superoxide inducing agent, blocks the hydroxyl radical formation in ram spermatozoa because of reduction in SOD activity after freezing (Santiani et al., 2014). Melatonin reduces the activity of caspase 3 and rate of apoptotic-like and also dead spermatozoa, and eventually improves the survival of spermatozoa after thawing. In fact, caspase 3 is activated after thawing and alters the outer layer of the sperm plasma membrane. It was shown that caspase 3 activity was decreased in the melatonin group in comparison with the control group (Najafi et al., 2018). Macromolecules are protected by melatonin against OS. This antioxidant has high lipid solubility and stimulates the activity of antioxidant enzymes such as glutathione peroxidase, glucose-6 phosphate dehydrogenase, SOD, glutathione reductase and nitric oxide synthase (Kaya, Aksoy, Baspınar, Yıldız, & Ataman, 2001). Our results showed the level of sperm DNA damage was decreased after the addition of antioxidant agents to cryomedium before freezing. Tempol, as a potent antioxidant, protects sperm DNA during cryopreservation. This is attributed to the role of tempol in reducing ROS (Varghese et al., 2005). Acosta et al. evaluated the impacts of tempo and tempol (two SOD analogues) on alpaca semen and showed that the rate of SDF after cryopreservation was similar to fresh group and significantly decreased in the frozen-thawed group without antioxidant supplementation. They concluded that tempol had the potential for maintaining DNA

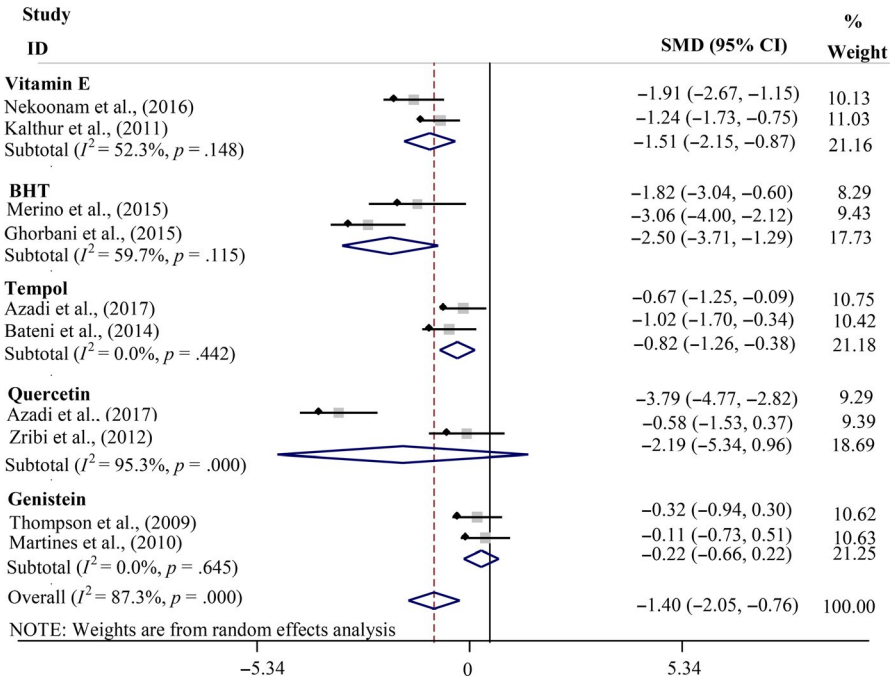

FIGURE 5 Subgroup meta-analysis of the effect of antioxidants on sperm DNA damage. SMD: standard mean difference: CI: confidence interval

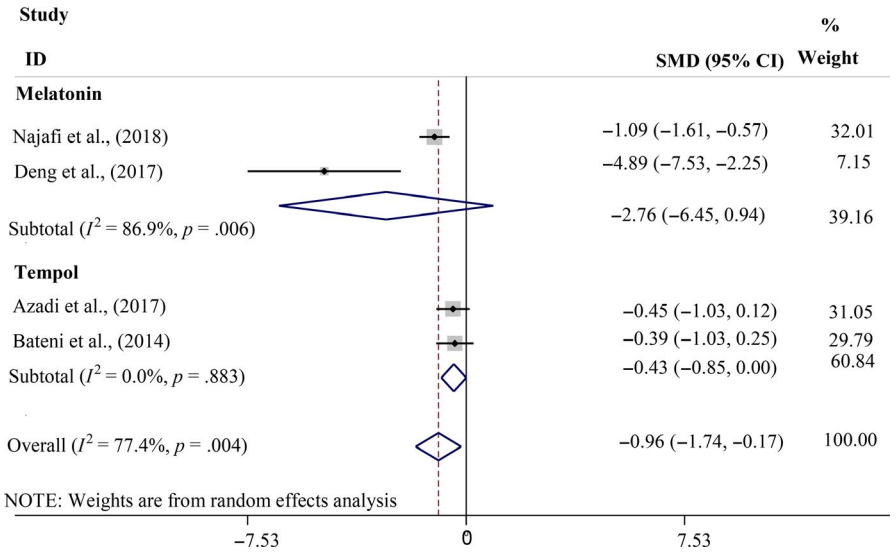

FIGURE 6 Subgroup meta-analysis of the effect of antioxidants on reduction in ROS level (H_2O_2). SMD: standard mean difference: CI: confidence interval

integrity during cryopreservation (Santiani et al., 2014). Vitamin E was another fat-soluble antioxidant that protects sperm DNA against the detrimental effects of cryopreservation. This antioxidant is located in the cell membrane, which neutralises and converts free radicals to alpha-tocopheroxyl radicals. Kalthur and colleagues reported that supplementing the cryomedium with vitamin E improved DNA integrity ($p < .001$) (Kalthur et al., 2011). Similarly, Taylor and coworkers investigated the impact of vitamin E on sperm quality and showed that vitamin E increased post-thaw sperm motility; however, it did not change the sperm viability and DNA integrity. They concluded that the positive impacts of vitamin E on men's fertility were dependent on men's age. Accordingly, men above forty years benefitted the most from vitamin E (Taylor et al., 2009).

BHT is another important scavenger of free radicals in freezing media responsible for reduction in SOD, glutathione peroxidase, and ROS production after cryopreservation and consequently protects sperm cells from DNA damage. Ghorbani et al. indicated that damage to human sperm reduced in the BHT group compared to controls (25% and 49%, respectively) (Ghorbani et al., 2015).

In our current study, tempol was found to be the intracellular scavenger of ROS even in the presence of an oxidative inducer. Many studies have indicated its potential for suppressing OS as measured by indirect methods such as malondialdehyde levels and DNA fragmentation (Mata-Campuzano et al., 2012). Melatonin independently neutralises many free radicals, but its role against free radicals has not been completely identified (Karimfar et al., 2015). Melatonin as an antioxidant decreases OS by scavenging O_2^- and H_2O_2. It also neutralises hydroxyl radicals more efficiently compared to glutathione. In fact, melatonin is also more effective than vitamin E in scavenging peroxyl radicals (Najafi et al., 2018; Reiter, 1995). However, our analysis failed to show any significant effect of melatonin.

5 | CONCLUSION

Subgroup analysis of our results indicated the positive impact of acetyl-l-carnitine/l-carnitine, melatonin, and CAT on progressive sperm motility without a significant impact of vitamin E. Tempol and melatonin had a positive impact on sperm viability. We did not find a significant impact of quercetin, vitamin E and acetyl-l-carnitine/l-carnitine on post-thaw sperm viability. BHT, tempol and vitamin E were found to be effective antioxidants in reducing post-thaw DNA fragmentation, although quercetin and genistein were not efficient antioxidant agents on protection of sperm DNA. Tempol was found to be the only antioxidant agent that was effective in reducing H_2O_2.

5.1 | Limitations

Small number of studies in each subgroup.

Small number of studies which assess the impacts of similar antioxidants on human sperm parameters.

5.2 | Recommendations

It is recommended that further studies be conducted with lower heterogeneity to evaluate more accurately the effectiveness of antioxidants on sperm parameters after thawing. A network meta-analysis is necessary in order to identify the most effective antioxidant in improving sperm parameters. Besides, a dose–response analysis is recommended to detect the impact of different antioxidant concentration on sperm parameters.

ACKNOWLEDGEMENT

We would like to thank Dr. Salehi-Abargouei and Dr. Gholami Fesharaki for their statistical consultation during the study.

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How to cite this article: Bahmyari R, Zare M, Sharma R, Agarwal A, Halvaei I. The efficacy of antioxidants in sperm parameters and production of reactive oxygen species levels during the freeze-thaw process: A systematic review and meta-analysis. *Andrologia*. 2020;52:e13514. https://doi.org/10.1111/and.13514