Iran Red Crescent Med J. 2019 November; 21(11):e94197. doi: 10.5812/ircmj.94197.
Published online 2019 December 1. **Research Article**

Protective Effects of Antioxidant Supplements on Sperm Parameters, Sperm DNA Damage and Level of Seminal ROS in RPL Patients: A Clinical Trial Study

Soheila Pourmasumi [1, 2], Nasrin Ghasemi [3], Alireza Talebi [4], Jalal Ghasemzadeh [4] and Parvin Sabeti [5, *]

[1] Non-Communicable Diseases Research Center, Rafsanjan University of Medical Sciences, Rafsanjan, Iran
[2] Clinical Research Development Unit (CRDU), Moradi Hospital, Rafsanjan University of Medical Sciences, Rafsanjan, Iran
[3] Abortion Research Center, Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
[4] Research and Clinical Center for Infertility, Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
[5] Department of Anatomy, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran

[*] *Corresponding author*: Department of Anatomy, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, Iran. Email: psabeti@yahoo.com

Received 2019 May 21; **Revised** 2019 October 07; **Accepted** 2019 October 09.

Abstract

Background: Recurrent Pregnancy Loss (RPL) is an important medical problem comprising approximately 15% of all pregnancies. Sperm DNA damage and level of seminal ROS may have a role in RPL.
Objectives: The main aim of the present study was to appraise the efficacy of antioxidants for improving these parameters in RPL patients.
Methods: In a clinical trial, 90 couples suffering from RPL were divided into two groups from April 2014 to December 2015 at the Yazd Research and Clinical Center for Infertility in Iran. Group I received supplemental vitamin E (400 μg) daily in combination with Se (200 μg) and the other group received vitamin E plus zinc for three months. Sperm parameters, sperm DNA damage, and seminal ROS were evaluated before and after treatment and compared between the two groups. Data were analyzed by SPSS and P values of ≤ 0.05 were considered statistically significant.
Results: Our results revealed normal sperm parameters in RPL patients. Sperm parameters substantially improved in the two treated groups ($P \leq 0.05$). The ROS level dramatically reduced in the two groups (P = 0.01). The evaluation of chromatin integrity with AB staining did not show any remarkable changes in group 1 (P = 0.3), but it showed a considerable rate reduction in group 2 (P = 0.04). Also, it showed significant decreases with TB, CMA3, and TUNEL assays in both groups (P = 0.001). However, we did not find any significant difference between the two groups.
Conclusions: Supplemental vitamin E in combination with selenium or zinc may improve semen quality by decreasing the level of seminal ROS and sperm DNA damage in RPL patients. We advocate their use for improving the quality of the men's sperm DNA.

Keywords: Reactive Oxygen Species, Recurrent Pregnancy Loss, Selenium, Sperm, Vitamin E, Zinc

1. Background

Recurrent Pregnancy Loss (RPL) is defined as three or more miscarriages in the first or second trimester of pregnancy; in Russia, North America, and Western Europe, it is defined as two or more consecutive miscarriages (1). It occurs in approximately 15% to 25% of clinical pregnancies but its prevalence increases with maternal age (2).

In the diagnosis of RPL, medical researchers and scientific physicians have been attentive to maternal aspects and less attention has been directed to the possible effects of paternal factors in this regard (3). Scientific reports show that male factors including sperm parameters and sperm DNA integrity may play important roles in RPL (4, 5). Previous studies have investigated the roles of different sperm parameters such as concentration, motility, morphology, viability, acrosomal status, the presence of leukocytes in seminal plasma, lipid peroxidation of sperm plasma membranes, the antioxidant capacity of seminal plasma, and sperm DNA integrity (5, 6).

Reactive Oxygen Species (ROS) are produced during oxygen metabolism. A normal physiological level of ROS is essential for sperm capacitation, acrosome reaction, and sperm-oocyte fusion, but the enhancement of intracellular or seminal ROS can be the cause of male subfertility or infertility (7). Embryo development is affected by both sperm and oocyte genomes (8). Brahem et al. reported that sperm chromatin quality and DNA integrity were related to

the outcome of reproduction, mainly RPL (9). Reactive oxygen species is one of the most important reasons for sperm DNA damage. The evaluation of sperm DNA fragmentation in the semen of RPL cases is a logical approach and the estimation of ROS in semen samples can indirectly provide the DNA quality of sperm cells (10).

Numerous studies have been planned to assess the effect of oral antioxidant therapy on improving sperm parameters and decreasing the percentage of sperm with DNA damage. Vitamin E is a free radical scavenger that protects the cellular membrane against lipid peroxidation of ROS (11). In a study, scientific researchers focused on the relationship between oral supplementation of vitamin E plus vitamin C and sperm chromatin quality. They reported an effective role for the antioxidants to decrease damage to sperm DNA. Moreover, the antioxidants had positive effects on sperm DNA quality (12). In another study, sperm motility, morphology, or both improved with selenium supplements in combination with vitamin E in idiopathic oligoasthenoteratozoospermia (OAT) men (13).

Zinc plays a critical role in the spermatozoa physiology and it cannot be stored in the body. It can affect sperm membrane stabilization, capacitation, acrosomal reaction, and embryonic implantation (11). Hadwan et al. found that treatment with 440 mg zinc sulfate daily for three months led to a significant improvement in semen volume, sperm count, and progressive sperm motility in asthenozoospermic men (14).

Selenium (Se) is an essential element for reproductive functions such as testosterone metabolism and sperm formation (15). Safarinejad et al. administered an oral order of selenium (200 μg) and N-acetyl-cysteine (600 mg) for 26 weeks for infertile men and showed a positive significant association between the concentration of this antioxidant in the seminal fluid and the improvement of sperm parameters including sperm count, motility, and normal morphology (16).

Sperm DNA damage is a major cause of RPL. As mentioned earlier, oxidative stress plays an important role in creating damage to sperm DNA and antioxidants can decrease the extent of this damage. However, studies are scarce on the effect of antioxidants on sperm parameters and DNA degradation in infertile men. This is the first study conducted on the Iranian population to investigate and compare the effects of two combined antioxidants concerning sperm parameters, DNA damage, and seminal ROS level in RPL patients.

2. Methods

This clinical trial was performed from April 2014 to December 2015 at our Research and Clinical Center for Infertility. The research and clinical techniques used in the present studies were approved by the Ethics Committee of the Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Yazd, Iran, and registered in IRCT (IRCT201704195261N). Idiopathic RPL cases from an abortion clinic were enrolled in the study. Written consent was obtained from all individuals participating in this study.

2.1. Patients

From among 133 couples who referred to the abortion clinic, we selected 90 couples with a history of recurrent abortion provided that the male partner had at least two normal semen analyses. In the first step, all patients were evaluated by a clinician at the abortion clinic for male and female factors and then, they were selected for the study.

2.2. Exclusion and Inclusion Criteria

Patients with at least two prior abortions at below 20 weeks of gestational age were chosen. The cases with an identified cause of RPL were excluded and just patients with unknown causes were selected. Patients who were taking antioxidant medications in the past months before the study and men with varicocelectomy, smoking, and alcohol consumption were excluded from the study. For assessing maternal and paternal chromosomal abnormality, the karyotypes test was used. Male cases were referred to an andrology clinic and semen samples were collected by masturbation after abstinence from sexual intercourse for three to seven days. Samples were incubated at 37°C for at least 30 min to be liquefied and then, semen analysis, sperm chromatin condensation, and ROS measurements were done for all patients. Sperm morphology was evaluated using the Papanicolaou staining method.

In the next step, the patients were randomly allocated to two groups (45 cases in each group). In group I, patients took 400 IU synthetic vitamin E (α-tocopherol) plus selenium daily and in group II, patients received 400 IU synthetic vitamin E (α-tocopherol) plus zinc daily.

After three months, semen samples were collected again and all parameters were re-examined. Data were collected before and after antioxidant therapy and imported into a computer system and analyzed by statistical software to distinguish between-group differences (Figure 1).

2.3. Semen Analysis (SA)

Semen samples were collected in wide-mouth sterile non-toxic containers and transferred to an andrology lab. After semen liquefaction, a conventional semen analysis was done according to the WHO guidelines (2010) (17).

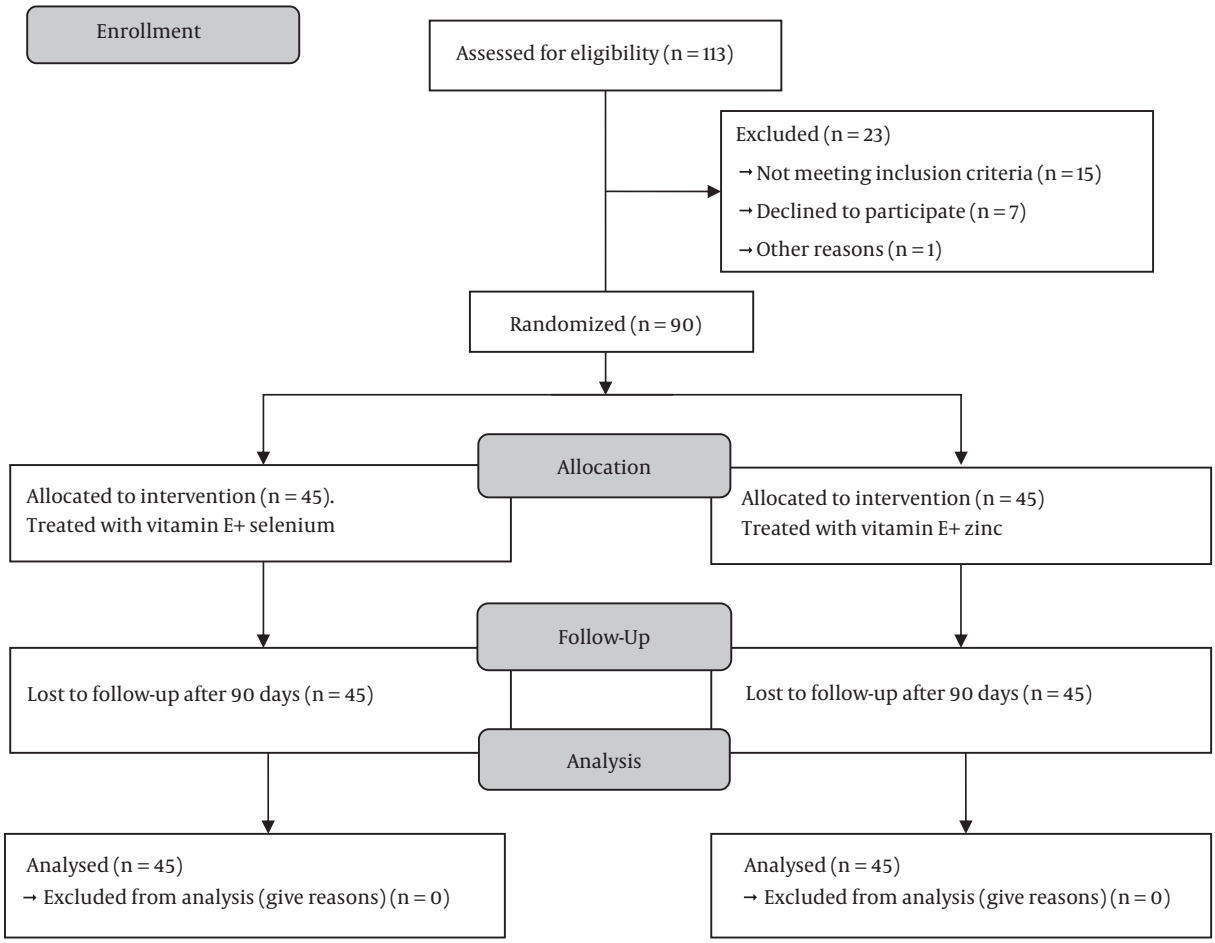

Figure 1. CONSORT flow diagram

Mackler chamber (Sefi Medical Co., Haifa, Israel) and phase-contrast microscope (Carl Zeiss, Germany) were used at 200X magnification for the evaluation of sperm parameters (sperm concentration and motility). The percentages of progressive, non-progressive, and immotile spermatozoa were calculated for sperm motility. Papanicolaou staining was applied and 200 spermatozoa based on Kruger strict were seen for morphological assessment by considering any abnormalities in the head, neck, or tail. A practiced technician who blinded to the study analyzed all samples.

For other sperm tests including DNA fragmentation, AB, and TB, the samples were washed and stored. In this step, the seminal fluid was washed two times with 8 mL Phosphate-Buffered Saline (PBS, pH 7.4; Sigma, St. Louis, MO) and centrifuged at 400 g for 5 min. The plates with washed spermatozoa were fixed with 5 mL acetic acid/methanol mixture (Merck, Darmstadt, Germany) for at least 30 min at 4°C. Then, 40 - 50 μL of this suspension was smeared on slides and frozen at -20°C until use for the DNA fragmentation test. For the sperm chromatin structure assay, 100 μL raw semen was kept at -80°C.

2.4. Measurement of Reactive Oxygen Species (ROS)

The liquefied semen was used for the investigation of the seminal ROS level. Seminal plasma was removed by centrifugation at 300 g for 7 min. The PBS solution (Sigma Chemical Co., USA) was added to the pellet and re-suspended in the same media. Then, 10 ml of luminol (5-amino-2, 3 dihydro-1, 4 phthalazinedione; Sigma Chemical Co., USA) was used as a probe and was added to the aliquot. Negative control was prepared by adding 10 μL PBS. The ROS level was analyzed by a chemiluminescence assay with an Autolamat LB 935 Luminometer (Berthold Technologies, Bad Wildbad, Germany) in the integrated mode for 15 min. The results were expressed as Relative Light Unit (RLU) per 20×10^6 spermatozoa (18).

2.5. Evaluation of Sperm Nuclear DNA Damage

The sperm DNA integrity and chromatin condensation were evaluated by Aniline Blue (AB), Toluidine Blue (TB), Chromomycin A3 (CMA3), and TUNEL tests.

2.6. Aniline Blue Staining

The AB dye was used to separate histones lysine-rich and arginine/cysteine-rich protamine. It is a valuable test for determining the remaining histones in the structure of sperm chromatin. For this test, previously washed semen samples were smeared on slides and fixed by buffered glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 30 min at room temperature. Slides were blotted by 5% AB solution (pH 3.5; Sigma Aldrich, USA) for 5 min. For the determination of marked and unmarked sperms, 200 cells in each slide were counted under a light microscope (Carl Zeiss Axiostar plus, Germany). Normal sperms under the light microscope showed unstained or pale blue-stained heads while abnormal sperms showed dark blue-stained heads (19).

2.7. Toluidine Blue Staining

This test is a direct and simple method for metachromatic staining of sperm chromatin. This metachromatic dye can bind to DNA strands' phosphate groups (20). For TB staining, washed sperms were smeared, dried in air at room temperature, and then fixed in fresh 96% ethanol-acetone (1:1) solution for 30 min at 4°C. Next, slides were hydrolyzed in 0.1 N HCl for 5 min at 4°C. In the next step, the slides were washed in distilled water three times of 2 min each. In the final step, the slides were stained with 0.05% TB (Sigma Aldrich, USA) for 10 min at room temperature. Then, they were mounted in the DPX medium. For the determination of stained and unstained sperms, 200 cells in each slide were counted under a light microscope (Carl Zeiss Axiostar plus, Germany). Normal sperms with good chromatin under the light microscope showed light blue-stained heads (TB-) while abnormal sperms showed dark blue to violet or purple-stained heads (TB+) (21).

2.8. Chromomycin A3 Staining

Chromomycin A3 is a guanosine-cytosine-specific fluorochrome. In human sperm, protamine deficiency can affect the chromatin packaging process. When chromatin is packaged poorly, CMA3 is a useful test to show this protamine deficiency in sperms by indirect imagining. For this test, semen samples were smeared on slides and fixed in methanol-glacial acetic acid (3:1) for 10 min at 4°C and then dried at room temperature for 20 min. The slides were stained with 100 μL CMA3 solution (Sigma Aldrich, USA) for 20 min. In the final step, the slides were washed with buffer and mounted with 1:1 PBS-glycerol. For the determination of stained and unstained sperms, 200 cells were randomly counted in each slide under a fluorescent microscope (Olympus BX51, Japan). Normal sperms under the fluorescent microscope showed dull yellow (CMA3-) and abnormal sperms showed bright yellow (CMA3+) (19).

2.9. DNA Fragmentation by TUNEL Staining

The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was applied for the determination of breaks in sperm DNA strands. For this test, we used the ApopTag Apoptosis Detection Kit (Qbiogene, Paris, France). In the first step, the semen samples were smeared on slides and incubated in a mixed solution of PBS and 1% Triton X100 (Sigma) for permeabilization. The next steps of this technique were done based on the kit manufacturer's instruction. In brief summary, slides were double-washed in PBS 1X. After 10 s of equilibration, the terminal deoxynucleotidyl transferase (TdT) solution was added to the slides and incubated in a dark, moisty box at 37°C. After one hour, the slides were double-washed in PBS and incubated with an anti-digoxigenin antibody in a dark, moisty box for 30 min. Peroxidase was revealed with diaminobenzidine (DAB). Then, Harris' hematoxylin (RAL, Martillac, France) was added to slides and mounted with Faramount mounting (Dako, Carpinteria, CA). For the determination of sperms with fragmented or healthy DNA, 500 cells in each slide were counted under a light microscope (Carl Zeiss Axiostar plus, Germany) with a 100X magnification lens. Sperms with fragmented DNA under the light microscope showed brown-colored nuclei (TUNEL+) and healthy sperms showed blue-gray nuclei (TUNEL-). For confirming the reproducibility check, negative and positive controls were used with each slide group (22).

2.10. Statistical Analysis

The data were expressed as mean $\pm$ SD. The independent-samples *t*-test and Mann-Whitney U test were used as appropriate. The paired-samples *t*-test was used to compare pre- and post-treatment parameters. Two-tailed P values of < 0.05 were considered statistically significant.

3. Results

The mean age of men was 32.97 ± 5.16 years in the vitamin E + Se group and 32.17 ± 5.46 years in the vitamin E + Zn group (Table 1). This parameter was not significantly different between the two groups. As reported in Table 1, the age of women and abortion frequencies were similar between

Table 1. Demographic Characteristics in Study Groups[a]

	Vitamin E + Selenium	Vitamin E + Zinc	P Value[b]
Male age (y)	32.97 ± 5.16	32.17 ± 5.46	0.97
Female age (y)	28.57 ± 4.08	29.55 ± 4.92	0.30
Miscarriage frequency (n)	2.97 ± 1.03	3.28 ± 1.47	0.24

[a]Values are expressed as mean ± SD.
[b]The Independent-samples *t*-test was used to compare variables between the two groups. P values of < 0.05 were considered statistically significant.

the two study groups and there was no statistically significant difference.

Table 2 shows sperm parameters and chromatin quality at pre- and post-treatment in the two groups. All sperm parameters, chromatin quality, and seminal ROS level significantly changed after treatment in the two groups.

The results of the comparison between the two treated groups can be found in Table 3 to assess the treatment efficacy. No significant difference was observed in all parameters between the two treated groups.

4. Discussion

The role of sperm factors in early embryogenesis has not been widely examined in RPL cases following spontaneous conception. However, recent studies (23) have highlighted that sperm DNA damage (DNA fragmentation or denaturation) and additionally, chromosomal aneuploidies may be associated with early embryonic development and outcome in RPL (23, 24). At present, in RPL cases, the male partner is only considered for the existence of chromosomal abnormalities, but chromosomal aberrations are the causal factors only in a small percentage of cases experiencing RPL (25).

Patients who included in the present study had normal sperm parameters. According to Leach et al. report in 2015, some men with RPL had normal sperm parameters with no sign of oxidative stress but in these patients, sperm chromatin abnormalities and sperm chromatin defects may be involved in oxidative stress consequences (26).

Carlini et al. in 2017 showed that sperm DNA fragmentation was more in RPL patients than in normal men (27). Moreover, Simsek et al. in 1998 (28) and Agarwal et al. in 2005 (29) reported a high level of seminal ROS and sperm chromatin damage in couples with a history of RPL, which were accompanied by oxidative stress. Also, they indicated an association between oxidative stress and abnormal sperm chromatin, possibly leading to recurrent abortions in these couples.

Any effort to decrease ROS would be beneficial (30). Antioxidants are the main supplements to eliminate the harmful effects of ROS (31). Many studies tried to assess the effect of antioxidants on sperm parameters (32, 33). All of the previously published studies were conducted on infertile patients and this is the first study to estimate the effect of two groups of antioxidants on RPL patients. In the present study, we used vitamin E plus selenium and vitamin E plus zinc in two groups of RPL men. Our results showed that combination treatment with antioxidants for 90 days could make a significant difference in sperm parameters and chromatin quality. Moreover, the antioxidants could significantly recover sperm concentration, motility, and morphology after treatment.

Similar to our study, Moslemi et al. in 2011 reported that 200 μg selenium plus 400 IU vitamin E had positive effects on sperm concentration, motility, and morphology in infertile patients (13). Similarly, Abad et al. in 2013 reported that the combination of antioxidants including vitamin E, zinc, and selenium for 90 days could improve sperm parameters (34).

In contrast to our results, Raigani et al. in 2014 concluded that 16 weeks of treatment with zinc and folic acid had no significant effect on sperm parameters (35). In a clinical trial published in 2005, researchers reported that antioxidants were not effective to improve sperm parameters. They found no statistically significant difference in all sperm parameters such as sperm count, motility, and morphology after 90 days of antioxidant therapy but in contrast to simple sperm analysis, they observed improvements in DNA fragmentation and chromatin quality after antioxidant therapy (12). In another study, Singh et al. in 2016 treated 40 infertile patients with a mixture of antioxidants for three months. The results of their study showed that sperm parameters and seminal ROS levels improved after antioxidant therapy (36).

In the present study, we found high levels of ROS in the semen of RPL patients. Our result is similar to the results obtained by Singh et al. (36), Yumura et al. (37), and Homa et al. (30). In contrast to our results, Suleiman et al. demonstrated that the level of seminal ROS was not related to sperm count and motility (38).

This study also revealed a significant reduction in seminal ROS levels and sperm DNA fragmentation after antioxidant therapy in the two groups. Also, we observed that sperm chromatin quality increased after antioxidant therapy in RPL patients but there was no significant difference between the two groups.

It is well known that spermatozoa membranes are rich in polyunsaturated fatty acids but their cytoplasm is poor in antioxidant enzymes (39). Therefore, spermatozoa are susceptible to oxygen-induced damage and this induction can lead to sperm membrane lipid peroxidation and damage to sperm mitochondrial and nuclear DNA. Recently, the

Table 2. Comparison of the Pre- and Post-Treatment Seminal Parameters, Chromatin Quality, and ROS Level in Study Groups[a]

Sperm Parameters	Vitamin E + Selenium		P Value[b]	Vitamin E + Zinc		P Value[b]
	Pre-Treatment	Post-Treatment		Pre-Treatment	Post-Treatment	
Volume	3.34 ± 1.78	3.74 ± 1.42	0.03	3.12 ± 1.56	3.43 ± 1.22	0.01
Count ($\times 10^6$/mL)	93.62 ± 21.87	102.73 ± 21.56	0.001	98.04 ± 22.36	105.80 ± 18.18	0.0001
Motility (%)						
Progressive	55.97 ± 8.13	63.60 ± 9.07	0.0001	57.51 ± 8.78	64.37 ± 9.23	0.0001
Non-progressive	12.13 ± 3.85	9.22 ± 4.06	0.002	11.20 ± 4.20	9.57 ± 4.43	0.09
Immotile	31.42 ± 6.87	27.17 ± 7.40	0.009	31.26 ± 7.70	25.93± 7.56	0.002
Morphology (% of normal)	33.82 ± 7.14	40.31 ± 7.99	0.001	33.95 ± 7.57	38.20 ± 8.47	0.009
ROS level	212.60 ± 209.41	141.50 ± 172.50	0.01	240.98 ± 241.92	184.48 ± 220.59	0.01
AB	50.33 ± 20.48	47.80 ± 17.78	0.3	52.97 ± 17.50	45.44 ± 18.39	0.04
TB	66.20 ± 15.38	54.64 ± 17.60	0.0001	67.33 ± 17.47	54.11 ± 19.31	0.0001
CMA3	39.33 ± 10.08	31.62 ± 7.28	0.0001	43.93 ± 10.56	35.91 ± 8.90	0.0001
TUNEL	32.9 5± 5.26	26.86 ± 5.70	0.0001	32.33 ± 5.91	27.40 ± 5.49	0.0001

Abbreviations: AB, Aniline Blue staining; CMA3, Chromomycine A3 Staining; TB, Toluidine Blue staining.
[a]Values are expressed as mean ± SD.
[b]Paired-samples *t*-test was used to compare dependent variables. P values of < 0.05 were considered statistically significant.

Table 3. Comparison of Seminal Parameters, Chromatin Quality, and ROS Level Between Treated Groups[a]

Sperm Parameters	Vitamin E + Selenium	Vitamin E + Zinc	P Value[b]
Volume	3.74 ± 1.42	3.43 ± 1.22	0.26
Count ($\times 10^6$/mL)	102.73 ± 21.56	105.80 ± 18.18	0.46
Motility (%)			0.68
Progressive	63.60 ± 9.07	64.37 ± 9.23	
Non-progressive	9.22 ± 4.06	9.57 ± 4.43	
Immotile	27.17 ± 7.40	25.93± 7.56	
Morphology (% of normal)	40.31 ± 7.99	38.20 ± 8.47	0.22
ROS level	141.50 ± 172.50	184.48 ± 220.59	0.30
AB	47.80 ± 17.78	45.44 ± 18.39	0.53
TB	54.64 ± 17.60	54.11 ± 19.31	0.89
CMA3	31.62 ± 7.28	35.91 ± 8.90	0.21
TUNEL	26.86 ± 5.70	27.40 ± 5.49	0.65

Abbreviations: AB, Aniline Blue staining; CMA3, Chromomycine A3 staining; TB, Toluidine Blue staining.
[a]Values are expressed as mean ± SD.
[b]The Independent-samples *t*-test was used to compare dependent variables. P values of < 0.05 were considered statistically significant.

role of ROS has been extensively investigated in male infertility (40). Oxygen is recognized as an essential material in cell metabolism, which can compromise cell viability in the case of extensive metabolism via oxidative stress. Oxygen metabolism results in the production of activated particles, known as ROS. These molecules are extremely reactive with other molecules (6, 41). The overproduction of ROS and reduction of total antioxidant activity (TAC) may lead to oxidative stress, which, in turn, culminates damages to sperm DNA with negative effects on the efficiency of sperms for reproductive function. This study suggests that supplementation with antioxidants can reduce ROS generation, prevent sperm DNA fragmentation, prevent the loss of sperm parameters, and increase sperm chromatin quality in RPL patients.

The careful selection of couples and the evaluation of antioxidant effects on sperm chromatin by three methods, especially tunnel assay, a very expensive assay, can be the strong points of this study and the limited number of patients is one of the weaknesses of the study.

4.1. Conclusions

The present study highlights that recurrent miscarriage might be due to the increased ROS level causing oxidative stress-induced damage to cellular macromolecules including DNA. The fertilization of an oocyte with a DNA-damaged sperm cell may result in implantation failure and pregnancy loss. Therefore, we advise that couples with idiopathic recurrent pregnancy loss must routinely test for the level of seminal ROS and sperm chromatin abnormality. Our results clearly demonstrate that vitamin E combined with zinc or selenium can improve sperm parameters by decreasing the level of seminal ROS and sperm DNA damage and increasing sperm chromatin quality in RPL

patients. Nevertheless, we propose further large randomized controlled studies to find the best antioxidants to reduce oxidative stress along with assessing pregnancy rates in these patients.

Acknowledgments

The authors especially thank the Andrology Laboratory staff for skillful technical assistance in the course of research. This study was supported by a grant from the Research and Clinical Center for Infertility, Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

Footnotes

Authors' Contribution: Data collection and English writing of the paper: Soheila Pourmasumi; patient selection: Nasrin Ghasemi; study supervision: Alireza Talebi; lab data collection: Jalal Ghasemzadeh; manuscript revision: Parvin Sabeti.

Clinical Trial Registration Code: IRCT201704195261N.

Conflict of Interests: There are no financial disclosure and conflict of interest in this study.

Ethical Approval: Ethics Committee reference number 340 (2014-09-14, 1393/06/23).

Funding/Support: The study was supported by the Infertility Clinic of Yazd University of Medical Sciences.

Informed Consent: Written consent was obtained from all individuals in the study.

References

1. Carp H. Immunotherapy for recurrent pregnancy loss. *Best Pract Res Clin Obstet Gynaecol*. 2019;**60**:77–86. doi: 10.1016/j.bpobgyn.2019.07.005. [PubMed: 31521575].
2. El Hachem H, Crepaux V, May-Panloup P, Descamps P, Legendre G, Bouet PE. Recurrent pregnancy loss: Current perspectives. *Int J Womens Health*. 2017;**9**:331–45. doi: 10.2147/IJWH.S100817. [PubMed: 28553146]. [PubMed Central: PMC5440030].
3. Rai R, Regan L. Recurrent miscarriage. *Lancet*. 2006;**368**(9535):601–11. doi: 10.1016/S0140-6736(06)69204-0. [PubMed: 16905025].
4. Puscheck EE, Jeyendran RS. The impact of male factor on recurrent pregnancy loss. *Curr Opin Obstet Gynecol*. 2007;**19**(3):222–8. doi: 10.1097/GCO.0b013e32813e3ff0. [PubMed: 17495637].
5. Gil-Villa AM, Cardona-Maya W, Agarwal A, Sharma R, Cadavid A. Assessment of sperm factors possibly involved in early recurrent pregnancy loss. *Fertil Steril*. 2010;**94**(4):1465–72. doi: 10.1016/j.fertnstert.2009.05.042. [PubMed: 19540481].
6. Gupta S, Agarwal A, Banerjee J, Alvarez JG. The role of oxidative stress in spontaneous abortion and recurrent pregnancy loss: A systematic review. *Obstet Gynecol Surv*. 2007;**62**(5):335–47. quiz 353-4. doi: 10.1097/01.ogx.0000261644.89300.df. [PubMed: 17425812].
7. Dutta S, Majzoub A, Agarwal A. Oxidative stress and sperm function: A systematic review on evaluation and management. *Arab J Urol*. 2019;**17**(2):87–97. doi: 10.1080/2090598X.2019.1599624. [PubMed: 31285919]. [PubMed Central: PMC6600059].
8. Aitken RJ, Koppers AJ. Apoptosis and DNA damage in human spermatozoa. *Asian J Androl*. 2011;**13**(1):36–42. doi: 10.1038/aja.2010.68. [PubMed: 20802502]. [PubMed Central: PMC3739394].
9. Brahem S, Mehdi M, Landolsi H, Mougou S, Elghezal H, Saad A. Semen parameters and sperm DNA fragmentation as causes of recurrent pregnancy loss. *Urology*. 2011;**78**(4):792–6. doi: 10.1016/j.urology.2011.05.049. [PubMed: 21813165].
10. Pourmasumi S, Sabeti P, Rahiminia T, Mangoli E, Tabibnejad N, Talebi AR. The etiologies of DNA abnormalities in male infertility: An assessment and review. *Int J Reprod Biomed (Yazd)*. 2017;**15**(6):331–44. [PubMed: 29177237]. [PubMed Central: PMC5605854].
11. Alahmar AT. The effects of oral antioxidants on the semen of men with idiopathic oligoasthenoteratozoospermia. *Clin Exp Reprod Med*. 2018;**45**(2):57–66. doi: 10.5653/cerm.2018.45.2.57. [PubMed: 29984205]. [PubMed Central: PMC6030611].
12. Greco E, Iacobelli M, Rienzi L, Ubaldi F, Ferrero S, Tesarik J. Reduction of the incidence of sperm DNA fragmentation by oral antioxidant treatment. *J Androl*. 2005;**26**(3):349–53. doi: 10.2164/jandrol.04146. [PubMed: 15867002].
13. Moslemi MK, Tavanbakhsh S. Selenium-vitamin E supplementation in infertile men: Effects on semen parameters and pregnancy rate. *Int J Gen Med*. 2011;**4**:99–104. doi: 10.2147/IJGM.S16275. [PubMed: 21403799]. [PubMed Central: PMC3048346].
14. Hadwan MH, Almashhedy LA, Alsalman AR. Study of the effects of oral zinc supplementation on peroxynitrite levels, arginase activity and NO synthase activity in seminal plasma of Iraqi asthenospermic patients. *Reprod Biol Endocrinol*. 2014;**12**:1. doi: 10.1186/1477-7827-12-1. [PubMed: 24383664]. [PubMed Central: PMC3882288].
15. Hadwan MH, Almashhedy LA, Alsalman AR. Oral zinc supplementation restores high molecular weight seminal zinc binding protein to normal value in Iraqi infertile men. *BMC Urol*. 2012;**12**:32. doi: 10.1186/1471-2490-12-32. [PubMed: 23145537]. [PubMed Central: PMC3503568].
16. Safarinejad MR, Safarinejad S. Efficacy of selenium and/or N-acetylcysteine for improving semen parameters in infertile men: A double-blind, placebo controlled, randomized study. *J Urol*. 2009;**181**(2):741–51. doi: 10.1016/j.juro.2008.10.015. [PubMed: 19091331].
17. World Health Organization. *WHO laboratory manual for the examination and processing of human semen*. Cambridge University Press; 2010.
18. Moein MR, Ghasemzadeh J, Talebi AR, Halvaei I, Nabi A. Evaluating the level of reactive oxygen species in seminal fluid of men with recurrent pregnancy loss; does it have any role? *Iran J Reprod Med*. 2014;**12**(6):42.
19. Talebi AR, Moein MR, Tabibnejad N, Ghasemzadeh J. Effect of varicocele on chromatin condensation and DNA integrity of ejaculated spermatozoa using cytochemical tests. *Andrologia*. 2008;**40**(4):245–51. doi: 10.1111/j.1439-0272.2008.00852.x. [PubMed: 18727735].
20. Pourmasumi S, Khoradmehr A, Rahiminia T, Sabeti P, Talebi AR, Ghasemzadeh J. Evaluation of sperm chromatin integrity using aniline blue and toluidine blue staining in infertile and normozoospermic men. *J Reprod Infertil*. 2019;**20**(2):95–101. [PubMed: 31058054]. [PubMed Central: PMC6486564].
21. Sabeti P, Amidi F, Kalantar SM, Sedighi Gilani MA, Pourmasumi S, Najafi A, et al. Evaluation of intracellular anion superoxide level, heat shock protein A2 and protamine positive spermatozoa percentages in teratoasthenozoospermia. *Int J Reprod Biomed (Yazd)*. 2017;**15**(5):279–86. [PubMed: 28744523]. [PubMed Central: PMC5510581].
22. Pourmasumi S, Nazari A, Fagheirelahee N, Sabeti P. Cytochemical tests to investigate sperm DNA damage: Assessment and review. *J Family Med Prim Care*. 2019;**8**(5):1533–9. doi: 10.4103/jfmpc.jfmpc_35_19. [PubMed: 31198709]. [PubMed Central: PMC6559112].

23. Shamsi MB, Venkatesh S, Pathak D, Deka D, Dada R. Sperm DNA damage & oxidative stress in recurrent spontaneous abortion (RSA). *Indian J Med Res*. 2011;**133**:550–1. [PubMed: 21623044]. [PubMed Central: PMC3121290].
24. Venkatesh S, Shamsi MB, Dudeja S, Kumar R, Dada R. Reactive oxygen species measurement in neat and washed semen: Comparative analysis and its significance in male infertility assessment. *Arch Gynecol Obstet*. 2011;**283**(1):121–6. doi: 10.1007/s00404-010-1645-4. [PubMed: 20814688].
25. Rubio C, Simon C, Vidal F, Rodrigo L, Pehlivan T, Remohi J, et al. Chromosomal abnormalities and embryo development in recurrent miscarriage couples. *Hum Reprod*. 2003;**18**(1):182–8. doi: 10.1093/humrep/deg015. [PubMed: 12525464].
26. Leach M, Aitken RJ, Sacks G. Sperm DNA fragmentation abnormalities in men from couples with a history of recurrent miscarriage. *Aust N Z J Obstet Gynaecol*. 2015;**55**(4):379–83. doi: 10.1111/ajo.12373. [PubMed: 26201831].
27. Carlini T, Paoli D, Pelloni M, Faja F, Dal Lago A, Lombardo F, et al. Sperm DNA fragmentation in Italian couples with recurrent pregnancy loss. *Reprod Biomed Online*. 2017;**34**(1):58–65. doi: 10.1016/j.rbmo.2016.09.014. [PubMed: 27838218].
28. Simsek M, Naziroglu M, Simsek H, Cay M, Aksakal M, Kumru S. Blood plasma levels of lipoperoxides, glutathione peroxidase, beta carotene, vitamin A and E in women with habitual abortion. *Cell Biochem Funct*. 1998;**16**(4):227–31. [PubMed: 9857484].
29. Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. *Reprod Biol Endocrinol*. 2005;**3**:28. doi: 10.1186/1477-7827-3-28. [PubMed: 16018814]. [PubMed Central: PMC1215514].
30. Homa ST, Vessey W, Perez-Miranda A, Riyait T, Agarwal A. Reactive Oxygen Species (ROS) in human semen: Determination of a reference range. *J Assist Reprod Genet*. 2015;**32**(5):757–64. doi: 10.1007/s10815-015-0454-x. [PubMed: 25749739]. [PubMed Central: PMC4429439].
31. Menezo YJ, Hazout A, Panteix G, Robert F, Rollet J, Cohen-Bacrie P, et al. Antioxidants to reduce sperm DNA fragmentation: An unexpected adverse effect. *Reprod Biomed Online*. 2007;**14**(4):418–21. doi: 10.1016/s1472-6483(10)60887-5. [PubMed: 17425820].
32. Atig F, Kerkeni A, Saad A, Ajina M. Effects of reduced seminal enzymatic antioxidants on sperm DNA fragmentation and semen quality of Tunisian infertile men. *J Assist Reprod Genet*. 2017;**34**(3):373–81. doi: 10.1007/s10815-013-9936-x. [PubMed: 23354588]. [PubMed Central: PMC5360677].
33. Keskes-Ammar L, Feki-Chakroun N, Rebai T, Sahnoun Z, Ghozzi H, Hammami S, et al. Sperm oxidative stress and the effect of an oral vitamin E and selenium supplement on semen quality in infertile men. *Arch Androl*. 2003;**49**(2):83–94. doi: 10.1080/01485010390129269. [PubMed: 12623744].
34. Abad C, Amengual MJ, Gosalvez J, Coward K, Hannaoui N, Benet J, et al. Effects of oral antioxidant treatment upon the dynamics of human sperm DNA fragmentation and subpopulations of sperm with highly degraded DNA. *Andrologia*. 2013;**45**(3):211–6. doi: 10.1111/and.12003. [PubMed: 22943406].
35. Raigani M, Yaghmaei B, Amirjannti N, Lakpour N, Akhondi MM, Zeraati H, et al. The micronutrient supplements, zinc sulphate and folic acid, did not ameliorate sperm functional parameters in oligoasthenoteratozoospermic men. *Andrologia*. 2014;**46**(9):956–62. doi: 10.1111/and.12180. [PubMed: 24147895].
36. Singh A, Jahan N, Radhakrishnan G, Banerjee BD. To evaluate the efficacy of combination antioxidant therapy on oxidative stress parameters in seminal plasma in the male infertility. *J Clin Diagn Res*. 2016;**10**(7):QC14–7. doi: 10.7860/JCDR/2016/15597.8159. [PubMed: 27630911]. [PubMed Central: PMC5020199].
37. Yumura Y, Takeshima T, Kawahara T, Sanjo H, Kuroda SN, Asai T, et al. Reactive oxygen species measured in the unprocessed semen samples of 715 infertile patients. *Reprod Med Biol*. 2017;**16**(4):354–63. doi: 10.1002/rmb2.12052. [PubMed: 29259489]. [PubMed Central: PMC5715895].
38. Suleiman SA, Ali ME, Zaki ZM, el-Malik EM, Nasr MA. Lipid peroxidation and human sperm motility: Protective role of vitamin E. *J Androl*. 1996;**17**(5):530–7. [PubMed: 8957697].
39. Sabeti P, Pourmasumi S, Rahiminia T, Akyash F, Talebi AR. Etiologies of sperm oxidative stress. *Int J Reprod Biomed (Yazd)*. 2016;**14**(4):231–40. [PubMed: 27351024]. [PubMed Central: PMC4918773].
40. Fortis MF, Fraga LR, Boquett JA, Kowalski TW, Dutra CG, Goncalves RO, et al. Angiogenesis and oxidative stress-related gene variants in recurrent pregnancy loss. *Reprod Fertil Dev*. 2018;**30**(3):498–506. doi: 10.1071/RD17117. [PubMed: 28825972].
41. Halliwell B, Gutteridge JM. Free radicals and antioxidant protection: mechanisms and significance in toxicology and disease. *Hum Toxicol*. 1988;**7**(1):7–13. doi: 10.1177/096032718800700102. [PubMed: 3278973].