Original Article

Alpha-Lipoic Acid Supplementation for Male Partner of Couples with Recurrent Pregnancy Loss: A Post hoc analysis in Clinical Trial

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Abstract

Background: Increased sperm DNA damage is known as one of the causes of recurrent pregnancy loss (RPL) which can be due to increased levels of oxidative stress. Therefore, the aim of this study was to assess the effect of alpha-lipoic acid (ALA) on sperm parameters and sperm functions in couples with a history of RPL.

Materials and Methods: In this post hoc analysis in clinical trial study, a total of 37 couples with RPL (n=12 and n=25 for placebo and ALA groups, respectively) were considered. Men were treated with ALA (600 mg/day) or placebo for 80 days. Semen samples were acquired from the participants before initiation and after completion of the medication course and assessed regarding conventional sperm parameters, chromatin damage/integrity, intracellular oxidative stress, lipid peroxidation, and seminal antioxidant characteristics. Individuals were further followed up for twelve months for pregnancy occurrence and outcomes. Finally, after excluding patients with no history of RPL, the data was analyzed.

Results: No significant differences were observed between the baseline measures of the aforementioned parameters except for seminal volume. After the intervention, the mean sperm DNA damage, protamine deficiency, and persisted histones were significantly lower in the ALA group than in placebo receivers ($P<0.05$). A decrease in the mean of seminal total antioxidant capacity ($P=0.03$), malondialdehyde ($P=0.02$), and sperm DNA damage ($P=0.004$) as well as an increase in sperm total motility ($P=0.04$) after treatment with ALA was noticed. In addition, the mean of protamine deficiency and persisted histones were declined post-ALA therapy ($P=0.003$ and 0.002, respectively). The percentage of spontaneous pregnancy in the ALA group (4 of 25 cases; 16%) was higher than in the placebo group (1 of 12, 8.3%).

Conclusion: ALA-therapy attenuates sperm DNA damage and lipid peroxidation while enhancing sperm total motility and chromatin compaction in the male partner of couples with PRL (registration number: IRCT20190406043177N1).

Keywords: Alpha-Lipoic Acid, DNA Damage, Oxidative Stress, Recurrent Pregnancy Loss

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Introduction

"Recurrent pregnancy loss (RPL)" is usually defined as the spontaneous failure of two or more clinical pregnancies before 24 weeks of gestation, affecting 0.8-1.4% of couples. From the pathophysiologic standpoint, RPL encompasses a vast number of etiologies ranging from explicit anatomic anomalies to chromosome abnormalities (1). As of today, the literature instinctively predominantly has focused on female-related etiologies. However, nearly 50% of RPL cases remain unexplained (2). Recent findings have shifted the attention toward male-related factors. As the sperm contributes to half the genomic content of the resultant zygote, rationally, male chromo-

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somal abnormalities could lead to the conditions opposing the embryo's developmental competency. As a result, maintaining the male-partner karyotype has turned into a routine RPL survey (1, 3). Moreover, paternal genes play a leading role in the regulation of peri-implantation embryogenesis and placental formation, emphasizing the importance of spermatozoa's chromatin and DNA intactness (4-6). In line with this, recent evidence has indicated an association between higher levels of sperm DNA damage and increased RPL occurrence (7).

Abortive apoptosis, defective chromatin maturation, and most notably oxidative stress (OS) are known as principal mechanisms involved in male infertility (8). OS sketches the existence of a homeostatic imbalance between oxidizing and reducing agents within seminal plasma or sperm resulting in an overabundance in reactive oxygen species (ROS), occurring whether as a consequence of ROS overproduction or the absence of efficient ameliorating antioxidants (9, 10).

Results of a study on the influence of antioxidant supplementation on the level of OS and its impacts on male fertility indicated beneficial outcomes in favor of sperm motility, cellular OS mediated damage, and DNA fragmentation (11). In addition, the positive effects of alpha-lipoic acid (ALA) on oocyte maturation, fertilization, embryo development, and reproductive outcomes were previously presented (12).

Possessing various intensive antioxidant characteristics in aqueous and lipid ambiance, ALA (also known as α-thiotic acid) works as a physiologic co-enzyme to the Krebs cycle. Besides its reduced form (dihydrolipoic acid [DHLA]), ALA is also able to utilize ROS scavenging tasks as well as regenerating enzymatic (glutathione and superoxide dismutase) and non-enzymatic (vitamins E and C) antioxidants (13). Furthermore, ALA and DHLA are putative metal chelators for namely Cu, Mn, Zn, Ars, and Hg (13, 14). Recent findings showed that ALA supplementation is associated with reinforced seminal antioxidant characteristics, reduced testicular oxidative damage, and androgen equilibrium (15-17). Moreover, evidence highlights that ALA supplementation is associated with lower sperm DNA damage both in animals and humans (18-21). In a recent study, we noticed that individuals with varicocele could gain more from an adjuvant three-month post microsurgical oral ALA supplementation course in comparison with sole surgical repair in terms of sperm motility and DNA damage (18).

To date, the limited documentation on the effect of antioxidants on sperm quality in couples with RPL has led to controversial results. While implementing an antioxidant-rich diet showed non-significant improvement in seminal parameters, a recent trial indicated that supplementation with Zinc and vitamin E could enhance the quality of sperm parameters and sperm DNA integrity in patients (22, 23). However, the effect of antioxidant medication on the clinical aspects of RPL has not been yet described. Therefore, we hypothesized that oral antioxidant supplementation with ALA could enhance sperm DNA and chromatin integrity, leading to improved embryonic development and, consequently, lower miscarriage rate in couples with a history of RPL. This preliminary study aims to evaluate this hypothesis based on a triple-blind randomized placebo-controlled clinical trial previously conducted by our group.

Materials and Methods

Study design

Conducted between July 2018 to June 2020, this clinical trial study is to report preliminary results of a randomized, triple-blind placebo-controlled clinical trial evaluating the effect of oral ALA supplementation in couples whose male partners exhibited high sperm DNA damage. Our primary analysis highlighted a notably higher prevalence of RPL among these couples compared with the general population (72 vs. 3%) (1). Considering a number of original papers, systematic reviews, and meta-analysis studies a significant correlation between sperm DNA damage with RPL was demonstrated. Therfore, the high level of sperm DNA fragmentation could be the reason for the high level of RPL (7, 23). In the light of these considerations, we aimed to assesspulation to explore the association between ALA supplementation and standard semen parameters, seminal and intracellular OS, the level of sperm DNA damage/chromatin integrity, pregnancy, and alive birth outcomes in couples with a history of RPL.

This study is approved by Royan Institute's Ethics Committee for Research Involving Human Subjects (IR.ACECR.ROYAN.REC.1397.108) and the Iranian Registry for Clinical Trials (IRCT20190406043177N1).

Patient recruitment

Male partners of couples with a history of RPL with a high level of sperm DNA damage were eligible to participate in our study. The DNA damage was assessed by applying sperm chromatin structure assay (SCSA) or terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method with 30 and 15% as the cut-off values, respectively (24, 25).

Patients and their partners were investigated for pre-existing fertility-deteriorating conditions and were consequently excluded from the study if present. Individuals with recent/ongoing history of varicocele, leukocytospermia, chemoradiation, cytotoxic medication, and malignancies were excluded from the study.

Patients were informed of the purpose and the rationale of the study, treatment groups, and randomization and instructed on sample collection/delivery to our laboratory.

It was elucidated that participation and medication were free of cost for all the subjects, and their right to access the test results was preserved. Participants were surveyed for data on their age, anthropometrics, and medical as well as medication/supplementation history was provided from each of the participants. Finally, signed written consent was acquired from each patient.

Interventions

In the case group, individuals were given 600 mg of ALA (Raha company, Iran) and controls received the identical placebo both daily for 80 days (17,18). ALA and placebo packaging was the same, and medications were given to the patients according to the simple randomization.

Participants were designated to provide us with a sample prior to the treatment and another one after 80 days of supplementation. Masturbation subsequent to 2-7 days of sexual deprivation was determined as our sample recruitment method of choice (26). After delivery to our laboratory, samples were weighed for assessment of semen volume and left to liquefy and, afterward, assessed for viscosity and liquefaction with the use of a wide-bore pipette.

Conventional semen analysis

Concentration

For assessment of sperm concentration, 10 µL of the semen sample was put on the sperm counting chamber (Sperm meter, sperm processor, Garkheda, Aurangabad, India). Afterward, a proficient technician counted cells with the use of a light microscope (LABOMED CxL; 20x) and eventually reported the observation as million sperms/ml (26).

Motility

Ten µl of semen was set on a sperm counting chamber and a coverslip (depth: 20 µm) was placed on top. Sperm motility was evaluated using an optical microscope (LABOMED CxL) and computer-assisted sperm analysis (CASA). A minimum of five microscopic fields (≥200 spermatozoa evaluated per field) were investigated. Sperm motility was determined as rapid progressive, slowly progressive, non-progressive, and immotile. Finally, percentages of total and progressive sperm motility were reported (26).

Abnormal sperm morphology

Applying Tygerberg criteria, two smears were prepared and fixed by methanol-dissolved triarylmethane dye. Afterward, the smears were stained with eosinophilic xanthene and basophilic thiazine solutions (Diff-Quick staining). Applying high magnification (1000x), a skilled technician assessed abnormalities in the head, neck, and tail regions and reported the morphologically abnormal spermatozoa per sample (26, 27).

Viability

Briefly, eosin Y (color index: 45380) and nigrosine (color index: 50420) dyes were resolved in distilled water (1:100 and 1:10, respectively) by applying mild heat. One drop of semen was blended with an equal amount of eosin Y. After 15 seconds, two drops of nigrosine were added to the mixture, followed by fine stirring. Promptly, 10 µm of the mixture was placed on a slide, smeared, air-dried, and cover-slipped. The smears were assessed using a bright-field optic microscope (LABOMED CxL; 100x) (200 spermatozoa/slide at least) (26).

Sperm DNA integrity/damage evaluation

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

Samples were washed with phosphate-buffered saline (PBS, Sama Tashkhis, Iran) and fixed in 4% methanol-free paraformaldehyde for 30 minutes. After re-rinsing with PBS, samples were permeabilized using 0.2% Triton X-100 (5 minutes). DNA fragmentation was determined by applying a detection kit (Apoptosis Detection System Fluorescein, Promega, Mannheim, Germany) followed by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA, USA).

Sperm chromatin structure assay (SCSA)

Two million spermatozoa were isolated and buffered to 1 ml with a mixture of TNE, NaCl, and EDTA. Afterward, 400 µl acid-detergent solution was added to 200 µl of the buffered sample. The blend was then stained by applying 1200 µl of acridine orange solution (Sigma, St. Louis, USA). Using a flow cytometer (FACSCalibur Becton Dickinson, San Jose, CA, USA), a minimum of 10000 sperm were assessed in each sample and the percentage of cells with DNA fragmentation was reported (28).

Aniline blue staining (AB)

Briefly, samples were washed with PBS, and two smears were prepared for each sample. Afterward, the slides were fixed with glutaraldehyde (2.5%) and later stained with aniline blue (5 in 4% acetic acid). The slides were then dried by consecutive ethanol bathing (70, 96, and 100%, respectively) and immersed in xylol for 5 minutes. Ultimately, the smears were mounted by Entellan mounting medium. A minimum of 200 sperm was evaluated using an optical microscope (LABOMED CxL) (29).

Chromomycin A3 (CMA3)

Briefly, two smears of sperm were prepared, washed, and fixed with Carnoy's solution per sample. Next, the smears were dyed with CMA3 solution (200 µ; 0.25 mg/ml) and washed with 1x-PBS three times. A minimum of 200 sperm was monitored by recruiting an epifluorescence microscope (Olympus, Japan) with proper filters (460-470 nm; 100x). Protamine-deficient and sufficient spermatozoa stained light yellow and dark yellow, respectively (30).

ROS generation

Intrinsic superoxide: Dihydroethidium and dichloro-dihydro-fluorescein assays

Briefly, two tubes were prepared. Samples were PBS-washed and approximately 5 million sperm were isolated in each tube later 1 ml of PBS was added to each. Afterward, 0.005 ml of Dihydroethidium (DHE) and dichloro-dihydro-fluorescein (DCFH) were separately added to a tube. The DCFH/DHE-stained samples were then incubated (37°C, 40 and 20 minutes, respectively). Finally, the samples were analyzed by applying flow cytometry (Becton Dickinson FACScan, San Jose, CA). The percentage of DCFH- or DHE-dyed sperm was recorded.

Lipid peroxidation, total antioxidant capacity evaluation, and superoxide dismutase activity

Malondialdehyde (MDA) formation to gauge the lipid peroxidation process using a commercial colorimetric MDA assay kit was studied (ZellBio GmbH, Ulm, Germany). The detection range was between 0.78-50 μM, according to the manufacturer. Seminal total antioxidant capacity (TAC) and superoxide dismutase activity (SOD) activity were evaluated by applying colorimetric kits from an identical supplier. The manufacturer's guidelines were followed and the colorimetric wavelength for TAC and SOD activity was set for 490 nm and 420 nm, respectively.

Pregnancy outcome

The participants' partners were followed up for an average of 12 months and surveyed regarding pregnancy occurrence/outcome. Consequently, the couples' spontaneous pregnancy and live birth rates were recorded.

Statistical analysis

The data were analyzed with the use of the Stata/IC 13.0 for Mac (StataCorp, USA). Given the normal distribution of the variables according to histograms, the intergroup pre-and post-intervention values by independent samples t test were compared. Further, paired samples t test was used to compare the values before and after measures in both ALA and placebo groups. Results were mentioned as mean ± standard error of the mean. $P<0.05$ is considered significant.

Results

In the current study, 35 individuals were allocated to each interventional group and randomized using permuted blocks. Subsequently, one subject in the ALA group and six placebo receivers were lost to follow-up (sample size: 34 and 29 ALA- and placebo-receivers). From the total of 63 left patients, 37 met the ASRM diagnostic criteria for RPL (two or more consecutive/non-consecutive abortions), confining our ultimate sample size to n=12 for controls and n=25 for cases (Fig.1).

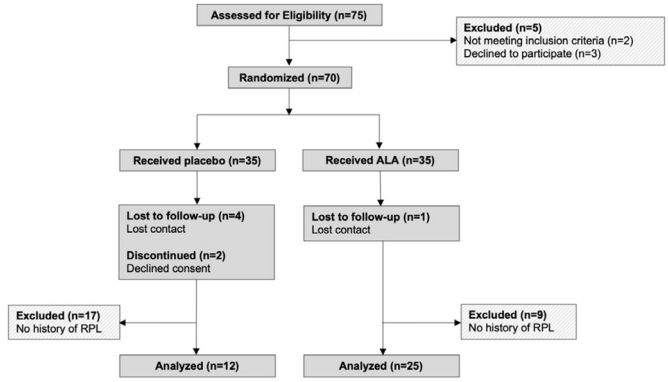

Fig.1: Study flow diagram and patient allocation. ALA; Alpha lipoic acid and RPL; Recurrent pregnancy loss.

Our analysis revealed no significant difference between the placebo and ALA groups in age (37.64 ± 2.31 vs. 40.12 ± 1.31, P=0.32), weight (88.3 ± 7.45 vs. 79.35 ± 2.69, P=0.16), height (174.7 ± 1.33 vs. 172.13 ± 1.17, P=0.21), and body mass index (28.76 ± 2.21 vs. 27.02 ± 0.82, P=0.36).

Except for semen volume (3.35 ± 0.30 vs. 4.52 ± 0.35, P=0.047), mean baseline levels of conventional sperm parameters consisting of concentration (78.54 ± 24.07 vs. 60.96 ± 9.71, P=0.42), total motility (51.91 ± 7.05 vs. 49.62 ± 4.20, P=0.77), viability (83.20 ± 6.41 vs 78.91 ± 3.66, P=0.54), and abnormal morphology (97.01 ± 0.86 vs. 98.07 ± 0.35, P=0.18) exhibited no statistically significant difference between placebo and ALA groups. Likewise, any significant difference in pre-interventional measures obtained from sperm DNA damage indicators, including TUNEL assay (18.80 ± 3.28 vs. 16.88 ± 2.47, P=0.67), SCSA (29.46 ± 5.49 vs. 36.87 ± 3.14, P=0.23), as well as chromatin integrity representatives [AB (17.36 ± 3.61 vs. 18.58 ± 2.40, P=0.78) and CMA3 staining (51.20 ± 3.64 vs. 55.16 ± 2.72, P=0.42)] between the placebo and ALA groups was not detected (Table 1).

Mean endline levels of conventional sperm parameters consisting of semen volume (3.56 ± 0.42 vs. 3.93 ± 0.33, P=0.55), sperm concentration (61.00 ± 8.57 vs. 58.45 ± 7.52, P=0.84), total motility (45.45 ± 6.49 vs. 58.45 ± 5.04, P=0.13), viability (83.13 ± 6.66 vs. 58.33 ± 2.65, P=0.71), abnormal morphology (97.9 ± 0.43 vs. 98.2 ± 0.23, P=0.39), and SCSA (31.18 ± 4.48 vs. 25.30 ± 3.46, P=0.32) exhibited no statistically significant difference between placebo and ALA groups. Meanwhile, the mean of TUNEL-assayed sperm DNA fragmentation level (14.19 ± 2.06 vs. 24.40 ± 3.66, P=0.01), CMA3 (41.35 ± 2.02 vs. 50.11 ± 2.73, P=0.02), and AB staining results (6.94 ± 1.39 vs. 13.22 ± 3.9, P=0.04) were significantly lower among ALA receivers compared to the placebo groups.

Unlike the placebo group, a significant increase in the mean percentage of sperm motility after ALA therapy (P=0.04) was noticed. However, the remaining semen parameters (sperm concentration, viability, and abnormal morphology) did not change significantly in either of the interventional groups (P>0.05, Fig.2).

Table 1: Pre- and post-interventional comparison between ALA and placebo receivers regarding conventional sperm parameters, sperm DNA damage and chromatin integrity, and seminal/intracellular oxidative stress representatives

Variable	Before (SE)		After (SE)	
	Placebo	ALA	Placebo	ALA
Abstinence (days)	3.09 (0.16)	3.4 (0.31)	3.15 (0.26)	3.37 (0.21)
	P=0.53	P=0.54		
Volume (ml)	3.35 (0.3)	4.51 (0.35)	3.56 (0.42)	3.93 (0.33)
	P=0.047	P=0.55		
Concentration (10^6 spermatozoa/ml)	78.54 (24.08)	60.96 (9.71)	61 (8.57)	58.45 (7.52)
	P=0.41	P=0.84		
Total motility (%)	51.91 (7.05)	49.62 (4.2)	45.45 (6.49)	58.45 (5.04)
	P=0.77	P=0.13		
Abnormal morphology (%)	97.09 (0.87)	98.08 (0.31)	97.09 (0.43)	98.29 (0.22)
	P=0.19	P=0.39		
Viability (%)	83.20 (6.4)	78.91 (3.67)	83.13 (6.66)	85.33 (2.65)
	P=0.54	P=0.71		
DNA damage (SCSA) (%)	29.46 (5.49)	36.87 (3.14)	31.18 (4.48)	25.30 (3.46)
	P=0.23	P=0.32		
DNA fragmentation (TUNEL) (%)	18.8 (3.28)	16.88 (2.47)	24.4 (3.66)	14.19 (2.06)
	P=0.67	P=0.01		
CMA3 (%)	51.2 (3.64)	55.16 (2.72)	50.11 (2.73)	41.35 (2.02)
	P=0.42	P=0.02		
AB (%)	17.36 (3.61)	18.58 (2.4)	13.22 (3.9)	6.94 (1.39)
	P=0.78	P=0.04		
SOD (AU)	24.14 (9.21)	13.64 (1.86)	12.8 (2.99)	11.58 (1.72)
	P=0.11	P=0.83		
TAC (AU)	0.46 (0.06)	0.37 (0.04)	0.37 (0.08)	0.27 (0.04)
	P=0.25	P=0.25		
MDA (AU)	34.24 (6.11)	34.82 (5.27)	27.19 (7.43)	25.68 (4.59)
	P=0.94	P=0.86		
DHE (%)	75.19 (5.65)	71.82 (3.45)	69.98 (6.47)	80.68 (3.59)
	P=0.6	P=0.13		
DCFH (%)	62.78 (7.83)	63.66 (5.17)	71.35 (10.61)	77.96 (4.56)
	P=0.93	P=0.5		
	P=0.15	P=0.32		

Represented as means and standard errors. ALA; Alpha-lipoic acid, SE; Standard error, ml; Milliliter, SCSA; Sperm chromatin structure assay, TUNEL; Terminal deoxynucleotidyl transferase dUTP nick end labeling, CMA3; Chromomycin A3, AB; Aniline blue, SOD; Superoxide dismutase, TAC; Total antioxidant capacity, MDA; Malondialdehyde, DHE; Dihydroethidium, DCFH; Dichloro-dihydro-fluorescein diacetate, and AU; Arbitrary unit.

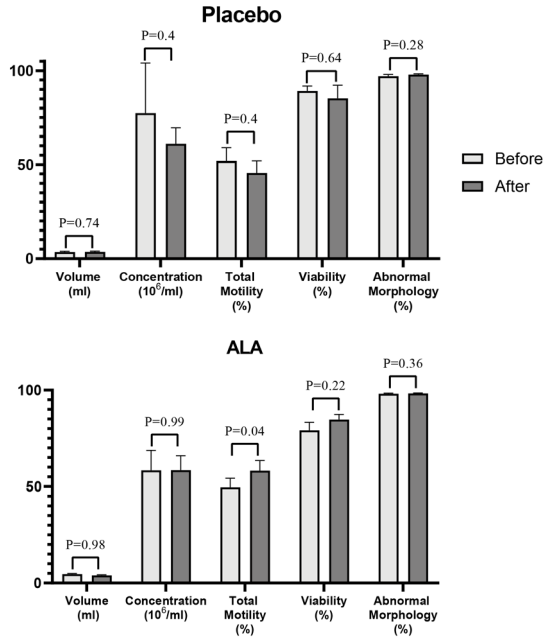

Fig.2: Comparison between sperm parameters before and 80 days after treatment with ALA or placebo. Represented as means and standard errors. ALA; Alpha lipoic acid.

According to SCSA, sperm DNA damage exhibited a significant decline post-ALA supplementation (P=0.004), but not after treatment with placebo (P=0.54, Fig.3). However, TUNEL results underlined statistically significant differences regarding sperm DNA fragmentation in neither of the interventional groups after treatment (P>0.05, Fig.3).

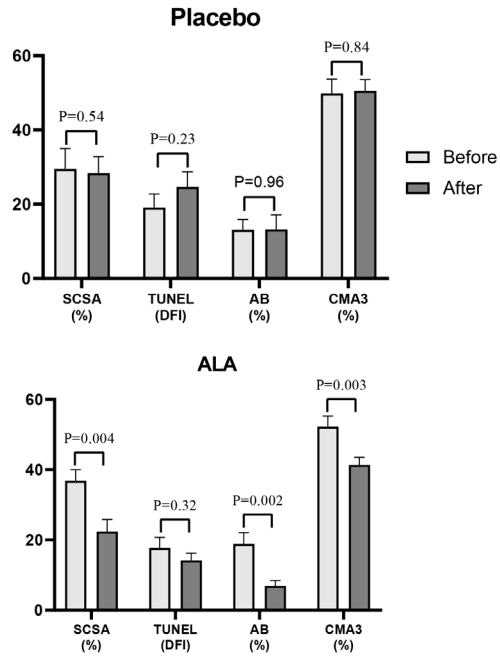

Fig.3: Comparison between the average sperm DNA damage (TUNEL and SCSA assays), percentage of sperm with persisted histones (AB staining) and protamine deficiency (CMA3 staining) before and 80 days after medication either with ALA or placebo. Represented as means and standard errors. SCSA; Sperm chromatin structure assay, TUNEL; Terminal deoxynucleotidyl transferase dUTP nick end labeling, ALA; Alpha lipoic acid, AB; aniline blue, and CMA3; Chromomycin A3.

Both CMA3 and AB tests revealed significantly lower levels after ALA-therapy (P=0.003 and P=0.002, respectively) while indicating no significant difference in the placebo group (P>0.05, Fig.3).

The analysis in the current study showed an insignificant rise in SOD (P=0.19) while a significant reduction in mean TAC and MDA levels after ALA-therapy was indicated (P=0.03 and P=0.02, respectively). In contrast, we did not witness any statistically significant difference between pre-and post-interventional measures of SOD, TAC, and MDA in the placebo group (Fig.4).

Finally, the percentage of spontaneous pregnancy in the ALA group (4 out of 25 cases, 16%) was insignificantly (P=0.5) higher than placebo (1 out of 12, 8.3%) group. A higher live birth rate in the ALA group than the placebo group was also observed, although not attaining statistical significance (ALA: 25.93% and placebo: 7.14%, P=0.15). Moreover, the mean frequency of executed ART cycles showed no significant difference between the two experimental groups (3.66: ALA and 2.87: placebo, P=0.85).

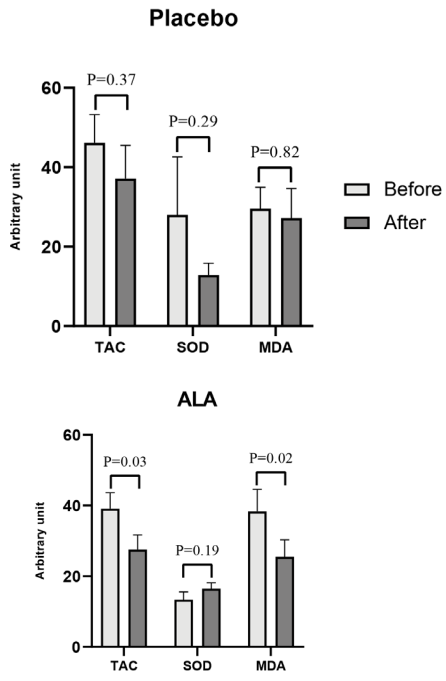

Fig.4: Comparison between baseline and endline mean measures of seminal oxidation and antioxidant indicators in both placebo and ALA groups. Represented as means and standard errors. TAC; Total antioxidant capacity, SOD; Superoxide dismutase, MDA; Malondialdehyde, and *; $\times 10^6$.

Discussion

Accumulating evidence supports the association between sperm DNA damage and RPL (7, 31, 32). Despite controversies, oral antioxidant supplements have relatively established their reputation as a safe empirical remedy for male infertility among reproductive specialists (33). However, their efficacy in terms of DNA damage alleviation is still arguable. To our knowledge, documentation on the effect of such products on sperm DNA damage/chromatin in male partners of the couples facing RPL is exceptionally scarce (22, 23). This study is the first to investigate the impact of antioxidant therapy on sperm DNA status and its relevance to pregnancy outcomes in couples with a history of RPL. Through the randomized placebo-controlled clinical trial, the beneficence of daily intake of ALA against placebo was weighed in regards to seminal OS, sperm nuclear integrity and DNA intactness, and eventually to pregnancy outcomes.

With a focus on sperm DNA fragmentation as the male-related factor to RPL, male partners of the couples with a history of RPL and high levels of sperm DNA damage were studied. The results indicated a significant decrease in mean sperm DNA damage (SCSA), nuclear protamine deficiency (CMA3 and AB staining), lipid peroxidation (MDA), and total antioxidant capacity of the seminal fluid following 80 days of oral supplementation with ALA.

The sperm DNA integrity status represents the degree of chromatin compaction obtained by several mechanisms, most notably through substituting histone nucleoproteins-plentiful in somatic cells with protamines (34). Protamines are in charge of maintaining the ideal compression within sperm chromatin by forming intra-/inter-DNA disulfide bridges making it less susceptible to nicks and fragmentations (34, 35). The evidence of the current study indicates a correlation between the extent of protamine deficiency and sperm DNA damage (36, 37). An increase in the mean of sperm nuclear protamine content and a simultaneous decrease in the level of remaining histones among ALA receivers were also observed, according to CMA3 and AB staining results, respectively. However, protamine-preserving characteristics of ALA in sperm nuclei have never been reported elsewhere. Such a feature may be attributable to ALA's capability to augment cellular cysteine content by means of upholding cysteine reuptake and active cytoplasmic cystine-cysteine reduction. The rise in cytoplasmic cysteine levels reinforces one-carbon metabolism, which in turn supplies spermatozoa with precursor methyl groups necessary for disulfide bonding, prompting the eventual robust sperm chromatin compression (38).

As evidenced, optimal chromatin compression protects sperm from DNA insults (37). Likewise, a statistically significant decrease in the mean level of sperm DNA damage as outlined by SCSA staining, while TUNEL assay results exhibited a non-significant incline. These findings are compatible with a previous study by our group, in which we found that men treated with ALA post-varicocelectomy experienced a significant decrease in their mean sperm DNA damage (18). Nevertheless, performing various sperm DNA testing enabled our study to thoroughly investigate the mechanisms involved in ALA-induced sperm DNA damage alleviation: TUNEL assay quantifies the present single and double-strand DNA breaks, while SCSA measures sperm DNA's susceptibility to single-strand nicks induced by acid denaturation (39). In this regard, our results rendered a notable decrease in

sperm DNA's predisposition to damage; however, ALA therapy did not compel the existing damage to resolve significantly.

In our study, after ALA supplementation, a significant decrease in mean TAC level was observed. TAC reflects the total ROS-scavenging capability of the present antioxidants in the seminal plasma, while SOD is an intrinsic enzymatic antioxidant capable of scavenging superoxide anion (11, 16). The significant decrease in seminal TAC content potentially depicts a steady ROS scavenging process in the individuals receiving ALA.

Analysis of data in this study indicated a statistically significant decrease in the mean level of MDA among ALA-taking individuals. MDA is primarily the end-product of lipid peroxidation, which is triggered by intracellular ROS overproduction leading to impairment in sperm's cytoplasmic membrane integrity/fluidity, ion-specific permeability, and enzymatic receptors' function (9).

As of today, our study is the second to investigate the correlation between antioxidant supplementation and sperm DNA damage/integrity in the male partner of the couples with RPL. A clinical trial on 60 men whose partners have encountered RPL in the past, has indicated the efficacy of antioxidant medication in chromatin quality promotion. Their results suggested that daily oral intake of Zinc and vitamin E for 90 days significantly improves semen parameters and DNA integrity in such couples (23). In our study, however, we further observed a decrease in DNA damage in addition to a similar enhancement in the chromatin integrity post-medication. Further, our study is the first to address pregnancy outcomes in this regard. We noticed that patients who underwent ALA supplementation tend to have higher rates of pregnancies lasting longer than 20 weeks of gestation compared to the controls (25.93% vs. 7.14%); however, such predominance did not attain statistical significance. Recent reviews and meta-analyses have confirmed the improvement of live birth/pregnancy rates and ART outcomes following antioxidant therapy (11, 40). However, a recent Cochrane meta-analysis assessed the effect of antioxidants on male subfertility, and the main outcomes included clinical pregnancy rate, live birth, and miscarriage. They concluded that administration of antioxidants may significantly lead to high live birth and clinical pregnancy, while there was no difference in miscarriage rate due to only three papers reported on this result (40). Nevertheless, further studies with adequate sample sizes and refined designs may shed light on this matter.

Conclusion

Daily supplementation with 600 mg of ALA improves sperm motility and DNA damage in the male partner of the couples with a history of RPL. Also, ALA therapy ameliorates sperm lipid peroxidation, leading to higher levels of DNA compaction through augmenting the sperm nuclear protamine content. Moreover, such intervention may decrease the rate of spontaneous pregnancy loss before the gestational week of 24; however, further investigation is needed to explain such effect satisfactorily.

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Authors' Contributions

M.H.N.-E., M.T.; Contributed to the conception, design, and coordination of the study, revised the manuscript, and performed the final scientific manuscript revision. M.H., Z.F.Z., V.E.; Were in charge of patient recruitment and drug prescription, executed the laboratory analysis, and analyzed the data. B.A.; Analyzed the data, performed literature research, drafted the manuscript, revised the manuscript, and the final scientific manuscript revision. A.Sh., M.A.S.G.; Were in charge of patient recruitment and drug prescription. All authors ead and approved the final manuscript.

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