

Original Article

The Effect of Vitamin E and Selenium on Sperm Chromatin Quality in Couples with Recurrent Miscarriage

Soheila Pourmasumi¹Ph.D., Nasrin Ghasemi^{2*}M.D., Ph.D., Ali Reza Talebi¹Ph.D.
Mehrnaz Mehrabani³Ph.D., Parvin Sabeti⁴Ph.D.

¹Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

²Recurrent Abortion Research Center, Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

³Physiology Research Center, Institute of Neuropharmacology, Kerman University of Medical Sciences, Kerman, Iran.

⁴Department of Anatomical Sciences, Kurdistan University of Medical Sciences, Sanandaj, Iran.

ABSTRACT

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Key words

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Background and Aims: Due to the paucity of studies, association between the morphology and function of sperm and recurrent miscarriage (RM) is not yet completely known. Increased reactive oxygen species and decreased antioxidant levels in men have been shown to be associated with RM. Recently it has been accepted that antioxidant therapy can approve sperm parameters. The goal of this study was to evaluate the effect of paternal factor and antioxidant therapy on sperm parameters in the couples with RM.

Materials and Methods: Sixty ejaculate samples with RM patients were analyzed before and after 3 months of vitamin E and selenium therapy. Sperm chromatin assay was assessed by cytochemical tests including aniline blue, chromomycin A3, and toluidine blue. To measure DNA fragmentation index, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) test was used. Data were analyzed by SPSS software.

Results: Patients had significantly higher percentage of sperm parameters ($p < 0.001$) compared to the time before treatment. TUNEL positive spermatozoa were decreased in post treatment compared to pre-treatment phase ($p < 0.0001$).

Conclusions: Our study demonstrates that antioxidants can improve sperm parameters and chromatin condensation in recurrent miscarriage male partner.

Introduction

Recurrent miscarriage (RM), also known as repeated pregnancy loss (RPL) and habitual abortion, is defined as three or more repeated spontaneous miscarriages [1]. The overall frequency of RM was estimated from 1% to 3% in women in their reproductive age [2]. The exact prevalence of RM depends on its definition. To date, there is no consensus on the definition of RM with regard to the number of the previous miscarriages and the gestational age of RM. The American Society for Reproductive Medicine defines the number of previous miscarriages in RM as two or more whereas Europe Society of Reproduction and Embryology defines it as three or more [3]. In some studies only pregnancy losses in the first trimester (≤ 14 weeks) were included whereas in others, pregnancy losses of the second trimester (≤ 24 weeks) were also investigated [4]. In some studies, the gestational age of repeated miscarriages was not clarified [5, 6]. Therefore, it is possible to study different populations in the forthcoming studies. However, the incidence of RM derived from these studies may not be comparable.

RM is a heterogeneous condition and it is unlikely that only a single pathological factor is attributed to RM [7]. Different factors may be involved in the etiology of RM including chromosomal abnormalities, anatomical anomalies, genetic disorders, psychological, thrombotic and immunological defects [8] while between 50% to 60% of cases remain to be idiopathic. The remaining RM cases are classified as idiopathic [7]. Hence, further

research is essential to explore other possible underlying causes of RM. To date, the identifiable causes of RM have been categorized as parental, fetal, environmental and psychological [9].

The clinicians have mostly focused on female factors and less attention has been paid to probable role of male factor in diagnosis of RM. There are some evidences proposing that sperm factors may have some role in inducing RM [10]. Due to the scarcity of studies, association between the morphology and function of sperm and RM is not yet known [10]. Some preliminary studies suggest that abnormal integrity of sperm DNA may increase the risk of miscarriage [11, 12]. However, these studies focus more on sporadic miscarriages rather than RM. An *in vitro* study assessed sperm functional parameters, such as hypo-osmotic swelling, acrosomal status, and nuclear chromatin decondensation between men of partners with unexplained RM and men who had recently conceived a child. It found a significant reduction in sperm function in the case group compared with the control group, suggesting that impaired sperm function may play a role in RM [13]. Carrell et al. also found a significant increase of sperm chromosome aneuploidy, apoptosis, and abnormal sperm morphology in men with partners having a history of RM [11]. Increased levels of reactive oxygen species (ROS) and decreased antioxidant levels in men have been shown to be associated with RPL [14]. Embryonic development is affected by both sperm and

oocyte genome [15]. It has been reported that the sperm DNA quality has been associated with reproductive outcome especially with RM [12]. Sperms in seminal plasma with endogenous antioxidant protect themselves from oxidative stress [16]. Recently it has been accepted that antioxidant therapy can approve sperm parameters [16, 17].

Sperm DNA damage may be a major leading cause of RM when male factor is involved, and oxidative stress is one of the main reasons of sperm DNA damage as stated before. The aim of this study was to evaluate the effect of paternal factor and antioxidant therapy on sperm parameters in couples with RM. So we focused on sperm parameters and sperm DNA damage in couples with RM before and after antioxidant therapy.

Materials and Methods

Study design

Patients

Sixty couples with two or more RM history in the first or early second trimester were chosen from recurrent abortion clinic in Shahid Sadoughi university of medical sciences of Yazd in Iran. Our study proposal was approved by Ethics Committee of Research and Clinical Center for Infertility, Shahid Sadoughi, University of Medical Sciences Yazd, Iran and was registered in Iranian registry for clinical trial by number: IRCT201704195261N3.

Idiopathic RM cases were enrolled for the study from February 2014 to May 2016. These cases were selected after detailed work for male and female factor by the clinicians. Selection criteria included a history of at least two prior

pregnancy losses at <20 week of gestation. All known causes for RM were ruled out and idiopathic cases were chosen. In brief, female partners of the couples had normal haemogram with normal anatomical status, normal ovarian function, and had normal endocrinological parameters. Other causes for RM like thrombophilia and antiphospholipid were also screened and male partners having normal semen parameters only were included in the study. Also the karyotypes of both partners were analyzed to factor out any chromosomal abnormality. Informed consent was obtained from all of the cases.

Semen samples were obtained by masturbation in a sterile container after 3-4 days of sexual abstinence. After liquefaction at 37°C for 30 minutes, basic semen parameters (volume, concentration, and total motility) were performed in terms of the World Health Organization guidelines. Sperm morphology was evaluated using the gimsa staining method. All patients were asked to take one tablet of vitamin E and selenium per day for 3 months and after 90 days, semen samples were collected and analyzed. Data obtained from pre- and post-antioxidant therapy were then compared.

Semen Preparation

After semen analysis, spermatozoa from fresh semen were washed twice in 8 ml of phosphate-buffered saline (PBS, pH 7.4) (Sigma, St Louis, MO) by centrifugation at 400 g for 5 min. Then the final pellets were fixed with 5 ml of acetic acid/methanol mixture (Merck, Darmstadt, Germany) for at least 30 min. at 4°C. Aliquots of the resulting suspension of nuclei were

smear on slides. Slides were kept frozen at -20°C to be later used for chromatin structure assay.

Detection of DNA fragmentation

The presence of DNA strand breaks in spermatozoa was evaluated by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay, using the ApopTag Apoptosis Detection Kit (Qbiogene, Paris, France). For cell permeabilization, slides were incubated in PBS with a solution of 1% Triton X100 (Sigma). The later phase of the procedure was carried out according to the manufacturer's instruction. Briefly, the specimens were washed twice in PBS, equilibrated with the equilibration buffer at room temperature for 10 seconds, and were incubated in a dark, moist chamber at 37°C for one hour with the terminal deoxynucleotidyl transferase (TdT) solution to allow DNA elongation. For each batch, a negative control without adding TdT enzyme and a positive control with DNase I treatment were always included to ensure the reproducibility of the assay. After stopping the enzyme reaction, the slides were washed twice in PBS and the DNA elongation was identified by incubation of the cells with antidigoxigenin antibody coupled with peroxidase for 30 min. in a dark, moist chamber. The peroxidase was revealed with diaminobenzidine. Slides were then counterstained with Harris' hematoxylin (RAL, Martillac, France) and finally mounted using Faramount mounting (Dako, Carpinteria, CA). Slides were observed under a microscope (Zeiss, Oberkochen, Germany) equipped with an x100 magnification lens. Spermatozoa with

fragmented DNA had brown-colored nuclei whereas the other cells were blue-gray (counter coloration with Harris's hematoxylin). On each slide, approximately 500 cells were counted, and the percentage of spermatozoa with fragmented DNA (DFI) was calculated.

Aniline blue staining

The aniline blue stain discriminates between lysine-rich histones and arginine/cysteine-rich protamines and has been used to reveal residual histones in sperm chromatin structure [18]. For this staining, air-dried slides were prepared from washed semen samples and were fixed in 3% buffered glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 30 min. at room temperature. Smears were stained for 5 min. in 5% aqueous aniline blue (Sigma Aldrich CO., USA) (pH 3.5). The percentage of spermatozoa stained with aniline blue is determined by counting 200 spermatozoa per slide under light microscopy (Carl Zeiss Axiostar plus, Germany). Sperm heads with unstained or pale blue stain were considered as normal and those with dark blue stained were rated as abnormal spermatozoa [19].

Toluidine blue staining

Toluidine blue is a basic nuclear dye used for metachromatic staining of sperm nuclear chromatin via binding to phosphate groups of DNA strands [20]. In brief, smears were air-dried, fixed in freshly 96% ethanol-acetone (1:1) at 4°C for 30 min. and then hydrolyzed in 0.1 N HCl at 4°C for 5 min. The slides were rinsed three times in distilled water for 2 min. Finally smears were stained with 0.05% toluidine blue (Sigma Aldrich Co., USA) for 10 min. at room temperature. Toluidine blue

staining buffer consists of 50% citrate phosphate (McIlvain buffer, PH=3.5). Afterwards, smears were mounted in DPX medium (Thermo scientific, UK). In light microscopic study, sperm heads with good chromatin integrity stain light blue (toluidine blue negative) and those of diminished integrity stain from dark blue to violet or purple (toluidine blue positive) [21].

Chromomycin A3 (CMA3) staining

CMA3 is a guanosine- cytosine- specific fluorochrome that reveals chromatin that is poorly packaged in human spermatozoa via indirect visualization of protamine deficient DNA [22]. For CMA3 staining, semen smears were first fixed in Carnoy's solution (methanol/glacial acetic acid, 3:1) at 4°C for 10 min. and then air-dried at room temperature for 20 min. The slides were treated for 20 min. with 100 µl CMA3 solution (Sigma, St Louis, MO, USA). CMA3 solution consists of 0.25 mg/ml CMA3 in McIlvain's buffer (pH 7.0) supplemented with 10 mMol Mg/Cl₂. The slides were rinsed in buffer and mounted with 1:1 PBS-glycerol. A total of 200 spermatozoa were randomly evaluated on each slide. Evaluation of CMA3 staining was performed by distinguishing spermatozoa that stain bright yellow (CMA3 positive) from those that stain a dull yellow (CMA3 negative) under fluorescent microscope (Olympus BX51, Tokyo, Japan) [19].

Statistical analysis

Data were analyzed using statistical package for the social sciences 20.0 (SPSS, SPSS Inc, Chicago, Illinois). Continuous data were presented as mean±SD and assessed by

independent Student's *t*-test. Paired sample *t*-test was used whenever appropriate. P-value less than 0.05 was considered as statistically significant.

Results

The mean age of the male patients was 32.16±4.80 while for their female partners it was 28.13±4.41 years (Table 1).

As shown in table 2, sperm quality of the pre-treatment group was compared with that of the post treatment group. There was a significant difference in all sperm parameters between pre and post treatment. Indeed, sperm concentration was found to be normal (>20×10⁶/ml) in all cases. Statistical analysis demonstrated significantly higher semen volume of pre-treatment in comparison to post treatment patients.

Mean value for the sperm concentration in the pre-treatment group was 95.26±21.91 which improved to 105.83±24.04 (10⁶/ml) after treatment with Vitamin E plus Selenium. The number of sperms with abnormal morphological decreased after antioxidant therapy. The results revealed a significant difference in morphology of sperm in patients from pre-to post-treatment. The results of the chromatin condensation assay and TUNEL assay is shown in table 3. There was a higher number of aniline blue positive in pre-treatment patients compared to post-treatment which was statistically significant. For the whole study population, the average values of toluidine blue positive sperm were analyzed (65.90±16.70 vs. 54.33±19.11) and the difference appeared to be statistically significant (p=0.001). A significant difference was also observed in the percentage

of CMA3+Sperm in pre and post treatment groups (40.30 ± 11.89 vs. 32.16 ± 8.37). The mean number of sperm cells with fragmented DNA was $32.36\pm 5.02\%$ in the pre-treatment

group compared with $26.96\pm 5.48\%$ in the post-treatment group. This decrease in DNA fragmentation was statistically significant. (Table 3).

Table 1. Demographic parameters in all study patients

Parameter	Mean±SD	Min	Max
Male age (yr)	32.16±4.80	24	45
Female age (yr)	28.13±4.41	19	35
Miscarriage time (n)	3.03±1.09	2	6

Table 2. Pre and post treatment seminal parameters in study patients

Semen parameters	Pre-treatment	Post treatment	P-value
Volume (ml)	3.40±1.76	3.91±1.56	0.04
Count ($\times 10^6$ /ml)	95.26±21.91	105.83±24.04	0.006
Motility (%)			
Progressive	55.16±8.17	64.53±9.36	0.001
Non progressive	12.30±3.74	9.00±3.99	0.004
Immotile	31.86±7.12	26.46±8.39	0.005
Morphology (%normal)	33.73±7.11	39.06±7.56	0.01

Values are presented by mean±SD. P-values obtained from difference between means were tested for significance by one way ANOVA on ranks.

Table 3. Sperm nuclear maturity tests in pre and post treatment patients

Chromatin integrity tests	Pre-treatment	Post treatment	P-value
Aniline blue	52.43±20.06*	45.20±19.45	0.01
Toluidine blue	65.90±16.70	54.33±19.11	0.001
Chromomycin A3	40.30±11.89	32.16±8.37	0.0001
TUNEL	32.36±5.02	26.96±5.48	0.0001

*Values are presented by mean±SD ($\times 10^6$ /ml). Paired sample t-test was used to compare dependent variables. TUNEL= Terminal deoxynucleotidyl transferase dUTP nick end labeling

Discussion

The results showed that treatment with vitamin E and selenium improves all sperm parameters and is statistically different from pre-treatment status. Although there was a positive effect on the seminal parameters with pre-treatment, the factors had significant difference between pre-

and post-treatment groups. Structure of sperm DNA is highly compacted. This form is the main factor to protect genomic substance [23]. ROS in high level is harmful for sperm DNA. When antioxidant capacity in sperm is below the cut off value, ROS level raises in cells [24].

Studies have shown that imbalance between antioxidant capacity and ROS level is important to predict cell damage. Researchers have shown that ROS level in semen is elevated in RM patients [25]. This elevation can raise DNA damage in sperm and subsequently can effect sperm fertilization ability, embryo development and pregnancy outcome. This process adversely affects embryogenesis and is a cause of RM. In the past, many studies have recommended that when DNA of sperm is fragmented, pregnancy can not occur, but now the role of paternal factor in RM is being under research [26-28]. Oxidative stress is the major origin of DNA damage especially in sperm cells and antioxidants can protect cell from ROS dangers [16]. Several clinical trials have examined the potential of antioxidants in reducing the level of free radicals and have concluded that antioxidant supplementation is beneficial to reduce oxidative stress in the infertile seminal fluid [17, 24, 29].

In a pioneering study by Maiorino et al. in 1980s, the effect of vitamin E on the improvement of sperm parameters was investigated [30]. Later, general studies were carried out to explore the effect and mechanism of antioxidant on sperm. The studies confirmed that vitamin E improves the concentration and motility of sperm in infertile men and increases pregnancy rates in infertile couples [31-33].

It has been reported that selenium improves sperm parameters by reducing malondialdehyde, a major product of lipid peroxidation [34], which can validate our findings. Some researchers believe that selenium may protect sperm by antioxidant

role against seminal oxidative stress [35, 36]. Brown et al. showed that selenium is essential for spermatogenesis [37]. Agarwal and his colleagues also reported the positive effect of selenium on the quality of sperm parameters, especially concentration, motility and morphology. They believe that the presence of selenium in seminal fluid can prevent free radicals effects and improve sperm function [38]. In a study by Gil-Villa et al. in 2009, it was reported that an antioxidant-rich diet improves parametric and functional status in the sperm of the RM patients [39]. In another study, Ko et al. identified that antioxidant-rich diets cannot improve sperm parameters; there was no significant change in the sperm parameters before and after the treatment [40]. Scott et al. reported that administration of 30-40 µg selenium daily improves sperm quality and fertility [41]. In another study, Agarwal et al. indicated that oral administration of selenium for 3 months can significantly increase sperm motility [38]. In line with the previous studies around the effect of antioxidant on sperm parameters, our result also showed that sperm concentration, motility, and morphology progressed significantly after antioxidant therapy.

This is the first study to evaluate the effect of antioxidant supplements on sperm chromatin in RM patients.

Today, the basic sperm analysis cannot reflect the sperm chromatin status and sperm with normal morphology and mobility can also have abnormal chromatin or DNA defect [42]. Studies have demonstrated that infertile men with normal sperm parameters can show a high

level of DNA defect [42, 43]. Based on our findings, aniline blue positive, toluidine blue positive and CMA3 positive sperms, after treated by vitamin E plus selenium, showed statistically significant reduction compared with the pre-treatment phase.

Our results also showed that DNA fragmentation decreases in post-treatment patient significantly. Measurement of fragmented DNA by TUNEL assay displayed negative association between antioxidant therapy and level of sperm DNA fragmentation. In the post-treatment group, TUNEL positive sperms statistically decreased compared with the pre-treatment group.

Chromatin condensation assay in our study group showed that histone to protamine transition had defect and their sperm chromatin structure had residual histone. In other words, the quantity of protamine was indirectly decreased; therefore, it can be said that sperms have irregular condensation or chromatin density. Talebi et al., as well as Kazerooni et al., in two separate studies examined the sperm chromatin condensation in RM patients. They reported defect in chromatin density of their patients [44, 45]. In 2003, Carrell and his colleagues found that abnormalities in the sperm nuclei were widely present in the patients with RM [11]. Our study results were parallel with all of these studies.

Since many reproductive researchers and geneticists believe that mother's role is very critical in RM, and the role of paternal factor in recurrent abortion is still mysterious, much more investigations are needed in this regard. Our study is the first one to investigate the effect of antioxidant supplements on sperm

parameters and sperm chromatin condensation in RM. In the studies conducted on antioxidants effects, scientists have focused on infertility, and most studies have assessed the effect of antioxidants on sperm parameters of infertile compared with fertile men. Today, researchers attempt to understand the link between RM and sperm DNA integrity and quality. The results of these studies are controversial. So far, the effect of the integrity of the sperm DNA in patients with RM is still not completely known. Perhaps the important cause for the difference between the results of previous studies is the number of patients included or different evaluation techniques. On the other hand, researchers suggest that the effect of the paternal factor on RM, compared with that of the maternal one, is so slight that it can be neglected.

Conclusions

According to the results, it is clear that in RM patients, there are reduction in sperm parameters such as decreased motility, increased abnormal morphology, abnormal sperm chromatin packaging and DNA fragmentation. The reason for this can be due to the effect of oxidative stress in the sperm. Poor chromatin packing or DNA damage can be the cause for the inability of the sperm nucleus to develop the embryo in post-fertilization stages. Therefore, abortion in patients who have no female factor with abnormal sperm parameters can be explained by our findings.

Conflict of Interest

The authors have no financial or nonfinancial conflicts of interest.

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