Original Article

Comparison between sperm parameters and chromatin in recurrent pregnancy loss couples after antioxidant therapy

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Abstract

Background and Aims: Recurrent Pregnancy loss (RPL) is a heterogeneous disease. The role of maternal factor is clear but the relationship between the paternal factors remains uncertain. It has been shown that increase the level of Reactive Oxygen Species (ROS) and decrease the antioxidant levels in men can lead to RPL. New researches show treatment with antioxidant can improved sperm parameter. The aim of the present study was to assess the effect of vitamin E plus Zinc therapy on sperm parameters and chromatin quality in couples with RPL. **Methods:** In this clinical trial study, 60 RPL patients were selected from RPL clinic. Standard sperm parameters were analyzed and then male partners were intake vitamin E and Zinc in antioxidant therapy plan for 90 days. After that, sperm parameters were analyzed again. Sperm chromatin assay was reviewed before and after vitamin E and Zinc therapy by cytochemical assay including aniline blue (AB), chromomycin A3 (CMA3), toluidine blue (TB). To assess DNA fragmentation index, TUNEL test was used. Data were analyzed and compared before and after treatment. **Results:** data analysis showed all sperm parameters significantly improved after treatment ($P < 0.001$). The number of AB^+ and TB^+ sperms were decreased significantly after vitamin E and Zinc therapy ($P = 0.0001$). Decrease in DNA fragmentation in post treatment group in comparison to pre-treatment was statistically significant. **Conclusion:** Supplemental of vitamin E plus Zinc may improve sperm parameters chromatin quality and decrease sperm DNA fragmentation in RPL patients.

Keywords: Antioxidant, chromatin, recurrent pregnancy loss, sperm

Introduction

Recurrent pregnancy loss (RPL), referred to the happening of three or more repeated pregnancy losses; though, the American Society of Reproductive Medicine (ASRM) has newly redefined recurrent pregnancy loss as two or more pregnancy losses.[1]

Several factors can cause RPL such as abnormalities in chromosome structure, genetic anomalies, abnormal anatomic structures, psychological problems, hormonal disorders, thrombotic and immunological defects. In half of the cases the etiology of RPL remains unexplained.[2] In recurrent pregnancy loss cases, primary care can play an important role. Here, simple testing or treatment the physicians by safely primary care can prevent the next miscarriage. Recently, clinical researchers have focused on the effect of male factors on RPL. In the past, clinicians believed that the maternal factor is the cause of abortion. However, now, the results of research have shown that when a woman has successfully conceived, but it has then ended by miscarriage, it may be due to a male factor.[3]

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In many cases, abnormal sperm with fragmented DNA can lead to pregnancy but in next step abnormal embryo development can terminate by miscarriage. Some studies suggested that abnormal sperm DNA integrity may increase the risk of abortion.[4,5] In a study, researchers evaluated sperm function by functional tests and sperm chromatin de-condensation between men whose partners were diagnosed with RPL and healthy men who had a child newly. They reported a significant decrease in the sperm function in the RPL cases compared with the healthy men.[6] Carlini and her colleagues in 2016 found in Italian men whose partners have a history of RPL sperm DNA fragmentation and abnormal sperm morphology were significantly increased.[7]

One of the factors that is harmful for sperm and can create damage to sperm DNA is Oxidative stress (OS).[8] There is a theory in men, elevation in Reactive Oxygen Species (ROS) level and demotion in antioxidant levels can disrupt balance in antioxidant status and maybe in couples lead to RPL.[9] Recently, researchers showed male antioxidant therapy can improve sperm parameters,[10] but in our review of the literature, we did not find any article that evaluates the effect of antioxidant supplementation on chromatin quality in RPL patients and this was the novelty of study.

The aim of this study was to investigate the antioxidant effects on male factor in couples suffering RPL. In this study we assessed male fertility parameters including sperm parameters and chromatin quality before and after antioxidant therapy to establish any paternal contribution to the RPL.

Methods

Procedure

This clinical trial study was approved by Ethics committee of Research and Clinical Center for Infertility, Yazd Reproductive Sciences Institute, Shahid Sadoughi University of Medical Sciences, Yazd, Iran. 60 patients with RPL history (2 or more repeated, unexplained miscarriages in the first or early second trimester) were selected from the abortion clinic, our Reproductive Sciences Institute, from February 2014 to May 2016. Written informed consent was obtained from all of the cases.

Our study cases were included after checkup women and male partner by the clinician. Female and male factor was checked and RPL cases with unknown cause cases were selected. On the other hand women in our study had normal anatomical, hormonal and physiological status. Patients with age older than 45 years old, the history of thrombophilia, antiphospholipid factor and other causes of RPL were excluded and male partners having normal semen parameters. Also, males and females were analyzed and were normal chromosomal karyotypes.

Male referred to andrology laboratory and sterile container give to them and wanted to obtain semen by masturbation after 3-7 days of sexual abstinence. After liquefaction at 37°C for 30 min, basic semen parameters including count, motility and morphology were performed as per the World Health Organization (WHO 2010) guidelines.[11] Sperm morphology was evaluated using the papanicolaou staining method.

In this step all patients were given 400 IU synthetic vitamin E (α-tocopherol) daily in combination with Zinc (10 mg) for 90 days and after this for the second time semen samples were collected and analyzed. Data from before and after antioxidant intake were entered into the computer system, and were analyzed and compared by statistical software. [Figure 1]

Semen preparation

For cytochemical tests 100 μ of the fresh semen was stored at -80°C till to assay. After semen analysis, fresh semen prepared and in first step sample mixed by of phosphate-buffered saline (PBS, pH 7.4) (Sigma, St Louis, MO) and centrifuged at 400 g for 5 min 2 times. In the next step, pellets mixed with 5 mL of acetic acid/methanol (Merck, Darmstadt, Germany) for at least 30 minutes at 4°C for fixation. Then 40-50 μL of this fixed suspension were smeared on slides and were kept frozen at -20°C until use for chromatin structure assay.

Aniline blue (AB) staining

The histone protein contains a large amount of lysine amino acid, which reacts with acidic colors such as AB. Therefore, when sperms after condensation in their chromatin have residual histone, are characterized by AB. To perform this test, after smear extraction from sperm samples and drying in air for 30-60 min, fixation was performed by 3% glutaraldehyde in phosphate buffer (pH 7.2) for 30 min at room temperature. In the next step, fixed slides were stained for 10 min with 5% AB (Merck, Germany) (PH = 3.5). After washing with distilled

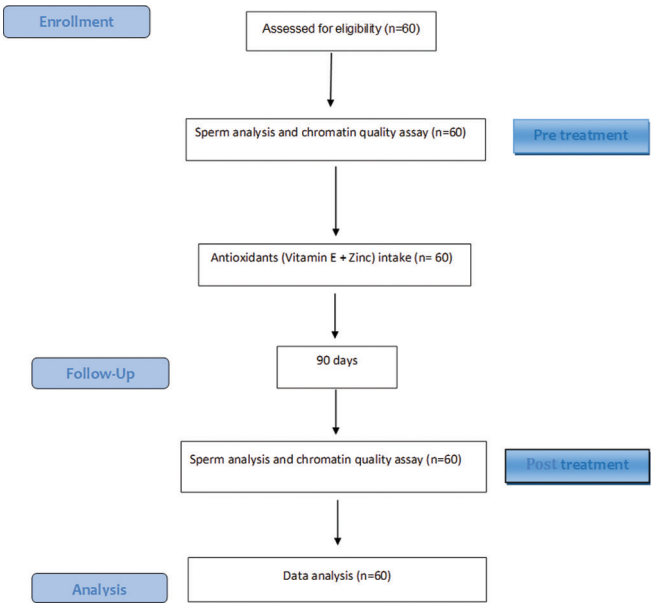

Figure 1: CONSORT Diagram of study

water and filtration using a Mont-DPX (Merck, Germany) solution, the slides were examined under light microscope (X-100 magnification), and counting 200 sperms, the percentage of colorless (mature) and blue (immature) sperm in each sample was determined. Sperm heads with unstained or pale blue stain were considered as normal spermatozoa and those stained dark blue were rated as abnormal spermatozoa.[12]

Toluidine blue (TB) staining

Toluidine blue is a metachromatic color. In the low-density chromatin, phosphate groups are uncovered and in this condition there was high trends to reaction by TB, so a range of bright blue (natural chromatin sperm), dark blue (mild chromosomal abnormal), purple (sperm with sever chromatin abnormality) and ultimately purplish (sperm with a very sever chromatin abnormality) can be detected. For this staining air-dried slides were fixed by freshly 96% ethanol–acetone (1:1) at 4°C for 30 minutes and then hydroxylation were done by 0.1 hydrochloric acid (HCl) at 4°C for 5 minutes. In the next step, the slides were rinsed in distilled water for 3 times (each time 2 minutes). Finally smear staining by 5% TB (Sigma Aldrich, USA) solution in McAllowin buffer 50% for 10 min at room temperature. Slides were mounted by mount-DPX (Merck, Germany), the slides were examined under light microscope (X-100 magnification), and counting 200 sperms.[13]

Chromomycin A3 (CMA3) staining

The fluorescent color of CMA3 is competing with protamine molecule for binding to a small DNA cavity and therefore indirectly shows the amount of protamine deficiency in the structure of sperm chromatin. So sperms that have protamine deficiency are colored by CMA3 and are detected in a fluorescent microscope with a bright yellow color. These sperms are defined as sperm with immature chromatin. For CMA3 staining, the slides were fixed in a refrigerator for 10 minutes by Carnoy's fluid is the fixative (methanol and acetic acid glacial 1:3) and then, in a dark room, 100 µl of CMA3 solution (Sigma-USA) was added on to each fixed slide for 10 minutes. In the next step, the slides were washed twice with a McIlvain's buffer (pH 7.0). Slides were mounted by mount-DPX (Merck, Germany), the slides were examined under fluorescent microscope (Olympus BX 51, Japan) and counting 200 sperms was done by distinguishing spermatozoa that stain bright yellow (CMA3 positive) from those that stain a dull yellow (CMA3 negative).[14]

Detection of DNA fragmentation

This technique directly shows the fracture percentage in one or two strands of DNA in the sperm. For this staining, at first slides were placed in 100% methanol for 4 min to fix the slides and then using ApopTag Apoptosis Detection Kit (Qbiogene, Paris, France).

After passing all step of kit protocol, slides were observed under a microscope (Zeiss, Oberkochen, Germany) with an X100 magnification. 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.[14]

Statistical analysis

Statistical analysis was done using the SPSS 20.0 software (SPSS Inc., Chicago, IL). The data distribution was normalized with K-S test. Result presented by mean ± SD. Independent sample *t*-test and Mann-Whitney U test were used whenever appropriate. Paired sample T test was used to comparison pre and post treatment parameters. Two tailed *P* value less than 0.05 considered to have statistically significant outcomes for the measured cases.

Results

Data analysis showed the mean age of the male and female partner were 32.10 ± 5.24 years respectively [Table 1].

Sperm parameters

Sperm parameters were analyzed in pre- and post-antioxidant therapy and results defined in Table 2. All sperm parameters such as count, motility, and morphology improved after treatment statistically significant.

There was increase in semen volume in pre-treatment than post treatment patients. However, this changes were not statistically significant. Mean values for the sperm concentration in the pre-treatment group were 96.56 ± 21.00 and enhanced after treatment with vitamin E in combination with zinc to 107.03 ± 18.07. The numbers of sperm with normal morphological shape increased after antioxidant therapy.

Sperm chromatin quality

Analysis of chromatin condensation showed higher number of AB^+ TB^+ sperms in pre-treatment patients compared to post treatment and this difference was statistically significant ($P = 0.0001$). A significant difference was observed in the percentage of

Table 1: Background factors in study cases

Factor	Mean±SD	Min	Max
Men age (years)	32.10±5.24	24	45
Women age (years)	29.60±5.21	19	35
Miscarriage time (n)	3.10±1.21	2	6

Table 2: Standard sperm parameters in pre and post antioxidant therapy groups

Semen parameters	Pre-treatment	Post treatment	*p*
Volume (ml)	3.09±1.64	3.49±1.28	0.1
Count ($\times 10^6$)	96.56±21.00	107.03±18.07	0.0001*
Motility (%)			
Progressive	57.03±9.00	65.06±8.72	0.001*
Non progressive	11.03±3.77	8.80±4.38	0.05*
Immotile	31.93±8.45	26.13±8.03	0.01*
Normal Morphology	33.90±6.40	41.26±6.93	0.0001*

Data was presented by mean±SD. Paired sample t-test was used to compare dependent variables. *P* values ≤0.05 considered statistically significant

CMA3+ sperm in pre and post treatment groups. The mean number of sperm cells with fragmented DNA in pre- and post-treatment were 32.03 ± 6.06% vs. 27.20 ± 5.64%, respectively. There was statistically significant decrease in DNA fragmentation [Table 3].

Discussion

Our result showed a significant increase in the motility, number, and normal morphology after 3 months of antioxidant therapy with Vitamin E plus Zinc. In addition, sperm chromatin quality was significantly increased after treatment. Several studies showed a relationship between sperm chromatin integrity and implantation failure or a rise in abortion risk. Numerous studies demonstrated any increase in ROS level in sperm is harmful for DNA and has negative effect on embryo formation, implantation and pregnancy development.[15,16] Although antioxidants can protect sperms, in some conditions, an increase in ROS level and decrease in antioxidant capacity lead to imbalance between ROS and antioxidants. This is a harmful condition for sperm chromatin. Studies have shown in patient with history of RPL, ROS level in semen is raised abnormally.[17] In a study, Carlini and her colleagues in 2016 studied sperm parameters and chromatin in 112 RPL patients and compared them with 114 fertile and 114 infertile men. They showed sperm DNA integrity in RPL patients is better than infertile patients. However, sperm DNA fragmentation was significantly higher in RPL patients than in fertile and parallel to infertile patients.[5]

Overall studies were arranged to find out the mechanism effect of the antioxidant on sperm parameters and chromatin quality. The studies confirmed that zinc improved in sperm count motility and normal morphology in infertile men and increases pregnancy rates in infertile patients.[18,19]

Related to our result, Omu and his collogues in 2008 reported that sperm parameters improved after 6 months of treatment with zinc in male infertile patient. In their study 45 asthenozoospermia men randomly divided into four groups: Zinc only, zinc + vitamin E, zinc + vitamins E + C and control group. They reported treatment with zinc only or plus vitamin E or vitamin E plus C demonstrated improved sperm parameters and decreased apoptosis and fragmentation of sperm. They discussed maybe increase in expression of Zn-Cu superoxide dismutase and anti-apoptotic factor (Bcl-2) is the main mechanism to repair DNA defects in sperm.[20]

Some researchers believe that Zinc deficiency is the cause of high level of sperm DNA fragmentation apoptosis and treatment with oral zinc therapy can improve sperm parameters via its antioxidant role against oxidative stress.[21,22]

Table 3: Sperm nuclear maturity and chromatin quality tests in pre and post antioxidant therapy groups

Chromatin integrity tests	Pre-treatment	Post treatment	*p*
Aniline blue (%)	52.30±16.87	41.50±14.90	0.004*
Toluidine blue (%)	66.76±17.11	50.86±19.45	0.0001*
Chromomycin A3 (%)	42.00±9.86	33.36±8.74	0.0001*
TUNEL (%)	32.03±6.06	27.20±5.64	0.001*

Data was presented by mean±SD. Paired sample t-test was used to compare dependent variables. TUNEL=Terminal deoxynucleotidyl transferase dUTP nick end labeling. *P* values ≤0.05 considered statistically significant

Gharagozloo in 2011 published a review paper on assessment of the effect of oxidative stress on sperm in male infertility cases and oral antioxidant therapy. In the report, it was discussed that, in 19 out of 20 clinical studies reported a reduction in reactive oxygen species and improving sperm parameters after antioxidant therapy.[23] Based on the Gharagozloo paper, we hypothesized that the treatment with antioxidants can produces an extra appropriate condition for production of sperm by reduction in level of ROS. Here, we confirm that treatment of male partner in RPL patients by antioxidant can significantly improve total sperm parameters and sperm DNA integrity.

In a clinical trial study, researchers by 90 days of antioxidant therapy including zinc and vitamin E for infertile men showed treatment with antioxidants has positive effect on improving sperm parameters and decreases DNA fragmentation.[24] In contrast to our result, Greco and his colleague reported that 3 months of treatment with antioxidants cannot make any change in sperm parameters compared before treatment, his report also showed sperm DNA fragmentation decreased in their study patients after treatment with antioxidants.[25]

We recently demonstrated that sperm parameters improve after treatment with vitamin E plus selenium in RPL patients. Our study was the first study to evaluate oral antioxidant supplements effects on male factor in RPL patient.[10] However, in a study, researchers used antioxidant-rich diet and assessed the effect of diet on sperm parameters in RPL patients. Similar to our result the result of their study showed antioxidant-rich diet can improved sperm parameters.[26] But they only focused on antioxidant-rich diet and in our study we treated patients with antioxidant supplements. In contrast to this result, a research published in 2012 showed that antioxidant-rich diets do not have any positive effect on sperm parameters in their study population, there was no significant change in the sperm parameters before and after treatment.[27] Today, clinical researchers struggle to find the relation between sperm DNA integrity and RPL. Nevertheless, the effect of the sperm DNA integrity in RPL patients is still ambiguous. Maybe the different case study number and the treatment duration of antioxidants are the main factors leading to controversial results in the research line.

Study limitations

In this study, we don't have control group and maybe this was limitation for study, but our study was pre-post study where we compared the data from cases after antioxidant therapy by pretreatment to match the study case.

Conclusion

Based on our finding, medical therapy of RPL patients with zinc + vitamin E can improve quality of sperm chromatin and parameters. We advocate their use prior to assisted reproductive

techniques (ART), it might improve success rates. The major limitation of this study was the lack of placebo-controlled group.

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Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form, the patient (s) has/have given his/her/their consent for his/her/their clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity.

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Conflicts of interest

There are no conflicts of interest.

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