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Research Article

Vitamin E Improves Sperm Quality of *Chlamydia trachomatis* Infected Mice

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Abstract

Chlamydia trachomatis (CT) is the most dominant sexually transmitted infection of the male genital tract which impairs semen quality and male fertility. Regarding to high DNA fragmentation of sperm in CT infection, it has been proposed that using the antioxidant agents such as vitamin E may ameliorate semen quality of CT infected patients.

The aim of this study is to investigate the effects of vitamin E administration on sperm quality improvement in CT infected mice.

Twenty four adult mice were distributed in four groups: control, CT infected, CT infected + vitamin E (100 mg/$kg_{body\ weight}$/day), and vitamin E (100 mg/$kg_{body\ weight}$/day) groups. Five days after infection induction, animals were treated with vitamin E for 3 weeks. Then animals were killed and the testicles were removed for sperm analysis, gene expression and western blot experiments. Furthermore, serum levels of sexual hormones were evaluated.

CT infection caused a significant reduction in testes weight, testosterone level, and sperm parameters including number, motility, and vitality. Moreover, follicle-stimulating hormone (FSH) level and expression of p53 were significantly higher in CT infected mice in compared with the control group ($p < 0.05$). Our results showed that sperm parameters, p53 expression, and sexual hormones levels in CT infected + vitamin E group had no significant difference compared with the control group.

Vitamin E can be an effective therapeutic agent to improve sperm parameters in CT infected patients possibly via its antioxidant properties.

Keywords: *Chlamydia trachomatis*; Sperm Parameters; Vitamin E

Abbreviations

CT: *Chlamydia trachomatis*; FSH: Follicle-stimulating Hormone; RT-PCR: Reverse Transcription Polymerase Chain Reaction; PMS: Progressive Motile Sperm; NPMS: Nonprogressive Motile Sperm; IMS: Immotile Sperm; WHO: World Health Organization, DSP: Daily Sperm Production; PBS: Phosphate Buffered Saline; LH: Luteinizing

Hormone; cDNA: Complementary Deoxyribonucleic Acid; GAPDH: Glyceraldehyde-3-phosphate Dehydrogenase; SD: Standard Deviation; ROS: Reactive Oxygen Species

Background

Infertility is a global problem affecting about 15% of the couples at reproductive age [1]. Male factors is responsible for approximately half of all infertility cases, and it can be divided into non-obstructive, obstructive and coital factors [2]. Testicular infection is one of the non-obstructive factors with adverse effects on sperm parameters [3]. *Chlamydia trachomatis* (CT) is the most dominant sexually transmitted infection of the male genital tract with nearly 100 million new cases per year [4,5]. It is well accepted that CT infection is related to prostatitis, urethritis, orchitis and epididymitis in males [6]. Moreover, CT infection can damage epithelial cells of seminiferous tubules and consequently cause a reduction in spermatogenesis. The CT infection also induces immunological reactions in male reproductive tract that can destroy or hinder spermatozoa [7]. CT infection can impair sperm quality and male fertility by DNA fragmentation and phosphatidylserine membrane translocation [8]. In this regard, Hosseinzadeh., *et al*. reported that incubation of sperm with CT could decrease sperm motility and viability [9]. Such a negative effects of CT on sperm can be a result of membrane lipid peroxidation in CT infected patients [10]. The main therapeutic strategy for treatment of CT infection is using of anti-biotic agents, such as tetracyclines, rifampin, macrolides, sulfonamides, and clindamycin [11]. However, overuse of the antibiotics can lead to evolution of new resistant microorganism [12]. Therefore, finding new strategies to reduce antibiotic consumption is necessary. Regarding to lipid peroxidation following CT infection, it seems that using of antioxidant agents such as vitamin E may prevent this process and improve semen quality of CT infected patients. Vitamin E or α-tocopherol has an ability to protect sperm membrane form oxidative stress-induced damages such as lipid peroxidation [13]. Sufficient similarities between spermatogenesis in mouse and man, made mouse as a great model of human spermatogenesis for biological experiments. This study was designed to investigate effect of vitamin E administration on improvement of sperm quality in CT infected NMRI mice. In addition, to explore molecular mechanism by which CT infection causes apoptosis in spermatozoa, protein and gene expression levels of P53 were investigated.

Methods

Animals and experimental design

This experimental study was performed on 24 adult NMRI male mice weighting 35 ± 2g. The animals were purchased from Pasture's Institute of Iran and housed in plastic cages at 12-h light/dark cycle and 24 ± 2°C temperature with freely available water and food. All animal process was performed following the protocols authorized by National Institutes of Health guide for the care and use of laboratory animals and all procedures were approved by the Animal Ethical Committee of Hamadan University of Medical Sciences (P/16/35/10/165, ec.umsha.ac.ir). Animals were distributed in four groups randomly (n = 6 for each group): control, CT infected, CT infected + vitamin E, and vitamin E groups. Five days after infection induction, treatments were performed with intraperitoneal injection of 100 mg vitamin E/$kg_{body\ weight}$/day for 3 weeks. At the end, the animals were weighed, sacrificed under deep anesthesia by ketamine. The testicles were removed and cleared from fat tissues and weighted. The right testes were transferred to liquid nitrogen and stored for real time-PCR (RT-PCR) and western blot analyses while the left testes were used for sperm parameters analysis. In order to retrieval sperm, epididymis was cut into small slices and released sperms were collected.

Infection

Infection process was performed according to the protocol published by Bernstein-Hanley., *et al.* [14]. In brief, at first the rats were immunosuppressed by cyclosporine A (10 mg/kg; Sigma) administration for 6 days [15]. Then, mice were injected into the tail vein with *C. trachomatis* L2 in 200 µl of sucrose-phosphate-glutamic acid buffer (SPG, 220 mM sucrose, 12.5 mM phosphate, 4 mM l-glutamic acid, pH 7.5). To confirm testicular infection, histological evaluation was performed after sacrificing animals. For this purpose testis tissue was fixed with Bouin' fixative (Sigma), dehydrated, embedded, sectioned (7 µm) and stained with haematoxylin/eosin (Merck). Testis section between groups were compared.

Sperm number and motility

In order to assess the sperm concentration, 1 ml of the sperm suspension was mixed with 9 ml formaldehyde as a fixative. Then, a droplet of solution was transmitted into a Neubauer hemocytometer chamber and number of sperm was counted under a microscope and was expressed as the number of sperm per ml. For

motility assessment, 10 µl of the sperm suspension was placed on pre-warmed Mackler chamber. Five microscopic fields with at least 200 sperm were assessed to evaluate sperm motility. Sperms were classified as progressive motile sperm (PMS), nonprogressive motile sperm (NPMS), and immotile sperm (IMS).

Sperm vitality

Sperm vitality was assessed by eosin-nigrosin staining according to World Health Organization (WHO) instruction (Organization 2010). In brief, 0.67 gr of eosin Y, 0.9 gr of sodium chloride and 10 gr of nigrosin were dissolved in 100 ml of purified water. 50 µl of sperm sample were mix with an identical volume of eosin-nigrosin suspension. After 30 seconds, a thin smear on a glass slide were prepared and observed under a light microscope at 1000X magnification. In this method, colorless spermatozoa considered as viable and red stained spermatozoa considered as nonviable.

Determination of daily sperm production

Daily sperm production (DSP) was determined according to instruction outlined by Sadeghzadeh., *et al*. [16]. In brief, the capsule of left testes was removed and homogenized in physiological phosphate buffered saline (PBS). An aliquot of the homogenate was evaluated by Neubauer hemocytometer for the number of homogenization-resistant sperm heads. In mouse, developing spermatids typically spend 4.84 days [17]. Thus, DSP can be valued by dividing the number of spermatids per testes by 4.84.

Assessment of hormonal levels

Blood samples were taken from the heart of the anesthetized mice and the serum was separated by centrifugation. The level of testosterone was measured using commercial testosterone ELISA kit (DRG EIA- 1559), and expressed as ng/mL, while follicle stimulating hormone (FSH) and luteinizing hormone (LH) concentration were analyzed using Monobind kit (Costa Mesa, California, USA) and expressed as IU/L. In all cases, serum hormone analysis was done based on the manufacture's protocol.

Evaluation of genes expression by RT-PCR

Expression levels of P53 gene was investigated in testes using RT-PCR. In brief, total RNA was extracted from testes using RNEasy mini kit (Qiagen, CA, USA) according the manufacturer's instructions. Then, extracted RNA was qualified and quantified by nanoDrop (ND-1000, NanoDrop technology, Australia) and transcribed to complementary DNA (cDNA) using first Strand cDNA synthesis Kit (thermos scientific, Schwerte, Germany). Then, 1µL (10PM/µL) of primer set (Table 1), 1 µL of cDNA sample, 7.5 µL SYBR Premix EX TaqTM (Takara Bio Inc, Japan), and 5.5 µL sterile water were mixed and the targeted gene was amplified using real time rotary analyzer (Rotor-Gene 6000, Corbet Life Science, Australia). Cycle threshold of P53 was standardized based on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression as the house keeping gene for each sample. Relative gene expressions were calculated using the Pfaffl method.

Gene	Sequence	Product size (bp)	NCBI accession No.
P53	F 5'-CCCCTGAAGACTGGATAACTGT-3' R 5'-TCTCCTGACTCAGAGGGAGC-3'	75	NM_030989.3
GAPDH	F 5'-AGGTCGGTGTGAACGGATTTG-3' R 5'-TGTAGACCATGTAGTTGAGGTCA-3'	123	NM_017008.4

Table 1: Sequences of the primers used for analysis of P53 and glyceraldahyde-3-phosphate dehydrogenase (GAPDH) expression.

Western blot analysis

RIPA buffer (Sigma Aldrich, USA) and protease inhibitors were used for protein isolation. Equal amounts of isolated protein (50 mg/lane) from each sample were run in 10% w/v sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were moved to a methanol-preactivated polyvinylidene fluoride (PVDF) membrane. Then membranes were blocked for 65 min by 3% w/v dried non-fat milk in TBS plus 0.1% Tween-20 and incubated with the first antibody against P53 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight. The membrane was washed and incubated with secondary antibody (anti-rabbit IgG-horseradish peroxidase, A6154, Sigma-Aldrich, USA) for one hour at 4°C. In

order to visualize the bands on the membrane Clarity™Western ECL Substrate (Bio-Rad, USA) was used. A commercial molecular weight marker (Thermo-Scientific™, USA) was used as control and Image J software was applied for analysis of the protein relative density.

Statistical analysis

All the analyses were performed triplicate and obtained values were presented as the means ± standard deviation (SD) for six animals per group. In order to assess the statistical significances among groups, statistical analyses were carried out using one-way analysis of variance (ANOVA) followed by Tukey's post- hoc test.

Results

Body and testes weight

All six mice in all four groups were included in the study and there was no excluded mice from the study. Results showed that there was no significant difference in the body weight among the groups, while testes weight significantly decreased in CT infected mice in compared with the control group ($p < 0.01$) (Table 2). As shown in table 2, testes weight in CT + vitamin E group was significantly higher than the CT group ($p < 0.001$). However, in vitamin E treated group, no significant difference was observed in testes weight in comparison to control group.

Groups	Control	CT infected	CT + Vit E	Vit E
Body weight (gr)	50.10 ± 1.21	52.83 ± 2.81	53.20 ± 2.71	54.16 ± 2.40[a]
Testes weight (gr)	0.13 ± 0.016	0.08 ± 0.011[a]	0.12 ± 0.06[b]	014 ± 0.072[b]
Sperm number ($\times 10^6$)	6.01 ± 0.54	3.33 ± 0.41[a]	4.66 ± 0.40[b]	6.080 ± 0.20[b]
Progressive motility (%)	50.36 ± 0.57	36.26 ± 0.30[a]	46.97 ± 0.21[b]	51.79 ± 0.9[b]
Non-progressive motility (%)	30.31 ± 0.48	18.63 ± 0.19[a]	27.43 ± 0.53[b]	30.71 ± 0.65[b]
Vitality (%)	50.39 ± 0.77	30.52 ± 0.72[a]	44.17 ± 1.29[b]	51.34 ± 1.22[b]
DSP (Count Production Sperm/g)	24.66 ± 0.40	16.17 ± 0.29[a]	20.97 ± 0.59[b]	25.01 ± 0.19[b]

Table 2: Body weight, testes weight and Sperm parameters of samples. Data are expressed as mean ± SD.

[a]: Statistically significant versus control; [b] Statistically significant versus CT infected group.

CT: *Chlamydia trachomatis*; Vit E: Vitamin E.

Histology of testis

Histological evaluation of testis tissue showed that CT infection causes significant reduction in number of Spermatocyte, Leydig cells, Sertoli cells, seminiferous tubule and interstitial tissue (Figure 1). Treatment with vitamin E decreased testis damage in the infected rats compared to the control group (Figure 1).

Figure 1: Testis histological sections were investigated under a light microscope in different experimental groups. (CT, *Chlamydia trachomatis*; vit E, vitamin E).

Evaluation of sperm parameters

We observed that CT infection significantly decreased sperm number, motility, vitality and daily production ($p < 0.001$, Table 2). Results also demonstrated that daily injection of vitamin E could improve the sperm parameters of CT infected mice. However, there was no significant difference in the sperm parameter between control and vitamin E groups ($p > 0.05$).

Level of hormones

As shown in table 3, no significant difference was observed in LH level between CT infected and control groups ($p > 0.05$). Results also indicated that the serum levels of testosterone and FSH in CT infected group were significantly decreased and increased in compared with the control group, respectively ($p < 0.01$) (Table 3). Furthermore, we found that vitamin E administration caused a significant increase in testosterone levels of the infected group ($p < 0.001$).

Groups	Control	CT	CT + Vit E	Vit E
Testosterone (ng/ml)	0.719 ± 0.056	0.279 ± 0.022 [a]	0.650 ± 0.041 [b]	0.782 ± 0.041 [b]
FSH (IU/L)	0.618 ± 0.029	0.810 ± 0.168 [a]	0.640 ± 0.083 [b]	0655 ± 0.025 [b]
LH (IU/L)	0.894 ± 0.047	0.921 ± 0.063	0.821 ± 0.127	0.914 ± 0.047

Table 3: Level of hormones in mice infected with *Chlamydia trachomatis* (C.t.) and treated with vitamin E (vit E, 100 mg/kg).

CT: *Chlamydia trachomatis*; Vit E: Vitamin E.

[a]: Statistically significant versus control; [b]: Statistically significant versus CT infected group.

P53 gene and protein expression

As illustrated in figure 2A, CT infection significantly increased the gene expression of p53 ($p < 0.05$). The RT-PCR data also demonstrated that vitamin E could prevent p53 upregulation in CT infected group. Protein levels of P53 almost were in agreement with the gene expression changes. As shown in figure 2B, a significant increase was observed in the levels of P53 protein in testes of CT infected mice in compared with the control group ($p < 0.05$). In CT + vitamin E group the P53 protein levels were significantly lower when compared to the CT infected group ($p < 0.01$).

Discussion

Our results showed that CT infection caused a significant reduction in testes weight, testosterone level, and sperm parameters including number, motility, and vitality. In parallel with our findings, Wolff., *et al*. [18] and Keck and colleagues [19] reported negative effects of CT infection on sperm parameters. The p53 is a tumor suppressor and has pivotal roles in apoptosis induction and normal spermatogenesis [20,21]. The apoptosis is essential for elimination of excess or abnormal germ cells during spermatogenesis. However, excessive apoptosis level may have negative influence on some sperm parameters such as morphology, motility and viability [22]. It has been seen that a high level of p53 was correlated with low motility of sperm [23]. Our data showed that CT infected mice had an increased level of P53 at mRNA and protein levels. In conforming with our result Eley., *et al.* [7] reported CT infection could be induction of spermatozoa apoptosis by CT's lipopolysaccharide. Also, Gallegos., *et al*. [24] represented that patients with genitourinary infection by CT had an enhanced sperm DNA fragmentation. However, there are several studies that have shown no effect of CT on sperm parameters [25,26]. Interestingly, our study revealed that administration of vitamin E could improve the sperm parameters in CT infected mice. Previous studies also supported positive effect of vitamin E on testes and sperm parameters via reducing oxidative stress damages [27-29]. Suleiman., *et al*. [30] reported

Figure 2: Effects of *Chlamydia trachomatis* infection and subsequent vitamin E administration on expression of p53 mRNA and protein levels in testes tissue. *Chlamydia trachomatis* infection causes significant increase of P53 mRNA and protein level, which in turn lead to excessive apoptosis of spermatozoa. The results were considered as the mean ± standard deviation. Letter a and b showing significant differences versus control and CT infected group respectively ($P < 0.05$) (CT, *Chlamydia trachomatis*; vit E, vitamin E).

that oral supplementation of vitamin E reduced Malondialdehyde (end product of the lipid peroxidation) levels in seminal plasma. We suggest, the beneficial effects of vitamin E on sperm motility in CT + vitamin E group could be related to the antioxidant effect of this vitamin and inhibiting sperm lipid peroxidation. As well as Momeni., *et al*. [31] demonstrated that vitamin E improved the activity of sperm antioxidant enzymes such as glutathione peroxidase, superoxide dismutase and catalase, which consequently led to increase in sperm viability and motility. Our results also indicated that vitamin E administration could decrease levels of P53 in CT infected mice and consequently increase their sperm quality; possibly through neutralizing CT-induced ROS. It should be mentioned that, despite of vitamin E beneficial effects, its high dose can act as pro-oxidant instead of an antioxidant and so has negative effect on sperm [32,33]. Therefore, further human studies are needed to confirm our findings and also find optimum daily dose of vitamin E for CT infected patients.

Conclusion

These results showed that CT infection could negatively affect sperm parameters and also induced P53 levels in the testicular tissue. Furthermore, vitamin E administration could improve the sperm characteristics in the infected mice and also normalize P53 levels. It can be postulated that vitamin E can be considered as a potential therapeutic agent along with antibiotics for improving the sperm parameters in CT infected patients.

Ethics Approval and Consent to Participate

All animal process was performed following the protocols authorized by National Institutes of Health guide for the care and use of laboratory animals and all procedures were approved by the Animal Ethical Committee of Hamadan University of Medical Sciences (P/16/35/10/165, ec.umsha.ac.ir).

Consent for Publication

Not applicable.

Availability of Data and Material

All data are included within the manuscript.

Competing Interests

The authors declare that they have no conflict of interest.

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Authors' Contributions

AB and MN designed, supervised the study, and prepared the manuscript. NK and HH designed and performed animal study, sperm analyzing, hormone detection, and writing the manuscript. SDM, NN and MR contributed in gene expression and western blot experiments. All authors have read and approved the final draft of the manuscript.

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