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Alcohol 45 (2011) 403–409

ALCOHOL

Effects of ethanol consumption on chromatin condensation and DNA integrity of epididymal spermatozoa in rat

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Received 13 December 2009; received in revised form 30 September 2010; accepted 12 October 2010

Abstract

Alcohol abuse is considered as one of the problems associated with poor semen production and sperm quality. Both acute and chronic alcohol consumption may affect spermatozoal chromatin disorders through apoptosis. Therefore, for the first time, this experimental study was performed to evaluate the effect of ethanol consumption on sperm parameters and chromatin integrity of spermatozoa aspirated from cauda epididymis of rats. Twenty adult Wistar rats were divided into ethanol consumption and control groups. Access to ethanol and water was provided ad libitum for experimental and control animals, respectively. The cauda epididymal spermatozoa were aspirated for analysis of sperm parameters and sperm chromatin integrity with aniline blue (AB), chromomycin A3 (CMA3), toluidine blue (TB), and acridine orange (AO) assays. Sperm progressive and nonprogressive motility of ethanol-consuming rats were significantly decreased compared with control animals ($P < .05$). In addition, the rates of AB-reacted spermatozoa were similar in both groups ($P > .05$). However, with regard to CMA3, AO, and TB stainings, there was a significant increase in ethanol group when compared with the controls ($P < .05$). The majority of TB+ and AO+ spermatozoa were higher than ''cut-off'' value in ethanol group, whereas the mean rates of CMA3+ spermatozoa was below the ''cut-off'' value in both groups. The results showed that ethanol consumption disturbs sperm motility, nuclear maturity and DNA integrity of spermatozoa in rat. Therefore, ethanol abuse results in the production of spermatozoa with less condensed chromatin, and this may be one possible cause of infertility following ethanol consumption.

Keywords: Chromatin condensation; DNA integrity; Rat; Spermatozoa; Ethanol consumption; Cytochemical tests

Introduction

Infertility is a major problem in up to 15% of the sexually active population and male factor is responsible in 50% of these cases (World Health Organization, 1999). Recently, the substances in the environment that can disturb male fertility have been increased. Ethanol is among the most widely abused drug, which can suppress reproductive function and sexual behavior in laboratory animals and humans (Abel, 1980; Fadem, 1993). The lack of sexual desire in long-term alcohol users has been reported from 31 to 58% (Gümüş et al., 1998; Jensen, 1984; Whalley, 1978). Whalley (1978) reported that, about 54% of hospitalized alcoholic men and 24% of healthy controls had erectile dysfunction. Alcohol is toxic for testes and causes fertility disturbances through low sperm count and motility in men (Maneesh et al., 2006). In addition, Van Thiel et al. (1975, 1980) showed that chronic ethanol exposure decreased plasma testosterone level and caused testicular atrophy. Irregular diameter of the seminiferous tubules and high amount of death cells in the lumen of alcoholic men were also noticed by Martinez et al. (2009).

Sperm nuclear DNA in mammalians is organized around protamine molecules during testicular spermiogenesis. In this phase, sperm-specific histones are replaced by protamines, which are rich in cysteine and other basic amino acids. During epididymal transit, the cysteine-thiol group of protamine molecules are oxidized to disulphide bonds (S–S), which are necessary for stability of sperm chromatin (Poccia, 1986; Said et al., 1999). In the cases of reduction in the number of S–S bonds between protamine molecules, the chromatin will be more susceptible to denaturation. In rats, spermatozoa contain approximately 84% of total SH and S–S groups in the epididymal caput as thiols. This is decreased to 14% in spermatozoa obtained from the cauda epididymis (Said et al., 1999). This difference indicates that during transit between the two epididymal regions, about 1.5 billion S–S bonds are formed per spermatozoa. Therefore, spermatozoa become highly resistant to a variety of agents such as acids, proteases, DNase and detergents such as sodium dodecyl sulfate after nuclear chromatin condensation (Mahi and Yanagimachi, 1975). Evenson et al.

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0741-8329/$ - see front matter
doi: 10.1016/j.alcohol.2010.10.005

(1999) reported that sperm chromatin condensation is a complex process, which is directly related to the capacity of sperm to fertilize an oocyte. Indeed, sperm DNA damages, such as DNA fragmentation, abnormal chromatin packaging, and protamine deficiency, have been correlated with the reduced ability of spermatozoa to fertilize oocyte in the context of assisted reproduction techniques and normal fertility (Ahmadi and Ng, 1999; Cho et al., 2003; Filatov et al., 1999; Lopes et al., 1998; Sakkas et al., 1996).

Regarding to alcohol consumption, although there is a significant association between alcohol intake and increasing frequency of sperm nuclear aneuplody (Robbins et al., 1997), but little is known about the impact of ethanol toxicity on sperm chromatin and DNA integrity. Zhu et al. (2000) reported that ethanol exposure enhanced the testicular germ cells apoptosis and the increased expression of Fas ligand was remarkable in rats, which were fed ethanol chronically. Moreover, both acute and chronic alcohol exposure can increase production of reactive oxygen species (ROS) (Wu and Cederbaum, 2003). Alcohol metabolism produces the reduced form of nicotinamide adenine dinucleotide (NADH), which enhances activity of the respiratory chain and ROS formation. In addition, one of the by-products of alcohol metabolism, acetaldehyde, interact with proteins and lipids to form ROS. It should be noticed that if levels of ROS rise above the body's antioxidant defense system, oxidative stress (OS) occurs (Agarwal and Prabakaran, 2005; Goverde et al., 1995).

It is generally accepted that plasma membrane and DNA molecules are the major targets of ROS in sperm and other cells. In addition, there is a positive correlation between the level of ROS and sperm apoptosis (Moustafa et al., 2004). So, in the cases of alcohol consumption, sperm nuclear anomalies and apoptosis may be expected due to the OS.

Alcohol drinking is increasing and it is considered as a common social problem. In Australia, one study found that 9% of 14–19 years old participants consumed five (females) or seven (males) drinks or more at least 1 day per week (Hargreaves et al., 2009). In addition, if the ethanol consumption is determined to affect male reproduction through DNA damaging, men who wish to be fertile, should be specially warned of this matter. According to our knowledge, there is no report on effects of ethanol on sperm chromatin condensation using cytochemical assays. Therefore, the aim of this study was to assess the possible detrimental effects of ethanol consumption on sperm DNA integrity and chromatin quality in rat as an experimental model.

Materials and methods

Animals

Twenty 10-week adult male albino rats of Wistar strain weighting 200–225 g were used. Rats were kept in clean cages in an air-conditioned, temperature-controlled room (25°C) with 12-h light: 12-h dark at least 2 weeks before experiment. Animals were divided randomly into two following groups. Control group included 10 rats allowed free access to rat chow and water. Experimental group included 10 rats with free access to rat chow and 5% ethanol (99%, vol/vol; Merck, Germany) in the same volume (50 cc daily) as controls that received water.

This prospective study has been approved by the animal ethics committee of Research and Clinical Center for Infertility, Yazd University of Medical Sciences and was done according to the National Institute of Health Guide for the care and use of laboratory animals.

Epididymal sperm preparation

Because one cycle of spermatogenesis in rat is approximately 45 days, 50-day surviving animals were anaesthetized with intramuscular injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). The distal end of the cauda epididymis was dissected and placed in 1 mL of prewarm T6 culture medium (37°C, 5% CO_2) for 10 min. Gentle agitation along with tearing of the tissue was applied to make spermatozoa to swim out into the culture medium placed in a falcon culture dish (Kempinas et al., 1998).

Semen analysis

In this study, sperm progressive and nonprogressive motility (%) and also normal morphology (%) were evaluated for 100 sperms from each animal. Sperm motion analysis was performed using Makler chamber (Sefi Medical Co., Haifa, Israel) and phase-contrast microscopy (Olympus Co., Tokyo, Japan) at ×200. Motility was expressed as percentage of progressive (grades "a" and "b") and nonprogressive spermatozoa. Moreover, the percentage of normal morphology of 100 spermatozoa per rat was assessed using Giemsa staining and light microscopy at ×400 (Seed et al., 1996). All analyses were performed by one experienced technician blinded to the study.

Cytochemical tests

Sperm chromatin condensation and DNA integrity were assessed using four different cytochemical tests including aniline blue (AB), chromomycin A3 (CMA3), toluidine blue (TB), and acridine orange (AO). In this study, we did not have any negative or positive controls, but before experiments, we tested the efficacy and quality of stains with and without acid denaturation of some specimens and they were considered as positive and negative controls, respectively.

1. AB staining

For these staining air-dried smears from fresh sperm samples of each study participant, fixed in 3% buffered glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 30 min at room temperature. Each smear was stained with 5% aqueous AB stain in 4% acetic acid (pH 3.5) for 5 min. Using light microscopy (Olympus Co, Tokyo,

Japan), 200 spermatozoa were counted in each slide and unstained or pale-blue stained were considered as normal spermatozoa, whereas dark blue stained as abnormal spermatozoa (Talebi et al., 2007, 2008).

2. CMA3 staining

Smears were first dried and then fixed in Carnoys solution (methanol/glacial acetic acid, 3:1) at 4°C for 10 min. Each slide was treated with 100 μL of CMA3 (Sigma, St Louis, MO, USA) (0.25 mg/mL in McIlvain buffer; 7 mL citric acid, 0.1 M + 32.9 mL Na_2HPO_4 $7H_2O$ 0.2 M, pH 7.0 containing 10 mM $MgCl_2$) for 20 min. At the end, the slides were washed in buffer and mounted with buffered glycerol (1:1). Chromomycin A3-reacted spermatozoa (CMA3+) were bright yellow-stained, and nonreacted were (CMA3−) yellowish green-stained spermatozoa when viewed under axiplane fluorescent microscope with a 460-nm filter (Zeiss Co., Jena, Germany).

3. TB staining

For this staining, the air-dried smears were fixed in fresh 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 then rinsed thrice in distilled water for 2 min and finally stained with 0.05% TB for 10 min. The component of staining buffer was 50% citrate phosphate (McIlvain buffer, pH 3.5) (Erenpreiss et al., 1997; Talebi et al., 2008). In light microscopic study using 100× eyepiece magnification, the chromatin quality of spermatozoa was determined according to metachromatic staining of sperm heads with following scores: score 0 = light blue (good chromatin), score 1 = dark blue (mild abnormal chromatin), score 2 = violet and purple (severe chromatin abnormality) (Rosenborg et al., 1990). So, the sum of spermatozoa with scores 1 and 2 were considered as TB+ or abnormal chromatin, whereas score 0 as TB- or sperm with normal chromatin.

4. Acridine orange test (AOT)

The smears were first air-dried and then fixed overnight in Carnoy's solution. Each sample was stained for 10 min in fresh prepared AO (0.19 mg/mL) in McIlvain phosphate-citrate buffer (pH 4) for 5 min. Smears were evaluated on the same day with the aid of fluorescent microscope (460-nm filter). The duration of illumination was limited to 40 s/field. The percentage of green (normal double-stranded DNA) and orange/red (abnormally denatured DNA) fluorescence spermatozoa per sample were calculated (Khalili et al., 2006; Talebi et al., 2007, 2008).

Statistical analysis

The Statistical Package for the Social Sciences 15.0 software was used to analyze data. Data were expressed in mean ± standard deviation Differences between variables were analyzed using nonparametric Mann–Whitney test and Kruskal–Wallis test. P value of ≤.05 was considered as statistically significant.

Results

Sperm parameters

The results showed that 31% of spermatozoa retrieved from the cauda epididymis were motile in control animals, and total motility (grades "a" and "b" and "c") did not differ statistically significant between groups ($P > .05$). This rate was slightly decreased in experimental rats (26.97%). The progressive motility (grades "a" and "b") was significantly higher in control group than ethanol-consuming rats (18.57 vs. 8.49%). With regard to the nonprogressive motility (grade "c"), a significant difference was also found between cases and controls ($P < .05$). In addition, the results did not show any significant alteration in sperm morphology (Table 1).

AB test

The sperm nuclear integrity of both groups was presented in Table 2. The rates of AB-reacted spermatozoa (AB+) were similar in both groups of animals under investigation ($P = .974$).

CMA3 test

In regard to CMA3 staining, although the difference between the two groups was statistically significant, but there was a slightly higher rate of CMA3-reacted spermatozoa in alcohol-treated animals than the controls (2.25 vs. 0.5%, $P = .030$). In other words, the percentage of CMA3+ sperm cells in both groups were below 30%, which considered as "cut-off" value (Sakkas et al., 1998) for this staining (Table 2).

AO test

Sperm nuclei from experimental group showed a significant evidence of DNA damage with regard to AO test (increase in orange–red fluorescence) when compared with those without ethanol consumption ($P = .000$). Animals

Table 1
Effect of ethanol consumption on some sperm parameters in rats

Variables	Control ($n = 10$) Mean ± S.D.	Case ($n = 10$) Mean ± S.D.	P value
Progressive motility (%) (grade "a" + grade "b")	18.57 ± 8.40	8.49 ± 2.78	.034[a]
Nonprogressive motility (%) (grade "c")	12.52 ± 1.78	19.28 ± 5.85	.039[a]
Total motility (%) (grade "a" + grade "b" + grade "c")	31.43 ± 9.31	26.97 ± 3.69	.293
Normal morphology (%)	64.67 ± 6.30	57.80 ± 12.13	.349

S.D. = standard deviation.
[a]The differences were statistically significant.

Table 2
Effect of ethanol consumption on the characteristics of sperm nuclear integrity

	Control (n = 10)	Case (n = 10)	
Variables	Mean ± S.D.	Mean ± S.D.	P value
AB+ (%)	0.80 ± 1.03	0.83 ± 1.02	.974
CMA3+ (%)	0.50 ± .52	2.25 ± 2.30	.030[a]
AO+ (%)	3.50 ± 3.20	40.75 ± 24.81	.000[a]
TB+ (%)	10.80 ± 6.89	59.66 ± 17.37	.000[a]

S.D. = standard deviation; AB = aniline blue; CMA3 = chromomycin A3; AO = acridine orange; TB = toluidine blue.
[a]The differences were statistically significant.

(58.3%) in experimental group and none of the control animals showed the rate of AO+ above 50%, which is defined as "cut-off" value in this test (Virant-Klun et al., 2002; Fig. 1).

TB test

There was a significant difference between control and experimental groups regarding to TB staining (P = .000). The percentage of animals showed more than 45% TB-reacted sperms, which considered as "cut-off" value (Tsarev et al., 2009), were remarkable in ethanol group (75 vs. 0% in controls) (Fig. 1).

Discussion

In this study, we evaluated sperm parameters including morphology and different kinds of motility in alcohol-consuming and control rats. The result showed that sperm progressive motility decreased significantly in case group, but there were no differences regarding sperm total and nonprogressive motilities and also sperm morphology between the two groups.

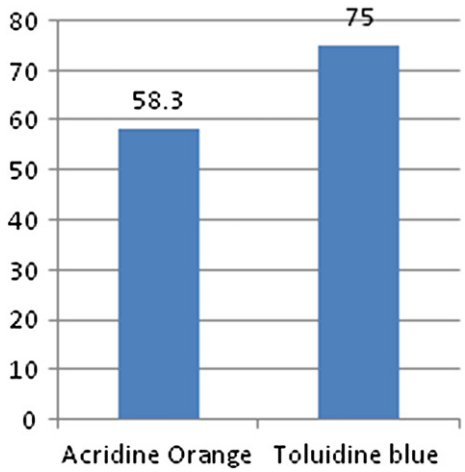

Fig. 1. The percentages of samples that are above the "cut-off" value in case group regarding to acridine orange (AO) and toluidine blue (TB) staining. These percentages were zero in controls. Seventy-five percent of TB+ samples were above the 45% ("cut-off" value); and 58.3% of AO+ samples were above 50% ("cut-off" value).

Long-term effects of chronic alcohol abuse come along with gynecomastia, impotence, testicular atrophy, and loss of libido, as it had been reported before (Boyden and Pamenter, 1983; Buffum, 1983; Whalley, 1978). Ethanol consumption also produces a significant decrease in the percentage of motility, concentration (Maneesh et al., 2006), and normal morphology in human and animal spermatozoa (Anderson et al., 1983; Nagy et al., 1986).

Martinez et al. (2009) reported histological abnormalities in testicular tissue of alcoholic animals. These included intense intercellular spaces, irregular diameter of the seminiferous tubules, and high amount of necrotic cells in the lumen compared with controls. In addition, they showed that the epididymal sperm motility is decreased in ethanol-treated rats.

In another study, Srikanth et al. (1999) demonstrated that the rate of spermatozoa with forward motility that were extracted from cauda epididymidis, significantly decreased in ethanol-consuming rats. They also showed that ethanol consumption in male rats may decrease pregnancy rate (to 60%) in their cohabitated female rats and number of pups delivered (to 50%).

Brzek (1987) also reported that a reduction of semen volume, density, and motility were caused by alcohol consumption. In addition, it was reported that daily alcohol consumption decreases normal sperm morphology (Goverde et al., 1995). Carlsen et al. (1992) found that ethanol users showed a significant decrease in mean sperm count, ranging from 113 to 66 million/mL. Our findings were also in agreement with the aforementioned investigations that have shown that epididymal sperm motility was significantly decreased in rats following ethanol consumption. However, our results did not show any significant difference regarding to the sperm morphology between the two animal groups.

Another sperm abnormality that may affect the outcome of fertility is sperm nuclear chromatin damage. One of the responsible factors for spermatozoal chromatin disorders is apoptosis (Sakkas et al., 2003; Saleh et al., 2003). It is known that ethanol induces apoptotic cell death in testicular germ cells and suppresses spermatogenesis (Rosenblum et al., 1985); in contrast, Maneesh et al. (2005) showed that testicular atrophy may be due to increased germ cell apoptosis. Thus, we considered that ethanol induces sperm nuclear chromatin and DNA damage through apoptosis. In other words, because sperm nuclear DNA damage has a positive correlation with apoptosis, increased apoptosis may be expected in this study.

On performing chromatin analysis in different groups of animals, significant differences were found for chromatin damages. The authors could not find any reports on staining of sperm cells with CMA3, AB, AO, and also TB in alcoholic men or in experimental models.

The sperm chromatin condensation was shown with application of two assays of AB and CMA3. AB staining is a cytochemical test for detection of residual histones. It

shows the lower amounts of protamines in the sperm nucleus indirectly and detects sperm chromatin condensation anomalies (Auger et al., 1990). Nuclei that are rich of residual histones have been stained with AB, whereas protamin-rich nuclei with moderately low levels of lysine have not been took up the blue stain (Hammadeh et al., 2001). Our results showed that the rates of AB-reacted spermatozoa (AB+) were similar in both ethanol-treated and control groups. Therefore, it can be concluded that alcohol consumption did not increase the rate of spermatozoa with residual histones.

In many studies, the chromatin-packaging quality of spermatozoa in connection with fertility status has been assessed by staining the sperm cells with CMA3 fluorochrome. CMA3 is a polymerase inhibitor fluorochrome specific for guanosine cytosine-rich sequence, which evaluates the degree of protamination of mature spermatozoa (Lolis et al., 1996). There was a correlation between abnormal sperm chromatin packaging as assessed by CMA3 staining and the presence of DNA strand breaks, decreased sperm penetration, the absence of sperm decondensation within the oocyte, and in vitro fertilization/intracytoplasmic sperm injection (IVF/ICSI) failure (Bianchi et al., 1993; Nasr-Esfahani et al., 2001; Razavi et al., 2003; Sailer et al., 1995). However, in this study, the rate of CMA3 positive sperm cells within the control samples was significantly less than the samples with ethanol consumption, but this difference was not much considerable. If we consider the ''cut-off'' value of 30% for the CMA3 staining (Sakkas et al., 1998), the mean percentage of CMA3+ spermatozoa was below this rate in both groups. So, it should be noticed that ethanol consumption cannot affect the sperm protamine status.

There was a significant evidence of increased red fluorescence spermatozoa with AO staining, which is a biomarker of the sperm nuclear DNA susceptibility to in situ acid-induced denaturation by shift of AO fluorescence from green to red. Present study used cytochemical assays for evaluating sperm DNA damage and among cytochemical tests, the AO can detect DNA abnormalities through DNA denaturation. In addition, AOT is a simple, reliable, and inexpensive test that can use as an alternative to expensive methods such as terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling and sperm chromatin structural assay (Evenson et al., 1999; Hoshi et al., 1996; Lopes et al., 1998). Therefore, we considered AO as a detecting test for sperm apoptosis through DNA denaturation. The increased red fluorescence showed the DNA damage or altered chromatin structure in sperm cells from ethanol user group when compared with controls. It means that ethanol may increase sperm apoptosis through DNA denaturation.

Regarding the AO staining, the percentage of sperm showing more than 50% red fluorescence was considered as ''cut-off'' value to characterize an abnormal chromatin status, which can negatively affect the fertilization rate (Virant-Klun et al., 2002). In this study, the rate of specimens that had more than 50% red fluorescence spermatozoa was 58.3% in ethanol-consuming rats. In addition, the percentage of AO positive sperm cells, which were above ''cut-off'' value was remarkable in ethanol group compared with control animals. It demonstrated that AO is an effective test for detection of denatured or single-stranded DNA in spermatozoa of ethanol users.

For measuring the rate of DNA fragmentation, TB staining was also applied. Toluidine blue is a basic nuclear dye for metachromatic and orthochromatic staining of chromatin, which measures the rate of sperm nuclear chromatin condensation via binding to phosphate groups of DNA strands. This stain is a sensitive structural probe for DNA structure and chromatin packaging (Rosenborg et al., 1990). In this study, the spermatozoa with dark blue and also violet and purple heads were significantly higher in experimental rats than controls. It confirmed that alcohol consumption decreased sperm chromatin condensation and also increases DNA fragmentation.

Carlsen et al. (1992) showed that the threshold for TB test is 45%, which provide 92% specificity and 42% sensitivity. The chromatin immaturity that is detected by high incidence (75%) of specimens with more than threshold TB-reacted spermatozoa showed that this staining is another effective cytochemical test for sperm immaturity measurement in alcohol users.

The significant differences in sperm chromatin and DNA status between the two groups were observed in this study. These differences have possible explanations including apoptosis and increased ROS in alcohol users. As it was mentioned earlier, ethanol enhances apoptosis of testicular germ cells including both spermatogonia and spermatocytes (Koh and Kim, 2006; Maneesh et al., 2005; Rosenblum et al., 1985; Zhu et al., 2000).

One study suggested that ethanol induces apoptotic cell death via the suppression of survival kinase activation and the phosphorylation of their downstram targets in rat testes (Koh, 2007). In addition, another study reported that ethanol reduced dose dependently the proliferation and induced apoptosis in cultured rodent and human mast cells (Nurmi et al., 2009).

Another possibility that would explain the increasing nuclear DNA damage is high concentrations of ROS (Wu and Cederbaum, 2003) and reduction in antioxidant defenses (Lecomte et al., 1994) in both acute and chronic alcohol exposure.

ROS is the major source of DNA damage, causing strand breaks, removal of nucleotide, and a variety of modifications of the organic bases of the nucleotides (Wu and Cederbaum, 2003). OS can contribute to cell damage and may play an important role in alcohol-induced DNA damage in the germ cells, and increased OS is a well-accepted mechanism of alcohol-induced tissue injury, particularly in testis (Emanuele et al., 2001). Amanvermez et al. (2005) showed that chronic ethanol

consumption induces OS in rat kidney, ovary, lung, and testis through high levels of lipid peroxidation and protein oxidation. They also showed that antioxidant supplements can partially protect tissues from ethanol-induced damages caused by ROS. Additionally, Ganaraja et al. (2008) demonstrated that alcohol consumption may cause a reduction in sperm normal morphology and count. In contrast, pretreatment with vitamin C as an antioxidant can improve sperm parameters in alcohol-treated rats.

However, no study to date has directly examined the correlation between alcohol intake and sperm oxidative damages. In fact, the relationship between seminal ROS and sperm chromatin abnormalities form a good topic for future studies.

In conclusion, our study with using cytochemical tests indicated possibly for the first time, that ethanol can significantly increase the epididymal spermatozoa DNA and chromatin damage in rats. Thus, the low rate of fertility in alcoholic men may be the result of damage to the sperm nucleus. These men should be advised to change their life style, avoid chronic alcohol consumption and use antioxidant regularly if they want to have a normal reproductive life.

Acknowledgments

The authors specially thank Maryam Nayeri for her skillful technical assistance during the course of this research. This study was supported by a grant from the Research and Clinical Center for Infertility, Shahid Sadougi University of Medical Sciences, Yazd, Iran.

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