Iran J Reprod Med Vol. 11. No. 11. pp: 925-932, November 2013

Original article

Seminal bacterial contaminations: Probable factor in unexplained recurrent pregnancy loss

Ali Nabi M.Sc., Mohammad Ali Khalili Ph.D., Iman Halvaei Ph.D. Candidate, Jalal Ghasemzadeh B.Sc., Ehsan Zare B.Sc.

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

Corresponding Author:
Mohammad Ali Khalili, Research and Clinical Center for Infertility, Safayeh, Bou-Ali ave., Yazd, Iran. POX: 89195-999
Email: Khalili59@hotmail.com
Tel: (+98) 351 8247085

Received: 16 September 2013
Accepted: 6 October 2013

Abstract

Background: It is estimated that about 50% of causes of recurrent pregnancy loss (RPL) cases remain unknown. Sperm factors are suggested to have probable role in cases with RPL.

Objective: The goal was to determine the possible relationship between semen bacterial contaminations with unexplained RPL. Also, the correlation between number of bacterial colony and sperm chromatin condensation was examined.

Materials and Methods: This study consisted of 30 fertile men (group A) and 30 infertile (group B) men with unknown RPL. Semen collection and analysis were done according to WHO manuals. Sperm count and motility were evaluated by Makler chamber. Eosin-Nigrosin and Papanicolaou staining methods were applied for viability and morphology assessment, respectively. The semen samples from both groups were cultured for aerobic bacteria. Aniline blue (AB) and toluidine blue (TB) staining methods were applied for evaluating sperm chromatin condensation.

Results: The numbers of colonies were significantly higher in group B when compared to group A. Also, S. aureus and E. coli contaminations showed significant differences between two groups. Both AB+ and TB+ sperm cells showed significant increase in group B compared to group A. There was a significant negative correlation between colony number and progressive motility (p=0.01), and sperm viability (p=0.007). In addition, positive correlations were found between colony number and AB^{+} (p=0.001) and TB+ (p=0.004) as well.

Conclusion: Bacterial contaminations in semen of men from RPL couples had significantly higher levels when compared to fertile controls. Presence of microorganisms in semen may be correlated with irregular sperm parameters and quality.

Key words: *Recurrent pregnancy loss, Bacteria, Semen.*

Introduction

Infection in the urogenital tract is one of the causes which can lead to male infertility. It is estimated that about 15% of infertile couples suffer from male urogenital tract infection (1). It appears the main mechanism induced by different microorganisms to cause infertility is not fully understood because diagnosis of many infections maybe difficult due to lack of any symptoms during infection. Numerous microorganisms including bacteria and viruses can lead to infection of genital system in men. Chlamydia trachomatis (C. trachomatis) is considered as the most common organism involved in acute non-bacterial prostatitis and urethritis also can infect epididymis, vas deferens and testis, as well (2).

Recurrent pregnancy loss (RPL) is the abortion of at least two consecutive pregnancies in the first or early second trimester of gestation (3). RPL may have different etiologies like anatomical, genetic, psychological, thrombotic and immunological defects. It is estimated that about 50% of RPL cases remain unknown, maybe due to different factors involved in RPL (4). Sperm factors are suggested to have probable role in RPL cases (5, 6). Many researchers tried to pinpoint the probable causes of sperm factors related to RPL, such as conventional sperm parameters (e.g. concentration, motility, morphology, viability) as well as acrosomal status, presence of leukocytes in seminal plasma, lipid peroxidation of sperm plasma membranes, antioxidant capacity of seminal plasma, and sperm chromatin integrity (6-10).

Ombelet *et al* designed prospective study in order to evaluate sperm parameters between fertile and infertile patients (11). They found that the rate of bacteria in seminal fluid in infertile men could be similar to fertile, and it seems that the clinical significance of presence of microorganisms in semen is still matter of debate. Talebi *et al* evaluated sperm chromatin packaging and DNA integrity in couples with unexplained recurrent spontaneous abortion (12). They stated that sperm cells from recurrent abortion have less chromatin condensation compared to fertile group. Also DNA integrity had better quality in fertile group compared to recurrent abortion group. There are several techniques in order to assess sperm DNA integrity (13-14). Aniline blue (AB) and toluidine blue (TB) staining methods are cytochemical assays which can detect sperm chromatin condensation anomalies.

We hypothesized that the bacterial contamination may have negative impact on sperm function and quality. To our knowledge, there are rare studies about the probable correlation of bacterial contamination and RPL. The main goal was to determine the possible relationship between the results from semen bacterial contaminations and unexplained RPL. Also, we compared the sperm chromatin packaging (using AB and TB tests) in RPL cases with fertile controls. Finally, the correlation between detected colony number and progressive motility, normal morphology, viability and DNA packaging tests were addressed.

Materials and methods

Patients

This case control study involved 60 individuals which were divided into two groups of A (fertile men, n=30) and B (infertile men with RPL, n=30). This study was done from November 2012 to May 2013. Inclusion criteria for groups A and B were to have a child within the last two years and to have at least two recurrent miscarriages in last two years, respectively. Exclusion criteria were smoking and variciselectomy. The semen samples from fertile group were obtained from men who referred to Akbari Clinic, Yazd, Iran for vasectomy.

The specimens were entered into study chronologically. Comprehensive examinations for group B were done and it was showed that all criteria are normal and the patients were considered as idiopathic cases. Individuals in both groups had no clinical signs, neither symptom of infections in their lower genital tract. The semen samples (group B) were obtained from patients who were referred to Yazd Research and Clinical Center for Infertility. All men were Muslim and had circumcised during their childhood. All the patients were asked to sign the consent forms. This study was approved by our institute ethic committee.

Semen collection and analysis

Ejaculates were collected by masturbation in a wide-mouth sterile container. Abstinence period was considered 3-7 days. Semen analysis was done according to WHO (2010) manuals (15). Sperm count and motility were evaluated by Makler chamber as described previously (16-17). Eosin-Nigrosin staining test and Papanicolaou staining were applied for viability and morphology assessment, respectively (Figure 1 and 2).

Semen culture

For assessment of bacterial infections, 10µl of semen sample was divided into two culture media: blood agar and eosin methylen blue (EMB) for detection of gram positive and gram negative microorganism, respectively. After 24 h of incubation at 37^{o}C, the presence of microbial and bacterial colonies were evaluated. After gram staining, differential tests were used for detection of microorganism species. Catalse test was applied for gram positive cocci in order to determine streptococcus and staphylucus. Gram positive cucci, with both coagulase and manitol fermentation tests positive, were considered as *S. aureus*. The number of colonies were counted using colony counter.

Aniline blue (AB) staining

Aniline blue (AB) staining, as a cytochemical staining, stains lysine-rich histones and is considered as a standard test for detection of defects in condensation of sperm DNA chromatin (18). The air-dried smears from fresh semen were fixed in 3% buffered glutaraldehyde in 0.2 M phosphate buffer (pH=7.2) at room temperature. After staining of each smear with AB stain (in 4% acetic acid, pH=3.5), the slides were checked

for presence of normal and abnormal spermatozoa using light microcopy (Olympus Inc., Tokyo, Japan). Unstained or pale blue stained cells and dark blue cells were considered as normal (AB-) and abnormal spermatozoa (AB+), respectively. At least 200 sperm cells were evaluated in each slide and the normal and abnormal spermatozoa were reported as percentage (12) (Figure 3).

Toluidine blue (TB) staining

TB stain can bind to phosphate groups of DNA strands and reveal the chromatin condensation status (19). Briefly, the air-dried smears from fresh semen were fixed in 96% ethanol-acetone (1:1) at 4^{o}C. The slides were then put in 0.1 NHCl at 4^{o}C for hydrolysation. After rinsing with distilled water, the slides were stained with 0.05% TB for 10 min. Pale blue sperm cells were considered as normal and dark blue or violet or purple spermatozoa were categorized to abnormal cells. At least 200 spermatozoa were checked for each slide and the normal (TB-) and abnormal (TB+) sperm cells were reported as percentage (20) (Figure 4).

Statistical analysis

The data were shown as mean±SD. Independent samples T test and Mann-Whitney U test were used as parametric and non-parametric tests, respectively, in order to compare the quantitative data between groups. Fisher's exact test was applied for comparison of qualitative data between two groups. Spearman test was used in order to find out the correlation between detected colony number and progressive motility, normal morphology, viability and DNA packaging tests (AB and TB). All hypotheses were considered two tails and significant level was set at 0.05.

Results

Of total of 35 and 47 semen samples in group A and B, 30 and 30 specimens were met inclusion criteria, respectively. There were no significant differences for men age, semen volume and sperm concentration between two groups. But progressive motility, normal morphology as well as sperm viability was statistically higher in group A compared to group B. Regarding sperm DNA integrity status, both AB+ and TB+ sperm cells showed significant increase in group B compare to group A (Table I). Table II shows semen culture outcomes between two groups. A total of 3 and 6 different types of bacterial species were detected in groups A and B, respectively. Only 3 samples in group B showed negative culture while no bacteria were grown after semen culture in 22 samples in group A.

The most prevalent bacteria in recurrent abortion and control groups were *S. aureus* and S. *epidermidis*, respectively. The numbers of colonies were significantly higher in group B when compared to group A (117666.67± 90551.31 vs. 28666.67±58768±0.06). In addition, *S. aureus* and *E. coli* in groups A and B were 0 vs. 9/30, and 0 vs. 6/30, respectively. There was a significant negative correlation between colony numbers and progressive motility (p=0.01, correlation coefficient=-0.31), and sperm viability (p=0.007, correlation coefficient=-0.34). Whereas, positive correlations were found between colony number and AB (p=0.001, correlation coefficient=0.41) and TB (p=0.004, correlation coefficient=0.37) as well.

Table I. Comparison of different parameters between two groups of recurrent abortion and control

Parameters	Control (group A)	Recurrent abortion (group B)	p-value
Male age	31.43 ± 7.00	31.97 ± 4.45	0.16*
Semen volume (ml)	3.21 ± 1.37	3.23 ± 1.25	0.95#
Sperm count (×10^{6}/ml)	109.73 ± 53.05	95.33 ± 54.62	0.22*
Round cell (×10^{6}/ml)	0.53 ± 0.81	1.03 ± 1.73	0.58*
Progressive motility (%)	64.27 ± 6.4	51.3 ± 12.36	<0.0001#
Normal morphology (%)	40.33 ± 9.92	25.53 ± 15.37	<0.0001*
Viability (%)	85.43 ± 4.77	70.2 ± 12.81	<0.0001#
TB+ (%)	34.3 ± 8.3	61.23 ± 20.5	<0.0001*
AB+ (%)	36.93 ± 10.16	65.67 ± 21.55	<0.0001*

The data were presented as mean±SD.
TB: Toluidine blue AB: Aniline blue
*: Statistical analysis using Mann-Whitney U test #: Statistical analysis using independent samples T test

Table II. Comparison of prevalence of bacteria detected from two groups of recurrent abortion and control

Parameters	Control (group A) (n=30)	Recurrent abortion (group B) (n=30)	p-value	OR (95% CI)
Colony (CFU/ml)	$2.8 \times 10^4 \pm 58768 \pm 0.06$	117666.67 ± 90551.31	<0.0001*	
Negative culture	22	3	<0.0001#	0.04 (0.009-0.1)
S. aureus	0	9	0.001#	26.9 (1.4-488.6)
S. saprophyticus	3	4	1#	1.3 (0.2-6.7)
S. epidermis	4	5	1#	1.3 (0.3-5.4)
E. coli	0	6	0.02#	16.1 (0.8-301.8)
Streptococci beta hemolytic	0	2	0.4#	5.3 (0.2-116.4)
Pseudomonas Sp.	0	1	1#	3.1 (0.1-79.2)
Enterococcus Sp.	1	0	1#	0.3 (0.01-8.2)

OR: odds ratio CI: confidence interval CFU: colony-forming unit
*: Statistical analysis using Mann-Whitney U test #: Statistical analysis using Fisher's exact test

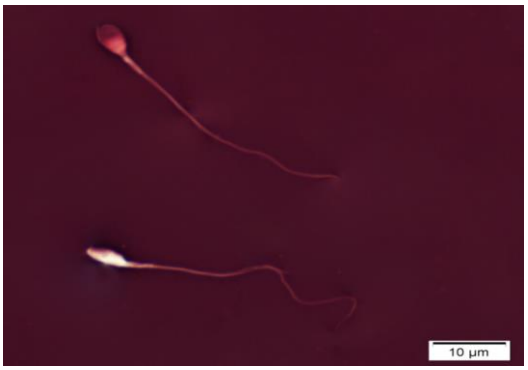

Figure 1. Evaluation of human sperm viability using eosin-nigrosin staining. a: unstained (white) is alive spermatozoon, b: stained (red) spermatozoon is dead.

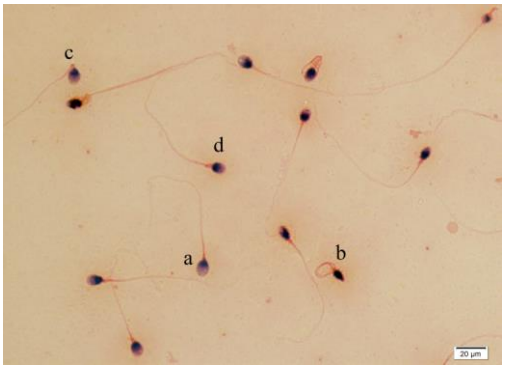

Figure 2. Papanicolaou staining for sperm morphological evaluation. a: normal sperm cell, b: coiled neck sperm cell, c: bent neck sperm cell, d: thick neck sperm cell.

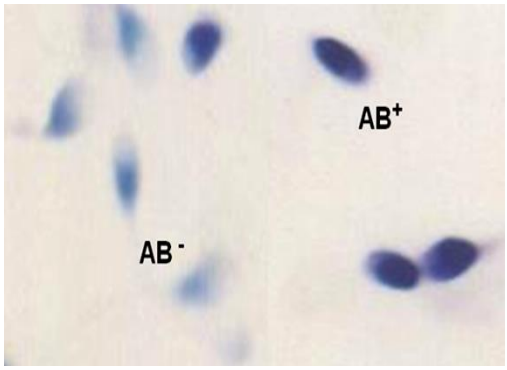

Figure 3. Aniline blue staining method. For sperm morphological evaluation. Light blue sperm heads show normal DNA and dark blue sperm heads show sperm DNA damage.

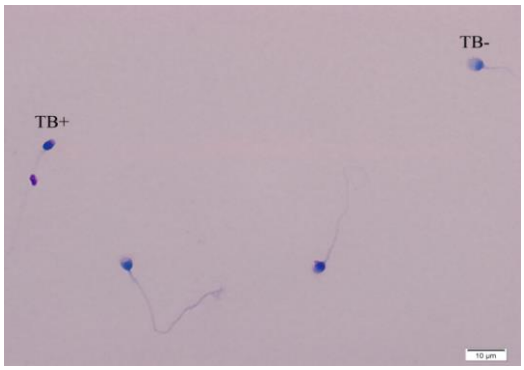

Figure 4. Toluidine blue staining method for sperm morphological evaluation. Light blue sperm heads show normal DNA and dark blue sperm heads show damage to DNAA.

Discussion

Unknown recurrent abortion could have different etiologies. In this study, it was shown that bacterial infection in semen might be related to this phenomenon in infertile couples. Our results showed that some bacterial species were significantly higher in recurrent abortion group compared to fertile controls. We found that sperm progressive motility, viability and normal morphology were significantly higher in group A compared to group B, while sperm count and semen volume were the same between groups. The relationship between conventional sperm parameters and recurrent abortion is matter of debate in literature (12, 21).

It was shown that microorganisms in semen can impair sperm function via agglutination of motile spermatozoa, impairment in acrosome reaction, morphology, and induction of apoptosis (22, 23). Some bacteria may affect sperm function via their pili. Gram positive bacteria, like enterococcus, can bind to mannose receptor in sperm surface and induce sperm damage (23, 24). It is shown that there is an inverse correlation between presence of some bacteria in semen with sperm count and motility (25).

In vitro studies using scanning electron microscopy and light microscopy have shown that bacteria (i.e. *E. coli* and mycoplasmas) may lead to alteration in sperm morphology (26, 27). It was stated that bacterial infection in semen of infertile men can affect the semen quality (28). Prabha *et al* have demonstrated an immobilizing factor production by *E. coli*. They showed that incubation of sperm with *E. coli* can impair sperm motility and morphology (26).

In our study, *E. coli* was detected in 20% of specimens in group B which is similar to previous report by our laboratory (29). Previously we found that *E. coli* in 22.2% of infertile samples resulted in significant reduction in progressive motility, normal morphology as well as sperm viability. It has also been proposed that the reduction of sperm motility by leukocytes and *E. coli* could be due to depletion of adenosine triphosphate (30). It has been reported that in vitro incubation of sperm with *E. fecalis*, *E. coli* and *S. aureus* may induce sperm apoptosis. This could be due to adhesion of bacterial pilus or flagellum to the sperm, resulting in activation of caspases followed by DNA damage (23).

In addition, bacteria toxin has been indicated as another mechanism for sperm apoptosis. Round cells are routinely checked at andrology labs, which are representatives of germ cells or leukocytes (15). It was stated that presence of leukocytes in semen can impair semen quality and sperm function (31). Our data showed that the rate of leukocytes had increasing trend in group B. The presence of leukocytes in semen could be a source of producing reactive oxygen species (ROS). It is well known that ROS can induce sperm membrane and DNA damage.

Our data showed that round cells, as a representative of leukocytes, had increasing trend in group B which was also more than WHO cut-off point of $\times 10^6$/ml. Presence of leukocytes in semen could be subsequently due to presence of microorganisms in semen. Therefore, bacteria in semen can indirectly induce ROS production and finally impair sperm viability and DNA integrity before fertilization and embryo development in later stages. TB can bind to phosphate groups of DNA strands and shows the rate of sperm nuclear chromatin condensation. Our results were in line with others that TB+ spermatozoa could be detected higher in recurrent abortion cases compared to fertile controls (12).

This could verify paternal role, especially the role of sperm DNA in etiology of abortion. Presence of microorganisms in semen may alter sperm DNA status directly or indirectly. It was shown that sperm chromatin condensation and DNA integrity status can affect fertility potential. When the sperm chromatin is less condenses, the susceptibility of sperm to environmental factors is more. AB staining test shows extra lysine-rich histone proteins. Our results showed that the rate of AB+ spermatozoa was significantly higher in group B compared to A which was in agreement with others (12, 21). In agreement to our results, Talebi *et al* found that AB+ spermatozoa had significant increas in unexplained recurrent spontaneous abortion group compared to controls (12).

The data generated from this study showed that the rate of TB+ sperm cells had significant increase in group B. Our data showed that about 61% of spermatozoa were TB-reacted in group B. This rate was 82% in Talebi *et al* study (12). While, Rybar *et al* did not find chromatin impairment (using SCSA) in semen samples contaminated by chlamydia, ureaplasma and mycoplasma strains compared to non-contaminated semen samples (32). Gallegos *et al* demonstrated that sperm DNA fragmentation (using SCD) will be increased in contamination of semen with mycoplasma and Chlamydia (33).

Since different strategies have been used for evaluation of integrity of DNA, it has become difficult to compare these studies. In this study, we have tried to detect only aerobic microorganisms. On the other hand, the clinical significance of the presence of anaerobic microorganisms in semen is still controversial issue. This has further exacerbated due the difficulties associated with the anaerobic culturing of semen. Probable role of anaerobic bacteria on semen quality and fertility potential needs to be further elucidated.

Also Chlamydia, as intrinsic microorganism, needs to be clarified by HELLA test which was not feasible to do in this study and is suggested to evaluate in future investigations.

Conclusion

This study showed that number of positive bacterial culture and colony number were significantly higher in semen of men from infertile couples due to recurrent abortion compared to fertile controls. In addition, it was shown that *S. aureus* and *E. coli* had significant increase in RPL cases. It could be suggested that comprehensive bacteriological examinations with subsequent antibiotic sensitivity assay and antibiotic therapy would be necessary before admission of infertile men into infertility treatment, especially in RPL cases.

Acknowledgements

The author would like to thank Mr. Razi, and Mr. Torki for their kind help during the study.

Conflict of interest

There is no conflict of interest in this article.

References

1. Pellati D, Mylonakis I, Bertoloni G, Fiore C, Andrisani A, Ambrosini G, et al. Genital tract infections and infertility. *Eur J Obstet Gynecol Reprod Biol* 2008; 140: 3-11.
2. Purvis K, Christiansen E. Infection in the male reproductive tract. Impact, diagnosis and treatment in relation to male infertility. *Int J Androl* 1993; 16: 1-13.
3. ASRM. Definitions of infertility and recurrent pregnancy loss: a committee opinion. *Fertil Steril* 2013; 99: 22.
4. Gupta S, Agarwal A, Banerjee J, Alvarez JG. The role of oxidative stress in spontaneous abortion and recurrent pregnancy loss: a systematic review. *Obstet Gynecol Surv* 2007; 62: 335-347.
5. Puscheck EE, Jeyendran RS. The impact of male factor on recurrent pregnancy loss. *Curr Opin Obstet Gynecol* 2007; 19: 222-228.
6. Nanassy L, Carrell DT. Paternal effects on early embryogenesis. *J Exp Clin Assist Reprod* 2008; 5: 2.
7. Hill JA, Anderson DJ, Polgar K, Abbott AF, Politch JA. Pregnancy: Seminal white blood cells and recurrent abortion. *Hum Reprod* 1994; 9: 1180-1183.
8. Sbracia M, Cozza G, Grasso J, Mastrone M, Scarpellini F. Semen parameters and sperm morphology in men in unexplained recurrent spontaneous abortion, before and during a 3 year follow-up period. *Hum Reprod* 1996; 11: 117-120.
9. Saxena P, Misro MM, Chaki SP, Chopra K, Roy S, Nandan D. Is abnormal sperm function an indicator among couples with recurrent pregnancy loss? *Fertil Steril* 2008; 90: 1854-1858.
10. Gil-Villa AM, Cardona-Maya W, Agarwal A, Sharma R, Cadavid Á. Assessment of sperm factors possibly involved in early recurrent pregnancy loss. *Fertil Steril* 2010; 94: 1465-1472.
11. Ombelet W, Bosmans E, Janssen M, Cox A, Vlasselaer J, Gyselaers W, et al. Semen parameters in a fertile versus subfertile population: a need for change in the interpretation of semen testing. *Hum Reprod* 1997; 12: 987-993.
12. Talebi A, Vahidi S, Aflatoonian A, Ghasemi N, Ghasemzadeh J, Firoozabadi R, et al. Cytochemical evaluation of sperm chromatin and DNA integrity in couples with unexplained recurrent spontaneous abortions. *Andrologia* 2012; 44: 462-470.
13. Halvaei I, Sadeghipour Roodsari HR, Naghibi Harat Z. Acute Effects of Ruta graveolens L. on Sperm Parameters and DNA Integrity in Rats. *J Reprod Infertil* 2012; 13: 33-38.
14. Nabi A, Khalili M, Halvaei I, Roodbari F. Prolonged incubation of processed human spermatozoa will increase DNA fragmentation. Andrologia 2013 . DOI: 10.1111/and.12088 available at: http://onlinelibrary.wiley.com/doi/10.1111/and.12088/.
15. WHO. WHO laboratory manual for the Examination and processing of human semen. 5th Ed: Cambridge University Press; 2010.
16. Khalili MA, Mojibian M, Sultan AM. Role of oocyte morphology on fertilization and embryo formation in assisted reproductive techniques. *Mid East Fertil Soc J* 2005; 10: 72-77.
17. Halvaei I, Khalili MA, Soleimani M, Razi MH. Evaluating the role of first polar body morphology on rates of fertilization and embryo development in ICSI cycles. *Int J Fertil Steril* 2011; 5: 110-115.
18. Auger J, Mesbah M, Huber C, Dadoune J. Aniline blue staining as a marker of sperm chromatin defects associated with different semen characteristics discriminates between proven fertile and suspected infertile men. *Int J Androl* 1990; 13: 452-462.
19. Rosenborg L, Rao K, Björndahlt L, Kvist U, Pousette A, Ákerlof E, et al. Changes in human sperm chromatin stability during preparation for in-vitro fertilization. *Int J Androl* 1990; 13: 287-296.
20. Talebi A, Moein M, Tabibnejad N, Ghasemzadeh J. Effect of varicocele on chromatin condensation and DNA integrity of ejaculated spermatozoa using cytochemical tests. *Andrologia* 2008; 40: 245-251.
21. Kazerooni T, Asadi N, Jadid L, Kazerooni M, Ghanadi A, Ghaffarpasand F, et al. Evaluation of sperm's chromatin quality with acridine orange test, chromomycin A3 and aniline blue staining in couples with unexplained recurrent abortion. *J Assist Reprod Genet* 2009; 26: 591-596.
22. Tremellen K. Oxidative stress and male infertility-a clinical perspective. *Hum Reprod Update* 2008; 14: 243-258.
23. Villegas J, Schulz M, Soto L. Bacteria induce expression of apoptosis in human spermatozoa. *Apoptosis* 2005; 10: 105-110.
24. Fujita K, Yokota T, Oguri T, Fujime M, Kitagawa R. In vitro adherence of Staphylococcus saprophyticus, Staphylococcus epidermidis, Staphylococcus haemolyticus, and Staphylococcus aureus to human ureter. *Urol Res* 1992; 20: 399-402.
25. Nunez-Calonge R, Caballero P, Redondo C, Baquero F, Martinez-Ferrer M, Meseguer M. Ureaplasma urealyticum reduces motility and induces membrane alterations in human spermatozoa. *Hum Reprod* 1998; 13: 2756-2761.
26. Prabha V, Sandhu R, Kaur S, Kaur K, Sarwal A, Mavuduru RS, et al. Mechanism of sperm immobilization by Escherichia coli. *Adv Urol* 2010; 2010: 240268.
27. Rose B, Scott B. Sperm motility, morphology, hyperactivation, and ionophore-induced acrosome reactions after overnight incubation with mycoplasmas. *Fertil Steril* 1994; 61: 341-348.
28. Khalili M, Sharifi-Yazdi M. The effect of bacterial infection on the quality of human's spermatozoa. *Iran J public Health* 2001; 35: 62-67.
29. Khalili M, Pourshafie M, Saifi M, Khalili M. Bacterial infection of the reproductive tract of infertile men in Iran. *Mid East Fertil Soc J* 2000; 5: 126-131.
30. De Lamirande E, Gagnon C. Reactive oxygen species and human spermatozoa. II. Depletion of adenosine triphosphate plays an important role in the inhibition of sperm motility. *J Androl* 1991; 13: 379-386.
31. Trum JW, Mol BW, Pannekoek Y, Spanjaard L, Wertheim P, Bleker OP, et al. Value of detecting leukocytospermia in the diagnosis of genital tract infection in subfertile men. *Fertil Steril* 1998; 70: 315-319.
32. Rybar R, Prinosilova P, Kopecka V, Hlavicova J, Veznik Z, Zajicova A, et al. The effect of bacterial

contamination of semen on sperm chromatin integrity and standard semen parameters in men from infertile couples. *Andrologia* 2012; 44: 410-418.
33. Gallegos G, Ramos B, Santiso R, Goyanes V, Gosálvez J, Fernández JL. Sperm DNA fragmentation in infertile men with genitourinary infection by Chlamydia trachomatis and Mycoplasma. *Fertil Steril* 2008; 90: 328-334.