Original Article

Assessment of accessory reproductive gland and oxidative stress in male partners of idiopathic recurrent pregnancy loss couples

Kavitha Papanna, Sreenivasa Gopalappa, Chaithra P. Thammaiah, Malini S. Suttur

Department of Studies in Zoology, Molecular Reproductive and Human Genetics Laboratory, Manasagangotri, Mysore, Karnataka, India

ABSTRACT

Background: Etiologies behind recurrent pregnancy loss (RPL) in half of the cases remains idiopathic even after an extensive investigation. The role of oxidative stress (OS) has been implicated in the pathogenesis of many human diseases including male fertility, but its role on male partner of RPL couple is limited. **Materials and Methods:** Semen samples from fifty male partners of the female with the history of three or more idiopathic primary RPL and fifty controls were obtained and analyzed according to the WHO guidelines along with sperm function tests. Functioning of the accessory gland was evaluated selecting one marker for each gland such as citric acid and fructose. OS markers such as reactive oxygen species, total antioxidant capacity, lipid peroxidation, and superoxide dismutase levels were estimated. Statistical analysis was done using independent samples *t*-test using statistical software SPSS version 14. **Results:** In RPL group, significantly lower scores were observed for sperm function test than the control group. Fructose and citric acid levels did not show any significant difference between the groups. Among RPL group, 2% and 4.5% of individual have a lower value for fructose and citric acid, respectively. The malondialdehyde concentration and ROS levels were significantly higher in RPL group. While the activity of superoxide dismutase and total antioxidant level was significantly lower in RPL males than in the control group. **Conclusion:** This study highlights the importance of evaluation of male factors for sperm function tests, OS markers, and accessory gland assessment along with the conventional parameters. This may help for better pregnancy outcome through proper evaluations and management.

Keywords: Pregnancy loss, reactive oxygen species, sperm function, total antioxidant capacity

INTRODUCTION

The term recurrent pregnancy loss (RPL) varies from infertility. The fertility rate is decreased or it results in total lack of fecundity due to impaired reproductive function. In case of RPL fecundity is maintained but fails to complete the full term of gestation.[1] RPL is classically defined as three of more consecutive pregnancy losses before the 20th week of gestation.[2] In general population, about 1-2% of couples experience RPL.[3] In adverse reproductive outcome, the effect of female factors is well-known in spite of which in the majority of the recurrent miscarriage cases the etiology remains unrevealed. Assessment of paternal factors in RPL is restricted to chromosomal and basic semen parameters;

Address for correspondence:
Dr. Malini S. Suttur,
Department of Studies in Zoology,
Molecular Reproductive and Human Genetics Laboratory,
Manasagangotri, Mysore, Karnataka, India.
E-mail: kavitharsh@zoology.uni-mysore.ac.in

Access this article online

Quick Response Code:

Website: www.fertilityscienceresearch.org

DOI: 10.4103/2394-4285.180486

Cite this article as: Papanna K, Gopalappa S, Thammaiah CP, Suttur MS. Assessment of accessory reproductive gland and oxidative stress in male partners of idiopathic recurrent pregnancy loss couples. Fertil Sci Res 2015;2:10-4.

however, the role of sperm factors has been ignored. The role of sperm factors is being considered as a major factor with the advent of assisted reproductive failures. Fertility potential of male is mainly based on the evaluation of semen which includes a number of spermatozoa that are present in the ejaculate, sperm motility, and their morphology. However, this is not so absolute that determines the fertility status, since their functional capability plays a major role.[4] This is because every sperm cell consists of different subcellular compartments with different functions which are helpful in capacitation, acrosome reaction and binding to the egg surface which are the most important mechanism of fertilization.[5] Human semen is a mixture of components produced by several different glands, and each gland produces a substance specific for accessory reproductive gland which can be used as markers to determine glandular function. The prostate produces citric acid, acid phosphatase, inositol, calcium, zinc, and magnesium, whereas fructose, ascorbic acid, prostaglandins are contributed by seminal vesicles.[6] Fructose and citric acid are involved in the specific role of energy metabolism, through glucose utilization. The role of the secretory products has been extensively studied in infertile males, and no reports are available among RPL males. Minimal concentration of reactive oxygen species (ROS) is needed for hyperactivation and capacitation of spermatozoa but in higher concentrations it causes damage by lipid peroxidation (LP) of plasma membrane, germ cell apoptosis, and DNA strand damage.[7] Plasma membrane of the spermatozoa is rich in polyunsaturated fatty acids; hence, they are susceptible for ROS, and low concentration of scavenging enzymes is present in the cytoplasm and thus has a limited capacity of DNA repair.[8] Oxidative stress (OS) has been implicated in the pathogenesis of many human diseases[9] including male fertility, but its role on male of RPL couple is limited.

Hence, this study was designed to investigate the role of sperm factors, functional status of accessory gland, and OS in idiopathic RPL couples. In this study, semen samples were subjected for routine analysis mainly focusing on the sperm function tests, functioning of the accessory gland by selecting a marker for each gland such as citric acid for prostate and fructose for seminal vesicle. Further OS markers such as ROS, total antioxidant capacity (TAC), LP and superoxide dismutase (SOD) were estimated. The results obtained were compared with the control group.

MATERIALS AND METHODS

Study group

Fifty male partners of the female who experienced three or more idiopathic primary RPL and fifty men who had fathered child/children without the history of RPL and with normal sperm parameters were enrolled as cases and controls, respectively. This study was approved by the Institutional Ethical Committee (Ref: IHEC-UOM No.52/Ph.D/2011-12). The cases and controls were informed, and written consent to participate in the study was obtained from them. Lifestyle factors such as smoking, alcoholism, and details regarding the consumption of medications were recorded both in the study and the control group. Antioxidant supplementation was not taken by any of the subject as well as the control group. Both subjects and the control group belongs to the same socioeconomic strata.

Semen samples were collected by masturbation after an abstinence period of 48-72 h. After liquefaction, routine semen analysis was performed according to the WHO guidelines (2010) to measure sperm motility, morphology, and count. Sperm function tests like nuclear chromatin decondensation (NCD) was performed using a protocol described by Gopalkrishnan *et al*.[10] and hypo-osmotic swelling (HOS) by Jeyendran *et al*.[11]

Exclusion criteria

Couples of which the females reported with anatomical, immunological, endocrinological, and infectious problems were not included. Individuals with acute illness within the last 3 months, smokers, alcoholics, and subjects under any antioxidant supplementation were not included. Transrectal ultrasound scanning was performed, and the subjects with anatomical abnormalities and varicocele were excluded for the present study. The subjects with leukocytes more than 1×10^6/ml were not included for the study.

Estimation of fructose

The level of fructose was estimated as described by Karvonen and Malm.[12] Semen plasma was separated by centrifugation and deproteinized by adding $ZnSO_4$ and NaOH incubated at room temperature for 15 min. The supernatant was mixed with indole reagent which contains benzoic acid and indole. Further, 32% of HCl was added and incubated at 60°C for 20 min. After cooling, the absorbance was recorded at 470 nm against blank.

Estimation of citric acid

The level of citric acid was estimated according to Polakoski and Zaneveld.[13] Semen plasma and trichloroacetic acid were mixed in equal proportion and cooled in ice bath. Supernatants were mixed with anhydrous acetic anhydride and incubated at 60°C for 10 min using water bath. Further dry reagent grade pyridine was added and again incubated at 60°C for 40 min. After cooling, the absorbance was read at 400 nm against blank.

Measurement of reactive oxygen species

Levels of ROS were measured in washed sperm suspensions by chemiluminescence assay using luminometer. Liquefied semen was centrifuged at 300 g for 7 min, and the seminal plasma was separated. The pellet was washed with phosphate-buffered saline (PBS) and resuspended in the same saline at a concentration of 20×10^6 sperms/ml. Luminol was prepared as 5 mM stock in dimethyl sulfoxide. It was added to the mixture and served as a probe. The negative control was prepared by adding PBS and luminol. Levels of ROS were assessed with the luminometer (Varioskan Flash Multimode Reader, Thermo Scientific) in the integrated mode and values were recorded. The result was expressed as relative light unit/20×10^6 sperms/ml.

Measurement of total antioxidant capacity

TAC was measured by the spectrophotometric method of Prieto *et al*.[14] Samples were mixed with 5% trichloroacetic acid as a reducing agent and incubated at room temperature for 10 min. To the supernatant, 1 ml of TAC reagent (0.6 M H_2SO_4, 28 mM sodium phosphate, and 4 mM ammonium molybdate) was added, incubated in water bath at 95°C for 90 min. After cooling, the absorbance was read at 695 nm against blank and expressed as μg/ml.

Measurement of superoxide dismutase activity

Super oxide dismutase (SOD) assay was estimated according to the protocol of Das *et al*.[15] In which, 1.4 ml of mixture was prepared which contains phosphate buffer pH 7.4, 20 mM α-methionine, 100 mM hydroxylamine hydrochloride, 50 µM ethylenediaminetetraacetic acid, 1% triton X and added to 100 µl of sample. About 100 µM of riboflavin was added and incubated for 10 min inside the SOD box. After incubation, Griess reagent was added (mixture of 0.1% Napthyl ethylene diamine and 1% sulphanilamide in 5% orthophosphoric acid) and absorbance was measured at 543 nm using spectrophotometer. The SOD activity was measured as units of SOD/mg of protein.

Measurement of malondialdehyde levels

Malondialdehyde (MDA) is one of the aldehyde products of LP. It was assessed by measuring levels of MDA formation using thiobarbituric acid (TBA).[16] TBA reagent was prepared using 8% SDS, 20% acetic acid, 0.8% TBA, and 0.8% butylated hydroxyl toluene, 0.2 ml of sample was added and incubated at 95°C in water bath for 95 min. After cooling, the mixture was centrifuged at 2000 rpm for 10 min and the supernatant was read at 532 nm using a spectrophotometer. The result was expressed as nMol MDA/mg protein.

Estimation of protein

A level of protein was estimated for the purpose of calculation of SOD and LP. Protein content was determined by a method of Lowry *et al*.[17] using bovine serum albumin as standard. To the sample, alkaline solution was added which consists of 4% sodium carbonate, 2% copper sulfate, and 1% sodium potassium tartrate, and incubated in dark for 15 min Folin and Ciocalteu's phenol reagent and water was added in the ratio of 1:1. Again this mixture was incubated in dark for 30 min and absorbance was measured at 660 nm using a spectrophotometer.

Statistical analyses

The obtained data were expressed in mean and standard deviation. Independent samples *t*-test was used to find out whether the significant mean difference exists between case and control subjects using statistical program SPSS (version 14.0, IBM). $P < 0.05$ was considered as significant.

RESULTS

Semen specimens from 100 individuals, which includes fifty male partners of female with the history of three or more consecutive idiopathic pregnancy loss and fifty control males, were examined. The mean age of RPL group was 34.3 ± 6.1 ranging from 21 to 45 years. The mean age of control group was 31.1 ± 6.4 ranging from 23 to 45 years with no significant difference ($P = 0.977$). The results of semen profile and sperm function test were depicted in Table 1. No significant differences were observed for volume, sperm count, and motility. With respect to sperm function test significantly lower scores were observed between the group for NCD as well as HOS. For NCD 30% and for HOS 25% of the RPL individual scored subnormal values. Functional capacity of accessory gland showed no significant difference for fructose and citric acid levels between the groups [Table 2]. Among RPL group, 2% of individuals showed lower fructose value (<13 µM/ejaculate) and 4.5% individual with lesser citric acid value (<13 mg/ejaculate).

Table 1: Comparison of semen profile and sperm function test between RPL and control group

	Volume (in ml)	Motility (grade a)	Count	NCD	HOS
Control ($n = 50$)	2.5±1.0	33.9±15.2	57.5±29.2	77±7.3	74.7±8.9
RPL ($n = 50$)	2.4±1.4	29.9±19.9	47.6±28.9	60.3±18.5	64.8±16.1
t-value	0.865	0.934	1.662	5.824	3.708
p value	0.568	0.353	0.100	0.001*	0.001*

n: number of subjects, RPL: recurrent pregnancy loss, NCD: Nuclear chromatin decondensation test, HOS: Hypo osmotic swelling test

Table 2: Values of fructose and citric acid between RPL and control group

	Fructose (≥13µmole/ejaculate)	Citric acid (≥13mg/ejaculate)
Control ($n = 50$)	132.4±50	27±12.2
RPL ($n = 50$)	114.3±71.98	25±15.5
t-value	1.434	1.380
p value	0.155	1.169

n: number of subjects, RPL: recurrent pregnancy loss

The MDA concentration in seminal plasma of RPL group is significantly higher than in the control group ($P = 0.001$). While the activity of antioxidant enzyme SOD in seminal plasma of the control group was significantly increased than in the seminal plasma of RPL group ($P = 0.001$) [Figure 1]. The mean ROS levels in RPL males was significantly higher when compared to the control group ($P = 0.001$). Whereas, the total antioxidant level was significantly lower in RPL males than in the control group ($P = 0.001$) [Figure 2].

DISCUSSION

The male factors are considered as a major contributing factor to infertility, but this has been totally ignored with respect to RPL though half of the genetic material is contributed by paternal genome to embryo. The paternal examination was restricted to chromosomal studies alone until date. Nevertheless, RPL evaluation is targeted only toward female and even after extensive diagnosis about 40-50% cases etiologies remains as idiopathic. The relation between standard semen parameters and RPL were controversial until now. Few studies were unsuccessful in determining a relation between standard semen parameters and recurrent miscarriage.[18-20] The plasma membrane integrity was evaluated by HOS test, the correlation between HOS test and recurrent miscarriage was previously described by few researchers and they found significantly lower score for HOS test between RPL and control group.[21,22] Chromatin decondensation of spermatozoa and pronuclear formation is vital for fertilization and normal embryonic development. Failure in decondensation results in chromatin damage leading to loss of fertilization potential and poor embryo quality which result in embryonic loss. The relation between abnormal chromatin condensation and RPL was reported by Gopalkrishnan *et al*. and Absalan *et al*.[23,24] While few researchers reported subnormal scores for all the function tests in RPL group.[25,26] The present findings accord with previous results, with decreased scores for NCD and HOS test in RPL group than the control group.

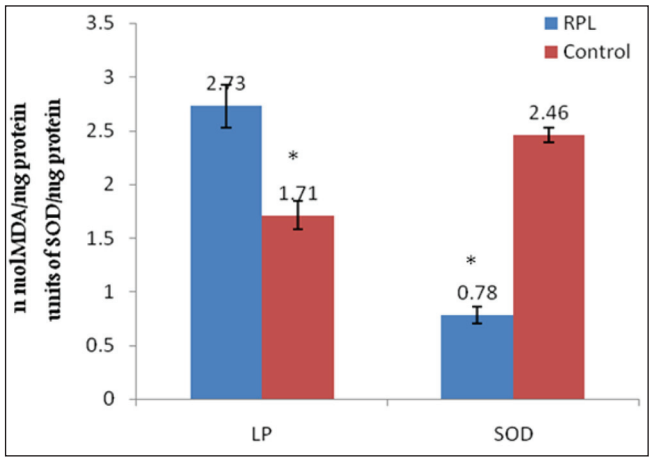

Figure 1: Melondialdehyde (LP) and superoxide dismutase (SOD) levels in seminal plasma of recurrent pregnancy loss (RPL) and control group. The values are expressed as a mean ± standard error, lipid peroxidation unit = nM malondialdehyde/mg of protein, superoxide dismutase unit = units/mg of protein (superoxide dismutase: $P = 0.034$*, $t = 2.155$ and lipid peroxidation: $P = 0.001$* $t = 3.778$)

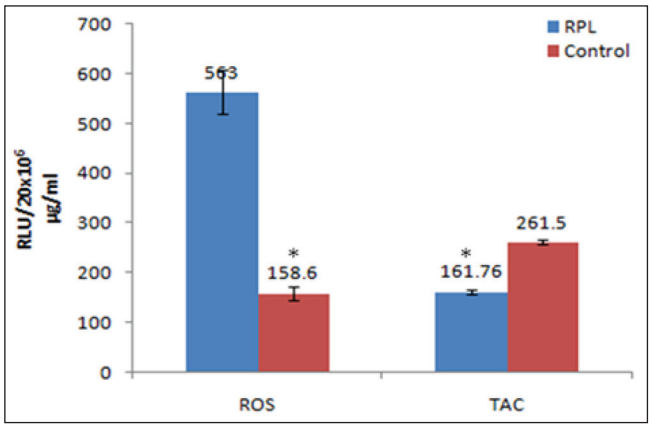

Figure 2: Reactive oxygen species (ROS) and total antioxidant capacity (TAC) levels in recurrent pregnancy loss (RPL) and control group. The values are expressed as a mean ± standard error, reactive oxygen species unit = Relative light unit/20 × 10^6. Total antioxidant capacity unit = µg/ml (reactive oxygen species: $P = 0.001$*, $t = 8.803$; total antioxidant capacity: $P = 0.001$*, $t = 10.031$)

Functional status of accessory glands was studied extensively in infertility and least focused on RPL. Fructose is considered as a measure of seminal vesicle function.[27] It is essential for sperm metabolism and motility which serves as a major energy source for spermatozoa.[28] Decreased fructose levels may be due to inflammation in the prostate or seminal vesicle or structural abnormality of seminal vesicles and their ducts.[29] In the current study, 2% of the RPL individuals scored less value for fructose. From the semen profile in these cases, lesser motility and abnormal score were observed for both the sperm function tests. Citric acid is a product of prostate and secreted by the prostatic epithelial cells. It plays a vital role in the prostatic function and maintains seminal pH.[30] The other possible role of citric acid in semen is it may act by chelating ions, thus keeping the calcium ion concentration of semen low. This activity will thus prevent premature capacitation of sperm and minimize premature activation of the acrosome reaction.[31] In this study, 4.5% of the RPL individual had a subnormal level of citric acid resulting abnormal liquefaction and few scored subnormal sperm function values. Though the frequency of abnormalities was less in this study further analysis and extrapolating to all RPL cases is needed, and it may be helpful for the precise diagnostic and proper treatment in RPL cases.

Seminal plasma contains effective antioxidant system that protects the spermatozoa against OS under normal condition. OS is as a result when ROS level exceeds than the TAC levels. ROS is one of the major contributing factors for the DNA damage, ensuring pre-post implantation pregnancy loss and poor assisted reproductive technique (ART) success rate.[32] Abnormal white blood cell (WBC) scores[33] and cigarette smoking is one of the major causes of increased ROS production in seminal fluid.[8] Hence, individuals with abnormal WBC scores and smokers were excluded from the study. Increased ROS levels cause pronuclear block and impair cleavage and leads to the formation of morphologically abnormal blastomere.[34] Superoxide anion, hydroxyl radical, and hydrogen peroxide are some of the major ROS present on the seminal plasma. In human spermatozoa, hydrogen peroxide is a major ROS product, and high concentration of hydrogen peroxide induces LP.[35,36] SOD, an enzymatic antioxidant, which scavenges both extra and intracellular superoxide anions and prevents LP of the plasma membrane. Hence, the levels of LP and SOD along with ROS and TAC were analyzed. The present findings revealed the elevated levels of MDA concentration and ROS while the concentrations of SOD and TAC were significantly lower in RPL compared to control group. This positively correlates the association of OS in RPL males, and this may be the one of etiology in idiopathic RPL cases. This problem can be overcome by the antioxidant therapy which can reduce the sperm DNA damage which is caused by the OS.[37]

CONCLUSION

The present study emphasizes the evaluation of male factor in idiopathic RPL cases. The data suggest the positive association of abnormal sperm functioning and OS on RPL males. Subnormal levels of fructose and citric acid were reported, where these parameters are totally ignored in the case of RPL subjects and never treated in this prospect. These analyses would aid in providing detail understanding of the problem and advising appropriate treatment for the RPL couples. Selection of the functionally superior sperm may assist in the successful outcome of the couples who opt for assisted reproductive techniques (ART's) like *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI). Antioxidant supplements and modification in lifestyle can be advocate in the early stage for the desired positive outcome in these couples. Further, these studies are needed in a large cohort to potentiate these results in cases of idiopathic RPL cases.

Acknowledgment

The authors wish to acknowledge all the study participants and gynecologist for their cooperation. KP is thankful UGC-RFSMS (BSR) for providing the stipend.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

REFERENCES

1. Kubo H. Epidemiology of infertility and recurrent pregnancy loss in society with fewer children. J Jpn Med Assoc 2009;52:23-8.
2. Ford HB, Schust DJ. Recurrent pregnancy loss: Etiology, diagnosis, and therapy. Rev Obstet Gynecol 2009;2:76-83.
3. Stephenson MD. Frequency of factors associated with habitual abortion in 197 couples. Fertil Steril 1996;66:24-9.
4. Aitken RJ. Sperm function tests and fertility. Int J Androl 2006;29:69-75.
5. Jeyendran RS, Van der Ven HH, Perez-Pelaez M, Crabo BG, Zaneveld LJ. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J Reprod Fertil 1984;70:219-28.
6. World Health Organization. Laboratory Manual for the examination of Human semen and Sperm Cervical Mucus Interaction. Cambridge University Press. New York 1999;4
7. Agarwal A, Said TM. Role of sperm chromatin abnormalities and DNA damage in male infertility. Hum Reprod Update 2003;9:331-45.
8. Saleh RA, Agarwal A, Sharma RK, Nelson DR, Thomas AJ Jr. Effect of cigarette smoking on levels of seminal oxidative stress in infertile men: A prospective study. Fertil Steril 2002;78:491-9.
9. Agarwal A, Prabakaran SA. Mechanism, measurement, and prevention of oxidative stress in male reproductive physiology. Indian J Exp Biol 2005;43:963-74.
10. Gopalkrishnan K, Hinduja IN, Kumar TC. *In vitro* decondensation of nuclear chromatin of human spermatozoa: Assessing fertilizing potential. Arch Androl 1991;27:43-50.
11. Jeyendran RS, Van der Ven HH, Zaneveld LJ. The hypoosmotic swelling test: An update. Arch Androl 1992;29:105-16.
12. Karvonen MJ, Malm M. Colorimetric determination of fructose with indol. Scand J Clin Lab Invest 1955;7:305-7.
13. Polakoski KL, Zaneveld LJD. Biochemical examination of the human ejaculate. Techniques of Human Andrology Biomedic Press 1977. p. 256.
14. Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of Vitamin E. Anal Biochem 1999;269:337-41.
15. Das K, Samantha L, Chainy GB. A modified spectrophotometric assay of superoxide dismutase using nitrite formation by superoxide radicals. Indian J Biochem Biophys 2000;37:201-4.
16. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351-8.
17. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265-75.
18. Homonnai ZT, Paz GF, Weiss JN, David MP. Relation between semen quality and fate of pregnancy: Retrospective study on 534 pregnancies. Int J Androl 1980;3:574-84.
19. Hill JA, Abbott AF, Politch JA. Sperm morphology and recurrent abortion. Fertil Steril 1994;61:776-8.
20. Sbracia S, Cozza G, Grasso JA, 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;11:117-20.
21. Buckett WM, Luckas MJ, Aird IA, Farquharson RG, Kingsland CR, Lewis-Jones DI. The hypo-osmotic swelling test in recurrent miscarriage. Fertil Steril 1997;68:506-9.
22. Carrell DT, Wilcox AL, Lowy L, Peterson CM, Jones KP, Erickson L, *et al*. Elevated sperm chromosome aneuploidy and apoptosis in patients with unexplained recurrent pregnancy loss. Am Coll Obstet Gynecol 2003;101:1229-35.
23. Gopalkrishnan K, Padwal V, Meherji PK, Gokral JS, Shah R, Juneja HS. Poor quality of sperm as it affects repeated early pregnancy loss. Arch Androl 2000;45:111-7.
24. Absalan F, Ghannadi A, Kazerroni M, Parifar R, Jamalzadeh F, Amiri S. Value of sperm chromatin dispersion test in couples with unexplained recurrent abortion. Gamete Biol 2012;29:11-4.
25. 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;90:1854-8.
26. Kavitha P, Malini SS. Positive association of sperm dysfunction in the pathogenesis of recurrent pregnancy loss. J Clin Diagn Res 2014;8:OC07-10.
27. Nun S, Musacchio I, Epstein JA. Variations in seminal plasma constituents from fertile, subfertile, and vasectomized azoospermic men. Fertil Steril 1972;23:357-60.
28. Mann T, Lutwak-Mann C. Male Reproductive Function and Semen: Themes and Trends in Physiology, Biochemistry and Investigative Andrology. New York: Springer-Verlag; 1981.
29. Schirren C. Textbook of practical Andrology. Schireng A, G. Hamburg. Germany. 1983. p. 17-31.
30. Said L, Galeraud-Denis I, Carreau S, Saâd A. Relationship between semen quality and seminal plasma components: Alpha-glucosidase, fructose and citrate in infertile men compared with a normospermic population of Tunisian men. Andrologia 2009;41:150-6.
31. Jequier MA. Male Infertility: A Clinical Guide. 2nd ed. Cambridge clinical guides. Cambridge University Press; 2011.
32. Aitken RJ, De Iuliis GN. Value of DNA integrity assays for fertility evaluation. Soc Reprod Fertil Suppl 2007;65:81-92.
33. Aitken RJ, Buckingham D, West K, Wu FC, Zikopoulos K, Richardson DW. Differential contribution of leucocytes and spermatozoa to the generation of reactive oxygen species in the ejaculates of oligozoospermic patients and fertile donors. J Reprod Fertil 1992;94:451-62.
34. Goto Y, Noda Y, Narimoto K, Umaoka Y, Mori T. Oxidative stress on mouse embryo development *in vitro*. Free Radic Biol Med 1992;13:47-53.
35. Kemal Duru N, Morshedi M, Oehninger S. Effects of hydrogen peroxide on DNA and plasma membrane integrity of human spermatozoa. Fertil Steril 2000;74:1200-7.
36. Misro MM, Choudhury L, Upreti K, Gautam D, Chaki SP, Mahajan AS, *et al.* Use of hydrogen peroxide to assess the sperm susceptibility to oxidative stress in subjects presenting a normal semen profile. Int J Androl 2004;27:82-7.
37. Gil-Villa AM, Cardona-Maya W, Agarwal A, Sharma R, Cadavid A. Role of male factor in early recurrent embryo loss: Do antioxidants have any effect? Fertil Steril 2009;92:565-71.