EVIDENCE OF INCREASED OXIDATIVE STRESS AND DNA DAMAGES IN WOMEN WITH RECURRENT ABORTIONS

Sumitha Prabhu PS, Aneesh P, Jiju J S, Reshma P and Dinesh Roy D

Abstract— Recurrent pregnancy loss is defined as 3 consecutive pregnancy losses prior to 20 weeks from the last menstrual period, and it affects approximately 1% to 3% of women. A total of 37 consecutive patients with recurrent miscarriages and 25 age matched healthy women were involved in this study. The subjects were in the age group 21 to 45 years. The present study was undertaken to assess the effect of increased oxidative stress and DNA damages in women experiencing recurrent pregnancy loss. Malondialdehyde test is performed to detect the frequency of oxidative stress in patients with recurrent pregnancy loss and the extent of somatic DNA damage is quantified by Cytokinesis Block Micronuclei assay. The study demonstrated that both the MDA value and micronuclei frequency are significantly elevated in the study subjects. The results were correlated with various demographic, lifestyle and clinical aspects of the patients. Increase in maternal problems such as history of infection, increased duration of married life, thyroid disorders, diabetes etc can also lead to foetal loss. Modification of life style along with proper medication for teratogenic infection and awareness of the role of Genetics in the etiology of RPL will help in reducing the risk for recurrent pregnancy loss.

Index Terms— Chromosomal abnormalities, DNA damages, Malondialdehyde, Oxidative stress, Reactive Oxygen Species (ROS), Recurrent pregnancy loss

1 INTRODUCTION

Recurrent pregnancy loss (RPL), also referred to as recurrent miscarriage or habitual abortion, is historically defined as 3 consecutive pregnancy losses prior to 20 weeks from the last menstrual period [1]. Recurrent spontaneous miscarriage (RSM), affects 1-3% of fertile couple [2]. Thirty percent pregnancies are lost between implantation and sixth week of gestation [3]. The incidence of recurrent pregnancy loss should be approximately 1 in 300 pregnancies. However, epidemiologic studies have revealed that 1% to 2% of women experience recurrent pregnancy loss [4]. It is estimated that approximately 8% to 12% of all cases of recurrent pregnancy loss are caused by endocrine diseases [5]. Upto 50%, however, no defined cause can be detected [6].

RPL have examined factors related to genetics, age, antiphospholipid syndrome, uterine anomalies, thrombophilias, hormonal or metabolic disorders, infection, autoimmunity, sperm quality, and lifestyle issues [7]. Certain environmental factors equally impact negatively on the human reproductive system that includes the heavy metals and poor maternal diet in pregnancy [8]. More recently, environmental pollutants including pesticides have been implicated in the pathogenesis of reproductive disorders [9] and infertility [10]. These factors, together or independently, may not only result in failure of the woman to conceive, but in some cases may result in the conception of non-viable embryos [11].

- *Sumitha Prabhu PS, Aneesh P and Jiju J S, Research Scholar, Meenakshi University, West KK Nagar, Chennai – 600078, TN, India.*
- *Reshma P and Dinesh Roy D, Genetika, Centre for Advanced Genetic Studies, Pettah P O, Thiruvananthapuram- 695024, India*

In a healthy body, ROS (reactive oxygen species) and antioxidants remain in balance. When the balance is disrupted towards an overabundance of ROS, oxidative stress (OS) occur [12]. At higher levels, OS can cause indiscriminate damage to biological molecules, leading to loss of function and even cell death [13]. Most ROS are formed as a consequence of the mitochondrial respiratory chain, but can also be formed by exogenous exposures such as smoke and environmental pollutants [14]. Lipid degradation occurs, forming products such as malondialdehyde (MDA) and ethane that are commonly measured as end products of lipid peroxidation. Lipid peroxidation is of particular significance in miscarriage which plays a key role in the pathogenesis of subfertility in both males and females [15]. Pregnancy complications such as spontaneous abortion, recurrent pregnancy loss, and preeclampsia, can also develop in response to OS [16].

Evidence exists supporting the role of oxidative stress in male infertility, including decreased sperm motility, sperm number, and sperm ovum fertilization [17]. The sperm cells and oocytes provide half of the nuclear embryo DNA; it may be assumed that both males and females could be involved in either infertility or RPL [18].

OS influences the entire reproductive span of women's life [19]. OS plays a major role in the female reproduction with involvement in the pathophsiology of pre-eclampsia, hydatidiform mole, free radical induced birth defects and other situations such as abortions [20]. Concentrations of ROS may also play a major role both in the implantation and fertilization of eggs [21]. Recently, OS has been reported to have an important role in the normal functioning of the female reproductive system and in the pathogenesis of female infertility [22]. Due to the formation of large number of ROS, which results in the breakage of double strands DNA in sperm and oocyts, may leads to recurrent miscarriages. Hence the present

study was undertaken to assess the effect of increased oxidative stress and DNA damages in women experiencing recurrent pregnancy loss. Thus this may help to aware the people about the factors which causes the recurrent abortion and the role of cytogenetics and aware the people how to reduce the recurrent pregnancy wastages by changing the lifestyle.

MATERIALS AND METHODS

Thirty seven couples suffering with varying degrees of pregnancy loss were selected for this study. All of these couples have atleast one year duration of married life. Twenty five age matched healthy women with atleast one child were selected as control for the study. These couples were referred from various infertility clinics and maternity centers of Kerala for genetic testing to Genetika, Centre for Advanced Genetic Studies, Trivandrum, Kerala. Detailed demographic and lifestyle characteristics were recorded using proforma.

Eight ml of venous blood was collected aseptically from all the subjects (both the husband and wife) by venepuncture after overnight fasting. 4ml was transferred into the vacuutainer containing sodium heparin to perform lymphocyte culture and CBMN assay. The remaining 4ml was transferred into plain tube and allowed to clot. The level of the serum lipid peroxide marker, malondialdehyde (MDA) was determined using thiobarbituric acid as main reagent and the values are measured on a semi- autoanalyser at 540nm. Malondialdehyde test is performed to detect the frequency of oxidative stress in couples with recurrent pregnancy loss.

Two parallel cultures were set up for each sample, culture A & B. The culture A was for detecting constitutional chromosome anomalies by using peripheral blood lymphocyte culture method described by Moorhead et al (1990), and GTG banded karyotypes were prepared according to ISCN pattern 1995. The culture B was for quantitating the extent of somatic DNA damages by Cytokinesis Block Micronuclei (CBMN) assay.

The lymphocytes were cultured in sterile bottles using 10 ml RPMI 1640 medium containing 15% foetal calf serum, 100Units/ml penicillin, 100Units/ml streptomycin and 1% phytohemagglutinin. At 44th hr after initiation, cells were blocked in cytokinesis by adding Cytochalasin B (Sigma, final concentration, 4.5µg/ml). The total incubation time for all cultures was 72 hr. After incubation, the cells were fixed in 3:1 methanol/glacial acetic acid, dropped onto clean microscopic slides, air dried, and stained with Giemsa stain. For each sample, 1,000 binucleated cells were scored at 100X magnification. The number of micronuclei per 1,000 binucleated cells was recorded.

RESULTS

Thirty seven women suffering with varying degrees of recurrent pregnancy loss were selected for the study. The age of the subjects ranged from 21 – 45 years with a mean age of 28.8. Twenty five age matched healthy women with atleast one child were selected as control for the study. Various demographic, clinical, lifestyle and physiological condition were recorded and correlated with the extent of DNA damages and oxidative stress.

The Cytokinesis block micronuclei assay revealed that there is a statistical significant increase in the mean CBMN frequency among the study subjects (13.47) than the control subjects (10.48). In the case of MDA value, it was found to be 1.63 in study subjects and 0.66 in control subjects. The present study observed a significantly high level of MDA in study subjects when compared to the control subjects (Table: 1).

TABLE 1:

DISTRIBUTION OF MEAN CBMN FREQUENCY AND MDA VALUE AMONG STUDY AND CONTROL SUBJECTS

Variables	Number	Mean CBMN Frequency	MDA value
Study Subjects	37	13.47	1.63
Control Subjects	25	10.48	0.66

The distribution of CBMN frequency and MDA value according to karyotype of the study subjects were given in the table: 2. Chromosome analysis of women with RPL revealed that among the 37 study subjects, 94.59% are of normal karyotype and 5.40% are of abnormal karyotype. The mean CBMN frequency of women with normal karyotype was 13.46 and that of abnormal karyotype was 13.70. The present study revealed that the mean CBMN frequency of abnormal karyotype was found to be higher than the mean CBMN frequency of subjects with normal karyotype. All the male subjects showed normal karyotype.

TABLE 2:

DISTRIBUTION OF MDA VALUE AND MEAN CBMN FREQUENCY ACCORDING TO KARYOTYPE OF THE STUDY SUBJECTS

Category	Variables	Number	Percentage	MDA Value	Mean CBMN Frequency
Karyotype of Wife	Normal	35	94.59%	1.62	13.46
	Abnormal	2	5.40%	1.90	13.70

The demographic characteristics of the study subjects were studied. The maternal age showed significant relationship with mean CBMN frequency and MDA value i.e., as the age increases the micronuclei frequency and MDA concentration was found to be increased. The study did not observe any significant relation with the paternal age and CBMN frequency.

The lifestyle characteristics of the study subjects were also studied. The duration of married life of couples with RPL were observed and among them those who had a duration >10

years showed highest CBMN frequency of 13.5 and highest MDA value of 2.47. This indicates that there was a significant relationship between the mean CBMN frequency, MDA value and increased duration of marriage life. Study subjects having history of infection, fever, urinary tract infection, frequent allergy, etc were reported with increased incidence of abortions. The histories of chronic illness among the subjects were also observed. The illnesses include polycystic ovarian syndrome, APLA syndrome, hypothyroidism, and hyperthyroidism. The present study observed an increased micronuclei frequency among the subjects with hypothyroidism and APLA syndrome.

According to the physiological characteristics, aubjects suffering with more than 3 times of spontaneous abortions and also those experienced with multiple number of medical terminations of pregnancies (MTP) were observed with increased micronuclei frequency and increased oxidative stress. Thus the present study observed a positive correlation between the increased extent of DNA damages and increased oxidative stress with various demographic, clinical, lifestyle and physiological characteristics among the study subjects with recurrent abortions.

DISCUSSION

The purpose of the present study was to evaluate the extent of somatic DNA damages and oxidative stress in subjects experiencing recurrent pregnancy loss. Human peripheral blood lymphocytes were used for quantifying the extent of somatic DNA damages. Many studies have linked excess generation of reactive oxygen species (ROS) with cellular damages and RPL. Malondialdehyde, a decomposition product of autooxidation of polyunsaturated fatty acids, is used as an index of oxidative damages. The present study observed a significantly high level of oxidative stress and DNA damages in the form of increased MDA value and mean CBMN frequency in study subjects than control subjects.

Recurrent Pregnancy Loss (RPL), also referred to Recurrent Miscarriage or Habitual Abortion, is a distinct disorder defined by two or more failed clinical pregnancies, and upto 50% of cases of RPL will not have a clearly defined etiology. Approximately 15-20% of clinically recognizable pregnancies end in spontaneous abortion. Oxidative stress-induced damage has been hypothesized to play a role in spontaneous abortion and idiopathic recurrent pregnancy loss [23]. Probably the most common cause of any pregnancy loss is a chromosome abnormality in the conception. Couples with recurrent miscarriage (RM) are facing an increased risk of being carriers of a structural balanced chromosome abnormality. The incidence of carrier status is 0.7% in the general population worldwide and increases to 2.2% after one miscarriage, 4.8% after two miscarriages and 5.2% after three miscarriages [24].

Many studies reported that increasing maternal age is a risk factor for infertility and miscarriages [25]. Maternal age and previous miscarriage rates increases the risk of subsequent miscarriages. De et al, [19] reported that women experience an age dependent increase in various adverse reproductive events such as infertility, pregnancy complications. In the present study also observed an increased mean CBMN frequency among older mothers. These results clearly indicate that the maternal age is also a factor determining pregnancy loss and the recurrent risk increases with the increase in maternal age.

Advancing paternal age has been recognized as a contributing factor for increasing the risk of producing aneuploid gametes. Abnormal DNA fragmentation may be seen in the setting of advanced paternal age or may result from correctable environmental factors, such as exogenous heat, toxic exposures, varicoceles, or increased reactive oxygen species in semen. The probability of producing aneuploid offspring [26] is increased in older men and there are higher frequencies of sperm chromosome aberrations [27]. Numerous studies have shown that abortion is closely associated with other confounding factors, such as smoking, alcohol, drug use, promiscuity and venereal disease. The occupational exposure, such as heavy metals, organic solvents, and ionizing radiation, affect semen quality, concentration, motility, viability, and morphology, as well as sperm chromatin status. The current study did not showed any significant relationship between paternal age and DNA damages.

It has been estimated that 40% of pregnancies in women with PCOS will result in spontaneous loss. Palacio et al [28] reported that polycystic ovary syndrome is also associated with decreased antioxidant concentrations, and is thus considered an oxidative state. Essah et al [29] reported that a high proportion of PCOS patients have a greater risk for spontaneous abortion due to the individual anatomic defect or the confounding factors associated with it. In the present study, 10.81% of subjects were found with PCOS and also the mean CBMN frequency and MDA value was found to be higher among them.

Poorly controlled diabetes mellitus results in increased risk for foetal loss. Studies show that patients suffering from this clinical condition run a significantly increased risk of spontaneous abortion, preterm labor, hypertensive disorders and operative deliveries [30]. Subjects with diabetes mellitus in the current study were observed with increased oxidative DNA damages.

Abalovich et al [31] reported that specific adverse outcomes associated with maternal overt hypothyroidism include increased risks for premature birth, low birth weight and miscarriage. The present study observed an increased mean CBMN frequency and MDA value in women with hypothyroidism, suggesting a positive correlation between thyroid abnormalities and recurrent miscarriages.

Infections are known cause of late foetal losses and logical causes of early foetal losses. Microorganisms associated with spontaneous abortion include variola, vaccinia, Salmonella typhi, and Vibrio. Women suffered with any kind of infection during their gestation period were observed with increased CBMN frequency and malondialdehyde concentration in the present study. This indicates a positive role of infections in causing recurrent miscarriages.

The major cause of spontaneous abortion is foetal chromosomal abnormalities, contributing to about 50~60% of the cases. The most prevalent abnormality of Spontaneous abortion is chromosomal aneuploidy. Other etiologic factors of Spontaneous abortion are anatomic anomalies, endocrine or hormonal problems, coagulation protein defects, and nutritional and

environmental factors [32]. In the present study, subjects with more than 3 spontaneous abortions showed higher mean CBMN frequency and MDA value. Several types of genetic problems like parental structural chromosomal abnormalities and recurrent aneuploidies may be associated with recurrent miscarriage. Approximately 15–20 % of all pregnancies in females end up in spontaneous abortions with 50 % of these being associated with chromosomal abnormalities. Elghezal et al [33] reported that parental chromosomal abnormalities are detected in about 2-8% of couples with recurrent miscarriages. The present study reveals that, out of the thirty seven female subjects only 5.40% were found to have structural abnormalities and they showed higher mean CBMN frequency and MDA value. This result indicates that there is a positive correlation between frequency of micronuclei, oxidative stress, and chromosomal abnormalities in women experiencing recurrent abortions.

CONCLUSION

The couples who had reported for the recurrent pregnancy loss showed a higher CBMN frequency and MDA value. The extent of DNA damages was found higher in those couples with an increased age, increased duration of married life, increased number of gestations, increased number of spontaneous abortions, increased number of MTPs, women with any history of infections, history of chronic illness. This study has shown that the incidence and distribution of chromosomal abnormalities among couples with repeated foetal loss is comparable to that reported worldwide. So, the risk of two consecutive losses can be reduced by assessing the reason through cytogenetic and biochemical analysis. Tests for DNA damage should be considered as part of the diagnostic and treatment pathways for those suffering from recurrent pregnancy loss. Further research is required into the mechanisms responsible for and preventing the DNA damage including antioxidant therapy.

REFERENCES

[1] Pratip Chakraborty., 'Recurrent Pregnancy Loss in Polycystic Ovary Syndrome: Role of Hyper homocysteinemia and Insulin Resistance', 2013, 8(5).

[2] Toth B, Jeschke U, Rogenhofer N, Scholz C, Würfel W et al., 'Recurrent miscarriage: current concepts in diagnosis and treatment', J Reprod Immunol 85, 2010, 25-32.

[3] Nybo Andersen AM, Wohlfahrt J, Christens P, Olsen J, Melbye M., 'Maternal age and fetal loss: Population based register linkage study', BMJ, 2000;320:1708–12.

[4] Stephenson MD, 'Frequency of factors associated with habitual abortion in 197 couples'. Fertil Steril., 1996, 66:24-29.

[5] Smith ML, Schust DJ., 'Endocrinology and recurrent early pregnancy losses', Semin Reprod Med 29, 2011, 482-49.

[6] Rai R, Regan L, 'Recurrent miscarriage', Lancet 2006, 368:601–611.

[7] Stephenson MD, Kutteh W., 'Evaluation and management of recurrent early pregnancy loss, 'Clin Obstet Gynecol 2007; 50:132–45.

[8] Coulam CB, Stephen M, Stern JJ, Clark DA., 'Immunotherapy for recurrent pregnancy loss: Analysis of results from clinical trials', Am. J.Reprod. Immunol 1996, 35: 352-359.

[9] Abdollahi M, Ranjbar A, Shadnia S, Nikfar S, Rezaie A., 'Pesticides and oxidative stress: a review', Med Sci Monit 2004, 10:RA141–RA147.

[10] Wells PG, Bhuller Y, Chen CS, Jeng W, Kasapinovic S, Kennedy JC, Kim PM, Laposa RR, McCallum GP, Nicol CJ, et al: 'Molecular and biochemical mechanisms in teratogenesis involving reactive oxygen species'. Toxicol Appl Pharmacol 2005, 207:354–366.

[11] Martenies SE, Perry MJ, 'Environmental and occupational pesticide exposure and human sperm parameters: a systematic review', Toxicology. 2013; 307(10):66–73.

[12] Ashok Agarwal, Sajal Gupta and Rakesh K Sharma., 'Role of oxidative stress in female reproduction', Reproductive Biology and Endocrinology, 2005, 3:28

[13] Graham J. Burton ,Eric Jauniaux., 'Oxidative stress', Best Practice & Research Clinical Obstetrics and Gynaecology 25 ,2011, 287–299.

[14] Lavranos, M. Balla, A. Tzortzopoulou, V. Syriou, R., 'Angelopoulou, Investigating ROS sources in male infertility: a common end for numerous path- ways', Reproductive Toxicology (Elmsford, N.Y.) 34(3), 2012, 298–307.

[15] Ahmed M Issa, Balsam G Hassan, Asmaa K. Gatea, 'Relationship of Nitric Oxide and Malondialdehyde to Miscarriage', Medical Journal of Babylon 9:4 , 2014.

[16] Ashok Agarwal, Anamar Aponte-Mellado, Beena J Premkumar, Amani Shaman and Sajal Gupta., 'The effects of oxidative stress on female reproduction: a review', Reproductive Biology and Endocrinology, 2012, 10:49.

[17] Tremellen K, Miari G, Froiland D, Thompson J., 'A randomised control trial examining the effect of an antioxidant (Menevit) on pregnancy outcome during IVF-ICSI treatment'. Aust N Z J Obstet Gynaecol 2007; 47:216–221.

[18] Lewis SE, Simon L., 'Clinical implications of sperm DNA damage', Hum Fertil (Camb), 2010, 13(4): 201–207.

[19] de Bruin JP, Dorland M, Spek ER, Posthuma G, van Haaften M Looman CW, te Velde ER., 'Ultrastructure of the resting ovarian follicle pool in healthy young women', Biol Reprod 2002,66:1151-1160.

[20] Lagod L, Paszkowski T, Sikorski R, Rola R., 'The antioxidant prooxidant balance in pregnancy complicated by spontaneous abortion', Ginekol Pol 2001, 72:1073-1078.

[21] Sharma RK, Agarwal A., 'Role of reactive oxygen species in male infertility', Urology 1996; 48:835–850

[22] Bedaiwy MA, Falcone T, Sharma RK, Goldberg JM., et al., 'Prediction of endometriosis with serum and peritoneal fluid markers: a prospective controlled trial', Hum Reprod, 2002, 17:426–431.

[23] Gupta S, Agarwal A, Banerjee J, Alvarez J.G., ' The role of oxidative stress in spontaneous abortion and recurrent pregnancy loss', a systematic review, fifth ed., In: Obstetrical and Gynecology Survey, , Vol. 62 Lippincott Williams & Wilkins, 2007 pp. 335–347.

[24] Van den Boogaard, E., et al. "Selective karyotyping in recurrent miscarriage: are recommended guidelines adopted in daily clinical practice?." Human reproduction 26.8 (2011): 1965-1970.

[25] Natinal Centre For Health Science Statistics, 1998.

[26] Griffin DK, Abruzzo MA, Millie EA, Sheean LA, Feingold E, Sherman SL,Hassold TJ., ' Non-disjunction in human sperm: evidence for an effect of increasing paternal age', Hum Mol Genet 1995,4,2227–2232

[27] Sartorelli EM, Mazzucatto LF, de Pina-Neto JM., ' Effect of paternal age on human sperm chromosomes, Fertil Steril 2001,76,1119–1123

[28] Palacio JR, Iborra A, Ulcova-Gallova Z, Badia R, Martinez P., ' The presence of antibodies to oxidative modified proteins in serum from polycystic ovary syndrome patients', Clin Exp Immunol 2006, 144,217–222

[29] Essah P A,Cheang K I,Nestler J E., 'The Pathophysiology Of Miscarriages In Women With Polysistic Ovary Syndrome,Review And Proposed Hypothesis Of Mechanisms Involved,Hormones,2004,3,221-227.

[30] Melamed N and Hod M , 2009 Perinatal mortality in pregestational diabetes. Int J Gynaecol Obstet 104: Suppl 1: 20-24.

[31] Abalovich M, Gutierrez S, Alcaraz G, Maccallini G,et al., 'Overt and subclinical hypothyroidism complicating pregnancy', Thyroid, 2002,12, 63–8.

[32] Ji Hyae Lim; Cell-Free Fetal DNA and Cell-Free Total DNA Levels in Spontaneous Abortion with Fetal Chromosomal Aneuploidy; 2013, 8(2): e56787.

[33] Elghezal H, Hidar S, Mougou S, Khairi H, Saad A. Prevalence of chromosomal

abnormalities in couples with recurrent miscarriage. Fertil Steril. 2007; 88:721–723