Original Research Paper Medical Science

IMMUNOLOGIC AND GENETIC ASPECTS OF RECURRENT PREGNANCY LOSS

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ABSTRACT Recurrent pregnancy loss (RPL) is a heterogeneous condition that has many possible causes including genetic, hormonal, metabolic, uterine anatomical, infectious, environmental, occupational and personal habits, thrombophilia or immune disorders. There is 10% to 20% of women experience a miscarriage throughout their reproductive period. Primary infection caused by TORCH and other infectious agents can cause RPL. The aim of the study was to find out chromosomal abnormality and quantify the extent of DNA damage by Cytokinesis Block Micronuclei (CBMN) assay along with evaluating role of infectious agent in pregnancy loss. The present study was carried out in 44 couples suffering from 2 or more pregnancy loss as study subject and 22 age matched healthy couples with atleast one child were selected as control for the study. The mean CBMN frequency of subjects was correlated with various risk factors. A significant increase in mean CBMN frequency among the study subjects along with risk factors is suggestive of increased risk towards recurrent pregnancy loss. The study demonstrated the role of microorganism in DNA damage and its effect on reproduction. Hence, all cases with RPL should be routinely screened for TORCH and other infection as early diagnosis and appropriate intervention will help in proper management of these cases.

KEYWORDS : Recurrent pregnancy loss, TORCH infection, Karyotype and Cytokinesis Block Micronuclei assay

INTRODUCTION

Recurrent pregnancy loss (RPL), defined as two or more consecutive pregnancy losses before 20th week of pregnancy (Pratip Chakraborty, 2013). Approximately 15 percent of pregnant women experience sporadic loss of a clinically recognized pregnancy. Just 2 percent of pregnant women experience two consecutive pregnancy losses and only 0.4 to 1 percent has three consecutive pregnancy losses (Salat-Baroux, 2000).

RPL is a multifactorial disorder resulting from genetic factors, anatomic factors, immunologic factors, endocrine dysfunction, thrombophilia, lifestyle factors and maternal infections. However, an increasing risk of foetal loss with increasing maternal age has been documented in women aged more than 30 years (Andersen et al., 2000). No apparent causative factor is identified in 50% to 70% of couples with RPL (The Practice Committee of the American Society for Reproductive Medicine, 2012).

Chromosome abnormalities are the leading known cause of RPL (Robert Mueller and Ian young, 2001). It has been reported that 50% of spontaneously aborted foetuses have chromosomal abnormali ties. In approximately 3–5% of couples with recurrent miscarriage, one of the partners is affected by a chromosomal translocation, as oppose to 0.2% in the normal population. This may repeatedly produce an unbalanced gamete, resulting in the recurrence of spontaneous miscarriage (Hirshfeld Cytron et al., 2011). 60% of balanced translocations are reciprocal translocations that are formed by exchange of segments between two non- homologous chromosomes, and 40% are Robertsonian translocations that are formed by the fusion of two acrocentric chromosomes at centromere with the loss of short arms (Van Niekerk et al., 2013; Ayse seyhan et al., 2011).

Maternal infections play a critical role in pregnancy wastage and their occurrence in patients with bad obstetric history (BOH) is a significant factor (Chopra et al., 2004). Certain infections including Listeria monocytogenes, Toxoplasma gondii, rubella, herpes simplex virus (HSV), measles, cytomegalovirus and coxsackie viruses are known or suspected to play a role in sporadic spontaneous pregnancy loss. These infection causing organisms produce toxic metabolic by products. Pregnancy loss due to infectious agents includes direct infection of the uterus, foetus, or placenta, placental insufficiency, and chronic endometritis.

The cytogenetic and immunologic analysis of recurrent pregnancy loss provides valuable insight into the cause of miscarriage which can eliminate further costly testing. In addition, recurrent risk estimates for subsequent pregnancies can also be determined. Hence the present study was undertaken to aware people about immunological and genetic aspects of recurrent pregnancy loss.

MATERIALS AND METHODS:

The study conducted with 44 couples with two or more pregnancy loss or abortions as study subjects and 22 age matched healthy couples with atleast one child were selected as control. All the couples were referred by various maternity centers to Genetika, Centre for Advanced Genetic Studies, Trivandrum for genetic testing. Relevant information's were collected from each subjects using proforma. Two parallel cultures were set up for each sample, culture A & B. The culture A for detecting constitutional chromosom e 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.

Lymphocyte culture: The fresh blood was collected by venipuncture and transferred into vacutainer containing sodium heparin as anticoagulant. Added 5 to 6 drops of whole blood samples to a vial containing 10ml of RPMI 1640 medium supplemen ted with 15% foetal bovine serum. Then phytohaemag glutinin (PHA, 100µg/ml) was added to proliferate the lymphocyte cells and incubated at 37°C for 72 hrs. At the 70th hour to the culture added a drop of colchicine (0.04µg/ml) to arrest the cell division at metaphase, then mixed gently and kept in incubator at 37°C for 2 hours. After incubation they were treated with a hypotonic solution (0.075M KCl) for 20 min and fixed in fresh fixative solution (methanol: acetic acid, 3:1). The cells were dropped onto slides and

the slides were air dried and stained with 10% Giemsa. Washed the slides in distilled water and observed under a research microscope through 100x objective. For karyotyping and detecting the structural anomalies, GTG banding technique was performed. To detect numerical and structural abnormalities 20-25 metaphases were analyzed and 5-6 metaphases were karyotyped.

Cytokinesis Block Micronuclei 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). Cells were harvested after 72th hr incubation, and they were treated with a hypotonic KCl solution (0.075M KCl) for 10 min and fixed in fresh fixative solution (methanol: acetic acid, 3:1). The cells were dropped onto slides and the slides were air dried and stained with 10% Giemsa. Micronucleated cells were analyzed under light microscopy at 100X magnification. The number of micronuclei is not less than 1000 binucleated cells were scored and the distribution of micronuclei among binucleated cells was recorded.

OBSERVATIONS AND RESULTS

Table 1: Distribution of mean CBMN frequency according to study and control subjects

Variables	Couples	Mean CBMN frequency of Husband	Mean CBMN frequency of Wife
Control subjects	22	10.24	10.38
Study subjects	44	12.28	11.91

In study subjects, mean CBMN frequency of husband and wife was 12.28 and 11.91. Mean CBMN frequency of control couples were showed 10.24 and 10.38 respectively. Study couples have highest mean CBMN frequency than control couples.

Table 2: Distribution of mean CBMN frequency according to demographic characteristics

Category		Variables	Number (Percentage)	Mean CBMN frequency
Age of husbands (Years)		<30	15 (34.09%)	11.82
		30-40	24 (54.54%)	12.43
		>40	5 (11.36%)	12.95
Age of wives (Years)		<30	33 (75%)	11.58
		30-40	10 (22.72%)	12.32
		>40	1(2.27%)	12.86
Duration of married life	Husband	<10	40 (90.90%)	12.25
		>10	4 (9.09%)	12.56
	Wife	<10	40 (90.90%)	11.85
		>10	4 (9.09%)	12.53

Demographic characters of the subjects were given. Age of the subjects were grouped into <30, 30 to 40 and >40 years. Highest mean CBMN frequency was showed by subjects with advanced age (both husband and wife). Increased married life of subjects showed increased mean CBMN frequency.

Table 3: Distribution of Mean CBMN frequency according to clinical characteristics

Category	Variable		Number (%)	Mean CBMN frequency
Number of pregnancies		≤3	35 (79.54%)	11.84
		>3	9 (20. 45%)	12.2
Number of abortions		≤3	35 (79.54%)	11.84
		>3	9 (20.45%)	12.2
H/o drug intake		Yes	25 (56.81%)	12.01
		No	19 (43.18%)	11.8
H/o UTI during pregnancy		Yes	20 (45.45%)	12.07
		No	24 (54.54%)	11.79
H/o any other infection during pregnancy		Yes	8 (18.18%)	12.35
		No	36 (81.81%)	11.82
Cytogenetic analysis	Husband	Normal	41 (93.18%)	12.21
		Abnormal	3 (6.81%)	13.16
	Wife	Normal	41 (93.18%)	11.84
		abnormal	3 (6.81%)	12.88

Clinical characteristics of subjects were showed in table 3. Subjects having increased number of pregnancy and increased number of abortions were showed highest mean CBMN frequency. Out of 44 couples, 41 couples have normal karyotype and 3 couples with abnormal karyotype. Couples with abnormal chromosome pattern were showed highest mean CBMN frequency.

Table: 4 Distribution of mean CBMN frequency according to type of infection

In table 4, 13 subjects were CMV positive and these subjects having highest mean CBMN frequency. 14 subjects showed positive result towards Toxoplasma infection. 13 subjects showed positive result with herpes simplex virus, 8 subjects with rubella positive. All the subjects with positive results towards infection showed highest mean CBMN frequency. 25 subjects have IgM antibody positive with mean CBMN frequency of 11.94. Highest mean CBMN frequency was showed in 22 subjects with IgG antibody positive.

Category	Variable	Number (%)	Mean CBMN frequency
CMV infection	Positive	13 (29.54%)	12.12
	Negative	31 (70.45%)	11.83
Toxoplasma infection	Positive	14 (31.81%)	12.05
	Negative	30 (68.18%)	11.85
HSV infection	Positive	13 (29.54%)	12.14
	Negative	31 (70.45%)	11.82
Rubella infection	Positive	8 (18.81)	12.17
	Negative	36 (81.81)	11.86
IgM antibody	Positive	25 (56.81)	11.94
	Negative	19 (43.18)	11.88
IgG antibody	Positive	22 (50%)	11.99
	Negative	22 (50%)	11.84

DISCUSSION

Primary infection caused by TORCH (Toxoplasma Gondi, Rubella virus, Cytomegalo virus, and Herpes simplex virus) and other infectious agents can cause DNA damage which leads to RPL (Al-Hilli et al., 2014). The present study also showed mean CBMN frequency highest in subjects with TORCH positive infection.

Maternal age and number of previous miscarriages are two independent risk factors for a further miscarriage. Advancing maternal age is associated with a decline in both the number and quality of the remaining oocytes. Advanced paternal age has also been identified as a risk factor for miscarriage. Previous reproductive history is an independent predictor of future pregnancy outcome. The risk of a further miscarriage increases after each successive pregnancy loss, reaching approximately 40% after three consecutive pregnancy losses, and the prognosis worsens with increasing maternal age (Nybo et al., 2000). Here advanced age of couples was showed highest mean CBMN frequency. Those women with increased number of abortions were also showed highest mean CBMN frequency.

Trkov et al., (2000) studied the micronucleus frequency in 31 couples (62 patients) with a history of two or more spontaneous abortions of unexplained etiology and 19 couples (38 individuals) with a history of infertility. The micronucleated cell rates were found to be

increased in lymphocytes of patients with infertility and two or more spontaneous abortions (P value >0.0001) in comparison with the rate of micronuclei frequency of 15 fertile couples who were included in the study as controls, suggesting a possible role of chromosomal instability in reproductive failure.

CONCLUSION

Based on the findings of the study, it is concluded that previous history of pregnancy wastage and the immunological reactions for TORCH infections during current pregnancy must be considered while managing RPL cases to reduce the adverse foetal outcome. If an infection occurs, it needs to be treated aggressively to prevent transmission to the foetus for further complication. Hence, all cases with RPL should be routinely screened for TORCH and other infection as early diagnosis and appropriate intervention will help in proper management of these cases.

In the case of chromosomal abnormality, clinicians should underst nd the importance of chromosomal analysis in these couples and refer them for karyotyping after two miscarriages to rule out the possible genetic cause of RPL, which can help further in proper prognostic assessment and genetic counseling of the concerned couple. Need more researches to indentify the etiology and risk factors to be easily to make standard guidelines for early diagnosis and treatment of this disorder.

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