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BIOCHEMICAL AND MOLECULAR INSTABILITIES IN PREMATURE OVARIAN FAILURE

Medical Science

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ABSTRACT

Premature ovarian failure (POF) is a common cause of infertility in women, characterized by amenorrhea, hypoestrogenism, and elevated gonadotropin levels in women under the age of 40. POF is diagnosed on the basis of two levels of serum FSH ≥40mIU/mL taken 6 weeks apart. It affects one in 10,000 women by age 20, one in 1,000 women by age 30 and one in 100 women by age 40. Thepresent study was undertaken to evaluate biochemical and molecular instabilities on subjects with premature ovarian failure. Thirty five female subjects with premature ovarian failure and 16 healthy women without any chronic illness were involved in this study. Reproductive hormones namely leutinizing hormone (LH), follicle stimulating hormone (FSH) and estradiol were estimated in each subjects after obtaining their informed consent. Cytokinesis-block micronuclei (CBMN) assay was also carried out in the lymphocytes of the subjects to assess the somatic DNA damage. The study demonstrated that the micronuclei frequency significantly elevated in the study subjects than control subjects. Women with various risk factors such as increasing age, BMI, family history of infertility, family history of cancer, delayed menarche, endometriosis, etc. can lead to increased genetic instabilities.

KEYWORDS:

Premature ovarian failure, genetic instabilities, DNA damage and Cytokinesis-block micronuclei Assay

Introduction

The end of a woman's reproductive lifespan is marked by the occurrence of menopause, defined as being the last menstruation that occurs for a woman, but is caused by the exhaustion of the ovarian reserve1. In general female population, across many ethnicities and over recent human history, the average age of natural menopause has remained at 50–52 years. Premature ovarian failure (POF) is a common cause of infertility in women, characterized by amenorrhea, hypoestrogenism, and elevated gonadotropin levels in women under the age of 402. POF is diagnosed on the basis of two levels of serum FSH ≥40mIU/mL taken 6 weeks apart. It affects one in 10,000 women by age 20, one in 1,000 women by age 30 and one in 100 women by age 403. Ovarian failure encompasses disorders in which the ovary is deficient in germ cells and those in which the germ cells are resistant to follicle stimulating hormone (FSH)4. Ovarian failure manifests itself between 30-39years in 82.5% cases5. POF accounts for 5-10% cases of secondary amenorrhea6, 7 and 10-28% cases of primary amenorrhea4. Most of the cases of POF are idiopathic, accounting for 60-80% of total cases3, 8, 9. Wide varieties of genes are implicated in POF. These include a mutation in the FMR1,FMR2,BMP15, FOXL2, FSHR,LH receptor, FSH beta variant, LH beta, Inhibin A, GALT, AIRE, EIF2B2, -4, and -5, NOGGIN and POLG. Apart from this some candidate genes also seems to be responsible for causing POF. Other causes of POF include autoimmune, iatrogenic and those resulting from environmental insults10.

Various genetic mechanisms implicated in the pathogenesis of POF include reduced gene dosage and non specific chromosome effects that impair meiosis. These can lead to ovarian failure by causing a decrease in the pool of primordial follicles, increased atresia of the ovarian follicles due to apoptosis or failure of follicle maturation. Management of POF needs to address the two major medical issues such as hormone replacement therapy (HRT) and infertility. Women also require personal and emotional support to deal with impact of diagnosis on their health and relationships. In addition, associated pathology needs to be assessed and managed so that long-term follow-up is essential to monitor HRT and for healthsurveillance11. Assisted reproductive technology used as a best fertility treatment for women with POF. Cryopreserved embryos have also been used to achieve pregnancy in women affected with POF. Since POF is very common disorder striking a large number of women, understanding the molecular and biochemical aspects of POF will help in the accurate management of the disease, hence the present study was undertaken to evaluate biochemical and molecular instabilities in subjects with premature ovarian failure.

MATERIALS AND METHODS

35 subjects with premature ovarian failure were selected for the study. 16 age matched subjects were selected as control for the study. The samples were referred from various gynecology departments and infertility centers of kerala to Genetika, Centre for Advanced Genetic Studies, Thiruvanathapuram, Kerala. Demographic, physiologic and lifestyle characteristics were recorded using proforma. In this study, Cytokinesis-block Micronuclei (CBMN) assay was carried out in each subject. CBMN assay was performed by using Cytochalasin B for quantitating the extent of somatic DNA damages.

Five ml of blood sample was collected aseptically by venipuncture and transferred 2 ml of blood into a heparinized vacuum container and the remaining blood was transferred to a plain tube and allowed to clot, serum was separated immediately. Follicle stimulating hormone (FSH), Luteinizing hormone (LH) and estradiol were measured by the chemiluminescence immunoassay (CLIA).

5-6 drops of heparinized blood was added to a culture tube containing 10 ml of RPMI 1640 media supplemented with 15% of fetal bovine serum and 10µg/mL phytohaemagglutinin. Cytochalasin B was added to the cultures at a final concentration of 4.5µg/mL after 44th hours of initiation of cells with phytohaemagglutinin. Cells were harvested after 72 hr incubation, and they were treated with a hypotonic solution (0.075M KCl) for 1 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 a microscope 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 among the study and control subjects

Category	Number	Mean CBMN frequency
Control subjects	16	9.8
Study subjects	35	12.08

Distribution of mean CBMN frequency among the study subjects and control subjects were given in table 1. The mean CBMN frequency of the control subjects was 9.8 and study subjects was 12.08. The present study frankly demonstrated that the mean CBMN frequency among the study subjects was greater than the control subjects.

Table 2: Distribution of mean CBMN frequency according to various demographic and anthropometric characteristics of the study subjects

Category	Variable	Number	Percentage (%)	Mean CBMN Frequency
Age (Years)	<25	4	11.42	11.65
	25-35	20	57.14	12.05
	>35	11	31.42	12.29
Birth order	<6	29	82.85	12.04
	≥6	6	17.14	12.26
Residence	Coastal	15	42.85	11.91
	Rural	6	17.14	12
	Urban	14	40	12.29
Religion	Christian	10	28.57	11.86
	Hindu	14	40	12.12
	Muslim	11	31.42	12.22
Occupation	Non sedentary	17	48.57	11.78
	Sedentary	18	51.43	12.36
Socio economic status	High	24	68.57	11.97
	Average	8	22.86	12.29
	Poor	3	8.57	12.44
Age at menarche (Years)	<15	31	88.57	12.03
	≥15	4	11.43	12.45
Last menstruation occurred	<1 month	14	40	11.96
	>1 month	21	60	12.15
Menstrual period	Regular	19	54.29	11.88
	Irregular	16	45.71	12.32
Height (m)	≤1.5 m	27	77.14	12
	>1.5 m	8	22.86	12.33
Weight (Kg)	<60	8	22.86	11.89
	60-70	13	37.14	12.19
	>70	14	40	12.24
BMI (Kg/m2)	<25	5	14.28	11.97
	25-30	7	20	12.13
	>30	23	65.71	12.48
Abdominal circumference (cm)	<85	3	8.57	11.90
	85-100	12	34.29	12.20
	100-140	20	57.14	12.34
Parental consanguinity	Yes	7	20	12.26
	No	28	80	11.65
Obesity	Yes	24	68.57	12.28
	No	11	31.43	11.99

Distribution of mean CBMN frequency according to various demographic and anthropometric characteristics of the study subjects is given in the table 2. Age of the study subjects were grouped into <25, 25 to 35 and >35 years. Subjects with >35 years showed a mean CBMN frequency of 12.29. As the age increases the mean CBMN frequency also increases. Subjects with birth order ≥6 had a high mean CBMN frequency of 12.26. 18 subjects who had sedentary type of occupation and showed higher mean CBMN frequency (12.36) than subjects with non-sedentary type of occupation. Subjects with age at menarche ≥15 years had a high mean CBMN frequency of 12.45 compared to other subjects whose age of menarche were <15 years.Subjects those who had menstruation during last time was grouped into <1 month and ≥1 month with mean CBMN frequency of 11.96 and 12.15. Sixteen study subjects had irregular menstrual periods with high mean CBMN frequency (12.32). Out of 35 study subjects, 14 subjects with >70 Kgof weight showed high mean CBMN frequency (12.24) and 23 subjects with >30 Kg/m2 of BMI showed highestmean CBMN frequency (12.48). 20 of the study subjects had abdominal circumference in between 100-140 cm and showed high mean CBMN frequency (12.34). Parental consanguinity was observed in 7 of the study subjects and they showed a higher mean CBMN frequency (12.26). Out of the 35 study subjects, 24 were obese and had a higher mean CBMN frequency of 12.28.

Table 3: Distribution of mean CBMN frequency according to the lifestyle characteristics of the study subjects

Category	Variable	Number	Percentage (%)	CBMN Frequency
Diet	Non-Vegetarian	26	74.29	12.19
	Vegetarian	9	25.71	11.75
Smoking	Yes	8	22.86	12.46
	No	27	77.14	11.97
Alcohol	Yes	8	22.86	12.55
	No	27	77.14	11.94
Daily water intake	Average	11	31.42	12.07
	Good	2	5.71	12.06
	Low	22	62.85	12.13
Regular exercise	Yes	13	37.14	11.79
	No	22	62.85	12.25
Physical activity	Average	26	74.29	12.03
	Good	5	14.28	12.15
	Low	4	11.43	12.29
Frequency of fruit consumption	Average	17	48.57	11.93
	Good	7	20	12.01
	Low	11	31.43	12.49

Distribution of mean CBMN frequency according to lifestyle characteristics of the study subjects is given in the table 3. Out of the 35 study subjects, 26 study subjects were non-vegetarians and they showed high mean CBMN frequency of 12.19. The study subjects with the habit of smoking showed higher mean CBMN frequency (12.46) than non smokers. Alcoholism was observed in 8 of the study subjects and they had high mean CBMN frequency (12.55). Subjects with low water intake showedmean CBMN frequency (12.13). Subjects without doing regular exercise showed high mean CBMN frequency of 12.25 compared to those who doing regular exercise.Majority of the study subjects belonged to average physical activity (26%) and subjects with poor physical activity had higher mean CBMN frequency (12.29). Subjects with low fruit consumption had high mean CBMN frequency (12.49).

Table 4: Distribution of mean CBMN frequency according to various clinical characteristics of the study subjects

Category	Variable	Number	Percentage (%)	Mean CBMN Frequency
H/o Diabetes	Yes	16	45.71	12.18
	No	19	54.29	12.04
H/o Hypertension	Yes	18	51.43	12.11
	No	17	48.57	12.09
H/o dyslipidemia	Yes	11	31.43	12.10
	No	24	68.57	12.09
H/oChronic illness	Yes	6	17.14	12.10
	No	29	82.85	11.97
H/o Thyroid	Yes	5	14.28	12.13
	No	30	85.71	11.94
Family h/o CAD	Yes	17	48.57	12.24

	No	18	51.43	11.98
Family h/o Cancer	Yes	1	2.86	12.18
	No	34	97.14	12.09
Mental stress	Yes	6	17.14	12.10
	No	29	82.85	11.95

Distribution of mean CBMN frequency according to clinical characteristics of the study subjects is given in the table 4. Among 35 study subjects those with H/o diabetes showed high mean CBMN frequency (12.18). 18 of the study subjects had H/o hypertension with high mean CBMN frequency (12.11). H/o dyslipidemia was reported in 11 study subjects with mean CBMN frequency of 12.10. 6 subjects had H/o chronic illness with mean CBMN frequency of 12.01. The mean CBMN frequency of 17 subjects with Family h/oCAD was higher (12.24) than subjects without the Family h/oCAD. Only one subject showed Family h/ocancer withmean CBMN frequency of 12.18.6 subjects showed mental stress and their mean CBMN frequency was 12.10.

Table 5: Distribution of mean CBMN frequency according to various Biochemical characteristics of the study subjects

Category	Variable	Total	Percentage (%)	CBMN Frequency
FBS (mg/dl)	<100	18	51.43	12.01
	100-200	15	42.85	12.17
	>200	2	5.71	12.18
Total Cholesterol (mg/dl)	<200	23	65.71	12.05
	200-240	10	28.57	12.08
	>240	2	5.71	12.23
HDL (mg/dl)	<40	15	42.85	12.19
	40-60	20	57.14	12.03
LDL (mg/dl)	<100	1	2.86	12.05
	100-160	26	74.29	12.07
	>160	8	22.86	12.08
TG (mg/dl)	<150	25	71.43	12.01
	150-300	10	28.57	12.14
Fibrinogen (mg/dl)	<150	3	8.57	11.97
	150-400	20	57.14	12.02
	>400	12	34.29	12.17
FSH (mlU/ml)	<25	2	5.71	11.90
	25-30	3	8.57	12.11
	>30	30	85.71	12.33
LH (mlU/ml)	<25	14	40	11.90
	45-60	15	42.85	11.98
	>60	6	17.14	12.34
Estradiol (pg/ml)	<25	14	40	12.27
	25-75	21	60	11.99

Distribution of mean CBMN frequency according to biochemical characteristics of the study subjects is given in table 5. Subjects with fasting blood sugar value >200 mg/dl showed high mean CBMN frequency (12.18). 2 subjects with total cholesterol >240 mg/dl showed a high mean CBMN frequency of 12.23. The mean CBMN frequency was studied according to HDL, LDL and TG levels. Subjects with HDL level <40mg/dl showed high mean CBMN frequency of 12.19. Subjects with LDL level >160 mg/dl had high mean CBMN frequency of 12.08. 10 subjects were having TG between 150 to 300mg/dl and they showed highmean CBMN frequency of 12.14. Mean CBMN frequency was high in subjects having fibrinogen level >400 mg/dl (12.17). Subjects showing FSH level >30 mlU/ml had higher mean CBMN frequency of 12.33. 6 subjects had LH level >60 mlU/ml and also had higher mean CBMN frequency (12.34). 14 subjects showed estradiol value between <25pg/mland they have highmean CBMN frequency of 12.27.

Discussion

A diagnosis of premature ovarian failure has a great impact on the health of affected women. Both primary and secondary forms of ovarian failure are biochemically characterized by low levels of gonadal hormones (estrogens and inhibins) and high gonadotropins (LH and FSH) (hypergonadotropic amenorrhea). The elevation of FSH is usually more marked than that of LH and FSH value >30 mlU/mlis indicative of ovarian failure3. In the present study the FSH level of study subjects >30mlU/ml showed high mean CBMN frequency (12.33) and elevation of LH level also showed high mean CBMN frequency (12.34). In the present study, subjects with elevated estradiol showed high mean CBMN frequency of 12.27.

Around and after menopause, women experience unfavourable changes in plasma lipids and lipoproteins. HDL cholesterol levels were borderline or significantly lower in women with POF12. The current study showed elevated level of TC, LDL and TG with high mean CBMN frequency. Subjects with low HDL have high mean CBMN frequency of 12.19. Total cholesterol level >240 mg/dl had the highest mean CBMN frequency (12.23) and LDL level >160 mg/dl also had the highest mean CBMN frequency of 12.08. Current study recorded high TG level among the affected women with a mean CBMN frequency of 12.14. The mean CBMN frequency was high in subjects with fasting blood sugar level >200 mg/dl. Subjects with fibrinogen level >400 mg/dl showed highest mean CBMN frequency of 12.17.

Irregularity in menstrual cycle is the most common symptom of POF. Behavioral changes, such as quite smoking should be accomplished primarily in order to reduce the risk of premature ovarian failure13. Irregularity in menstrual cycle was showed by 16of the study subjects and had high mean CBMN frequency (12.32). In the study subjects, the mean CBMN frequency of those who had the habit of smoking was higher (12.46) than non smokers. Females with age of menarche ≥15 years were more risky to develop infertility than those with age of menarche less than 15 years. The mean CBMN frequency of the study subjects having age at menarche ≥15 years was high (12.45).

Age, smoking, age of maternal menopause, parity, social class, meat and alcohol consumption were all independently associated with an early natural menopause. In the current study, subjects those who had menstruation during last time was ≥1 month showed high mean CBMN frequency of 12.15. Those who had poor socio-economic status had highestmean CBMN frequency (12.44) than others. Non vegetarians among the study subjects also showed high mean CBMN frequency (12.19).

Hypothyroidism is the most common associated autoimmune disorder with POF (25-60%) and coincidence with diabetes mellitus is 2.5%14] In the current study subjects suffered from both thyroid and diabetes showed high mean CBMN frequency. Those with H/o thyroid disorder had a high mean CBMN frequency of 12.13 and those with H/o diabetes showed mean CBMN frequency of 12.18.

Women who experience premature menopause (before age 40 years) or early menopause (between ages 40 and 45 years) experience an increased risk of overall mortality, cardiovascular diseases, neurological diseases, psychiatric diseases, osteoporosis, and other sequelae15.

CONCLUSION

The present study involves biochemical and molecular instabilities in premature ovarian failure. The distribution of mean CBMN frequency according to demographic, clinical and biochemical characteristics of the study subjects was observed. Age at menarche, menstrual cycle, H/o CAD, H/o cancer and H/o thyroid disorder had a major role in causing premature ovarian failure. FBS, total cholesterol, FSH, LH, estradiol were also found to be significantly elevated in these subjects. Early diagnosis and management of POF is crucial.In some cases, early diagnosis by genetic investigation may lead to advice for early conception or oocyte harvesting and preservation. Hormone defect may be substituted by estrogen/progestin preparations. The only solution presently available for the fertility defect in women with absent follicular reserve is represented by ovum donation.

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