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### RESEARCH ARTICLE

#### CYTOGENETICS AND MOLECULAR EVIDENCE OF ANOVULATORY INFERTILITY.

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#### Abstract

Anovulation is probably the major cause of human infertility and it affects between 6% and 15% of all women of childbearing age. One of the cardinal signs of anovulation is irregular or absent menstrual periods. The aim of the present study was to investigate the cytogenetics and molecular evidence of anovulatory infertility. The present study was carried out in 52 subjects suffering from anovulatory infertility and 18 age matched subjects as control. The cytogenetic and molecular analysis of study subjects were correlated with various demographic, clinical and lifestyle aspects. Lymphocyte culture and cytokinesis-block micronuclei (CBMN) assay was also carried out in each subject. The study demonstrated that the micronuclei frequency significantly elevated in the study subjects than control subjects. Anovulatory women with various risk factors such as advancing age, duration of married life, occupation, BMI, age at menarche, family history of PCOS and family history of infertility etc. can lead to increased genetic instabilities. Subjects having abnormal level in reproductive hormones were also showed abnormal chromosome pattern. Subjects with increased genetic instabilities and abnormal karyotype may leads to severity of infertility. Change in lifestyle such as food habit, exercise, weight management, reducing psychological stress etc. is the best treatment options for reproductive health.

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#### Introduction:-

Infertility is a worldwide health problem, with one in six couples suffering from this condition (Arkierupaia, 2015) and it affects 15% of couples that have unprotected sexual intercourse (Sharlip et al., 2002). Anovulation is the major factor causing infertility in women, is defined as the failure of the ovaries to produce, mature and release ova over a period of time generally exceeding 3 months. It affects between 6% and 15% of all women of childbearing age. One of the cardinal signs of anovulation is irregular or absent menstrual periods (Legro, 2003).

Chronic anovulation is classified by World Health Organization (WHO) criteria, originally determined by Insler et al., (1968) and Rowe et al., (1997). In Group 1 anovulation, the levels of LH and FSH are below the range necessary to stimulate follicle development due to hypothalamic-pituitary causes such as tumor, inflammation or any other destruction (ESHRE Capri Workshop Group, 2006). In Group 2 anovulation is always associated with

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Polycystic Ovarian Syndrome (PCOS) (Broekmans et al., 2006). Type 3 anovulation occurs due to premature ovarian failure (Van, Hop and Faucer, 1997).

Anovulation is the prime factor in infertility (Laven et al., 2002). Disorders of anovulation account for about 30% of infertility and often present with irregular periods (oligomenorrhoea) or an absence of periods (amenorrhoea) (Hamilton and Taylor, 2006). Premature ovarian insufficiency, which is characterized by cessation of menstruation before 40 years (Santoro, 2003; Timmreck and Reindollar, 2003) and genetic abnormalities like Turner's syndrome (45, X), in which underdeveloped (streak) ovaries result in primary ovarian failure (premature menopause) (Diana and Alison, 2003) plays important role in anovulation. Along with the prevalence of overweight women, there is an increase in women with anovulatory infertility (Christiane et al., 2016). Women who are underweight as a result of illness, anorexia nervosa, or over exercise also become amenorrhoeic (Adam and Anthony, 2007). Thyroid disease is a common cause of menstrual cycle irregularity (Koutras, 1997). Oligomenorrhoea and amenorrhoea occur in 58% of patients with hyperthyroidism (Koutras, 1997).

Anovulation can be diagnosed by measuring the amount of LH and FSH. Investigation of prolactin and thyroid stimulating hormone concentration are also diagnostic measures. A transvaginal ultrasound scan of the pelvis and BMI measurement will confirm polycystic ovarian syndrome and karyotyping is the method for identifying genetic abnormalities.

Infertility is the major reproductive problem all over the world. Based on the survey performed in developed countries, World Health Organization (WHO) estimates that female infertility accounts for 37% causes in infertile couples, male infertility for 8% and both male and female infertility for 35%. The most common cause of female infertility is ovulatory disorders. 20-25% of female infertility is due to anovulation. Hence the present study was undertaken to evaluate the cytogenetics and molecular evidence of anovulatory infertility.

### **Material and Methods:-**

52 subjects suffering from anovulatory infertility and 18 ages matched healthy control subjects were also selected for the study. The samples were referred from various maternity centers of Kerala to Genetika, Centre for Advanced Genetic Studies, Thiruvananthapuram, Kerala. Demographic, physiologic and lifestyle characteristics of subjects were recorded using proforma.

Eight ml of venous blood was collected aseptically from all the subjects by venipuncture. 4ml was transferred into the sodium heparin vacutainer to perform lymphocyte culture and CBMN assay. The remaining 4ml was transferred into plain tube and allowed to clot. With the serum, sugar and lipid profile were estimated by enzymatic method using semianalyser. Lutinizing hormone (LH), Follicle stimulating hormone (FSH), Prolactin and Estradiol were measured by the Chemi Luminescent Immuno Assay (CLIA) using Beckman Access 2 fully automated hormone analyzer. Quality control was performed by participating in the Bio-rad EQAS.

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 10 $\mu$ g/mL phytohaemagglutinin. Cytochalasin B was added to the cultures at a final concentration of 4.5 $\mu$ g/mL after 44th hour. Cells were harvested after 72 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 air dried then 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 recorded.

### **Observations and Results:-**

In the present study, 52 females subjects between the age of 19 to 35 years and their mean age was 26.44 years. Control subjects between the age range of 17 to 35 years with average age of 27.4 years. Birth order of subjects ranged from 1 to 7. Most of the females from rural area and rest were from urban and coastal area. 52 study subjects were showed mean CBMN frequency of 12.29 and 4 of them were showed abnormal karyotype. 18 control subjects were showed mean CBMN frequency of 9.85. Study subjects were showed highest mean CBMN frequency than control subjects.

**Table 1:-** Distribution of Mean CBMN frequency according to demographic characters of subjects

Category	Variables	Number	Percentage (%)	Karyotype		Mean CBMN Frequency
				Normal	Abnormal	
Age (Years)	≤25	22	42.31	22	0	11.69
	26-30	21	40.38	20	1	12.45
	> 30	9	17.31	6	3	13.36
Birth Order	<4	43	82.69	39	4	12.28
	≥4	9	17.31	9	0	12.31
Residence	Coastal	4	7.69	4	0	12.14
	Rural	30	57.69	28	2	12.19
	Urban	18	34.62	16	2	12.48
Education	Primary	8	15.38	8	0	12.43
	Secondary	6	11.54	5	1	12.35
	Higher secondary	23	44.23	21	2	12.19
	Graduation /PG	15	28.85	14	1	12.34
Occupation	Non-sedentary	30	57.69	27	3	12.26
	Sedentary	22	42.31	21	1	12.33
Duration of Married life (Years)	≤5	44	84.62	41	3	12.16
	>5	8	15.38	7	1	12.98
Economic status	Low	6	11.54	6	0	11.99
	Medium	35	67.31	31	4	12.28
	High	11	21.15	11	0	12.49
Parental Consanguinity	Yes	9	17.31	7	2	12.54
	No	43	82.69	41	2	12.24
BMI (kg/m <sup>2</sup> )	<25	21	40.38	21	0	11.75
	25 to 30	23	44.23	21	2	12.49
	> 30	8	15.38	6	2	13.1

Demographic characteristics of subjects were given in table 1. Age of the subjects were grouped into ≤25, 26 to 30 and >30 years. Majority of subjects were in the age range of ≤25 years. Advancing age of the subjects was showed highest mean CBMN frequency of 13.36 and 3 of them having abnormal karyotype. Subjects having higher birth order (≥4) showed a mean CBMN frequency of 12.31. Rural and urban residing subjects were showed abnormal karyotype. 22 subjects have sedentary type of occupation. Highest mean CBMN frequency was shown by subjects having sedentary type of occupation and one of them were showed abnormal karyotype. Subjects having increased duration of married life were showed highest mean CBMN frequency. Most of the subjects with medium socio economic status with mean CBMN frequency of 12.28. BMI of the subjects were grouped into normal, overweight and obesity. 23 subjects were overweight with mean CBMN frequency of 12.49. Obese subjects have highest mean CBMN frequency (13.1) and 2 of these obese subjects were showed abnormal karyotype. 9 subjects having parental consanguineous marriage and 2 of them showed abnormal karyotype.

**Table 2:-** Distribution of Mean CBMN frequency according to demographic characters of subjects

Category	Variables	Number	Percentage (%)	Karyotype		Mean CBMN Frequency
				Normal	Abnormal	
Family history of PCOS	Yes	49	94.23	45	4	12.29
	No	3	5.77	3	0	12.13
Family h/o of infertility/ subfertility	Yes	11	21.15	11	0	12.4
	No	41	78.85	37	4	12.26
Age at Menarche (Years)	≤14	21	40.38	20	1	12.13
	>14	31	59.62	29	3	12.39
Endometriosis	Yes	4	7.69	3	1	12.81
	No	48	92.31	45	3	12.24

About the clinical characteristics of subjects, 49 subjects have family history of PCOS and 4 of these subjects were showed abnormal karyotype. Subjects with family history of infertility/subfertility were showed highest mean CBMN frequency. 31 subjects achieved their menarche after the age of 14 years and they showed highest mean CBMN frequency (12.39). 4 of the subjects having endometriosis with highest mean CBMN frequency and one of them were showed abnormal karyotype.

Subjects were distributed based on their biochemical and hormonal characteristics. Biochemical characteristics such as fasting blood sugar, total cholesterol, high density lipoprotein, low density lipoprotein and triglyceride. Abnormal levels of biochemical characters of subjects were showed highest mean CBMN frequency. High FBS level possessed a risk of abnormal karyotype. Subjects with total cholesterol above the normal range had a high mean CBMN frequency of 12.48 where as it is 12.14 for female with normal total cholesterol. Abnormality in karyotype also occurred in females with high level of total cholesterol. Increase in mean CBMN frequency was shown by subjects with low HDL level, 4 females who suffered from abnormal level of HDL was reported as abnormal karyotype. There was an increase in mean CBMN frequency when the level of LDL exceeds the normal range. 12.43 was the mean CBMN frequency of subjects with LDL level of  $\geq 130$  mg/dl and some of them possessed abnormal karyotype. Subjects with  $\leq 150$  mg/dl of TG had a mean CBMN frequency of 12.25. In hormonal characteristics of subjects, increased FSH, LH, prolactin and estradiol level were showed highest mean CBMN frequency with abnormal karyotypes. Based on lifestyle characteristics, majority of the subjects were non vegetarians and having highest mean CBMN frequency. 14 subjects were used contraceptive drugs and 2 of them were showed abnormal karyotype.

### **Discussion:-**

A study of Moran et al., (2008) illustrated that DNA of anovulatory infertile subjects showed significant damage by increased mean CBMN frequency in lymphocytes. Report suggests the presence of genetic abnormality in PCOS subjects (Nersesyian et al., 2006). The current study also showed that significant DNA damages had been occurred in study subjects. According to Romero et al., (2008) advanced age was a significant risk factor associated with women's infertility. In the current study it was observed that there was an increasing mean CBMN frequency along with increasing age had been reported in the current study.

From the study of Arun William (2016), it was clear that birth order also had a relation with micronuclei frequency. In the current study, subjects with birth order ranged from 1-7, a highest mean CBMN frequency (12.31) could be observed in subjects with  $\geq 4$  birth order. According to Ajeet, (2014) demographic characteristic of the couples is one of the factors affecting fertility. The majority of cases with primary infertility were from urban area. The present study also reported a highest mean CBMN frequency of 12.48 in subjects from urban area. So from this study it was suggested that urbanization and change in lifestyle could mediate the progress DNA damage.

From the current study it was clear that sedentary occupation had an indirect role on micronuclei frequency. Subjects from the sedentary occupation background possessed a mean CBMN frequency of 12.33. An increasing micronuclei frequency with sedentary life had also been observed in the study of Arun (2016). In this study, a highest mean CBMN frequency of 13.28 was observed in subjects who attained menarche between 16-18 years. Mokhtar et al., (2006) revealed that females with age of menarche more than 15 years were more risky to develop infertility than those with age of menarche less than 15 years. According to Ajeet, (2014) menstrual irregularities in the form of any deviation from normality like, oligomenorrhea, hypo or hypermenorrhea were also significant risk factors for primary infertility. Present study clarified that irregular menstruation had resulted in increasing micronuclei frequency.

Biochemical and hormonal investigations were also showed a positive correlation between CBMN frequency. Current observations were consistent with the findings of Minsa, (2016) who found increased micronuclei frequency for abnormal level of biochemical and hormonal investigations. The cytokinesis-block micronuclei assay revealed increased micronucleus frequency in couples with infertility or two or more spontaneous abortions, suggesting a possible role of chromosomal instability in reproductive failure (Trkova et al., 2000). The current study also observed a higher micronuclei frequency among study subjects than the control subjects. More karyotype abnormalities were also found in study subjects than control subjects.

**Conclusion:-**

The present study showed an increased mean CBMN frequency in relation with various demographic, clinical, biochemical, endocrinological and lifestyle characteristics. The study demonstrated a positive correlation with anovulatory infertility and the extent of somatic DNA damages and abnormal karyotype. Medical treatments aim to manage and reduce the symptoms or consequences of anovulation. Medication alone has not been shown to be any better than healthy lifestyle changes.

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