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Androgen Excess and Genetic Instabilities in Anovulatory Infertility

KEYWORDS: Anovulation, Androgen Excess, Genetic Instability and Cytokinesis Block Micronuclei Assay

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ABSTRACT *Anovulation is the failure of the ovaries to release ova over a period of time generally exceeding three months. Anovulation is one of the major causes of infertility. Among the causes of infertility, 20-25% accounts for anovulation. The present study was undertaken to evaluate the androgen excess and genetic instabilities in anovulatory infertility by investigating the various anthropometric and clinical aspects of the subjects. Twenty four subjects with anovulatory infertility and 19 healthy women without any chronic illness were involved in this study. Reproductive hormones namely leutinizing hormone (LH) and follicle stimulating hormone (FSH) 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. Anovulatory women with various risk factors such as history of chronic illness, family history of cancer, increased duration of married life, endometriosis, hypercholesterolemia etc. can lead to increased genetic instabilities and the severity of infertility. Lifestyle management should be used as the primary therapy for the treatment of metabolic complications and improvement in ovulatory function and pregnancy.*

INTRODUCTION

The normal function of ovary to releases one ovum every 25–28 days. This average time between ovulation events is variable, especially during puberty and the perimenopause period (StreeRoga, 2010). Anovulation is the failure of the ovaries to produce, mature and release ova. This condition may result from ovarian immaturity or post maturity, altered ovarian functions, primary ovarian dysfunction or disturbed interaction of the hypothalamus, pituitary gland and ovary, caused by stress or distress (Mosby's Medical Dictionary, 2009).

Anovulation or irregular menstrual cycle is the hallmark of polycystic ovary syndrome (PCOS) and remain a central part of the consensus diagnosis. PCOS is probably the most common cause of anovulatory infertility (Rotterdam, 2004; Jakubowski, 2005). PCOS affects 5–10% of women of reproductive age (Mastorakos, 2006). There is high prevalence of PCOS in women with anovulatory infertility (83%) (Kousta, 1999). Androgen excess is one of the most common endocrine disorders of reproductive aged women, affecting approximately 7% of population (Asuncion et al., 2000). Anovulation among regularly menstruating women may be associated with androgen levels and endocrine changes similar to those seen in women with PCOS (Sunni, 2013). The prevalence of PCOS is excess (5%) with the combination of anovulation and hyperandrogenism (ESHRE, 2012). Such ovulatory dysfunction also frequently represents foetal or perinatal exposure of females to androgen excess in many mammalian species.

Anovulatory Androgen Excess (AAE) is a condition in women that usually develops in adolescence and is diagnosed in about five of every 100 women of any race and any country of origin. It is diagnosed by a combination of abnormal cycles (amenorrhea, oligomenorrhea or irregular cycles) and evidences that male like hormones called androgens are either too high or too active causing hirsutism, acne and androgenetic alopecia (Azziz et al., 2009).

Anovulation is one of the major causes of infertility (Padma, 2013). Among the causes of infertility, 20-25% accounts for anovulation. One of the most common identifiable factors that accounted for female infertility was ovulatory disorders (25%). Other reports describe ovulatory disorders are responsible for more than half of the causes of female infertility (Unuane et al., 2011).

Female infertility is also associated with genomic instability. There is a high incidence of genomic instability in lymphocytes of women with PCOS (Milosevic, 2012). Moreover, couples with a history of spontaneous abortions and idiopathic infertility tend to have an increased micronuclei (MN) frequency in lymphocytes (Trkova et al., 2000).

Antioxidants (including vitamins C and E) and antioxidant cofactors (such as selenium, zinc, and copper) are capable of disposing, scavenging or suppressing the formation of reactive oxygen species (ROS) (Ruder, 2009). ROS is also believed to play a role in the different phases of the endometrial cycle. Late luteal phase is characterized by elevated levels of lipid peroxide and a decrease in the antioxidant, superoxide dismutase. ROS stimulates the secretion of Prostaglandin 2F-α *(PG2Fα)* through activation of nuclear factor-kappaα (NFkα). Disruption in physiological levels of ROS leads to female reproductive dysfunction and in

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some cases, to unexplained infertility. Oxidative stress (OS) in female reproduction has been associated with PCOS and endometriosis. These pathologies negatively affect pregnancy rates and in vitro fertilization (IVF) outcomes (Gupta, 2014).

Management of anovulatory infertility is still a difficult medical task because of the difficulty in the diagnosis and treatment. Anovulation can sometimes be treated with medical or surgical induction, but it is the cause of anovulation that will determine whether ovulation induction is possible (Hamilton et al., 2006). There is now a greater focus on the management of the metabolic consequences of anovulatory infertility, primarily through lifestyle intervention to achieve weight loss and increase physical activity. No systematic studies were reported regarding the genetic instability, especially the DNA damage in anovulatory infertility. Hence, the present study was undertaken to evaluate androgen excess and genetic instabilities in anovulaory infertility.

MATERIALS AND METHODS

Twenty four subjects with anovuatory infertility were selected for this study. The samples were referred from various gynecology departments and infertility centers of Kerala to Genetika, Centre for Advanced Genetic studies, Thiruvananthapuram, Kerala. Nineteen healthy subjects without any chronic illness were also selected as control for this study. Detailed anthropometric and clinical characteristics were recorded using proforma. Both the study and control subjects reported normal Karyotype. In this study Cytokinesis Block Micronuclei (CBMN) Assay was also 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 by venipuncture and transferred two ml of blood into sodium heparinized vacuutainers for quantifying the extent of somatic DNA damages by cytokinesis-block micronuclei (CBMN) assay. The remaining three ml of blood was transferred into a plain tube. Blood was allowed to clot, serum was separated immediately. Luteinizing hormone (LH) and Follicle stimulating hormone (FSH) were measured by Chemiluminescence Immunoassay (CLIA).

Two ml blood was added to a culture tube containing 10 mL RPMI 1640 supplemented with 100units/mL penicillin, 100µg/mL streptomycin, 15% fetal bovine serum and 100µg/mL phytohemagglutinin. Cytochalasin B was added to the cultures at a final concentration of 4.5µg/mL (Sigma) after 44thhours 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 light microscopy at 100X magnification. The number of micronuclei in not less than 1000 binucleated cells were scored and the distribution of micronuclei among binucleated cells was recorded.

RESULTS

The subjects were grouped according to their demographic and anthropometric characteristics such as age, birth order, residence, religion, occupation, parental consanguinity, duration of married life, economic status and BMI. Among the 24 study subjects, 14 subjects (58.33%) were belonged to below the age of ≤25 years and showed a mean CBMN frequency of 12.68. The remaining 10 subjects (41.67%) with the age of >25 years showed a mean CBMN frequency of 13.34. The birth order ranged from 1 to 7 and majority of the subjects were belonged to first birth order. The highest mean CBMN frequency (13.8) showed by subjects with >6 birth order. Majority of the study subjects were belonged to rural area (70.83%) followed by coastal area (16.67%) and urban area (12.5%) and the highest mean CBMN frequency was observed in urban area (13.87). Highest mean CBMN frequency showed in the Muslim religion (13.5) followed by Hindu religion (13.23) and Christian religion (12.5). Sixteen study subjects had non-sedentary type of occupation with mean CBMN frequency of 12.82 and only 8 individuals have sedentary type of occupation with mean CBMN frequency of 13.59. Parental consanguinity was reported only in 2 out of 24 study subjects and showed mean CBMN frequency of 13.19. The duration of married life of these subjects ranged from 1 to 5 years with a mean duration of married life of 3 years. Subjects who have >3 years of married life showed highest mean CBMN frequency of 13.35. Those subjects, with high economic status showed highest mean CBMN frequency 13.65. On the basis of BMI, 20 to 25 Kg/m^2 showed mean CBMN frequency of 12.96 and >30 Kg/m^2 showed mean CBMN frequency of 14.2. Highest mean CBMN frequency showed by subjects with BMI of >30 Kg/m^2.

History of chronic illness was reported in 2 out of 24 study subjects with mean CBMN frequency of 13.5. Only one reported family history of cancer among 24 study subjects with mean CBMN frequency 13.25. Only one had endometriosis with mean CBMN frequency of 14.2. Consumption of contraceptive drugs was reported in 3 out of 24 study subjects and their mean CBMN frequency was 13.2. Majority of study subjects (n=20; 83.33%) attained menarche between 13 to 15 years of age and the remaining 4 subjects attained menarche between 16 to 18 years. Those who attained menarche between 16 to 18 years of age showed highest mean CBMN frequency of 13.28.

Normal serum total cholesterol was reported only in 8 (33.34%) study subjects and the remaining 16 subjects were hypercholestrolimic (>200 mg/dL). The mean CBMN frequency of hypercholestrolimic subjects were13.38. The study subjects showed FSH value 31 to 40 mIU/ml had higher mean CBMN frequency (13.53) compared to 11 to 20 mIU/ml (13.03) and 21 to 30 mIU/ml (13.07). Study subject with LH level >80 mIU/ml showed highest mean CBMN frequency of 13.4.

Table 1:- Distribution of mean CBMN frequency according to various demographic and anthropometric characteristics of the study subjects

Category	Variables	Total	Percentage (%)	Mean CBMN Frequency
Age range	≤25	14	58.33	12.68
	>25	10	41.67	13.34
Birth order	1 to 3	20	83.3	12.2
	4 to 6	3	12.5	13.15
	>6	1	4.17	13.8
Residence	Coastal	4	16.67	13.02
	Rural	17	70.83	13.27
	Urban	3	12.5	13.87
Religion	Christian	2	8.33	12.5
	Hindu	20	83.33	13.23
	Muslim	2	8.33	13.5

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Nature of occupation	Sedentary	8	33.34	13.59
	Non sedentary	16	66.67	12.82
Parental consanguinity	Yes	2	8.33	13.19
	No	22	91.67	13.15
Duration of married life	<3	11	45.83	12.68
	3	5	20.83	13.28
	>3	8	33.33	13.35
Economic status	High	2	8.33	13.65
	Medium	21	87.5	13.17
	Low	1	4.17	12.7
BMI (kg/m^2)	20 to 25	11	45.83	12.96
	26 to 30	12	50	13.32
	>30	1	4.17	14.2

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

Category	Variables	Total	Percentage (%)	Mean CBMN frequency
History of chronic illness	Yes	2	8.33	13.5
	No	22	91.67	13.16
Family history of cancer	Yes	1	4.17	13.25
	No	23	95.83	11.9
Endometriosis	Yes	1	4.17	14.2
	No	23	95.83	13.15
Contraceptive drugs used	Yes	3	12.5	13.2
	No	21	87.5	13.1
Age at Menarche (years)	13 to 15	20	83.3	12.72
	16 to 18	4	16.67	13.28
Total cholesterol (mg/dL)	<200	8	33.34	13.09
	≥200	16	66.66	13.38
Follicle stimulating hormone (FSH) (mIU/ml)	11 to 20	8	33.33	13.03
	21 to 30	9	37.5	13.07
	31to 40	7	29.17	13.53
Luiteinizing hormone (LH) (mIU/ml)	21 to 40	4	16.67	12.95
	41 to 80	19	79.17	13.23
	>80	1	4.17	13.4

DISCUSSION

Anovulation or irregular menstrual cycles are hallmarks of polycystic ovary syndrome (PCOS) and remain a central part of the consensus diagnoses (Zawadzki and Dunaif, 1992; Rotterdam, 2004). PCOS is one of the most common endocrine disorders amongst women of reproductive age. It is characterized by hyperandrogenism, menstrual disturbances, infertility due to chronic anovulation and polycystic ovaries (Sirmans and Pate, 2013). Cytogenetic studies have shown that women with PCOS have increased damage in their genetic material (Moran et al., 2008; Nersesyan et al., 2006; Yesilada et al., 2006).

The present study illustrated that DNA of anovulatory infertile subjects showed significant damage, by increased mean CBMN frequency in lymphocytes, confirming some reports that deal with this issue (Moran et al., 2008). These reports suggest the presence of genetic abnormality in PCOS subjects (Nersesyan et al., 2006; Yesilada et al., 2006; Hamurcu et al., 2010).

In the present study showed increased mean CBMN frequencies with advancing age were consistent with the results of international collaborative projects (Bonassi et al., 2003). In the current study it was observed that micronuclei frequency (MN) was highest in the age group >25 years, there seems to be relation between age and micronuclei frequency.

The increase in genomic damage in obese subjects and the positive correlation between genomic damage and BMI in total over-weight/obese subjects indicate that obesity increases genomic damage (Donmez-Altuntas, 2014). The present study showed BMI >30 kg/m^2 have highest mean CBMN frequency of 14.2.

According to Balen, (2007) the endocrine abnormalities in women with polycystic ovary syndrome include raised concentrations of luteinising hormone (LH; seen in about 40% of women), testosterone and androstenedione in association with low or normal concentrations of follicle stimulating hormone. The present study observed an increased FSH value, increased LH value as well as an increased total cholesterol level. All these characteristics showed a significant increase with mean CBMN frequency among the anovulatory subjects.

Genetic instability can have very serious consequences for PCOS patients. It has been proved that chromosomal abnormalities (structural, numerical) are associated with increased risk of cancer and early miscarriages (Migliore and Coppede, 2002). There are studies supporting the existence of these two phenomena in PCOS patients (Gadducci et al., 2005). In this study a positive correlation exists between the increase in mean CBMN frequency and subjects with family history of cancer. The study subjects with family history of cancer showed mean CBMN frequency of 13.25. It is generally accepted that PCOS is associated with gynaecological malignancies such as endometrial cancer and less with ovarian cancer. The present study showed increased mean CBMN frequency in subjects with endometriosis (14.2).

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.

CONCLUSION

In short, the present study involves androgen excess and genetic instabilities in anovulatory infertility. The distribution of mean CBMN frequency according to demographic, biochemical and endocrinological factors of the study subjects was observed. Age, birth order, parental consanguinity, BMI etc. showed increased level of CBMN frequency. The level of mean CBMN frequency was higher among those who have the family history of cancer and history of chronic illness. Total cholesterol, FSH and LH were also found to be significantly elevated in study subjects. These findings suggest that the women with anovulatory infertility have a high incidence of genomic instability and androgen excess does not cause morbidity or mortality, but it was associated with insulin resistance, dyslipidemia, hypertension, and vascular diseases; therefore, it is a forerunner of cardiovascular disease. The deep rooted origin and detrimental effects of PCOS on the biochemical, reproductive and metabolic functions of the body and its life-threaten-

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ing consequences warns the early recognition and management of this syndrome. It may be impossible to reverse the genetic programming of the syndrome. It can be managed by modifying various lifestyle factors. A wider range of treatment options have become available to infertile couples like medical treatment, surgical treatment and different assisted reproduction techniques.

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RESEARCH ARTICLE

CYTOGENETICS AND MOLECULAR GENETICS ON FEMALE INFERTILITY WITH SPECIAL EMPHASIS ON POLYCYSTIC OVARIAN SYNDROME

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Abstract

Female infertility is defined as the inability to conceive naturally or to carry a pregnancy to full term. It occurs for almost 15% of all women worldwide. The etiology of infertility is an important criterion for recognition and characterization of infertile women. The common factors for infertility in females are anovulatory disorder, tubal factors, endometriosis, uterine and cervical factors. Infertility affects 40% of women with PCOS and is the most common cause of female infertility. The present study was undertaken to evaluate the cytogenetics and molecular genetics on female infertility with special emphasis on polycystic ovarian syndrome by investigating the various anthropometric and clinical aspects of the subjects. Seventy five female subjects with infertility and 50 healthy women without any chronic illness were involved in this study. Reproductive hormones namely leutinizing hormone (LH), follicle stimulating hormone (FSH), prolactin 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. Infertile women with various risk factors such as increasing age, BMI, family history of infertility, family history of cancer, menarche, endometriosis etc. can lead to increased genetic instabilities and the severity of infertility. Lifestyle modification with diet and exercise will reduce the risk for infertility.

Introduction:-

Female infertility is defined as the inability to conceive naturally or to carry a pregnancy to full term (Gaware et al., 2009). The incidence of female infertility is rising and varies from 10 to 20% (Moghadam et al., 2013). Female infertility is caused by genetic, hormonal, or environmental factors. In addition, pelvic inflammatory disease, uterine fibroids, age-related factors, tubal blockage, and hostile cervical mucus can cause infertility in females (Olooto, 2012). Female infertility occurs in about 37% of all infertile couples (Unuane et al., 2011).

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The etiology is an important criterion for recognition and characterization for infertile subjects. Advanced age, high body mass index, age of onset of sexual activity, prior pelvic surgeries and stress were the most significant risk factors associated with women's infertility (Romero et al., 2008). Problems regarding menstruation (ammenhorroea, menorrhagia polymenhorroea, dysmenhorroea) along with insanitation are the major danger alarms (Maeda and Tsukamura, 2006). Any cause leading to irreversible or non-compensable damage to the genital tract, uterus, fallopian tubes or ovaries may cause inability to conceive (Jain et al., 2004; World Health Organization, 2003).

The polycystic ovary syndrome (PCOS) affects 7 to 8% of women (Azziz et al., 2004) and may be the most common cause of female infertility (Norman et al., 2002). Women with PCOS have an increased risk of miscarriage, gestational diabetes, preeclampsia and preterm labour (Boomsma et al., 2006).

Accumulating evidence suggests that genetic factors contribute to the etiology of female infertility in humans (The ESHRE Capri Workshop Group, 2008; Matzuk and Lamb, 2008). Genes involved in meiosis are also good candidates for genes contributing to female infertility (Sanderson et al., 2008). Karyotype analysis is also performed in women presenting with primary amenorrhoea, premature menopause, and recurrent pregnancy loss (ESHRE Capri workshop group, 2000). The sex chromosome aberrations and the presence of constitutional inversions, translocations, or small supernumerary marker chromosomes (sSMC) can lead both to infertility and repeated abortions (Liehr et al., 2004; Shah et al., 2003). Diminished ovarian reserve (DOR) is a primary infertility disorder characterized by a reduction in the number and/or quality of oocytes, usually accompanied by high follicle-stimulating hormone (FSH) levels and regular menses (Broekmans et al., 2007). Cytogenetic studies of female patients enrolled in an intracytoplasmic sperm injection (ICSI) programme reviewed by Gekas et al., (2001) have shown an unexpectedly increased incidence of abnormal karyotypes, ranging from 1.1 to 9.8% when cases with low level sex chromosome mosaicism were included.

As many as 20% of women with infertility problems (including fecundability and early pregnancy loss) have been diagnosed with PCOS (Diamanti et al 1998). PCOS is the most common cause of menstrual irregularity that leads to infertility. There is now a greater focus on the management of the metabolic consequences of PCOS, primarly through lifestyle interventions to achieve weight loss and increase physical activity (Sevendsen et al., 2005). The investigation and management of female infertility can be done by changing lifestyle, regular exercise, dieting etc. and this is one of the debated topics. No serious attempts were made earlier to correlate DNA damage and female infertility with polycystic ovarian syndrome. Hence the present study was undertaken to correlate various cytogenetics and molecular genetics on female infertility with polycystic ovarian syndrome.

Materials and Methods:-

Seventy five study subjects with a clinical diagnosis of infertility referred from various infertility centers of Kerala to Genetika, Centre for Advanced Genetic studies, Trivandrum. Fifty healthy subjects without any chronic illness were also selected as control for this study. Detailed demographic, clinical and biochemical characteristics of the subjects 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.

Seven ml of blood sample was collected by venepuncture. Two ml of blood was transferred into sodium heparinized vacuutainers for quantifying the extent of somatic DNA damages by Cytokinesis-Block Micronuclei (CBMN) assay. The remaining five ml of blood was transferred into a plain tube. Blood was allowed to clot, serum separated immediately. Blood sugar and lipid profile were estimated using semi-automated clinical chemistry analyzer.

Two ml blood was added to a culture tube containing 10 mL RPMI 1640 supplemented with 100units/mL penicillin, 100µg/mL streptomycin, 15% fetal bovine serum and 100µg/mL phytohemagglutinin. Cytochalasin B was added to the cultures at a final concentration of 4.5µg/mL (Sigma) 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 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.

Results:-

In the present study 75 individuals were taken as the study subjects and 50 healthy individuals were taken as the control subjects. The study subjects showed a mean CBMN frequency of 13.18 while the control subjects showed a mean CBMN frequency of 10.63. This difference in mean CBMN frequencies showed a statistically significant difference.

Table 1:- Distribution of mean CBMN frequency according to various demographic characteristics

Category	Variables	Total	Percentage (%)	Mean CBMN Frequency
Age (Years)	20-28	40	53.3	13.01
	29-36	35	46.6	13.38
Birth Order	<3	51	68	13.02
	4 to 6	20	26	13.46
	7 to 9	4	5.3	13.76
Residence	Coastal	8	10.6	12.98
	Rural	52	69.3	13.11
	Urban	15	20	13.54
Parental consanguinity	Yes	8	10.6	13.45
	No	67	89.3	13.15
Duration of married life (Years)	1 to 5	52	70	13.14
	6 to 11	23	29.3	13.27
Social Status	High	9	12	13.69
	Low	2	2.6	13.11
	Medium	64	85.3	13.11
BMI (Kg/m^2)	<20	1	1.33	12.06
	20 to 25	42	56	13.02
	>25	32	42.66	13.33

The subjects were grouped on their demographic characteristics such as age, birth order, residence, parental consanguinity, duration of married life, social status and BMI (Table 1). Among the 75 study subjects, 40 subjects (53.3%) were between the age of 20 to 28 years and showed a mean CBMN frequency of 13.01. The highest mean CBMN frequency of 13.38 was shown by 35 subjects (46.6%) of age between 29 to 36 years. The highest mean CBMN frequency (13.76) shown by subjects between 7 to 9 birth order. Majority of the study subjects were belonged to rural (69.3%) followed by urban area (20%) and coastal area (10.6%). The highest mean CBMN frequency was observed in urban area (13.54). Subjects with parental consanguinity showed highest mean CBMN frequency of 13.45. Subjects having 6 to 11 years of married life showed highest mean CBMN frequency of 13.27. From the study subjects, 9 (12%) subjects were showed a high social status with a highest mean CBMN frequency of 13.69. 64 subjects (85.3%) were belonged to middle social status and showed a mean CBMN frequency (13.11). On the basis of BMI, subjects with <20 Kg/m^2 showed mean CBMN frequency of 12.06 and 20 to 25 Kg/m^2 showed mean CBMN frequency of 13.02. Highest mean CBMN frequency (13.33) showed by subjects with BMI >25 Kg/m^2.

Table 2:- Distribution of mean CBMN frequency according to various clinical characteristics

Category	Variables	Total	Percentage %)	Mean CBMN Frequency
Family H/o infertility or subfertility	Yes	6	8	13.19
	No	69	92	13.18
Family H/o cancer	Yes	3	4	13.22
	No	72	96	13.18
Family H/o chronic illness	Yes	71	94.6	13.19
	No	4	5.3	12.93
H/o X-ray exposure	Yes	70	93.3	13.19
	No	5	6.6	13.05
Menstrual periods	Irregular	38	50.6	13.21
	Regular	37	49.3	13.16

Menarche (years)	13 to 15	69	92	12.87
	16 to 18	6	8	13.21
Endometriosis	Yes	9	12	13.22
	No	66	88	12.91
Contraceptive drugs used	Yes	12	16	13.18
	No	63	84	13.16
Clinical conditions	Abortion	30	40	13.35
	Infertility	45	60	13.07

The subjects were grouped on their clinical characteristics such as family history of infertility or subfertility, family history of cancer, family history of chronic illness, history of X-ray exposure, menstrual periods, menarche, endometriosis, contraceptive drugs used and clinical condition (Table 2). Subjects with family history of infertility/subfertility (8%) showed highest mean CBMN frequency of 13.19. Subjects with family history of cancer showed higher mean CBMN frequency (13.22) than subjects without family history of cancer. Family history of chronic illness was reported in 71 out of 75 study subjects with mean CBMN frequency of 13.19. Subjects with irregular menstrual periods showed high mean CBMN frequency of 13.21. Majority of study subjects (n=69; 92%) attained menarche between 13 to 15 years of age and the remaining 6 subjects attained menarche between 16 to 18 years. Those who attained menarche between 16 to 18 years of age showed highest mean CBMN frequency of 13.21. Nine subjects had endometriosis with mean CBMN frequency of 13.22. Consumption of contraceptive drugs was reported in 12 out of 75 study subjects and their mean CBMN frequency was 13.18.

Table 3:- Distribution of mean CBMN frequency according to various biochemical and endocrinological characteristics

Category	Variables	Total	Percentage (%)	Mean CBMN Frequency
Fasting blood sugar (FBS) (mg/dl)	70 to 100	7	9.33	13.17
	101 to 126	30	40	13.19
	>126	38	50.6	13.20
Total Cholesterol (mg/dl)	<200	26	34.6	13.04
	≥200	49	65.2	13.2
HDL (mg/dl)	21 to 31	18	24	13.27
	32 to 42	44	58.6	13.22
	43 to 51	12	16	12.95
LDL (mg/dl)	<100	6	8	13.13
	100 to 150	32	42.6	13.18
	>150	37	49.3	13.19
TG (mg/dl)	<150	53	70.6	13.04
	150 to 250	19	25.3	13.10
	>250	3	4	13.22
Follicle stimulating (FSH) (mIU/ml)	<25	41	54.6	13.09
	25 to 30	12	16	13.24
	>30	22	29.3	13.37
Luteinizing hormone (LH) (mIU/ml)	<45	16	21.33	13.04
	45 to 60	41	53.33	13.18
	>60	19	25.33	13.30
Estradiol (pg/ml)	<25	8	10.6	12.84
	25 to 75	36	48	13.20
	>75	31	41.3	13.24
Prolactin (ng/l)	<25	24	32	13.02
	≥25	51	68	13.53

The subjects were grouped on their various biochemical and endocrinological characteristics such as FBS, total cholesterol, HDL, LDL, TG, FSH, LH, Estradiol and Prolactin (Table 3). Normal FBS was reported in 7 (9.33%)

subjects and remaining subjects were pre-diabetic and diabetic. The mean CBMN frequency of pre-diabetic subjects and diabetic subjects were 13.19 and 13.20. Normal serum total cholesterol was reported only in 26 (34.6%) study subjects and the remaining subjects were hypercholestrolimic (>200 mg/dl). The mean CBMN frequency of hypercholestrolimic subjects were 13.2. Subjects with triglyceride value <150 mg/dl showed mean CBMN frequency of 13.04. Highest mean CBMN frequency (13.22) was shown by subjects with triglyceride value >250 mg/dl. The study subjects showed FSH value >30 mIU/ml had higher mean CBMN frequency (13.37) compared to <25 mIU/ml (13.09) and 25 to 30 mIU/ml (13.24). Study subject with LH level >60 mIU/ml showed highest mean CBMN frequency of 13.30. Subjects with >75 pg/ml value of estradiol showed highest mean CBMN frequency of 13.24. Majority of the subjects have prolactin level ≥25 ng/l and showed highest mean CBMN frequency of 13.53.

Discussion:-

The prevalence of female infertility is varies worldwide ranging from 3% to 7%. Polycystic ovarian syndrome is a common condition estimated to affect 4-18% women in the reproductive age. PCOS is associated with reproductive, psychological, metabolic and cardiovascular diseases (Boomsma et al., 2006).

According to Zlotogora, (2006) in communities with a high level of consanguineous marriage, diagnosis of a recessive disorder in one or more members of the same family is generally indicative of a recent mutation, whereas the presence of a rare disorder in several families suggests an older mutational event or previous admixture through marriage with a person from another community. In the present study, subjects with parental consanguinity showed highest mean CBMN frequency.

Mokhtar et al., (2006) revealed that females with the age of menarche more than 15 years were more risky to develop infertility than those with age of menarche less than 15 years. In the present study, the subjects with advancing age of menarche were showed a high mean CBMN frequency.

BMI is a common feature in women with PCOS, with prevalence of 30-70%. Certain single nucleotide polymorphism associated with obesity and it contributed to elevate the body mass index in PCOS (Ewens et al., 2011). The present study showed a high CBMN frequency among the subjects with obesity.

According to Wijeyaratne et al., (2005) those with PCOS had significantly higher median BMI. Regarding BMI, 69.2% of overweight/obese patients had polycystic ovary morphology. The higher incidence of overweight may be linked to the lack of exercise amongst women and fatty food habits. Increasing BMI was significantly related to an increasing trend in the proportion of women with the metabolic syndrome. PCOS is one of the most common risk factor among female infertility. Thus in the present study it is showed that mean CBMN frequency increases with increasing the BMI.

According to Guastella et al., (2010) patients with polycystic ovaries have statistically significant higher LH levels and LH/FSH ratios than patients with normal ovaries. In the present study, majority of the subjects were shown hormonal disturbances and also increased mean CBMN frequency.

Conclusion:-

The present study involves Cytogenetics and Molecular Genetics on Female Infertility with Special Emphasis on Polycystic Ovarian Syndrome. The distribution of mean CBMN frequency according to demographic, clinical and biochemical characteristics of the study subjects was observed. Age, birth order, parental consanguinity, and BMI etc. showed increased level of mean CBMN frequency. The level of mean CBMN frequency was high among those who have the family history of infertility, family history of cancer and family history of chronic illness. FBS, total cholesterol, FSH, LH, prolactin and estradiol were also found to be significantly elevated in study subjects. These findings suggest that the women with PCOS have a high risk for infertility. While PCOS cannot be prevented or cured, it can be controlled, with varying degrees of success, by maintaining a healthy diet and by exercising. Healthy lifestyle factors, including exercise, are associated significantly with reduced DNA damage.

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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 anovuatory infertility. The present study was carried out in 52 subjects suffering from anoulatory 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.

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 amenorrheic (Adam and Anthony, 2007). Thyroid disease is a common cause of menstrual cycle irregularity (Koutras, 1997). Oligomenorrhea and amenorrhea 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, Thiruvanathapuram, 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 10µg/mL phytohaemagglutinin. Cytochalasin B was added to the cultures at a final concentration of 4.5µg/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/m²)	<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 ≥130 mg/dl and some of them possessed abnormal karyotype. Subjects with ≤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 (Nersesyan 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 ≥ 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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EVIDENCE OF INCREASED OXIDATIVE STRESS AND DNA DAMAGES IN OLIGOSPERMIA

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Abstract— Oligospermia is defined as a sperm density (count) less than 20 million/ml. Infertility affects approximately 15% of couples worldwide, among them 20–25% of reproductive problems being contributed to male factor. Male infertility is a relatively common condition affecting approximately 1 in 20 of the male population. Defective sperm function is held to be the largest, single and defined cause of human infertility. The excessive generation of reactive oxygen species (ROS) by abnormal spermatozoa has been defined as one of the important etiologies for male infertility. Generation and persistence of ROS in seminal fluid and sperm increase the rate of lipid peroxidation of sperm membrane which is manifested by a high MDA level. The present study was undertaken to evaluate the role of oxidative stress by measuring the level of oxidative stress marker, Malondialdehyde (MDA), in the sera of males suffering with oligospermia. The extent of somatic DNA damage in these subjects was quantified by using Cytokinesis Block Micronuclei Assay. These investigations were carried out in 56 subjects suffering with oligospermia and 15 healthy fertile men as control subjects. The MDA value and the micronuclei frequency was significantly elevated in study subjects as compared with that of control subjects. The finding of increased oxidative stress marker level may indicate that oxidative stress may be involved in the pathogenesis of sperm DNA damage leading to oligospermic condition as well as infertility in male subjects. These individuals can be better informed about the extent of somatic DNA damages, oxidative stress and genetic risks. This may help in preventing the sufferings of infertile subjects with oligospermia.

Index Terms— DNA damages, Double strand breaks (DSB), Infertility, Malondialdehyde (MDA), Oligospermia, Oxidative stress, Reactive oxygen species (ROS), Sperm DNA integrity

1 INTRODUCTION

Infertility is defined as the failure to achieve a pregnancy within one year of regular (at least three times per month) unprotected intercourse [1]. It affects approximately 15% of couples worldwide, among them 20–25% of reproductive problems being contributed to male factor [2]. Male infertility is a relatively common condition affecting approximately 1 in 20 of the male population. In a vast majority of infertile subjects sufficient numbers of spermatozoa are generated to initiate a pregnancy; however, the functionality of the spermatozoa has been compromised. As a result, defective sperm function is held to be the largest, single and defined cause of human infertility [3].

Oligospermia is defined as a sperm density (count) less than 20 million/ml [4] which may leads to male infertility [5]. 'Male factor' infertility is seen as an alteration in sperm concentration and/or motility and/or morphology [6].

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Semen analysis remains the cornerstone in the evaluation of male infertility or oligospermia condition [5]. The primary causes of defective sperm function are undoubtedly multifactorial, involving a range of primary genetic, lifestyle and environmental factors, acting alone or, more frequently, in combination. At the level of the gamete, it is the oxidative stress that impairs the functional and structural integrity of these highly differentiated cells like sperm [7].

The oxidative stress not only disrupts the integrity of sperm DNA but also limits the fertilizing potential of these cells as a result of collateral damage to proteins and lipids in the sperm plasma membrane [7]. Oxidative stress is caused by an imbalance between pro-oxidants and anti-oxidants. This ratio can be altered by an increased level of reactive oxygen species or a decreased antioxidant defence mechanism [8]. Recombination is triggered by the generation of a DNA double strand break (DSB) within an amplicon. The occurrence of such lesions are particularly frequent in the male germ line, owing to the fact that spermatogenesis requires multiple cell divisions in an oxidative environment with depleted DNA repair enzymes [9]. Studies have also shown that elevated rate of DNA nicks and double strand breaks in sperm of infertile men could lead to infertility and 50% of miscarriages; this means that this individuals have a background of genetic instability that can be caused by their inability to repair DNA damage and are susceptible to mutagenic and clastogenic agents [10]. Sperm DNA damage is strongly associated with sperm function and infertility [11].

The fertilizing potential of sperm depends not only on the functional competence of spermatozoa but also on sperm DNA integrity [12]. Classical semen analysis, which include sperm concentration, motility and morphology gives an approximate evaluation of the functional competence of spermatozoa, but does not always reflect the quality of sperm DNA. Men with normal sperm grams may still be infertile; the cause could be related to abnormal sperm DNA [13]. Sperm DNA integrity has an important role not only for fertilization but also for normal embryo and foetal development [14]. Sperm with compromised DNA integrity, regardless of the degree of DNA damage, appear to have the capacity to fertilize oocytes at the same rate as normal sperm [13]. However, the embryos produced by fertilization of an oocyte with DNA damaged sperm cannot develop normally [15].

The genetic integrity of the male gamete is essential for a successful and healthy pregnancy. Data in the literature suggest that the frequency of sperm cells with massive DNA fragmentation is a marker of sperm quality and a potential predictor of fertility [16]. Sperm DNA fragmentation (SDF) could be a result of unrepaired DNA breakage produced during the process of chromatin remodeling or could be a consequence of an apoptotic like DNA degradation process [17]. Oxidative stress may also induce SDF, when reactive oxygen species (ROS) generation overcomes the antioxidant scavenging activities of ROS [18].

The excessive generation of reactive oxygen species (ROS) by abnormal spermatozoa has been defined as one of the important etiologies for male infertility. Generation and persistence of ROS in seminal fluid and sperm increase the rate of lipid peroxidation of sperm membrane which is manifested by a high MDA level [19].

Strong evidence suggests that high levels of ROS mediate the occurrence of high frequencies of single and double strand DNA breaks commonly observed in the spermatozoa of infertile men [7]. Several studies reported that accumulation of oxidative DNA damages may leads to defective DNA repair capacity and spermatogenic failure. Hence the present study has become important to evaluate oxidative stress-induced nuclear DNA damage and its effects on sperm quality and to prevent this oligospermia condition.

2. Materials and Methods

Fifty six males suffering from varying degrees of oligospermia were selected for this study. All these samples were referred from various infertility clinics for genetic testing to Genetika, Centre for Advanced Genetic Studies, Thiruvananthapuram, Kerala. Fifteen asymptomatic healthy fertile men formed the control group, and the detailed demographic, clinical history, lifestyle characteristics, and other relevant informations were recorded using proforma.

8ml of venous blood was collected aseptically by venepuncture from all these study subjects and from that, 4ml of blood was set up for culture A and B. The culture A was for detecting constitutional chromosome anomalies by peripheral blood lymphocyte culture method [20] and banded with GTG banding technique. The culture B was for quantitating the extent of somatic DNA damages by Cytokinesis block micronuclei (CBMN) assay [21]. The remaining 4ml of blood was transferred to plain tube and allowed to clot, serum separated immediately. The level of the malondialdehyde, the end product of lipid peroxidation, was determined using thiobarbituric acid as main reagent and measuring these values on photoelectric colorimeter at 540nm.

For detecting constitutional chromosome anomalies, cell division was arrested at metaphase and Giemsa stained as well as GTG banded. GTG banded slides were observed under microscope and good quality metaphases were photographed. From the prints each chromosome was cut down and pasted according to the size, position of centromere and the banding pattern. Karyotypes were prepared according to ISCN (1995) pattern.

The lymphocytes were cultured in sterile bottles using RPMI 1640 medium and were prepared for each subject. The media is supplemented with 100 units/ml penicillin, 100 units/ml streptomycin, 10% fetal bovine serum and 1% phytohemagglutinin. At 44 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. The data was computed and analyzed using SPSS 11.3 for Windows.

3. Results

TABLE 1:

COMPARISON OF MEAN CBMN FREQUENCY AND MDA VALUE AMONG THE STUDY AND CONTROL SUBJECTS

Category	Number	Mean CBMN frequency	MDA Value
Study Subjects	56	14.08	2.13
Control Subjects	15	11.16	1.08

Fifty six subjects with oligospermia, suffering from varying degrees of infertility and 15 healthy fertile subjects were selected and analyzed; the results were recorded. Detailed demographic, lifestyle and clinical characteristics were studied. The age of the study subjects ranged from 21 to 50 with a mean age of 34.83. The CBMN assay was performed to

quantify the extent of DNA damage and the malondialdehyde (MDA) was estimated to evaluate the level of oxidative stress among these study subjects. The mean CBMN frequency of the study subjects was higher (14.08) than the control subjects (11.16). The MDA value was also higher (2.13) in study subjects, when compared to that of the control subjects (1.08) [Table 1]. The mean CBMN frequency and the MDA values were statistically increased among the study subjects than the control subjects.

The demographic characteristics among the study subjects were studied. The age range of the study subjects ranged from 21-50 and the highest mean CBMN frequency was observed among the age group 41-50 (14.20). The birth order of the study subjects ranged from 1 to 9 and the highest mean CBMN frequency was shown by the birth order less than or equal to 3 (14.12). This study demonstrated that the mean CBMN frequency decreases with increased birth order. Majority of study subjects belonged to the religion, Hindu (n=37, 66.07%), Muslim (n=10, 17.85%) followed by Christian (n=9, 16.07%). The highest mean CBMN frequency was recorded in Christian (14.12) and Muslim (14.12). Majority of the study subjects (64.28%) belonged to rural area followed by urban (28.57%) and coastal (7.14%) and an increased mean CBMN frequency was shown by those residing at rural area.

The life style characteristics were also considered in this study. Only 3 among the 56 study subjects had the family history of infertility/sub fertility with the highest mean CBMN frequency of 14.3. Only 4 had the family history of cancer among first or second degree relatives with high mean CBMN frequency of 14.08 and only 2 showed the history of chronic illness with highest mean CBMN frequency of 14.75. History of smoking was reported in 21.42% subjects and 3.57% of study subjects had habit of alcoholism with high mean CBMN frequency of 14.12 and 14.55 respectively. Parental consanguinity was reported in 14.28% study subjects with a highest mean CBMN frequency of 14.13. This clearly indicates a significant relationship between the mean CBMN frequency and the history of infertility, cancer, chronic illness, smoking, alcoholism and parental consanguinity, etc. Simultaneously, a higher MDA value was observed among those subjects with higher mean CBMN frequency which suggests that lifestyle modifications can minimize the extent of oxidative stress.

Among 56 study subjects 87.5% showed normal karyotype and 12.5% showed abnormal karyotype. The mean CBMN frequency was found to be higher (14.41) in those subjects who had an abnormal karyotype (Table 2). This study revealed that the abnormal karyotype shows an increased mean CBMN frequency. An abnormal karyotype was found in 12.5% of the men with oligospermia. Thus the chromosomal abnormalities are more frequently observed in population of oligospermic males than the general population.

TABLE 2:

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

	Variable	Number	Percentage	MDA Value	Mean CBMN Frequency
Karyotype	Normal	49	87.50%	2.09	14.03
	Abnormal	7	12.50%	2.36	14.41

4. DISCUSSION

Oligospermia (less than 20 million spermatozoa m/L) is the condition with reduced number of sperms in males [22] and may lead to infertility condition. Infertility is a problem for many couples wishing to conceive. According to World Health Organization, it affects approximately 15% of couples worldwide, among them 20–25% of reproductive problems being contributed to male factor. Male infertility is a global health problem and affects one man in 20 in the general population [2].

Several studies reported that oxidative stress is a major factor in the etiology of male infertility [23]. Oxidative stress induces un-repairable DNA damages. It is suggested that normal persons are more resistant to mutagens compared to patients with various chromosome instability syndrome and cancer. Through cytogenetic analysis, variation in susceptibility to mutagen induced genomic damage can be quantitated.

The purpose of the study was to evaluate the role of oxidative stress and DNA damage in the development of oligospermia. 56 subjects with varying degree of oligospermia risk markers were selected for this study and MDA test and CBMN assay was performed. In this study, the demographic, lifestyle factors and clinical characteristics were concerned. The study demonstrated that the mean CBMN frequency of oligospermia subjects shows a positive correlation with advancing age, family history of infertility, family history of cancer, history of chronic illness, smoking, drinking, parental consanguinity and karyotype, and the mean CBMN frequency was varied according to birth order, religion, residence, education and occupation.

The older men seem to produce more sperm with DNA damage, which derives from three potential sources: oxidative stress, abortive Fas-mediated apoptosis or deficiencies in natural processes such as recombination and chromatin packaging that induce DNA strand breaks [7]. In this study a positive correlation exists between the increase in CBMN frequency and advancing age among the study subjects. The highest mean CBMN frequency was reported in the age group 41 to 50.

Occupational chemical exposure has also led to oligospermia and non-motility and those occupational exposures include heavy metals, solvents, and fumes (notably welding

fumes). More refined analysis of each semen parameter confirmed the higher risk of asthenospermia in subjects exposed to heavy metals [24]. The present study showed increased mean CBMN frequency in non sedentary occupation.

In cases of azoospermia or severe OAT (oligo-astheno teratospermia), there may be deletions in the azoospermic factor (AZF) region of the Y chromosome. The presence of a Y deletion means that the defect will be passed to sons, who will then also be affected by spermatogenic disturbances or failure [25] present study also observed that the subjects suffering from oligospermia with family history of infertility shows high mean CBMN frequency.

Alcohol has been shown to have a deleterious effect on all levels of male reproductive system. Alcohol induced reduction in levels of testosterone, LH and FSH not only hampers their normal morphological development and maturation of spermatozoa (producing significant teratozoospermia), but also slows down the sperm production by testicular germ cells, especially in heavy alcoholics [26]. In the present study it has been found that the subjects with alcohol consumption reported to have highest CBMN frequency. Thus the study suggests alcohol abuse significant risk factor for oligospermia. Previous studies conducted on fertile male smokers showed reduction in semen volume in comparison to nonsmokers and this reduction in semen volume was in proportion to the number of cigarettes smoked [27]. The present study also observed cigarette smoking as a risk factor for oligospermia.

If the parents were first cousins, both sperm counts and motility parameters were significantly reduced when compared with the others [28]. In the present study the study subjects with parental consanguinity showed high mean CBMN frequency. The reduction in the number of sperm or function may be caused by either a chromosomal or a single gene disorder. Higher frequencies of chromosomal abnormalities ranging from 5% to 27% are found in infertile males than in general male population. Various chromosomal abnormalities reported are numerical or structural abnormalities of sex chromosomes, Robertsonian translocation, paracentric inversions of autosomes and marker chromosomes 9. With decreasing sperm counts, there is a progressive increase in the frequency of chromosomal abnormalities which are more common with severe oligospermia [29]. In this study the abnormal karyotype showed a high mean CBMN frequency suggesting relatively high range of DNA damage than others.

5. Conclusion

The subjects who had reported for the risk factors such as smoking, alcohol consumption showed higher values of both mean CBMN frequency and MDA. The level of mean CBMN frequency was higher among subjects who have the family history of cancer, chronic illness and infertility. Overall men population seems to be more predisposed to infertility. This is mainly due to lifestyle changes and sometimes occurs as hereditary problems. Increasing awareness of the role of genetics in the etiology of diseases and its overall impact on the burden imposed on individuals, families and society has led to the emergence of modern clinical cytogenetics. These individuals can be better informed about extent of somatic DNA damages, oxidative stress and genetic risks. This may aid in preventing the sufferings of infertile subjects with oligospermia.

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ORIGINAL RESEARCH PAPER

Biological Science

OXIDATIVE STRESS AND DNA DAMAGES IN BAD OBSTETRIC HISTORY

KEY WORDS:

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ABSTRACT

Bad obstetric history (BOH) implies previous unfavourable foetal outcome in terms of two or more consecutive spontaneous abortions, history of intrauterine foetal death, intrauterine growth retardation, stillbirth, early neonatal death and/or congenital anomalies. Twenty couples suffering from bad obstetric history were selected as study subjects. Fifteen healthy age matched couples with one or two live children were selected as control subjects for the present study. The study was undertaken to assess the effect of increased oxidative stress and DNA damages in couples experiencing BOH. Malondialdehyde (MDA) test was performed to detect the oxidative stress in patients with BOH and the extent of somatic DNA damage was quantified by Cytokinesis Block Micronuclei assay. The results of mean MDA concentration and the mean CBMN frequency showed a statistically significant difference between the study and control subjects. Subjects with increased hormone level (FSH and LH) also showed increased MDA value and mean CBMN frequency. Similar result was observed among subjects with TORCH infections. Male study subjects having the unhealthy lifestyle habits and abnormal semen parameters showed increased MDA value and mean CBMN frequency. The results were correlated with various demographic, lifestyle and clinical aspects of the patients. Modification of life style, by changing the dietary habit and sedentary life style will help to reduce the oxidative stress. Moreover, the diagnosis of chromosomal anomalies at the exact time can lead to prevention of future birth of affected babies and also pregnancy loss due to suspected genetic loss can also be reduced in the society to an extent.

INTRODUCTION

Recurrent miscarriage (RM), also known as recurrent pregnancy loss, is a distressing condition affecting around 1% of couples trying to conceive. It can be very frustrating for both clinicians and patients as, despite intensive workup, no clear underlying pathology is forthcoming in at least 50% of couples (Homer, 2019). Bad obstetric history (BOH) implies previous unfavourable foetal outcome in terms of two or more consecutive spontaneous abortions, history of intrauterine foetal death, intrauterine growth retardation, stillbirth, early neonatal death and/or congenital anomalies (Namrata Kumari et al., 2011). The causes of BOH may be genetic, hormonal, abnormal maternal immune response, maternal infection and anatomical (Nickerson et al., 2012). Common established causes include uterine anomalies, antiphos pholipid syndrome, hormonal and metabolic disorders and cytogenetic abnormalities. Other etiologies have been proposed but are still considered controversial, such as chronic endometritis, inherited thrombophilias, luteal phase deficiency and high sperm DNA fragmentation levels (Hachem et al., 2017). However, an increasing risk of fetal loss with increasing maternal age has been documented in women aged more than 30 years (Andersen et al., 2000). Primary infection caused by TORCH (Toxoplasma Gondi, Rubella virus, Cytomegalo virus and Herpes simplex virus) are the main cause of BOH (AlHilli et al., 2014). Gestational diabetes mellitus (GDM) forms the most common medical complication of pregnancy (Ambroise et al., 2000).

Oxidative stress (OS), which is defined as an imbalance between pro-oxidants and antioxidant capacity, has been implicated in suboptimal reproductive performance from the earliest stages of development to labour and delivery. Oxidative stress occurs when there is an imbalance between the production of free radicals and the body's ability to counteract their damaging effects through neutralization with antioxidants (Poston et al., 2011). Oxygen radicals and Reactive Oxygen Species (ROS) play both a physiologic and pathologic role in the female reproductive tract. It includes various mechanism like lipid damages, inhibition of protein synthesis and depletion of ATP. There by it affects physiological function in female reproduction such as oocyte maturation, ovarian steroidogenesis, ovulation, implantation and embryo development (Ruder et al., 2008).

ROS generation is mainly due to electron leakage from the mitochondrial membrane, but also due to exogenous exposures such as smoke and environmental pollutants. At higher levels, OS can cause indiscriminate damage leading to loss of function and even cell death. Several structures such as membranes, proteins, lipids and nucleic acid are prone to damage by superoxide ions (Kong et al., 2010). Lipid degradation occurs, forming products such as malondia ldehyde (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 (Alahmar et al., 2019). Pregnancy complications such as spontaneous abortion, recurrent pregnancy loss and preeclampsia, can also develop in response to OS (Agarwal et al., 2012). 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 (Bedaiwy et al., 2002). Due to the formation of large number of ROS, breaking of DNA (double strand breaks) in sperm and oocytes occurs which may leads to BOH.

BOH is the common complication of pregnancy, affecting

approximately 15% of all clinically recognized pregnancies in the general population. Perinatal mortality remains a challenge in the care of pregnant women worldwide, particularly for those who had history of adverse outcome in previous pregnancies. The risk factor for pregnancy is increasing day by day. Recent studies showed that environment, genetic and life style factors contribute to BOH, still the relation between DNA damage related BOH is unclear. Hence the present study was undertaken to assess the evidence of increased oxidative stress and DNA damages in couple experiencing BOH. The specific objectives are to estimate the level of oxidative stress and the extent of somatic DNA damages, if any, in couples experiencing BOH by, Cytokinesis-block micronuclei (CBMN) assay in couples experiencing BOH.

MATERIALS AND METHODS

Twenty couples suffering from varying degrees of bad obstetric history were selected as the test subjects and fifteen healthy age matched couples with one or two live children were selected as control subjects for the present study. Study subjects were referred from various infertility centres of Kerala to Genetika, Centre for Advanced Genetic studies, Trivandrum. Detailed demographic, physiological and life style characteristics of the subjects were recorded using proforma. Eight ml of venous blood was collected aseptically from all the subjects by venepuncture. Cytokinesis Block Micronuclei (CBMN) assay to quantifying the extent of somatic DNA damages and Peripheral blood lymphocyte culture (PBLC) was carried out to evaluate the chromosome aberrations, if any, among these subjects. Malondialdehyde (MDA) was performed based on the reaction of malondial dehyde (MDA) with thiobarbituric acid (TBA); forming a MDA- tba2 adduct according to a modified version of Sato (1979) methods. The physiological characters like height, weight, BMI, obesity, etc were recorded and other clinical parameters like Family history of (FH/o) infertility, H/o Diabetes, H/o Hypertension, H/o Dyslipidemia, H/o Chronic illness, H/o Thyroid disorder, H/o Endometriosis, H/o UTI and H/o infections were also evaluated. History of X-ray exposure, H/o drug intake, consanguinity, smoking habit, alcohol consumption, water intake per day and dietary pattern were also included as lifestyle characteristics.

OBSERVATIONS AND RESULTS

The age of the female study subjects ranged from 22 to 41 years with a mean age of 28.1. The age of the male study subjects ranged from 29 to 51 years with a mean age of 35.95. The duration of married life of these sub-fertile subjects ranged from 1 to 16 years with a mean duration of 4.6 years. The number of previous abortion of study subjects reported as 1 to 5 with a mean of 2.65.

Oxidative stress marker (MDA), showed a statistically significant increase among the study subjects than the control subjects. Subjects with increased age, birth order, increased duration of married life, delayed menarche, irregular menstruation, uterine abnormalities, etc. showed increased MDA value than the subjects without these characteristics. The mean CBMN frequency was also found higher in couples with increased age, increased duration of married life, increased number of pregnancies, increased number of previous abortions, PCOS, uterine abnormalities, smoking, alcohol consumption etc.

Out of the 20 female study subjects 3 showed abnormal karyotype and out of 20 male study subjects, 4 showed abnormal karyotype. Subjects with abnormal karyotype showed increased MDA value and mean CBMN frequency. Moreover, subjects with increased hormone level (FSH and LH) also showed increased MDA value and mean CBMN frequency. Similar result was observed among subjects with TORCH infections. Male study subjects having the unhealthy lifestyle habits and abnormal semen parameters showed increased MDA value and mean CBMN frequency. The incidence of abnormal karyotypes was also higher among subjects with abnormal semen parameters.

DISCUSSION

Overall incidence of BOH in literature showed large etiological heterogeneity. Age, obesity and high parity have been shown to be independent risk factors for RPL and stillbirth and incidence of BOH was found to be 5.27%. Another term related to BOH is RPL (recurrent pregnancy loss) or Habitual Abortion is a distinct disorder defined by two or more failed clinical pregnancies, and up to 50% of RPL will not have a clearly defined aetiology.

A large prospective study conducted by Andersen et al., (2000) reported that, the risk of a spontaneous abortion was 8.9% in women aged 20-24 years and 74.7% in those aged 45 years or more. High maternal age was a significant risk factor for spontaneous abortion irrespective of the number of previous miscarriages. The risk of an ectopic pregnancy and stillbirth also increased with increasing maternal age (Andersen et al., 2000). The present study was conducted in 20 couples and it was observed that the mean CBMN frequency gradually increases with increased maternal age as well as paternal age. The mean CBMN frequency of women with below the age of 30 was 11.6 and above the age of 40 showed a mean CBMN frequency of 12.79. Paternal age is also a risk factor for recurrent fetal loss. Highest mean CBMN frequency showed in subjects above 33 years of age (13.24) and comparatively lower for those below 33 years (11.59).

An increasing risk of fetal loss with increasing maternal age has been documented in women aged more than 30 years in a prospective study by Singh (2010). At 42 years of age, more than half of all pregnancies resulted in a spontaneous abortion, ectopic pregnancy or stillbirth. After three or more spontaneous abortions, the proportion of pregnancies ending in spontaneous abortion increased to 44.6% in nulliparous women and 35.4% in parous women. In the present study number of abortions increased with increased age in the study subjects.

Cytogenetic analysis of previous miscarriages is an important component in the assessment of the couples with BOH. Identifying a cytogenetic cause for BOH can be psychol ogically important to overcome grief and loss. According to Anderson et al., 2000 there are many reasons for spontaneous abortion including chromosomal abnormalities, maternal age, medical-illness, infections etc among these chromo somal abnormalities it is believed to be the most common etiological factors behind spontaneous abortion and may account for up to 50% of miscarriages.

In a study conducted by Cigaril et al., (2005) reported that, catalase (CAT), superoxide dismutase (SOD) and lipid peroxide (LPO) levels were increased in pregnant women compared with non-pregnant women. CAT, SOD activities and LPO levels were increased from the rst trimester to the third trimester in pregnancy without UTI. However, CAT and SOD activities were decreased; LPO levels were increased from the First trimester to the third trimester in pregnancy with UTI. Pregnancy causes oxidative stress and also UTI during pregnancy may aggravate oxidative stress (Ciragil et al., 2005). In the current study an increased mean CBMN frequency along with peaked percentage of karyotype was observed in subjects with history of UTI.

According to a study done by Ghneim (2016), the levels of MDA production, were significantly increased in RM patients when compared to those in healthy patients (HP) women. Similar trend was observed in the placental tissue MDA levels of RM patients were significantly increased when compared to those obtained for HP women (Ghneim et al., 2016). MDA levels increased significantly in the women with spontaneous

abortion compared to the healthy pregnant women (Torkz ahr ani et al., 2019).

Generation of ROS is a consequence of metabolically active cells and it is likely that threshold levels of oxidative stress exist for promoting conception. The best available evidence suggested a varied diet with regular use of multivitamins, limited in caffeine and alcohol and maintenance of a healthy body weight may promote fertility (Ruder et al., 2008).

OS plays in modulating a range of physiological functions and its role in pathological processes affecting the female reproduction. OS modulates a host of reproductive pathol ogies affecting natural fertility in a woman's life and also menopausal transition and post-menopausal years. The role of OS is becoming increasingly important as there is new cumulative evidence suggesting that oxidative stress is involved in conditions such as abortions, preeclampsia, hydatidiform mole, fetal teratogenicity, preterm labor and intrauterine growth retardation, all of which lead to an immense burden of maternal and fetal, morbidity and mortality (Gupta et al., 2009). In the present study, MDA levels increased significantly in the study subjects compared to control subjects. Thus significant MDA level indicates increased lipid peroxidation which leads to increased oxidative stress in the study subjects.

It has been reported that hyper-secretion of basal LH with or without polycystic ovaries is a risk factor for miscarriage. Raised follicular phase serum LH levels increase the risk of miscarriage following either spontaneous conception or assisted conception. Elevated urinary LH excretion has been reported in 57% of women with recurrent miscarriage. Deleterious effects of high LH can be reversed by LH suppression using gonadotropin-releasing hormone analogs (Kaur et al., 2016). Endocrine disorders play a major role in approximately 8% to 12% of recurrent pregnancy loss (RPL). Endocrine abnormalities, including thyroid disorders, luteal phase defects, polycystic ovary syndrome, hyperpro lact inaemia and diabetes have to be evaluated in any case of RPL. Moreover, elevated androgen levels and some endocr ino logical aspects of endometriosis are also factors contributing to RPL (Pluchino et al., 2014).

According to Trout (2000), women with unexplained RPL have a greater incidence of elevated serum FSH and E (2) -Estradiol levels. When combined, FSH or E (2) levels, or both, were elevated in 58% of the unexplained RPL group and 19% of the control group. According to the present study FSH and LH levels are observed to be higher in study subjects with increased percentage of abnormal karyotype than control subjects.

CONCLUSIONS

It is important to understand the ways in which lifestyle behaviours may benefit or harm fertility in order to minimize complications and to maximize fertility outcomes. By understanding the impact of lifestyle on reproductive health, and by actively modifying lifestyle behaviours, men and women are capable of controlling their own fertility potential. The study demonstrated a positive correlation with karyotypic abnormality and various risk factors associated with BOH. The diagnosis of chromosomal anomalies at the exact time can lead to prevention of future birth of affected babies also pregnancy loss due to suspected genetic loss can also be reduced in the society to an extent. Further research is required into the mechanisms responsible for and also preventing the DNA damage including antioxidant therapy.

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Original research paper

Biochemical and endocrinological aspects of female infertility: Descriptive comparative study

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Abstract

Infertility is a disease of the reproductive system and its treatment can affect all aspects of people's lives, which can cause various psychological-emotional disorders or consequences including frustration, hopelessness, depression, guilt, anxiety and feelings of worthlessness in life. In order to evaluate Biochemical and endocrinological aspects of female infertility, a test-control study was designed. For the study 150 clinically diagnosed infertile female subjects and 150 age matched healthy females with one or more children were involved in the study as control. Level of uric acid concentration was comparatively higher among test group (5.5mg/dL) than the control subjects (4.4mg/dL) ($p<0.005$). The inflammatory marker (hsCRP) demonstrated a very high concentration among the test subjects than the control group (1.0±0.59) ($p<0.005$). It was evident that, study group expressed an FSH concentration of 30±10 and control with a FSH level of 12.8±6.00 ($p=<0.005$).

Keywords: Biochemical, endocrinological aspects, female infertility

Introduction

"Infertility is a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular unprotected sexual intercourse" [World Health Organization (WHO), (2020)]. Meanwhile the WHO's epidemiologic definition of infertility as "women of reproductive age at risk of becoming pregnant who report unsuccessfully trying for a pregnancy for more than two years" (WHO 2006) [1].
Cwikel *et al*. (2004) and Hämmerli *et al.* [2] (2009) reported that, "infertility is not a disease, it and its treatment can affect all aspects of people's lives, which can cause various psychological-emotional disorders or consequences including frustration, hopelessness, depression, guilt, anxiety and feelings of worthlessness in life". Studies done by Inhorn and Birenbaum-Carmeli and Greil *et al*. suggested that, "the infertility-related complexities and life experiences are highly influenced by the socio-cultural context in which the infertile person lives, so any comprehensive study on the subject with disregard to this context is pointless" [3].

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Parikh *et al*. (2012) [4] suggested in study that, "infertility shares some common pathways with Cardiovascular Diseases (CVDs)". Parikh *et al.* (2012) add on that, "Hypertension, thyroid dysfunction, diabetes, endocrinological factors and lifestyle related problems (obesity) are all known to be associated with CVD". Bevc *et al*. (2008) mentioned that, "CVD is the one of the most common causes of morbidity and mortality in the world and atherosclerosis is known to be the main reason for increased cardiovascular risk. Moreover, it is widely known that inflammation plays an important role in atherosclerosis" [5].
In 2017, Zegers-Hochschild *et al.* [6] mentioned in a study that, "infertility is further categorized as primary or secondary. The primary infertile female is a woman who has never been diagnosed with a clinical pregnancy and meets the criteria of being classified as having infertility". Vander and Wyns (2018) [7] illustrated that, "secondary female infertility applies to a woman unable to establish a clinical pregnancy but who has previously been diagnosed with a clinical pregnancy".
Hart (2016) suggested that, "the most powerful negative predictive factor of fertility is increasing women's age at conception, other factors including lifestyle and environmental factors are believed to play an increasing role". Study by Vander and Wyns mentioned that, "factors influencing fertility will be presented as gender specific or not". Mascarenhas *et al*. reported that, "there are 48.5 million couples are suffering with infertility worldwide" [8].

Methodology

A case-control study layout was adopted for the present study to identify and evaluate Biochemical and endocrinological aspects of female infertility. The test subjects were referred from various infertility clinics were chosen for the study. Demographic, physiological and lifestyle features were noted using Proforma. Venous blood samples were collected and used to measure CBMN assay, mutagen sensitivity analysis, hormonal assay and biochemical assay. Observations and outcomes were analyzed using the SPSS statistical software.
For the study 150 clinically diagnosed infertile female subjects and 150 age matched healthy females with one or more children were involved in the study as control.

Inclusion criteria

1. **Patients:** Clinically proven patients with infertility by a Gynecologist were included in the study.
2. **Controls:** Subjects without history of infertility, dyslipidemia, hypertension, diabetes, renal disease or other cardio vascular disease were not included as Controls.

Exclusion criteria

1. Neither the patients nor the controls should be suffering from any acute or chronic illness, cancer or on prolonged medication are excluded.
2. Subjects above the age of 45 and below the age of 18 are excluded.

Results

Table 1: Comparison study-FBS concentration among study and control subjects

FBS	Mean	SD(±)	p
Study Subjects	101	23.2	<0.005
Control Subjects	95.5	10.4	

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Table 2: Comparison study-total cholesterol concentration among study and control subjects

Total Cholesterol	Mean	SD (±)	p
Study Subjects	220	30.0	<0.005
Control Subjects	180	21.1	

Table 3: Comparison study-uric acid concentration among study and control subjects

Uric Acid	Mean	SD (±)	p
Study Subjects	5.5	1.25	<0.005
Control Subjects	4.4	1.00	

Table 4: Comparison study of hSCRP concentration

hSCRP	Mean	SD (±)	p
Study Subjects	3.0	0.70	<0.005
Control Subjects	1.2	0.59	

Table 5: Comparison study of TSH concentration among study and control subjects

TSH	Mean	SD (±)	p
Study Subjects	5.0	2.00	<0.005
Control Subjects	2.5	1.3	

Table 6: Comparison study of FSH concentration between study and control subjects

FSH	Mean	SD (±)	p
Study Subjects	30	10	<0.005
Control Subjects	12.8	6.0	

Table 7: Comparison study of LH level between study and control subjects

LH	Mean	SD (±)	p
Study Subjects	30.7	11.5	<0.005
Control Subjects	15	5.0	

Table 8: Comparison study of progesterone level among study and control subjects

Progesterone	Mean	SD(±)	p
Study Subjects	6.0	4.99	<0.005
Control Subjects	12.1	4.00	

Table 9: Comparison study of estradiol level between study and control subjects

Estradiol	Mean	SD	p
Study Subjects	90	29.8	<0.005
Control Subjects	115	41.12	

Table 10: Comparison of prolactin level among study and control subjects

PRL	Mean	SD	p
Study Subjects	36.10	8.54	<0.005
Control Subjects	13.34	5.20	

Table 11: Comparison of MDA level among study and control subjects

MDA	Mean	SD	p
Study Subjects	2.99	1.70	<0.005
Control Subjects	1.46	0.89	

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Table 12: Comparison of MCBMNF among study and control subjects

mCBMNF	Mean	SD	p
Study Subjects	13.00	1.25	<0.005
Control Subjects	9.00	0.75	

Table 13: Comparison of mean b/c value among study and control subjects

Mean b/c value	Mean	SD	P
Study Subjects	0.890	0.058	<0.005
Control Subjects	0.599	0.054	

Discussion

In 2014, Sirmans and Pate reported that, "the complex coordination of hormones that affects the reproductive cycle in women can be modified by certain conditions that cause altered ovulation. Polycystic ovary syndrome (PCOS) is the most common endocrine disorder of reproductive-aged women that affects 5% to 10% of women ages 15 to 44 years in the United States" [9]. Later, Bergh *et al*. (2016) pointed out that, "risk factors for PCOS in adults include type 1 and type 2 diabetes and gestational diabetes. Insulin resistance affects 50% to 70% of women with PCOS, which can lead to comorbidities including metabolic syndrome, hypertension, dyslipidemia, glucose intolerance, and diabetes" [10].

According to Sirmans and Pate (2014), "polycystic ovary syndrome (PCOS) has a cardiovascular effect that includes increased coronary artery calcium scores and increased carotid intima-media thickness. Additionally, women with PCOS are at increased risk of mental health disorders including depression, anxiety, bipolar disorder and binge eating disorder". Deyhoul *et al*. in 2017 reported that, "there are many hormonal disorders that cause infertility. Hypothyroidism, hyperprolactinemia (high male hormone levels) and luteal phase defect (low progesterone) are a few examples of these disorders. Hormonal disorders are a major cause of infertility in women. The inability of women at ovulation and regulation of hormone levels leads to too high or too low production of hormones" [9].

According to Meneses and Holland (2014) [11], "these hormonal disorders are characterized with symptoms such as irregular menstrual cycles, excessive bleeding, or very little bleeding, pelvic and abdominal cramps, absence of menstruation or long menstruation and excessive weight loss or weight gain". Deyhoul *et al*. (2017) mentioned that, "these following factors may cause hormonal disorders: gland problems such as thyroid gland, pituitary gland and hypothalamus gland problems. These preliminary glands are responsible for the production of sex hormones. Birth control pills, stress and some diseases such as hypothyroidism affect these glands. If any of these glands encounter any problem, a disorder can prevent from the full process of ovulation, and thereby, pregnancy will become difficult" [12].

In study done by Meneses and Holland (2014) it was pointed out that, "some treatments can cause hormonal disorders. Targeted cancer therapies can cause anatomical and hormonal changes which negatively affect the breast cancer patient's sexual potential. There are large differences in the evidence-based interdisciplinary treatment and management of breast cancer young patients who are treated and are fertile now and there are concerns about pregnancy after cancer treatment" [11].

According to Shahini *et al*. (2013), "MS is a very common disease in Western countries and includes multiple endocrine disturbances, such as overweight, altered levels of hepatocytolysis, arterial hypertension, obesity, dyslipidemia, and IR. MSi samaj or social health problem, particularlyin developed nations such as the United States but also in Europe, with a prevalence of 20 and 30%, respectively" [13].

Study done by Silvestris *et al*. in 2019 reported that, "several factors have been implicated and primarily include the hypercaloric diet in association with deregulated dietary habits,

sedentary lifestyles, increased age and augmented BMI. MS is also suspected to play a definite role in carcinogenesis, particularly in the gastrointestinal tract" [14].
Studies done by Iñiguez *et al*. (2008) and Livshits and Seidman *et al*. (2009) had demonstrated that, "females with MS, inadequate metabolic control and primary or secondary amenorrhea show low levels of LH and FSH, associated with a lack of residual insulin secretion".
Vujkovic *et al*. (2010) [15] reported that, "abnormalities of GnRH pulse generator, as well as a decrease in numbers and amplitude of LH pulses in patients with diabetes and amenorrhea compared to patients with normal menstrual cycles. On the other hand, IR, hyperinsulinemia and related metabolic abnormalities in MS may exert a role in the progress of the PCOS". According to Hammiche *et al*. (2011), "all therapeutic approaches used for the correction of insulin homeostasis in obese and MS patients, such as Thiazolidinedione's, Metformin, lifestyle modification for weight reduction or bariatric surgery have been proven to produce restoring effects on ovulation and hyperandrogenemia" [16].

Conclusion

FBS, total cholesterol, uric acid, hsCRP, TSH, FSH, LH, progesterone, estradiol, prolactin, MDA, mCBMNF and mean b/c value showed a statistical significance difference among study and control.

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ORIGINAL RESEARCH PAPER

Health Science

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ROLE OF CYTOGENETICS IN FIRST TRIMESTER ABORTION USING PRODUCT OF CONCEPTION AND EXTENT OF SOMATIC DNA DAMAGES IN MATERNAL BLOOD

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ABSTRACT

Spontaneous abortion (miscarriage) is most common during the first trimester of pregnancy. A fetal loss is occurring when the fetus has died within 8-12 weeks of gestation without heart activity has been recorded. Miscarriages affect 15% of women, primarily in the first trimester. Forty two women suffering with first trimester abortion were referred from various maternity centres of Kerala to Genetika, centre for advanced genetic studies, Trivandrum for chromosome analysis were taken as study subjects and twenty age sex match healthy women are selected as control subjects. The present study was undertaken to evaluate the role of cytogenetics in first trimester abortion using product of conception and extent of somatic DNA damages in maternal blood using Cytokinesis Block Micronuclei (CBMN) assay.

The results were computed and correlated with various other demographic, physiological, clinical and lifestyle characteristics. Regarding the karyotype of POC, 69% showed abnormal karyotype and 31% showed normal karyotype. The mean CBMN frequency of study subjects was 12.05 and control was 10.02 and showed a statistical significance. The study revealed that, the chromosomal abnormalities play an important role in first trimester pregnancy losses and these abnormalities were associated with various demographic, physiological, clinical and lifestyle characteristics. Thus, cytogenetic study should be offered to all couples especially for mother with two or more abortions. Patients who have had an unexplained pregnancy loss should be offered genetic counseling with an option for karyotype analysis for finding the role of cytogenetic in pregnancy losses. There by proper management of pregnancy losses in the future can be avoided.

INTRODUCTION

Spontaneous abortion (miscarriage) is most common during the first trimester of pregnancy. A fetal loss is occurring when the fetus has died within 8-12 weeks of gestation without heart activity has been recorded (Quenby, 2007). Miscarriages affect 15% of women, primarily in the first trimester (Clark et al., 2001). Among the heterogeneous causes of miscarriage, chromosomal abnormalities, mainly aneuploidies, occur most frequently. Although in the great majority of cases they are *de novo* mutations and their impact on the risk in subsequent pregnancies is compound. Identification of the cause of miscarriage plays an important role in genetic counseling (Massalska et al., 2017).

There are many reasons associated with higher rate of pregnancy loss. This include chromosomal abnormalities which are found in more than half of embryos miscarried in the first 13 weeks (Stephenson and Kutteh, 2007). A chromosomal abnormality derived from one parent or the recurrence of a numerical abnormality might be a cause of recurrent abortion. In about 50–70% of abortion, a chromosomal abnormality is identified in the products of conception (Hogge et al., 2003; Shawky and Kamal, 2012). Percentage of prenatal loss of chromosomally abnormal fetus is different according to type of aberration and it was estimated to be 100% loss for autosomal monosomy and tetraploid, 96.5% for autosomal trisomy and structural rearrangement constitutes up to 53.4% (Pflueger, 1999). Cytogenetic evaluation of product of conception (POC) is essential to determine the cause of pregnancy loss and aid the prenatal diagnosis of subsequent pregnancies. POC helps to profile cytogenetic abnormalities, their relationship with maternal and gestational age.

Globally chromosomal abnormalities have been rapidly increasing. Unexplained and spontaneous abortion affects approximately 1% of women; unexplained miscarriage remains a frustrating problem. There are several reasons may leads to an abortion but the genetic/core reason behind spontaneous abortion still remain unknown. Genetic study with product of conception on first trimester abortion can help to find out the reason behind spontaneous abortion. Due to high incidence and complex etiology of spontaneous abortion, it is of great importance to investigate the subsequent pregnancy outcomes in order to ensure an effective perinatal care.

More than one third of the approximately 205 million pregnancies that occur each year around the world is unplanned and about 20% of them end in induced abortion (Geetha Balsarkar et al., 2015). Miscarriages affect 15% of women, primarily in the first trimester period of pregnancy (Clark et al., 2001). Approximately 50% of all cases of early pregnancy loss are due to fetal chromosomal abnormalities (Alijotas-Reig et al., 2013). The frequency of clinically recognized early pregnancy loss for women aged 20–30 years is 9–17% and this rate increases sharply from 20% at age 35 years to 40% at age 40 years and 80% at age 45 years

(American Society for Reproductive Medicine, 2012). Several types of genetic problems like maternal structural chromosomal abnormalities and recurrent aneuploidies may be associated with recurrent miscarriage (Elghezal et al., 2007). Many risk factors are thought to be associated with early pregnancy loss is still unclear. Hence the present study was undertaken to evaluate the role of cytogenetics in first trimester abortion using product of conception and extent of somatic DNA damages in maternal blood using Cytokinesis Block Micronuclei (CBMN) assay.

MATERIALS AND METHODS

Forty two women suffering with first trimester abortion were referred from various maternity centres of Kerala to Genetika, centre for advanced genetic studies, Trivandrum for chromosome analysis were taken as study subjects and twenty age matched healthy women were selected as control subjects. Demographic, physiological, clinical and life style characteristics were collected using proforma. In this study, Cytokinesis Block Micronuclei (CBMN) assay was carried out in each subject for quantitating the extent of somatic DNA damages using maternal blood samples. Chromosome analysis was performed using product of conception. Chromosome preparation was done by taking tissue from product of conception and banded with GTG banding techniques (Seabright, 1971).

OBSERVATION AND RESULTS

Forty-two women with first trimester abortion and their products of conception were selected as study subjects and twenty age matched healthy subject were selected as control subjects. The maternal age was ranged from 20-45. The gestational weeks of study subjects were ranged from 7-15 weeks and most of them were of 12 weeks old at the time of abortion. The duration of married life of the study subjects was ranged from 1-19 with a mean duration of 4.79 years and number of gestations was ranging from 2-7 years. Among the 42 subjects, 34 subjects had number of spontaneous abortions. There are 11 study subjects reported with family history of thyroid disorder and 25 study subjects with history of illness and 18 subjects with a history of infection.

The results were computed and correlated with various other demographic, physiological, clinical and lifestyle characteristics. Regarding the karyotype of POC, 69% showed abnormal karyotype and 31% showed normal karyotype. The higher incidence of abnormal karyotype was found among subject with advanced maternal age. The incidence of abnormal karyotype was higher among subjects belonged to urban area.

The mean CBMN frequency of study subjects was 12.05 and for control subjects it was 10.02 and this showed a statistical significance difference with $p<0.05$. The physiological characters such as height, weight, BMI shown to have a direct correlation with mean CBMN frequency. Subjects with regular exercise and good physical activity showed decreased mean CBMN frequency as compared to subject with irregular exercise and poor physical activity. The study subject with history of infection showed higher incidence for abnormal karyotype and mean CBMN frequency. The abnormal karyotype and mean CBMN frequency was higher in subject with increased duration of married life. The abnormal karyotype and mean CBMN frequency was higher among subject with increased number of gestations.

DISCUSSION

Abortion is primarily a health concern of women but it is increasingly being governed by patriarchal interests which more often than not curb the freedom of women to seek abortion as a right. Findings indicate that, the abortion rate declined significantly in the developed world, but not in the developing world, between 1990 and 2014 (Sedgh et al., 2007). The present study also observed that, the increase in maternal age shows a high risk of pregnancy loss that is, the higher incidence of abnormal foetal karyotype were found among subjects with increase maternal age.

In the present study, it was observed that, the incidence of abnormal foetal karyotype was increased with increasing gestational weeks. In a study conducted by Kashanian et al (2006), it has been reported that, women whose first pregnancy resulted in miscarriage are at a higher risk of having miscarriage in the second pregnancy when, compared with women who had a live birth. Kiss et al (2009) found that, chromosome abnormalities were found in 5% of the couples with a history of two abortions, in 10.3% with three abortions and in 14.3% with four or more abortions. In a Patient with a history of two miscarriages, the subsequent risk of pregnancy loss was estimated about 25%, whereas subjects with history of 3 abortions, the subsequent risk of miscarriage were estimated to 33% (Dubey, 2005). The present study also demonstrated that, the incidence of abnormal karyotype was increased with increase in number spontaneous abortions and MTPs.

Infection may affect the placenta and there by harm the developing baby - cause premature labor, or lead to birth abnormalities. According to the National Institutes of Health (NIH), infections that are known to harm the developing baby including, bacterial vaginosis, which might cause preterm labor, contagious diseases, such as hepatitis, syphilis, herpes and HIV - which can infect the fetus. Chlamydia can cause eye infections. Pneumonia and gonorrhea, which can contaminate the amniotic fluid and cause preterm labor there by leads to eye infections and possible blindness. Fifth disease, which can trigger a miscarriage or cause fetal anemia, group B streptococcus, which can cause severe complications in newborns and in rare cases can be fatal, toxoplasmosis, which can cause birth abnormalities and intellectual disabilities. *Listeria* which can cause miscarriage, stillbirth and birth abnormalities. Cytomegalovirus which is often harmless but can also cause birth abnormalities and intellectual disabilities (Villines, 2019). In the present study also demonstrated that the incidence of abnormal karyotype was observed higher among subjects with history of infection.

Maternal obesity has been associated with adverse pregn ancy outcomes. Increased BMI increases the incidence of induction of labor, caesarean section, pre-term labor and macrosomia. The BMI of women in the first trimester of pregnancy is associated with the risk of adverse pregnancy outcome (Shahla Yazdani et al., 2012). In the present study also demonstrated that, the incidence of abnormal karyotype was observed among subject with increase BMI.

Drugs intake by the pregnant mother can affect the fetus in several ways. They can act directly on the fetus causing damage or abnormal development leading to birth defects or death. About 2-3% of all birth defects results from use of drugs. Risk of miscarriage almost doubles for the women who drink alcohol in any form during pregnancy. And birth weight of the babies is substantially below normal. This syndrome includes inadequate growth before or after birth, facial defects, a small head, mental retardation and abnormal behavioral development (Sullivan, 2004). The current study also observed that the prolonged use of drugs by women were leads to miscarriage and also showed that the incidence of abnormal karyotype were higher among subjects with history of drug intake.

To assess the various environmental exposures require data or assumptions regarding mother's location throughout pregnancy (Bell et al., 2012). In the present study it was demonstrated that, the incidence of abnormal karyotype was observed higher in urban area than rural and costal area. Environmental conditions in the area of mother's residence

during pregnancy have direct association with pregnancy and childhood health outcomes. These include exposure to ambient air pollutants during pregnancy that affects fetal growth and risk of birth defects.

CONCLUSION

The current study concluded that the chromosomal abnormalities play an important role in first trimester pregnancy losses and these abnormalities were associated with various demographic, physiological, clinical and lifestyle characteristics. Thus cytogenetic study should be offered to all couple especially for mother with two or more abortions. Patient who have had an unexpected pregnancy loss should be offered genetic counseling there by an option for karyotype analysis for finding the role of cytogenetics in pregnancy loss. The diagnosis of chromosomal anomalies at the exact time can lead to the prevention of future birth of affected baby and hence the incidence of chromosomal abnormality can be reduced to a certain extent in the society.

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Health Science

CYTOGENETIC AND MOLECULAR CYTOGENETIC STUDIES ON ABORTED FOETUSES

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ABSTRACT Miscarriage is spontaneous or induced interruption of pregnancy until 20 complete weeks. Miscarriages occur in approximately 15% of diagnosed pregnancies. A chromosomal abnormality derived from one parent or the recurrence of a numerical abnormality might be a cause recurrent abortion. The present study was conducted on thirty two medically terminated foetuses with gestational week between 14 to 20 week were included as test group and eighteen healthy children of the age ranged from 2 months to 2 years as control subjects. All these aborted foetuses were referred from various infertility clinics and maternity centers of Kerala to Genetika, Centre for advanced genetic studies, Trivandrum for chromosome analysis. Various demographic, physiological, clinical and life style characteristics of the couple (parents) were collected using proforma. Karyotype analysis was performed using intracardiac puncture blood sample of the aborted foetuses and venous blood samples were collected from the control subjects to detect chromosome abnormalities, if any. Cytokinesis-Block Micronuclei (CBMN) Assay was also performed on each sample by using cytochalasin B for quantitating the extent of somatic DNA damages. Regarding the foetal karyotype analysis, 52.94% (n=18) of the study subjects showed abnormal karyotype and 47.06% (n=16) showed normal karyotype. The mean CBMN frequency of study subject was greater than that of control subjects. The higher incidence of abnormal foetal karyotype and increased mean CBMN frequency was found among subjects with advanced maternal and paternal age. Chromosomal analysis is an important etiological investigation in couples with repeated spontaneous abortions as it helps in genetic counseling and deciding about further reproductive options. Modifying lifestyle habits and proper medication will help to avoid future pregnancy loss.

KEYWORDS : Recurrent abortion, Foetal karyotype, Chromosome abnormalities, DNA damage

INTRODUCTION

Miscarriage is spontaneous or induced interruption of pregnancy until 20 complete weeks. Miscarriages occur in approximately 15% of diagnosed pregnancies (Stephenson et al 2002) and although they are common, most women who have miscarriages give birth to a healthy child in later life. The probability for a couple to have two consecutive miscarriages ranges from 2.2 to 4%. A chromosomal abnormality derived from one parent or the recurrence of a numerical abnormality might be a cause recurrent abortion (Pflueger 1999).

Globally over 42 million abortions are performed annually and 10-15 percent of it takes place at second trimester that is between 13-28 weeks and third trimester abortions relatively less. The complication rate of abortions (second and third trimester abortions) is 13 times higher than that of first trimester abortions. Abortion-related complications account for approximately 13% of maternal deaths worldwide, roughly estimated as 47000 deaths per year. Second trimester abortion carries a higher risk of morbidity and mortality as compared to first trimester abortion especially in developing countries. Currently, there are conflicting reports regarding the rates of chromosomal abnormalities between recurrent and sporadic pregnancy losses. Some studies related to second trimester abortion are done previously still the genetic reason behind the second and third trimester was remaining unclear. It is necessary to understand the genetic reason behind spontaneous abortion for the diagnostic and therapeutic approaches of assisted reproduction.

Second trimester abortion is the termination of pregnancy in a period from 13 to 28 weeks of gestation, which again is subdivided into early period between 13 and 20 weeks and late period between 20 and 28 weeks (Lalitkumar et al 2007). Globally, over 42 million abortions are performed annually and 10–15% of the cases take place in second trimester period, over half of which are considered unsafe and disproportionately contribute to maternal deaths (Facts on Induced Abortion Worldwide 2007). In 2008, there were 29 abortions per 1,000 women aged 15–44 years in developing countries, compared with 24 per 1,000 in the developed world (Sedgh et al 2012). As researches showed, the prevalence of induced second trimester abortion was as high as 25%–30% in India (Mulat et al 2015). Chromosomal abnormalities in the foetus are the major causes of abortions. Detailed investigations are necessary to rule out the genetic basis of these types of abortions, so that proper solutions can be instituted. Hence the present study was undertaken to evaluate the cytogenetics and molecular cytogenetics study on aborted foetus. The specific objectives of the study are to evaluate the chromosomal abnormalities, if any, present in aborted foetuses by Karyotyping and to measure the extent of somatic DNA damage, if any, in aborted foetuses by Cytokinesis Block Micronuclei (CBMN) assay.

MATERIALS AND METHODS

The present study was conducted on thirty two medically terminated foetuses with gestation week between 14 to 20 week were included as test group and eighteen healthy children of the age ranged from 2 months to 2 years as control subjects. All these aborted foetuses were referred from various infertility clinics and maternity centers of Kerala to Genetika, Centre for advanced genetic studies, Trivandrum for chromosome analysis. Various demographic, physiological, clinical and life style characteristics of the couple (parents) were collected using proforma. Karyotype analysis was performed using intracardiac puncture blood sample of the aborted foetuses and venous blood samples were collected from the control subjects to detect chromosome abnormalities, if any. Cytokinesis-Block Micronuclei (CBMN) Assay was also performed on each sample by using cytochalasin B for quantitating the extent of somatic DNA damages.

OBSERVATIONS AND RESULTS

The karyotype of 32 study subjects were analyzed, among them paternal age of study subjects were ranged from 26-45 years with a

mean age of 33.5. The maternal age was ranged from 22-40 with a mean age of 29.58. The gestational weeks of study subjects were ranged from 14-20 weeks and most of them were of 14 weeks old at the time of abortion.The duration of married life of the study subjects was ranged from 2-11 with a mean duration of 4.79 years and number of gestations was ranging from 2-5 years. Among the 34 subjects, 19 subjects had family history of abortions and 15 subjects without family history of abortion. The number of spontaneous abortions among study subjects was grouped into two as ≤1 and ≥2. Majority of the study subjects belonged to ≥2.

The physiological characters included gestational weeks, H/o illness, no. of previous abortions, number of gestations and family history of abortions. Majority of the study subjects showed congenital abnormalities. The torch infections were observed among the study subjects. The Cyto Megalo Virus (CMS), Toxoplasmosis, Rubella Virus, Herpes Simplex Virus (HSV) infections and other infections were observed among the study subjects. History of illness and history of infection have significant role in this study so those are taken as another variable. The lifestyle characters included drug intake. Drug intake was taken as lifestyle variable. The clinical characters are included in this study are cleft lip with no cardiac pulsation, congenital anomalies, dysmorphism, depressed nasal bridge, multiple anomalies and down syndrome.

Regarding the karyotype analysis, 52.94% (n=18) of the study subjects showed abnormal karyotype and 47.06% (n=16) showed normal karyotype. While considering the foetal karyotype analysis, 53% showed abnormal foetal karyotype and 47% showed normal foetal karyotype. The mean CBMN frequency of 34 study subjects was 12.0. Among them 16 (47.06%) had normal chromosomal pattern and 18 (52.94%) had abnormal chromosomal pattern. The mean CBMN frequency of 18 control subjects was 9.98. The mean CBMN frequency of study subject was greater than that of control subjects.

The higher incidence of abnormal foetal karyotype was found among subjects with advanced maternal and paternal age. Moreover, the results showed significant relationship between paternal age and the mean CBMN frequency. As the paternal age increased the mean CBMN frequency has also increased. The abnormal karyotype was higher among subjects with increased duration of marriage and the mean CBMN frequency also increased along with increased duration of marriage. The abnormal karyotype was higher among subjects with higher number of gestation and increased gestational weeks and the mean CBMN frequency also increased along with increased number of gestations and increased gestational weeks. This is true regarding the incidence of abnormal karyotype was higher among subjects with increased number abortions and the mean CBMN frequency also increased along with increased number of abortions.

The study subjects with history of family abortions, infection, illness and drug intake showed high incidence of abnormal karyotype and the mean CBMN frequency was also increased on study subjects that had history of infection, illness and drug intake. The mean CBMN frequency was increased on study subjects having TORCH group of infections.

DISCUSSION

According to the study done by Tavokina et al (2006) it was observed that, "the frequency of chromosomal abnormalities among spontaneous miscarriages of the first trimester of pregnancy makes 50-60%". In 2005 Pflueger reported that, "pregnancy loss is quite common, with 15–20% of recognized pregnancies resulting in failure. The majority of these occur early in gestation, although losses in the second and third trimester are not rare. Approximately 2–5% of women will experience two or more losses. The majority of pregnancy failures are associated with cytogenetic abnormalities, with over 50% of early miscarriages and as many as 5% of stillbirths exhibiting abnormal karyotypes". In the current study it was observed that, the incidence of abnormal foetal karyotype was 52.94%. Moreover the incidence of abnormal foetal karyotype was more among subjects with increased number of previous abortions.

Stephenson et al (2002) suggested that, "miscarriage may occur in approximately 15% of diagnosed pregnancies and, although they are common, most women who have miscarriages give birth to a healthy child later in life". In a previous study done by Gardo (1993) reported that, "the probability for a couple to have two consecutive miscarriages ranges from 2.2 to 4%. Among the main causes for miscarriage are chromosome anomalies (whether numerical or structural), mostly represented by trisomies, by polyploidies and by the monosomy of sex-determining chromosome X". Morton et al (1987) observed that, "most miscarriages occur in the first trimester, generally between eight and 12 weeks, and half of these are caused by chromosome anomalies. On the other hand, approximately 99% of pregnancies with chromosome anomalies evolve to miscarriage".

Capalbo et al (2015) observed that, "birth defects have been observed in 3% of the live births, with a significant proportion of these defects (20%) related to chromosomal abnormalities or gene mutations. Some of the aneuploidy, including anioplose, 13, 18, 21, X, and Y chromosomes can lead to the birth of the baby alive and abnormal". Marquard et al in 2010 reported that, "more than 50% of couples who have a history of recurrent abortions are referred to as unspecified or idiopathic causes". Studies done by Handyside et al (2010) and Feichtinger et al (2015) showed that, "chromosomal analysis can determine the cause of 80% of cases of repeat unexplained abortions in women over 35 years of age". In previous study done by Hassold et al (2001), "mother's age is the most important factor that directly affects the frequency of chromosomal abnormalities in the embryo". In the present, study subjects with history of family abortions, infection, illness and drug intake showed high incidence of abnormal karyotype and the mean CBMN frequency was also increased on study subjects that the rest.

Maternal age also plays a significant role in the incidence of recurrent miscarriage. This suggests that pregnancy abnormality is a significant contributory factor to miscarriage given that the incidence of pregnancy abnormality increases with maternal age (Quenby and Farquharson, 1993). The risk of miscarriage for women younger than the age of 24 years is 9.5%. With age this risk rises, it increases to 11% by the age of 30 and reaches 33% in women aged 40. The incidence increases dramatically to 53% in those women over the age of 44 (Quenby and Farquharson, 1993). In the present study, the incidence of abnormal foetal karyotype was more among foetuses having increased maternal and paternal age. Moreover, it was also revealed that as paternal age increases there was increase in abortion rates and the mean CBMN frequency was high in higher age group.

In 2013 Hoffman et al reported that, "Congenital heart disease (CHD) is the most common congenital birth defect. CHD accounted for nearly one third of all birth defects, and the prevalence rate reached to 8 to 12 per 1000 live births worldwide". Jenkins et al (2007) have demonstrated that, "a number of genetic and environmental factors have been associated with the development of CHD in the foetus. Furthermore, some risk factors have been identified, such as phenylketonuria, rubella, retinoic acid, and the use of certain specific drugs. Moreover, mothers infected with a virus during pregnancy may be at a higher risk of developing CHD in offspring". Ye et al (2019) suggested that, "the risk of CHD in offspring was significantly increased among mothers with viral infections, the reasons are not clear, which was rarely discussed in previous studies". In a previous study by Waldorf et al (2013) have shown that, "rubella virus, herpesvirus, and cytomegalovirus were human teratogens that could cause a spectrum of birth defects, including blindness, deafness, CHDs, mental retardation and central nervous system complications, if the viral infection is acquired in the early months of pregnancy". In the present study also it was observed that, maternal infection showed an important role in abnormal growth of foetus.

Griffin et al (1995) reported that, "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". According to the study by Sartorelli et al (2001), "the probability of producing aneuploid offspring is increased in older men and there are higher frequencies of sperm chromosome aberrations". In the present study an increased micronuclei frequency was observed among study subjects with increased paternal age.

SUMMARY AND CONCLUSIONS

Various lifestyle and environmental factors are directly responsible for higher extent of DNA damage that causes abnormal foetal development. The present study concluded that molecular, cytogenetic and immunological factors play an important role in spontaneous

abortions. Chromosomal analysis is an important etiological investigation in couples with repeated spontaneous abortions as it helps in genetic counseling and deciding about further reproductive options. The diagnosis of chromosomal anomalies at the exact time can lead to the prevention of future birth of affected baby and hence the incidence of chromosomal abnormalities can be reduced to a certain extent in the society. Modifying lifestyle habits and proper medication will help to avoid future pregnancy loss.

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Original research paper

A study on relation between hormonal parameters and risk markers in infertility

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Abstract

One in every four couples in developing countries is affected by infertility. The magnitude of the problem calls for urgent action, particularly when the majority of cases of infertility are avoidable. Of 60-80 million couples suffering from infertility every year worldwide, probably between 15 and 20 million (25%) are in India alone. A case-control study layout was adopted for the present study on relation between hormonal parameters and risk markers in infertility the test subjects were referred from various infertility clinics were chosen for the study. Out of 150 study subjects, 100 infertile women showed increased FSH concentration and they revealed increased MDA concentration, mean b/c value and mCBMNF. Infertile women with low concentration of estradiol demonstrated an elevated MDA concentration, mCBMNF and mean b/c value than others with increased level of estradiol. Out of 150 study subjects, 115 individuals showed an increased PRL concentration and they revealed increased values of MDA concentration, mCBMNF and mean b/c value.

Keywords: Hormonal parameters, risk markers, infertility

Introduction

WHO (1991) reported that, "infertility is a serious health issue worldwide, affecting approximately 8%-10% of couples worldwide". According to Poongothai *et al*. (2009), "of 60-80 million couples suffering from infertility every year worldwide, probably between 15 and 20 million (25%) are in India alone". Mascarenhas *et al*. (2012) suggested that, "one in every four couples in developing countries is affected by infertility. The magnitude of the problem calls for urgent action, particularly when the majority of cases of infertility are avoidable" [1].

As per the report of WHO, Calverton (2004) mentioned that, "the overall prevalence of primary infertility ranged between 3.9% and 16.8%". In a previous study by Talwar *et al*. (1986) it was estimated that, infertility vary widely among Indian states from 3.7% in Uttar Pradesh, Himachal Pradesh and Maharashtra, to 5% in Andhra Pradesh, and 15% in Kashmir.

In 2007, Kumar reported, "the prevalence of primary infertility has also been shown to vary across the tribes and castes within the same region in India". According to the study by Shamila and Sasikala (2011) it was reported that, "the prevalence of female infertility was 45.67% in Kanyakumari, 44.24% in Thirunelveli and 41.91% in Thiruvananthapuram" [2].
Sadock and Sadock (2011) observed that, "40% of infertility cases were related to men, 40% of women and 20% of both genders". According to a multicentric study conducted by WHO (1987) it was point out that, "from 1982 to 1985, 20% of cases were attributed to male factors, 38% to female factors, 27% had causal factors identified in both partners and 15% could not be satisfactorily attributed to either partner" [3].
In 2011, Unuane *et al*. suggested that, "female infertility occurs in about 37% of all infertile couples". In a study by Kumar and Singh (2015) mentioned a report on the status of infertility in India i.e., "nearly 50% of infertility is related to the reproductive anomalies or disorders in the females. In addition, over 25% of infertility cases, no detectable cause can be traced after routine tests, which leaves the case as unexplained infertility"[4].
According to Domar *et al*. (1990), "female infertility accounts for up to 70% of these cases, largely due to the very complex processes involved in the female reproductive system". In 2013, Direkvand-Moghadam *et al*., "the incidence of female infertility is rising and varies from 10 to 20%". Agarwal and Allamaneni (2004) suggested that, "infertility is a common problem; treatment is sometimes inadequate because the aetiology is not fully understood" [5].
In 2005, a study by Agarwal *et al*. mentioned that, "the absolute number of couples seeking infertility services has increased dramatically". Olooto *et al*. (2012) reported that, "female infertility is caused by genetic, hormonal, or environmental factors". In addition, Olooto *et al*. (2012) add on that, "pelvic inflammatory disease, uterine fibroids, age-related factors, tubal blockage and hostile cervical mucus can cause infertility in females" [6].

Methodology

A case-control study layout was adopted for the present study on relation between hormonal parameters and risk markers in infertility. The test subjects were referred from various infertility clinics were chosen for the study. Demographic, physiological and lifestyle features were noted using proforma. Venous blood samples were collected and used to measure CBMN assay, mutagen sensitivity analysis, MDA and hormonal assay. Observations and outcomes were analyzed using the SPSS statistical software.

Study variables

- **Hormonal parameters:** Serum PRL, Serum Progesterone, Serum LH, Serum FSH, Serum TSH, Serum Estradiol.
- **Genetic instability and oxidative stress parameters:** CBMN assay, mutagen sensitivity analysis and MDA.

Study subjects

In order to evaluate the role of OxS and genetic instabilities among subjects with female infertility, a test-control study was designed. For the study 150 clinically diagnosed infertile female subjects and 150 age matched healthy females with one or more children were involved in the study as control.

Inclusion criteria

- **Patients:** Clinically proven patients with infertility by a Gynaecologist were included in the study.

- **Controls:** Subjects without history of infertility, dyslipidemia, hypertension, diabetes, renal disease or other cardio vascular disease were not included as Controls.

Exclusion criteria

- Neither the patients nor the controls should be suffering from any acute or chronic illness, cancer or on prolonged medication are excluded.
- Subjects above the age of 45 and below the age of 18 are excluded.

Results

Table 1: Comparison study of risk markers and TSH level

TSH	Number	MDA	mCBMNF	Mean b/c value
≤4	75	2.4	12.10	0.750
>4	75	3.00	12.50	0.799

Infertile females within creased TSH level showed a higher MDA concentration, elevated mCBMNF and mean b/c value.

Table 2: Comparison study of risk markers and FSH level

FSH	Number	MDA	mCBMNF	Mean b/c value
≤21.5	50	2.00	12.47	0.742
>21.5	100	2.95	12.50	0.800

Out of 150 study subjects, 100 infertile women showed increased FSH concentration and they revealed increased MDA concentration, mean b/c value and mCBMNF.

Table 3: Comparison study of risk markers and LH level

LH	Number	MDA	mCBMNF	Mean b/c value
≤12.5	10	2.80	12.12	0.735
>12.5	140	3.08	13.10	0.780

Infertile women with elevated LH showed comparatively higher MDA concentration, mCBMNF and mean b/c value than the rest.

Table 4: Comparison study of risk markers and progesterone level

Progesterone	Number	MDA	mCBMNF	Mean b/c value
≤20	137	2.90	13.00	0.810
>20	13	1.72	12.39	0.768

Infertile women with reduced progesterone levels reported higher MDA, mCBMNF and mean b/c value than those with higher progesterone concentration.

Table 5: comparison study of risk markers and Estradiol level

Estradiol	Number	MDA	mCBMNF	Mean b/c value
≤120	124	2.95	12.80	0.801
>120	26	2.50	12.62	0.789

Infertile women with low concentration of estradiol demonstrated an elevated MDA concentration, mCBMNF and mean b/c value than others with increased level of estradiol.

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Table 6: Comparison study of risk markers and PRL level

PRL	Number	MDA	mCBMNF	Mean b/c value
≤29	35	2.70	12.42	0.777
>29	115	2.98	12.59	0.780

Out of 150 study subjects, 115 individuals showed an increased Prolactin concentration and they revealed increased values of MDA concentration, mCBMNF and mean b/c value.

Discussion

According to Szczepańska *et al*. (2003), "Oxidative Stress (OxS) biomarkers have been found in various sites in the female reproductive tract, suggesting their role in various physiological functions". In 2001, Polak *et al*. suggested that, "ROS are involved in various causative factors of infertility, i.e. tubal factor, peritoneal factor, endometriosis and unexplained infertility" [7].

Van Langendonckt *et al*. (2002) explained that, "the scientific basis of unexplained infertility remains a challenge and OxS may have a role in its pathophysiology. The role of OxS in infertility is not completely ascertained". Szczepańska *et al*. (2003) point out that, "a number of studies have evaluated the role of OxS in tubal factor infertility, endometriosis and peritoneal factor infertility [8].

The tubal and peritoneal microenvironments influence fertilization and early embryonic development". Agarwal *et al*. (2003) suggested that, "elevated concentrations of ROS in these environments may have detrimental effects on the spermatozoa, oocytes, sperm oocyte interaction and embryos both in the fallopian tube and the peritoneal cavity". In 2005 another study by Agarwal *et al*., "activated macrophages have been implicated in the pathogenesis of endometriosis. These macrophages are the source of increased generation of ROS in the peritoneal environment associated with endometriosis" [6].

According to Kumar (2007), "infertility is a global health issue, affecting approximately 8-10% couples worldwide". According the report of "World Health Organization", Adamson *et al*. in 2011 estimated that, "60 to 80 million couples worldwide currently suffer from infertility". Vander and Wyns (2018) reported that, "from 1950 to 2010 and projections to 2050, as measured by the average number of births over a woman's lifetime" [9].

Parikh *et al*. (2012) has been suggested that, "infertility may share some common pathways with CVD. Polycystic ovarian syndrome (PCOS), obesity and thyroid dysfunction are all known to be associated with CVD". In a study by Sotiriadis *et al*. (2007) explained that, "Hypercoagulable states or thrombophilia may contribute to early miscarriages, a potential unrecognized cause of subfertility" [10].

In a previous study by Andrews *et al*. (1991) point out that, "women with fertility have also increased levels of psychological stress, as manifested in conditions such as depression and anxiety, which may contribute to CVD" [11]. In 2012 Agarwal *et al*. explained that, "OxS, which has an important role in the development of CVD, is also increased in infertile patients with conditions such as endometriosis, PCOS, obesity and unexplained infertility". However, the association between female infertility and CVD was not yet studied [6].

According to Martin (2008), "DNA damage is a form of cell stress and injury that has been implicated in the pathogenesis of many neurological disorders". In 2003, Andreassi explained that, "DNA damage is caused by multiple endogenous and exogenous factors such as OxS, age, smoking, hypertension, hyperlipidemia and diabetes mellitus". In another study by Andreassi *et al*. (2005) it was reported that, "diabetes is a major determinant of somatic DNA instability". Simon *et al*. (2011) has been proved that, "OxS can provoke extensive oxidative DNA damage, DNA strand breaks and chromosomal aberrations" [12].

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Conclusion

- Hormonal Parameters such as, TSH, FSH, LH and Prolactin showed positive correlation with mCBMNF, MDA and Meanb/c value.

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