Cytogenetics and Y Chromosome Microdeletion in Azoospermic Males - A Retrospective Cohort Study from a Tertiary Care Hospital in Kerala

Sreejith Pongillyathundiyil Sasidharan[1], Sheila Balakrishnan[2], Krishna Govindan[3], K.R. Chandramohanan Nair[4]

[1]Multi-Disciplinary Research Unit, Government Medical College, Thiruvananthapuram, Kerala, India.
[2]Department of Reproductive Medicine, Government Medical College, Thiruvananthapuram, Kerala, India.
[3]Department of Pathology, Government Medical College, Thiruvananthapuram, Kerala, India.
[4]Department of Anatomy, Government Medical College, Thiruvananthapuram, Kerala, India.

ABSTRACT

BACKGROUND

Infertility is the inability to become a parent of a child even after one year of intercourse involving male and female partner without any contraceptives. There are many causes for infertility, Y chromosome microdeletion is one among that. Partial or complete deletion of the proximal Yq region, which contains azoospermic factor (AZF) locus, leads to infertility. Along with genetic and biochemical factors the ethno-geographical reasons also play an important role in infertility. The present study was done to identify the association of genetic and hormonal factors in the development of infertility in azoospermic males of southern Kerala.

METHODS

Retrospectively screened the medical records of 2100 infertile males of the Department of Reproductive Medicine of Sree Avittom Tirunal Hospital, Government Medical College, Thiruvananthapuram for a period from January 2017 to December 2019. Stringent inclusion criterias were taken to select patients for the molecular study and finally 46 were selected. Structural and numerical chromosome abnormalities were detected using karyotyping and microdeletion was identified using polymerase chain reaction. Electro-chemiluminescence immunoassay method was used for the quantification of reproductive hormones. Demographic data of selected patients were collected from the medical records.

RESULTS

The cytogenetic results showed that among the selected patients, 10.86 % had Klinefelter syndrome and one person had De la Chapelle syndrome. Partial microdeletion in AZFa, b or c regions have been observed in 13.63 % of the patients. The hormonal analysis showed significant change in concentration of reproductive hormones irrespective of genetic defects. Demographic data showed that the majority of participated patients are unskilled/skilled laborers, economically poor and are from urban areas.

CONCLUSIONS

The study concludes that among the selected patients, 24.49 % have clinically significant chromosomal abnormalities like Klinefelter syndrome, De la Chappelle syndrome and partial microdeletion on the AZF region. Irrespective of genetic defects, significant changes in the concentration of reproductive hormones are also observed.

KEYWORDS

Azoospermia, Infertility, Karyotyping, Y Chromosome Microdeletion

Corresponding Author:
Dr. K.R. Chandramohanan Nair,
Former Senior Scientific Officer,
Department of Anatomy,
Government Medical College,
Thiruvananthapuram, Kerala, India.
E-mail: chandramohan1964@yahoo.co.in

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BACKGROUND

Infertility is the inability to become a parent of a child even after one year of trying to conceive by the partner without any contraceptives. Among the many causes, defective spermatogenesis is a major factor for infertility. In humans as well as animals, spermatogenesis is an intricate developmental procedure which leads to the formation of mature spermatozoa. The process of spermatogenesis is regulated by the synchronized activities of genetic factors, reproductive hormones and ethno-geographical conditions. A variation in any one of these will cause accumulation of error and end up with impaired spermatogenesis and leads to infertility.[1] Male infertility is defined as the inability of a male to cause pregnancy in a fertile female. It accounts for 40 – 50 % of total infertility cases in human. The male infertility can affect 7 % of all men and can be due to many factors. About 25 – 55 % of the male infertility cases with serious testicular defects (sperm maturation arrest and hypo-spermatogenesis and sertoli cell only syndrome) and 5 – 25 % patients with oligozoospermia/azoospermia are owing to genetic deletions.[2]

Y chromosome related infertility is a situation which directly influences the formation of sperm and leads to male infertility and causes the patient not to become father of a child. In men, the Y chromosome contains a region called MSY (male-specific region of the Y chromosome) which is an integral part for spermatogenesis. The MSY regulate a series of vital functions viz. transcription, regulation of gene expression, maintenance of microtubule ubiquitination etc. The most common genetic cause for defective spermatogenesis is deletion of a specific genetic fragment in MSY known as Y chromosome microdeletion. In 1976, a terminal genetic deletion in the long arm (11q) positioned in MSY was discovered in azoospermia men. Based on phenotypic correlation of patients with genetic deletions, a hypothesis was formulated and the vital function of the region was identified in spermatogenesis and the region was mentioned as the azoospermia factor (AZF).[3] Y chromosome microdeletion is one such important factor, where partial or complete removal of the proximal Yq region can occur.[4] Studies on extensive chromosomal deletion mapping has revealed a correlation of five different genetic deletions on proximal Yq region with infertility.[5] These genetic locations on AZF locus are termed *AZFa* (proximal), *AZFb* (central) and *AZFc* (distal) regions, which contain multiple genes for spermatogenesis.

The *AZFa* region carrying DDX3Y and USP9Y (ubiquitin specific peptidase 9, Y-linked) genes, are connected with azoospermia and sertoli cell–only histology. The AZFb region, accommodating many copies of *RBMY* and *PRY* (PTPM13-like, Y-linked) genes, are related with severe oligozoospermia and germ cell arrest at primary spermatocyte stage and hypo-spermatogenesis. AZFc region contains DAZ (deleted in azoospermia) gene which is responsible for germ cell arrest and hypospermatogenesis.[6] Deletion on the above specific genes on AZF region will cause corresponding effects on spermatogenesis and leads to infertility.

In infertility clinics, screening of deletion on AZF region has now become part of regular diagnostic practice.[7,8] Compared to other genetic abnormalities, microdeletions are too small to be identified through karyotyping. The European Academy of Andrology (EAA) and the European Molecular Genetics Quality Network (EMQN) have published guidelines to eliminate inaccuracies and misleadings in the genetic diagnosis.[9] The guideline has dramatically uplifted the diagnostic and prognostic value of microdeletion in infertility treatment. Earlier studies have reported population specific variations in the prevalence of Yq microdeletions ranging from 0 - 28 %.[8] The genetic identity of south India population is distinct from their northern and western counterparts. Therefore, region wise molecular studies are essential for unraveling the prevalence of microdeletion patterns in Indian population. There is no major study available from the southern Kerala population to identify their microdeletion pattern in azoospermic males. Hence the present study was done to identify the percentage occurrence of genetic and hormonal variations in azoospermic males of southern Kerala.

Objectives

The primary objective of the study was to identify the pattern of *AZF* gene microdeletions in patients with azoospermia. The secondary objective was to identify the variations in concentrations of major reproductive hormones in patients with azoospermia and genetic defects.

METHODS

In the present retrospective study, we have screened medical records of 2100 infertile males aged 20 to 47 years. All selected individuals were consulted at the Department of Reproductive Medicine of Sree Avittom Tirunal Hospital, Government Medical College Thiruvananthapuram, India, over a period of three years from January 2017 to December 2019. Majority of them were from the four southern districts of Kerala viz., Thiruvananthapuram, Kollam, Pathanamthitta and Kottayam.

Out of the total infertile males, only azoospermia patients were selected for the present study. Patients with known genetic abnormalities, pyospermia, history of chemotherapy or radiotherapy, recent febrile illness, recent androgen administration, absence of vas deferens, hypogonadism or low volume ejaculate were excluded. Among the 2100 infertile males, 202 were azoospermic. They were contacted over telephone and 46 had participated in the molecular study. All experiments in human samples were carried out after obtaining the informed consent of the patients and clearance from the institutional human ethics committee (IECNo.06/06/2016/MCT. 17/11/2016).

Cytogenetic analysis: All the selected individuals were investigated cytogenetically by somatic karyotyping. From each patient 5 ml blood was collected for the analysis. The peripheral lymphocytes were cultured and stimulated with Phytohemagglutinin (PHA) for 72 hrs in Roswell Park Memorial Institute (RPMI) 1640 medium with 10 % fetal

bovine serum. Standard cytogenetic techniques were used for chromosomal slide preparation.[10] G banded metaphase chromosomes were observed by microphotography. Karyotypes were described according to the International system for chromosome nomenclature (ISCN).[11] Minimum of 20 metaphase cells were counted for each individual and analyzed. The karyotyping analysis was done at child developmental centre, SATH, Government Medical College Thiruvananthapuram.

Polymerase chain reaction (PCR); Genomic deoxyribo nucleic acid (DNA) was extracted from 200 µl peripheral blood samples of the selected patients using QIAamp DNA Mini Kit (Quiagen, Hilden, Germany). Quality and quantity of extracted genomic DNA was determined using a biophotometer at 260/280 nm (eppendorf Hamburg, Germany). According to the EAA/EMQN guidelines, six sequence tagged sites (STS), sY84, sY86, sY127, sY134, sY254 and sY255 were selected as targets for PCR.

Each STS primer was amplified in a specific region of the AZF locus located in the long arm of Y chromosome as specified in Table 1. Singleplex PCR method was used for the detection of microdeletion on *AZFa, AZFb* and *AZFc* region. In each PCR reaction, genomic DNA of one fertile male and female was kept as positive and negative control respectively. *SRY* and *ZFX* genes were included as standard references.[12]

The PCR assay was repeated twice in case of patients with microdeletion in AZF loci for confirmation. The PCR reaction was performed in a reaction volume of 20 µl consisting of 5ng gDNA and ready to use PCR master mix (Quiagen, Hilden, Germany). The PCR condition involved, initial denaturation at 94^{o}C for 10 min, followed by 35 cycles of denaturation at 94^{o}C for 30 sec, annealing at 58^{o}C for 30 sec, extension at 72^{o}C for 1 min and final extension at 72^{o}C for 10 min. The results were analyzed using 2 % agarose gel electrophoresis of the amplified product. The presence or absence of amplified PCR products depended on the occurrence of AZF region in the Y chromosome.

Analysis of reproductive hormones; Concentration of serum reproductive hormone viz, follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone and prolactin were screened following electro-chemiluminescence immunoassay method (Roche - cobas e 411analyzer, Roche Diagnostics, Mannheim, Germany). The normal range of the selected hormones is FSH: 1 - 12 mIU/mL, LH: 1.5 – 10 mIU/mL, testosterone: 2.45 – 10.0 ng/mL and prolactin: 3.1 – 16.5 ng/mL.

Collection of demographic data; Medical history, demographic and socioeconomic data including education status of the individuals were collected from the medical record of SATH.

Statistical Analysis

Categorical variables were expressed as frequency and percentage. Continuous variables were expressed as mean, standard deviation, minimum and maximum.

RESULTS

Karyotyping

Results of the study showed that among the selected individuals 10.86 % exhibited Klinefelter syndrome, Figure 1 and one had De la Chapelle syndrome (46XX male).[13] Autosome chromosomal abnormalities like polymorphism, translocation and inversion were observed in 8.69 % of the selected individuals. Previous studies attributed that the prevalence of chromosomal abnormalities in azoospermic males depends on the studied population and the range varied between 15 to 25 %.[14] The present study also supports previous results on correlation of azoospermia and Klinefelter syndrome.

PCR Analysis

The incidence of Y chromosome deletion in a population depends on the severity of genetic defects. In the present study, polymerase chain reaction method confirmed partial microdeletion on selected STS in AZF locus of the study subjects. The study revealed that 9.09 % of the patients have Y chromosome microdeletion in sY84 region (*AZFa*), 2.27 % of the patients have sY127 deletion (*AZFb*) and 2.27 % of the patients have sY254 deletion (*AZFc*). Microdeletion in other AZF gene locus on sY86, sY 134 and sY255 were not observed in this study, Figure 2.

Hormonal Analysis

The results of hormonal analysis are depicted in table 2. Present study showed that 72 % of the patients have FSH concentration higher than the reference value.

Sl. No.	STS	Primer Sequences
1	sY84	F5′AGAAGGGTCCTGAAAGCAGGT3′ R5′GCCTACTACCTGGAGGCTTC 3′
2	sY86	F5′GATGTCAAGGCTGCAGATC 3′ R5′GCCCAGTCTTTGGGATTTC 3′
3	sY127	F5′CCTTATATGGGTGAGCCAGATG 3′ R5′ACACAGACAGGGAAATCTCCAG 3′
4	sY134	F5′GTCTGCCTCACCATAAAACG 3′ R5′ACCACTGCCAAAACTTTCAA 3′
5	sY254	F5′GGGTGTTACCAGAAGGCAAA 3′ R5′GAACCGTATCTACCAAAGCAGC 3′
6	sY255	F5′GTTACAGGATTCGGCGTGAT 3′ R5′CTCGTCATGTGCAGCCAC 3′
7	SRY	F 5′GCTGGTGCTCCATTCTTGAG 3′ R5′ GAATATTCCCGCTCTCCGGA 3′

Table 1. Primer Sequences Used for PCR Analysis of Sequence-Tagged Sites (STS)

	Minimum Value	Maximum Value	Median	Mean	SD
Testosterone (ng/ml)	0.60	4.85	3.35	3.09	1.23
FSH (mIU/ml)	1.72	61.63	21.50	24.41	17.32
LH (mIU/ml)	2.20	28.60	9.05	11.96	7.84
Prolactin(ng/ml)	3.00	17.60	6.10	6.94	3.35

Table 2. Hormonal Concetration in the Selected Patients (Mean and SD Values)

Another major variation observed was in the case of Luteinizing hormone, wherein 41 % of patients showed higher than normal range. A similar trend was shown by 13

% of patients in case of testosterone and 4.5 % in prolactin, Figure 3.

Demographic Data

Socio economic status of selected individuals showed that the patients approaching SATH for infertility treatments are economically poor and are mainly from urban areas. Majority of the patients are unskilled/skilled laborers with basic education. It has also been noticed that the majority of the patients have done preliminary clinical investigations like blood tests, hormone analysis and scanning. But none of the patients has done cytogenetic or molecular analysis even before moving on to advanced treatments like in vitro fertilization (IVF), testicular sperm extraction (TESE), intracytoplasmic sperm injection (ICSI) etc.

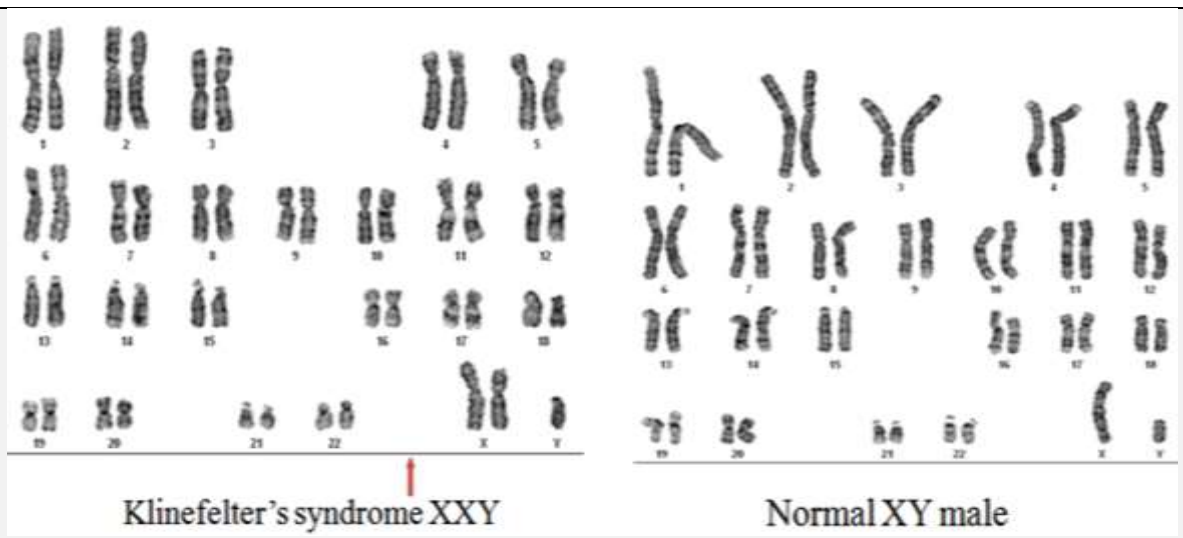

Figure 1. Chromatogram of Patients with Normal and Klinefelters Syndrome

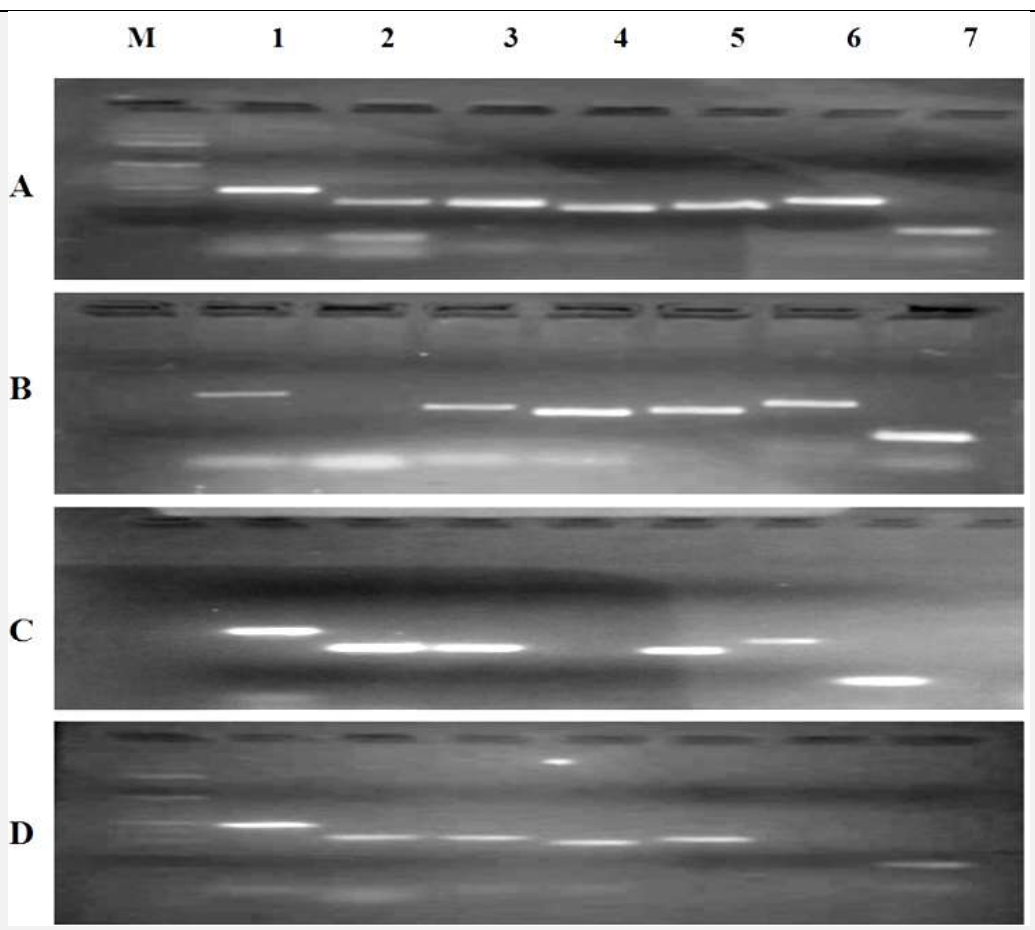

Figure 2. Amplified Products of STS Primers in 2 % Agarose Gel. Lane M. 100bp Marker, 1. SRY (472 bp), 2. sy 84 (320 bp), 3. sy 86 (326 bp), 4. sy 127 (274 bp), 5. sy 134 (301 bp), 6. sy 254 (400 bp), 7. sy 255 (126 bp). A Normal, B. sy 84 Deletion, C. sy 127 Deletion, D. sy 254 Deletion

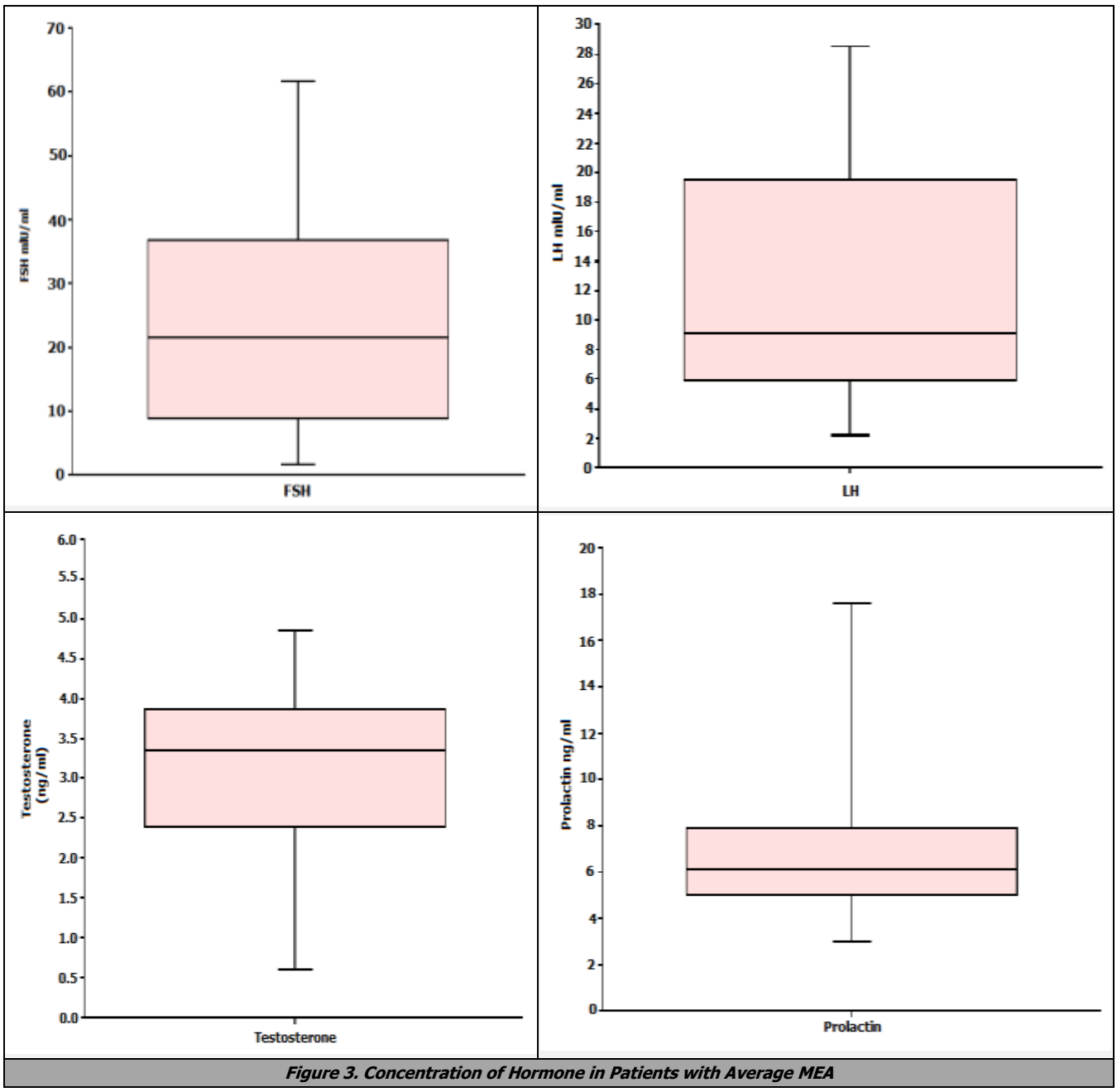

Figure 3. Concentration of Hormone in Patients with Average MEA

DISCUSSION

Among the various etiological factors, genetic defects have a key role in the development of male infertility. The frequency of genetic abnormalities vary from 2.1 to 28.4 % in infertile male compared to less than 1 % in the general male population.[15] Among the sex chromosome abnormalities, Klinefelter syndrome is the commonest, and Y chromosome deletions are the second major genetic alterations.[16] In infertility treatment, the screening of Yq microdeletion has now become a standard test in several countries.[8] Deletion of genes located in AZF locus on Yq chromosome can cause a spectrum of phenotypes ranging from azoospermia to normospermia. However, the amount of genetic material lost is a determining factor behind those phenotypic changes.[17] Microdeletion of Y chromosome causes partial, complete or combinations of AZF a, b or c gene losses. The type and pattern of microdeletion is not only a diagnostic tool but also an important prognostic protocol. It helps to determine the success of sperm retrieval and even for foreseeing the percentage of success in assisted reproduction.[18] Deletions of different AZF genetic regions are phenotypically or functionally correlated with diverse capacities of sperm production. Up to 75 % of infertile men with Y chromosome microdeletion in the *AZFc* region will have sperm in the ejaculate and can be retrieved by TESE. Whereas infertile men with microdeletions in *AZFa* or *AZFb* regions always have azoospermia and may not have viable sperm.[19]

The study subjects showed an age range of 28 to 47 years with a mean age of 35.2 ± 4.5. Majority (53.6 %) were in the age group of less than 35 years and 42.9 % were between 36 to 40 years. In the present study structural and numerical chromosome abnormalities in all the selected individuals were screened by karyotyping and PCR. The results showed that among the selected patients 10.86 %

exhibited major chromosome abnormality, Klinefelter syndrome, whereas few patients showed autosomal genetic defects.

The study revealed that 9.09 % of the selected patients had *AZFa* partial deletion at sY84. The semen analysis data showed all patients with *AZFa* microdeletion were azoospermic as well. Hypospermatogenesis is the most common observable character among the partial *AZFb* deleted patients.[20] In general, testicular phenotype of patients with *AZFb* microdeletion will encompass maturation arrest during spermatogenesis and it can be correlated with the testicular histology.[21-23] The results of the study showed that among the study subjects, 2.27 % had *AZFb* partial deletion at sY127. Detailed examination of the medical record of the patient showed a significant level of testicular failure. Among the AZF locus, the *AZFc* deletion exhibits phenotype ranging from azoospermia to severe oligospermia.[24] In general, testis of men with *AZFc* deletion will have sperm in their testis and can be retrieved for assisted reproductive methods.[4] But the transmission rate of *AZFc* microdeletion to male offspring will be 100 %. Therefore, the couple needs to be aware about the possibility of becoming infertile if the progeny is a male. In the present study it was observed that 2.27 % of the study subjects have *AZFc* partial deletion at sY254. Physical and ultrasound diagnostics results of the patients have shown small sized testes bilaterally and testicular biopsy demonstrated seminiferous tubules with maturation arrested spermatozoa.

Like western populations, many north Indian populations also show frequency of microdeletion in the pattern of *AZFc > AZFa > AZFb > AZFb+c.* The prevalence of higher *AZFc* deletion in south Indian population has also been reported.[25] In contradiction to the western pattern and other studies from India, our data has identified a lower frequency ($1/6^{th}$) of *AZFc* microdeletion is southern Kerala population, whereas *AZFa* deletion is almost double ($4/6^{th}$). The variation in the frequency may be either due to the stringent patient selection criteria or due to the ethnic background of the study population. The present study has discovered a significantly higher amount of genetic defects among the selected patients, even from the low number of samples. The genetic makeup of the selected population may differ from the population in other regions. Hence detailed and systematic survey in this group is important to identify the genetic fingerprint of the population.

Concentrations of serum reproductive hormones like FSH, LH, testosterone and prolactin generally affect the standard evaluation and also assist the diagnosis as well as treatment of infertile males. Higher concentration of reproductive hormone is a predictive factor of quality of spermatogenesis.[26] Many contradictory findings are available regarding the correlation between azoospermia, genetic defects, and concentration of reproductive hormones. Here the hormonal analysis data has shown significant change in concentration of reproductive hormones in many cases, irrespective of genetic defects. Serum reproductive hormones are important factors for maintaining spermatogenesis.

One of the hardest challenges a man can face in his life is the diagnosis of his infertility, and facing the reality of not becoming a biological parent of a child in his life. Today the advancement of assisted reproductive technologies has become hope for many. But accurate diagnosis is mandatory to avoid unnecessary wastage of time and effort. The demographic data showed that in infertility treatment, economic status of patients and lack of infrastructure are the primary road blockers in selection of molecular diagnostic measures even before advanced treatments in infertility care. The outcome of this study emphasizes that there is a need to implement the cytogenetic and molecular analysis tools as a routine diagnostic protocol in infertility care and for proper genetic counseling. The protocols should be practiced and made available to the society irrespective of their socio-economic status. Further, it also points towards the need for undertaking the study in a large population for more conclusive data about the Kerala population.

CONCLUSIONS

The present study has identified significant structural and numerical genetic abnormalities in the selected populations. Among the selected patients, 24.49 % have clinically significant chromosomal abnormalities like Klinefeltor syndrome, De la Chapelle syndrome and partial microdeletion on the AZF region. The study also observed a considerable alteration in the concentration of reproductive hormones, irrespective of any genetic defects. Even though the sample size is low, the data emphasizes the need for practicing the cytogenetic and molecular analysis in infertility care as a routine clinical diagnosis and for genetic counseling prior to assisted reproduction.

Data sharing statement provided by the authors is available with the full text of this article at jebmh.com.
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