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Y-chromosome Microdeletions Analysis in the AZFc Region and Oxidative Damage Profiling of Oligoasthenoteratozoospermic Patients of South Indian Cohort

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ABSTRACT This work aims to analyze Y-chromosome microdeletion in AZFc region of Oligoasthenoteratozoospermic (OAT) patients and oxidative damage profile in idiopathic OAT patients. Peripheral blood, serum, and semen samples of 57 OAT men and 57 fertile controls samples were collected for analyzing YCMD in the AZFc region using polymerase chain reaction and gel electrophoresis and to understand the oxidative damage level, total antioxidant capacity (T-AOC), Human 8-Hydroxy-deoxyguanosine (8-OHdG), Lipid peroxidation (LPO) and nitric oxide (NO) profiles were used. Out of the 57 OAT men, nine (15.7%) were observed with AZFc deletion. The remaining 48 idiopathic infertile men samples were analyzed for oxidative damage. The T-AOC levels were significantly reduced inpatient samples than in the control ($p<0.05$). This study shows that testing YCMD and oxidative stress damage analysis is necessary before undergoing Assisted Reproductive Technology (ART).

INTRODUCTION

Male infertility contributes to almost half of all infertility cases (Akinsal et al. 2018; Mirfakhraie et al. 2011).Various multifactorial lifestyle factors contribute to male infertility.In around 50 percent of infertility cases, male factors alone or incombination with female factors were responsible. Male infertility is a multifactorial syndrome with genetic to non-genetic causes (Mirfakhraie et al. 2011; Mukherjee et al. 2022a, b). Up to 10 percent of men with azoospermia and 5 percent with severe oligospermia have Y-Chromosome Microdeletions (YCMs), deletions in the male-specific region of the Y-chromosome. Differentiating between these microdeletions is possible by determining where part of the Y chromosome is missing, and so the terms "proximal," "middle," and "distal" are used (Rabinowitz et al. 2021). Genetic factors such as Y chromosome microdeletions are responsible for around 10 percent of male infertility,which corresponds with azoospermia or severe oligozoospermia (Dong et al. 2012; Plaseska-Karanfilska et al. 2012; Yousefi-Razin et al. 2016). After Klinefelter syndrome (KFS), Y chromosome microdeletions are the most widely recognized reason for genetic abnormality in infertile men (Tiepolo et al. 1976). Cytogenetic analysis revealed that the Y chromosome has azoospermia factor (AZF) regions on the long arm of the Y chromosome that was required for spermatogenesis regulation (Chandley et al. 1989; Totonchi et al. 2012). The AZF area is divided into three sub-non-overlapping regions: AZFa, AZFb, and AZFc (Akinsal et al., 2018). Deleting these regions independently or in combination will impair spermatogenesis (Akinsal et al. 2018; Ge et al. 2008; Balachandar et al. 2010a).

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Many factors control the process of spermatogenesis. Here genes play an essential role (Lin et al. 2006), among which six genes are predominant. They include Hs.12670, Hs.553658, Hs. 274135, Hs. 268122, Hs. 521701, and Hs.171130, which encode proteins with predictable functional domains (Vogt et al. 2005). 14 protein-coding genes were located on the AZF locus that is critical for spermatogenesis (Hopps et al. 2003; Skaletsky et al. 2003; Vogt et al. 2005). According to previous studies, the most predominant type of microdeletion is AZFc region deletion, followed by AZFa, AZFb, and AZFbc, while AZFabc region deletions were observed.

Idiopathic male infertility has no identifiable cause, studied as a multifactorial disorder due to genetic, environmental, and hormonal factors (Aktan et al. 2013; Tirlapur et al. 2013). Though the basis is unknown, oxidative stress could be among them (Aktan et al. 2013; Tremellen et al. 2008). Reactive oxygen species are a potential contributor to male infertility, correlated with excess ROS in human semen. According to recent studies,male infertility is attributed to ROS in about 30 percent to 80 percent of cases (Bui et al. 2018; Wagner et al. 2018). Free radicals are chemical species that are very unstable and react quickly because of their unpaired electron. They need to become stable (Jensen et al. 2003). These are obligatory metabolic products of aerobic cells, maintained at low levels by antioxidant systems (O'Flaherty et al. 2017). ROS can either have beneficial or causative effects on the function of sperm based on its nature, concentration, location, and length of exposure. The fertile male reproductive tract maintains an equilibrium between ROS production and antioxidant counteracting. ROS is required for capacitation, acrosomal reaction, and oocyte fertilization. An imbalance in this equilibrium, referred to as oxidative stress, can be detrimental to normal spermatozoa and cause DNAdamage (Barbato et al. 2015). Sources of ROS include both intrinsic (spermatic leukocytes, specific redox reactions in metabolism, individuals with varicoceles, inflammation and infections, hyperglycemic patients, high testicular temperature, apoptosis in germline, imbalance in hormones, old age) and extrinsic factors (industrial exposure to heavy metals, pesticides, smoking, alcohol, radiation, xenobiotic effects) (Wagner et al. 2018; Bui et al. 2018). To overcome this, sperm cells depend on antioxidants present in semen naturally. Enzymatic antioxidants like superoxide dismutase (SOD) and glutathione peroxidase (GPX) and non-enzymatic antioxidants such as vitamins E, A, and C, glutathione, zinc, melatonin, folic acid, selenium, and carnitine, which quench oxidative stress causing agents (Asadi et al. 2017; Sheweita et al. 2005). Antioxidant supplements (low molecular weight components such as zinc, vitamins C and E, selenium melatonin, and cytochrome C) enhance spermatogenesis and fertility (Asadi et al. 2017; Balachandar et al. 2010b).

Human 8-Hydroxy-deoxyguanosine (8-0HdG) is a marker of oxidative stress and DNA damage. The mutation associated with 8-0HdG directly causes DNA damage (Balachandar et al. 2008a). Studies reported that the level of 8-OHdG in spermatozoa is linked with sperm motility and morphology (Ni et al. 1997). Variation in ROS production and T-AOC in seminal fluid specifies oxidative stress correlated with male infertility (Pasqualotto et al. 2005; Pasqualotto et al. 2000; Pasqualotto et al. 2008). As ROS elevates, the system's availability of Total antioxidant capacity (T-AOC) decreases. This contributes tothe loss of sperm function (Mahfouz et al. 2009). Diminishing fertilization potential is caused due to increased lipid peroxidation, which harms the male reproductive system and sperm (Ben et al. 2009; Balachandar et al. 2008b). Nitric oxide concentration significantly affects sperm motility, capacitation, and binding with zona pellucida (fertilizing ability) (Balercia et al. 2004). If exceeded, reactive nitrogen species (RNS) leads to nitrosative stress, causing effects on the male reproductive system, DNA synthesis inhibition, mitochondrial respiratory dysfunction, and in association with ROS causes, increased tissue injury (Türkyilmaz et al. 2004; Balachandar et al. 2007; Famurewa et al. 2022).

This study analyzes the Y chromosome microdeletions in the Vellore cohort of OAT patients. The researchers analyzed the deletions specific to the AZFc region by performing molecular diagnosis using STS primers (sY254 and sY255) by polymerase chain reaction (PCR) technique and oxidative damage in OAT patients determined using total antioxidant capacity (T-AOC), Lipid peroxidation and nitric oxide (NO) profiles (Balachandar et al. 2008c).

Objective

The chief objective of this study is to analyze Y-chromosome microdeletion in the AZFc region of patients and the oxidative damage profile in idiopathic OAT patients.

MATERIAL AND METHODS

Study Population

The researchers collected blood samples from Sandhya hospital, Vellore, Tamil Nadu, in the present study. All subjects alluded to for assessment of infertility met the primary male infertility criteria. Furthermore, subjects exposed to gonadotoxins in radiation or cancer treatment were excluded. Subjects were enlisted in the study concerning the fertility status of their female partners. They went through physical and clinical examinations. For this study, 57 samples were taken from primary infertile men. An equal number of fertile male samples with proven fertility (without any ART) were taken for the analysis of YCMD in the AZFc region and oxidative damage analysis.

Ethical Approval

The Human Ethical Committee of Vellore Institute of Technology (VIT), Vellore, Tamil Nadu, approved this study. Written informed consent was obtained from the participants who volunteered in this study. Volunteers collected data about their family history, age, bodymass index (BMI), lifestyle, and chemical exposure.

Semen Analysis

The participants semen samples were collected in a sterile container following 4-6 days of sexual abstinence.The participants were analyzed and selected according to the semen analysis by the World Health Organization (WHO) 2010 guidelines (Lu et al. 2010).

Blood Sample Collection

4mL peripheral blood samples were collected in red-topped vacutainers from idiopathic infertile patients and control, centrifuged at 1000g for 15 min after coagulation; supernatant serum was collected and stored at -40°C for further analysis.

DNA Extraction

For DNA extraction, blood samples were obtained from the intravenous vein of subjects and collected in EDTA vacutainers. The genomic DNA was extracted according to the standardized lab protocol (Tremellen 2008) and stored at -20! for further analysis. DNA samples were quantified using Nanodrop and Thermo Scientific, and qualitative analysis was done using 1% agarose Gel electrophoresis.

PCR Analysis

For diagnosing microdeletions in the AZFc region of the Y chromosome,the researchershave used sY254 (sY254F -52 -GGG TGT TAC CAG AAG GCA AA-3 and sY254R- 52 -GGG TGT TAC CAG AAG GCA AA-3) and sY255 (sY255 F - 52 - GTT ACA GGA TTC GGC GTG AT-32 and sY255R 52 -CTC GTC ATG TGC AGC CAC-32) STS markers. The product size of the sY254 was 380bp, and sY255 was 123bp (Wagner et al. 2018). In this study, male samples were used as positive controls, respectively. Standardized PCR conditions were followed, as mentioned in Tables 1 and 2. The products of PCR were then analyzed for Y chromosome microdeletion by 1% Agarose Gel Electrophoresis. The absence of any STS markers specified the microdeletion compared with the control. The procedures were repeated if any sample was deleted to confirm the result.

Table 1: Concentrations for PCR master mix amplification of STS primers in the AZFc region

	Quantity	*Total*
Master Mix	6 µL × 49	294 µL
Forward Primer	1 µL × 49	49 µL
Reverse Primer	1 µL × 49	49 µL
MiliQ Water	8 µL × 49	392 µL
Total Quantity	16 µL × 49	784 µL
DNA Template	4 µL each + 16 µL master mix	20 µL

Table 2: Optimum temperature and duration for amplification of STS markers in the AZFc region

Steps of PCR	*Conditions*
Initiation	95°C for 5 minutes
Denaturation	94°C for 1 minute
Annealing (Standardized at)	61°C for 1 minute
Elongation (Initial extension)	72°C for 1 minute
Final elongation	72°C for 10 minutes
Hold	4°C for infinity (“)

8-Hydroxy-desoxyguanosine Assay

Using Human 8-0HdG ELISA kit (KINESIS Dx), the levels of 8-0HdG in were measured. The standard dilutions were prepared. 40 µL of a serum sample and 50 µL of the standard were mixed. Additionally, 10 µL of Biotin conjugate and 50 µL of HRP conjugate were added. The plate containing this mixture was covered and incubated for 1 hour at 37°C in the incubator. After incubation, the plates were washed with 1X wash buffer and dried off. Later 50 µL of Substrate A and Substrate B were added to each well and incubated for 10min at 37°C in the dark. Finally, 50 µL of Stop solution was added (wells should turn yellow to blue). The absorbance was read at 450 nm within 15 minutes after adding stop solution blanking on the zero standards. The principle behind this method is the Sandwich ELISA method. The final obtained 8-0HdG levels were compared with the standard curve. Final levels of 8-0HdG were expressed in ng/ml.

Total Antioxidant Capacity Assay

The Human Total Anti-Oxidant Capacity ELISA kit (KINESIS Dx) was used to measure the total antioxidant capacity (T-AOC) level according to the manufacturer’s instructions. The standard dilution was prepared. 40 µL of serum sample and 50 µL of standard solution were mixed. Additionally, 10 µL of Biotin conjugate and 50 µL of HRP conjugate were added. The plate containing this mixture was covered and incubated for 1 hour at 37°C in the incubator. After incubation, the plates were washed with 1X wash buffer and dried. Later 50 µL of Substrate A and then Substrate B was added to each well and incubated for 10 min at 37°C in the dark. Finally, 50 µL of Stop solution was added (wells should turn yellow to blue). The absorbance was read at 450 nm within 15 minutes after adding stop solution blanking on the zero standards. The principle behind this method is the Sandwich ELISA method. The final concentration was calculated by comparing it with T-AOC standard curve. The final T-AOC levels were expressed in U/ml.

Lipid Peroxidation Assay

Thiobarbituric acid (TBA) assay method was used to study Lipid peroxidation (LPO) levels in patient serum. 30µl sample was mixed with 150µl of homogenization buffer and 150ìl TBA reagent.To initiate lipid peroxidation artificially, 2µl of butylated hydroxytoluene (BHT) was added and incubated at 100°C for 15 min, followed by thawing at room temperature. To the above mixture, 0.3 ml of n-Butanol solution was added and centrifuged at 15,000 RCF for 3min. Using an enzyme-linked immunosorbent assay (ELISA) plate reader (ELx800 from BioTEK), fluorescence was measured at 530 nm. The obtained LPO levels were tallied following MDA (lipid peroxidation product) standard curve, expressed in µM (Eroglu et al. 2014).

Nitric Oxide

Nitric oxide (NO) was estimated in the patient serum using the Griess reagent. The absorbance of the samples was quantified at 540 nm. The final concentrations were compared with the standard sodium nitrate curve. Final values were expressed in µM/mL (Benedetti et al. 2012).

Statistical Analysis

Statistical values were obtained from GraphPad version 5.0 (GraphPad Software, San Diego. CA). Values acquired were expressed as mean ± standard deviation (SD). For evaluating pair-wise collations in TAC, 8-OhDG, MDA, and NO values of subjects were derived by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison (post hoc) test. Pearson’s correlation analysis was implemented to conclude coefficients of correlations. If the *p*-value was <0.05, then it is statistically significant.

RESULTS

Out of the 57 OAT men, nine (15.7%) were observed with AZFc deletion. The remaining 48 idiopathic infertile men samples were analyzed for oxidative damage. The researchersfound that the LPO, NO, and 8-OHdG concentrations were significantly higher in patient samples than in controls ($p<0.05$). In contrast, the T-AOC levels were significantly reduced in patient samples than in the control ($p<0.05$). The mean age (years) of fertile controls (30.16 ± 7.7) (mean±SD) and OAT men (33.62 ± 5.94) did not differ significantly ($p>0.05$); however, the control group was found to be younger than OAT patients. Subjects with leukocytopspermia, exposure to environmental factors, medication, infections, subjects exposed to gonadotoxins in radiation or cancer treatment, cigarette smoking, and alcohol consumption were excluded from study.

Y Chromosome Microdeletion Analysis of AZFc Region in OAT Patient Sample 3

The "DAZ gene family" is a multigene family consisting of four DAZ genes in two clusters; cluster 1 consists of DAZ1 and DAZ2, whereas cluster 2 consists of DAZ3 and DAZ4. These are the candidate gene found in the AZFc region of the human Y chromosome (Foresta et al. 2001; Saxena et al. 2000; Vogt et al. 1997). Elimination of all DAZ genes or absence of any one of the DAZ genesis associated with the impairment of spermatogenesis. It causes severe testiculopathy (Foresta et al. 2001).The STS markers, such as sY254 and sY255, were used to detect the AZFc regions' deletions that cause men's infertility (Wagner et al. 2018).

Previous studies concerning Y chromosome microdeletion in the AZFc region showa discrete percentage of deletion among different parts of India. Broadly, in North India, on average, 46.5 percent (Dada et al. 2003; Mahanta et al. 2011; Thangaraj et al. 2003), likewise 45.34 percent of average in Central India (Ambulkar et al. 2015; Ambulkar et al. 2014) and closely 44.85 percent in South India (Papanna et al. 2015; Vijesh et al. 2015; Vineeth et al. 2015) have been reported. These results show that the AZFc region has a crucial role in the proper functioning of spermatogenesis, resulting in infertility, which is inheritable in upcoming generations.

The Oxidative Damage Analysis in Idiopathic OAT Patient Sample

The above-performed oxidative damage analysis assays can be easily demonstrated in clinical laboratories to measure the oxidative stress level in infertile men. Sperm parameters like sperm concentration, motility, and morphology were significantly different in patient samples than in controls. This study revealed that in the patient sample (48/57), the LPO, NO, and 8-OhDG concentrations were significantly increased (48/57) (54.60±4.64, 17.89±2.74, and 47.94±2.26 respectively) than in controls (40.47±2.64, 10.96±1.91 and 4.85±0.59 respectively) ($p<0.05$). The T-AOC levels (3.94±0.43) were significantly reduced in patient samples (48/57) than in control (43.05±1.64). Table 3 demonstrates LPO, NO, 8-OhDG, and T-AOC levels in the blood of fertile and infertile samples.

Pearson's correlation analysis was done to understand the correlation between oxidative stress profile in blood and conventional semen parameters. In Pearson's correlation analysis, LPO levels in serum were significantly negatively correlated with sperm concentration, motility, and morphology ($r=-0.499, p<0.001$; $r=-0.459, p<0.001$; $r=-0.460, p<0.001$, respectively). NO and T-AOC levels in the blood plasma were insignificant with sperm parameters ($p>0.005$). Contrarily, 8-OhDG levels in blood were significantly positively correlated with sperm morphology ($r=0.377$, $p<0.001$).

Table 3: LPO, NO, 8-OhDG, and T-AOC levels in the blood of fertile and infertile sample

Covariates	*LPO(μM)*	*NO (μM/mL)*	*8-OHdG (ng/ML)*	*TAOC (U/mL)*
Control	40.47±2.64	10.96±1.91	4.85±0.59	43.05±1.64
Patients	54.60±4.64*	17.89±2.74*	47.94±2.26*	3.94±0.43

Values have shown in mean and standard deviation
*: $p<0.05$

The above-obtained results show that the increased oxidative stress leads to DNA damage, cross-linkage, and chromosomal rearrangement. DNA damage in sperm is related to male infertility.

DISCUSSION

This study has examined Y-chromosome microdeletion in the AZFc region of patients with Oligoasthenoteratozoospermia (OAT) and the oxidative damage profile in idiopathic OAT patients. To analyze YCMD in the AZFc region using polymerase chain reaction and gel electrophoresis, peripheral blood, serum, and sperm samples were collected from 57 OAT men and 57 fertile controls. To determine the level of oxidative damage, T-AOC, 8-OHdG, LPO, and NO profiles were also Nine (15.7%) of the 57 OAT men were found to have AZFc deletion. The remaining 48 samples of men with idiopathic infertility were examined for oxidative damage. The amounts of LPO, NO, and 8-OHdG were substantially more significant in patient samples than in control samples ($p<0.05$).

In contrast, T-AOC levels in patient samples were substantially lower than in control samples ($p<0.05$). Before undertaking Assisted Reproductive Technology, testing for YCMD and oxidative stress damage analysis is required, according to this study (ART). These observations were associated with spermatogenic factors, which cause infertility. Figure 1 shows the qualitative analysis of DNA samples on 1% agarose Gel electrophoresis. In this study's Y chromosome microdeletion analysis of the AZFc region, four OAT patients have detected sY254 and sY255 microdeletion compared to the control samples. An example of sY255 PCR products is shown in Figure 2.

A previous case report of an OAT patient mentioned that the correlation between AZFc microdeletion and oligoasthenoteratozoospermia might be correlated with oxidative stress (Singh et al. 2012). This study's results were contradicted by other population studies (Choi et al. 2013), which agreed with the previously reported statement as mentioned that the geographic and ethnicity of populations might impact the frequency of the Y chromosome microdeletion genotype (Kuroda-Kawaguchi et al. 2001). Therefore, the researchers needan additional sample size to confirm their results.

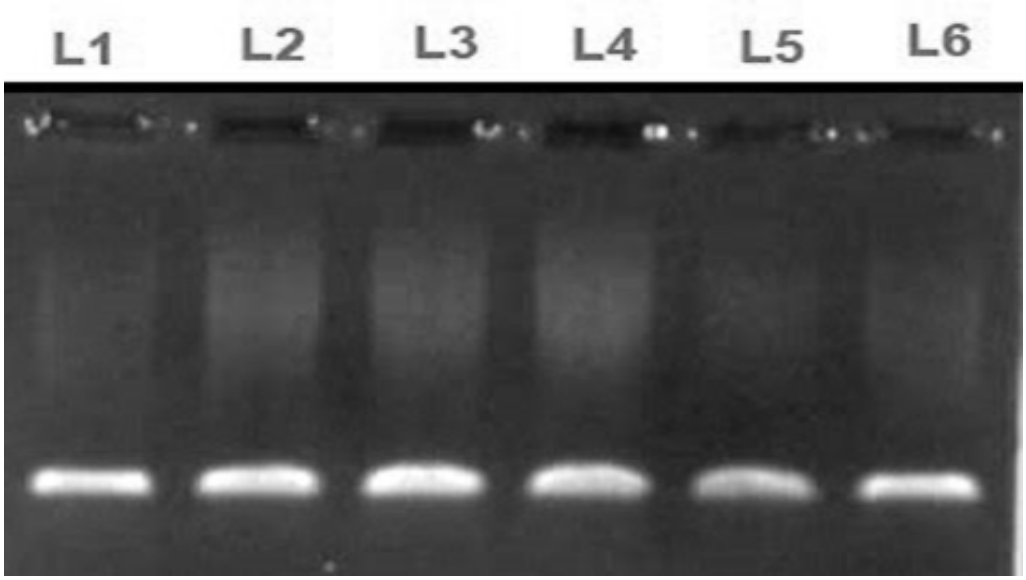

Fig. 1. Qualitative analysis of extracted DNA of OAT patients on 1 % agarose gel electrophoresis
L1- Sample YCM1, L2- Sample YCM2, L3- Sample YCM3, L4- Sample YCM4, L5 Sample YCM5, L6- Sample YCM6

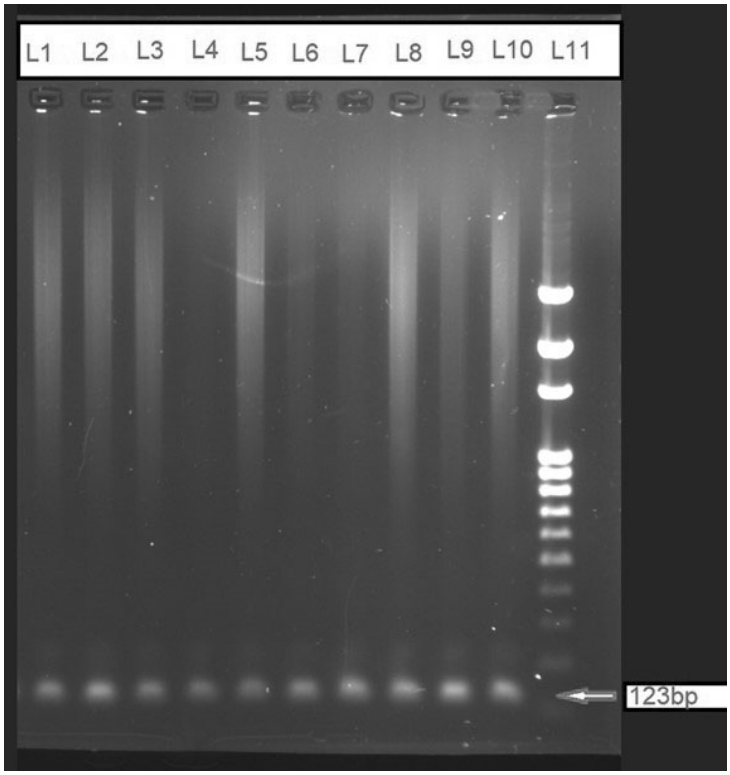

Fig. 2. PCR products on a 1% agarose gel electrophoresis for sY255 (product size-123bp)
L1- Sample YCM1, L2- Sample YCM2, L3- Sample YCM3, L4- Sample YCM4, L5- Sample YCM5, L6- Sample YCM6, L7- Sample YCM7, L8- Control 1, L9- Control 2, L10-Control 3, L11- 100bp DNA ladder

CONCLUSION

The incidence of Y chromosome microdeletions in the AZFc region among OAT men is comparatively less compared to oxidative damage in the idiopathic infertile men sample than in fertile controls. A high incidence of oxidative damage was observed. This study's results recommend Y chromosome microdeletion and investigation of oxidative stress damage in infertile couples before proceeding with any IVF treatments.

RECOMMENDATIONS

This study shows that testing YCMD and oxidative stress damage analysis is necessary before undergoing Assisted Reproductive Technology (ART).

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CONFLICTS OF INTEREST

The authors declare that this study does not have conflicts of interest.

AUTHOR CONTRIBUTIONS

Conceptualization, A.A., H.K.G., S.S.R.; resources and data curation, A.A., A.B., H.K.G., S.S.R., K.R., A.G.M., P.B., R.M., U.R.W.; writing- original draft preparation, A.B., H.K.G., S.S.R., K.R.; writing-review and editing, K.R., A.G.M., U.R.W.; visualization, A.V.G., K.R.; supervision, A.V.G., K.R.;

project administration, A.V.G., K.R.; All authors have read and agreed to the published version of the manuscript.

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