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EDITOR'S NOTE

Males contribute to about half of the infertility cases. Research on male infertility has come a long way. Y-deletion analysis is close to becoming a routine investigation in the assisted reproduction laboratories. However, still more than 80% of infertility cases remain idiopathic, and normozoospermic infertile individuals are of particular interest, as despite a normal semen picture, infertility remains perplexing. In such cases, molecular abnormalities in sperm could be at the core of the problem. This makes molecular investigations very interesting and futuristic ideas. In the near future, molecular analysis of sperm could answer many questions that puzzle infertility specialists today. Sperm carry DNA, RNA and a full epigenome for delivery to the oocyte. It is this combination of molecular conjuncture that sets the molecular milieu required for post-fertilization development. The discovery of the molecular specialities, modalities and abnormalities would need deeper insights into the molecular composition of sperm in complex with those of the oocyte. In this special issue of IFS newsletter on male infertility, the articles mainly focus on the sperm's molecular contribution towards fertility, particularly post-fertilization development. Molecular events induced by sperm and the developmental events that ensue would make an advanced line of investigations that could transform the way infertility management is handled at present. We have tried our best to bring the latest updates on this to the readers of this newsletter. Thank you very much for your interest in the activities of the Indian Fertility Society. Happy Diwali and stay safe !

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CRACKING THE "GENETIC CODE" FOR SPERM MEDIATED Ca^{2+} RISE IN THE OOCYTE

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ABSTRACT

The precise order of events activating the oocyte upon sperm entry into a well-equipped ooplasm is known. The inactive oocyte undergoes more complex and intimate interactions once sperm expels out certain mediators to switch "on" the dormant oocyte for completing fertilization and to begin embryogenesis. Alterations in any step lead to failed oocyte activation. The efforts to unravel this ever-challenging phenomenon are ongoing. Oocyte activation involves not only the oscillating calcium levels shaking the meiotic machinery but the real picture is well beyond this saga. The step ahead technologies have fostered new hopes for better understanding of this complex phenomenon. Dynamism of gene expression waves in the oocyte as a welcome note is worth studying. This review focus on deciphering the mechanisms of genomic regulation of sperm mediated Ca^{2+} rise within the oocyte.

INTRODUCTION

Mammalian reproduction demands a functional sperm just like a mature oocyte as an essential prerequisite. Both the cells undergo multiple steps of development shaping them to have an optimal gametic association. Sperm matures first to be able to contribute itself to release the oocyte under metaphase II arrest. A fully mechanized sperm after crossing through all the surrounding barriers heads towards the oocyte plasma membrane. Sperm-oocyte membrane fusion enables sperm to release its triggering factors in the ooplasm in order to activate the downstream signalling pathway leading to meiotic resumption.

The model that is apt to understand the activation process is intracytoplasmic sperm injection (ICSI). In clinical scenario as well ICSI remains at forefront for treating infertile males. Despite of having fertilization rates of 70-80%, total fertilization failure still occurs in 1-3% cases of ICSI. The most common cause attributed to failed oocyte activation is lack of Ca^{2+} oscillations. While investigating the genetic causes in infertile males without associated medical or surgical problems, one of the potential causes could reside during the meiotic regulation. Errors in meiotic progression in absence of a male factor deficiency may lead to reduced oocyte activation and fertilization rate.

THE EVENTS

Oocyte activation is the series of morphological and biochemical events beginning with repeated Ca^{2+} oscillations that lead to meiotic resumption, cortical granules exocytosis and block to polyspermy. The subsequent events are decondensation of sperm nucleus, recruitment of maternal RNAs, second polar body extrusion, pronuclei formation, DNA synthesis and process of cleavage (Xu et al., 1994; Schultz and Kopf, 1995).

The arrested oocyte is released by sperm triggering or oocyte activating factors causing inositol 1,4,5-trisphosphate (IP3)-mediated Ca^{2+} release from endoplsasmic reticulum (ER). Over the last few decades, a number of sperm oocyte activating factor (SOAF) have been implicated in the generation of Ca^{2+} release such as phospholipase C zeta (PLC-ζ), oscillin, post-acrosomal sheath WW domain binding protein (PAWP), TR-KIT etc. but none of these candidate proteins have been shown to elicit the repetitive Ca^{2+} release in mammalian oocytes with controversial results in different studies.

OOCYTE UNDER ARREST

Oocyte MII arrest is maintained by the 'Cytostatic Factor' (CSF) that blocks the exit of arrested oocyte until the sperm breaks it by generating oocyte cytoplasmic Ca^{2+} oscillations. CSF is not a single molecule but it comprises of an activity that inhibits cell division and keep the oocytes arrested in MII (Tunquist and Maller 2003). CSF maintains arrest by preventing loss in Maturation-Promoting factor (MPF) and Emi2 (Early mitotic inhibitor 2) mediated inhibition of Anaphase-Promoting Complex/Cyclostomes (APC/C) (Madgwick and Jones 2007). MPF is a complex of two subunits cyclin dependent kinase 1(CDK1)/cyclinB. At the time of meiotic arrest, there is high activity of MPF due to CSF. Egg specific protein Emi2 is a substrate for polo-like kinases (plk). APC/C is a ligase that helps in polyubiquitination of key cell cycle proteins such as cyclin B, targeting them for immediate proteolysis and degradation. Another kinase WEE1B phosphorylates CDK1, inhibits MPF activity and maintain meiotic arrest (Oh et al. 2011).

There is also role of spindle assembly checkpoint (SAC) proteins suggested in maintaining CSF activity. The SAC proteins inhibit APC/C (Shah and Cleveland 2000). The suggested proteins are Bub1, Mad1, Mad2. These SAC proteins are the downstream effectors of c-Mos/MEK/MAPK pathway. c-Mos is a kinase belong to proto-oncogene family regulating cell growth and development (Hunter 1987). c-Mos activates mitogen-activating protein kinase (MAPK) kinase MEK1 that further activates MAPK which subsequently activates SAC proteins (Schwab et al 2001). The c-Mos/MEK/MAPK signalling pathway is an ideal CSF candidate as it has shown to stabilize and activate MPF (Palmer et al 1998). Thus, oocyte meiotic arrest is maintained by the CSF activity which comprises of MPF stabilization and APC/C inhibition through Emi2, WEE1B and SAC proteins.

OOCYTE RELEASE

During fertilization sperm releases SOAFs such as PLC-ζ in the oocyte that hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) into IP3 and diacylglycerol (DAG). Subsequently, IP3 binds to specific receptors InsP3Rs present on smooth endoplasmic reticulum (SER) that leads to

release of Ca^{2+} in the ooplasm. Ca^{2+} is loaded back in the lumen of SER by Ca^{2+} ATPases (SERCA pumps). During signalling this stored Ca^{2+} is released into the cytosol by Ca^{2+} release channels. The spike and fall of Ca^{2+} levels in the ooplasm elicit Ca^{2+} oscillations (Machaty 2016). Ca^{2+} oscillations switch on the CaMKII (calcium/calmodulin-dependent protein kinase II). Activated CaMKII in turn phosphorylates the oocyte-specific protein Emi2 and WEE1B leading to its inactivation. The inactivated Emi2 no longer will be able to inhibit APC/C. APC which was inactive during arrest is now liberated from the inhibition by CSF. APC activation causes a decline in MPF activity and therefore the concomitant release from the MII arrest (Nixon et al 2002). The other way by which CAMKII regulate MPF activity is through phosphorylation of the protein kinase WEE1B (Oh et al. 2011). Phosphorylation of WEE1B deactivates MPF and leads to resumption of meiosis.

CONSTITUENTS OF GENETIC CODE REGULATING CA^{2+} SIGNALLING PATHWAY

Triggers: Till now PLCζ has been most widely accepted as SOAF and is located in the acrosomal, equatorial, post-acrosomal region of sperm head. It belongs to phospholipase C family. Role of PLCζ as SOAF has been proved in multiple studies. Male mice lacking PLCζ failed in spermiogenesis (Ito 2010). PLCζ knockdown in sperm leads to a reduction in Ca^{2+} oscillations at fertilization (Knott et al. 2005). Infertile males also showed reduced levels of PLCζ (Kashir et al. 2011). Patient with repeated failed ICSI had PLCζ gene mutations on both alleles (Kashir et al. 2012). These mutations involved catalytic domain of PLCζ that lead to loss of its ability to cause Ca^{2+} oscillations in eggs (Kashir et al. 2012, Escoffier et al. 2015). Whole genome sequencing in patient with repeated ICSI failure showed that there was only one gene that had a homologous mutation that was predicted to be disruptive, and that gene was PLCζ (Escoffier et al.2016). This study has been the strongest to date that PLCζ is the critical protein for causing Ca^{2+} oscillations and egg activation at fertilization.

The Machinery: The oocyte cytoplasmic as well as nuclear maturity status is as critical as the sperm triggers. Oocyte meiotic machinery must be responsive to the expelled-out sperm triggers. There have been multiple genes essential for meiotic progression. Majority of results are based on studies done in mice and Xenopus but effects can be reflected to humans in the homologous genes. Meiotic failure is observed in mutations affecting cell cycle genes such as Cks1, Cks2, Mos etc (Kim et al., 2015; Spruck et al., 2003; Araki et al., 1996). Both Cks1 and Cks2 modulate the cell cycle by binding to Cdk1 and Cdk2 (Egan and Solomon 1998). Inhibition of Wee2 results in failed pronuclear formation and fertilization (Oh et al., 2011). WEE2 mutations result in oocyte fertilization failure and injection of WEE2 effectively resulted in fertilization (Sang et al., 2018).

Eggs from CAMKII knockout mice fail to show any signs of meiotic resumption at fertilization (Backs et al. 2010). Fertilization of oocytes from WEE1B knockdown oocytes show no signs of meiotic resumption (Ducibella and LeFevre 1997; Oh et al. 2011). Oocytes from Mos-null mice also fail to arrest at MII and undergo high rates of spontaneous fragmentation (Colledge et al., 1994). SERCA, the ion pumps at ER membrane redirects cytosolic Ca^{2+} into ER (Ullah et al., 2007). There are 3 encoding genes (SERCA1, SERCA2 and SERCA3) and up to 14 SERCA-transcripts that are formed by alternative splicing (Periasamy and Kalyanasundaram, 2007). The associated mutations have not been reported in humans but are potential source of oocyte activation deficiency (Petersen et al 2001). Studies have shown the gene expression wave in MII oocyte getting changed in activated oocyte. MII oocytes majorly showed genes linked to cell cycle and proliferation whereas activated oocytes were associated with cell development (Yoko and Tomohiro 2008).

CONCLUSION

There have been many mind-boggling questions which remain unanswered despite of continuous cutting-edge research targeting gametic associations especially in the field of assisted reproduction. These questions are often encountered while dealing with repeated failures in IVF/ICSI cycles. Answer to many of these questions is unraveled by concept building and thriving on the newer evolving genetic technologies.

Mammalian oocytes undergo cell cycle arrest and resumption by Ca^{2+} homeostasis. The key players in the process are the sperm triggering factors, oocyte machinery constituting molecules of cell cycle and calcium signalling. Many milestones have been attained but once all the critical genes are identified and their aberrant function is identified by genetic studies in cases of failed fertilization, it will be easier to structure the components of the "genetic code" of sperm mediated oocyte Ca^{2+} rise.

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SPERM RNAS AS MARKERS OF MALE FERTILITY

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Assisted reproductive technology is the widely used technique to treat male infertility, yet the percentage of successful pregnancies is quite low i.e. only 30%. Previous studies have revealed that a spermatozoa contains an impressive population of coding and non-coding RNAs (Ostermeier et al. 2002; Krawetz et al. 2011). These RNAs are delivered to the oocyte and are important for embryonic development. Their abundance and importance in embryo development makes them interesting candidates to be used as sperm quality biomarkers for in-vitro fertilization. Several studies have reported dysregulated expression of mRNA and miRNAs in men with different sperm anomalies or even in normozoospermic infertile individuals. Although substantial work has been done with respect to sperm miRNAs and their role in fertilization and embryo development; however, one study has reported that sperm RNA elements (SREs) are important for natural conception (Jodar et al. 2015). It was observed that patients expressing all SREs were more likely to achieve live birth (LB) by TIC/IUI than those with one or more SREs absent. These findings suggested that in those patients lacking at least one of the SREs, the earlier use of ART would avoid unsuccessful IUI cycles. These SREs were found in the sperm of patients achieving live birth after TIC (timed inter course)/ IUI (Intra-uterine insemination). Till date, two sequencing studies have been undertaken to identify miRNA profile in sperm with respect to the fertilization rate and embryo quality (Hua et al. 2019; Hua et al. 2020). Hua et al. (2019) identified five miRNAs (miR-132-3p, miR-191-3p, and miR-520a-5p) that were downregulated, and two miRNAs (miR-101-3p and miR-29a-3p) that were upregulated in the L-GQE (low rate of good quality embryos) group as compared to H-GQE group (high rate of good quality embryos), suggesting that sperm miRNAs may be used as a potential biomarkers for the assessment of sperm quality in IVF. Evidence have shown that sperm miRNA may not be essential for fertilization; however, their dyregulation have led to arrest in embryo development, suggesting their indispensable role in fertility (Yuan et al. 2016). This study showed that partial deletion of miRNA biogenesis machinery (Drosha and Dicer) leads to reduced embryo development; however, introduction of miRNA from wild type sperm has rescued the outcome (Yaun at al. 2019), suggesting that in future one can think of injecting an embrogenesis related sperm miRNA to the oocyte/zygote at the time of ICSI to achieve successful pregnancy.

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MITOCHONDRIAL GENOME AND INFERTILITY IN ASTHENOZOOSPERMIC MALES

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Infertility is the lack of pregnancy after one year of regular unprotected intercourse. Infertility severely affects psychological harmony, sexual life and social status of the couple. The couple desiring to have a child but not able to conceive develops inferiority complex, as this is a social stigma. In our country, though over-population is a problem at the national level, childlessness for majority of the couples is a curse. They fear their own heritage coming to an end. Childlessness may cast a shadow on the physiological and social adequacy of the female and in the case of the males social standing may be diminished".

A multicentric study by WHO found that in about 20% of infertile cases, the problem lies predominantly in the male (pure male factor), in 38% female (pure female factor), in 27% in both partners and in the remaining 15% no clear cause of infertility could be identified (unexplained infertility). The identification and classification of male infertility still relies on the results of semen analysis, but recent studies have shown that infertile men with normal or low sperm count have sperm with DNA damage. For normal fertility, a male requires sperm with intact normal morphology, motility and DNA integrity. Sperm are produced through a complex series of events called spermatogenesis and based on this; causes of infertility can be classified as secretory or excretory.

Infertile men can be affected by azoospermia, oligozoospermia, asthenozoospermia, teratozoospermia or by combination of any of these. The aetiology of impaired sperm production and function could be due to different factors acting at pre-testicular, post-testicular and directly at the testicular level. Amongst the most important causes of male infertility are endocrinological, environmental and genetic factors. Genetic factors are important cause of irreversible spermatogenic arrest. It can be partial or complete spermatogenic arrest and result in oligozoospermia to azoospermia. Genetic factors can be single gene disorders, chromosomal or mitochondrial disorders. Chromosomal anomalies may be numerical or structural and are usually de-novo events, resulting from mutations in the parental germ cells. Till date, genetic analysis only provided aetiology for idiopathic infertility but today in era of assisted reproduction it is both an important diagnostic and prognostic marker. Even after cytogenetic and Yq microdeletion analysis of idiopathic male infertility, in about 30-40% cases no aetiology can be identified. These cases may have mutations in autosomes or in the mitochondrial genome. Recent advances in assisted reproductive techniques can result in iatrogenic transmission of these mutations or deletion to the next generation. Since the embryo is the product of the union of the egg and the sperm, problems within the embryo can come from abnormalities either within the egg or the sperm. Abnormalities within the embryo are most often characterized by the presence of abnormal chromosomes.

The mitochondrial genome is thought to deteriorate with age, through accumulation of point mutations and rearrangements (deletions) in most tissues at a rate much faster than the nuclear genome. This is because replicative intermediates are single stranded for prolonged periods during mitochondrial DNA (mt.DNA) replication and partly because of a lack of double stranded DNA repair mechanism. The mutation fixation rate of mt.DNA is 15-20 times greater than rate for nuclear DNA and this difference is attributed to increased DNA damage from elevated concentrations of endogenous ROS produced by products of oxidative phosphorylation (OXPHOS). Excessive production of ROS may play a role in the etiology of male infertility.

The mutations in mt.DNA genes that impair the expression of one or more proteins encoded in the mt.DNA can promote diseases in humans. Since these diseases are very often severe, not very much attention has been paid to the sperm quality of patients with mitochondrial diseases. However, since sperm have few mt.DNA copy no. (40-100) as compared to ova (approx. 1 lakh) and other somatic cells mt.DNA, mutations produce early phenotypic defects and may manifest as spermatogonia or spermiogenesis. An increasing number of mt.DNA point mutations or deletions have been reported in various diseases. However, point mutations in t-RNA genes and large deletions of the mt.DNA are predominant compared with point mutations in the rRNAs and the Respiratory Chain (RC) enzyme encoding genes. An important feature of the genotype/phenotype relationship in mt.DNA pool is that mutants coexist with wild-type mtDNA. This condition is called heteroplasmy. As consequence, the mitochondrial defect will greatly depend on the type of the mutation but also on the proportion of both mutant and wild type DNA. The respiratory chain (RC) is made up of five mtDNA encoded polypeptides (except complex II, which is exclusively encoded by the nucleus. The mitochondrial RC ensures the process of electron transport from reducing equivalents (e.g. NADH, succinate) to molecular oxygen with a very large loss of free energy, much of which is conserved by the phosphorylation of ADP to yield ATP, in the process of OXPHOS. Energy in the form of ATP is produced by the OXPHOS system, which consists of five multiprotein enzyme complexes that release the energy stored in the form of a proton gradient across the inner mitochondrial membrane.

The mt.DNA is transmitted through the maternal line via the mitochondria contained in the ooplasm. Maternal transmission is also a hallmark of mtDNA-related diseases. Mature human oocytes contain between 100,000 and 600,000 mitochondria and mt.DNA copies. This is in contrast to sperm cells which have been reported to contain between 10 and 700 copies mt.DNA. The mt.DNA content of the spermatozoon decreases five to six-fold during spermatogenesis, probably because of a down-regulation of the mitochondrial Tfam. During spermatid development, ubiquitin binds to the mitochondria, which makes the sperm mitochondria prone to proteolysis, resulting in the loss of paternal mt.DNA molecules. In another study, t-tpis, a testis specific translocator, belonging to the translocator of mitochondrial outer membrane (TOM) complex, has been identified as a sperm mitochondria-specific factor, which incorporates an elimination factor

present in the oocyte . The elimination factor is not yet identified, but it probably activates an endonuclease system. The ubiquination process is thought to follow the selective digestion of sperm mt.DNA by endonucleases . Elimination of sperm mitochondria in the mouse can be inhibited by treatment with anti-tpis. Recently, transmission of paternal mt.DNA was detected in skeletal muscle of a patient, but this is an infrequent phenomenon. Paternal transmission has also been studied in ICSI and IVF embryos and offspring. In these cases, low amounts of paternal mt.DNA were detected in 16 of the 32 abnormal polyploid embryos but not in offspring normal embryos. Mitochondria of the mammalian spermatozoon are restricted to the midpiece of the flagellum . The elongated mitochondria wrap in a helical fashion around the outer dense fiber-axoneme complex to form the cylinder-shaped mitochondrial sheath. Within the sheath, adjacent mitochondria associate end to end and along their lateral surfaces. This positioning of a concentrated array of mitochondria adjacent to the flagellum is believed to represent an efficient mechanism for the provision of energy required for flagellar motility .

Absence of or abnormal mitochondria have been described in asthenozoospermic or akinetozoospermic men. The spectrum of midpiece abnormalities ranges from irregular and disorganized mitochondria to a decreased number or absence of mitochondria. For instance, spermatozoa with shorter midpieces were found in asthenozoospermic subjects, while midpiece width and tail length were comparable to controls. At the ultrastructural level, the asthenozoospermic subjects demonstrated significantly fewer mitochondrial gyres than their fertile counterparts. The reason for this can be that fewer mitochondria are initially formed or there is a faulty migration system where an excess of mitochondria is lost as the peripheral cytoplasm is shed. Sperm mitochondria are further modified during sperm maturation in the epididymis, the disordered arrangement of the midpiece mitochondria could represent a failure of this process . It is also possible that both a reduction in mitochondria and a failure of bond formation in the midpiece plays a part in the poor quality of movement of spermatozoa in these subjects. Asthenozoospermia due to a complete absence of mitochondria has been reported in several subjects. The spermatozoa from a patient in some studies revealed the mitochondria with increased matrix, thickening of membranes, parallization of cristae and lipid inclusions, which are characteristic findings in mitochondrial disorders. Abnormal mitochondria were also found in spermatids, suggesting that the ultrastructural changes of the mitochondria are primary rather than secondary to the degeneration of the spermatozoa . The highest frequency of occurrence of the 4977-bp mt DNA deletion were found in sperm in the fraction with the lowest motility and a higher incidence of the deletion was found in patients with asthenozoospermia, oligozoospermia and primary infertility compared with normal individuals . Considering the importance of mitochondria in spermatogenesis, many groups have studied the properties of mitochondria from infertile or elderly male and hypothesize that some forms of infertility may be explained as premature aging of the testes.

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ROLE OF OXIDATIVE STRESS AND GENETIC FACTORS IN MALE INFERTILITY

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ABSTRACT

Male infertility is responsible for almost 50% of all cases of infertility and affects ~7% of men worldwide. About 30 to 40% men in the reproductive age group have a qualitative or a quantitative defect in sperm production and several factors acting at pre-testicular, testicular and post-testicular levels may impact fertility and are classified as secretory or excretory causes. Over 2000 genes play a role in the regulation of male reproductive health, development and differentiation of male reproductive system and the male gametes. It results from insufficient or no production of sperm, lack of structural and genomic integrity and defective motility which affects transport of sperm to the site of fertilization, fertilization as well as the outcome of fertilization. Spermatogenesis is a complex process involving many cellular processes as well as a large number of genes in humans. Underlying causes of male infertility have been shown to be genetic as well as non-genetic or a combination of both. Genetic aberrations associated with male infertility range from chromosomal anomalies, microdeletions, monogenic aberrations, gene polymorphisms, copy number variations (CNVs) and mutations. As Y-chromosome was thought to be largely responsible for sex determination, mutations in the AZF and SRY regions of the Y-chromosome are the earliest known changes responsible for male infertility. With advances in genomics, reports on the disruption of autosomal genes associated with male infertility are continuously emerging. Among non-genetic factors responsible for male infertility, oxidative stress plays a big role. Physiological levels of ROS are essential for capacitation and hyperactivation of sperm and many redox sensitive reactions. Due to limited anti-oxidant capacity and a highly truncated DNA repair mechanism, sperm are unable to protect themselves against various environmental insults which disrupt genomic integrity, the chief among them being oxidative stress. Oxidative stress has been shown to affect structural, mitochondrial and nuclear genome integrity by several mechanisms including lipid peroxidation of mitochondrial membrane leading to a vicious cycle of ROS generation which has been shown to induce mutations in the nuclear and mitochondrial DNA by oxidative damage. Thus oxidative stress affects integrity & fertilization potential of sperm as well as outcome of pregnancy. Oxidative DNA damage also induces de novo germ line mutations and post-zygotic mutations in the embryo and increases the spontaneous mutation rate. Accumulation of oxidative DNA adducts especially at the CpG islands induces methylation alterations and thus impacts the epigenome.

Thus oxidative stress and oxidative DNA damage not only impact the reproductive health of the individual but impacts the health trajectory of the offspring conceived using sperm with high levels of DNA damage. This review discusses recent developments regarding the role of genetic factors and oxidative stress in male infertility, which has substantial significance in the determination of strategies for infertility management.

INTRODUCTION

Globally about 70 million couples are infertile. Inability to conceive can be due to male factors, female factors or combination of both. On an average, infertility affects around 15% of couples (De Kretser DM 1997). In almost 50% of cases infertility is due to male factor and about 30 to 40 % cases are idiopathic. Male infertility is attributed to genetic, non-genetic and unexplained factors. In a large number of male infertility patients the aetiology of disease is not known. Understanding of genetic basis of infertility is a growing field and the unexplained factors responsible for male infertility can be an interplay of unknown predisposing genetic factors coupled with a facilitatory environment biologically precipitating into male infertility.

Causes of male infertility can be classified as pre-testicular, testicular & post-testicular. Pre-testicular male infertility has its origin in endocrine disturbances which include disorder of Gonadotrophin-releasing hormone production and secretion, disorders of LH, FSH & androgen function and other genetic/acquired endocrinopathies affecting testicular functions. Male infertility of testicular origin can be due to varicocele, cryptorchidism, testicular injury/infection, Gonadotoxin/prior chemothreapy exposure, smoking, alcohol abuse or due to genetic causes affecting spermatogenesis. Male infertility of post-testicular origin results from genitourinary tractobstruction, auto-immune conditions affectingviability, ejaculation disorders & erectile dysfunctions etc.

Male infertility w.r.t spermatogenesis can be broadly categorised into two categories: quantitative defects, qualitative defects and a combination of both these defects. Quantitative defects can range from azoospermia to cryptozoospermia (<1 million sperms/ml) to oligozoospermia (<15million sperms/ml). Azoospermia can be of excretory origin (obstructive), azoospermia and secretory origin (non-obstructive) azoospermia. Obstructive azoospermia can be due to obstruction or dysfunction of genitourinary tract affecting sperm release. Non-obstructive azoospermia can result from endocrine disturbance of the hypothalamic-pituitary axis origin or due to other causes like spermatogenesis arrest, infection, inflammation and autoimmune disorders, etc. Qualitative defects affect the ability of sperm to reach site of fertilization, ability to fertilize the ova due to structural and/or functional defects irrespective of the quantitative defects. The structural defects are defects in head, mid-piece or tail of sperm which affects sperm motility and ability of sperm to travel to site of fertilization are collectively termed teratozoospermia. Sperm motility defects resulting from defects in sperm flagella, metabolic deficiency are collectively called asthenozoospermia. WHO from time to time has defined normal ranges of seminal parameters like sperm count, motility,

semen volume etc. in context of evaluation of male fertility but a male having all his semen parameters in the normal range may still be infertile. Thus conventional sperm parameters are poor predictors of fertility potential. Where no cause of male infertility can be identified as all sperm parameters are normal and all relevant clinical and diagnostic evaluations, such condition is called unexplained male infertility.

Male infertility can result from genetic or non-genetic factors. Non-genetic factors responsible for male infertility include environmental factors, endocrine disorders, oxidative stress, infections, varicocele, auto-immune disorders, lifestyle etc. This paper focuses on the role of oxidative stress and genetics in male infertility.

GENETIC FACTORS

Clinical presentation of male infertility spans a wide spectra like its genetic basis and with advent of next generation sequencing, genetic landscape of male infertility has expanded further. New genes which play role in male fertility are emerging. Genetic aberrations associated with male infertility range from chromosomal anomalies, microdeletions, monogenic aberrations, gene polymorphism, copy number variations (CNVs) to point mutations. Karyotypic abnormalities and deletions of the azoospermia factor (AZF) regions of the Y chromosome are most prevalent sub-microscopic deletions and earliest known genetic causes of spermatogenesis failure. Microscopically visible alterations of Y-chromosome affecting male fertility include Ring-Y chromosome, truncated or isodicentric Y-chromosome.

Aneuploidy of sex chromosomes are most prevalent chromosome anomalies in infertile men (Gekas J et al. 2001). Klinefelter's syndrome (47, XXY) is one such aneuploidy highly prevalent in infertile men who present with hypogonadism and azoospermia.Extra dosage of genetic and epigenetic alterations in Klinefelter syndrome, besides androgen deficiency, is thought to be reason for clinical manifestations in Klinefelter syndrome (Sharma A et al., 2015; Zitzmann M et al., 2015; Belling K et al., 2017). Robertsonian translocations, inversion and reciprocal translocation are more frequently observed structural anomalies of autosomes in Oligozoospermic men (Vincent MC et al., 2002). Chromosome structural and numerical anomalies have been hypothesised to cause spermatogenesis failure and male infertility by interfering with chromosome synapsis during meiosis. Asynapsed regions of chromosome have been shown to trigger meiotic checkpoints resulting in spermatogenesis failure (Sun F et al., 2007). Besides structural rearrangements have also been proposed to affect structural integrity and thus expression of genes involved in spermatogenesis (Harewood L & Fraser P, 2014) resulting in male infertility.

Long arm of Y-chromosome was predicted almost half a century ago to carry genes essential for spermatogenesis (Tiepolo L & Zuffardi O, 1976). Later, AZF regions on long-arm of Y-chromosome were proposed to be essential for normal spermatogenesis (Vogt PH et al., 1996; Skaletsky H et al., 2003). Genetic anomalies of AZF regions are most frequent cause of Azoospermia and Oligozoospermia and are present in up to 30% of Azoospermic men. Complete deletion of Azoospermia Factor (AZF) regions of Y-chromosome are found in upto 20% of infertile men with severe oligozoospermia or Azoospermia (Krausz C et al., 2003). AZF region on Y-chromosome includes AZFa, AZFb & AZFc sub-regions. Complete deletion of AZFc is most common, deletion of AZFb less common and deletion of AZFa least common in infertile men with oligozoospermia or azoospermia. Incomplete or partial deletion of AZF regions is also present in infertile men with spermatogenic failure. Partial deletion coupled with duplication of AZFc region has been shown to be a risk factor for spermatogenic failure (Lin YW et al., 2007). Partial deletion of AZFa and AZFb with natural transmission of AZFc resulting in Oligozoospermia are comparatively much less reported in event of natural pregnancy resulting from presence of a fertile female (Krausz C & Casamnoti E, 2017). gr/gr deletions are other common deletion of AZFc region associated with Oligozoospermia and result from non-allelic homologous recombination and affect dosage of several multicopy genes like BPY2, CDY1 and DAZ which are involved in spermatogenesis (Repping S et al., 2003; Krausz C et al., 2014). Deletion of AZF region &consequent instability of the Y chromosome may also lead to mosaicism (46XY/45XO) which decides the severity of phenotype in case of Y-chromosome anomalies in offspring. Thus the child may inherit same or larger deletion.AZFc deletions are also associated with progressive decline in sperm count and thus the infertile couple must be explained the need of sperm cryopreservation at the earliest after puberty. Mutations and translocation of Sex determining region of Y chromosome (SRY), a master regulator gene responsible for initiation of testis development and differentiation, are found in XY females (Swyer syndrome) and XX males (de la Chapelle Syndrome) (Quinn A and Koopman P, 2012).

Current clinical practice of identifying genetic determinants of male infertility is largely limited to screening of chromosomal deletions, translocations etc., with Androgen Receptor mutations being the only standard monogenic factor screened in this process. Mutations in Androgen Receptor (AR) gene lead to male infertility due to androgen insensitivity.Monogenic causes of male infertility are quite understudied, thus are not well characterized and much less standardised. Since ~2000 genes are involved in spermatogenesis, knock-out studies in mice suggest that a large number of genes (>400) can result in male infertility of monogenic origin (Jamsai D & O'bryan MK, 2011). Advances in genomic technologies like Comparative Genomic Hybridization, Genome-wide Association Studies &Exome Sequencing etc. have immensely increased power to identify and characterize genes involved in male fertility. A recent literature survey study listed 78 new genes moderately linked to male infertility (Oud MS et. al., 2019). Alhathal N. et al., 2020identified another 36 novel variants in 33 candidates genes responsible for male infertility using exome sequencing. Variants in these genes were not previously linked to male infertility.Many of these novel candidate genes are functionally involved in meiosis. Mutations of CFTR genes are associated with Male Infertility. CFTR is a transmembrane conductance regulator involved in transmembrane chloride ions transport and is expressed in epithelial cells. CFTR mutations lead to male infertility by virtue of congenital bilateral absence of vas deferens (CBAVD) or congenital unilateral absence of vas deferens (CUAVD). Similarly, mutations in SPATA16 (spermatogenesis associated 16), PICK1 (protein interacting with PRKCA 1) and DPY19L2 (DPY-19 like 2) have been shown to cause Globozoospermia (De Braekeleer M et al., 2015), a condition in which spermatozoa are unable to bind to zona-pellucida and lead to fertilization due to deficiency of Phospholipase C zeta.Homozygous recessive mutations in Aurora Kinase C, a p.Y248* non-sense mutation & c.144delC homozygous frameshift mutation, have been shown to be responsible for Macrozoospermia, a condition characterised by presence of high percentage of spermatozoa with enlarged heads and multiple flagella (De Braekeleer M et al., 2015). Due to involvement of various genes in sperm head formation and its coupling with sperm tail, mutations in many genes like Polyaminemodulated factor 1 binding protein 1 (PMFBP1), testis specific 10 (TSGA10), Sad1 and UNC84 domain containing 5 (SUN5), bromodomain testis associated (BRDT) and centrosomal protein 112 (CEP112)result in production of acephalic

spermatozoa. Due to involvement of ~2000 genes in spermatogenesis, mutation in any one of these genes have potential to lead to male infertility.

Some SNPs have also been shown to be strongly associated with infertility, and such association may become more stronger in context of facilitatory genetic background and pre-disposing environment. SNP arrays were used to identify SPATA16 and DPY19L2 as candidate genes for Globozoospermia (Dam, A. H. D. M. et al., 2007; Harbuz R et al., 2011). Polymorphisms of CYP2D6 and CYP1A2 have been proposed to have a role in idiopathic male infertility (Hekim N et al., 2019). Protamine2 polymorphisms G398C and A473C have been shown to be associated with the teratozoospermia (Dehghanpour F et al., 2019). Genotype CC of SNP rs4045481 in RNF212 gene has been proposed to be a risk factor of azoospermia (Yu CH et al., 2018). Similarly, GWAS studies based on SNP arrays by Hu Z et al., 2011 & Zhao H et al., 2012, identified some SNPs associated with non-obstructive azoospermia but they have not been yet validated by further studies.

Copy Number Variations (CNV) contribute to phenotypic diversity as well as onset of several diseases possibly by defective recombination, meiotic failure and cell death.Comparative Genomic Hybridization platforms enabled identification of CNV67, a recurrent X-chromosome linked CNV, having effect on spermatogenesis and male fertility (Lo Gizacco D et al, 2014; Shen Y et al., 2017). E2F1 CNV have been shown to contribute to spermatogenic disruption and cryptorchidism by increasing susceptibility to heat stress (Rocca MS et al., 2019). Similarly, CNV of cation channel of sperm (CATSPER) affects fertilization potential of sperm by impairing penetration ability & acrosome reaction without disturbing semen parameters (Luo T et al., 2019).

Despite all the advances in understanding the genetic landscape of male infertility, current diagnostic approach to screen for genetic aberrations responsible for male infertility have yield of only 4% (Tüttelmann F et al., 2018).Studies in our lab have shown that infertile men have higher number of variants spread throughout the genome.

ROLE OF OXIDATIVE STRESS IN MALE INFERTILITY

Oxidative stress refers to supraphysiological levels of reactive oxygen species (ROS) which are defined as highly reactive oxygen containing molecules in free radical form or non-radical formswhich upon reaction extract an electron from reactants. ROS being metabolic intermediates are also essential part of various biochemical reactions.Obesity, Diabetes, Smoking,psychological stress, poor nutrition, excessive alcohol consumption, chronic inflammation & varicocele etc. are some of the common causes of oxidative stress. ROS include oxygen radical (O2-.), hydrogen peroxide (H2O2), hydroxyl radical (OH.) as well as products generated from their reaction with biomolecules which in turn are also reactive like organic hydroperoxides (ROOH), Peroxyl radicals (ROO.) and alkoxyl radicals (RO.) etc. ROS are either generated during various cellular reactions or canbe of extracellularoriginand when ROS generation overwhelms the antioxidant defence of cells or extracellular space, it results in oxidative stress. Being highly reactive, overwhelming ROS can result in their undesired reactions with biomolecules like DNA, proteins & lipids present in their immediate vicinity leading to adduct formation thus resulting in genetic & structural damage. Research from our lab suggests that mild oxidative stress seems to have beneficial effects in terms of maintaining sperm telomere length (Mishra S et al., 2016). Though physiological levels of reactive oxygen species (ROS) are required for sperm capacitation and hyperactivation to acquire fertilizing capacity, supraphysiological levels of ROS overcome the antioxidant defences of the cell and result in damage to cell. Electron transport chain in mitochondria, peroxisomes and several cellular enzymes like are main endogenous sources of free radicals in somatic cells. Sperm mitochondria,spermatozoa enzymes like NADPH oxidase, lipoxygenase & L-amino acid oxidase & activated leukocytesin seminal plasma are main sources of free radicals in human semen.

Leukocytes presence in semen above leukocytospermic threshold of >1 million/ml is source of oxidative stress. Leukocytespresence, mainly Neutrophils in activated state, in semen above this threshold is mostly due to reproductive tract infection, auto-immune reaction, trauma or surgery etc. (Aitken RJ et al., 1994; Saleh RA et al., 2002). Similarly leukocyte presence in washed sperm preparations or IVF culture media is damaging to sperms during assisted reproduction procedures due to lack of anti-oxidant properties of seminal fluid and limited anti-oxidant capacity of IVF culture media (Krausz C et al., 1994; Sukcharoen N et al., 1995). Being specialised cells, sperm cells have limited cytoplasm and comparatively limited defence mechanisms and capacity to counter oxidative stress and consequent structural-functional damage resulting from oxidative stress (Cristian O'Flaherty, 2014). Supraphysiological levels of ROS have been shown to harmfully affect sperm membrane fluidity and permeability as well as damage sperm DNA (Kodama H et al., 1997; Aitken RJ et al., 1998). Sperm membrane is enriched with polyunsaturated fatty acids (PUFA) which contain several carbon-carbon double bonds which can react with reactive oxygen species to generate peroxyl (ROO*) and alkoxyl (RO*) radicals which further generate other lipid radicals by sequestering hydrogen from neighbouring carbon atoms (Koopers AJ et al., 2010). These reactions cause further peroxidation of membrane lipids finally culminating in production of low molecular weight aldehydes malondialdehyde, acrolein and 4-hydroxynonenal which react with each other to form dimers. The dimers so formed are mutagenic and can react with sperm genome to form DNA adducts and cause mutations in sperm genome (Marnett LJ 2002; Cejas P et al., 2004; Ayala A et al., 2014) which unlike somatic cells have limited DNA-repair potential. Oxidative damage to sperm genome have post-fertilization consequences also because ova &zygote before first cleavage divisionhavepoor expression of Base-excision repair enzyme OGG1 resulting in potential propagation of oxidative stress induced mutations in to zygote and subsequently to embryo& offspringaffecting their health and survival (Smith TB et al., 2013; Aitken RJ et al., 2001; Aitken RJ 2017). Besides genomic damage, oxidative stress also damages telomeres and centrioles of the sperm, these structures being highly vulnerable to oxidative stress (Lu Y et al., 2017; Darmishhonejad Z et al., 2020). Telomeres are paternally inherited structures and if a sperm with oxidatively damaged centrioles and telomere fertilizes an oocyte, it has potential to adversely affect embryonic development, implantation and progress of embryo to term.Oxidative stress not only damages the genome but also the RNA and normalization of levels of free radicals post yoga based lifestyle intervention improves DNA integrity of mitochondrial and nuclear genome but also normalizes levels of sperm transcripts which are critical for early embryonic development.

As oxidative stress is well known to damage sperm genome there are about 9000 regions with are hotspots for oxidative damage . It has been observed that except few mutations which affects fertility associated genes, most of the mutations affecting fertility don't localise to oxidative stress induced mutation hot-spot on chromosome 15, which largely harbours genes associated with neurological development (Aitken RJ & Baker MA 2020). These genes have paternal inheritance pattern and as advanced paternal age is associated with increase oxidative stress and DNA damage in spermatozoa, neurological disorders like Autism, bipolar disease, spontaneous schizophrenia, epilepsy, Marfan syndrome &

attention deficit hyperactivity disorder etc. are more associated with conception at advanced paternal age. Similarly use of assisted reproduction techniques is also associated with increase oxidative stress and consequent damage to sperm genome, risk for autism & Prader-Willi syndrome was observed to be higher in case of ICSI, than IVF (Kissin DM et al., 2015; Hattori H et al., 2019; Aitken RJ & Baker MA 2020). Similarly smoking being associated with higher oxidative stress induced DNA-damage in spermatozoa due to 8-OHdG lesions, a significant observation was found between cancer incidence in offspring and paternal smoking (Heerema NA et al., 2020; Lee KM et al., 2009; Aitken RJ & Baker MA 2020)

Besides genetic aberrations, oxidative stress also leads to epigenetic changes. Methylation of DNA at Cytosine nucleotide in CpG islands sequences is one of the major epigenetic mark. DNA methylation is done by DNA methyltransferases (DNMTs), mostly at cytosine nucleotide in CpG island sequences leading to heritable changes in gene expression without modification of DNA sequence. Methylation of CpG islands which are situated in or near promoter region of a gene usually leads to downregulation of expression of that gene. Both cytosine and guanine nucleotides are susceptible to oxidation. Guanine nucleotide is most sensitive to oxidation and its oxidation leads to formation of 8-oxo-2'-deoxyguanosine (8-oxodG). 8-oxodG can be removed by OGG1 DNA glycosylase followed by gap filling by DNA Base Excision repair machinery. But OGG1 recruitment to DNA can also lead to DNA demethylation by TET1(Zhou X et al., 2016). Thus supraphysiological/uncontrolled ROS has potential to induce loss of DNA methylation in form of global hypomethylation through TET1. Similarly, oxidation of Cytosine by ROS leads to formation of 5-hydroxymethylcytosine and several other oxidation products (Ménézo Y et al., 2010). Subsequent deamination of the said oxidation products of cytosine and guanine can lead to their conversion into Thymine. This results in hypomethylation of CpG island sequences which in turn can cause overexpression/relocalization of concerned DNA methyltransferases and subsequent aberrant hypermethylation of undesired sequences. Thus pathological levels of unmanaged ROS observed in idiopathic male infertility, in long term has potential to change the epigenetic landscape of cell.

Oxidative stress also affects mitochondria, one of its own source, at both structural and genomic levels (Koppers AJ et al., 2008). Reaction of oxygen with electrons leaked from electron transport chain in mitochondria leads to generation of superoxide anion along with ROS generation from activation of intrinsic apoptotic pathway which also get activated during senescence of sperms in vitro(Koppers AJ et al., 2011; Aitken RJ et al., 2012a). cis-unsaturated fatty acids, common preservative parabens, bisphenol A, cryopreservation, radiofrequency electromagnetic radiation, UV radiation, heat and lipid aldehydes generated due to lipid peroxidation are some of the stimulants of ROS generation from mitochondria in spermatozoa. Lipid aldehydes like Acrolein & 4-hydroxynonenal (4HNE) are particularly notorious in initiating self-perpetuating chain of ROS generation from mitochondria of spermatozoa by binding to mitochondrial electron transport chain proteins (Aitken RJ et al., 2012b; Moazamian R et al., 2015). NAPDH Oxidase (NOX5) is another mitochondrial enzyme involved in ROS generation in spermatozoa(Musset B et al., 2012) and has role in sperm capacitation but is unusually high in Asthenozoospermic men with high DNA damage&in Teratozoospermic men (Ghani E et al., 2013; Vatannejad A et al., 2019). Lipoxygenase is another enzyme upregulated in spermatozoa with high residual cytoplasm content (Fischer KA et al., 2005) and has potential to generate lipid hydroperoxides by deoxygenation of polyunsaturated fatty acids (PUFA) present in spermatozoa membrane. These lipid peroxides in presence of transition metals iron & copper can generate lipid peroxidation cascade culminating in generation of lipid aldehydes like 4-HNE which further react with key sperm proteins affecting their function (Aitken RJ et al., 1993).Lipoxygenase inhibitor has been shown to reduce 4-HNE levels and improve sperm function (Walters JLH et al., 2018). L-amino acid Oxidase is another spermatozoal enzyme which may be involved in ROS generation using aromatic amino acids (Houston B et al., 2015).Mitochondrial genome encodes only for ~1% of proteins required for its function and rest of the mitochondrial proteins are nuclear encoded and transported to mitochondria after their synthesis. Nuclear and Mitochondrial genome communicate with each other through several messengers which include metabolic intermediates which act as either inhibitors or activators of epigenetic machinery enzymes e.g., alpha-ketoglutarate, an intermediate of Krebs cycle, is an co-factor of TET demethylases and succinate and fumarate, two other intermediates of Krebs cycle, are inhibitors of TET demethylases (Laukka T et al., 2016; Tahiliani M et al., 2009). Succinate is also an intermediate in ROS generation in mitochondria. Thus any effect of oxidative stress on mitochondrial function, by affecting nuclear-encoded mitochondrial proteins through enzymes regulating nuclear epigenetics, will affect mitochondria in return, thus forming a feedback loop between nucleus and mitochondria. Oxidative stress has been shown to result in activation of both mitochondria-dependent and mitochondria-independent pathways of apoptosis (Sinha K et al., 2013). Oxidative stress can lead to mutations in mitochondrial DNA. High mitochondrial mutation load can impair mitochondrial energy production leading to meiotic arrest during spermatogenesis followed by apoptosis of spermatocytes. Research from our lab as well as other labs has shown that low levels of mitochondrial mutations affecting ATP production in spermatids can lead to motility defects or impaired morphology in turn leading to oligospermia or asthenozoospermia (Kao SH et al., 1998; Shamsi B et al., 2008; Ambulkar PS et al., 2016). Further, mitochondrial mutations in spermatogonial cells lead to meiotic arrest of spermatogenesis which can result in hypospermatogenesis or maturation arrest phenotypes.

Oxidative stress adversely affects spermatozoa motility, ability to undergo acrosome reaction, capacity to fuse with oocyte vitelline membrane and genetic integrity of spermatozoa. Thus oxidative stress affects spermatozoa at structural, functional and genetic levels with adverse consequences at pre-fertilization, fertilization and post-fertilization stages.Thus it is important to maintain optimal seminal free radical levels for normal functioning of redox sensitive processes but also to prevent DNA damage to both the mitochondrial and nuclear genome and lower the burden of genetic, epigenetic disorders in the offspring.

THE WAY FORWARD

There is compelling evidence for etiological role of oxidative stress in male infertility, which is both a bad news and a good news, bad news from the perspective that oxidative stress is largely ignored in terms of not being inclusive in clinical workup towards management of male infertility and good news from the perspective thatunlike genetic factors it can largely bemanaged if found involved etiologically in male infertility in case specific manner. Standard clinical workup should be inclusive of oxidative stressassessment& its possible causes in a given case. Antioxidantsas therapy (reviewed in Showell MG et al., 2014) and work in our lab on effect of Yoga-based lifestyle intervention on oxidative stress (Dhawan V et al., 2018; Bisht S et al., 2020) has shown to be helpful in

management of oxidative stress associated with fertility and in other complex disorders.While yoga-based lifestyle intervention is an ages old practice and has no known adverse effects but antioxidant overuse in absence of oxidative stress or after abatement of oxidative stress has potential to induce equally damaging reductive stress (Peris E et al., 2019).Yoga not only improves integrity of the mitochondrial and nuclear genome, it normalizes transcripts and causes hypomethylation of genes which maintain genomic integrity.It can reduce the incidence of childhood cancers and other autosomal dominant disorders and complex neuropsychiatric diosrders by decreasing the oxidative DNA damage which induces both germ line and post zygotic mutations due to persistence of damaged sperm DNA post fertilization. Besides patients can be counselled about avoidance of oxidative stress inducers like poor diet, obesity, alcohol overconsumption, smoking,sedentary life style etc. besides identifying potential causes of oxidative stress by studying occupation, medical history & lifestyle of patient.Going a step ahead to measure DNA damage suffered by spermatozoa due to oxidative stress will also help in evaluation of potential risk to future offspring and to help patient make informed choices. Genetic landscape of male infertility is still largely a no-man's land. Monogenic causes of male infertility are still not well understood. Genetic lesions responsible for sub-fertility also largely pass undetected to next generation. While not much can be done therapeutically in most cases of male infertility of genetic origin but improved understanding of genetic lesions leading to male infertility can help patients make informed &better choices.As oxidative stress is a major player in inducing mutations and epimutations both which cause infertility and affect health of offspring if such sperm with damaged DNA are used it is of vital importance to assess levels of seminal free radicals and quantify oxidative DNA adducts in couples with male factor infertility opting for assisted conception.

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