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Paternal Factors - Role in Idiopathic Recurrent Pregnancy Losses

Review Article

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Introduction

Recurrent pregnancy loss (RPL) or habitual miscarriage is the loss of three or more consecutive pregnancies before the 20th week of gestation [36]. The World Health Organization (WHO) has defined a miscarriage as the loss of a fetus or embryo weighing ≤500 g, which would normally be at 20−22 weeks of gestation. RPL affects approximately 1 in 300 pregnancies. However, epidemiologic studies have revealed that 1% to 2% of women experience recurrent pregnancy loss [68]. There are several leading causes of RPL, among them are uterine anatomical defects, (intrauterine adhesions, uterine fibroids or polyps and cervical incompetence), genetic factors, infectious, immunological, environmental and blood dyscrasias. However, despite extensive investigation of female partners in a large number of cases (40%−50%) no cause has been identified and such cases are classified as idiopathic. It is possible that in such cases, the male partner may harbour sperm abnormalities beyond abnormalities in standard semen parameters. Evaluation of male factor in RPL has not been done extensively. Paternal chromosomal analysis is the only evaluation which is done and sperm molecular factors like oxidative stress, DNA damage and sperm transcripts are not analysed. With the advent of advanced assisted micromanipulation procedures, the role of sperm factors is being increasingly realized as routine semen parameters do not provide much information regarding sperm functional competence and reproductive potential. Thus, this review is written with the aim to discuss the role of sperm factors namely oxidative stress, DNA damage, dysregulation in sperm transcripts in aetiology of recurrent pregnancy losses.

Increased life expectancy, advanced age of marriage, various socio-economic factors and an overall change in role of women in society has led couples delay parenthood. Also the increased accessibility to assisted reproductive techniques has increased the chance of older parents with poor pregnancy outcomes to conceive children, hence, increasing the average paternal age at first childbirth. In comparison to 1993, the paternal age of fathers has increased by 15% in a period of ten years [16]. Increased paternal age affects testicular function [41], reproductive hormones [33], sperm parameters [1, 14], sperm DNA integrity [51], telomere length [18], de novo mutation rate [22], chromosomal structure [51, 58] and epigenetic factors [23].

The paternal contribution to an embryo plays an important role in understanding early developmental processes and their effect on the health of a child. Sperm cells are highly differentiated, polarized and specialized, containing only the constituents required during and after fertilization (early embryonic development). The contribution of the male gamete to embryogenesis has not been well investigated. During fertilization, the sperm transmits not only nuclear DNA but also oocyte activation factor (OAF) (critical for fertilization), centrosomes (critical for cell division) [70], and a population of messenger RNA (mRNA), non coding RNA that are of critical developmental importance [54]. It has also been established that non-genetic modifications (DNA methylation, histone tail modifications, targeted histone retention and protamine incorporation into the chromatin) during embryonic development have a significant influence on the sperm cell develoment. Damage to genetic constituents and perturbations in the maintenance of these mechanisms have been demonstrated to affect fertilization potential and the early development of the embryo [42].

DNA Fragmentation

Semen analysis is an important first step in the laboratory evaluation of the infertile male. It includes the assessment of the ejaculate volume, sperm concentration, motility, and morphology using WHO criteria [75]. As routine semen analysis has low predictive value for reproductive outcome and sperm functional assessment [46], sperm DNA testing has been increasingly used as an adjunct to the routine semen analysis [3, 21, 30, 78]. Sperm DNA integrity is one of the important determinants of normal fertilization and

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embryo development though several studies indicate that they play a major role 2 days post fertilization with activation of paternal genome. Many studies have shown that high sperm DNA damage is associated with numerous reproductive processes, e.g. impaired fertilization, disrupted preimplantation embryo development, miscarriage, and birth defects in the offspring [20, 30, 78]. Zini et al., (2008) and (Robinson et al., 2012) [78, 59] have observations and concluded that sperm DNA damage was not only associated with a significantly increased risk of pregnancy loss after IVF and ICSI but also after spontaneous conception. Kumar K et al., documented that a DFI greater than 30 is associated with failure to conceive spontaneously and DFI >24 and less than 30 is associated with spontaneous conception but recurrent spontaneous abortion.

Sperm DNA damage is thought to be induced by several mechanisms: (1) apoptosis during the process of spermatogenesis; (2) DNA breaks generated during the remodeling of sperm chromatin during the process of spermiogenesis; (3) post-testicular DNA fragmentation, which is induced by oxygen radicals, including the hydroxyl radical and nitric oxide, during sperm transport through the seminiferous tubules and storage in the epididymis; (4) DNA fragmentation induced by endogenous caspases and endonucleases; (5) radiotherapy and chemotherapy; and (6) environmental toxicants and xenobiotics, (7) smoking and alcohol and (8) advanced paternal age [8, 60]. Sperm carrying damaged DNA can complete the initial process of fertilization but oxidative stress and various environmental factors target the peripheral nucleohistone compartment which has telomeric DNA and promoters of several genes of key developmental importance. Oxidative damage to telomeres is associated with accelerated shortening and accumulation of highly mutagenic base 8 hydroxy 2 deoxyguanosine and genomic instability.

DNA fragmentation as a result of single or double strand breaks can be measured by two common methods i.e. sperm chromatin structure assay (SCSA) [28, 31], or by the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay [67]. TUNEL however cannot differentiate between apoptosis and necrosis. Abortive apoptosis like features in immature/abnormal sperm include remnants of cytoplasm and poor chromatin packaging and/or damaged DNA Abortive apoptosis is initiated during spermatogenesis. Spermatozoa marked for elimination escape at ejaculation in what is called abortive apoptosis and contribute to poor sperm quality. DNA damage in ejaculated spermatozoa cannot be explained by apoptosis alone [61-63] but can also be due to aneuploidy as well as mutations, chromosomal disjunction and meiotic segregation [14, 15, 50]. DNA damage can also result from activated PARP and activated caspase3 and PARP-1 has been implicated in DNA damage and apoptosis as it activates apoptosis during increased DNA repair and damage. Mishra S et al., 2015 documented that sperm of infertile men have lower levels of PARP and as this is key enzyme which is recruited whe their are DSB lower levels are associated with incomplete and partial repair and persistence of DNA damage.

In the sperm nucleus, the chromatin is organized into loop domains that are attached to a proteinaceous structure, termed the nuclear matrix, every 20-120 kb. This organizes the chromatin into functional loops of DNA that help regulate DNA replication and gene transcription [74]. Several studies have demonstrated a functional role for the sperm nuclear matrix during early embryogenesis. These data suggest two roles for the sperm nuclear matrix [64, 76]: to facilitate the proper association of DNA with the nuclear matrix (required for paternal pronuclear DNA replication). A study by Carrell (Carrell et al., 2003a) [19] reported that recurrent miscarriage is related to higher levels of sperm DNA damage, and Evenson (Evenson et al., 1999) [29] observed that pregnancy loss rate increased significantly with sperm DNA damage. Animal studies [2, 32, 55] regarding the possible mechanism underling the association between sperm DNA damage and miscarriage showed that sperm DNA damage can lead to abnormal embryo development and impaired embryo implantation.

Oxidative Stress

"Oxidative stress" is a state of homeostatic imbalance associated with cellular damage induced by increased oxygen and oxygen-derived oxidants (reactive oxygen species) which overwhelm the antioxidant defence mechanisms [47]. Oxidative stress in sperm is the result of imbalance between ROS generation and the scavenging antioxidant potential. Sperm exist in a state of oxygen paradox as they require oxygen for ATP production but are thus exposed to high ROS levels which damage both mitochondrial and nuclear DNA [65]. When oxidative stress occurs, reactive oxygen species react with molecules in various biological systems, causing extensive cell damage and disruption of cell function. The excessive production of ROS might have serious implications on sperm structure and functionality, because spermatozoa are particularly susceptible to damage induced by ROS, because their plasma membranes contain large quantities of polyunsaturated fatty acids, their cytoplasm contains low concentrations of scavenging enzymes, and sperm have a limited capacity to repair its DNA [37].

It has been reported that spermatozoal membrane is rich in polyunsaturated fatty acids and it is poor in cytosolic antioxidants. Therefore, sperms are vulnerable to oxygen induced damage which leads to lipid peroxidation and mitochondrial and nuclear DNA damage. Increased reactive oxygen species (ROS) and reduced TAC leads to oxidative stress which culminates in sperm DNA damage impairing the reproductive functional efficiency of the sperm. ROS being highly reactive has a tendency to react with sperm biomolecules as proteins, lipids and DNA. Increased free radical levels damage the nucleohistone component of the sperm genome. This component which maintains its nucleosomal structure has genes which are transcribed and are critical for early embryonic development (HOX and, HSP genes). ROS induces damage to this component and thus severely affects early embryonic development.

It has been reported that majority of couples experiencing RPL are infertile and a large number of couples experiencing assisted and spontaneous conception failure may have underlying sperm factor(s) (sperm mitochondrial and nuclear DNA damage and oxidative stress). A previous study from our laboratory (Shamsi et al., 2009) [66], documented that infertile men with normal/or abnormal sperm parameters had raised ROS and decreased antioxidant levels. However, it is difficult to predict increased ROS levels and DNA damage based on standard semen parameters. Thus tests for seminal oxidative stress and DNA damage should be included in diagnostic workup of idiopathic cases of RSA. Therefore, the attention has now shifted from analysing standard

semen parameters to studying/evaluating molecular aspects of spermatozoa, among these are sperm chromatin structure assay, free radical levels, sperm transcript and telomere length.

Spermatozoal Transcripts

In early years of 20th century, the role and the presence of sperm RNAs was controversial and questioned by many. It's the late in 1990s, different studies documented the presence of different types of RNAs in human spermatozoa. These mRNAs were assumed to be residues left over from spermatogenesis, but there is evidence that the spermatozoa deliver a unique set of mRNAs to the oocyte. Three different types of sperm mRNA have been discovered in human spermatozoa [13]. The first group of mRNAs has a specific function during spermatogenesis but does not exhibit an obvious function post-fertilization. The presence of this set of remnant mRNAs could serve as a diagnostic tool with which to follow the fidelity of the later phases of spermatogenesis [49]. A second group of RNAs (e.g., mRNA coding for PLC-z) also originates from the testicular germ cells and may have an additional role in fertilized oocyte. A third not yet extensively studied group of sperm mRNAs (e.g., mRNA coding for clusterin) may originate from a non-testicular source and, after incorporation into the sperm, could be introduced into the oocyte during fertilization. A recent report also demonstrated that some mRNAs can be translated de novo, supporting the hypothesis that a population of mRNAs may have a function during or beyond the process of fertilization [40]. As there is no evidence that the proteins encoded by the majority of mRNAs found in mature spermatozoa are also present in sperm [25], these mRNAs may be viewed as potential contributors to development in early embryonic period.

The repackaging of DNA into a torroid conformation, which is approximately 20 times more condensed, enables the complete shutdown of the spermatid nucleus [7, 17]. The gradual shutdown of RNA transcription begins during meiosis, when the paired sex chromosomes are accommodated in the male germ cell, leading to the repression of gene expression on the X and Y chromosomes [26, 35]. The shutdown of transcriptional activity in human spermatozoa has been confirmed [39] strengthening the previous observations. The retention of mRNAs in spermatozoa begins to occur during the early stages of spermatogenesis. Any alteration in the amount or composition of sperm mRNAs may indicate abnormalities in spermatogenesis, which may later affect embryo development. The mRNA fingerprints of normozoospermic and teratozoospermic men have been shown to differ [56]. Additionally, variations in the expression of two sperm RNAs coding for LDHC transcript variant 1 and TPX1 have been reported in men with poor sperm motility [73]. A study (Ostermeier et al., 2002) [53] detected over 3,000 mRNA species in ejaculated spermatozoa through microarray analysis.

The presence of different mRNA transcripts in human spermatozoa has previously been reported [77]. Based on microarray data using 8x60K Agilent chip we selected genes which showed significant fold change and investigated the expression of some important genes (*WNT5A, HSP90, PRM2 TOMM7, RBM9, RPS6, RPL10A, EIF5A, AKAP4, STAT4, SOX3* and *FOXG1*) that have been postulated to have a critical role in early development. The PRM2 and HSP90 expression patterns were significantly altered in the male partners of couples with idiopathic recurrent pregnancy loss, while no association with recurrent pregnancy loss was found for WNT5A. Gene expression of TOMM7, RBM9, RPL10A and AKAP4 were up regulated by around one fold, FOXG1, SOX3 and STAT4 were up regulated more than one fold as compared to EIF5A and RPS6 in the male partners of idiopathic RSA couples compared to the controls. In our study we observed increase in the gene expression of genes which are important for normal fetal development. High levels of HSP90 and significantly lower PRM2 levels in the male partners of couples may be due to higher levels of free radicals causing oxidative stress, which is compensated for by increased HSP90 expression. High free radical levels in spermatozoa may cause a pronuclear block, impair cleavage and lead to blastomere fragmentation and poor-quality blastocysts. While high-throughput technologies have provided a glance at the mRNA population contained in spermatozoa, future studies should focus on the functional aspects of these RNAs in the growing embryo. The results from such studies will further strengthen the correlation between the mRNA fingerprint of sperm and embryogenesis.

Telomere Length

Telomeres are highly conserved hexameric nucleotide repeat sequences (TTAGGG). Telomeres cap the ends of eukaryotic chromosomes and confer genomic stability and aid in maintainence of genomic integrity. Their primary role is to preserve genomic structure and maintain its stability [12]. With each successive cell division, and hence with aging, the telomere length in somatic cells undergoes progressive shortening [6, 11, 28]. In somatic cells, the guanine rich telomere DNA is maintained by telomerase, a reverse transcriptase enzyme [5]. With each cell division, due to end replication problem the telomeres are shortened. But telomerase extends telomere by adding TTAGGG repeats. With increasing age, the incomplete DNA replication leads to telomere shortening [5] and oxidative stress further accelerates this shortening. Compared to somatic cells, sperm (germ cell) telomere length (STL) was found to increase with increasing age [4, 34, 44]. Although such rare mechanism of telomeres extension remains unclear and poorly understood, it might be explained as kind of a biological resistance against the aging process. This molecular resistance expressed by human species against aging might be necessary to boost the chances of perpetuation of the species. Further studies are required to confirm this discrepancy of telomerase extension observed in testis.

We have previously shown high levels of free radicals to be associated with DNA damage in sperm, as well as shorter telomeres in the male partner of infertile couples and couples experiencing idiopathic recurrent pregnancy loss. As telomeres are histone-bound and located in the periphery of the sperm nucleus, telomeres are highly susceptible to oxidative damage [72]. Sperm with shorter telomeres show segregation abnormalities and thus are at an increased risk of being aneuploid. Shortened telomere can increase the incidence of offspring with major or minor congenital malformations, childhood cancers, perinatal morbidity, developmental delay, and failure to thrive. Shorter telomeres can be regarded as putative cause of impaired spermatogenesis and slow cleavage and impaired blastocyst development.

Other Factors

Mutations

Spermatogenesis occurs continuously throughout reproductive lifetime and hence spermatozoa can acquire de novo single nucleotide variants or mutations because of the continuous ongoing process of spermatogenesis that involves multiple asymmetric pre-meiotic spermatogonial divisions and the testicular environment is more prone to toxic effects of oxidative stress in ageing men [22]. The paternal contribution to offspring de novo mutations was estimated to increase by 4% per year [45]. At the age of 20, a sperm would have undergone 150 chromosomal replications, and at the age of 50, it would have gone through 840 replications [22, 27] and thus accumulated several nucleotide changes with each cell division. This increases the probability of replication errors in the germ line leading to the accumulation of mutations and hence increased de novo mutation rate in spermatozoa. On average, the rate of de novo mutation increases by two base pairs every successive year [45].

Chromosomal Aneuploidy

Chromosomal aneuploidy is the presence of an abnormal number of chromosomes in a cell. Chromosomal aneuploidy arises during meiosis especially in sperm with shorter telomeres. Most of the aneuploid embryos die in-utero and hence chromosomal aneuploidy is the leading cause of failed pregnancy [71]. On average, 10% of sperm cells of healthy male population have chromosomal aneuploidies and include chromosome 21 and 22 [57]. However, this number increases with paternal age [38]. The incidence of chromosome disomy 18 significantly increases among older men (>50 years) when compared to younger men [38]. McIntosh et al., reported increased risk of up to two fold among fathers of 50 years and older when compared to the fathers of age group 25–29 years [48].

DNA Methylation

Epigenetics is stable heritable modification on histone tails but not the DNA sequence that leads to altered gene expression. Unlike DNA mutations, epigenetic patterns can be disrupted or silenced by various environmental and endogenous factors such as nutrition, age, drug/toxin exposure and phenotypic variation. Therefore, both spermatogenesis and spermiogenesis processes are marked by successive steps of epigenetic reprogramming of the male gamete which is influenced by several environmental factors. These epigenetic events may impair or inhibit key steps of fertilization, implantation and/or the embryo development [24]. Both the complex path of sperm production and the delicate balance of epigenetic and genetic factors during sperm maturation contribute to the formation of a mature sperm with the ability to fertilize an oocyte and contribute to the developing embryo. It has been proposed that the level of DNA methylation in human sperm could be linked to their ability to initiate a pregnancy in an assisted reproduction procedure [10]. It has been found that DNA methylation plays an important role in mammalian development and influences different processes like X-inactivation [9] genomic imprinting and embryo development as soon as the zygote is formed [43]. The sperm cell has a highly differentiated and specialized morphology, and the epigenome of human sperm is unique, elegant and essential to embryogenesis. Epigenetic factors suggest that sperm play diverse and critical roles in regulating embryogenesis. Alterations in sperm epigenome due to aberrant methylation can profoundly impact blastocyst development and oxidative stress induces genome wide hypomethylation and increases genomic instability with unmasking of repetitive elements. Oxidative stress also induces slippage errors and may thus induce microsatellite instability and may lead to pre and post implantation losses.

Conclusions and Future Directions

Paternal factors chiefly oxidative stress, DNA damage, dysregulation in levels of sperm transcripts, shorter telomeres and oxidative stress induced changes in sperm epigenome adversely impact embryonic development and are key determinants in embryonic development and health of offspring. The assay of oxidative stress and integrity of the sperm DNA is essential in couples with iRSA following natural and assisted conceptions. Early diagnosis of oxidative stress should warrant prompt antioxidant supplementation and life style modifications. Studies from our lab have shown a significant decline in seminal oxidative stress (10 days) and decline in oxidative DNA damage(6months) in sperm following practice of yoga and meditation and upregulation in levels of telomerase and improvement in quality of male partners of couples experiencing RPL. It is believed that the mRNAs are transcripts for key developmental genes and that the small non-coding RNAs modify post-fertilization events. Thus analysis of paternal factors are critical in diagnostic workup of couples with idiopathic RPL.

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