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# The effect of epigenetic sperm abnormalities on early embryogenesis

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## Abstract

Sperm are a highly specialized cell type derived to deliver the paternal haploid genome to the oocyte. The epigenetic, or gene regulatory, properties and mechanisms of the sperm assist in preparation of the paternal genome to contribute to embryogenesis and the genome of the zygote. Many recent studies have addressed the issue of altered epigenetic processes in the sperm. This review evaluates the current understanding of DNA damage, chromosome aneuploidy, reduced telomere length, malformations of the centrosome, genomic imprinting errors, altered mRNA profiles, and abnormal nuclear packaging in the sperm prior to fertilization and the observed effects on embryogenesis. Attention has also been given to understanding the underlying etiology of sperm with altered epigenetic mechanisms in humans. (*Asian J Androl* 2006 Mar; 8: 131–142)

**Keywords:** embryogenesis; DNA damage; imprinting; aneuploidy; telomere; centrosome; nuclear packaging; male infertility

## 1 Introduction

Genetic contributions from sperm are vitally important to embryogenesis. In addition to contributing a high fidelity DNA codon sequence, epigenetic control, defined as regulation of DNA transcription, translation, or chromosome function, are required. Recent studies indicate that sperm with damaged DNA or abnormal DNA regulation retain the ability to fertilize an oocyte, but that the embryos created might be negatively affected.

This review evaluates several mechanisms by which genetic and epigenetic sperm abnormalities might contribute to poor embryogenesis. Included are sperm DNA

damage, chromosome aneuploidy, reduced telomere length, malformations of the centrosome, genomic imprinting errors, an altered mRNA profile, and abnormal nuclear packaging.

## 2 DNA damage

Nuclear DNA damage in mature sperm includes single strand nicks or double strand breaks [1]. DNA damage likely occurs during spermatogenesis in distinct pathways. Abnormal spermatocytes might undergo caspase dependent apoptosis, possibly induced by associated Sertoli cells [2–5]. If caspase activity is initiated, the affected sperm are removed via phagocytosis of the supporting Sertoli cells [4]. DNA damaged sperm are also found in testicular biopsy samples, but this damage is reported to be independent of the classical caspase-activated apoptosis pathway [2, 3, 5]. Therefore, the damage identified in mature sperm is likely to result from one or a

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combination of three sources: oxidative stress [6–9], errors in chromatin rearrangement during spermatid elongation [10], or caspase independent apoptosis [11–13].

Mature sperm DNA damage is assayed by several different methods. The susceptibility to DNA damage can be measured by the sperm chromatin integrity test, which is based on the acridine orange stain measuring DNA nicks and breaks. Whereas the TUNEL assay also measures both nicks and breaks, the COMET assay measures only breaks.

It is noteworthy that detection of DNA damage by any of these methods correlate to one another [14], all indicating that increased levels of sperm DNA damage during *in vitro* fertilization (IVF) correlated with decreased embryo morphology at early cleavage stages [15], decreased pregnancy rates [9, 16, 17], increased spontaneous abortions [18], and failure to progress to the blastocyst stage in culture [19, 20]. Interestingly, in a study by Virant-Klun *et al.* [15], extra embryos derived from semen samples with high DNA fragmentation are shown to arrest at the six- to eight-cell stage, which coincides with full activation of the embryonic genome. It is also notable that in a population of 24 couples with recurrent spontaneous abortion, DNA fragmentation in the husband's semen sample was significantly higher than the rate seen in control populations [18].

Although these studies indicate a strong association between sperm DNA damage and embryogenesis, causation has not been demonstrated. DNA damage might be secondary to other early spermatogenic abnormalities, as would be expected in sperm undergoing caspase independent apoptosis. DNA damage is also not likely to be the sole effect of errors in chromatin rearrangement or environmental stress.

The use of sperm with increased DNA fragmentation highlights the question of whether the zygote or embryo carries the capacity to compensate or correct for sperm DNA damage following fertilization. It was determined in the early 1970s that mature sperm show an increased incorporation of labeled nucleotides into the nucleus following mutagenic exposure [21–23]. This implies that some level of DNA damage repair in the sperm nucleus is possible, even after the process of DNA condensation has been initiated [24].

In addition to nucleotide incorporation, which suggests a DNA repair mechanism in sperm, Pederson and Brandriff [25] show that mammalian pronuclear embryos express DNA excision proteins active in DNA damage

repair. Therefore, a mechanism that provides for DNA damage repair in the male pronucleus has been shown to be present. Indeed, later investigations show that during pronuclear decondensation sperm protamine replacement by histones inherently causes DNA breaks [26, 27]. The discovery that zygotes show an increase in DNA synthesis and excision repair in the male pronucleus when fertilized with sperm that have artificially induced DNA damage followed soon after Pederson's work [28, 29]. The upregulation of these repair mechanisms in the presence of sperm with increased DNA damage indicates it is likely that there is a response mechanism to male pronuclear DNA damage in the fertilized oocyte.

Investigations by Harrouk *et al.* [30] suggest that embryos created from sperm with cyclophosphamide treatment show a limited increase in DNA repair protein expression by the one-cell stage, which continues to increase after activation of the embryonic genome. Recognizing that the embryo is still trying to repair DNA damage at this later stage might indicate that the increase in repair proteins might not be sufficient to compensate for the amount of damage induced, at least in the study by Harrouk *et al.* [30].

These studies have led to elegant investigations of cell cycle regulators and DNA repair mechanisms in the zygote [30, 31]. Investigation of the role p53 has in the developing embryo indicates damaged DNA in the male pronucleus signals activation of p53-responsive reporter genes [31]. Therefore, the embryo might tolerate some amount of DNA damage in the fertilizing sperm by upregulation of DNA repair mechanisms and cell cycle delay. In fact, Shimura *et al.* [31] reports a delay to S-phase in embryos created from X-irradiated sperm. This is not to say that cell cycle delay is beneficial to embryogenesis, but that this might be an explanation for the observed phenomenon.

It is clear that DNA damage in the fertilizing sperm is a factor in how an embryo develops and that the oocyte will try to compensate for DNA damaged sperm. Conversely, it is not clear how much damage in an individual sperm is enough to result in compromised embryo growth. Further investigation is needed to quantify how much DNA damage is in individual sperm. It is possible that current fluorescence-based assays used in sperm DNA damage detection could be adapted to allow this type of quantitative analysis. This development would be complemented by studies detailing the amount of DNA damage that the one-cell embryo can effectively com-

pensate for. Therefore, a threshold value for DNA damage in individual sperm that will allow normal embryogenesis would be identified. This work would ultimately culminate in the creation of preventative care for the reduction on sperm DNA damage and a sperm selection technique that allows for separation of sperm with tolerable levels of DNA damage.

### 3 Chromosome aneuploidy

During metaphase I or II of meiosis, nondisjunction can occur, resulting in sperm with an abnormal complement of chromosomes. Fluorescent *in situ* hybridization (FISH) in interphase sperm cells affords convenient evaluation of sperm chromosome ploidy [32] and has revealed that aneuploidy occurs in humans at a much higher rate than in other organisms [33-37]. In fact, classes of subfertile men have been shown to have increased sperm aneuploidy rates [38-44]. Men with azoospermia, severe oligospermia or teratospermia are most likely to be affected in this manner. Normally, when using probes for chromosomes 13, 18, 21, X and Y, the rates of aneuploidy in sperm are at relatively low percentages of the total sperm population, ranging from 1.2% in fertile donors to 4.0% in men with severely decreased sperm parameters [45]. Although these sperm aneuploidies are reported at a relatively low frequency, any increase in sperm aneuploidy is potentially important because chromosomal abnormalities are a leading cause of miscarriage [46].

Some studies have examined the potential of transmitting paternal aneuploidies to the developing embryo. Marchetti *et al.* [47] was able to determine that there is no selection against aneuploid sperm during fertilization or embryo development to the first cell division. He used male mice containing a Robertsonian translocation, resulting in high levels of chromosome 16 aneuploidy. Mating these males resulted in two-cell embryos with chromosome 16 hyperhaploidies at a rate almost identical to that of the sperm (20% vs. 21.8%) [47]. This provides strong evidence that the transmission of aneuploidies from the sperm to the embryo is possible and that there is likely no measurable effect on the formation of a two-cell embryo in mice. Correlative studies in the human also suggest the same. For example, Carrell *et al.* [48] reports an increase in the incidence of sperm aneuploidy in the partners of women experiencing recurrent miscarriage in IVF cycles where the best possible em-

bryos are transferred. Marquez *et al.* [49] finds that aneuploidy is most common in the most morphologically normal embryos after review of FISH analysis of 1255 discarded human embryos. Also, Burrello *et al.* [50] indicates that an increase in sperm aneuploidy correlated with decreased pregnancy in IVF patients. Escudero *et al.* [51] further validate these studies by combing preimplantation genetic diagnosis with sperm FISH to determine that, in cases of paternal Robertsonian translocations, the percentage of abnormal sperm present can predict the number of affected embryos by the equation:  $A = -55 + (1.9 B)$ , where A is the percentage of abnormal embryos and B is the percentage of abnormal sperm. Such studies emphasize the possibility that aneuploid sperm are capable of initiating embryo growth but seem to carry a decreased potential for live birth.

It is still unknown to what extent increased aneuploidies in sperm contribute to adverse outcomes in assisted reproduction and natural conception. The risk factors include the chance of spontaneous abortion or delivery of a child with a congenital abnormality. Screening men with severe morphological defects and severe oligospermia undergoing assisted reproduction, in combination with an investigation of the chromosomal status of embryos, will help clarify the likelihood of paternal transmission of aneuploidies and its effect on embryogenesis.

### 4 Reduced telomere length

The chromosome telomere is a series of 5'-TTAGGG-3' repeats found at the terminal region of a chromosome. The repeat sequence plays a vital role in DNA replication, the protection of the chromosome by inhibiting chromosomal fusions, and chromosomal localization in the nucleus [52-54]. The length of the telomere varies between chromosomes and is shortened with every cell division by 50 to 200 base pairs [55, 56]. Telomere length in cells is maintained by telomerase, a complex of mRNA and proteins [57]. Abnormal telomere shortening is implicated in many disease states [58], including male factor infertility [59], but how the sperm telomere relates to embryogenesis in a mammalian model has only recently come to attention.

The investigation of telomere length in the sperm was started by reports of knockout mice lacking telomerase expression [60]. Initially, normal telomere lengths pro-

tected animals from loss of fertility. By the third generation of offspring both telomere length and fertility is greatly reduced. Interestingly, Hemann *et al.* [61] report that these third generation telomerase null mice do not exhibit chromosomal fusions in mature sperm, a common pathology seen in other affected cell types. Instead, there is a large increase in germ cell apoptosis early in spermatogenesis.

The study by Hemann *et al.* [61] implies a mechanism for removal of spermatocytes with short or absent telomeres from the pool of developing sperm. Presumably, only sperm with a sufficient telomere progress through spermatogenesis. In fact, there is a severely reduced sperm concentration reported in these mice by the third generation. This targeted degradation of abnormal sperm might be acting as a checkpoint of spermatogenesis. It is possible that loss of fertility in these mice is not a result of sperm with shortened telomeres but another unresolved, yet related, mechanism that is resulting in poorly developed sperm and decreased sperm concentration.

The proposal that the reduced fecundity seen in these animals is not a direct result of shortened telomeres is supported by earlier work, indicating a severe atrophy of the testis and reproductive accessory glands in these mice [60]. This atrophy indicates that the supporting cells of the reproductive tract are also affected by telomere shortening. Therefore, the coordination of the reproductive tract in spermatogenesis might be compromised.

In contrast to the conclusion set forth by Hemann *et al.* [61], Liu *et al.* [62] report that spermatocytes at prophase I of meiosis produced from these mice do carry shortened telomeres with abnormal localization, and likely contribute to the pool of mature sperm. In fact, when wild-type oocytes were inseminated with sperm from these telomerase null mice the result was decreased fertilization, increased embryo fragmentation, decreased formation of blastocysts and an increase in apoptotic cells in the resultant embryos.

Taken together, the reports by Liu *et al.* [62], Hemann *et al.* [61] and others suggest that although mammalian germ cells with shortened telomeres are targeted for destruction early in development, some sperm complete maturation. This suggests that the presumed telomere checkpoint is leaky in spermatocytes. Additionally, fertilization with telomerase null sperm causes severe defects in the resultant embryos. The observations summarized here might indicate that these sperm are dys-

functional as a result of short telomeres in mature sperm. Unfortunately, the adverse effects of short telomeres in the supporting testicular cells and accessory glands confuse this conclusion.

Assessing the telomere length of mature sperm rather than just spermatocytes from telomerase null mice would allow for a direct measure of the telomerase effect. If telomeres were reduced in mature sperm, this would strengthen the argument for direct effects of reduced telomeres in sperm on embryogenesis. The investigation of sperm telomere length would also greatly benefit from a germ cell-specific knockout. This would remove the effect of degeneration on the rest of the reproductive tract.

To date, there are a few reports addressing the issue of abnormal telomeres in human infertility. In one such report no difference in telomerase activity was seen between oligospermic, non-obstructive azospermic, and obstructive azospermic men; unless there were no germ cells present [63]. This is not to say that there is no association of reduced or dysfunctional telomeres and human infertility. There are, as of yet, many unexplored areas of possible study in the human. The telomere plays a major role in chromosomal localization within the sperm nucleus [64]. It is possible that abnormal localization could cause meiotic errors resulting in sperm borne aneuploidies, because nuclear localization and telomere-driven pairing of homologues have both been shown to be important factors in recombination [65]. Abnormal pronuclear apposition and syngamy in the zygote might also be related to telomeres in the fertilizing sperm. Telomeres are associated with microtubule motors during meiosis in other model systems [66, 67], implying that the movement of the male pronucleus, via the sperm aster, is likely related to telomere binding [59]. The delineation of these molecular pathways and identification of human pathologies is still required to substantiate these proposed mechanisms.

## 5 Malformations of the centrosome

The sperm centrosome is a highly modified organelle that has been adapted to function in both the mature sperm and during fertilization in non-rodent mammals [68, 69]. At the onset of spermiogenesis, the sperm centrosome is identical to the somatic counterpart, containing two centrioles and a thick network of pericentriolar proteins. During sperm maturation, the distal centriole degenerates,

and much of the pericentriolar material is reduced to a small fibrous sheath and the outer black box surrounding the proximal centriole in the midpiece [68].

In the developing human embryo, fertilization, pronuclear apposition, chromosomal segregation and cell division are all dependent on the contribution of a functional sperm centrosome [69–71]. During the first of these processes, fertilization, the sperm centrosome gives rise to the sperm aster, which will provide for proper microtubule organization in the embryo. Thereafter, the sperm aster will coordinate pronuclear apposition and the first mitotic division during which the sperm aster gives rise to the somatic centrosome.

Electron microscopy of mature sperm is currently the gold standard for determining if the centrosome is properly formed, as outlined above, to function in fertilization. The shortcoming is that this is only a morphological assessment, and the ability to form a sperm aster is not assayed, and morphology and function have not been correlated. A new technique is emerging to evaluate the functional characteristics of the sperm centrosome [72, 73]. The technique involves injection of human sperm into mammalian oocytes with subsequent observation of sperm aster formation via fluorescent immunohistochemistry. This diagnostic technique has indicated that the sperm aster size predicts IVF outcome via comparison of embryo cleavage and sperm aster formation [73]. This assay might become an invaluable tool in diagnosing male infertility as a result of centrosomal dysfunction. However, further validation of this technique, including a proposal of standardized criteria for assessment of sperm asters and a comparison of results with ultrastructural examination of semen samples, is needed.

Embryos resulting from sperm with poorly developed, damaged or absent centrosomes might express varying levels of disorganization. This correlation has been reported by Rawe *et al.* [74] in a case study of a patient who demonstrated poor centrosomal morphology and a reduction in ability to form sperm asters. The first attempt at IVF resulted in no progression of zygotes past the pronuclear stage of development. The second attempt using more stringent sperm selection, choosing the most morphologically normal sperm, resulted in a pregnancy, but later spontaneously aborted.

Just as poor sperm centrosome formation affected embryogenesis in the case study reported by Rawe *et al.* [75], embryos created with testicular sperm have been

shown to have an increase of chromosomal mosaicism. It is interesting that the observed mosaic aneuploidies were more than double the mosaicism in control embryos (26.5% vs. 53%), and mosaicism likely occur at the first embryonic division where chromosomal segregation is under control of the sperm-derived centrosome [71]. It is possible that incomplete maturation of the sperm centrosome seen in testicular stage sperm results in poor chromosomal segregation in some cases [69, 76–78].

Although the sperm centrosome appears to be needed for chromosome segregation, two studies have shown that the oocyte might be able to compensate to some degree. Although freeze-drying sperm likely affects protein function, including the centrosome. Liu *et al.* [79] obtained a live birth using intracytoplasmic sperm injection (ICSI) with freeze-dried rabbit sperm. Another study reports a piglet obtained after intracytoplasmic sperm head injection [80]. During ICSI of this type, the sperm tail, containing the centrosome, is dissected away. If the removal of the tail portion of the sperm is complete, then there is obviously no paternal centrosomal contribution. On a related note, there is also no contribution of the paternal centrosome in nuclear transfer techniques [81]. In any nuclear transfer study to date, the nucleus is removed with a very small amount of cytoplasm, if any at all. Procedures using somatic cells for fertilization do not attempt to include the donor centrosome during transfer and if the donor cell is another oocyte, the maternal centrosome has been degenerated. The issue of how embryogenesis continues without a centrosome in the above cases is currently unresolved, but it is possible that the maternal centrosome components are able to compensate in rare circumstances that lead to viable embryo growth. The fact that they do work opens exciting avenues of investigation where a centrosome might be assembled from either remnants of the maternal centrosome or *de novo* in the oocyte by upregulation of centrosomal protein synthesis pathways. Further investigation into these protein levels will aid in elucidation of possible mechanisms of centrosomal function and redundancy.

## 6 Genomic imprinting errors

Genomic imprinting, a key form of epigenetic regulation, refers to the variable expression of a paternal or maternal gene. Imprinting occurs mainly through DNA methylation, but by definition never involves DNA se-

quence alterations [82]. DNA methylation is catalyzed by DNA methyltransferase and involves the addition of methyl units at the 5' position of cytosine residues in CpG dinucleotides. Cytosine residues can also be actively or passively demethylated. Active demethylation is a rapid process facilitated by enzymatic action, whereas passive demethylation results from a loss of methyltransferase activity. Passive demethylation is usually associated with DNA replication [83].

Imprinting patterns in sperm are modified during epididymal transit where global sperm DNA methylation is greatly reduced in preparation for fertilization via passive demethylation [84]. This reduction leaves the sperm hypermethylated compared to the oocyte [85]. After protamine removal, at fertilization, paternal gene copies are further modified by active demethylation [86, 87]. Subsequently, paternal and maternal genes undergo further passive demethylation through the morula stage [88]. Methyl transferase is then directed from the cytoplasm into the nucleus of the cells in the blastocyst inner cell mass where a new embryonic methylation pattern is established [89].

Imprinting errors in the developing fetus have been identified and shown to cause severe pathologies. Some studies also suggest that imprinting diseases might be increased in assisted reproduction [90–92]. For example, Marques *et al.* [93] determine that sperm from oligospermic men show an increase in abnormal methylation of the *H19* gene, which is associated with Beckwith–Wiedemann syndrome and early childhood cancers [94]. Although it is the methylation of the maternal allele that is altered in Beckwith–Wiedemann disease, there is still debate as to how the sperm might contribute to this altered state in assisted reproduction. The study by Marques *et al.* [93] implicates a pathway for paternal involvement of a methylation error that is reported to be increased in babies from assisted reproduction [94, 95].

In addition to disruption of methylation patterns causing known syndromes, a decreased genome-wide methylation in sperm has been identified with poor embryo quality in rats [96] and decreased IVF pregnancy rates in humans [97]. In 1996, Doerksen and Trasler [96] inhibited normal germ cell methylation in male rats by giving doses of 5-azacytidine, resulting in a normal level of fertilization from natural mating but severe embryo fragmentation after the first division. Although the study by Doerksen and Trasler [96] reports reduced sperm concentration, other sperm quality parameters, such as

viability, motility, DNA integrity or morphology, were not reported in study and control groups. In addition, there was no quantification of demethylation in mature sperm. Although the study by Doerksen and Trasler [96] has obvious limitations in establishing reduced methylation as the sole cause of the reported embryo quality, it establishes a link between disruption of methylation in sperm and embryogenesis.

Benchaib *et al.* [97] report the use of 5-methyl-cytosine immunostaining as an indicator of genome-wide, or global, methylation in sperm. Recently, this method has been used in his laboratory to determine that decreased global methylation in semen samples from normospermic men is related to a poor pregnancy outcome during IVF but not to fertilization rate or embryo quality at the eight to ten cell stage [98]. It is interesting that decreased global methylation was observed in men with normal semen parameters. This supports the evidence from Doerksen and Trasler [96] that global methylation status independently affects embryogenesis.

These data indicate either experimentally induced or naturally occurring paternal genome imprinting errors relate to abnormal embryogenesis and pregnancy failure. Indeed, two researchers independently identified little effect on fertilization rates but decreases in either embryo morphology or pregnancy when evaluating sperm with altered genome-wide methylation status [96–98].

It is also important to mention that many steps of genomic imprinting during spermatogenesis are well described [93, 99, 100], and show a correlation between sperm methylation prior to fertilization and normal embryogenesis. It is not known if paternal gene expression in the early embryo is regulated by methylation. The next step in understanding the effect of paternal imprinting on preimplantation embryogenesis is to identify if methylation of specific paternal gene copies are required during preimplantation embryogenesis, and, if so, which genes they might be. In addition, it is important to determine if the global reduction in methylation seen in some patients during IVF treatment is a causative factor for poor embryogenesis or a sign of poor spermatogenesis, not having any direct effect on the embryogenesis.

## **7 Altered mRNA profiles**

The mature, ejaculated sperm contains mRNA transcripts that have traditionally been classified as residual mRNA from spermatogenesis [101, 102] yet, during

fertilization, this paternal mRNA is deposited into the oocyte [103]. More recent data suggests that the transfer of these paternal mRNA transcripts during fertilization might have a significant role in embryogenesis [104, 105].

Ostermeier *et al.* [104] establish the profile of mRNA transcripts in mature sperm by comparing pooled sperm cDNA and individual ejaculate cDNA by microarray analysis of 27 016 unique expressed sequence tags. This comparison of cDNA isolated from individuals, compared to a pooled sample of nine men, indicated distinct mRNA fingerprints in individual men. Therefore, Ostermeier is suggesting that variation in fertility might be associated with the mRNA fingerprint. This observation will be strengthened when fertile and infertile men are compared. Indeed, others have shown that specific transcripts identified in men correlate to sperm quality [106]. It remains to be identified whether the variation in sperm mRNA content seen is directly affecting the mature sperm or is an indication of aberrant spermatogenesis.

Ostermeier *et al.* [105] report data in support of sperm mRNA influencing embryo growth. Interestingly, the mature sperm contains mRNA that code proteins required for early embryogenesis but are absent in the metaphase II oocyte. This infers that the newly fertilized oocyte could draw upon these transcripts for the initial phase of embryo growth. In a subsequent report another subset of paternally-derived mRNA that might participate in embryogenesis has been reported [107]. Small noncoding RNA that play an established role in somatic cell and oocyte epigenetic control were also found to be transferred to the oocyte. Specifically, a set of 68 sequences were identified that might have a significant role in gene silencing through RNA interference (RNAi) in the fertilized oocyte. The preliminary analysis of these 68 sequences showed several potential oocytic mRNA targets. These include dickkopf 2 (DKK2), a protein in the wingless-type signaling pathway, which is active in embryonic development. Work from this group shows that transfer of paternal mRNA is variable between men and that paternal mRNA transcripts might be required for embryogenesis.

In addition to the possible positive effect sperm mRNA might play, the presence of paternal transcripts deleterious to embryo growth has also been validated [108, 109]. Potentially harmful protamine transcripts were selectively eliminated from oocytes fertilized with round spermatids whereas other sperm derived tran-

scripts were retained [109, 110]. These studies indicate that there is selective and temporal reduction of sperm mRNA in the oocyte, which might play a role in preserving normal embryogenesis. This provides additional evidence that some paternal mRNA transcripts are beneficial to the developing embryo and are, therefore, preserved [111].

An important step in strengthening the correlation between the presence of sperm mRNA and embryogenesis, is to verify if sperm transmitting aberrant mRNA profiles yield embryos with decreased developmental potential. Such work has been initiated in a study examining the level of specific mRNA transcripts in sperm fractions from density gradient preparation and IVF outcomes [106]. Once the suggested correlation has been well established, the transmitted mRNA, either coding or interfering, will need to be shown to have activity in the embryo.

## 8 Sperm nuclear packaging

Nuclear organization in both somatic and germ cells is considered to be a method of epigenetic gene regulation. The sperm chromatin is compacted and largely quiesced by the sperm specific nuclear proteins protamine 1 (P1) and protamine 2 (P2). Protamines replace nuclear histones during maturation, organizing the chromatin into tightly packed toroid structures aiding in transcriptional inactivation and protection of the DNA [112]. The addition of P1 and P2 in a species specific ratio (P1 : P2) is emerging as a significant factor in male fertility [113]. The P1 : P2 ratio in human donors of known fertility lies close to 0.9 [114], ranging from 0.8 to 1.2 [115]. Perturbation of this ratio in either direction is implicated in the manifestation of poor semen parameters and a general decrease of fertility [39, 115, 116].

Studies are emerging that not only address the protamine-related decrease in semen parameters, but investigate the specific role of sperm DNA compaction via protamines in embryogenesis [114, 115, 117–119]. As an illustration, P2 haploinsufficiency in the mouse resulted in increased sperm apoptosis and early embryonic arrest after ICSI [118]. It is interesting that Rhim *et al.* [117] report the use of a transgenic mouse model in which native P2 was replaced with the rooster protamine, termed galline. This replacement induced a decrease in chromatin compaction but did not affect mouse fertility in natural matings. Moreover, IVF patients with a complete

absence of P2 suffered a striking reduction in natural fertilizing capacity, but when ICSI was used, fertilization, embryogenesis and pregnancy ensued normally [114]. Also, in humans, increases in the P1 : P2 ratio are associated with a decrease in fertilization capacity; but no change in embryo quality, whereas a drop in the ratio below 0.8 correlates with decreased fertilization and poor preimplantation stage embryo morphology [115]. Most recently, Mitchell *et al.* [120] show that when men undergoing testicular sperm extraction (TESE) for ICSI have decreased P1 transcripts present in developing spermatocytes, the pregnancy rates are decreased compared to men with a higher amount of P1 transcript.

The above research shows that epigenetic regulation of DNA via nuclear packaging in the sperm is related to the function of the mature sperm. This is expressed in a wide gamut of phenotypes depending on the experimental model, ranging from abnormal nuclear condensation during spermatogenesis [117] to loss of fertilization potential and a severe decrease in embryogenesis [115, 118]. These studies raise many interesting questions.

The studies listed examine protamine expression as a function of the whole ejaculate. It is possible that some sperm from a man with an abnormal P1 : P2 ratio might have normal sperm cells present and those sperm are selected during reproductive therapy, which causes a bias in some reports. The classification of protamine levels in individual sperm is currently underway and a preliminary report shows that heterogeneity is common [121].

The mouse model causing P2 haploinsufficiency identifies that loss of P2 in the mouse is detrimental to embryogenesis, but it does not adequately represent what is seen in humans. The predominant pathology seen in humans is an elevated P1 : P2 ratio [115]. This could be better modeled in the mouse by perturbation of the protamine expression using RNA interference technology in a mouse model where P1 or P2 expression is not fully ablated but reduced. Evaluating the fertility of such a model might help in mimicking the pathology seen in humans.

## **9 Conclusions and future directions**

Although much work has been done, our knowledge of genetic and epigenetic sperm factors contributing to poor embryogenesis is limited. The correlative studies reviewed here are important starting points but fall short of causation in many instances. There seems to be two

largely prevailing obstacles that overlap into all of the areas of sperm function discussed herein.

First, the creation of a population of fully functional sperm free of defects seems to have been selected against in many classes within the chordate phylum. The reduction in sperm quality has given way to production of large numbers of sperm to compete for fertilization [122]. This gives a heterogeneous population of sperm cells in the ejaculate. Some sperm are capable of providing all the requirements for fertilization and embryogenesis, whereas other sperm are capable of fertilization but result in abnormal embryos and still others are completely dysfunctional. The analysis of an entire ejaculate will, therefore, yield a composite picture of all of these sperm. Therefore, one might argue that single cell analysis would rectify the issue of a heterogeneous population, but analysis of this type is then biased by the selection of a single cell. Increasing the number of single cells evaluated will account for this bias, but analysis of enough cells is often impossible with the current techniques available.

Second, the cellular pathways and organelles within the sperm function as a whole. The effects of a testicular insult in either an acute or chronic fashion might affect a particular spermatogenic factor, but that factor will likely have a role in another pathway. For instance, the development of a varicocele leads to increased accumulation of heavy metals binding to spermatocyte DNA [123]. The increased DNA damage and changes in DNA accessibility will likely cause interference in protein synthesis capitulating effects in many areas of sperm maturation. Although this scenario is possible, the reality is that there are usually several environmental or epigenetic and genetic factors at play at the same time; some are beneficial to sperm production and others are detrimental.

Both the highly complex nature of sperm production and the delicate balance of epigenetic and genetic factors of sperm maturation come together to form a mature sperm with the ability to fertilize an oocyte and contribute to the developing embryo. When any of these functions fall short, the result might be simply defined as a known syndrome of male infertility or might be more complex in nature. The latter of these is likely the case in the majority of instances, considering most men that are diagnosed with some type of sperm abnormality have no known cause to attribute to the condition to [124–126]. This pursuit of a defined syndrome in subfertile men is complicated



by the fact that many of these sperm abnormalities seem to possess a multi-allelic genotypic pattern with variable penetrance [127, 128]. The future of sperm physiology lies in being able to parse out these underlying genetic and epigenetic causes of sperm abnormalities that have been shown to affect embryogenesis.

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