Within-Ejaculate Sperm Selection and its Implications for Assisted Reproduction Technologies

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Abstract

In most animals, males produce large numbers of sperm in each ejaculate, but only very few end up fertilising an egg. This bottleneck in sperm numbers from ejaculation to fertilisation offers an intuitive opportunity for selection to act and improve the fitness of the next generation. However, the general view that sperm phenotype is not linked to its haploid sperm genotype stalled further research into this idea until recently. Two studies in zebrafish *Danio rerio* now suggest that selection among sperm within the ejaculate of male may have far reaching consequences for the following generation(s). Selection for longer-lived sperm resulted in offspring that showed higher survival during embryo development and a reduced number of abnormally developed larvae, as well as increased reproductive success during adulthood. These effects have been linked to the haploid genotypes in the sperm. We here discuss the possible benefits of refined sperm selection based on sperm haplotypes in the use of Artificial Reproduction Technologies. Understanding the genetic processes occurring after meiosis until syngamy may provide insights that may help improve the existing methods and with that their success rates.

Introduction

Sperm are one of the most specialised and diverse cell types studied and vary across species, across individuals and also within individuals (Birkhead, Hosken, & Pitnick, 2009). Given the fundamental role of sperm in reproduction, it is surprising how little is known about the importance and possible causes and consequences of phenotypic and genetic variation among sperm within a male's ejaculate. Sperm defects are among the most well-known causes of male infertility (Hull *et al.*, 1985). While one male produces thousands to millions of sperm in a single ejaculate, many are immotile and exhibit abnormal behaviour and morphology. In fact, organisms that have sperm with a single flagellum have considerable morphological abnormalities including the presence of additional heads or flagella (Holt & Van Look, 2004). While for a long time the variation observed to differences during spermatogenesis and variation in male condition, more recent evidence suggests that the genetic variation present in sperm of every heterozygous male may contribute to the variation more than though so far. Even among perfectly fertile sperm, variation does exist and may affect not only sperm performance but also offspring fitness. With these recent insights in mind it may be a good time to assess inadvertent sperm selection during Assisted Reproduction Technologies (ARTs) and related methods such as cryopreservation.

The amount of phenotypic variation within a male's ejaculate may be affected by several factors (Holt & Van Look, 2004). The extent of inbreeding for example is negatively associated with sperm quality and positively associated with increased morphological diversity (e.g. (Gage *et al.*, 2006; Asa *et al.*, 2007; Fitzpatrick & Evans, 2009). In contrast, the intensity of sperm competition is negatively associated with variation in sperm morphometric traits, possibly due to stabilising selection for optimal sperm phenotypes (Calhim, Immler, & Birkhead, 2007; Immler, Calhim, & Birkhead, 2008; Stewart, Wang, & Montgomerie, 2016). In both scenarios mentioned above, sperm phenotype is assumed to be determined by the diploid male genotype and reflects the genetic quality and condition of the producing male (Yasui, 1997). More recent insights suggest that some sperm traits such as longevity

and other functional traits may be at least partially influenced by the haploid genotype in individual sperm (Alavioon *et al.*, 2017; Borowsky, Luk, & Kim, 2018; Rathje *et al.*, 2019; Bhutani *et al.*, 2019); reviewed in (Immler, 2019; Joseph & Kirkpatrick, 2004). In fact, two recent studies in the zebrafish suggest that selection on sperm phenotypes results in selection on sperm genotypes which in turn affect offspring performance and fitness throughout life (Alavioon *et al.*, 2017, 2019).

Regardless of the nature of sperm traits under haploid control, the mere fact that some sperm traits are affected by haploid sperm genotypes suggests that selection among sperm during this short period of time between ejaculation and fertilisation may play a key role in determining the genetic variation and composition of the following generations (Immler & Otto 2018; Immler 2019). Given these findings it may be the right time to consider the importance of sperm selection also in commonly practices ARTs.

The Importance of Sperm Selection

As mentioned above, recent studies have shown that selecting sperm by phenotype may affect offspring performance. In the Atlantic salmon *Salmo salar* and the marine ascidian *Styela plicata* for example, selection on sperm longevity affected the development and survival in the offspring (Crean et al., 2012; Immler et al., 2014). In the zebrafish *Danio rerio*, selection for sperm longevity among intact fertile sperm within an ejaculate resulted in longer-lived sperm siring embryos with higher survival and a reduced number of apoptotic cells (Alavioon *et al.*, 2017) . These fitness benefits observed early in life continued to persist throughout adult life in these same offspring, as adult male offspring sired by longer-lived sperm also showed higher reproductive success with more eggs being laid, more eggs being fertilised, more embryos surviving to 24 hours post fertilisation and an overall longer lifespan that offspring sired by the short-lived sperm of the same ejaculates (Alavioon *et al.*, 2019). More recently, a study in *Astyanax* cavefish showed that selection on sperm function under chemical stress selected for specific sperm haplotypes (Borowsky *et al.*, 2018). Similarly, in the Mexican tetra *Astyanax mexicanus* showed that sperm of offspring from crosses between two highly

inbred strains showed higher phenotypic variation in sperm swimming performance, than pure species offspring from either of the two strains suggesting that the increased genetic variation in the inter-strain crosses contributes to sperm phenotypic variation (Borowsky, Luk, & Kim, 2019). In contrast, a study in *Drosophila* fruit flies failed to show a role of the haploid sperm genotype in determining sperm length (Pitnick et al.), whereas a recent study One speculative view at this point could be that sperm morphometry largely is determined by the diploid male genome whereas sperm function may be at least partly based on the haploid sperm genotype (Immler, 2019). This is an interesting idea that deserves further testing.

While most of the above described studies were performed in externally fertilising fish, more recent evidence comes from domestic cattle bulls where X- and Y-sperm differed by eight proteins (Scott *et al.*, 2018). Similarly, the long-standing question about the sex ratio distortions caused by… in the house mouse *Mus musculus* has recently been explained by functional and genetic differences between X- and Y-sperm (Rathje *et al.*, 2019). Another study in the house mouse found a way to separate X- and Y- sperm in the house mouse for subsequent use in *in vitro* fertilisation. And finally, a comparative study including house mice and non-human primates found that between 29% and 47% of genes expressed in post-meiotic haploid spermatids show biased gene expression – a significantly higher number of genes than previously assumed (Bhutani *et al.*, 2019).

All these recently gained insights suggest that understanding the link between sperm phenotype and sperm genotype may be crucial for improving the currently available ARTs and to replace some of the common practices to obtain for example sex ratio biases in livestock breeding. Being able to make an effective selection will enable us to achieve the desired reproduction outcome in the next generations, eliminate or reduce the risk of certain diseases and also to understand the underlying mechanisms behind haploid selection in both animals and human.

Possible Steps of Sperm Selection in ARTs

ARTs have been successfully used to improve the health and quality of domestic animals and livestock (Verma *et al.*, 2012), for the conservation of endangered animal species (Wildt & Roth, 1997) and to circumvent fertility problems in human (Okun *et al.*, 2014). For efficient livestock production, it is essential to breed healthy animals with desirable phenotypes. Therefore, semen from high quality individuals is sold commercially, cooled or preferably frozen. For endangered animals, sperm also need to be prepared and stored to keep the genetic material from several individuals within species and to be able to be sent all around the world. In humans, many couples with known or unknown fertility problems or other conditions affecting the fertility (different types of cancer, etc.) are in need of semen preparation and preservation to be able to do ART at later stages in life when obtaining new samples may be unlikely, or in some cases impossible. In most cases, sperm cryopreservation is a necessary step.

Semen cryopreservation involves a number of key steps, each of which may affect sperm quality and function (Figure 1). The possible negative effects of cryopreservation on sperm quality are well known (e.g.(O'Connell, McClure, & Lewis, 2002; Zribi *et al.*, 2010), and include anything from changes in the membrane structure, molecular changes such as DNA fragmentation and RNA degradation as well as epigenetic changes, mitochondrial damage and reduced motility (e.g. Ugur et al., 2019). Such damages inevitably affect offspring development and fitness. In the rhesus macaque *Macaca mullata* for example, cryopreservation had no effect on sperm motility, fertilisation success pregnancy rate, but it may have a potential effect of offspring survival (Gabriel Sánchez-Partida *et al.*, 2000). However, the sample sizes in this study are too small to have statistically sound evidence for such an effect. A more recent study in wild brown trout *Salmo trutta* found that offspring sired by frozen-thawed sperm exhibited reduced growth rates compared to offspring sired by non-frozen sperm (Nusbaumer, Marques da Cunha, & Wedekind, 2019). However, which step of cryopreservation really contributes to this change in offspring phenotype is not clear. Future studies will need to

understand the effects of each of the steps involved in cryopreservation on sperm quality and also how selection for intact sperm may be achieved.

Fertilization success and successful pregnancy rate in ART is mostly determined by gamete quality (Morrell, 2006). It is difficult or rather unlikely to obtain decent quality embryos from poor quality oocytes and sperm. In humans, most ARTs therefore involve one or several steps to test sperm quality and sperm fertility and to identify intact and undamaged sperm. To assess overall semen quality and sperm performance, methods like swim up (Volpes *et al.*, 2016) and density gradient centrifugation (Bolton & Braude, 1984) are widely employed prior to human ART (Organisation, 1999). Similarly, tests to assure sperm DNA such as the comet assay (single cell gel electrophoresis), TUNEL (terminal deoxynucleotidyl transferase-nick-end-labelling) and SCSA (sperm chromatin structure assay) are widely used. These methods focus on selecting apparently normal sperm and removing damaged or non-functional sperm. They are used mainly when preparing sperm samples for Artificial Insemination (AI), Intra-uterine fertilisation, *in vitro* fertilisation (IVF) or Intracytoplasmic Sperm Injection (ICSI).

In contrast, the vast majority of semen samples used AI in animals are not subjected to any selection procedure, which may contribute to lower than optimal pregnancy rates. Different methods of sperm selection have been advocated at different times but they often lack documented evidence of efficacy. Individual clinics, breeding companies, farmers or producers cannot carry out extensive testing on each sperm sample to be used. The industry is therefore in need of reliable and simple methods for selecting highly fertile sperm and separating them from damaged sperm to be used in preparation for ART. One of the most common selection methods used for semen preparation prior to AI in animals is colloid centrifugation (Morrell, 2006). Colloid centrifugation has been extensively researched as far as basic sperm characteristics are concerned, and is known to select sperm that are motile, have intact membranes and good chromatin integrity. Magnetic Activated Cell Sorting (MACS) was introduced to the field more recently and is believed to allow for selection against apoptotic sperm (Sheikhi *et al.*, 2013). Although all

of these methods are efficient in selecting for normal looking and functional sperm, little work has been carried out to understand the potential consequences of such sperm selection for the following generation(s).

Possible Future Avenues

Natural selection generally offers a number of hurdles and selection steps for sperm to overcome (Birkhead, Møller, & Sutherland, 1993). This may result in a limited number of sperm actually reaching the site of fertilisation. Although the role of sperm selection at this stage has been repeatedly challenged, the recent body of evidence suggests that sperm selection at the intra-ejaculate level may be more important than assumed. It is therefore crucial to understand which sperm traits are reflecting the sperm genotype and what sperm traits are selected for under conditions of natural reproduction. We are currently only at the beginning of understanding this question, but if we gain insights into the genetic mechanisms occurring after meiosis and before syngamy, we will have the opportunity to improve many aspects of ART in animal and human reproduction. Not least may such insights improve the overall success rate of AI, IVFs and ICSI, but it may also have commercial value if we manage to take advantage of sperm selection for example to separate X- and Y-sperm for efficient production of domestic stock animals where desired sex ratios often are biased toward one of the two sexes (e.g. female-bias in egg-producing poultry and dairy cattle, male-bias in meat poultry and meat cattle etc.). It may also be more widely used to generally improve the health of the resulting offspring by selecting optimal sperm phenotypes by mimicking selective pressures occurring during natural selection.

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Figure

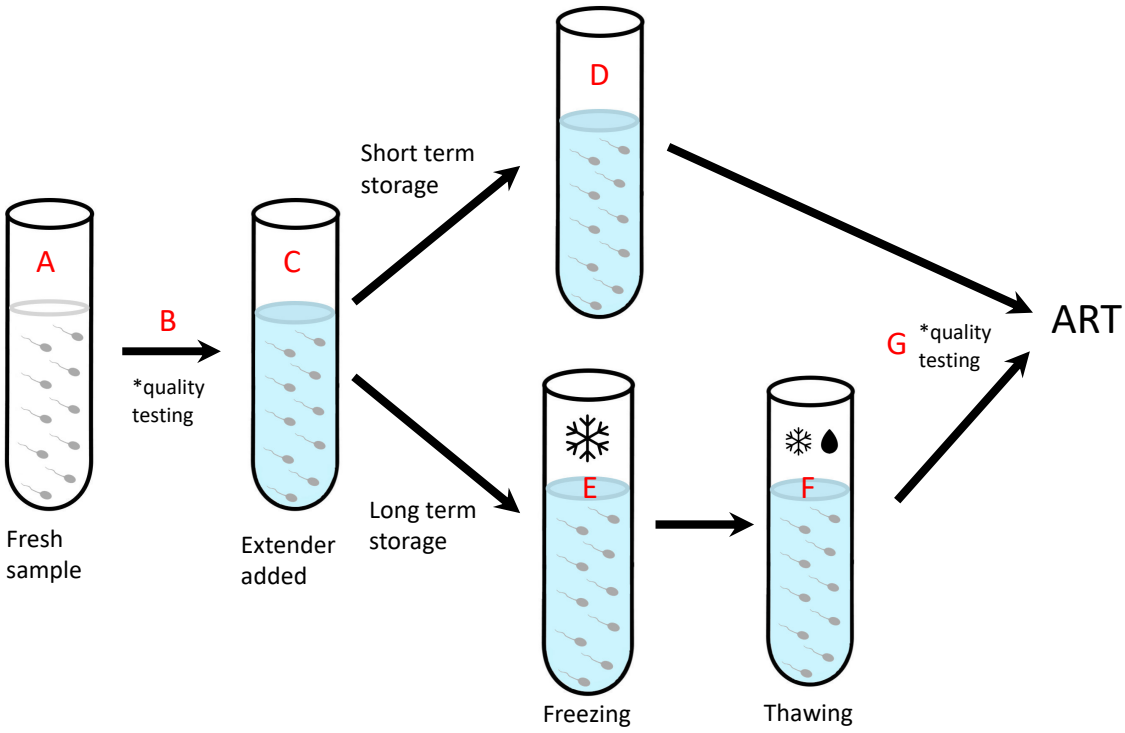

Figure 1: Major steps from sample collection to ART with putative effects on sperm presented in *italics* in brackets after each step (positive traits marked as +, negative traits marked as: –, or unclear marked as ±). **(A)** Sample collection occurring by natural stimulation (ejaculate should be in its natural condition +) or electro-stimulation (may alter ejaculate composition in terms of density ±, seminal fluid -); **(B)** tests on general ejaculate quality such as overall volume, sperm density, sperm motility and velocity, morphology (delay between ejaculation and insemination ±) **(C)** transfer of sample into a buffer/extender for short-term or long-term storage (alteration of seminal fluid composition ±, change in temperature -); **(D)** short-term storage without for up to hours/days freezing (fridge or room temperature change in temperature -; further delay between ejaculation and insemination ±); **(E)** Long-term storage by cryopreservation by freezing (freezing may cause damage in membrane, DNA and epigenetic marks as well as morphology -) and **(F)** subsequent thawing for use (rapid change in temperature -); **(G)** quality control for sperm overall quality including swim-up, DNA quality, morphology (physical handling and exposure to buffers ±).