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XIIIth International Symposium on Spermatology

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The XIIIth International Symposium on Spermatology was organized in special honour of Professor Björn Afzelius (1925–2008) and Professor Leif Plöen (1941–2003).

The 13th International Symposium on Spermatology

Lars Björndahl

Introduction to the Symposium

It was with great pride and joy we welcomed a wide range of scientists (Table 1) to the 13th International Symposium in Spermatology at the conference venue Skogshem and Wijk on the suburban island of Lidingö, just outside central Stockholm. The symposium took place on 9–13 May 2018, and focussed on any aspect involving the Spermatozoon (Fig. 1). Of special interest was the variability in solutions for basically the same task: to transfer half the genetic material of a new individual and to deliver this genetic material to a gamete of another individual. Looking at both animals and plants, there is a huge variability in challenges to accomplish the mission. Therefore, there was also a wide range of species represented (Table 2).

A Long Series of International Symposia on Spermatology

The series of Spermatology Symposia has a long history (Table 3) but always with the purpose to bring scientists from different fields together—to encourage discussions, interaction, networking and time to enjoy and contemplate. The proceedings attempts to summarise key points and also form a basis for young scientists for further exploration of the field of spermatology. One main point we know from the symposia is how much can be learnt from understanding differences and similarities between spermatozoa from different species where dissimilar challenges for reproduction have led to divergent solutions. The first 50 years of Spermatology Symposia is described by Professor Hideo Mohri in the next chapter of these Proceedings.

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Table 1 Origins (work address) of registered participants

Country	Number of participants	Country	Number of participants	Country	Number of participants
Japan	17	Poland	4	Catalan	1
United Kingdom	9	Switzerland	4	China	1
Spain	8	Australia	3	Colombia	1
Sweden	8	France	3	Finland	1
Norway	7	Ukraine	3	India	1
Russia	6	USA	3	Iraq	1
South Africa	6	Czech Republic	2	Israel	1
Canada	5	Italy	2	The Netherlands	1
Slovakia	5	Austria	1		
Germany	4	Belgium	1		

Fig. 1 The rural venue of the 13th International Symposium on Spermatology on the suburban island of Lidingö outside central Stockholm, Sweden. (Photo L. Björndahl)

A Personal Dedication of the 13th Symposium

For me personally, three Swedish dedicated scientists have been immensely important for my way into sperm science. Therefore, this symposium was dedicated to them.

Björn Afzelius (1925–2008; Fig. 2) generously took time to introduce me to the fascinating world of cilia and sperm tails when I did an advanced course in physiology on cilia in the human body (Björndahl 1980). His enthusiasm was contagious, and I still have a keen interest in the propeller of the sperm (Holmberg et al. 2018). Björn also facilitated my interest in sperm nuclear chromatin stability and zinc content by introducing me to Godfried M. Roomans and allowing me to work with X-ray microanalysis in his laboratory (Roomans et al. 1982).

Leif Plöen (1941–2003; Fig. 3) was not only an interested and thorough opponent at my public doctoral dissertation, but he also introduced me to a wider range

Table 2 Examples of species represented at the symposium

Human	Rooster	Sturgeon	Trout
Mouse	Turtle	Drosophila	*C. elegans*
Bull	Frog	Koala	Asian elephant
Boar	Crocodile	*Ciona intestinalis*	Malaria parasite
Sea Bass	Salmon	Tortoise	
Dog	Duck	Red Deer	

Table 3 Venues and hosts of past and next coming International Symposia on Spermatology

No	Year	Venue	Host
I.	1969	Siena, Italy	Baccio Baccetti
II.	1973	Stockholm, Sweden	Björn Afzelius
III.	1978	Boston, Woods Hole, USA	Michael Bedford
IV.	1982	Seillac, France	Jean André
V.	1986	Fujioshida, Japan	Hideo Mohri
VI.	1990	Siena, Italy	Baccio Baccetti
VII.	1994	Cairns, Australia	Jim Cummins
VIII.	1998	Montréal, Canada	Claude Gagnon
IX.	2002	Cape Town, South Africa	Gerhard van der Horst
X.	2006	Madrid, Spain	Eduardo Roldan
XI.	2010	Okinawa, Japan	Maki Morisawa
XII.	2014	Newcastle, Australia	John Aitken
XIII.	2018	Lidingö, Sweden	Lars Björndahl
XIV.	2022	Vancouver, Canada	David and Sharon Mortimer

of mammalian spermatology, electron microscopy with further X-ray microanalysis investigations (Björndahl et al. 1986, 1991; Björndahl and Kvist 1990), general science philosophy, and last but not the least, the philosophy of Piet Hein.

Last, but not the least, *Ulrik Kvist* (1947–; Fig. 4), my Ph.D. supervisor (Björndahl 1986), mentor and friend—for inviting me to the world of physiology, enticing me into the field of sperm biology and male reproductive medicine, introducing me to Björn and Leif and an ever-encouraging visionary inspiration to critical thinking and development. It is a great pleasure to have Ulrik as co-organiser and presenter at this Spermatology Symposium.

Structure of the Symposium

This symposium had 7 main themes with invited speakers, 17 free oral presentations and 44 poster presentations. Morning sessions were separated from afternoon sessions by a 2-hour lunch break to inspire spontaneous interaction among participants. The long, bright evenings of early May with generous weather also contributed to the intended atmosphere of scientific and social interchange.

Fig. 2 Professor Björn Afzelius (1925–2008). (Photo provided by the family)

Fig. 3 Professor Leif Plöen (1941–2003). (Photo provided by the family)

Themes

- Are Sperm at the Verge of Extinction?
- Sperm DNA—protection and delivery of a complete and undamaged genome
- Sperm Competition, Evolution and Sperm–Egg Interaction
- Genetic aspects of sperm production and performance and its effects on the offspring
- CASA—Advances and Challenges
- Challenges for Sperm Function In Vitro
- Heterogeneity of Sperm Morphology and Laboratory Techniques to Overcome Assessment Challenges
- Sperm Motility (Free Poster Theme)
- Fertility and Infertility (Free Poster Theme)

Fig. 4 Emeritus Associate Professor Ulrik Kvist. (Private photo)

Table 4 Commercial sponsors of the 13th International Symposium on Spermatology

Nidacon International AB www.nidacon.com	A Swedish company headquartered in Gothenburg, manufactures and markets medical devices, mainly for Assisted Reproduction Technologies (ART), with IVF, ICSI, artificial insemination (IUI) and vitrification solutions. *NidaCon* continually strives to improve the outcome of ART, with more pregnancies, by developing superior media systems for clinics, patients and the animal breeding industry.
MICROPTIC S.L. www.micropticsl.com	A company based in Barcelona, it is a world-leading company in the field of semen analysis. The main goal of its business is to produce high-quality products that are continuously improved, integrating the last innovative technology available.
Hamilton Thorne www.HamiltonThorne.com	A leading worldwide provider of precision instruments, consumables, software and services that reduce cost, increase productivity, improve results and enable breakthroughs in the ART field. Hamilton Thorne's CASA II software features modules for sperm motility and concentration, strict morphology, DNA fragmentation, viability and user-defined morphology. The IVOS II hardware platform utilises an automated and heated stage with a built-in optical system for fast and precise sperm analysis.
Nordic Cell www.nordiccell.com	Supplies Nordic gynaecologists and fertility clinics with disposables. We also help in upgrading IVF laboratories with equipment, plus we offer both consulting and complete turnkey laboratory solutions when new IVF clinics are being established. www.nordiccell.com.

Much Appreciated Support for the Meeting

The 13th International Symposium on Spermatology could not have been organised without the grant from the Swedish Research Council (grant 2017-06369) and the commercial sponsorship from Nidacon International, Microptic, Hamilton Thorne

and Nordic Cell (Table 4). Also, the full support from our ANOVA and its originator and director, Associate Professor Stefan Arver, is thankfully acknowledged.

ANOVA is a multi-disciplinary centre dedicated to Andrology, Sexual Medicine and Transgender Medicine. It is a part of the Stockholm Public Health within the Karolinska University Hospital and research wise part of the Department of Medicine Huddinge, Karolinska Institutet.

ANOVA performs investigations of men in infertile couples, men with hypogonadism or other endocrine disorders affecting male sexual and fertility functions. Among other responsibilities are investigations and medical treatments of erectile dysfunction and follow-up of vasectomy operations. ANOVA is also a certified Swedish Tissue Establishment with the commission to cryo store spermatozoa as a means of male fertility preservation.

ANOVA started to develop from a basic clinical semen laboratory in 1987, a few years later evolved into an Andrology Centre with a clinical andrology practice. The unit for Sexual Medicine was added to serve an increasing need for psychological and psychotherapeutic care for men with sexual problems. This unit now also investigates and treats women with sexual problems as well as individuals with risk behaviour of sexual violence and abuse of children. The name of the unit was changed to Centre for Andrology and Sexual Medicine (CASM). The Transgender Medicine unit was included in 2016 and handles psychiatric, psychological, social welfare matters, as well as endocrine and legal issues related to transgender problems—supporting individuals suffering from Gender Dysphoria. To celebrate the inclusion of Transgender Medicine, the name of the combined unit became ANOVA.

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Fifty Years of the Spermatology Symposium

Hideo Mohri

The foundation of the International Symposium on Spermatology (ISS) was made by Baccio Baccetti (1931–2010) of the University of Siena, Italy, and its first meeting was held in Siena and Rome in 1969. The term, spermatology, was used the first time for this meeting. In other words, Baccetti invented this term. He gathered eminent electron microscopists working with spermatozoa of various kinds of animals all over the world. Among them, from Japan, there were Jean Clark Dan who was the discoverer of the acrosome reaction and Gonpachiro Yasuzumi who worked with bird and snail spermatozoa and the superviser of Osamu Tezuka, a famous mangaka or animation creator. Tezuka was also a medical doctor. Almost 100% of the presentations at this meeting were morphological ones, revealed under the electron microscope. Charles Brokaw was the only one person working with sperm motility among the attendants of the first meeting which the author could not attend. The exact name of the symposium was the International Symposium of Comparative Spermatology. Thus the title of the proceedings book published from Academic Press was "Comparative Spermatology." Some articles were written in French. Since then this international symposium has been held every 4 years just like the Olympic Games (Figs. 1, 2, and 3).

Incidentally, in 1965, 4 years earlier, the author, together with veterinarians, gynecologists, and anatomists, etc., founded a similar symposium on spermatozoa in our country. This Japanese symposium has also continued until now repeating annual meetings. Baccio Baccetti was a good electron microscopist, but sometimes worked with biochemists in sperm motility or metabolism, and was even interested in AIDS. He, together with Björn Afzelius, published "The Biology of the Sperm Cell," a classic in spermatology, or more exactly in comparative spermatology.

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Fig. 1 Baccio Baccetti, the founder of the ISS and the organizer of first and sixth meetings

Fig. 2 Upper left to lower right: Björn Afzelius, the organizer of second meeting; Don Fawcett and Michael Bedford, the organizers of third meeting; J. André, the organizer of fourth meeting; Hideo Mohri, the organizer of fifth meeting and Jim Cummins, the organizer of seventh meeting

The second meeting was held in Stockholm in 1973, organized by Björn Afzelius (1925–2008). The author first met him at the Misaki Marine Biological Station, the University of Tokyo in 1958. He was famous in discovery of the details of so-called 9 + 2 structure of flagella and cilia in 1959, describing arms, now dynein arms, and spokes in the axoneme of sea urchin spermatozoa and also numbering the outer doublet microtubules. Furthermore, he had already suggested that sliding between the adjacent doublet microtubules is the fundamental mechanism of flagellar and ciliary movement, based on his morphological observation with the electron microscope. Later in 1975, he described the immotile cilia syndrome.

Fig. 3 Upper left to lower right: Claude Gagnon, the organizer of 8th meeting; Gerhard van der Horst, the organizer of 9th meeting; Eduardo Roldan, the organizer of 10th meeting; Masaaki Morisawa, the organizer of 11th meeting; John Aitken, the organizer of 12th meeting; and Lars Björndahl, the organizer of 13th meeting

As indicated by the title of the proceedings book, "The Functional Anatomy of the Spermatozoon," Pergamon Press, the functional aspects of spermatozoa was taken up together with morphological ones in this meeting, although the name of the symposium was again the International Symposium on Comparative Spermatology. Thus fertilization, sperm motility, etc. were added as the main subjects. The author, together with Ian Gibbons, the discoverer of dynein, attended as a motility person and talked about the comparison of the newly discovered tubulin and dynein with actin and myosin in muscle.

Among the attendants, there were Laura and Arther Colwin, who first described the fusion of egg and sperm membranes at fertilization, and Gerald Edelman, a Nobel laureate with his work on the chemical structure of antibody, who was interested in mammalian fertilization around that time. As a matter of fact another Nobel laureate, Yoshinori Ohsumi, who elucidated the mechanism of autophagy, was in Edelman's laboratory and once worked with mammalian fertilization. Both Colin Austin and Min Chua Chang who discovered independently the phenomenon called capacitation in 1951 were also present in this meeting.

The third meeting was held in Boston and Marine Biological Laboratory, Woods Hole, in the United States in 1978. The organizers were Don Fawcett (1917–2009) and J. Michael Bedford (1932–2018). Fawcett, as an eminent anatomist, made various excellent electron microscopical works as summarized in his book, "The

Mammalian Spermatozoon" and after retirement from Harvard, he devoted himself to studies on parasitic diseases in Africa. Bedford was the first postdoctoral fellow of M.C. Chang and has much contributed to maturation, capacitation, and fertilization of mammalian spermatozoa.

The name of this symposium was the International Symposium on the Spermatozoon. As the subtitle of the proceedings book, "The Spermatozoon," Urban & Schwarzenberg, also indicates, the scope of the symposium was further extended to Maturation, Motility, Surface Properties, and Comparative Aspects. Comparative and evolutionary aspects became the backbone of the ISS from the first meeting through this 13th meeting. As there were many motility people including Ian Gibbons and his wife Barbara, a very skillful biochemist, in the United States, the hot discussion was made on the then-current topics concerning the mechanism of sperm motility. Also, quantitative assessment of sperm motility was taken up as a workshop organized by Robert Rikmenspoel. Surface properties of spermatozoa in connection with fertilization, capacitation, and spermiogenesis were one of the main subjects. Human spermatozoa were also one of the targets in this meeting. Thus there was a tendency to include also applied fields from this meeting. The author revealed that the arms in the sperm flagella are really dynein molecules with peroxidase-conjugated anti-dynein antibody prepared by Kazuo Ogawa, who later determined the whole sequence of dynein heavy chain in 1991.

In this meeting, Ryuzo Yanagimachi, Yana, attended for the first time together with Claudio Barros of Chile. Yana succeeded in in vitro capacitation, and thus in successful in vitro fertilization of mammalian sperm. He also discovered hyperactivation and succeeded in ICSI and ROSI, etc. and reared many excellent reproductive biologists. He is 2 years elder than the author, i.e., 90, but is still actively working. Both Gibbons and Yana received the International Prize for Biology, which was established to commemorate the contributions that the late Emperor Showa (Hirohito) and the present Emperor, Akihito, of Japan have over long years made to biological sciences by themselves.

The fourth meeting was held in Seillac of the Loir district in France in 1982, organized by Jean André (1922–2017), who observed the genesis of sperm mitochondria under the electron microscope. "The International Symposium on Spermatology" was used the first time as the name of the symposium. The subtitle of the proceedings book, "The Sperm Cell," Martinus Nijhoff Publishers, was Fertilizing Power, Surface Properties, Motility, Nucleus and Acrosome, and Evolutionary Aspects. Together with the development of in vitro fertilization (IVF) and embryo transfer, we had to evaluate not only motility but also the fertilizing power of human and domestic and experimental animals' sperm. Changes in nucleus and acrosome were specifically discussed. Monoclonal antibody and evaluation of sperm motility using light scattering were introduced. It was impressive that many French colleagues spoke in French with the English summary on the slides. We took the pleasant outdoor lunch.

We can find the names of Jim Cummins, Claude Gagnon, Masaaki Morisawa, and David Mortimer who have been or will be the organizer of the ISS among the attendants of this meeting. David Phillips who published excellent electron

micrographs of spermatozoa in many kinds of animals was also included. In the Loir district, there are many old castles, so that we enjoyed the sightseeing very much. At that time, the members of international organizing committee were Jean André, Björn Afzelius, Baccio Baccetti, Michael Bedford, Don Fawcett, Gunther Meyer, and Hideo Mohri. After this meeting, Meyer's name disappeared from this list and new names of the organizers of succeeding meetings were added one by one, losing the names of persons who passed away.

The fifth meeting was held in Fujiyoshida, a small town in the foot of Mt. Fuji, in 1986, organized by the author. The reason why this town was selected as the venue of the meeting was that Bedford insisted that in the big city like Tokyo with many attractive places it would be difficult to keep all the attendants together for discussion. Of course, beautiful scenery of Mt. Fuji was another reason. The author planned this meeting for Jean Dan and Gonpachiro Yasuzumi, but unfortunately both of them had passed away before the meeting was held. In this meeting, sperm metabolism, male contraception, and separation of X- and Y-sperm were added as main subjects. The last was realized later by Laurence Johnson based on DNA difference, using cell sorting and flow cytometry. Unfortunately, the difference between X- and Y-sperm is quite small in humans, and furthermore now sexing of early embryo is available after in vitro fertilization, but its practical application has been made to cattles, etc.

Ties and scarfs with the cross sections of mammalian sperm flagellum were prepared for the attendants. At the beginning of banquet, Austin and the author broke the top of the container of sake with wooden hammers, the ceremony called kagamiwari in Japanese. Several attendants climbed up to the summit of Mt. Fuji. We can find young John Aitken, Claude Gagnon, and Eduardo R.S. Roldan who also organized the ISS meetings later among the attendants. The proceedings book, "New Horizons in Sperm Cell Research," Japan Scientific Societies Press, was published.

Incidentally, this year, 2018 is also the 50th anniversary of the naming of tubulin by the author. The paper proposing the name tubulin for the main constituent of microtubule appeared in a March issue of Nature in 1968. To tell the truth, the author asked Thaddeus Man of the UK, the author of "Biochemistry of Semen," for recommendation of his manuscript to Nature, and Jean Dan was the recommender of this name. Now about 1500 tubulin papers have been published every year, and the author is very happy as the godfather.

The sixth meeting was again held in Siena, organized by Baccetti in 1990. Celebrating the 20th year of spermatology, he used the name, International Congress on Spermatology, but this was only for that time, because spermatologists did not like a big name. Baccetti tried to summarize all the results obtained so far in the field of spermatology. Indeed, at the beginning of the meeting and of the proceedings book, "Comparative Spermatology 20 Years After," Raven Press, "History of Spermatology" was presented by Afzelius and Baccetti, and the book was quite voluminous, including all the about 200 presentations. In applied field, the number of gynecologists, andrologists or pathologist, etc., increased. Reactive oxygen species (ROS) and gene expression were among the topics. An award was given to Don Fawcett for his great contributions to spermatology.

Thaddeus Mann attended this meeting and talked about octopus sperm. As described above, he wrote "Biochemistry of Semen" and later its revised book "Biochemistry of Semen and of the Male Reproductive Tract" summarizing enormous data concerning sperm metabolism and related subjects. Since the author started his academic carrier with studies on respiration and lipid metabolism of spermatozoa, these books were bibles for him. Owing to his wife's health conditions, Mann could not attend the preceding meetings and unfortunately his attendance was limited only this one. Every night we enjoyed good Italian wine.

The seventh meeting was held for the first time in the Southern Hemisphere, in Cairns of Australia in 1994, organized by Jim Cummins. He spent several years in Yana's laboratory in Hawaii as a postdoc. Cummins made several experiments concerning maturation, capacitation, and fertilization and then greatly contributed to the development of reproductive biology and medicine in Australia. The proceedings book "Advances in Spermatozoal Phylogeny and Taxonomy," Museum national d'Histoire natureelle, summarized only the contributions in the field of phylogeny and taxonomy of spermatozoa, together with some articles of nonparticipants. Editors were Barrie G.M. Jamieson, a comparative spermatologist and one of the co-organizers of this meeting, Juan Ausio of Canada, and Jean-Lou Justine of France.

In this meeting, applied spermatology sections both in human and in animal science occupied great portions. SRY, sperm competition, and CASA were found among the topics. Technology of molecular biology was gradually and more and more used also in sperm researches. We presented some phylogenetic results concerning the number of subunits of outer arm dynein. The outer arm dynein in all the animal spermatozoa and cilia, both Protostomia and Deuterostomia, consists of two heavy chains and the outer arm looks like a hook, while that of other organisms such as *Chlamydomonas* and *Paramecium* consists of three heavy chains and their outer arm looks like a pistol. As reported at the preceding meeting in Newcastle, recently Kazuo Inaba showed that a flagellar protein regulating sperm motility, caraxin, has the same distribution, namely in uniconta, as two-headed outer arm dynein has among all the living organisms. This group, uniconta, would be keeping some ancient genes and characters, because three heavy chains were caused by duplication of a certain heavy chain.

We can find the name of Gerhard van der Horst among the attendants. We loved the Nature of Australia including the Great Barrier Reef very much. Fortunately, we could see many wild platypuses as well as kangaroos or wallabies and nests of termite on the way of excursion.

The eighth meeting was held in Montreal, Canada, in 1998 after a competition with the Worcester Foundation in the United States where the pill was developed By Gregory Pincus and M.C. Chang. Organization was made by Claude Gagnon (1950–2012). He was the head of urology laboratory and had spent several months in the author's laboratory in Tokyo. He studied the effects of ROS on spermatozoa and obtained sperm motility inhibitor, semenogelin, from the seminal plasma. This meeting was characterized by the title of the proceedings book, "The Male Gamete, from Basic Science to Clinical Applications," Cache River Press. Various topics

were discussed in relation to assisted reproduction. Male infertility was becoming a main problem.

At the beginning of the proceedings book, a tribute to Yves W. Clermont of Canada was presented for his long-term contributions to studies on seminiferous epithelium. Thus spermatogenesis was a main theme. Interactions with egg and egg coat, transmembrane and intracellular signaling, HIV, etc., were among the topics. One session was devoted to fish spermatozoa. To this meeting, the big three pioneers in reproductive biology and spermatology, namely Austin, Chang, and Yana attended.

The ninth meeting, the first in twenty-first century and also in African continent, was held in Cape Town of South Africa in 2002, organized by Gerhard van der Horst. He has examined the semen of more than 100 species of animals including endangered wild animals ultrastructurally and with CASA and has thus contributed to human and animal reproduction. The conservation of endangered wild animals is an urgent problem in Africa and was one of the main themes of this meeting. Criteria of semen quality were discussed in relation to assisted reproduction in addition to other main subjects.

At the banquet, we enjoyed the meat of crocodile, ostrich, a kind of antelope, etc., which we could not taste in other places. Of course, these are not the species going extinct. The venue was not far from the table mountain. We also visited the Cape of Good Hope and watched whales and cape penguins.

The tenth meeting was held in Madrid, Spain, more exactly at El Escorial in the suburb of Madrid, in 2006, organized by Eduardo Roldan. He has contributed sperm biology in general, including bioenergetics and signal transduction, and is much interested in sperm competition in relation to sperm evolution. Relief of endangered wild species is also his research subject. Thus, these themes were reflected in the sessions of this meeting. Various manipulations of sperm cells were discussed. There was one report on male contraception.

The proceedings book of this meeting, "Spermatology," Nottingham University Press, was also quite voluminous. For Afzelius, this became the last meeting to attend. Tim R. Birkhead, a debater of sperm competition, attended this meeting.

The 11th meeting was held in Okinawa, again in Japan, but with somewhat different atmosphere from that of the main islands of Japan, in 2010. The organizer was Masaaki Morisawa, who was the director of the Misaki Marine Biological Station and elucidated the signal transduction mechanism at motility initiation and in chemotaxis of spermatozoa. In this meeting, Björn Afzelius memorial symposium concerning motor proteins and sperm motility and Jean Clark Dan memorial symposium concerning molecular biology of the acrosome reaction were held together with the tributes to them. Sperm genomics, environmental impacts, male sterility were among the main themes. Several luncheon seminars and workshops were also provided.

The proceedings book, "Sperm Cell Research in the 21st Century: Historical Discoveries to New Horizons," Adthree Publishing, was published, including the obituary of Baccio Baccetti. Among the attendants, there was Gen Hoshi, the former president of the International Union of Biological Sciences (IUBS), who identified

the factors inducing the acrosome reaction in starfish. The presentation ceremony of the awards for the excellent posters took place at the end of this meeting. The sunset viewed from the venue where once held the so-called summit was very beautiful.

The 12th meeting was held in Newcastle, again in Australia, in 2014, organized by R. John Aitken. This was a joint meeting with AAAA, the Association for Applied Animal Andrology. He is also an eminent reproductive biologist and concerned with the effects of ROS and other factors on sperm functions from a long time ago. The meeting started with sperm biology in domestic animals. Paternal impacts on development were also discussed. As a new technology CRISPR/CAS system was introduced. There were lunch workshops concerning CASA and DNA damage, etc.

The proceedings book was not published after this meeting. Masaru Okabe, who made excellent work in molecular biology of spermatozoa concerning fertilization, gave us Thaddeus Mann Memorial Lecture. The awards for excellent talks were presented at the banquet. We enjoyed the excursion visiting some winery in the suburb.

In 2018, we had the 13th meeting in Stockholm, organized by Lars Björndahl and his colleagues. He worked with chromatin stabilization of human sperm with zinc and standardization of semen analysis. The presentations at this meeting are described in this proceedings book. At this meeting, Hagai Levine and his group reported a significant (50–60%) decline in human sperm counts in Western countries for these four decades, reconfirming the report by Skakkebaek's group in 1992. Based on this presentation, "Stockholm Male Reproductive Health Statement 2018" was compiled to call on governments, organizations, the scientific and medical communities, and individuals in the world.

In all the preceding meetings, we have enjoyed their friendly atmosphere. We have much learned and have been inspired from each other and have got new ideas for future researches through the mutual exchange, sometimes resulting in cooperative works. The next meeting in Canada organized by David Mortimer will be no exception.

In future, it is needless to say that molecular biology and information biology together with further improvement of image analysis would elucidate the basic problems concerning various facets of spermatozoa from primordial germ cells to fertilization or the onset of development. Male infertility and decline of sperm counts are serious problems not only for individuals suffering from it but also for the survival of human being as described above and of endangered wild animals in future. Induction of a single gene necessary for a certain step of sperm formation has already been tried in a special case lacking such a gene. Although asthenozoospermia could be overcome by ICSI, and even some of azoospermia by TESE, in vitro spermiogenesis, spermatogenesis in other animal's body or production of egg and sperm from the somatic cells such as iPS cells, etc., would be further improved or carried out. As the rapid increase in human population is also serious big problem, so we should pay more attention to male contraception. It was not often that this theme was taken up in the ISS. In fact, the author made some experiments on alpha-chlorohydrin and gossypol, once the candidates of the male pill, but

never talked about them in the ISS. Evolution of spermatozoa is always a fascinating problem. We need more evidences concerning sperm competition and other hypotheses.

Even living, in other word reproduction, in the space and the revival of once extinct animals like in the film "The Jurassic Park" would not merely dreams. Teruhiko Wakayama who first succeeded in cloned mice in Yana's laboratory told us the other day that freeze-dried semen samples of mice are now going around the earth in the space laboratory and that the injection of sperm nuclear DNA from the testis of the mouse frozen under the ground with mammoth gave offsprings or something. Finally, the author would like to recommend to include some researchers of male gametes of the organisms other than animals, for instance, plants and seaweeds, in this symposium. Recently, we had a research group on sexual reproduction in animals and plants in Japan, and have found that there are common phenomena and common genes among them concerning gamete recognition and fusion. Viva spermatozoa!

Spermatology in Sweden

Ulrik Kvist

Lecture given by Ulrik Kvist on June 3rd 2018, XIIIth International Symposium on Spermatology, 9th to 13th May 2018, Stockholm.

Gustaf Retzius,
Åke Franzén,
Björn Afzelius,
Lennart Nicander,
Leif Plöen.

Gustaf Retzius 1842–1919 (Fig. 1)

Gustaf Retzius was a physician, anatomist and also a member of the Swedish Academy 1901–1919 chair no 12, appointing the Nobel laureates in literature. He was Professor in Anatomy and Histology at the Karolinska institute, Stockholm, Sweden. His works on the sensory organs and the nervous system are particularly famous.

Gustaf Retzius retired early and took up a complete new field of research—comparative spermatology when he was in his sixties. Retzius financed all spermatology research with family money and his world-wide contacts. He examined spermatozoa from over 400 animal species from all six continents.

He was exceedingly productive; all the large volumes seen on his right side in the photograph were written and illustrated by him.

Retzius worked at his home in Stockholm or at the Kristineberg Marine Research and Innovation Center in Fiskebäckskil on the Swedish Westcoast (Fig. 2) where

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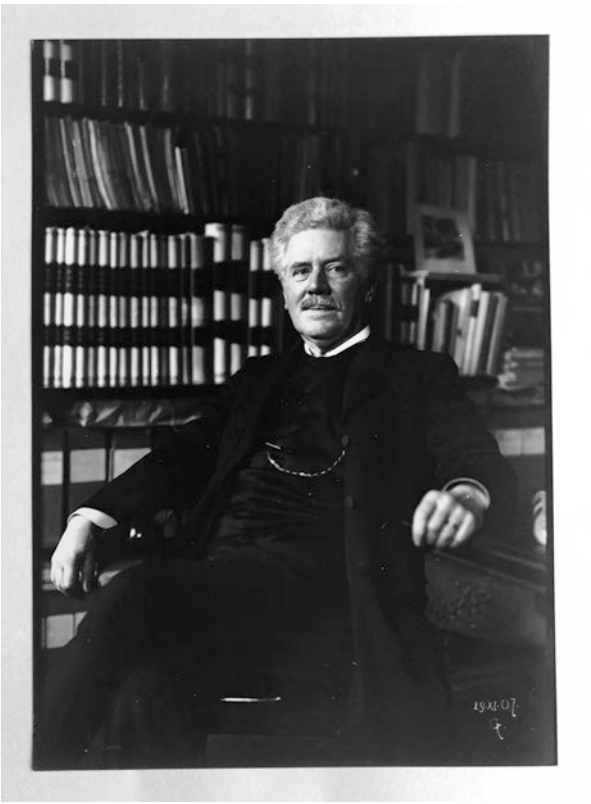


Fig. 1 Gustaf Retzius 1842–1919. Portrait 1907-11-21 (in Thomas Lindblad Collection), Photo 2021 by Anna Lantz, Hagstromer Library Karolinska Institutet, Stockholm, Sweden

Fig. 2 Kristineberg Center for Marine Research & Innovation. Fiskebäckskil, Sweden. Dronefoto by Eduardo Infantes. https://kristinebergcenter.com

Fig. 3 Retzius first Microscope by C. Verick eleve special de E. Hartnack, Rue de la Parcheminerie 2, Paris", Photo 2021 by Anna Lantz, Hagstromer Library Karolinska Institutet, Stockholm, Sweden

also two later Swedish Spermatologists, Åke Franzén and Björn A. Afzelius, spent summers as teacher and researcher.

Retzius instrument was the light microscopy. Initially he used the Verrick microscope (Fig. 3) and later a Zeiss microscope. He preferred to use sun rays and was thus dependent of favourable weather for drawing the spermatozoa he investigated.

Retzius and Technical Excellence

His investigations were at the top of what could and can be achieved by light microscopy.

Wherever possible he took his material from freshly killed animals, fixed their spermatozoa with osmium tetroxide or Zenker's fixative and performed the examination with a good Zeiss microscope provided with apochromatic lenses with good resolution and numerical apertures of 1.3 or 1.4.

Retzius stated in 1904: "In order to understand the fertilization process it is necessary to undertake a detailed investigation of the two parties at fertilization—the spermatozoon and the oocyte" (Retzius 1904–1921, cited in Björn 1995). Retzius

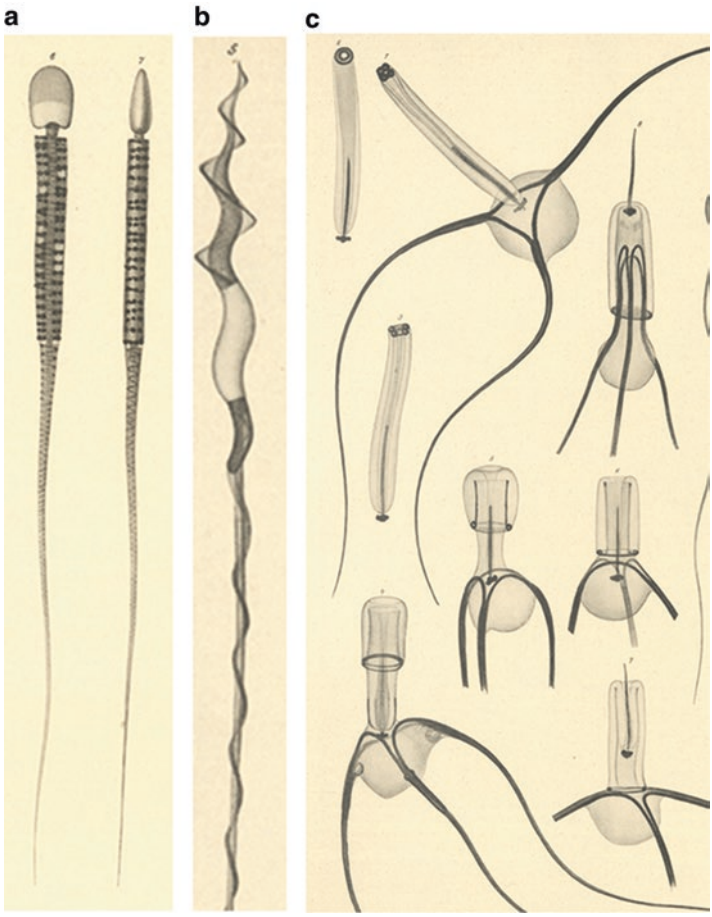


Fig. 4 (**a**) Pipistrelle Bat Vol XIII Table XXIX; (**b**) Passerine Bird Vol XIV: Table XXXVII; (**c**) Marine Crustacean Vol XIV: Table XII; Spermatozoa drawn by Gustaf Retzius. Photo and compilation from Biologische Untersuchungen, Neue Folge, Volumes XI–XIX. Photo 2021 Anna Lantz. For further descriptions see Björn A. Afzelius. Gustaf Retzius and Spermatology Int. J. Dev. Biol. 39: 675–685, 1955 and original paper in Biologische Untersuchungen, Neue Folge, hagstromerlibrary@ki.se

microscope and the original collection of Biologische Untersuchungen can be studied at the Hagströmer Library at the Karolinska Institute, Stockholm, Sweden (hagstromerlibrary@ki.se) (Figs. 4–6).

Like Carl von Linnaeus/1707–1778, another Swedish natural scientist Retzius made systematic comparisons. In contrast to Linnaeus, who believed spermatozoa to be parasites infecting drinking water Retzius realized their importance and opened up a new field of Comparative Spermatology. His systematic work of sperm morphology had mostly phylogenetic implications and is still a unique source for future researcher. Retzius also claimed it to be necessary to study spermiogenesis in order to be able to interpret the homologies.

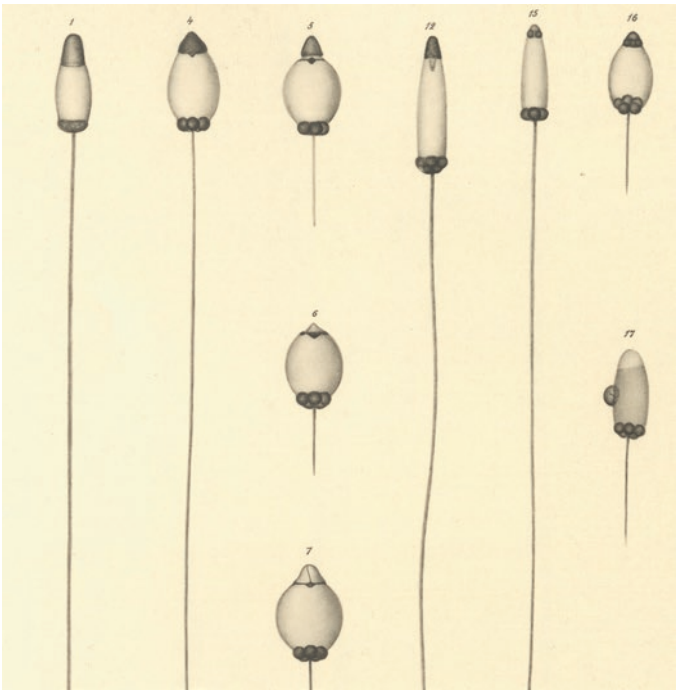


Fig. 5 Primitive spermatozoa. Drawing by Gustaf Retzius. Photo from Biologische Untersuchungen, Neue Folge, Volume XIII : Tabloid I. Photo 2021 Anna Lantz. For further descriptions see Björn A Afzelius. Gustaf Retzius and Spermatology Int. J. Dev. Biol. 39: 675–685, 1955 and original paper in Biologische Untersuchungen, Neue Folge, hagstromerlibrary@ki.se

Retzius noted the intraspecies variation in sperm size and shape in the case of the gorilla and the gibbon, just as it had been described both by him and by others in the case of the human ejaculate (Fig. 7). The gorilla was wild and not captured in a Zoo or Circus. This inherent pleiomorphism is still of importance when discussing the possible reasons for the poor semen quality in our species.

During 40 years 1974–2016, I had the privilege to introduce physicians to be into the field of male reproductive physiology in the Gustaf Retzius Lecture Hall at the Karolinska Institute Campus in Stockholm, Sweden, with photos of his drawings of various spermatozoa on the lecture hall walls (Figs. 8 and 9).

Retzius was the pioneer that introduced the concept of the primitive spermatozoon. The functional aspects with respect to fertilization was revealed some 50 years later by his Swedish successor Åke Franzén (Fig. 10).

Åke Franzén (1925–2011) was the director of the Invertebrate Department at the Swedish Museum of Natural History, retiring from this appointment in 1991.

Franzén showed that the primitive sperm described by Gustaf Retzius (Fig. 5) was found only in such animal species, where the semen is discharged in the ambient water, usually for external fertilization. With this enlargement of the concept, emphasis shifted from phylogeny to the mode of fertilization (Fig. 11).

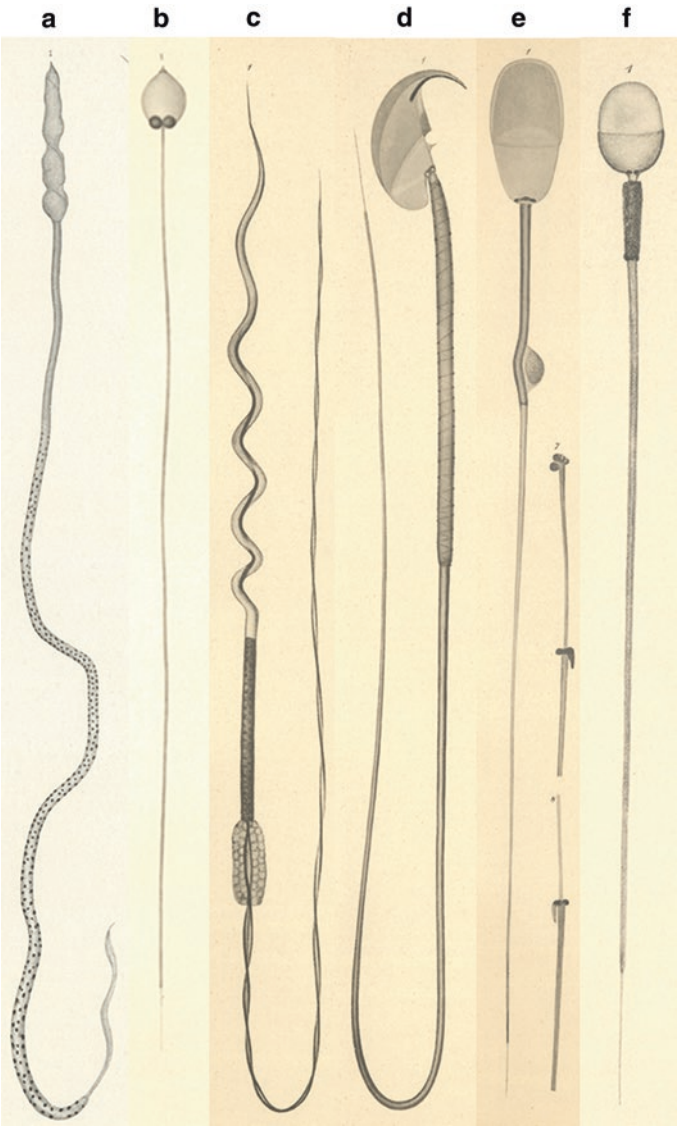


Fig. 6 Comparative spermatology. (**a**) Flat worm Vol XII: Table XIII, (**b**) Blue Mussel Vol XI: Table X, (**c**) Thornback Ray XIV: Table XXVIII, (**d**) House mouse Vol XIV: Table XVI, (**e**) Bull Vol XIV: Table VIII, (**f**) Human Vol XIV: Table LX. Selected drawings of various spermatozoa by Gustaf Retzius. Photo and compilation from Biologische Untersuchungen, Neue Folge, Volumes XI–XIX. Photo 2021 Anna Lantz. Hagstromer Library Karolinska Institutet, Stockholm, Sweden. For further descriptions see Björn A Afzelius. Gustaf Retzius and Spermatology Int. J. Dev. Biol. 39: 675–685, 1955 and original paper in Biologische Untersuchungen, Neue Folge hagstromerlibrary@ki.se

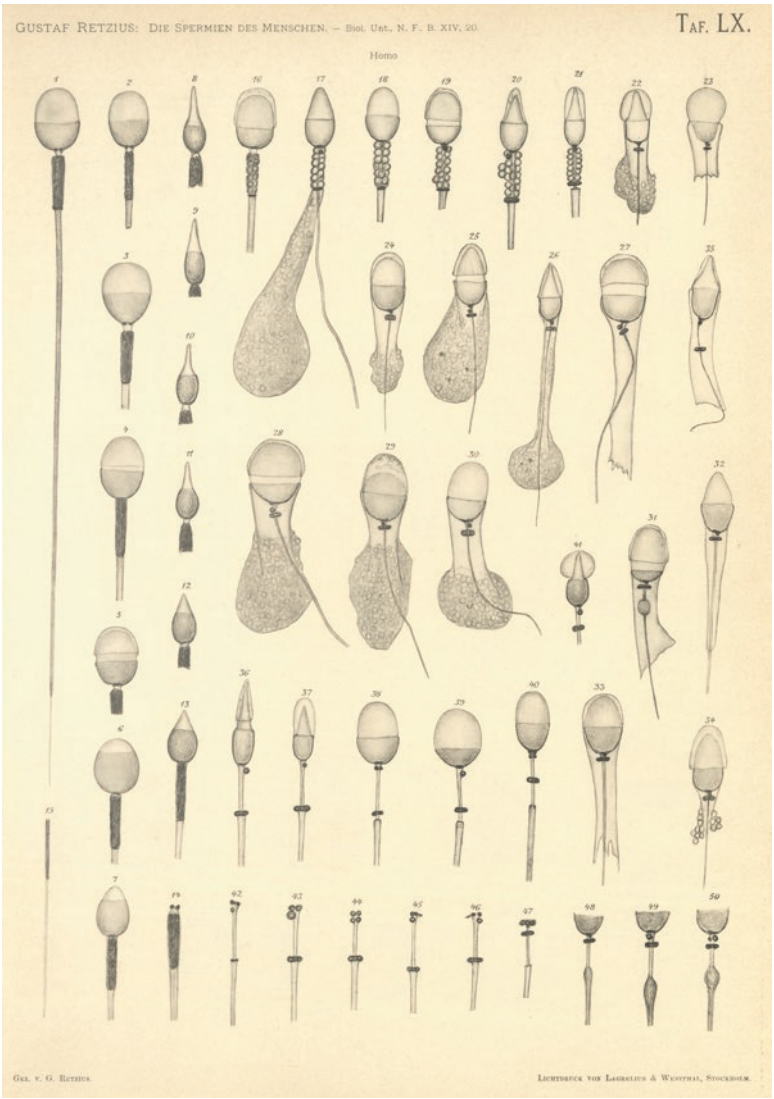


Fig. 7 The Pleiomorphism of human spermatozoa. Drawings by Gustaf Retzius. Photo from Biologische Untersuchungen, Neue Folge, Volumes XIV: Table LX. Photo 2021 Anna Lantz**.** For further descriptions, see Björn A Afzelius. Gustaf Retzius and Spermatology Int. J. Dev. Biol. 39: 675–685, 1955 and original paper in Biologische Untersuchungen, Neue Folge, hagstromerlibrary@ki.se

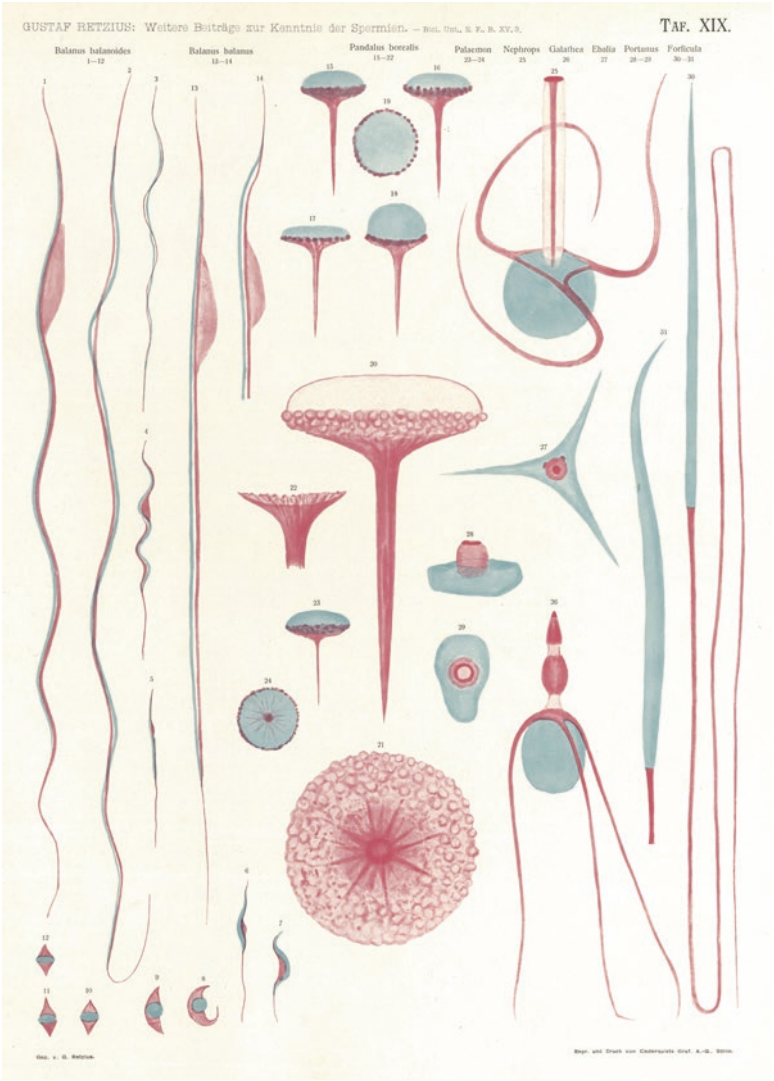


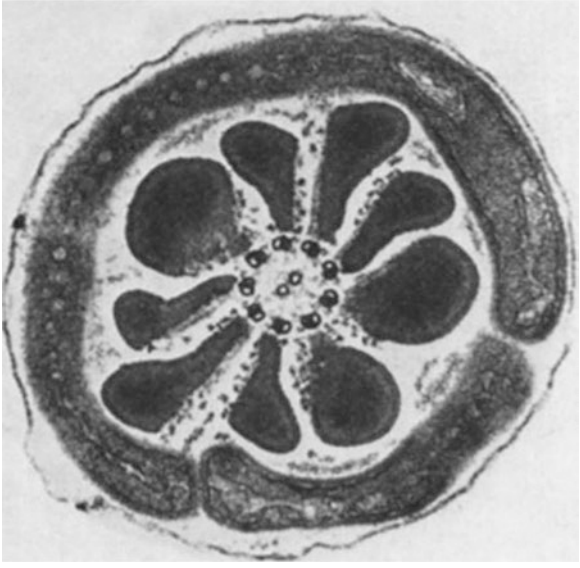
Fig. 8 Original of Wall Photo in Retzius Lecture Hall (Fig. 9 below) at the Karolinska Institute Campus, Stockholm, Sweden. Lecture hall for teaching Male reproductive Physiology by Kvist 1970–2016. Photo 2021 Anna Lantz, hagstromerlibrary@ki.se

Franzén pointed out the differences between sperm evolved from external to internal fertilization. At external fertilizing sperm has a small spherical head, large acrosome, a small midpiece with 4–5 mitochondria and a simple tail with a 9 + 2 axoneme. This is in contrast to sperm of internal fertilizing species which have evolved to be more complex with an elongated head, extended midpiece with many mitochondria and with nine solid outer fibres around the axoneme forming a 9 + 9 + 2 pattern (Fig. 11). This complex sperm could meet the challenge of elastic

Fig. 9 Retzius Lecture Hall at the Karolinska Institute Campus, Stockholm, Sweden. Lecture hall for teaching Male reproductive Physiology by Kvist 1970–2016

Fig. 10 Åke Franzén 1925–2011

Fig. 11 Electron microscopy section of Sperm tail illustrating Franzéns concept of the 9 + 9 + 2 pattern of the sperm tail organization



forces encountered during motility and endure the structural integrity in the viscous media of an internal fertilization environment.

Franzén used the phase contrast microscopy and later also the transmission electron microscopy. In 1955/1956, two notable research papers appeared in a study of some 200 mainly marine invertebrate species (internal and external fertilizers) where he showed that the fertilization environment was evolutionary very important to shape sperm form and Franzén (1955, 1956).

He continued to published his observations on sperm structure from 1956 to 2002 (van der Horst 2018).

Åke Franzén showed that sperm morphology and ultrastructure can serve as a guide to phylogenetic relationships (Franzén 1956). This inspired further phylogenetic research by Afzelius, Baccetti, Cummins, Dallai, and Jamieson (Cummins 1983).

Björn Afzelius (1925–2008) was professor in biological ultrastructure research at Stockholm University (Fig. 12).

In the 1950s, Björn Afzelius realized that microtubules effected flagellar motion by sliding past one another (Fig. 13).

In 1976, he described the human syndrome with immotile cilia due to loss of dynein arms and situs inversus (Afzelius et al. 1975).

Björn Afzelius wrote in 1967 a popular Swedish textbook called "Cellen"—the Cell—that has inspired many young students including myself. The Cell was translated into many languages. At 17 years, I was fascinated by the chapter "About mitochondria" and became eager to learn more—I turned the page and was immediately blessed! The next chapter was of course named "More about mitochondria".

At my dissertation party Björn had my mum at the table, an experience she never stopped talking about and with concern to locomotion, he danced all evening.

Björn encouraged and supported many young researchers, among them myself and Lars Björndahl to focus on Spermatology and facilitated us to take part in the International Symposia on Spermatology.

When I presented my interest in the sperm head chromatin and role of zinc, he smiled and said, "I think the tail is the most interesting part of the sperm—the head is just the passenger".

Björn made it possible for Björndahl and me to study zinc in sperm head in individual spermatozoa with Gottfried Roomans (Kvist et al. 1988; Kvist et al. 1980).

Lennart Nicander

Lennart Nicander was professor in Anatomy and Histology at the Swedish University of Agricultural Sciences 1958–1979.

I first met him at the Spermatology meeting in Seillac 1982. He was then professor in Anatomy and Histology at the veterinary university of Norway. His

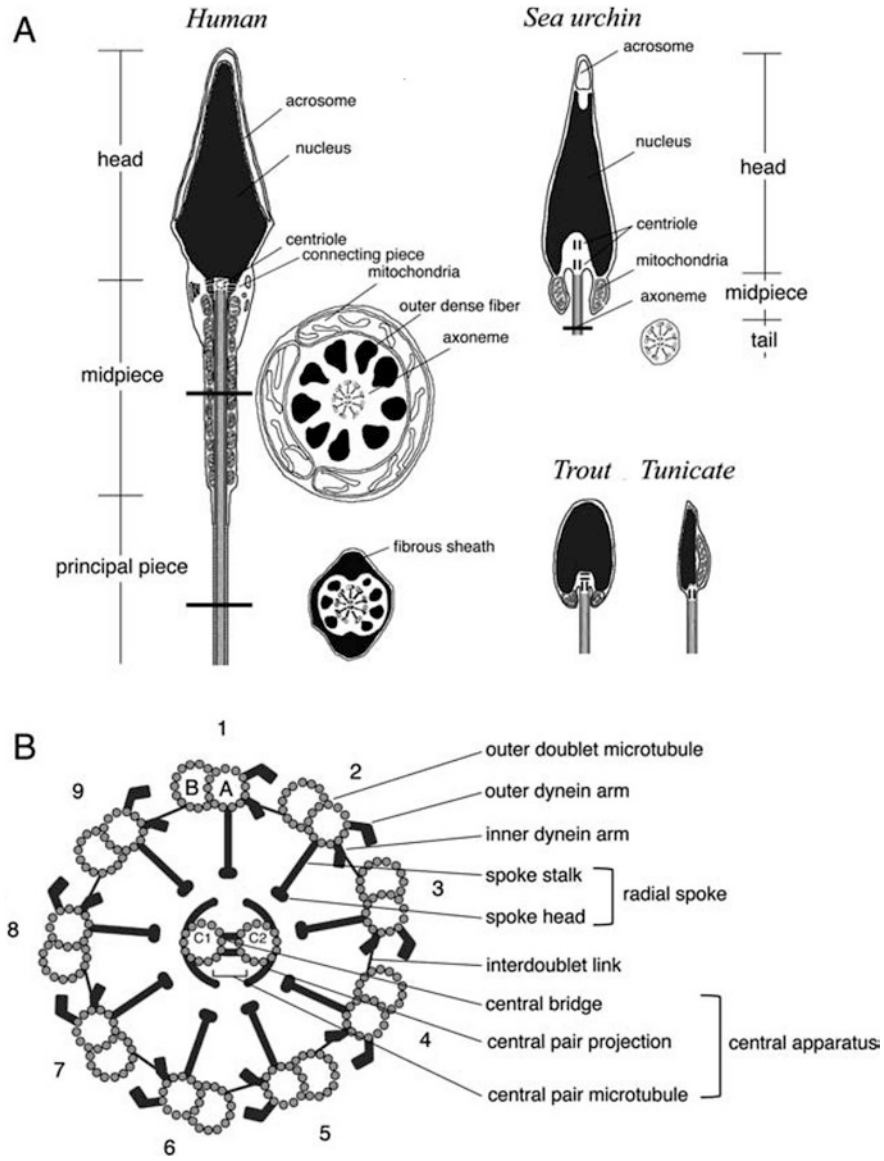


Fig. 12 Sperm tail organization illustrating Afzelius concept of dynein arms as essential for tail movement. Internal structure of sperm flagella. (**a**) Comparison of human sperm flagella and primitive sperm flagella. In human sperm, the axoneme is surrounded by ODFs, mitochondria, and plasma membrane, whereas in the principal piece, it is surrounded by ODF, an FS, and a plasma membrane. In sperm from sea urchins, tunicates, and teleosts, the axonemes are simply surrounded by a plasma membrane. (**b**) Substructures comprising flagellar axonemes. Axonemal structures are well conserved among invertebrates, lower vertebrates, and mammals. Figure 12 is reproduced with kind permission from professor Kazuo Inaba University of Tsukuba, Shimoda Marine Research Center. Zoolog. Sci. 2003 Sep;20(9):1043–56. https://doi.org/10.2108/zsj.20.1043

Fig. 13 Björn Afzelius 1925–2008

encouragement to my findings on sperm chromatin presented in Seillac never to be forgotten.

He contributed to our understanding of the functional organization of the epididymis and the Blood–testis barriers (Nicander 1967; Plöen and Setchell 1992).

Leif Plöen 1941–2003

Leif Plöen worked at the Department of Anatomy and Histology at the Swedish University of Agricultural Sciences in Stockholm and Uppsala, Sweden.

His supervisor was Lennart Nicander. Leif Plöen investigated the ultrastructure of spermatogenesis in the rabbit and he developed experimental models for cryptorchidism (Plöen 1972).

He succeeded Lennart Nicander as professor in Anatomy and Histology in 1979 and made important clinical contributions concerning testicular ultrastructure and methods for diagnostic testicular investigations.

I first met Leif Plöen 1980 as the opponent on my thesis On the sperm chromatin decondensation ability (Kvist 1980), and 6 years later he returned to Karolinska as Björndahls opponent (Björndahl 1986) (Fig. 14).

Plöen made possible for Andrology at the Karolinska Institutet and Karolinska University Hospital to run comparative research with the Swedish Veterinary University in Uppsala Sweden (Rodriguez-Martinez et al. 1990; Rosenlund et al. 1998, 2001) concerning spermatogenesis, spermatology and sperm chromatin organization (Fig. 15).

Fig. 14 Leif Plöen 1941–2003

Fig. 15 Opponent and respondent 1980. Leif Plöen and Ulrik Kvist at the Dissertation Celebration Party

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Part II
Are Sperm at the Verge of Extinction?

Following reports on declining sperm numbers in human, there is an interest in understanding if this a universal problem, affecting also other species. What is the role of environmental influence? The fate of endangered species is also a matter that might be linked to a general decline in sperm production.

Temporal Trends in Human Sperm Counts: Findings and Implications

Hagai Levine

In 1992 Carlsen et al. published their paper (Carlsen et al. 1992), claiming that there is a decline in sperm concentration, at least in Europe and the USA. This question remained controversial, even after 25 years. A definitive meta-analysis was critical given the predictive value of sperm count for fertility, morbidity, and mortality. In 2017, we published the first systematic review and meta-regression analysis on trends in human sperm count (Levine et al. 2017). We aimed to answer the question: have human sperm counts, as measured by sperm concentration (SC) and total sperm count (TSC), declined?

Following a pre-defined protocol, 7518 abstracts were screened and 2510 full articles reporting primary data on SC were reviewed. A total of 244 estimates of SC and TSC, sampled in 1973–2011, were extracted for meta-regression analysis. The slopes of SC and TSC were estimated as a function of sample collection year using both simple linear regression and weighted meta-regression models, adjusted for pre-determined covariates and modification by fertility ("unselected by fertility" vs. "fertile") and geographic group ("Western," including North America, Europe, Australia, and New Zealand vs. "other").

SC declined significantly between 1973 and 2011. There was a significant decline in SC between 1973 and 2011 among Unselected Western (−1.38 million/ml/year; −2.02 to −0.74; $p < 0.001$) and among Fertile Western (−0.68; −1.31 to −0.05; $p = -0.033$). Among Unselected Western studies, the mean SC declined, on an average, 1.4% per year with an overall decline of 52.4% between 1973 and 2011. Trends for TSC and SC were similar, with a steep decline among Unselected Western (−5.33 million/year; −7.56 to −3.11; $p < 0.001$), corresponding to an average decline in mean TSC of 1.6% per year and an overall decline of 59.3%. Results changed minimally in multiple sensitivity analyses, and there was no statistical support for the use of a nonlinear model and no sign of "leveling off."

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In summary, this comprehensive meta-regression analysis reports a significant decline in human sperm counts between 1973 and 2011, driven by a 50–60% decline among men unselected by fertility from Western countries.

The study was well accepted by the scientific community and it seems like there is now a consensus that sperm counts indeed declined, at least among Western men. However, the causes and the implications of sperm counts decline remained under discussion. This decline goes together with other disturbing male reproductive health trends (Skakkebaek 2017), such as the potential increase in congenital malformations of the male reproductive system, decrease in testosterone, and increase in testicular germ cell tumors as well decreased fertility and increases in miscarriage rates. In addition, low sperm count has been found to be associated with increased morbidity and mortality (Jensen et al. 2009).

The study received wide attention, as manifested by an Almetric score of 4021. However, this interest was not translated into further resources for research or in changes in public policy regarding male fertility, despite calls from the scientific community (Barratt et al. 2018; Levine et al. 2018). The contribution of sperm count decline to reduced fertility is not clear, as well as its reversibility and future trends (Skakkebaek et al. 2019).

Is there a chance that this decline would lead to the extinction of the human species? Given the extinction of multiple species, often associated with man-made environmental disruption, although unlikely, this is a possibility we must address. While the exact causes for the sperm count decline deserve further investigation, the United Nations noted the sperm count decline could be seen as a sign for the existential nature of our own toxification (www.ohchr.org/EN/NewsEvents/Pages/DisplayNews.aspx?NewsID=25232&LangID=E).

Our efforts should be, not primarily be focused on predicting the future, but on shaping it. The immediate need is a concentrated effort to understand the complex causes of human sperm count decline and its implications. Most importantly, we need to identify the concrete steps needed on both the global and local level, to target the root causes of the decline, reverse this trend, and secure our future. We must act now. With the implications of sperm disruption for future generations, these changes may well be irreversible. When the extinction of the human species is at stake can we really take the risk?

Conflict of Interest None.

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Is Decreasing Sperm Concentrations a Sign of a General Decay in Fertility Potential?

Lars Björndahl

Annoying Facts

Reports on imminent or ongoing catastrophes tend to rapidly attain general attention. Without diminishing the gravity of signs of severe changes in the global male reproductive potential, it is essential that the scientific community continuously consider and evaluate the underlying evidence and reasonable alternative explanations. It is also essential to keep in mind that statistical methods do not prove the existence of true differences or relations. The statistical tests only estimate the probability that random variability has caused the observed data: if the probability for random distribution of results to cause the found results is less than 5% it is usually considered statistically significant. Still, in 5 out of 100 random instances results are outside the "reference range." Furthermore, a reference limit based only on the distribution of results from healthy volunteers (or men with proven fertility) does not represent a limit between normal fertility and subfertility. The distribution of results from fertile men does not disclose anything about the distribution of results from subfertile men.

This chapter is dedicated to elucidating both factors that could constitute alternative explanations to a real decline in human sperm production and factors that could be possible contributors to a true general decline in human sperm production.

Among factors and facts to be considered when evaluating a possible decline in sperm counts is also the meaning of "semen quality," sperm production, sperm transport, ejaculation, and laboratory techniques. Without taking sufficient attention to such aspects, the risk for mistaken conclusions increases—both for false premonitions and false dismissals of true threats to male reproductive potential.

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What Is Semen Quality?

A very common simplification used in publications investigating male factors and semen analysis results in relation to fertility is the ambiguous term "semen quality." The meaning of "quality" is a distinguishing characteristic. However, sperm concentration or total sperm count cannot be said to distinguishing factor for a man's fertility potential in the entire range of results. It is true that men with few spermatozoa are less probable to contribute to a "spontaneous" pregnancy (Björndahl 2011), but no exact limit exists. For sperm numbers above, for example, 40 million, there is no direct relation between fertility and sperm number. For instance, 150 million sperm in the ejaculate does not mean a doubled fertility capacity compared to an ejaculate containing 75 million spermatozoa. The WHO semen manual (Cooper et al. 2010) is often misunderstood concerning the distributions of results and assumed reference limits: the distributions of results from fertile volunteers (in male contraceptive studies) only show those distributions, not limits that distinguish between subfertile men from fertile men.

Another misconception is the overuse of sperm concentration as a measure of quality, ignoring that this measure depends on two mainly independent functions: on one hand the production and transport of spermatozoa to the urethra and on the other the secretory function of primarily the seminal vesicles and the prostate. Variation in these functions can be considerable and if sperm production is the primary interest, the total number of spermatozoa is a better measure than the concentration.

Sperm Production

The starting point of spermatogenesis is related to the onset of puberty leading to gonadotrophins release from the pituitary causing high intratesticular androgen levels and the development of immature testicular "strings" into testicular tubuli seminiferi containing germ cells in different stages of development and "nursing" Sertoli cells. Once started, the process with high mitotic activity of spermatogonia and subsequent sperm production appears to continue without major changes even into high age provided the intratesticular androgen levels remain high. After several generations of mitotic divisions, some spermatogonia enter spermatogenesis by entering the meiotic division, where finally four gametes are formed from each immature germ cell. The quantity of sperm production is largely dependent on the number of stem cells and the number of generations of mitotic divisions—two factors that can be assumed to be genetically controlled. Based on the estimation of a magnitude of 50–100 million spermatozoa formed each 24 h, several hundreds of germ cell mitoses would occur every second. This would explain the wide range of sperm numbers that is considered as a sign of normal fertility potential.

From this point of view, possible factors threatening male reproductive potential would be interaction with the genetic control of spermatogenesis as well as normal pituitary and testicular endocrine functions (including use of anabolic steroids or dietary supplements with similar additions as well as hormone receptor mutations).

Sperm production being continuous requires a functional storage function which is believed to be one main function of the epididymides. The reservoir capacity is however, compared to other animals, limited. A maximum of 2 days of testicular sperm production is estimated to be stored in the cauda epididymis (Bedford 1994). Studies aiming at establishing baseline sperm production indicate that a maximum abstinence time ideally should be 42–54 h (at a maximum up to 60 h), corresponding to 1.75–2.25 days (or a maximum of 2.5 days), if the man has ejaculated every 42–60 h the week preceding ejaculate collection (Amann and Chapman 2009). Ignoring this could lead to difficulties establishing both the true baseline for sperm production and therefore create difficulties determining the effects of interventions like hormonal contraception (Behre et al. 2016).

Ejaculation

The process of sending spermatozoa to the gametes of another individual (i.e., oocytes) is a quite intricate and complicated function. Compared to the situation in water-living animals, mammals like humans have adapted to internal fertilization and fetal development. The process of ejaculations is effectuated by autonomous nerve signals stimulating secretomotor activity in accessory sex glands and contractions of smooth muscle cells in caudal epididymides, vasa deferentia, prostatic land acini, and seminal vesicles. Due to differences in the relation between lumen diameter, the thickness of smooth muscle tissue (wall thickness), and viscosity of fluids, the normal sequence of ejaculation is that spermatozoa are mixed with prostatic fluid and expelled before the emptying of the seminal vesicles (Fig. 1) (Björndahl

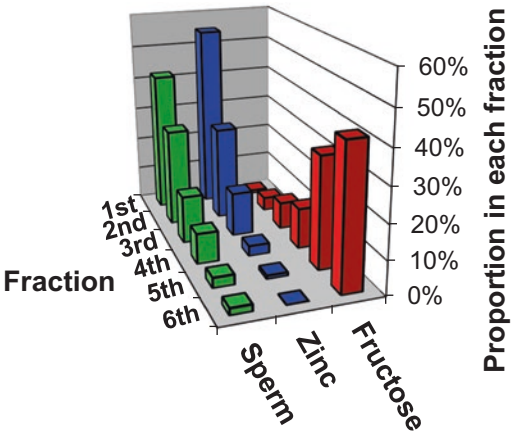

Fig. 1 Schematic representation of the relative contribution of spermatozoa (green), prostatic fluid (zinc, blue), and seminal vesicular fluid (fructose; red) in an ejaculate collected with the six ejaculate fractions separate (split-ejaculate technique) (Björndahl and Kvist 2003)

and Kvist 2003). In the natural situation, this is to the advantage of the spermatozoa, since the seminal vesicular fluid, in general, has a mainly negative impact on sperm function (Lindholmer 1973) and sperm DNA protection by chromatin stability (Björndahl and Kvist 1990). The quality of the ejaculation is apparently dependent on the duration and quality of sexual arousal (Pound et al. 2002).

The expulsion of the ejaculate is largely dependent on contractions of the striated muscle tissue surrounding the corpora cavernosa and thereby increasing the pressure in the urethra. The rhythmical contractions of the striated muscle tissue are also dependent on a proper erectile function to increase the urethra pressure and thereby execute an efficient ejaculation.

Any pharmaceutical treatment able to interact with autonomous nerve activity, smooth muscle, or striated muscle contraction is therefore likely to interfere with normal ejaculation. Psychoactive drugs like serotonin uptake blockers are suspected to be able to interact with normal ejaculation, as is antihypertensive drugs like α_1-adrenoreceptor blockers (Doxazosine) that cannot only increase the risk for retrograde ejaculation but also interfere with sperm transport and emptying of accessory sex glands leading to azoospermic ejaculation or even "dry ejaculation" (orgasm without antegrade ejaculation, often mistaken for retrograde ejaculation if the presence of sperm in postorgasmic urine has not been investigated). Peripheral nerve damage, not uncommon in poorly controlled diabetes mellitus, is also a possible cause for interference with normal ejaculation.

Laboratory Techniques

It is a strength of the recent study indicating a temporal decline in sperm numbers that attempts have been done to eliminate publications based on substandard laboratory techniques by only including studies based on haemocytometer measurements of sperm numbers (Levine et al. 2017). By doing this, a number of common sources of errors are minimized. Studies using low volume chambers have a higher risk for using subsampling with aliquots with poor representativity of the entire ejaculate. Furthermore, counting still motile sperm in undiluted ejaculates also increases the risk for technical errors in assessing the sperm number, and counting less than 400 spermatozoa increases the influence of random errors to result in errors in the results (Fig. 2). To reduce the influence of random errors it is essential to compare replicate counting of at least 200 spermatozoa (Björndahl et al. 2016).

With regards to the assessment of sperm motility, the assessment of progressive motility, a controlled and constant temperature is important. Room temperature is not a defined temperature and within the usual range of temperatures expected to be that temperature, the velocity of motile spermatozoa will vary. The recommendation is therefore to have a controlled temperature close to 37 °C.

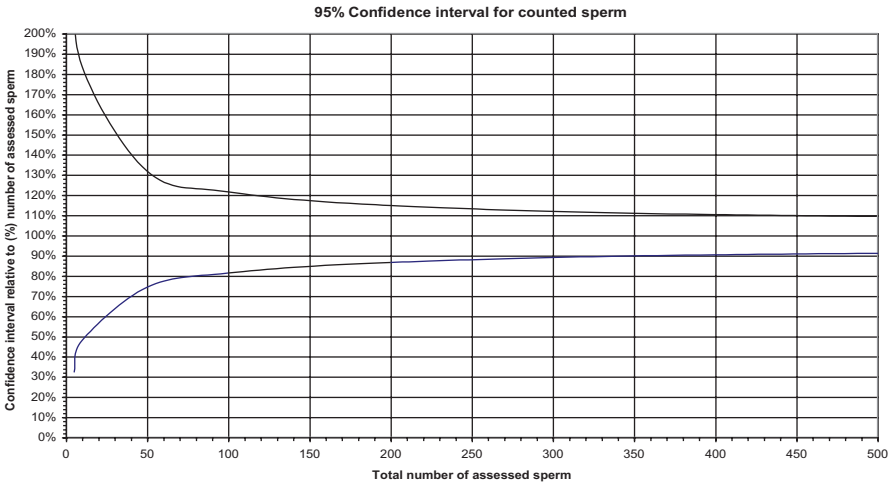

Fig. 2 Graph showing the uncertainty in results (95% Confidence Interval) depending on the total number of observations (spermatozoa counted) to obtain the result

Discussion and Conclusions

The ideal studies for global assessment of a possible decline in sperm numbers would be studies where investigated samples have been collected after 3 days of daily ejaculations to exhaust the epididymal sperm storages. Without doing that the obtained sperm numbers are not directly related to sperm production but a combination of the number or stored spermatozoa and the daily production. The time for sexual abstinence cannot replace systematic exhaustion of epididymal sperm storages, and mathematical adjustment of sperm counts based on sexual abstinence before sample collection cannot do that either. Furthermore, the mathematical adjustment is based on an average with uncertainty, and the adjusted value then is burdened with both the basic uncertainty augmented with the added uncertainty due to the variation in the adjustment.

Furthermore, what does it mean that the average number of sperm has decreased among investigated men? Since all included studies are based on investigations of men recruited under different circumstances from different populations, there is a possibility that variation in recruitment or population may be a contributing factor to the observed differences. There could also be a shift in participants' frequency of ejaculations before the investigated ejaculate, irrespective of the "days of abstinence" before the collection of the investigated ejaculate.

Few studies have followed the same men. There are indications that men without fertility problems may retain sperm production and functions better than men with fertility problems (Björndahl 2013). Following the same cohort of men could indicate age-related decline while examining birth-cohorts of men would be better to unravel temporal changes based on year of birth.

Although studies going back to early 1990s have indicated a possible ongoing decline in sperm production (Carlsen et al. 1992) contemporaneous studies of population fertility (based on Time-To-Pregnancy) have indicated that at that time a decrease in sperm number had not yet had an impact on the population fertility (Akre et al. 1999; Scheike et al. 2008).

From a global perspective, a decline was apparent in Western countries. In other parts of the world, a similar pattern of decline could not be shown. An important aspect of the latter is, of course, a lack of published investigations over time to the same extent as in Western countries. However, it still raises questions if a decline is mainly a problem in Western countries—or if it is a matter of changes of ethnical/genetical contributions to investigated populations in these countries. There is a general lack of knowledge if there are genetic differences (e.g., testicular size and thereby capacity for spermatogenesis) in different parts of the world, or if other factors like nutrition and exposure to gonadotoxic substances and pharmaceuticals may contribute both to differences and decline.

In conclusion, a general decline has been found in ejaculates examined in Western countries. If this is due to a true decline leading to a decline in fertility potential remains to be proven. To do that, more focused studies are needed, with a design to clearly define recruitment and status of participants, minimize bias by variability in sperm storage, and laboratory techniques.

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Environmental Toxicants and Sperm Production in Men and Animals

C. de Jager, S. M. Patrick, N. H. Aneck-Hahn, and M. S. Bornman

Introduction

Endocrine disrupting chemicals (EDCs) are ubiquitous in the environment and have the ability to interfere with, amongst others, hormone-dependent physiological processes through the interaction with hormone receptors (Bergman et al. 2013). Thus, EDCs can bind to receptors and elicit direct or indirect agonist or antagonist action, binding onto allosteric sites to yield unfavourable outcomes even at very low concentrations and hindering hormone production, transport, metabolism and degradation (Zoeller et al. 2012). Numerous studies are focused on the effects that both acute and chronic exposure to EDCs may have on humans and wildlife. These studies have shown that exposure to EDCs potentially impacts the growth and development of various bodily organs, bodily processes and fertility (Bornman et al. 2010; Colborn et al. 1993). Exposures to various EDCs have been reported to influence embryonic development, especially at the androgen-sensitive sex-determining programming windows during early gestation (Sharpe 2009). Changes in endogenous hormone regulation during embryonic development may result in impaired functioning of bodily systems, such as the male urogenital system (Schug et al. 2011). The impact of embryonic exposure may be identifiable at birth, as in the case of

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urogenital abnormalities, or later in adult life, such as poor semen quality or other reproductive disorders such as tumors. Humans are exposed to numerous known EDCs including polychlorinated biphenyls (PCBs), bisphenol A (BPA), used in the production of some plastics, and insecticides used for vector control (Diamanti-Kandarakis et al. 2009).

In 2018, the World Health Organization (WHO) estimated that 228 million cases of malaria occurred worldwide, with 93% of these malaria-related deaths occurring within the African region (WHO 2019). Although faced with climates conducive for malaria transmission and being susceptible to cross border transmission, South Africa (SA) has been able to control the spread of malaria through various initiatives since the 1930s and now malaria is currently endemic in three provinces, KwaZulu-Natal, Mpumalanga and Limpopo (Raman et al. 2016). The Vhembe district in the Limpopo province has consistently reported the highest malaria incidence rates in SA and vector control measures are essential to alleviate the malaria burden.

In 2002, SA ratified the Stockholm Convention and is therefore permitted to use organochlorines 1,1,1-trichloro-2,2-bis(4-chlorophenyl) ethane (DDT) and pyrethroids such as deltamethrin and cypermethrin DDT for malaria vector control (Bouwman 2004) through IRS programs (WHO 2006). The major concern with IRS programs stems from incorrect storage, application and contamination of the surrounding areas (Van Dyk et al. 2010) which poses a health concern for both animals and humans. In light of the use of DDT in IRS, the main exposure routes in humans include inhalation and ingestion of contaminated food and water sources (Van Dyk et al. 2010). Technical-grade DDT consisting of 65–80% of the active insecticidal ingredient *p*,*p*′-DDT and 15–21% of the less insecticidal *o*,*p*′-DDT (Turusov et al. 2002) is used for IRS. Both *o*,*p*′-DDT and to a lesser extent *p*,*p*′-DDT component have estrogenic properties (ASTDR 2002). Dietary and environmental exposures to *p*,*p*′-DDT and its metabolites result in bio-accumulation of these chemicals in adipose tissue and serum in the human body (Kirman et al. 2011). The DDT from the circulation is metabolized into 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene (*p*,*p*′-DDE) which is the persistent metabolite that bio-accumulates in fatty tissue. DDT and *p*,*p*′-DDE have the ability to cross the placenta with concentrations in cord blood being similar to concentrations in maternal blood (ASTDR 2002). *p*,*p*′-DDE is a potent inhibitor of androgen binding to the androgen receptor (Danzo 1997), androgen-induced transcriptional activity and androgen action in males during development and in adulthood (Kelce et al. 1995). This suggests that abnormalities in male sex development induced by *p*,*p*′-DDE may be mediated at the level of the androgen receptor (Kelce et al. 1995).

Genes dependent on androgens are subject to altered expression as a result of the competitive agonist behaviour of *p*,*p*′-DDE (Phillips and Tanphaichitr 2008). It has been reported that foetal, neonatal or pubertal exposure to DDT/DDE may result in impaired reproductive function as well as semen and sperm quality (Martenies and Perry 2013). Various studies have investigated associations between serum concentration levels of DDT and DDE, on semen and sperm quality parameters including sperm count, morphology, motility, concentration and semen appearance, volume,

pH (Aneck-Hahn et al. 2007). Although studies have yielded inconsistent results, there is growing evidence of the negative impact DDT/DDE exposure has on male reproductive function.

While exposure to EDCs have been associated with adverse effects on semen and sperm quality very few studies have focused on the effects of EDCs on sperm chromatin integrity. Sperm chromatin integrity is imperative for the successful transfer of paternal genetic information from one generation to the next (Sadeghi et al. 2009). The sperm chromatin structure assay (SCSA) evaluates DNA fragmentation as a result of low pH treatment (Evenson 2016). and provides two valuable results, DNA fragmentation index (DFI) and High DNA stainability (HDS), both measures are indicators of abnormal chromatin indices (Spano et al. 2005). DFI provides a measure of damaged DNA and HDS measures impaired chromatin condensation (Evenson 2016; Spano et al. 2005).

A limited number of studies have focused on the effects of EDCs on male reproductive parameters, hormonal disruption and sperm chromatin integrity, which is essential for the optimal pre-conception environment. Thus, the aim of the study was to investigate the impact of exposure to a complex mixture of EDCs, DDT pyrethroids and other agricultural pesticides, on seminal parameters, hormonal regulation and sperm chromatin integrity.

Methods

Study Design and Population

A cross-sectional study was conducted between 2003 and 2008 ($n = 544$, from three DDT-sprayed villages—$n = 310$, three non-DDT sprayed villages—$n = 234$) and 2012–2017 ($n = 431$ from three DDT-exposed—$n = 236$; three non-DDT exposed—$n = 195$). The Limpopo Province is situated in the north-eastern corner of South Africa and is divided into five districts, including the Vhembe District. The Vhembe district is a malaria-endemic area where housing comprises traditional mud dwellings with thatch (straw grass) roofs or brick and cement houses.

Recruitment and Sampling

The participants were from rural IRS villages or nearby non-IRS villages in the Thulamela Local Municipality recruited from 2003–2008 to 2012–2017. Participants from sprayed and non-sprayed villages volunteered, but we excluded men who had lived in study villages for less than a year, those younger than 18 or older than 40 years, those with neuropsychiatric disorders or those who appeared intoxicated. All participants provided informed consent and were interviewed using a structured questionnaire, which detailed their general history, personal use of insecticides,

diet, smoking and drinking habits, illegal substance use, exposure to other insecticides and fertility history. Physical measurements included participants' weight and height and the body mass index (BMI) was calculated.

Exposure Assessment

We used a Shimadzu GCMS-QP2010 gas chromatograph/mass spectrometer to measure 1,1,1-trichloro-2,2′bis(*p*-chlorophenyl)ethane (DDT) and 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) as reported (Aneck-Hahn et al. 2007). We could not detect *o*,*p*′-DDD, *p*,*p*′-DDD, *o*,*p*′-DDE in 66–75% of the men, and this report focuses, therefore, on DDT and DDE concentrations. Total cholesterol and triglycerides were determined by enzymatic methods (Aneck-Hahn et al. 2007) and the total plasma lipid level was calculated according to Rylander et al. (2006). The lower limit of detection (LOD) for DDT and DDE was 0.02 μg/g lipid.

Biochemical Analysis

We collected venous blood samples from participants between 08:00 and 10:00. The samples were centrifuged at 670 × *g* for 10 min at room temperature and stored in 500 μL aliquots at −20 °C on-site and during transport. At the University of Pretoria laboratory, samples were stored at −80 °C until analyzed. Hormones measured, in serum with the Cobas® 6000 analyser (Roche Products (Pty) Ltd Diagnostics Division) using ECL (ElectroChemi-Luminescence) immunometric detection, were: total testosterone (t-T; 05200067160) and human sex hormone-binding globulin (SHBG; 03052001160). We measured serum albumin on the general automated platform to calculate bioavailable testosterone (b-T) and free testosterone (f-T) using the calculator available at http://www.issam.ch/freetesto.html, following Vermeulen's formula.

Semen Analysis

Semen samples were collected into a sterile wide-mouthed container after the prescribed 72 h sexual abstinence period. The semen sample was incubated at 37 °C until liquefied and semen analysis and additional andrological tests were conducted following the World Health Organization's (WHO) 1999 standards and procedures (WHO 1999) while adhering to the quality control procedures of the European Society of Human Reproduction and Embryology (ESHRE 1998). After liquefaction, semen characteristics including appearance, ejaculate volume and semen pH were assessed (Mortimer 1994). Sperm motility was assessed on a wet preparation

following WHO (1999) motility classification using the classes "a" through "d" sperm progression rating, where "a" indicates rapid progressive motility and "d" indicates immotile sperm (NAFA 2002). Sperm morphology slides were stained by the Papanicolau method and scored according to the WHO (1999) classification.

Sperm Chromatin Structure Assay

Samples with concentrations of sperm greater than 20×10^6 cells/mL were used to conduct the SCSA, using previously described methods (Evenson and Jost 2000). A BD Accuri C6 Plus™ (Beckton Dickinson, New Jersey, USA), fitted with an air-cooled argon ion laser and standard optical filters to collect red and green fluorescence, was used for flow cytometry analysis. Prior to any analysis on each day, BD CS&T RUO beads (BD, Gauteng, SA), dyed with fluorochromes, were used to calibrate the instrument and the software. To visualize the analysed sample, a forward scatter (FSC) and side scatter (SSC) dot plot was generated with a linear scale. The FSC axis provided a measure of the size of the cell and the SSC axis provided a measure of the complexity of the cell. Flow cytometric data were analysed off-line using the FlowJo version 10 (FlowJo, LLC, Ashland, Oregon, USA). The DFI frequency histogram was calculated using the ratio between the red and total (red plus green) fluorescence. The DFI histogram provided the percentage of damaged sperm with detectable DFI (%DFI). High DNA sustainability (%HDS) was determined using the percentage of sperm with high levels of green fluorescence.

Animal Study

The animal study aimed to investigate the effects of in utero-, lactational- and direct exposure to select EDCs, found in a malaria area, in South Africa, on male reproductive health and parameters, testicular histology, associated hormonal changes and apoptosis in Sprague-Dawley rats. The study design was based on the Organization for Economic Cooperation and Development (OECD) One-generation reproductive toxicity study 415 protocol (OECD 1983). Pregnant females (P1) were randomly allocated to the four experimental groups and dosed with either cottonseed oil, DDT, DDE or a mixture of EDCs throughout the duration of their gestation and lactation period. The P1 females in each group were continually dosed with either cottonseed oil, DDT, DDE or a mixture of EDCs throughout the pregnancy. Following birth, the P1 females were dosed during the lactation period of 3 weeks. Thus, the pups (F generation) were indirectly exposed to the EDCs during lactation. Following the lactation period, the male (F1) pups from each of the experimental groups were kept in their respective groups. The F1 pups were directly dosed daily for 10 weeks until reaching sexual maturity at 13 weeks of age. The anogenital distance, which is the length of the perineum from the base of the genital tubercle to

the center of the anus (Fielden et al. 2002), was measured and recorded. Body weight was recorded and the testes, seminal vesicles, the right epididymis, prostate and the liver were removed and weighed. Any macroscopic abnormalities were recorded before organs were fixed in the relevant fixatives for further analysis. The left epididymis was used for determining the epididymal sperm count. Blood was used to determine total testosterone.

Statistical Analysis

To determine median differences between measured variables in IRS and unsprayed groups, Wilcoxon rank sum tests were used. General linear models were used to determine associations between exposures to malaria vector control pesticides *p*,*p*′-DDT, *p*,*p*′-DDE and pyrethroids and SCSA parameters. Logistic regressions tested for relationships between hormone concentrations (response variables) and DDT and DDE concentration (predictor variables) expressed as a dichotomous variable (uptake vs. no uptake) and as a categorical variable as described earlier. Multivariate logistic models were adjusted for age, BMI, personal use of other insecticides, and smoking. F1 males from the same litter share a common mother, a P1 female, and hence data analysis employed the survey command in STATA 12 (StataCorp, TX, USA) to deal with the dependence of data within litters (i.e. clusters). In total, 16 clusters of F1 males were analyzed using Survey Linear Regression. The exposed groups (groups B–D) were compared to the control group (Group A), at the 0.05 level of significance. Additionally, group B was compared to group D to assess the possible effect of exposure to a single chemical compared to exposure to the same chemical in a mixture. Furthermore, differences among the exposed groups were assessed using the adjusted Wald Test at the 0.05 level of significance. Groups were compared with respect to endpoint values of the study parameters. In the analysis of the endpoint AGD, the value at baseline and the body weight was adjusted for.

Results and Discussion

Changes in DDT/DDE Concentrations

In sprayed villages *p*,*p*′-DDE lipi adjusted exposure levels were significantly lower between 2012 and 2017 (mean ± SD: 5.80 ± 6.6 μg/g) compared to the 2003–2008 (216.9 ± 210.6 μg/g) period ($P < 0.001$). In the non-sprayed villages *p*,*p*′-DDE exposure levels were significantly lower between 2012 and 2017 (mean ± SD: 1.47 ± 3.68 μg/g) compared to the 2003–2008 (2.81 ± 4.26 μg/g) period ($P < 0.001$) (Table 1). The levels of DDT in the study population in the 2003–2008 sampling period was 3.5times higher than occupational spray workers in Limpopo, South

Table 1 Changes in DDT/DDE concentrations in non-sprayed versus sprayed villages in Limpopo Province, South Africa

	Non-sprayed villages		Sprayed villages	
	p′,p′-DDT	*p′,p′*-DDE	*p′,p′*-DDT	*p′*,p-DDE
2003–2008	2.9 (9.3)	2.8 (4.3)	90.2 (102.5)	216.9 (10.6)
2012–2017	0.21 (0.45)	1.5 (2.8)	1.8 (3.8)	6.3 (6.8)

Africa (Dalvie et al. 2004) and 5times higher where DDT was phased out in Chiapas, Mexico (de Jager et al. 2006a, b). Despite the lower exposure levels in the 2012–2017 cohort compared to the 2003–2008 cohort, significant effects were seen on seminal parameters (sperm viability, morphology and counts) when comparing the DDT-sprayed and non-sprayed villages. Changes in the spray program are evident in the lower levels of DDT/DDE found in the 2012–2017 group. This change can be as a result of various factors, for example, the severity duration of a malaria outbreak. The type of housing in the villages has changed from the traditional thatch and clay to brick houses. DDT is sprayed on traditional houses while pyrethroids are sprayed on brick houses for malaria vector control. With the fluctuation in malaria cases in South Africa, mainly attributed to changes in rainfall and cross-border movement (WHO 2019), IRS spray patterns have changed. We anticipate that the sudden increase in malaria cases from 2016 (4323) to 2017 (22,061) (WHO 2019) may result in the IRS vector control strategies being intensified.

Hormonal Changes in Young Men

Crude linear regression results comparing the highest category of exposure to the lowest three showed that men whose DDE concentrations were in the highest category (173–997 μg/g lipid), had mean total testosterone concentrations that were 4.8 (CI 95% 3.3, 6.3) nmol/L higher than men in the three lower categories (BDL—172 μg/g lipid). Likewise, men whose DDT concentrations were in the highest category, (77–519 μg/g lipid), had mean total testosterone concentrations, 5.9 (CI 95% 4.4, 7.4) nmol/L higher than men in the three lower categories ("BDL" – 172 μg/g lipid). Similarly, men with DDE concentrations in the highest category had significantly higher log transformed estradiol concentrations, and lower log transformed FSH concentrations (Bornman et al. 2018). Furthermore, DDT and DDE exposure were significantly positively associated with total-, free and bioavailable testosterone, nonsignificantly associated with lower FSH and LH, whereas little effect was seen on estradiol. Low LH and FSH concentrations in the current study will result in impaired spermatogenesis. It seems possible that both androgen receptor antagonism by DDE and diminished LH and particularly FSH can account for the low sperm count and function (Moline et al. 2000; Mortimer et al. 2013). Sperm motility seemed to be additionally impaired by direct toxicity,

possibly due to DDE exposure representing a non-genomic mechanism (Tavares et al. 2015).

Urine Metabolites

Analysis showed that 3,5,6-trichloro-2-pyridinol (TCPY), 1,2,3-benzotriazine-4-one (BTA) a herbicide and 3-(2,2-dichlorорvinyl)-2,2-dimethyl-cyclopropane-1-carboxylic acid (Trans/DCCA) were the most common metabolites. Unlike DDT/DDE, pyrethroids are metabolized rapidly in the environment and have short half-lives (Saillenfait et al. 2015). Mammals metabolize pyrethroids through oxidation and ester hydrolysis and then by conjugation, which yields hydrophilic metabolites that are readily excreted in urine (Kaneko 2011). Consequently, the metabolite, 3-phenoxybenxoic acid (3-PBA), is estrogenic (Mnif et al. 2011) and is a common metabolite of deltamethrin, permethrin, fenvalerate, cypermethrin and cyhalothrin (Dalvie et al. 2004). Studies have determined possible associations between 3-PBA and decreased sperm concentration and motility (Martenies and Perry 2013; Meeker et al. 2008; Saillenfait et al. 2015). Furthermore, 3-PBA is associated with increased concentrations of follicle stimulating hormone (FSH), luteinizing hormone (LH) and decreased levels of inhibin B in men (Meeker et al. 2009), adversely affecting spermatogenesis.

SCSA

From the general linear model used to test associations between exposures to malaria vector control pesticides, an increased relative risk of increased %DFI was found when participants were exposed to *p′*,*p*-DDT and *p′*,*p*-DDE in the 20122016 sampling period. The findings, however, were not statistically significant. This finding follows a trend with the most recent study conducted in the Vhembe district, which found a significant positive association between exposure to *p′*,*p*-DDE and %DFI (de Jager et al. 2009). The lipid adjusted concentrations of both *p′*,*p*-DDT, and *p′*,*p*-DDT detected in participants from this study were significantly lower than concentrations found in other studies interested in similar outcomes. During the 2003–2009 sampling period, conducted in the Vhembe district, concentrations of 83.9 μg/g of DDT and 177.8 μg/g of DDE were found (de Jager et al. 2009). Other studies focusing on environmental exposures to DDT/DDE have reported lipid adjusted concentrations ranging from 41 μg/g of DDE, reported in Chiapas Mexico (de Jager et al. 2006a, b), to 134 μg/g and 46 μg/g of DDT and DDE respectively, in Limpopo (Aneck-Hahn et al. 2007). In the 2012–2016 study period, concentrations of DDT and DDE, were approximately 645 times lower (0.13 μg/g *p′*,*p*-DDT) and 78 times lower (2.26 μg/g *p′*,*p*-DDE) compared to the 2003–2009 sampling period (de Jager et al. 2009). The nonsignificant associations may be attributed to the

significantly lower DDT/DDE exposure levels found more recently. Although the effect of EDCs is thought to cause significant effects at low concentrations, the very low concentrations of DDT and DDE suggest that perhaps these pesticides have not been sprayed in the area as assumed. This may suggest that other pesticides are used, as the participants reported that their homes had been sprayed at least once in the past year. A study included participants from various European countries and failed to show an association between DDE and SCSA parameters, despite mean DDE concentrations of 790 ng/g (Spano et al. 2005). Suggesting that a focus should also be placed on determining the associations between pyrethroids and other EDCs as sperm DNA fragmentation.

Animal Study

The mean anogenital distance (AGD) was significantly shorter in the mixture group ($P = 0.005$) when compared to the controls. The AGD is a sensitive marker of prenatal disruption of the development of the male reproductive system (Swan et al. 2015). The AGD in males is longer than in females—generally double the distance in females measured in multiple mammalian species, thereby suggesting that the AGD is under the hormonal influence (Hsieh et al. 2008). The mixture group (group 4) received technical grade DDT, DM, *p*-NP and phytoestrogens, all of which have estrogenic properties. Synergistic activity between chemicals (Silva et al. 2002) could have further enhanced the total additive estrogenicity, resulting in a shorter AGD of the mixture group (group 4) (Patrick et al. 2016).

After prenatal exposure to the anti-androgen DDE, the mean AGD was also shorter, but not significantly. A shorter anogenital distance in males signifies feminizing changes (Palanza et al. 2001), which might be related to lower/impaired androgen function during the hormone-sensitive male programming window (Sharpe 2009). However, not only androgen plays an important role during masculinization, but an optimal androgen-estrogen balance is also involved. Maintaining the appropriate androgen–estrogen balance is crucial for the normal development of the structure and function of the male reproductive tract. Disruption of the balance during early fetal development may lead to abnormal development of the male reproductive tract (Rhind et al. 2001; Svechnikov et al. 2014). Although the shorter AGD may seem a 'minor' phenotypic variant of a normal male, the longer-term implications may be more serious.

The mean prostate mass was significantly higher in the DDT group (group 2; 1.02 g, $P = 0.018$), compared to the control group (group 1; 0.83 g). Technical grade DDT has estrogenic properties mainly due to the *o*,*p*′–DDT isomer (Metcalf 1995). Estrogens have direct effects on the adult prostate gland and have been implicated in the etiology of the prostatic disease (Sikka and Wang 2008). It seems plausible that exposure to both endogenous and exogenous estrogenic and/or anti-androgenic compounds could interfere with prostate growth (Boberg et al. 2015). Exposure to a

mixture of chemicals may exert an additive or a synergistic effect, thus, effects may exist on a histological level, which was not studied.

In the testes, testosterone plays a vital physiological role and is essential for normal spermatogenesis (Nieschlag and Behre 1998) as it promotes the differentiation of spermatogonia by stimulating genes within the Sertoli cells (Russell and Griswold 1993). The mean total testosterone concentrations were significantly higher in the DDE (group 3; 28.12 nmol/L, $P = 0.038$) and mixture (group 4; 28.62 nmol/L, $P = 0.023$ compared to the controls. These findings confirmed the previous findings of higher testosterone concentrations following exposure to 300 mg/kg *p,p′*-DDE for 15 days (O'Connor et al. 1999). In a study investigating hormonal changes associated with DDT uptake in men (Bornman et al. 2011), exposure to estrogenic- and anti-androgenic compounds increased steroid hormone binding globulin (SHBG), but it was not measured in the present study.

Where Do We Go from Here?

The concurrent exposure to both DDT and DDE seemed to result from estrogenic and/or anti-androgenic effects in men living in malaria areas. The high testosterone concentrations in both the human and animal models may result from DDE blocking the androgen receptor. Testosterone gives negative feedback to the pituitary, reducing LH and FSH secretion, which will impair testicular function. Despite the decline in exposure levels over time, seminal parameters and chromatin integrity were still affected. Utero or early life exposures, as seen in the animal model, may therefore adversely affect reproductive health, due to the potential synergistic and additive effects of known EDCs in malaria areas. While still dependent on DDT for malaria vector control, a more sustainable approach is needed towards malaria elimination, involving innovation, education, communication and health promotion strategies targeting affected populations.

The United Nations 17 Sustainable Development Goals (SDGs) (Morton et al. 2017) aim to enhance access to basic services, promote environmental sustainability and support inclusive growth. The third goal (SDG 3) aims to provide health and well-being for all and is a key factor in measuring the progress and success of the SDGs. More specifically, SDG3.3: which states that by 2030, amongst others, to end the epidemics of malaria and neglected tropical diseases. Although there are various malaria control strategies in place, it has become evident that integrative approaches are required to achieve the malaria elimination agenda. The University of Pretoria Institute for Sustainable Malaria Control's (UP ISMC) vision and mission is anchored in using an integrative transdisciplinary approach towards safe malaria control and elimination. Current initiatives include: safer alternatives (innovation)—the use of new materials and environmental friendly larviciding; development of study material (education)—Sibo Fights Malaria; launching a mobile application (communication)—Malaria Buddy and health promotion (music, drama and nutrition)—to communicate malaria messages and create awareness amongst affected communities,

these are all used as sustainable approaches towards malaria elimination. The UP ISMC is geared towards education, health promotion, communication and innovative high impact research, which are key to address the SDGs, including the elimination of malaria, while reducing reliance on potentially harmful EDCs (SDG3.9) and limiting the effect environmental toxicants have on sperm production in men and animals.

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Part III
Sperm DNA: Protection and Delivery of a Complete and Undamaged Genome

The purpose of the spermatozoon is to deliver half of the genome of a new individual to the oocyte. The DNA must be protected from harmful influences and still be able to deliver the undamaged DNA at the right time.

Post-testicular Sperm DNA Oxidation: What to Be Afraid of?

Joël R. Drevet

Normal embryo development, as well as the health of the progeny, is partly dependent on gamete nuclear integrity. If sperm DNA fragmentation that could be in part due to oxidative nuclear alterations is known to impact reproductive success, no one ever considers the impact of mild sperm DNA oxidative damage. To analyze this situation, we have developed mouse models that display some level of post-testicular sperm DNA oxidative damage. The data presented will first focus on why mammalian spermatozoa are susceptible to DNA oxidation, and where these oxidative alterations are located in the mouse sperm nucleus and chromosomes. Translation to the clinic will then be made with on the one hand the characterization of human sperm DNA susceptibility to oxidative damage and, on the other hand, the development of an assay allowing a reliable and accurate evaluation of oxidative DNA damage in human sperm samples. Data will also be presented showing how frequent this situation is in male patients having difficulty to conceive. In addition, preliminary data will show that besides base oxidative alterations the sperm epigenetic information (with a particular focus on sperm DNA methylation status and the small noncoding RNA sperm pool) may be affected by post-testicular oxidative stress.

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Sperm as a Possible Source of Transgenerational Epimutations and Genetic Instability

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For the past 25 years, male infertility has been successfully treated with intracytoplasmic sperm injection (ICSI), while diagnosis and aetiology of the underlying problem have not been completely understood. The origin for the poor spermatogenesis can be explained by anatomical anomalies (e.g. cryptorchidism), iatrogenic causes (e.g. chemotherapy), lifestyle factors (e.g. smoking/drugs) or genetic anomalies (e.g. Klinefelter, Y-chromosome AZF deletions, de novo mutations, etc.) but still in almost 60% of cases, infertility is categorized as unexplained. The percentage of genetic causes involved in male infertility increases with the severity of the oligospermia, been highest in azoospermia.

Interestingly, while infertility is not a heritable condition, the massive use of ART techniques has made it possible to pass (epi)genetic abnormalities to the next generations. The professionals working in ART should focus on those risk factors arisen by using "infertile sperm" to treat infertility. Various approaches and different focuses might contribute to unravel the whole picture.

Sperm is one of the most complex cell types; its maturation includes two waves of intrinsic double strands breaks (DSB): the first one during meiosis (which allows recombination to take place), and later again during spermiogenesis. Nuclear elongation during spermiogenesis is accompanied by the transition of chromatin from a nucleosome-based structure to a protamine-based structure, reducing the nuclear volume and increasing chromatin compaction. When sperm is passing from the testis to the epididymis, a further compaction of nuclear structure is achieved by oxidation of the cysteine-rich protamines (SH groups), mainly in the caput epididymis. The replacement of histones by protamines is less complete in the human (85%) compared to other mammals and even more so in infertile males with OAT.

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Chromatin remodelling is therefore a huge intrinsic source for DNA damage. During gametogenesis, extensive epigenetic reprogramming also takes place; evidence is arising showing that both these processes (repair of intrinsic DSB and epigenetic markers) are prone to error when environmental or lifestyle factors are involved. Failure of the proper repair of DSB might induce de novo mutations. These de novo mutations might occur randomly in our genome or clustered. The amount and location of these de novo mutations depend on aspects like age, the fitness of the individual, exposure to drugs, etc. When the mutated/damaged sperm is used to fertilize an oocyte, several outcomes are possible: poor embryo development, increased miscarriages or pregnancy and birth of children with (increased) de novo mutations. Again, depending on where the mutation has taken place (introns/exons) and the type of mutation is the chance of disease. The mode of inheritance (paternal/maternal) and the increased number of de novo mutations have been studied in a pilot study using exome sequencing on trio-based approach (both parents and children born from ICSI-TESE treatment). As sperm in non-obstructive azoospermia is probably the most compromised type of sperm to use in ICSI, this approach can give an insight into the risks of using "infertile sperm" in ART.

In recent years, the feasibility of approaches such as targeted disease gene panel sequencing, whole exome- and even whole-genome sequencing has dramatically increased the potential of genetic approaches in male infertility. An overview of the different novel genetic techniques (targeted gene sequencing, WES, WGS), and the advantages and disadvantages of each methodology are to be discussed. At the present clinical workout, exome and genome sequencing can be used to identify novel disease genes, whereas targeted gene sequencing is useful to follow-up novel candidate genes in large cohorts.

Why should we focus on the diagnosis of male infertility when ICSI is still successful even in azoospermia or in cases with 100% abnormal sperm morphology? Increasing the knowledge of the underlying anomalies in the spermatogenesis pathways will be useful (1) to avoid using sperm produced which is unable to produce a healthy/viable embryo, (2) to predict and prevent the risk of passing on the infertility to the next generation, and (3) to learn more about normal and abnormal human reproduction in general, which may aid in developing novel treatment strategies.

Complex Population of Chromatin-Associated Proteins Identified in Mature Sperm of the European Sea Bass (*Dicentrarchus labrax*) Through High-Throughput Proteomic Analysis

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Background: The sperm chromatin is condensed by protamines in all mammals and many additional species. Protamines are small and extremely positively charged proteins (50–70% arginine), which are known to streamline the sperm cell and protect its DNA. In addition, it has also been shown in mammals that 2–10% of its mature sperm chromatin is also associated with a complex population of histones and chromatin-associated proteins differentially distributed in the genome. These proteins are transferred to the oocyte upon fertilization and may be involved in the epigenetic marking of the paternal genome and embryo development. However, little information is so far available on the additional potential sperm chromatin proteins present in other protamine-containing nonmammalian vertebrates, based on high-throughput mass spectrometry protein identification strategies.

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Main Questions: To characterize the proteome of the mature sperm of the European sea bass (*Dicentrarchus labrax*), a modern teleost fish chosen as a representative of protamine-containing nonmammalian vertebrate species, in order to determine whether its sperm chromatin also contains a complex population of histones and chromatin-associated proteins, similarly as it has been reported in mammals and some non-vertebrate species.

Experimental Design: Proteins were extracted from purified sperm cells and purified sperm nuclei and digested with trypsin. The peptides were separated using liquid chromatography (LC) and identified through tandem mass spectrometry (MS/MS). The criteria used for protein identification were ≥ 1 peptide per protein (at least one unique peptide) and an FDR of 1%.

Main Results: A total of 300 proteins have been identified. Of interest, 105 chromatin-associated proteins, of which nine are histone or histone variants, have been detected, in addition to the protamines. These results provide phylogenetically strategic information, indicating that the coexistence of histones, additional chromatin-associated proteins, and protamines in mature sperm is not exclusive of mammalian vertebrates, but it is also present in other protamine-containing vertebrate species. Additional novel aspects of the sperm structure and function are also identified through the comparative analysis of this sperm proteome together with that of other species.

Conclusions: The coexistence of histones and other chromatin-associated proteins in addition to protamines in the *D. labrax* mature sperm chromatin indicates that this complex composition is more conserved in vertebrates than previously thought. The availability of the sperm proteome also contributes to further functional insights of this cell.

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Does Cinnamtannin B-1 Protect or Destabilize Sperm DNA? Contradictory Results of SCSA® and TUNEL

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Background: Oxidative stress is known to interfere with the fertilization capacity of spermatozoa damaging sperm nuclear DNA and affecting the epigenetic profile of these cells. Cinnamtannin B-1 (CINB-1) is a naturally occurring A-type proanthocyanidin found in a limited number of plants including *Linderae umbellateae* and *L. nobilis*, which exhibit antioxidant properties. It has been proven its DNA protection by the Terminal dUTP Nick-End Labeling assay (TUNEL). The objective of this study is to evaluate the CINB-1 sperm DNA protection with the Sperm Cromatine Structure Assay (SCSA®) to evaluate the fragmentation after the oxidative stress produced by incubation at 37 °C (4 h).

Main questions: Does CINB-1 protects or destabilize sperm DNA?

Experimental Design: Ninety samples were evaluated from 15 sperm donors. Sperm samples were collected with a period of abstinence between 48 and 72 h and were incubated at 37 °C for 4 h with 0, 10, and 100 μM of CINB-1. DNA integrity was checked by measuring the index of sperm DNA fragmentation (DFI), for which TUNEL assay and the SCSA® were used.

Main Results: The TUNEL assay found significant differences when comparing the samples with CINB-1 with the control at 4 h, observing a positive effect when decreasing the percentage of DFI (Control 4 h 15.12 ± 1.15; 10 μM 10.08 ± 0.93; 100 μM 9.44 ± 0.74; ($p < 0.001$)). The SCSA® showed significant differences

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($p < 0.001$) between control and CINB-1 treatments (Control 0 h 21.91 ± 3.03; Control 4 h 18.69 ± 3.10; 10 μM 66.38 ± 9.38; 100 μM 92.85 ± 5.18 ($p < 0.001$)). We observe how the CINB-1 exerts a negative effect on the sperm, the greater the concentration of CNB-1, the greater the DFI.

Conclusions: The SCSA® and TUNEL techniques offer contradictory results regarding the protection of CINB-1 in fresh human semen samples. As other authors have already pointed out, it is probably that the TUNEL assay is a very insensitive methodology for assessing DNA damage in spermatozoa. This insensitivity has been related to the truncated base excision repair pathway in spermatozoa, lacking the abasic site endonuclease. These cells cannot create the 3′-OH termini that are required by the TUNEL assay.

Peripubertal Serum Dioxin Concentrations and Sperm Methylome of Young Russian Adults

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Background: Animal data have demonstrated that the sperm methylome is sensitive to endocrine disrupting compounds (EDCs) at specific windows of development; however, the association of exposure to EDC in the peripubertal period with sperm DNA methylation in human or animals is unknown.

Main Questions: Are peripubertal serum dioxin concentrations associated with genome-wide profiles of DNA methylation in sperm collected in young adulthood?

Design: The Russian Children's Study is a prospective cohort of 516 boys who were enrolled at 8–9 years of age and provided semen samples at 18–19 years of age. At enrollment, serum dioxins, including the potent dioxin congener, 2,3,7,8-te trachlorodibenzo-*p*-dioxin (TCDD), were measured. Whole genome bisulfite sequencing of sperm was conducted to identify differentially methylated regions (DMR) between highest (n = 4, median serum TCDD 10.4 pg/g lipid) and lowest (n = 4, median serum TCDD 0.39 pg/g lipid) peripubertal TCDD groups.

Main Results: The mean methylation across all CpG sites was significantly lower in the highest peripubertal serum TCDD group compared to the lowest group (49.1 ± 4.9% and 61.9 ± 5.2%, respectively; t-test, p = 0.01). We found 52 DMRs that distinguished two serum TCDD groups. One of the top scoring networks, "Cellular Assembly and Organization, Cellular Function and Maintenance, Carbohydrate Metabolism," identified estrogen receptor alpha (ESR1) as its central regulator.

Conclusions: Findings from our limited sample size suggests that peripubertal environmental exposures are associated with sperm DNA methylation in young adults. Funds: RSF #14-45-00065, parental study - US NIH #R01 ES014370

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Sperm DNA Fragmentation in Human Split Ejaculates

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Background: Standard semen evaluation according to WHO thresholds (2010) is still insufficient to disclose individuals with a diagnosis of idiopathic infertility. Sperm DNA fragmentation is considered a possible valuable marker, and flow-cytometry-based Sperm Chromatin Structure Assay (SCSA) analysis has been used. However, the method is cumbersome and time-consuming; hardly applicable in a clinical setting where split ejaculates are used for diagnostics and intervention. The present study explores an alternative method using conventional bright-field microscopy (HalospermG2, Halothech, Madrid, Spain) with Sperm DNA fragmentation (SDF) as end values. Analyses were done on split ejaculates (F1, sperm-rich fraction, mainly prostate-dominated versus F2, sperm-poor fraction, mainly seminal vesicle-dominated) of documented fertile donors and partners in a couple seeking for ART-intervention with a clinical diagnosis of idiopathic infertility (patients).

Main Question: Can DNA-fragmentation analysis by HalospermG2 be applied in a clinical setting for an idiopathic infertility survey?

Experimental Design: Consensual fertile semen donors (n = 14) and patients (n = 6) attending clinical setting (Reproduction Medicine Centre (RMC), Linköping, Sweden). Ejaculates were collected by masturbation in two consecutive fractions (sperm-rich F1 and sperm-poor F2). Samples were evaluated for volume, sperm concentration (manual counting), and motility (subjective) following WHO-criteria and also for proportions of DNA fragmentation (SDF) using a commercially available kit (HalospermG2; Halotech DNA, Madrid, Spain). Statistical analysis was performed in the R statistical environment (ISBN 3-900051-07-0). Data are expressed as means ± SD, (range).

Main Results: The restricted number of individuals hereby explored yield no differences between donors (SDF (%): F1, 19.8 ± 9.4 (7.87–42.15); F2, 23.2 ± 13.4

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(5.95–50.68)) (means ± SD, ranges between brackets) and the patients (SDF (%): F1, 17.7 ± 6.6 (9.66–28.98); F2, 20.8 ± 10.9 (10.60–43.18)), which was extensive to the fraction where spermatozoa were fortuitously recovered from. However, there was a wider range for F1 than for F2.

Conclusions: DNA fragmentation analysis using the HalospermG2 kit can be easily performed in an ART setting. HalospermG2 calculates a global ratio from the fragmented-degraded sperm divided by the total of spermatozoa counted. The limited number of individuals diagnosed with idiopathic infertility makes us refrain from other conclusions, but values are similar to data emanated from SCSA (Sperm Chromatin Stability Analysis) using flow cytometry on spermatozoa from the same individuals (Alvarez-Rodriguez et al. 2016), considering SCSA has an inbuilt threshold in the resulting data (a DNA-fragmentation index (DFI) lower than 25% is considered normal).

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Part IV
Sperm Competition, Evolution and Sperm-Egg Interaction

One aspect of spermatozoa important to sperm development in many species is sperm competition. Once at the site of fertilization the spermatozoon must find the key to the fertilization lock.

The Sexual Cascade: Evolutionary Dynamics of Sperm Competition

Geoff A. Parker

We currently classify sexual selection into pre- and post-copulatory components. In fact, pre-copulatory sexual selection (as envisaged by Darwin) represents the final stages of a cascade of events in the evolution of sexual strategy (the 'sexual cascade'), each stage arising as a consequence of the stage before. The cascade begins with the evolution of recombination and syngamy in ancestral eukaryotic unicells. As zygote provisioning becomes an important component of zygote fitness (e.g. during the evolution of multicellularity), anisogamy arises by gamete competition (ancestral sperm competition) and/or gamete limitation in a broadcast spawning ancestor—these two selective forces act in concert. Recent models show more clearly the conditions under which the evolutionarily stable strategy (ESS) is for one sex (isogamy) or two sexes (anisogamy): gamete competition is always the greater selective force favouring anisogamy if an average of just one (or more) spawning competitor is present. The primordial sexual conflict probably involved a form of gamete conflict. With the evolution of two sexes, selection would operate to generate a unity sex ratio. The earliest multicellular animals would have been immobile or weakly mobile organisms with high gonad masses in both sexes, their only outlet for their reproductive effort being expenditure on gametes, with high sperm expenditure maintained by sperm competition and/or sperm limitation. Gradually, once weak mobility developed (for whatever initial reason), the selective pressures of gamete competition/limitation favours female targeting (males moving towards females to release sperm), leading to increased expenditure on female targeting and reduced expenditure on sperm. This process ultimately drives the evolution of copulation and internal fertilisation and greatly reduces sperm expenditure as pre-copulatory sexual selection then leads to the evolution of Darwinian sex roles and sexual conflict. There is, therefore, an evolutionary shift from sexual selection by sperm competition and sperm selection to sexual selection by an increasingly

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pre-copulatory component (the form of sexual selection that Darwin envisaged). Sperm competition nevertheless remains an important component of sexual selection, and a rich diversity in sperm allocation patterns is shaped by evolutionary sperm competition games. Different forms of pre-copulatory competition may also affect sperm allocation, sperm form and function.

Genotype–Phenotype Associations in Relation to Evolution of Sperm Form and Function

Eduardo Roldan

Males exhibit an enormous diversity in reproductive traits. For example, they display wide variations in testes mass relative to body mass, testis architecture and kinetics of the spermatogenic cycle. These differences translate into considerable variations in relative sperm numbers. Males also show wide divergence in the morphology, size and function of spermatozoa. Sperm numbers and sperm design, which are key determinants of fertility, are likely to be under the influence of selective forces such as sperm competition and modes of gamete transport and fertilization. A general response to sperm competition is an increase in the number of sperm produced and transferred to the female tract. An overall improvement of sperm quality (e.g., high percentages of motile and normal spermatozoa) is also widely observed. Moreover, sperm swimming velocity, which is crucial to negotiate barriers in the female tract, reaches the site of fertilization and penetrates ovum vestments, is strongly influenced by the intensity of sperm competition. The velocity of spermatozoa is also affected by factors such as the shape of the sperm head, sperm dimensions (the longer the sperm, the faster their speed) and ATP levels (required to fuel cell propulsion). Genes coding for proteins involved in reproduction are also under strong selective pressure. Among these genes, protamines are important for chromatin condensation and, thus, for the determination of head morphology. Rodents and primates have two types of protamines (PRM1 and PRM2) and the proportion between them seems crucial for normal sperm formation and function. Sperm competition influences the evolution and regulation of these genes but in a very complex way.

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From Mouse to Human: New Aspects of Sperm Transport and Fertilization Using Cutting-Edge Technologies

Sabine Kölle

Successful fertilization only occurs if sperm are able to reach the oviduct and to survive in the female genital tract till ovulation takes place. Cutting-edge imaging technologies such as digital live cell imaging (LCI) and probe-based confocal laser endomicroscopy (pCLE) allow for the first time to visualize sperm transport, fertilization and early embryogenesis under near in vivo conditions, in real-time and on a cellular level. As shown by these novel imaging technologies, the cumulus-oocyte complex firmly attaches to the ampullar epithelium immediately after ovulation initiating a signal cascade leading the sperm to the site of fertilization. Spermatozoa then form a sperm reservoir in the oviduct in which they maintain their fertilizing capacity for 3–4 days in most mammals and humans, for 4 weeks in birds, 6 months in bats and up to 9 years in an Australian snake. In most mammals and mice, sperm bind at a tangential angle to the microvilli of the ciliated cells of the isthmus revealing strong flagellar beating. Binding of the sperm persists till ovulation occurs. Contrary to the human sperm, binding in the oviduct occurs only temporarily (1–90 s). Sperm bind to the microvilli of secretory cells and compete in binding to specific sites. As soon as an oocyte is in the ampulla, spermatozoa get hyperactivated, detach and quickly migrate to the ampulla. Although millions or billions of sperm are inseminated only very few sperm reach the oocyte. After penetration of one sperm, the zona pellucida proteins change the configuration so that no other sperm can get into the oocyte. After fertilization, the presumptive zygote detaches from the ampullar epithelium and migrates down to the uterus.

Male subfertility or infertility is often due to a lack of spermatozoa with proper fertilizing capacity. Our novel imaging technologies have shown that spermatozoa that lack fertilizing capability are (a) either not able to bind to the oviductal epithelium or (b) are not able to detach as soon as the oocyte has reached the site of fertilization or (c) are not able to find their way to the oocyte. If sperm are not able to

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bind to the tubal cells sperm, they will not survive. Alterations of the female genital tract such as inflammation result in sperm being stuck in mucus rendering them unable to detach and reach the oocyte.

In summary, the novel imaging technologies established in the female genital tract allow to analyse physiological behaviour of sperm under near in vivo conditions, in real-time and on a cellular level. In addition to that, these technologies allow us to gain deep insights into the effects of diseases as well as into the actions of medications on sperm transport, formation of the sperm reservoir and fertilization.

First Snapshot of How Sperm Binds the Egg at the Molecular Level

Luca Jovine

Egg–sperm interaction at fertilization marks the beginning of a new individual and allows the transmission of the genetic information to the next generation. Studied by scientists since the seventeenth century, this fundamental process has also long captured the attention of the public because of its direct relevance to reproductive medicine. However, how the female and male gametes recognize each other at the molecular level has until recently remained unknown. Using structural biology, we have found that—despite insignificant sequence identity—a common egg coat protein architecture mediates the initial interaction with sperm in mollusk and human, two organisms separated by 600 million years of evolution. Building upon this discovery, which revealed an expected link between invertebrate and vertebrate fertilization, we have determined the first three-dimensional structure of an egg coat–sperm protein complex. By visualizing the molecular details of the initial contact between gametes, this has both revealed how this interaction has made species-restricted and suggested a mechanism for sperm penetration through the egg coat (Raj et al. 2017). Together with recent data on a subsequent recognition step that ultimately triggers gamete plasma membrane fusion (Han et al. 2016; Ohto et al. 2016; Aydin et al. 2016; Nishimura et al. 2016), these studies started to unveil one of the most crucial moments of life at unprecedented detail.

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The Typical and Atypical Centrioles and Their Potential Roles in the Sperm and Embryo

Emily Lillian Fishman, Katerina Turner, Ankit Jaiswal, Sushil Khanal, Brooke Ott, Patrick Dusza, and Tomer Avidor-Reiss

Introduction

Centrioles are the fundamental building blocks of two unique subcellular organelles, the centrosome and the cilium (aka flagellum in sperm); the sperm alone, not the oocyte, provides them to the embryo upon fertilization. Centrioles are the only known structure that is exclusively paternally inherited. While this unusual pattern of inheritance has been known for over 100 years, many mysteries remain regarding the role of the centrioles in reproduction (Scheer 2014). Here we review the recent discovery of a sperm centriole with atypical structure and composition, which is surprising considering the high conservation of centriolar structure exhibited throughout evolution (Carvalho-Santos et al. 2010). We start with a general overview of the typical centriole structure. We then describe the centrioles in the sperm and zygotes of insects, where atypical centrioles were first discovered; next, centrioles in mammalian sperm and zygotes, where atypical centrioles were subsequently found; and, then the odd exception of mice, where sperm centrioles are not detected. Finally, we speculate why atypical centrioles may have formed and discuss the clinical relevance of sperm centrioles, methods to detect them, and their potential implications in infertility.

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General Overview: Typical Centriole Structure, Composition, Function, and Formation

The centriole is a complex subcellular organelle whose cellular role ranges from assisting in cell division to cilium formation for sensory functions and motility. Because of this wide range of functions, centrioles consist of many parts. The most basic, hallmark features of a centriole are its ninefold symmetric cylindrical microtubules and its strict number control; cycling cells contain exactly two centrioles at the G1 phase and have precise mechanisms to maintain this number. The two centrioles differ in age, structure, and function; one is referred to as the mother (mature) and the other is the daughter (immature). Neither centriole is immediately essential as a microtubule-organizing center for division, but they are necessary for maintaining proper centriole numbers, and they are fundamentally essential for cilia formation. Malformation or improper centriole numbers can cause developmental defects, cellular dysfunction, and cell death. Many tissues developing from the embryo need centrioles, so, the two functional centrioles are essential for the embryo. Therefore, this section will focus on the structure, components, and functions of typical centrioles in cycling cells, such as those arising from the differentiating embryo.

Typical Centrioles Are Cylindrical Cytoplasmic Structures Made of Nine Triplet Microtubules

Centrioles are cylindrical, proteinaceous structures found in the cytosol. Centrioles are heavily conserved across animal evolution; most animals studied have centrioles (with the notable exception of in planarians worms (Azimzadeh et al. 2012)). Since their structure is so iconic, electron microscopy has provided a structure-based definition that has been used nearly exclusively since centrioles were described using this technology in the mid-twentieth century (Burgos and Fawcett 1956).

The centriole's most distinguishing component is its nine microtubule triplets (Fig. 1). These microtubules form the centriole wall and are named the A, B, and C microtubules, from the innermost to the outermost, respectively. The A microtubule is round, whereas the B and C microtubules are incomplete and attached to the adjacent microtubules (reviewed by Winey and O'Toole 2014). The lengths of the microtubules are different, with the A and B microtubules being longer than the C microtubule (Fig. 1a). The diameter of the centriole is approximately 250 nm, and the length ranges from 200 to 500 nm, depending on what phase of the cell cycle the cell is in (reviewed by Avidor-Reiss and Gopalakrishnan 2013a). The centriole is a polar structure; the minus end of the microtubules is at the base of the centriole and the plus end is at the tip.

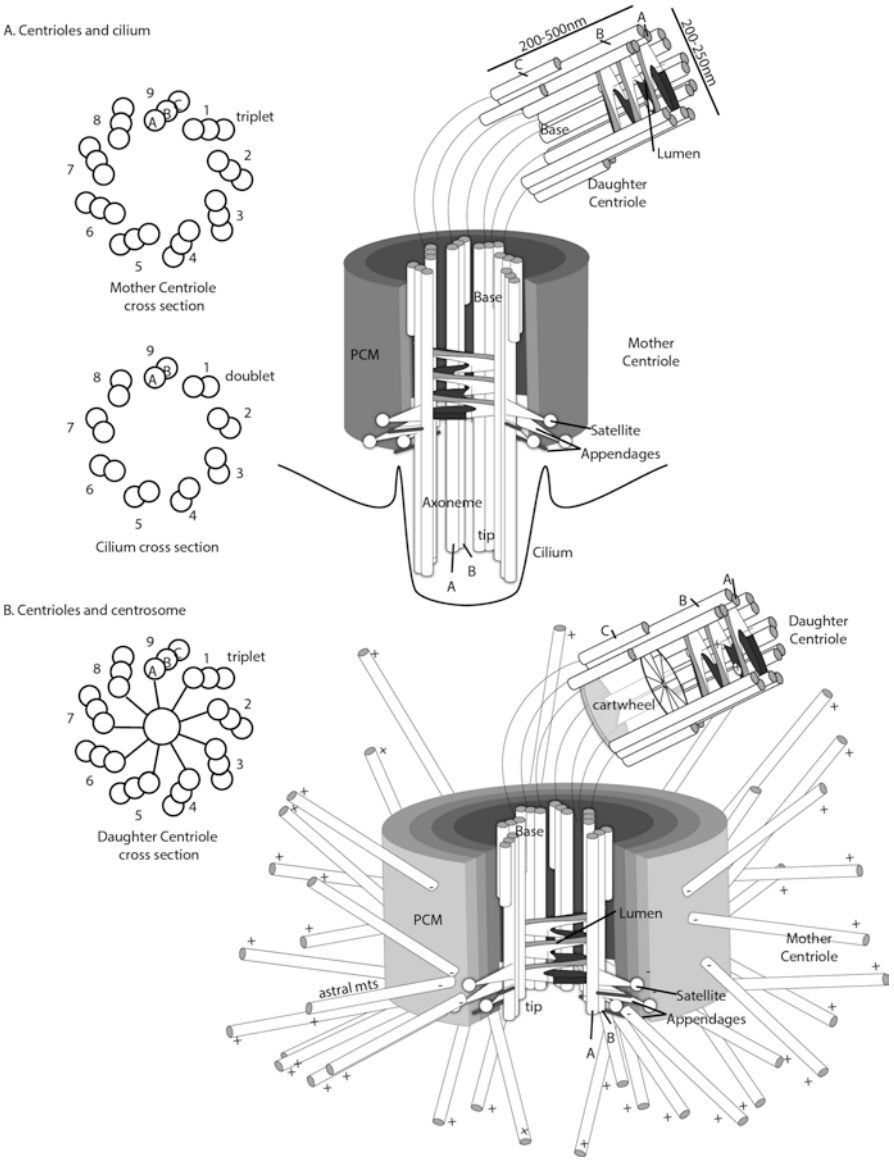

Fig. 1 The typical centrosome in cycling cells has two main functions. (**a**) To nucleate a cilium and (**b**) to act as a microtubule organizing center. *PCM* pericentriolar material

Typical Centrioles Often Have Associated Substructures that Correspond to the Centriole's Maturity

Centrioles often have associated substructures within the cylinder, in the lumen, and peripheral to the wall (reviewed by Winey and O'Toole 2014). Since the mother and daughter centrioles differ in maturity, they each have distinctive features that are used to identify them.

The mother centriole has nine pairs of appendages and can form a cilium and the centrosome (Fig. 1). The mother's distal appendages are fibrous extensions of filaments present at the centriole tip that anchor the centriole to the cell membrane, allowing primary cilia formation. Since the cilium is exclusively nucleated from the mother centriole, it is logical that these appendages are absent from the daughter centriole. The subdistal appendages appear as a triangular structure attached to the microtubule triplets, which anchor the microtubules that emanate from the centriole (Bowler et al. 2019; Kodani et al. 2013; Paintrand et al. 1992) (Fig. 1a).

The daughter centriole often has a cartwheel structure inside the base of the cylinder. The cartwheel is primarily made up of an evolutionarily conserved centriole-specific protein, Sas-6, and has nine spokes that connect to the microtubular wall. While the cartwheel is essential for the normal formation of the microtubular wall, it only partially mediates the ninefold symmetry of the microtubular wall (Hilbert et al. 2016). This cartwheel helps form the new centriole (Fig. 1b) (Cavalier-Smith 1974), but upon maturity, it is no longer needed. Therefore, the mother centriole usually lacks a detectable cartwheel (Lange and Gull 1996; Paintrand et al. 1992).

In both the mother and daughter centrioles, the tip of the lumen is filled with proteins. These proteins are evolutionarily conserved and centriole-specific. However, their precise role is unclear (Azimzadeh et al. 2009; Pearson et al. 2009). Despite these proteins not being well understood, electron microscopy has identified "helical disks" in the distal lumen (Ibrahim et al. 2009; Paintrand et al. 1992), and super-resolution microscopy suggests that the lumen is highly organized (Sydor et al. 2018).

A Typical Centriole Forms the Centrosome or the Cilium/Flagellum

Centrioles have two main functions; the first function is essential: the mother centriole forms a cilium for sensory functions and motility. The second, and perhaps more well-known function is to form a centrosome that nucleates a vast cytoskeleton that is important for intracellular transport and cell division.

The most essential function of the centriole is to template the polymerization and extension of the centrioles' A and B microtubules to create a doublet microtubule-based axoneme that acts as the skeleton of the cilium/flagellum. The cilium functions as a sensory and motility organelle (reviewed by Azimzadeh and Marshall

2010). For example, in the retina, the rod and cones are sensory cilia that recognize light (reviewed by Yildiz and Khanna 2012). In contrast, the motile cilium of the sperm cell, the flagellum, propels the sperm cell to the egg (Fawcett 1975).

The second function of the centriole, forming a centrosome, involves the recruitment and organization of pericentriolar material (aka PCM), a protein mass that surrounds the centriole (Fig. 1b) (Bornens 2002). From the PCM, the centrosome nucleates and anchors a star-shaped (aster) microtubule network, and therefore, the centriole initiates the formation of a significant component of the cytoskeleton (Bobinnec et al. 1998). The sperm centriole forming a centrosome is particularly important after fertilization because the large aster (aka the sperm aster) mediates the migration of the pronuclei (Navara et al. 1994).

While centrioles are considered the major microtubule organizing center of the cell, alternate and compensatory mechanisms exist for exceptional circumstances and specific cell type programs. For example, in oocytes, planarians, and plants, accurate division occurs in the absence of centrioles, in some circumstances, other organelles can adopt the microtubule organizing function of the centrioles. Additionally, in sick, centriole-lacking cells, *inaccurate* division occurs without centrioles (Vitre and Cleveland 2012). Therefore, in a cell type-specific manner, centrioles can be essential, nonessential, or any degree of importance in between, but because centrioles are essential for typical, cycling cells, we consider two centrioles to be essential unless alternate molecular pathways have been shown.

Centriole Maturation and Duplication Regulates Centriole Number and Structure

Centriole number is regulated by a precise mechanism of duplication, maturation, and segregation. When a cell prepares to divide, each centriole duplicates once; by coordinating centriole duplication with the cell cycle, a healthy cell guarantees that it forms only one new centriole near each preexisting centriole (reviewed by Avidor-Reiss and Gopalakrishnan 2013b).

In the typical centriole biogenesis pathway, a cell alternates between having two and four centrioles. During mitosis (M), each daughter cell receives two connected (engaged) centrioles as part of the centrosome at its spindle pole. After division, in Gap One (G1) phase in each daughter cell, the pair of centrioles migrates to the cell periphery, where the mother centriole docks to the cell membrane and forms a cilium. Once the cell receives a signal to move forward into Synthesis (S) phase, the DNA replicates while the cilium starts to disassemble. Furthermore, the centriole forms a centrosome, and each centriole forms a new centriole precursor (procentriole) perpendicular to its wall. Consequently, the cell ends the S phase with four centrioles organized into two pairs, each containing a new procentriole and an older centriole, either the mother or the now maturing daughter. As the cell progresses to the Gap two (G2) phase, the two pairs of centrioles separate from each other and the procentrioles begin to mature, becoming what is sometimes referred to as

granddaughter centrioles. Each pair of centrioles (mother and granddaughter, or maturing daughter and granddaughter) recruits PCM and assembles an aster. Next, the cell enters mitosis and the centrosomes push each other to opposite sides of the cells. Each centrosome localizes to one spindle pole and helps determine the axis of the spindle. During mitosis, the new centriole in each pair is disengaged but is kept next to the older centriole via a protein linker (Kuriyama and Borisy 1981; Vorobjev and Chentsov Yu 1982). During mitosis, each pair of centrioles localize to opposite poles of the spindle and segregates into different daughter cells (Tsou and Stearns 2006).

Because of this strict number-regulating pathway, a centriole needs two cell cycles from its initial formation to reach functional maturity. The procentriole, formed during the S phase as an immature centriole, will gradually gain distinct abilities in G2 and M; it then becomes a daughter centriole (G1 and subsequent S, M, and G2), and finally a mother centriole (G1). Only when a centriole enters the G1 phase for the second time does it become a mature, mother centriole and it is only when it has reached maturity that it can form a cilium for the first time.

This canonical centriole biogenesis pathway is used in most animal cell types studied, with a few notable exceptions. The first major exception is multiciliated cells; during differentiation, cells acquire supernumerary centrioles through alternate molecular pathways that are not tied to the cell cycle but are part of the cell's specific differentiation program (reviewed by Tang 2013). For example, ciliated epithelial cells have hundreds of centrioles (aka basal bodies) (Vladar and Stearns 2007). The other major exception to canonical biogenesis is de novo centriole formation. De novo centriole formation is well-established in two circumstances, mouse embryos, which we will address in the section titled "The Confusing Status of Murine Sperm Centrioles," and manipulated cell culture systems. When centrioles are ablated or excised, structurally normal centrioles are formed de novo; however, they form in variable numbers (Uetake et al. 2007). Likewise, de novo formation can be induced using overexpression of certain centriolar proteins, but it also results in supernumerary centrioles (Rodrigues-Martins et al. 2007). Supernumerary centrioles are known to cause aneuploidy (Ganem et al. 2009). However, over time some tissue culture cells with supernumerary centrioles can regain the proper number of centrioles (Wong et al. 2015).

Insect's Sperm and Zygote Have a Typical Centriole and an Atypical Centriole

Like sperm cells in most animal species, insect sperm consists of two major parts, the head and a tail (Fig. 2a). The sperm tail contains the axoneme, which is the cytoskeletal basis for sperm movement, and a long mitochondrial derivative that acts as a second structural element in the tail (Chen et al. 2017; Fabian and Brill 2012; Noguchi et al. 2012). The neck connects the head and tail and contains two

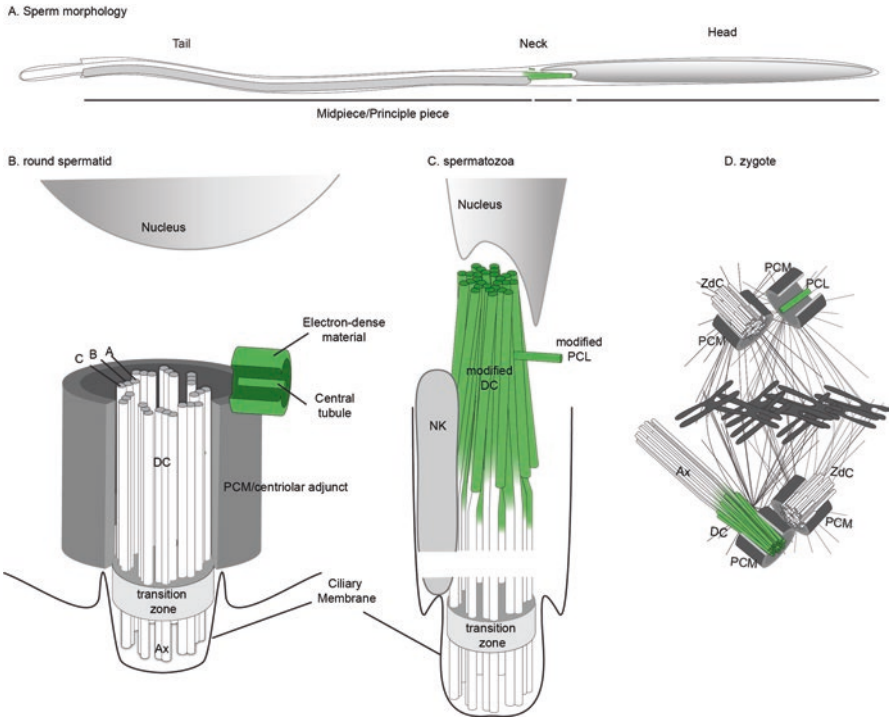

Fig. 2 *Drosophila* sperm centrioles. (**a**) An overview of sperm morphology. (**b–d**) Spermiogenesis begins as a round spermatid with a single centriole and a centriole like structure (PCL) (**b**). Then during spermiogenesis, the axoneme extends, nuclear morphology changes and the centrioles are remodeled to produce a (**c**) spermatozoa with two atypical centrioles. Both centrioles are contributed to the zygote (**d**) where they each recruit PCM, organize microtubules, act as platforms for the formation of a new, typical, daughter centriole, and form a bipolar spindle. Atypical centrioles are highlighted in green. *DC* distal centriole, *PCM* pericentriolar material, *PCL* proximal centriole-like, *Ax* axoneme, *NK* Nebenkern derivative (mitochondria), *ZdC* zygotic daughter centriole

centrioles in many animals. These centrioles are traditionally named the distal centriole and proximal centriole (Ritter 1919; Thomas Harrison Montgomery 1912). The distal centriole is farther away from the nucleus, closer to the cell membrane, and nucleates the axoneme. Alternatively, the proximal centriole is near the nucleus, and farther away from the cell membrane, and does not nucleate an axoneme. For a long time, it was thought that insects had only one centriole, the distal centriole (reviewed by Callaini et al. 1999; Fuller 1993; Phillips 1970; Riparbelli et al. 2010). However, more recently, it became evident that some insects have an additional second centriole that has an atypical structure and composition. The second, atypical centriole does not form the axoneme, and is, therefore, analogous to the proximal centriole; it is known as the Proximal Centriole-Like structure (PCL) (reviewed byAvidor-Reiss et al. 2015). Likely, a second centriole is also present in the sperm

of other insect species, but it has not been observed because the techniques utilized were not sensitive enough to detect the PCL, due to its very small size, easily disrupted structure, and irregular features.

Insect Sperm Often Has Two Atypical Centrioles

Extensive literature has been published on the fine ultrastructure of the sperm of various insects (reviewed by Jamieson et al. 1999). Because insects are one of the most diverse classes of animals, it is not surprising that these publications describe a wealth of different findings in the neck. Adding to this complexity, the varied centriole structure in sperm means that the literature is extremely varied in their identification. Recently though, as techniques have become more sensitive, it seems likely that in male insects, meiosis culminates with the sperm containing precisely two centrioles, although their structure and composition are varied (reviewed by Avidor-Reiss et al. 2015) (Fig. 2b).

During spermiogenesis, the spermatids undergo a series of morphological changes that produce an elongated spermatozoon with two centrioles. At the same time, both centrioles undergo a process termed centriole remodeling, which in some insects, leaves one detectable centriole, and one that is too small and unusual to be detected using standard techniques (Fig. 2c). Remodeling is presumably related to the function of the centriole in the mature sperm; however, the exact relationship of remodeling, sperm function, and embryonic development has not been fully evaluated yet.

The most easily identifiable centriole in insect haploid sperm cells is the distal centriole, which forms the axoneme. Centriole remodeling culminates in several atypical features in the distal centriole that vary heavily from species to species. In *Drosophila*, the centriole's diameter is reduced, causing the transition from a round lumen, to an elliptical shape (Khire et al. 2016). Some insects' (e.g., *Drosophila* and honey bees) distal centrioles have nine triplet microtubules (Hoage and Kessel 1968; Tates 1971), while others have doublets (e.g., *Tribolium* (Dias et al. 2015; Fishman et al. 2017). Sometimes the two central microtubules from the axoneme are observed permeating the centriole lumen (reviewed in Avidor-Reiss 2018). Despite the distal centriole's wealth of atypical features in various insects, it can be recognized using electron microscopy.

Where the distal centriole is recognizable, the structure of the second centriole in insects is less regular, both in spermatids and spermatozoon. In some insects, such as *Adalia*, the proximal centriole has singlet microtubules in spermatids but lacks microtubules in the spermatozoon (Dallai et al. 2017). In *Drosophila* and *Tribolium*, the second centriole is extremely difficult to recognize because it lacks microtubules at all stages (spermatids and spermatozoa) (Gottardo et al. 2015; Khire et al. 2016). However, because it is not connected to the axoneme, but still appears to perform centriolar functions, it is presumed to be the analog of the Proximal Centriole.

In *Drosophila* spermatids, the PCL is composed of an electron-dense material that forms a ring around a central tubule, which is not a microtubule (Fig. 2b) (Gottardo et al. 2015; Khire et al. 2016). The PCL in *Tribolium* spermatids also lacks microtubules; it is composed of an electron-dense structure surrounded by translucent material (Fishman et al. 2017). In *Drosophila*, during spermiogenesis, centriole remodeling modifies the structure of the PCL, the electron-dense material is wholly reduced, leaving it with only a central tubule (Khire et al. 2016) (Fig. 2c).

The PCLs in *Drosophila* and *Tribolum* went undetected for many years because they are minuscule and difficult to distinguish from cytosolic structures; they additionally lack centriolar hallmarks, such as microtubules. And yet, despite their atypical features, they were determined to be centrioles because they appear to follow the typical centriole-formation pathway. This conclusion is supported in *Drosophila*, where after fertilization, the PCL performs centriolar functions, namely recruiting PCM and acting as a platform for the formation of a procentriole (Fig. 2d) (Blachon et al. 2014).

The PCL appears to resemble the early procentriole, and therefore is thought to be a form of centriolar neoteny (reviewed by Avidor-Reiss and Turner 2019; Jo et al. 2019), where juvenile characteristics are retained during maturation. It is not well understood why a neotenic PCL benefits the sperm, but we speculate that it plays a role in motility, perhaps related to the small size of the sperm neck.

Zygote Centrioles

Upon fertilization, the spermatozoon provides the distal centriole and the proximal centriole (or PCL) to the zygote. Both centrioles, despite their atypical features, are essential for development (Fig. 2d) (Khire et al. 2015, 2016). After fertilization, they recruit the maternal PCM and form a centrosome and aster, which allows for male and female pronuclei congregation (Blachon et al. 2014; Riparbelli et al. 2000). Then, the distal centriole and the PCL duplicate and each acts as a platform for the formation of a new, typical procentriole. Thus, the resulting zygote forms two centrosomes, one with the distal centriole and its zygotic daughter centriole, and the other with the PCL and its zygotic daughter centriole. Despite the atypical structure of both sperm centrioles, they direct the formation of typical centrioles, suggesting that centriolar microtubules do not employ a template mechanism to form new centrioles (Avidor-Reiss et al. 2012).

In the zygote, the apparent role of the centriole is to form a centrosome and regulate centriole number. It is unlikely that the PCL, with its microtubule-lacking structure, can form a cilium without regaining its typical structure, and there is no evidence to suggest that it is ever regenerated into a typical centriole. Likewise, the distal centriole remains attached to the axoneme for several cell divisions which would logically impede its ability to form a cilium (Riparbelli and Callaini 2010). Thus, both the PCL and the distal centriole cannot form a cilium, but this is not an issue as the first cilia in the embryo forms much later in development.

Typical and Atypical Sperm Centrioles Function in the Zygote of Non-murine Mammals

Much like insects, mammalian sperm consists of two major parts, the head and the tail, which are connected by a neck that contains the two centrioles (Fawcett and Phillips 1969) (Fig. 3a). Unlike the PCL in insects, the proximal centriole is easily identifiable in most mammals studied (with the exception of murines, see section

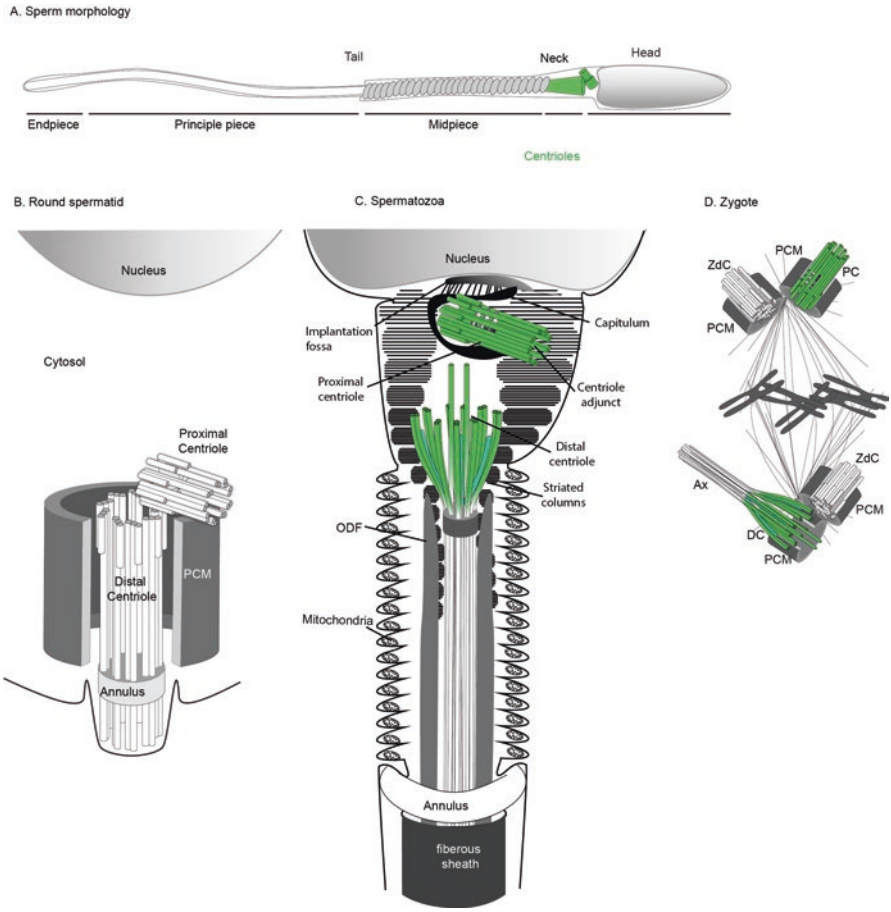

Fig. 3 Humans and bovine sperm centrioles. (**a**) Overall sperm morphology. Spermiogenesis begins with a round spermatid (**b**), with a typical proximal and distal centriole. The Annulus is the structure homologous to the insect transition zone (Avidor-Reiss and Leroux 2015; Basiri et al. 2014). Throughout spermiogenesis, centrioles are modified while the axoneme extends and the nucleus is reshaped to produce a spermatozoa (**c**). Upon fertilization, the spermatozoa contribute both centrioles to the oocyte to produce a zygote. After fertilization, these sperm centrioles recruit PCM, organize microtubules, act as a platform for the formation of two new, typical centrioles, and form a bipolar spindle (**d**). Atypical centrioles are highlighted in green. *PCM* pericentriolar material, *ODF* outer dense fibers, *PC* proximal centriole, *DC* distal centriole, *ZdC* zygotic daughter centriole, *Ax* axoneme

"The Confusing Status of Murine Sperm Centrioles") due to its prominent and characteristic centriolar microtubules; the distal centriole in mammalian sperm is the source of much confusion. The distal centriole can easily be found in early spermatids but was not identified for many years in the spermatozoon due to its modification via centriole remodeling during spermatogenesis.

Mammalian spermatogenesis occurs within the seminiferous tubules of the testes (reviewed by Hess and De Franca 2009; Wistuba et al. 2007). Spermatogonial stem cells are the stem cell niche from which sperm cells differentiate. They reside in the outer layer of cells along the periphery of the seminiferous tubule, adjacent to the epithelium (basal lamina). As they develop and differentiate, they move inwards, toward the lumen. Due to this development pattern, a single cross section of a seminiferous tubule contains several different stages of sperm, which allows for a parsimonious determination of developmental processes (reviewed by Roosen-Runge 1977). After several mitotic divisions, the spermatogonia enter meiosis as primary spermatocytes, and then when they enter meiosis II, they are termed secondary spermatocytes. Finally, after meiosis, they are termed spermatids. While no longer in a state of division, round spermatids begin to undergo dramatic morphology change in a process referred to as spermiogenesis. After the elongated spermatid releases excess cytoplasm, the spermatozoon is released into the seminiferous tubule's lumen. The spermatozoa continue to mature in the epididymis (reviewed by Neto et al. 2016).

During spermiogenesis, the distal centriole nucleates an axoneme and densely packed, specialized structural features form in the sperm's neck, including the striated columns, capitulum, and fibrous sheath. Interestingly, at the same time, the proximal centriole nucleates an axoneme-like structure known as the centriolar adjunct (not to be confused with insect sperm's PCM-like structure of the same name). The centriolar adjunct is a mysterious feature because its function and mechanism of formation are unknown. Furthermore, the proximal centriole has long been accepted by some researchers as the less mature daughter centriole, which should not be capable of nucleating an axoneme. This apparent disagreement with the known functions of the daughter centriole has prompted an investigation into the origin of the proximal centriole, and because the distal centriole abuts the side of the proximal centriole, which resembles the engaged orientation where the immature daughter centriole abuts the side of the mature mother, it has been proposed that the proximal centriole's origin is actually the mother centriole, and the distal centriole originates from the daughter (Alieva et al. 2018). Intriguingly, the adjunct is eliminated in the spermatozoa of most animals, including rhesus monkeys, but not in humans, indicating that it is a sign of relative immaturity (neoteny) of human sperm or that it has a unique function in humans (Manandhar et al. 2000b; Zamboni 1971).

Similarly, to centriole remodeling in insects, centriole remodeling occurs during spermiogenesis. During centriole remodeling, while structural changes occur, some proteins are reduced, while others are enriched. Centriole remodeling results in a spermatozoon with a mildly affected proximal centriole and a dramatically remodeled distal centriole, that is unrecognizable using standard criteria. Remodeling continues as the spermatozoa mature in the epididymis,

and may even continue beyond that (Simerly et al. 2016). However, the mechanism that controls centriole remodeling, and the purpose of remodeling process, is unknown.

Non-murine Mammalian Sperm Have Two Centrioles, One Typical and One Atypical in Structure

During centriole remodeling, the round spermatid's two typical centrioles (Fig. 3b) undergo a series of changes that disguise the centrioles. At this time, the PCM transforms into the striated column and capitulum (Fig. 3) (Fawcett and Phillips 1969) and typical PCM proteins, such as γ-tubulin, PCNT, CEP152, and CEP192, are reduced from the striated columns and capitulum (Fawcett and Phillips 1969). However, centriole proteins that are not part of the PCM of most cell types, RTTN and CEP295, are found in the capitulum and striated columns. Sperm specific proteins, SPAG4 (Shao et al. 1999), Speriolin (Goto and Eddy 2004), and SPATA6 (Yuan et al. 2015) also localize to these structures.

Remodeling changes occur in the proximal centriole, although it keeps its typical microtubule structure (Manandhar et al. 2000a; Schatten 1994) (Fig. 3c). The proximal centriole is marked by classical centriolar proteins such as CETN1, CEP135, CEP120, and CEP76, but it lacks centriole wall proteins CNTROB and RTTN (Fishman et al. 2017; Manandhar et al. 2000a). This unusual protein composition suggests that, even with a typical shape, the proximal centriole is partially remodeled.

The greatest enigma of the mammalian sperm neck has been the distal centriole. While the nine triplets of microtubules of the distal centriole are easily identified in spermatids, the identity or even the existence of the distal centriole has been debated for nearly half a century (Fawcett and Phillips 1969).

The Discovery of the Atypical Centriole in Non-murine Mammals

The historic inability to recognize the distal centriole lead to the degeneration hypothesis, which speculated that the distal centriole was reduced to a nonfunctional remnant during spermatogenesis, leaving behind the electron light region called the vault, and occasionally some disorganized microtubules (Manandhar and Schatten 2000; Manandhar et al. 2000b). The distal centriole lacks RTTN, CEP295, CEP135, and CEP120 (centriole wall proteins); CEP76 and CP110 (centriolar tip proteins); and CEP164 and CEP89 (appendage proteins) (Fishman et al. 2017). While the degeneration hypothesis was accepted for many years, it was recently

disproved when it was discovered that the distal centriole is present and functional, albeit with an atypical structure that prevented detection in the past.

Unlike the typical cylinder shape with triplet microtubules, the distal centriole has splayed microtubule doublets, and a restricted protein profile (Fishman et al. 2018) (Fig. 3c). These identification obstructions were overcome by three technological improvements: (1) improved electron microscopy and fixation/preparation, (2) highly specific antibodies against recently discovered centriolar components, and (3) super-resolution microscopy.

1. In the past, sperm centrioles were studied in chemically fixed samples. Part of the difficulty in identifying the distal centriole using the standard, chemical fixation electron microscopy is that the distal centriole's microtubules are masked by closely associated dark electron-dense structures. With new fixation techniques, namely high-pressure freeze-substitution preparation, the resolution is improved enough that eight to nine doublet microtubules are visible in a unique configuration; these microtubules are splayed out and flattened around the vault.
2. The distal centriole also went unnoticed because classic centriolar proteins were either undetectable or inconsistent. For example, CETN1 inconsistently showed unequal staining in the proximal and distal centriole, which was interpreted as a sign that the distal centriole degenerates partially or completely (Manandhar et al. 2000b). However, previously unstudied centriole lumen proteins such as POC1B and POC5 can be easily and consistently identified in both centrioles using immunofluorescence, as can CETN1, with improved fixation conditions. Furthermore, POC1B labels the distal centriole more prominently than the proximal, suggesting that the distal centriole is not degendered but rather remodeled into an atypical, almost unrecognizable structure.
3. With the recent advancement of super-resolution microscopy, the fine structure of the distal centriole was reexamined. The splayed doublets noted in the electromicrographs are flanked by rod structures labeled with POC5 and POC1B. With further improved resolution, Stochastic Optical Reconstruction Microscopy shows rods of POC5. The exact interactions between POC5, POC1B, CETN1, and microtubules are unknown. It is also possible that other interacting partners have yet to be identified.

To date, the atypical distal centriole has been meticulously described in human and bovine sperm. Although the remodeled distal centriole is expected to be present in most non-murine mammals, this remains to be tested. Interestingly, the atypical distal centriole appears to have a distinct size in different animals (smaller in humans as compared to bovine). It would be important to see size variation between non-murine mammals and to gain insight into the reason for size variability. The exact role of the remodeled distal centriole is unknown, but speculations exist (see section "The Evolution of the Atypical Centriole" below).

The Two Sperm Centrioles Function in the Zygote of Non-murine Mammals

Because centrioles are so important for accurate cell division and forming cilia (Bornens 2012) and the embryo is a cycling cell, it makes sense that centrioles are similarly important for the developing embryo. Abnormal centriole numbers can result in aneuploidy and, during development, can lead to serious birth defects or spontaneous abortion (Sathananthan et al. 1996; Vitre and Cleveland 2012). This logic has been used as the primary support for the idea that centrioles are essential for the embryo; experimental evidence of this in mammals, is limited, but the idea is universally accepted.

Since centrioles are essential for the embryo, it is interesting that the oocyte, which provides most of the organelles an embryo needs to develop, has no centrioles (Manandhar et al. 2005; Namgoong and Kim 2018; Szollosi et al. 1972). During oogenesis, the centrioles are eliminated through a centrosome reduction program that varies between animals. In mammals, centrioles disappear during the Pachytene stage (during prophase of meiosis I), meaning that the oocyte lacks centrioles during both the first and the second meiotic divisions, and likely does not contribute any centrioles to the offspring (reviewed in Manandhar et al. 2005). Therefore, if the embryo is to inherit centrioles to use as a platform for the formation of new centrioles or for centriole number control, it must inherit the centrioles from the sperm or form them de novo (Fig. 4).

The elimination of centrioles in the oocyte appears to be an active, dominant process, not simply the result of protein degradation over time. This is exemplified when centrioles were transferred during somatic cell nuclear transfer in pigs; CETN1/2 labeling showed degradation of the somatic centrioles (Manandhar et al. 2006). One interpretation of this observation is that the developmental program in the oocyte can affect the sperm centriole once introduced to the egg. If the oocyte has a dominant centriole reduction program that acts on sperm centrioles, it is possible that the sperm coevolved with postfertilization centrosome reduction to minimize the centriolar contribution (the passive hypothesis described in section "Atypical Sperm Centrioles May Have Passively Evolved Due to Lack of Necessity").

Because of the ethical considerations surrounding this work, and because mice are not a suitable model system for investigating the centriole's role in human reproduction, direct observations of the centrioles behavior in normal zygote is limited to small observational studies, mostly in nonviable embryos (Kai et al. 2015; Sathananthan et al. 1996). Although some of these studies recognize embryonic centrioles using electron microscopy or PCM-specific antibodies, it is difficult to determine whether or not their centrioles resemble the centrioles of healthy embryos. Furthermore, it is impossible to know if the centrioles were the cause of the embryo's defect, or if they were simply affected by defects in another process that also plagued the embryo. As a result, most studies on viable non-murine embryos, especially in recent years, use embryos from livestock species, such as cattle; more research on

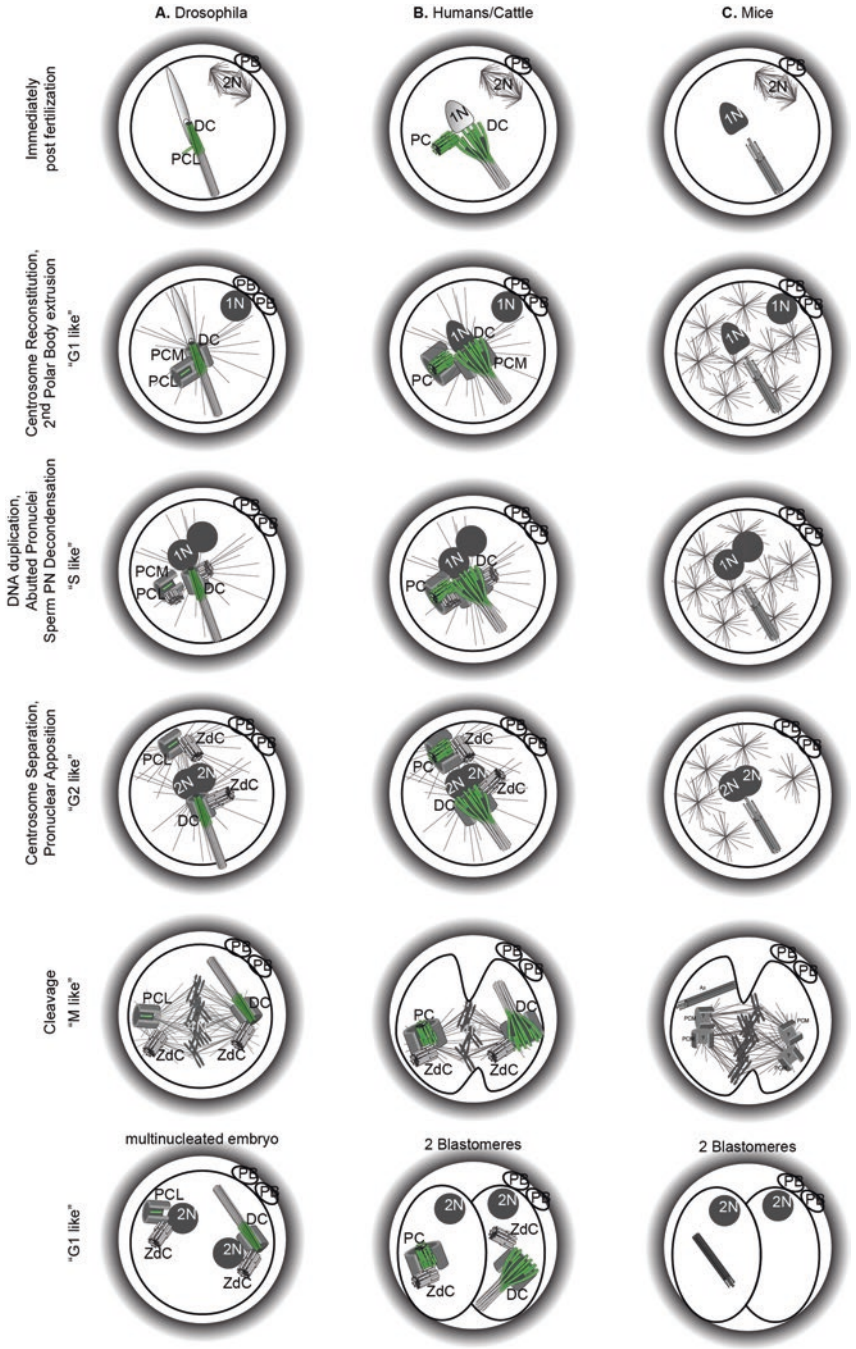

Fig. 4 Comparison of zygotic development in *Drosophila* (**a**), Humans/Cattle (**b**), and Mice (**c**). Known atypical centrioles are highlighted in green. *PB* polar body, *PC* proximal centriole, *DC* distal centriole, *ZdC* zygotic daughter centriole, *PCL* proximal centriole-like

these species will benefit the understanding of reproduction (Madeja et al. 2019; Polejaeva et al. 2016).

After fertilization, the sperm tail remains associated with the male pronucleus during decondensation, and recruitment of maternal PCM proteins suggests that the centrosome remains affiliated with the sperm tail (Wu et al. 1996). After the completion of meiosis II and the extrusion of the second polar body, the centrosome continues to recruit PCM proteins and forms the sperm aster, which congregates the male and female pronuclei (Fig. 4a). At this point in cattle zygotes, CEP152 can be detected in two foci, one of which is attached to the sperm axoneme, suggesting that both the proximal and remodeled distal centriole recruit PCM. Next, centriole duplication takes place. Prior to entry into mitosis, foci can be seen using anti-CEP152, two of which are co-labeled with anti-Sas6, indicating that they are newly formed centrioles (Fishman et al. 2018). This suggests that the sperm's centrioles, despite being atypical, are still able to perform key functions of centrioles, forming an aster and acting as a platform for centriole duplication.

After the formation of the new centrioles, the zygote enters mitosis, forms a bipolar spindle, and divides, providing each of the two resulting blastomeres with exactly two centrioles. Interestingly, throughout this first division, the distal centriole remains attached to the sperm's axoneme. This was observed originally because the axoneme appeared attached to one of the spindle poles during the first division, and later the centriole itself was identified in this pole. While the axoneme can be observed for several cell cycles, it is not clear if the centriole remains attached to it, or how long the centriole remains attached and thereby incapable of nucleating a cilium. The asymmetry of the zygote's centrosomes, due in part to the presence of the axoneme, means that the contents of the two blastomeres is inherently unequal. The implications, if any, of this unequal inheritance on the blastomeres or their eventual fates are unknown.

The Confusing Status of Murine Sperm Centrioles

For many reasons, including generation time, ease of breeding, and maintenance costs, mice have been the predominant model system for scientific inquiry across biology, including the fields of reproduction and development. However, it has become evident that mice do not adequately model human development, so interrogating these differences provides insight into the evolution of alternative mechanisms in mice and humans.

While human and murine sperm have the same major parts, murine sperm have three obvious differences (Fig. 5a), namely, (1) the head is elongated and has a hook shape of unknown function (Tourmente et al. 2016); (2) the tail is longer than most other mammals (Gomendio and Roldan 1991); and (3) the neck is attached to the side of the nucleus (Fawcett 1970). These differences could potentially be linked to the centrioles, as the centrioles of mouse sperm are also notably different when compared to those of humans and other mammals. Unlike in other mammals, the

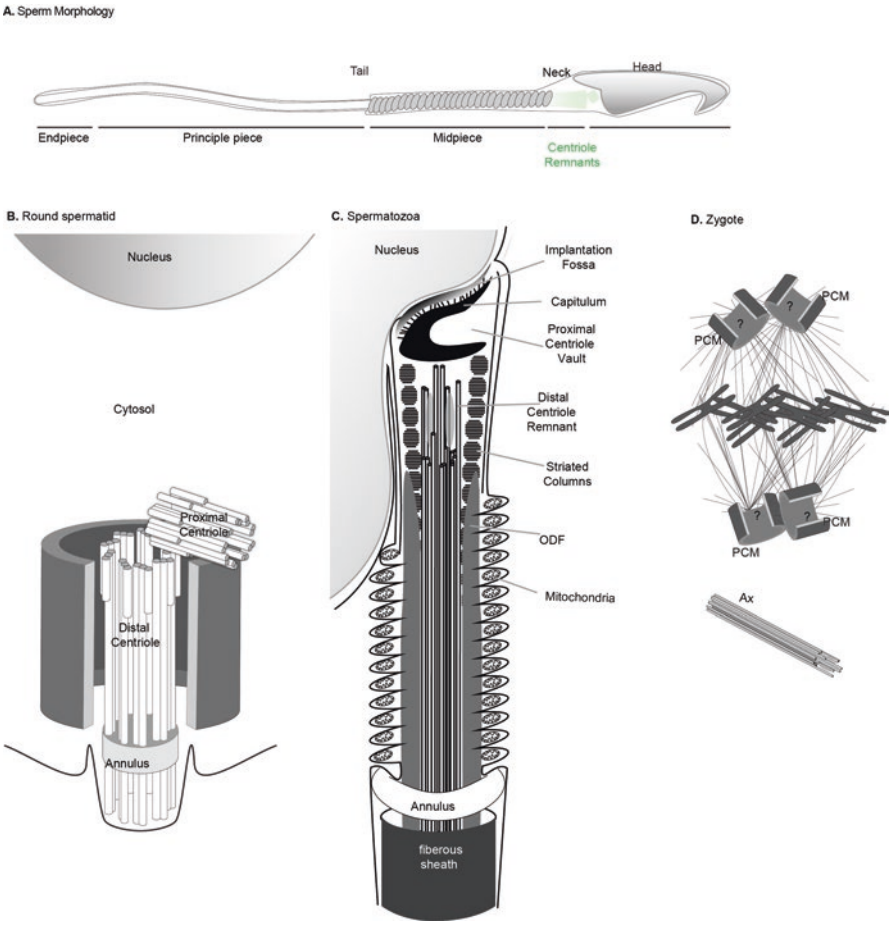

Fig. 5 Mice sperm centrioles. (**a**) Overall sperm morphology. Spermiogenesis begins with a round spermatid (**b**), with a typical proximal and distal centriole. Throughout spermiogenesis, these centrioles are extensively modified while the axoneme extends and the nucleus is reshaped to produce a spermatozoa that seems to lack centrioles completely (**c**). After fertilization, two bipolar spindles form that each has PCM, but no centrioles have been observed (**d**). It is accepted that the sperm, oocyte, and zygote lack centrioles altogether. Centrioles are not detected until the blastocyst stage. *PCM* pericentriolar material, *ODF* outer dense fibers, *Ax* axoneme

current dogma is that centrioles are absent in the spermatozoa of mice, rats, and hamsters (reviewed in Schatten 1981) (Fig. 5b), and therefore findings in mouse sperm and embryos have limited application to humans. However, the dogma needs additional validation in context of the discovery of an unexpected atypical centriole in humans. Reevaluating whether mice have or do not have sperm centrioles has major implications. If mice truly lack centrioles completely during early development, research would provide insight into how cells can function without centrioles, and if mice have extremely atypical sperm centrioles, it would open the door to

experiments on how and why atypical centrioles form—experiments that are difficult in humans or other larger model systems.

Mouse Sperm Centriole Degeneration Starts in the Testis and Continues in the Epididymis

Murine spermatogenesis appears similar to that of humans, with development taking place in the seminiferous tubules in the same general pattern. And similarly, murine sperm also go through a centrosome reduction program. In early spermatogenesis, CETN1/2 localizes to the proximal and distal centrioles—both of which have visible centriolar microtubules and PCM components such as γ-tubulin (Manandhar et al. 1998) (Fig. 5b). But in the subsequent elongated spermatid stage, the γ-tubulin disappears from both centrioles, indicating that the PCM undergoes reduction. Next, CETN1/2 disappears first from the distal centriole, and later from the proximal centriole in epididymal sperm (Manandhar et al. 1998; Schatten 2016) (Fig. 5c). The Centrosome Remodeling Program in humans and cattle has a Reduction and Enrichment subprogram (section "Typical and Atypical Sperm Centrioles Function in the Zygote of Non-murine Mammals"), but in mice, we speculate that the either the enrichment subprogram is attenuated or absent altogether, or the reduction subprogram is far more dominant. Regardless of the mechanism, the final product is an ejaculated spermatozoon, which seemingly lacks centrioles.

Mice Are Thought to Have No Functional Sperm Centriole

Several observations support the idea that mouse sperm lack centrioles and that centrioles form de novo from zygotic proteins. While this hypothesis is well accepted, the observations supporting it have some alternate explanations.

Studies that look directly for centrioles and conclude that they are absent due to several observations show that (1) triplet microtubules are not detected in the neck region of mouse spermatozoa by electron microscopy (Manandhar et al. 1998), nor are centrioles with typical ultrastructure detected after fertilization (Szollosi et al. 1972). (2) centriolar proteins (e.g., CETN1/2, and γ-tubulin) are not detected in the neck of spermatozoa (Schatten et al. 1985, 1986), or the embryo (Schatten et al. 1985); centrioles do not appear until the 32/64-cell stage (Simerly et al. 1993; Gueth-Hallonet et al. 1993; Coelho et al. 2013), (Bangs et al. 2015). (3) after fertilization, instead of forming a dominant sperm aster like other mammalian embryos that have paternal centriole inheritance, mouse embryos form many mini asters that appear random; there is no immediate dominant microtubule organizing center (Calarco 2000; Clift and Schuh 2015; Coelho et al. 2013; Schatten et al. 1985). These mini asters are organized by small electron dense aggregates and can be detected by anti-PCM antibodies (Calarco-Gillam et al. 1983; Hiraoka et al. 1989;

Houliston et al. 1987; Maro et al. 1985; Szollosi et al. 1972). Several antibodies against PCM proteins have been used to study the distribution of PCM including the human auto-antiserum 5051 (Calarco-Gillam et al. 1983), the monoclonal antibodies MPM-1 and MPM-2 recognizing mitotic phosphoprotein and γ-tubulin antibodies (Gueth-Hallonet et al. 1993). All these antibodies label the spindle poles in embryo, suggesting that these proteins participate in the function of the spindle poles (Calarco-Gillam et al. 1983; Hiraoka et al. 1989; Maro et al. 1985), but it should be noted that acentriolar spindle poles have PCM in the absence of centrioles (Debec et al. 2010), and therefore PCM alone is not inherently evidence of centrioles.

Alternatively, the inability of these studies to detect the centrioles can be explained by the use of older and insufficiently sensitive technologies (namely fixation methods and antibodies); inability to detect or visualize a centriole should not be confused with total absence. With the advent of new tools, such as High-Pressure Freezing–Freeze Substitution and Cryo Electron Microscopy, and Super-resolution microscopy (i.e., STORM), the sperm neck needs to be reexamined. Furthermore, with the discovery of the atypical centrioles in *Drosophila* and human sperm, future studies need to consider the possibility of functionally competent centrioles that do not resemble typical centrioles.

Two studies type that looks for centrioles indirectly by examining their microtubule organizing function and suggest that centrioles are absent. (1) The zygote spindle appears anastral and barrel-shaped (Schatten et al. 1985) (Fig. 5d). This barrel shape is different from the spindles of other mammalian embryos, but similar to the shape of the meiotic spindle, which lacks centrioles (Sakai et al. 2011; Simerly et al. 2019; Wu et al. 1996). (2) The sperm tail is not associated with the spindle pole in mice (Simerly et al. 1993) like it is in humans and other mammals (Navara et al. 1994; Wu et al. 1996). These two types of observations indeed suggest mechanistic differences between mice and non-murine mammals, but it is worth noting that after the formation of the mini asters, the embryo eventually does form two bipolar spindles (Reichmann et al. 2018). How the poles are selected from the mini asters is not known, but extremely atypical centrioles are one potential explanation.

Two studies type that looks at the requirement of the sperm or sperm tail suggests centrioles are absent. (1) Embryos made from injection of a sperm head with no tail, and thereby no centriole, result in viable offspring (Yan et al. 2008). (2) Parthenogenic activation of mouse oocytes, which guarantees no paternal centriole contribution, can result in healthy pups that develop into healthy, fertile adults (Kono et al. 2004). These two types of observations suggest that centrioles are not essential for early development. However, both experiments are *inefficient* in producing live pups. Regardless, it is known that cell culture cells can form centrioles de novo (Uetake et al. 2007) but most of the time, these cells will form the wrong number of centrioles and will develop aneuploidy (Vitre and Cleveland 2012). However, some of the time (Wong et al. 2015) they recover the correct centriole number and proliferate. It is unclear if the success of nucleus-only injection or parthenogenesis is due to a chance formation of exactly two centrioles or a compensatory mechanism, but it is expected that the embryo cells end up with the correct number of centrioles for the pups to be healthy.

In addition to these alternate explanations, there are experiments that can be interpreted to suggest that mouse sperm could have extremely atypical centrioles. (1) Some centriolar microtubules are observed using electron microscopy (Iwashita and Oura 1980; Manandhar et al. 1998); and (2) an experiment using injection of mouse sperm into a cat egg results in the formation of a sperm aster (Comizzoli et al. 2006; Jin et al. 2012). The latter experiment was conducted to determine whether the aster formation program was maternally or paternally derived; they assumed that when injecting a mouse sperm into a cat egg the resulting embryo would be acentriolar, and therefore the sperm aster that resulted must be a feature of a maternally derived program. However, there is an alternate explanation: the sperm contains a highly atypical, possibly inactive, but not incompetent centriole. This centriole, in the presence of the correct maternally derived program, would be able to facilitate, or at least act as a landmark for the formation of a dominant microtubule organizing center. This ability of atypical centriole is also supported by the discovery that overexpressing CETN2 in mice results in the retention of CETN2-enriched foci, which reside at the spindle poles during metaphase. It suggests that these CETN2-enriched foci are at least competent to act as platforms for the recruitment of microtubules (Simerly et al. 2018). It is unclear if the CETN2 foci simply stay out of the way, or actively orchestrate recruitment.

One way to reconcile all these observations is that very atypical centrioles are present in the mouse sperm, but they are not functional postfertilization because of the presence of mouse egg-derived inhibitory factors, but in the context of a different developmental program, they may be competent to orchestrate aster formation, or at least not interfere.

In conclusion, the dogma is that the sperm centrioles do not organize a functional centrosome in the mice zygote, and instead, centrioles are formed de novo from a maternally derived program, and there is plenty of evidence to support this idea. But having a maternally derived dominant developmental program is not mutually exclusive with the existence of a sperm centriole. The idea that mouse sperm lack centrioles altogether is widely accepted, but the discovery of a highly atypical centriole in non-murine spermatozoa, and the possible alternate explanations for these experiments necessitate a reevaluation of the dogma.

The Evolution of the Atypical Centriole

Across evolution, sperm centrioles execute essential centriolar functions in the zygote, regardless of atypical structure in some species. The ancestral “primitive” sperm had two typical centrioles in its neck (reviewed in Avidor-Reiss 2018), and the evolution of atypical centrioles in insect and non-murine mammalian sperm, as well as the loss of centrioles in murine sperm presumably occurred more recently. Furthermore, since many of the animals between insects and humans have two typical centrioles, it appears that atypical centrioles evolved independently in insects and mammals. But if atypical centrioles truly are the product of two evolutionary

events, this begs the question as to why atypical centrioles evolved. We propose two hypotheses: a passive and an active hypothesis.

Sperm Competition Drives Sperm Evolution, and May Have Actively Selected for Atypical Centrioles

The active hypothesis is based on the premise atypical centriole have advantages. Because sperm undergo such extreme competition, especially in polyandrous species, there is significant evolutionary pressure that drives sperm evolution. Because of this evolutionary pressure, sperm are some of the body's most evolved cells, with even closely related species having vastly different sperm morphologies (Firman and Simmons 2009). Postmating sexual selection causes spermatozoa to undergo a direct selection process and they undergo rapid evolution (reviewed in Birkhead and Pizzari 2002; Parker 1984).

Sperm competition exhibited when sperm from different males competes to fertilize the egg—often favors speed or progressive motility, so for insects, and maybe mice, having a very small atypical centriole could allow for a smaller sperm neck region, and thereby a faster, more hydrodynamic sperm.

Sperm selection also takes place due to female cryptic choice, when physical and chemical properties of the female tract provide an advantage to sperm from one male over others. Having a splayed distal centriole could give mammalian sperm more efficient planar movement to navigate the complex physical environment of the female reproductive tract. This idea is supported by the observation that structural symmetry in the flagellum correlates with fertilization types. In animals that use external fertilization (reviewed in Avidor-Reiss 2018), the flagellum has pseudo radial symmetry (ninefold symmetry). However, in many animals with internal fertilization, including the atypical non-murine mammalian distal centriole, the flagellum, and the centriole, have increased pseudo bilateral symmetry (twofold symmetry).

Additionally, it is possible that some zygotic processes or sperm/egg complementation, that we do not yet understand, benefit from an atypical centriole. The active hypothesis would speculate that there is some fundamental difference between sperm or zygote function of animals with atypical sperm centrioles and animals with typical sperm centrioles.

Atypical Sperm Centrioles May Have Passively Evolved Due to Lack of Necessity

The passive hypothesis is based on the premise that atypical centriole evolved because of relaxed centriolar requirement. This could be due to the relative independence of the oocyte, or because sperm centrioles do not form cilia in early

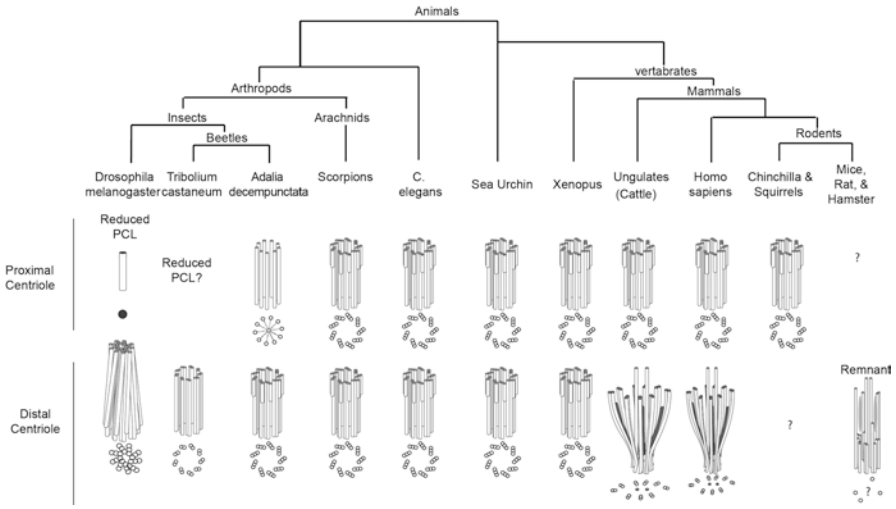

Fig. 6 Phylogenetic comparison of spermatozoa centrioles. The majority of animals studied have two centrioles that vary in structure, from PCLs to typical centrioles, to more recognizable atypical centrioles. Neither the type of modification nor the specific centriole that is modified, is completely conserved, suggesting that insects and mammals evolved atypical centrioles independently. Furthermore, the unusual lack of centrioles in murine sperm suggests that they extended their centriole degeneration program, using a similar, but more extreme, mechanism to that of other mammals. *PCL* proximal centriole-like, *SDC* sperm distal centriole

embryogenesis. Experiments showing centriolar reduction of somatic centrioles in a porcine embryo (Manandhar et al. 2006) suggest that the oocyte actively silences centrioles, and therefore there is no embryonic necessity for typical centrioles. Likewise, when human sperm was introduced to a hamster egg (whose developmental program resembles that of the mouse), no sperm aster was formed (Hewitson et al. 1997), supporting the idea that the hamster zygote does not use a centriole-based mechanism. Perhaps the oocyte contains most of the developmental program needed to direct divisions, and therefore the sperm does not need to provide typical centrioles but only a rudimentary centriole that recruits maternal proteins to execute other beneficial functions. Therefore, evolutionary drift has changed the centrioles with no major effect on fertility.

Centrioles appear not essential for division in early mouse embryos and since cilia appear only much later in the blastocyst, the main canonical roles of centrioles are not employed here (Simerly et al. 1993). They do appear to assist in bringing the two pronuclei in many species, and they may be essential only as a mechanism to control centriole numbers so that the tissues differentiating from the embryo have precisely two centrioles. Furthermore, this hypothesis would explain why centrioles vary so much even between species with atypical centrioles (Fig. 6).

Sperm Centrioles Contribute to Infertility and Reduced Male Fecundity

After one year of attempting conception, 10–14% of couples in the United States are unable to conceive (review in Chandra et al. 2013; Kelley et al. 2019; Kumar and Singh 2015) (Fig. 7). Out of these couples, 40–50% is due to male factor infertility (reviewed in Kumar and Singh 2015; Chandra et al. 2013; Kelley et al. 2019) and 15–30% of couples suffer from unexplained infertility (reviewed in Isidori et al. 2006; Quaas and Dokras 2008; Thonneau et al. 1991). These statistics show that both male factor infertility and unexplained infertility are prevalent.

The standard of care for infertility starts with timed intercourse, lifestyle modification (such as; weight loss), medication, intrauterine insemination (IUI), and ends with assisted reproductive technology (ART), usually in the form of Intracytoplasmic Sperm Injection (ICSI) (reviewed in Quaas and Dokras 2008). These latter treatments are expensive, with an average cost of $5894 for medication, $10,696 for IUI, and $61,377 for ART (Katz et al. 2011); depending on location, these treatments may not be covered by insurance. Also, ART involves multiple surgical procedures, which increase the risk of complications for the female partner compared to the other less invasive treatments (Wang and Sauer 2006). These treatments can overcome defects related to motility and sperm fusion with the egg; however, if the centrioles are defective after fertilization, current treatment options are unable to address defects (reviewed in Quaas and Dokras 2008; Van Blerkom 1996a). To improve patient care, it would be essential to identify men with centriole-based infertility. With a diagnosis, couples would likely save money on healthcare and reduce the number of invasive procedures.

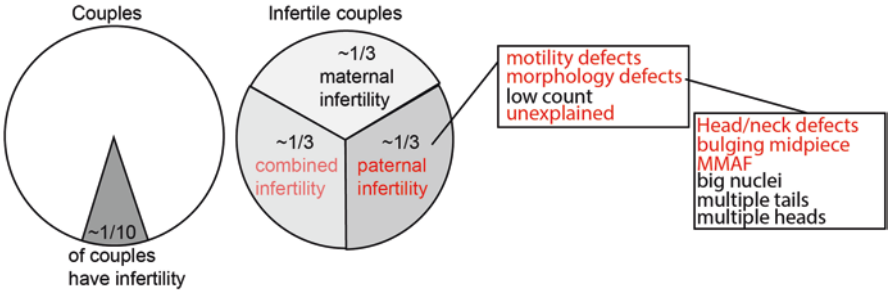

Fig. 7 Origins of infertility. About 1/10 couples experience infertility, and of those, about 1/3 experience male infertility. Male infertility is diagnosed because of low count, motility defects, or morphology defects—centrioles play a potential role in motility and morphology; specifically, we expect that they are a candidate cause of head/neck severing, bulging midpieces, multiple tails, and other MMAF disorders. The frequency of these conditions is not known. It is also possible that centriolar defects affect neither morphology nor motility, but affect the developmental outcome, meaning that individuals with centriolar defects could be mistakenly labeled as maternal infertility. Centrioles are a candidate cause for any disorder listed in red

A Link Between Centriole Defects and Male Infertility

A few studies indirectly associated a suspected sperm centriole defect with a zygote developmental defect (Chemes and Rawe 2003; Moretti et al. 2017; Rawe et al. 2008; Van Blerkom 1996b). However, these studies have a small sample size or are case studies (reviewed in Avidor-Reiss et al. 2019). It is important to note that these papers are not true experiments, because they are observational studies; as such, their findings are strictly limited to correlations. And yet, despite the lack of causative, direct evidence, it is accepted that centrioles are essential for, and heavily implicated in human fertility (reviewed in Avidor-Reiss et al. 2019; Palermo et al. 1997; Schatten 1994).

Studies that conclude that there is a connection between centriole defects and infertility can be separated into two categories, studies that describe a direct centriolar defect, and those that describe defects that indirectly could be associated with the centrioles, or centriole-associated structures.

There are two case studies that directly attribute a case of infertility to malfunctional centrioles. The first describes two infertile patients who exhibit centriolar adjunct defects (Garanina et al. 2019). This study found that the sperm of infertile men had a longer centriolar adjunct when compared to five fertile patients, suggesting that the centriole adjunct defect is correlated to infertility. Another study found a missense mutation (D455V) in the centriole protein, CEP135, in an infertile man (Sha et al. 2017). The mutated protein was mislocalized and formed ectopic aggregates, and the sperm had multiple morphological abnormalities of flagella (MMAF), resulting in immotility. When his sperm was used to fertilize using intracytplasmic sperm injection (ICSI), the embryo developed for several days, but the pregnancy failed, suggesting that centrioles play a role in embryo development after the zygote stage. This mutation in CEP135 is a candidate cause for a common clinical phenotype: most IVF embryos do cleave, but one of the major failure points during development occurs between the eight-cell stage and blastocyst formation. Because these embryos were implanted prior to blastocyst, it is not clear exactly when they died, but CEP135, and centrioles as a whole, could be candidates for the nearly 50% of embryos that fail to reach the blastocyst stage or the nearly 75% that fail to implant. Understanding even a fraction of this large group of embryonic failures could dramatically improve IVF efficiency.

The second category of studies that suggest an association between sperm centrioles and infertility focuses on broader neck defects. Because the centrioles are in the neck, which connects the head and tail, it is possible that the infertile men from these studies have centriolar defects too (Rawe et al. 2002). This category includes head–neck defects (sometimes called the Head Tail Coupling Apparatus, HTCA), where the head is severed from the tail, in varying degrees of severity (reviewed in Chemes 2012). This defect can be rescued by injection of the separated sperm head and tail in close proximity. Contrastingly, when a sperm head without the tail was injected into an oocyte, embryo development failed (Palermo et al. 1997). This suggests that, unlike in mice, the tail in human sperm is essential for fertilization

possibly because the centrioles are attached to the tail (Emery et al. 2004; Gambera et al. 2010).

Overall, only a few studies have observed defects in centrosome proteins, location, number, or structure in infertile men. These few studies have introduced the idea that centrioles may be important for fertility in humans, but due to their observational nature, any conclusions are limited to correlation and association. More comprehensive studies on the subject are needed to determine the degree of association between centrosomal defects and infertility, and experiments need to be conducted in nonhuman, non-murine models, such as livestock species (cattle, sheep, and pigs) to delineate the centrosomal role and mechanism in the embryo to ultimately determine causal candidates for centriole-based infertility. Conducting this work in livestock species will have major implications for humans, but will also help the agricultural industry breed more efficiently.

A Link Between Centriole Defect and Human Embryo Development

Because of the ethical and legal considerations surrounding human embryos, one of the most prevalent methods of examining the centrioles in human embryos is to look at arrested embryos. Most of these embryos have been tripolar, presumably due to polyspermy (Kai et al. 2015; Sathananthan et al. 1991). Inherently, polyspermic embryos have supernumerary centrioles, but it is impossible to tell whether the centrioles are the reason for their arrest or artifacts of the failed development. For this reason, currently, we do not know how to diagnose malformed centrioles by looking at the sperm, nor can we identify centriolar defects by looking at embryonic outcomes alone. Therefore, an important first step in the clinical application of reproductive centriole research is identifying centriolar defects that cause infertility or failed development, and fully characterizing the developmental phenotype.

More Research Is Needed for Effective Treatment of Centriole-Based Infertility

Currently, there is no treatment for centriole-based infertility (Pandruvada et al. 2021; Royfman et al. 2020; Turner et al. 2020). However, as we learn more about centriole defects and their effect on motility and the embryo, potential treatment avenues can be investigated. One potential treatment for centriole-based infertility would be to select sperm for their centriole quality. Since it is possible that the centrioles affect the sperm's motility or morphology, developing light-microscopy observable criteria that are associated with functional centrioles would allow clinicians to select for sperm with good centrioles. Identifying sperm with functional

centrioles would be especially helpful in patients with especially heterogenous sperm (Hinduja et al. 2010).

If the patient's sperm centrioles are homogenous and defective to the point that embryonic development fails, another option may be to replace the defective centrioles with centrioles from a donor. A few studies have demonstrated that a fertile embryo develops when the tail with the centrioles is injected near the sperm head (Emery et al. 2004; Gambera et al. 2010), using a donor's sperm tail and the patient's sperm head could potentially overcome the centriole defect while still producing a genetically related child. This idea is similar to mitochondrial replacement therapy (MRT), where the parental DNA is transferred from a diseased oocyte to an enucleated donor (Craven et al. 2010; Tachibana et al. 2018).

Methods of Studying Sperm Centrioles Past, Present, and Future

Centrioles are challenging to study because they are so small that their internal structure cannot be resolved by classic light microscopy, and all techniques currently used have limitations. Therefore, the study of centrioles is reliant on multi-approach techniques that are expensive, time-consuming, and not accessible for clinical laboratories. Ultimately, there is a need to develop biomarkers and techniques with higher throughput and specificity, such as super-resolution microscopy and image-based flow cytometry. Here we review and evaluate the many techniques that exist for studying centrioles.

Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) is commonly used to study centrioles as it provides the highest level of structural detail. Sperm used in TEM are fixed, either through chemical fixation (Oliveira et al. 2011) or through cryofixation (Ounjai et al. 2012). The sample is then mounted, sectioned, stained, and imaged. Although useful in viewing cellular structures, TEM does not provide information on specific protein distribution (Chemes et al. 1987; Moretti et al. 2017; Oliveira et al. 2011). TEM is extremely expensive and laborious, so it is inaccessible in most clinical settings, and unsuitable for large-scale studies (Chemes et al. 1987; Moretti et al. 2017).

Despite its incompatibility in clinical settings, TEM has been used in several studies researching sperm centrioles. Sperm centrioles in gametes, fertilized embryos, and in preimplantation embryos examined using TEM suggested that human centrosome inheritance is paternal and that inheritance of abnormal centrosomes can result in infertility (Sathananthan 1998). TEM was also used to evaluate the sperm of a patient with severe asthenoteratozoospermia, and it was determined that the observed alterations in the sperm head–tail junction and attachment were

due to an abnormal centriole resulting in improper aster formation and defective embryos (Rawe et al. 2002). Additionally, TEM was primarily used to discover that subjects with unexplained infertility have a significantly longer centriolar adjunct compared to healthy subjects (Garanina et al. 2019). Each of the studies described earlier examined no more than a few patients, demonstrating the limited capacity of this technique.

Western Blot

Western blots are commonly performed to detect specific proteins from a cell lysate. However, it only informs us indirectly about centrioles since it characterizes total protein in the cell. Because centriolar proteins are found largely in the cytoplasm, more so than in the centriole itself, it is difficult to evaluate the centriole-specific population of a given protein (Gavini and Parameshwaran 2019). This is especially true for embryos, which contain high levels of centrosomal proteins in the cytoplasm. One way to overcome this limitation is to separate the sperm centrioles biochemically and then analyze them via Western blot. However, these techniques are time-consuming and rely on purity that is difficult to achieve (Firat-Karalar et al. 2014).

Despite its limitations, Western blots have been used in several studies: higher levels of centrin were found in normozoospermic samples when compared to oligoasthenozoospermic (Hinduja et al. 2010). Additionally, expression of centriolar protein Tektin-2 (TEKT2) is decreased in cryopreserved human sperm when compared to fresh sperm, which may contribute to the loss of motility observed in cryopreserved sperm (Alshawa et al. 2019). An antisperm antibody that targets TEKT2 was also found in samples from infertile men (Zangbar et al. 2016).

Immunohistochemistry

Immunohistochemistry (IHC) is a staining technique that allows for the visualization of specific proteins within a tissue. IHC uses antibodies, similar to Western Blots; however, rather than labeling proteins within a lysate, samples are sectioned and mounted on a microscope slide (Erdogan et al. 2005). IHC allows the proteins to remain in their original distribution within cells, which enables the precise localization. However, IHC is a subjective technique that relies on high signal-to-noise ratio and quantitative methods are relative, not absolute, which makes these experiments difficult to replicate (Walker 2006). Furthermore, its use for analyzing sperm centrioles is very limited because of their size and IHCs diffuse staining, but the complete absence of centrioles and the axoneme was visible in spermatids with spermatogenetic arrest (Aumuller et al. 1987).

Immunofluorescence with Classic or Super-Resolution Microscopy

Similar to IHC, Immunofluorescence is a staining technique that allows for the precise localization of a protein in a cell and can determine if the protein is present in the centrioles, but it exhibits more focused staining and yields higher resolution images. Immunofluorescence also allows for quantification, although normalization is needed to account for experiment-to-experiment variation (Petrunkina and Harrison 2013). While immunofluorescence is technically and logistically feasible in most clinics, it is rarely used in diagnostic tests and standards have not been developed to diagnose sperm centriole defects. The lack of use in clinics is partially due to the reliance on highly specific, expensive antibodies that require optimization, especially in centrioles, due to the dense proximity of proteins and epitope masking. Using classic microscopy, immunofluorescence can be used to localize proteins to a centriole, but because of the small size of centrioles (~200 nm wide), it is difficult to determine the localization of proteins to specific substructures inside a centriole because the resolution of light microscopy is limited by the wavelength of the light (~400–700 nm, depending on the fluorophore used, and the size of the antibody). Super-resolution can circumvent this limit, and provide a higher resolution (up to 10 nm), but super-resolution microscopes are not yet widely available, they require extensive optimization, and they are much more time consuming than other light microscopy techniques.

Flow Cytometry

Flow cytometry is similar to immunofluorescence in that cells are fixed and stained with a fluorescently labeled antibody against a specific protein, but flow cytometry provides the ability to quickly quantify many cells. Stained cells are placed into a flow cytometer that passes each individual cell through a laser. The level of fluorescence within the cell is automatically measured and photos can be taken to allow for morphological analysis. Flow cytometry's main advantage is its speed and precision: millions of cells can be analyzed quickly and protein quantification and cell sorting can be automated, thus reducing error and subjectivity.

Flow cytometry has not been performed on sperm centrioles yet, but it has been performed in a post acrosomal bovine sperm protein (Kennedy et al. 2014). Sperm were categorized based on levels of fluorescence and then subcategorized based on morphology. This study was able to correlate protein levels with certain sperm morphology and conception rate, thus exemplifying how flow cytometry could be used for sperm diagnostics in the future.

Altogether, the techniques available for the fine localization and quantification of centriolar proteins are improving, but each technique has its own benefits and limitations. The combinatory approaches currently used are not feasible for regular clinical applications, so there is a significant need to develop these techniques to make them feasible for clinical applications. Finally, the described techniques

involve treatment (fixation or lysis) of the sperm that renders it unusable for fertilization, so these techniques are strictly able to determine correlation, which could improve diagnostics. The development of techniques that do not interfere with the viability of the sperm would allow for sperm selection and thus will become important as we learn more about what characteristics make one sperm better than another for IVF.

Final Remarks

Despite that sperm centrioles have been studied for more than two centuries, many aspects of their structure, function, and precise role during reproduction remain unclear. With recent technological advancements, centrioles are emerging as a hotbed of novel biology during reproduction and development that will have major implications for human reproductive health and agricultural production. As a result, there is a need for direct studies comparing the biology of sperm centrioles in various animal groups, especially with humans. It is essential to develop new and improved techniques to uncover the role of the centriole during sperm movement, fertilization, and embryo development.

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Adaptive Modifications of the Regulation of Sperm Motility in the Diversification of Reproductive Modes of Amphibians

A. Watanabe and E. Takayama-Watanabe

Background: In amphibians, morphologically and physicochemically diversified egg coats, such as egg jelly and foam nest, are significant in the evolution of various reproductive modes, while sperm motility and signaling path to regulate it should be adaptively modified to penetrate the egg coat with a species-specific nature. Hyposmolality of freshwater and/or a cysteine knot protein called SMIS initiates and enhances motility, allowing amphibian sperm to penetrate egg coat.

Main Questions: Purpose of the present work is to understand how signaling path for the regulation of sperm motility has been modified in adaptation to a specific reproductive mode in amphibians.

Experimental Design: Different responses to SMIS and hyposmolality were examined using an active site peptide of SMIS in the sperm of *Cynops pyrrhogaster*, *Buergeria japonica*, and *Rhocophorus species* that undergoes internal fertilization, external fertilization in freshwater, and that on a tree, respectively. RNA-seq of the spermatogenic testes and pharmacological study were performed to examine Ca^{2+} permeable channels that mediated the signaling path for the initiation and enhancement of sperm motility.

Main Results: Sperm of *C. pyrrhogaster* initiated and enhanced motility by SMIS in an isotonic solution, whereas the SMIS enhanced motility of *R. arboreus* sperm only in a hypotonic solution. *B. japonica* sperm initiated and enhanced motility only by hyposmolality. From RNA-seq, *C. pyrrhogaster* sperm possessed multiple Ca^{2+}-permeable channels including a T-type voltage-dependent Ca^{2+} channel, Cav3.2, and an osmolality-sensing cation channel, TRPV4. In pharmacological study in *C. pyrrhogaster* sperm, Cav3.2 and TRP channel were suggested to be critical for the SMIS-induced motility. TRP channel but not Cav3.2 is suggested to

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mediate the regulation of motility in *B. japonica* sperm. In *R. schlegelii* that undergo external fertilization on a tree, TRP channel was critical for hyposmolality- and SMIS-induced motility whereas T type voltage-dependent Ca^{2+} channel mediated only hyposmolality-induced initiation.

Conclusions: In the diversification of reproductive mode from external fertilization in freshwater to arboreal fertilization and internal fertilization in amphibian evolution, TRP channel is critical in the hyposmolality-triggered signaling for sperm motility and involves the SMIS-triggered one in the advanced reproductive modes. SMIS-triggered signaling seems to add a specific feature to the regulation of sperm motility, which contributes to the achievement of the advanced reproductive modes in amphibians.

Part V
Genetic Aspects of Sperm Production and Performance and its Effects on the Offspring

Advances in genetic methodology opens new possibilities to understand normal and abnormal sperm structure and function. Björn Afzelius discovered dynein arms in 1958 and described the immotile-cilia-syndrome 20 years later. Also other ciliary defects can cause infertility and structural defects should be traced to the genetic origins. Other aspects involve how selection of haploid germ cells can influence the offspring, and how.

When Cilia Go Bad: The Complex Genetics of Ciliopathies

Anna Lindstrand

Disruption of ciliary and basal body function has been associated with a growing number of human genetic disorders, collectively termed ciliopathies. Cilia can roughly be divided into motile or non-motile cilia. Motile cilia can be observed in sperm and airway epithelial cells, while immotile cilia are often referred to as primary cilia and are observed in photoreceptor cells or olfactory neurons. Although clinically distinct, ciliopathies manifest similar hallmark clinical features including retinal degeneration, renal and pancreatic cysts, liver fibrosis, polydactyly and other skeletal abnormalities, situs inversus, male infertility due to defective sperm locomotion or spermatogenic failure, and central and peripheral nervous system defects. More than 30 different ciliopathies have been reported and almost 200 ciliopathy-associated genes are known. Furthermore, genetic studies have indicated that many ciliopathy-associated genes can contribute to different ciliopathy disorders.

Utilizing a custom designed oligonucleotide array–CGH (aCGH) targeting the coding exons of ~2000 genes of interest we have studied the genetic contribution of copy number variations (CNVs) throughout a spectrum of ciliopathies. In this way, we have identified several novel genes such as ARMC4 underlying Primary Ciliary Dyskinesia (PCD, MIM244400), IFT74 in Bardet–Biedl syndrome (BBS, MIM 209900) and WDR63 in isolated encephalocele. Furthermore, we have shown that CNVs contribute pathogenic alleles to a substantial fraction of affected individuals both as primary disease drivers but also play a critical role in modifying disease penetrance and expressivity.

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

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Introduction

Sperm are one of the most specialised and diverse cell types studied and vary across species, across individuals and also within individuals (Birkhead et al. 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 and 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 and 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 and

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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 et al. 2007; Immler et al. 2008; Stewart et al. 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 et al. 2018; Rathje et al. 2019; Bhutani et al. 2019); reviewed in Immler (2019) and Joseph and 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 and 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 h 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 et al. 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. 2020), 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 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 humans.

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 and 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 (Fig. 1). The possible negative effects of cryopreservation on sperm quality are well known (e.g. O'Connell et al. 2002; Zribi et al. 2010), and include anything from changes in the membrane structure,

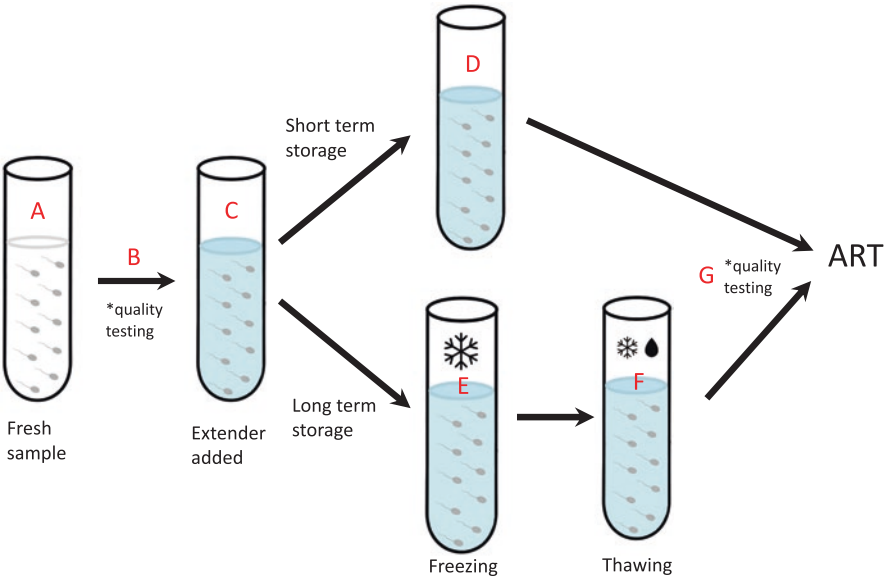

Fig. 1 Major steps from sample collection to ART with putative effects on sperm presented *in italics* in brackets after each step (positive traits marked as +, negative traits marked as: − or unclear marked as ±). (A) Sample collection occurring by natural stimulation (ejaculate should be in its natural condition +) or electro-stimulation (*may alter ejaculate composition in terms of concentration* ±, *seminal fluid* −); (B) tests on general ejaculate quality such as overall volume, sperm concentration, 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* ±)

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 on 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 et al. 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.

Fertilisation success and successful pregnancy rate in ART are 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 and Braude 1984) are widely employed prior to human ART (World Health 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 et al. 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 towards 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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Heads and Tails: Requirements for Informative and Robust Computational Measures of Sperm Motility

Gemma Cupples, Meurig T. Gallagher, David J. Smith, and Jackson C. Kirkman-Brown

Introduction

Motility is undoubtably essential for sperm to navigate the female reproductive tract and successfully reach the site of fertilisation. Despite this importance, routine clinical assessment of sperm motility is generally limited to a manual appraisal, with the aim of calculating the proportion of cells that fall into the categories of being progressively motile, non-progressively motile or immotile (as set out by the WHO V guidelines (World Health Organisation 2010)). Manual assessment is difficult for a number of reasons, not least because sperm are very small and fast moving (swimming at speeds of multiple body lengths per second), with their rapidly moving flagellum, which propels the cell, being impossible to assess in any quantitative way.

To address the difficulties in obtaining reproducible and accurate manual assessment, computer-aided sperm analysis (CASA) systems have been developed over the past few decades (Hiramoto and Baba 1978; Katz and Overstreet 1981; Holt et al. 1985), and then rapidly progressed by both academic and commercial entities. However, CASA systems are not considered sufficiently informative for clinical

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practice, in no small part due to the lack of evidence linking current motility measurements and prognosis (for a review, see Lewis 2007).

In order to develop a deeper, quantitative understanding of how sperm motility affects the fertilising capability of sperm, we must first address the significant variation in how CASA is used, including how samples are prepared and imaged, and how the resulting data are analysed. Without consistency of approach, the aim of calculating robust motility results will remain out of reach. Further, it may be that existing measures do not provide the physiological insight necessary for understanding sperm motility. Throughout this manuscript, we will highlight how the development of computational motility analysis to include tracking and assessment of the flagellar waveform may provide more insight into the links between kinematic motion and fertility.

Traditional CASA motility systems work by tracking the path traced out by the heads of sperm as they swim. In each frame, the pixels belonging to the head of each sperm are identified, the centroid assigned as the "location" of the cell, and centroids in subsequent frames are connected to form a path. From these paths, various characteristic measures can be calculated (see Fig. 1 for a sketch showing some of the most commonly used measures and Gallagher et al. (2019) for detailed explanations). While traditional CASA systems have many advantages over manual analyses for *motility* assessment (for comparisons between CASA and manual assessment for other types of analysis such as count or morphology, see Talarczyk-Desole et al. (2017)), the lack of agreed methods for calculating each of the CASA motility parameters remains a significant barrier to obtaining consistent CASA-calculated results across systems and centres.

In order to obtain more informative measures, systems need to go beyond the *what* of sperm locomotion (progressive velocity, path linearity, etc.) and investigate the *how*, through capturing the details of the rapidly varying flagellar waveform as a cell swims. Pioneered in the late 1970s (Hiramoto and Baba 1978), with significant recent advances (Hansen et al. 2019; Walker et al. 2019), flagellar analysis systems have been used to great effect to investigate the detailed beat of collections of tens of sperm, although numbers have been limited by the significant manual input that has been required. With the release of the FAST package (Gallagher et al. 2019), we can now perform high-throughput characterisation of populations of sperm resolved at the cell level and seek to improve the physiological relevance of computational measurements of sperm motility.

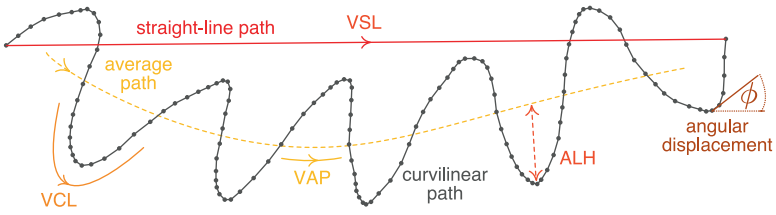

Fig. 1 A sketch highlighting some of the most common CASA measure terminology

Computational motility analysis of sperm has the potential to provide more insight than traditional manual analysis (van der Horst and du Plessis 2017), in addition to producing results that are significantly more reproducible and robust across operators and locations. In this paper, we will highlight how new techniques such as flagellar tracking can bring more informative measures to assessment, as well as the requirements needed for preparing, imaging and analysing samples to obtain robust computational motility results.

Preparation Matters

Human sperm encounter a wide range of fluidic environments on their journey to fertilisation; from within semen undergoing liquefaction, to penetrating cervical mucus, these varying environments can have a dramatic effect on motility. Perhaps the most significant factor is the viscosity of the surrounding fluid, which is well known to induce changes in the characteristic waveform of human sperm (Katz and Berger 1980; Smith et al. 2009b), and thus impact the yaw and amplitude of the resulting sperm track (see Fig. 2).

Preparing sperm in a high-viscosity mucus analogue may give the best practical indicator of whether sperm can swim progressively in the physiological environment (Ola et al. 2003). An open question is whether the ability to penetrate mucus can be predicted from motility characteristics in saline, for example through combining lower viscosity data with mathematical modelling. This capability could form an appealing basis for a clinically feasible test with greater diagnostic power. The ability of sperm to swim in semen may also be of interest, although due to the variation in seminal viscosity both within and between men, comparisons between results must be performed with care. In all cases, details of the preparation protocol should be reported alongside the computational motility results.

Control of sample temperature is important to ensure consistent motility results (Esfandiari et al. 2002). As such we suggest that samples and fluid media are kept in an incubator and, later, imaged on a heated slide at 37 °C for consistency. The ability of sperm to hyperactivate (a change in motility induced by capacitation) is also affected by temperature, with sperm populations imaged at 37 °C showing significantly increased rates of hyperactivation than those at 20 °C (Mafın-Briggiler et al. 2002).

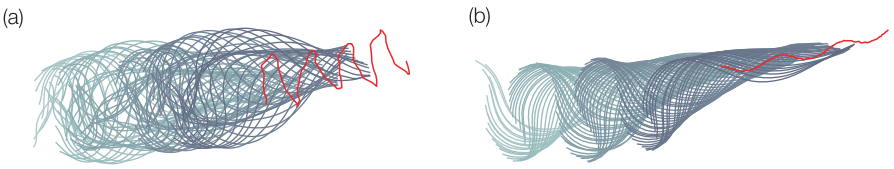

Fig. 2 Characteristic flagellar waveform and track for human sperm swimming in (**a**) low-viscosity media and (**b**) high-viscosity mucous analogue. In each panel, the red line shows the path traced out by the centroid of the sperm head as it swims

It is not only direct fluid effects that can hinder the accuracy of computational motility analyses. When imaging in semen (or diluted semen), the presence of excessive debris may lead to other objects being misidentified as sperm, which can also be a problem when trying to assess samples that are too concentrated. It is particularly important to consider the concentration of a sample when the aim of the assessment is to track the flagellar beat; the separation of crossed sperm flagella is a computationally difficult problem to solve and is beyond the reach of current flagellar tracking systems.

Imaging Matters

Sperm imaging for computational assessment of motility is typically performed in one of two types of observation slides: capillary-loaded 20 μm deep disposable chambers and drop-loaded reusable slides. The restricted depth of the former mitigates the amount of rolling of swimming cells. As the analysis performed by current systems assumes that sperm swim in a planar fashion, excessive rolling induces errors when calculating motility results, especially when attempting to track the planar projection of the waveform. When acquiring data to be used for flagellar analysis, shallower (10 μm deep) chambers constrain sperm to swim close to the walls (known to promote planar beating of cells (Nosrati et al. 2015)), thus enabling more accurate tracking of the flagellar waveform. However, the reduced volume in these chambers is a barrier to achieving accurate results for other types of analysis, such as sperm count (Tomlinson and Naeem 2018). Obtaining a characteristic sample from an inhomogeneous preparation must be done with care, and exactly how sampling effects are influenced by the small volume of fixed-depth chambers is unknown and warrants detailed investigation.

It is not only the preparation of cells for imaging that can affect results; variations in imaging protocols can have equally significant effects, particularly the choice of frame rate, imaging duration and image quality. Each of these parameters must be carefully reported alongside motility results to ensure that any comparisons within and between systems are fair and robust. While it is clear how image quality can impact results (the resulting images must have sufficient contrast for the objects of interest to be detected reliably, be that head or flagellum, and sufficiently low noise), it is also important to understand exactly how other factors can change the results of motility analysis.

An important quantity to be aware of when imaging oscillatory systems is the Nyquist rate (Nyquist 1928), defined as twice the maximum frequency of the object being imaged (in the present work, either the moving head or beating flagellum of a swimming sperm). The Nyquist rate sets a lower bound for recording sufficient information to reconstruct a signal using a Fourier series. Human sperm beat up to ≈30 Hz, so the image acquisition rate needs to be at least ≈60 Hz. It should also be noted that, while the flagellar beat frequency is the fundamental frequency driving movement, the details of a piecewise-linear reconstructed path will have higher

modes, necessitating the use of higher acquisition frame rates to capture the finer details. These are not new observations; the effect of varying frame rate on motility assessment has been known for many years (Mortimer 2000). Despite this knowledge, many systems image below this rate, with common sampling frequencies being 25, 30, 50 or 60 Hz (Bompart et al. 2018).

Figure 3 highlights the substantial effect frame rate can have on the apparent path traced out by a sperm head. In this figure, we show a section of the path traced out by a human sperm imaged in a 10 μm deep chamber at a frame rate of 169 Hz and analysed in FAST (Fig. 3a). In panels (b)–(d), subsampled paths highlighting what would have happened if acquisition was to be carried out at reduced sampling frequencies of 100, 60 and 30 Hz followed by piecewise linear construction are shown. At 100 Hz, there is very little noticeable difference in the reconstructed path, with the majority of the detail still visible at 60 Hz. When the sampling frequency is reduced to 30 Hz, below twice the BCF of the cell (21.4 Hz), only the gross structural details of the path remain. Such dramatic changes in the track can have implications for the calculation of all derived measurements.

Surprisingly, the duration of capture can be as impactful on the accuracy of the motility results as the acquisition frame rate. As an example, consider the straight-line velocity (VSL) of a swimming sperm. If the cell's track is relatively straight, then VSL will remain approximately constant for increasing imaging duration. If, however, a cell is circling (such as the example in Fig. 4), this is not the case. In Fig. 4b, we plot VSL for this circling sperm, calculated over periods of increasing duration up to 2.5 s. Ignoring the small amplitude, rapid oscillations due to the beating flagellum, we observe that the calculated value of VSL decreases almost linearly with sampling duration, from what could be considered to be a fast progressive sperm, with an apparent progressive velocity of 39 μm/s after 0.5 s of imaging, to a cell which could be considered as non-progressive, with a speed of 4.6 μm/s after 2.5 s. Such a significant change in this example cell (which is swimming at an approximately constant

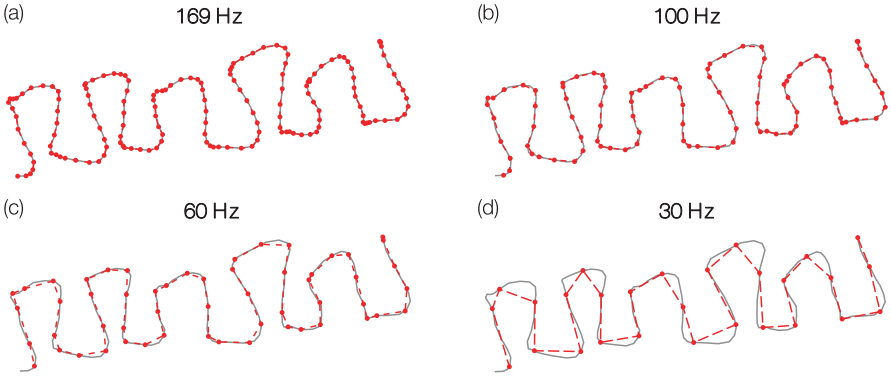

Fig. 3 Effect of acquisition frame rate on the path drawn out by a single sperm. In each panel, the location of the cells in each frame is shown as a dot, and the solid grey lines show the high-resolution path captured at 169 Hz

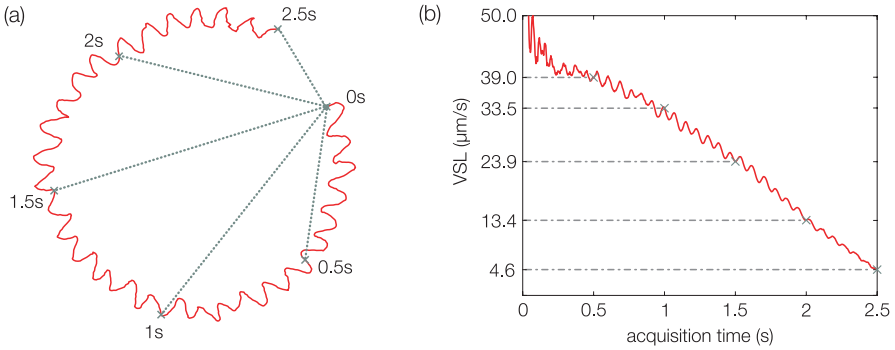

Fig. 4 (**a**) Actual path (red line) and straight-line displacement (grey lines) for a circling sperm at increasing half-second time increments. (**b**) Variation in VSL for a circling sperm against acquisition time

curvilinear speed) highlights the need for consistency when comparing results both within and between systems. When taken with the frame rate variations discussed above, these results render comparisons between CASA motility parameters, for data taken with varying frame rate and duration, largely impossible.

Data Analysis Matters

Achieving informative results relies on more than preparation and imaging; equally as important is the use of algorithms that are accurate, reproducible and robust to experimental procedures. In this section, we will highlight the significant effect that data analysis can have on subsequent motility measures, and how best to get consistency in calculation for robust results.

As discussed in section “Imaging Matters”, small changes to protocols can have a big effect on results (see acquisition frame rate and VSL calculation); the same is true of algorithmic changes, such as using a different method to approximate an average path from centroid data. While it is undeniable that individual CASA systems should be able to produce motility results that are significantly more consistent than those from a manual analysis, the lack of agreed methods for calculating kinematic measures means that comparisons between results from different systems are not straightforward.

The measurement that is most sensitive to the choice of computational method is the average path. As sperm swim, the track traced out by their head centroid can be decomposed into two components, a left–right oscillatory motion and a long-time progression (average path) of the cell, each easily discernible by eye. Once a reliable average path is calculated, many more measures of motility can follow including calculations of average path velocity (VAP), and the amplitude and rate of oscillatory motion (amplitude of lateral head displacement, ALH and beat-cross frequency, BCF, respectively).

In order to computationally extract the average path, we must first consider what a "good" reconstruction would be; our aim is to smooth oscillatory behaviour induced by the beating flagellum, while retaining long-term variation in the path. We do not, for example, wish to over-smooth and obtain the straight-line path connecting the first and last points in the track, nor should the average path follow exactly the curvilinear path of the head. The desired algorithm, therefore, is one that yields such a "good" reconstruction and is robust to variations in experimental protocol.

Many methods of calculating an average path can be very sensitive to a choice of parameters, the number of points available (acquisition frame rate) or the temporal variability in the path. A common algorithm used in some CASA systems is the moving average (first developed by Hooker 1901 to smooth yearly oscillations in marriage rates and reveal long-term trends). The moving average involves smoothing the path by replacing each point with the average location of itself and its surrounding neighbours. Given a tracked sperm path $\boldsymbol{p}$, the ith point on the smoothed path $\overline{p}_i$ can be calculated using an n-point centred moving average via

$$\overline{p}_i = \frac{1}{n} \sum_{j=i-(n-1)/2}^{i+(n-1)/2} p_j, \tag{1}$$

for a choice of odd-valued n, with appropriate conditions for calculating the first and last few points. If choosing an even-valued n, an additional point may be considered from the direction of either the start or end of the track. In practice, values of n in the range 3–15 are commonly used (Mortimer et al. 2015). The exact choice of n is significant in determining the path and must be selected with respect to both the acquisition frame rate and the oscillatory properties of the particular trajectory. The results of calculating the average path for two choices of n and four choices of acquisition frame rate are shown in Fig. 5, panels (a) (11-point smoothing) and (b) (30-point smoothing), whilst panels (d) and (e) show the results of the same smoothing algorithms on the 169 Hz track of a different sperm for comparison. It is clear from these figures that there must be a direct relationship between the number of

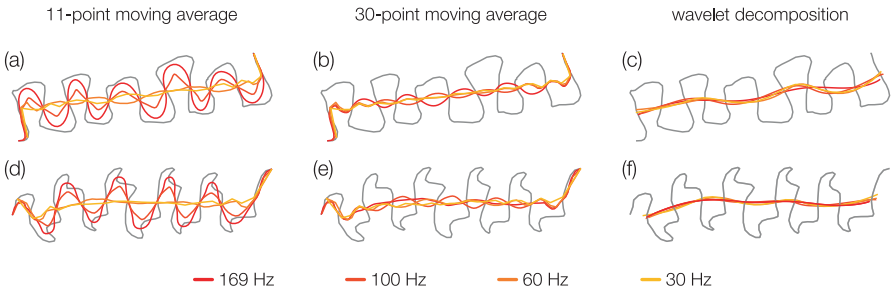

Fig. 5 Comparison of average path calculation methods for a selection of acquisition frame rates and sperm. Panels (**a**)–(**c**) show the effect of diminishing frame rate on the average paths using (**a**) an 11-point moving average smoothing, (**b**) a 30-point moving average smoothing and (**c**) a wavelet decomposition. Panels (**d**)–(**f**) show the same calculations for a different sperm track for comparison

points captured in the track and the choice of n to ensure the calculation of a path resembling that which would be picked out by eye. Neither choice of n in Fig. 5a, b, d, e manages to smooth out all of the path oscillations.

In FAST, an alternative method of calculating the average path is employed, that of wavelet decomposition. Wavelets provide robustness for analysis and have been used in a wide range of biomedical applications (Unser and Aldroubi 1996; Brechet et al. 2007; Rafiee et al. 2011), although they do not appear to have been widely employed for CASA. Using similar concepts to the well-known Fourier transform, which can be used to decompose a signal into constituent trigonometric functions in the frequency domain, the wavelet transform can be used to decompose a signal into translations and dilations of a given wavelet function (we will use the discrete Meyer wavelet, Fig. 6a). There are many works providing a comprehensive

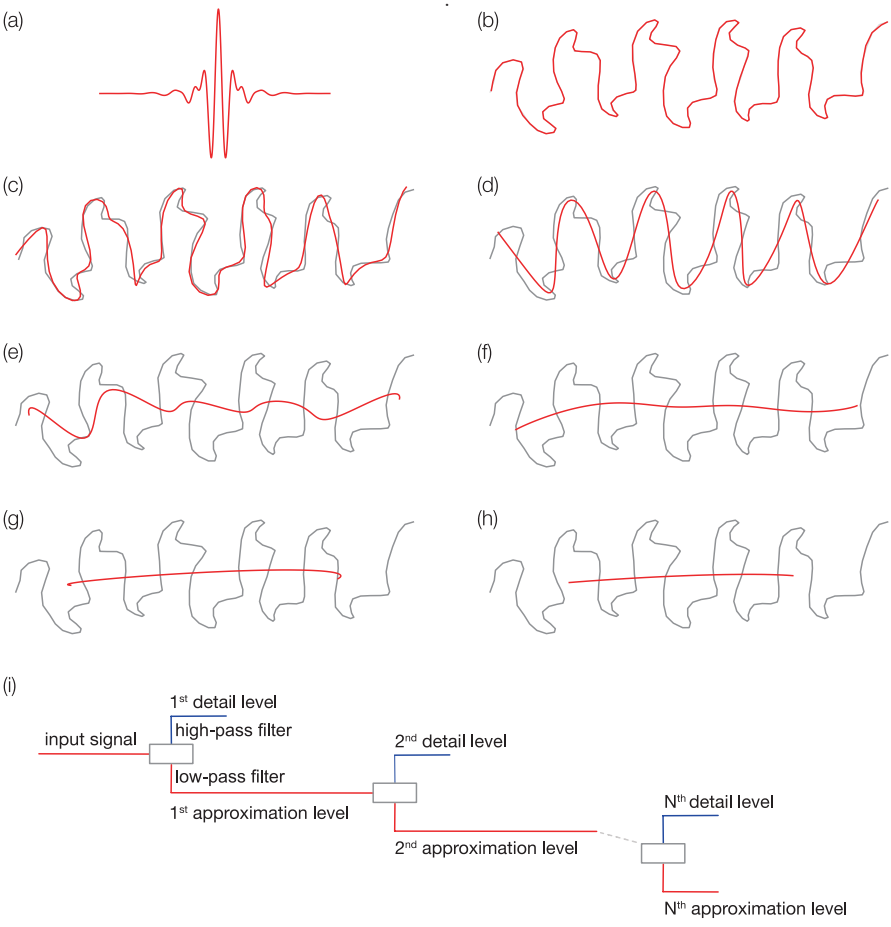

Fig. 6 The wavelet decomposition for extraction of the average path. (**a**) Shows the discrete Meyer wavelet and (**b**)–(**h**) show the reconstructed path with decreasing levels of detail associated with approximation levels 1–7. Finally, (**i**) contains a sketch of the steps in the discrete wavelet decomposition

Table 1 VAP calculations (μm/s) for the sperm tracks shown in Fig. 5 using 11-point moving average, 30-point moving average and wavelet decomposition

Cell	Smoothing type	169 Hz	100 Hz	60 Hz	30 Hz
i	11-point moving average	84.3	57.5	46.3	44.8
	30-point moving average	45.6	46.6	46.0	44.5
	Wavelet decomposition	35.5	36.9	37.5	37.8
ii	11-point moving average	136	98.8	71.2	69.7
	30-point moving average	73.6	77.3	73.0	70.3
	Wavelet decomposition	53.3	55.3	55.9	57.8

understanding of the wavelet transform and its uses (see the excellent textbook by Mallat 1999); here we shall endeavour to provide a brief overview focusing on how discrete wavelet decomposition can be used to achieve an average path that is robust to acquisition protocol and oscillatory frequency of the cell.

The discrete wavelet transform works by repeated application of N low- and high-pass filters to a signal, extracting high-frequency components (known as detail levels) and leaving low-frequency components (known as approximation levels). After filtering, the remaining signal is subsampled, with the new signal containing half as many points. This process can be repeated to achieve the desired N levels, providing the original signal has at least 2^N points. In the example in Fig. 6, we have decomposed a sperm track using the discrete Meyer wavelet over seven levels. The full wavelet approximation is shown in Fig. 6b, with panels (c)–(h) showing the reconstruction after removing the high-frequency components at levels 2–7 respectively. In this example, we see that discarding the first four detail levels, and reconstructing, yields a signal containing the long-term drift of the cell, but without the oscillatory behaviour. The number of levels can be set as the maximum available (recalling that an N level decomposition requires at least 2^N points), and discarding the four highest levels of detail provides results that are robust to the frame rates tested in this paper (see Fig. 5c, f).

Table 1 details the VAP calculations for the paths shown in Fig. 5. We see that the calculation of VAP using an 11-point moving average has the most significant dependence on frame rate, decreasing from ≈85 μm/s (169 Hz) to ≈45 μm/s (30 Hz), with the calculations using the 30-point moving average and wavelet decomposition being almost independent to changes in frame rate. While the VAP calculation appears to be robust for this cell, visual examination reveals a significant qualitative change in the average path for the 30-point moving average. The wavelet reconstruction, however, is robust *both* for VAP *and* for the qualitative nature of the resulting average path, providing a much-improved method for this purpose.

Mechanics Matters

The ability to directly track and analyse the flagellar beat of large numbers of cells, provided by FAST, for example, allows for measures of sperm motility with greater mechanistic, and potentially also physiological, relevance, which may be more

informative than head-track derived parameters. It has now been shown that the CASA measure BCF (beat-cross frequency), originally intended to be a proxy measure for flagellar beat frequency, does not agree, indeed *does not even correlate*, with the directly measured beat frequency of human sperm (Gallagher et al. 2019), highlighting the need for direct tracking of the flagellum. Further measures, such as the wave speed or wavelength of the flagellar beat, provide deeper insight into the propulsive mechanisms of sperm, with the direct links between fertility outcomes and these parameters being an area of active investigation.

Combining mathematical modelling with tracked waveform data from human sperm enables the calculation of a range of experimentally intractable quantities; one potentially important quantity is the metabolic requirements of motility. There are many methods for approximating the rate of working of a beating flagellum on the surrounding fluid, from simple, but reasonably accurate, implementations of the resistive force theory of Gray and Hancock (1955), to more involved numerical calculations using regularised stokeslet (Gillies et al. 2009; Gallagher and Smith 2018), boundary element (Phan-Thien et al. 1987; Smith et al. 2009a; Ishimoto et al. 2018), force coupling (Delmotte et al. 2015b) or immersed boundary methods (Dillon et al. 2003). Figure 7 shows the power exerted on the surrounding fluid by cells swimming in low- and high-viscosity fluids using resistive force theory.

As first noted by Machin (1958), the active force generation for sperm propulsion must be generated throughout the flagellum, rather than being localised to the head/neck junction. It has since been shown that this force generation is due to the activity of dynein motors acting on the flagellar ultrastructure, which consists of the well-known "9 + 2" arrangement of microtubules (Afzelius 1959; Fawcett and Porter 1954), which slide past each other to bend the flagellum (Satir 1968). Detailed experimental works have probed the function of these motors using very high resolution, fixed-cell procedures such as cryo-electron tomography to great effect, with recent findings highlighting that flagellar motility is driven by an asymmetric distribution of dynein activity on opposite sides of the flagellum, causing the characteristic bending waveform (Lin and Nicastro 2018). Light microscopy imaging can, in

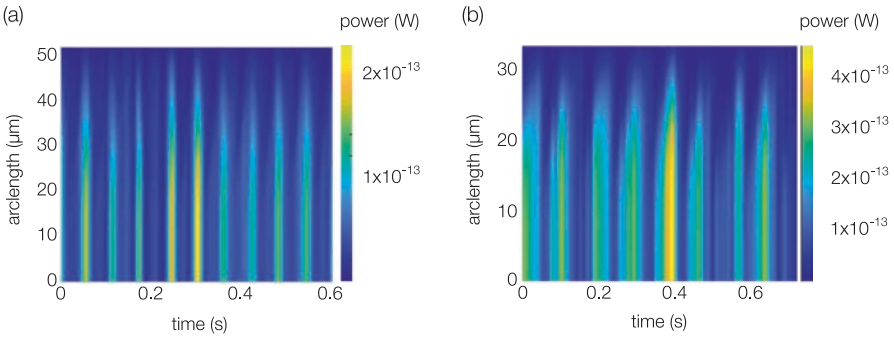

Fig. 7 The power exerted by the flagellum on the fluid by the section of the flagellum distal to the point in arc length chosen against time. (**a**) and (**b**) show a sperm swimming in low- and high-viscosity fluid environments respectively

combination with modelling, yield information on dynein activity and its dynamic modulation, such as through informing the application of active moments to induce sperm-like bending (Neal et al. 2020). Using methods designed to probe the elastic behaviour of filaments (Hall-McNair et al. 2019; Schoeller et al. 2021; Delmotte et al. 2015a), we can explore the internal workings of sperm motility, aiming to calculate parameters relating to good mechanical function directly from imaging data.

Most of the discussion above has focused on progressive motility, however as discovered by Yanagimachi (1970) in hamster sperm, a mammalian sperm's ability to hyperactivate is an essential component for successful fertilisation (Suarez 2008). This phenomenon has been widely studied both experimentally (Wolf et al. 1986; Stauss et al. 1995; Ohmuro and Ishijima 2006; Kaneko et al. 2007; McPartlin et al. 2009; Boryshpolets et al. 2015) and through mathematical modelling (Olson et al. 2011; Curtis et al. 2012; Ooi et al. 2014; Ishimoto and Gaffney 2016).

Many groups have investigated the use of head-tracking CASA technology to identify hyperactivated sperm (Cancel et al. 2000; Goodson et al. 2011; van der Horst et al. 2018), particularly through identifying subpopulations of cells via the fractal dimension of the tracked paths (Cancel et al. 2000; Goodson et al. 2011; van der Horst et al. 2018). However, the changes in these head tracks originate in a dramatic increase in asymmetry and amplitude of the flagellar beat.

The ability to capture and analyse the flagellar waveform of large numbers of motile cells will form the basis for robust characterisation of sperm that have hyperactivated motility. Additionally, the capability to track the waveform over long time periods allows for investigation into the behaviour of cells as they switch between *active* and *hyperactive* states (Achikanu et al. 2019), which may shed new light on the underlying causes that drive this biochemical process. A further desirable goal is the prediction of the potential of individual cells to undergo hyperactivation from characteristics of their activated (i.e. "pre-hyperactivated") progressive motility.

Conclusions

The use of computational analysis for sperm motility has great potential for assessing male fertility. However, in order to unlock this potential, it is essential that computational assessments are conducted with the same level of rigour as a manual analysis. In this chapter, we have highlighted what we consider to be the requirements for robust measures of computational sperm motility, including how samples must be prepared and imaged and how resulting data should be analysed, with particular focus on the opportunity afforded by the use of signal processing techniques such as wavelet analysis. As robust and informative measures of sperm motility are clarified, the parallel development and application of state of the art methods for data visualisation to convey the relative importance of the results, particularly in a clinical environment, will be of interest. One example is through specialised multivariate visualisation techniques such as glyphs (Duffy et al. 2013; van der Horst

et al. 2018). Finally, we believe that routine flagellar analysis will provided an integrated measure of sperm quality, encompassing more informative baseline measures of motility, and revealing details of cell metabolism and signalling that will have strong diagnostic power. For understanding the fertilisation capable, or even live-birth-suitable, sperm, this type of knowledge will provide a new stronger bedrock for future developments.

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Ethical approval: All donors were recruited in accordance with the Human and Embryology Authority Code of Practice (version 7) and gave informed consent (South Birmingham LREC 2003/239 and East Midlands REC 13/EM/0272).

Conflict of interest: The authors declare no competing interests.

Appendix

The characteristic sperm tracks presented in this paper are taken from semen samples provided by unscreened normozoospermic donors recruited at Birmingham Women's and Children's NHS Foundation Trust after giving informed consent. Full details of the experimental procedures for each cell shown in Figs. 1–7 can be found in this appendix.

Figure 1—Cells were suspended in supplemented Earle's balanced salt solution (sEBSS) without phenol red, and supplemented with 2.5 mM Na pyruvate and 19 mM Na lactate (06-2010-03-1B; Biological Industries, Kibbutz Beit HaEmek, Israel), and 0.3% weight/volume charcoal delipidated bovine serum albumin. Cells were imaged in a 10 μm depth chamber (10-01-04-B-CE; Leja Products B.V., Nieuw-Vennep, The Netherlands) using an Olympus (BX-50) microscope and negative-phase contrast microscopy (objective 20× 0.40 Ph1 BM ∞/0.17 WD 1.2), and recorded using a Hamamatsu Photonics C9300 CCD camera (pixel size 7.4 μm at 285.2 Hz).

Figure 2—(a) Cells were selected using a swim-up, where a 500 μL aliquot of supplemented Earle's balanced salt solution (sEBSS) without phenol red, and supplemented with 2.5 mM Na pyruvate and 19 mM Na lactate (06-2010-03-1B; Biological Industries, Kibbutz Beit HaEmek, Israel), and 0.3% weight/volume charcoal delipidated bovine serum albumin was placed in a 5 mL round-bottom tube (Corning, Falcon 352058). A 300 μL aliquot of semen was pipetted to the bottom of the tube, inclined and left in the incubator for 30 min at 37 °C. Cells were imaged in a 10 μm depth chamber using a Nikon (Eclipse 80i) microscope and

negative-phase contrast microscopy at 10× magnification (10× 0.2 Ph1 BM ∞/-WD7.0) and an Andor Zyla 5.5 (Andor, Oxford UK) microscopy camera at 200 Hz with pixel size 6.5 µm × 6.5 µm.

Figure 2—(b) Cells were suspended in supplemented Earle's balanced salt solution (sEBSS) without phenol red and supplemented with 2.5 mM Na pyruvate and 19 mM Na lactate (06-2010-03-1B; Biological Industries, Kibbutz Beit HaEmek, Israel), and 0.3% weight/volume charcoal delipidated bovine serum albumin, with the addition of 1% methylcellulose (M0512, Sigma-Aldrich, Poole, UK, specified so that an aqueous 2% solution gives a nominal viscosity of 4000 centipoise or 4 Pa s at 20 °C). The cells were loaded by capillary action into flat-sided borosilicate capillary tubes (VITROTUBES, 2540, Composite Metal Services, Ilkley, UK) with length 50 mm and inner dimensions 4 × 0.4 mm. One end of the tube was sealed with CRISTASEAL (Hawksley, Sussex, UK #01503-00). Cells were selected for imaging by immersing the open end of the capillary tube into a 1.5 mL Beem capsule (Agar Scientific, UK) containing a 200 µL aliquot of raw semen, within 30 min of sample production. Incubation was performed for 30 min at 37 °C in 6% CO_2. Cells were imaged at 2 cm migration distance into the capillary tube and in the surface accumulated layer 10–20 µm from the inner surface of the capillary tube at 10× magnification using a Nikon (Eclipse 80i) microscope and negative-phase contrast microscopy and an Andor Zyla microscopy camera (pixel size 6.5 × 6.5 µm) at 200 Hz.

Figure 3—The sample was counted according to WHO guidelines (WHO 2010) and diluted to a concentration of 10 M/mL in of supplemented Earle's balanced salt solution (sEBSS) without phenol red, and supplemented with 2.5 mM Na pyruvate and 19 mM Na lactate (06-2010-03-1B; Biological Industries, Kibbutz Beit HaEmek, Israel), and 0.3% weight/volume charcoal delipidated bovine serum albumin. Cells were imaged in a 10 µm depth chamber using a Nikon (Eclipse 80i) microscope and negative-phase contrast microscopy (objectives 10× 0.2 Ph1 BM ∞/-WD7.0), using a Basler Microscopy ace camera (acA 1300-200uc) at 169 Hz with pixel size 4.8 × 4.8 µm, using Pylon Viewer (v.5.0.11.10913, Basler AG, Ahrensburg, Germany).

Figure 4—See Fig. 1.

Figure 5—See Fig. 3.

Figure 6—See Fig. 5.

Figure 7—(a) See Fig. 2a.

Figure 7—(b) See Fig. 2b.

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Effect of Cryopreservation on the Genome of Sperm in Animals and Humans

Julia Kopeika

Cryopreservation has been widely utilised in assisted reproduction technology, agriculture and conservation programs for endangered species. Efficiency of freezing method is traditionally assessed by survival and fertilisation rate. However, less attention has been paid to the integrity of genetic material in reproductive cells after cryopreservation even though genetic integrity is one of the most crucial features for reproductive cells. The capability of stored reproductive cells to retain the capability for motility, fertilisation and commitment to further cleavages after cryopreservation can be unessential under the conditions when genome integrity is affected.

All data in the literature on the effect of cryopreservation on the sperm genome can be roughly systematised into three main groups of studies, where effects were studied at the molecular, cellular or organismal levels. It is interesting to note that different authors have presented contradictory results even though almost the same methods of analysis are applied. This paper aims to review the data available in the literature and discuss the controversy.

A number of studies suggest that cryopreservation of spermatozoa might be responsible for some imbalance in DNA–protamine interactions, change in the degree of condensation and/or DNA fragmentation level. The damaged by freezing sperm genome was even proven to be repaired after fertilisation by the oocyte machinery. As for long-term effect, theories arose that semen cryopreservation could be even used as a tool for aquacultural selection purposes not only for the fittest sperm but also for individuals having the maximum genetic variation to cope with their extreme artificial environment.

In summary, despite the growing evidence that cryopreservation affects the genetic integrity of sperm, there is still insufficient data available on the potential impact of these changes on future offspring. A number of factors are reported (including different cooling and warming regimes, antioxidants and stimulants of the DNA repair system) which might either reduce or reverse the side effects of

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sperm cryopreservation to improve outcomes. However, urgent studies are needed to establish and optimise the safety of currently used techniques. New perspectives for future research are proposed.

Introduction

Cryobiology remains to be a pivotal discipline in advancing reproductive medicine, agriculture, aquaculture and research. Establishment of methods and applications for spermatozoa has the longest history and is the most widely used in reproduction (Agca and Critser 2002). Samples of reproductive cells from different strains and lines of species that are important to biomedical research have been cryopreserved and stored in cryobanks (Critser and Mobraaten 2000). Furthermore, the banking of genome resources such as cryopreserved male, female and embryo germplasm would greatly help conservation efforts for endangered species and captive breeding programs (Johnston and Lacy 1995; Watson and Holt 2001). Application of artificial reproductive technologies using frozen-thawed gametes of rare and endangered species will become increasingly important in conservation biology programme.

Modern cryotechnologies also allow to overcome reproductive obstacles such as age, death (for human), extinction (for endangered species), non-synchronous maturation and the availability of male and female reproductive cells (in aquaculture).

However, if cryopreservation is applied with a view of storing gametes for future use, it is also important to understand if there is any "trade off" for this unique ability to suspend the time. If gametes are subjected to freezing thawed process, are they exactly the same afterwards? It is well known that not all cells will be able to withstand the freezing process with its range of biologically extreme factors.

The following factors such as "solution effect", intracellular ice, minimal cell volume and phase transition of the membrane were identified as main elements responsible for cryoinjury (Lovelock 1953; Meryman et al. 1977; Mazur 1963; Farrant 1977; Belous and Bondarenko 1982).

The basic principles of cryopreservation suggest that the major cause of cell destruction during freezing and thawing is the rapid formation of intracellular hexagonal ice crystals. Therefore, in an attempt to prevent this, one of the remedies will be an attempt to reduce the amount of intracellular water. Removal of too much water, however, and subsequent excessive dehydration are also detrimental. The freezing point of water which contains other ions and dissolved solutes is lower than 0 °C and dependent on the concentration of the dissolved solutes. When such a solution is cooled, some of the water molecules freeze first, increasing the liquid concentration of dissolved solutes and lowering the freezing point of the remaining solution further. The increased osmotic pressure of the remaining dissolved solutes can also cause damage to cells during cooling (referred to as the "solution effect") (Mazur et al. 1972). The objective therefore is to remove enough water from cells during cooling so survival is optimized by minimal ice crystal formation. This, together with the use of a solution that has an osmotic pressure whose damaging

solution effects are minimized, ensures maximum cell survival. It is now generally recognized that damage during cryopreservation is not low-temperature storage itself but the transitions through the temperature zones during cooling and thawing, when ice crystal formation is most likely to occur (Willadsen 1977; Mazur 1984; Shaw et al. 2000). The degree of dehydration within a cell and the subsequent chance of cell survival during cooling are dependent on the following factors:

1. The temperature at which slow cooling is terminated by direct plunging into liquid nitrogen. At a constant cooling rate, the lower the temperature the more time is allowed for dehydration.
2. The concentration of cryoprotectant used. The rate of movement of water across a cell membrane is proportional to the difference in concentration of solute molecules on the two sides of the membrane. The higher the concentration of cryoprotectant, the lower the freezing point of the solution, and this allows for more dehydration before all of the extracellular water freezes.
3. The permeability of the cell membrane to water and cryoprotectant.
4. The size of the cell. Smaller cells can be cooled faster because of their larger surface-to-volume ratio, and hence water can leave smaller cells faster than larger ones.

On thawing, the opposite processes to those that operate during cooling take effect. The basic aims during these processes are twofold: to rehydrate the cell and to remove cryoprotectant that has permeated the cells. Rehydration of the cells begins during warming as ice melts and turns into liquid water capable of permeating the cell membranes. Clear evidence that the rate of removal of cryoprotectant from thawed cells is very important to their subsequent. The cells swell and disintegrate due to the rapid influx of water. The method to avoid this osmotic shock is to remove the cryoprotectant gradually by a stepwise transfer of the cells through successively lower concentrations of cryoprotectant solution (Leibo and Mazur 1978). At each transfer, there is an increase in cellular volume to a maximum because of isotonic equilibration (Critser et al. 1988).

Cryopreservation causes extensive damage to membranes, resulting in decreased metabolism of cells, and disturbs the bioenergetic processes of cells by damaging the mitochondria. However, less attention has been paid to studies on the integrity of genetic material in reproductive cells after cryopreservation even though genetic integrity is one of the most crucial features for reproductive cells. Safety of cryopreservation and low-temperature storage are important from the viewpoint of maintaining the original genofund of the particular sample, individual or species. The capability of stored reproductive cells to retain the ability for motility, fertilisation and commitment to further cleavages after cryopreservation can be unessential under the conditions when genome integrity is affected.

Natural characteristics of sperm in mammal species such as small size, extremely low content of fluid, relatively good membrane permeability and low surface volume ratio give these cells natural advantage in cryo-resistance in comparison with other much larger cells like oocytes and embryos. However, this is not the case across all species. Human sperm were among the first cells to be successfully

frozen. Whereas, in other non-mammalian species, the cryopreservation of male gametes remained to be slightly more challenging. For example, male gametes of fresh-water fish species are very sensitive to osmotic changes, which make them extremely susceptible to cryopreservation process even at early stage of plunging them in cytoprotectant media.

Results

Main Pillars of Genetic Integrity

Genetic integrity is determined not only by sequence of nucleotides in deoxyribonucleic acid (DNA) but also by surrounding machineries of nucleoproteins, DNA repair system, methylations and spindles.

DNA

DNA is a long molecule composed of nucleotides in a linear order that constitutes the genetic information of cells capable of replicating itself and of synthesizing cellular proteins via an RNA intermediate translated from the DNA template. The function of DNA is to transmit the genetic message from generation to generation and to allow the expression of that message under appropriate conditions.

DNA is a long, unbranched high molecular weight polymeric compound, which on complete hydrolysis, yields pyrimidine and purine bases, a sugar component and phosphoric acid. DNAs from different species reveal wide variations in the molar proportions of bases although the DNA from different organs and tissues of any one species are essentially the same.

Once it was recognised that DNA is the informationally active chemical component of essentially all genetic material (with the notable exception of RNA viruses), it was assumed that this macromolecule must be extraordinarily stable in order to maintain the high degree of fidelity required of a master blueprint for an organism. It has been something of a surprise to learn that the primary structure of DNA is in fact quite dynamic and subject to constant change (Friedberg et al. 1995). Base alterations can commonly arise from the inherent instability of specific chemical bonds that constitute the normal chemistry of nucleotides under physiological conditions of temperature and pH. Cryopreservation process on the other hand exposes cells to non-physiological range of physical and chemical changes that could also potentially influence structural and functional stability of DNA (Kopeika et al. 2015).

Nucleoproteins

In eukaryotes, DNA is associated with both histone and nonhistone chromosomal proteins to form chromatin. The presence of the chromatin structure is an important warrant of the DNA stability and function (Ljungman and Hanawalt 1992). It has been shown that the presence of DNA-bound histones and the folding of the chromatin fibres into higher order structures can influence the frequency of induced DNA breaks (Ljungman and Hanawalt 1992). The mechanism of protection against, for example, hydroxyl radical-induced DNA strand breaks afforded by histones and the folding of the chromatin into higher order structures is not likely due to steric hindrance since the hydroxyl radicals are very small, uncharged molecules that should be able to move about even in regions of condensed chromatin (Ward 1993). However, the hydroxyl radicals are extremely reactive, making them very short-lived in cells. Thus, only those hydroxyl radicals formed in the close vicinity of the DNA helix would be able to cause DNA-strand breaks. Any proteins bound to the DNA would lower the frequency of DNA-strand breaks by scavenging the hydroxyl radicals (Ljungman and Hanawalt 1992). Therefore, the disturbance of chromatin structure might make DNA more susceptible to different reactive agents.

The main classes of proteins that are responsible for the structural and functional competence of the genetic information presented in cells are presented in Table 1. The expression of the genetic information is controlled predominantly through the interaction of proteins with DNA. So the normality of genome function is entirely dependent on the integrity of DNA–proteins complex. Sperm cells rely mainly on protamines responsible for its DNA packaging.

Table 1 Proteins that are responsible for the structural and or functional stability of the genome

	Proteins which interact with DNA
1	*Replication and repair* • DNA polymerases of different types • DNA unwinding proteins and others in the replication complex • Ligases and repair proteins • Nucleases and excision enzymes of the repair process
2	*DNA packaging proteins* • Chromatin: nucleosomes and histones • Proteins of the sperm, protamines and other proteins • Virus condensation proteins: internal and coat
3	*Transcription* • RNA polymerases and its various subunits • Repressor and regulation of the initiation of transcription • Cyclic AMP receptor proteins (CRP) • *Rho* factors in the termination of transcription
4	*Nucleases* • Restriction endonucleases • Exo- and endonucleases • Nucleases of hydrolysis
5 A	*DNA-modifying proteins* Methylases

Protamines

The chromatin of early spermatids is organised like that of somatic cells in the form of nucleosomes (McGhee and Felsenfeld 1980). However, during the middle and late stages of spermiogenesis, spermatid histones are replaced by transition proteins and finally these are replaced by protamines in which about 55–75% of the amino-acids correspond to arginine (Bellve et al. 1975).

While mature mammalian spermatozoa almost always possess protamines (human sperm chromatin contains 15% histones (Tanphaichitr et al. 1978)) the sperm nuclei of most species of teleost fish contain either protamines (P) or protamine-like proteins (PL) and histones (type H). These interspecies differences must be taken into consideration when cryoresistance of sperm is assessed, especially using methods that depend on DNA–protamine interaction (e.g. staining methods).

The main reason for replacement of histones by protamines during spermatogenesis appears to be the fact that protamines convert DNA molecules into a very compact state. The phosphate charges of the DNA present in the nuclei of eukaryotic organisms are always neutralised by proteins. This neutralisation is almost complete in those sperm nuclei which contain protamines, so that the basic amino acid/phosphate ratio is close to unity in such cells. This neutralisation results in a complete insolubilisation of the complex and only a relatively small change in its packing density is observed when the humidity of the sample is modified. In fact, a nucleoprotamine sample only accepts about 25% of its dry weight as water of hydration at 100% relative humidity (Fita et al. 1983). In contrast, the DNA in somatic nuclei is not fully neutralised by histones. The relatively low degree of neutralisation of DNA by histones makes the nucleohistone complex very sensitive to the ionic conditions and the presence of divalent ions. In many cases, the degree of hydration of DNA complexes has a strong influence on the conformation of DNA.

Fish protamines have been proposed to perform an additional function. It was suggested (Shimizu et al. 2000) that the physiological role of protamines for fish is to protect the ejaculated spermatozoa against disruption by low osmotic pressure. Nuclei without protamine more rapidly swelled than did those with protamine and completely broke down within 10 min, whereas more than 80% of the sperm nuclei with protamine resisted the disruption under similar conditions (Shimizu et al. 2000). Disruption by osmotic pressure is known to happen during cryopreservation.

Another factor that stabilises sperm chromatin is zinc. A positive correlation exists between zinc content and chromatin stability (Kvist et al. 1988). It has also been proposed that spermatozoal zinc protects an inherent capacity for decondensation and that zinc supplementation before storage of sperm reversibly inhibited spontaneous decondensation as well as EDTA-enhanced decondensation (Kvist and Bjorndahl 1985). Disulphide bonds provide another way to make the complex more stable. During sperm maturation in eutherian mammals, sulph-hydryl groups become oxidised, resulting in the formation of disulphide groups and chromatin stabilisation (Bottiroli et al. 1994).

A number of studies have demonstrated that the structural organisation of sperm DNA is important to the function of spermatozoa (Evenson 1989; Blow and Laskey 1986). The results suggested that the organisation of DNA within sperm head is necessary for proper DNA replication. These experiments imply that even though spermatozoa are transcriptionally inert and do not replicate their DNA, they are just as dependent as somatic cells upon the structural organisation of their DNA for proper function. As fertilisation occurs and ooplasmic segregation happens, other dynamic responses take place in the nuclei of both egg and spermatozoon: the resumption of meiosis, breakdown of the poreless nuclear envelope, reduction of disulphide bonds in protamines, decondensation of chromatin, replacement of protamines by histones, reformation of the nuclear envelope and DNA replication. So the success of these events depends on the structural integrity of the nucleus contributed by the spermatozoon and, as data suggest, even very subtle changes in the sperm nuclear structure may have a significant impact on embryo development (Ward et al. 2000).

Some researchers believed that the only truly essential component in the sperm head for embryogenesis is the DNA itself, and that all other sperm components evolved solely as the means of transporting the parental DNA into the oocyte, but data show that it might not be the case. Subtle variations in the proteinaceous nuclear structure can also affect paternal genome function in embryogenesis. It is also likely that disturbance of the sperm nuclear matrix seriously damages the paternal genome or its expression (Ward et al. 2000).

Cryo-Related Extreme Factors that May Damage the DNA-Protein Complex

Despite the chemical, physical and biological factors that stabilise the DNA–protein complex in the nucleus, it is still possible to destabilise this complex. Loss of native structure must involve disruption of factors responsible for its stabilisation. The factors are *hydrogen bonding*, *hydrophobic interactions*, *electrostatic interactions and disulphide bridging*.

Hydrogen bonds can be disrupted by heat or urea and guanidinium. These compounds contain functional groups that can accept or donate hydrogen atoms in hydrogen bonding. Hydrogen bonds of the alpha-helix will be replaced by hydrogen bonds to urea, for example, and the helix will unwind.

Agents that disturb *hydrophobic interaction* are organic solvents, such as acetone or ethanol-dissolve nonpolar groups. Well-known cryoprotectant ethylene glycol has been shown to affect hydrophobic interactions in chromatin (Schwartz and Fasman 1979). Detergents are known to dissolve nonpolar groups. Another phenomenon described is cold denaturation (Foguel and Silva 1994). Cold increases solubility of nonpolar groups of water. When a hydrophobic group contacts water, the water dipoles solvate it by forming an orderly array around it. The array is called an “iceberg”, because it is an ordered water structure, but not true ice. The ordering of water in an “iceberg” decreases the randomness (entropy) of the system and is energetically unfavourable. If hydrophobic groups cluster together, contact with

water is minimised, and less water must become ordered. This is the driving force behind hydrophobic interaction. (The clustering together of hydrophobic groups is also entropically unfavourable, but not as much as “iceberg” formation.) At low temperatures, solvation of hydrophobic groups by water dipoles is more favourable. The water molecules have less thermal energy and can “sit still” to form a solvation “iceberg” more easily. Cold denaturation is not a stabilising factor for all proteins; it is important in proteins that are highly dependent on hydrophobic interactions to maintain their native structure.

Ionic disbalance can bring as well to tremendous changes in the structure of proteins. For example, pH extremes are known to affect ionic status of the system. Most macromolecules are electrically charged. Ionizable groups of the macromolecule contribute to its net charge (sum of positive and negative charges). Bound ions also contribute to its net charge. Electric charges of the same sign repel one another. If the net charge of a macromolecule is zero or near zero, electrostatic repulsion will be minimized. The substance will be minimally soluble, because intermolecular repulsion will be minimal. A compact three-dimensional structure will be favoured, because repulsion between parts of the same molecule will be minimal. pH extremes result in large net charges on most macromolecules. Most macromolecules contain many weakly acid groups. At low pH, all the acidic groups will be in the associated state (with a zero or positive charge). So the net charge on the protein will be positive. At high pH, all the acidic groups will be dissociated (with a zero or negative charge). So the net charge on the protein will be negative. Intramolecular electrostatic repulsion from a large net charge on the protein will be negative. Intramolecular electrostatic repulsion from a large net charge will favour an extended conformation rather than a compact one.

Cell proteins often form additional covalent intra-chain bonds. Most notably, the formation of *disulphide bonds* (also called S–S bonds) between the two –SH groups of neighbouring cysteine residues in a folded polypeptide chain often serves to stabilise the three-dimensional structure. Disruption of bonds leads to nonfunctional protein. As it was mentioned above, there is evidence in literature already that cryopreservation causes disturbance in disulphide binding.

DNA Repair System

As it has been shown before, the integrity of cellular DNA is challenged constantly by endogenous and exogenous agents, resulting in an estimated $1–3 \times 10^4$ spontaneous lesions per cell per day (Loeb 1991). Cellular processes that occur in an attempt to deal with this genotoxic insult include direct DNA repair of the lesion, induction of gene transcription, cell cycle arrest and cell death (Carr and Hoekstr 1995). All cells and spermatozoa possess a variety of enzymatic mechanisms that repair damaged DNA (Ashwood-Smith and Edwards 1996). The repair system can be divided into three main classes (Eisen and Hanawalt 1999): (1) Direct repair (in which abnormalities are chemically reversed), (2) Recombinational repair (in which homologous recombination is used to repair abnormalities) and (3) Excision repair

(in which a section of the DNA strand containing an abnormality is removed and a repair patch is synthesised using the intact strand as a template). There are three distinct forms of excision repair: these are base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR).

DNA damage can be caused not only by environmental agents such as UV light but also by weak endogenous mutagens including reactive oxygen species, and metabolites that can act as alkylating agents. Very slow turnover of DNA consequently occurs even in cells that do not proliferate. Genome instability caused by the variety of DNA-damaging agents would be an overwhelming problem for cells and organisms if it were not for DNA repair.

If there are extreme factors that can potentially disturb the stability of the genome, the status of the repair system must be taken into consideration. In relation to reproductive cells it is must be acknowledged that mature spermatozoa do not have a repair system at all (Matsuda and Tobari 1989). Damage of the paternal genome might alter the expression of some, but not all, of the DNA repair machinery in early preimplantation embryos. In embryos fertilised by mutagen-exposed spermatozoa, DNA damage may act as a trigger to alter chromatin conformation and the regulation of transcription (Harrouk et al. 2000).

The DNA repair system is present in oocytes, and its activity can be influenced in various ways (Ashwood-Smith and Edwards 1996). The integrity of this system is crucial not only for the oocytes but also for the fertilised egg since it can repair the lesions in DNA contributed by spermatozoa. Therefore, in a study of the effects of extreme factors on the genome as a system, the DNA repair system must be ultimately taken into consideration. The intensity of the damage depends not only on the interaction of extreme factors and the genome but also on the reaction of the DNA repair system. Moreover, the same set of extreme factors might affect the DNA repair system directly, making cells more sensitive to mutagenic influences. Activation of the repair system can be used as evidence for the presence of DNA lesions. Estimation of the DNA repair system pathway involved in the repair process may help in solving the mechanisms of DNA damage.

Imprinting

Vertebrate development involves programmed changes in gene expression that promote the differentiation of totipotent embryonic cells into somatic cells that build the body of the animal. Epigenetic factors include genetic imprinting through DNA methylation. The epigenetic modification of genomic DNA by the addition of a methyl group to the 5′ position of cytosine is a common characteristic of organisms with relatively large and complex genomes (Bestor 1990). This is a type of epigenetic alteration in which, for some genes, only one allele (either paternal or maternal) is expressed during development. Imprinting differences between male and female are complementary and settle during gametogenesis, but occur also in males during epididymal maturation. Moreover, they are altered during embryogenesis (Monk and Grant 1990). This DNA methylation has been extensively examined in

mammals and it is thought to play a crucial role in a number of important mammalian developmental processes, including such things as X-chromosome inactivation and the more general silencing of specific genetic loci during the determination and differentiation of cells (Riggs and Pfeifer 1992). The epigenetic phenomenon of genomic imprinting is also known to occur among plants (Alleman and Doctor 2000).

The paternal pronucleus is critical for proliferation of cells of differentiated tissues (Surani et al. 1990). Disturbance of the normal DNA methylation could alter male germ cell development and function, resulting in alterations in fertilisation and early embryo development (Doerksen and Trasler 1996). A variety of imprinting effects is known including abnormalities and lethality in the early embryo, late foetus and neonate as well as prenatal and postnatal growth effects (Cattanach and Beechey 1997). Aberrant expression of imprinted genes can result in developmental failure, neurodevelopmental and neurobehavioral disorders and cancer (Murphy and Jirtle 2003). Although there are still many unanswered questions about epigenetic factors, the fact is that the disturbance of the natural distribution of methyl groups in reproductive cells or embryos has a huge effect on the progeny. It has been suggested that one of the mechanisms of preconception carcinogenesis involves imprinting changes, resulting in altered gene expression in the offspring (Auroux 2000). Any human manipulations with reproductive cells and embryos must be treated with caution. It has been shown that even a common manipulation such as in vitro embryo culturing might be responsible for the induction of imprinting errors (Young and Fairburn 2000).

In cryopreservation, some cryoprotectants (e.g. Me_2SO) appear to change imprinting (Ashwood-Smith 1986), which leads to the long-term effect. In another study, Me_2SO was shown to cause DNA hypomethylation, which was interpreted as a reason for the increase of some genes expression (Reboulleau and Shapiro 1983). Until today still a limited number of studies have shown the effect of cryopreservation on the imprinting (Bonduelle et al. 1998; Hao et al. 2002a, b).

Effect of Cryopreservation on the Genome

Cryodamage of Genetic Material in Spermatozoa

Spermatozoa are the male reproductive cells, the primary function of which is to deliver the paternal genome to the egg, and to initiate development by activating the oocyte, leading to the development of the normal embryo. From the earliest work on sperm cryopreservation, the main criteria evaluated for sperm viability have been motility and fertilising ability. The recovery of these functions after sperm cryopreservation and the production of morphologically normal offspring have been cited by some researchers to argue that cryopreservation does not influence the sperm genome. The relatively high rate of the motility after sperm cryopreservation together with morphologically normal offspring derived from these sperm encouraged researchers to use cryotechnologies almost straight away without further

in-depth studies on biological and physiological consequences. It is worth pointing out that in pathological development, malformations may occur at different levels (organ, cell, cell connection (e.g. synapses), organelle and also at the molecular level). If the problem is studied only at the level of the whole organism, changes that occur at the molecular level may go un-noticed and vice versa. In DNA lesions, the subsequent physiological consequences of these changes on the organism's capacity for growth, survival, development and reproduction can range from the undetectable to the profound (Friedberg et al. 1995).

New data that have emerged from recent literature may change previously established views about the effect of low-temperature storage on the genetic integrity of spermatozoa. All data in the literature on the effect of cryopreservation on the sperm genome can be roughly systematised into three main groups of studies, where effects were studied at the molecular, cellular or organismal levels. It is interesting to note that different authors have presented contradictory results even though almost the same methods of analysis are applied. This chapter reviews data available in the literature as well as analyses some contradictory results which have arisen in published work.

Study of the Sperm Genome at the Molecular Level

The first group of studies is where different staining methods were used on cryopreserved sperm to evaluate changes in the structure of deoxyribo-nucleoproteins. The methods are based on the fact that the emission spectra of dyes are sensitive to the status of DNA-associated protamines. For example, acridine orange (AO) binds to native (double-stranded) DNA as a monomer and emits green fluorescence, whereas denatured DNA emits red fluorescence. Using AO staining of human sperm, Royere et al. (1988), Royere et al. (1991), Hamamah et al. (1990), Pasteur et al. (1991) showed that the level of native DNA significantly decreased after cryopreservation. Hamamah et al. (1990), studying staining characteristics of human and boar spermatozoa, discovered that the boar spermatozoa are more cryoresistant. The percentage of native DNA in boar spermatozoa was not affected by cryopreservation, whereas in human sperm, it was subject to significant changes. It might be the case that the boar DNA–protamine complex is more resistant to freezing than that of human. Another factor that needs to be considered is that whereas the samples of boar spermatozoa were selected from a group of healthy animals, the human samples were taken from a group of men that were involved in an infertility programme. Therefore, the possibility of having sperm samples with some anomaly was theoretically higher for human samples. In fact, the percentage of native DNA in the human samples before any treatments (44–64%) was somewhat lower than what would be considered as the lowest normal margin (50–65.6%) (Sukcharoen 1995; Hoshi et al. 1996). Furthermore, Hoshi et al. (1996) showed that when the proportion of green AO fluorescence (native) was less than 50%, the spermatozoa of 61% of the patients completely failed to fertilise. It is generally agreed that success of

cryopreservation depends on the initial quality of cells (Hammadeh et al. 1999) and that low-quality spermatozoa are more likely to be less cryoresistant.

Very recent study using Tunel assay, once again showed sperm nuclear–DNA and mitochondrial–DNA damages were increased significantly after freeze-thawing of spermatozoa in fertile (14.85 ± 17.6% and 5.80 ± 11.59%) to (27.54 ± 19.74% and 7.33 ± 6.13%) and sub-fertile groups (19.84 ± 17.52% and 7.53 ± 8.56%) into (29.48 ± 16.97% and 10.21 ± 11.73%), respectively (Amor et al. 2018).

A lower cryoresistance may apply not only to the membrane and subcellular structures but also to the genetic structures. Hammadeh et al. (1999), using aniline blue, demonstrated that the percentage of condensed chromatin in human spermatozoa (aniline blue positive) after cryopreservation was significantly higher in semen samples from infertile patients than those of fertile donors. This indicates that chromatin in the fertile patients withstands cryodamage better than sperm chromatin obtained from infertile patients. Similar results were obtained by other authors (Donnelly et al. 2001a). In this study, sperm DNA integrity was determined using a modified alkaline single-cell gel electrophoresis (comet) assay which reveals single-strand breaks in DNA. DNA of semen and prepared spermatozoa from fertile men were found to be unaffected by cryopreservation. In contrast, spermatozoa from infertile men were significantly damaged by freeze-thawing. The ability of semen from fertile men to resist freezing damage may be due to some protective constituents in seminal plasma. Seminal plasma contains an abundance of antioxidant enzymes such as superoxide dismutase and catalase, which removes reactive oxygen species (ROS). The total antioxidant capacity of semen from fertile men has been found to be significantly greater than that for samples from infertile men (Lewis et al. 1995). It may be the case that only fertile samples have these antioxidants present in sufficient abundance to confer protection against the trauma of cryopreservation. Semen from infertile men is also known to possess a greater percentage of spermatozoa with fragmented DNA than semen from fertile men (Sun et al. 1997; Lopes et al. 1998). But there is evidence that even the DNA integrity of sperm from healthy donors can be affected by the cryopreservation procedure. It has been shown that sperm DNA integrity was reduced by 20% after cryopreservation of semen (Donnelly et al. 2001b), but it was suggested that some improvements in freezing outcome can be achieved by selecting out the subpopulation of sperm with best motility and DNA integrity.

Differences in interindividual cryosensitivity might not only occur between samples from healthy and infertile individuals but also between normal donors. Variables such as chromatin condensation are known to vary significantly not only between individuals (Huret 1986; Evenson et al. 1991) but also between spermatozoa from the same ejaculate (Bedford et al. 1973; Witkin et al. 1977; Rosenbush and Sterzik 1994) which is caused by differences in stabilisation by disulphide bonds (Blazac and Overstreet 1982). The mechanisms for changes in the percentage of the native DNA after cryopreservation are still unclear. It might be related to the disturbance in DNA–protamine interaction and redox-oxidative changes in disulphide bonds caused by cryopreservation. These changes are more likely to affect the future development of the embryos. It was shown previously that spermatozoa with

anomalies in sperm chromatin packaging, despite being morphologically normal, were common for subfertile men (Hammadeh et al. 1998; Highland et al. 1991; Sakkas et al. 1998; Esterhuizen et al. 2000; Bianchi et al. 1996) or to couples with unexplained infertility (Gopalkrishnan et al. 1999; Host et al. 1999). It is interesting to note that when rainbow trout was studied with the help of the comet assay, there were no significant differences between fresh and cryopreserved samples for some donors, but almost half of the frozen-thawed samples did have an increase in damaged nuclei (Labbe et al. 2001). Because the definition of subfertility or infertility is more obscure for non-human species than for humans, it is difficult to say whether the differences in cryoresistance between different trout individuals were in group of "normal" fish or were caused by the hidden subfertility of some studied fish.

Some work on the impact of cryopreservation on the genome has been carried out using Feulgen dye. A decrease of DNA–Feulgen content after cryopreservation was estimated and was explained as a result of overcondensation (Royere et al. 1988, 1991; Hamamah et al. 1990). If cryopreservation can cause overcondensation, then decondensation after fertilisation might also be affected. Pasteur et al. (1991), studying decondensation of human sperm chromatin in vitro with the help of the detergents sodium dodecyl sulphate (SDS) and dithiothreitol (DTT), demonstrated significant changes after cryopreservation. Although decondensation processes even in normal conditions are not fully understood (Pasteur et al. 1991), the key event is connected to the disruption of disulphide cross-linkages established between cysteine molecules of protamines (Perreault et al. 1984). Based on the fact that SDS and DDT were less likely to cause decondensation after cryopreservation it might be suspected that freezing–thawing of sperm induces changes in S–S bonds and/or causes denaturation that prevents the biochemical action of SDS on the proteins.

Summarising data obtained by DNA staining methods, some conclusions can be drawn. Cryopreservation of spermatozoa might be responsible for some imbalance in DNA–protamine interactions that lead to changes in the degree of condensation. Affects on the status of thiol bonds might be one of the possible explanations. Thiol status was investigated during cryopreservation of boar sperm (Courtens et al. 1989) and an increase in SH groups after cryopreservation was established. However, more investigations are needed to confirm the results for other species, since it was shown that parameters such as the degree of condensation (Perreault et al. 1988; Bedford et al. 1973; Mahi and Yanagimachi 1975) and type of protamines (Calvin 1976; McKay et al. 1985, 1986; Corzett et al. 1987; Hecht 1988) vary significantly between different species. It has been postulated that poor packaging and/or damaged DNA may contribute to the failure of sperm decondensation and, consequently, result in fertilisation failure (Sakkas et al. 1996). Another possibility for causing the disturbance in DNA after cryopreservation is the effect of the cryotechnique on the overall metabolism of the cell. Cryopreservation is known to disturb calcium transport through the cellular membrane (Bailey and Buhr 1993). An increase in intracellular calcium can raise the frequency of nuclear DNA breaks through nuclease activation (Epe 1993), together with the production of free radicals, that are known to be harmful via lipid peroxidation (Leibovitz and Siegel 1980; Rao and David 1984).

Development of Embryos Derived from Cryopreserved Sperm

The important question that rise from all above-mentioned observations if increase in DNA fragmentation has any impact on subsequent embryo development. More recent studies demonstrate that, for example, embryo development to the blastocyst stage following ICSI was only reached from rams whose sperm had higher level of intact DNA. Definitively, the impact of sperm DNA damage on embryonic development depends on the balance between sperm DNA fragmentation extent, fragmentation type (SSBs or DSBs) and the oocyte's repair capacity (Palazzese et al. 2018).

It is reasonable to assume that if cryopreservation of sperm causes damage to the DNA, then it could be repaired by the oocyte's intrinsic repair system following fertilisation. To test this idea, different inhibitors and stimulants of the DNA repair system were utilised in culture of freshly fertilized embryos of the loach (*Misgurnus fossilis*) derived using cryopreserved sperm. Embryo survival dropped significantly when an inhibitor of the DNA repair system (3-aminobenzamide) was added (Kopeika et al. 2004), and revived when embryos were exposed to caffeine (Kopeika et al. 2003). The original study was subsequently repeated by others in trout species using the same inhibitor (3-aminobenzamide) of the repair system and it was suggested that at least 10% of sperm DNA damage could be repaired by the DNA repair system of the oocyte after fertilization (Perez-Cerezales et al. 2010). A mammalian study also demonstrated the persistence of DNA aberration in murine embryos derived from sperm subjected to freezing–thawing (Yamauchi et al. 2012). Animal studies also showed that cryopreserved sperm not only contributed aberrant DNA into oocytes during fertilization but also altered gene expression in surviving embryos and increased telomere length (Perez-Cerezales et al. 2011). It was also interesting to see that both slow freezing and vitrification of human sperm cause DNA changes in key paternal genes involved in early embryo development (Valcarce et al. 2013). Therefore, to assess the effects of cryopreservation on sperm, it is essential to perform long-term follow-up studies on resultant children.

Study of the Sperm Genome at the Chromosomal Level

All studies mentioned above analysed the whole sperm sample after cryopreservation. As a result both viable and nonviable cells in the ejaculate were subjected to the staining. It is, therefore, difficult to know whether the measured percentage of native DNA was obtained from live and motile cells or dead and immotile cells. In this case, it is difficult to draw conclusions as whether cryopreservation causes changes in condensation directly in motile sperm, or it is a secondary change resulting from necrotic or apoptotic processes caused by the cryopreservation. To overcome this problem, other types of studies have been carried out using artificial insemination as a tool for separation of viable and nonviable sperm. The human sperm–hamster oocyte system was used and chromosomal abnormalities were studied after the first cleavage (Okada et al. 1995; Chernos and Martin 1989). Unfortunately, the factor evaluated in these studies is not sensitive enough to exclude

the presence of moderate effects of cryopreservation on the genetic structure. No changes in the level of chromosomal aberrations were registered in these studies after cryopreservation.

It is important to point out that the cleavage and proper distribution of chromosomes are driven by the centrosome apparatus (Sathananthan 1997) that is usually contributed to the oocyte by spermatozoon during fertilisation (Moomjy et al. 1999; Sutovsky and Schatten 2000; Colombero et al. 1999). For all organisms from sea urchin (Schatten et al. 1986) to human (Palermo et al. 1994, 1997), the centriole is contributed to the oocyte by the spermatozoon. The only known exception to this rule is found among rodents (Schatten et al. 1986; Sutovsky et al. 1996; Sutovsky and Schatten 2000) where the centriole is located in the oocyte and spermatozoa are not responsible for delivering this organelle. Therefore, rodents are considered in these circumstances as a poor model (Sutovsky et al. 1996). In the studies cited above (Okada et al. 1995; Chernos and Martin 1989), fertilisation was carried out between species that have different principles of centriole organisation. If it was presumed that cryopreservation caused some damage to the centriole of human spermatozoon, then it would not be noticed in the human sperm–hamster oocyte system. Unfortunately, no literature has been found to study the impact of cryopreservation on the centriole of sperm or other reproductive cells to date, although elements of the centriole such as microtubules and spindle are known to be affected by some cryoprotectants and cooling in the oocytes (Pickering and Johnson 1987; Magistrini and Szollosi 1980).

Long-Term Study of the Effect of Cryopreservation on the Sperm Genome

Another group of studies was carried out on offspring derived from cryopreserved spermatozoa. One study was carried out to determine whether the cryopreservation of African catfish (*Clarias gariepinus*) spermatozoa has an effect on the survival of specific genotypes (Van der Bank and Steyn 1992). Using an enzyme, glucose phosphate isomerase, as a genetic marker, these authors undertook a comparative genetic study of domesticated and wild catfish populations. Significant deviations from expected Hardy–Weinberg proportions occurred when the offspring were obtained by using cryopreserved milt. The authors concluded that semen cryopreservation could be used as a tool for aquacultural selection purposes—not only for the fittest sperm but also for individuals having the maximum genetic variation (heterozygotes) at their disposal to cope with their artificial environment. However, these results were not confirmed in another almost identical study carried out by the same investigators (Van der Walt et al. 1993). The second study showed that cryopreservation of semen did not influence selection for specific allele combinations. The authors concluded that the differences were due to different cooling rates used in different studies (5 °C/min was used as opposed to 8 °C/min in the previous study). According to the authors, 5 °C/min is responsible for 10% higher sperm survival compared to the cooling rate 8 °C/min. The lower sperm survival obtained by using

a freezing rate of 8 °C/min could have possibly been responsible for the high degree of selection induced by using this specific freezing rate (Van der Walt et al. 1993).

More studies have addressed the survival of fish embryos derived from cryopreserved sperm. Neifakh et al. (1988) reported a slight but significant increase in the mortality of diploid embryos of carp (*Cuprinus carpio*) and loach (*Misgurnus fossilis*) derived from cryopreserved sperm. The dominant lethal mutation test has been widely used as a means of estimating genetic effects of a mutagen (Matsuda et al. 1989). The increased mortality of embryos after the stage of late blastula is considered as a reliable indicator of genotoxic effects on the reproductive cells before fertilisation in aquaculture (Cherfas and Zoy 1984). Long-term analyses, not only during the period of embryo development, but even longer including postembryonic and late adulthood, can be quite sensitive for the detection of some genetic-related effects. Unfortunately, for human and other mammal species as opposed to fish, the investigations of long-term effects are difficult and concern only relatively small population samples.

The survival of androgenetical haploid embryos was evaluated for carp after sperm cryopreservation and fertilisation of X-ray inactivated oocytes (Kopeika et al. 1994). Embryonic mortality was significantly higher after fertilisation with cryopreserved sperm than in controls; this was interpreted as evidence of genetic failure.

The possible genotoxic effect of cryopreservation has not been considered until now from the point of view of the induction of direct changes in the structure of specific genetic elements. But the influence on the genome could be achieved even without direct damaging effect to the genome itself. An effect on the genome could be realised indirectly through selective pressure on the population of cells. Even the optimum protocol of cryopreservation brings death to some cells in the suspension. The survival of cells during cryopreservation is not necessarily a random process and there are specific criteria that make cells more or less cryoresistant. There are many reports in the literature about the differences in cryoresistance between species (Holt 2000), between different strains within one species (Tada et al. 1990; Schmidt et al. 1987; Dinnyes et al. 1995; Pomp and Eisen 1990; Songsasen and Leibo 1997; Nakagata and Takeshima 1993) and even between different individuals within one population (Beatty 1976; Thurston et al. 2002). Such heterogeneity (Curry 2000) in any frozen object whether it is sample of spermatozoa, oocytes or embryos, will lead to the survival of those cells that possess the “better” structure. This may cause selection on the basis of definite sets of characteristics. However, what exactly will be selected is not yet clear. It was shown that rooster spermatozoa with better cryoresistance have better membrane permeability (Ansah and Buckland 1983; Fujiara et al. 1995). Thus, investigations are needed on whether this type of selection would affect some other phenotypic characteristics of future progeny. Although there are already some data about morpho-functional changes in the progeny derived from cryopreserved reproductive cells or embryos (Dulioust et al. 1995; Savushkina and Zvetkova 1994; Savushkina 1996; Van der Bank and Steyn 1992; Van der Walt et al. 1993; Nakatsukasa et al. 2001), the mechanism of this is still not clear.

Conclusion

Some conclusions can be drawn from studies carried out so far: (1) Cryopreservation of sperm can cause some disturbance in the degree of chromosome condensation, but it varies between different species and different individuals. Spermatozoa from healthy donors tend to be more cryoresistant and in most cases do not show any changes. Changes in the degree of condensation are more like to be connected to the imbalance in DNA–protamine interaction. More investigations are needed to study this factor in more species, to determine the exact mechanism of the imbalance in condensation, to study whether the changes in condensation caused by cryopreservation are reversible or whether they play any significant role in the further development of embryos. (2) Based on the comet assay study, it can be speculated that some single-strand breaks in the DNA are possible. Other methods need to be applied to study this further. It seems that single-strand breaks do not necessarily happen and their production is more likely to happen in samples from individuals with fertility or sub-fertility problems. More direct studies on DNA damage after cryopreservation need to be done. (3) Some chromosomal aberrations, such as trisomy, have been reported, and again more studies are needed on different species. There has not been any research carried out on the effect of cryopreservation on the sperm centriole, damage of which may cause faulty distribution of the chromosomes at cell division. (4) Conflicting results have been obtained with embryos derived from cryopreserved sperm. More studies are needed on such embryos because it is important not only to find changes in the genome of spermatozoa but also to assess whether these changes contribute to the future offspring. Evaluation of embryos can be used as a tool to separate the viable and motile spermatozoa from the non-viable ones in the same ejaculate.

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Mutations in the CFAP-Coding Genes Lead to Male Infertility with Multiple Morphological Abnormalities of the Sperm Flagella

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Background: Sperm motility is vital to human reproduction. Malformations of sperm flagella can cause male infertility. Men with multiple morphological abnormalities of the flagella (MMAF) have abnormal spermatozoa with absent, short, coiled, bent, and/or irregular-caliber flagella, which impair sperm motility. Genetic factors are involved in the pathogenesis of MMAF.

Main Questions: The known MMAF-associated genes are only responsible for approximately half of human cases. Novel pathogenic mechanisms still remain to be elucidated in MMAF.

Experimental Design: We conducted genetic analyses using whole-exome sequencing and genome-wide comparative genomic hybridization microarrays in a multicenter cohort of 80 Chinese men affected by MMAF. Furthermore, the genome editing technology of CRISPR-Cas9 was employed to generate knockout mouse models for further functional investigations on our identified novel genes for MMAF.

Main Results: Biallelic mutations in the genes encoding cilia and flagella associated proteins (CFAPs) were identified in 15 (19% out of 80) cases with MMAF, including 7 in *CFAP43*, 2 in *CFAP44*, 4 in *CFAP65*, and 2 in *CFAP69*. These CFAP-coding genes are specifically or preferentially expressed in testis. Knockout mouse models of these four genes manifest short, coiled flagella and other phenotypes consistent with human MMAF.

Conclusions: Our experimental observations on human subjects and mouse models strongly suggest that biallelic mutations in *CFAP43*, *CFAP44*, *CFAP65*, and *CFAP69* can cause sperm flagellar abnormalities and impair sperm motility.

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The Mouse-Specific Gly-to-Cys Mutation in Mammalian Acrosin is a Cause of Impairment in Proteolytic Activity

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Background: Among eutherian mammals, only mouse has an additional cysteine residue (Cys162) next to the conserved Cys163 of acrosin, and the mouse acrosin shows exceptionally low proteolysis. Acr–nul male mice are fertile and yield litters in size comparable to wild-type ones, whereas acr–nul rats yield litters much smaller in size and show in vivo sperm penetration of egg ZP at a frequency much lower than the wild type. No recombinant acrosin has been obtained so far in the mature and active form.

Main Questions: Why does mouse acrosin show exceptionally low proteolytic activity? Could such low activity of mouse acrosin be ascribed to the additional Cys162 residue?

Experimental Design: The Cys-to-Gly and Gly-to-Cys mutants as well as wild types of mouse and rat acrosin were prepared using pMT/Bip-V5-His insect cell expression system. Proteolytic activity was evaluated using gelatin–zymography, while amidase activity was determined using peptide–MCA fluorometric substrates.

Main Results: Like native ones, recombinant mouse and rat acrosins showed almost no and remarkable proteolytic activities, respectively. Reverse mutation of Cys162-to-Gly in mouse acrosin resulted in the recovery of proteolytic activity comparable to rat acrosin. Conversely, Gly162-to-Cys mutation of rat acrosin markedly impaired the proteolytic activity. Interestingly, all of these acrosins and mutants showed clear amidase activity equally. On the other hand, mutation of the conserved Cys163 to Gly severely impaired not only proteolytic activity but also amidase activity of the mouse Cys162-to-Gly acrosin.

Conclusions: Mouse may have evolved sperm by somehow adapting to acrosin activity impairment.

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Chemical and Genetic Approaches to Identify *Caenorhabditis elegans* Spermiogenesis-Related Factors

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Spermiogenesis is the final phase of spermatogenesis in which round spermatids transform into spermatozoa. However, the molecular basis of spermiogenesis is largely unknown in many species. One of the reasons why we use *Caenorhabditis elegans* to study spermiogenesis is that spermatids from this organism do not require any hormones and accessory cells in vitro to become spermatozoa. *C. elegans* spermiogenesis consists of two pivotal events, the pseudopod extension from spermatids and the fusion between the plasma membrane and the intracellular membranous organelles (MOs). In vitro activators such as Pronase (Pron) can induce these two cytological reactions in a simple, chemically defined medium. This advantage enables us to explore compounds that up- and downregulate spermiogenesis, which would be powerful tools for basic research and be the seeds of future drugs for infertility and contraception.

Our aim in this study is to obtain compounds that exhibit significant effects on *C. elegans* spermiogenesis and to clarify how those compounds are involved in the spermiogenesis pathway. Therefore, we screened a chemical library to find out compounds that block Pron-induced spermiogenesis and eventually obtained DDI-1, an interesting compound that blocks the pseudopod extension, but not the MO fusion. Moreover, we introduced random mutations into the *C. elegans* genome with ethyl methanesulfonate, isolated mutant males, and tested if spermatids from those males can activate into spermatozoa by Pron stimulation in the presence of DDI-1. By this genetic screening, we isolated several mutant strains in which the inhibitory activity of DDI-1 is neutralized. Currently, we are trying to figure out the mutated genes that show the suppressive effect.

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Part VI
CASA: Advances and Challenges

There have been two major consensus papers about CASA – the first from the Spermatology meeting in Cairns in 1994, the second from an ESHRE SIGA campus meeting in San Miniato in 1998 – but many of the recommendations from those meetings are still overlooked or ignored.

The aim of this session is to revisit these recommendations and decide which are still relevant today, in other words, what is still left to do to bring CASA to the point where it is the "go to" solution for all relevant work. The concept of this session is to consider what CASA is currently being used for in the lab, and what it could be used for if it had the capacity.

Routine Application of CASA in Human Clinical Andrology and ART Laboratories

David Mortimer and Sharon T. Mortimer

Introduction

The fundamental biological significance of Eutherian sperm motility has been reviewed extensively elsewhere (Mortimer 1997) and its assessment is a key component of a semen analysis (Björndahl et al. 2010). But it is not just the proportion of the spermatozoa that show flagellar motility that is important, rather it is the quality of their movement that is key. As long ago as 1951, Macleod and Gold noted that "The quality of sperm motility is a prime factor to be considered in semen analysis. Achievement of intra- and inter-observer standardization is essential in any method used to assess sperm motility, and observers must be properly trained" (MacLeod and Gold 1951). Patterns of sperm movement are described using kinematic measures, and determining these "kinematics" cannot be performed objectively by eye beyond basic subclassifications into classes of progression (rapid, slow, and nonprogressive).

Early objective methods for determining sperm kinematics were based on microcinematography (Rothschild 1953; Rikmenspoel and van Herpen 1957), multiple-exposure or timed-exposure photomicrography, and later videomicrography (Overstreet et al. 1979; Katz and Overstreet 1981); for historical reviews see Mortimer (1990) and Mortimer (1997). The basis of current day human sperm kinematics was the microcinematographic work from the laboratory of Professeur Georges David in Kremlin-Bicêtre, Paris (David et al. 1981). While cine- and videomicrographic technologies were powerful research tools they were laborious, requiring manual frame-by-frame tracking of the spermatozoa and manual analysis of the sperm tracks. As such, they were not amenable to routine clinical applications, but the development of personal computer technology in the early 1980s led to the introduction of computer-aided (or -assisted) sperm analysis technology

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("CASA") in the late 1980s. Unfortunately, misguided marketing strategies whereby these early CASA systems were advertised and sold as "computer-automated semen analyzers" led to extensive criticism of the technology since it was simply unfit for this purpose at that time (e.g., Mortimer 1994a; Mortimer et al. 1995; Mortimer and Mortimer 1998). As a consequence, CASA has never realized its true potential in human clinical andrology and is still viewed with substantial skepticism by many scientists and clinicians alike.

A workshop organized by the European Society for Human Reproduction and Embryology's Andrology Special Interest Group in San Miniato (Italy) in April 1997 resulted in consensus guidelines on the application of CASA technology, with particular relevance to human spermatozoa (ESHRE Andrology Special Interest Group 1998). In particular, these guidelines identified limitations in the use of CASA for analyzing human semen samples (see also Mortimer 1994a). Lack of substantial functional improvements in CASA technology (i.e., beyond simple developments in computer/imaging technology) since that time has meant that those guidelines are largely still pertinent today (Mortimer et al. 2015b).

In this paper, we review how we have used CASA in routine clinical andrology over the past two decades, including how CASA is valuable for identifying functional subpopulations of spermatozoa both as part of a more functional approach to semen analysis, and in washed sperm populations (sperm hyperactivation) as a sperm function test. We also consider the current limitations of CASA technology for human sperm morphological analysis, as well as the conditions necessary for moving CASA technology forwards in clinical andrology by integrating standard semen analysis quality control aspects into its use, and how CASA technology needs to be validated by the manufacturers before selling their systems to users.

CASA and Human Semen Analysis

Table 1 summarizes the main issues why human semen is such a difficult material to analyze in this regard: indeed, human semen is probably the most difficult type of specimen to analyze by CASA, and many patient samples are "the worst of the worst." While the limitations of CASA technology for identifying human spermatozoa in semen and reliably differentiating them from the debris were discussed more than 20 years ago (Mortimer 1994a, 1997; Mortimer and Mortimer 1998), and remain of concern today, technology improvements have reduced their severity, especially when the more common positive–low (PL) phase contrast optics are used instead of negative–high (NH) phase contrast optics (whereby the sperm heads, as well as many other objects appear as bright objects against a dark gray/black background, very similar to dark ground illumination). Many modern CASA systems allow the operator to edit/correct the objects identified within the analysis field to correct for missed or spurious spermatozoa, but this is quite time consuming and, in real-world practice, many operators simply do not bother to do this—causing

Table 1 A comparison of biological and technical issues in the analysis of human and domesticated animal semen samples using CASA

Criterion	Domesticated animals	Humans
General fertility/ fecundity	Animals such as bulls and boars have been selected over many generations for high fertility	Mankind is generally a low fecundity species (maximum 28%)
Semen cleanliness (presence of other cells, debris, etc., in the semen)	Generally "clean" in terms of other cells, debris, etc.	Typically very "dirty" ejaculates, containing lots of particles and cellular and other debris (large amount of background noise)
Semen viscosity	Most species have relatively low viscosity semen	Generally, high viscosity, with micro-heterogeneity, making accurate sampling very difficult
Possibility for dilution prior to analysis	High (e.g., many domesticated and wildlife species > 1000 M/mL); samples are typically highly diluted with an "extender" before analysis	Generally < 200 M/mL, often very low (< 25 M/mL) Dilution requires homologous seminal plasma to preserve motility kinematics
Proportion of motile spermatozoa	Typically (very) high proportions of motile spermatozoa, often well above 60%	Typically lower (often < 50%) Lots of dead spermatozoa with aggregation ("clumping")
Sperm morphology	Highly consistent in very many species, although in some groups (e.g., carnivores) there can be many types of abnormalities (and the naked rodent mole has spermatozoa that are even more pleomorphic than man)	Highly pleiomorphic morphology

Adapted from Mortimer et al. (2015b)

inaccurate results. An ideally suited CASA system should not require human input in such a basic process step.

Because of these issues, the CASA-determined concentration of spermatozoa in a human semen sample is often incorrect—and perhaps only actually correct when the various sources of error such as missed and clumped spermatozoa on the one hand, and spurious spermatozoa on the other, cancel each other out. In "clean" semen samples these issues are insignificant, but in routine clinical andrology laboratories, such samples are often a rarity. Obviously if the total sperm concentration is incorrect then any proportions of motile spermatozoa will perforce also be incorrect.

Another key issue with human semen is that it is effectively impossible to mix such a viscous fluid to achieve a completely random distribution of the cells throughout the sample, there is always a degree of "micro-heterogeneity." The standard volumetric sampling and dilution of human semen for haemocytometry employs a 50 µL aliquot of semen taken using a positive displacement pipette (Mortimer 1994a; Björndahl et al. 2010, 2016), which is based on Mortimer et al. (1989). Although not discussed in the 1989 paper, the reason we settled on a standard 50 µL

aliquot was because when we tried using a 25 μL volume substantially more assessments had to be repeated due to nonagreement of the replicates (original data no longer available). Clearly the inherent sampling variability when using aliquots of just 2–5 μL to load fixed-depth slide preparations such as are used for CASA, or Makler chambers, will be far greater, undermining the likelihood of comparable replicate analyses using such small aliquots of human semen.

In general, any CASA system should be able to identify moving objects and identify those that are spermatozoa (as opposed to, e.g., objects showing Brownian motion, or false motion caused by specimen drift, collisions, or "stirring"). Therefore, CASA should provide accurate results for the *concentrations* of motile spermatozoa, especially those that are defined as progressively motile, when fixed-depth preparations are used in the absence of artifacts such as the Segré–Silberberg effect that affects capillary-loaded slide chambers (Douglas-Hamilton et al. 2005a, b). Nonprogressive motility (NPM) remains an issue for most CASA systems since the human classification of these cells is based on their showing flagellar movement but no "space gain"—but CASA analysis is based upon tracking sperm head movement. The consequence of this is that when comparing a three- or four-category motility assessment performed by a properly trained human operator with a CASA analysis, the NPM subpopulation of cells will typically differ. While this is an issue for simplistic data comparisons (e.g., as part of software validation), we need to recognize that these spermatozoa are clinically irrelevant since they can never penetrate the cervical mucus in vivo (and hence have no chance of contributing to a conception)—and therefore focus on the progressive spermatozoa only. This will be key when performing proper validations of CASA software in the future.

The "gold standard" methods for determining total sperm concentration remain flow cytometry and careful haemocytometry by properly trained operators (Björndahl et al. 2010, 2016). Although PL phase contrast-based CASA can come close to such results, no system has yet been validated against reference methodology as per the criteria defined by Björndahl et al. (2016). But how often is the total sperm concentration important? Most modern clinical situations do not rely on this information, for example, in all forms of assisted reproductive technology (ART). Even the vaunted updated WHO Lab Manual fifth edition ("WHO5") clinical cutoff of 15 M/mL (Cooper et al. 2010; World Health Organization 2010) has little value here (see Björndahl 2011) because, as derived, its clinical interpretation is that if a man's semen sample shows < 15 M/mL then there is a < 5% chance that he has fathered a child in the past 12 months—but if he is sitting with his partner in an infertility specialist's office then we already know that! Therefore, in many clinical situations, CASA could be used to analyze the concentration of progressively motile spermatozoa in semen, which any system should be able to do with good accuracy.

Going further, in biological terms, only those progressively motile spermatozoa in semen that are capable of penetrating into and migrating within the cervical mucus are of clinical importance for fertility. Based on historical experimental evidence (e.g., Aitken et al. 1985; Mortimer et al. 1986), these spermatozoa are actually a subpopulation of the "rapid progressive" subpopulation—which is why the

ESHRE Andrology SIG has rejected the assessment of a single "progressive" category as per WHO5 (Barratt et al. 2011). Indeed, based on studies of sperm-mucus interaction performed in the 1990s, and adapted for evolving sperm kinematics analysis technology, we have been identifying a "good mucus penetrating" fraction of motile human spermatozoa as part of a more functional approach to routine semen assessment for over 20 years (Mortimer and Mortimer 2005, 2013). For the Hamilton Thorne IVOS and IVOS-II platforms operating at 60 Hz imaging frequencies and measured at 37 °C the Boolean sort algorithm for this subpopulation is:

$$\text{VAP} \geq 25\,\mu m/\text{s AND STR} \geq 80\% \text{AND ALH} \geq 2.5\,\mu m$$

To avoid including spermatozoa whose kinematics have been compromised by ROS-induced damage, which can cause a quasi-hyperactivation pattern of movement, a fourth term of ALH < 7.0 μm can also be included.

CASA and Washed Sperm Preparations

Human sperm populations prepared by either density gradient centrifugation or direct swim-up from semen should be very clean (i.e., minimal contamination with dead or immotile sperm and other cells or debris), with reference values for competence and benchmark of 90% and ≥ 95% progressively motile, respectively (ESHRE Special Interest Group of Embryology and Alpha Scientists in Reproductive Medicine 2017). Obviously such specimens will be very amenable to CASA analysis for the determination of both total and motile sperm concentrations as well as for sperm kinematics. Of special interest in such preparations is where the spermatozoa are incubated under capacitating conditions and the subpopulation that show hyperactivated motility can be analyzed (Mortimer 1997; Björndahl et al. 2010; Mortimer and Mortimer 2013).

Hyperactivation in Eutherian spermatozoa is due to a change in the flagellar beat pattern (Mortimer 1997). Boolean sort criteria for human spermatozoa have been validated for the Hamilton Thorne IVOS and IVOS-II platforms operating at 60 Hz imaging frequency and measured at 37 °C based on hyperactivating spermatozoa identified using flagellar beat criteria (Mortimer et al. 1997; Mortimer 2000):

$$\text{VCL} \geq 150\,\mu m/\text{s AND LIN} \leq 50\% \text{AND ALH} \geq 7.0\,\mu m$$

The Hamilton Thorne CASA-II software (available on the IVOS-II and CEROS-II platforms) also includes the kinematic measure of fractal dimension ("D"), allowing the use of a more robust definition of human sperm hyperactivation that should be independent of track smoothing which is otherwise needed to derive ALH values (Mortimer et al. 2015b):

$$\text{VCL} \geq 150\,\mu m/\text{s AND D} \geq 1.20$$

HA motility assessment of washed sperm populations can be used for the prediction of fertilization failure, with reduced levels of HA associated with reduced fertilizing ability in vivo and in vitro (Johnston et al. 1994; Alasmari et al. 2013).

Issues of Non-comparability of Kinematics Between CASA Systems

When publishing a kinematic definition, it is critical that the imaging frequency and software version/CASA instrument are defined. Imaging frequency affects the appearance of the track, with lower sampling rates giving a simpler track (Mortimer et al. 1988b), resulting in reduced curvilinear velocity (VCL) values and increasing the amplitude of lateral head displacement (ALH) values, thereby affecting the proportion of tracks that meet any predefined criteria. The reason that VCL is affected is relatively straightforward: fewer points along the track means fewer deviations from a straight-line path, which is the shortest distance between two points. Why ALH is affected, particularly using CASA, is a little more complicated.

As originally defined, ALH is an expression of the width of the track. When manual track analysis was being used, the operator could see the "peaks and troughs" of the track and simply measure the distance between them. This is not possible with CASA, which calculates kinematic values from the *(x,y)* coordinates of track points, and so the peaks and troughs are identified by comparing the lengths of "risers"—the distance between points on the curvilinear path and their corresponding points on the smoothed "average" path (Fig. 1). The algorithm used for the derivation of the average path is largely CASA instrument-dependent, so different types of instruments will produce slightly different average paths. The most

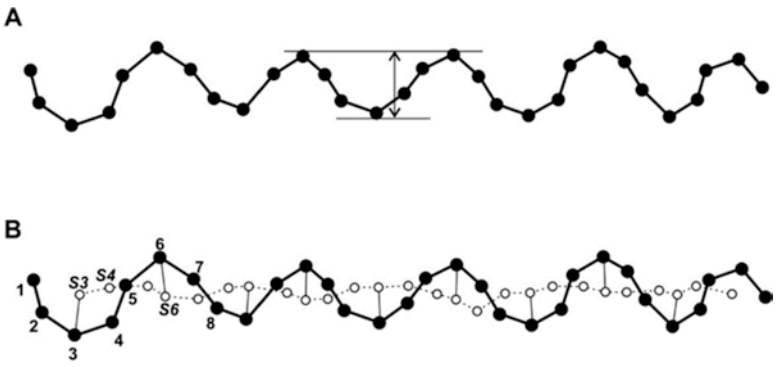

Fig. 1 Derivation of ALH values via manual analysis (**a**) and CASA (**b**). Note that ALH describes the track width (rather than the distance between the point of inflection and the peak). For CASA derivation, the ALH is determined using riser length—the local maxima of the distance between a point on the curvilinear path and its corresponding point on the average path. The riser lengths are then doubled for the calculation of ALH

commonly used method for defining the points on the average path is to use Tukey windows or smoothing. Smoothing is achieved by taking the mean of a group of *(x,y)* coordinates from the curvilinear path. So, for example, with 5-point smoothing the coordinates of the first five points on the curvilinear path are averaged, giving the first point on the smoothed path (this point is the "averaged" value for the middle of the five points on the curvilinear path, i.e., track point 3; Fig. 1). This calculation then continues along the track, with the next point on the smoothed path being the mean *(x,y)* coordinates of points 2–6 on the curvilinear path, and so on. Once the riser values are determined for a track, the local maximum values are identified and these are taken to represent the track peaks or troughs. The degree of smoothing that is used can have a significant effect on the riser length, and hence on the derived ALH values (Fig. 2). Different CASA systems use different smoothing algorithms, some with different fixed-point (e.g., 5-, 7-, 9-, or 11-point) Tukey windows applied to all tracks, while others use "adaptive smoothing" algorithms that optimize the smoothing of each track. Hence, the average paths will differ, resulting in different values for VAP and for the risers used to calculate ALH.

Another area of difference between CASA instruments is how the derived riser values are used to calculate the ALH value of a track. Firstly, some instruments do not conform to the consensus definitions for ALH (originally set by a Working Group at the American Society of Andrology meeting in Houston in 1988), and only present the riser value (i.e., a classical wave amplitude value), rather than the full

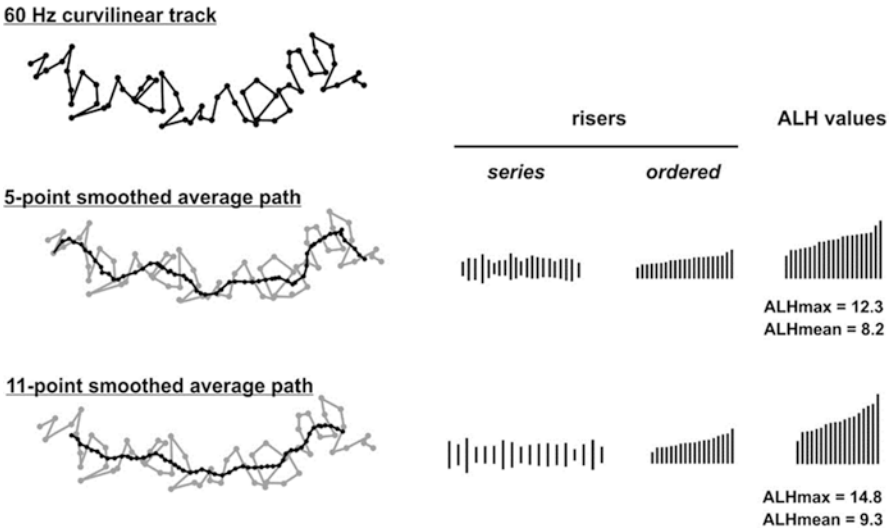

Fig. 2 The figure shows an example of a 60 Hz curvilinear track of a washed human spermatozoon, along with the derivation of its average path using fixed 5- and 11-point smoothing. Alongside each of the smoothed tracks are the local risers derived from that track arranged first in series order (i.e., their sequence along the track) and then in ascending order. In the right-hand column, the ALH values derived from these riser values (ALH = 2× riser) are shown for comparison between the two smoothed tracks (arbitrary units)

track width, which would be 2× the riser value(s). Secondly, some instruments present track-averaged values (ALHmean), while others present the maximum value for the track (ALHmax).

Clearly, then, this information is critical in being able to conduct a meaningful comparison between the results of studies using different CASA instruments. Moreover, a sort definition validated for one instrument's software will not be valid for a different system if there are differences in imaging frequency, track smoothing, or ALH calculation.

Sperm Functional Assessment: The "SFA"

In the mid-1990s we realized that trying to develop the ultimate "assault course" for testing spermatozoa in the andrology laboratory in an attempt to identify the underlying diagnostic cause(s) of male factor infertility was effectively a lost cause. This was due to the increasing availability and success of ICSI, with the concomitant loss of interest by clinicians in identifying or explaining sperm dysfunction, and lack of interest in many patients in having such a diagnosis when all they really wanted was to achieve a pregnancy using ART as soon as possible. In response to this realization, we developed the concept of "structured management" (Mortimer 1994b, 1999) whereby information from simple diagnostic testing is used in a progressive manner to stream the management of infertile couples using ART. After initial investigations, couples for whom simple insemination-based treatment would have a good chance of achieving a pregnancy could embark upon such treatment, while couples for whom such treatment would be contraindicated on the grounds of likely impaired gamete approximation would proceed directly to IVF—unless severe sperm dysfunction had been identified, in which case they would proceed directly to ICSI. Hence such management protocols determine the appropriate level of medical intervention required to achieve a reasonable chance of pregnancy according to available diagnostic information. "Appropriate" is judged in terms of cost (both healthcare resource use and to the patient), the likelihood of a successful outcome (especially in consideration of the female partner's age and associated risk factors such as patient morbidity) and be dependent, to a greater or lesser degree, upon local circumstances reflected in the availability, accessibility, and affordability of the various diagnostic and therapeutic services.

The andrology testing necessary for this sort of management can be packaged into a simple "sperm functional assessment" or "SFA," comprising:

- a comprehensive semen analysis (as per the ESHRE Andrology SIG rather than WHO5 since its morphology assessment needs to include the Teratozoospermia Index or "TZI")
- CASA assessment of the seminal sperm population to determine the "good mucus penetrating" fraction
- an investigation for sperm surface-bound antisperm antibodies ("ASABs")

- a "trial wash" using an optimized two-layer density gradient technique and
- CASA assessment of the washed sperm population for hyperactivation using the "HAmax" assay which includes both a control assessment and one using a combination of progesterone and pentoxifylline as an agonist to induce maximal levels of hyperactivation following only a 1 h incubation under capacitating conditions.

Detailed methods for all these components are available in Björndahl et al. (2010). Unfortunately, with the unavailability of IMMUNOBEAD reagents in recent years, a simpler assessment of ASABs than that described by Rowe et al. (2000) has had to be accepted, considering only a high prevalence of head-directed ASABs as indicating the need for ICSI.

Clinical Value of the SFA

Combining these two CASA analyses with other prognostic markers of sperm function, especially sperm morphology including the TZI, has been found to be an effective approach in identifying causes of infertility, and in designing personalized treatment strategies that bypass couple-specific problematic functional aspects. In one center, among 485 new referrals, 103/266 (39%) men with "normal" WHO semen analyses had abnormal results in other SFA components, causing ICSI to be recommended: yet the overall prevalence of ICSI decreased from 60% to 40% alongside the development of a very active IUI treatment stream with a concomitant decrease in failed IVF fertilization (Mortimer et al. 2002; see Table 2). Moreover, clinical outcomes among a cohort of couples for whom IUI was recommended based on the SFA, were significantly higher than those among a contemporaneous group of couples who had not been prescreened: fecundity rates of 22% c.f. 11% per cycle, and pregnancy rates of 41% c.f. 18% per couple (Mortimer et al. 2002; see Fig. 3).

In another center where the SFA was routinely performed prior to ART treatment, during the period 2006–2014 the prevalence of failed fertilization and low (<25%) fertilization rate among the IVF and ICSI cases was investigated (Mortimer et al. 2015a; see Table 3). Further investigation of the failed/low IVF fertilization cases revealed that at least half of these abnormal outcomes could be attributed to atypical stimulation response, primarily unusually high numbers of COCs retrieved

Table 2 Change in performed treatment modalities at one center as a result of introducing the SFA (Mortimer et al. 2002)

	Treatment cycles per year			
Period	IVF/ICSI	IUI	IVF: ICSI	Low (< 25%) or failed IVF fertilization
Before SFA	550–600	Very few	40: 60	6%
With SFA	650–700	ca. 600	60: 40	1%

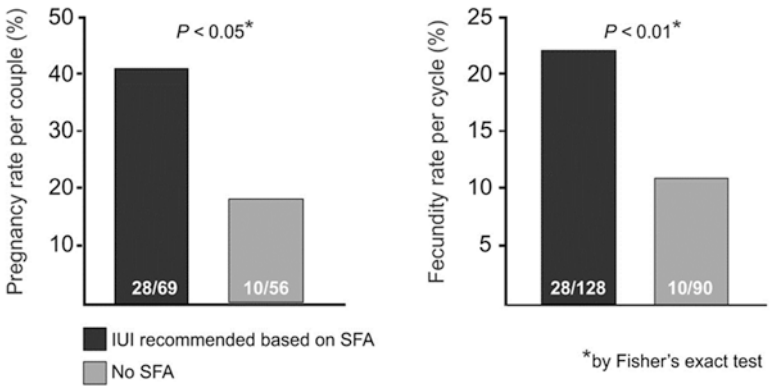

Fig. 3 Treatment outcomes in a population of 69 good prognosis patients identified through SFA pre-screening compared to a contemporaneous population of 56 patients who were not pre-screened (128 and 90 IUI treatment cycles respectively). Note that clomiphene or FSH was only used in 28% of the cases. (From Mortimer et al. 2002)

Table 3 Prevalence of low and failed fertilization cases during the period 2006–2015 in a center where the SFA is used routinely prior to ART treatment (from Mortimer et al. 2015a)

Fertilization	IVF	ICSI	IVF vs. ICSI
Low (< 25%)	16 (1.9%)	34 (3.0%)	−1.1% ($P = 0.148$)
Failed	14 (1.7%)	27 (2.4%)	−0.7% ($P = 0.339$)
Cases	830	1129	42.4%: 57.6%

and poor oocyte maturity: leaving a probable sperm-based risk of IVF fertilization failure of < 1% (Table 4). Therefore, when sperm functional potential is carefully evaluated prior to treatment, failed or low IVF fertilization occurs with a very low prevalence (total of 3.6%). Extrapolating from these findings, a "poor" semen analysis, even if several characteristics fall below WHO reference values for recently fertile men, does not seem adequate justification for using ICSI.

Strategies to Validate CASA Technology for Clinical Applications

During the early years of CASA technology (late 1980s through the 1990s), many researchers attempted to validate CASA technology for human semen analysis applications from the basic technology aspect and the clinical applicability (diagnosis/prognosis) perspectives (reviews: Mortimer et al. 1995; Mortimer and Mortimer 1998). Unfortunately, much of this work suffered from fundamental technical issues that undermined the quality of the data, and hence the clinical value of CASA semen analysis results.

Table 4 Clinical background of the low and failed IVF fertilization cases from Table 3 (from Mortimer et al. 2015a)

	Fertilization	
Stimulation response	Failed (0%) (n = 14)	Low (<25%) (n = 16)
Normal	2	3
High (>15 COCs)	1	8
Low (<5 COCs)	6	0
Poor maturity (<4 MIIs)	7	5

At that time even fewer andrology laboratories than today performed clinical semen analyses robustly using properly implemented methodology performed by adequately trained operators working within a quality-managed environment (see Björndahl et al. 2016). Consequently, when CASA results were compared against those obtained in a reference andrology laboratory there was very poor comparability. This was especially so when data were analyzed using appropriate statistical methodology such as "discrepancy analysis" (Bland and Altman 1986)—rather than by simple linear regression with no consideration of slope or intercept—revealing the poor accuracy of CASA results for sperm concentration and the percentages of motile or progressively motile spermatozoa in semen (e.g., Mortimer et al. 1988a). Obviously, studies that attempted to use such inaccurate, poor quality clinical data against diagnostic or prognostic endpoints were thereby severely compromised or even foredoomed. Clearly, CASA manufacturers and vendors were very frustrated by this, but none of them performed or supported rigorous validation studies, typically saying they were too expensive. In the late 1980s, one vendor stated that he couldn't understand why we were so concerned with the "right answer" when the results from their CASA system were so much more reproducible than their experience with many andrology laboratories (D Mortimer, personal communication): clearly failing to grasp the vital importance of accuracy compared to precision (akin to clustering gunshots closely together, a substantial distance from the bullseye). However, within the modern regulatory environment for in vitro diagnostic devices, which covers all automated analyzers used in clinical laboratories, it is now incumbent on the manufacturer to establish that a device performs to an adequate standard, including operational quality control aspects of the device or instrument, prior to marketing it for such purposes or use; for example, for the US FDA, see www.fda.gov/MedicalDevices/DeviceRegulationandGuidance/IVDRegulatoryAssistance/ (accessed February 1, 2021).

Appropriate accuracy and quality control aspects for semen analysis for andrology laboratories operating within the context of ISO 15189 ("Medical Laboratories—Requirements for Quality and Competence" International Standards Organization 2012), including the expectation of being able to state the uncertainty of measurement for reported results (measurement error should not exceed ±10%), are now well-established (Björndahl et al. 2016). This includes the need for rigorous staff training and the objective establishment of competence, internal quality control, external quality assurance, and the routine use of replicate assessments and establishing their reproducibility.

Applying these fundamental principles to validate the use of any CASA system for human semen analysis means that:

- A meaningful number of clinical specimens (sufficient for robust statistical comparison, ideally 120 as a minimum) must be analyzed in parallel by an expert andrology laboratory using reference methodology within a proper quality-managed operational environment, and the CASA system.
- If the CASA analyses included human intervention to correct the detection and recognition of spermatozoa within the image analysis process this must be stated, and such intervention must become a required step in the subsequent routine clinical use of the instrument.
- All assessments must be performed in at least duplicate, with verification of the adequate reproducibility of the replicates before calculating the final result.
- Expression of results to an appropriate degree of precision based on the methodology (i.e., no "false precision").
- The CASA-derived result must have an established measurement uncertainty or measurement error, that is, it needs to fall within ± 10% of the reference method's result.

Clearly, results can only be reported if the actual measurement was made. Hence an instrument described as an "automated sperm quality analyzer" that might report the proportion of spermatozoa with normal morphology based entirely on an algorithm that derives the morphology value indirectly from associated sperm concentration and motility data must be questioned as there is no reference value against which the sperm morphology result can be validated.

Without proper validation, no claims of suitability for purpose (e.g., "automated semen analyzer") should be made. Hopefully, the modern regulatory environment will improve the performance of CASA systems for the future—but this will only happen if the validation studies are performed in expert andrology laboratories in strict accordance with the abovementioned criteria.

Although we have not considered computer-aided/-automated sperm morphology assessment in detail, the same sort of validation must be applied there as well. However, currently, this is effectively impossible since human observers assess sperm morphology by evaluating the head, neck, midpiece, tail, and cytoplasmic residue regions, while CASA sperm morphology systems only look at the head morphology and morphometry, and perhaps neck symmetry. Consequently, for this application CASA morphology systems must become more sophisticated and consider the whole cell, ideally using color imaging to take proper advantage of polychromatic staining techniques. Without this sophistication, even the simplistic "*%* normal [ideal] forms" is not possible, much less determination of the Teratozoospermia Index ("TZI," see Björndahl et al. 2010).

Conclusions

Key messages of this review are:

1. CASA technology still has significant limitations as to its applicability for human semen analysis. These are mainly related to imaging and reliable discrimination between immotile spermatozoa and other cells and debris.
2. Without proper validation, total sperm concentration and the proportions of motile and progressively motile spermatozoa in semen cannot be determined by CASA; criteria for robust validation have been provided, along with the necessary uncertainty of measurement. Values for the concentrations of motile and progressive spermatozoa should be reliable, provided that the spermatozoa have been properly differentiated from spurious motile spermatozoa and other cells.
3. The reporting of population-averaged values for kinematic measures of human spermatozoa in semen is biologically meaningless due to coexisting heterogeneous subpopulations of spermatozoa; rather, functional subpopulations should be identified and quantified, for example, those which show the necessary kinematics for penetrating cervical mucus.
4. CASA technology is ideally suited to the analysis of washed populations of human spermatozoa: reliable values for sperm concentration and the proportions of motile and progressively motile spermatozoa are readily obtainable.
5. Population-averaged values for kinematic measures of washed human sperm preparations are biologically meaningless due to coexisting heterogeneous subpopulations of spermatozoa. Instead, functional subpopulations should be identified and quantified instead, for example, hyperactivating spermatozoa.
6. Human sperm hyperactivation can be reliably determined from head centroid-derived tracks using current CASA technology. However, hyperactivation classification criteria validated for one system must not be used with another system unless the systems employ identical track analysis algorithms and/or the classification criteria have been specifically revalidated for the other system.
7. It must be the responsibility of the CASA instrument vendor to validate their system for each application for which suitability is claimed. This needs to include a statement of the achievable measurement error, and the validation needs to be published (at least on the vendor's website) including proper statistical analysis of the data obtained by properly trained personnel working in an expert laboratory against appropriate reference materials.

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Processes and Data Management of Computer-Aided Sperm Analysis in Human and Animal Spermatozoa

Gerhard van der Horst

CASA Parameters Quantified and Pitfalls

Despite the advances in automation of Computer-Aided Sperm Analysis (CASA), there are four important aspects that need consideration. Firstly, which parameters are measured? Secondly, how do we eliminate/control the many variables in methodology such as handling of semen, preparing sperm for various assessments, and microscopic techniques? Thirdly, which parameters are the most important in terms of sperm functionality, and finally how do we statistically analyze and interpret this vast amount of data to make biological sense? The main aim of this paper is to show systematically how to resolve problems surrounding the four aforementioned aspects indicated and present a good basis for the different quantitative and qualitative approaches.

Table 1 lists the main motility parameters that are measured in an advanced CASA system including data of Saanen goat spermatozoa in phosphate-buffered saline. More than 50 parameters are quantitatively assessed and divided into motility percentage groupings and kinematic parameters. Sperm functional aspects can also be assessed by using sort functions to define sperm mucous penetration and hyperactivation, for example. For the percentage groupings, sperm subpopulations such as rapid progressive, medium progressive, and nonprogressive are measured and the same applies to the eight kinematic parameters. In addition, sperm movement can also be expressed on the basis of speed only or just progression and even in terms of rapid nonprogressive sperm. This last category has been ignored by all CASA systems (high velocity and low linearity) and may prove to be the spermatozoa that are more likely to penetrate and fertilize the oocyte.

Table 2 lists the main morphology parameters that are quantified by CASA. By careful selection of large number of morphometric parameters (Table 3), it is

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Table 1 The range of CASA motility parameters that can be measured including both motility groupings (e.g., percentage rapid progressive) and kinematics and kinematic groupings (e.g., VCL of medium progressive). The specific values represent an example of Saanen goat sperm in phosphate-buffered saline (unpublished data)

	Total	Percentage (%)	M/mL	M/Sample	VCL (μm/s)	VAP (μm/s)	VSL (μm/s)	STR (%)	LIN (%)	WOB (%)	ALH (μm)	BCF (Hz)
Motile	764	79.8	1756.8	3513.6	166.1	104.1	88. 5	81.8	54.3	64.0	3.4	28.8
Progressive	561	58.6	1289.8	2579.9	189.1	121.1	106.5	87.8	58.3	65.3	3.6	30.9
Rapid progressive	211	22.1	485.2	970.4	237.4	153.8	143.4	87.8	60.6	64.5	4.5	28.7
Medium progressive	350	36.5	804.1	1609.2	160.0	101.8	84.6	84.4	60.0	65.7	3.1	32.3
Non progressive	203	21.2	466.8	1.9	102.5	58.36	38.6	65.4	43.0	60.4	2.6	22.8
Immotile	90	21.2	1.4	933.6								
Rapid	278	29.1	639.3	1278.5	238	149.4	128.5	83.5	54.2	63.1	4.6	25.6
Medium	383	40.0	880.7	1761.4	141.9	92.5	79.4	83.3	56.6	65.7	2.7	29.6
Slow	103	20.2	443.8	887.5	62.7	45.1	38.7	80.5	61.8	71.8	1.32	18.6

Additional parameters: sort functions for mucus penetration test, hyperactivation, and several other sort functions present further possibilities

Table 2 Parameters for measuring the percentage of abnormal sperm in terms of head, acrosome, midpiece, and tail

Parameters (%)	Head specifics		Midpiece specifics	Tail specifics
Normal		Normal shape	Normal	Normal
Abnormal		Tapered	Normal size	Short
Head defects		Thin	Abnormal insertion	Absent
Midpiece defects		Round	Abnormal angle	Irregular
Tail defects		Pyriform		Rolled
Cytoplasmic droplets		Amorphous		Multiple
		Nuclear craters		Angulation
	Acrosome	Normal/abnormal		

Table 3 Morphometric parameters determined by CASMA such as SCA including the three sperm indices indicated below, representing combinations of head, midpiece, and tail abnormalities

Head parameters	Midpiece parameters	Tail
Length (μm)	Width (μm)	Length
Width (μm)	Area (μm)	
Area (μm)	Insertion distance	
Perimeter (μm)	Insertion angle (degrees)	
Ellipticity		
Elongation		
Roughness		
Regularity		
Vacuoles/craters (%)		
Acrosome cover (%)		

Additional parameters: teratozoospermic index (TZI), multiple anomalies index (MAI), and deformity index (DI)

possible to classify the percentage of morphologically normal spermatozoa and the various abnormal sperm morphology forms very accurately as indicated in Table 2. In addition, the abnormalities per spermatozoon or in the sperm population are expressed as various indices such as the teratozoospermic index (TZI), the multiple anomalies index (MAI), and the deformity index (DI) (WHO manual 2010). To calculate these abnormalities may take a considerable amount of time manually but in CASA systems such as Sperm Class Analyzer (SCA) is performed objectively within seconds. The different morphological percentages presented in Table 2 as well as the morphological indices give a much more comprehensive view of sperm morphology in animal species such as rats and dogs (van der Horst et al. 2017; Morselli et al. 2019).

There are various other sperm parameters that are quantified using CASA such as vitality, fragmentation, acrosome reaction, and others. The potential number of parameters being measured objectively by CASA thus far exceeds 100 and most of these cannot be assessed manually. However, care needs to be exercised in terms of three major aspects:

(a) Have all potential and practical variables been minimized when performing motility, morphology, and other CASA analyses?
(b) Are all these variables useful and which ones need to be focused on?
(c) Which are the most appropriate data analyses to be performed involving many approaches including both formal statistical procedures and multivariate visualizations, particularly when comparisons need to be made where no clear-cut statistical differences exist.

The most important variables that can affect the analysis

- Methods of collection, sampling vials, and semen handling
- Species considerations: ideal sampling and analysis conditions for a species
- Time elapsed from sampling to analysis
- Type of pipette used: positive displacement pipette and semen
- Media selected: species specific for wash, capacitation, and hyperactivation
- Temperature control during capturing: microscope stage heating
- Microscope conditions such as optics: Köhler and critical illumination
- Type of phase-contrast optics: positive for human sperm in SCA and negative phase for all animals
- Type of chamber (e.g., Leja, HT, and GoldCyto, chamber depth, and filling method and time)
- Field selection: random and middle of chamber
- Sperm concentration: control numbers to avoid collisions during motility analysis
- Stain and staining protocol for morphology analysis
- Number of sperm analyzed: at least 200 motile sperm but usually many more

These variables are largely self-explanatory and cannot all be discussed in this paper as it warrants a separate publication. However, some of these will be elaborated in more detail. For determination of accurate sperm concentration, it is essential to use a positive displacement pipette mainly to counteract the effect of spermatozoa sticking to the side of the pipette tip. Microscopic conditions need to be optimal which means correct setting of critical illumination and even background illumination (Köhler). For example, it is not only the object that needs to be sharply focused but also that the light needs to be sharply focused on the object (critical illumination). Most CASA systems use negative phase contrast for human spermatozoa but this can lead to inaccurate determination of sperm concentration as all particles have the same white appearance against a black background. Positive-phase contrast largely excludes non-sperm particles during analysis since they show as either black or dark grey and the sperm heads appear white. However, negative-phase contrast is essential for sperm motility analysis of mammalian sperm, other vertebrates, and invertebrates.

During CASA motility analysis, the number of spermatozoa captured in one field needs to be very accurately monitored and controlled. If there are too many collisions among spermatozoa, it will severely affect sperm kinematic parameters and even the percentage of sperm motility (an immotile sperm may be pushed by a motile sperm and will be recorded as motile).

Sperm morphology can only be accurately assessed using CASA provided that sperm fixation and staining are optimal and imply that the staining protocol neither swells nor shrinks the sperm. In a landmark paper, it has been shown that Papanicolaou shrinks sperm and DiffQuik swells sperm, while SpermBlue is isosmotic and isotonic with semen (Maree et al. 2010). For almost all stains commonly used to assess animal sperm, very little or almost no chemical or physiological information exists and it is accordingly not surprising that about 38 stains are in use for assessing animal sperm morphology (Chenoweth and Lorton 2014). There is accordingly a great need for standardization at least in terms of using the same stain for the same species/breed.

CASA Parameters and Sperm Subpopulations Which Needs to Be Focused on

Once experimental procedures are standardized, variables minimized (indicated above) and data collection trusted, which are the most relevant parameters to be used for data analysis? Many research articles report averages for most parameters, including sperm kinematics. However, for a parameter such as curvilinear velocity (VCL), individual values may range from 25 to 286 μm/s in a sperm sample and this usually results in a very large standard deviation, and accordingly, the average value has no scientific or biological meaning.

What is then the best way to select and group the raw kinematic data for analysis? Analysis of subpopulations seems to be the best option. In Table 1, percentage groupings are shown for a Saanen goat motility analysis and the average percentage motile is 79.8% and the VCL is 166 μm/s. However, the rapid progressive percentage is 22.1% but the VCL is 237 μm/s. If only the percentage of rapid sperm is considered, then almost 30% swim rapidly and implies there are rapid sperm that swims less progressively at about 190 μm/s. CASA systems do not seem to incorporate fast but nonprogressive sperm that may indeed be the ones that will eventually become hyperactivated.

Other subpopulations of sperm calculated by CASA (so-called sort functions) are sperm mucous penetration (VAP, ALH, and STR cutoff values) and hyperactivation (VCL, ALH, and LIN but also other kinematics in goat used as cutoff values) and they are species specific. Our comparative spermatology group at the University of the Western Cape calculated hyperactivation cutoff values for African elephant and Tankwa goat and these are currently submitted for publication. Figure 1 is an example of the typical hyperactivated pattern of Tankwa goat sperm compared to sperm incubated in phosphate-buffered saline.

There are many possibilities to combine several kinematic parameters to express various aspects of sperm functionality. Two further examples will be indicated: vigor of motility relates to VCL, VSL, VAP, and BCF, and DANCE (VCL × ALH) is an expression of the two-dimensional area occupied by a sperm swimming for

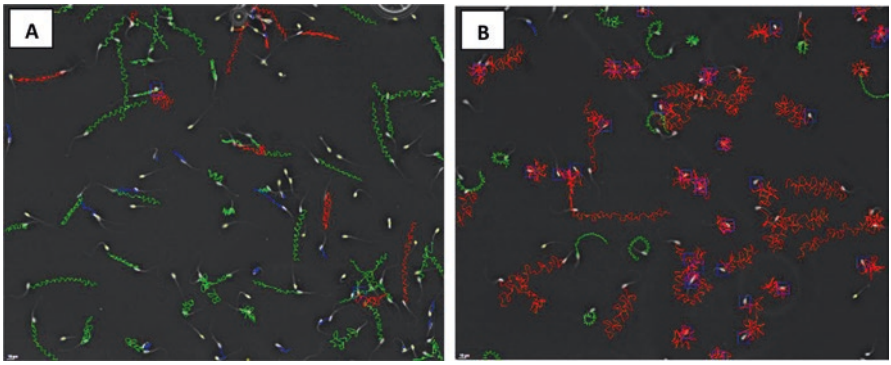

Fig. 1 (**a**) and (**b**) showing the importance of pattern recognition and analysis by CASA. (**a**) Tankwa goat sperm in phosphate-buffered saline medium. Sperm showing predominantly forward progression in terms of rapid progressive (red), medium progressive (green), and slow (blue). (**b**) Tankwa goat sperm in 2 mM procaine hydrochloride showing predominantly hyperactive sperm with high velocities and low linearity (red) and medium progressive sperm (green); (**a**) and (**b**) accordingly show how CASA is capable of identifying and classifying each sperm in terms of predetermined cutoff values

1 s. The calculations of these latter parameters can be performed as part of the sort functions of a CASA system. However, there are many further possibilities to combine specific parameters and analyze these using various statistical procedures (see later). The focus in the above section was on mammals but essentially all the above parameters and subpopulations can be determined in all other vertebrates such as fish, amphibians, reptiles, and birds. For example, Mafunda et al. (2017) published a paper on penguin sperm showing the value of the motility subgroups. Fewer studies have been published on CASA motility of invertebrate sperm but a recent paper elaborated on very detailed CASA motility studies and many applications in invertebrate broadcast spawners (van der Horst et al. 2018). In most marine broadcast spawners, sperm seem to swim in a helical pattern (van der Horst et al. 2018). Insect sperm with tail lengths exceeding 220 μm and a long thin head barely wider than the tail still provides major challenges for CASA motility analysis.

Which parameters are important in terms of morphology and CASA? Similar to motility, it is of little value to only use average values for the different morphometric parameters or even just the percentage of normal sperm morphology purely based on manual assessment. Van der Horst et al. (2017) showed how normal sperm morphology can be determined by using the percentile distributions of morphometric parameters of the domestic rat using CASMA and this method has also been applied for Tankwa goat (Ngcauzele 2018). Moreover, CASA morphology systems can calculate TZI, MAI, and DI rapidly. The value of these indices is that they add another important dimension to abnormal sperm morphology. All these indices in different ways indicate the number of abnormalities per sperm or abnormalities in the sperm population. Accordingly, when the percentage of normal sperm morphology is borderline for a particular species a TZI of 2.0 (more than two abnormalities per sperm)

may assist to show that there is actually a deeper problem associated with morphology and potentially functionality (fragmentation?).

In conclusion to "which parameters should be used," the focus needs to be on those sperm subpopulations as defined by using CASA sort functions and ones that relate to sperm functionality and potentially relating to fertility success. Flagellar analysis using devoted programs such as FAST (Gallagher et al. 2019) provides in conjunction with traditional CASA new opportunities for defining sperm function and hopefully potential fertilization outcome. It is also important to realize that sperm diversity among different animal species is enormous and requires species-specific cutoff values for aspects such as the percentage of rapid progressive sperm and many other facets. In this regard, in some CASA systems, the configuration menu allows the user to make user-friendly changes to align analysis to a specific species.

Most Appropriate Quantitative and Qualitative Statistical Approaches for CASA

Standard statistics such as Student's *t*-tests, Anova, Kruskal–Wallis, and many other appropriate tests will always be relevant and should be used. However, care needs to be taken to test for normal distributions where averages and standard deviations will feature, and in the case of skewed distributions medians need to be used.

When different methodologies (e.g., manual morphology analysis vs. CASA) are compared, the gold standard is Bland–Altman plots. However, there is a chance element in this analysis and it needs to be verified by using Passing and Bablok analysis. If Passing and Bablok show $p > 0.05$, the Bland–Altman plot can be accepted (Mortimer et al., 2015). Figure 3 shows an example of sperm morphology analysis manually assessed using WHO criteria compared to CASA. In this case, it is inappropriate to use *t*-tests. In contrast, Dearing et al. (2019) used Bland and Altman plots and convincingly showed that manual analysis is much more inconsistent when compared to automated CASA.

Receiver-operating characteristic curves (ROC curves) as shown in Fig. 2 are one of the most useful methods to establish cutoff values to distinguish one sperm population from another. In Fig. 2, one kinematic parameter (VCL) is shown with a very high level of sensitivity and specificity to distinguish between hyperactivated and non-hyperactivated sperm. In combination with several other kinematic parameters, ROC cutoff values for hyperactivation have been accurately defined in Tankwa goat (Ngcauzele 2018). This is one example to show that ROC curves have become the gold standard to distinguish between different sperm populations.

Multivariate visualizations are most useful when comparing many CASA parameters in different treatments, for example, and this analysis method has been largely ignored despite several publications in this context (van der Horst et al. 1991, 1995). Andrews plots, sunray plots, and Chernoff faces (Figs. 4, 5, and 6) are useful when

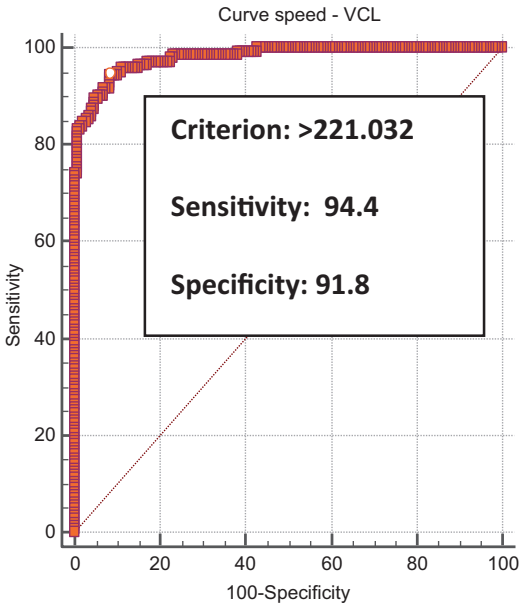

Fig. 2 Receiver-operating characteristic (ROC) curve to show the cutoff value for VCL for hyperactivation with a high level of sensitivity and specificity in Tankwa goats (Ngcauzele 2018)

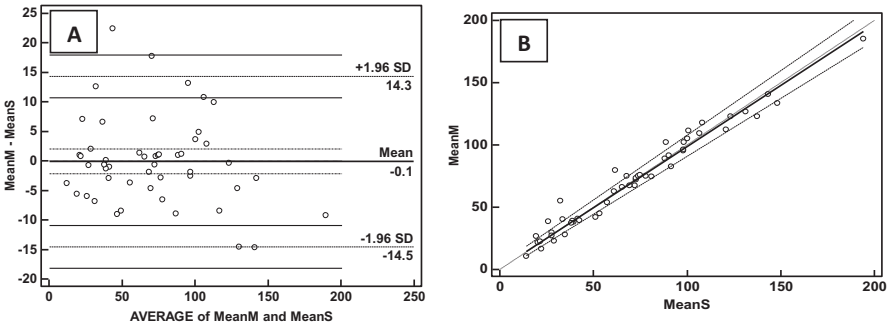

Fig. 3 (**a**) Bland–Altman plot for comparison of manual versus automated CASA morphology analysis showing statistically that it is basically similar. (**b**) Passing and Bablok analysis confirming the validity of Bland–Altman analysis in (**a**)

there are mostly no statistically significant differences between controls and treatments for many parameters. Multivariate visualizations are acceptable statistical approaches and the fact that they are qualitative tests cannot be ignored merely because the differences show no *p* value significance. Accordingly, multivariate methods may assist to clarify minor differences among controls and treatments or sperm morphometric parameters in sperm competition studies.

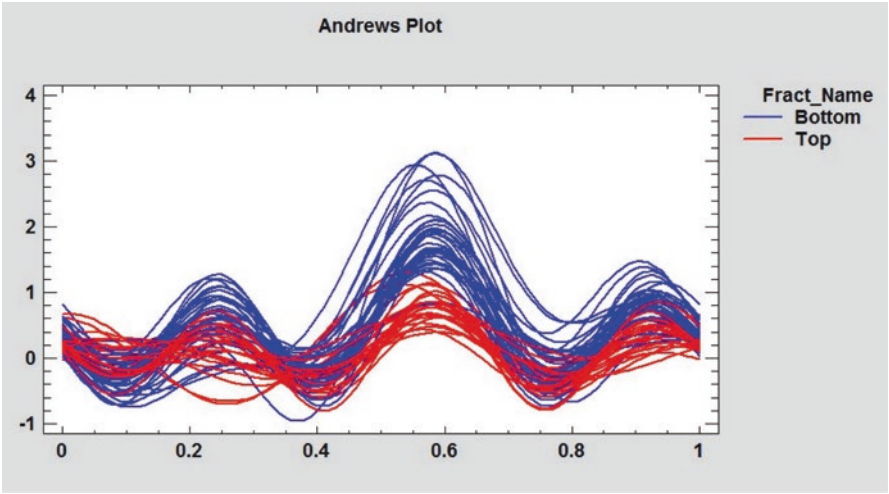

Fig. 4 Andrews plot comparing two sperm fractions (top and bottom) of the same sample for the percentages of total motility, rapid progressive sperm, rapid sperm, BCF of rapid population, DANCE of rapid population, and acrosome-induced reaction. Bottom fraction in blue and top fraction in red (Keyser et al. 2016)

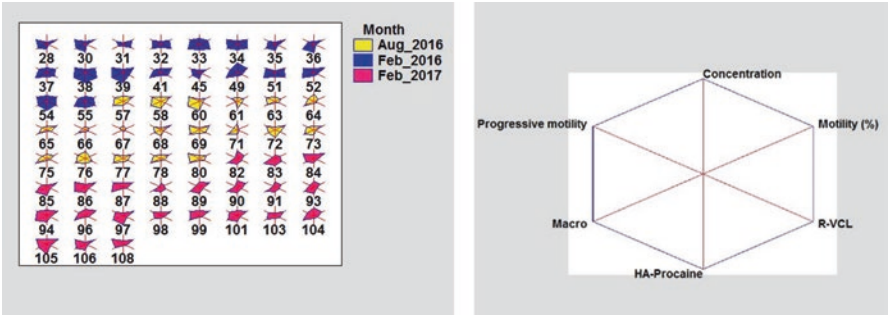

Fig. 5 Star symbol—sunray plots on left based on six sperm parameters on right for Tankwa goats over two seasons (unpublished data and Ngcauzele 2018)

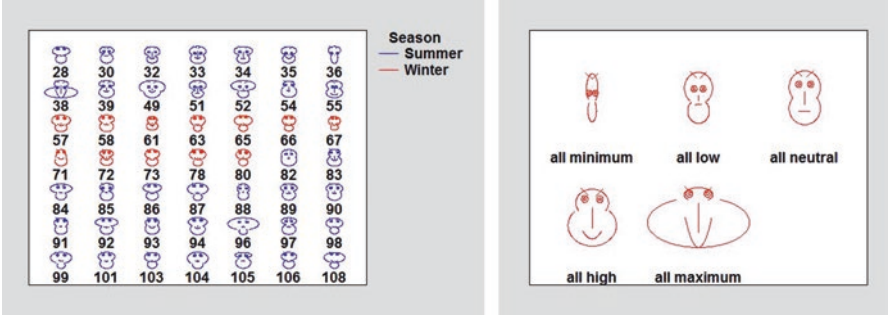

Fig. 6 Chernoff faces based on six sperm parameters (mainly motility and kinematics as in Fig. 5) for Tankwa goats over two seasons (unpublished data and Ngcauzele 2018)

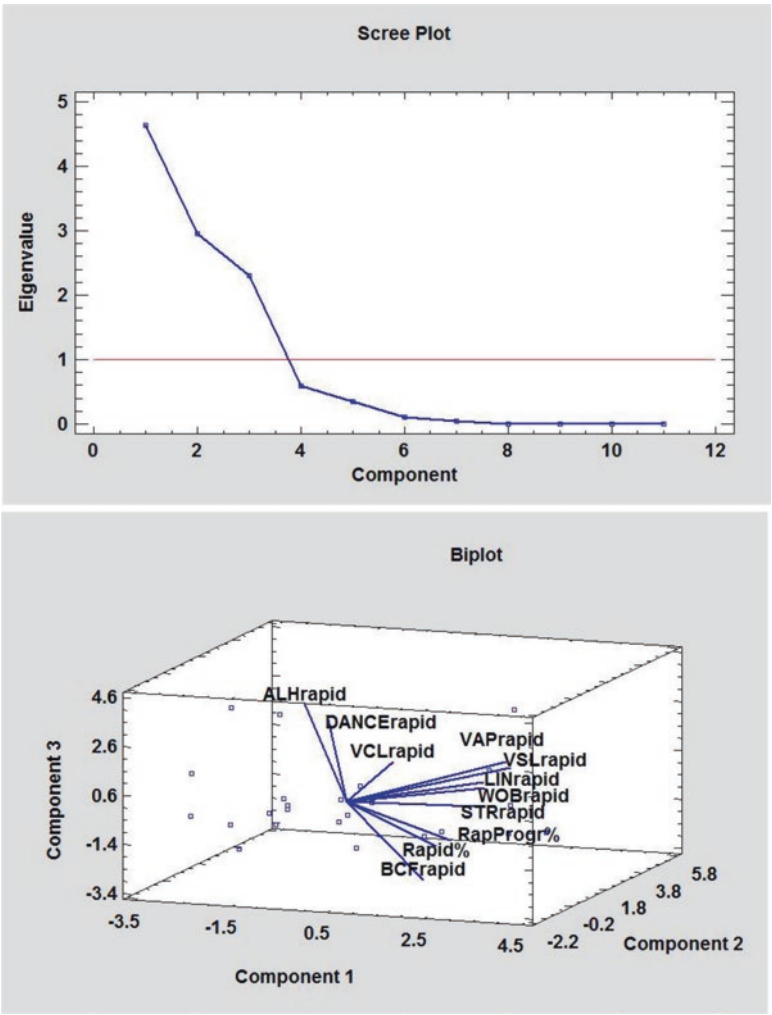

Fig. 7 Principal component analysis of 11 CASA kinematic parameters. (**a**) Plot of Eigenvalues identifying the three most important groupings or components. (**b**) Subsequent three-dimensional bi-plot showing the different kinematic relationships. Here there appear to be strong positive relationships among many rapid sperm categories

There are many other statistical methods to provide further clarification of CASA parameters. Principal component analysis, for example, provides a further method to enhance the relationship among parameters within large data sets. Figure 7 shows an example of using a multitude of parameters after applying redundancy analysis and the most important factors emerge that relate positively or negatively.

Conclusion

It is evident that CASA provides a quantitative basis for assessing sperm motility, sperm functionality, and sperm morphology without the subjective bias of manual assessment. Furthermore, CASA makes the analyses of a large number of parameters possible provided that great care is exercised to minimize methodology variables and that those parameters selected best describe sperm functionality and may be of value in potential fertility assessment. The value of multivariate visualizations is demonstrated in addition to more formal statistical approaches such as ANOVA.

Despite the major advances in CASA, the results generated using these automated systems will only receive better credibility once all or most variables are excluded such as incorrect optical settings and the quantitative data analyzed using a wide range of statistical approaches predicting what is biologically important.

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Relationship Between Flagellar Movement and Head Trajectory at Higher Frame Rates: Is This Still a Valid Approach for CASA?

Sumio Ishijima

Introduction

It has been established that the basis of sperm flagellar motility is ATP-dependent sliding between adjacent outer doublet microtubules (Summers and Gibbons 1971). However, the details of the mechanism by which active sliding along each of the nine outer doublet microtubules generates and modulates sperm flagellar motility have remained to be determined for more than 45 years. Our recent observations of oscillatory sliding movement of the activated doublet microtubules and the flagellar bend formation revealed that metachronal sliding and two types of synchronous sliding of the outer doublet microtubules generate and modulate sperm flagellar motility (Ishijima 2015, 2016; Takei et al. 2017). The metachronal sliding propagates around the axoneme circumferentially from one doublet to another along the axoneme, whereas the two types of synchronous sliding take place synchronously throughout an extended region along the doublet microtubules: oscillatory synchronous sliding occurs between most pairs of the doublet microtubules, while non-oscillatory synchronous sliding occurs between a specific pair of the doublet microtubules. Moreover, sperm hyperactivation is the conversion mode of synchronous sliding from the non-oscillatory synchronous sliding to the oscillatory synchronous sliding under a constant rate of microtubule sliding. One of the most important features of the hyperactivated sperm motility is a low beat frequency caused by the large microtubule displacement of the oscillatory synchronous sliding.

The circumferential propagation of the active sliding around the nine doublet microtubules of the metachronal sliding suggests that it is easier for the flagellum to

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generate helical waves than planer waves. In fact, most spermatozoa beat with three-dimensional flagellar waves; e.g., bull and human spermatozoa have relatively large three-dimensional components, although structures such as the central pair microtubules in 9 + 2 sperm flagella and the fusion of fibrous-sheath and 3-, 8-doublet microtubules in mammalian sperm flagella partition the nine outer doublet microtubules into two groups and generate somewhat planar flagellar waves. Accordingly, the bull and the human spermatozoa rotate around their long axes as they swim. This swimming behavior illustrates the difficulty in analyzing sperm motility based on the head trajectory. Increasing the image sampling frequency to more than 100 frames per second improves the accuracy of motility analysis of the spermatozoa rotating around their long axes and is necessary to develop a new method for analyzing three-dimensional movement of sperm motility. These topics are also discussed in more detail in several papers (Owen and Katz 1993; Mortimer and Swan 1999; Ohmuro and Ishijima 2006; Castellini et al. 2011; Mortimer et al. 2015).

Microtubule Sliding Mechanism in Sperm Flagellar Motility

It has been found that metachronal and synchronous microtubule sliding generate and modulate sperm flagellar motility (Ishijima 2015, 2016; Takei et al. 2017). Observations of the rhythmic sliding of the microtubule fibers extruded from the sperm flagella and flagellar bend formation have clarified the microtubule sliding mechanisms underlying flagellar bend formation (Ishijima 2016). In these experiments, three different microtubule sidings have been shown to generate and modulate the flagellar motility (Fig. 1). The first of these is the metachronal sliding associated with propagating flagellar waves (Fig. 1a). The second is the oscillatory synchronous sliding occurring synchronously throughout an extended region along the length of the flagellum (Fig. 1b), in which most pairs of adjacent doublet microtubules are able to slide; thus, the oscillatory synchronous sliding generates bends on both sides of the flagellum. The third sliding is the non-oscillatory synchronous sliding occurring synchronously throughout an extended region along the length of the flagellum (Fig. 1c), in which a specific pair of doublet microtubules slides; thus, the non-oscillatory synchronous sliding generates bends on only one side of the flagellum. Based on these observations, schematic diagrams showing the metachronal and synchronous sliding were constructed (Fig. 1d–f). Further confirmation of these two types of sliding and their dynamic characteristics were found by analyzing the microtubule sliding of flagellar movement of the hyperactivated spermatozoa (Ishijima 2015; Takei et al. 2017).

Detailed analyses of flagellar movements of monkey, golden hamster, and Suncus spermatozoa showed that the microtubule sliding velocity was constant within each species (Ohmuro and Ishijima 2006; Kaneko et al. 2007; Ishijima 2007; Takei et al. 2017). Figure 2 shows plots of beat frequency against shear angle of flagellar waves of golden hamster spermatozoa both before and after the hyperactivation. The

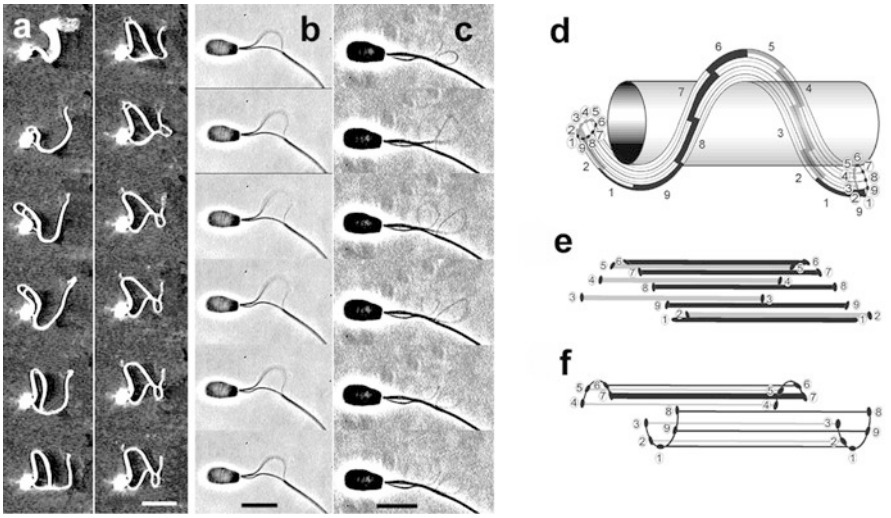

Fig. 1 Three types of microtubule sliding underlying the sperm flagellar motility. Observations of metachronal sliding (**a**), non-oscillatory synchronous sliding (**b**), and oscillatory synchronous sliding (**c**). Schematic diagrams show metachronal sliding (**d**), oscillatory synchronous sliding (**e**), and non-oscillatory synchronous sliding (**f**). The numbers in the circles indicate the number of the doublet microtubules. (**d**) The local sliding propagates circumferentially around the axoneme from one doublet to another along the axoneme. The numbers indicate the sliding of corresponding doublet microtubules. (**e**) The sliding occurs synchronously throughout an extended region along the length of the flagellum between most pairs of doublet microtubules. (**f**) The sliding occurs synchronously throughout an extended region along the length of the flagellum between a specific pair of doublet microtubules (doublets Nos. 7 and 8 in this figure). Bars: 15 μm in (**a**) and 10 μm in (**b**) and (**c**). Modified from Ishijima (2016) and Takei et al. (2017)

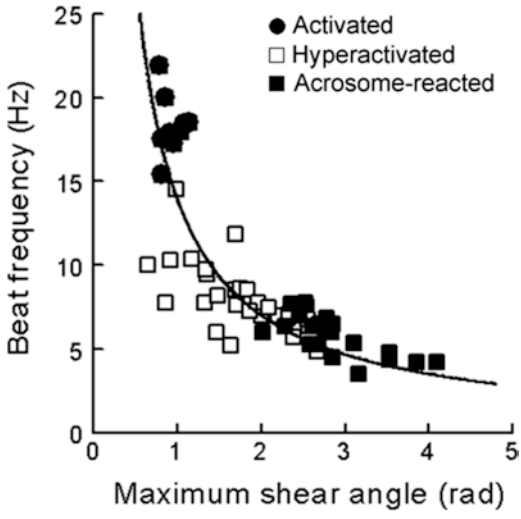

Fig. 2 Change in beat frequency of flagellar movement of golden hamster sperm during hyperactivation. The curved line is a least-squares regression line. Modified from Mohri et al. (2012)

regression line shows that the beat frequency is inversely proportional to the shear angle, demonstrating that the sliding velocity of the microtubule sliding is constant because the shear angle is proportional to the displacement of microtubule sliding. Therefore, the regulation of beat frequency and flagellar waveform are fundamentally dependent upon each other. The sliding-constant flagellar movement of the spermatozoa has been universally utilized and reported in various species of animals, such as fish (Ishijima 2012), golden hamster (Ohmuro and Ishijima 2006; Takei et al. 2017), monkey (Ishijima 2007), sea urchin (Ishijima 2007), and Suncus (Kaneko et al. 2007). The plots of beat frequency against shear angle in Fig. 2 also make several important aspects of the sperm flagellar motility clearer. The activated (non-hyperactivated) spermatozoa barely change the shear angle, but does change beat frequency to a limited extent, indicating that the microtubule sliding of the activated spermatozoa remains constant for keeping a constant waveform to ensure the high-speed swimming of the spermatozoa. In contrast, the acrosome-reacted spermatozoa beat with a constant beat frequency, suggesting that the flagellar movement of the acrosome-reacted spermatozoa with a low beat frequency has an advantage in attaching the surface of zona pellucida and generating the shearing force required for passage through the zona pellucida (Ishijima 2011).

Effect of Three-Dimensional Flagellar Waves on Sperm Motility

The flagellar movement of spermatozoa exhibits three-dimensional waves to a greater or a lesser extent (Table 1). Spermatozoa with large three-dimensional flagellar waves rotate around their long axes when they swim (Fig. 3a), whereas spermatozoa with relatively planar flagellar waves do not rotate (Fig. 3b). The head trajectory of monkey spermatozoa swimming with fairly planar waves is a smooth sine curve (Fig. 4a). In contrast, the head trajectory of human spermatozoa with large three-dimensional waves is a zigzag path because the rotational movement of

Table 1 Three-dimensional geometry of sperm flagellar waves

Species	3D component (%)[a]	Handedness of flagellar wave[b]
Bull	33	Left-handed wave (73%)
Human	20	Left-handed wave (57%)
Golden hamster	6	Left-handed wave (76%)
Monkey	3	Left-handed wave (100%)
Sea urchin	0	Right-handed wave (95%)

[a]The ratio of the lengths of the semiaxes of the elliptical cross section of the three-dimensional waves
[b]Most sperm flagella of freshwater animals beat with left-handed waves, whereas those of marine animals beat with right-handed waves. The chirality of the three-dimensional waves is regulated by intracellular concentrations of Ca^{2+} (Ishijima and Hamaguchi 1993; Ishijima 2013). Modified from Ishijima et al. (1992)

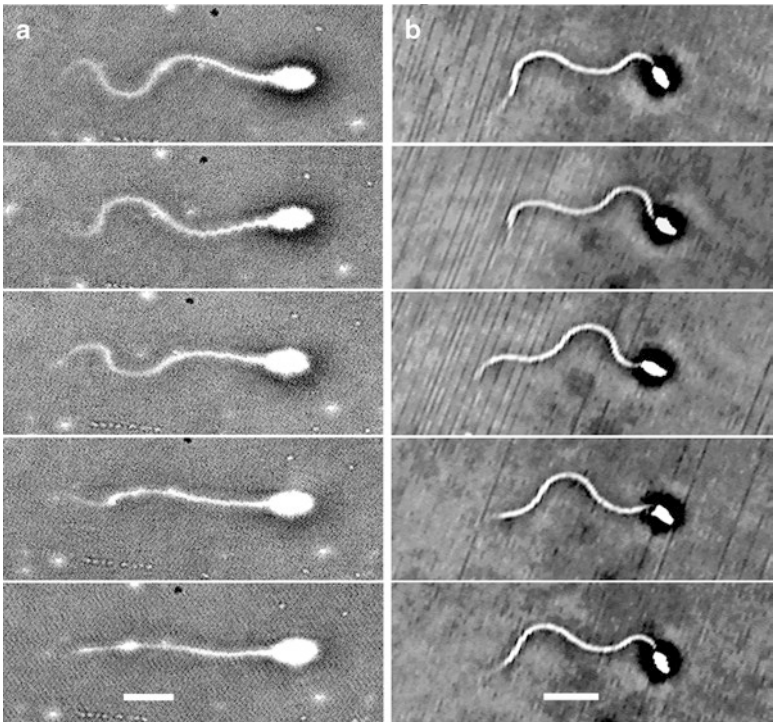

Fig. 3 Flagellar movements of human and sea urchin sperm. Human sperm rotate around their long axes during swimming freely (**a**), whereas sea urchin sperm do not rotate (**b**). Bars: 10 μm

spermatozoa around their long axes is superimposed on the yawing motion (the side-to-side movement) of the sperm head (Fig. 4b). This difference in the swimming behavior of spermatozoa illustrates the difficulty in analyzing sperm motility based on the head trajectory.

The hyperactivation of spermatozoa changes their flagellar waves (Ishijima et al. 2002; Ohmuro and Ishijima 2006) through the changes in sliding behavior of the nine outer doublet microtubules (Ishijima 2015; Takei et al. 2017). The change in flagellar waves of the hyperactivated spermatozoa also induces sperm rotation around their long axes, hence creating a zigzag head trajectory (Ishijima et al. 2002, 2006). Accordingly, the hyperactivated spermatozoa also create the difficulty in analyzing sperm motility based on the head trajectory, e.g., there is a higher value of beat cross frequency (BCF) of the hyperactivated spermatozoa (Mortimer et al. 2015).

A simple method for analyzing the spermatozoa rotating around their long axes is reported by Rikmenspoel (1965). The observed zigzag head trajectory is the result of a yawing motion modulated by the rotational movement of the spermatozoa. Therefore, fundamental motility parameters of these spermatozoa, e.g., sperm rotation frequency, beat frequency, and amplitude of sperm flagellar movement, are

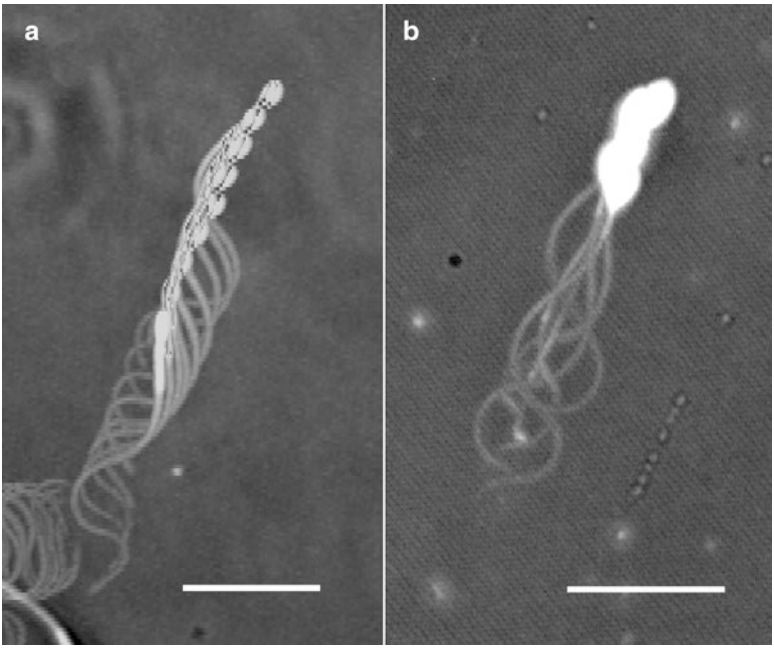

Fig. 4 Head trajectories of monkey and human sperm. Intact monkey sperm have planar waves, and their head trajectories are smooth curves (**a**), whereas human sperm rotate around their long axes and have head trajectories that are zigzag curves (**b**). The images are recorded at 200 frames per second. Bars: 20 μm

obtained using a fast Fourier transform (Brigham 1974). The CASA system with a software program for calculating the fast Fourier transform may be useful for eliminating the problem of the sperm rotation around their long axes caused by large three-dimensional flagellar waves. For this calculation, a high image-sampling rate is necessary to obtain accurate values of sperm motility parameters (Brigham 1974).

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CASA: A Suitable Tool for Epidemiology and Reprotox Studies

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Introduction

As early as 1678 von Leeuwenhoek used a light microscope to visualize sperm. This did not change until shortly before the Second World War when European opticians developed phase-contrast optics. Andrology laboratories in the 1950s used phase-contrast microscope to assess living sperm and today it remains the primary instrument used in Andrology lab (Amann and Katz 2004). As phase-contrast optics allows high-contrast visualization and good edge detection of each translucent cell, they are an essential part of the current computer-aided sperm analyser (CASA) systems (Amann and Katz 2004).

Early publications on quantifying sperm motion found their way into scientific journals from the 1940s to 1970s (as summarized by Boyers et al. (1989) and Mortimer et al. (2015)). These early studies often used manual cartography and in the 1970s progressed to photo- and cine micrography to micro-cinemicrography and in the early 1980s videomicrography on video cassettes replaced the former technology (Amann and Katz 2004; Mortimer et al. 2015). This presented a substantial cost savings over prior methods, applications and technologies were applied to the microscopes. The development of the personal computer in the 1980s and the development of digitization of video images for automated reconstruction allowed

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the analysis of individual sperm tracks—the first CASA instruments were developed (Amann and Katz 2004; Mortimer et al. 2015).

The term CASA refers to an automated system, which consists of hardware and software that visualizes and digitizes successive images of sperm. It processes and analyses the information of these images to provide accurate, precise and meaningful information on the kinematics of the individual sperm cell. The system should also supply a summary of population statistics (Amann and Katz 2004). The goal was to develop a CASA system that provides objective data on sperm motion or kinematics, i.e. characteristics that describe sperm movement as opposed to the proportion of motile sperm in a detailed manner, based on the well-defined velocity thresholds classify motile and immotile sperm (Amann and Katz 2004; Larsen et al. 2000; Mortimer et al. 2015). An advantage of a CASA systems over a manual analysis if the settings of the CASA are correct then the high precision provision of quantitative data on sperm kinematics (Hirano et al. 2004). Although CASA systems have developed relatively fast over the last few decades and the system's technology has improved greatly, their reliability still relies on training, ability and the experience of the user. Many of the guidelines for the use of CASA that were published in 1998 still apply today (Amann and Katz 2004; ESHRE Andrology SIG. 1998; Mortimer et al. 2015). Terminology and definitions of various kinematic criteria were resolved in 1988 (Mortimer 1990) and were included in the WHO third edition of their lab manual in 1992 (WHO. 1992).

One of the first systems developed was the CellSoft™ system (CRYO Resources Ltd) which sold a number of systems in 1985 (Amann and Katz 2004). Over the next period, there were a number of studies and publications using the system to evaluate various mammalian sperm (bull, human, mouse and rat sperm) reviewed in Amann and Katz (2004). This system used a free-standing phase-contrast microscope with a heated stage and conducted real-time video capture and provided unattended analysis after image capture (Amann and Katz 2004). The second commercial system was the HTM-2000® (Hamilton Thorn Research) known as a "system in a box" was launched in 1986 (Amann and Katz 2004). In 1992, the Sperm Motility Quantifier (SMQ) was developed. This system was one of the first systems to allow routine analysis at a 50 Hz frame rate (Mortimer et al. 2015). Over the years, a number of systems have been developed, for example, the Sperm Quality Analyser (SMA) by Medical Electronic Systems, the Danish system Copenhagen Rigshospitalet Image house sperm Motility Analysis System (CRISMAS) and the Sperm Class Analyzer® (SCA) CASA system developed by Microptic SL, in Spain. Microptic has kept improving their systems and at the time of writing this, they are up to SCA 6. Hamilton Thorn has brought out a "second-generation" Intergrated Visual Optical System (IVOS), the IVOS-II and the CASA-II software can be run with an external microscope as a CEROS-II (Mortimer et al. 2015).

A limitation of many of the early systems was the use of CASA in the clinical settings. These limitations were addressed at three consensus meetings in 1994, 1995 and 1997 (Mortimer et al. 2015) and this ultimately leads to the "Guidelines on the application of CASA technology" in 1998 (ESHRE Andrology SIG 1998; Mortimer et al. 2015) that are still relevant today. The advances on computer

technology have resulted in the steady improvement in CASA systems and software algorithm that have cemented it as a research tool. Providing improved, rapid, reliable and objective quantitative assessments of sperm on a structural and functional level (van der Horst et al. 2018a). CASA has the potential to detect the effects of environmental and occupational hazards on sperm function (Amann and Katz 2004; Toth et al. 1989). It has been used as a reliable and objective research and diagnostic instrument in the medical, veterinary, laboratory animal and wildlife fields (Holt et al. 2007; van der Horst et al. 2018a). Over the past 30 years, the number of publications related to the use of CASA in human and animal sperm analysis has increased steadily. They have become more user-friendly producing vast amounts of data in short periods of time, the type of measurements have also evolved and are no longer just related to sperm concentration, motility (%) and kinematics and morphology, they are now able to assess a number of parameters related to sperm functionality (van der Horst et al. 2018a). However a word of caution, it is important that the correct methodology, quality control and data interpretation should be carefully adhered to, to ensure correct and accurate analysis (Amann and Waberski 2014).

Research Applications: Epidemiological Studies

One of the research areas that CASA is suited to are epidemiological studies. Especially those that are detecting the effects of environmental and occupational hazards on sperm function. There are a number of publications for example listed on the Hamilton Thorne website going as far back as 1997 (www.hamiltonthorne.com/index.php/48-publications/ivos?start=20). At the University of Pretoria, the Department of Urology's interest in effect of endocrine-disrupting chemicals started around this time. One of the first studies associated with our department was an epidemiological, cross-sectional study that took place in Chiapas, Mexico. Mexico used DDT for malaria vector control and from 1997 to 2000. By 2000, they had phased out the use of DDT and used alternative methods for malaria vector control (De Jager et al. 2006). de Jager et al. 2006 investigated seminal parameters associated with environmental DDT exposure and *p,p′*-DDE concentrations in young healthy men aged between 18 and 40 years old ($n = 116$; mean age 27 ± 8.2). Semen analyses were conducted according to WHO (WHO 1999), plasma *p,p′*-DDT and *p,p′*-DDE exposure levels were determined. Sperm kinematics using the Hamilton Thorn HTM-2030; 6A software at 30 Hz, were measured. Dilutions were made of samples to a count of approximately 40×10^6 sperm/mL in Earle's Balanced Salt Solution (EBSS). The percentage motile sperm, progressive motility, linear velocity and curvilinear velocity were all analysed in duplicate. Using a Makler chamber (depth of 10 μm), 5 μL sample, 20 fields and a minimum of 150 sperm were analysed at 37 °C (De Jager et al. 2006). Morphology was not assessed using CASA as the software was not available at the time of the study. Pearson correlations were conducted between each transformed continuous seminal parameter and *p,p′*-DDE levels. The results showed that there was a weak negative correlation for VCL

($r = -0.23$; $p = 0.02$), VSL was significantly correlated with *p,p′*-DDE concentration of 45±31 µg/g ($r = -0.19$; $p = 0.05$). Implying that *p,p′*-DDE exposure decreases the VCL and VSL of the sperm, thereby negatively affecting sperm motility (De Jager et al. 2006).

In 2003, the Department of Urology initiated another epidemiological study on male reproductive health based on the Mexico study. Malaria is still one of the major non-infectious diseases that South Africa (SA) has to cope with and DDT is used for malaria vector control. This study assessed non-occupational exposure to DDT and semen parameters in young healthy men, in a rural area in the Limpopo province, SA. Between 2003 and 2005 men ($n = 311$) aged between 18 and 40 years (mean age 23 ± 4.7 years) were recruited from three villages in Venda that were annually sprayed with DDT. A semen analysis was done according to the WHO (WHO. 1999), and sperm kinematics were assessed using a Hamilton Thorn, HTM-IVOS Version 12, at 60 Hz. Leja slides with a depth of 20 µm were used, 30 frames were captures and a minimum of 150 sperm were analysed in duplicate at 37 °C (Aneck-Hahn et al. 2007). Samples that had counts of more than 40×10^6 sperm/mL were diluted with cell-free seminal plasma from the same participant. The percentage of motile sperm, progressive motility, average path (VAP), straight-line velocity (VSL), amplitude of lateral head displacement (ALH), VCL and beat cross-frequency (BCF) was assessed. The data were analysed as described in Aneck-Hahn et al. (2007). The results showed CASA parameters that had a statistically significant negative association with *p,p′*-DDT were VSL ($\beta = -0.002$; $p = 0.03$), ALH ($\beta = -0.0003$; $p = 0.03$) and BCF ($\beta = -0.01$; $p = 0.000$). The CASA mean motility (cubed) had a significant negative association with *p,p′*-DDE ($\beta = -0.02$; $p = 0.001$). The mean CASA motility (48.53% ± 18.6%) compared well with the manual mean motility (50.1% ± 15.8%). The BCF (28.68 ± 6.3 Hz) is negatively associated with *p,p′*-DDT concentrations, which indicate that higher levels of DDT can cause in increase in the flagellar beat pattern. This will have an adverse effect on sperm motility. The BCF is useful in determining the changes in the flagellar beat pattern (Mortimer 2000).

The Department of Urology had purchased the Sperm Class Analyser (SCA)—research package from Microptic Automatic Diagnostic Systems SL in Spain (www.micropticsl.com/). The research package includes a number of modules including human motility, morphology, DNA fragmentation and the research package includes domesticated and other animal species, including the rat model.

Further studies in the Vhembe district in Limpopo province investigating the association between environmental toxicants, which included DDT, DDE and seminal parameters using a new CASA system. This study forms part of a much larger study that is investigating the effects of organochlorines on paternal epigenetic effects in men from villages that are sprayed and reference villages that are not sprayed. Preliminary CASA data were analysed from two sampling periods in 2017. Leja slides with a depth of 20 µm depth. A Bassler camera was used at a frame rate set at 50 frames per second (fps), ×10 positive phase for human sperm, on a heated stage at 37 °C of an Olympus CH2 microscope. It is important to remember that

numerous factors can affect sperm motility and kinematic results and should be standardized (van der Horst et al. 2018a), particularly for reliable objective results. Issues to bear in mind are sample type (fresh neat or washed/processed samples), processed sperm, has media been added to test for capacitation or hyperactivation, temperature and also the CASA settings for motility (van der Horst et al. 2018a).

Fifty-two participants qualified for statistical analysis (i.e. all the data for the semen parameters, exposure levels and CASA data were available), 34 stayed in sprayed villages and 18 in unsprayed villages. A principal component analysis (PCA) using statistical software R was performed to summarize the biological information and most important biological variables. Hierarchal clustering on the PCA results gave profiles of biological information on the sperm. DDT, DDE and age were categorized by using cut-off values for DDT and DDE (0.07 μg/g and 1 μg/g respectively), median age 25 years and spray status was not sprayed. Biological profiles for each category (DDE/DDT, age and spray status) were performed using multinomial logistic regression with $\alpha = 5\%$. The clustering on the PCA results gave three profiles of sperm characteristics (Table 1). Using Profile 2 as a "good" reference and compared it with Profile 1 and 3 throughout each category using multinomial logistic regression (Fig. 1).

The results showed that a participant from a sprayed village was 6 times more likely to be in Profile 1 (poor sperm characteristics), than those living in a non-sprayed village Profile 2 (neither good nor poor characteristics). Participants with a DDT level of above 0.07 μg/g were four times more likely to be in Profile 1 compared to Profile 2 (DDT < 0.07 μg/g) (Table 2).

It is important to note that these are the results of a portion of the data collected over a period of 4 years. There are clear limitations due to the small sample size and the wide confidence intervals (CIs). Some associations are not significant, which may change once we have analysed to all the participants' data. However, clearly some exposure effects on sperm kinematics need further elucidation. Fortunately, the SCA CASA system does allow adjustment of certain settings and reanalysis. This snapshot of data from the larger project was to show an example of the type of statistics that can be used for large data sets to draw out information on subpopulations, which may be useful in epidemiology studies. As described in van der Horst et al. (2018a), the use of multivariate pattern analysis, PCA and clustering methods is able to demonstrate the existence of motile sperm subpopulations in several species (van der Horst et al. 2018a).

Research Applications: Reproductive Toxicology Studies

One of the major impacts of CASA has been its use in the area of reproductive toxicology. Early in the 1980s, the first studies were being published about the effect of chemical exposure (occupational and environmental) on reproductive health, specifically motility (Amann and Katz 2004). CASA is used in reproductive toxicology because some of the motility parameters are sensitive to toxins (ESHRE Andrology

Table 1 The description of three profiles of sperm characteristics and the significance of the characteristics

Profiles (size)	Biological characteristics	*P* value
Profile 1: (n = 27)	Non-progressive type C percentage	<0.0001
	Straightness index STR medium	<0.0001
	Immotile type D percentage	0.0009
Profile 2: (n = 19)	Average value VAP slow	<0.0001
	Oscillation index WOB slow	<0.0001
	Linear speed VSL slow	<0.0001
	Linearity LIN slow	<0.0001
	Curve speed VCL slow	<0.0001
	Beat frequency BCF average	<0.0001
	Medium progressive type B percentage	0.0003
	Beat frequency BCF medium	0.0015
	Oscillation index WOB medium	0.0016
	Straightness index STR slow	0.0022
	Rapid progressive type A percentage	0.0027
	Mucous penetration percentage motile	0.0029
	Linearity LIN rapid	0.0164
	Beat frequency BCF rapid progressive	0.0167
	Linear speed VSL rapid	0.0257
	Oscillation index WOB rapid	0.0343
	Average value VAP rapid	0.0382
Profile 3: (n = 6)	Hyperactive percentage motile	<0.0001
	Amplitude lateral head ALH average	<0.0001
	Curve speed VCL medium	<0.0001
	Amplitude lateral head ALH rapid progressive	<0.0001
	Amplitude lateral head ALH medium	<0.0001
	Curve speed VCL rapid	<0.0001
	Linear speed VSL medium	<0.0001
	Average value VAP medium	<0.0001
	Average value VAP rapid	<0.0001
	Linear speed VSL rapid	<0.0001
	Circular tracks percentage motile	0.0002
	Medium progressive type B percentage	0.0037
	Beat frequency BCF average	0.0165

Profile 1—poor sperm characteristics; Profile 2—neither bad nor good characteristics (+/− good); Profile 3—poor yet interesting characteristics

SIG. 1998). The papers published by Maree and van der Horst (2013) and van der Horst et al. (2018a) on quantification and identification of subpopulations with species-specific cut-off values and the current perspectives of CASA applications, respectively, pay attention to the diversity of mammalian spermatozoa. The research findings show that the CASA system if programmed correctly with the relevant

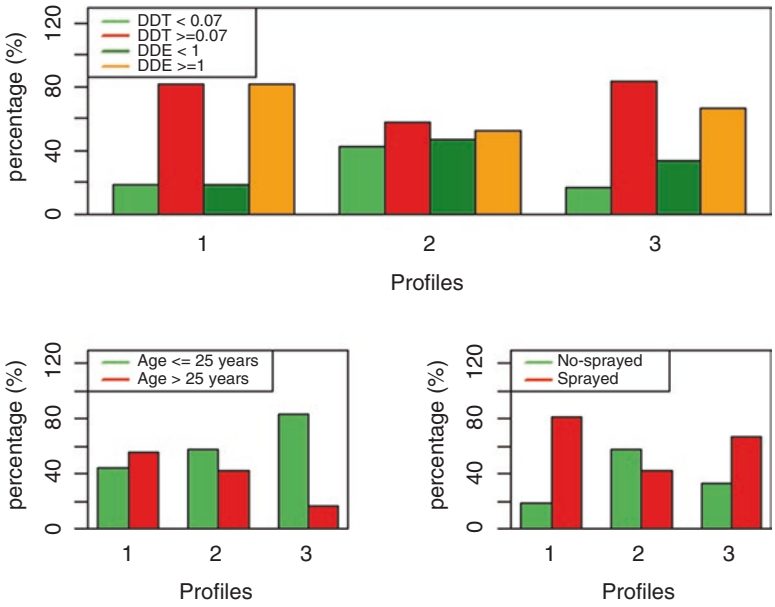

Fig. 1 Comparison of three profiles of sperm characteristics following clustering using principal component analysis (PCA)

Table 2 The association between factors, profiles, odds ratio (OR), confidence interval (CI) and *p* value

Factors	Profiles	OR	CI 95%	*p* value
Sprayed (ref = non-sprayed)	Profile 1 vs. Profile 2	6.05	[1.6–22.94]	0.0080**
	Profile 3 vs. Profile 2	2.75	[0.4–18.74]	0.3033
Age above 25 (ref = below 25)	Profile 1 vs. Profile 2	1.72	[0.53–5.56]	0.371
	Profile 3 vs. Profile 2	0.27	[0.03–2.83]	0.278
DDE above 1 (ref = below 1)	Profile 1 vs. Profile 2	3.2	[0.84–12.1]	0.0868.
	Profile 3 vs. Profile 2	3.63	[0.35–37.43]	0.2779
DDT above 0.07 (ref = below 0.07)	Profile 1 vs. Profile 2	3.97	[1.05–15.07]	0.0417*
	Profile 3 vs. Profile 2	1.8	[0.26–12.31]	0.5488

settings for the species (human, primates, rodents, domestic and wildlife animals) involved can provide rapid, reliable and objective quantitative assessments of sperm motility (van der Horst et al. 2018a). There are numerous reproductive toxicology studies listed on the Hamilton Thorne (www.hamiltonthorne.com/; accessed 10 June 2020) and the Microptic SL (www.micropticsl.com/; accessed 10 June 2020) websites.

CASA systems today may have default setting for a variety of animal species, but some of the systems allow the user to adapt the settings and optimize for new species. The RatTox system was designed to investigate the effect of various substances/toxicants on both sperm function and form. Because these systems are

constantly undergoing improvement which enables them to measure more parameters and better able to define the quality of a sperm sample quantitatively and objectively. For example, the SCA 6 has an integrated sperm functional assessment using several sort functions that can measure sperm cervical penetration, capacitation and hyperactivation. It also includes a module for DNA fragmentation using the Halosperm® Test which is also being implemented in the Hamilton Thorne IVOS-II system (Mortimer et al. 2015).

An important publication as far as CASA morphology is concerned in terms of reproductive toxicology is the paper by van der Horst et al. (2018b) describing the cut-off values for rat sperm morphology. For our reproductive toxicology laboratory, this proved extremely useful. In 2012, we were doing a study to investigate the association between exposure to a relevant mixture of environmental toxicants (endocrine-disrupting chemicals (EDCs)) and the effects on male reproductive health in Sprague–Dawley rats (Patrick et al. 2016), there was no information on normal parameters for rat morphology. At the time of during sample collection from the rats, caudal sperm were collected from the different exposure groups to determine sperm counts and at the same time, morphology slides were made and stored in the hope that there would be an opportunity in the future to analyse the morphology using an improved Casa morphology module.

A morphologically normal rat sperm was clearly defined by van der Horst et al. (2018b) (Figs. 2 and 3). This was the first study to define normal and abnormal sperm in two strains of rats.

The outcomes on rat sperm morphology allowed us to continue and analyse the rat morphology of our reprotox study, albeit retrospectively. Our study originally investigated the effects of life-time exposure (in utero-, lactational- and direct) to a mixture of environmentally relevant concentrations of a mixture of EDCs found in the Vhembe district of SA on the reproductive health of Sprague–Dawley rats (Patrick et al. 2016). After dosing for 104 days, histological assessments and

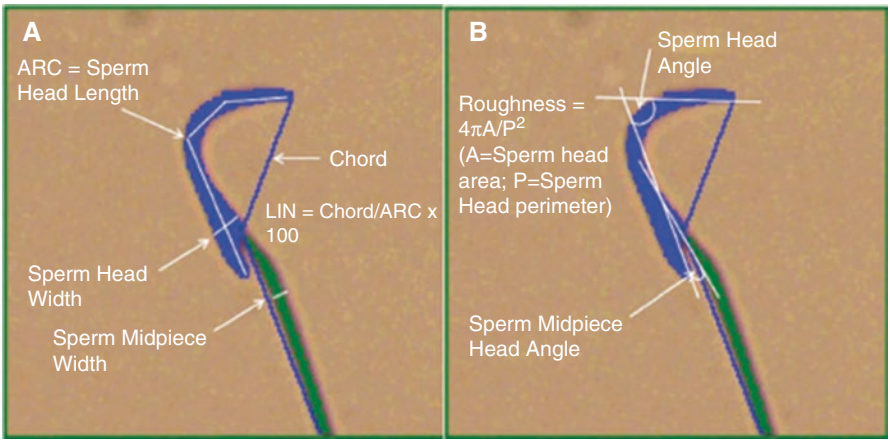

Fig. 2 Stained rat sperm thresholded for head (blue) and mid-piece (green) showing various morphometric features, the derivatives, e.g. linearity, which expresses Chord/ARC × 100%, and angles of sperm head and mid-piece (LIN = Linearity), taken from van der Horst et al. (2018b)

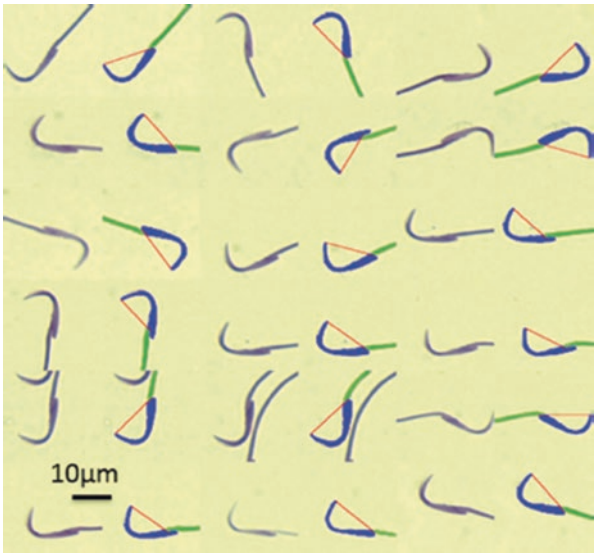

Fig. 3 SpermBlue showing the chord measurements taken from van der Horst et al. (2018b)

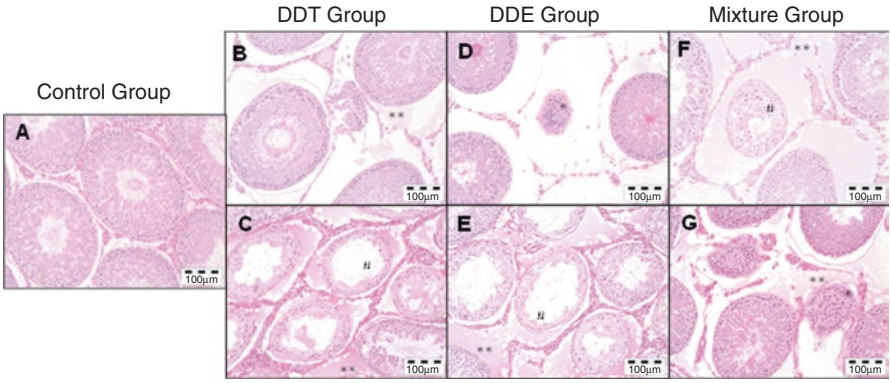

Fig. 4 Testicular histology of F1 males at PND 90—Normal testicular histology in the control group (**a**), abnormal testicular histology in DDT-exposed group (**b**, **c**), DDE-exposed group (**d**, **e**) and in the mixture-exposed group (**f**, **g**); small seminiferous tubule diameter with no lumen (*), necrosis in the interstitium (**), disorganization of the seminiferous epithelium (#). (Taken from Patrick et al. 2016)

reproductive endpoints were assessed. Histology of the testes showed that selected seminiferous tubules contained dilated tubular lumens. There was a marked detachment of the seminiferous tubule, necrosis in the interstitium with marked disorganization of the seminiferous epithelium and only a few germ cells present. The seminiferous tubule diameter was reduced with no lumen, absent seminiferous tubules and decreased cellularity of the seminiferous epithelium in the exposure groups (groups b–d) (Fig. 4). In the exposure groups, the changes in the size of the seminiferous tubule diameter, epithelium thickness and lumen diameter per stage of

the spermatogenic cycle differed from the control (Fig. 4). Evaluation of spermatogenic stages showed that all stages were present. The seminiferous tubules had thinner epithelium thickness indicating reduced germ cell layers, which is indicative of Sertoli cell toxicity resulting in altered fluid retention.

Figure 5 shows an example of the rat sperm morphology from each respective dosing group (Cottonseed oil; Group B: 35 mg/kg DDT; Group C: 35 mg/kg DDE; Group D: 0.5 mg/kg deltamethrin; 35 mg/kg DDT; 2.5 µg/kg *p*-nonylphenol; 2.5 µg/kg phytoestrogens (genistein, coumestrol and zearalenone)).

Applications for Chemical Exposures

The advances in CASA technology have made CASA useful for investigating exposure effects of chemicals on sperm kinematics, motility, concentration and morphology. The latest generation of CASA systems also includes quantitative modules to measure sperm DNA fragmentation, vitality, acrosome reaction and intactness and

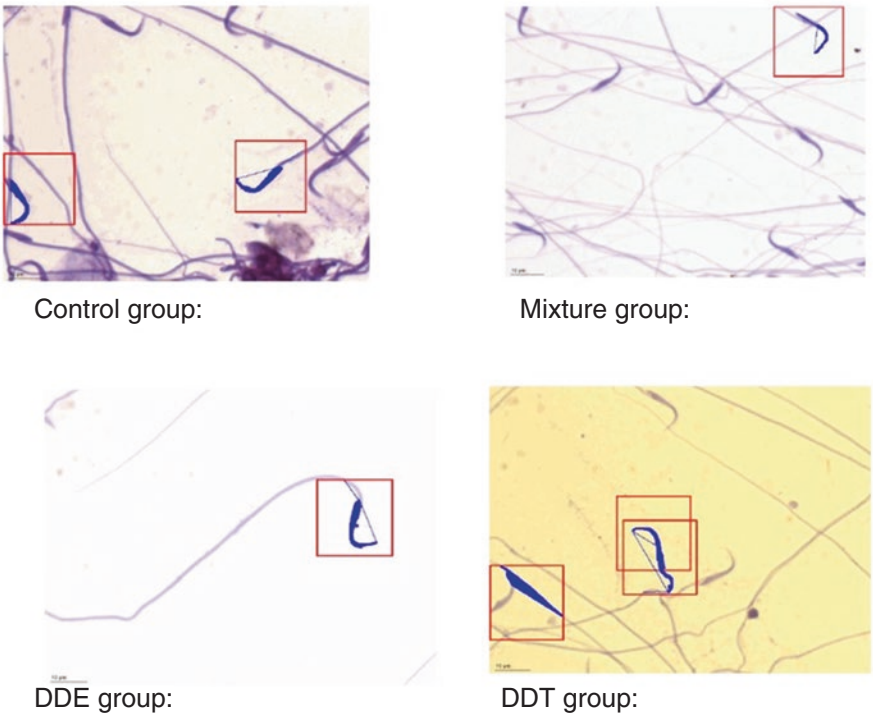

Fig. 5 Example of the rat sperm morphology from each respective dosing group (Control group: Cottonseed oil; DDT Group: 35 mg/kg DDT; DDE Group: 35 mg/kg DDE; Mixture Group: 0.5 mg/kg deltamethrin; 35 mg/kg DDT; 2.5 µg/kg *p*-nonylphenol; 2.5 µg/kg phytoestrogens (genistein, coumestrol and zearalenone))

hypo-osmotic swelling. Additional sperm functional tests like sperm mucous sperm penetration and hyperactivation (van der Horst et al. 2018a). There has been increasing evidence that environmental factors have an impact on male reproductive health, with semen quality deteriorating markedly over the last few decades (Mukhopadhyay et al. 2010).

There are a number of studies investigating the exposure effects of pharmaceuticals on semen quality. Smarr et al. (2017) investigated the relationship between paracetamol and its metabolite *p*-aminophenol in urine and semen quality using the Hamilton Thorne IVOS system and IDENT™ stain for sperm concentration, motility was assessed on the HTM IVOS semen analysis system and sperm morphometry using the IVOS METRIX system. Urinary paracetamol was associated with a reduction in BCF and increased DNA fragmentation. The urinary *p*-aminophenol was associated with a reduction in sperm head area (Smarr et al. 2017).

In another study by Aitken et al. (2016), the effects of phenolic compounds on the functional integrity of sperm cells were investigated. To reflect the assisted reproductive technique (ART) setting, human sperm were incubated for 2–24 h with a selection of polyphenols (25–200 μM) that have been previously used in mammalian sperm studies. Sperm kinematics were measured on an HTM IVOS, settings are described in Aitken et al. (2016). The results showed that after a 2 h at 37 °C exposure to epigallocatechin-3-gallate (EGCG) there was a highly significant dose-dependent suppression of all aspects of sperm movement including total motility, % progressive, % rapid motility, VSL, VCL and VAP (Aitken et al. 2016). For more information on chemical exposure, refer CASA manufacturer's websites (www.hamiltonthorne.com/; (www.micropticsl.com/, accessed 10 June 2020).

Discussion

Currently, there are numerous methods available to examine semen or sperm, ranging from a simple visual assessment to a detailed molecular analysis; despite this, there is no single method to determine male fertility (Amann and Waberski 2014; Luther et al. 2020). Manual motility assessment as described in the WHO manuals 1999 and 2010 provides limited information about the population of motile sperm for semen quality assessment (Luther et al. 2020; WHO 1999, 2010). Unless a trained and experienced person is performing the analysis, it may still provide a subjective measurement (Maree and van der Horst 2013; Mortimer et al. 2015; van der Horst et al. 2018a). Many of the CASA systems and technologies that are available today can provide vital information on sperm movement patterns, which cannot be assessed using manual evaluation. The systems are also able to objectively and accurately measure more than one semen parameter, sperm concentration, motility subpopulations and sperm morphometry for many mammalian species, but also other species like fish, frogs and birds (Luther et al. 2020; Maree and van der Horst 2013; van der Horst et al. 2018a).

The advantage of using CASA for epidemiological and reproductive toxicology studies was that semen quality of men exposed to putative occupational and environmental hazards could be accurately assessed. Particularly in the 1980s, the Environmental Protection Agency convened a meeting to discuss the need to use objective methods to determine sperm motion (Amann and Katz 2004). The first studies using CASA were retrospective and lacked dosimetry data, despite this the studies provided evidence of the suitability of CASA for reproductive toxicology in humans and a consensus approach paper was published by Schrader et al. (1992). Since then, a number of human epidemiology studies using CASA systems to determine semen quality (motility, kinematics, concentration and morphology) have shown the suitability of CASA in epidemiological studies. The cross-sectional population-based study done by Rahban et al. (2019) on semen quality, sampled young men from six centres across Switzerland. Identical CASA systems were used in all study locations and very strict instructions were followed for recording sequences. Strict quality control procedures were followed to ensure that the data were accurate and reliable. For comparison of CASA-generated data between research laboratories and with previously published studies it is extremely important that all capturing techniques and analytical methods are standardized (Maree and van der Horst 2013).

Rather than relying on mean values for the sperm population that oversimplifies the analysis and negatively affects the usefulness of the data, sperm subpopulations should be evaluated (Abaigar et al. 1999; Maree and van der Horst 2013; Martinez-Pastor et al. 2005). Mean values for sperm motility can mask the effects of drug treatment or chemical on spermatozoa, especially in species with heterogeneous ejaculates, as a subpopulation of the total population may be more responsive or susceptible (Maree and van der Horst 2013). Successfully using CASA technology Maree and van der Horst (2013) identified three subpopulations of mammalian sperm and the cut-off values for sperm-swimming speed in different mammalian species. These subpopulations represent sperm in different physiological states (Abaigar et al. 2001). According to Holt and Van Look (2004), only a small percentage of sperm possess all the mechanisms required for synchronous cell functions during transport, capacitation, hyperactivation, acrosome reaction and egg penetration. A CASA analysis provides a large amount of data on motility-related parameters, finding a suitable statistical method, e.g. multivariate cluster analysis, regression analysis or principal component analysis to select the motility parameters that best-identified sperm subpopulations in an ejaculate may be the appropriate way to analyse the data (Maree and van der Horst 2013). A number of studies highlight the importance of VCL and VSL to define sperm subpopulations and their response to drug treatments (Maree and van der Horst 2013).

Studies that involve potential reproductive toxicants (e.g. metals, pesticides, flame-retardants and pharmaceuticals) should include morphological assessment of the sperm. Abnormal sperm morphologies should be quantified and additional cut-off points developed. When manually assessing morphology it is difficult to discern differences between macro and microcephalic rat sperm unless they are in the same field (van der Horst et al. 2018b). Furthermore, subtle differences in borderline

forms make subjective manual assessment nearly impossible compared to CASMA using objective cut-off points (Perreault and Cancel 2001). The publication by van der Horst et al. (2018b) is the first to describe cut-off values for evaluating the percentage normal sperm in Sprague–Dawley and Wistar rat strains using automatic analysis mode in the SCA CASA system. Sperm morphometry should always be combined with sperm motility measurement and sperm functionality tests, for example, acrosome intactness and reaction, DNA fragmentation and sperm hyperactivation (van der Horst et al. 2018a, b).

Hyperactivation may be used as a marker to predict pregnancy. Additionally, hyperactivation is also a sperm function parameter, which can be used to assess pharmacological compounds administered to treat male infertility. However, increased exposure to environmental pollutants, like pesticides (e.g. DDT/DDE), pharmaceuticals (paracetamol) (Smarr et al. 2017) and other compounds that can mimic natural hormones like estrogens or androgens may have detrimental effects on sperm function, like hyperactivation (Aneck-Hahn et al. 2007; Kay and Robertson 1998; Mortimer et al. 2015; van der Horst et al. 2018a).

Is CASA a suitable tool? There are a number of good studies and reviews which have been highlighted in this paper show that CASA is an extremely useful tool for epidemiological (human), reproductive toxicology (animals, laboratory, domestic and wildlife) and a measure of drug/chemical effects on sperm. CASA data and multivariate approaches for specific sperm functional parameters will make it possible to detect invaluable differences that might be missed with a manual assessment (Luther et al. 2020). The future of CASA looks bright with systems improving as technology (e.g. cameras, computer software and hardware) and data processing improve.

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Part VII
Challenges for Sperm Function In Vitro

Studies of spermatozoa as well as clinical routines for treatment and cryopreservation often means that spermatozoa are taken out of their natural environments. This session aims at outlining in vitro challenges for spermatozoa in vitro and what can be done to overcome them.

Common Challenges for Sperm In Vitro: Causes and Consequences

Ulrik Kvist

Before the human spermatozoon reaches the ovum in the ART Laboratory, it experiences various challenges. This paper will focus on two major unphysiological challenges that may affect its full fertilizing potential.

These are (1) sperm exposure to seminal vesicular fluid and (2) sperm exposure to an osmotic roller-coaster.

Exposure to seminal vesicular fluid results in chromatin zinc deficiency and a vulnerable chromatin.

Exposure to the osmotic roller coaster results in sperm ATP depletion, tail coiling, impaired sperm motility and low sperm density affecting sperm functional properties and sperm selection procedures by swim up and density gradient centrifugation.

These two unphysiological challenges are unintendedly forced onto the spermatozoa when collected as a whole ejaculate and then subjected to diagnostics and sperm selection for ART.

Physiology tells that the fertilizing spermatozoon is normally not exposed to the later expelled seminal vesicular fluid.

Thus, at intercourse, the man transfers spermatozoa in *prostatic fluid* onto the cervical mucus and progressively motile spermatozoa can immediately proceed towards the oviducts (Kremer 1968; MacLeod and Gold 1951). The later expelled ejaculate fractions are dominated by seminal vesicular fluid and become directly entrapped by the seminal gel as it forms.

In contrast, **in vitro**, the spermatozoon is also exposed to the **seminal vesicular fluid.** This occurs during liquefaction and after liquefaction before diagnostic procedures and selection for assisted reproduction takes place.

Physiology tells that the transfer from prostatic fluid into the cervical mucus does not add any major osmotic challenges to the spermatozoa.

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Thus the prostatic fluid and the cervical mucus are both isotonic to body fluids (Fig. 1).

In contrast, **in vitro**, the spermatozoon is exposed to **an osmotic roller coaster** with osmolality first going up, then steeply down.

Thus, in vitro the sperm experience two new major unphysiological challenges, the causes and consequences to be considered in order to improve our skills in diagnostics and assisted reproduction.

Sequence of Ejaculation

Sperm Exposure to Seminal Vesicular Fluid: An Unphysiological Challenge During Sperm Preparation for ART

The sequence of ejaculation was described in the 1940s. Collection of split ejaculates revealed that spermatozoa are predominantly expelled together with the zinc-rich prostatic fluid and that the seminal vesicular fluid dominated the later expelled ejaculate fractions (Björndahl and Kvist 2003; Eliasson and Lindholmer 1972; Lundquist 1949; MacLeod and Hotchkiss 1942) (Fig. 2). Spermatozoa in prostatic fluid swim freely and are not entrapped in the gel. They reveal better motility and vitality than spermatozoa expelled in later fractions with seminal vesicular fluid (Amelar and Hotchkiss 1965; Eliasson and Lindholmer 1972; Valsa et al. 2012). Furthermore, spermatozoa in prostatic fluid retain high level of chromatin zinc and

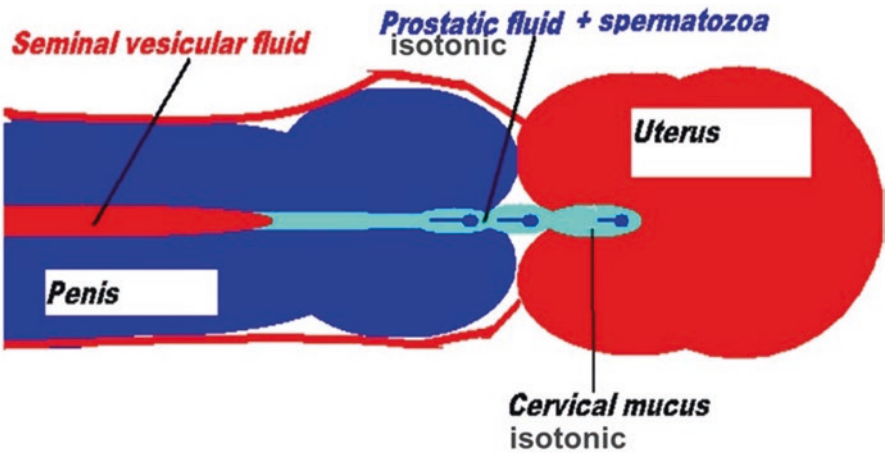

Fig. 1 Physiology of sperm transfer. Man offers woman sperm in the zinc-rich isotonic prostatic fluid onto the isotonic cervical mucus. The later expelled ejaculate fractions are dominated by seminal vesicular fluid. The fertilizing spermatozoon is normally not exposed to seminal vesicular fluid or to osmotic challenges. Drawing by Ulrik Kvist

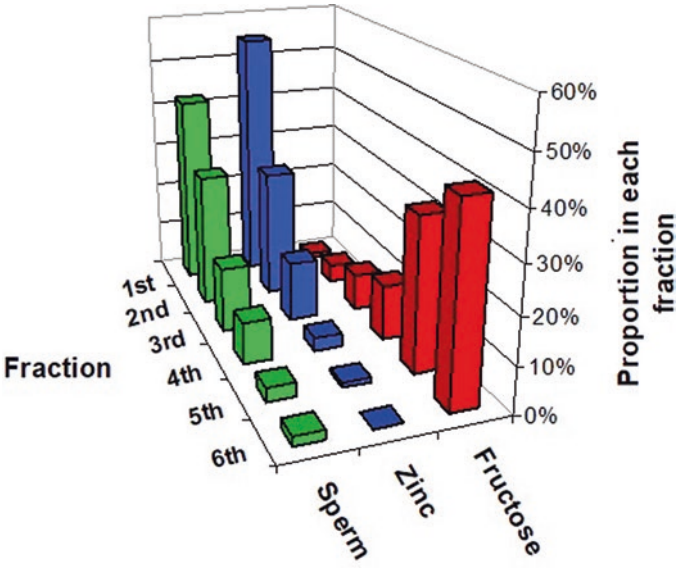

Fig. 2 Sequence of ejaculation. Spermatozoa and zinc-rich prostatic secretion (low pH). Seminal vesicular fluid (high pH, rich in zinc chelators and oxidizing compounds) (Björndahl & Kvist 2003) . Diagram by Lars Björndahl

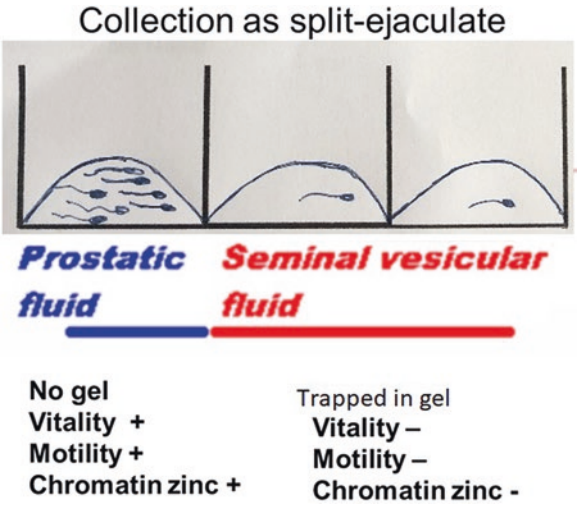

Fig. 3 Collection as split-ejaculate. Drawing by Ulrik Kvist

show a reversible zinc-dependent type of chromatin stability (Björndahl and Kvist 1990; Björndahl and Kvist 2010; Kvist 1980a, b, c) (Fig. 3). Spermatozoa residing in seminal vesicular fluid fractions loose chromatin zinc and change rapidly a chromatin stability dependent on zinc to a chromatin stability dependent on S–S cross-links (Björndahl and Kvist 2010).

In vivo, spermatozoa are normally not exposed to the seminal vesicular fluid. However, infertile men with inflammation of the prostate may have a delayed

emptying of the prostatic fluid, and in these cases spermatozoa may primarily be expelled with and therefore exposed to the seminal vesicular fluid in the first expelled portion (Björndahl et al. 1991; Kjellberg 1993). In addition, Amelar and Hotchkiss (Amelar and Hotchkiss 1965) found that some 7–8% of infertile men had the majority of spermatozoa expelled in the later expelled fraction.

In vitro, collection of whole ejaculates means that spermatozoa always and to a various degree will be exposed to the seminal vesicular fluid. Thus, during liquefaction and while the sample is on hold for diagnostic procedures and selection for ART individual spermatozoa will be at risk to lose chromatin zinc to zinc-binding proteins of seminal vesicular origin.

Emission and Ejaculation

The process by which spermatozoa and fluids are emptied into the urethra is the emission. Preceding emission, the sympathetic nervous system releases noradrenalin as transmitter, acting on alfa-1-receptors of smooth muscles in the walls of the epididymal cauda, the vas deferens, the seminal vesicles, the 20–25 prostatic glands and the inner sphincter of the bladder neck. This leads to contractions that propel sperm and fluid into the urethra and at the same time closing the way up into the bladder. Substances interacting with α_1-receptors may interact with emission and therefore be used to enhance emission, e.g. in diabetic neuropathy (Björndahl et al. 2010).

The sequence of emission is worth discussing. It does not appear to be a fine-tuned process by the sympathetic nervous system which is more of an on-and-off procedure concomitant with orgasm. Instead, it appears that the geometry that have evolved of the various organs is of major importance for the sequence of emptying. In Fig. 4 the lumina and the wall thickness of the various organs are illustrated. As

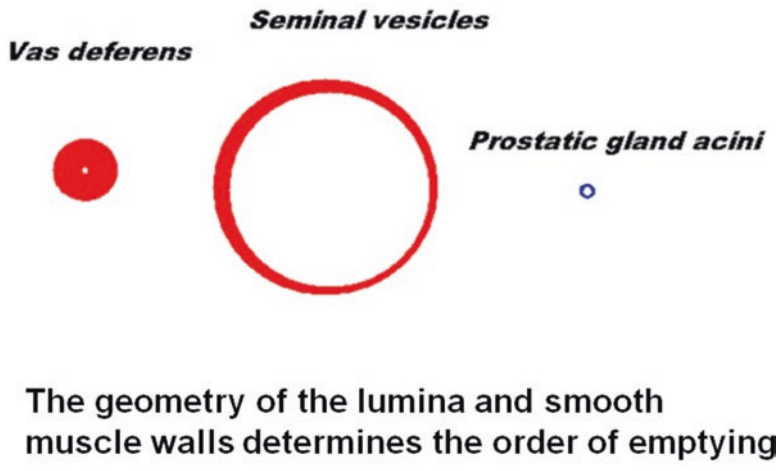

Fig. 4 Geometry of sperm and fluid emission. Drawing by Ulrik Kvist

shown a muscular contraction in the vas deferens and the prostate would have a more prominent effect on the diameter and pressure in these lumina than in the huge lumen of the bag-shaped seminal vesicles. Laplace's Law gives that lumina with big diameter like the seminal vesicles will end up with a higher wall tension than lumina with a small diameter for a given pressure. This also means that it would take more power and time to overcome the wall tension of the seminal vesicles than that of the lumina of the vas deferens and the prostatic acini and ducts. In case of prostatic inflammation, oedema and increased diameter of acini and ducts would correspondingly delay the emptying of the prostatic glands until the wall tension in diluted ducts is overcome. In addition to the geometry also the viscosity of the various fluids will influence on when the structure would empty.

The WHO Ejaculate Is an Artefact

It is a widespread misunderstanding that the human ejaculation produces a homogeneous fluid with constant environment for the spermatozoa (Björndahl and Kvist 2003). Collection of the whole ejaculate in one container as done for diagnostic reasons (World Health Organization 2010) creates an artefact that does not exist as a physiological entity in nature. The ejaculate is not under homeostatic control. Its composition is highly variable both between men and within the same man due to the random variation in the contributions from the different glands for every emission and ejaculation. Moreover, its composition varies conspicuously with time after ejaculation. Thus, spermatozoa collected in a whole ejaculate for diagnosis, treatment or research purposes face various challenges in the form of light, high oxygen concentration, low carbon dioxide concentration, increased pH and changed bioavailability of zinc and calcium and extreme variations in osmolarity in the man-made laboratory product called whole ejaculate. The whole liquefying ejaculate is with respect to the individual spermatozoon more and less todays happening and can probably not be standardized and controlled with respect to sperm functionality.

Sperm Chromatin and Zinc

At ejaculation, the spermatozoon appears to have a chromatin stability dependent on zinc that rapidly is overridden by a second type of stability dependent on S–S crosslinks. In the whole ejaculate**,** chromatin **salt bridges** with zinc (S–Zn–S) appears to loose zinc during and after liquefaction and the liberated thiols rapidly turn into **covalent disulphide bridges** (S–S) (Björndahl 1986; Björndahl and Kvist 1982, 2010; Kjellberg 1993; Kvist 1980a, b, c; Kvist and Björndahl 1985; Kvist et al. 1988; Roomans et al. 1982).

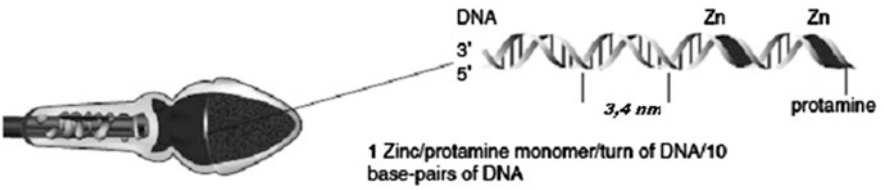

Fig. 5 Human sperm chromatin composed of one protamine chain and the two strands of the DNA double helix. One zinc for every protamine monomer for every turn of DNA, i.e. for every 10 base pairs. Drawing compiled by Ulrik Kvist

Incorporation of Zinc into the Chromatin

Zinc is incorporated into the protamine of the chromatin in the developing spermatids when histones finally are exchanged for protamines. The sperm nucleus consists of one zinc for every protamine monomer, i.e. one zinc for every five sulphur/ for every turn of the DNA and thus for every 10 base pairs of DNA, Fig. 5 (Björndahl and Kvist 2010). Zinc appears to have evolved to be a structural part of the chromatin, which temporarily stabilizes the chromatin until delivery into the ooplasm.

Zinc interacts with protamine thiols forming salt bridges of zinc (S–Zn–S). Since there is one zinc for every protamine monomer and every five sulphur it appears that zinc links the monomers together forming intermolecular salt bridges. In addition, the protamine monomers also have intramolecular disulphide bridges engaging other thiol residues of the protamine-cysteine residues (Björndahl and Kvist 2010). In the whole ejaculate, chromatin depletion of zinc results in a rapid oxidative re-stabilization that depends on oxidation of the liberated thiols into S–S bridges (Björndahl and Kvist 2010; Kvist 1980a, b, c).

Sperm Chromatin Zinc and Prostatic Fluid

The prostatic fluid in which spermatozoa normally are expelled at ejaculation is conspicuously rich in zinc (4–5 mM). Zinc is accumulated in the prostate under normal testosterone conditions. It is secreted in the prostatic fluid as free zinc ions Zn^{2+} and as bound to Citrate, Fig. 6 (Arver 1982). Citrate has 1–3 sites that can bind zinc. In the prostatic fluid, the bioavailability of zinc is high, and spermatozoa remaining in the prostatic fluid retain a high and normal level of zinc in their chromatin (8 mM) Fig. 6 top (Kvist et al. 1985), and in other zinc-rich parts as the sperm neck and tail. Thus, the prostatic fluid normally preserves the sperm chromatin zinc during the transfer to the cervical mucus.

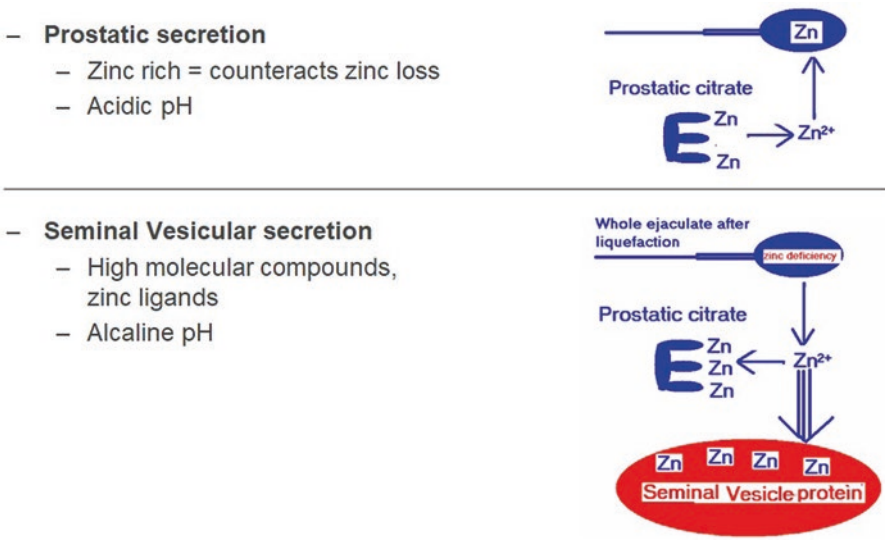

Fig. 6 Chromatin zinc and semen. Drawing by Ulrik Kvist

Sperm Chromatin Zinc and Seminal Vesicular Fluid

The fertilizing sperm in vivo is not normally exposed to the seminal vesicular fluid as is the situation in vitro. The seminal vesicular fluid contains high-molecular-weight proteins (HMW proteins) (Arver 1982) and seminogelins (Lilja et al. 1989) that can bind zinc with high affinity. When prostatic fluid and the seminal vesicular fluid are mixed, the HMW proteins bind up free zinc, and zinc bound within the sperm is released and lost to the surrounding fluid (Fig. 6 bottom). When the slightly alkaline seminal vesicular fluid is mixed to the acidic prostatic fluid, the pH increases. Increased pH also increases the available binding sites of citrate for zinc enhancing the loss of zinc from the sperm. Thus, seminal vesicular fluid may cause loss of zinc from the sperm by direct binding of zinc and indirectly by increasing the binding of zinc to citrate (Fig. 6 bottom).

Also Zinc-Rich Semen Can Cause Sperm Chromatin Zinc Deficiency In Vitro

The ejaculate can, in spite of a high and various zinc concentration 2–4 mM, act as a zinc-chelating solution and cause zinc deficiency in spermatozoa, Fig. 6. Thus, due to the presence of zinc-binding proteins of seminal vesicular origin in the ejaculate, the human spermatozoon is at risk to lose chromatin zinc at ejaculation, during liquefaction and afterwards when handled in the diagnostic and the ART laboratories (Arver and Eliasson 1982; Kjellberg et al. 1992; Kvist 1980a, b, c; Kvist and Eliasson 1980).

Zinc Bound to Seminal Vesicular HMW Proteins Varies Between Fertile and Infertile Men

The relative amount of zinc bound by seminal vesicular HMW proteins was 2–67% among 115 infertile men (Kjellberg et al. 1992), whereas 13 fertile donors had <10% of zinc bound to HMW proteins. Arver (1982) studied fertile men ($N = 20$) and found mean HMW-Zn of 13%. Thus, a high proportion of zinc bound to HMW proteins is more common among infertile men than among fertile men and sperm donors and reflects a higher risk for chromatin zinc deficiency. It appears that men with signs of inflammation in the prostate have lower secretion of zinc and end up with relatively more zinc bound to HMW proteins as measured 4 h after ejaculation. Moreover, men with signs of inflammation of the prostate also showed delayed emptying of the prostatic fluid meaning that spermatozoa might be expelled primarily with zinc-binding seminal vesicular fluid and loose zinc directly during ejaculation.

Zinc Bridges and Disulphide Bridges in the Human Sperm Chromatin: A Model

Figure 7 illustrates a model of how zinc by a single mechanism can form salt bridges between adjacent thiols groups and exert multiple functions: (1) temporarily stabilize the sperm chromatin structure and at the same time (2) prevent the thiols from oxidation into covalent disulphide bridges and thereby (3) preserve an inherent mechanism for rapid decondensation induced by zinc removal. Removal of zinc at the right moment can result in a rapid decondensation. However, premature removal of zinc at or after ejaculation would result in a destabilization and tentatively leave a vulnerable DNA and a risk for oxidative and other attacks that may damage the DNA and also give permanently covalent closure and S–S–crosslinking of the sperm chromatin. Figure 8 illustrates two spermatozoa exposed to SDS-EDTA. One resisted the treatment and the other decondensed and unravelled the chromatin. The figure also illustrates that the sperm nuclear chromatin has two complementary roles, to close and protect the genome during transfer and to open and deliver the genome in the oocyte.

The "Chemical" Resistance of the Mammalian Sperm Chromatin

The question why ejaculated mammalian sperm resist to be dissolved in vitro has been dealt with since 1878 when Friedrich Miescher (1878) suggested that the resistance of the mammalian sperm nucleus was because of the presence of a vulcanized protein he named sulfonuklein (i.e. the Cysteine-rich protamines and DNA) and that boiling in urea for hours was needed to dissolve the nuclear content, Fig. 9 top.

A 100 years later, Bedford et al. (Bedford et al. 1973) also focused on the sulphur and suggested that the resistance of the sperm chromatin towards decondensing

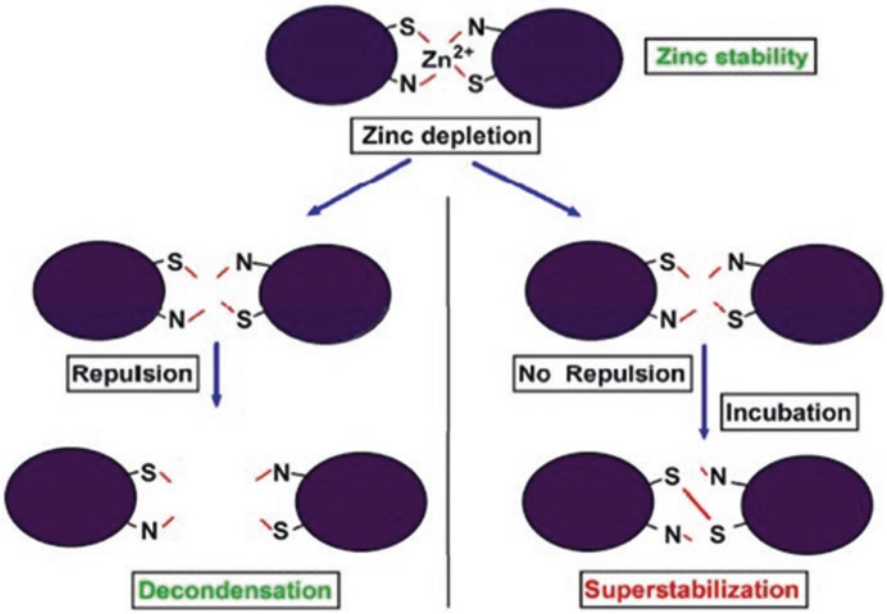

Fig. 7 Zinc-bridges—one mechanism multiple effects: Zinc (1) stabilizes (S–Zn–S) bridges and (2) prevents S- from oxidation into S–S and thereby (3) preserves an inherent mechanism for decondensation by zinc withdrawal. Removal of zinc gives two possibilities: immediate decondensation otherwise superstabilization (S–S). Drawing compiled from Björndahl & Kvist 2011

with DTT was due to stabilizing S–S bridges formed during the epididymal passage (Calvin and Bedford 1971) (Fig. 10 top). However, the fact that DTT besides being a S–S bridge cleaving agent also is a powerful zinc chelator was not taken into consideration. Therefore, the role of zinc in the chromatin was missed and not elucidated. Forty years later, Björndahl and Kvist (2011) and Ekwurtzel et al. (2011) showed that the major resistance towards DTT in spermatozoa from liquefied semen samples was effectuated by chromatin zinc bridges (S–Zn–S) since EDTA eliminates the resistance to decondense the chromatin in low concentrations of DTT.

Delivery: Decondensation of the Chromatin

In contrast, to the sperm chromatin resistance to open up shown by Miescher and Bedford, the sperm chromatin decondenses normally immediately upon sperm entrance in vivo in the oocyte, Figs. 9–11. Also, when studied within 5 min after ejaculation 90% of human ejaculated spermatozoa decondenses rapidly their chromatin if exposed to zinc chelating EDTA and detergent, Fig. 11 (Björndahl and Kvist 1985, 2010). Thus, zinc withdrawal at ejaculation revealed that the human spermatozoon at ejaculation has an inherent mechanism for decondensation of the chromatin that could be initiated by zinc withdrawal and concomitant repulsive

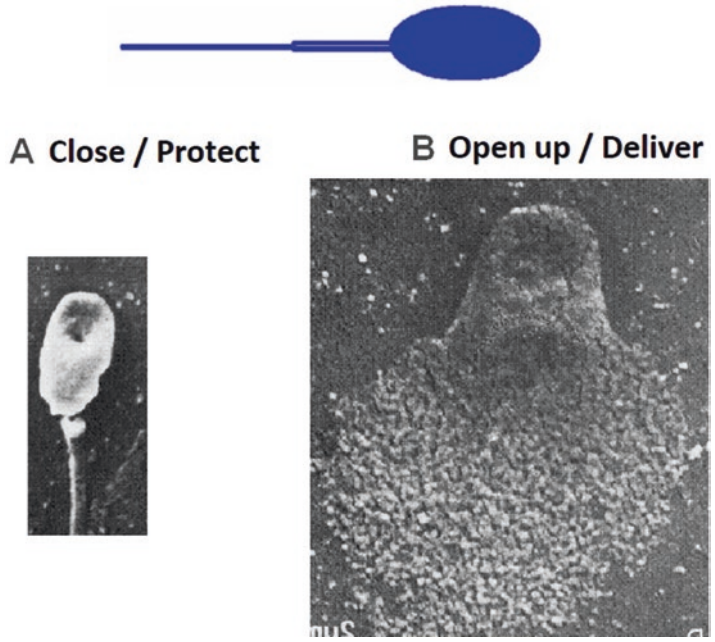

Fig. 8 The sperm nuclear chromatin has dual roles. A and B are Scanning electron microscope (SEM) images of human spermatozoa exposed to SDS with 6 mM EDTA; in A the sperm head as an intact superstabilized nucleus that does not decondense in SDS-EDTA, while in B. the sperm head is grossly swollen and decondensed. Sperm preparation by Ulrik Kvist, 1980; SEM images by the Lennart Nilsson

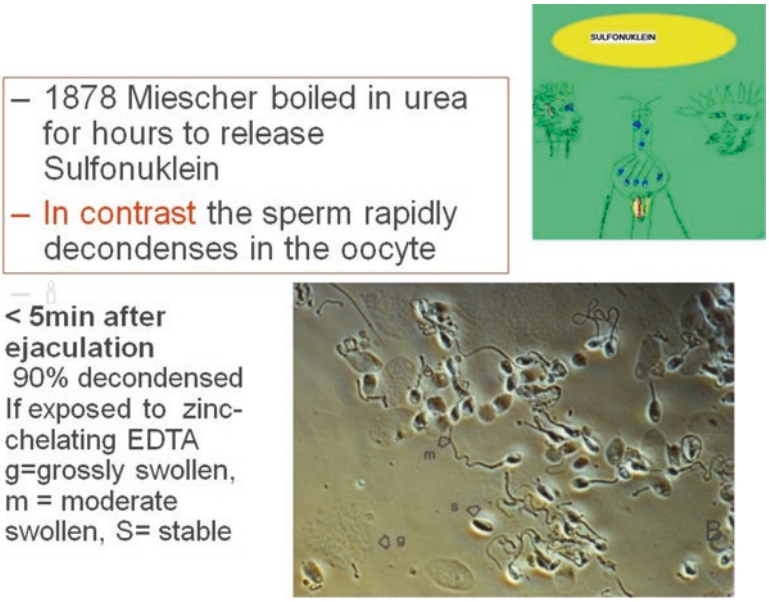

Fig. 9 The sperm chromatin has dual roles: close/protect and open up/deliver. Drawing by Ulrik Kvist, micrograph by Ulrik Kvist

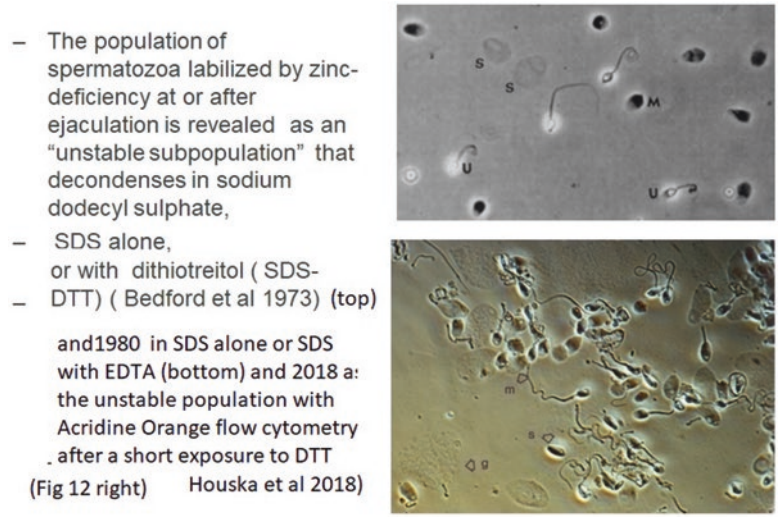

Fig. 10 The population of spermatozoa labilized by zinc deficiency at or after ejaculation is revealed as an "unstable subpopulation" that decondenses in sodium dodecyl sulphate, SDS alone, or with dithiothreitol (SDS-DTT) (Bedford et al. 1973) (top). In the 1980s in SDS alone or SDS-EDTA (bottom) and in 2018 also as an "unstable population" with Acridine orange flow cytometry after short exposure to DTT (Fig. 12 right) (Houska et al. 2018). Figure compiled by Ulrik Kvist from Bedford et al. 1973) (top) and photo by Ulrik Kvist (bortom)

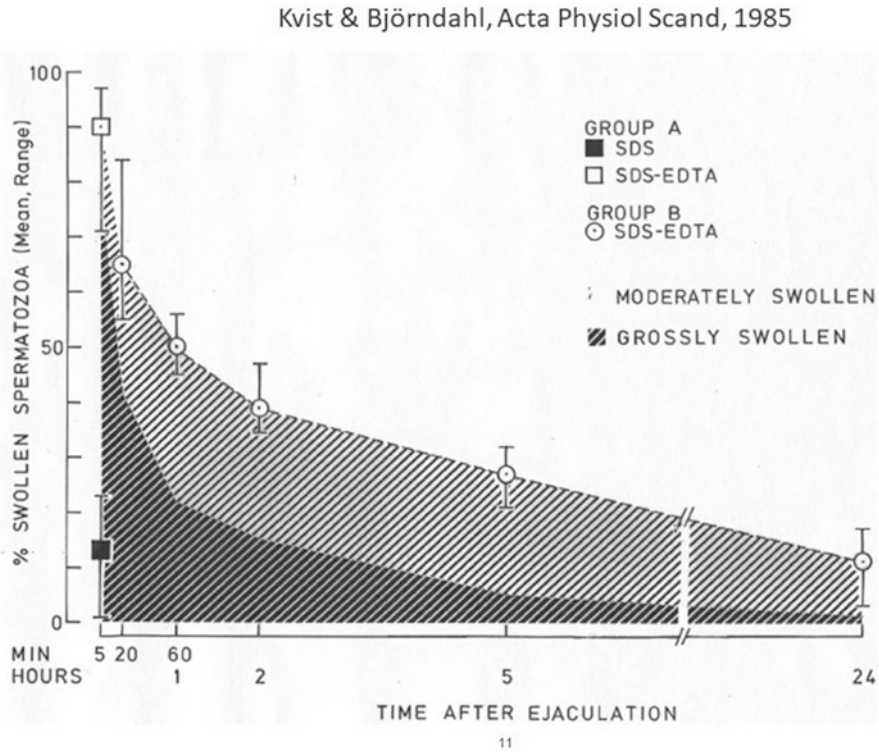

Fig 11 Loss of zinc-dependent stability in semen. Drawing by Lars Björndahl. Compiled from Kvist & Björndahl 1985

forces—here exerted by detergent, Figs. 9 and 10. However, this inherent mechanism for decondensation is rapidly lost in spermatozoa in contact with the liquefying ejaculate (Fig. 11). The zinc-dependent chromatin stability relying on salt bridges with zinc is rapidly lost in vitro and overridden by a covalent S–S-dependent stability that resisted the repulsive forces exerted by detergent, Fig. 11 (Björndahl and Kvist 1985, 2010).

Sperm Chromatin Stability in Liquefied Semen

Thus, in liquefied human semen, chromatin stability is dependent on both, zinc bridges and disulphide bridges. To overcome the secondary stability, a disulphide bridge cleaving agent like dithiothreitol (DTT) is needed (Björndahl and Kvist 1985; Huret 1986; Huret and Miquereau 1984). The remaining chromatin stability is dependent on zinc and can quickly be eliminated by EDTA-induced zinc withdrawal (Ekwurtzel et al. 2011).

The sperm chromatin stability and sperm chromatin zinc content differ between fertile men and men in barren unions (Kjellberg et al. 1992; Kvist et al. 1988). However, the relation between the type of structural stability of the sperm chromatin and the integrity of the sperm DNA as measured by the acridine orange (AO) flow cytometric test (AO flow cytometry) has not yet been thoroughly studied (Barratt et al. 2010).

The Zinc-Deficient Spermatozoa

It has long been known that a certain population of spermatozoa achieve zinc deficiency at or after ejaculation.

Bedford et al. (1973) showed that a certain population of human ejaculated spermatozoa decondensed in SDS alone, Fig. 10 top. In the 1980s it was shown that the samples with a high proportion of unstable spermatozoa swelling in SDS alone emanated from men with relatively low secretory contribution of prostatic fluid and relative abundance of seminal vesicular fluid (Eliasson and Kvist 1976). (Kvist 1980a, b, c; Kvist and Eliasson 1978, 1980). It was also demonstrated that zinc-binding EDTA increased the proportion of spermatozoa swelling in SDS (Figs. 9 and 10), if exposed to SDS directly **but reduced** the proportion swelling in SDS if spermatozoa were stored without repulsing SDS after EDTA exposure (Kvist 1980a, b, c).Thus, in the 1980s it was obvious that spermatozoa had an inherent zinc-dependent stability and that zinc removal enhanced the development of another disulphide-bridge-dependent stability (Figs. 8 and 11).

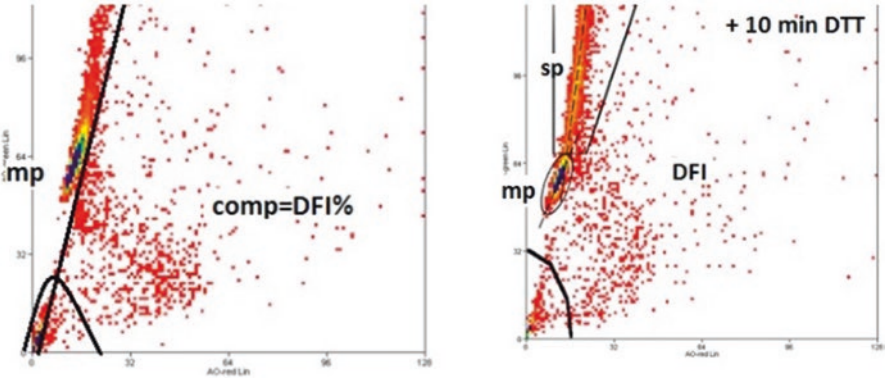

Fig. 12 DTT exposure revealed a new subpopulation (sp) of spermatozoa with unstable zinc-deficient chromatin after acridine orange (AO) flow cytometry (Petr Houska et al. 2018). Left: AO-scattergram control. *X*-axis red fluorescence. *Y*-axis green fluorescence. Right: AO-scattergram after DTT exposure. *mp* main population, *comp* cells outside the main population, *DFI* DFI population, *sp.* second population. Spermatozoa were kept in salt solution with Zn to prevent S–S–superstabilization before DTT exposure. Flow cytometry diagram of human spermatozoa by Petr Houska, compiled from Houska, Björndahl & Kvist 2018

DFI and Chromatin Stability

Zinc bridges (S–Zn–S) and disulphide bridges (S-S) both contribute to chromatin stability.

Strand breaks in the sperm DNA can be assessed by AO cytometry (Ringertz et al. 1970). The AO flow cytometric test (e.g. SCSA®) identifies a main population of spermatozoa with native DNA. Cells outside the main population (COMPαt), later called "DNA fragmentation index" (DFI) spermatozoa, have DNA strand breaks (Fig. 12, left side).

In recent years, studies have been published indicating a relation between impaired sperm DNA integrity (as measured by Sperm Chromatin Structure Assay—SCSA) and decreased in vivo and in vitro fertilization (Bungum et al. 2011; Evenson 2016; Faduola and Kolade 2015). The clinical usefulness for ART is other discussion (Castilla et al. 2010).

Gaps of Knowledge

Besides SCSA, also Comet, TUNEL and Sperm Halo test aim to identify spermatozoa with broken DNA, and a defragmentation index with various meaning is also given as a result from these tests. It seems that high DFI values are bad signs indicating many sperm with damaged DNA. On the other hand, low DFI values may be due to low access to the sperm chromatin structure thus being a false-negative observation from TUNEL (From Björk et al. 2009) and AO flowcytometry (Björndahl and Kvist 2010; Pettersson et al. 2009). For none of the above methods, it has been

thoroughly explored how the prevailing type of chromatin structural stability may interact with the method and the results for DNA damage obtained.

Searching for the Zinc-Deficient Spermatozoon

Given the fact that:

1. The major resistance to decondense the sperm chromatin in SDS-DTT is mediated by chromatin zinc (Ekwurtzel et al. 2011).
2. It follows that spermatozoa that have achieved un unstable chromatin due to significant zinc deficiency at or after ejaculation and thereafter had undergone a partial oxidation of some of the freed thiols into disulphide bridges must be more susceptible to DTT than spermatozoa that had not undergone significant zinc deficiency.

Recently, the problem to identify and characterize the unstable spermatozoa that had achieved significant zinc deficiency at or after ejaculation was addressed. The main findings were reported in a poster at the XIIIth International Symposium on Spermatology, 9th to 13th May 2018, Stockholm (Houska et al. 2018).

The two main findings are as follows:

1. In every human ejaculate the unstable spermatozoa showed up after DTT exposure as a new second subpopulation besides the main population and the COMP-(DFI) population.
2. The size of the unstable population after DTT covaried with the sample DFI% values as obtained before DTT exposure.

And the overall conclusion is as follows:

Samples with many unstable, zinc-deficient spermatozoa are the samples with the highest risk to develop DNA damage.

All samples were run with AO flow cytometry after short exposure to DTT. DFI values were obtained before DTT exposure when samples were run in TNE buffer as normally used for AO flow cytometry.

In Fig. 12 (right side) the new, not earlier identified subpopulation of spermatozoa with unstable chromatin is shown (SP in Fig. 12 right).

Note that this population is distinct and different from the main population (MP) and from the population of spermatozoa defined as outside the main population which are the spermatozoa with excess DNA damage—the DFI spermatozoa (Fig. 12 left and right, COMP). Thus, the unstable spermatozoa with tentative zinc deficiency did not belong to the DFI spermatozoa. These two types of spermatozoa constitute at the time of measurement two different sperm populations. Moreover, the obtained DFI values were similar whether run before or after DTT exposure. This emphasizes that the DFI sperm population is present in the samples and detected, whereas the samples are run before or after 10 min DTT exposure. However, samples with high DFI values also had many zinc-deficient spermatozoa as revealed by DTT. The explanation is most probably that the samples with many

unstable spermatozoa are the samples exposed to abundance of seminal vesicular fluid that induces both zinc deficiency and increased risk for oxidative damage mediated by the oxidative properties of the seminal vesicular fluid, c.f. Bungum et al. (2011).

The unstable spermatozoa in the second population are *localized above and slightly to the right of the main population* in the AO scattergram. This tells that they had a more open chromatin structure that was more accessible for the Acridine Orange than the remaining main population. Moreover, the slope in the scattergram for the second population is steeper than that for the main population. This means that the second population appears "healthier" than the main population since a steep slope means a lower ratio between red single-stranded and green native DNA.

Moreover, they had far less single-stranded DNA than the DFI spermatozoa, Fig. 12 right.

The size of the unstable population covaried significantly with the % DFI of the samples.

In Fig. 13 are shown histograms of the red fluorescence and the obtained values for DFI%. After DTT exposure a new second peak appears to the right of every main peak and the size of the second peak varies between samples and notably covaries with the sample DFI values.

How Can this Make Sense?

In every human ejaculate there is a variable subpopulation of spermatozoa that achieved zinc deficiency at or after ejaculation. This subpopulation was earlier in the 1980s revealed as swelling in microscopy with detergent SDS with or without DTT and here as the second population by Houska with the AO flow cytometry. In every semen sample there is also a DFI subpopulation with excess of DNA damage. The question is if and how these two populations are related? At the time of analysis, the spermatozoa are separated into two different populations that do not overlap. However, they have a common connection saying that the semen samples that developed the highest values for DFI also are the samples that had most spermatozoa that achieved with zinc deficiency. The results presented here therefore seem to be in concordance with a suggestion that men delivering semen samples where many spermatozoa become zinc-deficient are at risk to be affected by DNA oxidation. Oxidative damage to DNA can result in strand breaks. Oxidation of zinc liberated free thiols leads into secondary stabilizing S–S bridges.

The oxidative damage to DNA that led to strand breaks can later be revealed as DFI-positive spermatozoa. Zinc-deficient spermatozoa with oxidatively stabilized chromatin can later be revealed by short DTT exposure as the second population.

It is therefore suggested that men with samples where many spermatozoa will experience zinc deficiency at or after ejaculation initially have a less stabilized chromatin due to achieved zinc deficiency and therefore are more susceptible for (1) oxidative damage of the DNA and (2) oxidative crosslinking of free chromatin thiols into covalent S–S bridges.

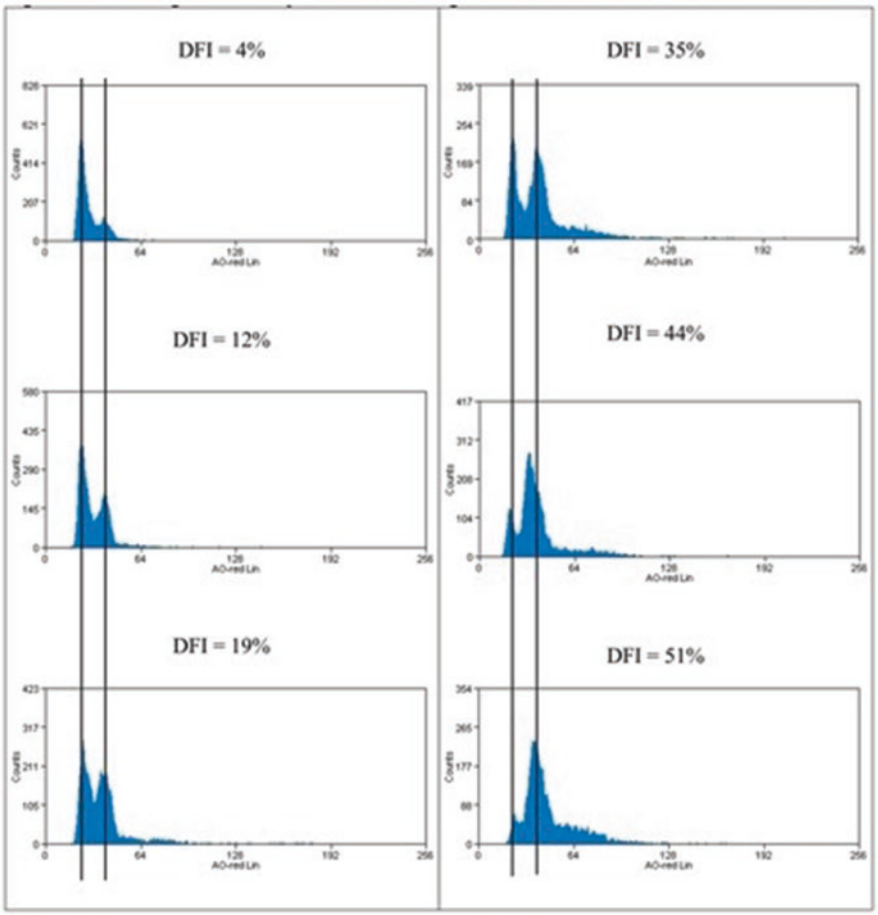

Fig. 13 The size of the zinc-deficient second population covaries with DFI%. Histograms of red fluorescens (*X*-axis) after acridine orange flow cytometry of spermatozoa pre-exposed 10 min to DTT. To the right of the first, main peak is seen a new second peak/population of variable size of red fluorescens corresponding to the second population in Fig. 12 right. To the right of the second peak are spermatozoa with high red fluorescence tentatively belonging to a population rich in single-stranded, i.e. defragmented DNA. Note that the size of the second population after DTT exposure covaries with the size of the DFI% measured separately before DTT exposure. Thus, samples with many zinc-deficient spermatozoa have also many spermatozoa that have acquired DNA strand breaks. Diagrams by Petr Houska 2018

In Conclusion

One of the consequences of sperm exposure to seminal vesicular fluid is sperm chromatin zinc deficiency.

Sperm exposure to zinc-chelating seminal vesicular fluid creates a subpopulation of spermatozoa with improper zinc stabilization that in the 1980s was revealed by microscopy with detergent or without DTT.

In the 2020s samples with the many zinc-deficient spermatozoa can be revealed by a significant second population in AO flow cytometry after DTT exposure.

The zinc-deficient spermatozoa have an increased risk for:

1. Oxidative DNA strand breaks that is manifested as high values for DFI and.
2. Oxidation of zinc-freed thiols into S–S bridges that can be opened by short exposure to DTT (Houska et al. 2018).

Consequences of chromatin zinc deficiency for sperm cryopreservation is to be studied.

The Sperm Osmotic Roller Coaster: Another Unphysiological Challenge During Sperm Preparation for ART

As indicated in Fig. 1, the human sperm will not experience any osmotic challenge in vivo when transferred from the isotonic prostatic fluid (Cooper et al. 2005; Holmes 2020; Holmes et al. 2019a, b; Holmes et al. 2018; Keitel and Jones 1956; Rothschild 1960) into the cervical that is isotonic to body fluids (Rossato et al. 1996).

In contrast, when handled in vitro the human spermatozoa not only have the challenge of exposure to seminal vesicular fluid but also experience an **osmotic roller coaster** with osmolality going first up and then down.

Osmolality and Consequences for Cellular Functions

In multicellular organisms like the human body, there are control mechanisms to maintain extracellular conditions for the cells (Andersson 1977). The spermatozoon is unique in that it leaves the initially controlled environment in the prostatic fluid and enters another controlled environment in the female body, the cervical mucus before it on hitherto unknown ways reaches the controlled environment in the crypts in the isthmus part of the fallopian ducts.

In the whole ejaculate in the diagnostic and ART lab the spermatozoon is left in an osmotically uncontrolled environment as outlined below. It must therefore rely entirely on its own mechanisms for regulation of cellular volume and intracellular electrolyte concentration. After experiencing an initial change in cell volume, the mammalian spermatozoon activates processes to restore the cell volume. In mammals, spermatozoa appear to acquire this ability during the transfer through the epididymis (Cooper and Yeung 2003; Petrunkina et al. 2005; Yeung et al. 2006). However, these processes are dependent on the supply of ATP. Osmotic challenge can therefore affect other cell functions negatively by reducing the available energy in the cell. There are reports indicating that exposures to both hypertonic and hypotonic challenges can have negative effects on human sperm motility (Makler et al. 1981; Rossato et al. 2002; Velazquez et al. 1977).

Osmolality Going up

When semen is collected as whole ejaculate the prostatic fluid rich in enzymes (Quinlivan 1972) mixes with the epididymal fluid rich in substrates like phosphorylcholine and the seminal vesicular fluid rich in proteins (Mann 1964). There is an almost immediate enzymatic and temperature-dependent activity resulting in a rapid increase in semen osmolality (Holmes 2020; Holmes et al. 2019a, b Mann 1964; Mann and Lutwak-Mann 1981; Pourian et al. 2000; Rothschild 1960). Osmolality increases from close to isotonic within 5 min after ejaculation to 322 (280–362) mOsm/kg after liquefaction at 1 h and to 379 (292–454) mOsm/kg 3 h later (Fig. 14). Note the huge individual variability in osmolality that spermatozoa from various men are challenged with (Fig. 14). The hyperosmotic challenge means that the cell starts to lose water and shrink. Individual spermatozoa are then forced to adjust their volume to the increasing osmolality by pumping out osmolytes accompanied by water. The hyperosmotic challenge is therefore likely to exhaust the ATP reserves of individual sperm and leave less ATP for other energy-depending processes like motility, Fig. 14.

Osmolality: Going Steeply Down

Osmolality then drops when sperm in hypertonic seminal fluid are exposed to isotonic sperm selection media (Fig. 15 left and middle). This hypotonic challenge forces water to move into the cell and the sperm swells. Swelling induces sperm tail

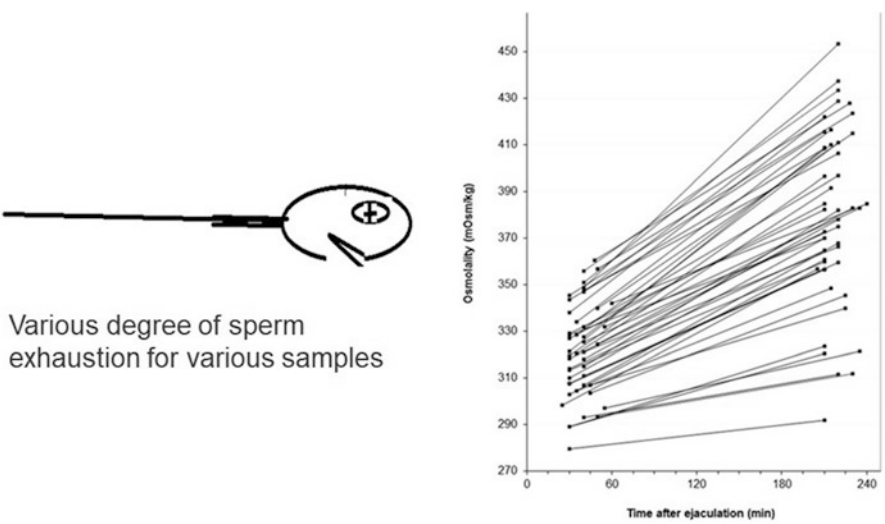

Fig. 14 Osmolality goes up during and after liquefaction. *X*-axis time after ejaculation (h). *Y*-axis Osmolality (mOsm/kg). Drawing by Ulrik Kvist and diagram by Emma Holmes

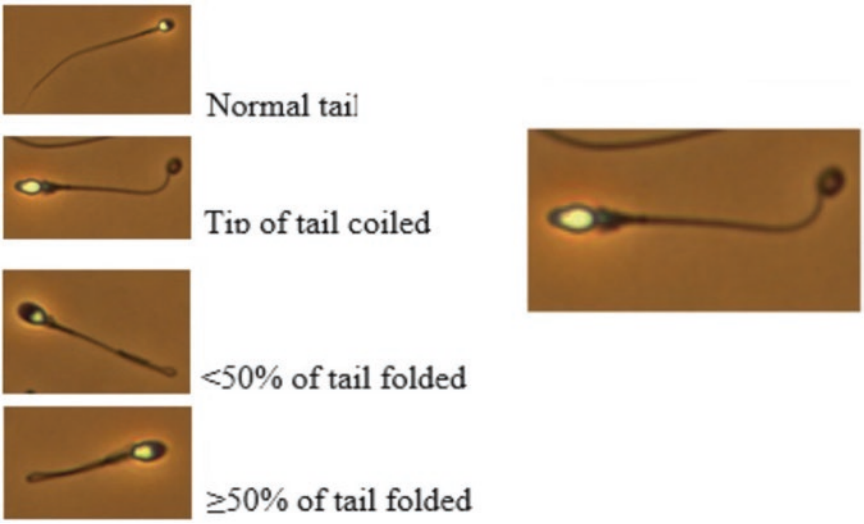

Fig. 15 The types of sperm swelling seen at sperm selection at a hypotonic jump of 20–110 mOsm/kg. Phase contrast micrographs by Emma Holmes 2011

coiling which affects sperm functional morphology, sperm motility and sperm density (Fig. 15). This would in turn affect sperm selection both by motility and by density. Thus, sperm selection by swim up and by density gradient centrifugation may to various degree be affected (Fig. 18).

It was Albert von Kölliker who in 1841 described spermatogenesis, and 15 years later Kölliker (1856) described how water inserted under the cover glass resulted in coiling of the sperm tail followed by cell death. Later, the Swedish group at the Veterinarian University in Uppsala with Lindahl, Drevius and Eriksson explained the mechanism behind hypoton tail coiling: the sperm becomes spherical by swelling when it takes up water, and since the membrane cannot expand, the tail is forced to coil or bend inside the sperm membrane. This morphological change of the sperm tail is irreversible unless the sperm is not re-exposed to hypertonic media again (Drevius 1963; Drevius and Eriksson 1966; Lindahl and Drevius 1964).

Spermatozoa with coiled tails are also commonly found in human ejaculates without prior hypoton exposure (Yeung et al. 2009).

The various forms of tail tip coiling and tail folding seen after hypotonic fall up to 110 mOsm/kg has been described (Fig. 15) (Holmes 2020). Water uptake thus affects sperm tail morphology by tail tip coiling and tail folding. This affects in turn sperm motility and can thereby reduce the yield after sperm selection with swim up. Water uptake also reduce, by definition, the sperm density and can thereby affect the yield after procedures by which sperm are selected due to their density, i.e. density gradient centrifugation (Fig. 18).

As evident from Fig. 16 (left and middle) that spermatozoa in individual samples undergo varying osmotic jumps at sperm selection and note that the jump increases

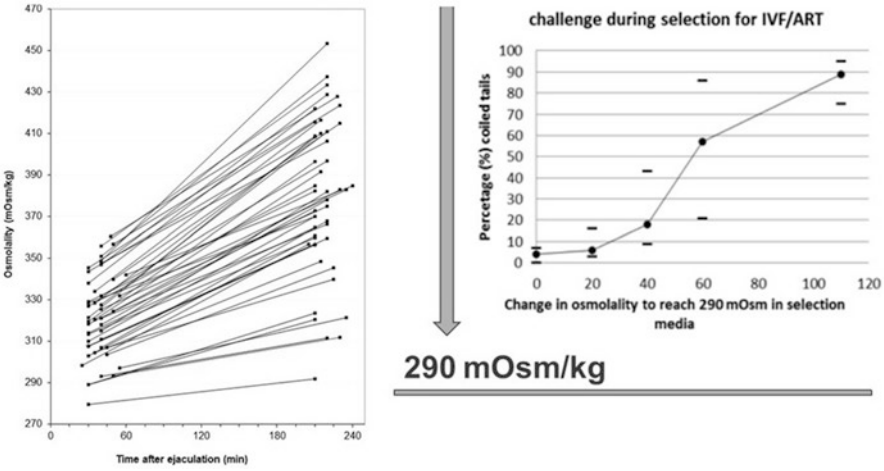

Fig. 16 The hypotonic to be at sperm selection for ART. Left: the increment in osmolality during and after liquefaction before selection for ART. Middle the arrow, the actual osmotic jump down to isotonic 290 mOsm/kg various between samples and with time after ejaculation. Right: the range of sperm undergoing severe hypotonic challenge with swelling at various osmotic jumps. Diagrams by Emma Holmes, compiled and modified by Ulrik Kvist

with time after ejaculation that pass before the sperm is transferred to the selection medium. The percentages of spermatozoa with tail tip coiling and the jump in osmolality varied in a dose–response manor (Fig. 16 right). In addition, for every given jump the tail coiling response varied between individual samples (Fig. 16 right) (Holmes et al. 2020).

Hitherto, temperature and pH are acknowledged as basic conditions that should be controlled for according to Guidelines for Good Practice in the IVF Laboratories (Eshre Guideline Group on Good Practice in IVF Labs, et al. 2016). Osmolality is of outmost importance for the function of spermatozoa and other cells. However, osmolality is acknowledged in these guidelines, and little is therefore known to what extent the increase in osmolality before selection and the drop in osmolality at selection affect the spermatozoa during the IVF procedures.

It seems although that many of ART semen samples might be at risk for hypertonic exhaustion and hypotonic chock affecting sperm selection by swim up and density gradient centrifugation (Makler et al. 1981; Rossato et al. 2002; Velazquez et al. 1977). From Makler et al. (1981), From Fig. 17, it is evident that 2/3 of all ejaculates have an osmolality above 330 mOsm at 1 h after ejaculation and thereby osmolality jumps over 40 mOsm/kg which affects sperm selection by swim up and density gradinet centrifugation Fig. 18.

As evident from Fig. 16, right, an osmotic jump of 40 mOsm/kg from 330 to 290 will affect motility and density of 18 (9–43%) of the spermatozoa and a corresponding jump of 60 mOsm/kg from 350 to 290 would affect 57% (21–86%) of the spermatozoa. Thus, osmotic challenges not controlled for may affect spermatozoa in a majority of ART semen samples.

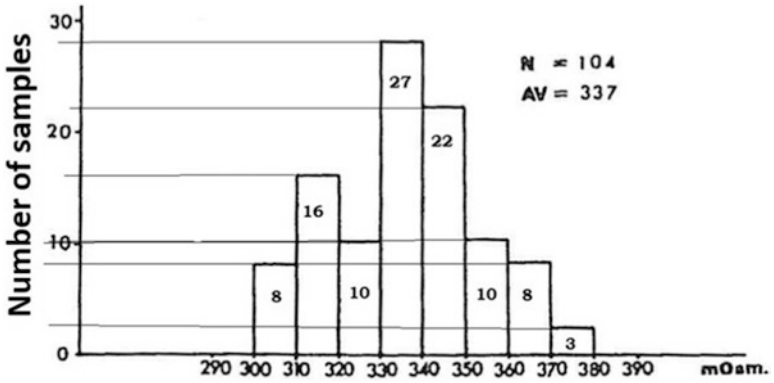

Fig. 17 Distribution of semen samples (N = 104) with respect to semen osmolality at 1 h after ejaculation. Redrawn after Makler et al. (1981). Note that a majority of samples will experience a significant hypo-osmotic jump at sperm selection for ART at 1 h after ejaculation. Compiled and modified by Ulrik Kvist

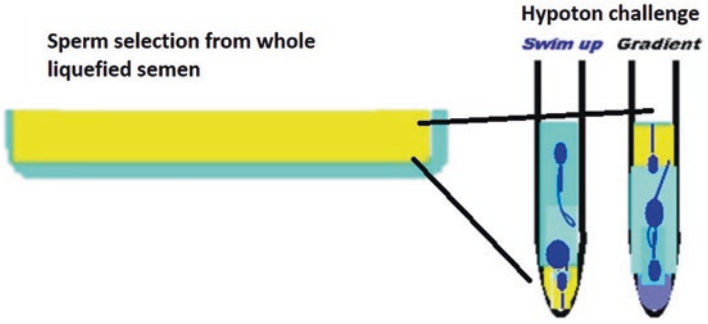

Fig. 18 Sperm selection from whole liquefied ejaculate causes hypotonic challenges when spermatozoa from hypertonic semen are exposed to isotonic media at swim up and density gradient centrifugation. Drawing by Ulrik Kvist

Routine monitoring of semen osmolality would be of limited value and just reveal what hereby already is known as the progressive increase in osmolality with time after ejaculation (Holmes 2020).

One may wonder why Rothschilds observation (Rothschild 1960) that human semen osmolality increases after ejaculation and possible consequences thereof for ART were not recognized by the reproductive science. Nevertheless, this dual osmotic sperm challenge is likely to occur every day in most semen sample used for diagnostics and sperm selection in the ART laboratories around the globe. It would have been revealed if the reasons for low yield after swim up and density gradient centrifugation had been searched for. However, there has been at least one report that the yield was doubled when gradient centrifugation was performed with a hypertonic gradient corresponding to the increased osmolality of semen, 350 mOsm/kg (Kvist et al. 1994). It may be that, with ICSI at hand, a low sperm yield is seen as a minor problem.

One can argue that spermatozoa with tail tip coiling are never seen by the ART staff since they are excluded by the gradient. Looking for them in the supernatant is beyond the scope of routine sperm selection. Anyhow they may appear disguised in the pellets. Swollen sperm are fragile, and their membrane will easily burst upon centrifugation (Drevius and Eriksson 1966). If so, the most fragile spermatozoa may lose their membrane and end up in the pellet as uncoiled non-moving dead sperm. Thus, the only evident sign of a hypotonic sperm trauma would be a low yield of spermatozoa. The corresponding finding after swim up would also be a low yield of spermatozoa. Actually, the hypotonic effect reported here is an immediate effect and would only be seen in the microscope if examined at the start of the swim up or gradient procedure. The phenomenon of coiled tail tips and folded tails became obvious when the laboratory introduced the Picsara system by which still pictures of the video recordings taken directly at the presumed hypotonic challenge was studied (Holmes 2020). That spermatozoa swim in a "jerky way" after hypotonic exposure which is likely due to coiled tail tips and folded tails (Holmes et al. 2020).

These signs of initial swelling (tail tip coiling and partly folded tail) were seen in the osmotic jump interval from 0 to −110 mOsm/kg.

This is a different picture to what seen in the hypoosmotic swelling test (Jeyendran et al. 1992) where most "living cells" react with more complete sperm-head tail coiling. The test solution of the hypoosmotic swelling test has an osmolality of about 175 mOsm/kg, and exposing one part of semen (330–350 mOsm/kg) with one part of the test solution (175 mOsm/kg) would give a hypotonic jump of 165–175 mOsm/kg which is well above the jump seen at sperm selection by swim up and gradient centrifugation. Therefore, there was no awareness to look for minor tail-tip coiling as signs for hypotonic chock in connection with sperm swim up and density gradient centrifugation.

This increase in osmolality of whole semen is apparently beyond physiological control and minimize the time the spermatozoon is exposed to increasing osmolality in the ejaculate rather than measuring osmolality. Thereby, both the initial hypertonic and the subsequent hypotonic challenges and their negative effects on the sperm would be minimized.

There are various ways to mimic physiology. In Fig. 19 is depicted to the left the sperm selection in vivo and to the right possible future approaches for sperm selection that by-passes both (1) sperm exposure to seminal vesicle fluid and (2) the osmotic roller coaster.

One approach is to collect the first, sperm-rich ejaculate fraction of a split ejaculate (Amelar and Hotchkiss 1965; Björndahl and Kvist 2003; Holmes 2020) followed directly by sperm selection. This physiological approach is already used in veterinary medicine and was practised by the pioneers at Bourne Hall in the childhood period of IVF to improve the chances of success of IVF (Cohen et al. 1985). Another approach to minimize the effects of seminal vesicular fluid exposition and the osmotic roller coaster would be early dilution of the ejaculate, ejaculation in buffer or collection of the ejaculate in a buffer followed by immediate sperm selection (Holmes 2020; Holmes et al. 2019a, b; Zollner et al. 2001).

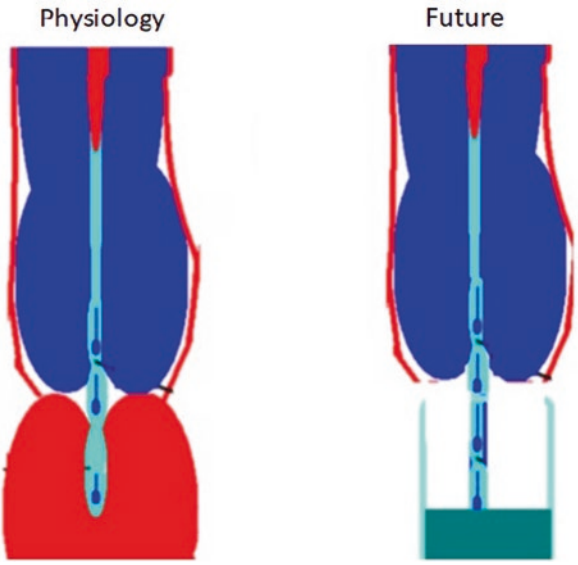

Fig. 19 Sperm selection for fertilization. Left: Present, physiological immediate selection from zinc-rich isotonic prostatic fluid. No exposure to seminal vesicular fluid. No osmotic challenges. Right: Future selection for fertilization at ART in order to prevent chromatin zinc deficiency and the osmotic roller coaster. Early collection and selection from the first sperm and zinc-rich ejaculate fraction of a split-ejaculate. Early dilution at ejaculation or ejaculation in buffer followed by immediate selection. Drawing by Ulrik Kvist

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Main Effects of In Vitro Manipulation of Human Spermatozoa

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Introduction

The development of assisted reproduction techniques (ARTs) started in 1978 when the first baby was born after in vitro fertilization (Steptoe and Edwards 1978). Since then, several laboratories worked hard to modify the techniques with the aim of ameliorating the performance, increasing the outcomes, reducing the costs, and simplifying the procedure. The use of ARTs has also led to the development of strategies to preserve fertility in patients undergoing therapies or surgical procedures potentially damaging the reproductive function. All these procedures foresee in vitro manipulation of gametes, reproductive tissues, and embryos exposing them to several noxious conditions that may be present in a laboratory setting, such as reactive oxygen species (ROS), contaminants present in the media or cryoprotectors, light, radiation, centrifugation steps, temperature, and others. Possible damages include alterations of DNA (fragmentation, oxidation, mutations, epigenetic modifications), membrane (peroxidation, phospholipid modifications), morphology, mitochondrial function and, in case of spermatozoa, decrease/loss of motility, or acrosome function.

The present chapter reviews past and current literature regarding possible damage due to in vitro manipulation of spermatozoa occurring during sperm selection or incubations for ARTs and cryopreservation (Fig. 1). In particular, the chapter focuses on sperm DNA fragmentation (sDF), a parameter that negatively impacts reproductive outcomes after ARTs (Cissen et al. 2016; Simon et al. 2017).

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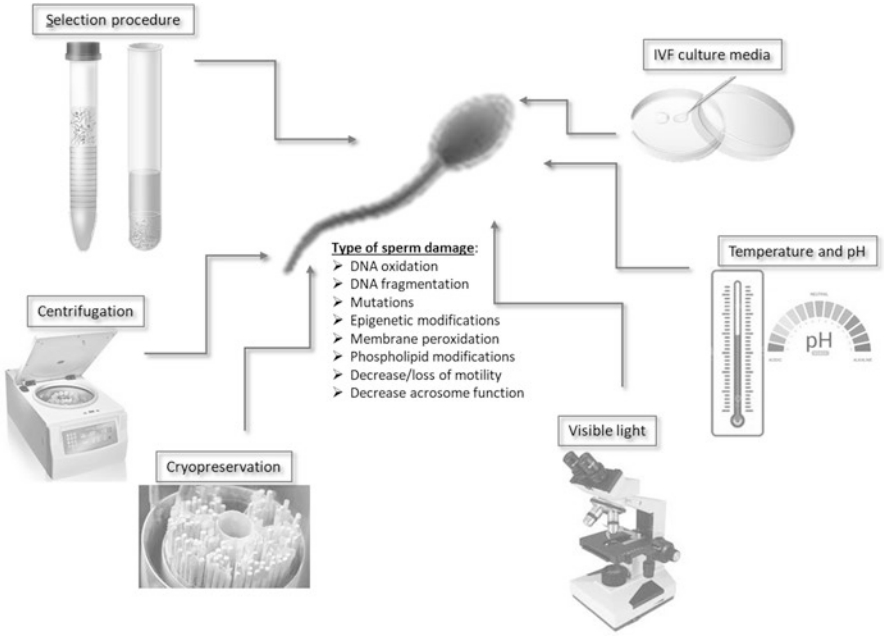

Fig. 1 Schematic representation of the possible inducers of damage to spermatozoa during in vitro manipulation for cryopreservation or assisted reproduction techniques

In Vitro Manipulation during Sperm Selection for ARTs

For in vitro fertilization (IVF/ICSI) purposes, spermatozoa are usually selected from whole semen employing several techniques (Jeyendran et al. 2019). Most used techniques are density gradient centrifugation (DGC) and direct or indirect swim up (WHO 2010). Such techniques allow the selection of highly motile and, likely, morphologically normal spermatozoa to be used to inseminate the oocyte. They have been designed to mimic as much as possible the natural conditions, where spermatozoa undergo an intensive quantitative and qualitative selection process within the female reproductive tract, to assure that only the best gametes reach the oocyte.

In the DGC technique, spermatozoa are forced to cross a gradient made of colloidal silicon and are separated based on their density, which is usually higher (>1.10 g/mL) in morphologically normal, motile, and mature spermatozoa. Percoll gradient, composed of polyvinylpirrolodone-coated silica particles, has been long used in ART settings until 1996, when its use was discontinued because of concerns regarding its toxicity (Mousset-Siméon et al. 2004). Presently, several DGC media, based on silane-coated silica colloid, are commercially available, and the most popular ones are PurSperm and Isolate.

The other popular method to select sperm for ARTs is swim up, during which motile spermatozoa are allowed to migrate up against gravity in a medium

containing bicarbonate and serum albumin. Although both procedures yield sperm populations with higher motility and better morphology with respect to whole semen, whether such techniques improve also sDF levels are less clear. Indeed, studies reporting average sDF levels before and after DGC demonstrated a decrease (Gandini et al. 2004; Morrell et al. 2004), an increase (Zini et al. 2000; Aitken et al. 2014), and no modifications of the parameter (Ebner et al. 2011; Oguz et al. 2018; Muratori et al. 2016). Regarding swim up, most studies reported a decrease of average sDF levels after the procedure (Zini et al. 2000; Younglai et al. 2001; Stevanato et al. 2008; Jackson et al. 2010; Parmegiani et al. 2010; Muratori et al. 2019), although the average levels of decrease were different. Heterogeneity of these studies may depend on several aspects, including patient selection, method used to evaluate sDF, the considered sperm population (live or total), and data analysis. Regarding the important issue of the different methods used to evaluate sDF (reviewed in Agarwal et al. 2016), it should be noted that they are very heterogeneous, evaluate different types of damage, and give different values and cutoff levels to define fertile and infertile men (Muratori and Baldi 2017). Among them, TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) and SCSA (Sperm Chromatin Structure Assay) are the most used and their results are highly correlated (Agarwal et al. 2016). Despite such correlation, the study by Aitken et al. (2014), reporting an increased fragmentation of DNA in spermatozoa after DGC, demonstrated that SCSA, at a difference with TUNEL, cannot detect this damage. Another important issue regards data analysis for this type of study. As mentioned, most papers report average sDF levels before and after selection (with DGC or other methods) possibly masking effects present in single samples. In a recent study, our group reported that DGC provokes an increase in sDF in about 50% of infertile couples treated by IVF/ICSI despite no variation in average levels after selection (Muratori et al. 2016). Most importantly, we found that subjects increasing sDF levels after DGC experienced a 50% lower pregnancy rate after ART (Muratori et al. 2016). Recently, we showed that in some subjects, sDF may increase even after swim up (Muratori et al. 2019) despite the observed decrease in average levels. Finally, an important issue concerns the sperm population where sDF is evaluated. Indeed, in performing such studies, we should consider that selection procedures eliminate poorly motile and/or dead spermatozoa. Thus, the comparison of sDF in samples before and after DGC is performed in two different populations, the first formed by dead and viable sperm (whole semen) and the second by virtually only viable spermatozoa (selected samples). We should consider that the elimination of dead, DNA fragmented spermatozoa during selection could partially mask the eventual increase of DNA damage in the viable cells. Our group demonstrated that by evaluating sDF by TUNEL only in live spermatozoa more subjects undergoing an increase of damage after selection with DGC and swim up can be unmasked with respect to traditional method (Muratori et al. 2019). Although in subjects undergoing an increase of sDF after swim up, the post-selection levels were much lower with respect to those observed after DGC (Muratori et al. 2019), our study raised concerns regarding both procedures to select sperm for ARTs. It should be mentioned that a recent study (Zhao et al. 2016) reported that selection by swim up and

DGC leads to spermatozoa with longer telomeres compared to unprocessed samples and that DGC was more efficient in selecting spermatozoa with longer telomeres compared to swim up. Longer sperm telomeres are associated with better reproductive outcomes (Liu et al. 2002).

Which is the mechanism leading to sDF increase during DGC and swim up? Aitken et al. (2014) demonstrated that metal contaminants present in colloidal particles may induce a localized oxidative attack in turn promoting DNA breaks in samples selected by DGC. Although swim up does not use colloidal particles, heavy metal contamination of media cannot be excluded as a source of damage after this procedure. However, the fact that the increase of sDF is not observed in all the treated samples (Aitken et al. 2014; Muratori et al. 2016, 2019) indicates that also intrinsic characteristics of spermatozoa of the subject are important.

In Vitro Sperm Incubation During IVF/ICSI Procedures

During laboratory procedures for ARTs, semen samples may remain long in the incubator waiting for the insemination procedure because insemination and ovulation are often not synchronized (Suarez and Pacey 2006). In addition, selecting sperm with advanced technologies, such as by binding to hyaluronic acid (the so-called PICSI—preselected intracytoplasmic sperm injection), or during selection using high magnification microscopy (where spermatozoa are selected on the basis of sperm morphology after high magnification) (Berkovitz et al. 2005) or birefrangence microscopy (selecting spermatozoa on the basis of birefrangence emission) (Gianaroli et al. 2008) may require time in order to find the right spermatozoon to be used in ICSI. During these procedures, spermatozoa may be exposed to light-ionized radiation, environmental ROS, pH and temperature modifications, and other possible insults potentially inducing damages in their DNA or compromising their functions. Cicaré et al. (2015) demonstrated that oxidative metabolites and oxidative signs at the membrane increase significantly in selected spermatozoa incubated for 22 h at 37 °C in 95% air and 5% CO_2 for capacitation, but an increase, although not significant, was observed for both parameters also after 3 h of incubation. Other studies, evaluating sDF with different types of assays, demonstrated an increase of this parameter both in selected spermatozoa incubated in IVF media (Muratori et al. 2003; Gosálvez et al. 2009a; Nabi et al. 2014) and whole semen (Toro et al. 2009) after short time (1–4 h) incubation. Interestingly, the increase of sDF during such incubations does not appear to reflect any decrease in sperm viability as the two parameters were not correlated in spermatozoa from donor subjects (Gosálvez et al. 2009b). Indeed, Gosálvez et al. (2009b) demonstrated that the dynamic of loss of viability is different with respect to that of the increase of DNA fragmentation index suggesting the possibility that different insults can produce damage to the membrane and the DNA during in vitro incubations. In addition, the study demonstrated that not all the samples behave in the same way during such incubations, identifying two groups of subjects with different rates and extent of development of the damage

(Gosálvez et al. 2009b), again indicating that intrinsic individual characteristics render the sample more or less susceptible to the damage, as it happens during sperm selection procedures for ARTs (see above). Finally, a recent study reported that spermatozoa bearing X chromosome can survive longer during incubation for long time or following modifications of pH and temperature, compared to those bearing Y chromosome because X spermatozoa are more resistant to apoptosis (You et al. 2017). Whether the prolonged survival of X spermatozoa might lead to shifts in the ratio of male-to-female births in ARTs is presently unclear. At present, conflicting data exist regarding male-to-female sex ratio in ART (Al-Jaroudi et al. 2018).

Overall, damage occurring during short- or long-time incubation and the selection of spermatozoa may represent one of the several factors involved in reducing the efficiency of ART procedures (Sakkas et al. 2015).

In Vitro Manipulation During Sperm Cryopreservation

According to the recent Clinical Practice guideline of ASCO (American Society of Clinical Oncology, Oktay et al. 2018), sperm banking should be offered to all postpubertal males receiving cancer treatment. Semen should be collected before the initiation of therapies because the quality and the integrity of DNA of the sample can be compromised even after a single treatment. Sperm cryopreservation should be offered also to other conditions, such as oligozoospermia (as there is evidence that semen quality may deteriorate with time), testicular traumas (which may provoke alterations in semen quality), immunological disorders (as therapies offered to these patients may alter testicular function), spinal cord injuries, and ejaculatory problems. Although sperm banking has been proven an efficient technique to preserve fertility, as cryopreserved spermatozoa can be used in ARTs with success, it is well established in the literature that spermatozoa can be damaged during the cryopreservation process. Indeed, several studies reported decreased motility, viability, and even normal morphology following sperm cryopreservation (reviewed in Hezavehei et al. 2018). To investigate whether the damage may be related to initial semen quality, our group (Degl'Innocenti et al. 2013) evaluated motility and viability before and after cryopreservation in 822 semen samples, collected from men who cryopreserved for different pathologies (cancer, oligozoospermia, ejaculatory disorders, autoimmune pathologies, and spinal cord injury) and that decide to dismiss sperm banking. The study demonstrated that motility and viability decreased in all samples irrespectively of the condition for which cryopreservation was performed, and, most importantly, there was a strict correlation with basal semen quality. In particular, if even a single parameter of semen analysis at the moment of cryopreservation was below the fifth percentile of WHO (2010) reference value, the possibility of recovering motile spermatozoa in post-thawed sample was very low, as in the case of oligo- or astheno-zoospermic men. Therefore, semen cryopreservation does not always guarantee recovery of motile/viable sperm for ARTs and, in these cases, the clinician should perform appropriate counseling to the patient about

the future possibility of fertility with cryopreserved semen (Degl'Innocenti et al. 2013).

Damage to spermatozoa due to cryopreservation may occur also at DNA (genetic, epigenetic, apoptotic, or oxidative damage), RNA (in particular mRNA degradation), and protein (degradation) level (Hezavehei et al. 2018; Kopeika et al. 2015). Damage at the level of nucleic acids or proteins may not be necessarily associated to loss of viability and motility, and, if a spermatozoon with such damages is chosen for fertilization it may produce a damaged embryo that will not implant. Regarding induction of DNA breaks with sperm after cryopreservation, there is a discrepancy in the literature which may be explained either by the difference in individual cryoresistence, methods of cryopreservation as well as the type of assay used to evaluate sDF (Kopeika et al. 2015). By using TUNEL assay, our group demonstrated that post-thawing sDF levels increase in the majority of subjects (Meamar et al. 2012; Tamburrino et al. 2017; Marchiani et al. 2014) although the percentage increase was variable among them, suggesting different vulnerability to cryodamage. Post-cryopreservation sDF levels were higher in patients cryopreserving for cancer with respect to those cryopreserving for other pathologies (Tamburrino et al. 2017), despite similar if not even better semen parameters at cryopreservation. Although in our study (Tamburrino et al. 2017) sDF levels at the moment of cryopreservation were not measured for ethical reasons, our study might suggest that in cancer patients damage due to cryopreservation may add to an already compromised sperm DNA (Ståhl et al. 2009; O'Flaherty et al. 2010; Beaud et al. 2019). Although further studies are required in order to define the entity of the damage, information about the possibility that cryopreservation may produce damage to DNA should be given during counseling of cancer (and non-cancer) patients wishing to preserve their semen for future fertility.

The good news is that damage due to cryopreservation does not depend on the extent of time spermatozoa remain in liquid nitrogen, as there are, apparently, no differences in ARTs outcomes following long-term storage (Huang et al. 2019). The critical steps are freezing and thawing, when spermatozoa are subjected to stressful conditions likely due to cold stress, ice crystal formation, osmotic changes, and generation of ROS during the procedure.

An important point concerns what to do in order to limit damage due to cryopreservation. In a recent review (Hezavehei et al. 2018), several studies performed in order to limit the damage have been considered. Most of these studies evaluated the effects of antioxidant agents (enzymatic and nonenzymatic), added to cryopreservation media, on sperm functions after thawing. Although some of these studies reported improvement of sperm parameters (such as viability, motility, acrosomal status), evidence is not conclusive (Hezavehei et al. 2018). There is also evidence that the extent of the damage may change depending on the freezing medium and the freezing method (slow, rapid, and ultrarapid). Some years ago, vitrification, an alternative method without the use of permeable cryoprotectants, has been introduced for sperm storage (Isachenko et al. 2011; Spis et al. 2019). This method foresees to plunge directly sperm suspension in liquid nitrogen to freeze them in an ultrarapid way. Using this method, Aizpurua et al. (2017) reported a reduced

damage to DNA (sDF) as well as higher acrosomal stability. Studies comparing vitrification versus conventional methods have been recently meta-analyzed (Li et al. 2019), reporting better motility recovery with vitrification methods. However, due to the high heterogeneity of the studies, results cannot be considered conclusive and further studies are required.

Another important issue concerns cryopreservation of testicular tissue or spermatogonial cells. Such procedures could be offered to prepubertal children who necessitate cancer treatments. At present, many laboratories offer this possibility (Valli-Pulaski et al. 2019) although it is necessary to remark to the patients and their families that the procedure is currently strictly experimental. Indeed, at present, reimplantation of testicular tissue or spermatogonial cells after puberty has been performed only in animal models (Neuhaus and Schlatt 2019). However, the fact that grafting immature monkey testis allowed the generation of sperm and then of offspring (Fayomi et al. 2019), reinforced the concept that, in the future, such procedures may be available also for humans. However, it must be evaluated whether epigenetic and genetic modifications may occur during in vitro processing and cryopreservation of the tissue and growth after grafting.

Conclusions

In conclusion, in vitro manipulation may produce damage to spermatozoa which may reflect in the outcomes of ARTs. Damage may occur during selection procedures, cryopreservation, or even simple incubation of spermatozoa. It may occur at several sperm levels, determining alterations of motility, morphology, mitochondrial function, ability to undergo acrosome reaction, or inducing damage to the DNA. Such damages may reduce the ability to fertilize the oocyte or may produce low-quality embryos with reduced probability of development and implantation. Among the possible inducers of damage during in vitro manipulation, reactive oxygen species, heavy metal contaminants of solution and cryoprotectants, light, radiations, and centrifugation steps have been shown to be involved. Clearly, further studies are needed in order to identify all the possible inducers of sperm damage in vitro, its consequences for embryo development and implantation, as well as to develop strategies to prevent it.

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Effect of Melatonin on Capacitation and Ca2+ Distribution in Red Deer Spermatozoa

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Background: Sperm capacitation is a complex process involving several changes in the spermatozoa, allowing to acquire the capacity to fertilize the oocyte: reorganization of the plasmalemma, protein tyrosine phosphorylation, and motility hyperactivation. Cytosolic Ca2+ concentration plays an important role in these events. In addition, melatonin is important for the functionality of the reproductive tract of mammals. In males, melatonin has a direct influence on testosterone levels and sperm quality (Li and Zhou, 2015, Clin Chim Acta 446:175–80), and it could regulate sperm capacitation, maybe by modulating ROS levels or by binding to its spermatic membrane receptors MT1 and MT2 (González-Arto et al., 2016, Theriogenology 86:1958–68).

Main Questions: The main objective was to elucidate the role of melatonin during sperm capacitation of the highly seasonal ruminant red deer (*Cervus elaphus hispanicus*), especially regarding intracellular calcium dynamics.

Experimental Design: The spermatozoa were recovered from the cauda epididymis of four red deers, extended in TALP-Hepes and incubated for 4 h at 39 °C and 5% CO_2 with melatonin (1μM, 10 nM, 100 pM, 1 pM), or no melatonin (control). The samples were analyzed at 0 and 4 h. We studied their capacitation status by the Ca2+ sensor chlortetracycline (CTC), identifying F pattern (all head, not capacitated), B pattern (only acrosome, capacitated), and AR pattern (no fluorescence or

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equatorial region, reacted sperm). Flow cytometry using propidium iodide, Fluo-4, and Merocyanine 540 yielded intracellular Ca2+ concentration ([Ca2+]i) and membrane disorder.

Main Results: Incubation promoted capacitation, significantly decreasing the F pattern (p = 0.035) except for 1 pM melatonin, which decreased pattern B with respect to the control (p = 0.010). Flow cytometry revealed an increase of [Ca2+]i after incubation, especially at 1 pM melatonin (p = 0.020). All groups presented higher membrane disorder (p = 0.017), more evident in 10 nM melatonin (p = 0.004).

Conclusions: This study reveals a modulatory effect of melatonin in sperm capacitation in this species. Picomolar melatonin seemed to modulate [Ca2+]i and its distribution, apparently decreasing capacitation, whereas nanomolar concentration could accelerate the process regarding membrane dynamics. This information might be useful for improving sperm conservation and in vitro protocols in this species.

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Unraveling the Signal Transduction Pathways of Novel Mitochondrial Peroxiporins in Activated Piscine Spermatozoa

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Recent studies of how ejaculated marine teleost spermatozoa survive the hyperosmotic shock of seawater (SW) have uncovered a key role of an aquaporin-8 type water channel, termed Aqp8bb, in an activated sperm. Aqp8bb alleviates oxidative stress by acting as a mitochondrial peroxiporin to facilitate the efflux of reactive oxygen species (ROS), thereby allowing ATP production and the maintenance of flagellar motility. It is not known, however, whether the ejaculated spermatozoa of freshwater (FW) teleosts, which experience an opposing hyposmotic shock during activation, display similar detoxification mechanisms, and whether conserved or divergent signaling pathways controlling Aqp8bb mitochondrial targeting, exist in the different lineages. To uncover the intracellular signaling cascade in marine teleosts, we initially used a repertoire of activators and inhibitors of different transduction pathways on SW-activated seabream sperm. These experiments suggested that c-Jun N-terminal kinase (JNK), protein kinase C (PKC), and glycogen synthase kinase-3 (GSK3) are involved in Aqp8bb phosphorylation and transport to the mitochondrion. Accordingly, inhibition of JNK and GSK3 caused ROS accumulation in the mitochondrion and a decline of sperm motility, and thus, established the importance of these kinases in the regulatory transduction cascade. To determine the molecular initiators of this signaling cascade, we incubated nonactivated sperm with a Ca2+ ionophore in the presence of external Ca2+. This induced the phosphorylation of GSK3 and of Aqp8bb, and the accumulation of the channel in the

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mitochondrion, all of which could be prevented with an inhibitor of PKC. In turn, exposure of immotile spermatozoa to ROS, or SW-activation of sperm in the presence of a Ca2+ chelator, promoted the induction of the JNK-GSK3 signaling pathway, with the resultant triggering of Aqp8bb phosphorylation and mitochondrial membrane trafficking. To identify the specific target residues that are phosphorylated in Aqp8bb, we heterologously expressed the seabream channel in human liver hepatocellular (HepG2) cells. These experiments coupled with site-directed mutagenesis of the identified residues confirmed that both intracellular Ca2+ and oxidative stress regulate Aqp8bb mitochondrial trafficking through the respective GSK3- and JNK-mediated phosphorylation of Ser16 and Thr24 in the Aqp8bb N-terminus. Intriguingly, investigations of FW-activated salmon spermatozoa revealed that Aqp8bb is also trafficked to the mitochondria, but is only regulated by the PKC-GSK3 pathway. In conclusion, these results reveal for the first time that Aqp8bb channels function as mitochondrial peroxiporins in the activated spermatozoa of both FW and SW teleosts, but that Ca2+-activated PKC is the primary signaling pathway in FW teleost spermatozoa, while both Ca2+-activated PKC and ROS-activated JNK are the primary signaling pathways that regulate the Aqp8bb-mediated detoxification mechanism in the spermatozoa of SW teleosts.

Prolonged Chilled Preservation and Preliminary Investigations of Energy Production of Koala (*Phascolarctos cinereus*) Spermatozoa

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Background: The current inability to successfully cryopreserve koala spermatozoa limits the capacity to maintain the genetic diversity of current and future koala populations by means of assisted breeding technologies (ABT). Consequently, this research focused on the prolonged chilled (5 °C) preservation of electro-ejaculated koala semen in order to facilitate artificial insemination of captive and wild populations.

Main Questions: This study was designed to confirm prolonged chilled preservation of koala semen samples over a 40-day period and to conduct a preliminary analysis as to the possible sources of energy production utilised by the sperm cell that might account for its remarkable longevity.

Experimental Design: Spermatozoa from 14 koalas were maintained for 40 days at 5 °C in Tris-citrate glucose (TCG) and assessed in terms of motility, plasma membrane integrity, mitochondrial membrane potential (MMP) and sperm DNA fragmentation. In a series of preliminary experiments, the metabolism of koala spermatozoa chilled for 24 h ($N = 2$) and 35 days ($N = 2$) was assessed to determine the possible mechanisms of survival and potential sources of energy production; assessment included the effect of mitochondrial reactive oxygen species (ROS) (MitoSox Red and Sytox Green assay), mitochondrial uncoupling (through the addition of carbonyl cyanide m-chlorophenyl hydrazine (CCCP)), cytosolic ROS

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production (CROS) and an evaluation of anaerobic glycolysis by addition of sodium oxamate to inhibit lactate dehydrogenase.

Main Results: Koala spermatozoa stored in TCG and left undisturbed for 40 days maintained a mean (±SEM) progressive motility of 40.5 ± 7.3%, rate of motility of 1.9 ± 0.4, percentage live of 35.8 ± 7.1%, high MMP of 24.7 ± 2.2% and only 3.0 ± 0.9% SDF. When spermatozoa from different koalas stored for 24 h and 35 days were compared, there was no statistical difference with respect to the percentage of spermatozoa staining MSR positive. CCCP at a concentration known to inhibit the motility of aerobically respiring mammalian spermatozoa had no effect on the percentage progressive motility, percentage live or percentage of MSR positive koala sperm stored for 35 days; however, the rate of motility significantly declined. While the addition of sodium oxamate had no effect on progressive motility or the percentage of live spermatozoa stored at 5 °C for 24 h, there was a significant reduction in the rate of motility.

Conclusions: Koala spermatozoa diluted in TCG and stored at 5 °C in an Eppendorf tube can maintain survival for up to 40 days, which coincides with the female's oestrous cycle. Koala spermatozoa also appear to maintain a similar level of motility when treated with reagents that typically uncouple aerobic metabolic mitochondrial activity or prevent energy production from anaerobic respiration, suggesting an alternate method of energy production.

Sperm Motion and Metabolism in Physiological Conditions

V. Magdanz, B. Eckel, and K. Reinhardt

Background: Sperm cells are assessed in an in vitro setting under conditions that are quite different from their natural environment: low viscosity media, high oxygen content, often in a two-dimensional setting. The natural environment often includes highly viscous fluids in branched microchannels, which changes the sperm motion significantly. Physical factors such as surface and fluid properties might have a significant impact on successful sperm migration and therefore in sperm selection procedures.

Main Questions: How do sperm move in body fluids? How can we emulate in vivo conditions in the laboratory?

Experimental Design: Bovine sperm cell motion is investigated in highly viscous media and isolated bovine oviduct fluid. Metabolic measurements are conducted with a Seahorse device for measuring oxygen consumption and extracellular acidification rates.

Main Results: Sperm cells swim with straighter paths and lower average lateral head displacement in high viscosity media and oviduct fluid. The metabolic rates of bovine sperm cells that have migrated in a swim-up procedure to the upper fraction are higher, while the ATP content of such sperm is lower. Swim-up sperm of the upper fraction tend to have longer flagella than sperm in the lower fraction.

Conclusions: It is worth investigating sperm behaviour in emulated body fluids to gain a better understanding of in vivo sperm migration.

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Gametes Collision in Freshwater Fish: Evidences of Guidance and Selection

S. Boryshpolets, V. Kholodnyy, H. Gadelha, and J. Cosson

Externally fertilizing species, especially freshwater fish, reproduce in an environment that is very harsh for gametes. They undergo the pressure of various external factors (temperature, water flow, pH, ion composition, viscosity, presence of ovarian fluid, etc.). Variability in cell responses to these parameters creates a wide spectrum of reproduction strategies. Existence of specific mechanisms for guiding and triggering the encounter of gametes would be highly expedient in these conditions, nevertheless, only scarce information exists on this issue. The existing data and observational studies performed by us support the idea that the factors, which are the part of ovarian fluid or released by the eggs, could significantly affect the behavior of male gametes and influence the outcome of fertilization. This could be made by the support of sperm motility traits on a certain level, attraction or repulsion of gametes with some predefined qualitative characteristics, and targeted promotion of sperm with proper genetic material to encounter the egg. The specific mechanisms supporting the potential selection by externally fertilizing females are unclear. In addition to chemical agents, there is evidence that some physical factors, e.g., presence of surfaces and their features, contribute to fertilization performance. All these phenomena, i.e., motility activation and progress, kinetic and tactic effects, possible selection, and promotion of gametes could be the elements in the guidance pattern. This is even more challenging for freshwater fish reproduction, considering the limited period of motility compared to marine species, and thus, it makes the need for specific support of gametes encounter even more apparent. This work suggests that guidance during fertilization is a rule, not a fortune, at least, because guidance is highly expedient during external fertilization in terms of environmental variability and stability of living matter.

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Lectin-Binding Pattern Changes on the Bovine Sperm After Differently Induced Process of Capacitation

P. Sečová, J. Jankovičová, K. Michalková, L. Horovská, M. Simon, and J. Antalíková

Background: Sperm capacitation is a complex process that undergoes mature sperm to acquire the fertilization ability. For in vitro fertilization, cryopreserved sperm are rather used instead of freshly ejaculated sperm. It was proved that cryopreservation procedures induced capacitation-like changes in bull spermatozoa that are associated with a different profile of phosphotyrosine-containing proteins compared to freshly ejaculated sperm after capacitation. The plasma membrane destabilization and reorganization caused by cryopreservation are sufficient to trigger the signalling cascade associated with physiological capacitation.

Main Questions: To monitor the possible differences in glycoprotein distribution on sperm plasma membrane provoked by cryopreservation using the various lectins.

Experimental Design: For our study frozen-thawed and freshly ejaculated bovine spermatozoa capacitated in a commercially supplied medium were used. Changes in membrane distribution of glycoproteins were analysed by the fluorescently labelled lectins: peanut agglutinin (PNA), wheat germ agglutinin (WGA), *Pisum sativum* agglutinin (PSA), *Lens culinaris* agglutinin (LCA) in sperm suspension.

Main Results: After thawing of cryopreserved sperm and capacitation of freshly ejaculated sperm, similar changes in PSA and LCA binding patterns were observed. In the case of PNA binding, we observed a moderate increase in the number of reactive sperm after thawing caused probably by cryoinjury. Different reaction pattern was recorded in freshly ejaculated compared to frozen-thawed sperm after WGA treatment. The changing reaction pattern of freshly ejaculated sperm after capacitation was accompanied by loss of fluorescence contrary to frozen-thawed sperm with changed pattern but preserved WGA signal.

Conclusions: WGA lectin seems to be a suitable for study of different glycoprotein distribution changes caused by cryopreservation procedure.

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Part VIII
Heterogeneity of Sperm Morphology and Laboratory Techniques to Overcome Assessment Challenges

There appears to be a difference in sperm morphology between species with competition on sperm level and those with competition on individual level. In species, like human, with pleiomorphic spermatozoa there is a special challenge describing and characterizing morphology aspects that are relevant to the function of the male reproductive organs and to male reproductive success. Categorization schemes must be based on relevant investigations of individuals of different reproductive success and be used consistently due to standardized training and techniques. Formal standardization could be a meaningful way of turning WHO recommendations into a proper universal standard.

Have We Conquered Sperm Morphology Analysis in Different Mammalian Species as Analysed by CASMA?

Gerhard van der Horst, Stefan S. du Plessis, and Liana Maree

Introductory Notes and Aims

Sperm morphology assessment has always been regarded as an important parameter in defining semen quality in both humans and animals (Auger et al. 2016; Maree et al. 2010; Valverde et al. 2016). Moreover, the percentage of morphologically normal spermatozoa and in particular sperm morphometry has been used with great success in a multitude of applications predominantly in animal species (invertebrates and vertebrates) (Legendre et al. 2019; Yániz et al. 2015). Sperm morphology and morphometry are species specific and are widely applied in taxonomy studies (van der Horst et al. 1995), sperm competition studies (van der Horst and Maree 2014) and toxicological research (Legendre et al. 2019). In view of the importance of sperm morphology/morphometry, the eminent challenge is to standardize the practice and methodology of morphology evaluation and simultaneously make the evaluation process more objective.

Historically, sperm morphology analysis has been used for fertility assessment. Cary (1916) was the first to define abnormal spermatozoa in humans by describing, for example, microcephalic spermatozoa and introduced the notion of sperm morphology in assessing human sperm quality. A sperm stain that differentially stains each sperm component was subsequently developed for semen and sperm morphology appraisal (Cary and Hotchkiss 1934). During the next five decades, the manual analysis of specifically human sperm morphology became better defined and characterized (Menkveld 2010). Since then, and in spite of it being recognized and

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widely used as a fertility marker, many studies have shown that manually performed human sperm morphology analysis is subjective and prone to numerous errors (Cooper et al. 2010; van der Horst and du Plessis 2017). Hancock (1952), Chenoweth and Lorton (2014), Nöthling and Irons (2008), Brito (2007) and many others have highlighted similar problems associated with manual analysis of animal spermatozoa and strongly advocated various measures that need to be taken in order to overcome inconsistencies.

Despite numerous publications describing manual sperm morphology analysis employing different types of stains, and using different classification systems in many animal species, it remains subjective and prone to many errors. Accordingly, there is a great need to develop standardized methods to analyse sperm morphology. Computer-aided sperm morphology analysis (CASMA, CASA-morphology) is one method that affords distinct advantages compared to manual assessment. CASMA eliminates human bias and therefore provides much greater objectivity, repeatability and a photo record for verification (Legendre et al. 2019; Maree et al. 2010; Valverde et al. 2016; van der Horst et al. 2018a, 2018b).

Visual or optical illusions show us that our minds tend to make assumptions about the world around us and what you think you see is often not reality. The Ebbinghaus illusion, for example, reveals that our brain makes judgements about size based on adjacent objects. This effect is clearly demonstrated in Fig. 1a. The orange circles are perceived to be of different sizes (top smaller than bottom) despite being the same size. The Ponzo illusion (Fig. 1b) illustrates that context is also fundamental for depth perception. It shows that identically sized lines can appear to be of different lengths when placed between converging parallel lines.

It appears that our visual system remains too limited to tackle all the information our eyes take in. Subsequently, our brains take shortcuts and select the most likely interpretation of what we see. While we know that different areas of the brain deal with colour, form, motion and texture, how the brain encodes and combines this information into a coherent picture remains poorly understood and can impact the manual analysis and interpretation of sperm morphology.

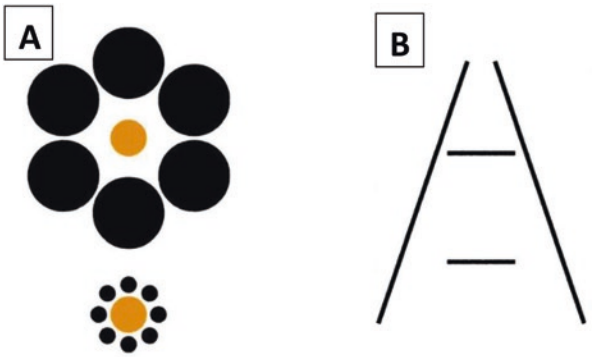

Fig. 1 (**a**) Ebbinghaus illusion and (**b**) Ponzo illusion (http://www.bbc.com/future/bespoke/story/20150130-how-your-eyes-trick-your-mind/)

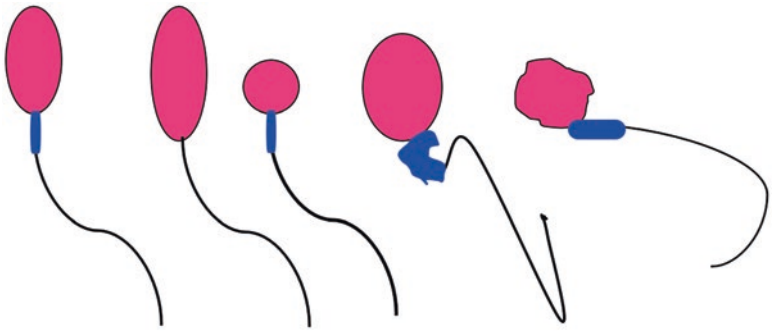

Fig. 2 Sperm cartoons depicting various easily identifiable sperm head (pink), midpiece (blue) and tail (black) deformities. Acrosomes are ignored for the sake of simplicity

Classical head morphological abnormalities (e.g. elliptical, micro, macro and amorphous) as well as various midpiece and tail defects are easily identifiable, even with the untrained eye, as depicted in Fig. 2. However, the problem is not distinguishing between these abnormalities but rather recognizing and classifying all potential intermediate forms. One of the most contentious of these is the so-called amorphous sperm. Vast differences in the interpretation of these intermediate forms during human sperm analysis for example relate to earlier versions of the World Health Organization (WHO) manual compared to the WHO 5 manual. The Tygerberg Strict Criteria for morphology classification of human sperm has been adopted by the WHO 5 and the basis of this set of criteria is that if there is any uncertainty of a spermatozoon being normal it should be classified as abnormal (Menkveld 2010). It is therefore clear that only the very experienced and trained eye may consistently classify correctly, and even then there is always an element of subjectivity. These concerns are well noted and shared in the realm of human sperm analysis despite a concerted effort to standardize manual sperm morphology assessment. In contrast, CASMA has contributed to a substantial improvement in the assessment of human sperm morphology in a relatively short period of time (Dearing et al. 2019; Maree et al. 2010; van der Horst et al. 2018a).

In animals we are faced with an even greater drawback. Not only are there large morphological differences between species and even amongst different breeds of the same species, but for a variety of reasons many different staining protocols (Yániz et al. 2015) and classification methods have been used to define normal morphology, even within the same species and breeds. Furthermore, a higher incidence of sperm abnormalities occurs in animals when there is a lack of sperm competition (Peirce et al. 2018; van der Horst and Maree 2014) compared to those where sperm competition is very high (van der Horst et al. 2018b).

The main aim of this paper is to address the aforementioned concerns and provide workable solutions to overcome these through current CASA morphology applications. As part of this objective, we will consequently demonstrate and discuss some of the latest technologies available in preparation, staining and

performing advanced but traditional CASA analysis of sperm morphology/morphometry for a wide range of mammalian species and indicate how this allows objective assessment of sperm morphology.

Materials and Methods

For the purpose of this study, only spermatozoa from species in which CASA morphology analysis was possible and successful will be discussed. Table 1 lists the 19 different species included in the study, representing laboratory animals, domestic species and wildlife species, as well as the methods employed for obtaining semen or epididymal sperm samples. In all instances, ethical clearance was obtained from the Ethics Committee of the University of the Western Cape. CASA morphology analysis of all these species will not be shown but rather representative examples under the different groupings as indicated in Table 1.

Table 1 Species included and methods used for obtaining semen/sperm in this study

Common name	Species name and breed	Method of collection
Laboratory animals		
Rat	*Rattus norvegicus albinus* (Wistar)	Cauda epididymis
Mouse	*Mus musculus*	Cauda epididymis
Rabbit	*Oryctolagus cuniculus*	Artificial vagina
Domestic animals		
Ram	*Ovis ovis* (Merino sheep)	Artificial vagina
Bull	*Bovis bovis* (Angus)	Electro-stimulation
Boar	*Sus scrofa* (Kolbroek)	Artificial vagina
Goat	*Capra spp.* (Tankwa)	Electro-stimulation
Horse	*Equus caballus* (Arabian)	Artificial vagina
Dog	*Canis familiaris* (Beagle)	Artificial vagina
Wildlife		
African elephant	*Loxodonta africana*	Prostatic stimulation and electro-stimulation
White rhinoceros	*Ceratotherium simum simum*	Electro-stimulation
Antelope	Oryx leucoryx (Arabian oryx)	Electro-stimulation
Lion	*Panthera leo*	Electro-stimulation
Cheetah	*Acinonyx jabatus*	Electro-stimulation
Wild dog	*Lycaon pictus*	Electro-stimulation
Bengal tiger	*Panthera tigris*	Electro-stimulation
Bottlenose dolphin (bottlenose)	*Tursiops truncatus*	Training collection method
Hyrax	*Procavia capensis*	Cauda epididymis
Manatee	*Trichechus manatus latirostris*	Training collection method

Sperm smears were prepared and stained with SpermBlue (SB—Microptic SL, Barcelona, Spain) or New SpermBlue (NSB) according to the procedures as described either by van der Horst and Maree (2009) or the updated protocol which can be found on www.micropticsl.com/products/disposables/morphology/spermblue/. In addition, a new version of SpermBlue is available (Microptic SL, Barcelona Spain) and has also been used in this investigation and quite extensively referred to by other users (Yániz et al. 2015). The fixative stain is combined in one solution, the NSB. The protocol for NSB basically entails making and air-drying of a sperm smear, staining with NSB for 45 s, brief immersion in distilled water and drying of the slide at a 60° angle. The entire fixing/staining process only takes about 1 min. The examples in this text reflect both the original SB and the NSB staining techniques.

The SCA 5.4, 6.3 and 6.4 software systems (Morphology module, Microptic SL, Barcelona, Spain) have been used to analyse sperm morphometry/morphology in the automated mode of captured microscope images. A Nikon E50i microscope (60 or 40× brightfield objective) fitted with either a Basler 312 fc camera, an SCA 1024 giga Ethernet camera or a Basler Ace ACA-1300-200uc camera (Microptic SL, Barcelona, Spain) were used to capture the microscopic images.

Assessing the percentage of spermatozoa with normal morphology for a species using CASA is based on the method described by van der Horst and du Plessis (2017). The process basically involves using the head, midpiece and tail measurements of several hundred spermatozoa per species and assessing them in terms of the distribution of each morphometric parameter for different percentile groupings. The Tankwa goat, bottlenose dolphin and laboratory rat will be used as representative examples to show normal and abnormal morphology determination based on this method. For all other species representative CASA morphology analyses will be depicted in figures, whilst morphometric measurements will be listed in tables. The SCA morphology module has the ability to measure and calculate eight different sperm head morphometric parameters as listed in Table 2. In addition, several midpiece and tail parameters such as insertion angle, midpiece width, surface area and tail length are also calculated. Combinations of these morphometric

Table 2 The various sperm head morphometric parameters/variables that are measured with the SCA morphology module

Variables	Formula
Length (μm)	L
Width (μm)	W
Perimeter (μm)	P
Area (μm^2)	A
Ellipticity	L/W
Elongation	$\frac{(L-W)}{(L+W)}$
Roughness	$4\pi \times (\frac{A}{P^2})$
Regularity	$\pi \times \frac{(L.W)}{(4.A)}$

characteristics have been used to calculate the percentage of morphological normal sperm for various species including Tankwa goat (Ngcauzele 2018), bottlenose dolphin (van der Horst et al. 2018b) and rat (van der Horst and du Plessis 2017). In addition, three sperm morphology indices are also calculated, namely the teratozoospermic index (TZI), multiple anomalies index (MAI) and deformity index (DI). These indices represent different combinations of more than one abnormality per spermatozoon or for the whole sperm population. Traditionally these indices have been used for human sperm but appear to have good applications for animal sperm (van der Horst and du Plessis 2017) including dog sperm (Morselli et al. 2019).

Results

By selecting SB and NSB as our preferred stain, it was possible to discriminate the individual sperm components from each other due to differential staining in different hues of blue. Such differential staining allows accurate thresholding of each sperm component by CASMA, irrespective of sperm morphology. Representative examples of CASA morphology analysis performed on spermatozoa from four species, namely dog, horse, bottlenose dolphin and bull (Angus), are shown in Fig. 3. With the current staining technique employed, the acrosomes of dog, horse and dolphin spermatozoa stain light blue (similar to human and other primates), while it stains dark blue in bull, ram, goat and boar. Despite this difference in colour, thresholding of the acrosome using the SCA CASA system appears to be highly accurate. Table 3 shows the morphometric parameters for acrosome, head, midpiece and tail for seven representative species of laboratory animals, domestic species and wildlife species. The smallest sperm head morphometric values among these examples are from dolphin and hyrax, whereas rabbit and bull show the largest morphometric values. The head length of bull spermatozoa is, for instance, twice that of dolphin spermatozoa. Interestingly, tail length in five of the species is remarkably similar (47.8–52.9 μm) and the exceptions seem to be bottlenose dolphin (67.7 μm) and bull (60.7 μm) having tail lengths exceeding 60 μm. What is even more evident is that sperm head shape varies largely among the species shown in Figs. 3 and 4. Despite these large differences in sperm form and size, it is possible to analyse the morphometry of virtually any mammalian spermatozoa by means of SCA CASMA, including spermatozoa with hooked sperm heads such as rats (Fig. 4c) and mice (not depicted).

By incorporating all these morphometric measurements into specific algorithms, CASMA has the ability to classify morphologically dissimilar spermatozoa from various species with great accuracy into morphologically normal and abnormal forms as shown in Fig. 4. Table 4 shows the cut-off values of the morphometric parameters for three morphologically distinct mammalian spermatozoa. In Tankwa goat spermatozoa the cut-off for sperm head length for normal morphology is 7.45 μm (minimum) to 10.87 μm (maximum). In rat spermatozoa with its much longer, slenderer and curved head shape, head length ranges from 19 to 23 μm. More detail specific to rat sperm morphology is shown in Fig. 5 including the

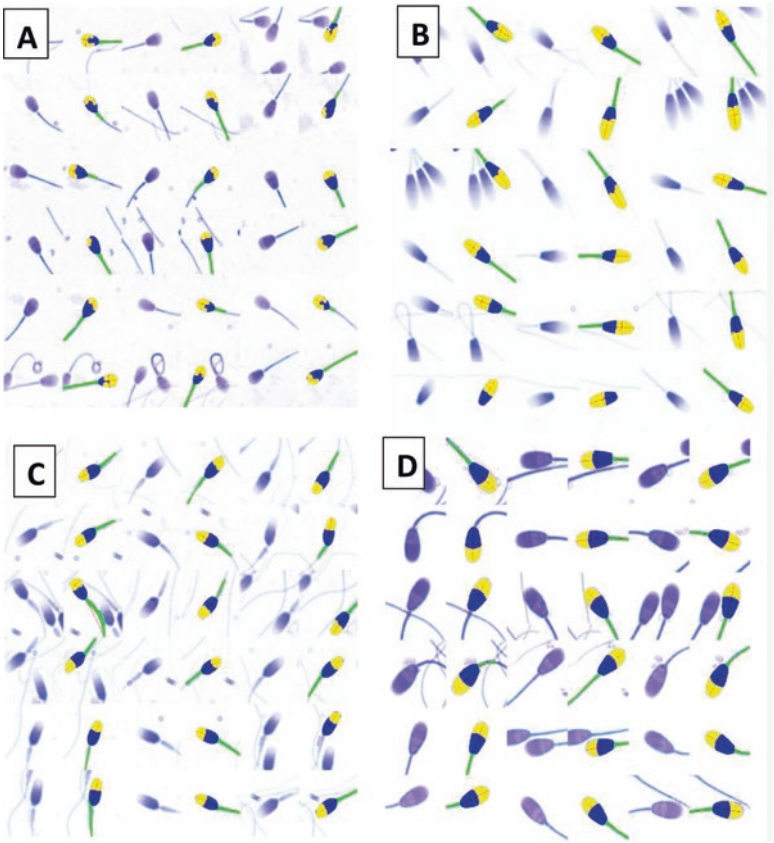

Fig. 3 Successful thresholding of the different sperm components in four representative species: (**a**) dog (beagle); (**b**) horse (Arabian); (**c**) dolphin; (**d**) bull. For each species, the sperm on right shows the analysis (acrosome yellow, head blue, midpiece green) of the sperm on immediate left

morphometric characteristics for individual normal (green block) and abnormal spermatozoa (red block). The abnormal spermatozoon (Fig. 5) shows a head length (Arc) and perimeter above the upper cut-off value for normal morphology and can be considered a macro head.

Discussion

The inconsistencies associated with manual evaluation of sperm morphology are apparent. Automated CASA morphology analysis may be the way forward for assessing normal sperm morphology objectively, but care should be taken of the many pitfalls which could result in incorrect analyses. One of the main issues is system related and based on the type of CASA/CASMA set-up to be used. In a

Table 3 Morphometric measurements of seven species across several mammalian taxa, including laboratory, domestic and wildlife species

Sperm	Parameter	Dog (Beagle)		Horse (Arabian)		Dolphin (Bottlenose)		Elephant (African)		Rabbit		Hyrax (Rock)		Bull (Angus)	
component		Av	±SD	Av	±SD	Av	±SD	Av	±SD	Av	±SD	Av	±SD	Av	±SD
Head	Length (µm)	6.07	0.25	6.39	0.48	4.93	0.41	6.7	0.5	9.17	0.29	5.63	0.21	9.58	0.47
	Width (µm)	3.81	0.14	2.60	0.22	2.61	0.21	3.2	0.2	4.89	0.22	3.84	0.15	4.83	0.23
	Area (µm^2)	26.12	3.6	20.39	2.84	10.81	1.38	19	2.56	41.8	3.21	17.89	2.65	45.05	4.04
	Perimeter (µm)	14.15	0.58	13.15	0.93	13.03	1.07	14.7	0.85	20.29	0.66	15.97	0.63	21.04	1.11
	Ellipticity	1.6	0.06	2.47	0.26	1.89	0.14	2.1	0.1	1.88	0.10	1.47	0.09	1.99	0.1
	Elongation	0.23	0.06	0.42	0.05	0.31	0.03	0.35	0.03	0.3	0.02	0.19	0.02	0.33	0.02
	Roughness	1.65	0.3	1.49	0.22	0.80	0.03	1.15	0.03	1.26	0.10	0.88	0.03	1.28	0.10
	Regularity	0.71	0.12	0.65	0.22	0.94	0.04	0.9	0.03	0.86	0.05	0.95	0.03	0.81	0.04
	Acrosome	57	2.77	54.3	3.26	42.5	5.03	38.5	0.75	57.01	1.94	56.2	1.95	57.4	1.93
Midpiece	Width (µm)	1.06	0.38	1.01	0.13	1.03	0.38	0.97	0.99	0.96	0.20	1.12	0.30	1.15	0.18
	Area (µm^2)	10.09	6.57	10.27	3.73	13.1	3.2	7.04	5.58	8.18	3.97	5.88	3.2	10.77	3.27
	Angle	1.85	1.48	1.74	1.69	7.02	8.95	1.76	1.76	2.15	1.28	15.47	1.3	0.23	0.23
Tail	Length (µm)	52.9	1.2	52.7	0.98	67.7	5.7	50.4	1.85	47.8	1.2	49.2	1.3	60.67	1.6

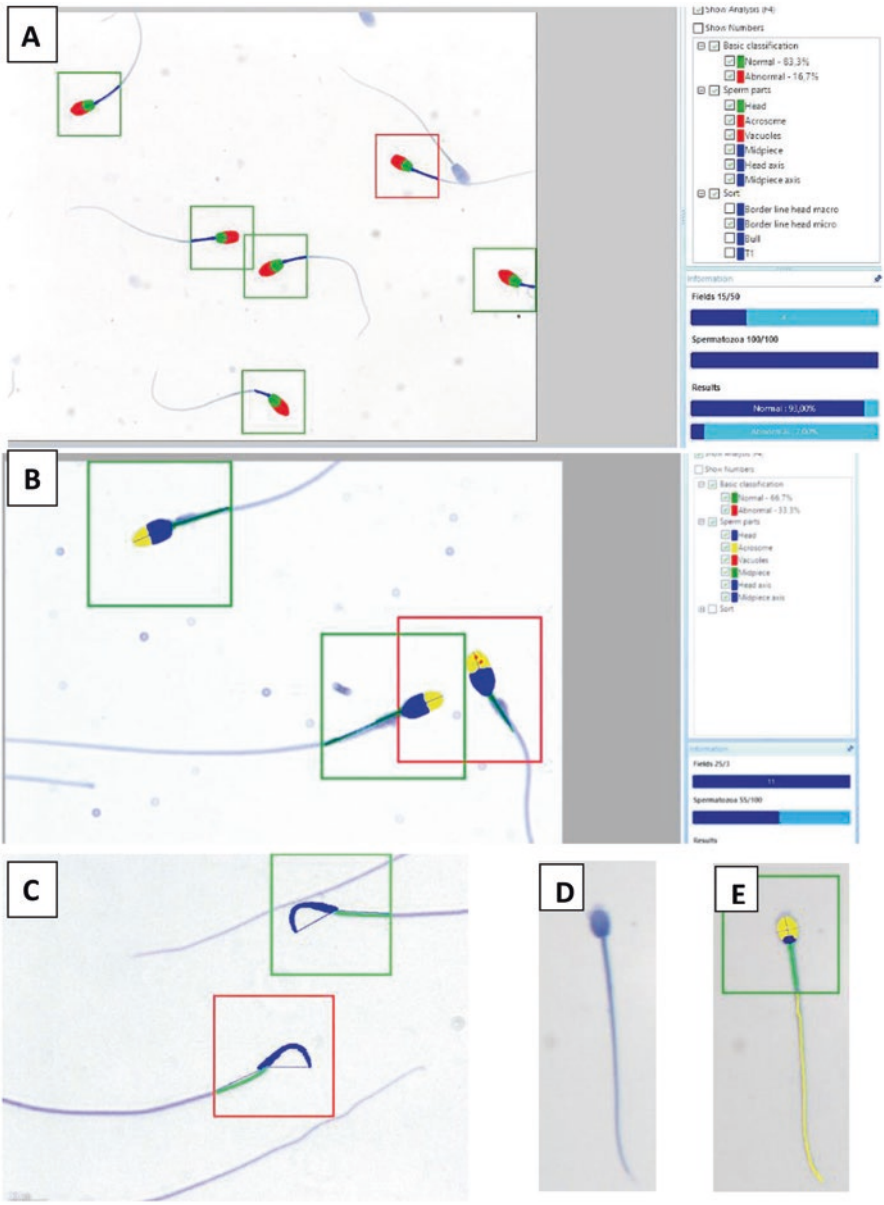

Fig. 4 Thresholding and identifying various components of sperm from four different species. Morphologically normal sperm are enclosed in green blocks, while morphologically abnormal sperm are enclosed in red blocks. (**a**) Tankwa goat sperm showing red acrosomes, green heads and blue midpieces; (**b**) Bottlenose dolphin sperm showing yellow acrosomes, blue heads and green midpieces. The red dots indicate nuclear craters/vacuoles; (**c**) Rat sperm showing blue head and green midpieces. Staining in this instance does not differentiate the acrosome; (**d**) Hyrax sperm (no mask); (**e**) Hyrax sperm (with mask) and green block showing yellow acrosome, blue head, green midpiece and yellow tail

Table 4 Various cut-off values calculated to determine the percentage of normal sperm for Tankwa goat, Wistar rat and Bottlenose dolphin

Sperm	Parameter	Tankwa goat		Wistar rat		Dolphin	
component		Min	Max	Min	Max	Min	Max
Head	Length (μm)	7.45	10.87	19	23	4.1	5.5
	Width (μm)	3.50	5.10	0.9	1.9	2.4	3.5
	Area (μm^2)	26.30	51.52	15	23	9.1	15.8
	Perimeter (μm)	16.67	23.59	40	50	11.5	15.5
	Ellipticity	1.78	2.53	1.1	2.2	1.78	2.53
	Elongation	0.28	0.43			0.12	0.50
	Roughness	0.79	1.75	0.08	0.3	0.7	1.32
	Regularity	0.61	0.97			0.8	1.15
	Acrosome	47	100			26	60
	Vacuoles (%)	0	20			0	
	Chord (μm)			8	14		
	H Angle (degrees)			50	87		
Midpiece	Width (μm)	0	1.99	0.4	1	1.0	1.6
	Area(μm^2)	0	27.50				
	MP Angle			0	60	0	60

Some parameters such as ellipticity and elongation are not sensitive parameters in rat, whereas chord is specific for rat. Blank spaces refer to not measured or determined or not applicable to specific species.

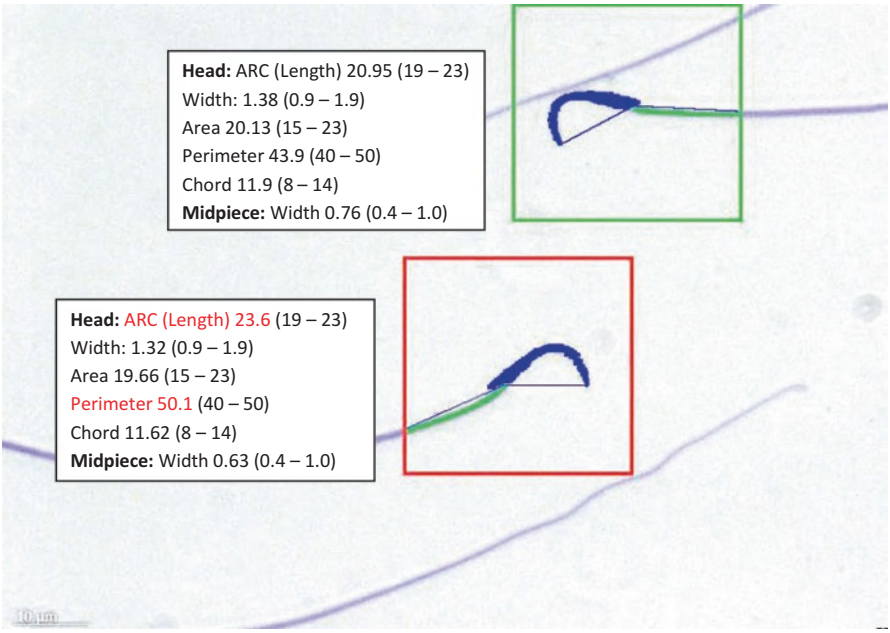

Fig. 5 Normal rat sperm (in green block) and all parameters on left in black fall within the cut-off ranges for these parameters. Abnormal sperm rat sperm (in red block) showing on left two parameters indicated in red that fall outside the normal cut-off points for those parameters and sperm accordingly classified as abnormal

recent review, Yániz et al. (2015) eluded to three types of CASA morphology analyses, namely traditional CASMA, CASMA based on negative phase contrast microscopy and CASMA via fluorescence microscopy. These three types of morphology analyses will be discussed as a basis before consideration of the results of this investigation in relation to traditional CASMA and its different applications.

Firstly, traditional CASMA involves the capturing and analysis of spermatozoa from a stained sperm smear using brightfield microscopy and specific software algorithms for detecting the various sperm components. Furthermore, these traditional systems, such as the SCA system used in this investigation, calculate 16 morphometric parameters relating to the sperm head, midpiece and tail. The use of cut-off morphometric values for a species/breed enables determining the percentage spermatozoa with normal morphology as well as some parameters relating to the percentage categories of, for example, elongated sperm or cytoplasmic droplets. In addition, the SCA system calculates three indices namely TZI, MAI and DI. These indices may prove to be a more powerful expression of sperm abnormality, as suggested for human spermatozoa since it combines more than one abnormality per spermatozoon in different ways. For example, when two patients/animals have similar percentages of morphologically abnormal spermatozoa but one of them has a much higher TZI than the other, it is conceivable that the patient/animal with more abnormalities per spermatozoon (higher TZI) is at a greater disadvantage in terms of overall fertility assessment. Recent investigations of dog and rat spermatozoa indicated that these indices show great potential as "adjuvant" morphology markers and should be pursued more rigorously in human and animal studies (Morselli et al. 2019; van der Horst et al. 2018a). The system employed for data capturing in this study, namely the SCA, has the ability to calculate more than 50 parameters during a typical traditional CASA/CASMA analysis. More than 95% of CASA systems use traditional CASMA for sperm morphometric/morphology analysis.

Secondly, negative phase contrast analysis (no staining) is currently only used by one group for CASMA analysis (Yániz et al. 2014). While phase contrast analysis of essentially live cells or fixed cells may provide the ideal answer (Maree et al. 2010), it is difficult to ensure that all spermatozoa are analysed in the same plane. Moreover, a 40× negative phase contrast objective is required and has three limitations: (a) sharp focus of all spermatozoa in the field of view; (b) the number of sperm that can be analysed within a short period of time and (c) the high cost of such an objective. These limitations might render this option not suitable to the industry for commercial and diagnostic purposes; however, it may lend itself to be useful in the research environment.

Thirdly, some CASMA systems have an option of analysis in fluorescence mode. The first application of this set-up was available in the Hamilton Thorne system to analyse rat sperm morphometry and morphology. In addition, Yániz et al. (2014) successfully employed an even more specialized system set-up with different animal spermatozoa by making use of nuclear stains that distinguish between live and dead spermatozoa. These nuclear stains are particularly useful in automatic analyses when there is background noise (non-sperm cells or debris). Similar to the disadvantages mentioned for phase-contrast CASMA, the cost of fluorescence makes

this option commercially less viable in the clinical set-up, but can be of value in the research environment.

In recent years, CASMA analysis in the human clinical setting has progressively received more credibility in the typical andrology laboratory. Many of the major fertility clinics in France, Belgium and the UK now routinely use CASMA in their total assessment of semen analysis. In the domestic animal and wildlife setting, CASMA is more complex for determining normal sperm morphology, as will be discussed below. In wildlife, there is another major obstacle seeing that the baseline morphometric values for determining normal sperm morphology virtually do not exist for most species.

The selection of a sperm stain is of foremost importance to assist in providing accurate capturing, thresholding and measurements by CASMA. Which stains have been used and what is their value in manual assessment as well as in CASMA? Chenoweth and Lorton (2014) reviewed some 38 stains that have been used for sperm morphology assessment in many mammalian studies and particularly among domestic animals, including DiffQuik, Hemacolour, Gentian Blue, Silver nitrate, SB and Nigrosin Eosin. These stains have all been used and/or suggested for manual analysis, while only few have been used in combination with CASMA. With the exception of SB, most staining combinations use a coagulative fixative such as methanol or ethanol for the sperm smear. These alcohols are good fixatives, but they have two disadvantages. Firstly, they not only fix the spermatozoa, but also anything else appearing on the slide. Such background stained particles adjacent to and even overlapping with spermatozoa are highly problematic for CASMA. Secondly, these coagulative fixatives cause shrinkage of spermatozoa, as was eloquently shown in the study by Maree et al. (2010). Furthermore, an ideal stain should be isotonic or isosmotic to freshly ejaculated semen, epididymal spermatozoa or spermatozoa exposed to a physiological medium (van der Horst and Maree 2009). Banaszewska et al. (2015) showed that SB staining resulted in more accurate measurements when three stains were compared in terms of head dimensions and detection of the acrosome with CASMA in stallion spermatozoa and is possibly a result of its isotonic/isosmotic characteristics.

Once spermatozoa have been stained, appropriate thresholding by CASMA software is imperative to accurately recognize the acrosome, head, midpiece and tail while providing detailed morphometrics of each of these components. It is noteworthy that spermatozoa from all 19 species included in this study were correctly detected and sperm components were accurately measured. Since the late 1990s, there were attempts to establish morphometric parameters of sperm stained predominantly with DiffQuik, Hemacolor and Haematoxylin using early CASMA versions of the SCA system, the Hamilton Thorne (HT), ISAS and several others (Boersma et al. 2001—HT—bull; Esteso et al. 2015—SCA—goat; Saravia et al. 2007—ISAS—boar; Yániz et al. 2015—ISAS—several species). From these outcomes, it appears that several CASMA systems are capable of capturing stained sperm images and performing detailed morphometric analysis.

One of the disappointing features of CASMA that is lagging is the use of automated systems to determine the percentage normal sperm morphology despite an

array of morphometric values being calculated. However, van der Horst et al. (2018c) suggested and developed a method to be able to establish normal sperm morphology in well-known breeds of laboratory rats. This method has been further elaborated to automatically analyse the percentage of morphologically normal spermatozoa of Tankwa goats (Ngcauzele 2018) as well as bottlenose dolphins (van der Horst et al. 2018b). Aspects of these three studies have been demonstrated in the current research paper.

The value of CASA morphology assessment is far-reaching and analyses of normal versus abnormal sperm morphology percentages are not only objective, reproducible and rapid, but also provide an array of morphometric parameters/variables for many applications as well as indices and a permanent record for verification and training. The major advantage of analysing very detailed morphometric features of sperm is that it can and has been directly applied in toxicology (Legendre et al. 2019) and shown correlations between progressive motility and sperm head length (Kochman et al. 2016).

However, great care needs to be exercised with basic preparation steps, selecting stains that will not affect sperm dimensions, using the correct hardware and software setups and implementing quality control. Despite some shortcomings, it seems that we have by and large conquered CASMA analysis in laboratory animals, domestic animals as well as in wildlife and this provides more objective tools for sperm morphology assessment and accordingly determining the percentage of normal spermatozoa.

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Progress with Sperm Morphology Evaluation After the Strict Criteria Prognosis Groups Era due to the Introduction of the New Lower Reference Limit Values for Semen Parameters of the 2010 WHO Manual

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Introduction

The introduction of the (Tygerberg) strict criteria (Menkveld 1987; Menkveld et al. 1990) by Kruger et al. in 1986 and 1988 introduced a new area for the role of sperm morphology as a prognostic tool for potential IVF fertilization outcome. Kruger et al. (1988) divided the sperm morphology results as obtained by the strict criteria method into three prognostic sperm morphology groups or so-called morphology patterns viz. the normal pattern (>14% normal forms), good prognosis pattern (5–14% normal forms) and the poor prognosis pattern (<5% normal forms). The status quo for these three prognosis groups has now changed drastically due to the introduction of the new lower limit values for semen parameters (variables) in the 2010 WHO semen evaluation manual to only 4% of morphologically normal spermatozoa (WHO 2010). This effectively ended the use and era of the three strict criteria prognosis groups. The new WHO (2010) low normal reference value also further contributed to the controversy of the current role of sperm morphology as a diagnostic semen parameter and its role in the decision-making process in assisted reproductive procedures (ART). Due to the current situation, the role of sperm morphology is continually questioned and many laboratories have totally excluded this semen parameter in their evaluation and treatment protocol. To compromise for this very low normal reference value and reassert its role of sperm morphology new ways have and are being explored to rectify the important role sperm morphology

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can play in the evaluation and decision-making process in the investigation and treatment of the infertile couple.

The aim of this lecture is therefore firstly to briefly discuss the development of sperm morphology evaluation methodology since the discovery by van Ham and description of the human spermatozoon by van Leeuwenhoek in 1676 through time up to the latest description of the evaluation methodology in the 2010 WHO manual (WHO 2010). The second aim is to explore new developments in the field of sperm morphology evaluation and reporting in order to reassert the role of sperm morphology in the current and future era.

Discovery and Description of the Human Spermatozoon

After van Leeuwenhoek's first letter in 1677 to the Royal Society in London with detailed descriptions and schematic drawings of the morphology of animal and the human spermatozoa (Kremer 1979), little attention was paid to sperm morphology evaluation, as such, for the next 100 years. In 1869, the importance of the presence of spermatozoa for fertilization was stressed by Sims (1869) who performed post-coital examinations on fluids of the vagina and the endocervix and stated that spermatozoa had to be present in the endocervical mucus for conception to occur. However, it was only as recently as the beginning of the twentieth century that the evaluation of sperm morphology became a focus of interest and that it was accepted that normal and pathological forms could simultaneously appear in a semen sample (Hotchkiss 1945; Joël 1971). In 1916, Cary summarized the existing literature and included drawings of atypical spermatozoa. Cary also stated that the fertilization potential was lowered through the presence of abnormal sperm and thereby was one of the first persons to associate abnormal morphology with the fertility potential of the human male (Cary 1916). Moench and Holt (1931, 1932) supported the statement made by Williams and Savage (1925) that the head of the spermatozoa was the biggest single source of information on the reproductive potential of spermatozoa and that, for the average male, the percentage of head abnormalities will be a good indicator of the reproductive capacity of an individual.

A statement made by Williams in 1937, and supported by Moench in 1940, saying "The difficulty in evaluating any semen specimen as to its fertilizing powers lies not in picking out definitely narrow, large, small cells, etc., but in knowing where to draw the line between normal and abnormal" still holds true for today.

The situation reflected by the statemen(s) of Williams (1937) and Moench (1940) could to a certain extend be attributed to the then-current morphology evaluation procedure in domestic animals. In contrast to the human, most domestic animal species generally reveal a morphologically homogeneous-looking sperm population. This homogeneous picture made it possible to use the appearance of the dominant spermatozoon forms, in proven fertile animals, as a model to describe the normal form for that specific species. This approach could not be adopted successfully for the human male due to the extreme heterogeneity of sperm morphology in the

human semen sample. With the animal system, normal spermatozoa were identified by default, as all spermatozoa that could not be identified as having an abnormality were considered as normal. This is reflected in the statement by MacLeod that "we are not prepared at this time to classify any but the most distorted forms as truly abnormal" (MacLeod and Gold 1951).

The disadvantage of this approach in animals was, therefore, that no specific criteria were put forward to describe morphologically normal spermatozoa. In the human, the so-called normal population would, therefore, contain the truly normal spermatozoa population and a fraction that, based on functional and biological evidence, is in fact, not morphologically normal. Therefore, the method for human sperm morphology evaluation also had the consequence that the later so-called borderline normal spermatozoa were considered and included as normal. In 1971, Eliasson made a very important contribution towards standardization of human sperm morphology evaluation by proposing standard measurements to identify sperm head abnormalities as too small or too large or to be within the normal range (Eliasson 1971). Eliasson also introduced the principle that the whole spermatozoon should be evaluated thus including midpiece and tail defects. Eliasson also stated that a spermatozoon should only be regarded as morphologically normal if the whole spermatozoon was normal and if not sure about the normality of a spermatozoon, that spermatozoon should be regarded as normal (Eliasson 1971). This approached, of identifying morphologically normal spermatozoa by exclusion, was later called the liberal approach by Comhaire et al. (1994). One of the consequences of the liberal approach was that this could lead to an incorrect classification of a specific male's fertility status, and couples could be deemed to present with unexplained infertility, in cases where the women were diagnosed as "normal" i.e., not presenting with any clinically diagnosed abnormalities (Oehninger et al. 1988).

Strict Criteria Approach for Sperm Morphology Evaluation

Although the current concept of the strict criteria sperm morphology evaluation methodology (Menkveld 1987; Menkveld et al. 1990) was already conceptualized in the late 1970s, the application was only published for the first time in the mid-1980s (Kruger et al. 1986, 1988). The new concept for the sperm morphology evaluation methodology for the description of a so-called morphologically "normal" spermatozoon was based on biological evidence for normality based on the sperm selective capability of the cervical mucus. This selective capability of cervical mucus allowing only certain morphological forms of spermatozoa to migrate through the endocervical mucus has been described in the literature since the early 1930s (Cary 1930). Cary stated that the morphology of human spermatozoa bears a definite relation to their success of migration through cervical mucus. This statement was repeated by Cary and Hotchkiss (1934) when they wrote "Abnormal forms may process motility but are rarely if ever found in the upper levels of

cervical mucus and we must consider them (abnormal forms) ineffectual for fertilization".

More recently studies by Fredricsson and Björk (1977), Mortimer et al. (1982), Fredricsson and Sennerstam (1984) and Hofmann et al. (1985) confirmed these observations. They all reported that a strong selection for certain morphological types of spermatozoa occurs with the migration of spermatozoa through cervical mucus, and that the morphological normality of these populations is significantly increased and of strong prognostic significance for expected in vivo fertilization.

The appearance of these spermatozoa, found in the endocervical mucus, has been used as a reference population to describe in detail the ideal "normal" spermatozoon (Menkveld 1987; Menkveld et al. 1990). To the best of our knowledge, this basic concept to define a morphologically normal spermatozoon has not been described in the available literature, at that time, before. The methodology for sperm morphology evaluation, based on the functionality of spermatozoa, became known as the (Tygerberg) strict criteria (Comhaire et al. 1994; WHO 2010). The principal of sperm morphology-based selection has been confirmed through the morphology-depended binding of spermatozoa to the zona pellucida of the human ova (Menkveld et al. 1991; Liu and Baker 1992). More recently, the morphological based role of spermatozoa has been confirmed in the migration of spermatozoa through the uterine cavity and fallopian tubes and through the corona radiata cells found around the ova (Yanagimachi 2011; Martyn et al. 2014; Castañeda et al. 2018; Tecle et al. 2019).

Strict Criteria for the Evaluation of Morphologically Normal Spermatozoa

According to strict criteria, the head of the spermatozoon must have an oval form with smooth contours. A clearly visible and well-defined acrosome must be present and should cover about 30–70% of the anterior part of the sperm head and exhibiting a homogeneous light-blue staining. For Papanicolaou-stained spermatozoa, the generally given measurements for a normal-sized spermatozoon of between 3–5 μm and 2–3 μm for length and width respectively, is probably too large as sperm head measurements are staining specific (Henkel et al. 2008). More accurate measurements for Papanicolaou-stained semen smears are now available in the literature (WHO 2010; Maree et al. 2010; Menkveld 2013). The principle piece of the tail should have a uniform calibre along its length, be thinner than the principle piece and should be about 45–50 μm long and without any sharp bends. Tails looped on itself are regarded as normal. The tail tip is very seldom visible. No cytoplasmic residues >30% of normal head size may be present at the neck/midpiece region or on the tail (Menkveld et al. 1990; WHO 2010).

The basic description for a morphologically normal spermatozoa according to strict criteria was (initially) primarily based on the work of Eliasson (1971) in so far that the whole spermatozoon should be evaluated and the use of the sperm

measurements as published by Eliasson in 1971. However, the strict criteria distinctly differed from the statement of Eliasson (1971) and other published papers (MacLeod and Gold 1951; WHO 1987) that so-called borderline normal spermatozoa should be regarded as abnormal. The reasoning behind this was to keep the variations allowed for sperm normality as small as possible. Menkveld (1987) and Menkveld et al. (1990) also stressed the importance of the coherence of the WHO manuals guidelines (WHO 1980, 1987) with regard to the preparation of semen smears, staining of these smears and the methodology for evaluating the spermatozoa on these smears. The thought with the introduction of strict criteria was that the existing rules for the process of sperm morphology evaluation should be applied stricter, not that the appearance of individual spermatozoa should be placed under more intense scrutiny.

Consequences of the Introduction of Strict Criteria

The theoretical advantages of the introduction of strict criteria were the expectations that better repeatable results between and within observers could be expected (Menkveld et al. 1990). The second was that the use of strict criteria could lead to a better prognostic diagnosis for in vivo and in vitro fertilization and pregnancy outcome and a better prediction of sperm functionality (Oehninger et al. 1988; Bernstein et al. 1995; Harrison and Harrison 1996; Molina et al. 1996). Oehninger et al. (1988) found that with the use of the liberal approach for sperm morphology evaluation, used by their institution at that time, their results indicated that 40.4% of couples were diagnosed with unexplained infertility compared to only 11.5% when the sperm morphology evaluation on the same samples was done according to strict criteria, thereby confirming the stronger predictive value for strict criteria for the in vitro fertilization procedure.

The most prominent expected outcome was of course an overall lowering of the percentage of morphologically normal spermatozoa for a sample evaluated by strict criteria compared to that of the liberal criteria methodology as illustrated in several publications (Oehninger et al. 1988; Bernstein et al. 1995; Morgentaler et al. 1995; Harrison and Harrison 1996; Molina et al. 1996; Menkveld et al. 2001, 2011; Mortimer and Menkveld 2001; Menkveld 2010a). However, over time a drift to a lower outcome of the percentages of morphologically normal spermatozoa, started to appear in the literature (Menkveld et al. 1986; Horte et al. 2001; Morbeck et al. 2011; Van den Hoven et al. 2015). The decline in the percentage of morphologically normal spermatozoa was caused by several factors, one of the most important factors being the continued stricter application or the stricter scrutineering of spermatozoa and thereby regarding the minutest aberration of a sperms form as an abnormality (Wang and Swerdloff 2014; Rothmann and Bort 2018; Sigman 2018). Negative environmental influences (Menkveld et al. 1986; Rolland et al. 2012) and the recognition and description of additional sperm morphology abnormalities also contributed to the decrease in percentage of morphologically normal spermatozoa

reported (WHO 1980, 1987, 1992, 1999, 2010; Menkveld 2013). More recently it has also been reported that a decrease in percentage of morphologically normal spermatozoa has been observed with a corresponding increase in male age (Maher et al. 2014; Van den Hoven et al. 2015; Danis and Samplaski 2019).

Evolution of Morphology Evaluation Criteria in Consecutive WHO Manuals

The first WHO manual for the performance of a standardized semen analysis was published in 1980. In this manual (WHO 1980), very little information was provided on the evaluation of sperm morphology. The instructions indicated that at least 100 spermatozoa with tails should be counted and classified according to the illustrations in the photographic plates provided. In total, nine categories were described. Normal spermatozoa should in general exhibit a regular oval-shaped head with intact midpiece and uncoiled tails. Sperm with nearly oval heads and no gross irregularities should also be regarded as normal as long as they fell in the normal range for sperm head measurements. Other categories mentioned are large oval heads, small oval heads, tapering heads, pyriform heads, duplicated heads, amorphous heads, tail defects and the presence of cytoplasmic droplets.

The second WHO manual was published in 1987 and followed a more or less similar approach as the 1980 edition, for the descriptions of normal and abnormal spermatozoa also based on sperm pictures in the plates provided. However, a little more detail was provided in so much that information on the evaluation of the midpiece and tail was also included as well as combinations of abnormalities (WHO 1987).

The third WHO manual was published in 1992. In this manual, much more detail on sperm morphology evaluation was provided with the statement that "strict criteria should be applied when assessing the normality of the spermatozoon". The basic standard description for morphologically normal spermatozoa was provided with normal measurements for Papanicolaou-stained spermatozoa. The necessity of the presence of a well-defined acrosome region, the absence of any neck, midpiece or tail defects and large cytoplasmic droplets are also indicated. However, it is then stated that "This classification scheme requires that all 'border-line' forms should be considered normal". The manual also indicated that four different categories of defects should be recorded viz. (a) head/size defects; (b) neck and midpiece defects; (c) tail defects and (d) presence of abnormal cytoplasmic droplets (WHO 1992).

The fourth WHO manual published in 1999 recommended that for assessing the morphological normality of the spermatozoon, strict criteria should be applied as described by Menkveld et al. (1990). It also stated, for the first time, that the classification scheme requires that all "borderline" forms should be considered as abnormal.

The fifth WHO manual published in 2010 maintained the same principals as the 1999 manual but provided much more details on the whole process of sperm morphology evaluation as one of the aims of the 2010 manual was to provide more evidence based guidelines and information for all aspects of the semen analysis procedure. In conjunction with this approach, the aim was also to provide evidence-based lower reference limits for normality of semen parameters compared to the different approaches in the previous manuals based, in general on semen parameter values obtained for so-called normal or fertile populations (Cooper et al. 2010).

Succession of the Normal Sperm Morphology Values in Consecutive WHO Manuals

In the first WHO manual published in 1980, the frequency for normal sperm morphology was indicated as 80.5%. At the time of the publication of the strict criteria (Menkveld et al. 1990), the normal cut-off value for sperm morphology was at ≥50% morphologically normal spermatozoa according to the 1987 WHO manual. This changed to ≥30% morphologically normal spermatozoa for the 1992 manual, with the suggestion that strict criteria should be used, and in the 1999 manual no cut-off value was indicated, but a note was added that with <15% normal forms a decrease in IVF fertilization rates could be expected when morphology was evaluated according to strict criteria. The 2010 WHO manual introduced the very low value of 4% as the lower normal limit for sperm morphology, as evaluated by strict criteria (see Table 1). Furthermore, the 2010 WHO manual also suggests that for the routine semen analysis procedure determining only the percentage of morphologically normal spermatozoa will be sufficient. This is an unfortunate statement as this further reduces the prognostic value of sperm morphology.

Results of the Introduction of the 2010 Manual Lower Reference Limit Value for Sperm Morphology

The introduction of the 2010 WHO lower normal values resulted in several negative effects. The first consequence was that the use of the morphology prognosis groups as introduced by Kruger et al. (1986, 1988), in essence, became irrelevant as only the poor morphology prognosis group now remains and the other two groups viz. the good (5–14% morphologically normal forms) and normal prognosis group ≥15% morphologically normal forms) are no longer applicable. Thus, this effectively brought an end to the era of the three strict criteria prognosis groups, with the consequence that opportunities could be missed for a correct or proper infertility assessment (Fugelsang et al. 2018).

Table 1 Normal and lower reference values for normal sperm morphology from consecutive WHO manuals

WHO edition and year published	Morphology (% normal)	Comments
1st (1980)	80.5	Frequency in normal ejaculate. Data from men who were fathers[a]
2nd (1987)	≥50	Criteria of normality. Criteria should be based on results of men who have recently achieved pregnancy, preferable within 12 month after partner ceasing contraception
3rd (1992)	≥30[b]	Normal values of semen variables. For normal semen variables, specimens should be evaluated from men who have recently achieved pregnancy, preferable within 12 month of the couple ceasing contraception
4th (1999)	(15)[c]	Reference values of semen variables. The reference ranges are given, based on the clinical experience of many investigators who have studied populations of healthy fertile men. Because these values are not minimum semen values needed for contraception, their categorization has been changed from "normal" to "reference" values
5th (2010)	4 (3.0-4.0)[d]	The reference value(s) have been generated from the results of several prospective, cross-sectional studies of semen quality and fertility. They were obtained by direct, retrospective selection of fertile men, defined as men whose partner conceived within 12 months after stopping use of contraception (Cooper et al. 2010)

[a]See data from MacLeod (1975) and Eliasson (1975)

[b]Although no clinical studies have been completed, experience in a number of centres suggests that the percentage of normal forms should be adjusted downwards when more strict criteria are applied. An empirical reference value is suggested to be 30% or more with normal forms

[c]Multicentre population-based studies utilizing the methods of morphology assessment in this manual are now in progress. Data from assisted reproductive technology programmes suggest that, as sperm morphology falls below 15% normal forms using the methods and definitions described in this manual, the fertilization rate in vitro decreases

[d]Lower reference limit (5th centiles and their 95% confidence intervals). Semen parameters that lie within the 95% reference interval do not guarantee fertility. Men whose semen characteristics fall below the lower limit(s) given are not necessarily infertile; their morphology (semen) characteristic(s) are below the reference range for recent fathers—as are, by definition, those of 5% of the fertile men who provided data used in the calculation of the reference range

A second consequence was that due to the loss in a strong predictive value (Morbeck et al. 2011; Wang and Swerdloff 2014) even more clinics exclude sperm morphology as a semen parameter to determine a male's fertility potential and as an indicator for the type of ART to be performed for a specific couple (Kovac et al. 2017; Kohn et al. 2018). Gatimel et al. (2017a, b) reported that sperm morphology had no relevance in the semen analysis anymore. In a combined report by the ESHRE Special Interest Group of Embryology and the Alpha Scientists in Reproductive Medicine (Vienna Consensus Report 2017a, b) it was stated "Sperm morphology assessment is subjective and so is dependent on consistent training. Since the Tygerberg Strict Criteria cut-off of 4% normal forms was derived in

relation to IVF success, it could be pertinent in differentiating between the need for IUI, IVF or ICSI (Mortimer and Menkveld 2001; Menkveld 2010a). However, the current visual evaluation of 200 or 400 spermatozoa used in the vast majority of laboratories to assess 'percentage of normal forms' has such a large uncertainty of measurement that it cannot be considered a reliable predictor for IVF success/failure for individual men (Kvist and Björndahl 2002; Björndahl et al. 2010). Unless determined using a more robust methodology, sperm normal forms should not be used to direct ART treatment options".

Investigations to Find Possible Corrective Measures to Compensate for 2010 WHO Low Lower Limit Value for Sperm Morphology

The statement in the 2010 WHO manual that "for the routine semen analysis procedure determining only the percentage of morphologically normal spermatozoa will be sufficient" must be regarded as an additional negative step in the reduction of the prognostic value of sperm morphology assessment. Indeed, by the introduction of the new low lower normal value of the WHO manual (WHO 2010) it is becoming of even greater importance that not only percentages of normal and abnormal spermatozoa should be reported but also that an in-depth analysis should be included to identify the types of sperm abnormalities that are present as this may add important additional information for selecting the type of clinical procedure to be adopted, being IUI, IVF or ICSI. In this regard, Menkveld (2010a, 2013) and Menkveld et al. (2011) suggested that together with sperm morphology evaluation by strict criteria new or refined sperm morphology "markers" should be sought for. This view is also supported in several other publications (Amman 2010; Auger et al. 2016; Rothmann and Bort 2018). Amman (2010) stressed the point that it is of more importance to identify sperm morphology abnormalities as this may identify spermatozoa defects occurring at spermiation. Amann recommends that spermatozoon be entered only once into the morphology evaluation results, categorized by the most important defect, in decreasing order of likely importance.

An extended sperm morphology analysis is also important for several other reasons. Firstly, as a morphologically normal spermatozoon does not necessary reflect or guarantee the normal sperm functional ability of such a spermatozoon (Menkveld 2010b). Secondly, it is known that certain sperm abnormalities are gene dependent (Carrell 2007; Krausz and Sassone-Corsi 2005; Golas et al. 2003; Dam et al. 2006) and also inheritable, especially in societies where interrelationship marriages can occur. Examples are the short tail syndrome (Khelifa et al. 2014; Amiri-Yekta et al. 2016) and the occurrence of globozoospermia (Kilani et al. 2004; Dam et al. 2007). Certain sperm abnormalities, like elongation, can be caused by "stress" and can be reversible when the cause of stress is over like in the case of a febrile illness

(Macleod 1951; Menkveld 2007) or the cause is treated in cases of leukocytospermia (Menkveld 2010b).

Several suggestions to overcome this adverse effect, of low percentage of morphologically normal spermatozoa, have been proposed. In order to obtain a higher cut-off value for normal morphology, Rothmann and Bort (2018) suggested a logarithm that approached the evaluation process to obtain the percentage of morphologically normal spermatozoa for a semen sample by means of an elimination process of abnormal spermatozoa. The questions are based on known sperm morphology defects included in the four main sperm abnormality classes referred to earlier. By asking a series of very specific question provided in the logarithm all spermatozoa with known abnormalities are excluded. The final conclusion on a sperms morphological normality is made based on the outcome of the final question. If a spermatozoon with no abnormality, or classified as a borderline normal spermatozoon, was identified, the spermatozoon is classified as normal. This renders a higher percentage of morphologically normal forms. Results obtained using this algorithm showed predictive value associated with fecundity in the National Institutes for Health (NIH) Fertility and the Environment (LIFE) study (Buck Louis et al. 2014; Rothmann and Bort 2018). In the LIFE study (Buck Louis et al. 2014), conducted in the United States, it is reported that couples who discontinued contraceptive use the male's semen analysis indicated that normal sperm morphology, determined with either strict criteria or more traditional methods, as still used in the US, amorphous, round, and pyriform heads, neck, and midpiece abnormalities, and coiled tails were significant predictors of time to pregnancy. Sperm head morphometry (width, elongation factor and acrosome area of heads) was also significantly related to fecundity rate. In a study were a simultaneous adjustment of multiple morphology parameters was used in a statistical model, only the percentage of spermatozoa with coiled tails was significantly related to fecundity (Wang and Swerdloff 2014).

Following the publications by Menkveld (2010a, 2013) and Menkveld et al. (2011), Auger et al. (2016) published an article named "Another look at human sperm morphology" with the question "can a standardized assessment of abnormal human sperm morphology provide additional useful information by identifying men with more severe disturbances in different types of (sperm) abnormalities?" Their reason being "that currently there are no baseline data for abnormalities of sperm morphology based on a standardised classification, in the general (fertile) or infertile population or other groups of men like those suffering with testicular cancers".

Auger et al. (2016) reanalysed their data of a study population recruited from 1987 to 1997 from four different cities. The method used by Auger et al. (2016) categorizes morphologically normal spermatozoa on the basis proposed by the WHO manual from the third edition onwards (WHO 1992, 1999, 2010). However, unlike the 2010 WHO manual guidelines, the classification used by Auger et al., considered all borderline aspects to be normal. Spermatozoa with morphology defects were divided into three categories viz. head abnormalities, with seven defect types, midpiece abnormalities with three defect types, and tail abnormities with five

defect types, resulting in a total of 15 types of defects. Each abnormal spermatozoon with more than one morphological defect was recorded into all the corresponding defect categories, allowing the calculation of the multiple anomalies index (MAI). For every type of sperm defect, three levels of sperm abnormalities were calculated and defined: a low level of abnormalities (LLA), below the 95th percentile of fertile men; a high level of abnormalities (HLA), above the 99th percentile in infertile men and an intermediate level of abnormalities (ILA) between the two percentiles mentioned. Auger et al. (2016) found that most sperm defects were significantly more frequent in the group of infertile men compared to the group of fertile men, with 20–30% of infertile men having higher frequencies, in 9 of the 15 sperm abnormality type groups, above the 95th percentile of the specific abnormality type group of fertile men. Head abnormalities were more frequent than midpiece and principle piece defects, mainly due to acrosomal or post-acrosomal region defects rather than head size abnormalities. Bent and coiled tails were the most frequent tail abnormalities and were observed in most semen samples of all three patient groups. For the men in the fertile group, it was observed that an abnormal spermatozoon generally had no more than two types of abnormalities. However, the broad overlap between the distribution profiles of the various morphological abnormalities in the fertile and infertile men precluded the definition of a single meaningful threshold for each category. In cases where the percentage of morphologically normal spermatozoa or an MAI is within the normal range the presence of HLA categories may be of a strong informative sign for an underlying abnormal clinical indication. Auger et al. concluded that, "definition based categorisation of sperm head, midpiece and tail defects has shown that sperm abnormalities are different in fertile and the other groups of men investigated, providing high and low thresholds, which can serve as a starting point for diagnosis or research purposes". However, the method suggested by Auger et al. (2016) is rather time-consuming and they therefore suggested that the method described should only be used for the first semen analysis of new patients and that it may be applicable for research and epidemiological studies. Thus the principal describe by Auger et al. can be considered as a new approach. However, due to the fact that, in contrast to the WHO 2010 manual suggestion, apparent borderline spermatozoa should be regarded as morphologically normal, the cut-off values provided for morphologically normal spermatozoa, of 23% (95% CI, 20–26%) by Auger et al. (2016) and ≥27% by Eustache (2017), may be too high.

fiore Study

The aim of this retrospective study was to investigate if a detailed sperm morphology evaluation may be able to identify if a single or combination of specific sperm morphology abnormalities which may improve the diagnostic role of sperm morphology in the evaluation of the fertility potential of the male partner of an infertile couple. For the purpose of this study, a literature search was performed to compile a list of all illustrations and/or descriptions of all single sperm morphology

abnormalities that could be found. These abnormalities were combined into a single data sheet. The sperm morphology evaluations were performed on semen smears from the first IUI procedure performed for a couple. The sperm morphology was performed by a single observer without the knowledge of the IUI outcome. Although in-depth evaluation records of the medical status of the male and female partners was available, only the outcome of the insemination results was taken into consideration for this primary investigation. Thus, the pregnancy outcome was recorded as pregnant yes/no.

The primary data presented here are very basic as the study is still ongoing. A total number of 77 different sperm abnormalities were identified. Number of semen samples analysed so far is 66 with pregnancies yes = 22 and no = 44. The mean (SD) percentage of morphologically normal spermatozoa for the overall group was 3.5(1.8)%, for the non-pregnant group (NPG) of 44 patients 3.0(1.7)% and for the pregnant group (PG) 4.4(1.7)%. The normal morphology values between the NPG and PG was significantly different ($P < 0.0034$). The overall percentage of amorphous heads was 8.1(3.8)%, 9.3(3.6)% for the NPG and 5.7(2.4)% for the PG, with $P < 0.0002$ for the mean difference between the NPG and regnant groups. For the overall group, the most frequent single abnormality was spermatozoa with small heads with a mean (SD) of 23.7(8.1)%, followed by spermatozoa with small acrosomes 17.8(7.6)% and spermatozoa with thicken midpieces with a mean incidence of 14.5(7.1)%. More details are provided in Table 2. The main impression is that small spermatozoa and/or spermatozoa with small acrosomes are the most prevalent abnormalities and may play an important role in male infertility and fertilization outcome.

Acrosomal abnormalities were found to be one of the most frequent sperm abnormalities in this small primary study. However, no single sperm abnormality manifested itself as a clear indicator of a potential sperm morphology related male infertility problem. Some of the expected or hypnotized sperm abnormalities thought to may be of clinical significance or to be associated with poor fertilization showed a higher, although small, incidences in the pregnant group but in most abnormality groups with a lower maxium range value. With including more patients in the study and thus more data available, the possibility may exist to still obtain a better profile of prognostic morphology patterns to serve as more precise indicators of a male's fertility potential.

Conclusions

The introduction of the 2010 WHO manual with a very low value for the lower reference limit for normal sperm morphology, of 4% morphologically normal spermatozoa, brought for all practical purposes an end to the area for the strict criteria sperm morphology prognosis groups viz. the poor, good and normal prognosis groups. These prognosis groups were already under pressure due to the continued downward trend of the percentage of morphologically normal spermatozoa reported

Table 2 Basic summary statistical results of most frequent occurring sperm abnormalities as calculated for all patients ($n = 66$) and subgroups of pregnant ($n = 22$) and non-pregnant ($n = 44$) couples after first IUI procedure

		All patients			Non-pregnant			Pregnant			
Abnormality class	Type of abnormality	*n*	Mean (SD)	Range Min–Max	*n*	Mean (SD)	Range Min–max	*n*	Mean (SD)	Range Min–max	*P*-value
Normal spermatozoa—Total of oval and v-shaped (%)		66	3.5 (1.8)	1–9	44	3.0 (1.7	1–7	22	4.4 (1.7)	2–9	0.0034
Head	– Amorphous (%)	66	8.1 (3.7)	1–17	44	9.3 (3.6)	2–17	22	5.7 (2.4)	1–10	0.0002
	– Small (%)	66	23.n (8.1)	3–38	44	22.5 (8.7)	3–38	22	25.9 (6.2)	11–36	0.0835
Acrosome	– Absent (%)	66	9.2 (6.3)	0–29	44	10.3 (6.9)	0–29	22	7.2 (4.1)	1–17	0.1250
	– Large + stain defects (%)	66	8.1 (4.5)	0–22	44	5.0 (4.1)	0–19	22	2.2 (2.0)	0–8	0.0018
	– Large + vacuoles (%)	66	7.8 (5.7)	0–31	44	6.1 (4.4)	0–20	22	11.1 (6.7)	0–31	0.0007
	– Small (%)	66	17.8 (7.6)	1–41	44	19 (7.8)	2–21	22	15.3 (6.8)	1–27	0.0554
	– Small + staining defects (%)	66	9.6 (6.7)	0–25	44	7.6 (4.9)	0–22	22	9.1 (3.4)	4–15	0.0694
	– Small + vacuoles (%)	66	9.6 (6.7)	0–25	44	7.2 (5.9)	0–25	22	14.6 (5.4)	5–24	<0.0001
Midpiece	– Thick (%)	66	14.5 (7.1)	2–32	44	15.6 (7.7)	3–32	22	12.3 (5.2)	2–24	0.0823
Tail	– Irregular (%)	66	8.0 (3.7)	1–18	44	8.6 (3.8)	1–18	22	7.0 (3.4)	1–16	<0.0001
ECR	– Neck medium (%)	66	6.0 (3.5)	0–16	44	7.1 (3.4)	1–16	22	3.8 (2.3)	0–8	0.0001

Mann–Whitney test for independent samples
ECR excess residual cytoplasm

in the literature, to such an extent that the role of sperm morphology in the diagnosis of male fertility potential is now regarded as of less importance, and in some centres totally disregarded, in the diagnosis of a male's fertility potential or the treatment options for the infertile couple.

In an attempt to improve the diagnostic role and status of sperm morphology, in the current time frame, several researchers have taken a new look at their sperm morphology data with the aim to possibly find new indicators for the diagnostic role of sperm morphology in the diagnosis of male fertility potential.

Unfortunately, none of these investigations were very successful although new perspectives have been put forward.

A new logarithm has been put forward by Rothmann and Bort (2018) increasing the resultant percentage of morphologically normal spermatozoa. However, "borderline" normal spermatozoa were included in the morphologically normal spermatozoa group. A study based on this model indicated that semen samples presenting with increased percentage of coiled tail were most significantly related with reduced fecundity (Buck Louis et al. 2014).

With a reanalysis of their data on sperm morphology, Auger et al. (2016) found that out of 15 different types of sperm morphology defects, the most frequently occurring type of abnormality in infertile males and males with testicular cancer were spermatozoa with post-acrosomal abnormalities.

In an analysis of preliminary data from an ongoing study from the fiore laboratory, St. Gallen, Switzerland, performed on semen smears from first time IUI patients, the most prevalent sperm abnormalities found were spermatozoa with too small heads and spermatozoa with small acrosomes. Unfortunately, maybe to the small number of smears analysed, there was an overlap in results from the pregnant and non-pregnant groups and more data is needed.

Taking the results of these studies into consideration, it is clear that the use or consideration of the percentage of morphologically normal spermatozoa only, for the diagnosis of male fertility potential based on sperm morphology is not sufficient. Much more attention to the type of sperm morphology abnormalities or patterns should be included in the basic semen analysis and work-up for the infertile couple, even if the significance of these abnormalities is not clear at the present moment as potential indicators for underlying testis pathology and as indicators for ART procedures.

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A Structured Assessment for the Assessment of Human Sperm Morphology

Susan A. Rothmann

Sperm morphology has historical value for predicting fertility outcomes, which has diminished in the last few decades. Ample data show that most labs using the Strict morphology scheme are overly critical with few normal sperm found even in fertile men. In many centers, morphology no longer correlates with success after assisted reproductive therapies. Many different interpretations of classification criteria exist as seen clearly in proficiency testing where intra-observer and interlaboratory variation typically exceeds acceptable and useful limits. The WHO reference ranges for normal Strict morphology encompass median and upper limits that many laboratories never attain.

The lack of a standardized method for the application of sperm classification criteria explains why so many different interpretations of the Strict classification scheme exist. In the 30 years of dissemination of the Strict scheme, subjective definitions of normal and borderline normal sperm largely replaced the original Strict definitions, making repeatability and training very difficult.

Our research to develop a more objective method included an extensive review of photographs and definitions of normal, borderline, and abnormal sperm from published papers and atlases. We obtained primary data by surveying the classification of 155 sperm by 99 international experts. Using well-established principles of pathology and taxonomy classification, we developed a dichotomous key algorithm with 12 queries of sperm shape and size. Borderline normal forms are classified as a separate category using definitions described by Menkveld in 1990. One hundred and forty-three archived morphology smears were analyzed with the algorithm and compared to original values using subjective analysis. Strict normal median with the algorithm was 18%, compared to the original median of 4% (WHO fifth reference medians for unscreened men = 14%, fertile fathers = 15%). The algorithm was used to analyze 436 archived smears from the NIH Life Study and the distribution of

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values was similar to WHO fifth reference ranges. Regression analysis of 170 smears showed excellent interobserver correlation (0.9). The method was also highly stable. An unexpected benefit was a reduced analysis time from 30 to 15 min. Because borderline sperm are classified independently, the algorithm can be used to determine percent normal forms for Traditional or Strict morphology schemes simultaneously or various indices. Participants at ASA Lab Workshops found the method was easy to use and adopt and typically became less critical using the algorithm. Supported by NIH Grant R43 HD044383-01.

On the Indispensability for Standardization of the Basic Examination of Human Semen

Lars Björndahl

Introduction

The problems linked to the lack of standardization of semen examination were recognized and documented already in the first half of the twentieth century (Harvey and Jackson 1945). In the 1950s, John MacLeod published a series of comprehensive studies on male factors related to fertility and infertility (MacLeod 1950, 1951; MacLeod and Gold 1951a, b, c, 1952, 1953a, b, 1957; Gold and Macleod 1956). Fundamental recommendations for proper examination of human semen were published in the 1970:ies by Rune Eliasson (Eliasson 1975, 1977, 1981, 2003, 2010). As a basis for studies on possible male contraception, initiated by the WHO, a first outline on recommendations for reliable semen analysis was published in 1980 (Belsey et al. 1980), followed by revised editions 1987–2010 (World Health Organization 1987, 1992, 1999, 2010). The importance of semen analysis for efficient investigation and treatment of men with infertility issues is well known (Bjorndahl 2016; Björndahl 2010; Björndahl and Barratt 2005; Tomlinson 2016). Still, the compliance with the WHO recommendations has been far from satisfactory (Barratt et al. 2011; Björndahl et al. 2016; De Jonge and Barratt 1999; Keel 2004; Keel et al. 2000; Riddell et al. 2005; Jequier and Ukombe 1983), in spite of further development of laboratory techniques (Mortimer 1994a, b; Bjorndahl et al. 2010; Kvist and Björndahl 2002), training Schemes (Barratt et al. 2011; Björndahl and Kvist 1998; Franken 2003; Punjabi and Spiessens 1998; Vreeburg and Weber 1998; Björndahl et al. 2002) and quality control programmes (Cooper et al. 1999; Dunphy et al. 1989; Franken et al. 2000a, b; Franken and Kruger 2006; Libeer et al. 1996; Palacios et al. 2012; Petersen et al. 2002).

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Details of how semen differs from other body fluids investigated in clinical routine have already been presented in the chapter "Is Decreasing Sperm Concentrations a Sign of a General Decay in Fertility Potential?" The focus of this chapter is therefore on semen examination standards—why it is important and what should be included.

Aim of the WHO Manual

The purpose of the WHO manual was originally to provide a laboratory standard for centres participating in male contraceptive studies (Belsey et al. 1980). The manuals have not been complete standard operating procedures (SOP). Furthermore, with the incorporation of more information, it appears that they have been increasingly difficult for ordinary laboratory staff to comprehend and transform into easy to understand and follow SOPs. In case the semen laboratory is run as appendage to an embryology laboratory with clinicians rather than laboratory scientist mainly responsible, it is easy to understand that the compliance with WHO recommendations sometimes is reduced to the reference limits described by WHO, without using the essential laboratory techniques.

The huge global interest in the WHO manual depends very much on the growth of the IVF industry. This has caused an imbalance for the potential focus of semen examination: it is not only a matter of choosing an appropriate module for assisted reproductive technologies (ARTs). It should even more be a matter of diagnostic techniques to discover and distinguish between different disorders in the male reproductive functions. Without the latter perspective, the diagnosis and treatment of male reproductive disorders will not advance.

Format for the WHO Manual

A WHO manual is developed by a committee of experts, suggested by scientific organizations by request from the WHO, often after obtaining funding by the scientific organization. More recent WHO procedures include both internal and external reviewers commenting on the committee's proposal. The final decision on publication is by the WHO administration. Revision of a WHO publication is very much dependent on the availability of funding.

Aim of the ISO Standard

An ISO standard is developed for the benefit of—in this case—health care and medical science in participating members (countries), to improve quality (safety and efficiency) in clinical routines and enhance scientific development by enabling

exchange of useful information. The aim is thus almost identical to that of the WHO. Therefore, the Technical Standard for Basic Human Semen Examination should be the same as the WHO manual section on Basic Semen Examination.

Is an ISO Standard Really Warranted When the WHO Manual Is Revised?

There is a need for a formal ISO standard, based on the same scientific evidence as the WHO manual. Basically, in many countries, the accreditation bodies require a formal standard to allow a complete accreditation. Even if the WHO manual becomes more succinct and without lots of variants, the ISO standard will be more structured and defined that makes it easier for individual laboratories to ensure that the correct procedures are followed. This also makes it less ambiguous for the accreditation bodies to inspect and verify that the procedures are correct. The specifics of ISO standards are that it should be possible to measure the qualities and compare assessments with golden standards.

Format of an ISO Standard Process

The process of developing an ISO standard is initiated by a member (country) and started if a sufficient number of other member states support the proposition. A project group is formed by experts nominated by the member states. The draft documents proposed by the expert group are distributed among member-state organizations for considerations and comments. When the expert group has final draft, this is sent to all member organizations of the Technical Committee for ballot. If approved, the document is then forwarded to the entire ISO for a ballot to be approved as an ISO standard. If accepted, the member countries decide if they will adopt the standard as a national standard, and within Europe, it can be adopted as an European Standard by the European Standards Committee (CEN). The final decision in each country thus lies in the local standardization organization.

Current Developments

Due to the current poor compliance with recommendations for reliable semen examination, the present WHO revision aims at recommending basic examination with primary focus on male reproductive organ functions. The implications for couple infertility are important but are naturally not only dependent on sperm factors meaning that the importance for couple fertility must be considered to be indirect (semen characteristics do not have very distinct “limits” between fully fertile and subfertile men).

The expectation is therefore that the sixth edition of the WHO will be easier to follow, with transparency regarding the recommended techniques that all laboratories should be able to use. The WHO manual must be much more accessible to general laboratory staff. Multiple parallel procedures will be avoided, recommending most reliable laboratory techniques. Alternative procedures are explained as extended procedures. In this section, there will also be description of assessment techniques where the scientific evidence of the clinical value is considered low, even if the tests may be popular and often requested. In short, all laboratories performing semen examination are supposed to be able to determine sperm concentration (and total number) (Björndahl et al. 2016), sperm motility (in four categories) (Björndahl 2010; Barratt et al. 2011), sperm vitality (when few spermatozoa are motile—to distinguish between live immotile and dead immotile spermatozoa) (Björndahl et al. 2003, 2004) and sperm morphology (Tygerberg strict criteria—as adopted by the WHO since 1992) including the ability to recognize abnormalities in all four "regions" (Björndahl et al. 2016; Mortimer and Menkveld 2001; Menkveld and Kruger 1995).

Simultaneously with this work, a Draft for an International Standard ("DIS") for the basic examination of human semen has been developed (to provide a framework for laboratory accreditation and improved safety, efficiency and quality in semen analysis globally). This draft is now (Summer of 2020) approved by the Technical Committee (TC212) for further enquiry to the member countries in ISO for approval as International Standard (ISO). There is already a general standard for general aspects of medical laboratories (15189) and the Technical Standard for Basic Semen Examination is supposed to work in addition to the ISO 15189.

Basic Principles for Reliable Testing

- Stable temperature—sperm motility (velocity) is temperature dependent (Mortimer 1994b; Björndahl et al. 2010).
- Replicate assessment with comparison to minimize the influence of random errors in taking aliquots for assessments—sampling for assessment of concentration and motility requires repeated assessments. Comparisons should identify when the two replicates are not similar enough (Mortimer 1994a, b; Björndahl et al. 2010; Björndahl 2013).
- To reduce the influence of random errors, sufficient numbers of cells must be counted (Mortimer 1994a, b; Björndahl et al. 2010; Björndahl 2013).
- Criteria for motility categories must be well defined (Björndahl 2010; Sifer et al. 2005; Bollendorf et al. 1996; Verheyen et al. 1999).
- Thin smears for morphology assessment—best staining results, low risk of losing material during staining process and the best basis for visual analysis in the microscope (Björndahl et al. 2010).
- Defined criteria for morphology assessment (Mortimer and Menkveld 2001; Menkveld and Kruger 1995; Menkveld et al. 1990, 1996, 2001).

- Training of staff (Mortimer 1994a, b; Björndahl et al. 2010).
- Frequent Internal and External Quality Assessment (Cooper et al. 1999; Dunphy et al. 1989; Franken et al. 2000a, b; Franken and Kruger 2006; Libeer et al. 1996; Palacios et al. 2012; Petersen et al. 2002).
- Proper equipment (Mortimer 1994b; Björndahl et al. 2010; Björndahl 2013).
- Patient information—preparation (abstinence time?), sample collection (complete?), time of collection?
- Initial handling of ejaculate—collected at laboratory or transported (temperature)? Time to controlled temperature? Mixing during liquefaction?

Discussion and Conclusions

The essential gain from using standardized laboratory procedures is multifarious. A primary return is reliable results for each individual investigated, thanks to the reduced influence of random errors. This also means that for most men repeated ejaculate examinations will not be necessary (Barratt et al. 2017). With standardized procedures, also training can be standardized using training material with known target values obtained using the standardized techniques. Another aspect is that it will increase the usefulness of transferring data between different centres. This also applies to the implementation of for instance decision limits derived from scientific publications, which is not safe if different techniques and standards are used in publishing and receiving centres.

From the scientific point of view, it is quite understandable that the advancement of both scientific and clinical andrology has been very slow without proper standardization in the basic laboratory work. Basing studies on men in infertile couples or other andrological disorders on laboratory techniques that allow a high degree of random errors is not likely to deliver results that are succinct. Contradictory results can then be expected from other studies with similar lack of standardized laboratory techniques (Group ECW 2018). Thus, both from a scientific and a clinical perspective, it can be considered highly unethical if standardized techniques with proper internal and external quality control are not used.

Although it may appear to be overkill to require a theoretical error margin of less than ±10%, many common laboratory practices (procedures and equipment) are performed without any awareness of the level of error associated with the chosen practice. The cost for errors in diagnostics is difficult to estimate, but there are examples of how unknown imprecision can cause unintentional suboptimal choice of treatment modality (Björndahl et al. 2016).

There must certainly be a balance between equipment cost, staff workload and patient benefit. However, before a laboratory can decide to abstain to use recommended standardized procedures and training programmes, the management must consider the level of uncertainty and risk for errors the alternative procedures bring about. The cost for such problems must be weighed in comparison with the cost of using procedures providing more reliable results.

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A Simple but Dramatic Technical Improvement in the Diff Quik Stain Protocol Used for Preparing Specimens for Sperm Morphology Evaluation (Improved Diff Quik Stain Protocol for Preparation of Sperm Morphology Evaluation Specimens)

F. Aono, K. Ochiai, T. Ueno, T. Okubo, and S. Teramoto

Background: Understanding an accurate semen analysis result is crucial for planning a couple's appropriate infertility treatment. In particular, assessment of sperm morphology is difficult but critically important to accurately ascertain. The Diff Quik stain protocol, which was introduced in the WHO semen analysis manual (5th edition), is simple and rapid, but the staining patterns are highly variable.

Main Questions: Does the Diff Quik stain protocol create a homogeneous stained sample, and can reproducibility be improved?

Experimental Design: We compared semen volumes of 3, 5, and 10 μL respectively for smear samples; duration in fixation solution of 10, 30, and 60 s in the stain solution II; and running water versus a tiny 150 μL volume of water, to eliminate the excess stain solution II.

Main Results: We obtained the most consistent results with a 3 μL semen smear sample volume, 30 s of duration in fixation solution, 30 s in the stain solution I, 60 s duration in staining solution II, and 150 μL of water for eliminating the excess stain solution II. We applied this improvement in the Diff Quik stain protocol to mouse Macaque monkey and human semen samples with the same consistent results.

Conclusions: We have established an improvement of the Diff Quik stain protocol for evaluation of sperm morphology, and this new protocol has made it possible to obtain reliable, and repeatable results with uniform consistency for precise evaluation of sperm morphology.

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Part IX
Fertility and Infertility

Several free communications have been submitted. Poster abstracts in the theme Infertility and Fertility are presented here, other under the specific themes.

Intracellular Viruses Identification in Sperm Assay of Patients with Fertility Problems

V. V. Ashapkin, M. J. Suhomlinova, A. Shakhov, and E. E. Bragina

Background: There are quite a number of viruses known that can infect the semen: human immunodeficiency virus, human papillomavirus, hepatitis B and hepatitis C viruses, herpes simplex virus (HSV), Epstein–Barr virus (EBV), and cytomegalovirus (CMV). For some of these viruses, intracellular localization in spermatozoa has been proven or discussed earlier. In our previous studies, we have detected intracellularly localized capsids of the Herpesviridae family viruses. But the role of vertical virus transmission for vertical dissemination of infection as well as for further abnormalities of embryo development is not clear up to now.

Main Questions: The main objectives of this study were to identify the viruses and to study the scale of viral infection in sperms from fertile patients and those from patients varying in infertility history.

Experimental Design: We examined semen of 75 fertile men (group I), 95 patients whose wives had a history of miscarriage (group II), and 67 patients with a complaint of no pregnancy for at least a year (group III). Patients with low spermatozoa quantity (less than ten million sperms/mL) were excluded from this study. We have undertaken transmission electron microscopy (TEM) analysis of spermatozoa with quantitative evaluation of the obtained data. DNA from human sperm samples with and without revealed by TEM viruses was isolated by a Chelex-100 method (Manuja et al. 2010). The viral DNA content was measured by quantitative SYBR Green PCR using three primer pairs specific for HSV1, CMV, or EBV DNAs. The results obtained were normalized by the dosage of a single-copy human gene (NOG)

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in the same sperm DNA samples. Since there is one copy of each single-copy gene per spermatozoid, these normalized values correspond to the average number of viral DNA copies per spermatozoid. Knowing the percentage of the spermatozoa containing viral particles from the TEM results, we could calculate the average viral content per infected spermatozoid.

Results: Sperm infection in group I was significantly lower than that of group II (3.12 ± 2.80 vs. 5.64 ± 3.65%, $p = 0.0004$) and lower than that of group III, but this difference was nonsignificant (4.33 ± 3.28%, $p = 0.05$). Detection rate of virus capsids was 24% (group I), 52% (group II), and 33% (group III). We did not find any difference in motility and sperm morphology between all examined groups. Viral capsids were detected in spermatozoa of normal morphology in each of the analyzed groups.

As concerning the results of viral DNA identification in sperm DNA samples investigated, we have observed the values from several copies to several 100 copies of CMV DNA per sperm, whereas HSV1 and EBV DNAs were never detected.

Conclusions: Quantities and detection rate of CMV revealed inside spermatozoa were higher among males whose wives had a history of miscarriage. The infected spermatozoa may be of normal morphology.

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Association Between Vitamin D Intake and Vitamin D Status with Semen Parameters Among Young Men in Southern Spain

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Background: We have previously reported that semen quality has drastically declined in Southern Spain during the last decade. Low serum vitamin D has been associated with low sperm motility in some epidemiological studies. In Spain, despite a high average sun exposure, population studies have shown that vitamin D serum levels are suboptimal and that a higher than expected percentage of the general population is vitamin D deficient. Changes in social mores and the perception of sun exposure as a health risk may have contributed to reduced exposure of unprotected skin to effective sun exposure.

Main Questions: Is there an association between vitamin D dietary intake and serum levels of vitamin D [25(OH)D2+3] and semen parameters in healthy young men?

Experimental Design: Cross-sectional study of 204 young university students (18–23 years old) recruited between 2010 and 2011 in Southern Spain.

Main Results: Only 2% of the participants were vitamin D deficient (< 30 nmol/L), and 18% had insufficient levels of vitamin D (<50 nmol/L). Sixteen percent of the men had low sperm concentration and 5% low progressive sperm motility. Varicocele was detected in 15% of the participants. In the analyses, semen quality parameters did not differ significantly by vitamin D status (all $p \geq 0.08$). Nonetheless, among men with values below the lower limit for semen parameters, especially sperm progressive motility, lower median 25(OH)D were observed ($p = 0.07$), although differences were not statistically significant. Among young men with predominantly sufficient serum 25(OH)D, there was no evidence for an association.

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Conclusions: This is the first study to examine this association in a random sample of Mediterranean young men unselected for fertility status. Further investigations are needed in order to confirm whether vitamin D deficiency is associated with low semen quality. It is unclear whether low semen quality may be improved by increasing 25(OH)D status in this population.

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Single-Cell Analysis of Intracellular Calcium Signalling of Patient Sperm and Its Relation to IVF Success

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Background: Progesterone (P4) influences human sperm function by stimulating an elevation of intracellular calcium concentration [Ca2+]i. Assessment of the P4 response in sperm populations indicates an association between poor [Ca2+]i increase and reduced fertilisation capacity. Analysis of P4 sensitivity single donor sperm demonstrates heterogeneity in responsiveness and dynamics of [Ca2+]i increase.

Main Questions: What is the nature of the P4-induced [Ca2+]i in single sperm from sub-fertile men and is there a relationship between the P4-sensitivity and intracellular Ca2+ dynamics to fertilisation outcome at IVF?

Experimental Design: Single cell intracellular calcium imagining was conducted on patient sperm that was surplus to requirement for ART treatment. Cells were studied on the day of treatment which allowed correlation of P4-sensitivity and ART outcome. The patients were grouped into three categories; ICSI, IVF failed fertilisation (IVF-FF), and IVF successful fertilisation (IVF +ve).

Main Results: All patient sperm samples were sensitive to P4. However, the percentage of patient cells that responded was significantly lower compared to donor sperm samples. IVF-FF sperm responded the least (98.1 ± 0.5 vs. 72.5 ± 7.7%, $P < 0.01$) and as a group had a mean peak P4 response (ΔFluorescence) that was significantly lower than that of donors ($P < 0.01$). ΔF of ICSI patient sperm was also significantly lower ($P < 0.05$). Analysis of absolute fluorescence revealed that IVF-FF sperm had significantly elevated basal fluorescence, therefore, linear regression analysis was conducted by plotting resting F versus F-increment for every cell for each sample. This analysis revealed a number of samples in all patient groups that had significantly impaired responsiveness to P4 and is correlated with

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fertilisation rate. Sperm samples that failed to generate [Ca2+]i oscillations in response to P4 were less likely to fertilise at IVF.

Conclusion: Impaired calcium signalling in response to P4 is a common feature of sperm from sub-fertile men and impacts on fertilisation potential.

Sperm from a Patient with a Homozygous In-Frame Deletion in CATSPERE Lack Functional CatSper Expression and Fail to Fertilise at IVF

S. G. Brown, P. V. Lishko, S. J. Publicover, C. L. R. Barratt, and S. M. Martins da Silva

Background: CatSper is a multi-subunit sperm-specific calcium channel that mediates the progesterone-induced increase in intracellular calcium. Evidence from CatSper subunit knock-out mouse models implicates it as essential for sperm fertilisation competence. Similarly, impaired or absent P4 response in patient sperm is associated with poor or loss of fertilisation at IVF. However, genetic evidence in a limited number of cases reported genomic deletions spanning a number of loci. We reported a unique case (Williams et al., 2014) of a man (patient 1) with sperm that lacked functional CatSper expression but he had no genetic errors in CatSper subunit coding regions. Subsequently, two new proteins have been proposed to be part of the mature complex (CatSper-epsilon and CatSper-zeta).

Main Questions: Does a patient with a reported defect in CatSper functional expression have a genetic defect limited to CatSper-epsilon and/or zeta?

Experimental Design: Exome data from patient 1 were analysed for genetic errors in CatSper-epsilon and CatSper-Zeta. Sanger sequencing was conducted to confirm the presence of a genetic defect.

Main Results: Patient 1 is homozygous for an in-frame 6-bp deletion in exon 18 (c.2393_2398delCTATGG, rs761237686) of CATSPERE that is predicted to be highly deleterious. Additional non-pathogenic intronic variations were also identified.

Conclusion: The reported mutation is the probable cause of loss of functional expression of CatSper in sperm from patient 1. However, further in vitro studies and population genetic analysis are necessary to confirm this hypothesis.

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Changes in Pattern of Protein Phosphorylation in Bull Testicular and Epididymal Sperm

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Background: The correct molecular "configuration state" created during the epididymal maturation is essential for the sperm's ability to capacitate and fertilize the oocyte. Whereas the mature spermatozoa (as specialized cells) are highly compartmentalized, transcriptionally inactive and thereby unable to synthetize new proteins, the importance of phosphorylation as a means of altering their function is greater than in many other cell types.

Main Questions: To monitor the bull-specific pattern of protein phosphorylation on testicular and epididymal sperm.

Experimental Design: The fluorescent pattern representing localization of tyrosine phosphorylated proteins was analysed using the anti-phosphotyrosine antibodies (P-Tyr-01 and 4G10) in spermatozoa isolated from different regions of epididymis—caput, corpus and cauda and in spermatozoa within the epididymal and testes tissue sections in immunofluorescence assay. Additionally, identification of the changes in tyrosine phosphorylated proteins pattern during the epididymal maturation of bull spermatozoa (phosphoproteomic study) (PY20) using western blot analysis was also referred.

Main Results: Six different staining patterns of both anti-phosphotyrosine antibodies (P-Tyr-01 and 4G10) in epididymal sperm samples were observed and their portion was segment-dependent. These findings were also confirmed by evaluation

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of tissue of cryosections. The results obtained by immunofluorescence were also supported by western blot analysis.

Conclusion: The increasing trend of tyrosine phosphorylation of proteins during the maturation of bull sperm in epididymis was consistently detected by all experiments. Although the post-testicular maturation of spermatozoa is a process common to all mammals, species-specific features of protein phosphorylation were found out upon bull sperm maturation in epididymis.

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Proteomics and Biomarker Identification in Improved Sperm Motility Parameters After 4 h of Ejaculatory Abstinence

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Introduction

Increased ejaculatory abstinence (EA) is commonly used as a cost-effective mechanism of increasing the total sperm count (T.S.C.) in the semen of patients providing sperm for assisted reproductive technologies (ART) (Bahadur et al. 2016). Therefore, manipulating the duration of EA has notable effects on basic semen parameters, including sperm concentration, motility and seminal plasma constituents. These factors remain primary predictors for male infertility diagnosis, despite interindividual variations in semen characteristics. As stated by the prescribed guidelines of the World Health Organization (WHO), subjects must remain abstinent for a minimum period of 48 h, but not longer than 7 days prior to collecting a sample for a standard semen analysis (WHO 2010), yet many publications referring to these recommendations are often contradictory (Comar et al. 2017; Raziel et al. 2001; Scarselli et al. 2019). Recent studies have contributed to the inconclusive and inconsistent nature of EA by suggesting EA periods of 4 h or less, as a strategy to improve

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sperm quality from a functional perspective (Alipour et al. 2015; Ayad et al. 2018b; Bahadur et al. 2016; Lehavi et al. 2014). In a previous study conducted in this laboratory, we observed enhanced sperm total motility and progressive motility after only 4 h of EA compared with 4 days (Ayad et al. 2018b). Similarly, Bahadur et al. (2016) found that only 40-min EA improved sperm motility and kinematics, and Alipour et al. (2017) also observed improvements in sperm motility, velocity, progressiveness and hyperactivation after only 2 h of EA. Additionally, Mayorga-Torres et al. (2015) observed that daily successive ejaculations over a 2-week period did not negatively influence sperm concentration, motility, morphology, vitality and other functional sperm parameters. Agarwal et al. (2016) found that normospermic men providing semen samples after 1, 2, 5, 7, 9 and 11 days of EA had lower levels of sperm DNA fragmentation as EA period was reduced.

These findings also extend into clinical ART where studies have shown significantly higher pregnancy rates when using sperm from men who abstained for 1–3 h (Li et al. 2018; Shen et al. 2019), 24 h (Borges Jr et al. 2018) and 2 days or less (Marshburn et al. 2010), despite the reduced sperm concentration, T.S.C. and semen volume compared to longer EA periods. Furthermore, Scarselli et al. (2019) found little difference in sperm concentration, motility and morphology yet it was translated into producing better quality blastocysts from a genetic perspective, with significantly higher euploidy rates when using sperm from men who abstained for 1 h compared to longer periods of EA. Borges Jr. et al. (2018) attributed these observations to the detrimental effects which EA periods of greater than 4 days have on sperm DNA, and how this directly reduces the pregnancy rates from intracytoplasmic sperm injection (ICSI) cycles. Marshburn et al. (2010) however, initially did not attempt to explain the physiological reasoning behind the observed increases in pregnancy rates from male partners who underwent less than 2 days of EA. However, a subsequent study performed by the same clinic did identify higher total antioxidant capacity (TAC) after shorter periods of EA (<1 day), which could diminish oxidative damage via an alternative mechanism which does not influence sperm membrane lipid peroxidation (Marshburn et al. 2014). This sentiment was reiterated by Shen et al. (2019), in explaining increased implantation, clinical pregnancy and live birth rates using sperm from male partners having undergone 1–3 h of EA.

Ejaculated spermatozoa are terminally differentiated cells; therefore, the main functional changes they undergo are invoked by the immediate seminal plasma microenvironment and the subsequent conditions of the female reproductive system (Elzanaty et al. 2005; Said et al. 2009). This seminal plasma microenvironment is imperative as sperm are the only cells which perform their functions outside of the body (Baccetti 1984). Consequently, epididymal and accessory sex gland secretions, which constitute the seminal plasma, are of particular interest in this study. The primary function of the prostate gland is to produce and release large amounts of ionised derivatives of citric acid in the form of citrate into the prostatic fluid (Kavanagh 1994). Furthermore, the prostate releases a variety of other molecules which play important roles in fertilisation. For example, prostate-specific antigen (PSA) is a glycoprotein enzyme capable of liquefying the seminal coagulum, thereby allowing sperm to swim freely (Balk et al. 2003). Zinc released by the

prostate aids in sperm DNA stabilisation, and low levels of seminal zinc are correlated with low fertility rates (Canale et al. 1986). The seminal vesicles serve as the main producers of fructose and glucose, which are largely accepted as being the primary sources of ATP for human spermatozoa (Mann 1946; Mukai and Okuno 2019; Westhoff and Kamp 1997). This has been reiterated by showing that glycolysis in the principal piece of the flagellum is critical for normal sperm motility (Turner 2003). Seminal vesicles also release amino acids, flavins, phosphorylcholine, prostaglandins, vitamin C and a range of proteins and enzymes (Mann 1946; Schoenfeld and Numeroff 1979). These molecules released by the seminal vesicles have a variety of roles in sperm maturation and in the acquisition of fertilising potential. The epididymis is primarily responsible for the release of neutral alpha-glucosidase (NAG) (Cooper et al. 1990), as well as proteins that prevent the premature onset of capacitation during epididymal transit and storage (Fraser et al. 1990). The relationship between NAG and sperm motility remains unclear because several studies have shown positive correlations between seminal concentrations of NAG and sperm motility (Elzanaty et al. 2002; Fourie et al. 1991; Viljoen and Du Plessis 1990) and others have reported negative correlations (Guerin et al. 1990; Krause and Bohring 1999). Consequently, changes in the ratios of selected epididymal and accessory sex gland biomarkers in seminal plasma have been widely used as clinical reflections of global changes in the volume of secretions of each of these glands (WHO 2010).

Apart from the above-mentioned constituents of seminal plasma, many studies have focused on identifying key proteins linked to infertility and poor basic semen parameters, however understanding the proteins involved in less-studied conditions, such as the seminal plasma microenvironment after 4 h of EA, may be significant in understanding the mechanisms involved in the impact of EA on basic semen parameters. A vast array of extracellular proteins have been identified in seminal plasma with diverse effects on sperm function pre- and post-ejaculation, including the modulation of sperm motility (Baas et al. 1983; Bernardini et al. 2011; Graham 1994), viability (Ashworth et al. 1994), response to reactive oxygen species (ROS) (Hamada et al. 2013; Sharma et al. 2013a, b) and overall function (Caballero et al. 2012; Mann and Lutwak-Mann 1981). With current advances in genomics and transcriptomics, proteomics technology has become a powerful tool in the research of human physiology (Li et al. 2018) to identify and categorize new biomarkers for diagnosis, prognosis and treatment (Aebersold and Mann 2003; Binz et al. 2003). Furthermore, proteins identified in seminal plasma are important in the capacitation of the spermatozoa, modulation of the immune responses in the uterus, the formation of the tubal sperm reservoir (Evans and Kopf 1998; Jansen et al. 2001) and ultimately in both the sperm–zona pellucida (ZP) interaction and the sperm and oocyte fusion process (Primakoff and Myles 2002; Yi et al. 2007). Despite reflecting sperm mitochondrial activity reduction, acrosome damage and DNA fragmentation, the seminal plasma proteome directly reflects spermatogenesis and epididymal maturation status in normozoospermic men (Intasqui et al. 2016). Approximately 10% of the proteins identified in seminal plasma are of testicular or epididymal origin, while the rest originate from accessory sex glands and other parts of the body

(Batruch et al. 2011). Studies have shown that epididymal proteins play a key role in sperm quality and that proteins directly associated with testicular function may be found in seminal plasma (Intasqui et al. 2013; Milardi et al. 2012; Pilch and Mann 2006; Wang et al. 2009). Due to the wide variation of seminal plasma protein origin and functions, seminal proteins could potentially be used for diagnosis and monitoring of pathways and interactions affecting male fertility, such as non-obstructive azoospermia, obstructive azoospermia, asthenozoospermia, varicocele and vasectomy (Batruch et al. 2011; Drabovich et al. 2011; Heshmat et al. 2008; Intasqui et al. 2013; Wang et al. 2009; Zylbersztejn and Fraietta 2013). Within the rapidly evolving field of proteomics, the establishment of extensive databases of proteins specific to spermatozoa and seminal plasma allows for advanced research into male infertility, extending to scenarios such as diagnostics and subsequent treatment approaches in idiopathic cases (Oliva et al. 2009).

For the purpose of the present study, basic semen parameters including sperm kinematics were selected as adequate measures of semen and sperm quality. Furthermore, citric acid, NAG and fructose were selected as key biomarkers of prostate, epididymal and seminal vesicular secretion and function, respectively. Additionally, possible direct effects of variations in the concentrations of these biomarkers on sperm functional improvements were explored along with the proteomic profile of the seminal plasma microenvironment and how these proteins may interact with the sperm in pre- or post-ejaculation when comparing samples after 4 h and 4 days of EA.

Materials and Methods

Prior to the beginning of the study, ethics approval was obtained from the Health Research Ethics Committee of the Faculty of Medicine and Health Sciences at Stellenbosch University. Informed written consent was provided by all subjects, and the study was performed in accordance with the Declaration of Helsinki (World Medical Association 2014). Freshly ejaculated semen samples were collected from 16 consecutive healthy donors (aged 19–25 years) at the laboratories of the Stellenbosch University Reproductive Research Group (SURRG). All samples were collected according to WHO (2010) guidelines in a location close to the laboratory and received within 10 min of collection. The first sample from each donor was collected after an EA period of 4 days; the second sample was collected from the same donor 4 h subsequent to the first collection. Samples were immediately placed in an incubator (37 °C, 5% CO_2, 30 min) and allowed to fully liquefy before further processing. Donor information including identification, age, date of semen collection, time of semen collection, abstinence duration, sample volume, pH, appearance, colour, odour, liquefaction, viscosity and agglutination was recorded. Sperm were isolated from the semen using double-wash method and seminal plasma was frozen and stored in liquid nitrogen for biochemical analyses. Sperm concentration, motility, kinematic and velocity parameters were determined using computer-aided

sperm analysis (CASA) with a Sperm Class Analyser version 5.4 (SCA 5.4, Microptic SL, Barcelona, Spain). The kinematic parameters (see Supplementary Table 1) measured were as follows: curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), linearity (LIN), straightness (STR) and Wobble (WOB).

Seminal plasma citric acid, fructose and NAG concentrations were measured using commercially available assay kits (Citric Acid, Fructose and Episcreen plus assay kits respectively; FertiPro) according to the manufacturer's instructions. Absorbance values were measured spectrophotometrically for citric acid and NAG at 405 nm and fructose at 470 nm using a FLUOstar Omega microplate reader (BMG Labtech). Citric acid and fructose concentrations are expressed in milligrams per millilitre, whereas NAG activity is expressed as milli-international units per millilitre.

A detailed description of the proteomic analyses is available as Supplementary Methods. The proteomic analyses of seminal plasma and the protein concentration of each of the 16 samples were measured using a Direct Detect® Infrared Spectrometer. Samples were then pooled in groups of four where each sample contributed an equal amount of protein (240 µg). SDS-PAGE was then performed on each group and gels were stained with "Coomassie brilliant blue" and subsequently washed with 25% methanol. Gels were sliced and de-stained with 200 mM NH_4HCO_3:acetonitrile 50:50 (Sigma-Aldrich, St. Louis, USA). In-gel digestion was performed as described in Supplementary Methods. LC-MS/MS was performed on a Thermo Scientific™ Ultimate™ 3000 RSLC nano and a Thermo Scientific Fusion Tribrid mass spectrometer equipped with a Nanospray Flex ionization source (Thermo Fisher Scientific, Waltham, USA).

Statistical Analyses

Comparisons between parameters measured after 4 days and 4 h of EA were performed using both Students paired *t*-test for normally distributed data sets and Mann-Whitney U-tests for nonparametric data sets, on GraphPad Prism© version 7.00 for Windows (GraphPad Software, La Jolla California, USA) and StatAdvisor™ (Statpoint Technologies, Inc., Warrenton Virginia, USA) for two sample paired comprehensive tests (*t*-test, signed-rank test, chi-square and Mann–Whitney (Wilcoxon) W-tests). All values are presented as mean ± SE. Statistical significance was set at $p < 0.05$. The raw files generated by the mass spectrometer were imported into Proteome Discoverer v1.4 (Thermo Fisher Scientific, Waltham, USA) and processed using the Sequest algorithm. The results files were imported into Scaffold 1.4.4 and identified peptides validated using the X!Tandem search algorithm included in Scaffold. Peptide and protein validation were done using the Peptide and Protein Prophet algorithms. Protein quantitation was performed performing a *t*-test on the paired data with the Hochberg–Benjamini correction applied. Protein quantitation was performed performing a *t*-test on the paired data with the

Hochberg–Benjamini correction applied. STRING.org interaction software database was utilized for pathway analysis and protein interaction of DEPs.

Results

As indicated in Fig. 1a–c, semen volume (P = 0.025), sperm concentration (P = 0.018) and subsequent T.S.C. (P < 0.001) were significantly decreased after 4 h compared with 4 days EA. A significant increase in the percentage of sperm total motility (71.8 ± 3.5 vs. 64.7 ± 3.5%, respectively; P = 0.03) and progressive motility (58.5 ± 3.4 vs. 47.7 ± 3.6%, respectively; P = 0.001) was observed after 4 h of EA, whereas no significant difference in the percentage of static (28.3 ± 3.5 vs. 35.5 ± 3.5% after 4 h and 4 days EA, respectively) or non-progressive (13.3 ± 0.9 vs. 16.2 ± 1.0% after 4 h and 4 days EA, respectively) spermatozoa was observed (see Fig. 1d). Kinematics assessment showed that VCL (91.8 ± 6.2 vs. 78.6 ± 4.0 μm s^{-1}, respectively; P = 0.035) and VAP (58.1 ± 2.897 vs. 49.6 ± 2.2%, respectively; P = 0.006) were also both significantly higher after 4 h versus 4 days of EA (see Fig. 1e, f). Furthermore, Table 1 shows that, after 4 h versus 4 days EA, there were significantly lower concentrations of citric acid (P = 0.037), NAG (P = 0.005) and fructose (P = 0.008). Additionally, when considering T.S.C, the absolute amount of fructose per spermatozoon was significantly lower after 4 h of EA (P = 0.037), whereas there was no significant effect of duration of EA on NAG or citric acid.

A total of 2889 proteins were identified in the seminal plasma of the entire study population, of which 22 extracellular proteins being significantly upregulated after 4 h of EA, and 2 extracellular proteins upregulated after 4 days of EA (see Table 2). The GO annotations revealed their involvement in carbohydrate binding, enzyme activator activity, protein binding, GTPase and ATPase activity. The variety of biological processes and cellular locations are presented as supplementary figures (Supplementary Fig. 1a, b).

Discussion

Figure 1a–c clearly shows that semen volume, sperm concentration and T.S.C. were reduced after 4 h of EA as expected. However, interestingly, total motility, progressive motility and several sperm kinematic parameters, including VAP, VCL and the proportion of rapid sperm were significantly higher after 4 h when compared to 4 days of EA (see Fig. 1d–f). These results are in line with two large studies performed by Comar et al. (2017) and Levitas et al. (2005), who found that the duration of EA had a significant direct relationship with sperm concentration and semen volume, yet a significant indirect relationship on sperm motility. The increase in sperm concentration and semen volume was attributed to the accumulation of sperm

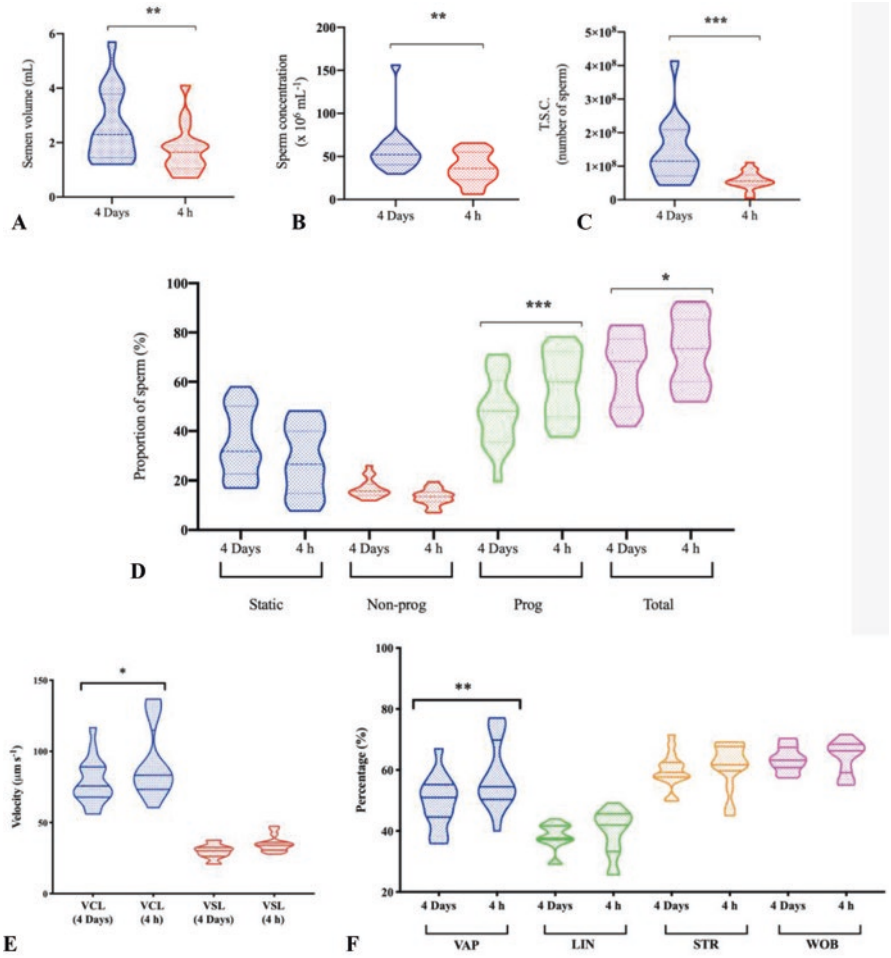

Fig. 1 Summary of basic semen parameters as measured with the Sperm Class Analyser (V5.4, Microptic) (**a**) semen volume, (**b**) sperm concentration, (**c**) T.S.C, (**d**) sperm motility parameters and (**e**, **f**) kinematics, after 4 days and 4 h of ejaculatory abstinence (EA). Data are the mean ± S.E.M. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; *T.S.C.* total sperm count. *Non-prog* non-progressive motility, *Prog* progressive motility, *total* total motility, *LIN* linearity, *STR* straightness, *VAP* average path velocity, *VCL* curvilinear velocity, *VSL* straight-line velocity, *WOB* wobble

reserves and the subsequent storage of these reserves in the epididymis, whereas the motility decreases were attributed to elevated levels of reactive oxygen species (ROS) and the ensuing DNA damage experienced by stored spermatozoa over longer periods of EA. Damaged spermatozoa (Iwasaki and Gagnon 1992) or infiltrating leukocytes (Kessopoulou et al. 1992) are the sources of ROS, which are directly correlated with reducing sperm motility parameters

Table 1 Summary of specific epididymal and accessory sex gland secretions per millilitre of seminal plasma and per spermatozoon after 4 days and 4 h of EA (Mean ± S.E.M)

Parameter	4 Days EA	4 h EA	Paired *t*-test (*P*-value)	Mann-Whitney *U*-test (*P*-value)
Concentration (mL^{-1})				
Citric Acid (mg mL^{-1})	8.01 ± 1.35	5.96 ± 1.23	–	0.037
NAG (IU mL^{-1})	29.32 ± 3.53	19.92 ± 3.61	0.005	–
Fructose (mg mL^{-1})	4.07 ± 0.52	1.66 ± 0.52	0.008	–
Absolute amount (spermatozoon^{-1})				
Citric Acid (ng $sperm^{-1}$)	0.165 ± 0.033	0.254 ± 0.059	–	ns
NAG (nIU $sperm^{-1}$)	0.056 ± 0.080	0.570 ± 0.130	ns	–
Fructose (ng $sperm^{-1}$)	0.045 ± 0.016	0.036 ± 0.011	–	0.037

EA ejaculatory abstinence, *NAG* neutral alpha-glucosidase

From the data shown in Table 1, it is clear that all the measured accessory sex gland secretions decreased after 4 h of EA, apart from citric acid concentration per millilitre and NAG per spermatozoon. These significant reductions in citric acid, NAG and fructose concentrations after 4 h of EA may be due to the insufficient time for the epididymis and accessory sex glands to produce their respective secretions, resulting in a decrease in semen volume associated with short abstinence (Goss et al. 2019; Levitas et al. 2005). When considering the quantity of each accessory sex gland secretion per spermatozoon, a significant reduction in fructose was observed. This observation contradicted the hypothesis that an increase in sperm motility should relate to an increased availability of glycolysable substrates. With variations in sperm concentration, available energy for sperm function may vary too. The primary energy source for human sperm appears to be glucose, metabolized and utilized via the glycolysis pathway, as well as pyruvate produced by this process, rather than oxidative phosphorylation. Visconti (2012) stipulated that ATP production in the individual sperm compartments is vital for various roles, such as hyperactivation and acrosome reaction. Seminal fructose and glucose are considered to be the main sources of energy by glycolytic breakdown in the principal piece of human spermatozoa, as well as being critical for normal sperm motility (Goodson et al. 2012; Mann 1946; Patel et al. 1988; Turner 2003). These glycolysable substrates are able to maintain high proportions of motile spermatozoa and increases in tyrosine phosphorylation, whereby midpiece mitochondria are supplied with pyruvate from the glycolytic breakdown of fructose and glucose (Goodson et al. 2012). It has been stated that human sperm motility relies largely, if not solely, on the glycolytic breakdown of sugars, yet the mere presence of mitochondrial machinery cannot be ignored (Rees et al. 1990; Williams and Ford 2001). Travis and Moss (1998) observed that spermatozoa maintain motility in the presence of oxidative phosphorylation un-couplers, indicating that mitochondrial respiration is essential to other processes and not primarily sperm flagellar movement. This observation was further reiterated by Miki et al. (2004). The balance between glycolytic energy production and mitochondrial oxidative phosphorylation is species-specific and

alternate between these two processes or function synergistically depending on substrate availability (Storey 2004).

As previously mentioned, these metabolic reactions are relegated to the principal piece of the spermatozoon, as the enzymes responsible for glycolysis and gluconeogenesis are concentrated in this portion of the flagellum (Eddy et al. 2003; Martínez-Heredia and Oliva 2006; Storey and Kayne 1975; Travis and Moss 1998). Oxidative phosphorylation in the midpiece also provides ATP with the ability to maintain low levels of tyrosine phosphorylation and hyperactivation in the presence of non-glycolysable substrates such as pyruvate, lactate and hydroxybutyrate and the absence of glycolysable substrates (Goodson et al. 2012).

Further analysis of seminal plasma using LC-MS/MS identified a total of 2889 unique proteins. Of these 22 extracellular proteins were upregulated after 4 h of EA, and 2 proteins upregulated after 4 days of EA (see Table 2). The large amount of

Table 2 Differentially expressed proteins from the comparison of samples from donors after 4 h and 4 days of EA (*Da* Dalton)

Accession number	Gene	Protein name	Molecular weight (Da)	*P*-value	Expression
Extracellular proteins					
Q6UW15	REG3G	Regenerating islet-derived protein 3-gamma	19,330	<0.0001	4 Days high, 4 h low
O00469	PLOD2	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 2	84,686	<0.0001	4 Days high, 4 h low
Q96DA0	ZG16B	Zymogen granule protein 16 homolog B	22,739	<0.0001	4 Days low, 4 h high
P62258	1433E	14-3-3 protein epsilon	29,174	<0.0001	4 Days low, 4 h high
P16035	TIMP2	Metalloproteinase inhibitor 2	24,399	0.00014	4 Days low, 4 h high
P09960	LKHA4	Leukotriene A-4 hydrolase	59,733	<0.0001	4 Days low, 4 h high
O14910	LIN7A	Protein lin-7 homolog A	25,997	<0.0001	4 Days low, 4 h high
Q99988	GDF15	Growth/differentiation factor 15	34,140	0.00028	4 Days low, 4 h high
Q8NFZ8	CADM4	Cell adhesion molecule 4	42,785	0.00042	4 Days low, 4 h high
Q8N5I2	ARRD1	Arrestin domain-containing protein 1	45,981	0.00025	4 Days low, 4 h high
Q8IYS1	P20D2	Peptidase M20 domain-containing protein 2	47,776	0.00031	4 Days low, 4 h high
Q9HBA9	FOLH1B	Putative N-acetylated-alpha-linked acidic dipeptidase	50,045	<0.0001	4 Days low, 4 h high
P11908	PRPS2	Ribose-phosphate pyrophosphokinase 2	34,769	<0.0001	4 Days low, 4 h high
P21291	CSRP1	Cysteine and glycine-rich protein 1	20,567	<0.0001	4 Days low, 4 h high

(continued)

Table 2 (continued)

Accession number	Gene	Protein name	Molecular weight (Da)	*P*-value	Expression
P19652	A1AG2/ ORM2	Alpha-1-acid glycoprotein 2	23,603	0.00013	4 Days low, 4 h high
P04433	IGKV3-11	Immunoglobulin kappa variable 3-11	12,575	<0.0001	4 Days low, 4 h high
P02511	CRYAB	Alpha-crystallin B chain	20,159	0.00013	4 Days low, 4 h high
P62979	RPS27A	Ubiquitin-40S ribosomal protein S27a	17,965	<0.0001	4 Days low, 4 h high
Q9H0R4	HDHD2	Haloacid dehalogenase-like hydrolase domain-containing protein 2	18,533	0.00029	4 Days low, 4 h high
Q8WUD1	RAB2B	Ras-related protein Rab-2B	24,214	<0.0001	4 Days low, 4 h high
O00754	MAN2B1	Lysosomal alpha-mannosidase	113,744	0.00038	4 Days low, 4 h high
P57735	RAB25	Ras-related protein Rab-25	23,496	<0.0001	4 Days low, 4 h high
P17066	HSPA6	Heat shock 70 kDa protein 6	71,028	0.00025	4 Days low, 4 h high
P20340	RAB6A	Ras-related protein Rab-6A	23,593	<0.0001	4 Days low, 4 h high
Intracellular proteins					
Q9H853	TUBA4B	Putative tubulin-like protein alpha-4B	27,551	<0.0001	4 Days low, 4 h high
P60891	PRPS1	Ribose-phosphate pyrophosphokinase 1	27,526	0.00023	4 Days low, 4 h high

proteins identified in seminal plasma highlights the wealth of information available in the constituents of the microenvironment to which post-ejaculatory sperm are exposed. Although this is essentially a snapshot into the physiological products and signals occurring in and around ejaculated spermatozoa, it may elucidate extrinsic influences and mechanisms by which sperm gain favourable traits such as improved motility and kinematics after successive ejaculations. Despite the direct availability of glycolysable substrates for sperm motility, sperm acquire motility and fertilizing capacity by interacting with a multitude of biologically active molecules throughout transit and storage in the male reproductive system. Supplementary Fig. 1a shows the biological processes of all the proteins identified by LC-MS/MS, whereby 145 and 142 proteins were involved in reproduction and reproductive processes respectively. Furthermore, a total of 815 proteins are involved in metabolic processes, whereas a total of 475 proteins are linked to developmental processes. Table 2 divides the DEPs into two distinct groups; those proteins primarily located intracellularly, which have limited scope in our analyses as they are most likely identified due to sperm and residual-cell lysis during the process of cellular preparation for analysis, and extracellular proteins such as those found in exosomes and those

released as paracrine signals in the male reproductive system, which are more important when considering the interaction between epithelial cells in the epididymis and the propagating sperm. These exosome-derived biomarkers are considered "treasure chests" filled with diagnostic molecules reflecting the status of the male reproductive system (Duijvesz et al. 2011; Poliakov et al. 2009; Simpson et al. 2009). Consequently, these secreted epididymal proteins have been extensively studied in order to improve outcomes of male infertility cases and conversely, an approach to novel modes of male contraception.

In order to explain the observed influence which EA has on basic semen parameters, we postulate four mechanisms functioning either independently or in unison, to improve sperm motility after 4 h of EA. First, the increase in sperm motility after 4 h of EA may centre on the preferable seminal plasma microenvironment created, whereby there is a reduced sperm concentration relative to the abundance of either glycolysable or non-glycolysable substrates for glycolysis and/or mitochondrial oxidative phosphorylation respectively. With fructose being significantly reduced, explaining this phenomenon from a metabolic standpoint must indicate either an abundance of another glycolysable substrate not measured (i.e. glucose) or an increase in oxidative phosphorylation of non-glycolysable substrates such as pyruvate or lactate. The ability of sperm physiology to select the preferred ATP source for maintenance of motility is not a foreign concept (Visconti 2012). Thus, with a decrease in glycolysable substrate availability in the seminal plasma, non-glycolysable substrates can still be converted to ATP by the mitochondria, thus fuelling the observed motility parameters (Bone et al. 2001; Goodson et al. 2012). Furthermore, the observed increases in sperm motility may not be due primarily to seminal plasma energy substrates, but rather the quality of the spermatozoon itself. Ayad et al. (2018a) suggested that the observed sperm functional improvements after 4 h compared with 4 days EA is due to the expulsion of degenerating and poor quality spermatozoa in the initial ejaculate when considering two successive ejaculates. In addition, as shown in Fig. 2a, it is clear that 10 of the 16 individual samples contributed more citric acid per spermatozoon after 4 h of EA, whereas Fig. 2b, c depicts a clearly lower proportional amount of fructose and NAG per spermatozoon: the contribution of 12 of 16 and 8 of 16 individual samples was proportionally less after 4 h of EA, respectively. The increases in citric acid per spermatozoon, although not statistically significant, cannot be ignored. The amount of citric acid per spermatozoon increased by a mean of 80%, indicating a possible explanation to the observed increases in sperm motility. In contrast, fructose decreased significantly by 20% after 4 h compared with 4 days EA**.** Elzanaty et al. (2005) found that shortened EA (2 days) is associated with decreased prostatic secretions and increased epididymal secretions**.** A previous study performed by Medrano et al. (2006) observed citric acid acting as a prominent substrate for energy metabolism in spermatozoa in addition to causing slight variations in seminal plasma pH; however, its relationship with and role in EA has not been adequately elucidated. An increase in the amount of available citrates for citric acid cycle utilisation in sperm metabolism may increase the energy available for sperm motility. As mentioned previously, spermatozoa use fructose originating from the seminal vesicles as carbohydrate

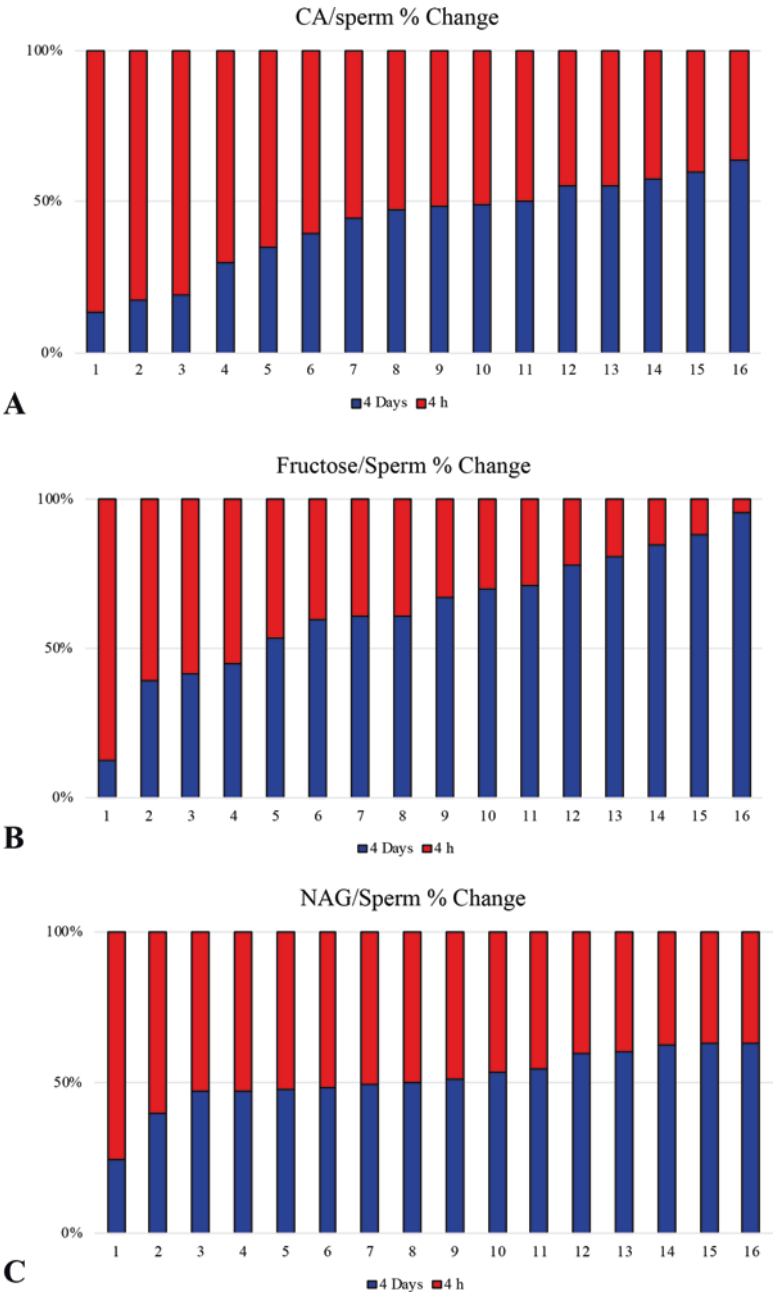

Fig. 2 Stacked columns showing percentage changes for each individual donor ($n = 16$) per spermatozoon in citric acid (CA; **a**), fructose (**b**) and neutral alpha-glucosidase (NAG, **c**). (Adapted from Goss et al. 2019)

source. Once absorbed by spermatozoa, the fructose is then metabolized into glucose and eventually converted to acetyl-coenzyme A (CoA), which drives the citric acid cycle, ultimately leading to the production of utilisable energy in the form of ATP, produced by the electron transport chain (Fig. 3). Furthermore, it has been shown that ATP production in spermatozoa preincubated in citrate increases without the addition of fructose (Medrano et al. 2006). It is believed that this occurs when citrates are converted to malate, an intermediary, that is subsequently used in the citric acid cycle in sperm mitochondria (Visconti 2012).

Finally, protein biomarkers may play an important, yet ancillary role in the observed improvements in sperm motility and kinematics after 4 h of EA when compared to 4 days. The protein interaction database analysis of DEP's in the seminal plasma from subjects after 4 h and 4 days of EA, was performed using STRING.org (see Fig. 4). Several clusters of protein interactions were identified and interestingly, of the four proteins involved in carbohydrate binding, two proteins being Zymogen granule protein 16 homolog B (ZG16B) and lysosomal alpha-mannosidase (MAN2B1) were upregulated after 4 h of EA. The other two proteins, regenerating islet-derived protein 3-gamma (REG3G) and procollagen-lysine,2-oxogluterate 5-dioxygenase 2 (PLOD2), were upregulated after 4 days of EA.

REG3G has been previously linked to acute immune response against gram-positive bacteria and not with any metabolic function in sperm (Mukherjee and Hooper 2009), and PLOD2 forms hydroxylysine residues in collagens, serving as sites for carbohydrate attachment important for the stability of collagen cross-links (Turpeenniemi-Hujanen and Kivirikko 1980). Interestingly, although ZG16B has only been linked to carbohydrate binding in the retina, MAN2B1 is pivotal in the catabolism of N-linked carbohydrates during glycoprotein turnover by cleaving all known alpha-mannosidic linkages (Gaudet et al. 2011). This may have implications in the fluidic mechanisms of carbohydrate utilization by sperm in the seminal plasma microenvironment and may highlight the ability of sperm to metabolize other monosaccharides as sources of ATP, including mannose (Mann 1967; Rodriguez-Gil 2006). Furthermore, Ras-related protein Rab-2B (RAB2B), Rab-25 (RAB25) and Rab-6A (RAB6A) are directly linked proteins which were upregulated after 4 h, these proteins are involved in GTP binding and facilitating protein transport in various cell types. These small GTP-binding proteins have been identified on human sperm acrosomal caps (Naz et al. 1992) and hamster sperm flagellum (NagDas et al. 2002). These proteins could be involved in flagellar motility through interaction with some downstream effectors, the extracellular signal-regulated kinase (ERK) pathway, protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K) (NagDas et al. 2002; Naz et al. 1992). These phosphorylation events, involving PKC (Rotem et al. 1990) and ERK1/2 (Lu et al. 1999), have been positively related to the acquisition and maintenance of sperm motility. Another protein upregulated after 4 h of EA is heat-shock 7 kDa protein A6 (HSPA6), which is a heat shock protein also linked to mitogen-activated protein kinase (MAPK) cascades including ERK1/2 (Shacoski 2012). Heat shock proteins are produced in response to cellular exposure to stressful conditions, and their expression has also been directly related to sperm motility in both porcine (Huang et al. 1999, 2000) and

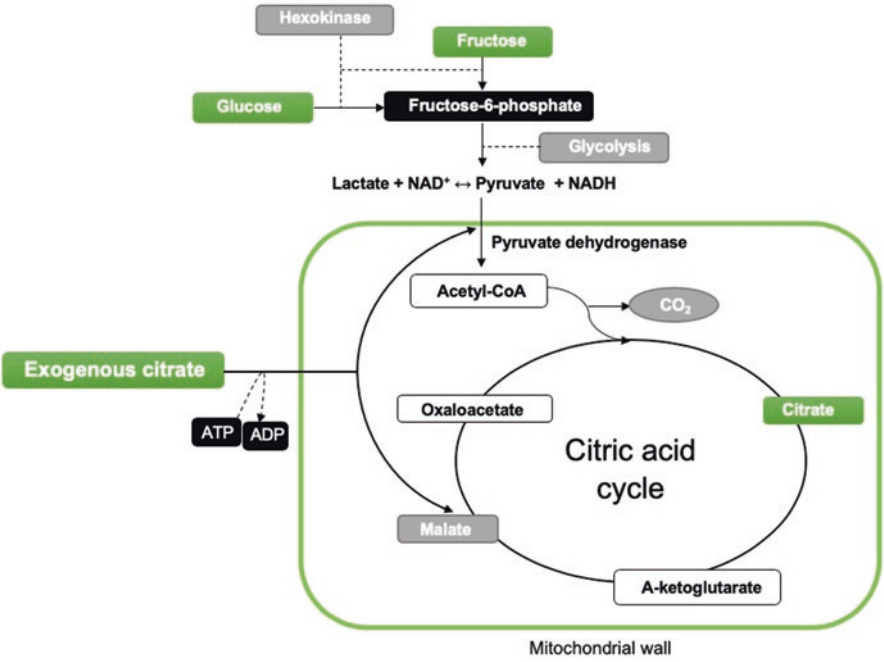

Fig. 3 Fructose and glucose enter the glycolytic pathway after phosphorylation by hexokinase and are subsequently converted to pyruvate via glycolysis to be used in the citric acid cycle. The entire citric acid cycle occurs within the inner mitochondrial wall. After entering the mitochondria through specific transporters, pyruvate can be oxidised to acetyl-coenzyme A (CoA) and CO_2. Acetyl-CoA then enters the citric acid cycle, which generates one high-energy bond in the form of GTP and four reduced compounds (three NADH and one FADH2) to be used in the oxidative phosphorylation process. Regarding citrate, when it is obtained from oxaloacetate inside the mitochondria as part of the citric acid cycle, exogenous citrate cannot permeate the inner mitochondrial membrane. Thus, before entering the cycle, it is first converted to malate and pyruvate by a series of enzymes using ATP. The electrons conserved in NADH and FADH2 are then used to reduce oxygen in the oxidative phosphorylation process. (Adapted from Visconti 2012 and Goss et al. 2019)

human (Cao et al. 2003) semen. With HSPA6 being upregulated after 4 h of EA, we may be observing an intrinsic response to epididymal storage of sperm from either the epididymal epithelial cells or the stored spermatozoa. Increased expression of heat shock proteins has been linked to ROS-related cell damage in sperm (Erata et al. 2008) and in other tissues (Dimauro et al. 2016; Mustafi et al. 2009; Zhang et al. 2018). Therefore, the increase in HSPA6 after 4 h of EA, may be due to the previous ejaculation, where levels of ROS-related sperm damage have been found to be higher than in periods of less than 1 day of EA (Agarwal et al. 2016; Gosálvez et al. 2011). This protection from ROS-related sperm damage may be contributing to the improved motility, which was seen after 4 h of EA.

To conclude, elucidating the mechanisms behind the improvement of sperm motility after EA periods of 4 h is clinically highly relevant in that a successive

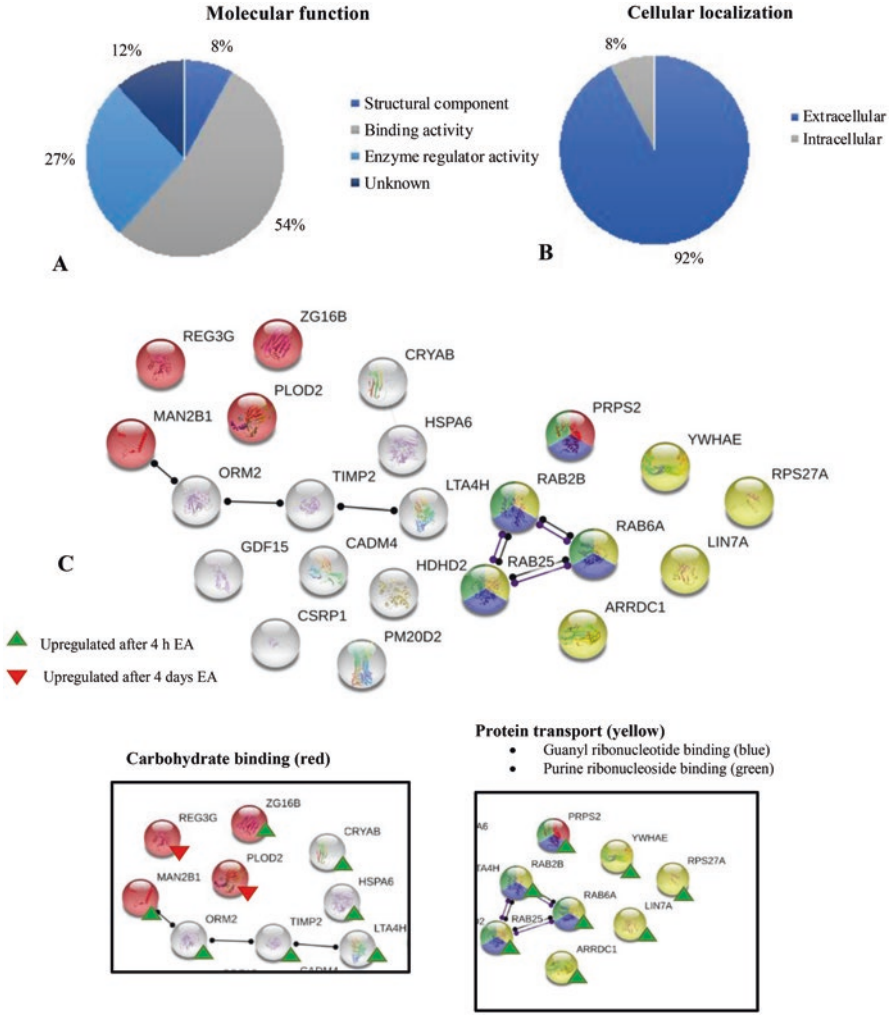

Fig. 4 GoMiner ontology analysis of molecular function (**a**) and cellular localization (**b**), protein interactions and clusters (**c**) revealed by STRING.org protein interaction database, of the 22 extracellular, differentially expressed proteins

ejaculation is often recommended in ART clinics after a poor sample is collected from a patient. Exploring the biochemical and physiological bases on which this practice is built upon, may serve to highlight the dynamic nature of sperm functional parameters in cases where EA is actively utilized and manipulated. This study highlights a gap in the understanding of how the seminal plasma microenvironment can impose notable changes on sperm motility and how the concentrations of the three major biomarkers of epididymal, prostatic and seminal vesicles secretion all decreased significantly after 4 h of EA, indicating reduction in the output of these

accessory sex glands, or a complex sperm selection mechanism relying on the physical positioning of sperm in the epididymis prior to ejaculation. A concomitant system of extracellular protein interactions has also been identified, giving an insight into the complex nature of this system and how many factors may influence sperm motility once sperm are ejaculated after variable EA.

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HSP90A May Control Spermatogenesis of Asian Elephant (*Elephas maximus*) Cryptorchid Testes

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Background: Cryptorchid testes in most mammals show the arrested spermatogenesis except a few mammals including elephants because of the heat. Induced stress by heat shock in the cryptorchid testes usually eliminates the germ cells by apoptosis or to protect the germ cells from the heat shock by an increase in the expression of heat shock factors (HSFs) and heat shock proteins (HSPs). Previously we showed that elephant spermatogenic cells conserve the same pathway to eliminate the defected germ cells as in the other animals although the ratio of apoptosis was low

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and they did not maintain the same pathway to protect cells through Hsp70 as shown in the other animals.

Main Questions: How can the elephant testicular cells survive under heat stress?

Experimental Design: To evaluate the heat shock responses in the elephant spermatogenic cells, testicular tissues were cultured under the experimental heat stress. To clarify the effects of the molecules, we performed inhibitor experiments using the same system. After culture, tissues were analyzed by immunohistochemistry and TUNEL using image analysis for investigation of the expression levels of heat stress related molecules.

Main Results: Here we show that HSP90A expression was initially increased after heat stress, and then HSP60 and ATP5A expression were increased in elephant spermatogenic cells. Surprisingly, proliferation and differentiation of elephant spermatogenic cells were also induced after heat shock. HSP90A inhibitor blocked these expressions. Furthermore, these molecules showed the unique localization in the elephant testis endogenously.

Conclusions: HSP90A may work as a key molecule for the regulation of the elephant spermatogenesis under heat stress.

Improved Sperm Function in Human Sperm Subpopulations: A Model for Studying Subfertility

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Background: Infertility is affecting one in four couples in developing countries, with the male factor being implicated in about half of these cases. In approximately 15% of the presumed sub- or infertile males, their semen analyses do not reveal obvious abnormalities (idiopathic), thereby conveying the importance of sperm functional testing. Furthermore, human semen is heterogeneous in nature, including subpopulations with different degrees of maturation and fertilizing ability. Evaluating the quality of such sperm subpopulations can reveal the functional capabilities of sperm in semen samples and may provide a model for studying sperm functionality in subfertile males.

Main Questions: This study aimed to determine and compare the functional characteristics of two sperm subpopulations found within the same human semen samples. Concomitantly, the improvement of such functional parameters was evaluated after exposure to various capacitating media and chemicals.

Experimental Design: Normospermic donor semen samples (n = 20) were separated into two fractions via 45% and 90% discontinuous AllGrad gradient centrifugation. These sperm fractions were assessed for their sperm functional characteristics, including evaluation of sperm vitality (BrightVit), CASA motility (SCA, Microptic SL, Barcelona), kinematics, hyperactivation (2 μM procaine, 5 μM caffeine), acrosome reaction (1 mM calcium ionophore), reactive oxygen species (ROS), and mitochondrial membrane potential (MMP) in human tubal fluid (HTF). Subsequently, sperm fractions were exposed to capacitating media, progesterone (1.98, 3.96, 19.8 μM), and myoinositol (11 mM), and sperm functionality was again assessed over time.

Main Results: AllGrad fractionation resulted in two distinctive sperm subpopulations, with the more motile (bottom) sperm fraction observed to have significantly higher percentages of sperm vitality, motility, swimming speed, hyperactivation,

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induced acrosome reaction, ROS negative, and intact MMP ($P < 0.05$) than the less motile (top) sperm fraction. After a 30 min exposure to capacitating media, progesterone, and myoinositol, there was a notable improvement in both sperm fractions' functional characteristics. Furthermore, after exposure, no differences were apparent between the two sperm fractions in terms of percentage sperm motility and hyperactivation, as well as sperm kinematics and acrosome reaction ($P > 0.05$) for most media.

Conclusions: Selected sperm functional parameters in lower quality sperm subpopulations could be improved to the level of a higher quality sperm subpopulation, separated from the same semen samples. If the lower quality sperm subpopulation mimics the sperm population in subfertile males, this can be used as a model to study such subpopulations and possibly broaden the potential treatment regimens and sperm isolation procedures for ART.

Absence of BSP do not Have an Important Effect on Male Fertility

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Background: Sperm encounter with various secreted proteins in epididymis resulted in gaining their motility and rearrangement of lipid/protein on the plasma membrane. A family of proteins named Binder of SPerm (BSP) expresses exclusively in male mammalian reproductive tract. Despite differences in the number of forms and concentration, they share analogs function and conserved structure in all the mammals. Studies have indicated an ambivalent function for these proteins in male reproductive tract by interacting with choline phospholipids (PC) on sperm membrane resulting in gaining motility and preventing premature capacitation. However, in the female genital tract, BPS proteins promote capacitation by interacting with components present in this environment. Recently, two homolog epididymal BSP proteins were identified in mouse (Bsph1 and Bsph2) and one in humans (BSPH1). In order to characterize and establish the role of these proteins in fertility knockout mice were produced and the fertility ability was assessed.

Main Question: What is the effect of lacking the Binder of SPerm proteins (BSPs) on male fertility?

Experimental Design: In order to define the role of BSP proteins in epididymis, the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated 9 (Cas9) was utilized to produce Bsph1 and Bsph2 knockout mice. The double knockouts were confirmed at mRNA (RT-PCR and qPCR) and protein (Mass spectrometry) level. The double knockout models were evaluated for animal fertility and several sperm parameters. Trio mating was performed to assess the fertility capability of the double knockout.

Main Results: Male mice without BSP proteins showed normal fertility, with no differences in litter size, motility, and sperm count compared to wild type. BSP null mice mated naturally and were healthy. Taken together, these data indicate that BSP

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proteins are not essential itself for sperm maturation and fertilization, at least in the mouse.

Conclusions: It seems that the absence of BSP protein does not have an important effect on male infertility.