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Taming Spermatozoa: From Their Selection to Their Use

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Abstract

The irruption of new technologies based on nanotechnology and new knowledge about the behavior of spermatozoa deriving from fluid mechanics are opening new pathways enabling specific cells or populations to be selected and subsequently monitored and led through a variety of systems. In the near future, spermatozoa will be "tamed" and used not only for reproductive purposes but also forth erapeutic ends. These advances will promote and improve the development of assisted reproductive technologies, enabling more successful results to be obtained. What follows is a review of the more interesting advances related to sperm monitoring, selection and utilities, in the form of a global overview of the potential future innovations in this field.

Introduction

Spermatozoa: historical and current interest

Spermatozoa have traditionally aroused great scientific interest, and many research papers in this field have sought to increase knowledge about their physiological characteristics in order to implement new and better cryopreservation procedures, to select particular types of cells, to improve their fertilizing ability and to understand their epigenetic and genetic mechanisms, among others. Leeuwenhoek ushered in the modern age of reproduction when, with the aid of a microscope, he first identified spermatozoa in 1677. Various the ories about the development of individuals were proposed at that time; these ranged from the perform is theory, which postulated that individuals were already preformed inside the spermatozoa and grew in the maternal uterus until birth, to the epigenist theory, which required the participation of both an ovum and a spermatozoon to create a new individual. However, the fertilizing role of spermatozoa was not scientifically confirmed until the research conducted by Dumas in1824 [1]. It is curious to note how classical philosophers such as Aristotle and Empedocles suggested that temperature variations and orientation in terms of points of the compass might have a bearing on the generation of males or females during sexual inter course. Subsequently, by a process of gradual accumulation, science has provided new knowledge about sperm physiology, behavior and mechanisms of action, with a stream of new insights being regularly found. Spermatozoa possess the genetic material (or DNA) that is transferred into the ovule during fertilization, conferring part of the information that will later give rise to the phenotypic characteristics of the individual. New technological advances allow spermatozoa to be captured, enabling separation by groups, selection in accordance with scientists' preferences or uses, and determination of how they should perform (similar to the way lions are required to perform in circuses or elephants are put to work as draft animals).

Depending on what we want to do with the spermatozoa, it is useful to determine whether the sample contains optimal spermatozoa, or whether it might be better to purify the sperm sample in order to choose only the better cells or, sometimes, just the single best cell. In addition, the cryopreservation of spermatozoa offers especially interesting possibilities in certain situations, such as the conservation of endangered breeds and exploitation for commercial use. In this context, it is important to ascertain the alternatives that exist and to be familiar with the way innovative procedures are implemented. Set against the background of all such new knowledge, this article aims to give an indication of how new disciplines and sciences, such as fluid mechanics and nanotechnology, are opening up new perspectives in reproductive science.

Reproduction is defined as the beginning of the life cycle and is subject to a significant degree of selection, not only in terms of choosing a mate, but also in terms of promoting the meeting of determinate gametes during fertilization. From this evolutionary perspective, it is interesting to consider the extent to which we are currently able to overcome reproductive difficulties to secure successful reproduction, in contrast to the situation in the past. Nowadays, factors such as age, gender, sexual orientation, etc. present no barrier to human reproduction. However, as suggests,

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scientists need to discuss how assisted reproduction might affect Tran's generational genetic composition, especially when gamete selection is usually carried out using arbitrary practices [2].

One notable feature of the current human reproduction and scape is that infertility in men already affects 8% to 12% of couples, which equates to about 50-80 million couples worldwide [3]. Humans have also experienced numerous difficulties in achieving successful gestation by a natural conception. It is estimated that the probability of a woman becoming pregnant during a natural cycle is only 20% [2], much lower than the rate occurring in domesticated mammalian species. Around 50% of this infertility is associated with defects attributable to men, especially owing to low sperm motility or concentration, or morphological abnormalities in spermatozoa [4]. When fertility is analyzed in domesticated ruminants, it is observed that around 20% of males are infertile or sub fertile, with the consequent negative impact on the profitability of farms [5]. And evaluation of equine and ovine sperm freezability,also shows that a high percentage of stallions (around 25%) do not successfully support freezing while in the case of rams, sperm survival rates after freezing are low [6,7].

Great spermatozoa variability exists within ejaculates

Vast heterogeneity can be found when comparing the spermatozoa of different species, and there is also considerable cell diversity within a single ejaculate. Each ejaculate thus features distinct sperm subpopulations differentiated by their ability to move (fast, medium or slow; short or long resistance; differences in trajectory; size) [8]. Recently, it has been reported that these phenotypes are related to the success of spermatozoa under normal conditions [9]. Many have argued that natural selection gives rise to individuals with better performance and traits, as demonstrated by studies carried out on animals that use external reproduction (salmon and marine invertebrates). These findings draw attention to the Trans generational consequences of reproductive technologies [10]. And in this context, environmental stress agents (among others) exert an epigenetic reprogramming effect over spermatozoa that increase the risk of the appearance of neuropsychiatric diseases, among other disorders, by modifying the hypothalamic-pituitary-adrenal axis of their offspring [11].

New assisted-reproduction technologies developed for overcoming infertility have led to the implementation of protocols for semen purification and sperm selection and enrichment [12]. Sperm purification seeks to identify and isolate superior cells (in terms of high motility and quality), thereby reducing the influence of the environment (for example, plasma, dead sperm, ROS and so on). However, as indicated above, it is not known whether this might have repercussions for future offspring. In this context, some participants in the area, rather than discussing ethical issues, are focusing on how reproductive technologies can affect offspring and how such effects may be measured and offset.

It is known that faster and more resistant (as well as larger) spermatozoa maximize their chances of naturally reaching the egg and have higher reproductive success. The spermatozoa that are currently being selected, by contrast, are the fastest specimens (not, however, the most resistant) and sometimes they are not even the fastest. It is suggested that the genetic information in the most resistant spermatozoa could be linked to the ability to transmit certain genes for positive selection, and therefore, the use of other sperm may reduce the chances of success in the new individual (referring to such different levels as embryonic development capacity, development of immaturity, lower resistance to certain diseases, etc) [2]. Oligospermia and teratospermia defects (which are both defects with high heritability) are not normally transmitted to the offspring [13], and yet today, science allows spermatozoa exhibiting such defects to secure reproductive success, something that would not otherwise occur. Faced with this problem, new studies aimed at securing greater understanding of what happens and how to select optimal spermatozoa for mimicking natural sperm selection are underway and promise to revolutionize this endlessly intriguing field.

Identifying sperm populations for diagnostic and therapeutic purposes

Sperm technologies are used during the selection of sperm for two main reasons: first, to as certain its viability (which will help to determine its capacity to achieve successful fertilization), and secondly to purify sperm populations for their sub sequent use in certain reproductive or therapeutic procedures.

For the purposes of determining sperm quality, image analysis systems have been developed to evaluate the characteristics of spermatozoa movement, revealing how fast they travel and what their trajectory is. Optical microscopes are connected to cameras that capture about 25 images per second (and more) to perform these calculations. Major advances have been made with this technology, and the prospect of new nanotechnological approaches promises to provide new image-based diagnostic alternatives [14,15].

Evaluation of spermatozoa to differentiate cell quality has also been carried out by classifying spermatozoa according to their affinity to stains. Such stains provide information about whether the sperm is alive and whether its acrosome is intact, among other things, but a new generation of substances (lecithins, carbocyanins and peptides), characterized by auto fluorescence or a capacity to be joined to fluorescent substances, and are able to adhere to specific spermatic structures, giving more precise information about their quality. Some of the most common uses of fluorochromes include evaluation of the status of the acrosomal membrane (using FITC-PNA or PSA), DNA fragmentation (using acridine orange or Hoescht), mitochondrial activity (using mitotracker or JC1), membrane integrity (using SYBR or PI) and indicators of oxidative stress (using a BODIPY probe). All these evaluations are carried out by fluorescence microscopy or by flow cytometry, although the latter is preferred for objective analysis of large cell populations.

Sperm selection also has a therapeutic or applicative aspect. Here, the best subpopulation or spermatozoon is chosen for use in assisted-reproduction techniques. An account of how these techniques has evolved and the progress that has been made in them is set out below.

Selection or enrichment of sperm populations

Washing or centrifugation is one of the first steps in sperm purification since it enables the removal of seminal plasma and pre-empts its capacitating effect [16]. With the same aim, the collection of rich spermatozoa fractions has been carried out in domesticated species (such as horses, rams and dogs) using fractioned ejaculation in order to avoid seminal plasma effects, although on some occasions this proves to be a considerable challenge. In male goats, sperm cryopreservation requires the elimination of the phospholipase a contained in the seminal plasma, and seminal plasma removal is currently a standard procedure for semen processing. This technique requires centrifuging semen samples at 500 g to 800 g for 10 min,

in order to reduce sperm injuries. Nonetheless, the cells may be negatively affected by this procedure and recently a colloid-based bottom cushion has been introduced (iodixanol is used, and is harmless to spermatozoa).

So when washing should be used? Prior to sperm freezing in goats; for cooling and/or freezing semen in species such as horses, dogs and pigs, when samples are of sufficiently good quality; and/or for preparing sperm doses for artificial insemination in domestic animals.

Colloids for sperm separation were initially used for selecting spermatozoa and their sub sequent use *in vitro* fertilization (IVF) [12]. This procedure combines centrifugation and the properties of colloids for separating different sperm subpopulations, based on their capacity for movement and their weight (which confers different degrees of buoyancy). Although these techniques were developed for IVF, they are been used in veterinary reproduction as a strategy for improving sperm cryopreservation. Substances such as Percoll (polyvinylpyrrolidone), Iodixanol (radio contrast agent with iodine) and silica-colloidal salts are used in various laboratories. These techniques are more restrictive or selective than centrifugation, since they enable the separation of seminal plasma, dead and non-sperm cells, bacteria, leucocytes, epithelial cells, immature and old spermatozoa, motile spermatozoa and progressive fast sperm. In accordance with the separation capabilities of these procedures, it is worth pointing out that they enable the purification and enrichment of sperm samples. A sample containing progressive, motile and morphologically normal spermatozoa (which are the most promising for fertilization purposes) and exhibiting low levels of ROS can thus be obtained.

Colloidal centrifugation is therefore advantageous in the following contexts: whenever spermatozoa are selected for IVF; for suboptimal semen samples that will be used in artificial insemination or for cooling or freezing; and/or for reducing the bacterial load of sperm samples.

Although the most frequently used method is the double gradient procedure (also known as double centrifugation layer or DLC), another easier procedure based on only one colloid layer has also been devised(known as single layer centrifugation, or SLC) [16]. Table 1 shows some examples of the use of colloidal separation in different species [17-28]. It is noticeable that the sperm recovery rate at the bottom of the tube or pellet (after centrifugation), which is considered to be the best population, reaches a percentage ranging from 30% to 60% in fresh semen in ruminants, while it is only 15% to 20% in frozen sperm samples.

Migration is another technique that enables the selection of motile sperm capable of swimming upward to the culture medium located above the semen sample [29]. The most frequently used technique is the so-called swim-up, and it is recommended for good quality sperm samples because its capacity for separation is comparatively low (approximately 10% to 20%). Nowadays, numerous rapid tests have been developed based on the migratory ability of spermatozoa, which are combined with other substances such as hyaluronic acid or heparin-binding proteins (HBP-30) [30].

Filtration through Sephadex or fiberglass is also used to retain dead sperm and leukocytes, thereby obtaining a better semen sample [12]. However, this procedure does not eliminate seminal plasma, and others techniques mentioned above are more-user friendly.

Magnetic activated cell sorting has been used to identify optimum spermatozoa, which are marked with annex in V and separated by magnetic fields [31]. Apoptotic spermatozoa have some phospholipids (the most common is phosphatidylserine) outwardly exposed. These phospholipids have a high affinity to annex in V, and the procedure is thus based on the incubation of spermatozoa and annex in V-labeling micro beads (50 nm diameter). Spermatozoa with damaged membranes and fragmented DNA bind to the micro beads, and they are then forced to pass through magnetic columns where the apoptotic spermatozoa are retained. This procedure allows the separation of motile, intact and complete DNA from the rest of the cells.

Selection of sperm containing X or Y sex chromosomes enables the gender of the offspring to be determined. While ethical regulations limit or prohibit its use in humans, it has been widely used in animals. Although several methods have been described in the literature, only flow cytometry has been found to separate X- and Y-spermatozoa efficiently. The effectiveness of the technique depends on the species of animal concerned, since the sexual chromosomes exhibit various levels of differences in their DNA contents. Bovine XY-sorted spermatozoa are commonly used in doses of frozen sperm, obtaining high fertility rates when they are released during artificial insemination. Although it has also been employed in other species, such as pigs and horses? [32,33], its commercial use is much less wide spread than in bulls.

Selecting the best (Hypothetical) spermatozoa

The sperm purification, separation and enrichment methods described above are usually used for artificial insemination, sperm preservation and IVF. However, more advanced technologies, for example ICSI, require the use of only one spermatozoon [34]. This in turn requires the use of new technologies to identify which spermatozoon is the most suitable. As far as ICSI technologies are concerned, new procedures such as IMSI, PICSI and separation by laser have been developed. IMSI is based on the ultra-magnification of spermatozoa, which enables the individual with the best morphological aspect to be selected for subsequent micro injection by ICSI [35]. Spermatozoa can be viewed up to a size of 6,000-8,000 magnifications, in order to discard those that present defects such as vacuoles or craters in the head. The PICSI (physiological ICSI) procedure has also been developed; this is based on the ability of mature spermatozoa to bind to hyaluronic acid, a phenomenon similar to the one that occurs during fertilization [36]. And recently laser technology has also been used to determine which sperm are most suitable for Microinjection by impacting sperm tails [37].

Nano science: New perspectives for reproductive science

More recently, fluid mechanics has started to provide new ways of selecting sperm using the behavior of cells in certain fluid circuits [38]. This science has been decisive in biomedical research in terms of enabling environments in the organism to be recreated. And the development of nano- and micro technologies (relating to nano devices, nano materials, etc) together with the aforementioned fluid mechanics are enhancing the success of assisted reproduction. New techniques based on chemo-attractive gradients, flow-through fluid and thermostatic forces as well as motility capacity are harnessing specific sperm characteristics.

Spermatozoa swim through the reproductive tract and fluids under the effects of temperature and other variables, moving near the walls (preferably by narrow channels similar to uterine crypts),

Table 1: Colloidal centrifugation protocols used by different authors for sperm selection in animals.

Specie	Type of centrifugation	Colloid	Sperm	Sample volume	Centrifugation	Sample concentration (mill/ml)	References
BULL							
	SLC	ANDROCOLL-B	Fresh	15 ml	300g x 20min	50	[17]
	SLC	OPTIPREP	Fresh	4 ml	1000g x 20min	30	[18]
	DLC (15%/35%)	OPTIPREP	Fresh	4 ml	1000g x 20min	30	[18]
BUCK							
	SLC	ANDROCOLL-B	Fresh, Frozen-thawed	4 ml	300g x 20min	100	[19]
	SLC	CAPRIPURE	Frozen-thawed	0.2 ml	300g x 20min	135	[20]
	DLC (45%/90%)	PERCOLL	Frozen-thawed	0.2 ml	300g x 20min	135	[20]
HORSE							
	SLC	ANDROCOLL-E	Fresh	4,5-25	300g x 20min	100	[21,23]
	SLC	PERCOLL	Fresh	1 ml	400g x 30min	-	[22]
PIG							
	SLC	ANDROCOLL-P	Fresh	15-150	300g x 20min	100	[23]
RAM							
	DLC (45%/90%)	PERCOLL	Fresh, Frozen-thawed	1.5 ml	600g x 20min	500	[24]
	DLC (45%/90%)	PERCOLL	Frozen-thawed	0.25 ml	900g x 15min	100	[25]
	SLC	OVIPURE	Frozen-thawed	0.25 ml	900g x 15min	100	[25]
	DLC (45%/90%)	PERCOLL	Frozen-thawed	0.15 ml	300g x 8min	-	[26]
	DLC (45%/90%)	PERCOLL	Frozen-thawed	0.15 ml	5000g x 5min	-	[26]
DEER							
	DLC (45%/90%)	PERCOLL	Frozen-thawed	1 ml	700g x 15min	400	[27]
	DLC *	BOVIPURE	Frozen-thawed	1 ml	700g x 15min	400	[27]
	DLC (45%/90%)	PURESPERM	Frozen-thawed	1 ml	700g x 15min	400	[27]
LLAMA							
	DLC (45%/90%)	PERCOLL	Fresh	0.75-1 ml	600g x 20min	-	[28]
	SLC	ANDROCOLL-E	Fresh	0.75-1 ml	600g x 20min	-	[28]

* According the manufactor's instructions.

and traveling as a group because this makes cells' progress more efficient in their journey toward the oviducts. Chemo-attractive substances such as hyaluronic acid, acetylcholine and cervical mucus are used for selecting spermatozoa, and currently these substances are being combined with micro fluidic environments in depth grooves, which simulate the endometrial crypts and favor sperm navigation. Furthermore, spermatozoa prefer to swim toward hot areas (thermo-attraction), something that has been used to create temperature-based- chips for separating spermatozoa. The design of more efficient sperm separation devices promises to emerge from the new findings about spermatozoa and the implementation of these new devices and technologies.

Nanotechnology opens up new perspectives on the biotechnology of reproduction, and as one of its pioneers, Richard Feynman, said more than 50 years ago, "There is plenty of the room at the bottom". Minuscule devices are being developed for moving through the body to perform different tasks. These devices consist of bars, tubes, propellers, spheres and cages the size of a cell that can travel through different organs (blood vessels, liver, stomach, reproductive tract), make a diagnosis, transport drugs or perform surgery [39,40]. At the moment it is relatively easy to guide them through a Petri dish, but it is more difficult to conduct them within biological fluids containing cells and proteins, or into complex channels and body cavities.

Nanotechnology is being used to implement new strategies and tests for fertility evaluation and semen purification. Some of these applications require the identification and validation of sperm quality biomarkers, such as the ligands and proteins located in various parts of the spermatozoa, which are potential indicators of fertility. Some of these proteins, which are mainly found on the surface of the spermatozoa, can be identified using magnetic nano particles to achieve fast and effective removal of defective sperm [41], using anti-ubiquitin antibody and with lectin peanut agglutinin, which bind to the surface of defective spermatozoa and to glycans exposed by acrosomal damage, respectively. Fertility tests based on nano particles

are being developed. The proteins bound to heparin secreted by the accessory glands are present in the sperm and seminal plasma. HBP-30 is the most abundant in the semen of high-fertility bulls and is known as the fertility-associated antigen (FAA). Bulls with FAA have been found to be 9% to 40% more fertile than others without FAA. A colloidal gold nano particle test (Reprotest) has also been developed for determining the quality of semen.

Micro- and nano motors have been developed for promoting the mobility of different particles, cells and drugs, based on chemical, physical or biological activity [42]. Various systems based on nano materials have been also designed to release drugs at different locations in the body, bypassing the immune system (which usually degrades and metabolizes these substances) and securing optimal therapeutic effect thanks to their high cellular and tissue penetration. Such systems include liposomes, polymeric nano particles, dendrimers, and polymersomes and nano emulsions [43]. Somewhat surprisingly, spermatozoa provide another vehicle for drug transportation, since they are motile cells that can be controlled using magnetic fields [44,45]. Spermatozoa have the ability to absorb high amount of proteins and hydrophilic drugs. Their membranes prevent the drugs contained within the spermatozoa from becoming diluted in corporal fluids, and also restrain the immune system effect and enzymatic degradation. Spermatozoa have the capacity to link up with other somatic cells, other than the oocyte during fertilization, and this could favor their penetration of various stargeted cells or tissues [46,47]. Moreover, the incomplete metabolic system of spermatozoa impedes the degradation of the drug being carried by them, indicating their potential as an optimal micro carrier for a range of substances. A study testing the use of doxorubicin-loaded spermatozoa shows that its effect can be extended to tumoral culture cells [48].

Many nano particles have been used for years, as in the case of colloidal gold particles, which can be bound to spermatozoa using lecithins or antibodies to obtain, for example, electron microscopy and atomic force microscopy images [49]. More recently, semi conductor nano particles of between 2 nm and 10 nm (called quantum dots) based on CdSe, CdS, CdTe, InP Zn S and PbS, among other materials, are being used because of their stability and intense brightness [14,15]. The bio safety of spermatozoa is currently being investigated and these nano particles offer significant potential for biomedical imaging in the study of pathological processes, both *in vivo* and *in vitro*.

The development of bio hybrid motors, based on the interaction of motile cells and artificial materials, offers mobile systems that make them ideal for biomedical applications. Spermatozoa are optimal candidates for these systems: they are small motile cells (50 to 70 microns) whose movement and velocity may be modified by varying their chemical, thermal and rheotaxis conditions. A micro tube (20 to 50 microns) has been developed to receive the sperm and allowing the flagellum to act as a propellant, displacing the micro tube at a speed of 5 to 30 microns/sc, depending on the depth of penetration, length of tube and temperature [45]. The electromagnetic properties of the microtubule enable it to be guided with a magneto or electromagnetic "coil". And the release of sperm is achieved with a thermo sensitive polymer incorporated into flexible micro tubes, which when heated above 28˚C makes the polymer stop spinning. More recently, a bio hybrid sperm has been developed by capturing a spermatozoon inside a micro helix [50]. With a laser, the micro helices move through electromagnetic fields as a consequence of the nickel and titanium bilayer coating. These allow the sperm to be released in the ovum, indicating its applicability to infertility treatments.

An engineering revolution based on micro fluidic and nanotechnology, among other sciences, is transforming the techniques used to manipulate spermatozoa. New protocols and particles are being implemented to determine the quality of spermatozoa, to ensure better separation and enrichment of sperm samples, to select spermatozoa displaying certain characteristics, to induce movement in immotile cells and to transport drugs borne by spermatozoa as a therapeutic tool. Nowadays, as this review has indicated, it is possible to determine which sperm population or individual spermatozoon should be used for different purposes; p scientists have been placed in the position of “sperm-tamers”, able to select the desired cells and to control their activity in order to obtain new approaches and higher reproductive success. The aforementioned processes will soon become available at the laboratory level, and will help to resolve cases of infertility in humans and animals.

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