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Review

Sperm preparation for ART

Ralf R Henkel* and Wolf-Bernhard Schill

Address: Department of Dermatology and Andrology, Justus Liebig University, Giessen, Gaffkystr. 14, Germany

Email: Ralf R Henkel* - ralf.henkel@derma.med.uni-giessen.de; Wolf-Bernhard Schill - wolf-bernhard.schill@derma.med.uni-giessen.de

* Corresponding author

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Abstract

The onset of clinical assisted reproduction, a quarter of a century ago, required the isolation of motile spermatozoa. As the indication of assisted reproduction shifted from mere gynaecological indications to andrological indications during the years, this urged andrological research to understand the physiology of male germ cell better and develop more sophisticated techniques to separate functional spermatozoa from those that are immotile, have poor morphology or are not capable to fertilize oocytes. Initially, starting from simple washing of spermatozoa, separation techniques, based on different principles like migration, filtration or density gradient centrifugation evolved. The most simple and cheapest is the conventional swim-up procedure. A more sophisticated and most gentle migration method is migration-sedimentation. However, its yield is relatively small and the technique is therefore normally only limited to ejaculates with a high number of motile spermatozoa. Recently, however, the method was also successfully used to isolate spermatozoa for intracytoplasmic sperm injection (ICSI). Sperm separation methods that yield a higher number of motile spermatozoa are glass wool filtration or density gradient centrifugation with different media. Since Percoll® as a density medium was removed from the market in 1996 for clinical use in the human because of its risk of contamination with endotoxins, other media like IxaPrep®, Nycodenz, SilSelect®, PureSperm® or Isolate® were developed in order to replace Percoll®. Today, an array of different methods is available and the selection depends on the quality of the ejaculates, which also includes production of reactive oxygen species (ROS) by spermatozoa and leukocytes. Ejaculates with ROS production should not be separated by means of conventional swim-up, as this can severely damage the spermatozoa. In order to protect the male germ cells from the influence of ROS and to stimulate their motility to increase the yield, a number of substances can be added to the ejaculate or the separation medium. Caffeine, pentoxifylline and 2-deoxyadenosine are substances that were used to stimulate motility. Recent approaches to stimulate spermatozoa include bicarbonate, metal chelators or platelet-activating factor (PAF). While the use of PAF already resulted in pregnancies in intrauterine insemination, the suitability of the other substances for the clinical use still needs to be tested. Finally, the isolation of functional spermatozoa from highly viscous ejaculates is a special challenge and can be performed enzymatically to liquefy the ejaculate. The older method, by which the ejaculate is forcefully aspirated through a narrow-gauge needle, should be abandoned as it can severely damage spermatozoa, thus resulting in immotile sperm.

Introduction

Since the birth of Louise Brown on 25 July 1978 and the subsequent onset of assisted reproduction in the human, scientists and clinicians were more and more urged to improve sperm separation techniques as the percentage of andrological cases increased rapidly. While the first *in vitro* fertilization (IVF) cases, including that of Louise Brown, were performed to treat tubal infertility, the increasing number of men showing poor semen quality prompted the development of a wide array of different laboratory techniques focusing on the selection and enrichment of motile and functionally competent spermatozoa from the ejaculate. The first sperm separation methods available only comprised of one or two washing procedures with subsequent resuspension of the male germ cells [1-3]. Mahadevan and Baker [4] then described a single wash followed by a swim-up procedure from the cell pellet. Following these first reports on human sperm separation, more sophisticated methods were developed to obtain sufficient amounts of motile, functionally competent spermatozoa for IVF. Eventually, methods were developed that improved sperm functions like motility, protected sperm functions and/or reduced detrimental effects from the environmental milieu like reactive oxygen species.

In this paper, we aimed at giving an update on the main sperm separation methods including their implications and importance for modern assisted reproductive technologies as well as an overview on different *in vitro* treatments of spermatozoa to improve their functional competence and to reduce detrimental effects.

Sperm separation techniques

Under *in vivo* conditions, potentially fertile spermatozoa are separated from immotile spermatozoa, debris and seminal plasma in the female genital tract by active migration through the cervical mucus [5]. During this process, not only progressively motile spermatozoa are selected, but male germ cells also undergo physiological changes called capacitation, which are fundamental prerequisites for the sperm's functional competence with regard to acrosome reaction [6,7].

The introduction of assisted reproduction, especially of IVF, during the 1980's, led to the development of a wide range of different sperm separation methods. Following the development of the classical swim-up method by Mahadevan & Baker [4], more complicated techniques were developed to increase the number of motile spermatozoa even in severe andrological cases. On principle, these techniques can be differentiated in migration, density gradient centrifugation and filtration techniques. For all migration methods, the self-propelled movement of spermatozoa is an essential prerequisite, while for density gradient centrifugation and filtration techniques the methodology is based on a combination of the sperm cells' motility and their retention at phase borders and adherence to filtration matrices, respectively. The migration techniques can again be subdivided into swim-up, under-lay and migration-sedimentation methods [8,9]. For density gradient centrifugation, separation media like Ficoll® [10], Nycodenz [11] and Percoll® [12,13] including the products (IxaPrep®, PureSperm®, Isolate®, SilSelect®) have recently been introduced to replace Percoll® [14,15]. The filtration methods like glass wool filtration [16,17] and filtration of spermatozoa on Sephadex beads [18] and membranes [19] are alternative techniques.

The ideal sperm separation technique should (i) be quick, easy and cost-effective, (ii) isolate as much motile spermatozoa as possible, (iii) not cause sperm damage or non-physiological alterations of the separated sperm cells, (iv) eliminate dead spermatozoa and other cells, including leukocytes and bacteria, (v) eliminate toxic or bioactive substances like decapacitation factors or reactive oxygen species (ROS), and (vi) allow processing of larger volumes of ejaculates. Since none of the methods available meets all these requirements, a variety of sperm separation techniques is mandatory in clinical practice to obtain an optimal yield of functionally competent spermatozoa for insemination purposes. Depending on the ejaculate quality, these methods have different efficiency and areas of use. In the conventional swim-up technique, functional spermatozoa can come into close cell-to-cell contact with defective sperm or leukocytes by centrifugation, thus causing massive oxidative damages of the sperm plasma membrane by ROS and consequently of sperm functions [20]. Therefore, the quality of the ejaculates has direct consequences on the choice of a sperm separation method.

Table 1: Advantages and disadvantages of the conventional swim-up method.

Advantages	Disadvantages
- easy to perform	- restricted to ejaculates with high sperm count and motility
- very cost-effective	
- usually recovery of a very clean fraction of highly motile spermatozoa	- low yield
	- spermatozoa can be massively damaged by reactive oxygen species
	- significant decrease of the percentage of normally chromatin-condensed spermatozoa

With regard to the possible risk of a seroconversion in women and in the offspring after performing ART with spermatozoa from a HIV-positive man initial concerns arose as HIV viruses reportedly can bind to and penetrate into spermatozoa [21,22]. Later research, however, showed that HIV-1 genomes found to an extend of 18% in seminal cells samples decreased to undetectable levels following a combined density gradient centrifugation with a subsequent swim-up [23]. No seroconversion of the virus could be observed after fertilization of oocytes by IVF-ICSI [24-26]. Other recent research [27] showed that HIV RNA and DNA could be detected in separated spermatozoa even in treated patients. Thus, viral validation of separated spermatozoa is necessary and should be performed even in treated patients. Additionally, only HIV-tested maternal serum or commercially available serum albumin, which is HIV-free due to its processing, should be used as protein supplement for culture media.

In order to give an overview, only the four most common techniques classical swim-up, migration-sedimentation, density gradient centrifugation and glass wool filtration are discussed.

Swim-up procedure

Apart from a simple wash and subsequent resuspension of the male germ cells, the swim-up from a washed pellet is the oldest and most commonly used sperm separation method. Originally described by Mahadevan and Baker [4], this method is still used largely in IVF laboratories around the world. Although its use among the male factor infertility group is very limited, the swim-up is still the standard technique for patients with normozoospermia and female infertility. Excellent fertilization rates were reported when these sperm preparations were used to inseminate human oocytes *in vitro*. However, as the indications for IVF were expanded beyond simple tubal factor cases to couples with idiopathic infertility and, ultimately, to male factor cases, the problem of fertilization failure appeared [28-30].

The methodology of this conventional swim-up is based on the active movement of spermatozoa from the prewashed cell pellet into an overlaying medium. Typically, the incubation time is 60 minutes. This technique is distinguished by a very high percentage (>90%) of motile sperm, preferred enrichment of morphologically normal spermatozoa as well as the absence of other cells and debris. Considering that the efficiency of the technique is based on the surface of the cell pellet and the initial sperm motility in the ejaculate, the yield of motile spermatozoa is limited. Many layers of cells in the pellet may cause potentially motile spermatozoa in the lower levels of the pellet never to reach the interface with the culture medium layer. In addition, a significant decrease in the percentage of normally chromatin-condensed spermatozoa has been reported after the swim-up procedure [31]. Another major disadvantage of this technique is the fact that for its use spermatozoa are pelleted, thus coming into close cell-to-cell contact with each other, cell debris and leukocytes, which are known to produce very high levels of reactive oxygen species (ROS) [32]. Due to the extraordinary high amount of poly-unsaturated fatty acids in the sperm's plasma membranes [33], these ROS cause lipid peroxidation and therefore a dramatic decrease in sperm functions, including motility [34]. Overall, although many men's spermatozoa may not be impaired to the extent of inhibiting fertilization, some couples' chances of successful IVF will certainly be compromised. It is therefore not reasonable to continue and to use a technique, such as swim-up from pelleted semen with the inherent potential to cause irrevocable damage to spermatozoa prejudicial to a desired functional endpoint. Eventually, this knowledge led to the development of other more gentle sperm separation methods that also allow a higher recovery of motile and functional spermatozoa. The advantages and disadvantages of the conventional swim-up are summarized in table 1.

An attempt to overcome at least the problems caused by ROS, the "swim-up" can be performed directly from the liquefied semen. During this procedure, several aliquots of liquefied semen are taken from a sample and placed in tubes underneath an overlay of culture medium. Round-bottom tubes or 4-well dishes should be used to optimize the surface area of the interface between the semen layer and the culture medium. The tubes may also be prepared by gently layering culture medium over the liquefied semen. The placing of semen underneath the culture medium, however, provides a much cleaner interface zone. A maximum recovery is obtained by using multiple tubes with small volumes of semen per tube, thus maximizing the combined total interface area between semen and culture medium. Mortimer [35] suggested the use of 250 μl semen and 500 to 600 μl culture medium per tube. After the incubation period, which is typically between 30 and 60 minutes, at 37°C, most of the upper culture medium layer is removed. This should be done with caution, working from the upper meniscus downwards, using a sterile pipette. Typically, 75 or 80% of the culture medium layer are removed and eventually combined, taking great care not to aspirate directly from the interface region. This procedure will also increase the total number of recovered spermatozoa, which can then also be used for ICSI [36].

Other swim-up methods include the swim-up of spermatozoa in a specially supplemented medium. Such substances can be SpermSelect™, which is a highly purified preparation of hyaluronic acid (Pharmacia, Uppsala,

Table 2: Advantages and disadvantages of the original migration-sedimentation method according to Tea et al. (1984).

Advantage	Disadvantages
- usually very clean fraction of highly motile spermatozoa	- the original method is restricted to ejaculates of high sperm count and good motility
- reactive oxygen species are reduced	- the original method has a very low recovery rate
- very gentle separation method	
	- special glass or plastic tubes are required
	- tubes are more expensive and relatively sensitive
	- for repeated use in IVF, glass and plastic tubes must be sterilized

Table 3: Advantages and disadvantages of density gradient centrifugation.

Advantages	Disadvantages
- usually clean fraction of highly motile spermatozoa	- production of good interphases between the different media is a bit more time-consuming
- spermatozoa from ejaculates with a very low sperm density can be separated	- a bit more expensive
- good yield	- potential risk of endotoxins
- leukocytes are eliminated to a large extent	- Percoll® may no longer be used IVF/ICSI
- reactive oxygen species are significantly reduced	

Sweden) with an average molecular weight of 3,000 kDa that was used at a final concentration of 1 mg/ml in culture medium. Compared with the traditional swim-up from a washed pellet [37], a swim-up directly from semen into a hyaluronic acid solution gave a significantly higher percentage of motile spermatozoa and, ultimately, the achievement of a higher pregnancy rate in a clinical IVF program [38]. However, highly purified hyaluronic acid is expensive and it has been shown to increase the calcium-influx into spermatozoa and therefore induce acrosome reaction [39]. Elevated local concentrations of hyaluronic acid in the cumulus oophorus have been shown to contribute to the acrosome reaction [40]. Thus, it seems rather questionable whether this substance is favourable for IVF. In addition, whether these improved results in sperm motility were specifically due to the use of the hyaluronate or to the use of a method, which did not involve the initial pelletting of unselected spermatozoa, has not been ascertained. On the other hand, however, hyaluronic acid has been regarded as an effective alternative to test sperm penetration into human cervical mucus [41-43].

Migration-sedimentation

A more sophisticated sperm separation technique is migration-sedimentation, which was developed by Tea et al. [8]. Principally, this method is a swim-up technique combined with a sedimentation step. Special glass or plastic tubes with an inner cone are used. In contrast to the conventional swim-up procedure, spermatozoa swim up directly from liquefied semen into the supernatant medium and subsequently sediment in that inner cone within an hour's time. Thus, this method is a highly gentle technique, especially if compared with methods that require centrifugation steps before the sperm separation like the conventional swim-up. In the original version, a fraction of highly motile and functionally competent spermatozoa can be obtained. Unfortunately, the yield is very low and therefore the original method did not find wide acceptance for IVF. The advantages and disadvantages of this method are summarized in table 2. Recently, Zavos et al. [9] proposed the use of a multi-chamber tube to retrieve functional spermatozoa for assisted reproductive techniques by means of a swim-up and sedimentation method. The assessment of its usefulness for IVF/ICSI, however, is still to be awaited.

Sánchez et al. [44] showed that after concentration of sperm cells in the ejaculate, even in cases with severe oligo- and/or asthenozoospermia a sufficient number of motile spermatozoa can be isolated for intracytoplasmic sperm injection (ICSI) after 2–3 hours of incubation. Compared with the density gradient centrifugation, these authors also demonstrated significantly better results for progressive motility, normal sperm morphology, chromatin condensation and reduction of the percentage of dead spermatozoa as determined by the eosin test. In addition, since spermatozoa isolated by this modified migration-sedimentation technique stick markedly less to the glass surface of the injection pipettes, this method has even an advantage over the density gradient centrifugation, which

is normally used for these cases. In this regard, the side migration technique that was recently proposed by Hinting and Lunardhi [45] is another interesting approach to obtain motile spermatozoa from very poor quality semen for ICSI as it also yielded better sperm quality.

Density gradient centrifugation

The typical methodology for the density gradient centrifugation comprised continuous [46] or discontinuous gradients [47]. With continuous gradients, there is a gradual increase in density from the top of the gradient to its bottom, whereas the layers of a discontinuous gradient show clear boundaries between each other. The ejaculate is placed on top of the density media with higher density and is then centrifuged for 15–30 minutes. During this procedure, all cells reach the semen sediment. However, highly motile spermatozoa move actively in the direction of the sedimentation gradient and can therefore penetrate the boundary quicker than poorly motile or immotile cells, thus, highly motile sperm cells are enriched in the soft pellet at the bottom.

A wide variety of methods using the principle of density gradient centrifugation to fractionate subpopulations of spermatozoa has been described in the literature. Ficoll® has initially been used as gradient material for preparing spermatozoa [48], but by far the most widely used substance for all methods of assisted reproduction (IUI, GIFT, IVF, ICSI, etc.) have been the polyvinylpyrrolidone (PVP)-coated silica particles Percoll®. Normal sperm function in terms of sperm fertilizing ability as assessed in the zona pellucida-free hamster egg penetration test [20,49], as well as in human IVF [13] and ICSI [50] was observed. In October 1996, Percoll® has been withdrawn from the market for clinical use in assisted reproduction [51]. This was because of the risk of contaminations with endotoxins [52-54], possible membrane alteration [55,56] and inflammatory responses that could be induced by the insemination of sperm populations contaminated with Percoll®. In addition, Percoll® adheres to the sperm membranes [57] and might alter them by removing coating envelopes [58]. Therefore, intensive washing of the spermatozoa after sperm separation with Percoll® was recommended [55]. This requires additional centrifugation and can again be detrimental to the spermatozoa because of the action of reactive oxygen species [20].

Another commercial product known as Nycodenz (Nyegaard & Co., Oslo, Norway) was also used as a density gradient material for preparing human spermatozoa. Nycodenz is the same molecule, iohexol, as used in the X-ray contrast medium Omni-paque. Studies revealed a low incidence of adverse reactions during angiography [11]. Both continuous and discontinuous Nycodenz gradients were evaluated, of which a four-layer discontinuous gradient was found to produce populations of highly motile spermatozoa with better yields and survival than either swim-up or Percoll® gradients from oligozoospermic and asthenozoospermic semen samples [11,59]. Compared with the conventional swim-up procedure from a pelleted sperm population, the use of Nycodenz also seems to be superior regarding sperm penetration into zona-free hamster eggs [60]. Thus, this technique has clearly great potential in the preparation of motile spermatozoa from poor quality semen for IVF use and warrants further investigation.

Other replacement products for Percoll® that were introduced into the market from the mid nineties and more commonly used in assisted reproduction are IxaPrep® (MediCult, Copenhagen, Denmark), SilSelect® (FertiPro N.V., Beernem, Belgium), PureSperm® (NidaCon Laboratories AB, Gothenburg, Sweden) or ISolate® (Irvine Scientific, Santa Ana, CA, USA). In contrast to Percoll®, which is a PVP-coated silica that can have deleterious effects on sperm membranes [56], all these replacement products contain silane-coated silica particles, are adjusted for the osmolarity with polysucrose and have very low toxicity. All these replacement products are non-irritating and are approved for human *in vivo* use. The results of sperm preparation using these new products compared with Percoll® regarding recovery rate, motility, viability, normal sperm morphology and velocity parameters like VAP or VCL vary considerably among different working groups. While Claassens et al. [61] and Söderlund and Lundin [62] did not find differences in the recovery rate between Percoll® and PureSperm®, Chen and Bongso [63], depending on the number of layers included for the density gradient, reported significantly higher values for PureSperm®. For IxaPrep® it is even more confusing because Yang et al. [64] found no difference to Percoll®, while Makkar et al. [14] found the replacement substance more effective. On the other hand, McCann and Chantler [65] as well as Ding et al. [66] found Percoll® superior. These authors attribute the better sperm quality obtained after the IxaPrep® preparation to a significantly decreased production of nitric oxide, which is regarded as sperm toxicant that reduces motility [67]. This could be due to an activation of guanylyl cyclase, thus increasing cGMP production, which inhibits sperm motility [68]. On the other hand, nitric oxide is also known to be a physiologic mediator for vasodilatation, immunosuppression, neurotransmission and cytotoxicity [69-72].

Regarding the other parameters such as motility, viability, normal sperm morphology or velocity parameters like VAP, the data currently available also vary considerably among different working groups. The reason for this can be attributed to the different conditions of the sperm separation, e.g. volume of semen to be separated, g-force,

Table 4: Advantages and disadvantages of the glass wool filtration.

Advantages	Disadvantages
- simple to perform	- a bit more expensive
- normally, recovery of spermatozoa with good motility	- the filtrate is not as clean as it is with other sperm separation methods
- spermatozoa from ejaculates with a very low sperm density can be separated	- remnants of debris are still present
- good yield	
- leukocytes are eliminated to a large extent	
- reactive oxygen species are significantly reduced	

centrifugation time or the number of layers of the gradient, and reflect the important role of the methodology. Moreover, this also shows that these data cannot be directly compared. Overall, the Percoll®-replacement products are good and reasonable alternatives, and this not only for the fact that Percoll® is no longer allowed to be used for clinical purposes in assisted reproduction.

Glass wool filtration

During glass wool filtration, which has already been described by Paulson & Polakoski in 1977 [17], motile spermatozoa are separated from immotile sperm cells by means of densely packed glass wool fibres. The principle of this sperm separation technique lies in both the self-propelled movement of the spermatozoa and the filtration effect of the glass wool. The success of this method is directly linked to the kind of glass wool used [73]. Thus, factors like the chemical nature of the glass (i.e. borate glass, silicate glass or quartz glass), the surface structure and charge of the glass wool, thickness of the glass wool fibres or the pore size of the filter have to be taken into consideration. In clinical practice, the glass wool, code number 112, from Manville Fiber Glass Corp. (Denver, CO, USA) or SpermFertil® columns from Mello (Holzhausen, Germany) have been tested extensively. Potential risks of the technique such as damages of the spermatozoa or the occurrence of glass wool fragments in the filtrate essentially depend on the kind of glass wool used and on the intensity of the washing prior to the filtration.

Compared with the swim-up or migration-sedimentation, glass wool filtration, just as density gradient centrifugation, is a technique that uses the whole volume of the ejaculate and therefore yields a significantly higher total number of motile spermatozoa [31,74]. Thus, it can also be used for patients with oligo- and/or asthenozoospermia [74]. Like density gradient centrifugation, glass wool filtration also provides the advantage that the sperm separation can directly be performed from the ejaculate. Only after the separation of the functional spermatozoa from the immotile ones, leukocytes and debris, a centrifugation step will be necessary to remove the seminal plasma. This is an important aspect as this procedure reduces cellular damage by reactive oxygen species. The advantages and disadvantages of this method are summarized in table 4.

By means of glass wool filtration, it is even possible to prepare motile spermatozoa from patients with retrograde ejaculation (Henkel et al., unpublished). In these cases, the procedure includes adjustment of the osmolarity of the patient's urine to values of about 350 mOsmol/kg by drinking water. Prior to the ejaculation, the patients are requested to urinate most of the urine in the bladder. The small amount of antegrade-produced ejaculate is collected in a plastic beaker, while the retrograde fraction of the ejaculate needs to be urinated immediately into a jar with 50 ml culture medium containing human serum albumin to dilute the urine, Finally, the urine/medium mixture has to be centrifuged, resuspended in 3 to 4 ml of fresh medium and filtrated on the glass wool column. As constituents of the urine can damage the spermatozoa, a speedy work-up of such ejaculates is mandatory.

In addition to the separation of spermatozoa, glass wool filtration has been shown to eliminate leukocytes to an extent of up to 90% [75]. Since leukocytes are frequent even in normal ejaculates [76] and produce 100-times more ROS than spermatozoa [32], this effect significantly contributes to a reduction of free radicals in the ejaculate [73,75]. This is of paramount importance for the functionality of spermatozoa because the male germ cells are particularly susceptible to oxidation by ROS because of their extraordinary high content of polyunsaturated fatty acids in their plasma membrane [33,77,78].

Another clinically interesting aspect related to glass wool filtration is chromatin condensation, which has repeatedly been shown to be predictive of fertilization *in vitro* [79-81]. Glass wool filtration [31] like the density gradient centrifugation with PureSperm® [82] or the migration-sedimentation technique [44] significantly selects normally chromatin-condensed spermatozoa, while conventional swim-up or Percoll®-centrifugation decrease this

sperm parameter. As human sperm chromatin condensation follows a seasonal rhythm, which even shows a shift of about half a year on the southern hemisphere [83], this might have a clinical impact on the results in IVF. Should a patient be examined in winter when the quality of sperm chromatin condensation is high [83] and referred to IVF in summer when the percentage of normally chromatin-condensed spermatozoa is significantly lower, IVF for this patient might fail. Thus, for these patients a sperm separation by means of glass wool filtration, PureSperm® or migration-sedimentation might be beneficial.

Glass beads

This method has been used for the preparation of hamster spermatozoa for *in vitro* capacitation [84] and resulted in an efficient, high yield selection of motile human spermatozoa from semen [85]. However, there were concerns regarding the possible spill over of beads into the insemination medium. As a result, the use of glass beads for effective sperm preparation for assisted reproduction has not widely been accepted.

Sephadex columns

In the early nineties sperm separation by means of Sephadex beads emerged [18] and a commercial sperm separation kit based on this principle (SpermPrep®) has become available. Compared to migration-sedimentation and swim-up from pelleted semen it produced significantly higher yields [86]. Moreover, morphologically normal sperm cells could be enriched in the filtrate after SpermPrep® separation as well as significantly higher pregnancy rates for intrauterine insemination as compared with the conventional swim-up method [87]. In a comparison between SpermPrep® method and Percoll® centrifugation, Percoll® separated spermatozoa showed a significantly higher percentage of normally chromatin-condensed and morphologically normal spermatozoa [88]. However, the fertilization rates reported by these authors were similar. López et al. [89] used a prepacked PD-10 column containing Sephadex G-25 particles (Pharmacia Biotechnology, Uppsala, Sweden), which is normally used to desalt proteins in solutions, to separate human spermatozoa and compared the results with the SpermPrep® method and Percoll® centrifugation. The PD-10 column and density gradient centrifugation in Percoll® yielded a comparable number of spermatozoa and showed similar percentages of morphologically normal spermatozoa after sperm separation. On the contrary, the SpermPrep® method resulted in significantly lower values of sperm count and morphology.

Transmembrane migration

Another alternative sperm separation technique that was also developed in the late eighties is migration/filtration of motile spermatozoa through a Nuclepore membrane filter. These filters are unusual because their pores are cylindrical and at right angles to the plane of the membrane [90]. The spermatozoa, therefore, have straight channels to swim through the membrane. Unfortunately, these membranes had a very low ratio of the total cross-sectional area of the pores to the overall membrane area. Consequently, the yield is extremely low. Primarily, this method was used for testing the motility of sperm populations treated with various pharmacological agents, but not as a preparation method for assisted reproduction [91].

Another approach of separating viable human spermatozoa by means of membranes was undertaken by Agarwal et al. [19] using a membrane which has been developed for selective removal of leucocytes (L4 membrane). Besides a significant increase of motility, ejaculates filtered through this membrane have been shown to contain fewer leukocytes. This fact is, of course, of importance in those cases that have increased numbers of leucocytes in the ejaculate as a result of infections. Moreover, this membrane seems to be selective for spermatozoa with normal membrane integrity [92,93] and sperm producing low amounts of reactive oxygen species [94]. However, despite these advantages of the membrane it has never come into practical clinical use for human assisted reproduction.

Improvement of sperm concentration in the fertilization well

Apart from the different sperm separation techniques to increase the number of functional spermatozoa for assisted reproductive techniques discussed above, trials have been made to improve fertilization rates by modifying the conditions of the co-culture of oocytes with spermatozoa. Micro-insemination techniques have especially been developed for patients with sperm counts or asthenozoospermia. One of the first who used such a method for assisted reproduction were Ranoux and Seibel [95]. These authors used a microvolume straw to co-incubate spermatozoa with the oocytes and incubated this straw intravaginally, thus they called the technique intravaginal culture (IVC). Giorgetti et al. [96] proposed a swim-across technique in a medium that contains human follicular fluid and found a significantly improved fertilization rate for patients with motile sperm counts less than 1×10^6 spermatozoa/ml of semen. Fishel et al. [97] reported critically on the microdrop IVF and preferred microinjection. On the other hand, Svalander et al. [98] presented encouraging results with a fertilization rate of 49.3% and a baby-take-home rate of between 20–24.7%. Although this is lower than the baby-take-home rate reported for conventional IVF (27–31.7%), these authors recommended the technique for moderate male factor infertility.

This success of the technique may be dependent on the volume of the microdrop as well as on the sperm concentration. In this regard, Özgür et al. [99] developed a predictive model to calculate the therapeutically optimal sperm concentration for a defined microvolume. This might be explained by data of Özgür and Franken [100] who showed that sperm-zona binding is dependent on the sperm-zona collision rates, which is dependent on the size of the microdrops itself. However, as the results of the microdrop IVF were conflicting, and successes sporadic, intracytoplasmic sperm injection (ICSI) has become the method of choice in the treatment of severe male factor infertility.

Immunological infertility and ART

Immunologic infertility due to sperm autoimmunity afflicts 5–10% of infertile couples [101]. Normally, male germ cells, which express surface antigens that are not present on somatic and pre-meiotic cells, are shielded from immuno-competent cells in the body by the blood-testis barrier. This prevents an immunologic reaction. However, in the event of a disintegration of the blood-testis barrier by infections, injuries or due to surgical treatments, these actually "strange" surface antigens can be exposed to the immune system. As a result, the body produces antibodies against its own spermatozoa. On the other hand, women can also produce anti sperm-antibodies. One possible target for such antibodies is the human sperm protein rSMP-B [102]. These antibodies can coagulate and immobilize the spermatozoa or can even be cytotoxic [103] and eventually lead to infertility as well as reduced fertilization rates in assisted reproduction [104]. However, contradictory results that the presence of sperm autoantibodies in female sera used for media in IVF does not affect the IVF result are also reported [105].

In the past, it was thought that anti sperm-antibodies could be eliminated just by washing them off. However, as normal antigen-antibody reactions usually have affinity constants between 10^7 and 10^9 l/mol, a simple washing step will not suffice for removing these antibodies from the sperm surface. As in men with sperm autoantibodies not all spermatozoa are affected by the antibodies, Hinting et al. [106] could significantly reduce the anti sperm-antibodies as tested with the mixed antiglobulin reaction test (MAR test) by using a swim-up technique with fetal cord serum. The cumulative pregnancy rates of treatments for intrauterine insemination (IUI) in cases of immunological infertility are reported between less than 10% [107] and up to 25% [108]. Today, the method of choice to treat severe cases of immunological infertility is IVF or even ICSI where pregnancy rate of 30–40% per cycle can be achieved.

In vitro treatment of spermatozoa

An alternative and/or complementary approach to sperm preparation for ART *in vitro* by means of the above discussed sperm separation methods is the idea to treat the spermatozoa *in vitro* in order to improve their functionality, i.e. motility, or to supply a protective environment with the purpose to maintain or improve their functional capacity for successful fertilization. Many substances including serum, peritoneal fluid and follicular fluid or other chemically defined pharmacological substances like progesterone, adenosine analogues or methylxanthins have been proposed to stimulate human sperm functions. The methodologies addressing the different aspects, motility and sperm functions, ROS, and the reduction of the visco-elastic properties of the semen are discussed below.

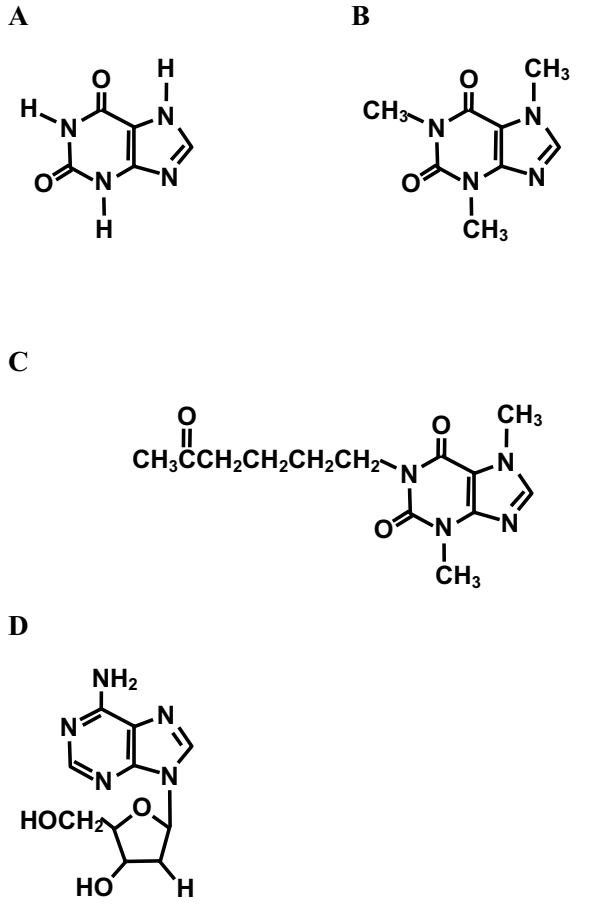

Figure 1
Molecular structures of xanthine (A), caffeine (B), pentoxifylline (C) and 2-deoxyadenosine (D).

Improvement of motility and sperm functions

CAFFEINE

The use of xanthine (Fig. 1A) derivatives like the methylxanthines caffeine (1,3,7-trimethyl-2,6-dioxopurine) (Fig. 1B) and pentoxifylline (Fig. 1C) for the pharmacological stimulation of sperm functions, especially for motility, is well known. Garbers et al. [109] already suggested stimulation of human sperm motility by caffeine. This substance is an inhibitor of the phosphodiesterase that leads to increased cellular levels of cAMP. Considering that a defective energy metabolism of the spermatozoa is also a potential cause of male infertility, the use of motility stimulating substances to improve sperm motility and therefore fertilization rates appeared reasonable.

Human spermatozoa can obtain energy from both glycolysis and mitochondrial oxidative phosphorylation [110]. Thus, motility parameters such as mode of progression, lateral head displacement or velocity of fresh and cryopreserved spermatozoa significantly increased after stimulation with caffeine due to augmented glycolysis and fructolysis [111-113]. A small study including 6 patients by Serres et al. [114] revealed that the stimulatory effect is most obvious in patients with asthenozoospermia. However, early reports documented not only a stimulating effect of caffeine, but also a time- and concentration-dependent detrimental effect on the sperm plasma membrane [115,116]. Moreover, spermatozoa pre-incubated with caffeine showed a significant decrease in the percentage of penetrated zona-free hamster oocytes [117]. The only study available [118] that investigated the effect of caffeine in assisted reproduction using 42 oocytes from 21 women showed an increase in sperm motility but a significant decrease in the fertilization rates and embryo development.

Direct incubation of mouse oocytes and embryos resulted in a time- and concentration-dependent artificial parthenogenic activation of the oocytes and a significant reduction of embryo cleavage rates [119]. As these data were more disappointing than stimulating for the clinical use of caffeine in assisted reproduction, it was further only used for research purposes. A very recent prospective study by Klonoff-Cohen et al. [120] investigated the daily caffeine consumption of 221 patients and its effects on the outcome of IVF/GIFT treatments. According to these authors, failed pregnancies were significantly associated with the female's caffeine consumption. In addition, gestational age at delivery decreased significantly in women who consumed more than 50 mg/day. Male caffeine consumption appeared to be a significant risk factor for multiple gestations but had no effect on sperm count, motility or normal sperm morphology. An earlier study [121], however, demonstrated a positive association between coffee consumption and sperm concentration, motility and abnormal sperm morphology in a total of 546 men. In combination with smoking as further risk factor, sperm motility and viability decreased significantly. Klonoff-Cohen et al. [120] confirmed the embryotoxic effect of caffeine causing delayed conception [122] or spontaneous abortions [123] and concluded that the caffeine consumption should be minimized prior to and during an assisted reproduction treatment.

PENTOXIFYLLINE

Pentoxifylline (Fig. 1C) is another methylxanthine derivative, which is also, like caffeine, a non-specific inhibitor of phosphodiesterase. In contrast to caffeine, the Food and Drug Administration (FDA) approved pentoxifylline for the administration to humans. The drug is used for systemic treatments of patients with cardio-vascular diseases (Trental® or Torental®). In addition, since its water solubility is higher than that of caffeine, this increases its usability [124].

The beneficial effect of pentoxifylline on sperm motility and motion characteristics like sperm velocity or hyperactivity has repeatedly been described for both fresh [112,125-129] and cryopreserved spermatozoa [128,130-132]. The results on the stimulation of sperm motility are conflicting. Yovich et al. [133], Rees et al. [112] and Lewis et al. [134] found no effect in normozoospermic patients, while others [133-136] observed a significant increase in motility and the number of progressively motile spermatozoa in patients with asthenozoospermia. This stimulatory effect can clearly be attributed to the increased intracellular levels of cAMP. In the absence of the drug, the cAMP content remained unchanged and correlated only with hypermotility and the amplitude of the lateral head displacement of the spermatozoa [137]. Cyclic AMP, in turn, is believed to stimulate a cAMP-dependent kinase [138], which itself induces sperm tail protein phosphorylation [139] with subsequent increase in sperm motility [140].

Apart from the effects on sperm motility, pentoxifylline is also reported to augment acrosome reaction [141]. Nassar et al. [142] demonstrated that this induction is not due to a Ca^{2+}-influx in the sperm cell, which is regarded to stimulate acrosome reaction [143]. The intracellular $[Ca^{2+}]_i$ even decreased following the pentoxifylline treatment. Since cAMP is intimately involved as a second-messenger in the induction of acrosome reaction [144], an unspecific inhibition of phosphodiesterases by this methylxanthine will increase the intracellular cAMP levels and therefore induce acrosome reaction. In addition, this drug also improves the spermatozoa's zona pellucida binding ability [139,145,146]. However, it appeared that this binding improvement to the zona pellucida is rather a result of the increase in the sperm velocity parameters straight-line

velocity (VSL) and average path velocity (VAP) as these parameters are indicators of progressive motility. Therefore, these spermatozoa represent a sperm population that did not initiate acrosome reaction with its characteristic change in movement characteristics yet [147].

The benefits of a treatment with pentoxifylline prompted its use in assisted reproduction programs. However, the results reported in the literature are rather conflicting. While Tasdemir et al. [148] and Tarlatzis et al. [149] found an improvement in the IVF rate and saw a promising development in small studies comprising 51 and 43 patients, respectively. Others did not find differences between the control and the treatment group in IVF and intrauterine insemination [150-152]. Thus, it was concluded that pentoxifylline should not be used indiscriminately [153]. On the other hand, pentoxifylline has been successfully used to increase fertilization rates in bovine *in vitro* fertilization [154] and as pre-treatment to stimulate epididymal and testicular sperm motility for ICSI [155,156]. Should the results of Numabe et al. [154] be confirmed, this could be a promising approach to improve fertilization rates especially for endangered species.

The conflicting results on the effectiveness of a pentoxifylline treatment raised the question of the embryotoxicity of this substance, especially since possible pentoxifylline-induced adverse effects on spermatozoa [157] and mouse embryo development [158,159] have been reported. In contrast, Lacham-Kaplan & Trounson [160] did not observe such negative effects on embryonic development after insemination of the oocytes with spermatozoa incubated in 3 mM pentoxifylline. Finally, short-term incubation of spermatozoa with subsequent washing of the male germ cells did not produce such adverse effects in intrauterine insemination or ICSI (Henkel et al., unpublished) [156]. An alternative approach to increase sperm motility or the number of motile spermatozoa was to administer the drug orally over period of 3 to 6 months [161]. In a placebo-controlled study including 47 normozoospermic men with idiopathic asthenozoospermia, Merino et al. [162] showed a significant increase in progressive motility in men who received 1,200 mg of pentoxifylline per day over 6 months. Clinical data about fertilization and pregnancy, however, are still not available.

Another important point that must not be underestimated in explaining the controversial effects of pentoxifylline is the fact that this drug is an unspecific inhibitor of the phosphodiesterase (PDE). Considering that eleven different families of this enzyme have been described [163], of which PDE-1 and PDE-4 are present in human spermatozoa and stimulate different sperm functions, i.e. acrosome reaction and motility, respectively [164], an unspecific inhibition of the PDE's will obviously result in both, stimulation of motility and acrosome reaction. Depending on the conditions and most importantly on the time of stimulation and the concentration of pentoxifylline in the medium, over-stimulation will definitely result in a too early acrosome reaction. Hence, over-stimulated spermatozoa for an IUI or IVF treatment will not fertilize oocytes because they are no longer able to bind to the zona pellucida. This dilemma might be overcome by the use of a non-embryotoxic PDE-4 inhibitor to stimulate sperm motility only. Unfortunately, to our knowledge, no further progress has been made in this regard. For ICSI, this problem is not relevant as spermatozoa bypass all physiological barriers because they are directly injected into the oocytes.

2-DEOXYADENOSINE

2-Deoxyadenosine (2-DA) (Fig. 1D) is an adenosine derivative that is not a phosphodiesterase inhibitor like pentoxifylline or caffeine. Like these substances, 2-DA is also a potent stimulant of sperm motility [165,166]. The molecular mechanism of motility stimulation, however, works via an A_2-receptor-mediated activation of adenylate cyclase [167], which in turn is thought to enhance the intracellular cAMP concentration [168,169]. Because of this characteristic, the use of 2-DA in assisted reproduction programs has been discussed. Other studies, however, failed to detect a responsive cAMP activity and either stimulatory or inhibitory G-proteins in spermatozoa [170,171]. In addition, Rivkees [172] could not detect A_2a- and A_2b-receptor gene expression in the rat testis. This would imply that these stimulatory receptors are either not present or, at least, at very low levels. On the other hand, the cAMP-inhibitory A_1-receptor has a capacitative effect on human spermatozoa when stimulated with an agonist [173]. While Imoedemhe et al. [174] in an internally controlled prospective study showed significantly higher fertilization rates after sperm stimulation with 2-DA and hence suggested further evaluation of the drug in assisted reproduction programs, Tournaye et al. [150] recommends a careful evaluation and selection of the patients before the treatment with motility stimulants.

Recently, 2-DA and pentoxifylline have been suggested for sperm stimulation in an *in vitro* culture of testicular tissue in order to obtain motile spermatozoa for ICSI [175]. However, it seems rather questionable whether a treatment of spermatozoa with 2-DA for the purpose to fertilize oocytes should be recommended because there is also evidence that it has adverse effects on embryos. In this regard, 2-DA significantly reduced the cleavage of mouse embryos beyond the 2-cell stage [160]. In addition, 2-DA and cAMP have been shown to exert cytotoxic effects by inducing G1 cell cycle arrest [176]. In view of this, a therapeutic clinical use of stimulants that increase the intrac-

ellular cAMP levels should be evaluated very carefully. At least, a very careful washing of the spermatozoa is mandatory.

KALLIKREIN

A substance, which has been discussed very controversially regarding its stimulatory effect on sperm motility and sperm functions during the past ten years, is kallikrein. Despite all components of the kallikrein-kinin system are present in the male and female genital tract [177,178] and the localization of the bradykinin B_2-receptor in rat testis [179,180], the function of the kallikrein-kinin system for male reproductive function is still unclear. The prostate-specific human glandular kallikrein, which is about 500-times less effective than tissue kallikrein, is present in human seminal plasma [181]. In addition, in vitro studies reported a positive effect of kallikrein and its cleavage products, the kinins, on sperm functions including motility [182,183]. This suggests an involvement of this enzyme or bradykinin in the male reproductive system. Gerhard et al. [184] reported a significant improvement of sperm motility when using kallikrein in an artificial insemination program where 172 patients were randomly assigned to the treatment and control group. However, the penetration distance of sperm in cervical mucus was significantly lower and was regarded as cause for the lower pregnancy rates in this group of patients. In contrast, Schill et al. [185] found an increased cervical mucus penetrability of human spermatozoa following treatment of with hog pancreatic kallikrein and bradykinin.

The results regarding ART are as contradictory as the results on *in vitro* stimulation of spermatozoa. Schill and Littich [186] reported an increased pregnancy rate in intrauterine insemination following stimulation of spermatozoa with kallikrein in a cross-over blind study in 48 asthenozoospermic and oligoasthenozoospermic therapy-resistant patients in 468 inseminations. All patients included in that study were shown to respond to this treatment beforehand. On the other hand, there are *in vitro* studies [187,188] and double-blind placebo-controlled studies [189,190] that did not show any effect of kallikrein and bradykinin on sperm motility or sperm count. Miska et al. [191] showed that porcine pancreatic kallikrein, which is taken orally, is absorbed in unaltered form by the intestine. In the light of the recent data by Monsees et al. [178-180] obtained in the rat, the kallikrein-kinin system in the male reproductive tract seems to play a role in the regulation of Sertoli cell function, the local regulation of spermatogenesis or in the function of the seminiferous tubules.

BICARBONATE

Another interesting approach to improve sperm motility and thus sperm recovery from the ejaculate that has not yet been used for clinical application in assisted reproduction is the stimulation of the sperm cell's kinematics by bicarbonate [192]. It is well known that bicarbonate is a major secretory component of the fallopian tube that stimulates sperm respiration [193], and is also postulated to be beneficial for fertilization [194]. The latter appears to be supported by its effect on capacitation, induction of acrosome reaction [192,195,196] and hyperactivated motility, which in turn is required for successful zona penetration in the hamster [197]. These physiological changes, especially acrosome reaction and hyperactivation, require the influx of Ca^{2+} into the spermatozoa. Acrosome reaction is absolutely dependent on the presence of extracellular calcium [195]. Recent work by Wennemuth et al. [198] showed that bicarbonate also facilitates the opening of voltage-gated Ca^{2+}-channels, which are eventually involved in the increase in flagellar beat frequency shortly after stimulation. Thus, bicarbonate is an important mediator of sperm cell function.

Therefore, media with enhanced levels of this anion might be helpful for sperm preparation and assisted reproduction. Henkel et al. [199] showed that sperm preparation by using a medium containing high levels of bicarbonate resulted in a significantly higher progressive motility as well as sperm recovery. In addition, the co-incubation of human spermatozoa with zonae in this medium resulted in a significantly increased zona binding of the spermatozoa. Jaiswal and Majumder [200] made similar observations in testicular and epididymal spermatozoa from goat and ram. While theophylline, a phosphodiesterase inhibitor, and epididymal fluid only induced a non-progressive flagellar movement of these initially immotile sperm, the addition of bicarbonate induced forward motility in 16 to 40% of the sperm cells. This motion stimulating effect of bicarbonate, which otherwise only appears in a subpopulation of the spermatozoa [201], is mediated by an activation of adenylate cyclase [202,203] with subsequent increased levels of cAMP, which in turn stimulate protein kinase A [204] and results in protein phosphorylation [205]. H89, a highly specific inhibitor of protein kinase A, significantly inhibited bicarbonate induced sperm motility and indicates the importance of this enzyme for sperm motility [201].

Considering motility and acrosome reaction as important sperm functions and bicarbonate as mediator as well as the aspect that this anion is non-toxic as other motility stimulants, it appears tempting to use a high bicarbonate medium not only for diagnostic purposes [199], but also in a clinical approach to improve sperm motility and functions for assisted reproduction. However, as elevated

pH levels can disturb the mitotic spindle, a treatment of oocytes with bicarbonate should be performed with high care.

CHELATORS

In a very recent approach, Wroblewski et al. [106] investigated the influence of different metal chelators, i.e. DL-penicillamine, 2,3-dimercaptopropan-1-sulfonate and meso-2,3-dimercapto-succinimic acid, on human sperm motility in vitro. These authors showed that the percentage motility and velocity, even of swim-up separated spermatozoa, can be enhanced by incubation with these chelators, and speculate of a possible future use of such a procedure to improve fertilization rates in IUI or IVF.

The proposed mechanism of this motility enhancement is the removal of the element zinc from the outer dense fibres (ODF), which are functionally essential substructures in the mammalian sperm flagellum. During spermiogenesis, cysteine and zinc are incorporated in spermatozoa [207]. In human spermatozoa, zinc is localized in the flagellum to an extent of 93–97% [208], especially in the ODF. When incorporated in the ODF, zinc is first associated with the sulfhydryl groups of cysteine by formation of relatively stable zinc-thiol complexes [209] in order to protect ODF from premature oxidation [210]. This trace element is later removed from the sperm to an extent more than 60% during epididymal sperm maturation [207,211]. Thereafter, the sulfhydryl groups are oxidized to disulphide-bridges that stabilize and stiffen the ODF [212], which in turn leads to a better energy conversion and therefore to better motility including forward progression [213,214].

Latest research from our working group, however, showed that at least DL-penicillamine and 2,3-dimercaptopropan-1-sulfonate are not suitable for clinical use as both chelators alter the sperm's responsiveness to the induction of acrosome reaction (Henkel et al., unpublished). Thus, it remains to be seen whether or not other chelators will not show such adverse effects.

PLATELET-ACTIVATING FACTOR (PAF)

Platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycerol-3-phosphocholine) is a biologically highly potent signalling ether phospholipid, which was first described by Benveniste et al. [215]. Apart from its multiple functions on circulation, inflammation, systemic vasodilatation, pulmonary bronchiole constriction, platelet and neutrophile activation, cardiac ischemia, tissue rejection, gastric ulcer and development [216-218], it is reported to be a cellular mediator in reproduction. Here, PAF appears to be involved in implantation [219] and may reflect embryo health and viability [220,221]. Moreover, PAF has been found in spermatozoa of different species like rabbit, mouse, pig, rhesus monkey and human [221-226].

Several authors reported positive effects on motility, capacitation, acrosome reaction and oocyte penetration [227-230]. PAF antagonists can inhibit its positive actions on sperm function [231]. The sperm content of PAF in the species investigated is positively related with the fertility status of the male [232]. Reinhardt et al. [233] provided evidence for a PAF receptor on human spermatozoa at the midpiece and proximal sperm head suggesting that the action of PAF is receptor-mediated, which does not result in increased levels of intracellular cAMP concentrations [234,235]. However, PAF binds to the surface receptor and activates a phospholipase, which in turn converts diacylglycerol (DAG) to inositol triphosphate (IP_3), and thus increases the intracellular $[Ca^{2+}]_i$ concentration that can either be released from intracellular stores or by an extracellular Ca^{2+}-influx via Ca^{2+}-channels. This intracellular Ca^{2+}-increase may thus be responsible for the induction of acrosome reaction [236]. However, although the exact molecular mechanism of the action of PAF has not yet been elucidated in detail, these positive effects led the use of PAF in assisted reproduction. Recently, in a prospective, randomized, blinded study in 143 patients, Roudebush et al. [237] presented data showing that pregnancy rates in intrauterine insemination were significantly improved after separating the spermatozoa with a medium containing PAF. The advantage of such a treatment is that PAF is a natural substance that is non-toxic as it is with many other motility stimulators.

Selection of live spermatozoa from a completely immotile sperm population prior to ICSI

An important aspect of sperm selection and the improvement of sperm functions are reflected by the selection of spermatozoa for ICSI from extremely poor semen quality. In this regard, special emphasis should be laid on the selection of spermatozoa from a completely immotile sperm population. For the success in ICSI it is important to inject viable spermatozoa. Normally, this distinction is made by the sperm's most obvious function, motility; a motile spermatozoon is live. However, in this particular group of patients this distinguishing mark is void and scientists were urged to find non-harmful methods that identify live spermatozoa in a population of immotile sperm and that are suitable for the use in ICSI, i.e. that do not harm the oocyte. To date, two different approaches for the distinction between live and dead spermatozoa have been pursued; (i) the initiation of motility as sign of vitality by means of stimulants and (ii) the identification of live spermatozoa according to their membrane integrity by means of the hypo-osmotic swelling test (HOS test).

The initiation of motility in an immotile sperm population is just a consequence of the idea to improve motility by means of the PDE inhibitor pentoxifylline as described above. As pentoxifylline stimulates motility without altering the sperm membrane [238] it appeared as an ideal substance to initiate motility in immotile spermatozoa. This method was successfully used to identify live testicular and epididymal spermatozoa and live births are reported [155,156,239].

The other option to identify spermatozoa suitable for ICSI is to detect their viability by means of their membrane integrity by the HOS test, which has originally been described by Jeyendran et al. [240]. To our knowledge, this method was first proposed by Pike et al. [241]. In a small study group of 7 patients these authors showed markedly improved fertilization rates when oocytes were injected with HOS test-positive sperm. Others who also demonstrated significantly higher pregnancy rates eventually confirmed these positive results [242-245]. Eventually, the basic technique has been modified and simplified. All of these modifications resulted in the same promising outcome, significantly elevated fertilization and pregnancy rates [246-249]. Buckett [250] suggested a combined HOS test with the eosin-nigrosin stain as a routine test in the andrological laboratory diagnosis to predict the spermatozoa's fertilizing ability in patients with severe and complete asthenozoospermia.

At this point, however, it is also important to mention the possible consequences of fertilization of oocytes with sperm by ICSI. As sperm motility is also significantly negatively correlated with the production of reactive oxygen species in the spermatozoa themselves and positively correlated with sperm DNA fragmentation [251], the probability to select such DNA-damaged spermatozoa for ICSI is far higher. According to present knowledge, sperm DNA fragmentation might not only cause an impaired embryonic development and early embryonic death [252-254], but also an increased risk of childhood cancer in the offspring [255,256]. The latter is due to the vulnerability of human sperm DNA during late stages of spermatogenesis and epididymal maturation. At this stage, DNA repair mechanisms have been switched off, resulting in a genetic instability of the male germ cells [257], especially on the Y-chromosome resulting in male-specific cancers [258]. Therefore, a careful examination and counselling of the patients seems mandatory, and fertilization with ICSI should not be performed at all cost.

Scavengers for reactive oxygen species (ROS)

In order to maintain cellular polarity and function, spermatozoa contain an extraordinary high amount of polyunsaturated fatty acids, particularly docosahexanoic acid, which has six double bonds per molecule in the plasma membrane. This, in combination with the sperm cell's lack of defence systems, which is due to the obvious deficiency in cytoplasm, and therefore its inability to repair membrane damages effectively renders spermatozoa particularly susceptible to oxidative stress [78]. As sperm functions are membrane functions, oxidative damage for any method of assisted reproduction should be reduced to a minimum in order to obtain a maximum of functionally competent male germ cells and to achieve fertilization and pregnancy. Apart from the different sperm separation methods (as discussed above), which also have a significant influence on the ROS production of the spermatozoa, another option to improve sperm functionality and motility is to reduce the detrimental effects of reactive oxygen species on spermatozoa in vitro or in vivo by means of scavenging these highly reactive molecules.

In vivo, spermatozoa depend on scavenging systems provided by the seminal plasma, which is the biological fluid that contains more antioxidant substances than anyone else does. The most important natural antioxidants in seminal plasma seem to be vitamin C and E [259,260], superoxide dismutase [261], uric acid [262], glutathione [263] or the polyamine spermine that acts directly as a free radical scavenger [264]. Patients with fertility problems show reduced levels of the antioxidative capacity of the seminal plasma [265-267]. Thus, in the context of oxidative damage of sperm function not only an excessive ROS production by either leukocytes [268] or the spermatozoa themselves [75,269], but also the provision of a sufficient protective antioxidative system in the male genital tract, including the seminal plasma and the female genital tract is of paramount importance. In attempts to tackle the problem of oxidative stress, spermatozoa are either treated with different antioxidants in vitro or the patients are treated with antioxidative drugs.

GLUTATHIONE / N-ACETYL-L-CYSTEINE

As sulfhydryl groups (SH-groups) play an important role in sperm metabolism and the antioxidative defence, and glutathione (γ-glutamyl-cystenyl-glycine) is a natural, highly effective reducing agent, this substance has been tried as a tool to treat male infertility. In a placebo-controlled, double-blind study, in which 20 patients were daily injected with 600 mg glutathione, Lenzi et al. [270] showed a significant increase in sperm motility, percentage of progressive motility and normal sperm morphology. For *in vitro* treatment of spermatozoa with glutathione during sperm separation, contradictory results have been published. Following swim-up preparation of human spermatozoa in the presence of glutathione Griveau & Le Lannou [271] found an improved acrosome reaction and 24 hours-motility on the same level as for Percoll® gradient centrifugation and suggest that glutathione has a therapeutic potential. In contrast,

Donnelly et al. [272] provided data indicating that this drug has no significant effect on progressive motility, neither by itself, nor in combination with hypotaurine. However, the treatment still afforded a significant protection against ROS-induced DNA damage. Unfortunately, so far only very few studies were undertaken in order to treat male infertility using glutathione *in vitro* or as a treatment for patients. In the human system, a medium based on human tubular fluid supplemented with glucose, taurine and glutathione failed to improve the clinical outcome, fertilization or pregnancy. The morphological quality of the embryos was even lower than in the same medium supplemented with glucose and without phosphate [273]. In the porcine and bovine system a positive effect on *in vitro* maturation of oocytes and blastocyst development, if the spermatozoa were treated with glutathione [274-276] was shown.

Another approach to treat oxidative stress-related male infertility was performed by Oeda et al. [277]. These authors used N-acetyl-L-cysteine (ACC), a water-soluble and non-toxic drug that is used in pulmonary diseases because of its strong effect in decreasing the viscosity of sputum by sulfhydryl-disulfide interchange reaction [278]. In addition, it was shown that the levels of glutathione in the epithelial lining fluid recovered to normal values after administration of ACC in patients with idiopathic fibrosis of the lung, suggesting that ACC may act as a precursor of glutathione and thus facilitate its biogenesis [279,280]. In the human ejaculate, ACC revealed a dose- and time-dependent significant reduction of the ROS production [277]. In addition, the substance significantly improved motility and did not have adverse effects on viability and acrosome reaction. On the other hand, Hughes et al. [281] demonstrated that the addition of ACC to a sperm separation medium induced sperm DNA damage. In a clinical study, Comhaire et al. [282] treated 27 infertile men orally with ACC and showed a significant reduction of ROS. Moreover, significant increases were found for acrosome reaction, the proportion of polyunsaturated fatty acids of the phospholipids and for the sperm count in oligozoospermic men. However, sperm motility, morphology and the pregnancy rate seemed to be unaltered. Whether or not this is due to the elevated acrosome reaction or the small number of patients included or to a reduced DNA integrity cannot be determined yet. This is a rational approach to treat male factor infertility, which should be followed up in a bigger study.

VITAMINS

Apart from glutathione, vitamins C (ascorbate) and E are natural antioxidants in cells and tissues where ascorbate scavenges highly reactive molecules in the watery phase, i.e. cytoplasm and surrounding liquids, and vitamin E is effective in the lipid phase, i.e. the membranes. In order to improve sperm functions in vitro, culture media were supplemented with ascorbate and vitamin E. While Verma & Kanwar [283] observed a dose-dependent improvement of motility and viability accompanied by a decrease in malondialdehyde production following vitamin E supplementation, Donnelly et al. [284] did not show a beneficial effect of neither ascorbate nor vitamin E on sperm motility. Different motility parameters like curvilinear velocity or linearity were even significantly decreased after the treatment. ROS production was significantly reduced whereas the baseline in DNA damage remained unaltered [285]. The simultaneous addition of vitamin C and E to the sperm preparation medium actually induced sperm DNA damage. These negative results of in vitro supplementation with vitamins on human sperm function appear plausible, as the lower levels of ascorbate in the seminal plasma in asthenozoospermic patients [266] can be regarded as a consumption of this antioxidant in vivo. Thus, the oxidative damage of the spermatozoa leading to reduced motility is most probably already set in the testis or epididymis and cannot be repaired by such treatment in vitro.

This consideration is supported by *in vivo* studies in rabbits, boars and in the human, where vitamin E supplementation resulted in improved sperm functions and a reduced production of free radicals [282,286-288]. However, there are also negative reports on the effect of vitamin supplementation. In the human, Rolf et al. [289] did not find changes in semen parameters of 31 asthenozoospermic and moderate oligoasthenozoospermic patients treated with high doses of vitamin C and E in a randomized, placebo-controlled, double-blind study. Likewise, no positive effects on semen volume, pH or sperm motility were observed following a dietary vitamin E administration in the cock [290]. The reproductive performance of the treated cocks was even negatively influenced. These contradictory results show that neither the exact mechanism and site of action, nor the indication for a successful antioxidative treatment of patients are clear. In addition, the action of genital tract inflammations has not yet been taken into consideration. As leukocytes produce enormous amounts of free radicals [32] and their specific impact on sperm function remains to be clarified, more research must be carried out in order to obtain a rational antioxidative therapy for male factor patients.

PENTOXIFYLLINE

Besides the effects of pentoxifylline on sperm motility and acrosome reaction discussed above, this PDE inhibitor has also effects on ROS. It has repeatedly been shown that pentoxifylline significantly reduces the superoxide release of human spermatozoa following phorbol myristate acetate stimulation [291,292]. This effect is possibly due to the reduction of the formation of endoperoxides as a

consequence of the elevated cAMP levels that inhibit the cyclo-oxygenase within the arachidonic pathway [292]. Contradictory results, however, were obtained regarding lipid peroxidation. While Gavella & Lipovac [293] found elevated levels of malondialdehyde after pentoxifylline treatment and rather warned of its use in assisted reproduction, McKinney et al. [294] could not confirm these findings. In a comparative study, Okada et al. [295] confirmed the ROS scavenging and motility stimulating effect of pentoxifylline *in vitro* in 15 patients and 18 controls, respectively. However, *in vivo* pentoxifylline at low dosages (300 mg per day) failed to decrease ROS generation and to increase motility. On the other hand, at high dosages (1,200 mg per day), motility and beat cross frequency were increased but the drug still did not have a beneficial effect on sperm fertilizing ability.

Reduction of visco-elasticity of the ejaculate

In most mammals, semen coagulates shortly after ejaculation and liquefies again later on. In the human, liquefaction takes place within 5–20 minutes after ejaculation. While so-called coagulation proteins like semenogelin or fibronektin, which derive from the seminal vesicle, promote coagulation, seminolysis is caused by a prostate-derived serine-proteinase, prostate-specific antigen (PSA) [296]. However, in some cases viscosity of the semen remains high and can be a cause for male infertility. It is also important to differentiate between ejaculates that have an excessive viscosity and such ejaculates that failed to liquefy [107]. As highly viscous semen can reduce sperm motility thus being a cause for male infertility, it is necessary in the clinical set-up of assisted reproduction either to reduce semen viscosity or to liquefy the ejaculate artificially. For this, a few methods are available. Mixing the semen with medium is the easiest way but will not work sufficiently in cases of excessive semen viscosity. Another method that was suggested in the past is to force the viscous seminal fluid through a narrow-gauge needle. However, considering the severe damage that this method causes to the spermatozoa including immotility, it cannot be recommended at all. It is therefore rather advisable to liquefy such ejaculates enzymatically by using a 0.2% solution of α-amylase. Several other enzymes like α-chymotrypsin, lysozyme or hyaluronidase have also been described for this purpose. Recently, a special trypsin-based dissolving solution, SpermSolute, has been reported [297]. However, as these enzymes can also damage spermatozoa, special care should be taken to remove the enzymes by washing procedures as soon as liquefaction is completed.

Concluding remarks

In conclusion, there are a number of different sperm separation methods available, which can be applied, even in combination with pharmacological substances to stimulate sperm functions or to protect the male germ cells from the detrimental influence of free radicals. Therefore, in patients with elevated ROS levels in the ejaculate or with proven or suspected genital tract inflammations, the conventional swim-up technique should certainly not be the method of choice, but rather more gentle methods like density gradient centrifugation, glass wool filtration or migration-sedimentation. A supplementation of the sperm separation medium with protective substances can be considered. It is also of paramount importance to discriminate between various patient groups including the consideration of seasonal changes of semen and sperm parameters. For the improvement of the percentage of normally chromatin-condensed spermatozoa, for instance, glass wool filtration or migration-sedimentation should be preferred. In order to liquidize viscous semen samples, one should absolutely refrain from forcefully aspirating semen through narrow gauge needles as this procedure severely damages the spermatozoa. Instead, an enzymatic liquidization with subsequent washing of the spermatozoa should be preferred. Thus, the sperm separation method must specifically be chosen in every individual case and consideration should be given for treating the patient pharmacologically *in vivo*. This obviously requires a careful andrological work-up and examination of male partners of infertile couples.

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