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Review

High quality sperm for nonhuman primate ART: Production and assessment

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Abstract

Factors that affect sperm quality can include method of semen collection, conditions for capacitation and whether or not agglutination is present. Media and procedures for sperm washing can also impair or improve sperm function in assisted reproductive technologies. For example, the removal of seminal fluid through large volume washing is required to eliminate decapacitation activity of seminal plasma. The forces involved with centrifugation and the metabolic stress of tightly pelleting sperm during washing procedures can have deleterious results. In contrast to human sperm, sperm from the most commonly used species of nonhuman primates, rhesus and cynomolgus macaques, do not spontaneously capacitate in vitro; rather, chemical activation with dibutryl cyclic AMP and caffeine is required. Recognizing motility patterns of non-activated and activated sperm can be accomplished with simple observation. After activation, sperm agglutination sometimes occurs and can interfere with sperm binding to the zona pellucida. Because nonhuman primate oocytes require a large investment to produce and currently, each animal can be hormonally stimulated a limited number of times, it is important to have means to evaluate quality prior to using sperm from a new male for in vitro fertilization. Methods for producing live, acrosome reacted sperm may also have application for ICSI. Because many genetically valuable males are now being identified, it may be necessary to individualize sperm preparation to accommodate male-to-male variation.

Introduction

The necessity of high quality sperm preparations may appear less important as ART advance in nonhuman primates. However, the study of intracytoplasmic sperm injection (ICSI) in humans and nonhuman primates has revealed that the use of randomly selected sperm for this procedure can result in various anomalies of sperm decondensation and embryonic development [1]. In a review of human clinical ICSI reports, chromosomal and genetic abnormalities are increased, but are most likely a result of underlying parental risk of the couples that require this procedure to achieve pregnancy [2]. These studies have underscored the importance of sperm quality for ART procedures.

The processing of rhesus monkey (Macaca mulatta) semen for recovery of high quality spermatozoa has its basis in methods developed more than 20 years ago when in vitro fertilization (IVF) was first achieved for rhesus monkey oocytes [3]. The medium used for washing spermatozoa from the seminal plasma as a modification of Tyrode's medium supplemented with bovine serum

albumin (BSA) that was originally developed by Bavister [4] for use in hamster IVF. This medium was improved with the addition of lactate and pyruvate, known as TALP, by Bavister and Yanigamachi [5] also for use in hamster IVF experiments.

Macaque spermatozoa do not spontaneously capacitate and acquire the ability to bind to the zona pellucida as do human and some other species of spermatozoa. It was critical to the development of methods for macaque IVF that sperm be capacitated and capable of completing fertilization. Therefore, the next important modification in macaque spermatozoa processing was the discovery that cAMP and caffeine were required for rhesus monkey sperm to acquire fertilizing ability [6]. This chemical method for capacitation is often referred to as "activation."

The development of the TALP medium and the addition of the cAMP and caffeine set the stage for successful IVF of macaque oocytes. The first successful IVF in the rhesus monkey [3] and the report of the first live rhesus infant from IVF and embryo transfer [7] both used the TALP medium for washing and incubation of sperm and the subsequent addition of cAMP and caffeine. The same basic method for sperm processing was also used for the first successful IVF in the cynomolgus monkey [8].

Methods for semen collection

The method used for semen collection can make a difference in semen quality. The earliest studies with rhesus semen usually utilized the rectal probe collection method [9,10]. Although the rectal probe method for semen collection has been used successfully in primates as well as many other species, the requirement for anesthesia, accurate probe placement, operator skill and the risk of burn injury limit the effectiveness of this technique [11,12].

The direct penile electro-stimulation method was pioneered by Mastroianne and Manson [13] using metal foil electrodes and square wave pulses and has been used successfully by other investigators [14]. This method was improved with the use of EKG gel electrodes, which greatly reduced the risk of burn injury [15]. Animals are not anesthetized for this procedure, rather are trained for chair restraint. Most reports of semen collected by this method indicate larger semen volumes and higher numbers of sperm per ejaculate compared to the rectal probe method. It may be that the direct penile method achieves better stimulation of the entire reproductive tract and fewer problems with urine contamination due to the lack of anesthesia.

Several factors are important to assure the best collection of semen with the direct penile electroejaculation method. Probably the most critical factor is the training and acclimation of animals to the chair restraint and the procedure. During the training process it is important that the operator maintain a calm, positive attitude. Settlage and Hendrickx [16] reported that semen collection can be performed several times in a row without ill effects and sperm numbers are maintained through several electroejaculations, but will eventually decrease with multiple collections. The experience at CNPRC has been that an early morning collection time produces the best samples. The time of day may be an important factor because later in the morning the male may have already masturbated to the point of depleting sperm numbers in the ejaculate. Finally, once the animal is trained and experienced with the procedure, he must be watched carefully for spontaneous ejaculation before any electro-stimulation is applied.

Typical values for rhesus ejaculate volume and total sperm per ejaculate are shown in Table 1; the range of values for both of these parameters for ten rhesus males are given.

Table 1: Ejaculate volumes and total sperm numbers of rhesus monkeys

	Ejaculate Volume (μl)			Total Sperm Count (×10⁶)		
Male #	Low	High	Average	Low	High	Average
1	200	1000	500	30	940	100
2	400	1200	1000	40	480	80
3	50	175	120	25	1260	100
4	50	800	200	10	210	100
5	30	1100	500	20	300	130
6	200	1200	500	50	990	200
7	200	1100	400	120	850	300
8	150	1100	300	30	300	200
9	30	600	200	6	400	30
10	20	500	150	10	500	120
Mean			387			136

All rhesus males used for the data in Table 1 were long-term semen donors at the California National Primate Research Center (CNPRC). When selecting a new donor, three males are trained and acclimated to the electroejaculation procedure and the male with the best semen parameters is chosen. In general, the percentage of motile, forward progressing sperm in rhesus semen is very high, usually above 85% [15]. Rhesus sperm are also relatively uniform in structure and morphology [15,17].

Other methods of semen collection that do not require electro-stimulation, such as artificial vagina and mounting dummies, have been investigated at the CNPRC without success. The artificial vagina method of semen collection has been successful for chimpanzees [18] and orang-u-tans [19], which may be due to their greater intelligence and ease of training to new behaviors. With further work, especially on methods for behavior modification in using an artificial vagina, successful semen collection might be possible. However, it is important that any method developed be reliable and repeatable for the majority of males because increasingly, individual males of genetic importance are being identified.

Semen washing and incubation

Once semen is collected into a 15 ml centrifuge tube it stands at room temperature for 30 minutes to allow the coagulum to exude the liquid fraction of the semen. The coagulum of rhesus monkey semen does not liquefy, but continues to contract and expel a liquid portion that contains the motile sperm. However, if the semen stands for longer than 30 minutes, the coagulum can reabsorb sperm and the total number of sperm per ejaculate and the percentage of motile sperm can decrease [16]. Therefore, it is important to carefully transfer the liquid fraction or remove the coagulum approximately 30 minutes after the semen is collected. Contamination of the liquid fraction with small pieces of coagulum can lead to agglutination problems after washing (see below).

Semen is washed three times by dilution with large amounts of medium and centrifugation. A modified Tyrode's medium with bovine serum albumin and lactate (TL-BSA) is currently used at CNPRC [20]. The large dilution, usually 200 to 500 μl of semen with 15 mls of medium, is necessary to assure the removal of a decapacitation factor that is present in rhesus monkey semen [21]. Macaque sperm are easily damaged by centrifugal force and must be centrifuged at no higher than 400 × g. For most semen samples, this washing procedure yields a sperm suspension that is over 85% motile, therefore further processing, such as centrifuging over a gradient, is not necessary because of the high percentage of motile sperm. Macaque sperm do not retain their motility when highly concentrated or pelleted for the extended time necessary to perform swim-up procedures.

Macaque sperm can be sensitive to the water used in the TL-BSA medium, so a quality control tested water is recommended. Soft plastics, such as disposable transfer pipets with attached bulbs have had detrimental effects on sperm quality. Care must also be taken when pipetting sperm suspensions because sperm can be damaged if sperm is moved quickly through a narrow opening.

Sperm capacitation and activation

The medium used for the initial washing and incubation of macaque sperm must contain bovine serum albumin. As will be discussed later, substitution of BSA with polyvinylalcohol (PVA) will not support the development of the ability of the sperm to bind to the zona pellucida and their subsequent acrosome reaction. Rhesus monkey semen has been shown to contain a decapacitation factor [21] and it may be that BSA in the washing and incubation medium either removes or neutralizes that factor.

As mentioned above, rhesus sperm will not spontaneously capacitate, but instead require dibutyryl cyclic AMP (dbcAMP) and caffeine to activate sperm [6]. The motility changes that typically indicate that activation has been successful include a marked increase in lateral head displacement and rapid changes in sperm swimming trajectory [22]. Although either of these chemicals should lead to an increase in intracellular cAMP, both are necessary to support activated motility changes and IVF. This method of chemical capacitation presents a problem of appropriate timing of insemination of oocytes. The chemicals that complete the capacitation process of sperm are also the same compounds that can prevent resumption of meiotic maturation of oocytes. Thus, the addition of activated sperm to the oocytes in culture must wait until resumption of meiosis, or germinal vesicle breakdown, has occurred.

Sperm-zona pellucida binding and subsequent acrosome reaction of the bound sperm can be a useful tool to determine the functional ability of sperm without requiring the use of expensive, developmentally competent oocytes [23]. Oocytes from non-stimulated ovaries are recovered at the time of ovariectomy or necropsy and intact oocytes are placed in medium with 4 M DMSO at -80°C. Upon thawing, only intact zona pellucida, with the cytoplasmic contents still inside, are used for the assays. Sperm are co-incubated with the zona pellucida for approximately 1 minute, then fixed and labeled. This technique has been useful in determining that dbcAMP and caffeine are both required for activation of macaque sperm and that each chemical has a separate effect on sperm [22]. It has also been used to demonstrate that macaque sperm are acro-

some intact at the time of binding to the zona pellucida, then quickly acrosome react after binding [24].

An alternative to using whole, intact zona pellucidae for the zona binding assay is the hemi-zona assay [25]. This assay has also been used to evaluate the quality of cryopreserved sperm [26] and the functional performance of sperm after treatment with a sperm acrosomal antigen [27]. Both of these assays that evaluate the ability of sperm to bind and acrosome react on the zona pellucida produce valuable information and are a reasonably inexpensive, quick alternative to IVF.

Agglutination of sperm

One of the real frustrations in dealing with macaque sperm is the ability of seemingly high quality sperm to agglutinate while being held for later in vitro fertilization, especially when the agglutination occurs only after the sperm have been activated. The causes of agglutination are largely unknown. Occasionally, small pieces of coagulum will be carried through the sperm washing process and will cause sperm to agglutinate in large numbers around the coagulum. These clumps of agglutinated sperm will often appear as mats of sperm when observed on a microscope slide under a cover slip. However, most agglutination occurs as small groups of 2 to 10 sperm with heads adhering to each other.

The exact causes of head-to-head agglutination is not known, but can be experimentally induced with anti-sperm antibodies [28,29]. Although agglutination is not well understood, the effects can be minimized. At CNPRC it has been noted that if animals are stressed during collection it will increase the occurrence of agglutination. The medium used for washing and incubating sperm may increase the effects of agglutination. Alterations in media components may be helpful. Recent data from CNPRC indicates that the substitution of PVA for the BSA in the washing medium may help avoid agglutination, particularly the type that occurs only after activation. Table 2 shows a comparison of Tyrode's medium containing lactate and either BSA or PVA with respect to the number of sperm bound to the zona pellucida and the percentage of bound sperm that acrosome react. Although sperm washed and incubated in TL-PVA and TL-BSA both exhibit activated motility, only the sperm samples washed in TL-BSA can bind to the zona pellucida (72 ± 45%) and acrosome react (14 ± 5.5%). In contrast, sperm samples washed in TL-PVA exhibit minimal zona binding (6 ± 4%) with a complete absence of the acrosome reaction.

BSA must be used in the washing procedure to assure that sperm attain the ability to fertilize oocytes, even though it may increase the incidence of agglutination. However, completing the initial washing steps for sperm in TL-BSA medium and then incubating sperm in TL-PVA medium has been successful in supporting IVF while reducing the occurrence of agglutination. In current experiments the use of other media for washing and incubation are being investigated. A wide variety of media, such as DMEM, BWW and Ham's F-12 will also support sperm motility and activation in nonhuman primates (unpublished observations). However, their ability to support zona binding and the acrosome reaction require further study.

Table 2: Macaque sperm-zona pellucida binding with and without BSA.

	No. sperm per zona	% acrosome reacted
TL-PVA	6 ± 4	0 ± 0
TL-BSA	72 ± 45[a]	14 ± 5.5[a]

[a]Significantly ($p < 0.05$) different than TL-PVA

Producing acrosome reacted sperm

There is increasing interest in methods to produce acrosome reacted sperm for injection into oocytes. As noted above, the use of randomly selected sperm for ICSI can result in various anomalies of sperm decondensation and embryonic development [1], which are mainly attributed to the acrosomal contents and the plasma membrane of sperm [30]. These structures are not usually present during the normal fertilization process because macaque sperm acrosome react at the time of binding to the zona pellucida [24]. In fact, there is increasing evidence in other species that the removal of the acrosome or at least its disruption, may improve the success of the ICSI procedure [31].

Many methods for producing acrosome reacted sperm are not effective for inducing acrosome reaction in macaque sperm. For example, it has been well established that progesterone induces the acrosome reaction of human sperm [32], however, similar concentrations of progesterone are not effective for macaque sperm (unpublished results). The acrosomes of macaque sperm can be removed by treatment with the calcium ionophone A23187 [24]. It is important to note that the procedure requires that sperm be pipetted vigorously so that the acrosomes will be removed. In macaques, as sperm acrosome react the fused vesicles bind tightly to each other and form a 'shroud" which is not easily removed from sperm in suspension unless subjected to mechanical treatment [24]. Once sperm have been treated with A23187, they will be immotile unless processed in a medium using egg yolk extender [33]. Because the effects of such media components on the oocyte are unknown, it would be advisable to induce the acrosome reaction in the standard washing and incu-

bation medium. Also, excess ionophore should be removed from the suspension to avoid any potential negative effects that inappropriately injected ionophore might have on oocyte activation.

Conclusions

Improvement in the handling of macaque sperm may help improve fertilization and embryo development in ART procedures. Semen collection methods that do not require electroejaculation but that would be reliable for all males should be a priority. Other areas of emphasis for future research should include alternative media for washing and incubation of sperm that will prevent agglutination and allow spontaneous capacitation without the addition of chemicals that can potentially inhibit oocyte maturation. Motility and morphology are not very predictive of IVF success, however assays for sperm-zona pellucida binding can be very indicative of function after activation. Methods for selecting sperm through gradients have potential for producing higher quality sperm for various ART procedures by eliminating abnormally motile sperm, but care must be taken that the procedure does not damage sperm by keeping them at high concentrations for more than a few minutes. Methods are needed for inducing the acrosome reaction in macaque sperm without chemicals that might be transferred into the oocyte with sperm injection. The success of ART procedures, including fertilization and subsequent embryo development, is partially dependent on sperm function and efforts to improve sperm quality should continue.

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