Significance of incorporating measures of sperm production and function into rat toxicology studies

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The rat is the preferred species for reproductive toxicity testing. The inclusion of measures of rat sperm quality, such as motility and morphology, into reproductive test protocols often increases the sensitivity of the test to detect effects, and provides the toxicologist and risk assessor with valuable information about the nature of the reproductive toxicity of the test substance. Technical advances in computer-aided sperm analysis have made it possible to evaluate motion characteristics of rat spermatozoa. This technology can provide an objective means of classifying the motion of rat spermatozoa as progressive or non-progressive, as required in test protocols. More specific tests of rat sperm function are being applied for the purpose of evaluating modes and mechanisms of toxicant action. Computer-aided sperm analysis can be used to evaluate sperm motion during cultures that support sperm capacitation and to identify hyperactivated spermatozoa. Under the same culture conditions, acrosome-specific stains can be used to identify effects of toxicants on the acrosome reaction. These approaches, in combination with *in vitro* fertilization in rats, can pinpoint sperm functional deficits and thereby assist the toxicologist in addressing hypotheses regarding the cellular–molecular bases of toxicant-induced male infertility.

Male reproductive toxicology is a relatively new field that has emerged in part as a consequence of reports in the late 1970s that male workers became infertile after being exposed to the pesticide dibromochloropropane (DBCP) (reviewed in Lahdetie, 1995). Epidemiological investigations involving the examination of semen samples provided by these men revealed that their infertility was due to azoospermia or diminished sperm counts. Thus, semen analysis, which had been an investigational tool for the infertility physician for many years, became a means for investigating the potential for drugs and chemicals to affect male reproductive health.

The widely publicized DBCP story served to alert regulatory agencies, as well as the public, about the potential for toxicants in the workplace or the environment to alter reproductive function in men. It also provided the rationale for adding direct evaluations of sperm production and quality to reproductive toxicology test protocols in which the rat is the preferred test species. Here we review the significance of including data on rat sperm production and function, such as epididymal sperm reserves, motility and morphology, in toxicology testing. In addition, we discuss the development and application of more specific tests of rat sperm function as may be needed to elucidate the

modes and mechanisms of toxicant action relevant to estimating reproductive risks in humans.

Sperm measures as endpoints in reproductive test protocols

It is important to understand the context in which sperm measures are used as markers of male reproductive effects in toxicology testing. Federal agencies, such as the Environmental Protection Agency (EPA) and the Food and Drug Administration (FDA), and international bodies, such as the Organization for Economic Cooperation and Development (OECD), provide a number of test protocols and guidelines for identifying adverse reproductive effects (reviewed in Blazak, 1997; Clegg *et al.*, 2001). These protocols and guidelines are used by industry to test pesticides, industrial chemicals, pharmaceuticals and food additives for potential reproductive toxicity in laboratory animals. The rat is the animal of choice for these tests, in part because there is a large toxicology database for this species. If a substance is identified as a reproductive toxicant in rats, further studies are conducted to determine the level at which it is toxic, the likelihood that it will be toxic to humans as well as to rats, and the extent to which humans might be exposed to the substance. All of this information is critical for performing a risk assessment on

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the chemical and, ultimately, for making regulatory decisions about the allowable uses of the substance as well as labelling requirements.

The most comprehensive test for reproductive toxicity is the multigenerational test, which is designed to detect adverse effects of exposure at all stages of the reproductive life cycle, as well as potential transgenerational effects (reviewed in Blazak, 1997; Clegg *et al.*, 2001). Young adult rats of both sexes (the parental or F_0 generation) are exposed to the test chemical daily for 10 weeks, a period sufficiently long to ensure that effects on germ cells exposed at any stage of spermatogenesis will be detected when that animal is mated. The animals are then mated with each other and pregnant dams are dosed through pregnancy, birth of the first (F_1) generation and lactation. After weaning, selected F_1 pups are dosed into adulthood and throughout breeding, pregnancy and lactation to give rise to a second (F_2) generation that is exposed until weaning. The multigenerational test relies heavily on measures of reproductive performance such as breeding, fertility and litter size, with additional information provided by reproductive organ weights and routine histology measured in each generation. Since both partners in breeding pairs have been treated, it is frequently impossible to ascertain the affected sex and, since treatment is prolonged, little information is provided regarding specific cellular targets. A variation of this test, the reproductive assessment by continuous breeding (RACB) test used by the National Toxicology Program to evaluate high priority chemicals, includes serial breeding during the treatment phase in the parental generation. The RACB test obtains additional information about the onset and severity of infertility and includes the option of crossbreeding with untreated animals to determine the affected sex (Chapin and Sloane, 1997; Clegg *et al.*, 2001).

In 1998, as part of an interagency harmonization effort and after much deliberation, the standard multigenerational test protocol was revised and updated to include direct measures of sperm production, motility and morphology (US Environmental Protection Agency, 1998; Chapin and Conner, 1999; Claudio *et al.*, 1999). These measures had been included in the RACB test, initially using mice but more recently using rats (Chapin and Sloane, 1997; Chapin *et al.*, 1997) and proved useful in numerous other reproductive toxicology studies in rats, especially those involving shorter-term exposures (Harris *et al.*, 1992; Linder *et al.*, 1992; Clegg *et al.*, 2001). This article focuses on the significance of sperm measures, but the harmonized guidelines also emphasize the importance of optimal fixation of the testis so that subtle changes in testis histopathology can be detected (Russell *et al.*, 1990; Chapin and Conner, 1999). The guidelines also include morphological measures of sexual differentiation and puberty that are sensitive indicators for environmental oestrogens and other hormone mimics (Claudio *et al.*, 1999; Clegg *et al.*, 2001). As currently structured, the harmonized multigenerational test and the RACB test are rec-

ognized as highly effective, apical tests for hazard identification when screening pesticides, drugs and industrial chemicals for effects on reproduction and fertility.

Spermatid counts in testes and epididymides

Information about sperm production and storage is obtained when rats are killed at the end of each generation in the multigenerational tests, or at specified time points in short-term protocols. In general, one testis and epididymidis is fixed for histologic evaluation, while the other is used for determining sperm measures. Sperm production by the rat testis is conveniently measured by counting the number of condensed spermatids in a testis homogenate, expressed as the number per testis or per gram of testis (Robb *et al.*, 1978). In turn, the number of spermatozoa per testis can be divided by 6.1 days (the period that spermatids reside in the testis after their chromatin condenses and they become resistant to disruption by homogenization or sonication) to derive the sperm production rate (number of spermatozoa produced per day). Likewise, mature spermatozoa are collected from the cauda epididymidis and counted to determine cauda epididymal sperm reserves. Computer-assisted counting of sperm nuclei stained with a DNA-specific fluorochrome (Strader *et al.*, 1996) can enhance the efficiency with which these measures are obtained.

Baseline data accumulated in control (untreated) rats indicate that these measures are dependent on age in young, pubertal rats, but are stable in adult rats (Robb *et al.*, 1978; Working and Hurtt, 1987; Blazak, 1997) and so make good indicators of impaired testis function or altered epididymal capacity. After reviewing the RACB data for 72 studies in mice, Chapin *et al.* (1997) concluded that epididymal sperm count was related linearly to fertility. When combined with data on either sperm motility or sperm morphology (both of which exhibit fertility thresholds), much of the variation in fertility could be explained ($r = 0.77$). Whether these relationships hold in rats will be determined as more data for these endpoints become available from multigenerational studies using the new protocol.

Numerous examples from both long- and short-term studies demonstrate that significant alterations in testicular and epididymal spermatid counts can occur at doses of toxicants that are lower than those associated with sterility (Linder *et al.*, 1992; Clegg *et al.*, 2001). Thus, when interpreted with other data from fertility and histopathological examinations, sperm counts are useful for corroborating anti-fertility effects, elucidating the mode of toxicant action, detecting effects at low doses and helping to determine the affected sex.

Evaluation of sperm motility in rats and the use of computer-assisted sperm analysis (CASA)

Sperm motility is a requirement for fertility. Therefore, it is often useful to examine epididymal spermatozoa to

determine whether changes in sperm motility account for changes in fertility status. The harmonized test protocols specify that epididymal spermatozoa will be examined to determine the 'percentage of progressively motile spermatozoa.' The intent of the test is to determine whether sperm samples recovered from treated animals differ significantly, with respect to their motion, from those recovered from untreated control animals.

The use of computer-assisted sperm analysis (CASA) makes it practical to evaluate the motion of a large number of spermatozoa in a short time. Microscopic images of spermatozoa are detected by video technology and a computer 'captures' each video image, records the location of the sperm head in each video frame and reconstructs the sperm path by connecting those images. Typically, today's instruments capture the maximum number of images possible according to the video standard in use (60 images s^{-1} in the US and 50 images s^{-1} in Europe), and then generate values for seven or eight characteristics for each track (for a technical review, see Boyers *et al.*, 1989), each of which provides information about one aspect of sperm motion, such as vigour, velocity or swimming pattern.

CASA technology was first applied to rat epididymal spermatozoa by Working and Hurtt (1987) and its potential advantages in terms of efficiency and objectivity were immediately attractive to the toxicology community, which began to explore the usefulness of this technology. It soon became clear that methods for sample collection, preparation and handling (for example, culture medium and temperature), as well as chamber depth and instrument settings, can give rise to a great deal of intra- and inter-laboratory variation in values obtained by CASA (Slott *et al.*, 1991; Chapin *et al.*, 1992). Workers using CASA convened to discuss these issues and published guidance on the conduct of CASA for rat spermatozoa (Seed *et al.*, 1996). Improvements in the optical systems and capture algorithms in CASA systems have increased the accuracy of rat sperm tracking, and more powerful computers have made it possible to track spermatozoa for longer periods, even continuously (Moore and Akhondi, 1995). These advances have improved the consistency of the data, and CASA is now used routinely to monitor rat sperm motion in many toxicology laboratories (Perreault, 1998).

Although Federal test guidelines do not require the use of CASA, the method is well suited for defining 'progressively motile spermatozoa' on the basis of selected CASA parameters. Freshly diluted cauda epididymal rat spermatozoa typically swim 'fast and straight'. Workers using CASA can set thresholds for two CASA measures, one for 'fast' and one for 'straight' and require that a spermatozoon exceed both to be considered progressively motile. In this regard, the measure called velocity of the average path or VAP, and the measure called straightness (derived from the ratio of straight-line velocity to VAP) can be combined in a bivariate analysis to identify and count progressively motile spermatozoa automatically. For example, we

have found that a VAP threshold of 100 $\mu m s^{-1}$ and a straightness threshold of 50 defines about 80% of cauda epididymal spermatozoa from untreated (control) rats as 'progressively motile' under our sample preparation and analysis conditions (S. Perreault, S. Jeffay and L. Strader, unpublished). The percentage of progressively motile spermatozoa was found to be the most valuable measure (when compared with individual CASA parameters) for detecting subtle changes in sperm motion induced by three model toxicants with different mechanisms of action (Horimoto *et al.*, 2000).

Information about the percentage of progressively motile spermatozoa is useful when a chemical affects the percentage of motile spermatozoa or the quality of sperm motion. In many cases, a testicular toxicant, applied for a long period, will produce testicular atrophy at the higher dosage, effectively shutting off the production of spermatozoa, with consequent infertility. However, at lower dosages, or if the chemical is being studied after short exposure times to track the development of the infertility, the adverse effect may show up as a decrease in the percentage of motile spermatozoa, the quality of the motion (fewer progressive spermatozoa), or both. This effect may occur in the absence of an effect on fertility. For example, several Sertoli cell toxicants produce testicular atrophy at high doses, but poor sperm quality, secondary to germ cell sloughing, at low doses (Richburg *et al.*, 1997; Li and Heindel, 1998). In such cases, information on sperm numbers, motility and morphology helps to determine the no adverse effect (and low adverse effect) doses. Such effects might be missed if fertility were the only outcome considered.

These measures are also valuable indicators of toxicity in short-term studies that have been proposed as more efficient and less expensive screens (Harris *et al.*, 1992; Linder *et al.*, 1992). After only a few days or weeks of exposure, the consequences of the toxicity may not be fully manifest, but the hazard may be identifiable by changes in spermatozoa that have reached the epididymis (Harris *et al.*, 1992). Likewise, sperm motility is a valuable indicator of toxicity that involves only the epididymis or the mature spermatozoa during epididymal transit (Perreault, 1997; Klinefelter and Hess, 1998; Perreault, 1998).

Although test guidelines call for only one measure of the quality of sperm motion (that is, the percentage of progressively motile spermatozoa), specific CASA outcomes, such as straightline or curvilinear (point-to-point) velocity or track amplitude, may provide additional information, individually or in multivariable analyses, on the pattern of motility and how it might be altered specifically by a given toxicant. These specific CASA outcomes are also useful for monitoring changes in sperm motion that occur during epididymal maturation *in vivo* and *in vitro* (Yeung *et al.*, 1992; Klinefelter, 1997). In these cases, the mean (or median) is calculated for a population of spermatozoa (usually 100–200) from each animal. Consideration of the distribution of each CASA endpoint within animals may also be important for detecting specific exposure-related

changes (Toth *et al.*, 1989), as well as changes in motion associated with the fertilizing spermatozoon in the female tract. However, it is important to consider that several of the routine CASA outcomes are highly correlated with each other and are not truly independent measures. A novel means for calculating three CASA outcomes, one that estimates straight line velocity, one that estimates linearity, and a third that indicates predictability of the path, has been introduced and demonstrated to comparably (and perhaps more reliably) describe rat sperm motion (Dunson *et al.*, 1999). Now that CASA technology has matured, more meaningful ways of handling the large amount of CASA data are emerging. Valid statistical evaluation will be critical for demonstrating the ultimate utility of sperm motion data in risk assessment.

Evaluation of sperm morphology in rats

Typically, rat sperm morphology is evaluated by examining either wet preparations of fixed epididymal spermatozoa using phase-contrast microscopy, or air-dried, stained smears using conventional light microscopy (Linder *et al.*, 1992; Seed *et al.*, 1996; Chapin and Conner, 1999). Untreated rats usually exhibit very few morphological abnormalities (< 2%) and examination of 200–500 spermatozoa per rat is recommended to detect changes in this outcome. Each spermatozoon is scored as normal or abnormal, and atypical forms are classified by abnormality in a manner that distinguishes head, midpiece and tail defects (Linder *et al.*, 1992). Head abnormalities described in the literature include: blunt hook, banana-head, amorphous head, pinhead, and double head (Seed *et al.*, 1996). However, it can be difficult to distinguish borderline forms with confidence. Automation of sperm morphology measurement using CASA is more difficult for rat spermatozoa, with their hook-shaped heads, than it is for round or oval headed spermatozoa. However, one system has been described (Davis *et al.*, 1994), and commercially available programs are currently being developed and validated.

As with motion analysis, sample preparation is critical. The spermatozoon must lie very flat to ensure accurate assessment of the hook. Any other orientation can introduce visual artifacts and result in misclassification of normal spermatozoa as abnormal. Subtle differences such as straightened hooks can be indicative of immaturity or premature spermiation, while blunted hooks can be indicative of abnormal acrosomes or acrosomal degeneration. More obvious forms, such as amorphous head shapes and double or fused spermatozoa, are easier to identify and may represent more biologically significant malformations (Linder *et al.*, 1997). As with sperm motility measures, sperm morphology data can provide valuable insights regarding the dose response to a chemical. In addition, morphological deficits, such as detached heads and abnormal tails, may correlate with sperm viability and be indicative of cell death after spermiation.

Interpretation of sperm measures in rats with respect to similar measures obtained in exposed humans

Several differences in methods and physiology need to be considered when extrapolating effects in rats to humans, or comparing the results of rat toxicology and human epidemiology studies. Rat spermatozoa are typically collected from the cauda epididymidis (or proximal vas deferens) whereas human spermatozoa are typically evaluated in seminal fluid (after ejaculation). Dilution of rat spermatozoa into culture medium could dilute any toxicant remaining in the epididymal fluid, and stimulation of sperm metabolism by energy substrates in the medium may overcome adverse effects of toxicants in epididymal fluid. These factors should be considered when making direct comparisons between sperm measures made in diluted rat spermatozoa and in human semen.

Rat spermatozoa are very homogeneous in comparison with human spermatozoa. In a typical sample from a control rat, most of the spermatozoa will be progressively motile, and nearly all will be morphologically normal. In contrast, human semen samples, even those from fertile men, typically exhibit a wide range of motility and a low but variable proportion of morphologically normal forms. Thus, a small change in a sperm parameter in a group of rats may be statistically significant as a result of the consistency of the measures in control rats, whereas a change of similar magnitude in a group of humans may be indistinguishable against the high background of abnormal spermatozoa. This is not to say that rats are more sensitive to the compound, but rather that detection of change may be easier in rats. Sensitivity is determined by many other factors.

Since many men with borderline semen quality are thought to be on the threshold of infertility, a small change in sperm production or function could shift them into the infertile status. However, it takes large shifts in sperm count or quality to render a rat infertile. Nevertheless, information about sperm quality in rats may allow the detection of adverse effects that occur at low dose, or after short-term exposure, especially in cases in which fertility is not affected.

For many years, clinicians have searched for the best endpoint in semen that could be used to predict fertility in individual men. Similarly, when CASA technology came into use, andrologists argued for a single best CASA parameter for predicting infertility. A study undertaken to address this question indicated that some sperm measures may be better correlated with fertility than others, especially under certain circumstances, but that information is lost by relying on a single outcome (Zinaman *et al.*, 2000). Likewise, it is naive to think that a single sperm measure in rats will be sufficient to detect reproductive toxicity from any chemical exposure. Different reproductive toxicants act by different mechanisms to produce an array of effects. Therefore, it is advantageous to consider all the evidence from sperm numbers, motility and morphology, as well as from reproductive organ histology, reproductive behaviour and fertility, to characterize the toxicity and estimate risk.

Fig. 1. Post-epididymal events required for fertility. At ejaculation, spermatozoa mix with the accessory sex gland fluid. During their journey through the female reproductive tract, sperm maturation changes and sperm selection occur. Spermatozoa must travel through the female tract where they attach to the oviduct epithelium and undergo capacitation. During the capacitation process, spermatozoa undergo changes in their tail and head membranes. Concomitant with capacitation, spermatozoa undergo changes in their motility and exhibit hyperactivation. Hyperactivation is thought to assist spermatozoa in detaching from the oviduct epithelium and penetrating the zona pellucida. Once spermatozoa bind to the zona pellucida of the egg (or, in some species, before binding to the zona pellucida), spermatozoa undergo the acrosome reaction. After the acrosome reaction, spermatozoa penetrate the zona pellucida and fuse with the plasma membrane and decondense in preparation for fusion with the oocyte genome.

However, evaluating multiple endpoints can lead to a dilemma. How does one interpret subtle changes in a single outcome, especially if it is only a single CASA parameter? The toxicologist looks for a change in the most sensitive end point and argues that this is the 'low effect level.' The regulator looks at this information and asks, 'Is this small change biologically significant? Is it adverse?' Such questions can be difficult to answer and may require judgement regarding the redundancy of outcomes. Just as it does not make sense to rely on a single outcome to predict fertility, it is important to look at all the outcomes to determine whether the effects of treatment are real and make sense in the context of all the information.

Although the clinician tries to predict fertility in an individual patient, the epidemiologist looks for convincing differences between groups of people. In epidemiology, it is not necessary to show that a decrease in sperm concentration or sperm motility in an exposed individual will result in infertility, but whether groups of men exposed to the same substance show significant and similar changes in semen quality. The same is true in toxicology when interpreting data from rats.

Development of functional tests for evaluation of modes and mechanisms of toxicity

At ejaculation, spermatozoa are released from the cauda epididymidis and mix with the accessory gland fluids to form semen for deposition into the female tract. Spermatozoa undergo further maturation changes during the journey through the female reproductive tract. These changes are termed capacitation and render spermatozoa capable of fertilization (Fig. 1). As spermatozoa undergo capacitation, they exhibit a specialized type of motion called hyperactivated motility (reviewed in Yanagimachi, 1994). Capacitation is also important in preparing the spermatozoon to bind to the zona pellucida and undergo the acrosome reaction, a prerequisite for fusing with the oocyte membrane and thereby completing the fertilization process. During these final stages of sperm maturation, spermatozoa may be susceptible to toxicants in the female tract. Importantly, these terminally differentiated spermatozoa cannot repair any damage (physiological or genetic) that may arise at this time.

Sperm measures described earlier provide valuable in-

Fig. 2. Representative tracks and individual computer-assisted sperm analysis (CASA) parameters of rat spermatozoa during culture *in vitro* under capacitating conditions. Initially, spermatozoa swim in a progressive manner but, as they capacitate, their motion becomes more vigorous and less progressive (intermediate). Finally, hyperactivated spermatozoa are characterized by whiplash motion with high vigour and low progression. Individual sperm tracks were reconstructed from the x,y coordinates of the sperm head at each video frame captured during CASA analysis. VAP, velocity of the average path (mathematically smoothed); VCL, curvilinear or point-to-point velocity along the sperm track; VSL, straight line velocity (total distance travelled divided by time); STR, straightness, or $\text{VSL/VAP} \times 100$; LIN, linearity, or $\text{VSL/VCL} \times 100$; ALH, maximum amplitude of lateral head displacement; BCF, beat cross frequency or the number of times the spermatozoon crosses its average path. Adapted from Cancel *et al.*, 2000.

formation about testicular and epididymal function, but they do not measure sperm functional competence or fertilizing ability. However, many *in vitro* fertilization tests and other assays for specific aspects of sperm function have been developed for clinical use and can be applied in toxicology (reviewed by Muller, 2000). Various strategies have been proposed for using such tests to evaluate rat sperm function after exposures either *in vivo* or *in vitro* (Perreault, 1989). However, their application in reproductive toxicology has been limited to date, due in part to the lack of repeatable, reliable assays *in vitro* in rats.

Sperm capacitation and hyperactivation assay

Sperm hyperactivation (HA) has been correlated with sperm fertilizing ability (Johnston *et al.*, 1994). Hyperactivated spermatozoa exhibit a whipping motion with high vigour but low progression. This whipping motion is believed to help spermatozoa detach from the

oviduct epithelium (Suarez *et al.*, 1991) and to provide increased thrust for penetration of the cumulus and oocyte zona pellucida (Suarez and Dai, 1992; Stauss *et al.*, 1995). Therefore, objective evaluation of sperm hyperactivation may be a prognostic end point to test sperm fertility (Mortimer, 1997).

The ability of rat spermatozoa to hyperactivate in the oviduct was first described by Shalgi and Phillips (1988). Few investigators have studied the movement of rat spermatozoa during culture *in vitro* (Jeulin and Soufir, 1992; Moore and Akhondi, 1995). Hyperactivated motility of rat spermatozoa under *in vitro* culture conditions was confirmed by Cancel *et al.* (2000).

Contemporary CASA methods allow spermatozoa with high velocity to be tracked accurately and classified as hyperactivated on the basis of measures of high vigour (for example, curvilinear velocity) and low progression (for example, low linearity, high amplitude) (Fig. 2). The ability to monitor a functional endpoint such as hyperactivation will make it possible to optimize culture media and conditions for *in vitro* capacitation and fertilization in rats, and will make it possible to evaluate the extent to which a specific toxicant or its metabolites may impair the ability of rat spermatozoa to undergo capacitation and thereby acquire fertilizing ability.

Sperm capacitation and acrosome reaction assays

The acrosome is an organelle that covers the rostral end of the sperm nucleus. During capacitation, the acrosome becomes capable of undergoing a lytic event called the acrosome reaction. Functionally, the acrosome reaction is important because only acrosome-reacted spermatozoa are able to penetrate the zona pellucida and fuse with the oocyte membrane (reviewed by Yanagimachi, 1994). The acrosome reaction may occur spontaneously in culture or may be induced by a specific protein in the zona pellucida. As with hyperactivation, spontaneous acrosome reaction can be monitored as an indicator that capacitation has occurred (Muller, 2000).

Limited information is available regarding *in vitro* capacitation and acrosome reaction of rat spermatozoa. Early studies demonstrated that rat spermatozoa can undergo *in vitro* capacitation as evidenced by their ability to fertilize rat oocytes *in vitro* (Toyoda and Chang, 1974), and can undergo spontaneous acrosome reaction during culture as evidenced by their ability to fuse with zona-free eggs *in vitro* (Hanada and Chang, 1976). As with other species of spermatozoa, a variant of the standard Krebb's Ringer bicarbonate-buffered medium supplemented with bovine serum albumin supports capacitation of rat spermatozoa *in vitro* (Toyoda and Chang, 1974). Subsequent modifications of the rat IVF medium include use of Hepes buffer and increased calcium content. Woods and Garside (1996) included caffeine, hypotaurine and heparin in the medium, and these three additives have been used individually to successfully stimulate sperm capacitation or the acrosome

reaction in other species. However, the addition of these three agents concurrently in the capacitation media did not significantly improve the fertilization capacity of spermatozoa. A review of the literature on IVF in rats reveals a great deal of variability with respect to rates of fertilization and indicates the need for a systematic effort to optimize IVF conditions for this species.

Spontaneous acrosome reaction has been monitored during sperm culture *in vitro* in many species (Cross and Meizel, 1989). In some, like hamsters and guinea-pigs, the acrosome is large and the acrosome reaction can be identified and monitored in motile spermatozoa using phase-contrast microscopy. However, in species with small acrosomes, such as humans and rats, it is necessary to fix and stain or label the acrosomes to see them. In rats, the acrosome fits tightly over most of the sperm nucleus and can be visualized directly only using electron microscopy (Fig. 3). Although this approach can be used to assess the acrosomal status of rat spermatozoa (Shalgi *et al.*, 1989), electron microscopy is technically difficult, labour intensive and expensive. Furthermore, spermatozoa must be at the right orientation for the acrosome reaction to be detected. Since all spermatozoa cannot be scored, the method is not well suited for quantifying acrosome reactions in a sperm population.

An alternative approach is to use fluorescent probes to label the acrosomal components. The antibiotic chlortetracycline (CTC) can be used to monitor the acrosome reaction and, although the exact mechanism is not known, it seems to interact with divalent cations (Ca^{2+}) attached to the membrane surface, with a related change in fluorescence. Oberländer *et al.* (1996) used the CTC staining pattern to evaluate the acrosome reaction during culture of rat spermatozoa *in vitro*, and described three patterns of CTC staining: (i) the entire head is labelled (which is thought to be indicative of uncapacitated spermatozoa); (ii) staining is over the acrosome only (the intermediate pattern is thought to be indicative of capacitated spermatozoa); and (iii) the sperm head is unstained except at its tip (indicating that it is acrosome reacted). In addition to being relatively easy to perform, this assay has the advantage of providing information on the dynamics of capacitation and the acrosome reaction. A potential problem is that the acrosome reaction is a continuous process so readings must be taken repeatedly over time in culture. In addition, care must be taken to score the cells quickly since the stain bleaches rapidly after exposure to the light. Furthermore, it is difficult to assign intermediate patterns to a specific category in an objective manner; in human spermatozoa up to eight patterns have been described.

Fluorescently tagged lectins have also been used successfully to label the acrosomes of many species of spermatozoa. The lectin peanut agglutinin (PNA) labels the rat sperm acrosome in developing spermatids (Martínez-Menárguez *et al.*, 1982). This lectin is being used successfully to monitor the rat acrosome reaction under *in vitro* culture conditions (Fig. 4; A. M. Cancel, S. Jeffay and S. D Perreault, unpublished). Similarly, antibodies raised against acrosomal

Fig. 3. Electron micrograph of a rat sperm head showing the characteristic hooked shape. The acrosome of the rat spermatozoon covers most of the sperm head (Yanagimachi, 1994). Scale bar represents 1.6 μm .

proteins can be used to label the rat sperm acrosome and monitor the acrosome reaction using immunofluorescence microscopy. Examples include anti-DE (antibodies raised against rat androgen-dependent secretory epididymal protein DE; Cuasnicú *et al.*, 1984) and anti-IP₃R (polyclonal antibody directed against inositol 1,4,5-trisphosphate receptor; Walensky and Snyder, 1995) antibodies.

Finally, acrosomal enzymatic activity has been used as an indicator of loss of the acrosome (Salzberger *et al.*, 1992). This method measures the release of acrosomal acid phosphatase from rat spermatozoa during prolonged incubation in rat fertilization media but does not distinguish between moribund spermatozoa that release the enzyme owing to cell death (known as the 'false acrosome reaction') and those undergoing a physiological or 'true' acrosome reaction.

Methods for labelling spermatozoa for both acrosome integrity and sperm viability would help overcome this problem. Such dual labelling should allow the simultaneous evaluation of sperm viability and acrosome integrity in individual spermatozoa, and thus identify dead spermatozoa that have undergone a 'false' acrosome reaction.

Any of these methods can now be applied to evaluate the potential effect of toxicants on rat sperm acrosome integrity and the ability to undergo an acrosome reaction *in vitro*. Interest in this potential mechanism of toxicant action has been raised by the report that cyclodiene insecticides inhibit the human sperm acrosome reaction *in vitro* (Turner *et al.*, 1997).

Only a few chemicals have been shown to target mature rat spermatozoa directly and thereby impair fertilizing ability (reviewed by Perreault, 1997). For example, the antifertility effects of ornidazole are thought to be due to inhibition of sperm metabolism by an active metabolite; spermatozoa from treated rats exhibit decreased motility and reduced ability to penetrate a viscous medium *in vitro* (Yeung *et al.*, 1995). These observations indicate that capacitation may be inhibited. When the acrosome reaction was monitored during culture *in vitro* using the CTC assay, no significant differences in the percentage of

Fig. 4. Confocal micrograph of two rat spermatozoa stained with fluorescein isothiocyanate–peanut agglutinin (FITC–PNA). FITC–PNA stains the apical acrosomal region of rat spermatozoa with intact acrosomes (AI). Spermatozoa that do not have an acrosome (AR) do not show the fluorescent signal. Scale bar represents 5 μ m.

spontaneously acrosome-reacted spermatozoa were found when comparing samples from control and treated rats (Oberländer *et al.*, 1996). Therefore, the mode of action appears to be through altered sperm motion and not inhibition of acrosome reaction.

***In vitro* fertilization in rats**

The first report of successful IVF of intact rat oocytes was published more than 25 years ago (Miyamoto and Chang, 1973). Unfortunately, although the rat is the animal model most extensively used for toxicology studies, limited advances have been made towards standardizing IVF in rats, and inconsistent rates of fertilization with high inter-experiment variability have been reported (Perreault and Jeffay, 1993). Therefore, it is likely that IVF conditions have not been optimized.

In vitro fertilization can be used to evaluate the effects of chemicals on the fertilizing ability of rat spermatozoa after exposures either *in vivo* or *in vitro*. If breeding studies indicate that fertilization did not occur, IVF assays can be used to identify specific deficits in sperm function and to distinguish between such effects and infertility due to impaired breeding or poor sperm transport.

Holloway *et al.* (1988) used rat IVF to evaluate the fertilizing ability of spermatozoa recovered from animals exposed acutely to the well-known testicular toxicant ethylene glycol monomethyl ether (EGME). Rat oocytes, obtained from immature female rats that had been treated with hormones to induce ovulation, were inseminated with rat spermatozoa and evaluated for fertilization after an overnight incubation. Fertilization (confirmed by the presence of a decondensing sperm head or male pronucleus and sperm tail inside the oocyte) was reduced in a dose–response manner in animals exposed to EGME (50–200 mg kg^{−1}). Similar effects were seen in male rats given a single exposure to a second testicular toxicant,

1,3-dinitrobenzene, and evaluated subsequently over time (Holloway *et al.*, 1990). Although the highest dose affected sperm numbers, the percentage of motile spermatozoa and IVF, the lowest dose inhibited IVF but did not alter sperm velocity. The same toxicants also inhibited fertilization of zona-free oocytes when administered to rats at similar and lower dosages to those that did not alter the percentage of motile spermatozoa (Berger *et al.*, 2000). Thus, IVF may be detecting subtle effects on sperm function.

Spermatozoa must undergo spontaneous acrosome reaction in culture and be capable of fusing with the oocyte membrane to fertilize zona-free oocytes. In contrast, spermatozoa must bind to the zona, undergo zona-induced acrosome reaction and be capable of penetrating the zona and fusing with the oocyte membrane to fertilize zona-intact oocytes. Thus, these two assays measure different aspects of sperm function. Evaluation of sperm fertilizing ability with both zona-intact and zona-free oocytes, coupled with CASA analysis to determine both the percentage of motile spermatozoa and the quality of sperm motion (including hyperactivation during IVF), may allow differentiation among antifertility effects due to alterations in capacitation, sperm motion (hyperactivation) or the ability to undergo the acrosome reaction *in vitro* (Perreault, 1989).

If they are carefully designed and properly standardized, IVF methods can be valuable in identifying the site of action of impaired gamete function, and distinguishing between effects on capacitation or zona penetration. However, these methods are costly in terms of labour and animal usage, and should therefore be reserved for answering specific questions. However, assays for sperm hyperactivation and acrosome reaction are easier to conduct, do not require the use of female rats, and can provide valuable information about the potential effects of toxicants on sperm capacitation and the acrosome reaction.

Artificial insemination

An *in vivo* fertility test using artificial insemination (AI) with limited numbers of spermatozoa has proven useful in evaluating the impact of low dose exposure to drinking water disinfection by-products on fertility in rats (Klinefelter *et al.*, 1994). This modified AI test is based on the premise that below a threshold number of spermatozoa, successful conception depends upon the quality of the spermatozoa inseminated. Insemination of the threshold number of spermatozoa (5.0×10^6) directly into the uterus provides optimal sensitivity for detecting toxicant-induced effects. Female rats must be inseminated near the time of ovulation. Oocytes can be recovered on the next day to score fertilization, or pregnancy can be allowed to become established so that implantation sites and fetuses can be evaluated later and fertility, defined as the proportion of implants, can be determined at mid- or late gestation.

An advantage of the AI test is that sperm fertilizing ability can be evaluated *in vivo* under physiological conditions in the female rat reproductive tract. As with IVF,

infertility or subfertility can be the result of insufficient numbers of healthy, viable spermatozoa inseminated (for example, if the treatment affects sperm viability) or of specific deficits in sperm function. Thus, this test is also sensitive to changes in sperm motility and survival, which makes it occasionally difficult to unscramble the mechanism or mode of toxicant action. However, when sperm motility is affected by treatment, the concentration of spermatozoa inseminated can be adjusted upward in the samples from treated rats so that equal numbers of *motile* spermatozoa can be inseminated for control and treated samples.

Genetic integrity of spermatozoa

Recent interest in and concern about toxicant-induced male-mediated developmental abnormalities makes it desirable to add tests of genetic integrity to reproductive toxicology studies (reviewed in Perreault, 1998). Tests for detecting alterations in sperm chromatin structure, DNA damage in spermatozoa (as induced by oxidative stress or DNA alkylating agents), and chromosomal breakage or aneuploidy in spermatozoa are being used to augment human studies (Perreault *et al.*, 2000) and are being adapted for use with rat spermatozoa. A detailed discussion of this subject is beyond the scope of this paper but it has been extensively reviewed by Evenson (1999).

Conclusion

Measures of rat sperm production and function are now being obtained routinely in multigenerational test protocols, as well as many short-term tests and toxicology studies. Their significance lies in the extent to which they improve the sensitivity of the overall test, and provide information about the site and mode of action and gender specificity of the test chemical. Specific tests of rat sperm function have been applied successfully in a number of toxicology studies, and their value with respect to identifying modes and mechanisms of toxicant action should become more evident as they are optimized and more widely used.

The information in this document has been funded by the US Environmental Protection Agency and the EPA/UNC Toxicology Research Program, Training Agreement CT 902908, with the Curriculum in Toxicology, University of North Carolina, Chapel Hill. It has been reviewed by the National Health and Environmental Effects Research Laboratory, US Environmental Protection Agency and approved for publication. Approval does not signify that the contents reflect the view of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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