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Spermatozoa Manipulation Techniques: A Current Assisted Reproductive Technology Tool Kit in Reproductive Physiology

[1]I.P. Ogbuewu, [1]U.E. Ogundu, [1]M.N. Opara, [1]I.C. Okoli,
[3]D.O. Umesiobi, [2]U. Herbert and [1]M.U. Iloeje

The application of technologies in the field of male reproductive physiology has advanced greatly during past couple of decades. Since, the advent of frozen semen and artificial insemination, reproductive physiologists have attempted to manipulate male reproductive rates of mammals, sometimes successfully or sometimes not. The past five decades has resulted in phenomenal expansion in the improvement spermatozoa with direct application to livestock and medicine. Spermatogenesis had been reported to be induced in male animals using synthetic fertility drugs and local plant extracts. Sperm production and function have been improved via the use of nutritional regiments, semen extender and semen pool admixture. Currently, the sex of animal has been predetermined with 85-95% accuracy by sexing spermatozoa. The current success recorded in male assisted reproductive techniques came about due to advances in computer science, biophysics, cell biology and genetic engineering. This review will briefly cover the developments and modifications of existing sperm manipulation techniques that have direct practical applications today and in the near future in animal agriculture and bio-medicine.

Key words: Spermatozoa, reproductive physiology, sperm manipulation techniques, biomedicine

[1]Department of Animal Science and Technology, Federal University of Technology, PMB 1526, Owerri, Imo State, Nigeria
[2]Department of Animal Production and Management, College of Animal Science and Animal Production, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria
[3]Field of Animal Reproductive Physiology, School of Agriculture and Environmental Sciences, Central University of Technology, Free State, Private Bag X20539, Bloemfontein 9300, South Africa

INTRODUCTION

A great progress has been made during the last 30 years the field of reproductive physiology through spermatozoa manipulation for improvement of male reproductive performance. Nutritional management (Herbert *et al.*, 2005; Ogbuewu *et al.*, 2009a), synthetic drugs (Herbert *et al.*, 2000), semen preservation and storage (Umesiobi *et al.*, 1998, 2002, 2004; Umesiobi, 2004) and semen pool admixture (Ogbuewu *et al.*, 2007) are some of the recent methods employed to improve male fertility. The ability to select superior sires is another way many scientists have improved male reproductive performance.

Semen freezing and insemination procedures have changed minimally in the past two decades. However, pregnancy rate averages have not dramatically improved and, at best, remain around 50 and 70% for timed artificial insemination (A.I.) and heat-detected breeding, respectively. Limitations in estrus synchronization and herd fertility account for inadequacies in pregnancy rates. The goal of scientists is to develop protocols that minimize costs and numbers of times animals are handled, yet increase pregnancy rates to A.I.

Intracytoplasmic sperm injection (ICSI) is another assisted reproductive technique of choice. As the name implies, the process involves collecting processing, maturation and injecting a single sperm into an oocyte. Intracytoplasmic sperm injection overcomes fertility problems associated with either the sperm or the egg. To accomplish ICSI, the oocyte is held by vacuum to a small glass tube. An individual sperm cell is then loaded into a micro syringe, which is carefully guided into the oocyte and the sperm is injected into the egg.

One of the newest reproductive research advances is the ability to transfer spermatogonia (sperm precursor cells) from one male to another. This technique has yielded offspring in mice. The principle of sperm transfer opens the door for the potential to use lower quality bulls as sperm carriers of high valued males. The procedure involves rendering the testis of the recipient male sterile and then re-populating the testis with spermatogonial stem cells from a donor male. This technology is currently impractical, but if it comes into fruition, it could revolutionize animal agriculture and the propagation of outstanding sire genetics.

SPERMATOZOA AND NUTRITIONAL MANAGEMENT

The efficiency of sperm production, libido and quality of sperm tend to remain uniform throughout the reproductive life of an animal but may be significantly altered by age, nutrition, environment and health status (Togun and Egbunike, 2006; Ogbuewu *et al.*, 2009a, b). Among these factors nutrition is the most prominent. The optimum performance of an animal is dependent on the availability of good nutrition (Togun and Egbunike, 2006; Ogbuewu *et al.*, 2009a, b, 2010a). Sexual maturity is known to be delayed by a poor nutrition regiment during growth (Omole, 1982). It is also affects the attainment of puberty and stimulation of hypothalamus to produce interstitial cells stimulating hormone that acts on the testicular tissue (Cogan *et al.*, 2004).

According to Luzi *et al.* (1996), restricted dietary protocol reduces libido and some sperm quality traits. However, the most important factor is not the amount of diet fed but its chemical characteristics. Diets with more than 15% of crude protein are suitable for sperm production (Nizza *et al.*, 2000; Fodor *et al.*, 2003). In mammalian spermatozoa, a very high amount of lipids are associated with the membrane fluidity and its competence (Apel-Paz *et al.*, 2003). Dietary addition of PUFA n-3 modified several traits of rabbit spermatozoa (Castellini *et al.*, 2003, 2004).

On contrary, high levels of cholesterol in the diet alter the metabolism of sertoli cells and the normal process of spermatogenesis (Yamamoto *et al.*, 1999). The high unsaturated levels of spermatozoa membrane makes these cells very susceptible to peroxidation (De-Lamirande *et al.*, 1997), which degrades sperm membrane structure, DNA integrity, sperm metabolism and sperm production (Jones *et al.*, 1979).

SPERMATOZOA AND SYNTHETIC AND NATURAL FERTILITY DRUGS

Semen quality has been reported to be enhanced by some fertility drugs such as Clomiphene citrate (Herbert *et al.*, 2000; Ndubeze, 2002) and the age at sexual maturity was reported by Chibundu (2005) to be shortened by the use of estradiol in buck rabbits. However, the high cost of these preparations made them totally unacceptable to the researchers in the study area.

The quest for naturally occurring compounds of herbal or plant origin that could be of benefit as contraceptive and fertility control agents is in the increase today especially in third world countries due high cost of these synthetic drugs (Atawodi *et al.*, 1995; Adedeji *et al.*, 2006; Ogbuewu *et al.*, 2009a, 2010a). Plants that were once considered of no value are now being investigated, evaluated and developed into drugs with little or no side effects (Adedeji *et al.*, 2006; Ogbuewu *et al.*, 2009a, 2010a). One of such plants is

Azadirachta indica, Glycine max and *Garcinia kola* (Adedeji *et al.*, 2006; Ogbuewu *et al.*, 2009a, 2010a). The observed increase in semen quality parameters of male animals administered local plant preparations could be attributed in part by the antioxidant activity of these plants (Ogbuewu *et al.*, 2010a, b).

SPERMATOZOA AND ANTIOXIDANT (TOCOPHEROL)

Several researchers have achieved beneficial results in male reproduction by applying vitamin E in their studies (Marin-Guzman *et al.*, 1997; Mbaegbu, 2004; Oguike, 2004; Osuagwu, 2004; Ogbuewu *et al.*, 2010c). Vitamin E can affect testicular or spermatozoa development and sperm motility (Marin-Guzman *et al.*, 1997). Deficiency of vitamin E may affect different aspects of the male reproductive processes and possibly the fertilization of oocytes in the female (Cooper *et al.*, 1987). Vitamin E is needed for the production of testosterone (Marin-Guzman *et al.*, 1997).

In semen, the trend of alpha tocopherol is even more complex than in plasma. The standard level of alpha tocopherol in rabbit's semen is relatively low (0.2-0.9 mmol L^{-1}) (Marin-Guzman *et al.*, 1997). Marin-Guzman *et al.* (1997) reported that alpha, beta, gamma and delta tocopherol is not homogeneously distributed in rabbit semen fractions. Alpha tocopherol is the main lipid antioxidant in all fractions, while gamma tocopherol, beta tocopherol and delta tocopherol are more abundant in germ cells and seminal plasma respectively (Marin-Guzman *et al.*, 1997).

It should be noted that alpha tocopherol in rabbits has a lower bio-availability than in humans (Gallis *et al.*, 2001) probably due to low fat, high fibre of rabbit diets which limit vitamin E absorption (Iuliano *et al.*, 2001). However, bioavailability of alpha tocopherol in semen and in blood is non linear (Castellini *et al.*, 2002).

SPERMATOGENESIS AND PHOTOPERIODISM

The artificial manipulation day length is one of factors influencing the quality and quantity of semen (Bodnar *et al.*, 2000). Photoperiodism affects the hypothalamus-pituitary axis and consequently hormonal release and spermatozoa production. According to Theau-Clement *et al.* (1994) a long light length (16L: 8D) program increases sperm production (qualitative and quantitative aspects) compared with a shorter light length (8L: 16D). By contrast, light intensity did not significantly affect semen characteristics (Besenfelder *et al.*, 2004).

SPERMATOZOA PRODUCTION AND FREQUENCY OF EJACULATION

Several authors have studied various semen collection rhythms that vary from one to four ejaculates per day (Bodnar *et al.*, 1996; Bunaciu *et al.*, 1996), on daily or weekly schedules varying from once weekly (Bencheikh, 1995; Bunaciu *et al.*, 1996) to daily collection (Bodnar *et al.*, 1996). Intensification of collection rates increased the quantity of semen per week but inevitably had an adverse effect on quality (Bodnar *et al.*, 1996). Semen collection rhythms are considered intensive when they entail two successive ejaculates (within 15-30 min) three times per week, extensive with only two successive ejaculates once a week and semi-intensive with two successive ejaculates two times per week (Bodnar *et al.*, 1996).

PRESERVATION OF SPERMATOZOA

Preserved sperm is an important integral component for the advancement of the science of reproductive physiology. By virtue of this technique sperm could be stored indefinitely, used widely and can be exported easily. The frozen sperm facilitates international exchange of genetic material, allows AI in both the reproductive and non-reproductive seasons and extends the effective reproductive life of a valuable male beyond its own life. Development of goat sperm cryopreservation, however, has progressed at a slower rate than the cattle and sheep (Abdullah *et al.*, 1997). The cryopreservation of sperm is a complex process, which involves balancing many factors for obtaining satisfactory results.

Cryopreservation of semen is technically challenging due to the presence of seminal plasma (bulbourethral gland secretions a lipases), which interact with some semen extender (egg yolk) to create substances that are toxic to sperm (Iritani *et al.*, 1964). This situation has not been observed in cattle semen extended with egg yolk (Baldassarre and Karatzas, 2004). Until now, the use of the Tris-egg yolk cryopreservation diluents, such as the one described by Salamon and Ritar (1982) is recommended, as it is easy to use. However, commercial extenders with no biological components have been developed to improve sanitary safety in semen processing (Hinsch *et al.*, 1997; Gil *et al.*, 2003). However, the adoption of these techniques in the third world countries has been hindered due to high cost of these preparations and poor infrastructural development. This has led the development of local semen extenders from herbal or plant materials in Nigeria (Umesiobi *et al.*, 1998, 2002; Umesiobi, 2004).

INTRACYTOPLASMIC SPERM INJECTION

Intracytoplasmic sperm injection is the mechanical insertion of a single sperm directly into the ooplasm of a matured, metaphase oocyte using a microscopic needle. It is a special type of *in vitro* fertilization (IVF) and one of the advanced assisted reproductive technologies that is now widely used to overcome male factor infertility in animal and humans. In this technology a sperm is picked up in a micro pipette and injected directly through the zona pellucida into the cytoplasm of the mature oocyte (Devroey and Van-Steirteghem, 2004). The fertilization rate is usually very high in these cases (Shawky *et al.*, 2006). The ICSI proved its efficacy in producing thousands of human babies since its first success in 1992 (Palermo *et al.*, 1992). There have been various studies comparing the pregnancy rates between conventional *in vitro* fertilization and ICSI (Shawky *et al.*, 2006).

INTRAUTERINE INSEMINATION

Intrauterine insemination was introduced in the 1980's as part of the treatment of infertile couples due to several problems including male factor infertility, cervical mucous factor, sperm antibody and unexplained infertility. In this technology the sperm is collected in a sterile container. The sperm is then washed and concentrated in 1-3 mL of the culture medium and is used for intrauterine insemination. The value of this technology is to have a high concentration of sperm by-passing the cervical mucus factor (Ombelet *et al.*, 2003). The sperm deposited in the uterus will be in close proximity of the tubal openings and therefore will have quicker accessibility to the oocyte. Certainly, washing the sperm increases the capacitation and helps the fertilization rate (Ombelet *et al.*, 2003). The pregnancy rate in these situations is between 30 and 40% (Ombelet *et al.*, 2003). The main objective of sperm preparation is to isolate the maximum number of motile sperm, eliminate dead sperm, amorphous cells, leukocytes and separate seminal plasma from the ejaculate without causing any damage to the sperm cells. Various methods of sperm wash preparation have been used including swim up, swim down, glass wool filtration and density gradient centrifugation (Henkel and Schill, 2003).

ARTIFICIAL INSEMINATION

Artificial Insemination (AI) is a term that covers a range of techniques, all of which involve the placing of sperm into the female genital tract artificially. Artificial insemination is the first generation of reproductive biotechnologies, both historically and in terms of numbers around the world. The introduction of AI in farm animals was forced by sanitary reasons and the first large scale applications with a commercial goal were performed in the late thirties of last century. Five techniques of insemination are used worldwide in animals (Leboeuf *et al.*, 2000). Such insemination may include intravaginal insemination, intrauterine insemination, intracervical insemination, intrafallopian insemination and intraperitoneal insemination.

SPERM SEXING

One area that has attracted significant research effort over the years is the possibility of developing technology that would allow sperm sexing in other to predetermine the sex of the young animal. In mammals, the primary information for sex determination is located on the short arm of the Y-chromosome (Sinclair *et al.*, 1990). The sex chromosomes are equally distributed among sperm cells and the chance to become a male or female offspring is random at 50: 50 quotes. A technology known as Beltsville Sperm Sexing Technology (Johnson, 1991) allows to identify X- or Y-chromosome-bearing sperm based on the relative difference in DNA contents and to determine the sex of offspring prior to fertilization. The Beltsville Sperm sexing technology has been validated on the basis of live births, laboratory re-analysis of sorted sperm for DNA content and embryo biopsy for sex determination (Johnson and Welch, 1999).

HISTORY OF SPERM SEXING

Many important advances in reproductive technologies occurred in the late 1970s and early 1980s. The first was accurate measurement of sperm DNA content using flow cytometry (Garner, 2001). In this measurement system, fluorochrome-stained sperm are made to flow through the system in a precise, single-file manner so that the dye that binds to each cell can be excited by a specific wavelength of light, thereby emitting a fluorescent signal. The fluorescence from each stained sperm is collected with a photomultiplier tube and the quantitative information is conveyed to a computerized system in which sperm can be categorized according to fluorescent intensity. Such a measurement system, which is capable of making precise, accurate measurements of fluorescent signals, was shown to be theoretically capable of measuring the small difference in DNA content between X- and Y- chromosome bearing sperm (Van Dilla *et al.*, 1977).

Early efforts to use this flow cytometric system to measure the DNA content of sperm were only marginally successful, in part because the highly condensed sperm nucleus makes quantitative fluorescence measurement difficult due to the unusual shape of the head and chromatin compaction (Van Dilla *et al.*, 1977). This problem was conquered in the early 1980s by instruments that either oriented sperm during measurement or whose coaxial measurement principal was essentially insensitive to cell orientation (Otto *et al.*, 1979; Pinkel *et al.*, 1982). A custom-made instrument used a beveled, flattened needle to hydro-dynamically force each spermatozoon into a similar orientation, thereby allowing accurate measurement of the fluorescence of the flat surface of most sperm nuclei (Fulwyler, 1977; Dean *et al.*, 1978). This instrumentation provided high-resolution measurements of the DNA content of mammalian sperm (Pinkel *et al.*, 1982).

One early piece of evidence that flow cytometric measurements might differentiate between X and Y cells was detection of a difference in DNA content of sperm from mice with a chemically induced genetic abnormality compared with those from normal mice (Gledhill *et al.*, 1982; Pinkel *et al.*, 1982). This unusual strain, called the Cattanach mouse, had a translocation of a piece of chromosome 7 to the X chromosome (Cattanach, 1961; Disteche *et al.*, 1981). The difference in DNA content between the bimodal peaks of sperm from the Cattanach mouse was 4.9%, whereas that of normal mice was 3.3% (Pinkel *et al.*, 1982).

The difference in genetic sex determination between mammals and birds provided another important clue. In mammals, sex is determined by which spermatozoon that fertilizes the ovum, the X- or Y-chromosome-bearing gamete. In avian species, however, sex is determined by the oocyte, not by the fertilizing spermatozoon. Avian species produce only Z - sperm. Flow cytometric analyses of cockerel sperm revealed a single peak, whereas those of mammals had two peaks (Garner *et al.*, 1983). The flow cytometric method was immediately applied to the sperm of domestic animals (Garner *et al.*, 1983).

The flow cytometric method was immediately applied to the sperm of domestic animals (Garner *et al.*, 1983). The DNA content differences in the bimodal populations of sperm from bulls, boars, rams and rabbits were 3.9, 3.7, 4.0 and 3.9%, respectively (Garner *et al.*, 1983). The methods of preparing sperm for flow cytometric analysis was destructive because the tails and most of the membranes and cytoplasm were removed, leaving the sperm biologically unusable (Gledhill *et al.*, 1984; Gledhill, 1988).

Flow cytometry was also used to test validity of claims that semen had been enriched in either the X or Y sperm by other methods. Using a variety of separation techniques for enrichment, including sedimentation, electrophoresis, filtration, centrifugation, albumin gradients and convection-counter streaming galvanization, galvanic and electrical charge differences, semen samples were analyzed to determine if either the X or Y sperm populations had been enhanced.

BASIC PHYSIOLOGY OF THE SPERM

Spermatogenesis in animal is a complex process consisting of several cell divisions which eventually separates the X and Y chromosomes into different spermatozoa. Each mammalian spermatozoon has an equal chance of carrying the X chromosome which will produce females or the Y chromosome which will produce males (Amann, 1989). Therefore, in mammals the sex of the offspring is determined at the time the spermatozoon enters the ovum.

It is well known that it is necessary to have a certain number of viable spermatozoa in order to achieve optimum fertility (Salisbury and Vandemark, 1961; Pace *et al.*, 1981). Any technique that removes or destroys either the X or Y bearing spermatozoa reduces the potential number of offspring that a male can sire by one half. This is because the number of viable spermatozoa needed to give optimum fertility still remains the same with sexed semen.

SPERM SEXING

The analytical flow cytometric system could identify the X and Y sperm populations, but this technology could not separate living sperm. The analytical system developed at Lawrence Livermore National Laboratory measurement system did not effectively separate intact X and Y sperm. To attain this goal, a Coulter EPICS V laser-based orthogonal flow cytometer/cell sorter at USDA in Beltsville, Maryland, was modified by beveling the sample injection tube tip and substituting a forward angle fluorescence detector and an optical fiber bundle for the light scatter detector so that fluorescence could be measured accurately (Johnson and Pinkel, 1986). This modified instrument was used to sort the first X- and Y-chromosome-bearing sperm nuclei (Johnson *et al.*, 1987b), separating the nuclei into X and Y populations at a purity of 95%. Stoichiometric staining of the sperm nuclei utilized severe treatments with dimethyl sulfoxide (DMSO) washes to remove the surrounding membranes, fixation in ethanol and protease digestion to ensure availability of DNA to interact with dye (Garner *et al.*, 1983; Johnson *et al.*, 1987b). Chinchilla sperm were the

gametes of choice due to a 7.5% difference in DNA content between the X and Y sperm (Garner *et al.*, 1983; Johnson *et al.*, 1987b). This sorting procedure also employed a new DNA dye, Hoechst 33342, which stained the DNA of the sperm more efficiently and accurately (Johnson *et al.*, 1987b) than previously used dyes. The Hoechst 33342 staining procedure soon was improved to make the process less damaging to sperm. Sonication of the sample for 10 sec before staining eliminated the need to prepare the sperm nuclei by exposure to DMSO, ethanol and proteolysis (Johnson *et al.*, 1987a, b; Johnson and Clarke, 1988).

Later it was found that intact sperm could be quantitatively stained with Hoechst 33342 without causing significant damage to cell morphology or motility (Johnson, 1990). The addition of propidium iodide to the staining mixture provided a means for eliminating dead and damaged sperm from the sorted product. Propidium iodide, which stains membrane-damaged sperm, later was replaced with food coloring. This was done because most food coloring quenches Hoechst 33342 safely, making the signal from Hoechst 33342 bound to DNA different in dead sperm than in live sperm (Johnson *et al.*, 1999; Rens *et al.*, 1999; Johnson and Welch, 1999).

SPERM SEXING AND SPEED

Sperm sorting speed is dependent on orientation of the sperm through the instrument. An improved system for orienting sperm during the sorting process was developed at USDA (Rens *et al.*, 1999; Johnson *et al.*, 1999; Welch and Johnson, 1999; Johnson, 2000). This nozzle design increased the proportion of sperm that could be correctly measured from 25% to more that 60% (Rens *et al.*, 1998, 1999). The new nozzle was adapted for a high-speed sorter and increased sorting rates from 2 to 6×10^6 sperm h^{-1} for each sex (Rens *et al.*, 1998; Johnson *et al.*, 1999). The USDA sperm-orienting nozzle was adapted for use with Cytomations SX MoFLo and its orienting ability enhanced by modifications developed at XY Inc. Implementation of these and other modifications to the SX MoFlo by XY Inc have significantly increased the efficiency of sorting X and Y sperm. This modified instrument is capable of sorting 15 $\times10^6$ bovine sperm/h for each sex (Seidel, 2000; Schenk, 2001).

LOW-DOSE INSEMINATION USING SORTED SPERM

Flow sorting had improved by 1996 so that it was possible to produce enough living sexed sperm that oviductal insemination, IVF, or Intracytoplasmic sperm

injection (ICSI) was feasible (Seidel *et al*, 1996a, b). The numbers of sperm available, however, were insufficient for routine artificial insemination because normal practice for insemination used on the order of 20×10^6 sperm dose^{-1}. This number seems unreasonable considering that only one sperm is needed to fertilize an oocyte. The problems associated with sperm successfully traversing the female tract of the sow were by-passed by inseminating sorted sperm directly into the isthmus of the oviduct (Johnson, 1992). The likelihood of placing fewer sperm at a site closer to where fertilization takes place in an animal led to an experiment to test the concept that fewer sperm per insemination dose could be used if the gametes were placed more optimally within the female tract. This was tested by depositing only $1\text{-}5\times10^5$ sperm/dose deep into the uterine horn of heifers using the traumatic, side-opening sheaths commonly employed in embryo transfer (Seidel *et al.*, 1996a, b, 1997).

These early pregnancies from shipped, sex-sorted sperm were followed by formation of XY, Inc and initiated the installation of a high-performance sperm sorter in Colorado so that it would be within reasonable driving distance from animal to be inseminated. The new instrument could sort 500-600 live sperm per second. Sufficient X-selected sperm were prepared so that 35 heifers could be inseminated with 3×10^5 unfrozen sperm/dose. Forty-two percent of the heifers inseminated with the sexed sperm became pregnant compared with a 54% pregnancy rate for controls inseminated with 3×10^5 motile unfrozen sperm (Seidel *et al.*, 1998). Eighteen out of the 19 animals born to X-sorted sperm were females (Seidel *et al.*, 1998). Pregnancy rates were 80% of what was achieved with unsexed sperm (Seidel *et al.*, 1998).

USE OF SEX-SORTED SPERM WITH *IN VIVO* FERTILIZATION

The report of live rabbit pups produced by flow-sorted sperm (Johnson *et al.*, 1989) was the biological evidence that confirmed the validity of earlier biochemical evidence. The bimodal peaks identified by flow cytometric analysis of DNA content were, in fact, the X and Y sperm populations. The X and Y - chromosome-bearing rabbit sperm were sorted according to their DNA content and then surgically inseminated into the uteri of rabbit does (Johnson *et al.*, 1989). Rabbit does inseminated with X-sorted sperm produced litters that were 94% female, whereas those inseminated with Y-sorted sperm produced litters that were 81% male insemination (Johnson, 1990, 1991, 1992). This demonstrated that phenotypic sex could be predetermined by sorting sperm into X and Y populations prior to insemination (Johnson, 1990, 1991, 1992).

The efficacy of the sorting procedure was also demonstrated with swine, but the efficiency was lower (Johnson, 1991). Intact boar sperm were sorted into relatively pure populations of Y sperm and inseminated into the isthmus of the oviduct of gilts, resulting in piglets that were 68% male (Johnson, 1992). Analysis of the sorted sperm sample predicted that 80% would be males. Samples sorted for X sperm with a purity of 80% were inseminated, resulting in 74% females. This is the first demonstration in large farm animals that the sex ratio of offspring could be significantly altered.

USE OF SEX-SORTED SPERM WITH *IN VITRO* FERTILIZATION

Sex-sorted spermatozoa were used for *in vitro* fertilization (IVF) to generate embryos from *in vitro* matured oocytes (Cran *et al.*, 1993). Sperm were sorted using a modified Becton Dickinson FAC Star Plus flow cytometer/cell sorter. The sperm were stained with Hoechst 33342 and sorted at a rate of 100 cells/second to obtain an X-sperm population of 79% purity and a Y- population of 70% purity (Cran *et al.*, 1993). Twin transfers of 9 sex-selected embryos produced 4 pregnancies, resulting in 3 males and 3 female calves (Cran *et al.*, 1993), all of the projected sex. These births demonstrated that sex-sorted sperm could result in living offspring. Further studies revealed that a sex ratio of 90% male could be achieved (Cran *et al.*, 1995). The skewed sex ratio of the calves born from these transfers was consistent with the predetermined sex as determined by the polymerase chain reaction assay of excised cells from similarly selected blastocysts (Cran *et al.*, 1993, 1995).

CRYOPRESERVATION OF SORTED SPERM

Success with sexed sperm maintained at near 5°C was followed by an attempt to cryopreserve sorted cells at 1×10^6 sperm per dose in 0.25 mL straws (Schenk *et al.*, 1999). This was twice the concentration that had been used successfully for unfrozen, sexed sperm to compensate for cell death due to cryopreservation and thawing. After sorting, sperm were concentrated by centrifugation to about 80×10^6 mL^{-1} so that they could be reconstituted to 20×10^6 mL^{-1} and placed into 0.25 mL straws. Pregnancy rates with sexed, cryopreserved sperm were identical, 52%, to that achieved earlier with a third more as many unfrozen sperm (Seidel, 1999a, b; Schenk *et al.*, 1999). With this advance, it became obvious that sperm sorting facilities should be located adjacent to semen collection sites (Amann, 2000) so that sexed sperm could be cryopreserved. The cryopreservation of sexed

sperm allows much greater flexibility in distribution of the product for insemination. Although the procedures for sexing sperm result in slightly lower post-thaw motilities and acrosomal integrities compared with control sperm, this damage is minor compared with that caused by routine cryopreservation (Amann, 2000).

The numbers of sex-sorted, cryopreserved sperm used for insemination have ranged from 1 to 6×10^6 sperm/straw (Seidel *et al.*, 1999). Normal insemination doses usually are 10-20×10^6 sperm. Sperm now can be sorted at rates approaching 4000 per second of each sex, but it is advantageous to use as few sperm per insemination as necessary to achieve acceptable pregnancy rates.

NORMALITY OF OFFSPRING

Numerous studies (Seidel, 1999a, b; Cran, 2000) have reported more than 1000 live births in six species from sexed sperm with no gross abnormalities being observed. Although, offspring born from pregnancies generated with sexed sperm appear normal from a general phenotypic stand point, rigorous epidemiological studies need to be conducted to verify and strengthen these observations. The concern is that the process of sexing sperm could damage the sperm DNA and, thus, could increase the incidence of genetic abnormalities. No increase in embryonic death between 1 and 2 months of gestation has been detected, with very few abortions occurring between 2 months of gestation and calving in cows (Seidel *et al.*, 1999).

THE FERTILITY PROBLEM ASSOCIATED WITH SORTED SPERMATOZOA

In spite of the remarkable technical expertise which has resulted in sperm technologies, the success rate, in terms of total numbers of live births is not as impressive. Key researchers acknowledge that fertility of sorted spermatozoa is somewhat lower (Seidel and Garner, 2002) than that of controls, but point out that results of studies showing this are confounded by using fewer sexed spermatozoa per insemination dose than normal procedures would dictate (Seidel and Garner, 2002).

One of the major limitations of flow cytometric sperm-sorting is the sorting rate, which means that animals fertilized with sex-sorted sperm tend to receive much lower doses of sperm than animals undergoing routine artificial insemination (AI). For example, in the horse, optimized flow-sorting allows approximately 10-20×10^6 sperm to be sorted per hour; thus it is impractical to obtain the 500×10^8 sperm that would typically be used to inseminate mares with unsorted sperm. However, when similar

numbers of sorted and non-sorted spermatozoa were used in an insemination trial in cows, pregnancy rates with sex-sorted spermatozoa continued to be 20- 40% lower than control, unsorted spermatozoa (Seidel *et al.*, 1999).

There also appear to be differences between species in the fertility rates using sex-sorted sperm, with some species (e.g., sheep; Hollinshead *et al.*, 2004) horses, (Buchanan *et al.*, 2000) pigs (Johnson *et al.*, 2005) having low pregnancy rates following insemination with sex-sorted sperm compared to insemination with unsorted sperm. However, such interspecies differences may reflect sub-optimal insemination procedures as well as problems with the fertilizing potential of sex-sorted sperm.

ATTEMPTS TO IMPROVE FERTILITY RATES IN SORTED SPERMATOZOA

Many of the industry's most recent initiatives appear to be targeted at improving fertilization rates. All aspects of the procedures are being scrutinized and analysed, including possible effects of the sorting process itself (Johnson *et al.*, 1999; Suh *et al.*, 2005), effects of transporting and storage of sperm (Lindsey *et al.*, 2005), optimal concentration of sperm (Xu *et al.*, 2006), possible effects of polyspermy (Wang *et al.*, 2003), mode and timing of delivery of the spermatozoa (Wheeler *et al.*, 2006; Wilson *et al.*, 2006) and insemination site (Buchanan *et al.*, 2000), but so far lower fertilization rates remain unexplained.

Lower pregnancy rates appear to be associated with the use of sex-sorted sperm at virtually every step in the process, i.e., low fertilization rates tend to be followed, in addition, by lower cleavage rates and lower rates of development to blastocyst stage (Xu *et al.*, 2006). Whatever is the cause or causes, the conclusion is similar across a number of studies, namely, that sex-sorting sperm negatively impacts the embryo's ability to develop normally (Wilson *et al.*, 2006).

Thus it could be argued that the procedures associated with the use of sex-sorted sperm are not yet fully refined; that results continue to improve (Xu *et al.*, 2006) and that, along with generalized sub-fertility, this is a sufficient explanation for the low fertilization rates. Whatever the final outcome, there is no doubt that this is currently a reasonable position to hold.

On the other hand, if the basic model of sex determination is inaccurate, there may be a way of interpreting the data which shows fertility following the use of sex-sorted sperm to be much more successful than currently viewed. The disadvantage of this new interpretation would be that contemporary and emerging technologies might not contribute as much to efficiency gains as expected.

IMPLICATIONS FOR SEX SELECTION BY MEANS OF SEX-SORTED SPERM

Many studies have been published showing that in spite of the most careful and consistent application of the techniques, fertility appears to be compromised. From this viewpoint, studies in which success rates are reported only in terms of numbers and sexes of live births, but do not mention the numbers of mothers that failed to become pregnant at all, do not give the information required to make a proper assessment of the efficacy of the procedures.

A further question arises from the current convention of reporting identifiable pregnancies instead of live births, since another unknown factor is the point at which an embryo of a particular sex might need to be compatible with, or possibly, ratified by, the uterine environment.

At present it is difficult to determine whether the low fertility rates are best attributable to factors associated with the sorting process (including damage to sperm), problems with insemination techniques, or low doses. Experiments using matched doses of sorted and non-sorted sperm attempt to address the issue of total numbers of sperm used, but even these experiments cannot control for the confounding effects of sperm damage (such as premature acrosome reaction (Moce *et al.*, 2006) that are almost certainly introduced by the sorting process. To perform a critical trial of sperm sorting, instead of comparing sorted and unsorted sperm, the fertilization rates of sorted and sorted/re-combined sperm should be compared.

If, on the other hand, the ovum has a role in sex allocation, then no matter how technically successful the sperm sorting techniques, fertility rates will remain low. That is to say, if an ovum is produced, each cycle, already adapted to receive an X- or a Y- sperm, exposure to any number of the other kind of sperm will result in failure to fertilize or failure to develop. This would mean that sex-sorted sperm were useful only when a client wished to have say, female offspring, or nothing. With further refinements to sperm-sorting procedures it is still possible that the fertilizing potential of sex-sorted sperm could be high. However, an alternative explanation for the low birth rates might be the one offered here, that the fault lies in assumptions about the model of fertilization, since the ovum may be playing a role in pre-determining the sex of the offspring. Controversy and doubt surround this suggestion, but finally it is a researchable question. Are fertility rates following sperm separation compatible with the hypothesis that there is a pre-conceptual maternal influence on the allocation of sex in mammals or will they, ultimately, confirm the chance model of sex determination?.

COMMERCIALIZATION

All the previous attempts to develop sexed sperm as a commercial product have ended in failure (Garner, 2001). This basic biological problem is highly attractive from both conceptual and monetary standpoints. However, potential differences between the X and Y sperm are relatively small and are not evident except for DNA. A variety of simplistic to very complicated methods were tried before the first repeatable success was reported in 1989 using flow-sorting for sexing sperm (Johnson *et al.*, 1989). This sexing procedure, which was originally developed for living sperm at the USDA Beltsville Agricultural Research Center by Johnson *et al.* (1989), was patented by USDA. The first commercial license was issued to Mastercalf Ltd and this group demonstrated that sexed animal could be produced by coupling the sperm sexing procedure with IVF and transferring the resulting embryos to recipients (Cran *et al.*, 1993, 1995). This approach, however, never became commercially viable. It is estimated that nearly $20 million has been invested in developing this technology from conceptualization in the early 1980s to the recent commercialization effort in 2000. Although, other approaches are currently being pursued (Johnson and Seidel, 1999), the only verified method for selecting sex offspring is flow cytometric sorting of sperm relative to DNA content. This sperm-sexing technology, which was patented by USDA, has been licensed by USDA for all nonhuman mammals through the Colorado State University Research Foundation, to XY Inc, a private company.

In 2000, the sale of sexed bovine sperm commenced in the United Kingdom. It will be interesting to see to what degree sexed sperm penetrate the semen market. This verified sexed product sets the stage for commercialization around the world in major animal producing countries. This commercialization of sexed sperm occurred nearly 20 years after technology for accurately determining the proportion of X and Y sperm in semen was first developed at Lawrence Livermore National Laboratory. It came about due to advances in both the hardware and software components of computer science, biophysics, cell biology and applied reproductive physiology plus efforts of innovative scientists. Many individuals have contributed to making semen sexing in animals a commercial reality since then research team of Bart Gledhill, Dan Pinkel, Duane Garner, Susan Lake and Larry Johnson began following up on the first flow cytometric studies on human sperm by Friedrich Otto, Wolfgang Gohde and Marvin Meistrich and since then many others has joined sexed sperm a commercial venture.

CONCLUSIONS

The present state of sperm technologies shows a great deal of promise. The last two decades have dramatically changed the field of reproductive physiology, through development and modification sperm sexing techniques. In line with the current review, some of the techniques for example sperm sorting, sperm cryopreservation, commercialization and the normality of offspring still have a large margin for improvement. It is essential to improve the efficiency of these sperm technologies and also to solve fertility problems associated with the use of sorted sperms.

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