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Integration of sperm sexing technology into the ART toolbox

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Abstract

Sex-sorting of mammalian spermatozoa has applications for genetic improvement of farm animals, in humans for the control of sex-linked disease, and in wildlife as a captive management strategy and for the re-population of endangered species. Considerable research has been undertaken worldwide on the Beltsville sperm sexing technology, the only effective method for pre-selection of sex of offspring. The combination of this method with assisted reproductive technologies has resulted in the birth of offspring in a wide range of animals, including cattle, the only livestock species in which sperm sexing is used commercially. Major improvements in the efficiency of sorting, in particular the development of high speed sorting (15 million X and Y spermatozoa per hour) have led to the production of offspring using conventional and low dose AI and the successful cryopreservation of sorted spermatozoa in cattle, sheep, horses and elk. A major limitation remains the short viable lifespan of sorted spermatozoa in the female genital tract, in most species necessitating sperm deposition deep in the uterus, and close to the expected time of ovulation, for acceptable fertility after in vivo insemination. Special deep uterine insemination technology has been employed to produce offspring in pigs and horses using low sperm doses. Considerable attention has been paid to reduction of the damage and capacitation-like changes to spermatozoa that result from flow cytometric sorting and from freezing and thawing. However, high-purity sorting of liquid-stored or frozen–thawed spermatozoa for immediate use, or re-cryopreservation for later use, does not reduce its fertilizing capacity in vitro, allowing its combination with in vitro fertilization or juvenile in vitro embryo transfer to produce blastocysts, and offspring in sheep and cattle after embryo transfer. Further research into sorting and preservation methods that incorporate strategies to prevent destabilization of sperm membranes may improve the fertilizing lifespan of flow cytometrically sorted spermatozoa. With continued improvement in sorting instrumentation and biological handling, sorting efficiency should reach a point where commercially

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acceptable pregnancy rates may be achieved in a number of species after conventional or deep uterine insemination.

Keywords: Sperm sexing; Artificial insemination; In vitro fertilization; Embryo transfer

1. Introduction

The only effective method for achieving sex pre-selection before conception requires separation of X- from the Y-chromosome bearing spermatozoa followed by their use for artificial insemination (AI) or for in vitro fertilization (IVF) with subsequent embryo transfer (ET). Production of embryos followed by sexing is practised to a limited extent but the wastage of the embryos of unwanted sex makes this approach inefficient.

Livestock production can benefit from sex pre-selection because inseminations can be planned to produce a specific sex. In addition to faster genetic progress there are other advantages for the management and efficiency of livestock production. These include the production of female replacement dairy heifers from the best cows using superior dairy bulls and the production of male and female crossbred lines in the pig industry. As with most assisted reproductive technologies (ART), the practical application of sexed spermatozoa depends on cost–benefit, fertility results, efficiency and ease of use. Results have been published world wide that demonstrate the effectiveness of the flow cytometric sexing process based on sorting sperm with differential DNA content as the X and Y sperm marker. However, the cost–benefit, efficiency and ease of use largely remain to be determined or achieved, and fertility is still lower than commercially acceptable levels in most species. There are also constraints on widespread application imposed by the inefficiency of the sorting process, with only limited numbers of viable spermatozoa produced from each ejaculate, particularly if the spermatozoa must be cryopreserved after sorting. This necessitates the combination of sperm sexing with other ART, such as IVF, which only require small numbers of spermatozoa.

In the first report utilizing DNA as a marker for separating X from Y chromosome bearing live spermatozoa (Johnson et al., 1989) more than 50 rabbits were born of the predicted sex. Since then, over 40,000 animals have been born worldwide using the Beltsville Sperm Sexing Technology (Johnson et al., in press). Most of these births have been from cattle, which is the only species where the technology is used commercially. The application of the method for sex pre-selection has been detailed in a number of reviews (Johnson et al., 1989; Johnson, 1991; Cran et al., 1993, 1995; Rath et al., 1997; Seidel et al., 1997; Johnson and Welch, 1999; Welch and Johnson, 1999; Garner, 2001; Seidel and Garner, 2002). This paper will describe the current status of this method and its integration with other ART in livestock and wildlife species.

2. Sperm sexing by flow cytometric sorting

The greater the difference in DNA content between X- and Y-chromosome bearing (X and Y) spermatozoa, the more efficiently the two populations can be resolved and

separated by flow cytometric sorting. Most domestic livestock have a DNA difference of 3.6–4.2% (Johnson, 1992; Johnson and Welch, 1999). Recently, the difference in DNA content between X- and Y-chromosome bearing spermatozoa has been determined for many wildlife species (O'Brien et al., 2002), including several primate (common chimpanzee: 3.3%; hamadryas baboon: 4.2%) and ungulate species (African elephant: 4.0%; giraffe: 4.4%). Generally, X and Y purities of more than 85% can be achieved in most species, depending on the rate of sorting.

The purity of X- or Y-enriched samples can be checked by several methods. Sex-sorted spermatozoa from species with a DNA content difference greater than 3.0% can be analyzed by flow cytometric DNA analysis or 'sort re-analysis' (Welch and Johnson, 1999). A sample of sorted spermatozoa is sonicated to remove sperm tails to improve orientation, then re-stained, incubated and re-analyzed through the flow cytometer. The proportions of X and Y spermatozoa are then determined by a curve-fitting mathematical model. For species with small differences in DNA content between X and Y spermatozoa (e.g., humans), purity can be determined by polymerase chain reaction (PCR; Welch et al., 1995) or fluorescence in situ hybridization (FISH; Johnson et al., 1993; Kawarasaki et al., 1998; O'Brien et al., 2001).

3. Developments in sperm sorting technology

Initial modifications to the standard orthogonal flow cytometer for sperm sorting were the addition of a second detector, that measured forward fluorescence (0° to the laser beam), and a wedge shaped injection tube, or beveled needle, that correctly orientated 20–40% of intact sperm to the laser beam (Johnson and Pinkel, 1986). These allowed high resolution between X and Y sperm populations in species with greater than 3.5% difference in DNA content. However, spermatozoa could only be sorted at approximately 350,000 per hour (Johnson et al., 1989). Consequently, due to the low sex-sorted sperm numbers available, the first offspring were produced by the combination of reproductive technologies such as surgical insemination, deep-intrauterine insemination, IVF and ET (Table 1).

The beveled needle was subsequently replaced by an orientating nozzle (Rens et al., 1998, 1999) which increased the proportion of correctly orientated spermatozoa to 70% (Johnson and Welch, 1999). This nozzle had an interior tapering ellipse to exert and maintain hydrodynamic forces on spermatozoa until they are released in front of the laser, providing less time for them to lose orientation compared to the shorter beveled nozzle. The nozzle was further refined by XY, Inc. (Fort Collins, Colorado) to incorporate an orientating ceramic nozzle tip (Cytonozzle™, an integral part of the MoFlo®SX sperm sorter). This high speed sorter operates under increased pressure (40–50 psi) and therefore faster flow rates (20–25,000 events per second) than the standard speed sorter (2–2500 events per second). In combination with the orientating nozzle and changes in the design of the sorter electronics, this allowed significant increases in sorting rates (15 million spermatozoa per hour) to be achieved while maintaining more than 85% purity. These developments led to the production of offspring in a number of species using conventional and low dose AI and the successful cryopreservation of sorted spermatozoa in cattle, sheep, horses and elk, bringing the sex sorting technology closer to practical application (Table 2). Despite these efficiencies, the

Table 1
Production of the first offspring of pre-determined sex derived from flow-cytometrically sorted spermatozoa on standard speed flow cytometers in combination with other reproductive technologies

Insemination method	Type of sorted spermatozoa	Dose ($\times 10^6$ total sperm)	Offspring species	Reference
Conventional AI	Fresh	5	Cattle and rabbit[a]	Morrell et al. (1988)
Surgical AI (uterine)	Fresh	0.3	Rabbit	Johnson et al. (1989)
Surgical AI (oviduct)	Fresh	0.3	Swine	Johnson (1991)
IVM, IVF, IVC, ET fresh embryos	Fresh	1.0 ml^{-1}	Cattle	Cran et al. (1993)
IVM, IVF, IVC, ET of fresh and frozen–thawed embryos	Fresh	0.02 per oocyte	Cattle	Cran et al. (1994)
IVM, ICSI, IVC, ET	Fresh	–	Sheep	Catt et al. (1996)
In vivo matured oocytes, IVF, ET	Fresh	–	Swine	Rath et al. (1997)
IVF, IVC, ET	Fresh	0.0025/0.81 μl droplet	Human	Levinson et al. (1995)
Conventional laparoscopic AI (uterine body)	Fresh	0.1	Sheep	Cran et al. (1997)
Deep AI (uterine horns)	Liquid stored fresh;	0.1–2.5	Cattle	Seidel et al. (1997)
(i) Conventional AI (uterine), (ii) IVF, IVC, ET; (iii) ICSI, IVC, ET	Fresh and Frozen–thawed	0.13 motile (AI)	Human	Fugger et al. (1998)

[a] Insufficient data for a shift in sex ratio.

time required to sort a single AI dose has limited the commercialization of the technology to cattle at present. The numbers of sex-sorted bull, ram, boar and stallion spermatozoa required for one insemination dose, and the time required to produce it using current technology, are summarized in Table 3.

Table 2
Production of the first offspring of pre-determined sex derived from flow-cytometrically sorted spermatozoa on high-speed flow cytometers in combination with other reproductive technologies

Insemination method	Type of sorted spermatozoa	Dose ($\times 10^6$ total sperm)	Offspring species	Reference
Conventional AI (uterine body) and deep AI (uterine horns)	Liquid stored and frozen–thawed	1.5–3	Cattle	Seidel et al. (1999a); Doyle et al. (1999)
Surgical AI (oviduct)	Frozen–thawed	0.4	Swine	Johnson et al. (2000)
Conventional laparoscopic AI (uterine body) and deep AI (oviducal)	Frozen–thawed	2–4	Sheep	Hollinshead et al. (2001)
Deep AI (tip of uterine horns)	Fresh	25	Horse	Buchanan et al. (2000)
Deep AI (hysteroscopic AI-utero-tubal junction)	Fresh and frozen–thawed	5 (motile)	Horse	Lindsey et al. (2002a)
Deep AI (non-surgical-anterior uterine horn)	Fresh	70–140	Swine	Vazquez et al. (2003)
Conventional AI (uterine body)	Frozen–thawed	5	Elk	Schenk and DeGrofft (2003)

Table 3
Total number of sex-sorted spermatozoa required for a single insemination, time to produce one insemination dose and resulting fertility in livestock species using current technology

Species	Total number of sperm inseminated ($\times 10^6$)	Type of semen	Pregnancy	Time to sort one AI dose[a]
Cattle	2	Frozen	10–20% lower than unsorted sperm and traditional AI[b]	8 min
Sheep[c]	20	Frozen	Similar to unsorted sperm and traditional AI[d]	1 h ± 20 min
Pigs[c]	50	Liquid frozen	4/12 (33%), 40–50% lower than traditional AI[e]	3 h 20 min
Horse[c]	5	Liquid	Similar to unsorted sperm and traditional AI[f] (13 to >50%)	20 min
	25	Frozen		1 h 40 min

[a] Sort speed of 15×10^6 sperm/h, one desired sex. Times are also dependent on sample, operator and machine.
[b] From Seidel et al. (1999a,b).
[c] For sheep, pigs and horses, hormonal synchronization of oestrus is needed when using only a single insemination. Data are from relatively small numbers of inseminations in the sheep, pig and horse studies.
[d] From Hollinshead et al. (in press-a).
[e] From Rath et al. (2003a).
[f] From Lindsey et al. (2002a,b).

4. Processing of spermatozoa for sorting and recovery after sorting

Depending on a number of factors such as male, ejaculate and flow cytometer operator, only about 30% of spermatozoa can be sex-sorted, the remainder being lost at various stages before, during and after sorting. The most significant loss (30%) is attributed to incorrect orientation. Future research to improve orientation will have a significant impact on sorting efficiency and commercialization. However, even with perfect orientation, the sperm sorters available today have an upper limit of 10,000 live spermatozoa of each sex sorted per second (Seidel and Garner, 2002).

The methods of preparing and handling of spermatozoa before, during and after sorting also have an important influence on their subsequent viability and fertility. The cells are subjected to many damaging processes, including high dilution, nuclear staining and incubation, mechanical forces associated with passage through the cell sorter, exposure to the UV laser beam and projection into the collection tube under high pressure (Maxwell and Johnson, 1999). To minimize this damage, constant temperature, osmolality of buffers, pH and sterility of staining, sheath, collection and cryopreservation media are essential. The collection medium, such as TEST-yolk (boar: Johnson, 1995) or Androhep® Enduraguard™ (Minitüb, Germany; ram: Hollinshead et al., 2003) or XY Talp and egg yolk (bull: Schenk et al., 1999) or glucose-skim milk extender (stallion: Buchanan et al., 2000), placed in the bottom of the collection tube, is usually supplemented with 2–20% purified egg yolk and, for some species, 1–10% seminal plasma. These additives provide some protection from the combined effects of dilution by sheath fluid and physical damage from projection into the collection tube (Maxwell and Johnson, 1999). Diluent requirements are not only specific to

sorted spermatozoa but are often species-specific, and a number of optimization studies have been carried out (bull: Schenk et al., 1999; ram: Catt et al., 1997b; Hollinshead et al., 2003; boar: Maxwell et al., 1997; stallion: Buchanan et al., 2000). After sorting, spermatozoa are centrifuged to remove the sheath fluid and concentrate the cells. They are then processed for immediate use, liquid storage or cryopreservation. These post-sorting processes may compound sperm damage that has occurred during sorting. However, to date it has not been possible to discern which of the various stresses imposed by the sorting procedure causes the negative effects on spermatozoa.

4.1. Potential damage to DNA integrity

All high-speed sperm sorters are fitted with an argon laser so that spermatozoa are not exposed to damaging low UV wavelengths that are absorbed by nucleic acids and proteins (Seidel and Garner, 2002). The condensed packaging of the chromatin in sperm DNA may make it more stable to the potentially mutagenic effects of UV light and H33342 than the DNA found in somatic cells. Nevertheless, there have been reports of mutations in actively mitotic cells after exposure to the bisbenzimide stain H33342 (Durand and Olive, 1982). However, Catt et al. (1997a) found no increases in endogenous DNA nicks after exposure of spermatozoa to high levels of H33342. Libbus et al. (1987) reported no effect of H33342 itself on sperm DNA but suggested that further investigations of embryos derived from sorted spermatozoa are warranted to assess chromosome damage. More recently, Seidel and Garner (2002) reported no difference in motility or DNA integrity, as determined by chromatin stability assay, between spermatozoa that had been passed through the sorter with or without staining. Similarly, after staining bull spermatozoa with either 149 or 224 μM H33342 there was no difference in post-sort and thaw motility (Schenk et al., 1999) and no difference was reported in cleavage (Merton et al., 1997) or blastocyst development rates (Zhang et al., 2003) after IVF with stained or non-stained spermatozoa. Laser power (125 compared to 25 mW) had no detrimental effects on in vivo fertilization with sorted boar spermatozoa or blastocyst development rates (Guthrie et al., 2002).

These in vitro and in vivo tests should also be considered in the light of births of over 20,000 calves after insemination with sex-sorted, frozen–thawed bull spermatozoa (Seidel, 2003). Offspring have also been produced from seven mammalian species including humans, without any apparent alteration to phenotype or genotype (Amann, 1999; Seidel and Johnson, 1999; Garner, 2001; Seidel and Garner, 2002; Vazquez et al., 2003).

4.2. Potential damage to sperm viability and fertility

Lower pregnancy rates after fertilization with sex-sorted compared to non-sorted spermatozoa have been reported in a number of studies (Johnson et al., 1989; Johnson, 1991, 1995; Cran et al., 1993; Seidel et al., 1999a; Hollinshead et al., 2002b,c; Seidel and Garner, 2002; Maxwell et al., 2003). However, whether the low fertility of sex-sorted spermatozoa was due to DNA damage and increased early embryonic loss, or other factors such as inseminate dose, timing of insemination relative to ovulation, manipulation of the female reproductive tract or reduced viability in the female reproductive tract is yet to be determined.

There is more published information on the effects of sperm processing associated with sex sorting on viability and in vitro fertility than on in vivo fertility. For example, Suh and Schenk (2003) found higher motility after thawing, and Campos-Chillon and de la Torre (2003) reported higher cleavage and blastocyst rates after IVF, with bull spermatozoa sorted at machine pressures of 40 psi compared to 50 psi. Ram spermatozoa undergo a 10-fold dilution prior to staining, a further two-fold dilution after staining and incubation, and a 200-fold dilution by sheath fluid during sorting. This results in the removal of many beneficial seminal plasma components such as anti-oxidants and proteins that maintain membrane stability. Capacitation-like changes have been observed after the sorting of both ram and boar spermatozoa (Catt et al., 1997b; Maxwell et al., 1997, 1998; Maxwell and Johnson, 1997, 1999), similar to those observed after freezing and thawing of ram spermatozoa (Gillan et al., 1997), reducing their fertilizing lifespan in vivo (Watson, 1995). However, the addition of 10% seminal plasma to the staining medium helped prevent undesirable membrane changes and, when added to the collection medium, helped restore the decapacitated state of both ram and boar spermatozoa after flow-cytometric sorting (Maxwell and Johnson, 1997). Centurion et al. (2003) suggested that heparin-binding proteins in boar seminal plasma might protect the spermatozoa from some of the negative effects of high dilution. Moreover, the beneficial effects of seminal plasma may be due to its protein component, rather than its ionic constituents, as replacement of the standard Tris-based sheath fluid with artificial seminal plasma containing no protein (Mortimer and Maxwell, 2004) temporarily improved progressive motility characteristics but severely decreased overall quality and longevity of sorted-frozen–thawed ram spermatozoa (de Graaf et al., 2004).

Unlike capacitation status, the motility and acrosome integrity of sorted spermatozoa are high. Prior to sorting, the cells are stained not only with H33342 but also with a food dye that penetrates the non-viable spermatozoa and quenches the intensity of their fluorescence, thus allowing them to be gated out from the live population. This process appears to select a population of highly motile and acrosome-intact spermatozoa in the ram, boar and stallion (Hollinshead et al., 2002a, 2003). This improved motility and acrosome integrity is maintained during post-sorting incubation of ram (Hollinshead et al., 2003) and stallion spermatozoa (Morris et al., 2003) but motility of bull and boar spermatozoa after sorting and freeze–thawing declines faster than unsorted controls in a thermo-resistance test (Rath et al., 2003b). This may reflect species differences, but fertility results for all species suggest a shorter lifespan in the female genital tract for sorted than unsorted spermatozoa.

5. Storage of sex-sorted spermatozoa

Pre-determination of sex by the sperm sexing technology requires use of a cell sorter at a fixed location which may be remote from donor males or females for AI. Therefore, liquid or frozen preservation and transport to the sorter would be advantageous. Standard storage processes, however, inevitably reduce the proportion of motile spermatozoa and cause degenerative changes to sperm membrane integrity, which ultimately reduce fertilizing capacity after AI (Maxwell and Watson, 1996). These changes may have an even greater impact on sex-sorted spermatozoa but they have not prevented the development of successful preservation protocols.

Sorted spermatozoa in the collection medium require centrifugation to concentrate the spermatozoa before re-dilution and processing for freezing. Apart from this additional step, most workers have adapted standard semen freezing protocols for cryopreservation of the sex-sorted spermatozoa. In the pig, for example, two widely used commercial freezing methods, the Beltsville pellet freezing method (Pursel and Johnson, 1975) and the German straw freezing method (Westendorf et al., 1975), have been adapted for freezing sex-sorted spermatozoa in 0.5 ml French straws. Offspring have been produced after artificial insemination with both liquid-stored-sex-sorted spermatozoa (bull: Schenk et al., 1999; Seidel et al., 1999a; stallion: Lindsey et al., 2002b) and sex-sorted-frozen–thawed spermatozoa (bull: Schenk et al., 1999; boar: Johnson et al., 2000; ram: Hollinshead et al., 2001; and stallion: Lindsey et al., 2002a). The latter pregnancies were obtained in the pig after surgical insemination into the oviduct (Johnson et al., 2000). Rath et al. (2000) established that 50 million fresh spermatozoa is the minimum necessary for normal fertilization to occur after deep uterine insemination of sows and the development of the Firflex® catheter (Magapor, Spain) raised the practical possibility of obtaining progeny after deep uterine insemination with low numbers of spermatozoa via the cervix (Johnson et al., in press). Pregnancy rates of 63.0% of 45 and 42.9% of 42 sows were reported by our group after deep uterine insemination with 250 million unsorted fresh and frozen–thawed spermatozoa, respectively (Bathgate et al., in press).

Early experiments on low dose insemination in the sheep indicated that fertilization could be achieved in superovulated ewes with fresh unsorted spermatozoa after deposition of as few as 100,000 in the oviduct or uterus (Maxwell et al., 1993). Deep uterine insemination has most readily been applied in cattle (Seidel et al., 1997, 1999a) where pregnancies can be achieved using as few as 200,000 spermatozoa per insemination with deep intrauterine AI to 2×10^6 spermatozoa for insemination into the body of the uterus. Since the benefit of inseminating into the uterine horn was only marginal, the latter is currently being used for most inseminations undertaken in commercial cattle production (Schenk et al., 1999). Such commercial development has yet to take place in the sheep industry.

6. Limitations to practical application of sex-sorted-frozen–thawed ram spermatozoa

The main limitations to the commercialization of sex-sorted and frozen–thawed spermatozoa in the sheep have been associated with their viable lifespan in the female genital tract. In vitro studies using chlortetracycline (CTC) staining and co-incubation with oviduct epithelial cell monolayers (OECM) carried out in our laboratory identified a greater proportion of sorted-frozen–thawed ram spermatozoa that had undergone capacitation-like changes and were released earlier from the OECM compared to non-sorted-frozen–thawed spermatozoa. In our previous studies, cryopreservation of non-sorted ram spermatozoa caused sperm membrane changes (Gillan et al., 1997) that were reflected in the pattern of attachment and release from the OECM (Gillan et al., 2001). Collectively, the sorting and freeze–thawing processes accelerate the maturation of sorted-frozen–thawed ram spermatozoa, reducing their fertilizing lifespan (Hollinshead et al., 2003, in press–b). Thus, for use in AI, in-

Table 4
The number of GnRH- and non-GnRH-treated ewes pregnant after insemination (%) with sorted and non-sorted (commercial control) frozen–thawed spermatozoa at 54, 58 and 62 h after sponge removal (adapted from Hollinshead et al., 2002c)

Sperm dose ($\times 10^6$)	Sperm type	Time of insemination				Total
		54 h (+GnRH)	58 h (+GnRH)	62 h (+GnRH)	54–58 h (−GnRH)	
1	Sorted	5/29 (17.2)	5/30 (16.7)	5/30 (16.7)	–	15/89 (16.9) b
4	Sorted	8/30 (26.7)	7/30 (23.3)	7/30 (23.3)	–	22/90 (24.4) ab
16	Sorted	11/30 (36.7)	12/30 (40.0)	5/30 (16.7)	–	28/90 (31.1) a
100	Commercial control	12/29 (41.4)	21/30 (70.0)	19/30 (63.3)	6/15 (40.0)	58/104 (55.8) c

Values with different letters differ ($P < 0.05$) within column (abc).

semination close to the site of fertilization and time of ovulation is critical for successful fertilization and ongoing pregnancy.

Standard laparoscopic intrauterine insemination of ewes with 2–4 $\times$ 10^6 sex-sorted-frozen–thawed spermatozoa resulted in a low pregnancy rate (25%; Hollinshead et al., 2002a,b). Treatment of ewes with gonadotrophin-releasing hormone (GnRH), in combination with progestagen sponges and PMSG, reduces the period over which ovulation occurs (Eppleston et al., 1991), thus, facilitating insemination closer to the time of ovulation. Despite inseminating ewes either just before or after ovulation with sorted-frozen–thawed spermatozoa, the pregnancy rate of ewes treated with GnRH 36 h after sponge removal did not differ from ewes not treated with GnRH (Table 4). Nevertheless, the number of animals was too low to detect differences in pregnancy rates after insemination of GnRH-treated ewes with the same doses of sorted and non-sorted (control) frozen–thawed ram spermatozoa (Table 5). The latter pregnancy results did not conclusively determine the minimum effective dose of sorted-frozen–thawed spermatozoa but it appears that 20 $\times$ 10^6 motile

Table 5
Pregnancy after intrauterine insemination of (i) GnRH-treated ewes with sorted and non-sorted (control) frozen–thawed ram spermatozoa 58 h after progestagen sponge removal and of (ii) non GnRH-treated ewes with non-sorted (commercial control) frozen–thawed ram spermatozoa 54–58 h after progestagen sponge removal (adapted from Maxwell et al., 2003)

Dose ($\times 10^6$ sorted and non-sorted control spermatozoa)	Sperm type	Number of ewes inseminated	Number of ewes pregnant (%)
5	Sorted	15	3 (20.0) a
	Control	15	7 (46.7) abc
10	Sorted	14	6 (42.9) abc
	Control	14	10 (71.4) c
20	Sorted	16	5 (31.3) ab
	Control	13	5 (38.5) ab
40	Sorted	11	8 (72.7) c
	Control	12	8 (66.7) bc
100	Commercial control (−GnRH)	13	7 (53.8) abc

Values with different letters differ ($P < 0.05$) within column (abc).

spermatozoa may be required, which falls within the minimum range recommended for unsorted-frozen–thawed spermatozoa (Evans and Maxwell, 1987).

7. Production of offspring from sex-sorted spermatozoa in livestock

The first calves produced from sex-sorted spermatozoa were reported by Cran et al. (1993, 1995; Table 1). At this time the process of sex-sorting was too slow for practical use with routine AI, and the offspring were produced by combining the technology with IVF and ET. Subsequently, sex-sorted spermatozoa were combined with ET after superovulation (Paranace et al., 2003). With the advent of high speed sorting, low dose AI deep in the uterine horn was effective with sex-sorted non-frozen (Seidel et al., 1997) and later with frozen–thawed spermatozoa (Seidel et al., 1999a; Seidel and Johnson, 1999; Table 2).

The first lamb from sex-sorted spermatozoa was produced by intra-cytoplasmic sperm injection (ICSI) with a fresh spermatozoon (Catt et al., 1996; Table 1). Lambs were subsequently produced after sex-sorting and conventional insemination by laparoscopy using either 10×10^6 non-frozen (Cran et al., 1997; Table 1) or $2–4 \times 10^6$ frozen–thawed spermatozoa (Hollinshead et al., 2002b; Table 2). The latter resulted in lower fertility compared with un-sorted frozen semen, with an insemination dose closer to 20×10^6 required to obtain commercially acceptable fertility levels after laparoscopic insemination with sex-sorted-frozen–thawed spermatozoa (Maxwell et al., 2003). Offspring have subsequently been produced by IVF from in vitro matured abattoir-sourced peripubertal lamb oocytes (Morton et al., unpublished results) and from oocytes aspirated from hormone-stimulated prepubertal lambs (Morton et al., 2004).

The first piglets were obtained after sex-sorting and surgical insemination of non-frozen spermatozoa (Johnson, 1991; Table 1). Subsequently, offspring were produced after sex-sorting and IVF of non-frozen spermatozoa (Rath et al., 1997, 1999; Abeydeera et al., 1998) and after surgical insemination with frozen–thawed spermatozoa (Johnson et al., 2000). Protocols for pig IVF and ET still require considerable modification before this approach can be used commercially. Only a non-invasive deep-uterine insemination (DIU) or ET method would currently be acceptable, both for economical and ethical reasons, for the commercial application of sex-sorted spermatozoa in pigs. To date, progeny have been produced after DIU of non-frozen bulk-sorted (Vazquez et al., 2003), sex-sorted non-frozen (Rath et al., 2003a), and sex-sorted frozen–thawed spermatozoa (Bathgate et al., unpublished results). These pregnancies have been achieved using the flexible Firflex® catheter for DIU in sows. While this catheter is only suitable for insemination in adult sows, offspring have been obtained after insemination of as few as 50×10^6 fresh spermatozoa in a volume of 2 ml (Rath et al., 2003a).

Buchanan et al. (2000) reported a 40% pregnancy rate in mares after AI of 25×10^6 spermatozoa in the uterine body or the uterine horn using sex-sorted non-frozen stallion spermatozoa. Pregnancies have been obtained with as few as 5×10^6 liquid or frozen–thawed spermatozoa using hysteroscopic insemination at the tip of the uterine horn (Lindsey et al., 2002a,b). Recently, a foal was born after hysteroscopic insemination of a mare with 10×10^6 sex-sorted non-frozen spermatozoa and subsequent surgical transfer of the in vivo derived embryo into a surrogate mare (Morris et al., unpublished results).

8. In vitro embryo production with sexed spermatozoa

With the exception of cattle, recent improvements in the efficiency of sex sorting have not increased the output of spermatozoa per hour to levels that would allow commercial application through AI. Nevertheless, IVF and ET provide alternative and potentially effective methods for the production of offspring of the desired sex. Combination of sexed spermatozoa with other ART such as JIVET (juvenile in vitro embryo transfer) and MIVET (mature in vitro embryo transfer) could further extend the use of sexed semen in livestock and facilitate increased production efficiency through better selection of breeding stock and rapid genetic gain. Production in vitro of pre-sexed embryos, followed by cryopreservation by freezing or vitrification and subsequent transfer, would further facilitate the commercial application of the technology.

Fresh sorted spermatozoa have been used to produce embryos and offspring using standard IVF (cattle: Cran et al., 1993, 1994, 1995; Seidel, 1999; sheep: Rhodes et al., 1994; pigs: Rath et al., 1997, 1999; Abeydeera et al., 1998; humans: Levinson et al., 1995; Fugger et al., 1998) and ICSI (sheep: Catt et al., 1996; human: Fugger et al., 1998; cattle: Lu et al., 1999).

In sheep and cattle, the application of high-speed sorting technology does not influence the proportion of oocytes derived from adult females and fertilized by ram and bull spermatozoa, or blastocysts forming as a proportion of mature oocytes cultured, after IVF compared to non-sorted spermatozoa. Recent developments in preservation of sex-sorted ram spermatozoa have facilitated the production of blastocysts after IVF with liquid stored-sorted-frozen–thawed ram spermatozoa (Hollinshead et al., in press-b). While the processes of sorting and storage (liquid or frozen) cause changes to sperm membrane integrity which reduce their fertilizing lifespan, they also bring the spermatozoa to a physiological state of readiness to participate in fertilization (Hollinshead et al., 2003). Importantly, high purity sorting of frozen–thawed spermatozoa for immediate use, or re-cryopreservation for later use, does not reduce the fertilizing capacity of spermatozoa. Thirty lambs of predicted sex were born after the transfer of both fresh and vitrified in vitro produced embryos, derived from frozen–thawed-sorted and frozen–thawed-sorted-re-frozen–thawed ram spermatozoa (Table 6). Preliminary studies have also been conducted with frozen–thawed-sorted and frozen–thawed-sorted-re-frozen–thawed bull spermatozoa, indicating that they can be efficiently sorted at high purities with retained functional capacity (Hollinshead et al., in press-a) and pregnancies have been obtained after ET of vitrified IVF cattle embryos derived from sex-sorted-frozen–thawed spermatozoa (Fry et al., 2004).

Frozen–thawed sex-sorted spermatozoa can also be successfully used for IVF of IVM oocytes derived from hormone-stimulated juvenile lambs (2–3-week-old; Morton et al., 2004) and calves (approximately 6-month-old; Fry et al., 2003). The proportion of lamb oocytes cleaving by 48 h post-insemination was significantly lower for sex-sorted sperm (X-sperm: 37.1%, Y-sperm: 31.05%) compared with the unsorted controls (52.4%), but the proportion of cleaved oocytes developing to the blastocyst stage did not differ (Table 7), and these embryos were capable of developing to term after transfer to recipients either fresh (Morton et al., 2004) or after cryopreservation (Morton et al., unpublished results). In the latter studies, transfer of 64 conventionally slow-frozen JIVET embryos derived from X and Y spermatozoa yielded 2 (6%) and 1 (3%) pregnancies, respectively, compared

Table 6
Pregnancy rate on day 20, day 60 and lambing and in vivo embryo survival of fresh and vitrified embryos, produced after in vitro fertilization of in vitro matured adult ewe oocytes with frozen–thawed, non-sorted (Control), frozen–thawed-sorted (FS) and frozen–thawed-sorted-re-frozen–thawed (FSF) spermatozoa, and transfer into synchronized recipient ewes (adapted from O'Brien et al., 2004b)

Type of semen	Type of embryo	Number of recipient ewes	Number of ewes pregnant on day 20 (%)[a]	Number of ewes pregnant on day 60 (%)[b]	Number of ewes lambing (%)[c]	Embryo survival (%)[d]
Control	Fresh	8	6 (75)	5 (62.5) a	4 (50.0) a	5/16 (31.3) a
	Vitrified	6	5 (83.3)	2 (33.3) b	1 (16.7) b	1/12 (8.3) b
FS	Fresh	9	7 (77.8)	5 (55.6) a	5 (55.6) a	6/18 (33.3) a
	Vitrified	8	3 (37.5)	2 (25.0) b	2 (25.0) b	2/16 (12.5) b
FSF	Fresh	17	14 (82.4)	13 (76.5) a	13 (76.5) a	17 /34 (50.0) a
	Vitrified	13	6 (46.2)	4 (30.8) b	4 (30.8) b	5/26 (19.2) b

Values with different letters differ ($P<0.05$) within columns (ab).
[a] Determined by peripheral plasma progesterone assay.
[b] Determined by ultrasound.
[c] Number of ewes that lambed between days 149 and 155 (fresh) or days 145 and 156 (vitrified)/number of recipient ewes × 100.
[d] Number of lambs born/number of transferred embryos × 100.

Table 7
Cleavage and development to blastocyst in vitro of in vitro matured ovine oocytes derived from hormone-stimulated juvenile (2–3-week-old) lambs and fertilized with unsorted (control) or sex-sorted ram spermatozoa (adapted from Morton et al., 2004)

Sperm type	Number of oocytes	Number of oocytes cleaving (%)		Blastocyst formation (%)		
		24[a]	48[a]	Day 6	Day 7	Day 8
Unsorted	632	217 (34.3) a	332 (52.4) a	62 (18.7) a	115 (34.6)	132 (39.8)
X	556	113 (20.3) b	206 (37.1) b	19 (9.2) b	56 (27.2)	68 (33.0)
Y	551	88 (16.0) b	171 (31.0) b	31 (18.1) ab	68 (39.8)	76 (44.4)

Values with different letters differ within ($P < 0.05$) columns (ab).
[a] Hours after insemination.

with 7/16 (44%) pregnancies after transfer of fresh JIVET embryos derived from unsorted spermatozoa.

9. Application of sperm sexing to wildlife management and conservation

In conjunction with ART, sex pre-selection of offspring through the use of sexed spermatozoa has great potential as a captive population management strategy for wildlife species, particularly those with single-sex dominated social structures (O'Brien et al., 2002). Producing predominantly female offspring also has the advantage of accelerating the re-population rate of endangered species with slow reproduction rates.

The development of species-specific methodologies is required for all aspects of sperm sorting including liquid storage (for transport to and/or from the sorting facility), sperm

staining, sorting and freeze–thawing. Among wildlife species, methodologies for both liquid storage of semen and sperm sorting have been developed in non-human primates (O'Brien et al., 2001, 2003) and in the bottlenose dolphin (O'Brien et al., unpublished results). Recently, the first pre-sexed offspring for any wildlife species was reported following insemination of female elk with sex-sorted-frozen–thawed spermatozoa (Schenk and DeGrofft, 2003).

The application of sperm sexing to breeding of wildlife is limited when the sperm sorter is located at a distance from the male(s) but would be facilitated by the sorting of frozen–thawed spermatozoa and re-freezing. In several non-human primates (hamadryas baboon, common marmoset, common chimpanzee, western lowland gorilla), high-purity sorting of frozen–thawed spermatozoa with recovery of progressively motile, acrosome-intact spermatozoa was possible after processing to remove cryodiluent (O'Brien et al., 2002, 2003).

10. Concluding remarks

Protocols are well advanced for the sex-sorting of mammalian spermatozoa using the Beltsville sperm sexing technology and offspring have been produced in several species by the combination of flow cytometric sperm sorting with a range of ART methods. A major limitation remains the short viable lifespan of spermatozoa after sorting, with fertility results in all species suggesting a reduced viability in the female genital tract compared with unsorted spermatozoa, making inseminations close to the time of ovulation necessary. There is a great need for the development of new assessment methods to investigate the effects of sex-sorting on the spermatozoa, other than the traditional sperm motility and viability tests. A detailed understanding of the effects of the sorting process requires the study of subtle changes in the spermatozoa at a molecular level, including the use of new probes for changes in membrane architecture and other in vitro sperm function tests (Gillan et al., in press).

At present, in vitro production of embryos from oocytes derived from juvenile or adult females seems to be the most promising method of generating offspring in some species, although encouraging results have been obtained with deep uterine insemination in cattle, sheep and pigs and hysteroscopic insemination in the horse. Sex-sorted spermatozoa have been successfully cryopreserved, and offspring produced from cryopreserved spermatozoa that have been thawed, sex-sorted, re-frozen, thawed and used for IVF. The latter achievement, currently only in sheep, has significant implications for wildlife breeding, where the sperm sorter is generally located some distance from the males.

Further research into sorting and preservation methods that incorporate strategies to prevent destabilization of sperm membranes, such as a reduction in sheath fluid pressure during sorting, the addition of seminal plasma at various stages during the transporting, sorting and freeze–thawing process and the development of new cryopreservation techniques, such as the multi-Thermal-Gradient freezing technology (Arav et al., 2002), may improve the fertilizing lifespan of flow cytometrically sorted spermatozoa. Together with continued improvement in sorting instrumentation and biological handling, sorting efficiency should reach a point where commercially acceptable pregnancy rates may be achieved in a number of species after conventional or deep uterine AI.

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