Artificial insemination in livestock production: the Vet's perspective

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Abstract

Artificial insemination (AI) can undoubtedly be considered as the oldest and most widely used technique in the assisted reproduction spectrum for livestock production. Following a brief introduction on the history of artificial insemination, an overview is given on available bovine assisted reproduction techniques. Subsequently, indications for use of AI in livestock production are discussed and the outlines of the cattle AI business are given. In addition, current and new applications of AI are described such as sperm sexing and the accompanying new interest in low dose and deep intra-uterine insemination

Key words: AI, cattle, artificial insemination, assisted reproduction, sperm sexing, deep intra-uterine insemination.

Introduction

Artificial insemination (AI), defined as the introduction of sperm in the female reproductive tract by means of an instrument, is undoubtedly the oldest technique within the assisted reproductive spectrum. Although many Arabian horse breeders banned assisted reproduction techniques until recently, it were the Arabs to first report on AI around 1322 (Verberckmoes et al., 2004b). Several centuries later, Anthony van Leeuwenhoek took the first step to what would be andrology later on by inventing a small microscope by which he was able to study gross movement of spermatozoa. While Spallanzani (1784) discovered that a bitch could be impregnated by 'cells' present in semen, we had to wait until 1900 when professor Ivanoff, hired by the Russian throne, started to develop AI procedures in horses. By 1922, he had AI procedures in place to be used in horses, cattle, sheep and swine (Ivanoff, 1922). The first commercial AI cooperative was founded in 1936 in Denmark, by Sørensen (Foote, 2002). Originally, AI was mainly performed with fresh semen, whereas from 1965 on, mostly frozen-thawed semen is used. Nowadays, AI is performed in all livestock species: cattle, horses, sheep, goats, pigs, poultry, rabbits etc... The evolution AI has gone through over the past decades is best illustrated by its use in cattle breeding business, as summarized by Vishwanath in 2003. Also in pig industry, AI became more widely used over the past decades with an increase in the number of AI matings of 5% in 1990 up to 60-70% in 2000 (Vyt et al., 2004).

The use of AI in animal reproduction was originally introduced for sanitary reasons. When frozen sperm became readily available later on, the economic advantages through improved fertility rates and accelerated genetically progress became fully clear. By lowering the insemination dose, the number of inseminations and offspring of highly valuable sires could be increased enormously. Since then, several interesting developments guaranteed and increased the importance of AI as an assisted reproduction technique (ART) such as sorting X- and Y-bearing spermatozoa by means of flow cytometry (Seidel, 2003), the development of new sperm diluters and the use of new AI techniques such as deep intra-uterine or utero-tubal junction insemination (Verberckmoes et al., 2002). This brief overview wants to comment on a few of these relatively new

prospects in the use of AI besides of giving a short description on the current state of the art of AI in livestock production, using the bovine model as an example.

(Bovine) assisted reproduction techniques

AI has become the tool of choice for rapidly spreading desired animal genetics in a given population, while emphasizing the male role in genetic selection. A substantial number of bulls have produced hundreds of thousands of insemination doses and numbers of offspring accordingly. However, in a next stage, breeders also wanted to use the valuable genetics on the female side in a more intensive way. This is why commercial embryotransfer (ET) business was established in the early 1970s (Hasler, 2003). Genetically valuable donor cows are superovulated and subsequently inseminated. Following fertilization, they carry on average between 4 and 10 embryos in the uterus until 6 to 7 days later. Through surgical (early 1970s) and later on non-surgical embryo collection (mid 1970s), on average 5 transferable embryos can be collected during uterine flushing and transferred into recipient cows (Hasler, 2003). This procedure can be repeated 4-5 times a year for the same donor cow, depending on its reaction to hormonal superovulation. The overall yield, expressed by the number of calves per donor cow per year could be increased to 15-20 calves born instead of only one through natural mating or AI. From the early 1980s on, effective methods to freeze embryos, utilizing dimethyl sulfoxide or glycerol as cryoprotectants, became available (Hasler, 2003). This way, a successful ET program was no longer dependent on the immediate availability of recipient cattle. Around 754.000 in vivo embryos are produced worldwide each year (Thibier, 2009). The next step to even increase the maternal contribution to the selection process was the introduction of in vitro embryo production (IVP) with the first IVP calf produced from an in vivo matured oocyte in 1982 (Brackett et al., 1982). A few years later, in vitro maturation, fertilization and culture were sufficiently optimized to result in the birth of a calf from the first totally in vitro produced embryo (Fukuda et al., 1990). Oocytes for IVP can be harvested from slaughterhouse ovaries (Hasler, 2003) or transvaginally aspirated from living donor cows through Ovum Pick-Up procedures (OPU) (for review: Bols, 2005). This way, nearly 331.000 IVP embryos were produced worldwide in 2008 (Thibier, 2009). Relatively new technologies include embryo sexing by PCR (Shea, 1999), marker assisted selection (MAS) (Georges, 1999) based on the identification of genetic markers for unknown alleles of valuable production traits, flow cytometric technology used to separate X- and Y-bearing sperm (see below) (Johnson, 2000), and cloning (Wilmut et al., 1997). Although reports are available on relatively large numbers of cattle clones produced (Faber et al., 2003), still several economical and practical hurdles need to be overcome before these high-tech techniques can be applied routinely in livestock production (Hasler, 2003).

Indications for the use of AI in animal (re)production

As stated above, the main reason for the introduction of AI in animal reproduction was out of a sanitary concern that bulls, which were used on different farms, would spread venereal diseases such as vibriosis and trichomoniasis. In addition, the transport of bulls would also facilitate the spread of non-sexually transmitted diseases such as (para)tuberculosis and brucellosis (Thibier and Guerin, 2000). Soon enough farmers understood that AI is the method of choice for the rapid dispersal of valuable genes in a given animal population in order to improve production traits in livestock (Vishwanath, 2003). By introducing AI in the pig, poultry and rabbit industry, a significant improvement in carcass quality could be obtained and breeding units could be expanded (Singleton, 2001). While the success of AI was largely based on the fact that the technique is simple, economical and has a high success rate, the introduction of freezing protocols – with glycerol as a cryoprotectant – for semen has enormously boosted the possibilities of AI. The use of frozen-thawed semen increased the speed with which valuable genes could be dispersed and made it possible to install testing and breeding programmes for promising bulls (Verberckmoes et al., 2004). Dairy bulls for example, will finally be selected based on the production and conformation characteristics of their female offspring, which is currently complemented by the genomics approach and marker assisted selection. However, freezing semen is not as successful in all farm animals as it is in cattle. Semen of horses, pigs, sheep, goats, poultry and rabbits is much more prone to freezing damage.

State of the art of AI in animal (re)production: the bovine model

As stated by Vishwanath (2003), the cattle dairy industry is the 'reference where technical advancement in AI and semen technology has been captured most successfully'. This is why most of the data referred to in this overview deal with cattle (re)production programmes, which does not mean it's easy

to retrieve reliable data when it comes to the worldwide use of AI. A 1980 survey reported a worldwide use of cattle AI doses of about 130 million (Bonadonna and Succi, 1980). Whereas an updated survey in 1995 reported a total number of doses of semen produced exceeding 200 million (Chupin and Thibier, 1995), the most recent available information mentions more than 260 million doses produced worldwide in 1998 (Thibier and Wagner, 2002), of which 95% was deep frozen. More than half of the frozen doses was produced in Europe. Fresh semen is produced only in limited amounts, more specifically in the Far East (Oceania) and Europe, about 12 million doses altogether. Some of the data of the most recent survey are summarized in Table I. The number of bulls enrolled in semen collection programmes is around 40.000 worldwide, of which 50% were located in Europe. The volume of international trade in frozen semen is given in Table II (Thibier and Wagner, 2002). Around 20 million doses of semen have been shipped around the globe (1998), which was approximately 10% of the total production. North America and Europe were the most important exporters while South America was the biggest importer. This worldwide trade in frozen semen reflects globalisation of animal breeding and a vast interest in the exchange of genetics between different countries and breeds. Obviously, as mentioned by Thibier and Wagner (2002) this raises concerns about animal health aspects because transfer of animal diseases through the use of AI cannot totally be ruled out. The same survey illustrated that about 20% of all breedable females in the world (about 550 million heads) were bred by AI. Large variations in this number exist between the different continents, being 61% in Europe, 25% in North America and the Far East, down to a marginal proportions of 1 to 4,5% in the Near East, South America and Africa. These low percentages can be attributed to the extensive production systems in these countries as well as to a lack of updated information. About 75% of AI's are performed in dairy cattle, the remaining 25% with semen from beef bulls.

It is assumed that, on average, 2 inseminations are needed to establish 1 pregnancy (Thibier and Wagner, 2002), which brings us to the efficiency of the AI procedure. As summarized by Vishwanath (2003), the main factors that determine the extent of the use of AI are the cost of the semen, the cost of the insemination procedure and the success of the technique. The contribution of these 3 factors to the overall cost depends largely on the country/ continent were the AI is used. This is mainly because other variables also play a steering role: commodity milk price, purchase prices of animals and the salvage value of an animal as meat. The success rate of AI can be expressed as the percentage of animals not returning to service within a defined period after first insemination referred to % Non Return Rates (%NRR). In most countries, these NRR's are within the range of 65-70% for 24, 60 or 90 days NRR's (Vishwanath, 2003). As mentioned above, AI demonstrated to be the tool of choice to accelerate genetic gain by using sperm of genetically superior bulls on a large scale within a certain population. Foote (1998) described the potential genetic contribution to genetic improvement of a single sire as the number of progeny of that sire multiplied by the genetic superiority. The number of progeny will be determined by the total sperm output of the bull, the number of sperm used per insemination and the percentage of cows calving to a single insemination. Several highly wanted bulls produced over a million doses of sperm during their lifespan.

Table I. — Partial data of the 'World statistics for artificial insemination in cattle' survey from 2002 (Thibier and Wagner).

Region	No. of bulls	No. of doses produced fresh (× 1000)	No. of doses produced frozen (× 1000)	Total (X 1000) (%)
Africa	646	55	1.484	**1.540 (0.6%)**
North America	9.627	0	43.270	**43.270 (16.4%)**
South America	530	0	5.917	**5.917 (2.2%)**
Far East	9.228	8.875	69.938	**72.812 (27.5%)**
Near East	268	31	2.559	**2.590 (1.0%)**
Europe	20.785	2.694	135.563	**138.258 (52.3%)**
Total	**41.084**	**11.656**	**252.733**	**264.390 (100%)**

Note: the addition of the total number of doses is made to the unit which explains the slight difference from the single addition from those figures here given to the thousand.

Table II. — Volume of international trade in frozen cattle semen, based on 'World statistics for artificial insemination in cattle' survey from 2002 (Thibier and Wagner).

Region	No. of doses imported	No. of doses exported
Africa	568.589	2.360
North America	1.366.100	13.564.500
South America	5.318.595	120.650
Far East	1.467.388	658.700
Near East	402.640	5.120
Europe	4.884.999	5.010.510
Total	**14.008.311**	**19.361.840**

Sperm sexing

Mainly determined by a specific breeding goal, it has always been the desire of cattle breeders to predict or determine the sex of the offspring before or as soon as possible following conception. When considering the possibility to determine the sex of offspring following conception, there are several methods described to sex embryos, which can be used in combination with Multiple Ovulation and Embryo Transfer (MOET) programmes. However, as stated by Seidel (2003), these are all time consuming, expensive and require biopsy of embryos which might possible cause damage and reduce pregnancy rates. In addition, these procedures are able to identify the sex of embryos, but do not predetermine sex, which means that half of the embryos produced are not of the desired sex. A possible alternative is to determine sex of the foetus during early pregnancy using ultrasonography (Müller and Wittkowski, 1986). This has already been successfully achieved in cattle and horses at 2-3 months of gestation. However, ethical problems can occur because the consequence of using this procedure is often that foeti with the undesired sex will have to be aborted. This implies that 2 or 3 months of gestation are lost and problems can occur with return to oestrus of aborted cows delaying the establishment of a subsequent pregnancy.

All these considerations forced scientists to concentrate on methods that predetermine sex, almost automatically leading to sperm sexing. Although over time, there has been a vast amount of papers published on different approaches to obtain this goal (for review: Garner and Seidel, 2008), most time and effort have been put in a method using flow cytometry and cell sorting after staining intact sperm with the membrane permeant bisbenzimidazole fluorescing DNA binding dye, Hoechst 33342 (Johnson and Welch, 1999). This technique is based on the difference in DNA content of X- and Y-chromosome-bearing sperm, which is approximately 3-5%, depending on the species studied (Moruzzi, 1979). Briefly, the sperm is stained with Hoechst 33342 after which the sperm cells are pumped into a stream in front of a laser beam at specific wavelengths (Garner and Seidel, 2008). The illuminated Hoechst 33342 stained sperm emit a very bright blue fluorescence which is measured using a photomultiplier tube. Subsequently, a high speed computer analyzes the relative fluorescence of the X- and Y-sperm populations as they flow in a fluidic stream. This stream is broken into individual droplets, many of which contain a sperm. The stained sperm is finally sorted by placing opposite charges on droplets containing X-sperm or Y-sperm. Positive and negative electrical fields will than sort out the different droplets into two different streams. A third stream of droplets that contain abnormal or no contents is not charged and discarded. This sperm sorting procedure is known as the Beltsville Sperm Sexing Technology and is patented by the USDA with Dr. Johnson as the inventor (US Patent #692958, 04/26/1991). Following optimization of the technique, AI of heifers with sexed sperm resulted in about 90% accuracy of sex of resulting offspring, although in some cases with reduced fertility (Seidel, 1999).

Although 90% is a pretty high accuracy, several hurdles need to be overcome before sperm sexing technology will become available for worldwide use. Apart from economical imperatives, which largely depend on local market conditions for meat and dairy production (Seidel, 2003), pure technical factors play an important role. The initial speed with which semen could be sexed and sorted was about 400,000 sperm/hour (Garner and Seidel, 2008), implying that it would take about 25 hours to sort one insemination dose of 10×10^6 sperm of each sex. Because sperm sexing can cause damage to the sperm (Garner, 2006; Garner and Suh, 2002), considerable debate is still going on about the minimal amount of sexed sperm needed for a successful

insemination (Garner and Seidel, 2008). While it has been determined that the insemination dose with nonsexed, frozen thawed sperm can be as low as 0.38×10^6 sperm to still obtain a NRR of 50% (Jondet, 1972), numerous other factors will determine the NRR, such as farm management, quality of sperm, production level and time of insemination, to name a few important ones. A study by DeJarnette et al. (2007) including data on 121 Holstein dairy herds, revealed an average conception rate of 44% following the use of sexed sperm. In 74% of these herds, the conception rates for sexed semen were at least 70% of those achieved with control semen.

To overcome the limitations imposed by the speed of analysis, alternative approaches were investigated, such as the use of sexed sperm for in vitro embryo production (Cran et al., 1993) and the use of alternative insemination techniques using lower numbers of sperm, such as deep intra-uterine insemination (see below). While the first strategy never became a financial success, because it also had to deal with additional drawbacks related to in vitro embryo production technology, the latter is still under investigation but slowly on the rise. As described by Gardner and Seidel (2008), several recent technical adaptations to the sperm sexing equipment have resulted in a considerable improvement in analyzing speed, which is now up to 20,000 sperm/second, sorting up to 6,000 sperm/second each of X- and Y-sperm at 90+% accuracy. In practical terms, this means that about 7 straws of sexed sperm with a sperm dose of 2×10^6 can be produced per hour.

Sexed sperm can be frozen using a conventional egg-yolk-Tris buffer (Schenk et al., 1999), so it can be collected and sorted at the same facility, which doesn't have to be the place where the actual inseminations take place. Sex sorted frozen sperm can be distributed, transported and subsequently offered to the farmers using the conventional distribution channels.

Given the fact that sperm is stained and subjected to laser light during the sexing process, a serious concern exists on the possible damage to sperm induced by this procedure. More specifically, sperm chromatin quality might be impaired by exposure to the Hoechst 33342 dye and the UV laser beam during flow sorting (Gardner and Seidel, 2008). However, the Sperm Chromatin Structure Assay (SCSA) revealed no obvious DNA damage with sex-sorted sperm (Garner et al., 2001), while genetic DNA damage can not yet completely be ruled out. Reassuringly, some large studies (over a 1,000 calves) are available on the characteristics of calves born following inseminations with sex-sorted sperm. These studies report no differences in gestation length, the occurrence of neonatal death, difficulty at calving, abortion rate (DeJarnette et al., 2007), birth and weaning weight or proportion of live births following AI with sexed semen compared to controls (Tubman et al., 2004).

In addition to cattle, sperm sexing technology has been applied to numerous other domesticated species including sheep (Hollingshead et al., 2004), rabbits (Johnson et al., 1989), horses (Buchanan et al., 2000), swine (Maxwell et al., 2004), cats (Pope et al., 2008) and dogs (Meyers et al., 2008).

Deep Intra Uterine Insemination

As mentioned above, the availability of sex sorted sperm in small quantities created the need for alternative insemination techniques. Whereas normal inseminations doses contain around 10 to 20×10^6 sperm, the concentration of straws containing sex sorted sperm usually is five to ten times less, around 2×10^6 sperm. In cattle, sperm is usually deposited into the uterine body when using conventional AI. This allows a strong 500-times reduction (Senger, 1993) of the number of spermatozoa needed compared to natural mating when the sperm is ejaculated into the vagina and the most caudal part of the cervix. However, in case of the use of sperm with limited commercial availability or sexed sperm, it has been proposed that deposition of sperm closer to the oviduct, at the utero-tubal junction (UTJ), could be an alternative to obtain acceptable pregnancy rates. Theoretically, the insemination dose as used for conventional insemination in cattle could be reduced by at least 100-fold if the sperm is deposited close to the UTJ (Hunter, 2001). This way, the loss of sperm through retrograde flow and phagocytosis can be minimized. In addition, sperm might survive for a longer time period in the sperm friendly environment of the isthmus (Suarez, 2001). In cattle, conventional insemination is performed under rectal guidance, with a stainless steel 'Cassou' insemination device. Already in the fifties, comparisons were made between the outcomes of insemination in the uterine body or deep intra-uterine insemination (IUI) in the uterine horns (Salisbury and VanDemark, 1951). In most of these studies, performed with high numbers of fresh semen, no differences between the two insemination techniques could be demonstrated. In most cases, a rigid insemination device was used for deposition of the sperm in the uterine horns. To enable deposition of semen near the UTJ, special insemination devices needed to be developed because a rigid device is not compatible with the curvatura of the uterine horns (Verberckmoes et al., 2004). Use of the 'Ghent' device for UTJ insemination with a flexible tip showed not to decrease pregnancy rates compared to conventional AI, even though a six fold

reduction in sperm dose was used (Verberckmoes et al., 2005).

Conclusions and future prospects

Although AI is the oldest technique in the spectrum of assisted reproductive techniques, it is still the most used one in livestock production as it is relatively simple, economical and easy to apply at the herd level to improve or introduce desired genetic traits. However, the choice between AI and natural mating will always depend on numerous factors related to production goals, market situation and farm management. While initially fresh semen was used, the development of freezing protocols and cryopreservation of sperm has introduced a huge trade activity with semen being transported intensively on international markets. While no substantial improvements have been made in the efficiency of cattle AI over the past decades, which is not only depending on sperm quality, several new developments in AI have been introduced. The ability to sex sperm and thus predetermine the sex of offspring before conception, has generated new interest in low dose and deep intra-uterine insemination protocols and equipment. The use of sexed semen has additional applications in embryo transfer programmes and in vitro embryo production. For some species, intracytoplasmatic sperm injection (ICSI) is already in use as the next step.

References

Bols PEJ. Puncture of immature ovarian follicles in bovine assisted reproduction. Verhandelingen. 2005;LXVII:3:177-202.

Bonadonna T, Succi G. World statistics for artificial insemination in cattle. Proc 9th Int Congr Anim Artif Insem. 1980; 5:655-7.

Brackett BG, Bousquet D, Boice ML et al. Normal development following in vitro fertilization in the cow. Biol Reprod. 1982;27:147-58.

Buchanan BR, Seidel GE, McCue PM et al. Insemination of mares with low numbers of either sexed or unsexed spermatozoa. Theriogenology. 2000;53:1333-44.

Chupin D, Thibier M. Survey of the present status of the use of artificial insemination in developed countries. World Anim Rev. 1995;82:58-68.

Cran DG, Johnson LA, Miller NGA et al. Production of bovine calves following separation of X- and Y-chromosome bearing sperm and in vitro fertilization. Vet Rec. 1993;132:40-51.

DeJarnette JM, Nebel RL, Meek B et al. Commercial application of sex-sorted semen in Holstein heifers. J Anim Sci. 2007;85:228.

Faber DC, Molina JA, Ohlrichs CL et al. Commercialization of animal biotechnology. Theriogenology. 2003;59:125-38.

Foote RH. Tracing 50 years of research. Foote RH, editor. Artificial insemination to cloning. Cornell University Resource Center. 1998;7.

Foote RH. The history of artificial insemination: selected notes and notable. Am Soc Anim Sci.2002;1-10.

Fukuda Y, Ichikawa M, Toyoda Y. Birth of normal calves resulting from bovine oocytes matured, fertilized and cultured with cumulus cells in vitro up to the blastocyst stage. Biol Reprod. 1990;42:114-19.

Garner DL, Schenk JL, Seidel GE. Chromatin stability in sex-sorted sperm. Andrology in the 21st century, proceedings of the VIIth international congress of andrology 2001:3-7.

Garner DL, Suh TK. Effect of Hoechst 33342 staining and laser illumination on viability of sex-sorted bovine sperm. Theriogenology. 2002;57:746.

Garner DL. Flow cytometric sexing of mammalian sperm. Theriogenology. 2006;65:943-57.

Garner DL, Seidel GE. History of commercializing sexed semen for cattle. Theriogenology. 2008;69:886-95.

Georges M. Towards marker assisted selection in livestock. In: Proceedings of the 15th Scientific Meeting of the AETE, Lyon, France 1999:97-103.

Hasler J. The current status and future of commercial embryo transfer in cattle. Anim Reprod Sci. 2003;79:245-64.

Hollingshead FK, Evans G, Evans KM et al. Birth of lambs of a pre-determined sex after in vitro production of embryos using frozen-thawed sex-sorted and refrozen-thawed ram spermatozoa. Reproduction. 2004;127:557-68.

Hunter RHF. New breeding opportunities with deep corneal insemination: exploiting modern sperm technologies in cattle. Reprod Dom Anim. 2001;36:217-22.

Ivanoff EI. On the use of artificial insemination for zootechnical purposes in Russia. J Agri Sci. 1922;12:244-56.

Johnson LA, Flook JP, Hawk HW. Sex preselection in rabbits: live births from X and Y sperm separated by DNA and cell sorting. Biol Reprod. 1989;41:199-203.

Johnson LA, Welch GR. Sex preselection: high-speed flow cytometric sorting of X and Y sperm for maximum efficiency. Theriogenology. 1999;52:1323-41.

Johnson LA. Sexing mammalian sperm for production of offspring: the state of the art. Anim Reprod Sci. 2000;60-61: 93-107.

Jondet R. Contribution to the assessment of the minimal number of frozen spermatozoa necessary to obtain fertilization in the cow. Proceedings of the 7th international congress on animal reproduction and artificial insemination 1972;2:1443-8.

Maxwell WMC, Evans G, Hollinshead FK et al. Integration of sperm sexing technology into the ART toolbox. Anim Reprod Sci. 2004; 82:79-85.

Meyers MA, Burns G, Arn D et al. Birth of canine offspring following insemination of a bitch with flow-sorted spermatozoa. Reprod Fertil Dev. 2008;20:213.

Moruzzi JF. Selecting a mammalian species for the determination of X- and Y-chromosome-bearing sperm. J Reprod Fertil. 1979;57:319-23.

Müller E, Wittkowski G. Visualization of male and female characteristics of bovine fetuses by real-time ultrasonics. Theriogenology. 1986;25:571-4.

Pope CE, Crichton EB, Gómez MC et al. Birth of domestic cat kittens of predetermined sex after transfer of embryos produced by in vitro fertilization of oocytes with flow sorted spermatozoa. Reprod Fertil Dev. 2008; 20:213-4.

Salisbury GW, VanDemark NL. The effect of cervical, uterine and cornual insemination on fertility of the dairy cow. J Dairy Sci. 1951;34:68-74.

Schenk JL, Suh TK, Cran DG et al. Cryopreservation of flow-sorted bovine sperm. Theriogenology. 1999;52:1375-91.

Seidel GE. Sexing mammalian spermatozoa and embryos – state of the art. J Reprod Fertil. 1999;54(suppl):475-85.

Seidel GE. Sexing mammalian sperm – intertwining of commerce, technology, and biology. Anim Reprod Sci. 2003;79: 145-56.

Senger PL. Site of semen deposition and its effect on fertility and sperm retention: a review. Reprod Fertil Dev. 1993;5: 659-63.

Shea BF. Determining the sex of bovine embryos using polymerase chain reaction results: a six-year retrospective study. Theriogenology. 1999;51:841-854.

Singleton WL. State of the art in artificial insemination of pigs in the United States. Theriogenology. 2001;56:1305-10.
Suarez SS. Carbohydrate-mediated formation of the oviductal sperm reservoir in mammals. Cells Tissues Organs. 2001; 168:105-12.
Thibier M, Guerin B. Hygienic aspects of storage and use of semen for artificial insemination. Anim Reprod Sci. 2000; 62:233-51.
Thibier M, Wagner HG. World statistics for artificial insemination in cattle. Livestock Prod Sci. 2002;74:203-12.
Thibier M. IETS Newsletter (International Embryo Transfer Society) September 2009.
Tubman LM, Brink Z, Suh TK, Seidel GE. Characteristics of calves produced with sperm sexed by flow cytometry. J Anim Sci. 2004;53:1029-36.
Verberckmoes S, De Pauw I, Van Soom A et al. New CEP-diluter improves storage capacity of fresh bovine semen. XXII World Buiatrics Congress, Hannover, Germany 2002:87-8.
Verberckmoes S, Van Soom A, De Pauw I et al. Assessment of a new utero-tubal junction insemination device in dairy cattle. Theriogenology. 2004a;61:103-115.
Verberckmoes S, Van Soom A, Dekruif A. Intra-uterine insemination in farm animals and humans. Reprod Dom Anim. 2004b;39:195-204.
Verberckmoes S, Van Soom A, Dewulf J et al. Low dose insemination in cattle with the Ghent device. Theriogenology. 2005;64:1716-28.
Vishwanath R. Artificial insemination: state of the art. Theriogenology. 2003;59:571-84.
Vyt P, Maes D, Dejonckheere E et al. Comparative study on five different commercial extenders for boar semen. Reprod Dom Anim. 2004;39:8-12.
Wilmut I, Schnieke AE, McWhir J et al. Viable offspring derived from fetal and adult mammalian cells. Nature. 1997;385:810-13.