Review Paper

Current Status of Sexing Bovine Spermatozoa In Indonesia

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Abstract

The government of Indonesia has launched a Program Self-Sufficiency for Beef and Buffalo in Year 2014 (PSDS/K-2014). The objectives of this program include increasing population, improving productivity and increasing beef production, and increasing farmer income and welfare. To achieve the increasing population, the availability of elite cows is required. Currently, the main problem of development cattle in Indonesia is limited number of elite cows in terms of both quantitative and qualitative. One thing that needs to be done to achieve the program is an increase in the genetic quality and capacity through improved livestock production and productivity of livestock including use sexing sperm for artificial insemination (AI).

The main application of sexed sperm to date has been to breed dairy heifers to produce female calves. To have calves with the sex according to the expectation, for example, female calve, we can perform the separation of sperm carrying the X chromosome so that the fertilized egg finally will become a female calve. Several methods have been performed to obtain optimal results and decent AI. Research Center for Biotechnology LIPI since 1999 until now, generate 70-90% of sperm Y. The results obtained by concentration of 5% and 10% is the ratio of the concentration of sperm that optimum separation column can be separated by 70-88% X sperm and Y sperm by 70-90%. Sperm motility before and after separation (sexing) is not too early for motility decreased from 76-80% to 67-80%. Difference increasing concentrations of BSA is expected to separate the spermatozoa that have a high motility (spermatozoa Y) will be able to penetrate the concentration of the more dense medium separation, whereas the X sperm will remain in the media that has a lower concentration. Generally indicates that, the separation of sperm quality after freezing is still fit for use for AI is characterized by sperm motility is still around 40-50%. After calves birth, offspring from sexed spermatozoa appear to have no more abnormalities than control spermatozoa. No significant effect on male calf and female calf at birth and weaning. Therefore, there appear to be no detrimental effect of sorting spermatozoa on resulting spermatozoa. Successful use of sexed sperm requires excellent management of cattle, careful handling of sperm and use of skilled inseminators.

Key words: Sperm; Sexing; Bovine; BSA column; AI (artificial insemination)

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Introduction

Hundreds of offspring calves have been born as result of artificial insemination (AI) with used sexed sperm. It is now possible to predetermine the sex of offspring from a number of species before fertilization with an accuracy of 85-95% (Seidel *et al*., 1999; Welch & Johnson, 1999; Johnson, 2000; Seidel & Garner, 2002). Several methods to sexing sperm have been attempted, but convincing results were not produced period to 1980. The major breakthrough was development of flow cytometry/cell sorting in the early 1980s (Garner & Seidel, 2003); the initial methods separated X- and Y-sperm effectively, but killed the sperm, making the procedure impractical. A major refinement to this procedure was making the system work without killing or damaging the sperm (Johnson *et al*., 1989). The currently successful method of

sexing sperm has been reviewed by Seidel & Garner (2002). The process of commercialization of sexed has accelerated recently. However, this technology is characterized by high cost, complexity of implementation and lower pregnancy rates than with control sperm. Nevertheless, sexed frozen bovine sperm are being produced commercially in many countries, although from a limited number of bulls. The main application of sexed sperm to date has been to breed dairy heifers to produce female calves.

The objectives of this review are to explain: (1) Morphologic, physiologic and biophysical differences between X-and Y-Chromosome-Bearing Spermatozoa; (2) Techniques employed for separation of X- and Y-spermatozoa based on spermatozoa are damaged during sexing procedures, effects on fertility, and (3) Application of sexed sperm for cattle in Indonesia including evaluation of the results and normality of calves resulting from sexed spermatozoa.

X-and Y-Chromosome-Bearing Spermatozoa

Sex is determined in mammal by whether the fertilizing spermatozoon contains an X-chromosome to produce a female or a Y-chromosome to produce male. Female animals have two similar sex chromosomes (X and X), whereas males have two different sex chromosomes (one X chromosome, and one smaller Y chromosome). The gametes (egg and sperm) are haploid cell containing either the X or the Y chromosome. Diploid somatic cells of female (homogametic cell) contain a pair of X chromosomes, but somatic cells of males (heterogametic sex) have XY sex chromosomes. The genetic sex is determined in the oviduct at the time of fertilization, and the sex of the offspring is determined by the sex chromosome within the spermatozoa (Hafez, 1987).

Extensive investigation have been carried out for pre-selection and complete separation of X- and Y-sperm before artificial insemination (Hafez, 1987; Seidel, 1999; Johnson, 2000; Tappa *et al*. 2000, Kaiin *et al*., 2004, Said *et al*., 2005). Sex of the offspring can also be predetermined in embryos arising from diploid or haploid nuclear transplantation into recipient ova, parthenogenetic activation of ova, or fusion of two oocytes. Different cytogenetic and cytologic techniques are used to examine the diploid cells at an appropriate stage of fertilization in order to diagnose the genetic sex of the embryo. For example, fluorescence microscopy is used to detect the presence of a Y chromosome. Chromosome analysis is performed by culturing leukocytes or fetal cells to study the individual chromosomes using karyotyping procedures.

Gametes Chromosomes

Chromosomes in every mammalian cell occur in pairs *(diploid)* except in gametes in which only one member of each pair is present *(haploid*). Each member of a pair is similar in size, shape and proportion to its partner *(homologous chromosomes*). The diploid number (2n) of chromosomes is constant in normal individuals within a species. One pear chromosomes in each cell is known as the sex chromosomes (XX in the female and XY in the male) whereas the non sex pairs are known as *autosomes*. The gametes are haploid and contain half the number of chromosomes (n) with the X chromosome in the female *(homogametic sex)* and either an X or Y chromosome in the male *(heterogametic sex)* (Hafez, 1987).

Cytogenetics of X-and Y-Spermatozoa

There are many potential differences between spermatozoa containing an X or a Y chromosome. Sex chromosomes are responsible for any differences in deoxyribonucleic acid (DNA) content. The presence of an X or a Y chromosome could cause a difference in size and shape of sperm, in weight, density, motility (type and velocity), surface charge, and in surface biochemistry or internal biochemistry (Foote, 1982). The degree of difference may also be affected by other factors such as age of semen, repeat breeding (possible differential embryo mortality) and use of bull that had been born co-

twin with heifers (and have circulating XX leukocytes).

There are species variations in the mass differential of the X and Y chromosome. The presence of a large X chromosome could result in greater weight and density of the X-containing sperm if size and other constituents are the same. It is possible that the shape of sperm head differs due to a haploid gene expression. It is not known if sperm with an X chromosome consistently differ in size or shape from those containing a Y chromosome (Foote, 1982).

Table 1. Some Differences Between X and Y Sperm.

Parameter	Difference	Evaluation
DNA	Less in Y sperm	Measurable and accepted
Size	X sperm is larger	Y sperm measured
Identify	Y chromosome fluoresces	Species specific
Motility	Y sperm faster	
Surface charge	X sperm migrate to cathode	
Cell surface	H-Y antigen; ion exchange columns	
Enzyme	LDH isozyme	

Ericsson & Glass, 1982.

How Sperm are Sexed

How sperm are sexed has been overviewed by Seidel, 2007. The basic principles are simple; the X-sperm contains more DNA than the Y-sperm (approximately 4 % more in the case of cattle). Although this difference is small, by attention to details, it is possible to measure DNA content of individual sperm with sufficient accuracy to distinguish between X- and Y-sperm with about 90% accuracy for 50% of the sperm. Therefore, about half of the sperm are discarded as unsexable, and there is a 10% error rate for those sexed with routine procedures (Seidel & Garner, 2002).

The DNA content of sperm is determined using a fluorescing dye, Hoechst 33342 that readily penetrates the sperm cell membrane and binds to the DNA. Thus, X-sperm ends up with about 4% more dye bound to their DNA than Y-sperm. This dye only fluoresces when exposed to a particular wavelength of light, and this is usually provided by an expensive laser. The fluorescence is measured by a detector and analyzed by computer. Since X-sperm have 4% more DNA, and therefore, bind more dye than Y-sperm, they give off 4% more fluorescence, which the computer can recognize. Note that we can also observe the fluorescence with a microscope, but our eyes and brains are not designed to discriminate a 4% difference in the amount of brightness of fluorescence, so X- and Y-sperm appear equal to us, even though they appear different to the instruments used for sexing (Seidel, 2007)

The principles just discussed are combined to make a system to sex sperm. The basic instrument used is a flow cytometer/cell sorter. It consists of a pump to move the fluid containing sperm past a detector of fluorescence. A laser provides the correct wavelength of light to cause fluorescence without damaging the DNA. A powerful computer also is needed to analyze the fluorescence.

The cell sorting part of the system works as follows: when the stream of fluid exist the flow cytometer, it is broken into little droplets by a vibrator, forming about 70,000 to 80,000 droplets per second. About one-third of the droplets contain a sperm and about two-third of the droplets contain a sperm and about two-third are empty; a few droplets contain two or more sperm. If a droplet contains a Y-sperm, a negative charge is added; and if the droplet contains no sperm, multiple sperm, damaged sperm, or sperm that are indistinguishable relative to DNA content, no charge is placed on the droplet. As droplets fall when they exit the nozzle of the flow cytometer (at a speed of about 80 km/h), they pass trough electric fields that are positive on one side and negative on the other. Since opposite electrical charges attract each other, the droplets with a positive charge (containing X-sperm) move toward the negative

part of the field, those with a negative charge more toward the positive field, and those with no charge continue straight down. Thus, three streams of droplet are produced that can be collected into three test tubes, thereby separating the X-from the Y-sperm. In practice, about 20% of sperm end up in the X-fraction, 20% in the Y-fraction and 60% are damaged or not sexable for one reason or another (Seidel, 2007).

Techniques of Sperm Separation

Techniques used to separate sperm are discussed in follows. Experimental attempts have been hampered by the lack of laboratory test to evaluate the degree of sperm separation. It appears that the presence of the F-body is associated with the Y chromosome. It is thought that the ratio F-body-positive and F-body negative spermatozoa can be altered by patented techniques (Foote, 1982). Most of the techniques employed for sperm separation are based on non equilibrium sedimentation (based upon velocity of fall) or upon equilibrium sedimentation on density gradient. These techniques use simple gravity or centrifugation (Hafez, 1987).

Only two laboratory methods for separation of animal and human X and Y sperm appear valid, reproducible and clinically applicable: albumin separation, which yields 75% to 80% Y sperm, Sephadex filtration, which yields 70 to 75% X-sperm (Beernink, 1986).

Gradient Separation Over Albumin Columns and Sephadex Filtration

Successful separation of X- and Y-chromosome-bearing human spermatozoa using an albumin gradient was first reported by Ericsson *et al*. (1973). The conceptual basis for this method is that Y-chromosome-bearing spermatozoa are smaller in size and exhibit a greater downward swimming velocity than X-chromosome-bearing spermatozoa within vertical columns of high density human serum albumin (Ericsson *et al*., 1973). A fraction enriched with Y-chromosome-bearing spermatozoa can be obtained by harvesting the first 22% of spermatozoa to swim to the bottom of the gradient, and discarding the remainder (Ericsson & Ericsson, 1999). Ericsson & Ericsson (1999) reported that the latest version of this technique increased the percentage of male children born 70-80%. However, the validity of sex pre-selection by this approach has been challenged repeatedly. This technique has never been shown to sex spermatozoa accurately from mammals other than humans (Beal *et al*., 1984; White *et al*., 1984). Another interesting aspect of the use this method is that when women are treated with clomiphene citrate to induce ovulation before insemination, the sex ratio is reversed, so that up to 73% females are born (Ericsson & Ericsson, 1999). Recently, Tappa *et al*. (2000) have attempted to sex cattle spermatozoa by gradient using column density Bovine Serum Albumin (BSA). This is column divided several fractions. Tappa *et al*. (2000) found that they could obtain fractions of up to 80% Y-chromosome bearing spermatozoa with reasonable viability using this procedure (Figure 1).

When semen is layered over columns of serum albumin, increased numbers of Y sperm are recovered from the albumin layers. Semen aliquots of 0.5 ml (diluted 1:1 with Tyrode's solution) are layered for 1 hour over a 7.5% solution of serum albumin in a glass column (8 × 75 mm), the initial sperm layer is then removed by pipette, the albumin centrifuged at 2,800 to 3,200 rpm for 10 minutes, and the sperm disc resuspended in Tyrode's solution. The resuspended sperm is then layered over a two-layer serum albumin column (12.5% at the top and 20% at the bottom). At 1 hour, the sperm layer is removed, and the after another half hour, the 12.5% layer is removed. The 20% serum albumin is then centrifuged for 10 minutes and the sperm disc resuspended in 0.25 ml of Tyrode's solution, which is then inseminated into the uterus (Beernink & Ericsson, 1982).

Sperm Sorting Based on Volumetric Differences

Spermatozoa containing an X chromosome are theoretically larger than those containing a Y chromosome. van Munster *et al*. (1999) recently

used interference microscopy and subsequent image analysis to demonstrate a difference in sperm head volume that matched differences in DNA content between X- and Y-chromosome-bearing bovine spermatozoa. A method based on this principle has been developed for sorting live spermatozoa by using interference microscopy optics with a flow cytometer (van Munster, 2002). Such a method, eliminates the need to use DNA-specific dyes, would be a highly attractive alternative method for sexing mammalian spermatozoa. Unfortunately, the potential purity of spermatozoa separated using volumetric measurements cannot exceed 80% purity of either sex based on theoretical consideration (van Munster *et al*., 1999) and recent effort to make this practical have not been encouraging (van Munster, 2002).

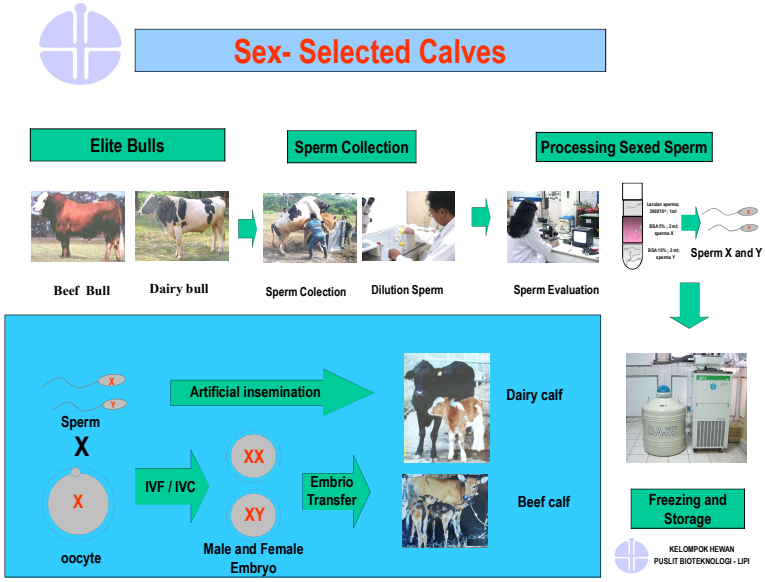

Figure 1. Processing Sexed Sperm by Gradient Swim-Down Procedure (Ericsson *et al*., 1973; Tappa *et al*., 2000)

Flow Cytometry and Cell Sorting

The major breakthrough was development of flow cytometry/cell sorting in the early 1980s (Garner & Seidel, 2003); the initial methods separated X- and Y-sperm effectively, but killed the sperm, making the procedure impractical. A major refinement to this procedure was making the system work without killing or severely damaging the sperm (Johnson *et al*., 1989).

With flow cytometers, high-speed measurements of components and properties of individual cells are made in liquid suspension. Several flow cytometers use a laser for monochromatic illumination of cells stained with fluorescent dyes. Light detectors sample the fluorescence produced by the interaction of light with dye and produce electrical signals proportionate to the intensity of fluorescent light from each object. It would appear that the use of flow cytometry gives reliable evaluation data for sperm for several species when analyses are conducted under carefully defined condition (Gledhill *et al*., 1982). This technique is useful in the evaluation of the degree of separation needed to produce population enriched in X or Y sperm (Foote, 1982).

The currently successful method of sexing sperm has been reviewed by Seidel & Garner (2002). The instruments currently used for sexing sperm are remarkable feats of engineering, capable of evaluating thousands of sperm per second. However, this is still slow relative to needs for routine AI. Therefore, for sexed semen to be practical, fewer sperm are used per insemination dose than are used normally, which means that applications for sexed sperm currently only fit systems of excellent management that result in high fertility.

Sperm Sorting Technology in Cattle Industry

Sex pre-selection is based on identifying differences between X & Y bearing sperm. The X chromosome contains about 4% more DNA in cattle and horses than Y chromosome. Therefore, this difference in DNA content can be used to distinguish and select X from Y bearing sperm. Flow cytometric sorting of mammalian spermatozoa based on DNA content is different than the process for other cell types. DNA contained within spermatozoa of mammalian animals is uniquely compact, resulting in a flat, paddle-shaped head in most species. When the DNA of a sperm is stained with a harmless fluorescent dye and subjected to flow cytometry, a brighter fluorescent is emitted from the edge of the spermatozoal head compared to the more transparent flat side. Therefore, proper sperm head orientation during cytometric evaluation is critical so differences in DNA content can be evaluated correctly. The X chromosome is glow brighter under illuminated laser beams than the Y chromosome.

Flow cytometric chromosome classification (flow karyotyping) has been routinely used in the analysis of mammalian karyotypes as well as to assess chromosomal abnormalities (Ashcroft & Lopez, 2000). Flow karyotyping and purification of chromosomes isolated from mammalian cell lines can be achieved using the MoFlo high performance cell sorter. The sorting set up of the MoFlo can be customized for rapid isolation of chromosome with high purity. With the MoFlo, chromosomes can be flow sorted at a data rate of approximately 8,000-12,000 chromosomes per second while still maintaining a good resolution of chromosome peaks. Following purification, DNA as well as proteins can be extracted from the flow sorted chromosome for genomic (Gribble *et al*., 2004) and proteomic (Wray & Wray, 1980) studies.

Formed in mid-1996, XY Inc.'s one of the company produced sexed sperm initial mission was to offer gender selection to the United States dairy industry by way of sorting sperm with a flow cytometer into "X" and "Y" bearing population, as an enhancement to artificial insemination (Anonimous, 2006). Today, XY Inc. has expanded its mission and now is the global leader in the research, development and commercialization of XY sex-selection technology for all relevant non-human mammals, including cows, horses, pigs, endangered species and more. XY Inc. is the master licensee in control of all sperm sorting in non-human mammals worldwide using technologies developed by U.S, Department of Agriculture, Colorado State University and DakoCytomation, a company that develops advanced flow cytometeres (Anonimous, 2006).

XY Inc.'s successes are many. For example, XY Inc. scientists produced "Call Me Madam", the world's sex-selected foal in 1998, the world's first sex-selected calf using frozen sexed sperm and artificial insemination in 1999; the world's first sex-selected lambs using frozen sexed sperm and artificial insemination in 2001; and the world's first foal, First Lady, produced from Call me Madam and the first sex-selected colt in 2003. XY Inc. began in 1996 as joint venture between Cytomation Inc., and Colorado State University Research Foundation. Cytomation provided the necessary access to MoFlo and cell-sorting technology, while Colorado State University provided advanced animal-biology techniques and studies. The successful partnership continues today. In 1997, XY Inc. acquired Master Calf, a British firm seeking to develop similar semen-sexing technologies. Currently, XY Inc. has commercial licenses in the UK,USA, Mexico, Argentina, Canada, Brazil and China (Anonimous, 2006).

Application of Bovine Sexed Sperm in Indonesian

The Indonesian Government has launched a Program Self Beef and Buffalo Year 2014 (PSDS/K-2014). The ultimate objective of the program includes increasing population, improving productivity and production of beef, and increasing farmer income and welfare. To achieve the required population, increasing the availability of elite cows is needed.

Currently, the main problem of development cattle in Indonesia is limited number of elite cows in terms of both quantitative and qualitative. Impact of land conversion in the

reduction of land for livestock forage so that the low reproductive performance of cattle to be characterized by high rates of Service per Conception (S/C > 2), Conception Rate (CR) of less than 70%, calving interval (CI) over 16 months, and estrus post parturition is still over 90 days. In addition, the mortality rate remains high, live weight and carcass weight is much lower than in cattle in developed countries. Live and carcass weight of cattle import (feeder) respectively 350 kg and 260 kg / head, higher compared with the local cattle with 250 kg live weight and carcass weight of 175.0 kg. One thing that needs to be done to achieve the desire of the above is an increasing in the genetic quality and capacity through improved livestock production and productivity of livestock including use sexing sperm for AI.

To have calves with the sex according to the expectation, for example, female calve, we can perform the separation of sperm carrying the X chromosome so that the fertilized egg finally will become a female calve. Separation of sperm carrying the male and female sexes has long done already (Johnson, 2000, Tappa *et al*., 2000, Seidel & Garner, 2002, Seidel, 2007). Separation of sperm with serum albumin column is based on: 1). The difference in weight, density or size of chromosome X and Y as the difference in the size of sperm components; 2). Differences in the expression of haploid chromosomes X and Y as a result of natural differences sperm components (Maxwell *et al*., 1984).

Several methods have been performed to obtain optimal results and decent AI. Before using BSA column, egg white albumin has been carried out using sperm separation column (Saili, 1999; Saili *et al*., 2000; Tappa *et al*., 2000). But, because of sperm quality post-separation is decreased and the quality of the egg white albumin is unstable, so do efforts to make the separation of sperm using bovine serum albumin (BSA) column. Successful use of BSA as a separation media have gradually been researched and applied in the Research Center for Biotechnology LIPI since 1999 until now, and it generate 70-90% of Y sperm (Tappa *et al*., 2000, Kaiin *et al*., 2004). The results showed that BSA concentration at 5% and 10% is the optimum ratio where the column separation resulted in 70-88% X sperm and Y sperm by 70-90%. Sperm motility before and after separation (sexing) showed a small decrease, from 76-80% to 67-80% (Kaiin *et al*., 2004, Said *et al*., 2005). By increasing concentrations of BSA, we expected to be able to separate the spermatozoa with high motility (Y spermatozoa). Higher motility spermatozoa would be more able to penetrate denser medium separation, whereas the X sperm will remain in the media that has a lower concentration.

According to Maxwell *et al*. (1984), the motility of spermatozoa are influenced by several factors such as: concentration of BSA, time and duration of spermatozoa through the solution of BSA and the concentration of spermatozoa to be separated in a liquid buffer. Previous study in the straw storage of liquid semen sexing in a solution of skim milk egg yolk buffer (SKT) at a temperature of 5°C showed that sexing sperm motility can be saved by maintaining a decent AI (45%) to 10 days of storage, whereas storage in Tris buffer medium yellow eggs (TKT) at the same storage temperature is not as good on the SKT, it only retained for a maximum of 5 days (Kaiin *et al*., 2004).

Testing of sperm sexing *in vitro* have been carried out by means of fertilization of the egg/oocyte. The results showed that the oocytes are fertilized by X sperm produced embryos 2 cell stages up to morula stage (27.3%-45.2%) and Y sperm fertilized oocytes that produce embryos 2 cell stage until the morula stage (27.6%-51.2%) compared with oocytes fertilized with sperm that are not separated by 51.7%. This is presumably related to the quality of the separation of sperm that have individual quality of sperm motility and that tends to be lower than the sperm that are not separated. Morula and blastocyst stage embryos produced are all stored in a frozen state. A total of six frozen embryos were transferred to six recipient cows owned by farmers in Konawe, Southeast Sulawesi. The results of embryo transfer using embryos fertilized *in vitro* with sexed sperm to the recipient female Bali cattle obtained results of the two pregnant recipients, one androgynous male calf was born corresponding to the sex of the sperm is used. Results embryo transfer male

calves born to cows Bali proved that the type of sperm derived from peranakan ongole (PO) and cow eggs derived from cow Brahman Cross (BX) (Said *et al*., 2005; Kaiin *et al*., 2008).

Testing of sperm sexing *in vivo* performed with artificial insemination (AI) sperm sexing to the acceptor dairy cows in the area of West Java, Simmental cattle in West Sumatra, Bali cattle in Southeast and South Kalimantan. Application AI with the use of sperm sexing in dairy cattle in KUD West Java acquired some degree of success that is born 17 of 21 births (CR = 80.09%) with sex as expected, or at 81% with the S/C 1.37 (Figure 2).

Figure 2. The calves were born by artificial insemination (AI) using sexed sperm in beef and dairy cows at farmer in West Java.

Application of sexed sperm in the province of West Sumatra conducted using male sperm Simmental cattle reared in the BIB Tuah Sakato. X and Y sperm motility after the separation process using a column of about 70-75% BSA. Under conditions of supply of liquid nitrogen as well as difficulties in the procurement of liquid nitrogen, the production of fresh straw sexing semen is a good alternative. The results with fresh sexed sperm showed that the percentage of calves born in the AI sexing as much as of 62.5%, abortion 12.5% and others 25%. Correspondence between the sexes was born by 83% (Tappa, 2006). While the AI results from sexed sperm in Bali cattle in Konawe, Southeast Sulawesi, produced sex-matched calves born at 90%. Generally, this study indicates that the separation of sperm quality after freezing is still fit for the AI and the sperm motility is still around 40-50%. After calves birth, offspring from sexed spermatozoa appear to have no more abnormalities than control spermatozoa. No significant effect on male calf and female calf at birth and weaning. Therefore, there appear to be no detrimental effect of sorting spermatozoa on resulting spermatozoa. The main constraints obtained in the field is the high level of AI acceptor stem mutation animals with sperm sexing, so many pregnant cows of his calve's birth is not tracked the data.

Conclusion

The main application of sexed sperm to date has been to breed dairy heifers to produce female calves. Successful use of sexed sperm requires excellent management of cattle, careful handling of sperm and use of skilled inseminators.

AI success using sperm sexing needs to be supported by many factors such as quality of bulls that are used, the production of sperm sexing procedure, sperm sexing straw quality, condition of AI acceptors, inseminator's skill, pregnant cattle raising, and many other factors. Therefore, the application needs to be done with sperm sexing AI widely, so it can be produced by the sex of the calf up to the expectations that are directed at the development of beef and dairy cattle in Indonesia. In addition, the application of AI with sperm sexing in the local cattle native to Indonesia is expected to increase the population.

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