Research Article

Minimum Number of Sex-Sorted Frozen Sperm per dose in Sahiwal (*Bos indicus*) Cattle

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Abstract | The aim of this study was to determine the minimum number of sex-sorted frozen sperm required for reasonable pregnancies in Sahiwal cow and heifers. Ejaculates from six Sahiwal bulls were processed according to Beltsville sperm sorting technology using a high speed cell sorter and sorted sperm were packaged in 0.25 ml straws with 1.5 million sperm per straw. Non-synchronized Sahiwal heifers (n= 82) and cow (n= 67) were inseminated with unsexed frozen semen (20 million) and sex-sorted frozen semen with 1.5, 3.0 and 4.5 million sperm per dose. Significantly ($P < 0.05$) lower pregnancy rate was recorded at 1.5 million (25.3%) and 3.0 million (32.5%) sperm compared to unsexed semen (45.4%) and sexed semen of 4.5 million (60%) sperm. No significant difference was found between sexed semen of 4.5 million sperm and unsexed semen. Pregnancy rates of 1.5 and 3 million sexed sperm were 56% and 72% of unsexed semen, respectively. Significantly ($P < 0.05$) lower pregnancy rates were observed in sorted X- semen (31.32%) and Y- semen (27.30%) compared to unsexed semen (45.40%). Pregnancy rates were significantly ($P < 0.05$) affected by the sire. The present work indicates that sexed semen as low as 3 million represents an optimal insemination dose and can be used to achieve reasonable pregnancy rates in Sahiwal cattle.

Keywords | Pregnancy rate, Sexed semen dose, Sahiwal, Sperm sexing, Artificial insemination

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INTRODUCTION

Separation of X- and Y- chromosome bearing sperm by flow cytometric sorting is the most successful assisted reproductive technique due to its added benefits of getting predefined male and/or female offspring. There is no alternative method currently available which can successfully replace the flow cytometric sperm sorting procedure (Seidel, 2012). Since the first report of birth of live offspring, this technique along with artificial insemination of fresh or frozen sperm got wide spread application in a number of species (Maxwell et al., 2004). However, its main drawbacks is the slow rate of recovery of sorted sperm and low pregnancy rates which is around 80% of control pregnancy rates (Seidel, 2012). It is reported that a high speed cell sorter can produce a maximum sort rate of 8000 sperms of each sex at 90% purity under ideal conditions (Sharpe and Evans, 2009). High speed cell sorter which is being used in India is capable of producing about 10 to 12 million sperms/h of each sex in Sahiwal cattle (Biswas et al., 2013). With this rate, it will take several hours to sort a bovine insemination dose containing 20 million sorted sperm. Thus, the alternative way of commercialization of sorted semen is the utilization of low dose insemination in field level programme (De-Jarnette et al., 2011). However, reducing sperm concentrations per dose have negative impact on fertility (De-Jarnette et al., 2011; Rath et al., 2013). It has been reported that fertility rate could be increased by increasing the sperm concentration, until the maximal point beyond which the fertility cannot be improved (Salisbury and Vandemark, 1961; Den-Daas et al., 1998; Saacke et al., 2000). Low dose artificial insemination of sex sorted frozen semen to get reasonable pregnancy rates in Indian breed like Sahiwal cattle has not previously been optimized. Hence, the present work was done to determine the minimum concentration of sexed sperm required for reasonable pregnancies by comparing the pregnancy rates after inseminating with sex-sorted frozen semen containing 1.5, 3.0 and 4.5 million sperm and 20 million unsorted sperm per dose in Sahiwal (*Bos indicus*) cow and heifers.

MATERIALS AND METHODS

Semen Evaluation and Staining

This study was carried out at Frozen Semen Bull Station and Bull Mother Farm, Paschim Banga Go-Sampad Bikash Sanstha Haringhata Farm, West Bengal, India during the period from April 2012 to December 2014. Out of 70 bulls in collection six Sahiwal bulls which had good initial motility of spermatozoa were selected for sperm sorting. Frozen semen laboratory where frozen semen straws produced regularly by conventional method, situated very adjacent to the semen collection yard. Neat ejaculates of six Sahiwal bulls were collected early morning following the standard method (GoI MSP, 2012). Neat semen having ≥ 500 x 10^6 sperm per ml, ≥ 70% initial motility and ≥ 80% viability were used for flow cytometric sperm sorting. Immediately after collection, half of the total volume of semen taken for sperm sorting and another half was used for frozen semen straw production by conventional method. The semen samples were kept at semen processing laboratory (20±2°C) for many hours between semen collection and completion of sorting process. Sperm preparation and staining was performed as per the method described previously (Schenk et al., 1999).

Flow Cytometric Sperm Sorting

Flow cytometric sperm sorting was carried out by a high speed influx (Becton Dickinson, BD Biosciences, San Jose, CA, USA) cell sorter at Frozen Semen Laboratory adjacent to semen collection yard following the method described earlier (Johnson et al., 1999). Briefly, just before actual sperm sorting, the stained sperm were finally diluted by modified TALP buffer to make 100 x 10^6 sperm/ml solution. The stained sperm were then kept at the sample station of cell sorter at 20°C until sorting of particular semen samples were completed. A tris buffer based sheath fluid (pH- 6.9) with an internal diameter of 70μ nozzle and 40 psi pressure was used during entire sorting process. Sperm stained with Hoechst 33342 excited by ultra violet laser of 355 nm wavelength (100 mW± 10 mW) and fluorescence intensity of sperm were measured by 460/50 nm and SSC detector channels. As X chromosome bearing sperm contain 4% more DNA and bind more Hoechst 33342 than Y chromosome bearing sperm and thereby glow brighter when exposed to ultra violet laser. The photo multiplier tubes set at right angles quantify the fluorescence, emitted by the sperm DNA and transfer that information to digital converter for processing. The bivariate dotplot of the cell sorter were analyzed by FlowJo (version 7.2.4). As sperm flow through flow cell of the sorter, the vibrating crystal piezo breaks the stream into small droplets at frequencies of 74 to 78 KHz and then pass through an electric field, where negatively charged droplets (containing X-sperm) were deflected towards the positively charged field and positive charged droplets (containing Y- sperm) were deflected towards the negatively charged field and ultimately both the -ve and +ve charged droplets were collected in two 15 ml sterilized tubes, previously filled with 2 ml of TRIS- base 30% clarified egg yolk (prepared as non-glycerol fraction). The sorted sperm fraction was mixed thoroughly with the clarified egg yolk to maintain the viability and motility of spermatozoa during the entire sorting process. As the sperm head is oval and flat, they flow through the laser beam in different orientations and about 30 % to 40% sperm cells were correctly oriented excluding other non-orientation and dead sperm by using this nozzle. Spigot (version 6.1.4, BD Biosciences) and FlowJo (version 7.2.4) software were used for sorting and histogram analysis of targeted sperm. Before actual sperm sorting the cell sorter was optimized for maximum efficiency. During sorting of sperm total event rate was 18000 to 20000/sec and nearly 3500 to 4000 live sperm/sec of each sex were sorted. With this rate the cell sorter was produced approximately 10 to 12 x 10^6 live sperms/h of each sex.

Cryopreservation of Sorted and Unsorted Sperm

After one hour of sorting of a particular semen samples, contents of two tubes were transferred to swing bucket centrifuge at 4°C and centrifuged (5810 R, Eppendorf AG, Hamburg, Germany) at 850 x g for 20 min. The supernatant was discarded from each tube and approximately 400 μL sperm pellet was diluted with 100 μL of TRIS-based 30% clarified egg yolk (without glycerol). Different batches were prepared by the same procedure and sorted sperm of different batches of a particular bull were diluted immediately with an equal volume of TRIS-based 20% egg yolk with 12.8% glycerol. The concentration of the sperm was measured by counting two haemocytometer chambers. The final concentration of each batch of each tube, containing TRIS-based 20% egg yolk and 6.4% glycerol, was adjusted to 6 x 10^6 sperm/mL. Extended sperm were packed into 0.25 mL "Top Bull" polyvinylchloride straws (IMV, France), with a concentration of 1.5 x 10^6 sperm in each straw by integrated system of filling, sealing and printing (IS4, IMV, France). Unsorted frozen semen containing 20 million sperm was packed in 0.25 ml straw after adding required quantity of dilutor measured by Accucell photometer (IMV, France) containing 6.4% glycerol. After keeping at 4°C in cold handling cabinet (IMV, France) for 4 to 6 hour of equilibration, both sorted and unsorted straw were cryopreserved by a programmable bio-freezer (IMV, France) as per standard procedure (GoI MSP, 2012). The sorted and unsorted frozen semen straws were preserved in liquid nitrogen container until used for artificial insemination.

Artificial Insemination

Non-synchronized Sahiwal heifers and cows of Bull Mother Farm at Haringhata Farm were selected for artificial insemination with unsexed semen (n= 269) and sex sorted X- semen (n= 83) and sorted Y- semen (n= 66). Frozen semen straws of unsexed and sorted semen of different batches were thawed at 37°C for 30 sec and semen was deposited into the body of the uterus by two skilled veterinarians. Unsexed semen contained 20 million sperm per inseminating dose (0.25 ml). Insemination with sorted semen at 3 million (n=40) and 4.5 million (n= 10) sperm per inseminating dose (0.25 ml) were carried out using two straws (1.5 million each) and three straws (1.5 million each) respectively to reach the desired final sperm concentration. Insemination with 1.5 million (n=99) sperm per inseminating dose (0.25 ml) was conducted using one straw per insemination. Pregnancy was diagnosed via trans-rectal palpation at approximately 60 days after insemination.

Statistical Analysis

Pregnancy rate was calculated as the number of heifers and cow diagnosed pregnant divided by the number of heifers and cow inseminated. Data on pregnancy rates with conventional nonsexed and sexed semen with different insemination doses were evaluated by the Chi- square test. A confidence level of $P < 0.05$ was regarded as being statistically significant.

RESULTS

The pregnancy rate in Sahiwal heifers and cows inseminated by unsorted semen (20 million) and sorted semen at inseminating doses of 1.5, 3.0 and 4.5 million are summarized in Table 1. Lower pregnancy rates ($P < 0.05$) were obtained with the sorted semen at 1.5, 3.0 million sperm compared to unsorted semen (20 million) and sorted semen doses at 4.5 million sperm. However, no significant difference in pregnancy rates ($P > 0.05$) was observed between unsorted (20 million) and sorted semen at 4.5 million sperm.

The results revealed lower (25.3%), intermediate (32.5%) and higher (60%) pregnancy rate at 1.5 million, 3.0 million and 4.5 million sorted sperm per inseminating dose, respectively. Though pregnancy rate by 3.0 million sperm was higher than 1.5 million sperm, however no differences ($P > 0.05$) were observed between sexed semen with sperm numbers 1.5 and 3.0 million. When the sperm number was increased to 4.5 million per dose, the pregnancy rate was significantly ($P < 0.05$) increased as compared to pregnancy rate obtained through using 1.5 million and 3.0 million sorted sperm. The results of the present study showed that pregnancy rates of 1.5 million and 3.0 million sexed sperm were 56% and 72% of unsexed semen, respectively.

Table 1: Pregnancy rates in Sahiwal heifers and cows inseminated with sex-sorted frozen semen and unsexed frozen semen

Status	Unsexed semen Inseminating dose of sexed semen			
	20×10^6	1.5×10^6	3.0×10^6	4.5×10^6
Insemination	269	99	40	10
Pregnant (%)	122(45.4)^a	25(25.3)^b	13(32.5)^b	6(60.0)^a
Non-pregnant (%)	147(54.6)^a	74(74.7)^b	27(67.5)^b	4(40.0)^a

Insemination expressed as number of animals inseminated; pregnant and non-pregnant are expressed as number of animals pregnant (%) and non-pregnant (%) respectively. ^{a,b}: Significantly different ($P < 0.05$)

Table 2: Pregnancy rates and calves born from Sahiwal cattle inseminated with X- and Y- sex-sorted frozen semen and unsorted frozen semen

Type of semen	Number of heifers and cows Inseminated (n)	Pregnant (%)	Calves born (n)
Unsexed semen	269	122(45.40)^a	94
Sorted X-semen	83	26(31.32)^b	21
Sorted Y- semen	66	18(27.30)^b	17

^{a,b}: Significantly different ($P < 0.05$)

The pregnancy rates obtained from unsexed semen and sexed semen is presented in Table 2. In the present study lower pregnancy rates ($P < 0.05$) were observed in sorted X- semen (31.32%) and Y- semen (27.30%) compared to unsorted semen (45.40%). However, no difference in pregnancy rate ($P > 0.05$) was recorded between X- and Y- sorted semen.

In the present study, average pregnancy rates obtained from the six bulls were 13/50 (26%), 9/34 (26.47%), 10/33 (30.30%), 7/20 (35%), 4/8 (50%), 1/4 (25%) using three semen doses. Pregnancies were affected by the sire ($P < 0.05$).

DISCUSSION

In the present work pregnancy rates were increased by increasing the AI dose from 1.5 million to 4.5 million total sorted sperm. This finding is in accordance with the finding where higher conception rates are recorded when sperm concentration is increased from lower concentration (De-Jarnette et al., 2011; Gaviraghi et al., 2013). Higher conception rates at 3.5 or 5.0 million over 2.0 million sorted sperm in virgin heifers are observed in the literature (De-Jarnette et al., 2008). In contrast, non-significant difference is also reported between the pregnancy rates of 1.5 million and 4.5 million doses of sexed spermatozoa (Seidel and Garner, 2002). Many authors concluded that lower pregnancy rate in sex-sorted frozen semen is due to low dose insemination (Gao et al., 2010). The present study also postulated that there was an advantage in increasing the sperm concentration from 1.5 million to 4.5 million sexed sperm. Lower pregnancy rates at 1.5 and 3.0 million sorted sperm relative to control can be compensable by increasing the sexed sperm numbers. Lower, intermediate and higher pregnancy rates at 1.5, 3.0 and 4.5 million sorted sperm might be the indicative of the result of sperm concentration. The present work also showed no advantage of 20 million unsexed sperm over 4.5 million sorted sperm. However, highest conception rates have been achieved by 4.5 million sorted sperm among four categories of frozen semen. From this study it can be interpreted that conception rates can be increased by increasing the sperm concentration until the maximal point beyond which improvement cannot be possible.

In general, fertility of sex sorted sperm should be 70-80% of unsexed sperm for commercial application of sperm sexing in cattle (Norman et al., 2010). In a well-managed dairy herd many authors reported the pregnancy rates with sexed spermatozoa usually have been 60-80% of unsexed control spermatozoa (Seidel et al., 1999; Doyle et al., 1999). Considering the advantage of sexed semen for cattle breeders 20% decrease in pregnancy rates can be tolerated especially in breeding virgin heifer (Schenk et al., 2009). Evidence from several published reports supports the pregnancy rates 72% of unsexed control at 3 million sexed sperm in the present study. Due to slow recovery rate low dose insemination in heifers and cow is recommended and which is the most practical and economically viable option for cattle (De-Jarnette et al., 2008; Schenk et al., 2009). Though pregnancy rates with 4.5 million sperm is higher than unsexed sperm in this study but due to slow recovery rates of sorted sperm this concentration may not be economically viable alternative for commercialization of sexed semen at field level. The results from the present study suggest that sexed semen at 3.0 million sperm may be the optimum concentration to get reasonable pregnancy in Sahiwal cattle until further qualitative improvement in terms of post thaw motility, membrane integrity and acrosomal intactness of sexed semen.

There is no literature available regarding pregnancy rates in sexed semen in indigenous cattle breed of India like Sahiwal cattle. Significant lower pregnancy rates in overall sorted sperm compared to unsorted sperm recorded in the current study is in agreement with previous studies in other breeds of cattle (Schenk et al., 2009; De-Jarnette et al., 2010; Gao et al., 2010; Gaviraghi et al., 2013; Abdalla et al., 2014). Many authors reported that conception rate for sexed semen is affected negatively by sexing technology (Norman et al., 2010). In the literature contrasting results have been found where no significant differences are observed between sexed and unsexed semen (Duarte et al., 2007; An et al., 2010; Lu et al., 2010). Results from this study suggests that semen that have undergone both sorting and cryopreservation causing un-compensable damage to sorted sperm might have lower pregnancy rates than the semen that is unsorted and frozen (Saacke et al., 2000; Gao et al., 2010).

In the present study, the pregnancies are affected by the sire ($P < 0.05$). The result of the present study is in agreement with other studies (Frijters et al., 2009; Hall et al., 2010; Sales et al., 2010; Abdalla et al., 2014). However, contrasting results have been reported in published literature where pregnancy rates are not affected by the sire (Gaviraghi et al., 2013). Bull-to-bull variation in conception rate in the present study might be due to the variation of tolerance of spermatozoa to sexing process (Frijters et al., 2009; Schenk et al., 2009) and variation in fertility among bulls (Rath and Johnson, 2008).

CONCLUSION

We have conducted different low dose insemination of sex sorted semen in Sahiwal cow and heifers. Reasonable pregnancies are achieved with low dose insemination of sorted semen. Lower (25.3%), intermediate (32.5%) and higher (60%) pregnancies are obtained with 1.5, 3.0 and 4.5 million sorted sperm respectively. Considering the slow recovery rate of sorted sperm low dose insemination above 1.5 million but less than 4.5 million could be economically feasible for commercialization of sexing technology in Sahiwal cattle. Low dose insemination at 3.0 million sexed sperm of fertile Sahiwal bull could be used at field level artificial insemination programme until further qualitative development of sexed semen. Nevertheless more research is required in terms of sorting procedure, sexed semen production, fertility of bulls and conception rate for successful implementation of sexing technology at field level.

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CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

AUTHORS' CONTRIBUTION

All authors have been involved in constructing the experimental design, analysing data and preparing manuscript.

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