Fractionation of X and Y Chromosome-Bearing Bovine Spermatozoa through Sugar Gradients for Sex Predetermination in Dairy Cattle

Nilani Kanesharatnam, Thampoe Eswaramohan, and Kandiah Balasubramaniam

Abstract—**Mankind has been interested in sex preselection since ancient times. It can be achieved either by sexing of early embryos or by separation of X and Y chromosome-bearing spermatozoa, but new separation techniques with better accuracy and low costs are necessary. The objective of this study was to fractionate X and Y-bearing bovine sperm using sucrose gradient. A discontinuous sucrose density gradient was prepared by layering successive decreasing sucrose solution upon one another. Finally 20µL semen sample was loaded on the top layer. Then it was centrifuged at 500 x g for 12 minutes at room temperature. After elution of fractions and centrifugation (at 700 x g for 5 minutes), sperm viability and acrosome integrity were assessed by using 0.4% Trypan Blue and 0.75% Giemsa stain. Other part of the pellet was stained with 2% orcein red for 30 minutes to obtain sets of chromosomes. Repeated measures analysis of variance (ANOVA) with Bonferroni's multiple comparison test was performed to compare the percentages of female sperms at every layer. Results have shown that means of percentage of X chromosomes increased from layer 1 (15.55 ± 2.939 %), layer 2 (14.0 ± 3.055%), layer 3(26.33 ± 0.881%) to layer 4 (31.85 ± 5.186), but there is a statistically significant difference between layer 2 and layer 4 ($P<0.05$). However it needs to perform further studies to obtain appropriate density gradient model. Our present study demonstrates that the discontinuous sucrose density gradients can be considered as low cost tool for sperm sexing of bovine semen.**

Index Terms—**Sexing, density gradient, centrifugation, karyotype**

I. Introduction

Mankind has been interested in sex preselection since ancient times. Sex preselection is now possible and is actually applied in both humans and livestock. It can be achieved either by sexing of early embryos or by separation of X and Y chromosome-bearing spermatozoa. Sexing of embryos is practiced in both humans and livestock, especially cattle, by performing the DNA polymerase chain reaction or in situ hybridization to detect Y specific DNA, in cells taken from the early embryo [1], [2]. Despite the success of embryo sexing, sperm separation is a more preferred method for sex preselection, because it would avoid the need for embryo manipulation and exclusion. In the livestock industry, it will allow a much more efficient use of the reproductive potential of the economically important livestock species. The economic and social benefits of such an accomplishment include selection of females for milk production whereas male offspring is more suitable for beef production in dairy cattle, and blocking male transmission of sex-linked genetic diseases, such as haemophilia [3]. Sperm sexing rouses great interestdue to extensive application in animal production and new separation techniques which present bothbetter accuracy and low costs are necessary. Flow cytometry separates X and Y-bearing sperms by

DNA content with an accuracy of 90% [4]. However this technique has disadvantages such as equipment costs, damage to sperm during sexing [5] and altered mRNA expression of embryos [6]. Using a simple methodology, the density gradient is capable to separate X and Y-bearing sperm with lower cost and without damages to sperm viability. Continuous Percoll and Optiprep density gradients can be used to separate X-bearing bovine sperm with an accuracy of 70% [7]. Sperm sexing with flow cytometry is not appropriate to Jaffna peninsula of Northern Province (NP), because of the high equipment cost and maintenance. The objective of this study was to fractionate X and Y-bearing bovine sperm using density gradient.

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II. Material and Methods

A. Artificial Vaginal Collection of Semen

Semen from Jersey bull was collected into an artificial vagina (Fig 1) using a teaser animal once a week since Oct 2010 from Artificial Insemination (AI) center at Thirunelvely. Before semen collection warm water (39-40 ℃) and air were injected into an artificial vagina to maintain the proper temperature and pressure for the ejaculation. The rubber part of the artificial vagina was lubricated by vaseline.

B. Establishment of Discontinuous Sucrose Gradient

Powdered sugar was placed in the oven at 60 ℃ for 2-3 hours. Weighed sugar powder by electronic balance was dissolved in warmed 2.9% sodium citrate buffer in the universal container in order to obtain appropriate mass percent of sugar. The densest layer (35%) was prepared by addition of 3.5g sucrose in 10ml sodium citrate buffer solution. Other layers were also prepared accordingly. Then the sugar solution was filtered through 0.2 µL filter units. Every layer (0.5 ml) was aspirated and loaded gradually into the eppendorf tube by the aid of the micropipette (Fig. 2). A discontinuous sucrose density gradient was prepared by

layering successive decreasing sucrose densities solution upon one another (35%, 30%, 20%, and 15%). The end of the yellow tip was allowed to make contact with the inside wall of the tube. Finally 20µL semen sample was loaded on the top layer. Layered eppendorf tube was then centrifuged at 500 x *g* for 12 minutes at room temperature to obtain the separation of X and Y bearing sperms. Immediately after the centrifugation the tube was removed from the rotor without any disturbance of the layers of sucrose.

Fig. 1. Artificial vagina used for the collection of bull semen

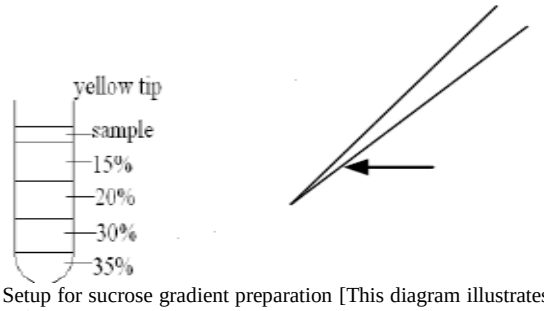

Fig. 2. Setup for sucrose gradient preparation [This diagram illustrates that the way of layering with various percentages of sucrose solutions]

C. *Assessment of Each Layer for 'X' Chromosome*

A tiny hole was introduced into the very bottom of the eppendorf tube using a fine needle (21 gauge size) which was burnt by sprit lamp. Fractions of equal volume (0.5 ml) were then collected in eppendorf tubes below the pierced hole. Each layer was occasionally aspirated by the micropipette into eppendorf tubes as another method for fraction collection. Then they were centrifuged at 700 x *g* for 5 minutes at room temperature. The supernatants were carefully aspirated and the sperm located at the bottom fraction were collected from the tubes. Sperm concentration was determined by using the hemocytometer (improved NEUBAUER). Motion characteristic of sperms was assessed with the aid of hemocytometer and stopwatch before and after centrifugation. Small aliquots of sperms were stained with Trypan blue (0.4%) and Giemsa (0.75%) to assess the viability and acrosome integrity. Other part of the pellet was stained with 2% orcein red for 30 minutes to obtain chromosomes. After staining the small part of the stained sample was placed on the slide over which small broken cover slip was placed. Then cover slip was pressed by the thumb to break the sperm cells. Cutex was applied along the edges of the cover slip. Then slides were analyzed under 100X oil immersion objective of the Olympus microscope to count the X and Y chromosomes of the sperms. Percentage of X chromosomes was determined by using 2% orcein red staining. The percentage of female sperms obtained at top, intermediate and bottom layers were compared to each other.

D. *Statistical Analysis*

Data was analyzed using prism 5.04 to compare the percentage of female sperms at top, intermediate and bottom sucrose gradient layers. Mean ± SEM was used to describe data. Repeated measures analysis of variance (ANOVA) with Bonferroni's multiple comparison test to compare the percentage of X chromosomes at every layer (Fig 3).

III. Results

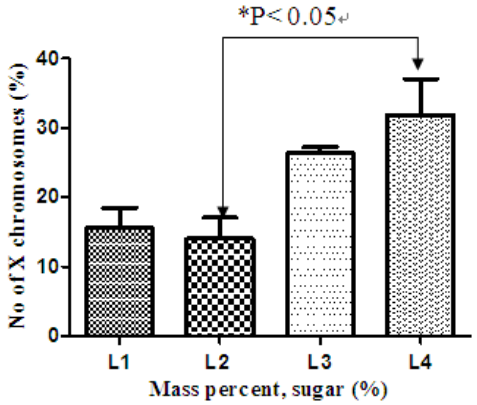

Fig. 3. Comparison of the percentage of X chromosomes in sucrose gradient layers. L1-Layer 1 (15%), L2- Layer 2 (20%), L3- Layer 3 (30%), L4- Layer 4 (35%)

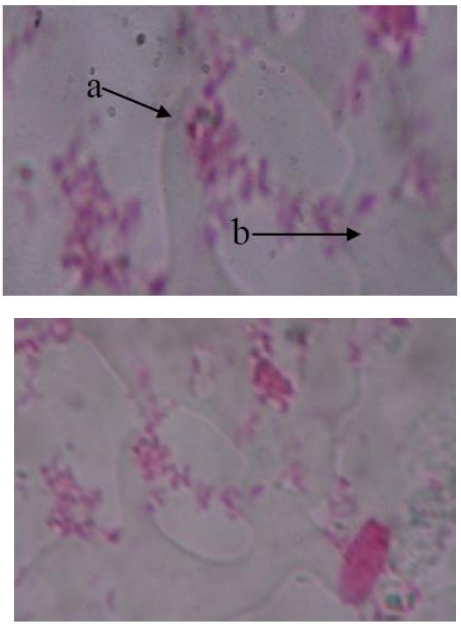

Fig. 4. Appearance of stained (2% orcein red) chromosomes of sperm cells in the Jersey bull under the Olympus microscope (X1000) a- sets of chromosomes in bull b- broken sperm head

Results have shown that means of percentage of X chromosomes increased from layer 1 to layer 4 (see Table 1), except layer 2, but there is a statistically significant difference between layer 2 and layer 4 (ANOVA, $P<0.05$) compared to other layers. However it needs to perform

further studies to obtain appropriate density gradient model.

TABLE I: THE PERCENTAGE OF FEMALE SPERMS IN PROGRESSIVELY INCREASING SUCROSE GRADIENT LAYERS

Sucrose gradient layers (W/V)	No of X chromosomes (%)
L1	15.55 ± 2.939
L2	14.0 ± 3.0550
L3	26.33 ± 0.881
L4	31.85 ± 5.186

IV. DISCUSSION

Sucrose is a commercial medium for the density-gradient centrifugation of cattle spermatozoa, because it is a readily available ingredients and low cost. The main disadvantages of sucrose solutions are some of their physico-chemical properties. Sucrose solutions in the concentration range have a high osmolality and are viscous. In our present study, ordinary sugar was used for preparation of discontinuous sucrose density gradient. Sugar can be used by sperm cells as the energy source during insemination of sexed semen [8]. However impurities in the sugar powder might change density of the solution. According to reference [7] the continuous Percoll and OptiPrep reagents can be used to separate X-bearing bovine sperm, but the Percoll and OptiPrep reagents were unavailable and high costs for our experiments. Before the preparation of the sucrose density gradient, sugar powder was placed in the oven at 60℃ to remove the moisture content until constant weight is obtained as higher temperatures (above 90℃) may cause caramelization. Desiccators can also be used to remove the moisture at room temperature, but this is time consuming method.

The discontinuous Percoll gradient made with 12 layers can be used for sexing in bovines [9], [10]. In our study the discontinuous density gradient with only 4 layers was prepared for sexing. The tiny hole should be just big enough to allow the sucrose solution to drip out at approximately one drop per second. For this purpose only tip of the needle must be introduced into the tube. However the fine needle may disturb the bottom fraction. If the hole is large, collection of fractions will be very difficult. After centrifugation sperm motility was lesser than initial assessments due to the viscosity of sugar solution and rough handling. Sperm viability did not differ significantly before (82%) and after (77%) sexing.

Warmed sodium citrate buffer was used to dissolve sugar readily. Buffer solutions don't alter the physiology of the sperms. The addition of buffering agents helps control the pH of the medium and regulation of the osmotic pressure [11]. Sugar solution was filtered through 0.2 μL filter units to remove the microbial contamination. Before loading the layers, tip of the micropipette or syringe must be washed with the sugar solution to be loaded to avoid the human error. Syringe or tip of the micropipette can be refilled that solution to load the layers. Gravity will feed the sugar solutions into the eppendorf tube slowly. During loading a steady application of the solutions by a clamed steady yields the most reproducible gradient.

Karyotype of cattle consists of 60 chromosomes, 29 pair of autosomes and 1 pair of sex chromosomes [12]-[14]. All of the autosomes are somewhat teardrop shaped with centromere at the end of the chromosome. The sex chromosomes have centromere in the middle of the chromosome. According to reference [3] X chromosome is larger than the Y chromosome; female spermatozoa should have more chromatin than male spermatozoa while the autosomes carried by X- or Y-bearing sperm are identical in DNA content. This difference in total DNA content ranges from 2.8% in humans to 3.6% in pigs and 3.8% in cattle [15], [16]. Reference [17] proposed that X and Y spermatozoa have 1 % difference in head radius, which would affect swimming and sedimentation velocities. Due to the relatively high DNA content of X chromosomes, female sperm might be occupied in the densest layer of the gradient (35%) in our experiments. Heavier spermatozoa should settle down faster than lighter spermatozoa, therefore centrifugation time could positively influence X-bearing sperm moving down the gradient. As sperm is centrifuged through increasingly dense layers of a solution, heavier X-sperm will sediment to the bottom, and lighter Y-sperm will migrate to top [18]. Sperms travel through the gradient until they reach the point in the gradient at which their density matches that of the surrounding sucrose.

Application of cutex along the margins of cover slip prevents the leakage of stained semen during pressing by thumb and oil intrusion inside the cover slip when viewing under oil immersion objective. When we pressed over the cover slip, some sperm heads were broken and expelled their contents including chromosomes (Fig 4). X chromosome was identified by its thickest appearance than other autosomes after staining. Although X chromosome is much larger than Y chromosome, identification of X chromosome was very difficult in each sets of chromosome. The development of DNA technology has led to more precise methods to estimate the percentages of X and Y sperm in different fractions [19]-[21]. Although reference [7] evaluated the sexing results by PCR analysis, it is not appropriate for cattle and animal breeders in developing countries due to the high cost. However our present study demonstrated that the discontinuous sucrose density gradients can be considered as low cost tool for sperm sexing of bovine semen in the developing countries.

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