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A Preliminary Study for Sperm Sexing by Using Sucrose Density Gradients in Jersey Bull at Artificial Insemination Centre at Thirunelvely (Northern Province of Sri Lanka)

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Abstract. Sperm sexing rouses great interest due to extensive application in animal production and new separation techniques which present both better accuracy and low costs are necessary. Thus the present study was experimented to separate X and Y-bearing bovine sperm using density gradient. To prepare discontinuous sucrose density gradient, sucrose solutions 35%, 30%, 25%, 20% and 15% were layered upon one another respectively into the eppendorf tube. 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 was used for quality control by 0.4% Trypan Blue and 0.75% Giemsa stain. Other part of the pellet was stained with 2% orcein red or 1% eosin for 30 minutes to establish karyotypes. The percentages of female sperms were counted at different layers and statistically compared with the Paired-T test. Results have shown that means of Percentage of X chromosomes increased from top layer (27.01 ± 7.501 %) to bottom layer (36.93 ± 3.316 %). But the difference are not statistically significant (P>0.05). However it needs to perform further studies to obtain appropriate density gradient model. Our preliminary study demonstrated that the discontinuous sucrose density gradients can be considered as low cost tool for sperm sexing of bovine semen.

Keywords: Sexing, density gradient, cattle breeding

1. Introduction

The separation of X- and Y-chromosome-bearing spermatozoa is aimed at controlling the sex of the offspring. The economic and social benefits of such an accomplishment include selection for females in dairy cattle, and blocking male transmission of sex-linked genetic diseases, such as haemophilia [1]. Sperm sexing rouses great interest due to extensive application in animal production and new separation techniques which present both better accuracy and low costs are necessary. Offspring sex predetermination has been a goal of livestock producers for generations. A technique which provides accuracy is the flow cytometry that separates, by DNA content, two populations of sperm (X and Y-bearing) with an accuracy of 90% [2]. However this technique has disadvantages such as equipment costs, damage to sperm during sexing [3] and altered mRNA expression of embryos [4]. 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% [5]. Sperm sexing with flow cytometry is not appropriate to Jaffna peninsula of Northern Province (NP), because of the high equipment cost and maintenance. Thus the present study was experimented to separate X and Y-bearing bovine sperm by using a discontinuous sucrose density gradient.

2. Material and Methods

2.1. Collection of semen

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The semen of Jersey was collected by means of artificial vagina once a week since Oct 2010 from Artificial Insemination (AI) center at Thirunelvely, NP of Sri Lanka.

2.2. Sucrose gradient preparation and centrifugation

Sugar was ground by grinder as the fine powder which was placed in the oven at 60°C for 2-3 hours. Required amount of sugar powder was weighed by using electronic balance accurately. Sugar powder was dissolved in warmed 2.9% sodium citrate buffer in the universal container. Then the sugar solution was filtered through 0.2 µL filter units. A discontinuous sucrose density gradient was prepared by layering successive decreasing sucrose densities solution upon one another. Every layer was loaded gradually into the eppendorf tube by the aid of the micropipette. The end of the yellow tip was allowed to make contact with the inside wall of the tube. The first solution applied was the 35% (W/V) sucrose solution (3.5g sucrose in 10ml sodium citrate buffer solution). Then 30%, 25%, 20%, 15% sucrose solutions were layered upon one another respectively. 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.

2.3. Elution of fractions

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 were then collected in eppendorf tubes below the pierced hole. 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. Small aliquots of sperms were stained with Trypan blue (0.4%) and Giemsa (0.75%) to assess the quality of the sperm and the other part of the pellet was stained with 2% orcein red or 1% eosin for 30 minutes to obtain karyotypes. 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 or eosin stain (1%) and the percentage of female sperms obtained at bottom layer (35% W/V of sucrose solution) was compared with the percentage of female sperms obtained at top layer (15% W/V of sucrose solution).

2.4. Statistical analysis

Data was analyzed using prism 5.04 to compare the percentage of X chromosomes at top and bottom sucrose gradient layers. Mean ± SEM was used to describe data. Means of the percentage of X chromosomes at top and bottom layers were compared by performing the Paired-T test.

3. Results

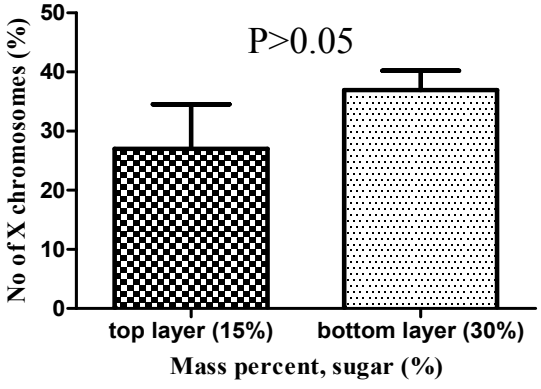

Figure 1: Comparison of the percentage of X chromosomes at top (15% W/V) and bottom (35%W/V) sucrose gradient layers.

Results have shown that means of percentage of X chromosomes increased from top layer (27.01 ± 7.501 %) to bottom layer (36.93 ± 3.316 %). But the difference are not statistically significant (P>0.05). However it needs to perform further studies to obtain appropriate density gradient model.

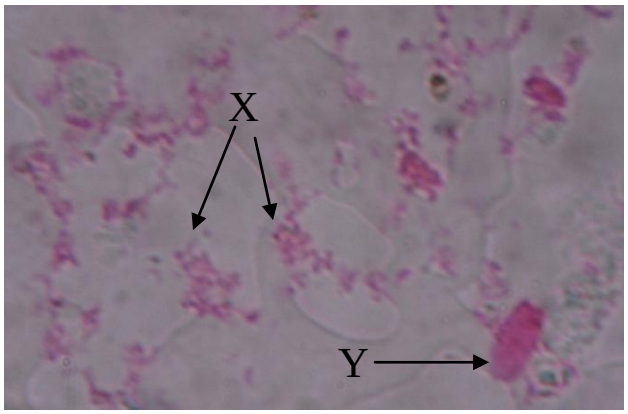

Figure 2: Appearance of stained (2% orcein red) chromosomes of sperm cells in the Jersey bull under the Olympus microscope (X1000)

X- Sets of chromosomes in bull

Y- Broken sperm head

4. Discussion

In our present study, ordinary sugar was used for preparation of discontinuous sucrose density gradient. Sucrose is a readily available ingredients and low cost. Sugar can be used by sperm cells as the energy source during insemination of sexed semen [6].

However impurities in the sugar powder might change density of the solution. Resende *et al*, (2009) used the continuous Percoll and OptiPrep reagents in attempt to separate X-bearing bovine sperm, but the Percoll and OptiPrep reagents were unavailable and high cost for our experiments. Before the preparation of the sucrose density gradient, sugar powder was placed in the oven at 60°C to remove the moisture content until constant weight is obtained as higher temperatures (above 90°C) may cause caramelization.

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 [7]. 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.

The discontinuous Percoll gradient made with 12 layers can be used for sexing in bovines [8, 9]. In our study the discontinuous density gradient with only 4 or 5 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 and after sexing.

Karyotype of cattle consists of 60 chromosomes, 29 pair of autosomes and 1 pair of sex chromosomes [10, 11, 12]. 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 Moruzzi, (1979) X chromosome is larger than the Y chromosome; female spermatozoa should have more chromatin than male spermatozoa. 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.

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 2). 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. Although Resende *et al*, (2009) evaluated the sexing results by PCR analysis, it is not appropriate for cattle and animal breeders in developing countries due to the high cost.

5. Conclusion

Our preliminary study demonstrated that the discontinuous sucrose density gradients can be considered as low cost tool for sperm sexing of bovine semen.

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