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Full Length Research Paper

Verification of X- and Y-spermatozoa separation by nested polymerase chain reaction (PCR), motility and membrane integrity in bovine

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The aim of the present study was to verify the presence of X- and Y-chromosome spermatozoa after separation with swimming speed using oestrus cows vagina mucus, Percoll discontinuous gradient (45 to 90%) and swim-up using TALP medium. The nested polymerase chain reaction (PCR) was used to determine X- and Y-chromosome bearing spermatozoa after separation. The primers for PCR were designed using amelogenin cDNA sequence with 329 and 266 bp for X- and Y-bearing chromosome spermatozoa, respectively. The motility was analyzed using computer assisted sperm analyser, whereas the membrane integrity was analyzed using hypo-osmotic swelling test (HOST). Results were confirm by the absence of single band, either for X- or Y-chromosome. Inversely, the double band indicating that the spermatozoa cannot be separated was observed. The percentage of X-chromosome bearing spermatozoa in the swimming speed using oestrus cows vagina mucus media, Percoll discontinuous gradient and swim up methods were 58.33, 44.33 and 50%, respectively. Statistically, both percentages were significantly different (P<0.001) as compared to the theoretical ratio (50:50). Spermatozoa motility, membrane integrity and concentration before and after separation were also significantly different (P<0.05). This study shows that although swimming speed using oestrus cow's vagina mucus media may be used to separate X- and Y-chromosome bearing spermatozoa in bulls, the results however, require further investigation.

Key words: Spermatozoa separation, nested polymerase chain reaction (PCR), motility, membrane integrity.

INTRODUCTION

Separation of X- and Y-chromosome spermatozoa has been used for genetic improvement in dairy and beef cattle industries. In animal production, the prospect of spermatozoa separation to detect sex ratio can increase the production and profit in the cattle farming (Farahvash et al., 2008). Furthermore, females are used for dairy and calving production, while males are desired for meat production (Hamano et al., 2007). It has been noted that the physical characteristics of spermatozoa such as size, weight density, electrical surface charges, surface macromolecular protein, different effects of pH and different effect of atmospheric pressure can be used to distinguish X- from Y-chromosome (Seidel, 1988; Yan et al., 2006). The separation of spermatozoa has been accomplished through many different methods, namely albumin gradient, sex-specific antibody binding, swim-up, Percoll gradient, free-flow electrophoresis and flow cytometry. Johnson (1995) reported that the composition of DNA on X- and Y-chromosome spermatozoa is in the

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range of 3.5 to 4.5%. Meanwhile, variation of DNA bovine for Holstein, Jersey, Angus, Hereford and Brahman bulls is 4.98, 4.24, 4.05, 4.05 and 3.75%, respectively (Garner and Seidel, 2008; Garner et al., 2001).

It has long been suggested that flow cytometry is the ideal technique for separation of spermatozoa and produces the best DNA purity (Johnson, 1995). However, the isolation of the X- and Y-chromosome using this technique involves the staining of spermatozoa with Hoechst 33342 in combination with the impact of an ultraviolet laser beam, which has potential for mutagenic induction (Yan et al., 2006). In addition, this technique also uses expensive equipment and may not be practical to operate (Seidel, 2003).

Majority of research on spermatozoa sexing require long duration to find out the complete result. One of the problems associated with this is probably the validation of the X- and Y-chromosome (Widsor et al., 1993). Therefore quick, effective and accurate method such as polymerase chain reaction (PCR) is essential to determine the purity of the sample and to predict the sex ratio of the offspring. On the other hand, it has been reported that motility and membrane integrity after sexing spermatozoa is also an important factor for cell survival and fertilizing ability in cattle (Correa and Zavos, 1994; Mehmood et al., 2008; Rota et al., 2000). Therefore, the aim of the present study was to verify the sexing of X- and Y-chromosome bearing spermatozoa using nested PCR as well as to determine the motility and membrane integrity after separation.

MATERIALS AND METHODS

Semen collection

Semen samples were collected using an electro ejaculator from four crossbred bulls aged 4 years old, from University Agriculture Park, Malaysia. Three ejaculates were collected from each bull and immediately after collection, the semen was put in a water bath maintained at 37°C and subsequently analyzed for volume, colour, pH and consistency. The spermatozoa concentration was counted using haemocytometer.

Assessment of motility

The motility of fresh spermatozoa from each ejaculate and after separation of spermatozoa was analyzed using the computer assisted semen analyser (CASA, HTM-IVOS-Ultimate; Hamilton Thorn Biosciences, Beverly, MA, USA). Briefly, freshly collected semen was diluted to obtain final concentration of 100×10^6 sperm/ml in normal saline. Approximately, 2 µl of the sample were placed in a 20 µl standard counting slide (SC20.01.FA; Leja, Niewvennep, The Nederlands). The slide was loaded into CASA and at least 20 fields were selected for motility analysis.

Semen sexing procedure

The separation of X- or Y-chromosome spermatozoa was carried using three different methods: (1) The swim up procedure according to Parrish et al. (1988). Briefly, in a centrifuge tube, 225 µl of fresh semen was layered carefully under 1 ml of equilibrated sp-TALP medium (containing 3.1 mM KCl, 100 mM NaCl, 25 mM $NaHCO_3$, 0.4 mM NaH_2PO4 $2H_20$, 0.5 mM $MgCl_2.6H_20$, 2 mM $CaCl_2.2H_2O$, 21.6 mM Na-Lactate, 10 mM Hepes, pen-strep stock 1 ml/100 ml, phenol red stock 50 µl/100 ml). After loading, the tubes were incubated at 39°C in water bath. After 40 min of incubation, 800 µl of the upper fraction of the sp-TALP (containing the selected spermatozoa) was collected, placed in 5 ml Sperm TALP medium, centrifuged for 10 min at 120 g, and the supernatant was discarded. (2) The swimming speed method. The method used was adopted from Garner and Seidel (2008) with slight modification. Briefly, straws with size of 0.5 ml (Medium straw, Kruuse) were used in this study. The first straws were filled with vaginal mucus from oestrus cows. Afterward, using Eppendorf, each of the straws was added with fresh semen with concentration of 12×10^6 sperm/ml. The straws were incubated at 37°C in humidified atmosphere of 5% CO_2. After 40 min, straw was cut into 4 equal parts and each cut was given a number starting from (place fresh semen) 1, 2, 3 and 4. Afterwards, the clippings of all number 4 were collected and subjected to DNA extraction for further analysis. (3) Percoll continuous gradients technique according to Parrish et al. (1995) was used. Briefly, 0.25 ml fresh spermatozoa from each bull were selected by centrifugation on a Percoll (Sigma – Aldrich Chemie GmbH, Germany) discontinuous gradient (45 to 90%). Initial attempt was made to make a 90% Percoll solution using a 9:1 mixture of Percoll and with a concentrated solution containing 2.0 mM $CaCl_2$, 0.4 mM MgCl, 21.6 mM lactic acid and 25 mM $NaHCO_3$. The 45% Percoll solution was prepared with 1 ml of 90% Percoll and 1 ml of sp TALP containing 31 mM KCl, 800 mM NaCl, 3 mM NaH_2PO4 and 100 mM Hepes. Samples were layered on Percoll gradient consisting of 2 ml of 45% Percoll gradient and 2 ml of 90% Percoll gradient, in a 15-ml centrifuge tube, and centrifuged at 700 g for 20 min at 30°C in a refrigerated centrifuge.

Assessment of integrity membrane spermatozoa

The membrane integrity was determined before and after separation using the hypo-osmotic swelling test (HOST) as described by Jeyendran et al. (1984). Briefly, 100 µl of semen was mixed with 1 ml of hypotonic solution (100 mOsm/kg H_2O) containing 13.51 g of fructose and 7.35 g of sodium citrate ($2H_2O$) in 1000 ml of distilled water. The mixture was incubated at 37°C for 60 min. Following incubation, 15 µl were placed on a slide, covered and observed under the compound microscope (Olympus CK2, ULWCD 0.30) at 400x. The spermatozoa were classified according to the presence or absence of swollen tail. At least, 200 spermatozoa were observed and recorded. The membrane integrity after HOST was classified into two groups: (1) Normal spermatozoa which displayed head coiled tails after HOST. (2) Abnormal spermatozoa which displayed no head coiled tails after HOST.

DNA extraction

DNA was isolated from unsexed, Y- and X-chromosome spermatozoa of each bull using a commercial DNA extraction kit (DNeasy Blood and Tissue Kit, Qiagen Inc. Germany), according to the manufacturer's instructions manual. Briefly, buffer X_2 containing 20 mM Tris, Cl (pH = 8.0, 10 mM), 20 mM EDTA, 200 mM NaCl, 4% SDS, 80 mM DTT and 12.5 µl/ml Proteinase K was prepared. Subsequently, DNA spermatozoa were extracted as follows: The same sample was added with buffer X_2 and incubated by shaking water bath at 56°C for 1 h until the sample dissolved. The mixture was subsequently added with buffer AL (Qiagen Inc. Germany), mixed thoroughly by overtaxing, adding ethanol (96 to 100%), and mixed again thoroughly. Afterward, the sample were placed into the

DNeasy Mini spin column in a 2 ml collection tube, centrifuged at ≥6000 xg (8000 rpm) for 1 min, discarded flow-through and collection tube. Subsequently, the sample mixtures were added with buffer AW1 (Qiagen Inc. Germany), centrifuge at ≥6000 xg (8000 rpm) for 1 min, and discarded flow-through and collection tube. Buffer AW2 were further added to the mixtures (Qiagen Inc. Germany), centrifuged for 3 min at 20,000 xg (14,000 rpm), discarded flow-through and collection tube, and finally added 50 to 100 µl buffer AE (Qiagen Inc. Germany). The sample were incubated at room temperature for 1 min and centrifuged for 1 min, at ≥6000 xg (8000). DNA was eluted using 200 µl of TE- buffer (10 mM Tris, 1 mM EDTA, pH 8.0) at 55°C.

Primers

The primers were purchased from First BASE Laboratories. The first primer sequences was F1: 5'-CATGGTGCCAGCTCAGCAG-3 and R1: 5'-CCGCTTGGTCTTGTCTGTTGC-3 produced 367 and 304 bp X- and Y-chromosome sperm amplicants, respectively. Second primer sequences was F1: 5'- CAGCAACCAATGA-TGCCAGTTC-3' and R1: 5'- GTCTTGTCTGTTGCTGGCC-3' Produced 329 and 266 bp X- and Y-chromosome sperm amplicons, respectively (Colly et al., 2008).

PCR amplification

PCR reactions were performed in a total volume of 50 µl, containing 2 µl of 2.9 ng /µl DNA sperm, 2 µl of 10 x Taq buffer, 2 µl of 25 mM MgCl, 0.5 µl of 25 mM dNTP, 0.5 µl of each primer , and 0.5 µl of 500 u Taq polymerase. The initial denaturation was 94°C for 4 min followed by 35 cycles at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 2 min. The reaction was accomplished by a final extension at 72°C for 5 min.

Statistical analysis

Statistical analysis was Fisher's exact test for the X-chromosome specific band. Furthermore, percentage of all data was expressed as mean values ± S.E.M. The statistical significances of the effects of motility and membrane integrity after separation were determined by ANOVA (S-PLUS Statistical Program, Insightful Corporation Seattle, WA, USA). p-Values <0.05 were considered to be significantly different.

RESULTS

Figure 1 shows the confirmation of X- and Y-chromosome spermatozoa using nested PCR after spermatozoa separation with swimming speed, Percoll gradient and swim up methods. Before the swimming speed, Percoll gradient and swim up separation, there were no differences in the percentage of spermatozoa with the Y- and X-chromosome in the semen. These results were confirmed by the absence of single band, either for X- or Y- chromosome. Inversely, the double band indicating that the spermatozoa cannot be separated was observed. However, after separation, the percentage of X-chromosome spermatozoa in the swimming speed method using vagina mucus media was 58.33%. On the other hand, 41.67% of the X-chromosome spermatozoa cannot be separated (double band), whereas, 42.01% of the Y-chromosome could be separated, while 57.99% were double band and could not be separated.

In the Percoll discontinuous gradient method, 44.33% of the Y-chromosome were separated, while 55.67% were double band and could not be separated; whereas, the percentage of X-chromosome was 43.03% separated and 56.97% double band and could not be separated. Statistically, both percentages were significantly different ($P<0.001$) as compared to the theoretical ratio (50:50). On other hand, after separation using swim up method, the proportion of X-chromosome was exactly the same with that of the control (before sexing spermatozoa) (50:50). The result was significantly different from the theoretical ratio (50:50).

The percentage spermatozoa motility before separation (74.78 ± 0.73%) and after separation were (57.24 ± 1.65%) for swimming speed, (83.63 ± 1.95%) for Percoll gradient and (56.71 ± 1.75%) for swim up, respectively. Comparing the result of spermatozoa motility before separation with the result after separation, it was found out that there were significant differences ($P<0.05$) among them. However, there were no significant differences ($P>0.05$) in the motility values between swimming speed and swim up method after separation.

The membrane integrity test before the spermatozoa separation was 71.22 ± 0.92% (Table 1) and after the spermatozoa separation with swimming speed, Percoll gradient and swim up were 50.80 ± 1.11, 80.64 ± 1.22 and 52.79 ± 1.38%, respectively (Figures 2 and 3). The result of membrane integrity before separation, when compared with the result after separation were significantly different ($P<0.05$) among three methods. Whereas, there were no significant differences ($P>0.05$) between the membrane integrity values, swimming speed and swim up method after separation. The spermatozoa concentration before separation was 25.55 ± 0.17 x 10^6 sperm/ml and after spermatozoa separation was 12.27 ± 0.46 x 10^6 for swimming speed, 9.44 ± 0.29 x 10^6 for Percoll gradient and 13.02 ± 0.47 x 10^6 sperm/ml for swim up, respectively. The result of concentration before separation, when compared with the result after separation were significantly different ($P<0.05$) among the three methods.

DISCUSSION

When compared with the theoretical ratio (50:50), the result of this study indicated that the percentage of X-bearing chromosome separated by the swimming speed using vaginal mucus media (58.33%) was higher than that of swim up (50%) and Percoll gradient (43.33%). The mechanism of separation of X- and Y-bearing spermatozoa by swimming speed using vaginal mucus media is not fully understood. It is suspected to be the presence of certain components in the vaginal mucus media which affect the DNA of spermatozoa during the process of separation. Klemm and Hawkins (1986) reported that the

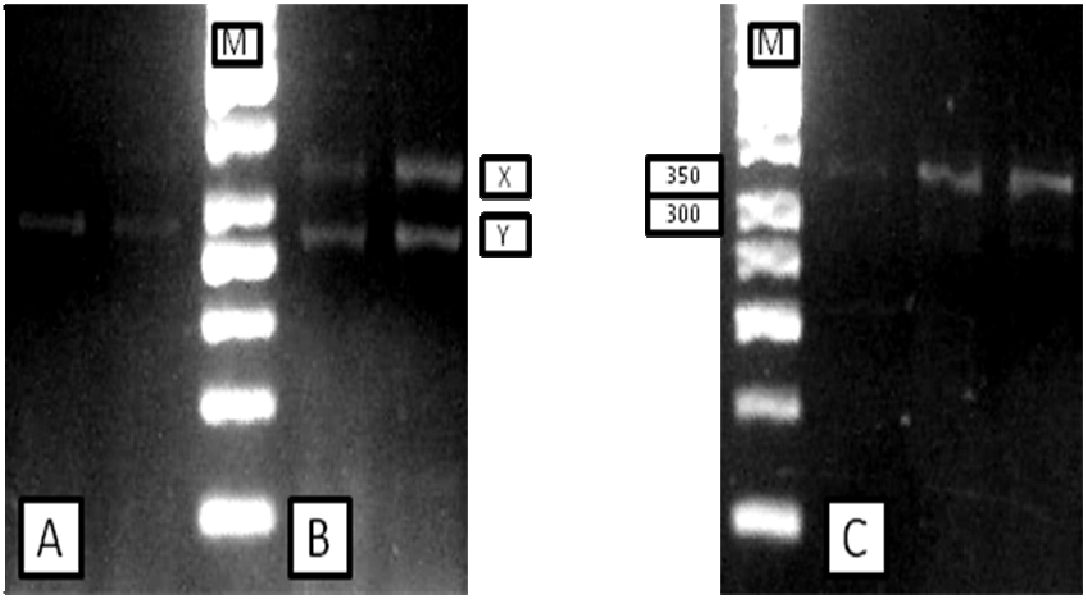

Figure 1. The conventional PCR analyzed for verification of X- and Y-chromosome after separation. Lane M: gel with molecular weight of 500 Da; Gel A: single band of Y-chromosome; Gel B: double band (could not be separated) and Gel C: single band of X-chromosome.

Table 1. Proportional percentage of motility, membrane integrity and concentration of spermatozoa before and after separation using different methods.

Sperm separation protocol	Motility (%)	Membrane integrity (%)	Concentration (sperm/ml)
Initial	74.78 ± 0.73	71.22 ± 0.92	$25.55 \pm 0.17 \times 10^{6}$
Percoll gradient (n = 12)	83.63 ± 1.95^{b}	80.64 ± 1.22^{b}	$9.44 \pm 0.29 \times 10^{6b}$
Swim up (n = 12)	56.71 ± 1.75^{a}	52.79 ± 1.38^{a}	$13.02 \pm 0.47 \times 10^{6a}$
Swimming speed (n = 12)	57.24 ± 1.65^{a}	50.80 ± 1.11^{a}	$12.27 \pm 0.46 \times 10^{6a}$

The proportional motility, membrane integrity, and concentration of sperm (mean ± S.E.M) and spermatozoa were obtained with CASA, HOST and Haemocytometer, respectively. Different letters in the same column is a statistical difference (P<0.05).

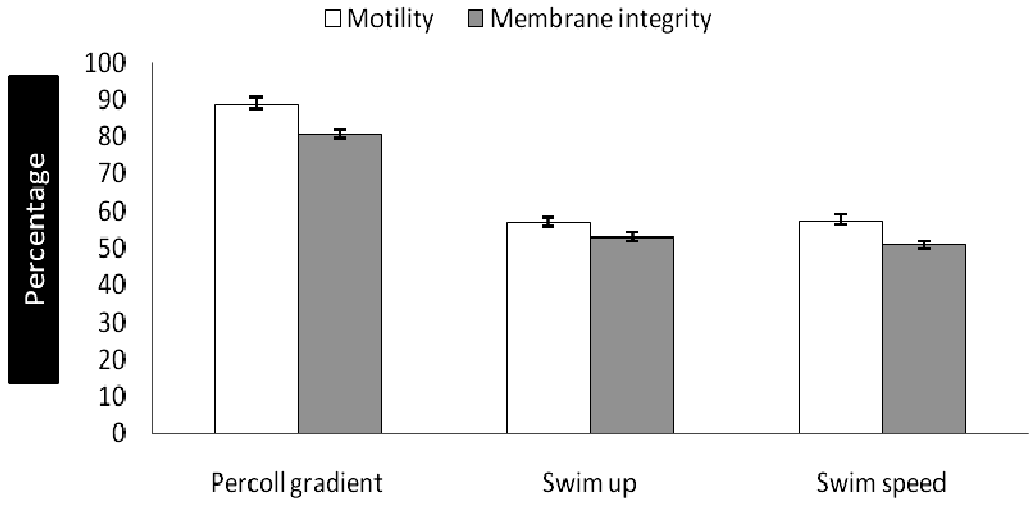

Figure 2. Profile percentage motility and membrane integrity after sperm separation using different methods

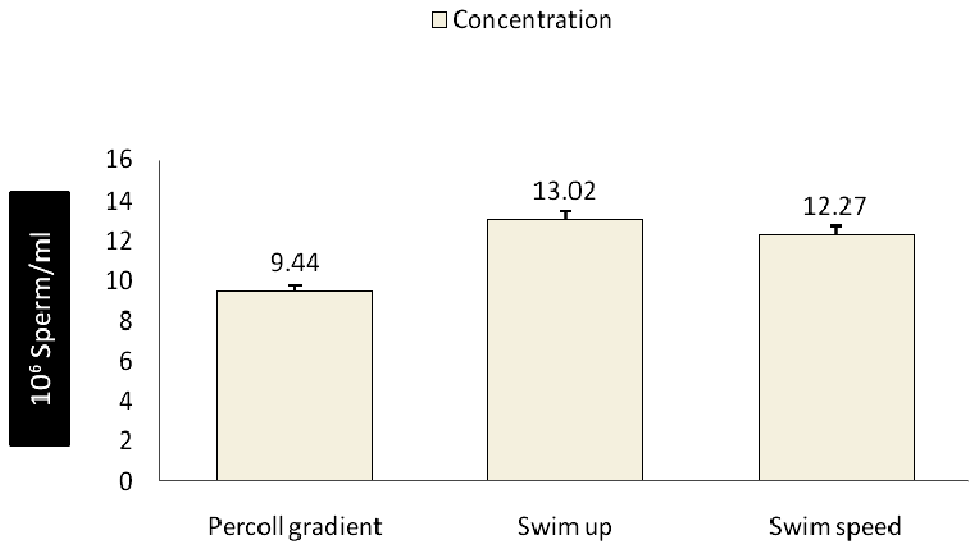

Figure 3. Profile concentration of spermatozoa after sperm separation using different methods.

main components in mucus vagina are alcohols, diols, alkenes, ethers, ketones, amines, esters and aromatic alkanes. These components could influence the denaturation of spermatozoa DNA, as described by Rosenkranz et al. (2002). It is reported that alcohol can affect DNA melting by disturbing the synthesis of RNA.

The DNA content of the X-chromosome bearing spermatozoa in bovine is higher than that of Y-bearing chromosome (Gerner et al., 1983; Johnson, 1995). During the X- and Y-separation process with vagina mucus media, DNA of X and Y-chromosome cause the paired strands of double-stranded DNA to separate into individual single strands. Finally, only X-chromosome spermatozoa could be detected by PCR. DNA of Y-bearing chromosome could not be detected because it contains the small Y-DNA. Analysis using nested PCR could not detect any Y-chromosome bands of 266 bp, whereas spermatozoa of X-chromosome, having band of 329 bp could be detected. These findings are in agreement with previous studies reported by Seidel et al. (1997), Seidel and Johnson (1999) and Seidel et al. (1998) confirming that the sex ratio (SR) of calves born from natural mating tends to be female and calves born from artificial insemination (AI) method tend to be male by SR of about 10 and 90% (in scale of 50% sex ratio).

In other parameter in this present study, after separation of spermatozoa by centrifugation on a Percoll discontinuous gradient (45 to 90%), the percentage of Y- and X-chromosome were 56.67 and 43.33%, respectively. These results are similar to those reported by Kobayashi et al. (2004), stating that percentage of Y-chromosome was 52.9% in bovine spermatozoa with Percoll gradient method. Furthermore, Wang et al. (1994) reported that centrifugation Percoll gradients were 52.5 to 55.7% population of X-chromosome in human sperm.

The mechanism of X- and Y-bearing spermatozoa by discontinuous Percoll gradient is not fully understood. Kanoko et al. (1987) suggested that the spermatozoa separation using Percoll gradients was as a result of different spermatozoa velocity sedimentation. The spermatozoa sedimentation velocity may be influenced by differences in spermatozoa head size or motility (Wang et al., 1994). However, there were no differences among the spermatozoa head dimensions in the top and bottom fractions.

On the other hand, when TALP media separation technique was used, the result showed that all X- and Y-chromosome spermatozoa could be detected, which is similar to the theoretical ratio (50:50). It can be stated that TALP cannot be reliably used for sexing sperm, because after confirmation by PCR, it showed double bands namely of X- or Y-chromosome. This finding is in accordance with the study reported by Yan et al. (2006), in which there were no significant differences among different swim up times in the ratio of X- and Y-chromosome in human.

In the present study, the percentage motility (74.78 ± 0.73%) and membrane integrity (71.22 ± 0.92%) before separation is higher than after the spermatozoa separation using swimming speed and swim up methods, this was suspected during the sexing spermatozoa, to require time and adaptation for each method. The consequence was reducing motility and membrane integrity after separation. The damage of spermatozoa can occur during the freeze-thawed process, due to several factors including cold shock, as well as osmotic and oxidative stress; these results are similar to those reported by Valciircel et al. (1996).

However, the proportion motility and membrane integrity with Percoll gradient was significantly different

($P<0.05$) after the spermatozoa separation. The proportion of the motility (74.78 ± 0.73) and membrane integrity (71.22 ± 0.92) before separation was lower than after separation, that is, 83.63 ± 1.95 and 80.64 ± 1.22 for motility and membrane integrity, respectively (Table 1). This is because during separation process by Percoll gradient, only motile and progressive spermatozoa have the ability to penetrate fractionation by density gradient centrifugation; consequently, the percentage of motility and membrane integrity was lower. This agrees with the report of Samardzi and Karadjole, (2006) that Percoll gradient fractionation clearly separates spermatozoa from external material such as extender particle, cells and bacteria. Furthermore, the Percoll continuous gradient (45 to 90%) centrifugation is widely used to increase spermatozoa motility (Alvarenga and Leão, 2002; Parrish et al., 1995; Suzuki et al., 2003).

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