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EFFECT OF SPERM SELECTION BY PERCOLL AND SWIM UP TECHNIQUES ON THE SEX RATIO OF RABBIT OFFSPRING

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ABSTRACT

This study was conducted to evaluate rabbit semen characterization before freezing and after thawing. Also, to determine the sex ratio of rabbit offspring's after artificial insemination (AI) using semen selected by Percoll or swim up method. Semen was collected from New Zealand white rabbit bucks (n=9) and 45 rabbit does in three groups (15 in each) were inseminated with frozen semen (control, G1), semen selected by 67.5% Percoll (G2) or swim up (G3) methods, respectively. Semen was processed in tris-buffer extender at a rate of 1:4. Results showed that percentage of progressive motility, livability and intact acrosome spermatozoa decreased ($P<0.05$) post-dilution, post-equilibration and post-thawing. Using semen selected by Percoll in does AI resulted in more females (65.8%) and less males (34.2%). Using semen selected by swim up method in AI produced more males (75%) and less females (25%). Control does produced sex ratio of 51.2% males and 48.8% females.
In conclusion, present results showed that, regardless sperm characteristics in post-thawed rabbit semen and fertility results, semen selected by Percoll density at 67.5% and 10 min centrifugation or swim-up can be used for determining the sex ratio of rabbits. This may control the desire to male or female production prior to conception.
Keywords: Rabbit, semen, Percoll, swim up, artificial insemination, sex ratio.

INTRODUCTION

Sperm separation procedures are able to significantly improve sperm quality with a high rate of progressive motility and morphological normal spermatozoa. Advances in DNA technology have enhanced the perspectives of genetic selection in domestic species to increase productivity (Garcia, 2001). Predetermining the gender of offspring in the dairy and beef cattle industry allows the breeders to plan their production toward a specific gender. The most effective way to achieve this goal is separating X- from Y-bearing spermatozoa (Stap *et al*., 1998).
In an attempt to develop a method for the separation of sperm cell populations based on their DNA content, Percoll density gradient centrifugation has been used on human and bovine spermatozoa (Kaneko *et al*., 1983 and 1984; Hossepian-de-Lima *et al*., 2000). Percoll is composed of colloidal silica particles (15-30 nm in diameter) coated with polyvinylpyrrolidone (Samardzija *et al*., 2006), which increases the specific gravity of the medium to 1.13 g/ml (Makler *et al*., 1998). It is used to isolate bacteria (Leuschner *et al*., 1999), neutrophils (Woldehiwet *et al*., 2003), viruses (Hanabusa *et al*., 2000) and subcellular particles (Swales and Wright, 2000; Domart-Coulon *et al*., 2001; Sheoran *et al*., 2005).

Parrish et al. (1995) applied these protocols for the *in vitro* fertilization with bull semen. Somfai et al. (2002) reviewed the results obtained with the

swim up and Percoll gradient methods by various authors and compared them. Majority of authors preferred the use of Percoll gradient centrifugation. However, Brandeis and Manuel (1993) reported that the swim up methods selected preferably motile sperm, while a higher percentage of sperm with intact acrosomes was obtained by Percoll.

Discontinuous 90-45% Percoll density gradient centrifugation is widely used to increase sperm motility (Parrish *et al*., 1995; Alvarenga and Leão, 2002; Suzuki *et al*., 2003).

Swim up spermatozoa may be selected on the basis of their vigor in swimming. This procedure permits the selection of the more motile spermatozoa. To date, this procedure is mainly developed for other species; however, it could be useful to gain further information on rabbit semen (IRRG, 2005). Artificial insemination (AI) in rabbits has been employed since the 1920 and resulted in similar or better pregnancy rates than natural breeding (Harkness and Wagner, 1983). However, there are still many disadvantages of AI such as the small amount of ejaculated semen volume (0.5-2 ml/head, Hong et al., 2012). Johnson (2000) observed that in species where the DNA content difference is greater, such as Chinchilla langier (7.5%), a 100% pure selection is possible, but in species where this difference is small, like human (2.8%), pureness decreases. In cattle the difference (3.8%) is close to the minimum necessary (3.5%) for separation to occur.

The aim of this study was to compare the ability of Percoll gradients centrifugation or swim-up for rabbits spermatozoa separation and assessment separation accuracy through the sex ratio of rabbit offspring after artificial insemination and parturition.

MATERIALS AND METHODS

This study was carried out in International Livestock Management Training Center and Sakha Animal Production Research Station belonging to Animal Production Research Institute during the period from March to June 2013.

Animals:

Nine adult bucks and 45 does (New Zealand White rabbits, NZW) aged 7–10 months and weighing 3-4 kg live body weight. All animals were housed individually in stainless-steel wire cages (50 x 50 x 30 cm).

Semen collection:

Semen was collected by means of an artificial vagina filled with a warm water (about 45°C). Bucks were previously stimulated before semen collection to increase sperm concentration by leaving a doe on top of the buck's cage for several minutes (Hong, et al., 2012).

Two males were placed in a large cage without a cover and when a male mounted the other and showed copulatory behavior an artificial vagina was immediately placed on the inguinal region. If ejaculation occurred then animals were separated. Two ejaculates were collected once a week (in a period of at least 15 min) to obtain good quality semen (Moce *et al.*, 2000).

Semen evaluation:

Semen was prepared according to Hong et al. (2012). As a general rule, conditions were carefully controlled to avoid contamination of the semen samples. Immediately after semen collection, volume was evaluated after remove gel mass. Within 5 min of collection, sperm quality parameters were evaluated before freezing (post-dilution and post-equilibration) and post-thawing for AI.

Sperm cell concentration was determined with a Thoma chamber according to Herak (1991). Percentage of sperm progressive motility was assessed using research microscope with warmed stage (37 ^{o}C) under the high power magnification (x400) according to Amman and Hammerstedt (1980). Sperm livability percentage was determined using eosin and nigrosin mixture stain according to Hachett and Macpherson (1965). Percentage of spermatozoa with intact acrosome was conducted as indicated by Watson (1975).

Semen was diluted with Tris-egg yolk extender consisting of 3.025 g Tris (hydroxymethyl amino methane), 1.675 g citric acid, 0.75 g glucose, 15 ml egg yolk, 7 ml glycerol, 0.005 g streptomycin, 0.25 g lincospectin and completed with bi-distilled water up to 100 ml. Semen was diluted at a rate of 1:4 with a buffer medium at the same temperature to avoid heat or cold shocks.

The extender was gently mixed with pooled semen at 37 ^{o}C in a water bath and cooled gradually in a refrigerator at 5 ^{o}C for 4 hours as an equilibration period. At the end of this period, the extended semen was loaded in 0.25 ml French straws ($20x10^{6}$ motile sperm/straw) using a semen filling machine. Straws were transferred into a processing canister and located horizontally in static nitrogen vapor 4 cm above the surface of liquid nitrogen for 10 minutes. The straws were then placed vertically in canister and immersed completely in liquid nitrogen for storage at -196 ^{o}C. For thawing, straws were dipped into a water bath at 38^{o} C for 30 seconds.

Semen preparation for insemination:

Frozen semen was prepared for three treatment groups. The 1st treatment frozen thawed semen (control). While the 2nd and 3rd treatments were frozen thawed semen prepared with Percoll and swim-up methods, respectively.

Percoll method:

A 90-45% Percoll density gradients was prepared with HEPES-TALP medium and used to prepare 67.5% Percoll density gradient for separate sperm from lipid droplets according to Gliozzi *et al*. (2003) and Somfai et al. (2002), by layering 1 ml of each Percoll fraction in a 15 ml Falcon (conical tube). For sperm selection, 500 µl of thawed semen was layered on the top of the gradient and centrifuged at 700 *g* for 10 min. The supernatant was discarded, the pellet recovered, suspended in 4 ml of HEPES –TALP medium and centrifuged at 300 *g* for 7.5 min to remove residual Percoll. Two-hundred microliters from the washed pellet was recovered.

Swim up method:

The self-migration (swim-up) described by Parrish *et al*. (1995) was used. Spermatozoa may be selected on the basis of their vigour in swimming. This procedure permits the selection of the more motile spermatozoa.

Basically, the procedure is adapted to the use of frozen/thawed semen. One ml TALP medium was poured into Falcons (conical tubes). Semen was thawed (38 ^{o}C, 30 s) and 100 µl were carefully transferred to the bottom of the Falcon so that the TALP medium and the semen extender do not become mixed. The Falcon tube is placed in an incubator for 45 min (37 ^{o}C) without any movement. After the swim up technique, 900 µl of the clear capacitation medium were introduced into a pipette and transferred to a tube for centrifugation at 200 *g* for 10-15 min. The liquid phase is removed and the remainder is re-suspended for estimation of sperm cell concentration. Two-hundred microliters from the pellet were recovered. In each method.

Fertility data and sex ratio:

Forty five rabbit does divided into three groups, 15 does in each. Does in the 1st group were inseminated with frozen thawed semen (control). While does in the 2nd and 3rd groups were inseminated with frozen thawed semen prepared with (Percoll) and swim-up methods, respectively.

Each doe was injected with approximately 0.5 ml prepared semen (20 x 10^{6} sperm/ml) using a glass pipette into the vagina of females. After semen injection, all does were injected with 100 IU of human chorionic gonadotrophin (hCG) (Sigma chemical Co, St, Louis, Mo) via the marginal ear vein to induce ovulation (Quintela et al., 2004). Pregnancy diagnosis was performed by abdominal palpation on day 12 post-mating. At parturition, borns were counted and number of males and females was determined.

Statistical Analyses.

Data were statistically analyzed using computer program of SAS (2002) Institute Inc., Cary, NC, USA (Version 9.0) to test the differences in sperm characteristics. The significant group differences were determined according to Duncan's Multiple Rang Test (1955). The group differences in percentages of male and female kids were analyzed using Chi-square test.

The percentage values were subjected to arcsine transformation before performing the analysis of variance. Means were presented after being recalculated from the transformed values to percentages.

RESULTS

Sperm characteristics in control semen:

Results presented in Table (1) revealed significant ($P<0.05$) reduction in all sperm characteristics including, percentage of motility, livability and intact acrosome during freezing processes (dilution and equilibration) and during thawing. This reduction was 32, 55 and 53%, respectively.

It is of interest to note that all sperm characteristics in post-thawed semen are within the normal ranges of good frozen semen used for AI.

Table (1): Sperm characteristics ofpost-dilution, post-equilibration and post-thawing.

Characteristics (%)	Post-Dilution	Post-eq uilibration	Post-thawing	Reduction rate (%)
Sperm motility	62.5±1.3[a]	53.1±1.2[b]	42.6±1.2[c]	32
Sperm iivability	68.2±3.1[a]	53.6±1.3[b]	30.8±0.5[c]	55
Sperm with intact acrosome	71.9±1.1[a]	59.4±1.1[b]	33.9±0.5[c]	53

a, b and c : Means within the same row with different superscripts are significantly different at (P<0.05).

Fertility and sex ratio of treated semen:

Data presented in Table (2) showed that pregnancy rate was higher for Percoll group (100%, 15/15) than for swim up and control groups (93.3%, 14/15 in each), but the differences were not significant. However, litter size was insignificantly higher for control (5.86/doe) than Percoll and Swim up (5.07 and 5.14/doe, respectively.

On the other hand, there was pronounced differences in sex ration among treatment groups (Table 2). Does in Percoll group produced significantly (P<0.05) more females (65.8%) and less males (34.2%) than the control does (48.8 and 51.2%, respectively). Meanwhile, does in swim up showed an opposite trend of Percoll grouo (25% females and 75% males). Such results indicated marked effect of treatments on sex ratio of rabbits, regardless the effect on pregnancy rate or litter size of does.

Table (2). Effect of treatment on pregnancy rate, litter size and sex ratio of rabbit does.

Item	Control	Percoll	Swim up
Inseminated doe (n)	15	15	15
Pregnant does:			
Number	14	15	14
%	93.3	100	93.3
Produced kids:			
Total borns (n)	82	76	72
Litter size/doe	5.86	5.07	5.14
Males (n)	42	26	54
Males (%)	51.2[b]	34.2[c]	75.0[a]
Females (n)	40	50	18
Females (%)	48.8[b]	65.8[a]	25.0[c]

a,b,c Different superscript letters within rows indicate significant difference (P < 0.05).

DISCUSSION

The present results in this study showed that rabbit sperm characteristics as percentage of motility, livability and intact acrosome spermatozoa decreased significantly (P<0.05) during freezing assessment beginning from dilution to post-thawing. Similar results on sperm motility were reported in human (Mathur et al., 1986), bull (Samardzija et al., 2006) and

rabbit (Hong et al., 2012). It is well known that semen preservation is a main limitation in rabbits artificial insemination. Although several authors indicated a reduction in motility and livability in frozen/thawed rabbit semen (Moce et al., 2003; Si et al., 2005; Castellini et al., 2006; Kashiwazaki et al ., 2006), others showed that fertilizing ability of rabbit semen can be maintained at post-thawing without deleterious effects on fertility (Roca et al., 2000; Lopez-Gatius et al., 2005).

Rabbit insemination using sexed semen has many merits summarized as the follow: Increase supply of replacement in the herds, increase availability of females for export, increase efficiency of progeny testing, increase efficiency of *in vitro* fertilization (IVF) programs, increase efficiency of superovulation (MOET) programs, accelerate genetic progress and increase number of superior bucks.

The main objective of this study was to compare the ability of Percoll gradients centrifugation or swim-up for rabbits spermatozoa separation and assessment separation accuracy through the sex ratio of rabbit offspring after AI and parturition. In this respect, treatment of rabbit semen with Percoll or swim up had a high selection intensity on sex ratio which was related to fertility. Reproductive rhythm had marked effect on seminal parameters of rabbits (Moce et al., 2005). Also, fertility varied according to the physiological stage of the does at the time of insemination (Brun et al., 2002) and type of semen preservation, fresh or frozen (Ragab-Ayat et al., 2012). Also, McEvoy (1992) mentioned that Y-bearing sperm is faster than X-bearing sperm.

According the results of this study, when semen was selected by Percoll density gradient centrifugation and used in AI of rabbit does, more females (65.8%) and less males (34.2%) were significantly ($P<0.05$) produced than the control (Table 2). These results are in disagreement with Johnson (2000) and Wolf et al. (2008) who showed no significant difference on the percentage of male and female embryos as a result of decreasing the amount (2 ml) of Percoll density gradient centrifugation, which makes the heavier spermatozoa reach the bottom faster. The use of larger volume of Percoll gradients in order to make spermatozoa penetration more difficult could be an alternative, while smaller volumes (1-4 ml) may not be enough to promote separation. However, larger volumes (>7 ml) and higher Percoll concentration (close to 90%) would turn the swimming down of lighter sperm more difficult (Johnson, 2000). On the other hand, Wolf et al. (2008) found that 67.5% Percoll density gradient centrifugation for 10 min showed a deviation ($P=0.015$) in the sex ratio toward female embryos (63%). However, the separation threshold between X- and Y- bearing bovine sperm is small because their difference in DNA content is less than 4%.

Contrary, using semen selected by swim up method in insemination of does in this study produced more males (75%) and less females (25%) than the control. Johnson (2000) reported that the Y-chromosome is lighter and smaller than the X-chromosome. The DNA content difference between them is quantified in 3.8% in cattle, which is close to the minimum necessary (3.5%) for separation to occur. In species where the DNA content difference is greater, such as the Chinchilla langier (7.5%), a 100 % pure selection is possible, but in species where this difference is small like human (2.8%),

pureness decreases. The present results regard to swim up method are in agreement with the results of Pegoraro et al. (2002), who observed a significant male and female ratio deviation (P<0.05) toward male embryos using swim-up favors Y- bearing sperm selection, which migrates faster to reach the media surface.

In conclusion, present results showed that, regardless sperm characteristics in post-thawed rabbit semen and fertility results, semen selected by Percoll density gradient at 67.5% and 10 min centrifugation or swim-up can be used for determining the sex ratio of rabbits. This may control the desire to male or female production prior to conception.

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تـأثير انتخـاب الحيـوان المنـوى بـالبيركول و بـالتعوييم علـى النسـبة الجنسـية لنتـاج الأرانب.

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معهد بحوث الانتاج الحيوانى – مركز البحوث الزراعية – الدقى – جيزة - مصر.

أجريت هذه الدراسة لتقييم صفات السائل المنوى للأرانب قبل التجميد وبعد الاسالة, أيضا لتقدير النسبة الجنسية لنتاج الأرانب بعد التلقيح الاصطناعى بالسائل المنوى المنتخب سواء بطريقة البيركول أو طريقة التعوييم. تم جمع السائل المنوى من عدد 9 ذكور أرنب نيوزيلاندى أبيض, وعدد 45 أنثى تم تقسيمها لثلاث مجاميع كل مجموعة تشتمل على 15 أنثى' المجموعة الأولى تم تلقيحها بسائل منوى مجمد (مجموعة مقارنة)' المجموعة الثانية لقحت بسائل منوى تم انتخابه بطريقة البيركول ' المجموعة الثالثة لقحت بسائل منوى تم انتحابه بطريقة التعوييم' واستخدم الترس فى تخفيف السائل المنوى بنسبة 1 : 4.

أوضحت النتائج أن النسبة المئوية للحركة التقدمية والحياتية وسلامة الأكروسوم للسائل المنوى انخفضت معنويا ($P< 0.05$) بعد التخفيف وبعد الموازنة وبعد التجميد والاسالة. باستخدام السائل المنوى المنتخب بطريقة البيركول فى التلقيح الاصطناعى لاناث الأرانب نتج عنه زيادة نسبة الاناث (65.8 %) وقلة نسبة الذكور (34.2%). عند استخدام السائل المنوى المنتخب بطريقة التعوييم فى التلقيح الاصطناعى للاناث نتج عنه نسبة أعلى من الذكور (75%) وقلة نسبة الاناث (25%). تلقيح الاناث فى المجموعة المقارنة نتج عنه نسبة جنسية (51.2 %) ذكور و (48.8 %) اناث.

أوضحت النتائج الحالية علاقة خصائص السائل المنوى للأرانب بعد التجميد والاسالة بنتائج الخصوبة. أيضا' انتخاب السائل المنوى بطريقة البيركول بنسبة 67.5 % لمدة 10 دقائق طرد مركزى أو بطريقة التعوييم يمكن استخدامها لتقدير النسبة الجنسية للأرانب, هذا ربما يقودنا للرغبة فى التحكم فى انتاج الذكور أوالاناث قبل حدوث الحمل.

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