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Development of a Sperm Preparation Method for Artificial Insemination in Rabbits

[1, 2]Jeong-Sup Hong, [3]Wook-Joon Yu, [2]You-Seok Kim, [2]Jin-Sik Cho, [2]Myeong-Kyu Park,
[1]Soo-Min Cho, [1]Hee Yi, [1]Hee-Jung Cho and [1]Ho-Chul Shin
[1]Department of Veterinary Pharmacology and Toxicology, College of Veterinary Medicine,
Konkuk University, Hwayang-dong 1, Kwangjin-gu, 143-701 Seoul, Republic of Korea
[2]Korea Testing and Research Institute 7-6, Gomak-Ri, Wolgot-Myeon, Gompo-Si,
415-871 Kyunggi-Do, Republic of Korea
[3]Korea Institute of Toxicology, 19 Shinseong-ro, Yuseong-gu,
305-343 Daejeon, Republic of Korea

Abstract: A novel method of sperm preparation by direct collection from the cauda epididymis and vas deferens of male rabbits is suggested and validated for improved artificial insemination efficiency in rabbits. Although, conventional methods in which sperm for artificial insemination are prepared using an artificial vagina have been well established in many laboratories due to its benefits over natural mating, this new method eliminates the existing disadvantages of the conventional semen preparation. To compare the quality of sperm collected using an artificial vagina with that retrieved directly from the cauda epididymis, motion parameters of sperm were analyzed with a computer-assisted sperm analyzer. Additionally, after 20 adult females were inseminated artificially with sperm derived from the cauda epididymis, all live dams were subjected to caesarean section on day 28 of gestation and their fetuses were examined for external, visceral and skeletal abnormalities. All data were compared to those of dams impregnated by the traditional artificial insemination method in which the semen was prepared with an artificial vagina. No significant difference was observed in any criteria examined between the new and traditional preparation methods. This new method yields the possibility of collecting more sperm usable in artificial insemination and reducing the time required for sperm preparation. The results of this study indicate that sperm preparation from the cauda epididymis for artificial insemination was faster and more efficient for impregnating rabbits than the traditional method.

Key words: Embryo-fetal development, rabbit, artificial insemination, breeding, sperm, vagina

INTRODUCTION

The rabbit is one of the species commonly used in embryo-fetal development studies because it is sensitive to thalidomide (Seller, 1962; Somers, 1962) and because many regulatory authorities have recommended the rabbit as an appropriate non-rodent species. To obtain pregnant rabbits used in embryo-fetal development studies, Artificial Insemination (AI) or natural mating is essential. AI in rabbits has been employed since the 1920s (Adams, 1961, 1972, 1976, 1981) and results in similar or better pregnancy rates than natural breeding (Harkness and Wagner, 1983). AI permits more controlled management and better planning than conventional natural mating, namely in batch parturition and batch weaning (Adams, 1987). In addition, AI offers the same benefits for rabbit breeding as in other species in the control of genetic diversity, rapid upgrading of stock, establishment of pregnancies in females that refuse to mate and avoidance of diseases such as pasteurellosis and *Treponema cuniculi* (Morrell, 1995). For these reasons, Artificial Insemination (AI) in rabbits has become well established in many laboratories conducting reproductive studies in breeding facilities and in rabbit farms. However, there are still many disadvantages to AI such as the small amount of ejaculated semen (0.5-2 mL/head), the procedural complexity, the time required for collecting semen, the impurities due to contamination with blood or urine and animal maintenance. Especially in the case of using male rabbits who are not accustomed to mounting another buck and then ejaculating semen into an artificial vagina, it takes a long time to collect semen or the collection of semen may be time consuming or even impossible. Moreover, in small-scale laboratories conducting reproductive studies, maintenance and space costs for male rabbits could be problematic. In this study, the

Corresponding Author: Ho-Chul Shin, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Medicine, Konkuk University, 143-701 Seoul, Korea

fertilizing capacity of spermatozoa was assessed during their passage through the male reproductive tract and by *in vitro* culturing of spermatozoa. Sperm retrieved from cauda epididymis become motile very rapidly *in vitro* and were able to fertilize eggs *in vivo* and *in vitro* (Overstreet, 1970; Cummins and Orgebin-Crist, 1971). In this study, researchers introduce a new sperm preparation method from the cauda epididymis and vas deferens for artificial insemination. To validate this new method, pregnancy status as well as embryo and fetus development were examined and the results were compared to those of the vehicle control groups used in embryo-fetal development studies conducted in this facility.

MATERIALS AND METHODS

Animals: Nulliparous female New Zealand White rabbits, aged 4-5 months, weighing on average 3.2~4.0 kg and males, aged 6-7 months, weighing on average 3.5~4.5 kg were obtained from Cheonan Yonam College (Cheonan, Republic of Korea) and used after 2 weeks of quarantine and acclimatization. The animals were housed in a room maintained at a temperature of 23±3°C and a relative humidity of 50±10% with artificial lighting from 08:00-20:00 and with 13-18 air changes/hour. Animals were housed individually in stainless-steel wire-mesh cages and were allowed sterilized tap water and commercial rabbit diet (Purina Korea Feed Co., Pyongtaek, Republic of Korea) *ad libitum*. All experiments were conducted in accordance with the regulation for the care and use of animals (revised adopted 2006/12/1 by Institutional Animal Care and Use Committee).

Semen preparation with an artificial vagina: Semen was prepared according to a method used in previous reports (Breederman *et al*., 1964; Chen, 1989; Kim *et al*., 1996). 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 maintained at 50°C was immediately placed on the inguinal region. If ejaculation occurred then animals were separated. Collected semen were pooled and diluted 10 fold with sterile physiological saline. Typically, 15-20 males were required as semen donors to conduct a single embryo-fetal development study in this facility using this method.

Sperm preparation from cauda epididymis and vas deferens: One adult male rabbit that did not have any experience in semen collection and did not show normal mounting behavior with other bucks was selected to impregnate females. The buck was anesthetized with thiopental sodium and then an incision was made on the left and right scrotum. Testes and Epididymides including some part of vasa deference were removed and testes were discarded. Left and light epididymides were dissected in 10 mL of sterile physiological saline maintained at approximately 38°C. In addition, sperm from the vas deferens were retrieved via flushing. Between 1 and 1.5 mL of sperm suspension was diluted to a total of 50 mL with sterile physiological saline (38°C). In this facility, animals for the sperm preparation are re-used for other studies.

Artificial insemination: An artificial insemination was conducted as follows Kaneda *et al*. (1993). Approximately 0.5 mL of the dilution was injected with a glass pipette into the vagina of females. After semen injection, all females were injected with 10 IU of human chorionic gonadotrophin (Sigma Chemical Co., St. Louis, MO) via the marginal ear vein to induce ovulation (Happer, 1961; Kennelly and Foote, 1965; Quintela *et al*., 2004). Sperm motility was examined microscopically twice, immediately before the start and end of artificial insemination (Cummins and Orgebin-Crist, 1971; Chen, 1989; Quintela *et al*., 2004). The day of artificial insemination was designated as day 0 of gestation.

Motion parameters of sperm in the cauda epididymis and semen: To compare motion parameters of sperms in cauda epididymis and in semen retrieved with an artificial vagina, three sexually mature adult males maintained for use as semen donors in this facility were selected. Semen was retrieved as described. Immediately after semen collection, bucks were anesthetized with zoletil (Virbac Lab, France) and sacrificed by exsanguination from the aorta. To measure motile parameters of sperm, bovine serum albumin (Sigma, St. Louis, MO, USA) was dissolved to a concentration of 0.5% in CO_2-independent medium (Gibco, Grand Island, NY, USA), the pH of the media was adjusted to 7.4 and media were incubated in a water bath at 37°C. Cauda epididymides were removed and an incision was made in cauda epididymis with a fine scissor to suspend sperm in 5 mL of medium. Retrieved semen was also diluted 10 fold with the same media. Sperm motion analysis was conducted at approximately 0.5, 1, 2 and 3 h after preparation using an HTM-TOX IVOS sperm analysis system (Version 12.1, Hamilton-Thorne Research, Beverly, MA, USA) and the following parameters were observed: motility: percentage of motile spermatozoa (%); Path Velocity (VAP); the average velocity of the smoothed cell path (μm sec^{-1}), Straight Line Velocity (VSL): the average velocity measured in a straight line from the beginning to the end of the track (μm sec^{-1}),

Curvilinear Velocity (VCL): the average velocity measured over the actual point to point track followed by the cell (μm sec^{-1}), Amplitude of the Lateral Head displacement (ALH): the mean width of head oscillation as the sperm cells swim (μm), Beat Cross-Frequency (BCF): the frequency of sperm head crossing the average path in either direction (Hz), Straightness (STR): average value of the ratio VSL/VAP (%) and Linearity (LIN): average value of the ratio VSL/VCL (%). Settings used were as follows: frames per second: 60 Hz; number of frames: 30; minimum contrast for motile cell detection: 80; minimum cell size of motile cell detection: 7 pixels; static intensity gates: 0.14-1.84; static size gates: 0.72-8.82; static elongation gates: 0-47; VAP cutoff for non-motile cells: 20 μm sec^{-1} and VSL cutoff for non-motile cells: 30 μm sec^{-1}.

Cesarean section: On day 28 of gestation, dams were anesthetized with thiopental sodium and subjected to caesarean section. The ovaries and uterus of each female were removed and examined for the number of corpora lutea and the status of all implantation sites, i.e., live and dead fetuses, early and late resorptions and total implantations. The uteri with no discernible implantations were placed in 2% sodium hydroxide solution for confirmation of early implantation loss by methods described by Yamada *et al.* (1985). Resorption was classified as early when only placental tissue was visible and as late when placental and embryonic tissue were visible at caesarean section. Live fetuses were weighed individually and evaluated for external morphological abnormalities. Based on these data, pre-implantation loss, post-implantation loss, fetal death and sex ratio were calculated.

Fetal examination: All live fetuses were examined internally for determination of gender and for visceral abnormalities using a fresh-tissue examination technique (Stuckhardt and Poppe, 1984). Evaluation of skeletal abnormalities was performed after clearing 95% ethanol-fixed fetuses with potassium hydroxide solution and staining the skeleton with Alizarin Red S using a modification of Dawson (1926)'s method. External, visceral and skeletal findings were classified as developmental malformations or variations. Researchers have used the terminology suggested in an internationally developed glossary of terms for structural developmental abnormalities in common laboratory mammals (Wise *et al.*, 1997).

Statistical analysis: Data from the treated group were statistically compared to those of the control group using the following methods. The data were analyzed for homogeneity of variance using the F test because the number of compared groups was two. If the F test showed no statistical significance then the data were analyzed by Student's t-test. If the F test showed statistical significance then the data were subjected to Welch's t-test. Data presented as frequencies such as gross findings, copulation index, fertility index and pregnancy index were analyzed by χ^2-test followed by the Fisher's exact test where necessary. A difference was considered statistically significant at $p<0.05$ or $p<0.01$. Statistical analyses were performed by comparing the treatment groups with the control group using the General Linear Model (GLM) procedure found in the SPSS 10.0 Software package (SPSS Inc., Chicago, Illinois, USA).

RESULTS

Motion parameters of sperms in cauda epididymis and semen: The results of the sperm analysis are shown in (Table 1). Motility was within normal ranges at 1 h after preparation and it was markedly decreased at 3 h after preparation in sperm collected from both semen (26.7±21.96%) and cauda epididymis (43.3±15.04%). In contrast, other motion parameters were increased or decreased within normal ranges until 2 (VAP, VSL, VCL, BCF) or 3 h (ALH, STR, LIN) after preparation indicating that motile sperm maintain their activity in spite of a decrease in motility.

Table 1: Motion parameters of rabbit sperm from semen and cauda epididymis

	Semen			
Time (h)	[1]MOT	[2]VAP	[3]VSL	[4]VCL
0.5	62.0±8.190	122.8±13.22	103.8±11.13	217.3±32.18
1.0	57.3±9.500	115.7±13.36	95.0±13.01	200.1±30.65
2.0	57.7±19.86	121.5±18.46	103.7±15.55	206.3±40.60
3.0	26.7±21.96	108.6±33.87	87.5±24.80	187.0±47.88
	[5]ALH	[6]BCF	[7]STR	[8]LIN
0.5	7.1±0.81	40.5±3.72	83.3±1.53	49.3±3.51
1.0	6.3±1.10	40.2±3.62	81.3±5.86	49.3±6.51
2.0	6.4±1.16	41.1±2.90	85.3±4.93	53.0±6.24
3.0	6.6±0.98	34.4±4.26	80.3±3.21	50.0±2.00
	Cauda epididymis			
	[1]MOT	[2]VAP	[3]VSL	[4]VCL
0.5	70.0±3.460	134.1±4.07	99.9±13.20	252.9±22.66
1.0	70.3±4.040	122.2±7.00	94.4±1.140	222.1±21.85
2.0	54.0±7.810	117.4±7.21	96.1±4.040	205.1±26.61
3.0	43.3±15.04	110.9±9.65	91.0±6.730	198.1±18.30
	[5]ALH	[6]BCF	[7]STR	[8]LIN
0.5	8.0±1.12	38.6±2.96	74.0±7.81	42.0±7.94
1.0	6.9±0.72	41.0±2.39	77.0±3.00	44.0±4.36
2.0	6.5±0.90	41.9±2.57	81.3±5.51	48.3±5.69
3.0	6.3±0.57	41.2±1.81	82.0±3.00	48.0±2.00

[1]MOT: Percentage of Motile sperm (%)/[2]VAP: Average Path Velocity (μm sec^{-1}); [3]VSL: Straight Line Velocity (μm sec^{-1})/[4]VCL: Critical Velocity or track speed (μm sec^{-1}), [5]ALH: Amplitude of Lateral Head displacement (μm)/[6]BC: Beat Cross frequency (Hz); [7]STR, VSL/VAP (%)/[8]LIN, VSL/VCL (%)

Table 2: Reproductive data of dams inseminated with sperm from the cauda epididymis and vas deferens or sperm collected with an artificial vagina

Parameters	Cauda epidi./Vas defer	Artificial vagina
No. of examined	20.00	63.00
No. of implantations	20.00	51.00
Conception rate*	100.00	80.95
No. of died (%)	-	1(1.59)
No. of aborted (%)	2 (10.00)	8 (12.70)
No. of early delivered (%)	0 (0.00)	3 (4.76)
No. of with live fetuses (%)	18 (90.00)	48 (76.20)

*No. of implantations/No. of examined ×100

All parameters except STR were increased in sperms collected from the cauda epididymis, compared to those in semen. Consequently, there were no significant changes in motion parameters of sperms collected from semen and the cauda epididymis.

Pregnancy data of artificially inseminated rabbits: Reproductive data from NZW rabbits artificially inseminated with sperm collected from the cauda epididymis/vas deferens and sperm collected using an artificial vagina is shown in Table 2. Twenty does were inseminated with cauda epididymal sperm and all females were impregnated. However, abortion was observed in 2 females on day 16 and 23 of gestation. In addition, no premature birth was found. Total 63 females were inseminated with sperm collected with an artificial vagina and 51 females were impregnated.

Death was observed in 1 animal and abortion was observed in 8 animals during the late gestation period. Premature birth was observed in 3 dams. Abnormal findings such as abortion, premature birth and animal death were considered to be spontaneous because these events have also been reported by other laboratories (Wilson, 1965; Wilson *et al*., 1965; Palmer, 1968; Hartman, 1974; Stadler *et al*., 1983; Morita *et al*., 1987; Nakatsuka *et al*., 1997; Gutierrez-Adan *et al*., 1999; Ujhazy *et al*., 2000; Rommers *et al*., 2006; Vega *et al*., 2008). Total 18 and 48 dams inseminated with sperms from cauda epididymis/vas deferens and sperms collected with an artificial vagina, respectively were subjected to caesarean section in this study.

Caesarean section: Following caesarean section (Table 3) conducted on day 28 of gestation, the number of corpora lutea in dams inseminated with sperm collected from the cauda epididymis/vas deferens and sperm collected with an artificial vagina were 11.2 and 11.8, the number of implantation was 9.1 and 8.9, the implantation rate was 80.8 and 76.5%, the post-implantation loss was 6.13 and 7.26%, the litter size was 8.50 and 8.21, the sex ratio was 0.46 and 0.46, the fetal weight of males was 37.26 and 36.26 g and the fetal weight of females was 36.45 and 36.30 g, respectively. There was no statically significant difference between the two preparation methods. At examination of external abnormalities, dwarfism was observed in 1 fetus of a dam inseminated with sperm of cauda epididymis and short tail, hematoma, dwarfism and umbilical hernia were observed in 1 fetus each of dams inseminated with sperms collected with an artificial vagina.

Table 3: Caesarean section data of dams inseminated with sperm from the cauda epididymis and vas deferens or sperm collected with an artificial vagina

Parameters	Cauda epidi./Vas defer	Artificial vagina
No. of examined	18	48
Corpora lutea: Mean±SD	11.2±1.76	11.8±2.49
	(9.44-12.96)	(9.31-14.29)
Implantation: Mean±SD	9.1±1.73	8.9±2.37
	(7.37-10.83)	(6.53-6.53)
Implantations to corpora lutea (%)	80.8%	76.5%
Fetal deaths		
Resorptions/Dead fetuses	8/2	29/2
Fetal deaths to implantations (%)	6.13%	7.26%
Live fetuses	153	394
Live fetuses per litter	8.50±1.34	8.21±2.47
	(7.16-9.84)	(5.74-10.68)
Male/Female	70/83	183/211
Sex ratio(♂/♀+♂)	0.46	0.46
Fetal body weight (g)		
Male	37.26±3.84	36.26±5.99
	(33.42-41.10)	(30.27-42.25)
Female	36.45±3.62	36.30±5.87
	(32.83-40.07)	(30.43-42.12)
External abnormalities	1 (dwarfism)	4 (short tail, hematoma dwarfism and umbilical hernia)

Fetal examination: The results of visceral and skeletal examinations completed on day 28 of gestation are shown in Table 4. One visceral malformation, misshapen kidneys was found in 1 fetus of dams inseminated with sperm collected with an artificial vagina. Other visceral variations including 1 fetus with a flexion ureter, 8 fetuses with a misshapen thymus, 1 fetus with a malpositioned left carotid artery and malpositioned kidney were observed from dams inseminated with sperm collected from the cauda epididymis/vas deferens, respectively and 2 fetuses with a dilated renal pelvis, 2 fetuses with a dilated ureter, 1 fetus with a flexion ureter, 27 fetuses with a misshapen thymus, 7 fetuses with a malpositioned left carotid artery and 2 fetuses with a malpositioned kidney were observed from dams inseminated with sperm collected with an artificial vagina. In dams inseminated with sperm from the cauda epididymis and vas deferens, no skeletal malformation was found although, skeletal variations

Table 4: Visceral and skeletal examinations of fetuses whose dams were inseminated with sperm from the cauda epididymis and vas deferens or sperm collected with an artificial vagina

Parameters	Cauda epidi/ Vas defer.	Artificial vagina
Litters examined	18.00	48.00
Fetuses examined	153.00	394.00
Visceral		
Litters with malformations	0 (0.00)	1 (2.08)
No. of fetuses with Visceral malformation (%)	0 (0.00)	1 (0.25)
Misshapen kidney	-	1.00
Litters with variations	7 (38.90)	23 (47.90)
No. of fetuses with Visceral variations (%)	11 (7.00)	41 (10.40)
Dilated renal pelvis	-	2.00
Dilated ureter	-	2.00
Flexion ureter	1.00	1.00
Misshapen thymus	8.00	27.00
Malpositioned left carotid artery	1.00	7.00
Malpositioned kidney	1.00	2.00
Skeletal		
Litters with malformations	0 (0.00)	2 (4.20)
No. of fetuses with skeletal malformations (%)	0 (0.00)	2 (0.50)
Fused sternebra	-	1.00
Fused rib	-	1.00
Litters with variations	16 (88.90)	45 (93.80)
No. of fetuses with skeletal variations (%)	108 (70.60)	287 (72.80)
Full supernumerary rib	102.00	268.00
Misshapen sternebra	5.00	13.00
Misaligned sternebra	-	1.00
Unossified maxilla	-	1.00
Short supernumerary rib	1.00	4.00
Litters with retardations	5 (27.80)	14 (29.20)
No. of fetuses with skeletal retardations (%)	7 (4.60)	23 (5.80)
Dumbbell ossification of thoracic centrum	2.00	7.00
Dumbbell ossification of lumbar centrum	-	1.00
Incomplete ossification of pubis	5.00	13.00
Bipartite ossification of sternebra	-	1.00
Hemicentric ossification of thoracic centrum	-	1.00

including 102 fetuses with a full supernumerary rib, 5 fetuses with a misshapen sternebra and 1 fetus with a short supernumerary rib were observed, respectively and retardation dumbbell ossification of the thoracic centrum and incomplete ossification of the pubis was observed in 2 and 5 fetuses, respectively. In dams inseminated with sperm collected with an artificial vagina, two skeletal malformations, a fused sternebra and a fused rib were observed in 1 fetus each. As for skeletal variations, 268 fetuses with a full supernumerary rib, 13 fetuses with a misshapen sternebra, 1 fetus with a misaligned sternebra, 1 fetus with a unossified mailla and 4 fetuses with a short supernumerary rib were observed, respectively and skeletal retardations such as dumbbell ossification of the thoracic centrum, dumbbell ossification of the lumbar centrum, incomplete ossification of the pubis, bipartite ossification of the sternebra and hemicentric ossification of the thoracic centrum were observed in 7, 1, 13, 1 and 1 fetuses, respectively. There were no tatistically significant differences in the incidence of visceral and skeletal malformations, variations and skeletal retardation between the two methods.

Time course of artificial insemination: In the case of the artificial insemination using an artificial vagina an average of 1~2 h (Kaneda *et al*., 1993; Kim *et al*., 1996) or more was taken from the male semen collection to the termination of artificial insemination. In contrast, in the case of the new method, all work was completed within 30 min. Furthermore, regardless of the state and condition of the animals, the time and period of the artificial insemination could be arbitrarily controlled.

DISCUSSION

Artificial insemination in rabbits is well established in many laboratories, breeding facilities and rabbit farms. The merits of artificial insemination compared to natural mating in rabbits are follows:

- Efficient use of semen
- Low disease transfer risk
- Facilitation of elite gene utilization
- Facilitation of batching
- Reduced labor requirements (time saving)
- Extended buck use
- Allowing reproduction from non-receptive females and ability to schedule pregnancy

In spite of these merits, there are several drawbacks to conducting semen preparation in facilities that do not conduct artificial insemination routinely. First, these facilities are not able to maintain males for a long time because the males are used once or twice and then sacrificed. Second, most males used in semen collection are not accustomed to mounting a teaser and ejaculating in an artificial vagina. Therefore, it takes a great deal of time to prepare semen for artificial insemination and some males cannot be used if they do not show any sexual behavior. However, these problems could be solved using the new preparation method.

The results presented here show that artificial insemination with sperm retrieved from the vas deferens and cauda epididymis of rabbits is viable and useful for impregnating female rabbits. Generally when conducting an embryo-fetal development study in rabbits, 80-100 mated females are needed. In this facility, 20-24 females per day are inseminated artificially and a total of 92 inseminated females obtained >4 days are used in one study. To obtain 92 inseminated females with semen prepared using an artificial vagina, 20 males are purchased from an animal supplier. Among these males, approximately 15 males are used because the remainder

failed in semen collection. Used males are sacrificed or are maintained for use in the next study. In contrast when females are inseminated with sperms prepared from the cauda epididymis and vas deferens, one male is able to inseminate a maximum of 50 females although, only 20 females were inseminated in this study. In addition, this new method markedly reduces the time required to prepare sperm for insemination compared to traditional methods although, the used animals are sacrificed. In fact, only approximately 30 min were required to inseminate 20 females using this new sperm preparation method whereas 2 h were required for semen preparation. The higher concentration of sperm yielded per head from the new method enables us to attain quantitatively and qualitatively better sperm from a minimal number of male rabbits which provides increased practicality in achieving maximum normal impregnation.

The method presented here provides economic benefits to facilities that conduct artificial insemination only occasionally. In spite of the merits of this method, there are some considerations that must be taken in applying this method to obtaining pregnant rabbits. It is possible to collect highly concentrated sperm from the cauda epididymis compared to that of semen. However, sperm in the cauda epididymis are not fully matured and therefore, might cause delayed fertilization, fertilization failure or abnormal growth of embryos and fetuses. To detect possible disorders in ova fertilized with sperm collected from the cauda epididymis *in vivo*, researchers examined embryo-fetal developments in dams inseminated with sperm collected from the cauda epididymis and in semen prepared using an artificial vagina. There were no differences in any criteria examined between sperm collected from the cauda epididymis and in semen indicating that sperm in the cauda epididymis are able to fertilize ova properly and have no negative effects on embryo-fetal development.

CONCLUSION

AI with sperm collected directly from the cauda epididymis and vas deferens in rabbits, effectively reduces the time, effort, space, cost and number of male rabbits required compared to sperm collected with an artificial vagina and is therefore, a better choice for artificial insemination in rabbits than the traditional method.

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