Fertility response evaluation of artificial insemination methods in sheep with fresh and frozen-thawed semen

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Abstract

The aim of this study was to evaluate the fertility response of artificial insemination (AI) methods with fresh and frozen sperm in sheep. *In experiment 1*, one hundred and fifty fat tailed Zandi ewes were assigned into 3 equal groups and inseminated with three AI methods consisting of vaginal, laparoscopic and trans-cervical AI with fresh semen. In experiment 2, a factorial study (3 AI methods×2 extenders) was used to analyze the effects of three AI methods and two freezing extenders containing soybean lecithin (SL) or Egg yolk (EY) on reproductive performance of 300 fat tailed Zandi ewes. Also, total motility, progressive motility, viability and lipid peroxidation of semen were evaluated after freeze-thawing in two extenders. In result, there was no significant difference among three AI methods when fresh semen was used. In experiment 2, the highest percentage of pregnancy rate, parturition rate and lambing rate were obtained in laparoscopic AI group ($P<0.05$). Although pregnancy rate, parturition rate and lambing rate in trans-cervical group were higher ($P<0.05$) than vaginal group, the results were not as high as laparoscopic group. No difference was observed between SL and EY extenders and their performance was close to each other. It can be concluded that although no difference was observed on reproductive performance for fresh semen, trans-cervical AI was more efficient than vaginal method when frozen-thawed semen was used, but its efficiency was not as high as laparoscopic method. Also, SL extender can be an efficient alternative extender to preserve ram sperm during cryopreservation procedure without adverse effects of EY.

Keywords: Artificial insemination; Frozen-thawed semen; Reproductive performance; Zandi ewes.

Introduction

AI in ewe is efficiently (40-60% conception rate) conducted with fresh semen through cervix [2,10]. However, there are several problems during using fresh semen such as small numbers of superior rams and crucial condition of fresh semen transportation which encourage farmers to use frozen semen. However, the post insemination conception rate with frozen-thawed semen is not realistic [21]. Special structure of ewes' cervix and lack of efficient AI method are the main reasons of this problem [21]. Long fibrosis tubular structure and interior rings of cervix are the most important obstacles against pipette in trans-cervical AI [14]. On the other hand, Laparoscopic AI has been used as an efficient method for AI, but it has some limitations including high cost and time consuming [8,31]. Therefore, using viable sperm with high quality requires an efficient strategy to improve the AI performance in sheep. Sperm may be provided for AI with two methods; the first method is to use fresh semen that has several problems such as short life, time limitation of transportation and hygienic risks. The second one is to use of frozen semen that has several advantages [11, 20]. Recently, results of sperm cryopreservation using a novel extender based on soybean lecithin have shown suitable effects on sperm quality [27]. These studies showed the ability of SL to increase the quality and fertility potential of post-thawed semen [26]. SL also increased the mitochondria potential and acrosome integrity of ram sperm [5] and decreased MDA formation of goat semen [22]. To our knowledge, no attempt has been made to evaluate the reproductive performance of trans-cervical AI using fresh and frozen semen (in SL and EY extenders) in ewe. Therefore, this study was conducted to compare the efficiency of trans-cervical artificial insemination with laparoscopic and vaginal AI using fresh and frozen-thawed semen (with two types of extenders containing SL and EY).

Materials and methods

72 *Experiments design*

73 Four hundred and fifty fat tailed Zandi ewes with 55 ± 5 kg weight and 3-4-year-old were
74 used in this study during breeding season. Two experiments were conducted to evaluate
75 reproductive performance of ewe with three AI methods using fresh sperm (experiment 1)
76 and frozen sperm (experiment 2).

77 *Experiment 1 (Fresh semen)*

78 One hundred and fifty ewes received CIDR (Easy-Breed™, CIDR®, New Zealand) for 12
79 days and after CIDR removal they received 500 IU eCG (Sanofi Animal Health, Libourne
80 Cedex, France) and were assigned into 3 equal groups (50 ewes per each group) for
81 insemination, 51 h after CIDR removal [2].

82 Semen was collected using artificial vagina from ten superior Zandi rams for AI. To
83 eliminate individual differences, the semen was pooled and then diluted 1:1 (v:v) with
84 extender and finally loaded in straws (0.25 mL). Semen was accepted for experiment if the
85 following parameters were observed: volume about 1.5 ml; sperm concentration of 3×10^9
86 sperm/ml and total motility of at least seventy percent.

87 First group of ewes was artificially inseminated via vaginal method; second group was
88 inseminated via laparoscopic method and in the third group, 100 IU oxytocin (Abureihan
89 Pharmacy; 10 IU/ml Oxytocin, Iran) was injected to each ewe 20 min before insemination
90 and the ewes were trans-cervically inseminated.

91 *Experiment 2 (Frozen-thawed semen)*

92 For this experiment, semen was collected using artificial vagina twice a week from ten
93 mature Zandi rams (3 and 4 years of age). Semen was evaluated after ejaculation and
94 accepted for experiment if the following parameters were observed: volume ranging between
95 1 - 2 ml; sperm concentration of 3×10^9 sperm/ml and the percentage of total motility of
96 sperm was higher than seventy percent. In order to eliminate individual differences, semen

samples were pooled and then aliquoted into two equal parts and diluted with 2 extenders (SL and EY) for freezing. The diluted semen was gradually cooled to 4°C for 90 min and subsequently loaded in 0.25 ml French straws (Biovet, L'Agile France). Then the straws were exposed to static nitrogen vapor (-70 °C) for 10 min, plunged into liquid nitrogen (LN₂) and stored in LN₂ until thawing and using for evaluation of sperm parameters and in vivo fertilization.

The basic cryopreservation medium was composed of 2.71 g Tris (Merck, Darmstadt, Germany), 1.0 g fructose (Merck), 1.4 g citric acid (Merck), 7 % glycerol (v/v), 100 IU penicillin and 1 mg streptomycin. The osmolality and pH were set at 320 mosm/kg water and 7.2, respectively. The plant origin extender (SL) consisted of basic medium supplemented with 1% soybean originated phosphatidyl choline. The animal origin extender (EY) as control extender was composed of basic medium supplemented with 20% egg yolk. Sperm which was frozen in these two types of extenders were used for different methods of artificial insemination, vaginal, laparoscopic and trans-cervical, respectively.

Frozen-thawed semen evaluation

Total and progressive motility of frozen-thawed semen in extenders containing SL and EY were analyzed using sperm class analyzer (SCSA). Sperm viability was evaluated via the eosin–nigrosine stain. The viability was assessed by counting 200 spermatozoa for unstained heads of spermatozoa (live) and/or stained/partial stained heads of spermatozoa (dead) under phase-contrast microscopy at 400 x [22]. MDA concentrations were measured as an index of lipid peroxidation according the study of Esterbauer and Cheeseman (1990). The quantification of thiobarbituric acid reactive substances as performed by comparing the absorption with the standard curve of malondialdehyde equivalents generated by the

acidcatalyzed hydrolysis of 1,1,3,3-tetramethoxypropane. The MDA concentration was expressed in nmol/ml.

Pregnancy diagnosis and other parameters

Pregnancy diagnosis was accomplished by an ultrasound unit (Falco 100, premedical) equipped with a 3.5 MHz sector probe on day 50 post insemination. Other parameters such as parturition rate, lambing rate and twinning rate were recorded after parturition.

Pregnancy rate = number of pregnant ewes / number of ewes inseminated x100. Lambing rate = number of lambs born / number of ewes inseminated x100. Parturition rate = number of ewes lambled / number of ewes inseminated x100. Twinning rate = number of twins / number of parturitions x100.

Statistical analysis

Pregnancy rate, parturition rate, lambing rate and twinning rate were analyzed using GENMOD procedure of SAS (9.1) and the significant difference between groups was tested via Chi-square. The T-test was used to compare the LSM for sperm parameters (motility, viability and MDA production)

Results

Table 1 showed the fertility results of ewe inseminated with different methods for fresh semen (experiment 1). There were no significant differences among three artificial insemination methods following AI with fresh semen. Pregnancy rate, parturition rate, lambing rate and twinning rate for vaginal method (62, 56, 62, and 10%), laparoscopic method (66, 60, 64, and 6 %) and trans-cervical method (64, 58, 62, and 6%) were recorded in table 1.

Fertility results of ewe inseminated with frozen-thawed sperm have been shown in table 2. The highest percentage of pregnancy rate, parturition rate and lambing rate were obtained by laparoscopic artificial insemination ($P < 0.05$). Although pregnancy rate, parturition rate and lambing rate in trans-cervical group were higher ($P < 0.05$) than vaginal group, the reproductive performance was not as high as laparoscopic group. Twinning rate was not affected by different insemination techniques.

Total and progressive motility of frozen-thawed semen in SL (44.83 ± 1.56 and 28.83 ± 1.40) and EY (44.33 ± 1.56 and 28.50 ± 1.40) extenders did not show any significant differences. Viability records of frozen-thawed semen showed no significant differences between SL (39.66 ± 1.70 %) and EY (39.16 ± 1.70 %) extenders. As related to MDA formation, no significant differences were observed between SL (4.00 ± 0.12 nmol/ml) and EY (4.14 ± 0.12 nmol/ml) groups (table 3).

Discussion

Artificial insemination improves the chance of using superior rams which helps to control genitally diseases. Our results showed that fresh semen had not significant different results among groups because fresh semen contains enough motile spermatozoa and enough spermatozoa will reach to fertilization site and AI method had no effect on fertility which this result is in agreement with Armstrong and Evans (1984) and Fair et al. (2007).

When we used frozen semen, the methods of AI were significantly different in case of reproductive performance. Technology of semen cryopreservation helps to preserve superior ram, but semen is sensitive due to cold shock and sperm motility will decrease after freezing-thawing process leading to lower fertility rate [6].

The deeper intra cervix insemination causes to more sperm reach to fertilization site and subsequently improve fertilization rate which leading to increase pregnancy rate in ewe [1,6]. For this purpose, laparoscopic AI has been used to improve fertility rate of frozen-thawed semen and this method was almost successful, but its limitations encouraged researchers to develop trans-cervical AI [32]. Our hypothesis was that quality of sperm after freeze-thawing is a crucial factor that can affect fertility results post insemination in three methods of AI. Although results of fresh semen were not significantly different using three methods of AI, frozen semen produced the higher results of pregnancy in trans-cervical compared to vaginal method. The higher efficiency of frozen sperm in trans-cervical method is mostly related to the unique structure of ewe's cervix which should be dilated for passing trans-cervical artificial insemination pipette [32]. Therefore, we injected oxytocin to ewes 20 min before insemination. Oxytocin initiates collagenolytic enzyme process which leading to cervical dilation [13] and it has been recorded that oxytocin caused to cervical dilation in ewes [15,17,18,23]. This effect is mediated by COX₂ mRNA expression and production of PGE₂ [4,28]. PGE₂ starts remodeling of cervix's extracellular matrix which cause to cervical dilation [16,30].

Moreover, oxytocin reduces the cervix injuries during insemination and transcervical embryo transfer and increases the functional speed during embryo recovery [29,32]. Also, oxytocin increases uterine constrictions without any deleterious effects on sperm transfer into the ampulla [23,24].

With regard to useful effect of oxytocin on cervical dilation, it was used for developing trans-cervical AI. When using frozen-thawed semen, pregnancy rate, parturition rate and lambing rate were higher in laparoscopic AI group than trans-cervical AI and vaginal AI. Because semen was deposited into uterine horns instead of cervix entrance for vaginal AI,

more sperm could reach to the fertilization place. But the efficiency of trans-cervical method was not as high as laparoscopic method.

Also, we applied two extenders (soybean lecithin and egg yolk based extender) for semen cryopreservation. In previous studies [12,19,26], SL had shown beneficial effects for protection of ram sperm against cryoinjuries because it can adhere to the sperm plasma membrane and preserve the sperm against ROS [26]. Moreover, several problems such as contamination, agglutination, and standardization of EY contained extenders lead to the increment of request for an egg yolk replacement in cryopreservation media [19, 25]. In some studies, SL had higher protection effects [5] compared to EY and in some others SL had equal effects compared to egg yolk [19]. Therefore, our hypothesis was which extender (SL or EY) is more efficient for cryopreservation of sperm during insemination via three AI methods. In laboratory parameters and fertility results, extenders had no significant effects on frozen-thawed sperm quality and they had equal preservation effects. Also, no interaction effect was observed between extenders and AI methods. However, regarding mentioned problems of egg yolk based extenders, we would rather use SL in cryopreservation media for frozen storage of ram semen.

Conclusion

Trans-cervical artificial insemination could be conducted in ewe by injecting oxytocin hormone. There was no difference on reproductive performance of vaginal, trans-cervical and laparoscopic artificial insemination when fresh semen was used, so vaginal artificial insemination is an efficient, cheap and applied method during using fresh semen. Trans-cervical artificial insemination was more efficient than vaginal method when frozen-thawed semen was used, but its efficiency was not as high as laparoscopic method. Regarding to the same effects of soybean lecithin and egg yolk based extenders on the performance of frozen-

thawed semen, soybean lecithin can successfully be used instead of egg yolk as a supplement for cryopreservation medium, without adverse effects on post-thawed sperm quality and fertility.

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Table 1, Effect of type of artificial insemination on reproductive performance of Zandi ewes with fresh semen

AI	Vaginal	Laparoscopic	Trans-cervical
Pregnancy rate (%)	62 (31/50)	66 (33/50)	64 (32/50)
Parturition rate (%)	56 (28/50)	60 (30/50)	58 (29/50)
Lambing rate (%)	62 (31/50)	64 (32/50)	62 (31/50)
Twinning rate (%)	10 (3/28)	6 (2/30)	6 (2/29)

The number in parentheses indicates the observation frequency in each group.

Table 2, Effect of type of artificial insemination on reproductive performance of Zandi ewes with frozen-thawed semen

AI	Vaginal		Laparoscopic		Trans-cervical	
Extender	SL	EY	SL	EY	SL	EY
Pregnancy rate (%)	4 ^c (2/50)	4 ^c (2/50)	44 ^a (22/50)	42 ^a (21/50)	30 ^b (15/50)	32 ^b (16/50)
Parturition rate (%)	4 ^c (2/50)	2 ^c (1/50)	42 ^a (21/50)	40 ^a (20/50)	22 ^b (11/50)	22 ^b (11/50)
Lambing rate (%)	4 ^c (2/50)	2 ^c (1/50)	42 ^a (21/50)	40 ^a (20/50)	22 ^b (11/50)	20 ^b (10/50)
Twinning rate (%)	0	0	0	0	0	0

The number in parentheses indicates the observation frequency in each group. Different superscripts (a, b, c) between groups indicate a significant difference ($P < 0.05$).

369 Table 3, total and progressive motility, viability and lipid peroxidation of frozen-thawed
 370 semen in extenders containing SL and EY.

	SL	EY	SEM
Total motility (%)	44.83	44.33	1.56
Progressive motility (%)	28.83	28.50	1.40
Viability (%)	39.66	39.16	1.70
Lipid peroxidation (nmol/ml)	4.00	4.14	0.12

371 Sperm samples were collected from 10 rams (5 rams per each group) for cryopreservation in two extenders
 372 (soybean lecithin and egg yolk extenders). Means $\pm$ standard error of the total and progressive motility, viability
 373 and lipid peroxidation were assessed after thawing.

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