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Effect of Ram Breed on the Efficiency of *in vitro* Development of Sheep Embryos

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The aim of this work was to investigate the impacts of ram breed on in vitro embryo development from fresh or frozen semen. Semen was collected from Najdi and Naimi rams and frozen; the mass and progressive motility of the spermwere assessed in each trial before and after freezing. Then, 970 oocytes in six replicates were fertilized with fresh and frozen semen in vitro. Different stages of sheep embryos were recorded. There were no significant differences in mass and progressive sperm motility of fresh or frozen ram semen between Najdi and Naimi,but there were significant differences between frozen and fresh semen within each breed. Our results showed significant ($P<0.05$) differences in 2-cell stage, 4-cell stage, 8-cell stage, morula, fragmented embryos, cleavage and blastocyst rates in the frozen semen group compared to fresh semen group in both breeds. In addition, significant ($P<0.05$) differencesbetween the two breeds were shown in 8-cell and16-cell embryonic stages.In conclusion, there were slight breed effects on the efficiency of in vitro development of sheep embryos.

Keyword: *In vitro* embryo production, Semen Cryopreservation, Naimi sheep, Najdi sheep.

The Najdi and Naimi sheep are two common breeds on the Arabian Peninsula. Both are fat-tailed sheep. The Najdi breed is black, and Naimi is white with a distinguished brown head. They are highly adapted to survive and reproduce under harsh conditions in the desert climate. However, the high temperature can stress the reproductive capacity of the ram (Aggarwal & Upadhyay 2013). Semen quality is the major factor that determines male reproductive efficiency, and it is influenced by breed, geographical location, and season (Karagiannidis *et al.,* 2000). Furthermore, clear differences in the semen characteristics were found among breeds and within individuals from one breed (Mert *et al.,* 2009). These differences may modulate semen composition and impair cryopreservation and in vitro fertilization procedures or even in vitro embryo development. Assessment of general features of the spermatozoa is the first step in animal reproduction labs to estimate the male fertility. Motility, membrane integrity, viability, organelle and DNA integrity of sperm are the parameters most commonly used to evaluate semen in vitro (Garcia-Alvarez *et al.,*

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2009). Nonetheless, the relationship between different sperm parameters and fertility varies greatly among studies (Hallap *et al.,* 2005; Hallap *et al.,* 2006; O' Meara *et al.,* 2008).

Several studies have developed techniques to assess male fertility. Zonapellucida penetration can test the fertilizing capacity of the sperm (Braundmeier *et al.,* 2002). However, an accurate method to assess the sperm efficiency is in vitro fertilization because it evaluates the interactions between spermatozoa and oocyte measuring different endpoints in the early development stages of the embryo (García-Álvarez *et al.,* 2009). Furthermore, many studies suggest that using frozen ram semen in IVF is a useful technique to test ram fertility (Byrne *et al.,* 2000; O' Meara *et al.,* 2005). However, to the best of our knowledge, there are no reports of in vitro embryo production to compare and use Najdi and Naimi fresh or frozen semen. Thus, this study investigated the effects of ram breed on the efficiency of in vitro development of sheep embryos.

MATERIAL AND METHODS

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich Co. (St. Louis, USA). Media were from Caisson Lab (USA) and Tryladil was from Minitübe Co. (Germany). This study was approved by the Research Ethics Committee of King Saud University.

Rams were housed at King Saud University, Faculty of Food Sciences and Agriculture, Riyadh, Saudi Arabia and were maintained under uniform nutritional conditions. Fresh water was provided ad libitum and natural feeding was also offered during this study. Semen samples were collected from five rams (proven fertility) of each breed with an electric ejaculator. Each ejaculate was immediately evaluated for volume, motility and concentration. Only semen with good quality (mass motility >80%, progressive motility >60%, concentration >2.5× 10^9 sperm/ml) was cryopreserved. Good quality semen was diluted in a solution of 5mL Tryladil, 15 mL distilled water, and 5mL (20%) egg yolk (v/v). After dilution, semen was packed in 0.25 mL (50× 10^9spz), sealed with polyvinyl alcohol powder and equilibrated (4°C) for 4 h. After equilibration, the straws were frozen in a freezing machine (Freeze-control-C1-8000) over 40 min and then submersed and kept in a liquid nitrogen (196°C). Semen straws were thawed in a water bath (37°C) for 30 s and were used for in vitro fertilization.

Ovaries were collected from the central slaughterhouse in physiological saline (0.9% NaCl) and transported to the laboratory within 1-2 hours. The ovaries were washed three times in physiological saline at 37°C. Oocytes were aspirated from antral follicles (2-8 mm in diameter) using 19 G needle attached to a 10 mL syringe filled with 0.5 mL collection media (TCM-199 Hank's salts) and supplemented with 10% FBS, 0.5 mM sodium pyruvate, 50 µg/mL gentamycin sulfate, and (140 µg/mL) heparin sodium salt. The aspirated follicular fluid was transferred into a 90 mm petri dish. The oocytes were surrounded by more than two layers of cumulus cells, and the homogeneous cytoplasm was selected. Oocytes were washed 2-3 times in collection media and then transferred into 50ìL of maturation medium under mineral oil. This maturation medium was TCM-199 Earle's saltssupplemented with 10% fetal bovine serum (FBS) (v/v), (0.5 mM) sodium pyruvate, 1µg/mL estradiol-17â, 0.02 IU/mL FSH, 0.023 IU/mL LH, 100µM cystamine, and 50 µg/mL gentamycin sulfate. Thiswas washed three times in maturation medium. Droplets containing oocytes were kept in an incubator at 38.5°C in a humidified atmosphere with 5% O_2 and 5% CO_2 for 24 hours.

Before in vitro fertilization, the total and progressive motility in fresh and frozen semen of two breeds was assessed by a Computer-Assisted Semen Analysis (CASA) in each experiment. Then, fresh and frozen-thawed semen were washed in capacitation medium (HEPES-Tyrode'salbumin lactate pyruvate) containing (6 mg/mL) BSA, (50 µg/mL) gentamicin, and (0.5 mM) Na-pyruvate. This was centrifuged twice at 1800 rpm for 5 min. For swim up, 0.5 mL of semen was kept under 1 mL of capacitation medium in a 15 mL conical Falcon tube at 38.5 ºC for one hour. Before transfer to fertilization drops, the oocytes were washed four times in IVF-Tyrode'salbumin lactate pyruvate with (6 mg/mL) BSA, (140 µg/mL) heparin and (50 µg/mL) gentamycin. Insemination used 2.0×10^6sperm into fertilization medium that included 10 oocytes per 50 µL fertilization drop in an atmosphere of 5% CO_2 and humidified air at 38.5ºC for 24 hours.

After in vitro fertilization, zygotes were removed from the cumulus cells by pipetting and washed in SOF medium supplemented with 1% (v/v) BME-essential amino acids, 1% (v/v) MEM nonessential amino acids, (50 µg/mL) gentamycin and (6 mg/mL) BSA. They were then allocated to a 50 µL drop of SOF medium (25 embryos/drop). The incubation conditions were humidified by 5% O_2, 5% CO_2, and 90% N_2 at 38.5 ºC. The culture continued for 8 days post-fertilization. Cleavage rates were recorded in fresh or frozen semen experimentsfrom the two breeds as the number of cleaved embryos per number of total oocytes. Embryo developmental rates were counted as the number of blastocysts per number of cleaved embryos on day 8.

Statistics anaylsis

A general linear model (GLM) statistical analysis system was used for analysis of variance (ANOVA). A factorial 2×2 design experiment was used to examine the effects of breed (Naimi and Najdi) and semen (fresh and frozen) on in vitro embryo production. Data were expressed as a mean ± standard error of the mean for all parameters. Least significant difference (LSD) used at a level of P-values<0.05.

RESTULS AND DISSICSSION

Here, two sperm parameters were used: 1) sperm motility to measure sperm ability to penetrate the oocyte and 2) progressive motility to measure the efficient penetration according to (Zarazaga *et al.,* 2009). Mass sperm motility and progressive sperm motility in Najdi and Naimi rams before and after cryopreservation are shown in Table 1. The results demonstrated no differences in mass and progressive sperm motility between Naimi and Najdi rams. This result was confirmed by Letsoaloet al., (2016) who found no differences in semen quality between Namaqua Afrikaner, Döhne Merino and Dorperbreeds. This

Table 1. Mass sperm motility and progressive sperm motility in Najdi and Naimi rams before and after cryopreservation

Breed	Semen	N	Mass motility (%)	Progressive motility (%)
Naimi ram	Fresh	30	(83.90±0.81)[a]	(60.87±1.55)[a]
	Frozen	30	47.53±1.41)[b])	15.73±0.52)[b])
Najdi ram	Fresh	30	82.07±1.06)[a])	57.77±1.21)[a])
	Frozen	30	43.13±2.05)[b])	(14.63±0.51)[b]

The means in each column with the same superscript(s) are not significantly different.

Table 2. Different in vitro development stages of sheep embryo after insemination by the fresh and frozen semen of Naimi and Najdi rams

	Naimi ram Fresh semen	Frozen semen	Najdi ram Fresh semen	Frozen semen
N. of oocytes (R)*	227 (6)	227 (6)	258 (6)	258 (6)
Degenerated oocytes (%)	31 (13.83±2.81)[b]	61 (26.87±2.72)[a]	35(13.47±2.15)[b]	60 (22.70±2.28)[a]
One cell stage (%)	41 (18.14±2.24)[b]	83 (36.25±1.97)[a]	40 (16.13±2.15)[b]	85 (32.67±3.81)[a]
2-cell stage (%)	22 (9.60±0.89)[b]	15 (6.75±1.62)[c]	37 (13.91±2.47)[a]	23 (8.81±0.85)[b]
4-cell stage (%)	24 (10.55±1.13)[a]	16 (6.86±1.08)[b]	30 (11.42±0.79)[a]	17 (6.56±0.38)[b]
8-cell stage (%)	20 (8.79±1.12)[a]	11 (4.80±0.76)[c]	17 (6.26±1.27)[b]	6 (2.40±0.93)[d]
16-cell stage (%)	1 (0.56±0.56)[c]	1 (0.45±0.45)[c]	8 (3.10±1.11)[a]	3 (1.21±0.77)[b]
Morula stage (%)	1 (0.42±0.42)[a]	0 (0.00±0.00)[b]	1 (0.31±0.31)[a]	0 (0.00±0.00)[b]
Fragmented embryos (%)	71 (31.44±1.53)[a]	37 (16.74±2.64)[b]	74 (29.45±3.44)[a]	62 (24.95±4.02)[a]
Cleavage rate (%)	68 (29.91±1.37)[a]	43 (18.85±1.14)[b]	93 (35.00±3.61)[a]	49 (18.98±1.07)[b]
Blastocyst rate (%)	16 (7.14±2.84)[a]	3 (1.30±0.87)[b]	(5.97±1.34)[a]16	2 (0.70±0.44)[b]

*Six replicates, means for each stage in the same row, with different superscript (a,b,c,d) are significantly different ($p < 0.05$)

disagreed with (Boland *et al.,* 1985) who reported a significant effect of breed on mass motility of sperm in Suffolk, Texel and Dorset Horn rams. Semen parameters are influenced by many factors such as nutrition and environment (Oyeyemi *et al.,* 2009). Furthermore, Miloud & Karima, (2016) reported that dietary supplementation could improve semen characteristics in three ram breeds. Our resultsfor mass and progressive sperm motility can be attributed to the similarity of nutritional and environmental factors of these breeds.

The results of the effect of fresh and frozen semen in Naimi and Najdi rams on in vitro embryo development are presented in Table 2. Our results showed lower significant ($P<0.05$) differences in the one cell stage, degenerated oocytes, 2-cell stage, 4-cell stage, 8-cell stage, morula, cleavage and blastocyst rates with frozen semen compared to fresh semen in both breeds. Although previous studies disagreed with these differences (Lehloenya *et al.,* 2010; Romão *et al.,* 2013), other results showed that semen cryopreservation impairs the efficiency of frozen ram semen by damaging the DNA structure of sperm (Peris *et al.,* 2004; López-Fernández *et al.,* 2010). Here, it was a clear that a lower of the number of embryos were found in the morula stage in all groups. *In vitro* sheep embryos arrest at 8-16 cells and undergo transition from maternal to embryonic genome (Telford *et al.,* 1990). Furthermore, the significant ($P<0.05$) differences between Naimi and Najdi breeds in the number of embryos in 8-cell and 16-cell stages may due to breed effects of the ram on embryonic genome activation.

The low cleavage and blastocyst rates after semen insemination from two breeds were similar to those obtained in Merino, Pedi and Zulu breeds (Mahoete 2010). Moreover, Morriset al., (2001) found large variability in the proportion of embryos produced in vitro using semen from four Dorset rams. Cleavage and blastocyst rates are affceted by many factors such as maternal nutrition (Grazul-Bilska *et al.,* 2012), season (Mara *et al.,* 2014), *in vitro* conditions (Romão *et al.,* 2013) and oocytes quality (Mahoete 2010). Fragmented embryos in this study were higher ($P<0.05$) in all groups compared to the Naimi frozen semen group. Furthermore, the *in vitro* abnormal development of early embryos may be affected by sperm integrity. In addition to breed effects, the harsh environmental conditions can stress the males. This might cause oxidative stress to the sperm and result in abnormal early cleavage and thus early embryo demise (Burruel *et al.,* 2014). Therefore, Najdi and Naimi rams may need modification and/or nutrition to decrease the impact of the arid environment stress before the use in IVF.

In conclusion, there were slight effects of ram breed on *in vitro* development of sheep embryos. More breeds are needed to confirm this observation. Moreover, the harsh environmental conditions may affect the *in vitro* production of sheep embryos, and this requires further studies to clarify these effects.

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