Research Article

Pregnancy and Lambing Rate Following Laparoscopic Artificial Insemination with Two Different Types of Diluent and Frozen-thawed Sperm Dose in Ewes

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Abstract | The effects with two different types of diluent (commercial Triladyl® and home-made tris based) and number of frozen-thawed ram sperm (100 × 10^6 and 50 × 10^6 sperm dose) on pregnancy and lambing rate following laparoscopic artificial insemination (LAP-AI) in indigenous ewes in Bangladesh were studied. A total of 78 non- pregnant ewes were synchronized for oestrus with two doses of 100 µg PGF2α (Cloprostenol) 9 days apart. The ewes were randomly assigned to one of four groups; G1 (n = 18; commercial Triladyl®/ 100 × 10^6 sperm), G2 (n = 21; commercial Triladyl®/ 50 × 10^6 sperm), G3 (n = 20; home-made tris based/ 100 × 10^6 sperm) and G4 (n = 19; home-made tris based/ 50 × 10^6 sperm). Ewes were inseminated within 18 h to 22 h after the onset of oestrus. The non-return rate (83.3%) and pregnancy rate (72.2%) were highest with commercial Triladyl®/ 100 × 10^6 sperm compared to other groups. The mean gestation period ranged from 149.50 ± 3.50 days to 153.75 ± 1.03 days. The lambing rate (100%) was highest with home-made tris based/ 50 × 10^6 sperm compared to other groups. The multiple birth rate was highest (66.7%) with commercial Triladyl®/ 100 × 10^6 sperm, home-made tris based/ 50 × 10^6 sperm compared to other groups. The multiple birth rate ranged from 61.5% to 66.7%. The single, twin and triplet lambing was higher 38.5%, 58.3% and 18.2% with commercial Triladyl®/ 50×10^6 sperm, home-made tris based/ 50 × 10^6 sperm and home-made tris based/ 100 × 10^6 sperm, respectively. The lambing size ranged from 1.69 ± 0.17 to 1.82 ± 0.20. In conclusion, the home-made tris based diluent and 100 × 10^6 sperm dose would be the most practical method for achieving high pregnancy and lambing rate following LAP-AI in Bangladeshi ewes.

Editor | Muhammad Abubakar, National Veterinary Laboratories, Park Road, Islamabad, Pakistan.
Received | July 27, 2020; **Accepted** | September 27, 2020; **Published** | October 16, 2020
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Email: drpankaj.np@gmail.com
Citation | Jha, P.K., M.G.S. Alam and F.Y. Bari. 2020. Pregnancy and lambing rate following laparoscopic artificial insemination with two different types of diluent and frozen-thawed sperm dose in ewes. *Veterinary Sciences: Research and Reviews*, 6(2): 143-149.
DOI | http://dx.doi.org/10.17582/journal.vsrr/2020.6.2.143.149
Keywords | Diluent, Sperm number, LAP-AI, Fertility rate, Bangladesh sheep

Introduction

Sheep farmers in Bangladesh are curiously looking for sustainable appropriate reproductive techniques. Frozen semen of superior ram and use of artificial insemination (AI) is commonly used worldwide to improve the sheep herd genetics through the selective breeding programs. Mainly two types of AI; transcervical (TC-AI) and laparoscopic (LAP-AI) are practiced in ewes. TC-AI using frozen semen is hardly practiced because of low pregnancy and lambing rates (Montoro *et al.*, 2002). The LAP-AI is the choice in ewes, results in an increased fertility rate by injecting semen directly into the uterine

horn (Fair *et al.*, 2005; Kumar and Naqvi, 2014). To establish LAP-AI in indigenous ewes in Bangladesh, different analogues and doses of prostaglandin are used to observe the effectiveness of oestrus response (Roy *et al.*, 2014; Zohara *et al.*, 2014), and pregnancy rate using frozen semen (Rekha *et al.*, 2016). The pregnancy rate following LAP-AI with frozen ram semen in Bangladeshi ewes varied widely 47% to 60% (Azizunnesa, 2016). The success of AI depends on the quality of semen, oestrus synchronization, types of insemination, the number of frozen-thawed sperm and timing of insemination (Montoro *et al.*, 2002; Kumar and Naqvi, 2014). LAP-AI is superior to TC-AI for pregnancy rate and requires a reduction in the number of spermatozoa per insemination. Different doses of frozen sperm 25 × 10^6 (Salamon and Maxwell, 2000; Khalifa *et al.*, 2013), 100 × 10^6 (Padilha *et al.*, 2012) and 250 × 10^6 (Anel *et al.*, 2003) have been documented to use for LAP-AI in sheep. Despite the above described, different types of diluents and frozen-thawed sperm dose, the influence on fertility in indigenous ewes in Bangladesh remains to be established. Therefore, the present study was designed to compare two different types of diluent (commercial Triladyl® and home-made tris based) and frozen-thawed sperm dose (100 × 10^6 and 50 × 10^6) on the pregnancy and lambing rate following LAP-AI in indigenous ewes.

Materials and Methods

All procedures were approved by the Animal Experimental Ethics Committee, Department of Surgery and Obstetrics, Bangladesh Agricultural University (AEEC/ DSO54 BAU/ 02/ 2015) and were carried out from November 2016 to December 2017.

Animals

Indigenous ram (n = 10), best selected based on semen quality evaluation were used as semen donors. Indigenous ewes (n = 78), non-pregnant were selected with the aid of ultrasound scanning (DRAMINSKI ANIMAL profi portable ultrasound scanner, Poland) using a 5.0 MHz transabdominal transducer. Both rams and ewes were belonged to a project funded by Bangladesh Academy of Science and United States Department of Agriculture (BAS-USDA; LS-02) and reared at Sheep Research Farm, DSO, BAU. Feeding, grazing, watering, and housing remained as routinely done in Sheep Research Farm, DOS, BAU (Jha *et al.*, 2018).

Frozen semen production

All chemicals used in this study were from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany). Semen collection and evaluation was performed as described by Jha *et al.* (2018). Briefly, semen was collected using an artificial vagina and semen samples were evaluated at 35˚C. Sperm motility (%; warming stage at 37˚C) was estimated subjectively by placing a drop of semen 5 µL (100 ×) using a coverslip. Sperm concentration (10^6/ mL) was calculated using a Neubauer counting chamber at a dilution of 1:200 (Semen: buffered formal saline, 200 ×). Only ejaculates with volume ≥ 0.5 mL, sperm motility ≥ 80%, and sperm concentration ≥ 2500 × 10^6/ mL were processed.

Two different types of diluent, commercial (Triladyl®) and home-made tris based were selected based on ram semen freezing experiment (Jha *et al.*, 2019). The semen sample was extended using a one-step and two-step protocol for commercial Triladyl® and home-made tris based, respectively. Individual ram semen ejaculates were extended to two different sperm concentration 400 × 10^6 and 200 × 10^6 spermatozoa/ mL to prepare final sperm dose 100 × 10^6 and 50 × 10^6 per 0.25 mL French mini straw.

The straws were frozen in liquid nitrogen (LN2) vapor in a Styrofoam box using 3 step freezing technique developed in the DSO laboratory, BAU (Jha *et al.*, 2019). Finally, the straws were plunged into the LN2 (-196 ˚C).

The semen straws were thawed in warm water at 37 ˚C for 20 sec. Batches with sperm motility ≥ 50% were stored in Cryocan for LAP-AI purposes.

Experimental design

The ewes (n = 78) were randomly allocated into 4 groups: G1 (n = 18; commercial Triladyl®/ 100 × 10^6 sperm), G2 (n = 21; commercial Triladyl®/ 50 × 10^6 sperm), G3 (n = 20; home-made tris based/ 100 × 10^6 sperm) and G4 (n = 19; home-made tris based/ 50 × 10^6 sperm). Ewes were inseminated within 18 h to 22 h after the onset of oestrus.

Oestrous synchronization

Oestrous synchronization was done according to Zohara *et al.* (2014). All ewes were given two doses of 0.4 mL (100 µg) intramuscular (IM) prostaglandin F2α (PGF2α) analogue (Cloprostenol, Ovuprost,

Bayer New Zealand Ltd., Auckland, New Zealand) 9 days apart.

Laparoscopic artificial insemination (LAP-AI)

Selected oestrus ewes were withheld from feed and water for at least 12 h. After proper clipping and shaving the lower abdominal region of the ewes, they were sedated with xylazine (Xylazin®, Indian Immunological Ltd, Hydrabad, India) @ 0.22 mg/ kg IM. Ewes were restrained in the laparoscopic cradle in dorsal recumbence with the rear legs lifted to an approximate 45° angle. Each leg fortified by a rope with a cradle hook. The operating area was scrubbed with antiseptic solution (7.5% w/v, Povisep®, Jayson Pharmaceuticals Ltd, Dhaka, Bangladesh) and 4 ml local anaesthetic lignocaine hydrochloride (Jasocaine®, 2%, Jayson Pharmaceuticals Ltd., Dhaka, Bangladesh) was injected SC on incision site; 5 cm to either side of the mid ventral line and 5 cm anterior to the mammary gland. Two small incisions (0.3 cm to 0.4 cm) was made through the skin to the left and right, approximately 3 cm to 4 cm of the midline and 4 cm to 5 cm cranial to the teats. Two trocars and cannulas, one (7 mm diameter × 150 mm length) and another (5 mm diameter × 100 mm length) were inserted into the peritoneal cavity through the left and right incision, respectively. The peritoneal cavity was inflated with CO_2 (LAPARO CO_2 PNEU insufflator, 2232, Richard Wolf, Germany). The trocars were removed from each cannula. A laparoscope tube (7 mm, 180°) fitted to a high-intensity fibre optic light source (Endolight led light sources, 4215, Richard Wolf Germany) was introduced into 7 mm cannula. With the help of laparoscopic tube and right-hand cannula, the reproductive tract was manipulated gently to position of the uterine horn for insemination. The loaded LAP-AI pipette (Robertsone pipette standard for LAP-AI in sheep with tube, Minitube, Slovakia) was introduced into right-hand cannula. One uterine horn (approx. 3 cm to 4 cm cranial to the cornual bifurcation) was punctured by the needle attached with the LAP-AI pipette. The semen, half of the 0.25 ml French semen straw, was directly injected intra-luminally of the uterine horn. The procedure was repeated on the other uterine horn. After insemination, the pipette, laparoscope tube and both cannula was removed. Excess gas if any was expressed and a sulphanilamide powder (Sumid-Vet®, Square Pharmaceuticals Ltd., Bangladesh) was applied at the wound sites. The skin wound was closed with silk 2-0 (non-absorbable) using interrupted suture. The sutured site was painted with Povisep® and applied a tincture benzoin cotton seal. Surgical instruments were drenched with povidone iodine solution (7.5% w/v, Povisep®, Jonson Pharma Ltd., Bangladesh), water and finally wiped with ethylene alcohol between LAP-AI of ewes.

Reproductive performance

The onset of oestrous was determined by monitoring every 6 h for 30 min to 40 min from 12 h to 96 h following the second PGF2α treatment, with the help of a mature ram wearing an apron. The non-return of oestrus was monitored 13 days to 21 days after insemination. The non-returned ewes were checked for pregnancy by ultrasound 40 days to 50 days after AI (Olivera-Muzante *et al.*, 2011). The oestrus response (number of ewes showing oestrus/ number treated × 100), non-return rate (number of ewes not returning to oestrus/ number inseminated × 100), pregnancy rate (number of pregnant ewes/ number inseminated ×100), lambing rate (number of lambing ewes/ number of pregnant ewes × 100), multiple birth rate (number of ewes lambing twin or triplet/ total number of lambing ewes × 100), lambing size (number of total lambs/ number of lambing ewes × 100) and the female or male lamb rate (number of female or male lamb/ total number of lambs × 100) were recorded (Turk *et al.*, 2008).

Data analysis

Excel (Microsoft Excel 2010) was used to record and calculate the frequency distribution of onset of oestrus, and SPSS (Version 20; IBM) was used for other data analysis. The gestation periods and lambing size were compared by one-way analysis of variance (ANOVA) followed by post hoc Tukey-HSD test. The other reproductive traits were compared by Chi-squared test. Data for gestation period and lambing size were presented as mean ± SEM and a value $p<0.05$ was considered significantly different.

Results and Discussion

Non-return rate, pregnancy rate and gestation period

The non-return, pregnancy rate and gestation period are presented in Table 1. The non-return rate (83.3%) and pregnancy rate (72.2%) were highest with commercial Triladyl®/ sperm dose 100 × 10^6 compared to other groups. However, the non-return rate and pregnancy rate ranged from 73.7% to 83.3% and 63.2% to 72.2%, respectively. The mean gestation period ranged from 149.50 ± 3.50 to 153.75 ± 1.03 days.

Table 1: *Effects of different types of diluent and frozen sperm dose on reproductive performance in ewes following LAP-AI.*

Reproductive parameters		Groups				
		Commercial triladyl®		Home-made tris based		
		100×10⁶ sperm dose	50×10⁶ sperm dose	100×10⁶ sperm dose	50×10⁶ sperm dose	P-value
Ewes (n)		18	21	20	19	
Non-return rate (%)		83.3 (15/18)	81.0 (17/21)	80.0 (16/20)	73.7 (14/19)	0.930
Pregnancy rate (%)		72.2 (13/18)	71.4 (15/21)	65.0 (13/20)	63.2 (12/19)	0.908
Gestation period (days) (mean ± SEM)	Single	153.0 ± 0.40	151.4 ± 1.88	152.25 ± 1.03	153.75 ± 1.03	
	Twin	153.16 ± 0.74	152.85 ± 0.70	152.4 ± 1.32	153.0 ± 0.65	
	Triplet	151.0 ± 3.0	156.0	149.50 ± 3.50	152.0	
Lambing rate (%)		92.3 (12/13)	86.7 (13/15)	84.6 (11/13)	100 (12/12)	0.749
Multiple birth rate (%)		66.7 (8/12)	61.5 (8/13)	63.6 (7/11)	66.7 (8/12)	1.000
Fecundity (%)	Single	33.3 (4/12)	38.5 (5/13)	36.4 (4/11)	33.3 (4/12)	0.993
	Twin	50.0 (6/12)	53.8 (7/13)	45.5 (5/11)	58.3 (7/12)	
	Triplet	16.7 (2/12)	7.7 (1/13)	18.2 (2/11)	8.3 (1/12)	
Lambing size (mean ± SEM)		1.82 ± 0.20	1.69 ± 0.17	1.81 ± 0.22	1.75 ± 0.17	
Female lamb rate (%)		45.5 (10/12)	59.9 (13/22)	60.0 (12/20)	38.1 (8/21)	0.409
Male lamb rate (%)		54.5 (12/22)	40.9 (9/22)	40.0 (8/20)	61.9 (13/21)	

Lambing performance

The lambing rate (100%) was highest with home-made tris based/ sperm dose 50 × 10^6 compared to other groups. However, the lambing rate was ranged from 84.6% to 100% (Table 1). The multiple birth rate was highest (66.7%) with commercial Triladyl®/ sperm dose 100 × 10^6, home-made tris based/ sperm dose 50 × 10^6 compared to other groups. However, the multiple birth rate ranged from 61.5% to 66.7%. The single, twin and triplet lambing was higher 38.5%, 58.3% and 18.2% with commercial Triladyl®/ sperm dose 50 × 10^6, home-made tris based/ sperm dose 50 × 10^6 and home-made tris based/ sperm dose 100 × 10^6, respectively. The lambing size ranged from 1.69 ± 0.17 to 1.82 ± 0.20.

LAP-AI is the method of choice for AI with frozen semen in sheep. Following LAP-AI, the pregnancy rate is comparable to natural service or fresh semen. The diluent home-made tris based and sperm dose 100 × 10^6 can be recommended for sustainable LAP-AI in indigenous sheep in Bangladesh.

The success of AI depends on quality of frozen semen and timing of AI (Hashemi *et al.*, 2006). The non-return rate was in agreement with Olafsson (1980) and Langford *et al.* (1979) who reported little difference in non-return rate after 25 days but Olafsson used unsynchronized ewes whilst Langford *et al.* (1979)used synchronized ewes. The non-return rate is associated with too early insemination; deleterious effects often occurred both before and after maternal recognition of pregnancy (Olivera-Muzante *et al.*, 2011).

The pregnancy rate (63.2% to 72.2%) was higher than Azizunnesa (2016) who reported pregnancy rate 47% to 60% using frozen semen in Bangladeshi ewes. This apparent difference may be due to semen quality and sperm dose and ease performance of the technique. The pregnancy rate was comparable with Moses *et al.* (1997) who reported 62.9%. The pregnancy rate was comparable with other authors who reported 60% to 66.6% using frozen sperm dose ranged from 30 × 10^6 to 100 × 10^6 per ewe (Fukui *et al.*, 2007, 2008, 2010). Nevertheless, it is higher than 45.0% as described by Anel *et al.* (2005) after AI with 250 × 10^6 frozen sperm per ewe. The variation in experimental designs among studies might be responsible for the disparity in results of fertility trials (Petrie and Watson, 2006). The pregnancy rate may vary with quality of frozen semen and nature of oestrus. Khalifa *et al.* (2013) reported pregnancy rate 46.2% and 71.0% with frozen sperm dose 100 × 10^6 which was produced after using AndroMed and BioXcell diluent. This difference might be due to quality of number of motile sperms per dose insemination. A recent study by Eppleston *et al.* (1994) has shown a fertility rate of 40.3% with 16 × 10^6 motile spermatozoa and 72.8% with 64 ×

10^6 motile spermatozoa. Evans and Maxwell (1987) found that 20 × 10^6 motile spermatozoa suffice for achieving a high pregnancy rate, while Eppleston *et al*. (1986) and Salamon *et al*. (1985) reported high fertility rate after insemination with 5 × 10^6 and 10 × 10^6 motile spermatozoa, respectively. Findlater *et al*. (1991) reported similar results with 13 × 10^6 motile sperm cells per uterine horn. Factors such as age, parity, lactation status, and body condition of the ewe could influence fertility rate (Anel *et al*., 2005; Fukui *et al*., 2010; Palacin *et al*., 2012). The lambing rate (84.6% to 100%) was higher than McKusick *et al*. (1998) who reported 75% following insemination with sperm dose 65 × 10^6. The lambing size 1.69 ± 0.17 to 1.82 ± 0.20 was in agreement with McKusick *et al*. (1998) who reported lambing size 1.89 ± 0.21. The lambing rate, fecundity rate and lambing size depend upon various factors like; genetic potential, plane of nutritional and timing of AI (Downing and Scaramuzzi, 1991).

Conclusions and Recommendations

The diluent home-made tris based and sperm dose 100 × 10^6 would be the most practical method for achieving high pregnancy and lambing rate following LAP-AI in Bangladeshi ewes. This study provides some preliminary and important results and suggest for further research with a greater number of ewes.

Acknowledgements

This work was supported by Bangladesh Academy of Science and United States Department of Agriculture (BAS-USDA; LS-02). The authors are grateful to Md. Rafiqul Islam Talukder (PhD student) and Nazmun Naher (MS student) for assistance in LAP-AI.

Author's Contribution

This publication is part of Mr. Pankaj Kumar Jha PhD degree thesis supervised by Professor Farida Yeasmin Bari and Professor Md. Golam Shahi Alam. Pankaj Kumar Jha conceived the idea, designed, carried out the study and prepared the manuscript for publication, while Professor Farida Yeasmin Bari and Professor Md. Golam Shahi Alam supervised the study and proofread the manuscript for publication.

Conflict of interest

The authors have declared no conflict of interest.

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