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Original Article

Different approaches of trans-cervical artificial insemination in oestrus synchronized indigenous field ewes using frozen ram semen

Mohammad Asaduzzaman[1, 2], Pankaj Kumar Jha[3], Amit Saha[2], Md. Golam Shahi Alam[2], and Farida Yeasmin Bari[2*]

[1] *Department of Livestock Services, Farmgate, Dhaka, 1215 Bangladesh*

[2] *Department of Surgery and Obstetrics, Bangladesh Agricultural University, Mymensingh, 2204 Bangladesh*

[3] *Animal Breeding Division, Nepal Agricultural Research Council, Lalitpur, 44703 Nepal*

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Abstract

The study aimed to determine the pregnancy rates of indigenous field sheep following different approaches to trans-cervical artificial insemination (TCAI). TCAI was performed in oestrus induced ewes using two straws of frozen-thawed semen. In group 1, single-time TCAI was performed on standing heat in traditionally/manually restrained ewes (n=20) and in lab cradle restrained ewes (n=20). In group 2, TCAI was performed in manually restrained ewes single-time after 46-48 h of PGF2a injection (n=20), and double-times at 46-48 h & 52-56 h after PGF2a injection (n=20). In group 3, TCAI was performed in manually restrained ewes single time at 52-56 h after PGF2a injection and 20 minutes after oxytocin injection (n=10), and 20 minutes after oxytocin-estradiol benzoate injection (n=11). The pregnancy rates were 35 % and 45 % in traditional and cradle restrained TCAI, 35 and 55 % in single-time and double-times TCAI, and 50 and 55 % in oxytocin and oxytocin-estradiol cervix-treated TCAI, respectively. Although the pregnancy rates did not differ significantly (p>0.05) by experimental group, the TCAI after cervical treatment and double-time TCAI importantly had more prengnancies than the other approaches to TCAI. Finally, it can be concluded that double-times TCAI would be an effective and acceptable approach for field sheep artificial breeding using frozen semen.

Keywords: sheep, oestrus synchronization, different approaches, transcervical insemination

1. Introduction

Animals are bred artificially by incorporating semen into a female's natural or synchronized oestrus genital tract using instruments. Artificial insemination (AI), without a doubt, is the oldest technique of assisted reproduction (AR) (Falchi, Taema, La Clanche, & Scaramuzzi, 2012) and it was initially used to avoid male injuries and to prevent sexually transmitted diseases (Hernández *et al.*, 2015). Soon it was recognized by farmers as a method of choice to add or spread superior genetic value of select males, enhancing the production of improved offspring with high productive traits and controlling contagious diseases in animal herds (Alvares, Cruz, & Ferreira, 2015; Evans & Maxell, 1987). It also facilitates maximum use of superior rams by lowering the sperm concentration in semen dose, increasing the number of inseminations and quality offspring.

The animal oestrus cycle can be manipulated to bring them to heat within a predefined time frame of 36 to 96 hours by one or several of the three hormones prostaglandins, progesterone, and GnRH. This enables to quickly synchronize a group of animals for efficient artificial insemination (AI) (Tsuma, Khan, Okeyo Mwai, & Ibrahim, 2015). Islam (2011) stated that the cyclic female shows oestrus within 2 to 6 days

*Corresponding author
Email address: faridabari06@gmail.com

following prostaglandin F2α (Estrumate®, In-Synch®). Gizaw *et al.* (2016b) described that when a single injection of prostaglandin was given to a flock of cycling ewes, 60 to 70 percent of the flock exhibited synchronized estrous beginning 30 to 48 hours later (Schoenian, 2012; Tsuma, Khan, Okeyo Mwai, & Ibrahim, 2015). Islam (2011) stated that, since not all stages of the estrous cycle were similarly receptive to treatment, a double injection system 7 days or 11 days apart to make sure that all functional CL regress, is the most widely used approach in small ruminants.

The AI of sheep is involved with appropriate knowledge and breeds (Fonseca, Souza & Camargo, 2010), female body condition, prolificacy, and oestrus status. In addition, farm conditions, time of year, season, the technique of AI, and technician skills also influence the fertility rate (Anel *et al.*, 2005). Apart from these, the structure of the sheep cervix is another crucial barrier for transcervical AI (TCAI) with its 4-7 convoluted rings that prevent the passage of AI pipette through to the body of the uterus (Evans & Maxell, 1987). The role of the cervical canal in regulating frozen sperm transport in sheep is still problematic, despite various inseminating equipment that has been tried with cervix manipulation for effective insemination (Aral, Temamogullari & Aral, 2011). Even though artificial insemination with laparoscopy and laparotomy techniques has been shown to have compelling performances, it also has a high cost, is time-consuming, and requires particular type of equipment as the main drawbacks hindering its wider adoption (Evans & Maxwell, 1987; Torres & Sevellec, 1987). To overcome all these issues, hormones, primarily oxytocin and prostaglandin E (PGE), were found to induce significant cervical dilatation (Khalifa, Sayre, & Lewis,1992; Masoudi *et al.*, 2017; Wulster-Radcliffe, Costine, & Lewis, 1999).

Azizunnesa, Bari and Alam (2014), Azizunnesa, Zohara, Islam, Bari, and Alam (2013), and Zohara, Islam, Alamc & Baric (2014) performed some baseline studies on sheep oestrus synchronization using different hormone protocols, ram semen preservation, and artificial insemination in the research station of Bangladesh Agricultural University, Mymensingh. Al Mansur *et al.* (2018) had little exposure to the performance of transcervical insemination in field indigenous ewes. The present study aimed to determine the pregnancy rates of indigenous field sheep on following different approaches to TCAI.

2. Materials and Methods

2.1 Study site, animals, and management

The experiment was carried out on Bangladeshi indigenous Jamuna basin sheep in different field sheep farms at Gopalpur and Bhoapur Upazilla under Tangail district and Mymensingh Sadar Upazila under Mymensingh district, during three subsequent breeding seasons from November 2018 to March 2021. All the ewes were mainly under the farmer's management systems, such as being maintained on pasture from morning to dusk with free excess of safe drinking water. The sheep were 2–4 years of age and/or at least one parity.

2.2 Sampling method and approval

Sample sizes were calculated using a formula, $n = N/1 + N(e)^2$ (Yamane, 1967: 886), where n is the sample size, N is the population size, and e is the level of precision. From 20 field sheep farmers, a total of 200 non-pregnant indigenous ewes were randomly selected as the hypothesized population size for the current study. At 95 percent confidence level and P = 0.5, the calculated sample size was 134 ewes. All experimental protocols followed the Animal Welfare and Experimentation Ethics Committee's (AWEEC) recommendations for animal care and use [No. AWEEC/ BAU/2019 (54), dated: 26/12/2019].

2.3 Experimental design

A total of 134 ewes were brought under the oestrus synchronization program, and oestrus was confirmed to 108 ewes. Transcervical artificial insemination was performed on animals divided into three experimental groups, where 101 ewes were provided with efficient artificial insemination, and 7 ewes were excluded for defective artificial insemination.

Group 1: Techniques of TCAI (n=40)

- Traditionally restrained, and TCAI on standing heat (n = 20)
- Lab cradle restrained, and TCAI on standing heat (n = 20)

Group 2: Time of TCAI (n=40)

- Single-time TCAI at 46-48 h after PGF2a injection (n=20)
- Double time TCAI at 46-48 h and 52-56 h after PGF2a injection (n= 20)

Group 3: Cervix treatment and TCAI (n = 21)

- TCAI at 52-56 h after PGF2a and 20 minutes of oxytocin (OT) injection (n=10)
- TCAI at 52-56 h after PGF2a and 20 minutes of oxytocin(OT) + oestradiol (OE) injection (n=11)

2.4 Semen preparation, thawing, and AI gun loading

The selected semen samples were frozen according to a conventional laboratory standard protocol by Jha *et al.* (2019). After thawing the frozen straw, the post-thaw seminal parameters were measured (37 °C for 30 seconds). While the production motility was at least 40 %, they were stored. During TCAI, the straw was thawed as before; the company end was fitted to an AI gun (®Inseminator for small ruminants, 0.25 ml straws, Minitube). The straw's sealed end was cut-opened with clean scissors and finally loaded into a pipette for TCAI.

2.5 Synchronization and detection of oestrus

Oestrus cycles of all groups were synchronized by prostaglandin (Gizaw *et al.*, 2016) using two injection protocols of Rekha, Zohara, Bari, and Alam (2016) at the unknown stage of the oestrus cycle of the non-pregnant ewes confirmed by ultrasonography (DRAMIŃSKI ANIMAL *profi*, Poland). Two injections of Ovuprost® (Cloprostenol sterile injection, BOMAC Laboratories Ltd., New Zealand) were given at 9-days interval i.m at the dose rate of 0.4 ml/ewe (Zohara, Alamc, & Baric, 2014) to make sure all functional CL regress. The heat was proved to be in oestrus while an aproned ram mounted her within 24 to 48 h of prostaglandin injection (Figure 1).

Figure 1. (A) Oestrus detection by aproned ram, (B) traditionally restrained TCAI, and (C) lab cradle restrained TCAI

2.6 Traditional and lab cradle restrained TCAI approach

In a traditionally restrained approach, the farmer or assistant stood over the ewe holding her, securing her two hind legs, and elevated the hindquarter as the ewe was in a slanting position. With firm and gentle pressure, the lubricated (KLY® Jelly) vaginal speculum was carefully inserted into the full depth of the vagina (10-13cm) following the slope of the female's rump. A light source (pen torch) was attached with a speculum to assist the visual inspection and locate and position the cervical os by pulling back or rotating the speculum slightly. The straw-loaded AI gun was inserted and plunged the semen into the cervical os. Two straws of frozen semen were used in artificial insemination containing 200×10^6 / 0.25 ml straw of frozen semen having at least 40 % motile spermatozoa. In the restrained cradle approach, the ewe boarded to cradle bed keeping her hindquarters elevated with head down position with the body in dorsoventral position. The hind legs were fixed with an upper rail of the cradle using ropes, and the front legs with two vertical rails. One assistant stood beside the cradle, holding and fixing the female head. The ewe was then inseminated following the traditional method.

2.7 Single-time and double-times TCAI approach

Single-time transcervical artificial insemination approach was performed at 46 - 48 h of induction of oestrus, and the double-time approach was performed at 46 - 48 h and 52 - 56 h of induction of oestrus, as it was close to Visser & Salamon (1974) who performed insemination at 12-14 h, and 23-25 h of onset of oestrus. During TCAI, the ewes were restrained traditionally, as in the previous description of group 1.

2.8 The cervix treatment approach of TCAI

The synchronized ewe cervix was ripened with oxytocin (OT) (Linda-S® DS, Synthetic Oxytocin USP 10 IU/ml, Nuvista Pharma Limited, Gazipur, Bangladesh) at a dose of 50 IU i/m per animal 20 minutes before TCAI in one group of animals. In other groups, the synchronized ewe cervix was ripened by treatment combining oxytocin (0.5ml, 2.5-10 IU/small ruminants) and oestradiol (1ml, 2mg/small ruminants) (Estradiol Benzoate Injection, QUANYU Sheng Wukeji, China) i/m per animal 20 minutes before insemination. The ewes were restrained by the manual or traditional approach before insemination.

2.9 Pregnancy diagnosis

The conception was detected by trans-abdominal ultrasonography 45 to 60 days after insemination using an ultrasound scanner, DRAMIŃSKI ANIMAL *profi*, Poland.

2.10 Data analysis

Data are presented as percentages. The Chi-square statistic in SPSS ver. 22 was used to assess differences among percentages of pregnancy rates for the alternative methods of transcervical artificial insemination. Significance was called for p-value < 0.05.

3. Results

The pregnancy rate of transcervical artificial insemination in the three experimental groups of animals, following different approaches, are presented in Table 1. The results for group 1 indicate the percentage of animals successfully impregnated was not affected by the restraining technique of animals during TCAI of indigenous ewes. The restraining techniques or methods had no impact on pregnancy rates ($p>0.05$). Although the pregnancy rate was higher in

Table 1: Pregnancy rates in Bangladeshi indigenous sheep following different approaches to artificial insemination

Group of animals	Approach to TCAI	Pregnancy rate (%)	Chi-square test (P value)
Group 1	Traditional	35 (7/20)	0.519
Restraining technique	Cradle	45 (9/20)	
Group 2	Single-time	35 (7/20)	0.204
Time of TCAI	Double-times	55 (11/20)	
Group 3	OT treatment	50 (5/10)	0.653
Cervix ripening treatment	OT + E2 treatment	55 (6/11)	

TCAI-trans-cervical artificial insemination, OT-Oxytocin, E2- Estradiol cradle-restrained animals than in manually or traditionally restrained animals, the difference was not statistically significant.

The results for group 2 contrast the pregnancy rates from single-time and double-times TCAI in indigenous field sheep at (46 - 48) h and (46 - 48) & (54 - 56) h after oestrus induction. Double-times TCAI had a higher pregnancy rate than the single-time TCAI. Although there was no significant ($p>0.05$) difference, double-times TCAI in field sheep showed an effective pregnancy rate higher than that from single-time TCAI.

The results for group 3 show the pregnancy rates of indigenous ewes following TCAI after treatment with cervical dilators: oxytocin (OT) or oxytocin and estradiol benzoate (E_2+OT) combined. The OT and E_2+OT treatment groups had higher pregnancy rates (50 and 55%, respectively) than the traditional restrained TCAI approach group (35%). Although there were no statistically significant differences ($p>0.05$) in the pregnancy rates, the combined dilator (E_2+OT) showed more effectiveness than the single dilator (OT).

4. Discussion

A technique or approach for successful artificial insemination has not yet been well established for sheep, due to their cervical-specific anatomy being more difficult than in horses and cattle (Halbert, Dobson, Walton, & Buckrell, 1990). The Guelph system for trans-cervical AI (GST-AI) has been developed; but it is not suitable for farmer's needs as it requires specially designed instruments and unique positioning of the ewe, and a trained and experienced inseminator. Moreover, cervical retraction and penetration rate, injury, abscesses, infections, and poor pregnancy rates are unappreciated factors associated with this technique (Buschbeck, 2020).

The current study performed TCAI in the farmer's field by traditionally or manually restraining the ewes. The lower pregnancy rate of 35% obtained from this approach was concordant with the observations of Gage (1994), Donovan, Hanrahan, Kummen, Duffy, and Boland (2004), and Salamon and Maxwell (1995) reporting 33, 34.8, and 36%, respectively. To obtain the maximum benefit of TCAI, we performed trans-cervical artificial insemination by restraining and boarding the ewes in the lab cradle and observed a comparatively higher pregnancy rate (45%) than with the manual restraining approach. The lower pregnancy rate in the manual restraining approach might be due to the difficulty of keeping the animal substantially immobile for a longer duration of AI. Sometimes, people fix the sheep less conveniently, and an instability in conditions can even lead to injuries, which lowers the success rate of artificial insemination. The higher pregnancy rate in the lab cradle restrained approach might be due to the stationary state of the animal, facilitating the operation of insemination, and lifting the sheep bed and buttocks to prevent semen backflow, simultaneously improving the success rate of insemination (Google Patent, 2012). However, such a higher pregnancy rate is in agreement with the observations of Donovan *et al.* (2001), Donovan, Hanrahan, Kummen, Duffy, and Boland (2004), and Maxwell and Hewitt (1986) reporting 42.2, 44, and 46%, respectively.

The time of AI is critical as it is related to ovulation time. Ovulation occurs at the end phase of oestrus. As such, AI needs to be done a sufficient time before the ovulation so that the sperm can migrate to the fertilization site, the ampulla. In tradition, oestrus is observed twice (morning and evening) daily. While oestrus is observed in the morning, AI is performed in the evening (around 10 h after oestrus onset). If oestrus is observed in the evening, then the insemination is done in the morning (about 14 h after oestrus onset). The fresh semen has a fertilizing lifetime of more than 24 h, but not more than 12 h for frozen semen in the female reproductive tract. Therefore, AI time needs to be adjusted according to semen type (Kumar & Naqvi, 2014).

Adjustment of the AI time is also essential in the case of oestrus synchronization. Fernandez-Abella, Preve & Villegas (2003) suggested that the best time of AI is at 46 h, at 48 to 72 h according to Karagiannidis, Varsakeli, Karatzas & Brozos *(*2001), and 58 to 63 h after the pessary removal according to Donovan *et al.*, (2001), and Donovan, Hanrahan, Kummen, Duffy, and Boland (2004). Single-time TCAI in the present study was performed at 46 - 48 h after oestrus induction and double-times TCAI at 46 - 48 and 54 - 56 h after oestrus induction. The pregnancy rate of single-time TCAI (35%) was in agreement with the reported pregnancy rates of Donovan, Hanrahan, Kummen, Duffy, and Boland (2004), Gage (1994), and Salamon and Maxwell (1995) reporting 33%, 22.2 - 34.8%, and 36%, respectively. The higher conception rate of double-times TCAI (55%) was in line with the findings of Halbert, Dobson, Walton, and Buckrell (1990), Stefanov *et al.*, (2006), and Wulster-Radcliffe, and Lewis (2002) who reported 53.33%v, 58%, and 59.3%, respectively. Similarly, Kumar and Naqvi (2014) found a higher pregnancy rate of transcervical artificial insemination with double-times insemination than with single-time insemination (26.4 vs. 20%). Maxwell (1980) found a 29% conception rate after insemination at 50 and 60 h intervals. Buckrell *et al.* (1992), and Halbert, Dobson, Walton, and Buckrell (1990C) performed transcervical artificial insemination at 54 h intervals of synchronization and found conception rates of 24.4 - 50.7 and 34.7 - 56.7 %, respectively. Similarly, Smith, Parr, Beaumont, and Oliver (1995) found 40% conception rate following trans-cervical artificial insemination at 50-56 h interval from the synchronization.

The deeper intra cervix insemination in ewes enables more spermatozoa to reach the fertilization site and consequently increases the fertilization and pregnancy rates (Anel *et al.*, 2006; Eppleston, Salamon, Moore, & Evans 1994). However, the sheep's cervical structure impedes these successful insemination functions (Wulster-Radcliffe, Costine, & Lewis, 1999). Some of the procedures have included treatments to dilate the cervix and reduce the difficulty of manipulating AI catheters through the cervix and into the uterus (Anel *et al.*, 2006; Candappa, Bainbridge, Price, Hourigan, & Bartlewski, 2009; Wulster-Radcliffe, Wang, & Lewis, 2004). These are physical-mechanical methods based on physical stress to the cervix or transfer of sperm by a flexible or semi-flexible pipette (Wulster-Radcliffe & Lewis, 2002; Wulster-Radcliffe, Wang, & Lewis, 2004) that may injure the cervix and produce some anti-sperm secretions leading to lower sperm viability and consequently lower conception rate (Hawk, 1983; Sayre & Lewis, 1997). Even though laparoscopic and laparotomy methods of artificial inseminations are more effective, they come at a high cost and require much time and specialized equipment (Evans & Maxwell, 1987; Torres & Sevellec, 1987). As a result, cervix treatment to improve sperm reach to the fertilization site by altering its physiological characteristics without causing injury is the most effective method of choice.

It has been shown that oxytocin and prostaglandin E (PGE) hormones effectively dilate the cervix and increase the pregnancy rate (Khalifa, Sayre, & Lewis,1992; Wulster-Radcliffe, Costine, & Lewis, 1999). In the luteal phase, they have no effect and result in minimal damage or injury from passage of an AI pipette into the cervix (Masoudi *et al.*, 2017). Pipette passage through the cervix is facilitated by oxytocin, allowing for an intrauterine supply of semen. Oxytocin aids the pipette's passage past the cervix and sperm deposit within the uterus. It was also found that the processes of transcervical (TC) embryo transfer (ET) in sheep improved considerably, increasing cervical dilatation, exogenous estradiol-17 beta (E2), and oxytocin (OT) (Wulster-Radcliffe, Costine, & Lewis, 1999). Masoudi *et al.* (2017) found the highest cervical dilations of 90% and 100% in OT and E2+OT groups with the highest pregnancy rates of 60 and 65% of fat-tailed ewes, respectively. These findings match our present results of 50 and 55% for OT and E2+OT groups, respectively. However, the level of variation between OT and E2+OT groups might be due to differences in sheep breeds, body sizes, and the sources and doses of dilators used. In the present study, we also observed higher pregnancy rates while using combined dilators (OT+E2) over the use of a single OT dilator, and this might be due to more penetration effects as described by Masoudi *et al.* (2017) for E2+OT (4.10 cm) and for OT (3.90 cm). While sheep cervix remains more dilated, deep cervical insemination produces higher fertilization. Fukui & Roberts (1978) also agree with this report and observed a 45% fertility rate for deep cervical insemination with frozen semen (300 million sperm/ dose). In another report, Falchi, Taema, La Clanche, & Scaramuzzi (2012) also stated that maximum cervical penetration in the preovulatory period with estradiol concentrations suggests the cervix's relaxation probably through an oxytocin-PGE mediated pathway.

5. Conclusions

With the application of frozen semen, the present research studied a more effective TCAI approach for artificial breeding in field ewes. Based on the findings, the following conclusions can be drawn:

i. TCAI with the traditionally restrained approach is straightforward, less time-consuming, and more suitable to apply in field perspective than TCAI with a lab cradle restrained approach. Although a slightly higher pregnancy rate was obtained in the latter of these approaches, it requires a particular cradle instrument that is not suitable for carrying and instant application at field farms.

ii. Oxytocin and estradiol treatment may permit routine transcervical artificial inseminations with a comparatively higher pregnancy rate. Still, the availability of these hormones, time-consuming administration, and smooth performance of insemination in many sheep may be significant negative issues hindering the adoption of artificial insemination.

iii. The time of TCAI was the most important factor among the alternatives tested. Double- times TCAI covered the ovulation and fertilization time and produced a higher conception rate than single-time TCAI, and might be the approach of choice in field sheep artificial breeding.

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