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Effects of cervical ripening treatments on pregnancy rates following transcervical artificial insemination in ewes: A preliminary study

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ABSTRACT

The study was aimed to identify the effective cervical ripening treatment to increase the pregnancy rates following transcervical artificial insemination (TC-AI) in indigenous ewes of Bangladesh. Three treatments schedules were compared, G1 (n = 6, control), G2 [n = 6, prostaglandin E1 (PGE1) as Misoprostol-Cytomis®] and G3 (n = 6, estrogen as Oestriol-Ovestin® + oxytocin as Linda-S® Vet). Ewes (n=18) were synchronized for estrus using two doses of 100 µg prostaglandin (PGF2α; Ovuprost®) 9 days apart. The cervical ripening treatments were administered just after the onset of estrus and left for 12 h before TC-AI. The cervical ripening was reflected with the depth of cervical penetration. The depth of cervical penetration in G1, G2 and G3 both before and after cervical ripening treatments were 0.33 ± 0.10 cm vs 0.43 ± 0.07 cm, 0.41 ± 0.09 cm vs 3.50 ± 0.26 cm and 0.43 ± 0.09 cm vs 1.54 ± 0.14 cm, respectively. The depth of cervical penetration increased significantly ($p<0.01$) in G2 and ($p<0.05$) in G3 compared with G1. Comparing the pregnancy rates, no significant difference was observed among the groups. However, the pregnancy rates were increased (66.67%) in G2 near to significant compared to G1 (33.33%). In conclusion, the depth of cervical penetration and pregnancy rates was deeper and higher with PGE1-Misoprostol treatment. This preliminary study enlightens the development of effective TC-AI techniques in indigenous ewes for its satisfactory pregnancy rates and, to accept by the Bangladeshi sheep farmers. Furthermore, this study provides some important results that suggest room for further research with a greater number of ewes.

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Introduction

Nowadays, sheep farming is gaining popularity among Bangladeshi farmers. Due to poor genetics, farmers are looking for sustainable and locally appropriate reproductive techniques to upgrade their sheep genetics. Artificial insemination (AI) in conjugation with estrus synchronization is extensively used in sheep breeding programs to disseminate genetic traits of interest and increase production. Achieving acceptable pregnancy rates is very important for uptake to AI technology by sheep farmers. To establish an AI program in Bangladeshi ewes, studies on estrus synchronization, AI techniques using chilled and frozen

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semen have been accomplished (Roy et al., 2014; Jha et al., 2020). However, the pregnancy rates reported 60 - 70% with fresh semen and 11 - 26% with frozen-thawed semen following transcervical insemination (TC-AI; Azizunnesa, 2016; Rekha et al., 2016, 2018; Jha et al., 2020), which is not acceptable for field AI purpose. This lower pregnancy rates in ewes are due to complex cervical anatomy (long, convoluted funnel-shaped cervix) that prevents the passage of AI pipette into the body of uterus, as a result, there is the reduction in a high proportion of the spermatozoa when they arrive the place of fertilization (Wulster-Radcliffe et al., 2004; Kershaw et al., 2005; Leethongdee, 2010). To overcome these difficulties, laparoscopic artificial insemination (LAP-AI) is practiced in ewes. In this insemination technique, semen is directly deposited into the uterine lumen and thereby results in higher pregnancy rates (75 - 80%). However, this technique is expensive, needs minor surgery, and skilled surgeon and, therefore it has limited acceptance in developing countries (Bari and Haresign, 1998; Yamaki et al., 2003; Anel et al., 2005; Leethongdee, 2011). For acceptable pregnancy rates, researchers are trying to increase the depth of cervical penetration using hormonal preparations for cervical ripening. Some common hormones like; estrogen, oxytocin, and prostaglandin were studied for cervical ripening to increase the depth of cervical penetration and, to deposit semen deep into the cervical canal to increase pregnancy rates (> 50%; Rickords and White, 1988; Khalifa et al., 1992; Salamon and Maxwell, 1995; Donovan et al., 2004; Leethongdee et al., 2007). No study on pregnancy rates in Bangladeshi ewes after using cervical ripening treatments in the TC-AI technique has been reported, therefore, the objective of this study was to identify the effective cervical ripening treatments on pregnancy rates following TC-AI in indigenous ewes.

Materials and Methods

The study was conducted at the Department of Surgery and Obstetrics (DSO), Bangladesh Agricultural University (BAU), Mymensingh during the period of January - December 2015.

Animal and management: Altogether ewes (n = 18; age 24 to 36 months, BCS 3 - 4, bodyweight 14 - 16 kg) and rams (n = 3; age between 26 - 32 months, BCS between 3 - 4, bodyweight 24 - 26 kg) were selected from Sheep Research Farm, DSO, BAU for this study. The animals were kept in a semi-intensive system and supplied 200 gm of concentrates per animal per day. The health management, feeding, grazing, watering, and housing remained as routinely done by Sheep Research Farm, DSO, BAU.

Experimental design: Ewes (n = 18) were synchronized for estrus using prostaglandin (PGF2α) before underwent cervical ripening treatment. Ewes were randomly allocated into 3 groups for cervical ripening treatments; G1 [n = 06, control), G2 (n = 06, prostaglandin E1 (PGE1) as Misoprostol-Cytomis®, Incepta Pharmaceuticals Limited, Bangladesh], and G3 (n = 06, estrogen as Oestriol-Ovestin® + oxytocin as Linda-S® Vet). The cervical ripening treatments were administered just after the onset of estrus and left for 12 h before TC-AI. The ewes were inseminated transcervically at 12 - 24 h of onset of estrus using fresh semen. Cervical penetration was measured before cervical ripening treatment and after cervical ripening treatment i.e. at the time of insemination using a modified iron rod graded in centimeter. The cervical ripening was reflected with the depth of cervical penetration.

Semen collection and evaluation: Best selected indigenous ram (n = 3) were used as semen donors to inseminat in ewes. To prove the ram's fertility, six ejaculates of each ram were collected at an interval of 7 days and evaluated for semen quality. Semen was collected using an artificial vagina (especially for sheep and goat, Minitub, Germany) as described by Jha et al. (2018). Before collection, the prepuce of the ram was wiped clean to prevent semen contamination. Rams were allowed at least 1 - 2 false mounts before the collection of each ejaculation. Soon after semen collection, the graduated collecting tube was separated and kept at 35°C for semen evaluation as described by Jha et al. (2018).

Semen volume was recorded from a graded collection tube. To evaluate the mass activity, a drop (0.5 µl) of semen was placed on a pre-warmed slide (35°C) without coverslip and examined under a microscope equipped with phase-contrast optics (100×). The mass motility was scored into 4 scales: 0 = no motion, 1 = motion is mostly weak and oscillatory, not progressive. 2 = movements are vigorous, but no waves and eddies, 3 = motion is vigorous but waves and eddies formed slowly across the field, 4 = waves and eddies are formed rapidly but not so vigorous as in excellent grade and 5 = movements are so vigorous that it is impossible to observe individual spermatozoon in undiluted semen. The sperm concentration (spermatozoa 106/ ml) was calculated by using a hemocytometer. Briefly, semen samples were diluted with distilled water (1:400) to fix the spermatozoa. A drop of diluted semen was placed on the hemocytometer counting chamber from the edge of pipette and spermatozoa were allowed to settle for 5 - 6 min before placing the chamber on the stage of the microscope and calculated. The motility was determined by eye-estimation of the proportion of spermatozoa moving progressively straight forward.

Briefly, a drop (0.5 µl) of semen diluted at 1:4 ratio with TRIS was placed on a clean pre-warmed slide (37° C) and covered with a coverslip and observed at higher magnification (400×) and expressed as a percentage. The mean values of semen parameters; volume, density, mass motility, concentration, and progressive motility % are shown in Table 1. The mean volume, mass motility, concentration and motility were 0.75 ± 0.14 ml, 3.4 ± 0.65, 3.5 ± 0.47, 3398.6 ± 562.47 × 106/ml and 80.10 ± 5.70%, respectively.

Estrus synchronization: All ewes were synchronized for estrus using two doses of prostaglandin F2α (Ovuprost®, Cloprostenol, Bayer New Zealand Ltd., Auckland, New Zealand) 0.5 ml deep im per ewe at an interval of 9 days (Jha et al., 2020). Animals were closely observed for estrus behavior using a teaser ram.

Cervical ripening treatment: The synchronized estrus ewes; G2 received prostaglandin E1 analog (PGE1; Misoprostol-Cytomis®, Incepta Pharmaceuticals Ltd., Bangladesh) @ 400 µg per ewe intra-vaginally in the form of cream. For doing this, two tablets were grinded using pastel and mortar until becoming fine powder and mixed with 1 - 2 drops of glycerin (for diluting grinded powder and embedding into foam). A sterile piece of sponge was modified according to the size of vaginal diameter and embedded in powdered misoprostol saturated with glycerin. The medicated vaginal sponge then inserted just in front of the cervical opening using an applicator. Similarly, G3 received estrogen analog (Ovestin® 1 mg/ gm vaginal cream; Organon Ltd., Dublin, Ireland) @1 mg/ ewe intravaginally and, after 20 min before insemination, all the ewes were treated with synthetic oxytocin (Linda-S® Vet 10 IU/ml; Nuvista Pharma Limited, Gazipur, Bangladesh) @ 50 IU/ ewe through iv route.

Cervical Penetration Measurement: In all groups, cervical penetration was measured at the time at first detected estrus detection (before cervical ripening treatment) and just before insemination using a modified iron rod graded in cm with the modification of Leethongdee et al. (2007), who used AI gun plunger for measuring penetration depth. The effectiveness of treatment was measured by recording the depth of cervical orifice during TC-AI in term of easiness of AI gun passes.

Transcervical artificial insemination (TC-AI): All estrus ewes were inseminated following the measurement of cervical penetration, using fresh semen. A commercially available AI gun (especially for sheep and goat, Minitub, Germany) was used for trans-cervical insemination. The evaluated semen was loaded into 0.25 ml mini straws and then loaded into the AI gun. The estrous ewes were kept dorso-ventrally on the laparoscopic cradle at 30°. The vulva of the ewe was cleaned with a dry paper towel and lubricated with non-spermicidal water-soluble lubricant. A vaginal speculum was introduced into the vagina and forwarded to locate the cervix guided through a torchlight, and the cervix was grasped with a modified vulsellum forceps. The inseminating gun was then inserted into the cervix and pushed forward as much as it easily passes through the rings. After deposition of semen, the gun was kept in position for a period of 2 min before withdrawn from the cervix. A similar procedure was applied for insemination in all heated ewes.

Pregnancy diagnosis: All inseminated ewes were monitored for non-return rates to estrus by the aid of a vasectomized teaser ram on the 15 - 17 days following insemination. The ewes which were in non-return to estrus were allowed to trans-rectal ultrasonography within 40 - 50 days of post insemination for confirmation of pregnancy.

Statistical analysis: The paired t test and ANOVA was used with a confidence interval of 95% and p values less than 0.01 and 0.05 were considered as a significant difference. The analysis was done using the SPSS software (IBM SPSS data editor, Version-12).

Results

Effects of Cervical Ripening Treatment on Penetration of AI Gun in TCAI in Ewes: The effects of cervical relaxation treatment are shown in Table 2. The Mean ± SE values for depth of cervical penetration of different groups were 0.33 ± 0.10, 0.41 ± 0.09 and 0.43 ± 0.09 cm before cervical ripening treatment and 0.43 ± 0.07, 3.50 ± 0.26 and 1.54 ± 0.14 cm following

Table 1. Characteristics of ram semen used in the TCAI program (Mean ± SE)

Ram ID	Volume (ml)	Mass motility (1 - 4)	Concentration ($\times 10^6$ spermatozoa/ml)	Progressive motility (%)
21	0.86 ± 0.11	3.80 ± 0.25	3877.5 ± 244.40	81.25 ± 4.78
23	0.73 ± 0.09	3.25 ± 0.50	3309.0 ± 341.27	81.50 ± 6.02
HM06	0.55 ± 0.07	3.25 ± 0.35	2620.0 ± 395.97	75.00 ± 7.07
Pooled	0.75 ± 0.14	3.50 ± 0.47	3398.6 ± 562.47	80.10 ± 5.70

Table 2. Depth of cervical penetration (before and after cervical ripening treatments)

Group	Drug and dose of treatment	Number of ewes	Before treatment; time at first detected estrus (cm; Mean ± SE)	After treatment; during TC-AI (cm; Mean ± SE)
A	Not received any treatments	6	0.33 ± 0.10^{a}	0.43 ± 0.07^{c}
B	PGE1; Misoprostol-Cytomis®	6	0.41 ± 0.09^{a}	3.50 ± 0.26^{a}
C	Estrogen; Ovestin® + Oxytocin; Linda-S®	6	0.43 ± 0.09^{a}	1.54 ± 0.14^{b}

cervical ripening treatment, respectively. There was no significant variation in AI gun passes (depth of penetration) among the two different treatment and control groups before treatment, however, mean values of cervical penetration were significantly different following treatment ($p<0.05$). Cervical penetration depth was significantly increased ($p<0.01$) in G2 and G3 ($p<0.05$) treatment compared with G1.

Effects of Cervical Ripening Treatment on Pregnancy Rates and Sex Ratio: Pregnancy rates and numbers of different sex's lamb kids in different treatment and control groups are presented in Table 3. Though there was a different ratio of pregnancy rates between groups, but this difference was not statistically significant ($p>0.05$). However, the pregnancy rates of PGE-Misoprostol (66.67%) was near to significant difference compared to control groups (33.33%). Furthermore, the pregnancy rates of estrogen + oxytocin (50%) were higher than the control group (33.33%). In this study, all kids born under control groups were female (n = 03), However, all kids born under PGE-Misoprostol were males (n = 05. Interestingly, the ratio of male and female kids was more or less similar (2 vs 3). But in terms of the total number of kids, the sex ratio of different kids were not significant (male vs female: 7 vs 6).

Discussion

In sheep, AI is a commonly used technique to speed up the genetic gain and increase production in a short time. The success of TC-AI depends on semen deposition into the cervix as deep as possible for increased pregnancy rates (>50%; Leethongdee, 2011). However, it is challenging due to the complex cervical anatomy of the ovine cervix. In regular TC-AI methods; without using cervical ripening hormonal treatments, the pregnancy rates results lower and not supposed to be acceptable for farmers. To increase pregnancy rates, hormones like; estrogen, oxytocin, and prostaglandin are used for cervical ripening and thereby increase the depth of cervical penetration and pregnancy rates (Salamon and Maxwell, 1995; Donovan et al., 2004; Leethongdee et al., 2007).

In this study, we had planned to evaluate the effect of cervical ripening treatments, depth of cervical penetration, and its effect on pregnancy rates. The depth of cervical penetration before cervical ripening treatments were similar among the groups, however, differed significantly following cervical ripening treatments. We observed pregnancy rates 33.33%, 66.67%, and 50% in the G1, G2, and G3, respectively. The pregnancy rates 33.33% in G1 are comparable to that of King et al. (2004) who found 20 - 40%. Another report showed 68.18% pregnancy rates in ewes with frozen semen after 3 - 4 h intracervical administration with 50 - 100 µg of PGE1 analog (misoprostol) for each ewe (Rashidi and Cedden, 2013) which is comparable to our result.

In G2 (PGE1 as Misoprostol-Cytomis), the penetration depth was deeper than other two groups (3.50 ± 0.26 cm vs 0.43 ± 0.07 cm and 1.54 ± 0.14 cm). The cervical lumen of G3 (estrogen + oxytocin) was comparatively more dilated (1.54 ± 0.14 cm) to pass an AI gun than G1 (0.43 ± 0.07 cm). Misoprostol (Cytomis 400 µg/ ewe) was applied locally as intra-vaginal embedding for 12 h before TC-AI and found cervical penetration 3.50 ± 0.26 cm; however, Leethongdee et al. (2007) found 5 - 6 cm cervical penetration with misoprostol treatment (1 mg/ ewe dose). The dose difference of misoprostol in the present study (400 µg/ ewe) and a previous study (Leethongdee et al., 2007; 1 mg/ ewe) might be due to breed variation. Leethongdee et al., (2007) used Welsh Mountain Sheep, comparatively larger breed whereas we used indigenous ewes which are smaller sized sheep. Post-treatment cervical penetration is significantly differing from pre-treatment cervical penetration within the groups. Moreover, in G2, a highly significant difference is shown in our study. In the control group, this change might be due to an increase of estrogen level and increased cervical mucus flow, but which was not sufficient to pass an AI gun for achieving a satisfactory result. The local intra-vaginal application of the PGE1 analog, misoprostol to non-pregnant sheep (Leethongdee et al., 2010) reported evidence of a PGE-mediated mechanism of

cervical relaxation during the periovulatory period. We used PGE1 analog (Misoprostol®) @400 µg/ ewe for dilatation of cervical lumen in G2.

In fact, the PGE1 in misoprostol increases cervical ripening by inducing collagen breakdown and softening of the cervical tissue structure, a mechanism known to be associated with local production of PGE and glycosaminoglycans (Ellwood et al., 1980; Ledger et al., 1983). The recent hypothesis proposed that PGE2 selectively binds to EP2 and EP4 (PGE2 receptors), stimulating hyaluronan (HA) synthesis, which may cause remodeling of the cervical extracellular matrix and culminating in cervical relaxation (Kershaw-Young et al., 2009). The effect of misoprostol is short-lived and suggested that the length of time for cervical ripening after misoprostol application is 4 - 6 h (Goldberg et al., 2001). For this reason, misoprostol was administered after the onset of estrus and kept for 12 h before AI. Estrogen (Oestriol 1mg/ ewe, ie. Ovestin®) for 12 h and followed by Oxytocin (Linda S-Vet) 40 IU iv/ ewe just 20 min before AI in G3.

In the present study, cervical ripening with estradiol and oxytocin treatment, the AI gun was able to penetrate through the cervical canal in comparison to control group. Estrogen had no effect on cervix dilation but the injection of estrogen in the combination of oxytocin can completely dilate the cervix of ewes, which partially goes with the present study. Exogenous oxytocin can dilate the cervix of the ewe (Khalifa et al., 1992; Stellflug et al., 2001); however, it is not with the agreement of another study (King et al., 2004), who found no significant dilation with oxytocin treatment.

The present study identified an effective cervical ripening treatment protocol for increasing pregnancy rates following TC-AI in indigenous ewes of Bangladesh. However, the experiment was not conducted in a field assay. The field trial would reveal the sustainability of the results of the present study to be used for farmer's ewe AI.

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