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Effects of Different Preparations of Gonadotropins (oFSH and pFSH) on Superovulatory Response and Embryo Yield in Indigenous Ewes in Bangladesh

Research Article

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ABSTRACT

Two different gonadotropins on superovulatory response and embryo yield in indigenous ewes were studied. Donor ewes (n=33) were synchronized for oestrus using vaginal sponges containing 45 mg flurogestone acetate (FGA) for 12 days. For the superovulation, different types of gonadotropins were used in ewes randomly allocated into group I [n=16; ovine follicle-stimulating hormone (oFSH), 2.2 mg] and group II [n=17; porcine follicle-stimulating hormone (pFSH), 25 mg]. Gonadotropin treatments started on day 10 of FGA treatment for four consecutive days. The superovulatory response and embryo recovery was performed by modified semi-laparoscopic method on day 5/6 following mating. The stages (very early morula, morula, early blastocyst, blastocyst and hatching blastocyst) and grades (grade 1 to 5) of embryos were calculated. The oestrus response rate was 100%, and had significantly earlier onset of oestrus with pFSH than oFSH (23.41±0.50 h *vs.* 26.15±0.46 h, respectively). The corpus luteum/donor was significantly higher with pFSH than oFSH (14.18±1.63 *vs.* 10.25±0.89, respectively). The embryos recovered/donor differed insignificantly with pFSH than oFSH (11.82±1.48 *vs.* 8.81±0.86, respectively). The embryo recovery rate was higher using oFSH than pFSH (85.04±2.93% *vs.* 82.75±3.31%, respectively). The proportion of morula and grade 1 embryos was differed insignificantly with both oFSH and pFSH (86.52% and 97.16% *vs.* 84.58% and 90.55%, respectively). In conclusion, both the oFSH and pFSH were equally efficient, giving consistent ovulation rate and embryo yield.

KEY WORDS corpus luteum, sheep, synchronization.

INTRODUCTION

Multiple ovulation and embryo transfer (MOET) are used to accelerate sheep genetics improvement through female's line by increasing ovulation rate, more transferable quality embryos, and thereby more lambs. Preliminary studies were done on oestrus synchronization (Roy *et al.* 2014; Zohara *et al.* 2014a; Zohara *et al.* 2014b; Zohara, 2016) and superovulatory response and embryo production (Zohara *et al.* 2014b; Zohara, 2016) using different preparations of gonadotropins and other hormones. In superovulatory regime, 45 mg Flurogestone acetate (FGA) was suitable for inducing oestrus in indigenous ewes (Zohara *et al.* 2014a; Zohara *et al.* 2014b; Zohara *et al.* 2017). Comparing the pFSH (porcine follicle-stimulating hormone) and eCG (equine chorionic gonadotropin), the former resulted in the best superovulatory rate (Zohara *et al.* 2017). However, induction of oestrus with oFSH, which is a specific gonadotropin used for superovulation in sheep (Forcada *et al.* 2000) was not done. For successful MOET, an accepted

embryo recovery rate was suggested above 85% (Bari *et al.* 2000; Cordeiro *et al.* 2003; Freitas and Melo, 2010). The choice of gonadotropins directly affects the superovulatory rate and embryos yield. A varieties of gonadotropins such as eCG, anterior pituitary extract from the horse (HAP), pFSH, ovine follicle-stimulating hormone (oFSH), and human chorionic gonadotropin (hCG) or human menopausal gonadotropin (hMG) have been used for inducing superovulation in donor ewes (Schiewe *et al.* 1985; Forcada *et al.* 2000; Bari *et al.* 2003; Ağaoğlu *et al.* 2012). Types and preparations of gonadotropins, animal origin, FSH/LH ratio, and superovulatory scheme can affect the ovarian response (Gonzalez-Bulnes *et al.* 2000a; Bari *et al.* 2003). Mainly two types of gonadotropins, oFSH and pFSH have been used for superovulation in donor ewes (Forcada *et al.* 2000; Bari *et al.* 2003; Ağaoğlu *et al.* 2012). It is reported that the efficiency of superovulatory response is increased using oFSH in sheep (Forcada *et al.* 2000). The present study was designed to evaluate the effectiveness of oFSH over pFSH on superovulatory response and embryo yield in indigenous ewes.

MATERIALS AND METHODS

All experimental procedures were approved by Animal Experimental Ethics Committee (AEEC) of BAU (Ref. no. AEEC/DSO-BAU/02/2015). The study was carried out at Department of Surgery and Obstetrics, Bangladesh Agricultural University (BAU), Mymensingh (N 24.73 latitude and E 90.44 longitude). The study was conducted during breeding season, between September 2016 and June 2018.

Oestrus synchronization and superovulation protocol of donor ewes

Apparently mature, healthy and cyclic donor indigenous ewes (were breed) aged 24 to 30 months, body weight 15 to 18 kg and body condition score 3.5 to 4.0 were synchronized for oestrus by inserting intravaginal sponges impregnated with FGA 45 mg (Syncrite-45® Vaginal Sponge, Animal Health Supplies, Ulladulla NSW 2539, Australia) for 12 days. To induce superovulation, donor ewes were induced with gonadotropins either oFSH (Ovagen™ ICP bio Reproduction, New Zealand) or pFSH (Folltropin®, Vetoquinol SA, France). The oFSH (2.2 mg) and pFSH (25 mg) were injected i.m. (intramuscular) single doses applied twice a day in 12 h interval beginning from day 10 of the induced oestrus or 48 h prior to sponge withdrawal for 4 consecutive days (Figure 1, Bari *et al.* 2003).

Ewes (n=33) clinically healthy and oestrus synchronized donor ewes were randomly allocated into two groups for superovulation with two different preparation of gonadotropins; group I (n=16) induced with oFSH and group II (n=17) induced with pFSH to evaluate the effectiveness of oFSH over pFSH on superovulatory response and embryo production in indigenous ewes.

Detection of oestrus and hand-mating of donor ewes

The vasectomized teaser indigenous ram (wera breed, n=2) was used to detect oestrus. The ewes were kept with teaser rams, and observed for signs of oestrus twice a day (0700 h and 1900 h) for 60 min in each observation from 12 h to 66 h following sponge removal. All detected oestrus donor ewes were naturally mated from selected rams (n=8) 6 h and 24 h after the onset of oestrus.

Media used for flushing and holding embryos

A commercial Emcare™ (0.4% albumin, 25 mg/L, kanamycin-sulphate; ICP-bio, Reproduction, New Zealand; Swelum *et al.* 2014) and Emcare™ (0.4% albumin, MOPS buffer, 25 mg/L, kanamycin-sulphate; ICP-bio, Reproduction, New Zealand; Blanco *et al.* 2003) was used as embryo flushing and holding media, respectively.

Counting of corpora lutea (CL) and embryo recovery from donor ewes

A modified semi-laparoscopic method was used to recover embryos from the donor ewes (Naqvi *et al.* 2006). The counting of CL and recovery of embryos were performed on day 5/6 following mating. Day 5/6 was considered from the time and day of standing oestrus and first successful mating.

Prior to laparoscopic procedure, the donor ewes were withheld from feed and water for at least 24 h. The abdominal area cranial to the udder was shaved. Anesthesia was induced firstly with xylazine hydrochloride (2%, Xyla®, Interchemiewerken, Netherlands) at 0.22 mg/kg body weight i.m. followed by G Ketamine® (Ketamine hydrochloride 50 mg/mL, Gonoshasthaya Pharmaceuticals Ltd., Dhaka, Bangladesh) 5 min later with the dose of 10 mg/kg body weight i.v. (intravenous). Anesthetized donor ewe was placed on a laparoscopic cradle in dorsal recumbence at 45˚ angle. The surgical area approximately 12 to18 cm cranial to the udder was scrubbed with povidone iodine (7.5% w/v, Povisep®, Jonson Pharma Ltd., Bangladesh). The abdomen was covered with a sterile drape attached with towel clip. Two small incisions (0.3 to 0.4 cm) were made through the skin to the left and right, approximately 3 to 4 cm of the midline and 4 to 5 cm cranial to the teats. Two trocar and cannula, 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).

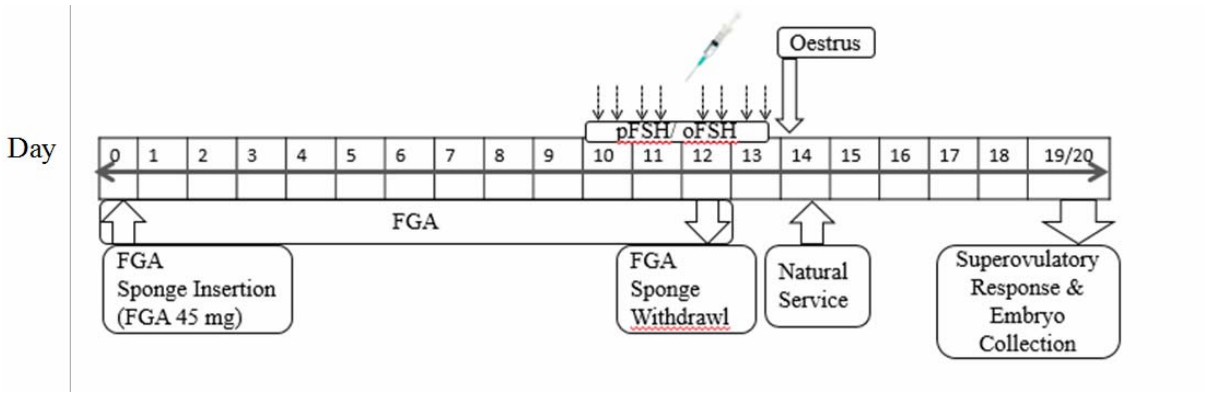

Figure 1 Schematic diagram of superovulatory treatment protocol of donor ewes

The trocars were removed from each cannula. A laparoscope tube (7 mm diameter, 180˚) fitted to a high intensity fibre optic light source (Endolight led light sources, 4215, Richard Wolf Germany) and a rotatable uterine grasping forceps (3.5 mm diameter, 240 mm length, Richard Wolf, Germany) were introduced into 7 mm and 5 mm cannula, respectively. The interior of the abdomen was viewed through the laparoscope and at the same time with the help of uterine grasping forceps, the number of CL was counted. Ewes showing more than three CL were considered as superovulated and were undertaken for further procedure. For embryo collection, the uterine grasping forceps were removed and the 5 mm cannula punctured wound was extended posteriorly (little bit bigger than the diameter of the uterine horn) using scalpel blade. The 5 mm cannula was replaced with a modified Babcock Forceps (teeth blunt). With the help of laparoscope tube, one uterine horn was grasped, exteriorized carefully and positioned with the help of finger for flushing embryos. A puncture hole was made at the base of the uterine horn about 3 to 4 cm below the external bifurcation with the help of 14-gauge needle (Monoject 14×1.5"). An 8 Ch/Fr Foley catheter (Bardex®, 2.7 mm, 3 mL, Malaysia) with an intubating stylet was inserted into the lumen through a puncture hole into the uterine horn. The stylet was pulled out and the balloon of the Foley catheter was inflated with 3 to 4 mL air using 5 mL syringe. The puncture wound was made in the tip of the uterine horn using a blunted 17-gauge needle attached to a 20 mL syringe loaded with 20 mL embryo flushing medium maintained at 37 ˚C. The flushing was performed slowly with continuous and gentle pressure. The flushing medium containing embryos was collected into a pre-warmed sterile 50 mL falcon tube. After flushing, the uterine lumen was completely evacuated by blowing air through 20 mL air using the same needle and syringe. The falcon tube containing flushed media was maintained into the water bath at 37 ˚C for sedimentation of the embryos.

The Foley catheter was carefully removed after deflation of the balloon. The exterior wall of the uterus was washed with 20 mL of heparinized saline and the uterine horn was replaced into the abdominal cavity. Similar flushing procedure was repeated for the other horn. Excess gas if any was expressed and a sulphanilamide powder (Sumid-Vet®, Square Pharmaceuticals Ltd., Bangladesh) was applied at the wound sites. The wound was closed by stitching muscle and fascia with chromic catgut 1-0 (4 metric) using continues suture and skin with silk 2-0 (non-absorbable) using interrupted suture. The sutured site was painted with Povisep® and a tincture benzoin cotton seal was applied. An i.m. injection of 0.4 mL (100 µg) Ovuprost®, a synthetic prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) analogue [Cloprostenol 250 µg/mL, Bayer New Zealand Ltd., Auckland, New Zealand] was given for regression of CL. The ewes were placed in sternal recumbency and allowed to remain undisturbed in pens for 2 to 3 h. All donor ewes were given 2 ml Streptopen® (Procaine benzyl penicillin 3 lac unit, Benzyl penicillin sodium 1 lac unit and Streptomycin 0.5 gm, Renata Animal Health Ltd., Dhaka, Bangladesh) i.m. as a precaution against infection for 5 to 6 days.

Searching and assessment of embryo quality

With the help of micropipette (1000 µL), the flushed media were aspirated from the Falcon tube starting from the bottom and transferred into 65 mm petri dishes. Searching of the embryos was carried out under the stereo microscope (20× to 30×, Olympus, SZX2-TR30, Tokyo, Japan) equipped with thermal plate at 37 ˚C. The second inspection of the petri dish was always carried out. Detected embryos were aspirated with a micropipette (0.5 to 10 µL) and transferred in a 35 mm petri dish containing a drop (50 to 100 µL) of holding medium maintained at 37 ˚C. Embryo assessment was carried out based on morphological aspects under the stereo microscope (20× to 40×, Olympus, SZX2-TR30, Tokyo, Japan).

They were counted and classified according to the stage of development; very early morula, morula, early blastocyst, blastocyst, and hatching blastocyst and given a quality grade on their gross morphological appearances (1 to 5 grades, Bari *et al.* 2000).

Statistical analysis

The data were analyzed using SPSS (2011) (20 Version). The time of onset of oestrus, duration of oestrus, total number of CL, total number of embryos collected, embryo recovery rate and rate of fertilized ova per donor between the superovulatory donor groups ewes were analyzed using independent sample t-test. The oestrus response rate, stages and grades of embryo between the superovulatory donor group ewes were analyzed using the Chi-square test followed by Fisher's exact test where appropriate. The variations were regarded as significant when P-values as less than 0.05.

RESULTS AND DISCUSSION

The oestrus response, time of onset of oestrus and duration of oestrus following induction with FGA along with superovulatory hormones (oFSH and pFSH) are shown in Table 1. The oestrus response rate was 100% in both groups. The mean time to onset of oestrus was significantly earlier (P<0.001) with pFSH compared to oFSH. However, the duration of oestrus differed insignificantly with oFSH than pFSH group.

The effects of two gonadotropins (oFSH and pFSH) on the number of CL, number of embryos recovered, embryo recovery rate, and fertilized ova per donor are shown in Table 2. The number of CL per donor was significantly (P<0.05) higher with pFSH than oFSH. The number of embryos recovered per donor was differed insignificantly with pFSH than oFSH. The embryo recovery rate was similar with both gonadotropins; however, the percentage was differed insignificantly with oFSH than pFSH. The fertilized ova with both gonadotropins were 100%.

Table 3 shows the stages of embryo development on day 5/6 after natural service following treatment with two gonadotropins oFSH and pFSH. There is not significant difference between the two gonadotropins on the stages of embryo. The stages of embryo were differed significantly (P<0.001) within each gonadotropin treatment group. The morula stage was differed insignificantly with both oFSH and pFSH compared with other stages of embryo.

Table 4 shows the grades of embryos following treatment with two gonadotropins. There is not significant difference between the two gonadotropins on grades of embryo.

The grades of embryo differed significantly (P<0.001) within each gonadotropin treatment group. The embryos with grade 1 was differed insignificantly with oFSH than pFSH. Different preparations of gonadotropins have been formulated to induce superovulation and produce high numbers of quality embryos in ewes. A 45 mg FGA and pFSH was found to be more effective for the synchronization of oestrus and superovulation within MOET program in indigenous sheep of Bangladesh (Zohara *et al.* 2014b; Zohara, 2016). In this study, about 45 mg FGA was used to synchronize oestrus along with two different preparations of gonadotropins, oFSH and pFSH as superovulatory treatments. In the present study, the oestrus response rates were 100% and it is in agreement with other workers (Akoz *et al.* 2006; Mayorga *et al.* 2011; Zohara *et al.* 2014b), but higher than reported by Gonzalez-Bulnes *et al.* (2002) and D'Alessandro *et al.* (1996) who reported a lower response (87-92% and 88.8%, respectively). The time of onset of oestrus and duration of oestrus following withdrawal of synchronizing agent (45 mg FGA) was 23.41 ± 0.50 h to 26.15 ± 0.46 h and 31.92 ± 1.95 h to 33.66 ± 2.28 h, respectively. The time of onset of oestrus with 45 mg FGA in donor ewes was similar to Zohara (2016) and D'Alessandro *et al.* (1996) who reported 20.5 ± 1.4 h to 28.2 ± 0.84 h and 25.7 ± 1.4 h, respectively. In contrast, time of onset of oestrus was lower than that of Gonzalez-Bulnes *et al.* (2002) who quoted 31.5 ± 1.5 h after using 40 mg FGA-oFSH, and Simonetti *et al.* (2000) who reported 55.94 h to 57.7 h with 40 mg and 60 mg FGA.

The time of onset of oestrus differed significantly between the two gonadotropins groups. This indicates that there may be some effect of gonadotropins on the onset of oestrus with these synchronizing agents influencing the follicular growth, development and maturation. Many studies explained that acceleration of the onset of oestrus may vary when using FSH concomitantly with or before progesterone removal. The discrepancies may be explained further due more to differences in doses of FGA, breed, age, and geographical location (Bari *et al.* 2003).

The number of CL per donor was significantly higher with pFSH than oFSH (14.18±1.63 *vs.* 10.25±0.89). This ensured the mean number of embryos recovered. This superovulatory response was excellent and higher than Zohara (2016) who reported 11.21 ± 0.14 after using pFSH, but lower than Gonzalez-Bulnes *et al.* (2002) who reported 18.2 ± 3.8 after using oFSH. The CL per donor are comparable with other studies (Bettencourt *et al.* 2008; Pampukidou *et al.* 2011), however it differs from other studies (Gonzalez-Bulnes *et al.* 2000a; De Silva *et al.* 2003; Lopes *et al.* 2006).

Table 1 Effects of superovulatory protocols of FGA + oFSH and FGA + pFSH on responses in donor indigenous ewes within multiple ovulation and embryo transfer (MOET) program

Superovulatory protocols	FGA + oFSH (n=16)	FGA + pFSH (n=17)	P-value
Oestrus response (%)	100	100	-
Time of onset of oestrus (h) (Mean±SEM)	26.15±0.46[b]	23.41±0.50[a]	0.001
Duration of oestrus (h) (Mean±SEM)	33.66±2.28	31.92±1.95	0.567

FGA: flurogestone acetate; oFSH: ovine follicle-stimulating hormone and pFSH: porcine follicle-stimulating hormone.
SEM: standard error of the means.

Table 2 Effects of ovine follicle-stimulating hormone (oFSH) and porcine follicle-stimulating hormone (pFSH) on responses in donor indigenous ewes within multiple ovulation and embryo transfer (MOET) program

Gonadotropins	oFSH (n=16)	pFSH (n=17)	P-value
Corpora lutea/donor (Mean±SEM)	10.25±0.89[a]	14.18±1.63[b]	0.045
Embryos recovered/donor (Mean±SEM)	8.81±0.86	11.82±1.48	0.090
Embryo recovery rate/donor (%; Mean±SEM)	85.04±2.93	82.75±3.31	0.610
Fertilized ova (%; Mean±SEM)	100±0.00	100±0.00	-

SEM: standard error of the means.

Table 3 Effects of ovine follicle-stimulating hormone (oFSH) and porcine follicle-stimulating hormone (pFSH) on percentage of embryos at different stages of development in indigenous donor ewes

Gonadotropins	oFSH (n=16)	pFSH (n=17)	P-value
Embryos yield (n)	141	201	
Very early morula	-	-	
Morula	86.52 (122/141)	84.58 (170/201)	0.886
Early blastocyst	9.93 (14/141)	9.95 (20/201)	
Blastocyst	2.13 (3/141)	6.61 (8/201)	
Hatching blastocyst	1.41 (2/141)	2.57 (3/201)	

Table 4 Effects of ovine follicle-stimulating hormone (oFSH) and porcine follicle-stimulating hormone (pFSH) on percentage of embryos at different grades in indigenous donor ewes

Gonadotropins	oFSH (n=16)	pFSH (n=17)	P-value
Embryos yield (n)	141	201	
Grade 1	97.16 (137/141)	90.55 (182/201)	
Grade 2	2.84 (4/141)	7.46 (15/201)	0.070
Grade 3	-	1.00 (2/201)	
Grade 4	-	-	
Grade 5	-	1.00 (2/210)	

The number of CL per donor was significantly lower with oFSH than pFSH. There is no suggestion that oFSH produces an increased superovulatory response relative to the 'foreign' hormone pFSH. The differences may be attributed due to differences in ovarian response (Cordeiro *et al.* 2003). It is believed that endogenous FSH concentrations are responsible for variation in ovulation rate between these groups of ewes (Fernie *et al.* 1994; Bari *et al.* 2001). Difference may be attributed due to difference in commercial preparations of FSH that may contain variable FSH/LH ratio between batches (Gonzalez-Bulnes *et al.* 2000a; Bettencourt *et al.* 2008). There are reduced ovulatory responses when FSH/LH ratio is increased or decreased. An elevated FSH/LH ratio might disrupt the sequence or balance of androgen and oestrogen production, which is critical for prevention of atresia of follicles, and may down-regulate its receptors on the theca and / or granulosa cells.

Therefore, an optimum of FSH/LH ratio is required for optimum responses in ewes (Bülbül *et al.* 2009).

Beside high superovulatory response, the success rate of MOET program depends upon embryo recovery rate resulting in maximum number of good quality embryos from each donor. The number of embryos recovered per donor and embryo recovery rate were 8.81 ± 0.86 to 11.82 ± 1.48 and 82 to 85%, respectively. These differences were not statistically significant. The embryo recovery rates was comparable with others authors (Bari *et al.* 2000; Baldassarre *et al.* 2002; Cordeiro *et al.* 2003; Lopes *et al.* 2006; Freitas and Melo, 2010).

However, the embryo recovery rates was higher than Zohara (2016) and Gonzalez-Bulnes *et al.* (2002) who reported 74% and 45.0 ± 3.8%. This could be due to refinement of the technique (modified laparoscopic) and ease in performance.

The difference in recovery rate may be due to gonadotropins treatment protocols. In this study the superovulatory treatment was given at constant dose which may lead to produce more embryos (Bari *et al.* 2003; Sánchez *et al.* 2013). The initial greater doses of FSH in a decreasing dose protocol can induce rapid and abnormal ovarian follicular development, desynchronization of oocyte-follicle growth reduction resulting in embryo recovery (Berlinguer *et al.* 2004).

More morulae (>85%) than other stages of embryo were observed in both groups of FSH. Embryos were flushed on day 5/6 after mating. Most of embryos developed to morula around day 5/6 after successful mating. Difference in stages of embryos may vary due to different sources of FSH. These may be explained by differences in FSH/LH ratio affecting both oestradiol and progesterone concentrations in blood, influencing oocyte catching by infundibulum, oocyte transportation, and quality of embryos produced (Gonzalez-Bulnes *et al.* 2000a; Gonzalez-Bulnes *et al.* 2000b). More grade 1 embryos (>90%) were recovered with both the FSH types. This result was comparable with several researchers (Cordeiro *et al.* 2003; Lopes *et al.* 2006; Larsson *et al.* 2007; Cueto *et al.* 2010). The differences in grades of embryos may be influenced by the preparations of gonadotropins used, permitting considerable progress in obtaining healthier superovulatory response for obtaining good-quality embryos.

CONCLUSION

In conclusion, both gonadotropins, oFSH and pFSH are suitable as superovulatory hormones due to excellent mean ovulation rate and embryo production in indigenous ewes. However, further trial may be performed to sustain this observation, since oFSH does not consider as a foreign hormone by the receptor of sheep ovarian follicle leading to increased superovulatory response compared to pFSH.

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