

Response of Superovulation by Using FSH (Follicle Stimulating Hormone) and Sex Determination of Embryos Using PCR in Pesisir Cows of West Sumatra

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Abstract. This study was conducted to determine the response of superovulation by giving 16 ml dosage of FSH hormone to female Pesisir cattle. The estrus schedule of 15 Pesisir cows was set by inserting CIDR (Controlled Internal Drug Release) into the vagina for 12 days. At day 10, all cattle were injected with FSH for three consequent days but with decreasing dosage. On the 3rd day, FSH injection was accompanied by PGF_{2α} injection and CIDR was removed. The detection of estrus was performed at day 13. Natural mating was proceeded after the estrus signs visible. Collection of donor embryos was done on the 6th and 8th day after mating. The variables measured were the response of superovulation, total number of corpus luteum, number of embryos and sex ratio. The results obtained were all Pesisir cows responded to superovulation. The average number of corpus luteum and embryos per cow were 5.93±3.17 and 6.00 (61.64 %), respectively, while the total of transferable embryos were 90, with an average of 6.00 or 61.64%. The sexing of embryos obtained in this study from 146 embryos were 76.03% males (111 embryos) and 23.97% females (35 embryos). Based on total of transferable embryos, there were 51.37% male embryos and 48.63% of females embryos. The result of this study showed that the sex ratio of male embryos was higher than female embryos.

Key words: Pesisir Cattle, response of superovulation, FSH, corpus luteum, total of embryos, PCR and sex ratio of male and female

Abstrak. Penelitian ini dilakukan dengan metode eksperimen untuk mengetahui respon superovulasi pada dengan level dosis 16 ml hormon FSH pada sapi Pesisir. Materi yang digunakan yaitu 15 ekor induk sapi Pesisir. Sapi Pesisir diatur jadwal berahinya dengan memasang CIDR (*Controlled Internal Drug Release*) pada vagina selama 12 hari selanjutnya pada hari ke-10 semua ternak diinjeksi dengan FSH. Injeksi FSH menggunakan dosis 16 ml FSH dan pemberian FSH dilakukan dengan dosis menurun selama 3 hari. Hari ke-3 injeksi FSH diiringi dengan injeksi PGF_{2α} dan pencabutan CIDR. Deteksi berahi dilakukan pada hari ke-13. Kawin alam dilakukan setelah tampak tanda-tanda berahi dari ternak. Koleksi embrio donor dilakukan pada hari ke-6 sampai 8 setelah perkawinan. Variabel yang diamati adalah respon superovulasi, jumlah korpus luteum, jumlah embrio dan *sex rasio* embrio. Hasil yang diperoleh adalah respon superovulasi sapi Pesisir adalah 100 %. Rataan dari jumlah CL dan embrio sapi Pesisir adalah 5,93±3,17 dan jumlah embrio yang layak untuk ditransfer adalah 90 buah dengan nilai rata-rata 6,00 atau 61,64 %. Jenis kelamin embrio yang didapatkan dari 146 embrio pada penelitian ini, terdapat 76,03% (111 embrio) dengan jenis kelamin jantan dan sebanyak 23,97% (35 embrio) dengan jenis kelamin betina. Dari total embrio yang layak transfer terdapat 51,37% embrio berjenis kelamin jantan dan 48,63% embrio berjenis kelamin betina. Hasil penelitian ini menunjukkan bahwa rasio embrio berjenis kelamin jantan lebih tinggi dibandingkan dengan embrio berjenis kelamin betina.

Kata kunci: sapi Pesisir, respon superovulasi, FSH, corpus luteum, jumlah embrio, PCR dan *sex rasio* jantan dan betina

Introduction

Pesisir cattle is one of the indigenous Indonesian cattle which is mostly bred by farmers in West Sumatera, especially in the South Pesisir Regency. They are found spread

over the coastal areas such as Padang Pariaman Regency, South Pesisir Regency, and Agam Regency (Anwar, 2004). Some desirable characteristics of this local cattle are its ability to adapt to low quality feeds and the traditional extensive production system, as well as the

ability to resist tropical the diseases and parasites (Adrial, 2010).

Pesisir Cattle contributed significantly towards the supply of meat for people in the West Sumatera province, especially for qurban (sacrificial animal) on Eid al-Adha (Mosleem Feast Day). The need for beef in the population has increased by 2.0 kg/capita/year during the last 10 years (Victorbuana, 2010). For the last few years, the population of Pesisir Cattle has continuously declined and may lead to the extinction of this germplasm in West Sumatera. Artificial insemination (AI) and embryo transfer (ET) technology are the practical options to solve this problem. ET technology is used not only to accelerate the number of cattle population but also to provide the opportunity for embryo manipulation by gender adjustment of the embryo to increase the number of descendants for one type of sex in preserved aattle population. According to Afriani and Lismanto (2015), to optimize AI, estrus synchronization is needed by giving 16 ml FSH + 200 mg GnRH injection. Furthermore, after the cow has successfully been induced for estrous, the AI process can be carried out. Then, on the sixth until eighth day after the AI, the flushing/embryo harvesting can be conducted followed by sex determination using PCR technique. Chen et al. (2007) mention that the best sex determination technique is by PCR, because the method is simpler, accurate and inexpensive. The advantage of sex determination of embryos is the increase on economic efficiency in embryo transfer program. In this study, male sex identification was conducted when applying ET to generate male cattle in order to increase meat production.

Materials and Method

Research Materials

The sample of the study are: 15 healthy Pesisir Broodstock cows, 47 months of age and 2 parity. Consumable chemical materials

includes some substances, such as CIDR (Control Internal Drug Release), FSH (Folltropin-V brand produced by Bioniche Animal Health Canada Inc), PGF_{2α} (Capriglandin Inj brand produced by Caprifarmindo Labs, Bandung Indonesia).

The materials for flushing media, such as lactate ringer, lydocain, gentamycin and 5% calf serum, male cattle sperm, petridish, micropipette, pastuere pipette.

PCR material consists of bunsen burner, stereo microscope, PBS solution, calf serum, gentamicin, lactat ringer, lydocain, cotton, alcohol 70 %, Primer BRY, Kit RTG (pure Tag Ready-to-Go PCR Beads (Product Booklet), agarose 2 % TBE solution 1x and ethidium bromide dye, loading dye (blue) double destilation water (ddH₂O).

The utilized tools are straw, bunsen burner, Eppendroff microtube size (1.5 ml; 0.5 ml and 0.2 ml), vortex, microcentrifuge, PCR machine, observation camera of gels electrophoresis result, petridish, wipes, cotton, aluminium foil, stereo microscope, foley catheter, pasteure pipette, rubber tube, needle, embryo filter, stir bars, gun AI set, CIDR applicator.

Research Prosedures

The procedure of the study was as follow;

A. Superovulation

The 15 Pesisir cattle were given CIDR (containing progesterone); CIDR was dispositioned in front of the cervix for 11 days, then on day 10, FSH injection was given with the optimum dose given at noon for three days. The cows was given 16 ml dose of FSH. On day 3, Gn RH was injected with the best dose at 200 mg GnRH based on a previous study. Once the estrus was detected AI was conducted on the cows.

B. Flushing of Embryos

On day 6th until day 8th after AI was conducted, flushing of embryos or harvesting was done with following procedures: the donor cattle was placed in a special restraining

cage with the anterior part higher than the posterior part by 10 to 20 cm. The reproductive organ was checked through rectal palpation, removal of excreta at the first place, followed by clinical examination of the cervix, uterus and ovaries and the number of corpus luteum on right and left ovary was counted. Moreover, epidural anesthesia with 2 % Lydocain in 4 – 6 ml was applied to ease the insertion of foley catheter and reduce defecation during the flushing process. Perineum, tail, vulva and the surrounding area are cleaned with water, lathered then rinsed with antiseptic solution and 70 % alcohol. After the preparation was completed, the cervix was opened. The tools used were prepared to be aseptic and sterile before insertion to avoid the contamination in reproductive organs and to maintain the quality of the embryos. The insertion of the dilators was done very carefully by using the right hand, while the left hand was used for the pre-rectal cervical fixation to guide the dilators inclusion. The cervix inclusion is proceed until the fourth cervical ring, until dilators reach caudal part of the corpus uterus. Then, after the cervix has fully included, the foley catheter attached to Stilette Cassou Insemination Gun to make it stiff was prepared.

Foley catheter was lubricated by jelly and the inclusion is proceed aseptically. The Foley catheter inclusion through cervix also proceeds manually, the inclusion is done with the right hand, meanwhile, the left hand is proceeded pre-rectal cervical fixation to guide the insertion of Foley catheter into the cervix. The Foley catheter was inserted until it reached half of cornua uteri.

C. Sex Determination of Embryo

The sex determination of embryo was performed by utilizing PCR with the following procedure: superovulated embryo was placed inside the petridish, then the biopsy by using microblade was carried out. The blastomeres which had been excluded from the embryo were placed inside the petridish , washed three

times with 10 µl of PBS solution. 1 (one) or 2 (two) blastomeres were placed into the RTG (Ready To Go) which had been added by 10 µl double distilled water (ddH₂O). Next, 5 µl BOV 97M primers was added into RTG. Then, the PCR proceeded with electrophoresis by using 2 % agarose which had been added with ethidium bromide. Further, DNA was added into the well using micropipette. The marker 1 kb is used, then buffer solution (TBE 1x) is added into the electrophoresis tools that is included agarose which contained DNA for 30 minutes. The result of electrophoresis was observed by utilizing UV rays (ultraviolet). Male embryo have two bands meanwhile female embryo have one band.

Research Variable

1. Response level of the superovulated donor cattle
The quantity of corpus luteum is dependent on the amount of corpus luteum in both ovary. The donor is considered as responding if it is contained CL greater than 1 (one) ($CL > 1$) and it is said as not responding if the CL is small or equal to 1 (one) ($CL \leq 1$).
2. Number of CL post superovulation
The number of CL in right and left ovary, the measurement is determined by rectal palpation.
3. The quality and quantity of the embryo
The number of embryo which was collected from Pesisir cattle. The total number of embryos which was collected after embryo harvest and is observed under stereo microscope. The embryos, were characterized based on the grade; grade A, B, C, Dg (degenerative) and Uf (unfertile).
4. Sex determination
 - a. The effectivity of sex determination in embryo was by observing the amount of detected embryo using PCR.
 - b. Observing the steps of embryo development in which sex determination can be effective

- c. Counting sex ratio of male and female in sex determination of Pesisir cattle.

Results and Discussion

The result of this showed that all (100%) cows exhibited the estrus response due to the administration of FSH followed by PGF_{2α}. It was based on the observation that the estrus indication appeared 24 hours after PGF_{2α} was injected to the Pesisir cows. Ovulation appeared approximately 24.4 hours after the GnRH treatment was given to virgin cows (81.5%) by injecting PGF_{2α} followed by GnRH 48 hours later (Kanitz et al., 2006). Heinonen et al. (1996) stated that estrus response in the cattle with intrauterine PGF 2α injection treatment was about 62.5 % while the cattle with intramuscular PGF_{2α} injection treatment was about 60.6 %. Siregar et al. (2013) reported that PMSG insertion to goats also showed 100% of estrus response. PGF_{2α} injection will lead the estrus phase of the cattle into follicular phase, meanwhile for cattles that have no response towards the PGF_{2α} have the possibility of already passing the follicular phase or early luteal (Siregar et al., 2010).

In Tabel 1 it can be seen that 100% respond rate is obtained by using FSH 16 ml dosages. This fact is related to Suradi's report (2004) that Simmental and Limousin cattles have 100% of superovulation respond. The respond of donor cattle that is obtained on FSH 16 ml dosage is higher than the response from the previous research (Nanda, 2012) with the response rate of 66.67% on the same treatment, Maret (2001) got 85.70% response rate on 46 mg FSH dosage, while Muawanah (2000) got higher response rate value on the same dosage. Rahman et al. (2014) reported that 100% response rate is obtained from the injection of 200 mg FSH to Boer goat. Lehloenya et al. (2008) also reported that the injection of 200 mg FSH to Boer goat also gave 100% response rate on both breeding season or non breeding season.

The main obstacle in embryo production in beef cattle is the variability in the cows ovulation response towards superovulation with FSH injection (Rico et al., 2009). Cushman (1999) suggested that ovarium response to the superovulation treatment depends on the amount of small follicular population inside the ovarium. Basically, it is known that 25 - 30% of donor cattle have no or less reaction toward superovulation treatment (Supriatna and Pasaribu, 1992). The reasons of the low superovulation response are the increase in cattle lifespan (Muawanah, 2000), inappropriate nutrition of donor cattle and reproduction organ interference (Suradi, 2004). Baril et al. (1993) and Gonzalez-Bulnes et al. (2004) stated that the reason behind variable response to superovulation depend on extrinsic factor (the authenticity and purity of gonadotropin hormon (FSH and LH) and superovulation treatment procedure (one or more dosage and hormon inclusion) during the gonadotropin hormon inclusion, season and ration influence) and intrinsic factor (cattle race, age and ovarium status at the treatment/mating moment). Ovary response towards superovulation can be different and it is strongly related to the variation of follicular development status during the treatment (Bo et al. 1995; Rajamahendran 2002; Sato et al. 2005).

The average of corpus luteum in Pesisir cattle after the injection of 16 ml dosage of FSH was 2.97 ± 1.69 , which was lower than that observed by Nilchuen et al. (2012) with the concentration of 200 mg and 250 mg FSH administered in Kamphaeng Saen beef breed cows and heifers showing number of CL 8.67 ± 0.98 ; 7.33 ± 0.98 ; 10.33 ± 0.98 and 13.00 ± 0.98 , respectively. Bülbül *et al.* (2013) got the CL average 8.4 ± 1.6 on twice a day injection of 400 mg FSH also 150 µg D-cloprostenol together with the fifth FSH induction to Brown Swiss cattle race. Ali et al. (2012) reported that in local cattle of Bangladesh the injection

of 320 mg FSH gave the best respond on the corpus luteum quantity 13.60 ± 0.51 meanwhile the lowest average is 8.60 ± 0.60 from the quantity of corpus luteum on 200 mg FSH. Abdullah et al. (2011) got the CL number of average 2.7 ± 0.7 on 8.8 mg FSH toward Boer goat. Rahman et al. (2014) reported that the injection of 200 mg FSH on the cross breeding goat (Boer x Katjang) got the CL average 6.40 ± 1.55 .

In cattle, CL existence can be examined through rectal palpation. Generally, functional CL will be palpable on ovarium surface. FAO (2005) stated that a well trained practitioner who does the rectal palpation to examine the ovary for CL mat find two until three CL. Sometimes, four to five embryos are found. However, not every part of CL always clearly appear on the ovarium surface, as sometimes there are also unfunctional CL that cannot be palpable. (Maidaswr, 2007). The differences of CL quantity which is produced by donor

cattle indicated that every cattle have different responses towards gonadotropin treatment (Hafez, 1987). In addition, Rocha (2005) mentioned that the response differences are strongly related to ovary status at the beginning process of superovulation. Yusuf (1990) showed that in cattle that were given superovulation treatment an average of 7 or more CL were obtained which is categorized as high, 3-4 CL as middle, and 0-2 CL average as low. Whereas, Donaldson (1985) categorized 12 CL per head into high category, 6-12 CL as middle category and 0-5 as low category. Based on the statement, the average of the CL quantity obtained from this research is categorized as middle. Saito (1997) enhanced that the level of ovary responses and embryo production has become two main parameters in analysing and interpretation of superovulation. The superovulation responde results in this study is shown in Table 1:

Table 1. Pesisir cattle superovulation respond after the injection of 16 ml FSH

Superovulation Response	Amount
The amount of treatment cattle (n)	15
Estrus respond (%)	100
Cattle that produce corpus luteum (n)	15
The amount of corpus luteum (n)	
Right	54 (1-6)
Left	35 (1-6)
Total	89
Average	2.97 ± 1.69
The percentage of cattle producing corpus luteum (%)	100
The number of cattle producing embryo (n)	15
The percentage of cattle producing embryo (%)	100
Total embryo	146
Recovery rate(%)	164%
The number of embryos according to grade	
Grade A	44
Grade B	46
Grade C	37
Grade D	6
Degenerative fertilized	13
Average	6.0
The percentage of proper transfer (%)	61.64

The donor livestock is the animal where the embryo source is harvested (Seidel and Elsdén, 1985). The value of the donor livestock generally can be seen only from the ability to produce milk and meat. The donor livestock should be healthy and good body condition because sick animals generally have no response toward the ovulation treatment. Over weight or under weight body condition of donor animals can reduce the fertility (Herren, 2000). In addition, Wright (1987) stated that donor cattle must be free from the disease and abnormality movements, have good productivity record and measured estrous cycle.

The average number of embryos obtained from this study was 9.73 per cow with 90 (61.65%) transfeable embryo from the total of 146 embryos with an average of 6.0. Compared to the research results of cattle embryo obtained by Arum et al. (2013) and Siregar et al. (2012), the number of embryos harvested in this study was relatively high, in which the averages gain of embryo in sequences are 1.00 and 2.00, respectively, and the transferable embryos are 1.00 and 1.10, respectively.

Compared to the number of CL, there were more embryos collected in this study, with 164% recovery rate. This recovery rate was higher than those obtained by Maret (2001) (108.3%) working on FH cattle, Maidaswar (2007) (105.81%) in non lactating FH cattle, Simmental and Limousin, and (Prasetyo, 2012) (94 – 100%) in FH Simmental, Limousin and Angus cattle. In Aceh cattle, Arum et al. (2013) obtained only 25% recovery rate. The reason for low recovery rate in this study was probably because of the small size of corpus luteum which render them undetectable during rectal palpation process (Maret, 2001). Moreover, it is said that non section embryo collection will cause 10% resulted embryo cannot be rinsed and it was still in the fallopian (Betteridge, 1980). Rensis and Scaramuzzi (2003) suggested that stress could also influence the quantity of

embryos harvested from the cattle. Furthermore, Merton et al. (2003) suggested that the number of subordinate follicles in follicular wave pool will determine the embryo quantity of the donor.

Polymerase chain reaction (PCR) is the easiest and fastest technique for sexing in broad scale, the primer is from variative specific-Y sequence which has been used to detect blood, meat and the blastomere of the samples (Zeleny et al., 2002; and Alves et al., 2003). This PCR technique is a DNA marker associated with the production of embryos according to the sex of the embryo genotype of the cattle (Peippo *et al*, 2007; Hirayama *et al*, 2008; Alonso *et al*, 2009). PCR is introduced as sexing molecular approach of embryo by using embryos cell after biopsy (Peura *et al*, 1991; Faber *et al*, 2003; Manna *et al*, 2003) which depends on the amplification of specific cromosom-Y with the sequence of DNA as the special indicator for male embryo and for an autosomal fragmen for both sexes male and female. Huhtinen et al. (1997) and Choi et al. (2009) reported that only embryos at the early stage (day 6.5, day 7, in the form of morula or early blastocysts) that has prognosis to stand on biopsy treatment.

The sex determination technique of embryo has economic benefit value because male cattle is more desired for beef than female cattle due to the better growth reason. Some researchers have published their research result that supports the suitability of the PCR method in sex determination for cattle based on the high accuracy and the speed in the presentation of the results of the sex ratio (Thibier and Nibart, 1995; Lopes *et al.*, 2001; Ekici *et al.*, 2006; Yu *et al.*, 2007).

Normal sex ratio transformation has been connected to various condition like climate, food, stress, pH in the female genital tract, maturation and/or illumination process of sex cells and the age (Lawrence, 1941; McPhee, 1942 in Salisbury and Vandemark, 1985).

pH condition inside the vagina control the sex determination where the acid condition influence the spermatozoa in some ways resulting in more the female calves while high pH will lead to male calves.

This study reveals that the percentage of male was 76.03% and female 23.97%. The total number of transferable embryo contains 51.37% male embryos and 11.28% female embryos. Lopatarova et al. (2010) confirmed that female embryos were between 44 – 45.9% of the total embryos harvested. Shea (1999) and Lacaze et al. (2008) reported the same result for female sex determination (44 – 48%) in every stage of embryo growth at day 6.5 and day 7.5. However, Hasler et al. (2002) reported the female embryo percentage (60.3%) washigher than that found in this study.

In in vitro studies, it has been shown that

the ratio of male in the embryos is higher than female (Kimura *et al*, 2008; Alonso *et al*, 2009). The number of male livestock arising from Artificial Insemination (AI) is generally higher than female livestock. This fact is in accordance with Payne’s (1970) who stated that generally the livestock produced from artificial insemination is male because principally the sperm carrying male gene is stronger than the sperm carrying female gene. It can be seen that the difference between X and Y chromosome is the size of the DNA (y is smaller and X is bigger), surface charge (X sperm moves to khatode) and motility (Y sperm is faster, Y chromosome fluorescence). Male embryo (XY) will be appeared in two stripes with 141-bp-long meanwhile for female embryo (XX) will be appeared in one stripe with 216-bp-long as shows in Figure 2.

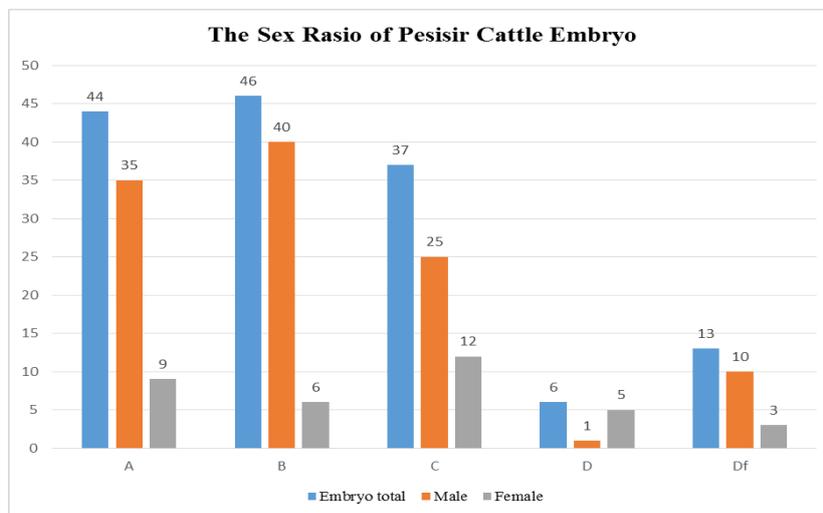


Figure 1. The diagram of the sex ratio of pesisir cattle embryo

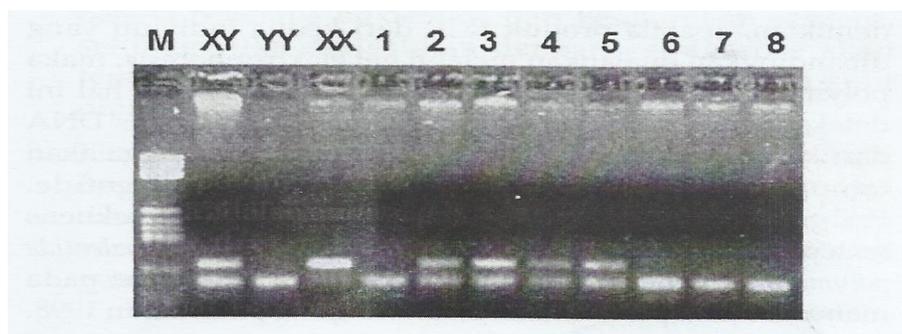


Figure 2. The appearance of a marking system of sex determination. M is the standard size while XX, YY and XY are the identification control (XY=male heterozygous, YY= male homozygous and XX= female). Source Jamsari (2007).

Conclusion

From the result of this study, it can be concluded that Pesisir cattle injected with 16 ml FSH dosage injection resulted in the total 146 embryos that were collected and determined for sex 76.03% (111 embryos) were males and 23.97% (35 embryos) were females, and the total transferable embryos, 51.37% were males and 11.28% were females. The result of the research shows that the ratio of the male embryo was higher than female embryos.

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