Assisted reproductive technology in tropical animals: Case in Pasundan cattle genetic conservation and utilization

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Abstract. Pasundan cattle are native Indonesian cattle located in West Java, Indonesia. These cattle have a superior trait in reproductive performance although reared in extreme environmental conditions with low-quality of feed, and resistance to parasite diseases than other cattle breeds. However, the ability to maintain Pasundan cattle genetic diversity, avoiding inbreeding, keeping the population healthy, and high reproduction efficiency are among the significant challenges currently occurred. The application of assisted reproductive technology (ART) seems promising to overcome those challenges and would impact the efficiency of genetic diversity conservation and preservation. This review aims to present the possibility to use the available ART in Pasundan cattle. The ART reviewed are the advanced technology of sperm cryopreservation, sex selection, artificial insemination, and in vitro embryo production. The sperm cryopreservation combined with sperm sexing is the most promising technology to maximize the use of genetic material of Pasundan cattle. Moreover, sexed sperm would have a promising impact on artificial insemination and/or embryo production to increase the cattle population. Furthermore, to protect genetic diversity, it is possible to apply the artificial reproductive technique and sperm or embryos conservation for genetic utilization in the future.

1. Introduction
Pasundan cattle is Indonesian native cattle and one of Sundanese iconic that live along the Southern coast of West Java and buffer zone areas along the Northern Priangan area. Pasundan cattle had a phenotypic characteristic that was similar to Bos indicus and Bos javanicus [1]. These cattle have reddish coat color with white color on the pelvis, tarsus, and carpus. Uniquely, the color will be changed from red to black due to the influence of the development of the androgen gland when the bull reaches sexual maturity [2]. Pasundan cattle have a relatively small body size, weighing around 220-240 kg and having a carcass proportion of 53-55% [1,3]. They are good at reproductive performance and relatively resistant to tropical diseases such as parasitism, extreme environmental changes, and poor feed quality conditions.
The females reach puberty at 19-24 months, while males at 18-30 months. On average, Pasundan cattle have post-partum estrus at 40-60 days, postpartum mating 65-85 days, days open 90-130 days, and calving interval of 13-15 months [3].

The population of Pasundan cattle decreased from 50,000 to 40,000 in a period of 3 years from 2013-2015 [4]. Currently, only around less than 50,000 head of Pasundan cattle exist in the farmer community along northern Priangan and then southern coasts [5]. According to that, the Indonesian Minister of Agriculture in 2014 decided to launch the program in improving its population number. To improve the population inefficiently way, the use of reproduction technology is a must to achieve that aim. This would be beneficial in preserving as well as utilize the Pasundan cattle genetic diversity sustainably.

In this review, we would like to start our discussion with gamete cryopreservation, mainly semen cryopreservation. This followed with the utilization of that preserved gamete for widely known reproductive technology namely artificial insemination and for in vitro embryo production. In the final part, we will discuss the pros and cons of this technology implementation to increase the population of Pasundan cattle.

2. Semen collection, sperm cryopreservation and sexing

2.1. Sperm collection
Sperm collection and preservation are the best options for long-term male genetic maintenance. In general, there are four main methods for sperm collection in a bull that is transrectal massage (TM), electroejaculation (EE), artificial vagina (AV), and post-mortem epididymal recovery [6]. The collection method must be carefully chosen because it can affect the quality of the ejaculate as well as the animal’s health and well-being. The use of an artificial vagina (AV) is considered a good technique in terms of sperm quality, less stress for the animal, and good quality for constant collection. Lastly, post-mortem sperm recovery can be used, both from the vas deferens and the epididymis are the possible methods for recovering material genetic from animals which dies unexpectedly or having problem to ejaculate with the normal method.

2.2. Sperm cryopreservation
The Pasundan bull sperm concentration was ranged between 725.20 x 10^6 and 856.60 x 10^6 mL^-1 and can produce frozen sperm of up to 167 straws per ejaculate or equal to 13,400 straw in a year [7]. The overall mean of Pasundan cattle sperm volume is 5.89 ml/ ejaculate, pH is 6.69, sperm concentration is 6.04 billion/ ejaculate, and individual sperm motility is 45.5 - 60.59% [8]. The Pasundan cattle spermatozoa were routinely cryopreserved in Artificial Insemination Centre (AIC) Lembang, West Java. The frozen sperm is then distributed to the local farmer for artificial insemination (AI). Achievements in cryopreservation of Pasundan cattle semen and different protocol of freezing-thawing have been reported. The using Tris-egg yolk (TEY), Tris-soy (TS), and AdroMed® as diluent resulted in a 33.27% decrease in Pasundan Cattle sperm motility during the freezing process [9]. The frozen Pasundan Cattle sperm yielded from Ciamis-West Java Regional Artificial Insemination Center (RAIC) have a 32.93-46.52% sperm progressive motility, 43.13%-59.43% sperm viability, 51.27%-59.02% sperm membrane integrity, and 86.83%-89.81% sperm DNA integrity post thawing [7]. The sperm dilution of TRIS (Trishydroxymethyl aminomethane), Citric acid, and Skim Milk with an addition 20% Egg yolk yielded 73.89, 69.85, and 64.80% sperm motility, respectively [10]. Another preservation using Tris-egg yolk with 1 mM L-Carnitin supplementation up to 108 hours shows 43.08% sperm motility [11].

Cryopreservation of gametes is an important tool in assisted reproduction programs. Long-term storage of oocytes or spermatozoa is required for future in vitro fertilization (IVF), artificial insemination (AI) or In Vitro Embryos Production (IVEP). When the geographical or temporal distance between donors and recipients results in no simultaneous availability of male and female gametes, cryopreservation is the only option [12]. Numerous studies have been conducted to increase the success rate of bull sperm cryopreservation. Even though cryopreservation of bull sperm has advanced beyond
that of other species, there are still significant gaps in knowledge and technology. The viability of sperm after thawing is still low and varies significantly between breeding bulls. With varying degrees of success, various extenders have been developed and supplemented with chemicals to reduce cryodamage or oxidative stress. [13].

2.3. Sperm sexing
Sperm sexing is another option for cattle reproduction technology that aiming to determine offspring sex before its birth. This technology brings benefits in arranging animal sex in a specific population for production efficiency purposes. For example, female is preferable in dairy cattle but in contrary with beef cattle which prefer male. The sperm selection is based on the difference in the characteristic of sperm morphology, amount of deoxyribonucleate (DNA), protein macromolecule, immunological properties, swimming ability, and a specific gravity between X and Y bearing sperm [14]. Methods developed for sperm selection that are percoll gradient [15], albumin separation [16], swim-up modification [17] and flow cytometry [18–20].

The sperm sexing also has been applied to Pasundan Cattle sperm in AIC Lembang, using the albumin column method. The recent study reported the sperm selection using 5 and 10 % Bovine serum albumin (BSA) for 45 minutes yielded abnormality of sperm X in the upper layer around 4.00 - 4.20% and at the lower layer was 4.10 - 4.40%. The longevity of sperm at the upper layer is 4.33 days and at the lower layer is 4.17 days, and the DNA integrity was around 97 - 98% [21]. For that, the mentioned procedure could be applied for cryo banking for genetic resources of Pasundan cattle. At this point, sperm sexing of Pasundan cattle would impact the structure population arrangement, for example, by aiming to have more females to deliver more offspring purposes through AI or embryo production.

3. Artificial insemination (AI)
Artificial insemination (AI) is one of the most promising and successful assisted reproduction technologies for increasing the livestock population as well as improve genetic gain. It can rapidly spread the genetic material, while at the same time minimizing the risk of disease spreading with minimal cost. To improve both population and genetic at the livestock population, the Indonesian government established AI centers that are responsible to produces and distributes frozen bull sperm in a high standards process. Here, the genetic quality of the male is also controlled by the productivity record of the pedigree. Currently, it is known that genetic improvement in the herd population is approximately 90% dependent on genetic improvement in the AI center.

The success of AI is dependent on several factors, including the understanding of the female reproductive cycle and its manipulation, the ability of estrus detection, and sperm quality [22]. The use of protocols to control follicular development and ovulation, also known as fixed-time AI (FTAI) protocols, give the advantage of allowing cows to be inseminated without the need for estrus detection [23]. The majority of these FTAI protocols depend primarily on exogenous progesterone (P4), gonadotropin-releasing hormone (GnRH), and prostaglandin PGF2a administration to induce ovulation and luteolytic (PGF2α) [24,25].

The procedure for FTAI in Pasundan cattle is similar to the standard procedure for cows in the other breeds. The recent procedure reported is the injection of PGF2α in combination with GnRH resulted in a higher concentration of estrogen and progesterone in Pasundan cows when compared to the injection of PGF2α (32.76 pg/ml and 0.22 ng/ml vs 28.83 pg/ml and 0.21 ng/ml, respectively). Moreover, the service preconception and calving rates were also significantly higher in the injection of PGF2α + GnRH compared to PGF2α alone [26].

4. In vitro embryos production
In vitro embryos production (IVEP) is likely to be the most effective technique to increase the animal population [27]. The IVEP technology has advantages over AI because by using IVEP, the flexibility in pairing between bulls and cows, the possibility of obtaining a greater number of embryos collection and allows the use of genetic material from females in critical situations such as reproductive pathologies or
for animals that have died. Despite all of these benefits, this technique is considered more expensive and consists of several steps such as obtaining oocytes and in vitro maturation (IVM), in vitro capacitation of sperm, in vitro fertilization (IVF), and in vitro development of embryos (IVD) [27]. Failures in one of these steps, such as the inability to obtain high-quality oocytes, lack of knowledge about optimal conditions for maturation gametes and embryos, and uncertainties about their subsequent transfer, are likely to have a connection with the low success rates of IVEP [28].

There are several applied procedures for collecting oocytes in cattle to produce embryos in vitro. Exogenous hormone therapy to stimulate multiple follicle development and maturation in conjunction with the laparoscopic ovum pick up (LOPU) or laparotomy technique for oocyte aspiration is a successful approach for obtaining oocytes. LOPU is the preferred method for oocyte collection because it promotes faster recovery and the ability to repeat the procedure in multiple animals while being less invasive than other methods. Slicing or aspiration of the ovaries is the most used method for collecting oocytes from unexpectedly dead animals.

Following collection, oocytes are transferred to IVM, where they go through a series of physical and chemical changes until nuclear, cytoplasmic, and molecular maturation is complete. Several factors influence the success of in vitro maturation, including follicle size, oocyte quality, media type, and additional substances added to the maturation media [29]. Gonadotropins, steroids, and growth factors are other substances found in culture media for in vitro oocyte maturation [30]. Estradiol is a steroid group that is frequently added to culture media for in vitro maturation, plays an important role not only in oocyte maturation but also in embryonic development. Estradiol in culture media does not affect the meiotic process, but it does increase fertilization and division rates [31]. The addition of epidermal growth factor (EGF) and gonadotropins to maturation media promotes cumulus expansion as well as nuclear and cytoplasmic maturation[32].

The IVEP requires fewer viable sperm for fertilization, it can make better use of male genetic material [33]. The number of motile sperm required for fertilization with conventional IVF is 5x10^4-1x10^5 per oocyte. In vitro fertilization technology also allows for the use of a single sperm to fertilize the oocyte via the intracytoplasmic sperm injection (ICSI) method. Furthermore, ICSI also allows for the use of low-quality, immotile sperm, as well as spermatids [27].

The final step in IVEP is to culture the fertilized oocytes in media that mimic in vivo conditions. A culture medium plays an important role in embryo development, cell number, and embryo implantability during this process [34]. Embryonic cell blastomeres will continue to divide from 2 cells to 4 cells, 8 cells, 16 cells, morula, and finally blastocyst. Cell proliferation and the early stages of differentiation occurred during blastocyst formation. The blastocyst differentiated into inner cell mass (ICM), which will become a fetus, and trophoderm (TE), which will become a placenta [35]. At this stage, ICM cells must be able to maintain pluripotent properties to differentiate into various tissues, whereas TE cells must be able to block these pluripotent signals to develop with specific functions to support embryo implantation [36].

Currently, to the best of our knowledge, there are no reports regarding the implementation of IVEP in Pasundan cattle. Though it could be implemented since a similar procedure in other cattle could be applied, but the application in such areas which do not have enough facility to support all the steps of embryo production became a hard obstacle to solve. Pasundan cattle are commonly kept by a farmer in relatively small numbers, therefore the application of IVEP would not a wise choice here. At this point, the use of AI much more has the advantage compared with IVEP, though in the genetic gain view over a generation, AI is relatively slower compared to IVEP. This due to, only at the male side AI was able to be inherited to the offspring.

5. Conclusion
The development and application of ARTs in the conservation and maintenance of genetic diversity in Pasundan cattle is a challenge, and their use in offspring production is still limited to semen cryopreservation, AI, and scientific research. The implementation of ART in this cattle need to be based on consideration of its price, efficiency, and also the support system available, though all ART can be
applied. Furthermore, it is necessary to conduct further study in the fundamentals of reproductive biology in conjunction with the use of ART in Pasundan cattle.

Acknowledgment
This study was supported by SEARCA through a Ph.D. full scholarship for Rini Widyastuti.

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