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Livebirth after artificial insemination using cryopreserved epididymal sperm recovered from the cauda epididymis of slaughtered non-descript bucks in the Philippines

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Abstract The study determined the viability of cryopreserved epididymal sperm (EpS) to full term pregnancy through AI technique in hormone responsive recipient goats. Cryopreserved EpS with post thaw motility score of ≥20% were used to inseminate estrus-synchronized (ES-AI) and ovulation-synchronized (FT-AI) recipient goats. The ES-AI goats had a mean hormone response rate (HRR) of 55.5% with a Pregnancy rate (PR) of 25% and full term delivery (FTD) rate of 20%. On the other hand, the ovulation synchronized FT-AI goats registered a mean HRR of 75%, with a PR of 58% and FTD rate of 54.2%. The use of either GnRH or Chorionic gonadotropin on day 9 in the FT-AI goats resulted in a mean hormone response rate, pregnancy rate and full term delivery rate of 72.2%, 61.2%, 58.3 % and 78.6%, 54.5% and 54.5%, respectively. These results validated the usefulness of cryopreserved EpS as an alternative source of germplasm for cryopreservation and utilization for offspring production. Moreover, the study can serve as a research platform for germplasm conservation *in vitro* and *in vivo* of genetically diverse native ruminant farm animals as well as endangered wildlife ruminants endemic in the Philippines through the use of Assisted Reproductive Technologies (ARTs).

Keywords: Livebirth of kids, Cryopreservation, Epididymal sperm, Artificial Insemination

Introduction

Assisted Reproductive Technologies (ARTs) such as artificial insemination (AI) and semen cryopreservation (SC) have persisted for decades as important approaches in genetic improvement and management of livestock (Vallet *et al*., 1992; Kikuchi *et al*., 1999; Whitley and Jackson, 2004; Amiridis *et al*., 2012; Cseh *et al*., 2012). In the Philippines, such technologies continue to

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consistently evolve with improvements to enhance the production efficiency of both small and large ruminants (Mamuad *et al*., 2005; Ocampo and Ocampo, 2015; 2016; Sumeldan *et al*., 2016; Atabay *et al*., 2020). AI is used in improving animal production through dissemination of selected male genetics of high quality of livestock ruminants. Quite recently, ovulation synchronization-AI also known as Fixed Time Artificial Insemination (FTAI) in water buffaloes has been used as an alternative option to improve the pregnancy outcomes for caracows that manifest breeding difficulties (Atabay, 2015). Likewise, SC have spurred the interest of developmental and conservation biologists focusing on mammalian gametes preservation as a means of protecting animal biodiversity (Moreno *et al.,* 2006, Saenz, 2007, Kozdrowski *et al.,* 2011 and Yulnawati *et al*., 2013). The said technique is being used as a research platform for the germplasm conservation of endangered ruminant species endemically found in the wild that fall prey to poachers.

The establishment of the PCC cryobank facility to keep a reserve pool of frozen germplasm from genetically superior commercial breeds have provided an impetus to include indigenous or native farm animals in the core collections. Animals of this nature are perceived as genetically diverse, truly prolific and possess climate resiliency traits. In some cases however, semen collection from native farm animals maintained by farmers can be difficult to achieve. Thus, an alternative approach is the use of epididymal sperm (EpS) recovered from a slaughtered male farm animal.

Our previous research work using the testes of slaughtered goats as the animal research model (2017 DA-BAR terminal report) resulted in the establishment of a workable EpS cryopreservation that can be useful for the future cryo-conservation of indigenous wildlife ruminants. An optimized method of epididymal sperm (EpS) recovery yielded good quality sperm from post-mortem goat testicles (Gautane *et al*., 2016) of which when resuspended in an appropriate semen extender, the EpS remain viable for up to three days when maintained at refrigeration temperature conditions. Also, EpS recovered within two (2) hrs before cryopreservation displayed modest but acceptable post-thaw survivability results (Bumanlag *et al*., 2017). Post-mortem EpS evaluated for fertility by IVF assay remained physiologically functional as demonstrated by their ability to penetrate an egg cell with male pronucleus formation (Ocampo *et al*., 2019a; 2019b). Nonetheless, the ultimate measure of success in any SC procedure is the offspring derivation upon its utilization. Hence, the objective of this study is to determine the post thaw motility of cryopreserved EpS and the subsequent fertilizing capability *in vivo* thru AI was performed in hormonally primed responsive recipient goats.

Materials and methods

Medium for sperm recovery and cryopreservation

The chemicals and reagents used for the preparation of cryopreservation solutions were manufactured by Sigma-Aldrich Co (3050 Spruce Street, St. Louis, MO, USA). The base medium (BM) for epididymal sperm recovery was 320 mM Tris hydroxymethylamino methane, 10 mM citric acid monohydrate, 3.0 mM fructose and 8.0 mM raffinose supplemented with antibiotics, 25µl/ml gentamycin. The cryopreservation solution labelled as TCFRYG consisted of the BM supplemented with 7 % glycerol (v/v) and 20 % (v/v) egg yolk.

EpS motility evaluation

Scrotal sac-intact goat testicles (n=30) collected from slaughtered non-descript bucks were transported to the laboratory within 45 min – 2 hrs upon death. Incisions were made on the cauda epididymis for 'sperm swim up' in the BM with certain modifications to avoid hemolyzed blood getting mixed in the sperm suspension as previously described (Gautane *et al.,* 2016). The collecting tube was subjected to a centrifuge spin at 5,000 rpm for 5 min to precipitate the sperm pellet. The sperm motility assessment was done by highly skilled technical personnel with a trained eye for sperm motility based on the criteria described elsewhere (Mamuad *et al*., 2005). Microscopic evaluation for visual motility was undertaken under medium (40x) and high power (100x) objectives using the inverted microscope (Nikon Eclipse Tx10i, Japan). EpS showing gross motility scores between 3-5 with at least a progressive or individual sperm motility assessment of ≥ 60 % were considered for further sperm processing.

EpS cryopreservation

The sperm pellet post centrifugation was gradually diluted with the TCFRYG solution as previously described to yield 200 x 10^6 sperm/ml concentration. Initially, the TCFRYG solution was added at a 1:2 ratio and then placed in the refrigerator for the gradual cooling process. The remaining extender solution was sequentially added in three consecutive steps and the mixture was left undisturbed for two (2) hours at 4-5°C in the refrigerator in order for equilibration to take place. After a pre- cryopreservation sperm motility analysis by conventional method along with the use of the Computer Assisted Sperm Analyzer (HTM IVOS II, Hamilton Thorne, Inc., Beverly MA,

USA), the sperm suspension was loaded in UV-sterilized plastic semen straws (0.5cc IMV, France) and sealed with polyvinyl powder. Cryopreservation was performed at a distance of 4 cm above the LN_2 level for 7 min in the styropore box. The frozen straws were then directly plunged into the LN_2 to complete the freezing procedure.

Post-thaw sperm motility evaluation

Thawing of the frozen straws was done either in a thawing jar or in the water bath with a pre-calibrated water temperature setting of 37±1°C for 15 sec. The contents of the straw were emptied in an Eppendorf tube. Visualization for post-thaw sperm motility (PTM) was performed under a microscope at 40-100x magnification. A PTM score of ≥20 % was considered acceptable for frozen EpS. Those with acceptable PTM values were kept indefinitely in LN_2 until further use. The EpS sperm motility before and after cryopreservation were compared and analyzed.

Fluorescence microscopy for plasma membrane integrity evaluation

The plasma membrane integrity of frozen-thawed EpS was determined by fluorescence microscopy using a commercial Live/Dead Sperm viability kit (Thermo Fisher Cat #L7011). The kit being a fluorescence based assay analyzed the viability and fertilizing potential of sperm. Following the manufacturer's instruction in the manual, the sperm were stained with the fluorescent dyes, SYBR: PI and were viewed in a fluorescence microscope with each of the dye's emission spectrum setting of 516 nm and 617 nm, respectively. Sperm head with green fluorescence are those that have intact cell membrane to indicate that they survived the cryopreservation procedure while those with red stained sperm head were classified as dead sperm because of the damaged cell membrane.

Experimental design

Recipient female goats with a Body Condition Score (BCS) of three (3) or better and with at least one (1) previous parity were used in the study. Ultrasonographic examination was performed in determining the reproductive status of the experimental female goats. Non-pregnant female goats were randomly allocated in either one of the two (2) hormone treatments to induce an equally onset of estrus before being subjected for AI as depicted in Figure 1. In Treatment 1 ES-AI (estrus synchronization–AI), a single intramuscular

injection (IM) injection of 2 ml (10 mg) of Prostaglandin F_2 alpha ($PGF_2\alpha$; Lutalyze, Pfizer, Inc, USA) was administered in thirty six (n=36) experimental female goats. AI was performed in the morning and afternoon in goats exhibiting overt signs of estrous manifestations with open os cervix. In Treatment 2, FT-AI (Ovulation synchronization or Fixed time AI), a Controlled Internal Drug Release: CIDR insert was initially placed with the use of an applicator stick (Eazi-Breed; Zoetis Inc., USA) on day 0 (day 0=start of hormone treatment) followed by GnRH: Gonadotropin Releasing Hormone injection (Cystorelin; Merial, USA) in thirty two (n=32) experimental goats. Removal of the CIDR insert was done on day 7 which was followed by an intramuscular injection (IM) of 2 ml (10 mg) $PGF_2\alpha$.

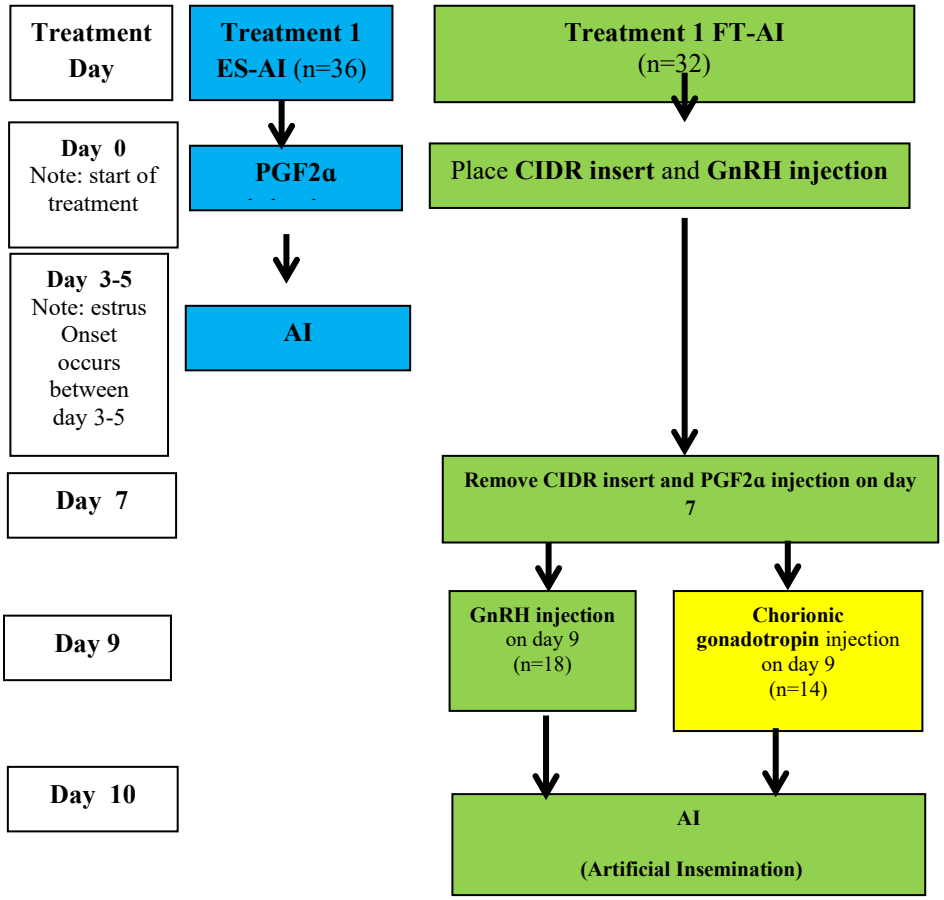

Figure 1. Hormone treatments: ES-AI and FT-AI used in the study

On day 9, the second GnRH injection (Cystorelin: Merial USA) was given separately in one group (n=18) as FT-AI treatment 2.1 whereas in the other group (n=14) , chorionic gonadotropin hormone (Chorulon: Merck, USA) was administered instead as FT-AI treatment 2.2. Fixed Time AI on day 10 was performed twice following the morning and afternoon schedule in goats with overt estrous symptoms and open os cervix. Pregnancy diagnosis was performed by ultrasonographic examination 30-35 days after insemination.

Statistical analysis

The data on sperm motility evaluation before and after ES cryopreservation were recorded as average ± s.e.m percentage (%) values. The hormone response rate (HRR), pregnancy rate (PR) and full-term delivery rate (FDR) in treatment 1 and treatment 2 were recorded in average percentage values and in mean ± s.e.m percentage (%) values. The gathered data in each of the two treatments were analysed using Student's T-test at 5% level of statistical significance.

Results

The percentage sperm motility values of EpS before and after cryopreservation are shown in Table 1. Using conventional or subjective method of assessment and the more objective approach using the CASA machine, the sperm motility significantly declined that was notable after EpS cryopreservation. Similar observation in the reduction of sperm motility values was found to be less pronounced among the progressively motile sperm (CASA PMOT) as assessed by the CASA machine. From all indications, the effect of cryogenic temperature on the sperm cryo-survivability after cryopreservation manifested a decrease in the proportion of remaining viable EpS. However, it is noted that the subpopulation of progressively motile sperm values (CASA-PMOT) did not statistically differ by Student's T-test ($p \geq 0.05$). The proportion of progressively motile sperm registered a mean ± s.e.m values of 20.5±2.9 percent before cryopreservation and 15.28±3.26 percent after cryopreservation.

Frozen-thawed EpS with PTM score of ≥20 % were subjected for plasma membrane integrity assessment using fluorescence microscopy. Using the Live Dead sperm assay kit, spermatozoa that survived the cryopreservation process emitted green fluorescence in their sperm head DNA. The EpS that did not survive showed sperm head emitting the red dye propidium iodide (Figure 2).

Table 1. Epididymal sperm (EpS) motility before and after cryopreservation

Sperm motility Parameters	Percent Epididymal Sperm Motility (%.) Before Cryopreservation	After Cryopreservation	Student's T-test
	Mean ±s.e.m.	Mean ±s.e.m.	$p \leq 0.05$
Sperm motility (%) by conventional/ subjective method of evaluation	64.2 ± 1.0[a]	19.16 ± 3.07[b]	0.024*
Sperm motility (%) by Computer Assisted Sperm Analyzer (CASA) method	75.5 ± 3.7[a]	32.75 ± 5.61[b]	0.01*
Progressively Motile sperm CASA PMOT (%)	20.5 ±2.9[a]	15.28 ± 3.26[a]	0.84[ns]

[a,b] Values with different superscripts within rows statistically differ at 5% level of significance by Student's Test.

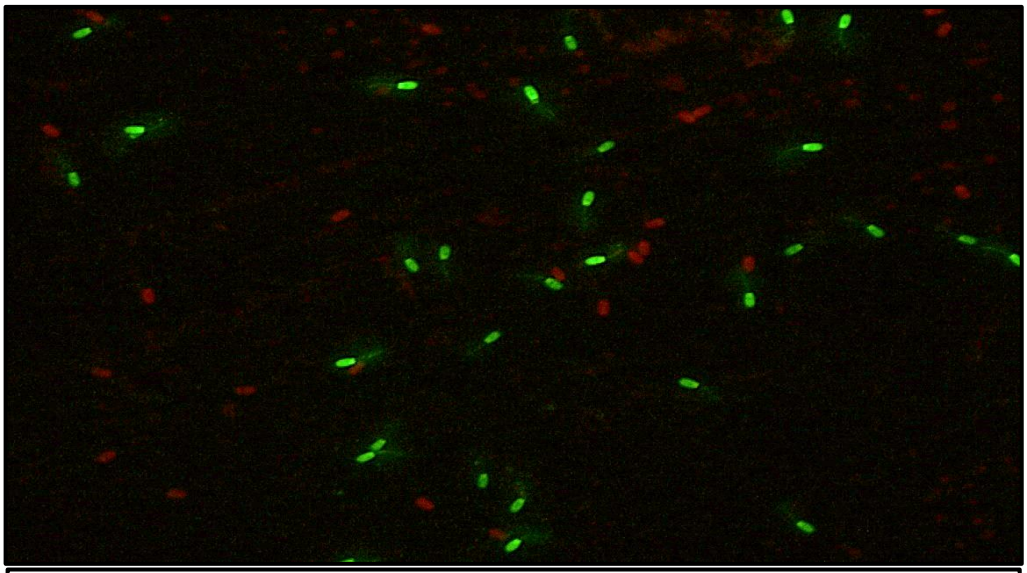

Fig 2. Fluorescence microscopy with commercial Live/Dead Sperm viability kit (Thermo Fisher Cat #L7011): Sperm head with green fluorescence are LIVE with fertilizing ability potential and Red stained sperm head are DEAD

Following this fluorescence viability assay, the fertilizing ability of the frozen-thawed EpS was subsequently validated by inseminating hormonally induced goats with results shown in table 2. In Treatment 1: ES-AI, the mean hormone response rate (HRR) was 55.5% with a pregnancy rate (PR) of 25.0% yielding a full term delivery rate (FDR) of 20.0%. In Treatment 2: FT-AI, the mean HRR was 75.0% with a PR of 58.0% and FDR of 52.4%.

The experimental results in FTAI goats (Table 3) were treated on day 9 separately in one group with GnRH as FTAI treatment 2.1 (n=18) and the other group was treated with Chorionic gonadotropin hormone as FTAI treatment 2.2 (n=14). The use of both hormone treatments on day 9 similarly enabled follicular ovulation thus ensuring the presence of egg cell(s) waiting to be fertilized upon insemination. Nonetheless, either ES-AI or FT-AI method confirmed that frozen-thawed EpS would terminate into full term delivery of kids upon utilization through assisted reproductive techniques (ARTs). Pregnancy diagnosis through ultrasonography showed conceptuses at 35 days post AI and the corresponding kids born thereafter (Figures 3-4).

Table 2. Results of Artificial Insemination using Cryopreserved epididymal sperm in ES-AI and FT-AI experimental goats

	Hormone Treatments	
	ES-AI	FT-AI
1. Number of female goats treated with hormones	36	32
2. Number of Hormone responsive goats (%)	20 (55.5%)	24 (75%)
3. Hormone Responsive rate (Mean ± s.e.m.)	**57.45 ±2.5[b]**	**76.86 ±3.3[a]**
4. Number of Goats Pregnant after AI (%)	5 (25%)	14 (58.3%)
5. Pregnancy Rate of Inseminated Goats (Mean ± s.e.m.)	**28.3 ± 5.0[b]**	**57.2 ±3.6[a]**
6. Number of goats with full term delivery (%)	4 (20%)	13 (54.2%)
7. Full Term Delivery rate (Mean ± s.e.m.)	**17.2±9.6[b]**	**54.6±2.9[a]**

[a,b] Mean ±s.e.m values with superscripts within columns differ by Student's T- test at 5% level of statistical significance

Table 3. Results of FTAI goats treated on day 9 with either GnRH and Chorionic gonadotropin hormone in the ovulation-synchronization procedure

Hormone treatment on Day 9	No. of Doe(s)			
	Treated	HRR	Pregnant post FTAI	Live Birth
Treatment 2.1 GnRH	18	13 (72.2%)	8 (61.2%)	7 (53.8%)
Treatment 2.2 Chorionic gonadotropin	14	11 (78.6%)	6 (54.5%)	6 (54.5%)

Legend: GnRH=Gonadotropin Releasing Hormone
HRR =hormone response rate

Discussion

Goat EpS cryopreservation was firstly reported in 2000 (Blash *et al.,* 2000) by putting the processed semen straws in the ultralow freezer at negative (-) 80°C for 10-15 min prior to LN_2 plunge. In this study, EpS cryopreservation was performed in a simple styropore box which was adopted from the works on ejaculated goat semen (Beltran *et al.,* 2013) with modifications on the semen extender solution to make it more suitable for EpS that lack seminal plasma normally produced by seminal fluid- producing accessory sex glands.

EpS cryo-survivability evaluated by CASA with special reference to the subpopulation of progressively motile sperm registered minimal differences before and after cryopreservation. The progressively motile sperms represented the subpopulation in the sperm suspension that is capable of moving from point A to point B loci. These are the spermatozoa considered capable of reacting to the stimulus targeting the female gamete cell for fertilization process to occur. This was confirmed by fluorescence microscopy examination that revealed the presence of live spermatozoa, those with fluorescing green sperm head DNA indicative of cryo-survivability and the eventual potential to fertilize *in vivo*. In this study, goat EpS with PTM score of ≥20% upon utilization for AI proved effective and manageable with either ES-AI or FT-AI methods. Similar CASA PMOT value s of 20.0% was reported in frozen epididymal sperm of the European Bison with a pregnancy rate of 22.0% in artificially inseminated heifers (Kozdrowski *et al*., 2011).

Goat estrus manifestations in treatment 1 after a single injection of 10mg $PGF_2\alpha$ were observed to occur variably within three to five days. In a normal

estrous cycle the secretion of $PGF_2\alpha$ from the uterus or injection of $PGF_2\alpha$ destroys the CL thus allowing female ruminants to come into heat. In water buffaloes a wide discrepancy on the onset of ovulation after a single injection with $PGF_2\alpha$ was observed after closely monitoring with an ultrasound machine and also revealed some caracows were not found ovulating at all as reported by Atabay *et al.* (2020). Other reports have used $PGF_2\alpha$ administration on Day 1 followed by second treatment on Day 10. In either treatment, the female goats will show estrus signs and can be inseminated by AI at either time (Whitley and Jackson, 2004) In this study, ES-AI treated goats had a HRR of 55.0% which was comparable with the HRR (55.0-65.0%) reported in Ethiopian ewes coming in heat 96 hr after a single injection of either 10.0 mg and 12.5 mg $PGF_2\alpha$ (Zeleke *et al*., 2016) but was lower when compared with other reports in goats given with 2 doses of $PGF_2\alpha$ 10 days apart at a range of 7.5 mg-15.0 mg per injection (Bowdridge *et al.,* 2013, Sumeldan *et al.,* 2016). In this study, the dosage used was 10 mg, whereas related reports used a range of 7.5 mg – 15 mg per injection with a more consistent and reliable results at 15 mg (Bowdridge *et al*., 2013) although lower doses at 5mg $PGF_2\alpha$ was reported 100% effective to induce estrus (Al Yacoub *et al*., 2011; Zeleke *et al*., 2016).

In Treatment 2, CIDR placed for 7 days was aimed at elevating circulating progesterone levels released from the insert so that once removed, the goat comes into estrus within 36 - 72 hrs. GnRH (Cystorelin) injection facilitates the release of FSH and LH that will induce a new follicular wave to yield a cohort of antral follicles. As they enter the remaining phases of their growth, two to three of these follicles enter the dominance phase. The second dose of GnRH given at day 9 in Treatment 2.1 induces an increase in the preovulatory LH peak to cause ovulation and finally the luteinization of follicular cells (Vallet *et al*., 1992). The beneficial effect of GnRH treated goats manifesting enhanced pregnancy results in FT-AI treatment 2.1 are in agreement with other reports (Al Yacoub *et al*., 2011; Parmar *et al.,* 2020; Pujar *et al*., 2016). Whereas in Treatment 2.2, chorionic gonadotropin hormone (Chorulon) induces ovulation because of its LH type of activity thus, when given on day 9 will induce ovulation by luteinization of follicular cells. Either protocol can lessen the fertility depression associated with longer term of CIDR use, resulting in good synchronization and fertility immediately after treatment, as evidenced by the higher HRR and resulting pregnancy rate observed in this study than when using $PGF_2\alpha$ alone. Ovulation synchronization with chorionic gonadotropin is similar with eCG and GnRH to cause ovulation from dominant follicles and therefore facilitates AI outcomes with much precision at a predetermined day (Rahman *et. al*., 2008; Fonesca *et. al*., 2015).

The pregnancy results resulting to live birth (Figure 3 and 4) obtained in this study are comparable to earlier reports using fixed time AI in goats (Whitley and Jackson, 2004; Holtz, 2008; Bowdridge *et al*., 2013) or higher than when using frozen thawed EpS obtained at necropsy from Spanish Ibex (*Capra pyrenaica hispanica*) through heterologous AI (Blash *et al*., 2000; Moreno *et al*., 2006). In other ruminants, a PR of 40-50% was reported using frozen thawed EpS when the female animal is treated with a single dose of 25 mg PGF2α or a double dose of PGF2α at 11 days apart (Yulnawati *et al*., 2013; Atabay *et al*., 2020). These findings substantiate further the preserved fertility of frozen-thawed EpS and its viability for use in AI. In other mammalian species, related studies have been reported to have produced live births (Kikuchi *et al*., 1999; Hori *et al*., 2004; Moreno *et al*., 2006; Guerrero *et al*., 2008; Okazaki *et al*., 2012), though with differing procedures to some extent.

Figure 3. Ultrasound image of goat conceptus and the resulting 'Epid

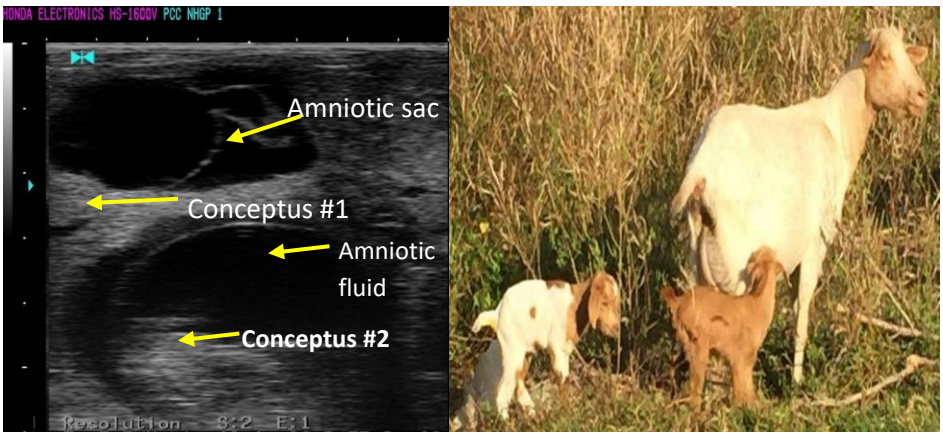

Figure 4. Two amniotic sacs detected and the resulting 'Epid twin kids'

In conclusion, our results have validated the potential of epididymal sperm (EpS) as a useful alternative for germplasm cryopreservation and utilization thru AI resulting to livebirth. EpS cryopreservation can be a useful tool for future wildlife conservation and eventual perpetuation of rare species in the country such as the Tamaraw (*Bubalus mindorensis*) or the Philippine brown deer (*Rusa Marianna*) before their population become decimated as a result of the uncalled-for poaching practices or by any unwanted weather disturbances brought about by global warming. AI use is more effective in conjunction with estrus or ovulation synchronization although the latter allows breeding at a fixed time during treatment protocol with higher PR. In this study, we report the first successful full-term delivery of kids through AI using frozen thawed EpS recovered from slaughtered non-descript/native bucks.

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