



SHORT COMMUNICATION

Birth of First IVF Calves in Sahiwal Cows of Pakistani Origin

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ABSTRACT

The present report accounts successful production of blastocysts and birth of calves following OPU-IVEP technique in Sahiwal cows of Pakistani origin. A total of 9 transvaginal OPU sessions were performed in twelve Sahiwal cows and 520 immature COCs were recovered using commercially prepared media. These COCs were placed in maturation medium for 24 h in an incubator at 38.5°C with 5% CO₂. The matured oocytes were co-incubated with sperm cells in IVF medium for 22 h. After fertilization, the presumptive zygotes were transferred to culture medium for 7 days at 5% CO₂. The results showed that the cleavage rate of IVF was 68.6% out of which 26.3% were developed up to blastocyst. A total of 2 blastocyst-stage fresh embryos were transferred in a single synchronized recipient cow. The pregnancy was confirmed one month after transfer and resulted in successful calving demonstrating the possibility of performing embryo transfer in elite native herds.

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INTRODUCTION

In Pakistan, livestock breeds require adequate attention for their genetic improvement through modern reproductive biotechnologies. Until now, Artificial Insemination (AI) has been solely used for the breed improvement of large animals (Wagner, 2012). AI offers inseminating large number of females by using superior male germplasm. Despite its advantages in reducing venereal diseases, and exploitation of superior sires, its lower coverage for cattle (32%) and breeding bulls of unknown genetic merit limits its significance (Wagner, 2012). For example, a previous report in Sahiwal cattle, one of the most important breeds of Pakistan with its population estimated to be around 2.7 million heads, identified almost zero genetic progress over the years (Khan *et al.*, 2008). Due to this, preference of exotic semen and direct animal import further threatens the population of local cows and over the past few years, import of exotic or cross-bred animals outnumbered Sahiwal cows mainly due to little attention towards its genetic improvement (Wagner, 2012).

With scanty availability of genetically superior bulls, genetic potential of Pakistani cattle breeds needs to be propagated through embryo transfer technology. In Pakistan, first bovine in-vivo embryo transfer was

reported by Anwar *et al.* (1989); however, studies on In-Vitro embryo production (IVEP) in local dairy cows are scanty. A sole abattoir-based study in Sahiwal cows reports beneficial effect of progesterone on oocyte recovery and development rate of 8-16 cells stage embryos (Saad *et al.*, 2019). Few studies on IVEP are available in Pakistani buffalo reporting 70-90% cumulus-oocyte complex (COC) maturation with 40-50% 2-cell cleavage rate, significantly higher maturation rate in oocytes recovered from >4 mm ovarian follicles (Yousuf *et al.*, 2003), contribution of bull's individual variations on success of IVF (Jamil *et al.*, 2007) and improved maturation and fertilization rate in the presence of cumulus cells and modified Ca⁺⁺free Tyrode, respectively (Jamil *et al.*, 2010). As general information is lacking in local cows, the primary objective of the present study was to establish a successful IVEP program in Sahiwal cows, keeping in view all essential steps including transvaginal ovum pick up, in-vitro embryo development and preparation of receipts along with implantation of embryos.

MATERIALS AND METHODS

Transvaginal Ovum pickup in Sahiwal cows: The study was conducted from January 2020 to October 2020 in

Embygen Animal IVF laboratory at Al-Haiwan Sires, Sahiwal. All the experiments were performed by using a commercial IVF media (Embriotec, Reproducao Animal, Anapolis, Brazil) pre-warmed for 24h and covered with mineral oil at each step. Twelve (n=12) Sahiwal heifer cows were selected as donor for oocyte recovery via transvaginal ovum pickup (OPU). These heifer cows were of 3-4 years of age with average body score of around 3-3.5. All OPU sessions were conducted at random stage of estrous cycle. Each OPU session consisted of 4 random cows, subjected to epidural anesthesia (4 ml of 2% lidocaine hydrochloride, Venus Pharmaceuticals, Lahore) and follicular aspiration was carried out in Normal Saline solution containing 1% Heparin by an OPU assembly (Embriotec, Reproducao Animal, Anapolis, Brazil).

In-vitro Embryo Production (IVEP): After follicular aspiration, COCs were searched separately for each animal, recovered in washing medium, transferred to four-well plate containing 500 μ L maturation medium (30 oocytes/ 500 μ L droplet) and kept in an incubator at 5% CO₂ and 38.5°C temperature. After 24 h, in-vitro matured COCs were washed three times with washing medium before transferring into fertilization medium (20 oocytes/ 70 μ L) droplet containing heparin (10 ng/mL, Sigma Aldrich) and 20 μ M D-penicillamine, 10 μ M hypotaurine, and 1 μ M epinephrine (PHE) for 16 hrs. Parallel with that, frozen semen of good quality Brahman bull was thawed at 35°C for 20 seconds and prepared for swim up procedure at manufacturer's instructions (Embriotec, Reproducao Animal, Anapolis, Brazil). After final washing, the sperm pellet was diluted with fertilization medium and a droplet (8 μ L) of capacitated semen was added to fertilization medium. The sperm cells and COCs were incubated together for 16h in the incubator at 5% CO₂ and 38.5°C. After 16 h, the presumptive zygotes were washed three times with washing medium. In the meantime, these zygotes were denuded by gentle pipetting, washed three times in culture medium and transferred to 4-well plate containing 500 μ L culture medium in conditions of 5% CO₂, 5% O₂ and 95% relative humidity. In-vitro embryo development was recorded on day 2, 5 and 7 after day of insemination.

Recipient cow preparation and Embryo Transfer: For recipient preparation, a single Sahiwal cow was selected for the embryo transfer. The heifer cow was about 2.5-3 years of age with average body score of 2.5-3. The cow was enrolled in standard Ovsynch synchronization program in which an injection of 0.0524 mg cirelin (Dalmalin, Fatro Pharmaceuticals, Italy) was administered on day 1 followed by an injection of 0.150 mg d-cloprostenol (Dalmazin, Fatro Pharmaceuticals, Italy) 7 days later. After 2 days, another injection of 0.0524 mg cirelin was administered. After 7 days following 2nd GnRH injection, 02 A grade blastocysts were washed thrice in washing medium and loaded into a clear plastic straw using transfer medium as per manufacturer's instructions (Embriotec, Reproducao Animal, Anapolis, Brazil) (Fig. 1). Later, these

blastocysts were transferred in the recipient Sahiwal cow using nonsurgical procedure into ipsilateral uterine horn using embryo transfer gun. Pregnancy diagnosis was performed by using ultrasound around day 40 after transfer.

RESULTS AND DISCUSSION

The data regarding oocyte recovery, in-vitro maturation, fertilization and formation of blastocyst is presented in Table 1. The results showed that following IVF, 68.6% cleavage rate was recorded with 26.3% blastocyst rate at day 7. After seven days following Ovsynch program, 02 A grade blastocysts were transferred while detecting corpus luteum (CL) for ipsilateral transfer of embryos, after which the cow was found pregnant with twin fetuses at day 40 of embryo transfer by ultrasonography (Fig. 2). The successful calving occurred on 17 October 2020, 274 days after embryo transfer with a healthy male (21 kg) and female calves (18 kg).

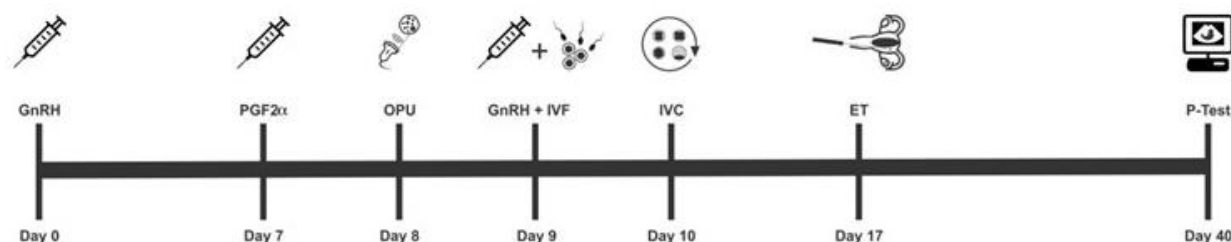
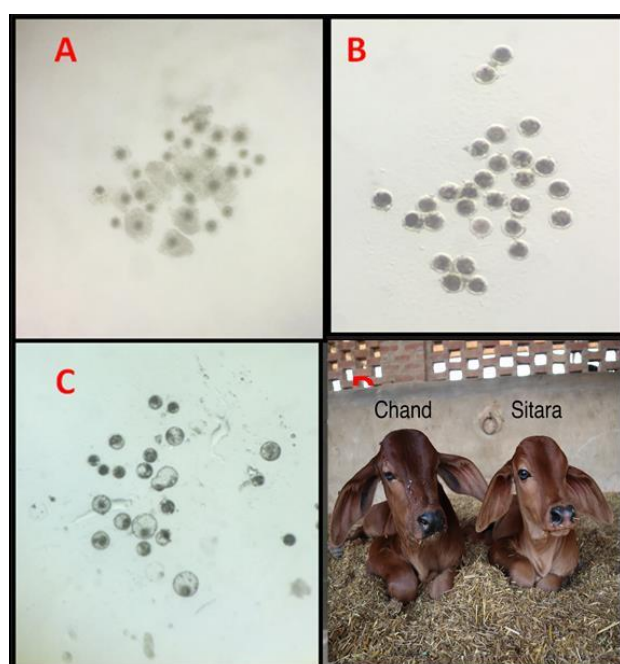
The primary aim of the present report was to evaluate the feasibility of OPU-IVEP program in local cows. To the best of our knowledge, this is the first report on the successful birth of calves by using a combined OPU/IVEP program in Sahiwal cows of Pakistani origin. A previous study reported the birth of first Sahiwal calf in India named "Holi" using similar technique (Saini *et al.*, 2015). The present study resulted in fairly decent number of oocyte recovery (~14.4 COCs/ cow) after OPU without using pre-hormonal treatments or wave synchronization, indicating Sahiwal cattle as good oocyte donor. The number of oocytes recovered in this study is fairly comparable to Holstein Friesian possibly due to higher adaptability of these animals in local climate (Watanabe *et al.*, 2017). It is expected that while manipulating superior milk producing Sahiwal cows, no hormonal treatment would be required probably with slight decrease in oocyte recovery, although this requires suitable experimental model especially during low breeding season. Similarly, results of the present report found significantly higher number of good quality (A+B) COCs. A sole study in abattoir-based oocyte recovery in Sahiwal cattle reported direct interaction of oocyte recovery with that of level of plasma progesterone which is consistent with our study as all of these donor cows were cyclic while enrolled in OPU program (Saad *et al.*, 2019). Our study further achieved comparable blastocyst rate as reported worldwide in other cattle species (Watanabe *et al.*, 2017). However, whether Sahiwal cows would result in similar COCs recovery rate in hot and humid summer or in low breeding season requires further investigations.

The present study reported "Ovsynch" as reliable synchronization program for preparation of recipient Sahiwal cows. In AI program, the Ovsynch resulted in 43% synchrony compared to CIDR (55%) in zebu cattle (Haider *et al.*, 2017; Shahzad *et al.*, 2019) and future studies are required on the use of CIDR-based synchronization in recipient preparation especially during low breeding season.

Table 1: Oocyte recovery, their quality and in-vitro developmental competence of embryos derived from Sahiwal cattle

No of oocytes	Grading				Cleavage rate	8-cell	16-cell	Morula	Blastocyst
	A	B	C	D					
520	35%182/520	35.8%186/520	11%57/520	18.2%95/520	68.6% 357/520	47.5%247/520	42%218/520	32.3%168/520	26.3% 137/520

Grade A: Oocyte having more than 5 layers of granulosa cells, Grade B: Oocyte having more 3–5 layers of granulosa cells, Grade C: Partially denude oocytes, Grade D: Completely denuded oocytes.

**Fig. 1:** A timeline of events depicting the synchronization of a recipient cow via Ovsynch program along with OPU, *in-vitro* embryo development, embryo transfer and pregnancy diagnosis in donor cows.**Fig. 2:** In-vitro embryo development and birth of IVF calves. A: In-Vitro matured COCs, B: Early developmental competence in the embryos C: Blastocysts after 7-days post culture D: newly born IVF calves named Chand and Sitara.

Conclusions: Successful OPU-IVEP program is presented for the Sahiwal cattle in this report which can successfully be used for the exploitation of superior cows of different breeds either for their conservation or favorable cross breeding. However, further studies should be carried out on a large herd to identify various variables that can improve IVEP program in local animals.

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