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Ashish Lakhera
Department of Veterinary Gynecology and Obstetrics, College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur, Madhya Pradesh, India

Nitin Bajaj
Department of Veterinary Gynecology and Obstetrics, College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur, Madhya Pradesh, India

SK Jain
Department of Veterinary Gynecology and Obstetrics, College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur, Madhya Pradesh, India

Brejesh Singh
Department of Veterinary Medicine, College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur, Madhya Pradesh, India

Amir Amin Sheikh
Department of Veterinary Physiology and Biochemistry, College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur, Madhya Pradesh, India

Rakshanda Bhagat
Division of Veterinary Medicine, F.V.Sc and A.H., Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, RS. Pura, Jammu, Jammu Kashmir, India

Correspondence
Ashish Lakhera
Department of Veterinary Gynecology and Obstetrics, College of Veterinary Science and Animal Husbandry, NDVSU, Jabalpur, Madhya Pradesh, India

Salvaging the reproductive potential of culled cows by *In-vivo* ovum pick up technique

Ashish Lakhera, Nitin Bajaj, SK Jain, Brejesh Singh, Amir Amin Sheikh and Rakshanda Bhagat

Abstract
The genetic potential of cows can be harvested by recent techniques like ultrasound guided ovum-pickup technique (*In-vivo*) and aspiration of oocytes from (SO) slaughter house ovaries (*In-vitro*) and subsequently production of embryo by IVF for transfer. However Unproductive, infertile or sterile genetically superior cows are regularly culled at farms. These cows do not have any commercial value and are ultimately sent to gaushalas. The aim of the present research was to study the maturation potential of immature oocytes recovered *in vivo* from unproductive cows by ultrasound guided ovum-pickup technique (OPU).

Keywords: OPU: ultrasound guided ovum-pickup technique, SO: Slaughter house ovaries

Introduction
Each ovary contains thousands of oocytes at birth, most are lost through atresia. This tremendous loss of genetic material could be reduced by harvesting oocytes from the ovary using ovum pick-up techniques [1]. With the initiation of *in vitro* fertilization (IVF) programs, a need of good quality oocytes have increased, the ability to produce oocytes from genetically valuable donors in a safe, repeatable manner would broaden the application of *in vitro* fertilization (IVF) procedure for beef and dairy cattle industry.
Traditionally, oocytes for IVF are obtained from slaughterhouse ovaries. The ovaries being collected at slaughter and transported to the laboratory. Follicles of 2 to 8 mm size on the ovarian surface are punctured for oocytes collection and culture in maturation medium, The main disadvantage of this technique is the lack of repeatability in genetically superior donors as there can be no further collection of oocytes once the cow has been slaughtered, Moreover cow slaughter is banned in india except in few states. Hence, numerous researchers have looked for alternative techniques to retrieve oocytes from living animal. A breakthrough was achieved with the adoption of human transvaginal ultrasound scanning procedures for ovum pick-up (OPU) in bovines. This technique is less traumatic for the animals and less invasive than other systems [1]. It has evolved as a valuable technology in breeding programmes because it can be applied to cattle in different reproductive stages and has been successfully applied in cycling, pregnant, senescent animals, and also in prepubertal calves. Harvesting of oocytes by OPU for *in vitro* embryo production has been a routine practice in advanced countries since its advent in 1996 [2]. However, in India, only few institutes have initiated the use of OPU technology with varying success [3].

Material and Methods
Apparently four healthy culled crossbred cows, reared under standard managemental conditions, maintained at College of Veterinary Science and A.H., Livestock Farm, Adhartal, Jabalpur comprised of the experimental animals for the present study. The cows with regular ovarian cyclicity and apparently normal genetalia and ovaries with no adhesion were selected for the study and their detailed reproductive history was recorded from the farm records. Before the cows were subjected to OPU session, the internal genitalia were palpated and their reproductive status was ascertained. The detailed history of individual cow and reasons for culling has been recorded.

Initiation of new cycle prior to OPU
The donors were synchronized with two prostaglandin $F_2\alpha$ (25 mg) treatment at 11-day interval. Ultrasound-guided ovum pick-up was performed 24 hours after the last $PGF_2\alpha$ injection. OPU was repeated once a week for four consecutive weeks followed by three weeks rest. OPU was then repeated twice weekly at three days interval for four consecutive weeks.

Preparation of animals: The animals were restrained in a suitably designed crate which allowed minimal movement. Before each aspiration session, faeces were removed from the rectum and the perineal area was cleaned with tap water and non irritant soap and smeared with 70% ethanol. Prior to follicle aspiration, each cow received caudal epidural anesthesia using 6 ml of 2% Lignocaine HCl (Xylocaine 2%) to decrease peristalsis and discomfort to the animal and to make the manipulations easy.

Instrumentation for OPU: OPU was performed using an ultrasound machine (Aloka SSD-500, Tokyo, Japan) with a 5 MHz transvaginal transducer having stainless steel dorsal needle guide and collection apparatus with a pressure of 50 mm Hg. The collection apparatus consisted of an 18 gauge, 60 cm long needle along with a needle guide attached to aspiration line of 100 cms length that was connected to a 15 ml centrifuge tube. Aspiration was performed using a suction apparatus (Eurovac AC/DC aspirator for paediatric use). The oocytes were aspirated in Dulbecco's phosphate buffered saline (DPBS without Ca^{++} and Mg^{++} ions) supplemented with 50 µg/ml gentamycin, 20 µg/ml heparin and 0.3% lyophilized BSA.

Transvaginal ultra sound guided oocyte recovery: The internal genitalia were palpated per rectally and the left or the right ovary was held with the fingers. The transducer was smeared with the ultrasound gel and inserted through the vagina, advanced to the fornix, and positioned just posterior to the ovary. By gentle manipulation of the ovary over the transducer, the image of the ovary was scanned on the ultrasound screen (Plate 5 and 6). The number and size of follicles from surface of each ovary was determined before puncture. Follicles from both ovaries were measured and classified according to diameter as small (3–5 mm), medium (6–9 mm) and large (≥10 mm). Follicles greater than 5 mm were positioned on the aspiration line and the needle was inserted through the needle guide with a grip and gently pierced through the fornix vagina. As soon as the tip of the needle was observed to be in the middle of the follicle, aspiration pressure was applied to aspirate the follicle. Simultaneously during aspiration, the needle was gently moved to and fro and from side to side to disrupt the granulosa cells ensuring proper detachment of the cumulus oocyte complex (COC). After aspiration of all follicles, an extra 20 ml of medium was aspirated to recover oocytes from the needle and silicone tubing. On completion of the OPU session, the contents of the centrifuge tube were transported to the laboratory at 37ºC. The recovered oocytes were searched under stereozoommicroscope, counted and morphologically graded into four categories. The number of aspirated follicles, the number of retrieved oocytes and oocyte recovery rate were recorded.

Searching and grading of oocytes: Oocytes collected by either OPU or slaughterhouse ovaries were searched under stereo zoom microscope at 110x magnification. All the oocytes were picked up by pipette and placed in 35 mm culture petri dishes containing 4-5 ml of washing medium for grading. Oocytes were evaluated by morphological appearance and graded as per the criteria outlined by [4].

Maturation of oocytes: After grading of oocytes, Grade I, II and Ill quality oocytes were used for *in vitro* maturation. Oocytes were washed thrice in oocyte washing medium (3 ml).

Procedure for maturation of oocytes: The 50 µl droplets of maturation medium were prepared in petridish (35 mm), oocytes were transferred in groups of 10-15 oocytes per droplet and the droplets were then covered with sterile pre equilibrated (38.5°C) mineral oil (Sigma) and incubated in CO_2 incubator with humidity >95 %, 5% CO_2 at 38.5°C for 27 hrs [5]. Following methods were carried out for assessment of IVM of oocyte: i.e. Cumulus expansion, ii. Identification of polar body.

Result and Discussion

The Follicles visualized, aspirated, recovery and maturation potential of oocytes recovered by ultrasound guided ovum pick up method are given in Table 1. Imaging of follicle in OPU and Ultrasound imaging of large size follicle are shown in Figure 1 and 2. whereas Grade II oocyte collected by OPU, Grade III and Grade IV oocyte collected by OPU and Cumulus expansion of oocytes collected by OPU is shown in Figure 3, 4 and 5.

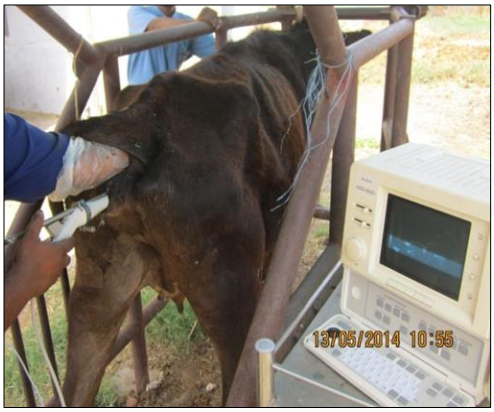

Fig 1: Imaging of follicle in OPU

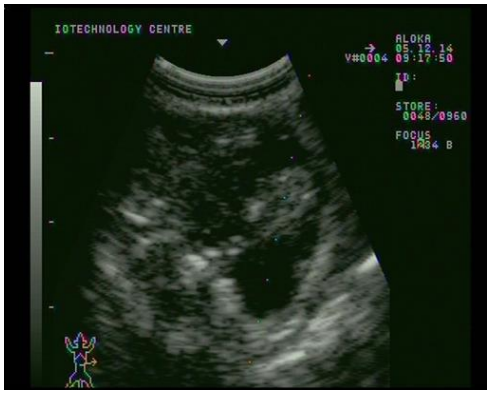

Fig 2: Ultrasound imaging of large size follicle

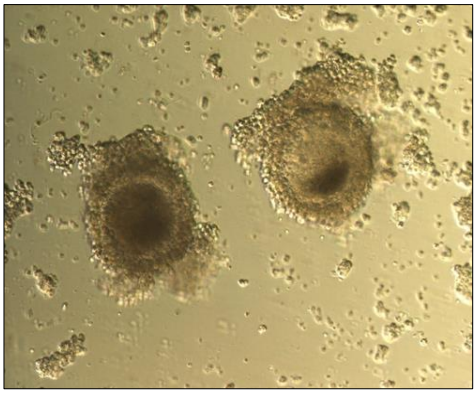

Fig 3: Grade II oocyte collected by OPU

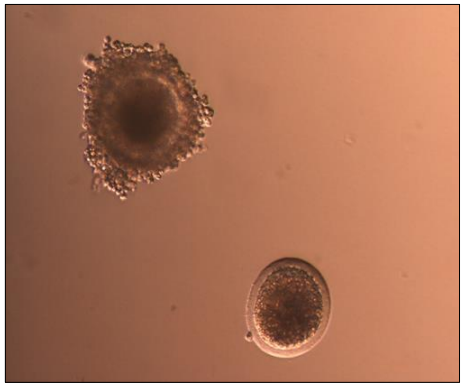

Fig 4: Grade III and Grade IV oocyte collected by OPU

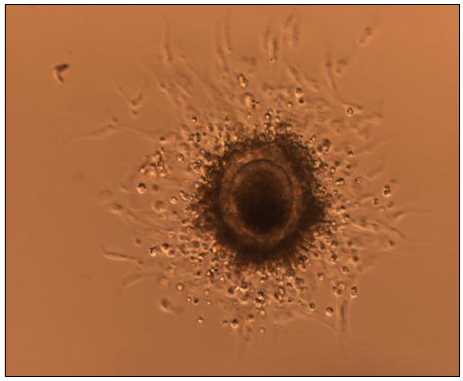

Fig 5: Cumulus expansion of oocytes collected by

Once a week OPU sessions: It is observed that in 18 sessions, 163 follicles (9.05 follicles per session) could be observed, of which 26 (15.95%) were large, 69 (42.33%) were medium and 68 (41.71%) were small follicles. Out of these 163 observed follicles, 91 (55.82%) *i.e.* (5.05 follicles per collection) could be aspirated. Cow wise, follicles per session ranged from 5.33 to 10.50, of which, 4.50 to 5.50 follicles per session could be aspirated. A total of 1 oocyte was recovered out of 18 sessions. The quality of recovered oocyte was grade IV.

Twice a week OPU sessions: It is observed that in 33 sessions, 287 follicles (8.69 follicles per session) could be observed, of which, 23 (8.01%) were large, 107 (37.28%) were medium and 157 (54.70%) were small follicles. Out of these 287 follicles observed, 185 (64.45%) *i.e.* (5.60 follicles per session) could be aspirated. Cow wise, follicles per collection ranged from 7.87 to 9.25, of which, 4.62 to 6.50 follicles per session could be aspirated. A total of 6 oocytes were recovered out of 33 sessions. The quality of recovered oocytes were grade II (3), and grade III (3) oocytes.

In 51 sessions irrespective of once and twice a week OPU sessions, 450 follicles *i.e.* 8.82 follicles per session were observed out of which 276(61.33%) follicles *i.e.* 5.41 follicles per session were aspirated. A total of 7 oocytes were recovered and the quality of recovered oocytes was grade II, III and IV.

It was evident from the results that the oocyte yield was better in twice a week OPU session as compared to that in once a week OPU session. Also, the number of follicles of different sizes (i.e. large, medium and small) available for aspiration in once or twice a week OPU session differed. The large and medium sized follicles were more in once a week session than twice a week OPU session. This variation in size of follicles between once and twice a week OPU session influenced the oocyte recovery. Therefore, higher number of oocyte (6 nos.) was obtained in twice a week session as compared to once a week session where only one oocyte could be recovered. [6]. also observed that heifers subjected to aspirations twice-weekly yielded a higher number of oocytes collected per session. The major benefit of a twice-weekly aspiration schedule is the prevention of the development of a dominant follicle, thus making a relatively homogeneous population of small follicles available that can be easily harvested.

Table 1: Follicles visualized, aspirated, recovery and maturation potential of oocytes recovered by ultrasound guided ovum pick up method

Animal number	Total number of sessions	Total number of follicles observed	Total number of follicles aspirated	Total number of oocyte retrieved from ovary					Total number of oocyte matured	
				Grade I	Grade II	Grade III	Grade IV	Total oocyte recovered	Cumulus Expansion	Polar body
CB 16	14	137	79	-	1	2	1*	4	2	-
CB1112	13	115	78	-	1	1	-	2	1	-
CB1149	12	105	62	-	1	-	-	1	-	-
CB1126	12	98	57	-	-	-	-	-	-	-
Total	51	450	276	-	3	3	1*	7	3	-

* Recovered oocyte in once a week OPU session rest all in twice a week OPU session

Conclusion

It is concluded that the reproductive potential of culled cows can be salvaged using oocytes obtained by ultrasound guided ovum pick up (OPU) techniue for conserving the lost genetic potential from such cows. However, the technique being precise needs high level of expertise and precision before it can be effectively applied. Oocyte yield was better in twice a week OPU session as compared to that in once a week OPU session. Low oocyte recovery by OPU in the present study may be due to uncontrolled pressure and a lower level of experience of the operators.

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