# BOVINE *IN-VITRO* EMBRYO PRODUCTION Part-1

The world food crisis and rise in demand for food of animal origin, competitive global markets for such foods and the negative effects of climatic change, especially in the marginal tropical areas [1], demand that innovative strategies and interventions be urgently employed [2]. The dairy sector in developing countries, especially in low input production systems, has the potential of providing cost effective animal protein requirements. To do this, such units need to be efficient, and the starting point is using appropriate genotypes. Many attempts aimed at improving the productivity of indigenous dairy cattle in developing countries through crossbreeding with temperate breeds have generally not been successful [3].

Many studies have shown that temperate dairy breeds best combine with local tropical breeds at 50% of each breed composition ($F_1$ cross). The $F_1$ is well adapted and produces reasonable amount of milk under the local conditions [3, 4].

Aggressive promotion of a few exotic cattle breeds in sub-Saharan Africa (SSA) has resulted in cattle genotypes that are not only poorly adapted to the production systems but are also commercially inefficient [5].

In order to meet the increasing demand for appropriate improved dairy cattle genotypes in the marginal dairy systems in the Eastern African region, more efficient production and delivery of improved germplasm is needed. Breeding strategies that utilize a combination of emerging reproductive technologies, especially the in-vitro embryo production (IVEP), ovum pick up (OPU) and semen sexing, offer prudent possible options [6]. For example, indigenous breeds such as the Boran that are inferior commercial milk producers would be valuable as oocyte donors as well as surrogates for crossbred embryos under IVEP-based systems.

Up to 80% of Kenya's landmass is arid or semi-arid (ASAL). The pastoralists who live in the ASAL depend heavily on livestock as their most viable source of livelihood. Among the Zebu breeds of livestock kept in these areas, the most commercially viable breed in Kenya is the Boran.

Conventional embryo transfer technology is based on the superovulation of a high-quality donor animal and the subsequent recovery of embryos by flushing the uterus a week after breeding to the bull of choice [7, 8, 9]. In cattle, this technology is well established. The technology can be advanced following further understanding of the reproductive physiology of the cow by the practitioners of the Embryo Transfer (ET) in Kenya so as to meet the demand for better bovine genetic improvement within Eastern African countries.

An alternative to avoid the more expensive conventional superovulation procedures during ET is *in-vitro* embryo production (IVEP) [10]. This technology allows for cheaper production of a predictable supply of embryos from ovaries of live or slaughtered cows. From live selected animals, repeated recovery of primary oocytes using ovum pick up procedures (OPU) is possible [7, 8]. To date in Kenya, our team has shown that IVEP and OPU technique have given considerable success in cattle, achieving a success rate ranging from 30 - 50% for development of pre-implantation stage embryo *in-vitro* and conception rates of up to 50%. The IVEP technology does not only offer optimization of high-quality dams (mothers), but also allows the preservation and rapid multiplication of genetically superior cows by making embryos available for sexing, cloning and nuclear transfer [11]. Use of such advances in technologies can be utilized to turn around the economic gains of farmers.

Utilization of the IVEP technology in Kenya can be used to undertake value addition to indigenous cows and improve household incomes and food security. Farmers keeping low-grade cows can enter into commercial contracts to provide these cows as surrogate recipients for production of heifer calves. In the process, owners of such cows will have a valued product

to sell for income and be left with milk for use by family members. This avenue can be used to raise household revenues and attract many more farmers to engage in farming, leading to improved national economic and food security. Kenya can utilize reproductive technologies in the dairy sector due to their massive potential benefits to revolutionize the sector for enhanced food security by increasing cattle productivity.

This paper highlights the practical ways of applying IVEP technologies to help turn around the economy of the Kenyan livestock farmers and boost food security. It discusses the technical aspects of the procedures involved in the *in-vitro* production of bovine embryos and embryo transfer, with special reference to the application of the techniques in Kenya.

## MATERIALS AND METHODS

Production of embryos in the laboratory involved four major steps: 1) Ovary collection, 2; Oocyte maturation, 3) in vitro fertilization and 4) Embryo culture.

In brief, ova were collected from live Boran cows in Kapiti Ranch using ovum pick-up (OPU) technology and transported to the laboratory in a warm saline solution (supplemented with 0.1g/L streptomycin). In some cases, ovaries from slaughtered animals were used. Pre-slaughter body condition scores (BCS) were assessed prior to slaughter at the holding area. Ova collected from cows were delivered to the laboratory within 4hrs of collection.

Cumulus oocyte complexes (COCs) were obtained by aspiration of 3 to 8 mm follicles using a 21-gauge needle attached to a 10 mL syringe and manipulated in Tyrodes Albumin Pyruvate (TALP) Hepes medium supplemented with 0.4% bovine serum albumin. The COCs were graded morphologically according to oocyte cytoplasm aspect and morphology of overlying zona pelucida cumulus cell layers; only grade A and B COCs were used for the work. These were COCs with compact cumulus cell layers and oocytes with homogenous (grade A) or slightly heterogeneous (grade B) cytoplasm. Standard *in vtro* oocyte maturation, fertilization and embryo culture procedures were conducted. Synchronization of the recipient cows was achieved by knocking off the corpous luteum using cloprostenol 500ug (2ml of lutealyase) and embryo transfer was done at day seven using *in vitro* produced blastocysts.

All chemicals used were from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise stated.

### Review of the Technical aspect of the Technology *In-vitro* Embryo Production (IVEP)

### Media and Stock Solutions

Media and stock solutions for *in vitro* embryo production need to be sterile, accurately done. The use of a Laminar flow Cabinet for sterility is indispensable to avoid any contamination. Using analytical balance with readability of at least 10 µg and accuracy of ±0.1 µg is very important. All components of the medium must be of the highest grade possible.

To achieve good standards on specifications of media and stock solutions, the following important factors need consideration.

#### i) Water

A Millipore-Q system or its equivalent should be used to produce high quality pyrogen-free water with a resistance of >18 megahms-cm. This study utilized this kind of water.

#### ii) Glassware

Glassware for media preparation should be designated solely for this purpose. Glassware to be used must be sterile and pass through the standard tissue culture cleaning procedure. This study used glassware that was double cleaned and sterilized through a glassware autoclaving system.

### iii) Disposable Materials

Disposable supplies such as Petri dishes, acrodisc filters, centrifuge tubes for media storage and pipette tips used during IVEP make the IVEP work seem expensive but the use of disposable materials is more beneficial and convenient in terms of eliminating contamination and ensuring purity and safety of the prepared media and the produced embryos. This study used disposable materials purchased from Nutricell Company in Brazil and all were stored within clean cabinets of the laboratory.

### iv) Filtration

The use of a 0.22µm filter is recommended, considering that the size of the smallest bacteria is 0.3µm. In this study, use of 0.22µm filter used and the first few drops were discarded to remove any contaminants from the filter and the container. The remaining volume was filtered again and stored in a refrigerator.

### v) Embryo Culture and Working Area

The culture media used for the growth of oocytes and embryos is highly prone to contamination. The 95-100% humidity and 38.5$^{o}$C temperature conditions for growth within the incubator are some of the factors that enhance the growth of any contaminating organisms present within the culture media. Sources of contamination could be air borne and/or from surfaces of the working area, or from any materials used in the preparation of the culture media. The following measures were implemented in this study to avoid contamination of the cultured oocytes and embryos.

a) **Working space** was separated, with various designated areas; dirty benches for aspirations, clean benches for media preparation and sterile spaces for oocytes and embryo handling and manipulations. The interior laboratory space containing the incubators was out-of-bounds for all external people, who were only allowed to view the procedures through a transparent glass wall.
b) **Workers** covered their hair, mouth and nose, and used disposable hoods whenever conducting oocyte and embryo culture techniques.
c) **Disinfectant** such as 70% alcohol was applied to the working surfaces before the preparation of the culture media and prior to any embryo production techniques.
d) **Hands** had to be washed first with clean water and sterilized with 70% alcohol before handling the materials for media preparation and embryo production.

### vi) Storage and Maintenance of Media and Reagents

In order to improve on IVEP results, this study ensured the expiry date of all reagents and media were noted and cross-checked at all times prior to use. Stock solutions and the media were kept in their manufacturers' prescribed storage conditions. To maintain the viability and efficacy of media and reagents, preparation of well labeled aliquots was done to avoid frequent open access to the stock solutions to eliminate any possibility of contamination. Storage containers were sealed securely to avoid any moisture contamination that tends to alter the pH or osmolality of the media. In our system, a folder/ file with all standard preparation procedures and also with documents showing important information on preparation and expiry dates for all media and reagents was available within the laboratory.

## Collection of Ovaries

Pre-slaughter record parameters were obtained prior to slaughter and or OPU to track genetics of embryos produced. Records were entered into a well-designed data collection form. Ovaries should be collected within 10 minutes after the slaughter of the animals, and kept in a sealed container containing physiological saline (0.9% sodium chloride with either 1ml/L Gentamycin or 100 µm/ml Streptomycin and 100 IU/ml Penicillin), at a temperature ranging from 28-33°C. Careful observation of the temperature of the saline is important to maintain the viability of the ovaries during the period of collection. In our system, the prepared saline was left to stand at 38°C overnight and the gentamycin was added in the morning of collection before transfer to a thermos flask. Temperature check-ups were done regularly at the slaughterhouse.

The time interval between animal slaughter and oocyte recovery from the ovaries, and the temperature of the holding medium is also important. In general, the time spent between ovary collection and oocyte recovery should be within 3-4 hours and a 30 - 33°C temperature range of the physiological saline should be maintained. However, in cases where the slaughterhouse is far distant from the laboratory, a decrease in the temperature is recommended, although the temperature should still be kept above 28°C to maintain the development potential of the oocytes. Prolonging the period of ovary collection may significantly affect the viability of the oocytes for IVEP. It is recommended that oocytes should be collected from the ovaries at most within a 6-hour period. Beyond six hours, development competence of the oocytes is greatly compromised. In our system, the slaughterhouses were located 10kms from the laboratory and the time taken between ovary collection and delivery to the laboratory was 3-4 hrs.

**Oocyte Recovery**

Before oocyte recovery, it is necessary to wash the ovaries in fresh sterile physiological saline (without antibiotics) to remove any contaminants. Thereafter, briefly rinse the ovary in 70% ethyl alcohol to eliminate surface organisms before beginning the oocyte recovery procedure. The ovaries are then dried lightly with sterile paper towels and primary oocytes are recovered from 1-6 mm vesicular follicles. It was important to avoid recovery of oocytes from big follicles (beyond 6 mm) because they contain secretions that tend to cause jell formation in the aspirates. In such cases the retrieval of oocytes during searching is negatively affected.

Recovery of the oocytes from the ovaries may be done by either of the following techniques:

- Oocyte aspiration using needles
- Slicing the ovaries using scalpel blade
- Follicle dissection from ovaries
- Ovum pick-up from live animals

For the ovaries collected from slaughtered animals, the aspiration method was commonly employed because of the convenience associated with it. Aspiration of oocytes was done using a needle attached to a 10-ml syringe. To avoid disruption of the surrounding cumulus cells, an 18-gauge needle was used. Possible toxicity associated with syringes containing rubber plungers and siloxane lubricants was avoided by using only sterilized syringes with plastic plungers.

One of the difficulties associated with the aspiration approach is the fact that oocytes may only be retrieved from about 60% of the punctured follicles. To minimize this effect, it was necessary to prime the needle and syringe with approximately 0.25-0.5 ml of aspiration medium. This is to provide the initial fluid that helps ensure the retrieved oocytes are within the fluid dispended from the syringe.

The aspiration medium used was Tyrosine-albumin-pyruvate (TALP)-HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) but also modified phosphate-buffered saline was used occasionally, especially, during training sessions. Aspiration medium can be prepared once, stored in a refrigerator and made available for one week's activity. However, it is very important to pre-warm the aspiration medium up to 38°C before use.

After aspirations are made from follicles of each ovary, the contents in the aspirating syringe are slowly transferred into a sterile centrifuge tube that is pre-moistened with TALP-HEPES and the oocytes are allowed to settle at the bottom of the tube that is placed into a rack within a warm water bath set at $38^{O}$C. The transfer of fluids containing the oocytes into the tube is done slowly with minimal disruption of the cumulus-oocyte complex (COC). When this is done from the last ovary of the day, the precipitate containing the oocytes that had settled at the bottom of the tube are selectively picked using a sterilized Pasteur pipette and transferred into sterile Petri dishes containing TALP-HEPES for subsequent searching, grading and counting of recovered

COCs. The recovery work of COCs should be done in a sterile environment within room temperature of 25°C.

Sometimes slicing of the ovaries is done in order to enhance retrieval of numbers of COCs. However, the method is more tedious and requires longer period of retrieval and is thus not recommended whenever manipulating a large number of ovaries because the extended period of oocyte retrieval would negatively affect oocyte competence. In this case, the method was used only when few ovaries were collected.

Follicle dissection is also a method that can be used. This method is effective in retrieving good-quality oocytes as reviewed extensively by Gordon [10]. However, it also increases the time needed for the retrieval process. In this study, this was only used during research.

Ovum pick up can also be done from live cows using ultrasound guided follicular aspiration through a needle passed trans-rectally as shown demonstrated in Fig 2.

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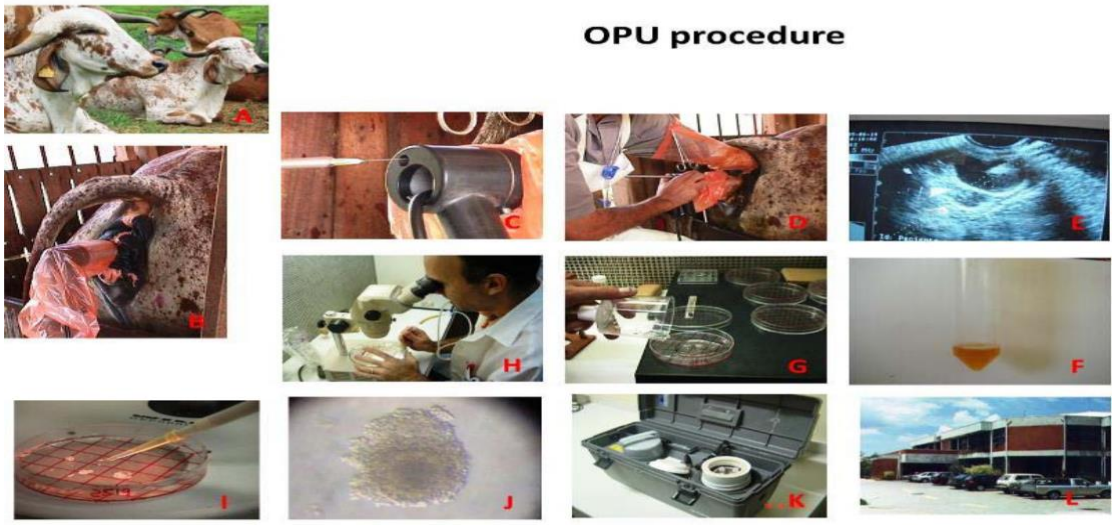


Figure 2: Photographic demonstration of the OPU procedure; donor cows selected (A), then ultrasound device inserted into the vagina (B), needle put into the device (C), the expert picks the ovary towards the intra-vaginal ultrasound device (D), the follicles are viewed through the ultrasound monitor (E), the follicles are aspirated into a tube (F), the follicles are poured into a petri dish (G), the follicles are searched (H), the follicles are recovered into another petri-dish (I), grade A COC (J), grade A COCs are loaded into thermos flask (K), the COCs are delivered to the laboratory (L)

This technique is used for repeated collection of COCs from top grade cows. The method was also used within the IVEP system reported here.