ADVANCES ON *IN-VITRO* PRODUCTION OF CAPRINE EMBRYOS

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INTRODUCTION

Since the past few decades, there is a growing interest in the methods of *in-vitro* embryo production in all species of farm animals. The development of IVEP methods and their commercial applications requires sufficient amounts of high quality oocytes capable of *in-vitro* maturation and fertilization. The numbers of high quality oocytes harvested from an ovary are an important consideration for the *in-vitro* production of embryos (Wani *et al.*, 2000).

The oocytes can be obtained from live animals as well as from slaughtered animals. If these are collected from immature live animals and slaughtered animals, transferred to the recipient animals the generation interval can be reduced. If the oocytes are collected from slaughtered animals, the utility of those animals even after slaughter are improved. Ovaries of slaughtered animals are the cheapest and most abundant source of primary oocytes for the large scale production of embryos through IVEP procedures (Agrawal *et al.*, 1995). *In-vitro* maturation of oocytes helps in the generation of embryos from the ovaries of high producing slaughtered animals, which are otherwise wasted (Nandi *et al.,* 2002, Jamil *et al.,* 2007).

So we can get high number of oocytes to obtained matured oocytes and fertilized embryos without disturbance of animals by surgical and non surgical technique**.** This formulates low cost supply of follicular oocytes which can be matured, cultured and fertilized *in-vitro* (Khillare, 2008). *In-vitro* embryo production in goat provides an alternate source of low cost embryos for transfer in breeding programs.

According to Paramio, (2010), *in-vitro* embryo production has the following advantages as;

(i) A significantly increased number of embryo from high genetic valued females,

(ii) Provides an excellent source of low cost embryos for basic research, embryo biotechnology studies (nuclear transfer, transgenesis, embryo sexing and stem cells) and all kinds of embryo research which need embryos for manipulation and

(iii) Used as a strategy for the rescue of some endangered animal species by inter-specific embryo transfer.

The IVEP system involves four steps (Vicente *et al.*, 2010)

(a) recovery of primary oocytes from antral follicles

(b) *in-vitro* maturation (IVM) of these oocytes

(c) *in-vitro* fertilization (IVF) of the matured oocytes and

(d) *in-vitro* culture (IVC) of the resulting embryos.

DEFINITION:

In-vitro production of embryos involves oocyte recovery, maturation, and fertilization with capacitated spermatozoa and culture of the resulting embryos (Malakar *et al*., 2007). *In-vitro* maturation, fertilization and culture (IVMFC) of embryos, which are collectively known as *in-vitro* embryo production (IVEP) (Rahman *et al.*, 2008). *In-vitro* embryo production involves a combination of the techniques of *in-vitro* maturation (IVM), fertilization (IVF) and culture (IVC) of oocytes (Kumar and Anand, 2012).

METHODS OF OOCYTES RETRIEVAL:

Recovery of good-quality oocytes is the first step for IVEP procedure. The oocytes can be retrieved by two ways as follows:

1.) Slaughter Animal:

Ovaries are collected from local slaughter house in collection vial containing 0.9% physiological saline kept in a thermos box at 25 to 30ºC and transported to the laboratory within 4 to 5 hours of slaughter (Mondal *et al.*, 2008). In the laboratory ovaries are freed from the surrounding tissues and overlying bursa. Ovaries are then treated with three washings in Dulbeccos Phosphate Buffer Saline (DPBS) and two washings in oocyte harvesting medium (DPBS+4 mg/ml BSA+50 IU/ml Penicillin) (Wani *et al.*, 2000; Wang *et al.*, 2007; Hoque *et al*., 2011). Ovaries are been Grade as follows in table no.1:

Table.1 Ovarian types and number of follicles and cumulus–oocyte-complexes (COCs) per ovary (Mondal *et al.*, 2008)

Ovarian type	Total number of visible follicles (mean)	Number of follicle aspirated (mean)	Collected COC's per ovary (mean)
Type I	5.25	3.15	1.96
Type II	3.94	2.57	1.54

Each ovary was processed individually and the oocytes harvested by one of the following three techniques:

i.) **Follicle Dissection:** Follicular dissection of ovaries was first used to recover follicular oocytes (Crosby *et al.*, 1981; Fukui *et al.*, 1988). Ovaries are placed in a petri-dish containing 5 ml of oocyte harvesting medium, they are held with the help of forceps and the whole ovarian surface was punctured with an 18'G hypodermic needle and oocytes are collected (Wani *et al.*, 2000; Hoque *et al.*, 2011).

ii.) **Slicing of ovaries:** Slicing of ovaries was used for obtaining oocytes (Wahid *et al.*, 1992a & b; Samake *et al.*, 2000). Ovaries are placed in a petri-dish containing 5 ml of the oocyte harvesting medium, held with the help of forceps. Incisions were given along the whole ovarian surface using a scalpel blade and oocytes are collected (Wani *et al.*, 2000; Hoque *et al.*, 2011).

iii.) **Oocyte Aspiration:** Aspiration of follicles are routinely used for oocyte recovery (Slavik *et al.*,1992, Watson *et al.*,1994).Visible follicles are aspirated using a 20'G hypodermic needle attached with a sterile disposable syringe containing 2 ml harvesting medium (Wani *et al.*, 2000; Hoque *et al.*, 2011).

The oocytes are then examined under an inverted microscope (Wang *et al.*, 2007, Majeed and Hoque *et al.*, 2011).

Table.2 Quality and Quantity of recovered oocytes per ovary in different collection methods (Wang *et al.*, 2007)

Methods of collection	No. of ovaries	No. of recovered oocytes per ovary		
		Good (%)	Fair (%)	Poor (%)
Follicle Dissection	35	55.20	25.80	19.00
Slicing of ovary	31	61.90	20.60	17.50
Oocytes Aspiration	32	58.10	22.60	19.30

2) Live animal:

For the oocytes retrieval from live animal super ovulation is the technique. Superovulation is induced by a combined injection of FSH and eCG. Super ovulation is induced by gonadotrophin treatment consisting of a total dose equivalent to 70 mg FSH and 300 IU of eCG administered intramuscularly 36 h prior to the LOPU procedure (Wang *et al.*, 2003).

FSH treatment prior to LOPU increased the population of 2–4 mm ovarian follicles and resulted in higher oocytes collection (Baldassarre *et al.*, 1994; Wieczorek *et al*., 2010). In the past, pregnant mare serum gonadotrophin (PMSG) alone (Samsul, 1997) or in combination with human chorionic gonadotrophin (hCG) (Hisham, 2006) was used to superovulate does. However, due to higher variability of stimulation and lower oocyte retrieval (OR) rate, a combination of recombinant caprine FSH and hCG as single doses was later introduced (Hisham, 2006; Phua, 2006; Amir, 2007) which has been improvised further (Rahman *et al.,* 2007a,b&c; Abdullah *et al.,* 2008; Rahman, 2008) and are still in use.

i) Laparoscopic Ovum Pick Up (LOPU):

Snyder and Dukelow first described LOPU in 1974. Oocytes recovered from live animal by a laparotomy, followed by aspiration of oocytes from ovaries using an aspiration pump are called as laparoscopic ovum pick-up (LOPU). The use of laparoscopy surgery enabled the laparoscopy ovum pick-up (LOPU) technique to be used in medium-sized farm animals such as sheep, goats and pigs (Baldassarre *et al.*, 1994; Stangl *et al.*, 1999; Wieczorek *et al.*, 2010). LOPU allows 50–80% of oocytes to be recovered from aspirated ovarian follicles and 30–90% of these are accepted for maturation and fertilization *in-vitro* (Wieczorek *et al.*, 2010). The use of this technique enables bloodless, safe and quick access to abdomen organs and easy stabilization of ovaries and aspiration of ovarian follicles (Kuhholzer *et al.*, 1997; Wieczorek *et al.*, 2010). Laparoscopic ovum pick-up (LOPU) in combination with *in-vitro* production (IVP) of embryos has the potential to improve the number of offspring produced by genetically valuable does (Tervit, 1996; Baldassarre *et al.*, 2002; Cognie *et al.,* 2003; Baldassarre *et al.*, 2004). LOPU are advantages technique to be employed for prepubertal or aged goats which would be unable to reproduce (Abdullah *et al.*, 2011). The efficiency depends on the good selection of donors, hormonal stimulation, frequency of oocytes recovery and technical factors (Stangl *et al.,* 1999; Kuhholzer *et al.*, 1997; Baldassarre *et al.*, 1994, 1996, 2002; Wieczorek *et al.*, 2010).

The method of laparoscopic ovum pick up is as follows:

The laparoscopy equipment consists of a 7 mm telescope, light cable, light source, 7 mm trocar for the laparoscope, an atraumatic grasping forceps, and two 5 mm trocars. The follicle puncture set consists of a puncture pipette, tubing, a collection tube, and a vacuum pump. The puncture pipettes are prepared by fixing a 20'G short bevel hypodermic needle into the tip of an insemination pipette with instant glue. The collection tube is a 50 mL centrifuge tube with inlet and outlet ports in the stopper. These ports are connected to the aspiration pipette and to a vacuum pump, respectively, with plastic tubing. The vacuum pressure is regulated with a flow valve and measured as drops of collection medium per minute entering the collection tube, and is adjusted to 50 to 70 drops per minute. The complete puncture set is gas sterilized and rinsed with collection medium prior to use. The collection medium is TCM 199 supplemented with 0.05 mg per ml Heparin and 1% (v/v) fetal calf serum; the collection tube contained 0.5 ml of this medium to receive the oocytes.

Anaesthesia was induced with intravenous administration of diazepam@0.35 mg/kg body weight and ketamine@5 mg/kg body weight and is maintained with isofluorane via endotracheal intubation. Oocytes are recovered by aspiration of follicular contents under laparoscopic observation (Baldassarre *et al*., 1996, 2003). Laparoscopic ovum pick-up is done under general anaesthesia. Animals are premedicated with intramuscular injection of atropine 0.05 mg/kg and xylazine 3–5 mg/kg. Anaesthesia was induced with intravenous injection of thiopentone natrium 5–10 mg/kg (Wieczorek *et al.*, 2010). The anaesthetized animals are restrained in a cradle in dorsal position. The goats are then suspended head down on a mobile surgery table at an angle of approximately 45° to the horizontal. A trocar for endoscopic camera is inserted into abdomen cavity in the left side of the abdominal midline and about 15 cm cranial to the udder. Next two trocars for atraumatic grasping forceps are inserted after filling abdomen cavity with filtered air. One is inserted in the right side of abdominal midline and about 15 cm cranial to the udder, the other in abdominal midline about 30–35 cm cranial to the udder. Three trocars are inserted into abdomen on an isosceles triangle plan with arms of 15–20 cm. The basis of triangle is 15 cm cranial to the udder. The ovaries are exposed by pulling the fimbria in different directions using the grasping forceps and the follicular contents are aspirated by puncturing follicles with the aspiration needle. At the end of the procedure, the surface of both ovaries is flushed with warm heparinized saline using an insemination gun introduced through one of the 5 mm trocars, in order to clean any

blood from the follicle puncture site (Kuhholzer *et al.*, 1997; Stangl *et al.*, 1999; Baldassarre *et al.*, 1996; Graff *et al.*, 1998; Wieczorek *et al.*, 2010).

Table3. Follicles Aspirated and Oocytes recovered by LOPU in different age (Koeman *et al.*, 2003)

Age & Number	Follicles aspirated	Oocytes Recovered	Recovery rate (%)
Prepubertal (23)	39	28.4	73
Adult (21)	19	15.9	84

ii) Transvaginal ultrasound-guided aspiration (TUGA):

Transvaginal ultrasound-guided aspiration (TUGA) technique used in live goats for recovery of oocytes (Graff *et al.*, 1999). The Transvaginal ultrasound-guided aspiration machine consists of ultrasound unit sector with a 5 MHz human transducer, a vacuum pump and aspiration needle system.

The goat is restrainted properly, and epidural anaesthesia is administered. The goat is placed in dorsal recumbency. The transvaginal transducer is inserted into the vagina and it is pressed into the fornix of the vagina next to the cervix. Each ovary is then held at the tip of the transducer; this allows the structures of the ovary to be viewed by the technician on the ultrasound monitor. The follicles of larger then 2mm are then perforated by a 60cm, 18'G aspiration needle, which is passed through a guide attached to the vaginal probe. The oocytes are extracted along with the surrounding follicular fluid and drawn into a collection container by vacuum aspiration with an electric suction pump (Paramio, 2010).

Classification of oocytes:

- The oocytes are then examined under an inverted microscope. The oocytes are classified into 4 grades as follows:
  1) Grade A: oocytes completely surrounded by cumulus cells;
  2) Grade B: oocytes partially surrounded by cumulus cells;
  3) Grade C: oocytes not surrounded by cumulus cells and
  4) Grade D: degeneration observed both in oocytes and cumulus cells.

Grade A and B are considered as normal COCs and grade C and D will be considered as abnormal (Khandoker *et al.*, 2001).

- The oocytes are graded as good, fair and poor on the basis of cumulus cells and cytoplasm.

  Good: Oocytes with many complete layers of cumulus cells and uniform cytoplasm.

  Fair: Oocytes with thin or incomplete layers of cumulus cells and uniform cytoplasm.

  Poor: Oocytes with few or no cumulus cells.

  Number of good, fair and poor oocytes is obtained from each ovary (Wani *et al.*, 2000; Majeed *et al.*, 2011).

- The oocytes are graded based on their cellular investments and cytoplasm uniformity, as follows:

  Grade 1: Multi-layered compact cumulus and evenly granulated cytoplasm.

  Grade 2: 1 to 3 layers of cumulus cells and evenly granulated cytoplasm.

  Grade 3: No cellular vestments or heterogeneous cytoplasm.

  Grade 4: Oocyte with abnormal shape & heterogenous oocyte cytoplasm.

  Oocytes of grades 1 and 2 are selected for subsequent uses (Wang *et al.*, 2003; Avelar *et al*., 2012).

In-vitro Maturation (IVM) of oocytes:

Maturation of mammalian oocytes is defined as the sequence of events occurring from the germinal vesicle stage to completion of the second meiotic division with formation of the second polar body (Blanco *et al.*, 2011).

The procedure for IVM was described by De Smedt *et al.* (1992). The good and fair oocytes are pooled and subject to two to three washings in maturation medium. The maturation medium consists of TCM199 with Earle's salts and 25 mM Hepes supplemented with bovine LH (0.02 U/ml), bovine FSH (0.01 U/ml), estradiol-17b (1 mg/ml), 0.2 mM sodium pyruvate, kanamycin (50 mg/ml) and 10% heat inactivated oestrous goat serum(Younis *et al.*, 1991; Wang *et al.*, 2003). Oocytes are placed in 2 ml maturation medium and incubated at 38.5^{o}C, under 5% CO_2 with a saturated humidity for 24-26 h. After the end of incubation a portion of oocytes is freed from the cumulus cells by continuously pippeting in and out of a capillary, so that completely denuded oocytes are obtained.

After maturation, the degree of cumulus cell expansion is determined according to Rahman *et al.*(2003, 2004) under microscope at 10x magnification as, Level-1: indicating less expansion of oocytes; Level-2: indicating moderate expansion and Level-3: indicating marked expansion of cumulus cells with a compact layer or chorona radiata. The denuded

oocytes are then placed on a glass slide, covered with cover slip, are fixed in an ethanol and acetic acid solution (3:1) for 24 h, stained with a 1% aceto-orecine stain and examined under a high power microscope. The maturation state of oocytes is evaluated on the basis of nuclear maturation.

The oocytes are classified as: germinal vesicle (GV); germinal vesicle breakdown (GVB); metaphase-I (M-I); metaphase-II (M-II); degenerated (Dg.) (Wani *et al.*, 2000; Hoque *et al.*, 2011).

Table4. Microscopic evaluation of COCs after 22h of IVM (Mondal *et al.*,2008)

Grade of COCs	Maturation rate(%)
A	71.70
B	51.52

In goats, the percentage of oocytes reaching metaphase II *in-vitro* was approximately 90% and high fertilization rates have been achieved from *in-vitro* matured oocytes (Younis *et al.*, 1991; De Smedt *et al.*, 1992).

Sperm Collection and Capacitation:

Procedures for sperm capacitation and IVF as described by De Smedt *et al.* (1992). Fresh semen was collected from one male into a Falcon tube using an artificial vagina on the day of IVF and was kept in the dark at 20 °C for 2 to 3 hr. The sperm are given two washings by centrifugation at 1500 rpm for 10 min using TCM-199, supplemented with 4 mg/ml BSA and 50 IU/ml Penicillin. 100 µl of the semen is covered by 2 ml of fertilization medium, composed of TCM-199 supplemented with 4 mg/ml BSA, 50 IU/ml Penicillin and 50 IU/ml. heparin, its pH is adjusted to 7.8 and kept in a 5% CO_2 incubator at 38.5^{o}C, at an angle of 45^{o}(Cognie *et al.*, 2003). The sperm concentration is adjusted to 10 x 10^6 sperm per ml and five µl are added to the fertilization drops containing the oocytes and final sperm concentration is made as, 1 x 10^6 sperm per ml. The sperm concentration was allowed to swim up for 2 h.

Defined medium was modified by addition of 6 mg/ml fatty-acid-free bovine serum albumin (BSA) and 100 µg/ml gentamicin sulphate (mDM) (Brackett & Oliphant, 1975). Percoll solution contained 90% Percoll (v/v) plus the following ingredients: 80 mM NaCl, 3.1 mM KCl, 0.29 mM NaH2PO4, 1.97 mM CaCl2, 0.39 mM MgCl2, 10 mM HEPES, 26 mM

lactic acid, 25 mM NaHCO3. The Percoll gradient was prepared by carefully adding 2 ml 45% Percoll solution (1 ml 90% Percoll solution diluted with 1 ml mDM) onto the top of 2 ml 90% Percoll solution in a 15 ml centrifuge tube. The sperm cells are washed with Percoll gradient. 100µl fresh semen is layered on top of 4 ml Percoll gradient followed by centrifugation at 500*g* for 30 min (Alexander *et al.*, 2010). Sperm capacitation medium containing 0.5mM 8-bromo-cAMP, 100nM ionomycin and 10 µg/ml heparin in 1 ml of mDM. The sperm capacitation mixture was incubated at 39° C for 15 min (B. Bhatia and C. L. Keefer, 2002).

***In-vitro* fertilization of the oocytes:**

In-vitro fertilization which means (fertilization in glass) sperm and oocyte join in a laboratory dish. First goat kid was born using the IVF techniques on ovulated oocytes in goat by Hanada (1985). Younis *et al.* (1991) was reported pregnancy in goats by embryo transfer after IVF of goat oocytes. Crozet *et al.* (1995) produced first goat kid from *in vitro* maturation and fertilization of goat oocytes.

In vitro fertilization of the oocytes is usually carried out following 24 h maturation in the IVM medium. Surrounding cumulus cells are removed by gentle pippetting and washing in fertilization medium and groups of 40-50 oocytes are placed in 4-well dishes in 500 ml of synthetic oviductal fluid (SOF) covered by 200 ml of mineral oil (Cognie *et al.*, 2004,Coppola *et al.*, 2007).

The matured oocytes are washed once in fertilization medium and then transferred to 2 ml of fertilization medium. The highly motile spermatozoa from the upper layers are added to the oocytes at the concentration of 1 x 10^6/ml approximately. The mixture of gametes was incubated at 39°C in a humidified atmosphere incubator with 5% CO_2 in air for 18-22 h (Baldassarre, 2003). The oocytes are fixed on slides in acid and alcohol for 24 h, stained with a 1% aceto-orecine stain and examined under high power microscope for the sperm penetration and fertilization. The sperm in perivitalline space or in vitellus, swollen sperm head in ooplasm or male and female pronuclei are observed (Wani *et al.*, 2000). The slides are examined at high magnification (100x) with emersion oil to observe pronuclear (PN) formation as- (i) Oocyte with male and female PN, normal fertilization; (ii) Oocyte with one PN, asynchronous PN development/parthenogenetic activation or one PN was obscured by lipid droplets and (iii) Oocyte with more than two PN, polyspermy (Hoque *et al.*, 2011).

***In-Vitro* Culture of Oocytes:**

During this stage, the newly formed zygote will need to be provided with the conditions necessary to start dividing and grow in the most similar way to what would be expected to be happening in the first few days of pregnancy.

There are several methods of in vitro culture of oocytes (Cognie *et al.*, 2003)

a. In vivo – fertilized oocytes are placed in the oviduct of a synchronized female and grown for 6-8 days;

b. Co-culture – fertilized oocytes are placed in culture drops containing cells from the oviduct to try to mimic the maternal environment;

c. Semi-defined medim – fertilized oocytes are placed in culture drops containing serum. It is called semi-defined medium because the composition of the serum is variable and usually unknown. This is the most common method of in vitro culture of oocytes in small ruminants;

d. Defined medium – fertilized oocytes are placed in culture drops where concentrations of all the components are known, including growth factors;

e. Sequential culture – the embryonic development needs change as it grows.

The idea of sequential culture is to place embryos in culture medium containing the nutrients necessary for one specific stage of development, mimicking the maternal environment.

Following *in-vitro* fertilization, embryos are washed several times and then placed in *in-vitro* culture (IVC) media for further development. The culture media play an important role in the development of *in-vitro* fertilized embryos. The most common culture media used *in-vitro* culture of oocytes was SOF (Synthetic Oviduct Fluid). These media contain items such as essential and non essential amino acids, antibiotics supplemented with BSA (Hoshi, 2003; Baldassarre, 2003, Coppola *et al.*, 2007). After 25 hours incubation, the fertilized ova are taken from the semen drops by using the appropriate micropipette. After that they are washed three times in pre-incubated medium (TCM-199) and are transferred to culture media SOF. The SOF (Synthetic Oviduct Fluid) consists of, Tissue Culture Medium-199 (TCM-199) supplemented with 2.5% Bovine serum albumin (BSA) + 10% goat follicular fluid (gFF). Embryos are cultured under paraffin oil and a humidified 5% CO_2 &, 5% O_2, 90% N_2, atmosphere at 38.5^{o}C. Embryonic development was monitored and 50% of the culture volume was replaced with fresh medium at 24-h intervals. Ova that did not progress to the next cleavage stage are removed from drops containing developing embryos at the time of

each change of medium. The development was checked every 48 hrs and the culture are continued for 6 to 7 days. Proportions of inseminated oocytes reaching the 2- to 4 cell stage are recorded at 48 h; morulae are recorded at 120 h, blastocysts at 168 h, and expanded blastocysts at 216 h post insemination, under 100x of magnification of microscope.

Table5. Rate of development of embryos after 7 days of IVC (Mondal *et al.*, 2008)

Number of zygote	**Development of embryos**	
	Number of morula(%)	Number of blastocysts(%)
Grade A	25.64	12.82
Grade B	6.89	3.45

Conclusion:

Recently, Laproscopic ovum pick-up and Transvaginal ultrasound guided aspiration are useful in the recovery of oocytes. The oocytes obtained from the slaughter animal can also be utilized effectively for the production of embryos and reducing the generation interval. The *in-vitro* embryo production helps in supplying the high quality embryos for the transgenic and chimera animal production. Hence, more number of good quality goats can be produced by in vitro embryo production.

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