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Understanding mechanism of *in vitro* maturation, fertilization and culture of sheep embryoes through *in silico* analysis

Dulam Sreenivas[1], Dowluru SVGK Kaladhar[2] *, A Palni Samy[3] & R Sangeeth Kumar[3]

[1]Chennai Fertility Centre, Chennai, [2]GITAM University, Visakhapatnam, [3]Dept of animal Biotechnology, Tamilnadu Veterinary, and Animal Sciences University, Chennai; Dowluru SVGK Kaladhar – Email: dr.dowluru@gmail.com; *Corresponding author

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Abstract:
Protein interations are presently required to understand the mechanisms of *in vitro* maturation, fertilization and culture of sheep embryoes through *in silico* analysis. The present work has been conducted on TCM-199 supplemented with epidermal growth factor (EGF), fetal bovine serum (FBS) or wheat peptones The maturation rate of oocyte was significantly higher in the FBS supplemented group when compared with BSA and wheat peptone supplemented groups. The *in silico* protein interaction studies has shown that the proteins EGFR (epidermal growth factor receptor), CCK (cholecystokinin)- a peptide hormone, Alb – a serum albumin, ESR- estrogen receptor 1, TGFA- transforming growth factor, STAT- signal transducer and FN1- fibronectin 1 has direct interaction and produces cell growth in *in vitro* culture. Alb is directly activates EGF and promotes MAPK3 that mediates diverse biological functions such as cell growth, adhesion and proliferation. Alb may also involve in stress response signalling and may be in cell cycle control.

Key words: *in vitro* embryo development, sheep, protein interaction

Background:
Protein - protein interaction network approaches in better understanding of protein function was initiated in the present approach, starting with defined biological processes. Most proteins interact with few partners, whereas a small but significant proportion of proteins, those hubs, interact with many partners **[1].** The interaction between hormone and derived factors such as kit ligand and oocyte secreted is essential for oocyte growth. As elementary constituents of cellular protein complexes and pathways, protein–protein interactions are key determinants of protein function **[2].** Thomas et al., 2008 proposed several target genes contained cAMP response elements (CREs), serum response elements (SREs), activator protein 1 (AP1) elements and GC-rich regions, but otherwise no common regulatory promoter element could be identified **[3].** Peptide hormones exert unique actions via specific G protein–coupled receptors; however, the therapeutic potential of regulatory peptides is frequently compromised by rapid enzymatic inactivation and clearance from the circulation **[4].** *In vitro* maturation (IVM) is one of the essential steps in the *in vitro* fertilization (IVF) process. In most of the experiments maturation media were supplemented with fetal bovine serum (FBS) **[5, 6],** horse serum (HS) or porcine serum albumin (PSA) **[6]** and albumin **[7]** as protein source and glucose/amino acids as an energy source **[8]** for embryo development Hormone supplementation varies in experiments as per the usage of these supplements.

Methodology:
Collection and transportation of ovaries
Morphologically normal ovaries from sheep, irrespective of age and body condition were collected immediately after slaughter from Chennai corporation abattoir. The ovaries were transported to the laboratory in a thermos flask containing normal saline maintained at 37°C and supplemented with 10µl/ml penicillin-streptomycin. Sheep oocytes were aspirated

from ovaries collected from a commercial slaughter house during the breeding and non-breeding seasons.

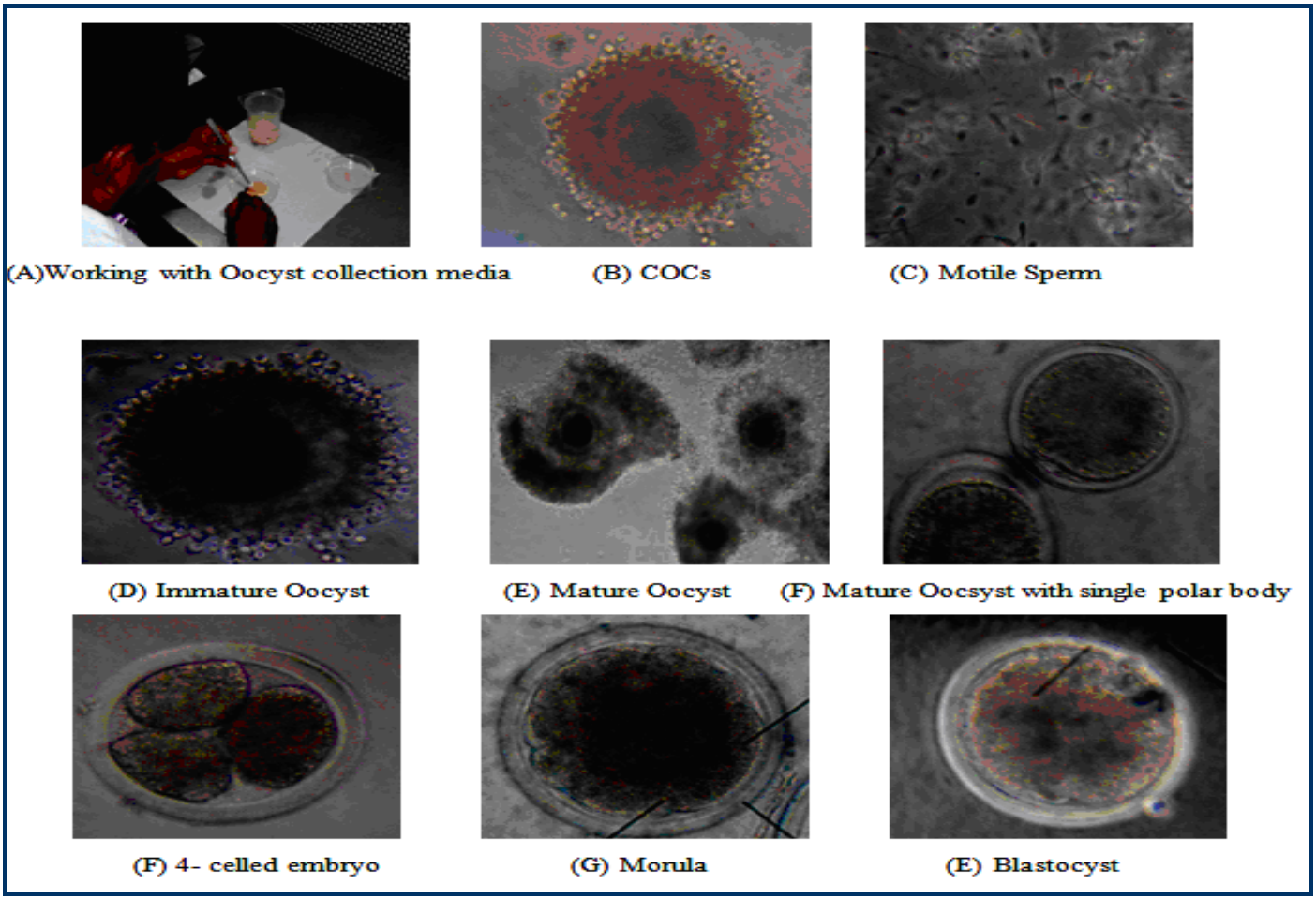

Figure 1: In vitro Embryo development of sheep

Retrieval of oocytes

The extra ovarian tissues were trimmed off and the ovaries were washed thoroughly under running tap water and rinsed five times in normal saline. Thereafter, the ovaries were kept in a sterile beaker containing normal saline at 37°C. Oocytes were retrieved by slicing technique as described by Wani *et al*. (2000). The cumulus oocyte complexes (COCs) were screened using a stereo zoom microscope and transferred into a 35 mm petridish containing fresh oocyte collection medium (TCM-199).

Effect of various protein supplements on in vitro maturation, fertilization and subsequent embryo development

This experiment was designed to evaluate animal protein supplements and its replacement with plant peptones during *in vitro* maturation, fertilization and subsequent embryo development of ovine oocytes. In each group six replicates were carried out.

Group I	10% FBS in maturation, fertilization and culture media
Group II	5mg/ml BSA-FAF in maturation, fertilization and culture media
Group III	0.18mg/ml wheat peptones in maturation, fertilization and culture media

***In vitro* maturation of oocytes**

The *in vitro* maturation (IVM) medium was TCM-199 supplemented with 1 µg/ml of follitropin (FSH), 0.02 IU/ml of luteinizing hormone (LH), 1 µg/ml of estradiol, 100 ng/ml epidermal growth factor (EGF) and 10 µl/ml penicillin-streptomycin. 10 per cent fetal bovine serum (FBS), 5 mg/ml bovine serum albumin (BSA) and 0.18 mg/ml wheat peptones were supplemented separately in maturation medium to assess the maturation rate. 50 µl IVM droplets were made in a 35 mm Petri dish and overlaid with mineral oil and pre-equilibrated in a CO_2 incubator for a minimum of 2 h at 38.5°C under 5 per cent CO_2. The graded and selected oocytes were washed four times in maturation medium and 10 COCs were transferred to each droplet and allowed to mature at 38.5°C in a humidified atmosphere under 5 per cent CO_2 for 24 h on a static platform.

Assessment of maturation of oocytes

Maturation was assessed based on the cumulus cell expansion by examination of oocytes under stereo zoom microscope.

Protein Interaction studies

To find out the protein interactions preliminarily, an interaction profile studies and experimental graphs has been studied using string database. The present work is conducted on STRING (version 9.0) and SignalLink databases for extracting protein-

protein interaction data. STRING is a database of experimentally known and predicted protein-protein interactions (Kaladhar *et al* 2012). The interactions will include direct (physical) and indirect (functional) associations derived from four sources: (1) genomic context; (2) high-throughput experiments; (3) co-expression (conserved) and (4) known knowledge. A STRING and SignalLink database quantitatively integrates interaction data from these sources for a large number of organisms, and transfers information between these organisms where applicable.

Results:

The result shows the different steps of *in vitro* production of sheep embryos up to the blastocyst stage in semidefined conditions: (1) oocyte maturation, (2) *in vitro* fertilization, and (3) *in vitro* development. The result of protein supplements are also be observed based on interactions using *in silico* approaches. There was no difference in efficiency of IVM of oocytes collected during the breeding and non-breeding seasons. The number and recovery rate of ovine oocytes following slicing technique are presented in **Table 1 (see supplementary material)**. Using slicing technique, a total of 1,407 oocytes were recovered from 341 sheep ovaries with an average yield of 4.13 oocytes per ovary. The percentage of different grades of oocytes recovered by slicing technique was presented in **(Figure 1)**. Among the total of 1,407 oocytes, 546(38.81%), 648(46.06%) and 213(15.14%) ooytes were classified as grade A, B and C, respectively.

Influence of protein supplements on maturation rate of ovine oocytes based on cumulus expansion

The effect of animal protein supplements and its replacement with plant peptones during IVM of ovine oocytes is presented in **Table 2 (see supplementary material).** In FBS supplemented group (group I), out of 206 oocytes cultured, 184 oocytes matured with a mean maturation percentage of 89.32±1.67. In BSA supplemented group (group II), out of 196 oocytes cultured, 157 oocytes matured with a mean maturation percentage of 80.10±1.64. In wheat peptone supplemented group (group III), out of 199 oocytes cultured, 108 oocytes matured with a mean maturation percentage of 54.27±1.81. The difference in oocyte maturation rate between groups was highly significant. The maturation rate of Oocyte was significantly higher in the FBS supplemented group when compared with BSA and wheat peptone supplemented groups.

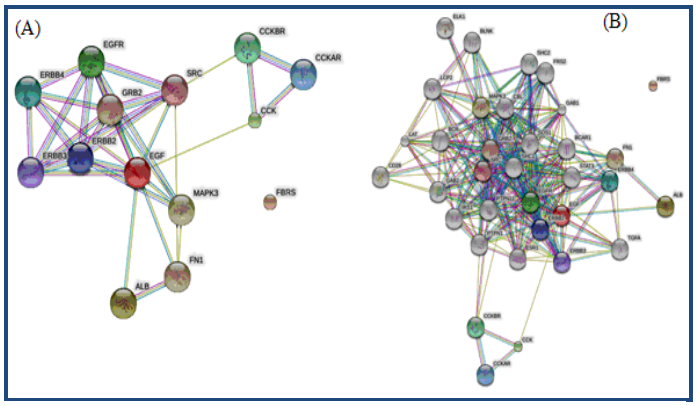

Figure 2: **(A)** Preliminary Search **(B)** Advanced search of protein interactions with EGF, Alb and CCK (Note: EGFR- epidermal growth factor receptor; CCK- cholecystokinin; a peptide hormone; Alb- Serum albumin; ESR- estrogen receptor 1; TGFA- transforming growth factor; STAT- signal transducer; FN1- fibronectin 1)

In silico interaction studies

The observed results has also shown that the proteins EGFR (epidermal growth factor receptor), CCK (cholecystokinin)- a peptide hormone, Alb – a serum albumin, ESR- estrogen receptor 1, TGFA- transforming growth factor, STAT- signal transducer and FN1- fibronectin 1 has direct interaction and produces cell growth in *in vitro* culture **(Figure 2 & 3).** Alb is directly activates EGF and promotes MAPK3 that mediates diverse biological functions such as cell growth, adhesion and proliferation. Alb may also involve in stress response signalling and may be in cell cycle control.

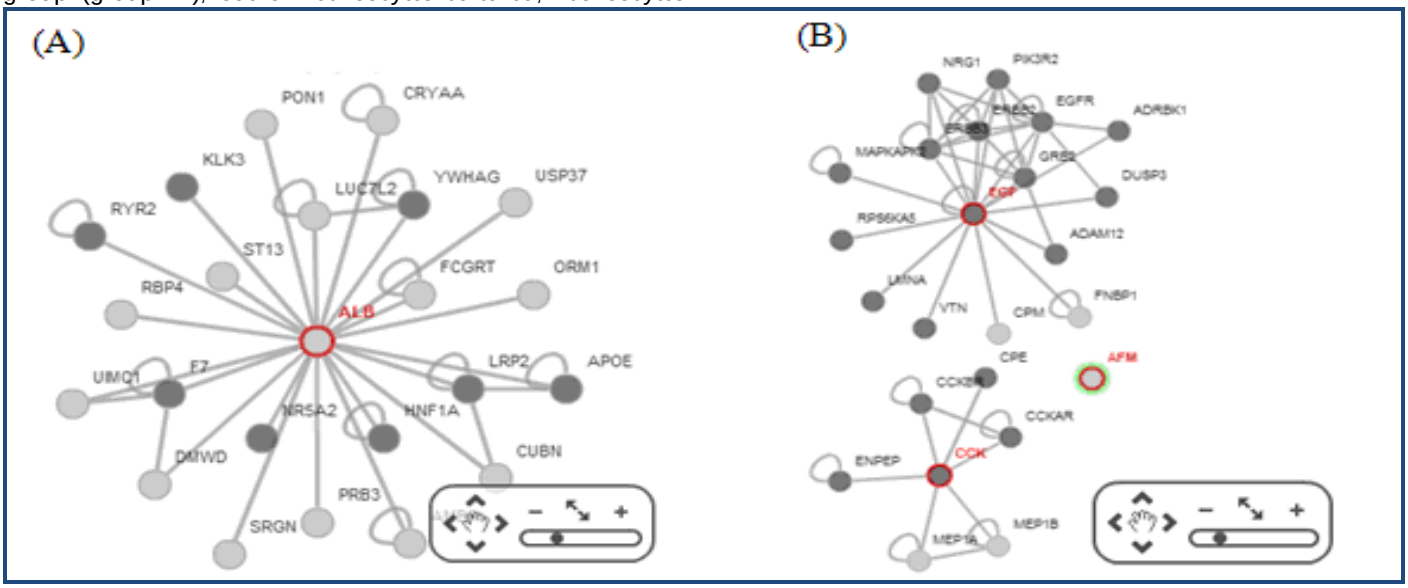

Figure 3: (A) Preliminary search**; (B)** Advance search**.** Pathwaylinker has also been shown the neighbor interaction of serum albumin with proteins such as Alb, CCK, AFM and EGF.

Discussion:

Rao *et al.,* 2002 has also proposed that there was no difference in efficiency of oocytes of IVM collected during the breeding and non-breeding seasons **[9].** IVM/IVF capability is associated with reduced animal numbers, reduced animal welfare concerns and reduced financial costs. Nuclear transfer cloning and most other reproductive biotechnologies are established in the pig and can generate close anatomical, physiological and biochemical parameters using *in silico* approaches **[10].** The widespread use of large mammal models in respiratory research, coupled to the limited availability by *in silico* methods can provide an opportunity for identification probes with detectable hybridization signal using probe level analysis **[11].** The combination of cellular, *in vivo*, and *in silico* modeling promises the eventual creation of a heterogeneous population of virtual patients for understanding the pathogenesis of this chronic inflammatory disease **[12].** Maternal MATER, ZAR1, GDF9, and BMP15 transcripts persisted during oocyte by *in vitro* maturation and fertilization and in preimplantation embryo, until the five- to eight-cell or morula stage, but transcription was not reactivated at the time of embryonic genome activation **[13].**

Recent progress towards understanding biological processes such as sperm maturation and fertilization indicates that the paternal contribution has been underestimated **[14].** Blastocysts derived from *in vitro* maturation, fertilization, and embryo culture protocols undergo apoptosis but that apoptotic levels are not greatly influenced by the oocyte maturation environment. EGF supplementation of oocyte maturation medium resulted in a concentration-dependent has increase in blastocyst development but did not influence blastocyst total cell number or apoptosis **[15].** According to Calder *et al*. 2005, a relative abundance of COC mRNAs is altered by serum in the maturation medium, which may signify long-term consequences for embryonic development **[16].** Endogenous c-Abl mediates endothelial apoptosis **[17]** and BSA significantly stimulated and accelerated development to the blastocyst and expanded blastocyst stages **[18].**

Conclusion:

The present research work has performed the embryo development of sheep through *in vitro* method. Various protein supplements are essential for cellular modifications and growth in various living systems. The present approaches are predicted need of protein supplements using protein interaction networks, that supports the interactions with cellular proliferation and MAPK networks.

Competing interests:

The authors declare that they have no competing interests.

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Supplementary material:

Table 1: Recovery rate of ovine oocytes by slicing technique

No. of ovaries sliced	No. of Oocytes retrieved	Recovery rate per ovary
341	1407	4.13

Table 2: Mean ± S.E. percentage of maturation rate of ovine oocytes in media supplemented with various proteins

Supplement	No. of replicates	No. of oocytes cultured	No. of oocytes matured	Maturation rate (mean% ± S.E.)
Fetal bovine serum (Group I)	6	206	184	89.32±1.67
Bovine serum albumin (Group II)	6	196	157	80.10±1.64
Wheat peptone (Group III)	6	199	108	54.27±1.81