International Journal of Cell Science and Biotechnology
E-ISSN 2320-7574
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Original Research Article

Isolation, Culturing and Cryopreservation of Putative Granulosa Stem Cells from Buffalo Ovaries

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Received 15 Aug 2015, Accepted 20 Sept 2015, Available online 11 Oct 2015, **Vol.4 (2015)**

Abstract

The current study was carried out to isolate, culture, characterize and cryopreserve the granulosa stem cells from buffalo ovaries. Granulosa stem cells were cultured in DMEM containing15% FBS, 1% penicillin/ streptomycin and 1%L-glutamine in 5% CO2 with humidified air at 38.5±0.5°C. After 3 days of culture different morphologies of cells were observed. The confluent monolayer should have been formed at the base of the droplet after 72 hrs of culture. The culture was harvested and cultured in a culture media. Followed by cryopreservation of the granulosa stem cells. This result may contribute towards establishing further studies of granulosa stem cells for various therapeutic and reproductive biotechnological applications.

Keywords: *Buffalo, Granulosa Stem Cells, Confluent Monolayer, Characterization and Reproductive Biotechnology*

1. Introduction

A granulosa cell or follicular cell is a somatic cell of the sex cord that is closely associated with the developing female gamete (called an oocyte or egg), in the ovary of mammals. In the primary ovarian follicle, and in later in follicular development (folliculogenesis), granulosa cells advance to form a multi-layered cumulus oophorous surrounding the oocyte in the preovaulatory or antral (or graffian) follicle.

Ovarian follicles begin their development as primordial structures, which consist of an oocyte arrested at the diplotene stage of the first meiotic division, surrounded by a few flattened granulosa cells. In the buffalos the follicle formation occurs during fetal period. Once the follicle reaches a species-specific size, it forms a fluid-filled space called an antrum. When this stage has been reached, follicles become acutely dependent on gonadotropins for further growth and development. The growth phase of the oocyte occurs during the preantral stage it is during this time that development of the zona pellucida occurs, as well as production of mRNA and proteins required for subsequent fertilization and early embryonic development. These factors must be stored within the oocyte, as resumption of meiosis results in transcriptional silencing. Oocyte developmental competence, defined as the ability of the oocyte to resume and complete meiosis, and support pre-implantation embryonic development after fertilization, is acquired gradually during folliculogenesis. Development of the ovarian follicle requires coordination of the processes of somatic cell proliferation and differentiation with oocyte growth and maturation. Paracrine interactions between the oocyte and surrounding granulosa cells are critical for ensuring this coordination by promoting integrated cellular functions. To the best of our knowledge, no work has been carried out on studies of these cells. Looking at scarcity of the information and usefulness of the granulosa stemcells the current study was designed.

2. Materials and Method

2.1 Chemicals and Media

All chemicals i.e. reagents, culture media and antibiotics used during the study were culture grade, obtained from Hi Media Laboratories, T-25 flask, 6 well tissue culture plates, and centrifuge tubes were procured from Tarsons product Pvt. Ltd. Membrane filters were from Advanced Micro devices. The culture media were reconstituted freshly as per instructions and filter-sterilized (a 0.22µm) prior to use.

2.2 Collection of sample

Buffalo ovaries were collected within 10 minutes after the slaughter of the animals, and kept in a sealed

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DOI: https://doi.org/10.14741/ijcsb/v.4.1.3

container containing physiological saline (0.9% NaC1 with 100 µm/ml Streptomycin and 100 IU/ml Penicillin), at a temperature ranging from 32-37°C. These ovaries were then placed in thermos flask and transported to the laboratory within 3 hrs.

2.3 Trimming of ovaries

The collected ovaries were washed several times with tap water so that the contamination is removed. These ovaries were then subjected from trimming by removing the extra tissues such as fallopian tubes and fimbriae. The trimmed ovaries were then kept in saline water (500 ml) and added with antibiotic solution in laminar air flow. Finally stored in refrigerator

2.4 Isolation of follicular fluid

The ovaries were dried lightly with sterile paper towels before follicular fluids are recovered from 2-8 mm vesicular follicles to avoid contaminating the aspirates during follicular fluid retrieval. It is important to remove the big follicles (beyond 9 mm) because they contain secretions that cause jelly formation in the aspirates. This may affect the retrieval of granulosa cells during centrifugation.

2.5 Procedure

Aspiration of follicular fluid was done using a needle attached to a 10-ml syringe. To avoid disruption of the surrounding cells, an 18-gauge needle was used. To minimize the loss of cells being punctured, it is necessary to prime the needle and syringe with approximately 0.25-0.5 ml of aspiration medium. This helps to ensure the retrieval of the follicular contents. After aspiration, the contents of the syringe were slowly dispelled into a sterile centrifuge or test tube with minimum disruption of the cumulus-oocyte complex (COC). Once the last ovary of a particular batch is processed, the granulosa cells were allowed to settle to the bottom of the tube for at least 5 minutes. The precipitate was taken using a sterilized micropipette. Sometimes the ovaries are sliced in order to retrieve granulosa cells. This method enhances the retrieval of a larger number of cells. But this method requires a longer period of retrieval and is not advisable when manipulating large number of ovaries. The third method used for slaughtered animals is follicle dissection. This method is also effective in retrieving good-quality follicular cells but, it also increases the time needed for the retrieval process.

The follicular fluid is searched for the oocytes. Once located in the Petri dish, oocytes were lifted in a sterile glass pipette with a bore diameter of about 400 µm and transferred into a dish of fresh pre-warmed washing medium (this may be the same as the aspiration medium). The glass pipette used must have a bore diameter wide enough to avoid disruption of the cumulus cells surrounding the oocytes. The cumulus cells surrounding the immature oocytes are required for successful in vitro, so they must be protected during oocyte retrieval.

2.6 Grading of oocyte

The collected oocytes were graded on the basis of their morphology as described below:

Grade A: Compact Cumulus Oocyte Complexes (COCs) with an unexpanded cumulus mass having ≥4 layers of cumulus cells, and with homogenous evenly granular ooplasm.

Grade B: COCs with 2-3 layers of cumulus cells and a homogenous evenly granular ooplasm.

Grade C: Oocytes partially or wholly denuded or with expanded or scattered cumulus cells or with an irregular and dark ooplasm.

Grade D: Oocytes without cumulus cells and with an irregular cytoplasm

2.7 Culturing of granulosa cells

Granulosa cells were collected from the aspiration medium from which the oocytes have been removed. The aspiration medium containing follicular fluid, granulosa cells and follicular debris is washed five times and centrifugation for 1 min at 1000 rpm. The final pellet of granulosa cells was suspended in DMEM medium supplemented with 15% FBS, antibiotic solution and L-Glutamine. The medium with granulosa cell were then passed 2 times through a 0.22 µm syringe filter attached to a 10 ml syringe to avoid contamination. The granulosa cells obtained were cultured in 15 ml of culture media (DMEM+15% FBS+ antibiotic solution+L-Glutamine) placed in three different T-25 flasks. The T-25 flasks are then placed in a CO2 incubator at 38.5°C, 5% CO2 for 72 hrs. The confluent monolayer should have been formed at the base of the droplet after 72 hrs of culture.

2.8 Cryopreservation of granulosa cells

Cryopreservation is a technique used to preserve specific tissue /organ for future use and long distance transportation. The freezing of granulosa cells has become a routine procedure. Cryopreservation is of two types:

2.8.1 Slow Freezing

It continuous cooling at very slow rate with help of freezing machines.

15% DMSO + 85% DMEM + granulosa cell

Method

Granulosa cells were taken in 500 µl DMSO and 4.5 ml DMEM and stored at -4°C for one day in refrigerator. The test tube containing cell was then removed from the refrigerator and transferred to deep freezer at -20°C for two days. This tube was then transferred at a lower temperature i.e. at -80°C.Test tube having cells are finally transferred to the cryocan containing liquid nitrogen at temperature -196°C.

2.8.2 Vitrification

Method consists of cooling a cell suspension to liquid nitrogen temperature without the formation of ice. Water is the primary constituent of biological fluids and is responsible for internal transport of essential fluids. The movement of water across the cell membranes help in determining whether the cell survives during freezing and thawing. As water in solution is a frozen, pure water forms crystal, leaving behind concentrations of other substances in solution which increases the osmotic pressure of remaining solute, thus damaging the cells. These events and their effects can be influenced by the levels and types of cryoprotectants and freezing rates

Cryoprotectants are divided into two groups

2.8.3 Permeating cryoprotectants

It has the ability to reduce the amount of water which freezes as ice at any sub-zero temperature. E.g. glycerol, dimethylsulphoxide (DMSO) etc.

2.8.4 Non-Permeating cryoprotectants:

Low molecular weight

It does not enter the cells before freezing but exerts effect by dehydrating the cells i.e. changes the water balance of cells by shrinking the cells before freezing. E.g. Sucrose.

High molecular weight

It closes the cell membrane defects that may occur during cryopreservation procedures and/or help repair damaged cell membrane post thawing.

2.8.5 Freezing rates

At slow freezing rates increased concentrations of salt and solute in the medium and cell causes injury to the cell (solution effect). At rapid freezing rates formation of intracellular ice causes injury to the cell. If concentration of cryoprotectant is low, fast freezing must be applied to minimise the exposure to high concentration of salts and other solutes. Raising the concentration of cryoprotectant has the effect of lowering the optimal freeing rate.

2.8.6 Seeding (induction of ice crystallization)

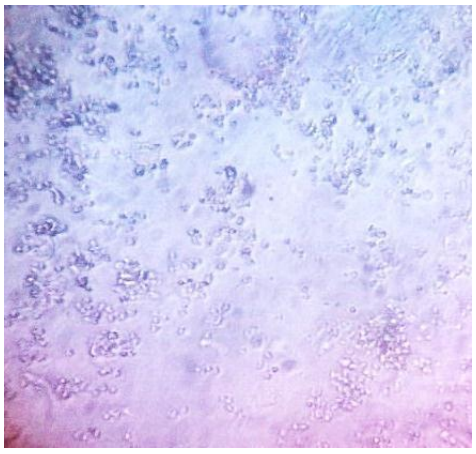
When any solution freezes it releases its latent heat of fusion at the temperature at which it freezes. During this the temperature in the solution rapidly rises as much as 30°C and rapidly falls to seeding temperature this rapid change in temperature is detrimental to embryos. By seeding, this heat of fusion of is overridden driving the temperature down at the precise temperature at which it would otherwise go up. Thus ice formation is induced without resulting increase in temperature.

2.9 Thawing

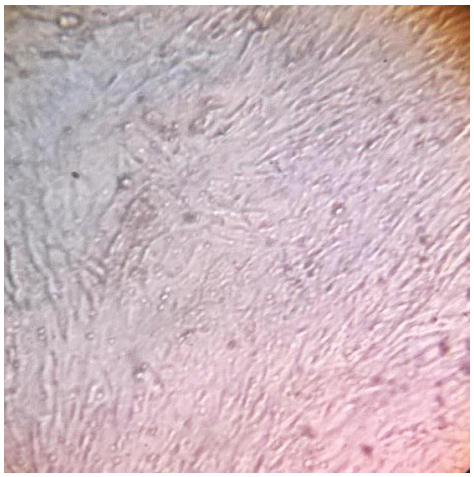
It is a rehydration process, applied to granulose stem cells according to size of cell frozen and volume reduction (dehydration) before transfer to liquid nitrogen. Cryoprotectant can reduce injury from solution effects but can themselves cause cellular injury by osmotic trauma, therefore the cryoprotectant is added and removed from the freezing medium in small amounts. Cryoprotectants are usually removed in stepwise manner similar to addition.

3. Results

After isolation the medium containing the granulosa cells was observed in inverted microscope. They are round shaped on the second day of growth period (a). The confluent monolayer should have been formed at the base of the droplet after third day of culture. At the fourth day of growth period, the round shaped cells were converted as cells having fibroblast like appearance (b).Finally the cells are harvested and cultured on a culture media. The granulosa cells are preserved by two methods, they are by vitrification and slow freezing. Finally they are stored successfully at -196 C in liquid nitrogen in a healthy condition.



(a)



(b)

Fig.1 (a) Round Granulosa cells at the second day of growth period. (b)Fibroblast cells are formed at the fourth day of growth period

4. Discussion

The major functions of granulosa cells include the production of sex steroids, as well as myriad growth factors thought to interact with the oocyte during its development. The sex steroid production consists of follicle-stimulating hormone (FSH) stimulating granulosa cells to convert androgens (coming from the thecal cells) to estradiol by aromatase during the follicular phase of the menstrual cycle. However, after ovulation the granulosa cells turn into granulosa lutein cells that produce progesterone. The progesterone may maintain a potential pregnancy and causes production of thick cervical mucus that inhibits sperm entry into the uterus. So the present study is a preliminary effort to investigate the granulose stem cells in buffalo.

However, cryopreservation of granulosa cells is used to prevent the damage caused by chemical activity. At low enough temperatures, any enzymatic or chemical activity which might cause damage to the material in question is effectively stopped. Cryopreservation methods seek to reach low temperatures without causing additional damage caused by the formation of ice during freezing it enable stocks of cells to be stored to prevent the need to have all cell lines in culture at all times. It is used to reduced risk of microbial contamination, cross contamination with other cell lines, genetic drift and morphological. The presence of cryoprotectants in freezing solution is necessary to prevent the cell damage during freezing and thawing. The cryoprotectants are DMSO, ethylene glycol, propylene glycol.

The DMSO is the intracellular agents, which penetrate inside the cells preventing ice crystals formation and membrane rupture (i.e., dimethyl sulfoxide (Me_2SO), glycerol, and ethylene glycol) and extracellular compounds that do not penetrate in cell membrane and act by reducing the hyperosmotic effect present in freezing procedure. Among them are sucrose, trehalose, dextrose, and polyvinylpyrrolidone. The main commonly used cryoprotectant, dimethylsulfoxide, provides a high rate of post freezing cell survival. DMSO penetrate inside the cell preventing the formation of ice crystals that could result in membrane rupture. There are few alternative cryoprotectants to DMSO use at the moment. Other substances such as glycerol, sucrose, or trehalose do not present cytotoxicity but still need to be more evaluated as cryoprotectants for granulosa stem cells.

Ethylene glycol (EG) has been successfully used a cryoprotectant for vitrification of granulosa cells due to its low formula weight and high permeation into cells compared with other cryoprotectants, including propylene glycol. They osmotically replace the intracellular water before cooling and especially during slow controlled cooling, reduce changes in the volume of the cell and prevent formation of intracellular ice. They are also thought to stabilize the intracellular proteins.

Moreover additives are also used in cryoprotectant solutions. Additives are compounds of various sizes that by themselves do not protect the cell from freezing damage but make the cell membrane more elastic, preventing effect of freeze damage to the cell, are used as supplements to cryoprotectant solution. This include sugars, other high molecular weight compounds, and proteins.

Sugars are polyhydroxyl aldehydes or ketones (carbon chains with terminals aldehydes for ketones and hydroxyl side chains). High levels of sugars and sugar alcohols are found in many polar plants, insects, fungi, and so on as non-toxic cryoprotectants. Various saccharides that have been used as supplements include mono-, di-, and trisaccharides. The monosaccharides used as cryoprotectants include fructose, glucose, and galactose, whereas the disaccharides include sucrose, trehalose, lactose, and trisaccharides include raffinose. Trehalose has been reported to be very effective as a cryoprotectant supplement for vitrification of oocytes. Sucrose is the most common sugar found in freeze- tolerant plants, some of which can increase their sucrose level ten-fold in response to low temperatures. Sucrose and trehalose inhibit the membrane mixing associated with chilling. Both sugars fit well in the cell membranes, binding to phospholipid head groups.

Proteins can also be used as supplements to cryoprotectant solutions. Examples include bovine serum albumin or foetal bovine serum. Another less common type of cryoprotectant supplement is thermal hysteresis proteins (THPs).

Large molecular weight compounds include PVP, hydroxylethyl starch, Ficoll, and polyethylene glycol (PEG). Although this compounds usually not protect the cells against freezing damage when used alone in solution, they will often enhances protective effect of

other low molecular weight cryoprotectants. PEG has also been used to supplement CPA solutions.

To the best of our knowledge, this is the first report on isolation, culturing and cryopreservation of putative granulosa stem cells. Further, their cauterization needs to be studied by stem cell specific markers using RT-PCR or western blotting.

Conclusion

It has been observed that buffalo granulosa stem cells were able to grow in invitro conditions. The granulosa stem cells which were initially round in structures, after 3 to 4 days of culture invitro. After cryopreservation the cells had almost 70% viability rate. The granulosa stem cells of buffalo can be cryopreserved successfully and can be used later on. These granulosa stem cells can be used in various purposes in future for various investigations. However, use of granulosastem cells in buffalo is therapeutic and assisted reproductive biotechnology needs further studies.

Acknowledgement

The authors are greatly thankful to Institute of Advanced Bio-Med sciences, kurukshetra, (Haryana) India for providing necessary facilities to carry out this work.

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