수의학석사학위논문

Development of In vitro Maturation Protocol for Rat Oocytes

래트 난모세포에 대한 시험 관내 성숙 프로토콜의 개발

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수의학과 수의산과·생물공학 전공

Ailia Muhammad Joan

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지도교수 장 구

이 논문을 수의학 석사학위논문으로 제출함

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부위원장 장 구 (인)

위 원 구 옥 재 (인)

Development of In vitro Maturation Protocol for Rat Oocytes

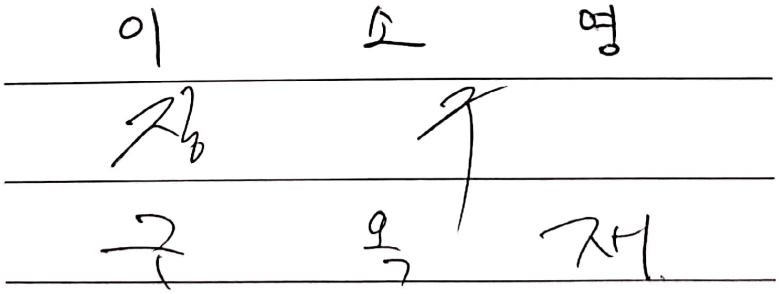
by Ailia Muhammad Joan

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF SCIENCE

in

Theriogenology and Biotechnology
Department of Veterinary Medicine, Graduate School
Seoul National University

We accept this thesis as confirming to the required standard



Seoul National University
June 2020

Declaration

This thesis is submitted by the undersigned for examination for the degree of Master of Science to the Seoul National University. This thesis has not been submitted for the purposes of obtaining any other degree or qualification from any other academic institution.

I hereby declare that the composition and experiment of this thesis and the work presented in it are entirely my own.

Ailia, Muhammad Joan

Abstract

Development of In vitro maturation Protocol in Rat Oocytes

Ailia, Muhammad Joan

Theriogenology and Biotechnology

The Graduate School

Seoul National University

Laboratory animals are an essential part of medical research and with emerging need to develop new medicines, vaccines, medical products need for laboratory animals increasing vastly. Initially, mouse models were developed and used majorly as their resemblance to human physiology and small size provided advantage of easy management. So, one can see very diverse and well-developed research on mouse lab models over the past 3 to 4 decades. But mouse models have limitations as well because of small size and short life span. Which made biologists rethink and go back to long-ignored rat models. Rats are excellent models because of their large size compared to the mouse so sampling, handling, and procedure performance are easy. Thus, rats are vastly used for cardiovascular, neurobiology, immunology, toxicology, physiology, pharmacology, nutrition, behavior, etc. studies. Increase demand for the rat model caused more focus on assisted reproduction technique studies in rats leading to many researchers finding new ways to increase production. Until now the major source of genetically modified rats' production is in vivo COC's

(cumulus-oocyte complex) and embryos. In this study, I tried to develop an in vitro maturation protocol for rat COC's and performed (Superovulation, in vitro Maturation, Parthenogenetic activation, Staining.)

First, Superovulation is an essential part as it enables us to obtain the maximum number of high qualities COC's. The most prevalent way of superovulation induction is using hormones equine chorionic gonadotropin and human chorionic gonadotropin.

Secondly, for in vitro maturation rats were anesthetized, and ovarian samples were collected surgically. Which is then first washed in PBS and COCs were obtained via crushing of ovaries using needle or scalpel blade. COC with a minimum of 3 layers of cumulus cells were selected and then washed in M2 media and cultured in prior made maturation media TCM 199 (Group A), TCM 199 Cumulus cell co-culture (Group B) for 24 hours. After 24 hours COC was analyzed and observed under the microscope.

Further, to assess the quality of in vitro matured COC, they are denuded using hyaluronidase and washed in M2 media. Then activated using different activation agents i.e. ionomycin, ethanol, zinc, etc. activated oocytes were then incubated for 4 hours in 2 mmol DMAP and then transferred in KSOM media for 16 hours. 2 cell embryos were then further incubated till blastocyst formation in MR1ECM media.

Lastly, to check the quality of blastocysts and activated oocytes Hoechst staining is performed. Blastocysts were washed, stained, and fixed on the slide and observed under a microscope. The number of cells was counted.

In conclusion, I tried to develop an in vitro maturation protocol with maximum efficacy and growth potential, I proved that oocyte cumulus monolayer co-culture is much better than simple culture, I also understand that oocyte maturation requires

many factors to develop properly and inter-cell communication, redox status, and cytoplasmic maturation are important for successful maturation of oocytes.

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Keywords: Ovaries, Oocytes, In vitro maturation, Parthenogenesis

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TABLE OF CONTENTS

LIST OF TABLES AND GRAPHS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ART	**Assisted reproduction technology**
COC	**cumulus–oocyte complex**
DMSO	**Dimethyl sulfoxide**
DNA	**Deoxyribonucleic acid**
eCG	**Equine chorionic gonadotropin**
ESC	**Embryonic stem cell**
FSH	**Follicle stimulating hormone**
h	**Hours**
hCG	**Human chorionic gonadotropin**
IP	**Intraperitoneal injection**
IV	**Intravenous injection**
N.B.	**Nota bene (Note well)**
RNA	**Ribonucleic acid**
SCNT	**Somatic cell nuclear transfer**
SD	**Sprague dawley**
WT	**Wild Type**

PUBLICATION LISTS

PUBLICATION PAPER

1. **Muhammad Joan Ailia**[1], Yun-Kyong Jin[2], Hee-Kyoung Kim[2], Goo Jang[1-3]: Development of In-vitro maturation protocol for rat oocytes, BMC Veterinary Research 2020 (Submitted)

ABSTRACTS and PRESENTATIONS

1. Bae H-S, Jin Y-K, Kim K-M, **Muhammad A-J**, Koo O-J, Lee J-Y, Jang G: Production of genome-edited rats by electroporation. The Korean Society of Animal Reproduction and Biotechnology 2017.
2. Jin Y-K, Kim H-K, Kim K-M, **Muhammad A-J**, Lee J-H, Koo S-H, Jang G: Generation of the transgenic rat by Sleeping Beauty Transposon System. Korean Society of Animal Reproduction and Biotechnology 2018
3. Jin Y-K, Kim H-K, Kim K-M, **Muhammad A-J**, Koo S-H, Jang G: Transgenic rat mediated by sleeping beauty transposon and development of embryo cryopreservation system. Transgenic Research 2019.

PART I. LITERATURE REVIEW

1. Rat as an ideal laboratory animal model

1.1 Rat as Disease Model

Murine are widely used laboratory animal because of their easy to handle size and fast growth rate. Rat is preferable because of its ideal size as compared to the mouse as well as its physiological more resemblance with humans thus used for many biomedical and genetic research[1]. Rat models have an edge because of its accurate representation of human diseases thus, widely used in studies like hypertension p, atherosclerosis [2], and Huntington disease [3], etc. Not only rat provides accurate pathological data but also rat has widely diverse genomic data [4] which provides a promising area that still needs to be explored, understands, and research. All this provides rat disease models edge over other lab animal models.

1.2 Transgenic Rat Models

With the advancement in genome engineering and diverse genomic data [4] of rat still pending to be explored the creation of humanized rats is one of the possible field areas yet to be studied completely. Since the development of mouse embryonic stem cells (ESC) [5, 6] a rapid advancement in the production of genetically modified mouse models can be seen over the past two decades [7, 8]. Thus, one can see a diverse and vast variety of mouse models available for research to date. But as I discussed earlier rat models have an edge on mouse models because of comparatively easy handling and more accurate physiological and pathological data representation. So, as a vacuum for transgenic rat production developed and need to produce genetically engineered rats increased. Over the years many precise genome modification tools and methods been developed for example; Targeted endonucleases, including zinc-finger nucleases (ZFNs) [9], transcription activator-like effector nucleases (TALENs) [10], and the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated (Cas) system [11].

2. In vitro maturation potential and scope

In vitro Maturation may be the answer to our problems and provides us the opportunity to maximize the potential of transgenic animal production[12] but with ease comes hurdles as well. One of the reasons in vitro maturation is long ignored and researchers still rely on conventional In vivo practices because the quality of IVM oocytes is lower than IVO (In vivo matured) counterparts[13, 14]. Nuclear maturation is evident but cytoplasmic maturation doesn't go that well[15]

To overcome these challenges many biologists tried to understand the physiology of COC maturation in rats and many important suggestions were advised over the past decade. Rat oocytes require special signaling from the cumulus cells, follicular somatic cells and bidirectional communication is necessary for successful maturation[16, 17]. Thus, many scientists tried to mimic in vivo conditions and advised co-culture for the proper maturation of oocytes[18], For example when porcine oocytes were cocultured with oviductal epithelial monolayer both cytoplasmic maturation and blastocyst development rate increased significantly [19] Glutathione is considered one of the major role player in cytoplasmic development[20] as it not only helps in male pronuclear formation but helps oocytes from oxidative stress[21]. Many scientists worked to overcome this challenge by adding a different kind of antioxidant supplements to the growth media among these supplements Cysteamine/Cystine addition to the maturation media or co-culture showed a significant increase rate of blastocyst formation in the IVM oocytes[22]

One of the most popular co-culture is the development of oocyte in cumulus clumps[23, 24]. cumulus and serum played an essential role in normal cytoplasmic maturation and subsequent developmental capability of rat oocytes[25]. Rat oocytes are one of the most difficult to manipulate and develop in vitro because unlike other mammalian species rat oocytes go for spontaneous activation soon after collection from oviduct[26]. Thus, to address the necessity of the development of IVM protocol, I tried to further evaluate and explore the in vitro maturation of rat oocyte

under co-culture, tried to assess their development via parthenogenetic activation using two activation chemicals ionomycin and 7% ethanol.

3. Parthenogenetic Activation

Parthenogenesis is a naturally occurring phenomenon in lower-class species like in amphibians, reptiles, and insects[27]. In mammal's artificial activation is necessary for the development of embryo post intracytoplasmic sperm injection (ICSI) and somatic cell nuclear transfer (SCNT). Also, parthenogenetic activation provides us with a haploid and tetraploid embryo, which can be used to produce parthenogenetic embryonic stem cells [28]. To the date, many parthenogenetic embryonic stem cells are derived e.g. mouse [29, 30], monkey[31], rabbit[32], and humans[33]. Thus, IVM oocytes can be used as a source of parthenogenetic embryos which can lead to the development of parthenogenetic embryonic stem cells.

There are many ways of parthenogenetic activation and can be induced both physically as well as chemically. The key is to mimic the physiology involved during the fertilization process i.e. Conveying Ca^{2+} signals to the metaphase-II arrested oocytes. For that purpose, many different modes of stimuli are being studied for example, Electric stimuli are long used to induce exogenous Ca^{2+} into the cytoplasm of mature oocytes[34]. Also, many chemical activation agents have been studied as well like calcium ionophore, ethanol, and ionomycin they all increase intracellular Ca^{2+}, which leads to activation of mammalian oocytes[35, 36].

Parthenogenetic activation opens a whole world of embryo development, either its SCNT, ISCI, or development of maternal genes only embryonic stem cells. It's a field yet to be explored and exploited. Specifically, concerning rat parthenogenetic activation and embryo development is least understood as well as developed. Thus, in these studies, I tried to study the development potential of In-vitro matured oocytes via parthenogenetic activation using two chemical agents ionomycin and ethanol.

PART II.

GENERAL METHODOLOGY

1. Materials

All reagents were purchased from Sigma-Aldrich Co. LLC. (Missouri, USA) unless otherwise specified.

2. Care and use of animals

SD rats used in this study were purchased by Orient-bio (Republic of Korea). All-female rats aged 3-4 weeks were maintained in 24 ± 2 ºC, 50% humidity, and 12:12 h light-dark cycle. All animal care and experiments were approved by the IACUC (No.160719a-2-7) and performed under the guideline of SNU.

3. COC collection

The rats were injected PMSG and hCG (HCG, Daesung microbiological labs, Gyenggi, Republic of Korea) 150 IU 48 and 16 hrs. prior dissection respectively. Rats were anesthetized using Aflaxan 0.3 ml/ rat. The surgical site is disinfected using ethanol 70%, an incision was given on linea alba, ovaries were retrieved and dissected and stored in pre-warm PBS solution. Later collected ovaries were sliced using a scalpel blade in M2 media. The released oocytes then collected in 4 well plate containing 500 ul M2 solution in each well. Only Germinal Vesicle oocytes which have 3 layers unexpended cumulus layers and balanced cytoplasm were collected. They are washed three times in M2 solution and then incubated in pre-prepared TCM 199 media for maturation.

4. In vitro maturation of oocytes

Oocytes were matured in TCM 199 which was supplemented with; Fetal calf serum 10% volume by volume, estrogen 1 ug/ml, EGF 10 ng/ml, Cysteamine 100uM, Sodium pyruvate 0.45mM, FSH 5 ug/ml. 4 well plate is used as maturation container. In each well 500 ul of TCM 199 plus supplementation is used. Oocytes

collected from ovaries were first washed 3 times in M2 media than one time in TCM 199. After that, they are incubated in above mentioned supplemented TCM 199 for 20 h at 37 °C temp. The quantity of 25-40 oocytes per/well is maintained.

5. Cumulus cell monolayer co-culture

Cumulus cell monolayer co-culture is used to mimic the in vivo physiological conditions. For Cumulus cell monolayer culture. cumulus cells after Denuding of oocytes are recovered in 1 ml Eppendorf tube. Then its Centrifuged for 10 seconds at 5200 RPM then supernatant solution was aspirated and replaced with PBS. The procedure is repeated 3 times to wash cumulus cells. pre-warmed DMEM supplemented with 10% volume by volume Fetal calf serum is used as culture media. Cells have grown until completion of monolayer the media was replaced every few days. Later this monolayer is used to culture freshly obtained oocytes from the ovaries.

6. Oocyte activation and culture

After 20 hours of incubation oocytes, growth and maturation were checked. The Cumulus growth and extrusion of the polar body is preliminary morphological quality determinants. The TCM 199 solution is sucked up using an aspirator and 0.1% hyaluronidase 500 ul is poured in each well to Denude the oocytes. The plate was incubated for 3 min for successful distribution and effective Denuding. The pipette is set to 50 ul and slowly pipetting is done to detached cumulus from oocytes. After Denuding oocytes were transferred to a fresh plate containing 500 ul of M2 after washing twice the oocytes were then activated.

For Activation 3 different type of activation chemicals are used;

Ethanol 7%: 70 ul 100% ethanol is mixed in 930 ul of M2 to make 7% ethanol solution. Oocytes were incubated in freshly made 7% ethanol solution for 3 min and then washed in M2 solution. After that incubated in DMAP 2 mM for 4 hours.

Ionomycin: Denuded oocytes were activated in ionomycin solution for 4 min. Ionomycin is sensitive to light thus the procedure was done in a dark room with minimum microscopic light. Later either wrap it with aluminum foil or incubate in the incubator for 4 minutes. Then wash activated oocytes in M2 and incubate in DMAP 2mM for 4 hours.

N.B: In all activation methods 25 oocytes/35 ul drop DMAP is used as standard. After 4 hours of incubation in DMAP, the oocytes were then transferred to KSOM media for 16 hours. After 16 h the 2-cell embryo was selected and grown in Mr1ECM for further development. The incubator settings were 37.5 °C with 5% O_2.

7. Hoechst staining

To determine the number of cells Hoechst staining was performed. For Hoechst selected oocytes were washed in 100 ul drop of M2 than transferred in 100 ul drop of Hoechst dye and incubated for 4 min. Hoechst dye is sensitive to light so it was performed in the darkroom. After 4 min the embryos washed twice in 100 ul M2 Drops. Glycerol (1 µl) drop placed on a glass slide in each drop 3 embryos were placed and covered with glass. Observed under the microscope in UV and counted the number of cells per embryo.

Part III.

In-vitro Maturation of rat oocytes

Chapter 1. In vitro maturation rat oocytes

1. Introduction

Rat is an important laboratory animal model, due to its close resemblance with human genomes, disease mimicking abilities, and easy handling of its model of choice nowadays [1]. As demand is increasing for transgenic rat models' researchers are looking for ways of maximum availability of rat embryos to modify[4]. Thus, a need for the development of rat in vitro maturation protocol is increasing with every passing day.

In vitro Maturation provides us the opportunity to maximize the potential of transgenic rat production but with ease comes hurdles as well. One of the reasons in vitro maturation is long ignored and researchers still rely on conventional In vivo practices because it is very hard to mature rat COC in vitro due to blockage at GI, GII stages of meiosis.

To overcome these challenges many biologists tried to understand the physiology of COC maturation in rats and many important suggestions were advised over the past decade. Rat oocytes require special signaling from the cumulus cells, follicular somatic cells and bidirectional communication is necessary for successful maturation[16, 17]. Thus, many scientists tried to mimic in vivo conditions and advised co-culture for the proper maturation of oocytes.

So, Rats oocytes were collected and matured in 2 types of culture conditions. Group A was matured in simple culture media containing TCM 199 with supplemental hormones i.e. FSH 0.05 IU / ml, LH 0.05 IU / ml hCG 15 IU/ml, Estrogen 1 ug/ml, EGF 10 ng/ml, Sodium Pyruvate 1 ug / ml, Cysteamine 100 uM. Group B was Co-cultured with a cumulus cell monolayer in TCM 199 with supplements.

2. Materials

All reagents were purchased from Sigma-Aldrich Co. LLC. (Missouri, USA) unless otherwise specified.

2.1 Care and use of animals

SD rats used in this study were purchased by Orient-bio (Republic of Korea). All-female rats aged 3-4 weeks were maintained in 24 ± 2 ºC, 50% humidity, and 12:12 h light-dark cycle. All animal care and experiments were approved by the IACUC (No.160719a-2-7) and performed under the guideline of SNU.

2.2 COC Collection

The rats were injected PMSG and hCG (HCG, Daesung microbiological labs, Gyenggi, Republic of Korea) 150 IU 48 and 16 hrs. prior dissection respectively. Rats were anesthetized using Aflaxan 0.3 ml/rat. The surgical site is disinfected using ethanol 70%, the incision was given on linea alba, ovaries were retrieved and dissected, and stored in a pre-warm PBS solution. Later collected ovaries were sliced using a scalpel blade in M2 media. The released oocytes than collected in 4 well plate containing 500 ul M2 solution in each well. Only Germinal Vesicle oocytes which have 3 layers unexpended cumulus layers and balanced cytoplasm were collected.

2.3 In vitro Maturation

TCM 199 media is used as a base for all 2 groups of the collected COCs. For group, A maturation media is made 4 hours prior collection to stabilize pH and temperature. TCM 199 is supplemented with FSH 0.05 IU / ml, LH 0.05 IU / ml hCG 15 IU/ml, Estrogen 1 ug/ml, EGF 10 ng/ml, Sodium Pyruvate 1 ug / ml,

Cysteamine 100 uM. 25 COCs per well containing 500 ul media are incubated for 20 h at 37.0 ºC, 5 % CO_2. IVM procedure can be seen in schematic diagram (Fig 1).

2.4 Cumulus cell monolayer Co-Culture

While for group B Cumulus cells after Denuding of oocytes are recovered in 1 ml Eppendorf tube. Then its Centrifuged for 10 seconds at 5200 RPM supernatant solution was aspirated and replaced with PBS. The procedure is repeated 3 times to wash cumulus cells. pre-warmed DMEM supplemented with 10% volume by volume Fetal calf serum is used as culture media. The cell has grown until completion of monolayer the media was replaced every few days. TCM 199 with Supplements is added to monolayer cumulus culture and freshly obtained COC's were co-cultured and incubated at 37.0 ºC, 5 % CO2 for 20 h. The whole procedure can be seen in the schematic diagram (Fig 2)

3. Results

Oocyte maturation was checked 20 hrs. post-incubation in TCM 199 plus supplementation. Oocytes were observed under a microscope and even growth of cumulus cell layers and cytoplasm, as well as extrusion of the first polar body, were set as initial markers of the development (Figure 4,5). Oocytes matured in Cumulus cell monolayer co-culture showed more even growth of cumulus cell layers they were well aligned, morphologically well developed, while upon denuding a significantly higher amount of extrusion of the first polar body was observed. While in the case of simple culture cumulus cell expansion was not that significantly improved and upon denuding the number of polar body extrusion was less than cumulus cell monolayer counterparts.

One can see a significant improvement of development post-activation in co-culture with 59% ± 4 two cell development while 23% ± 2 at the eight-cell stage as can be seen in Table 1 and figure 3.

Although results are of basic morphological and development based, they provide a basic understanding of physiology and chemical significance going on to assist the development of the oocyte in vitro. Many other parameters can be added like measurement of glutathione or molecular studies that can be done to further support the hypothesis as well as further additions that can be added to improve protocol further.

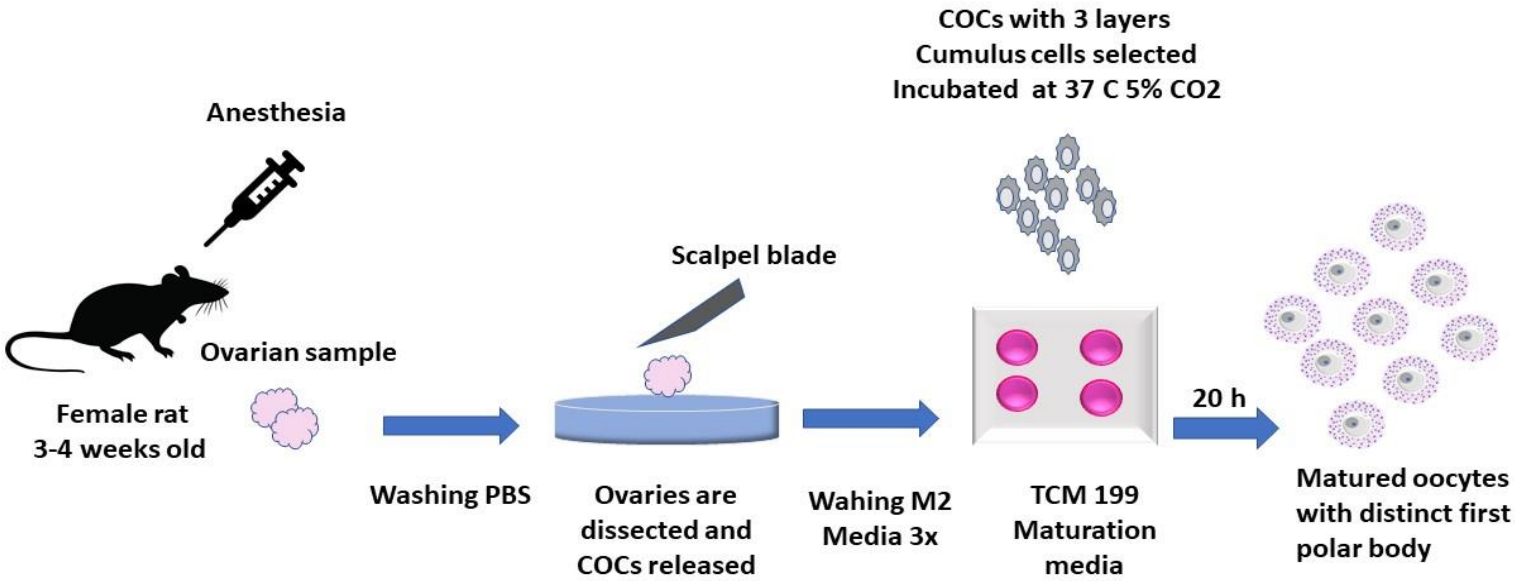


Figure. 1 Schematic diagram of procedure used for In vitro Maturation

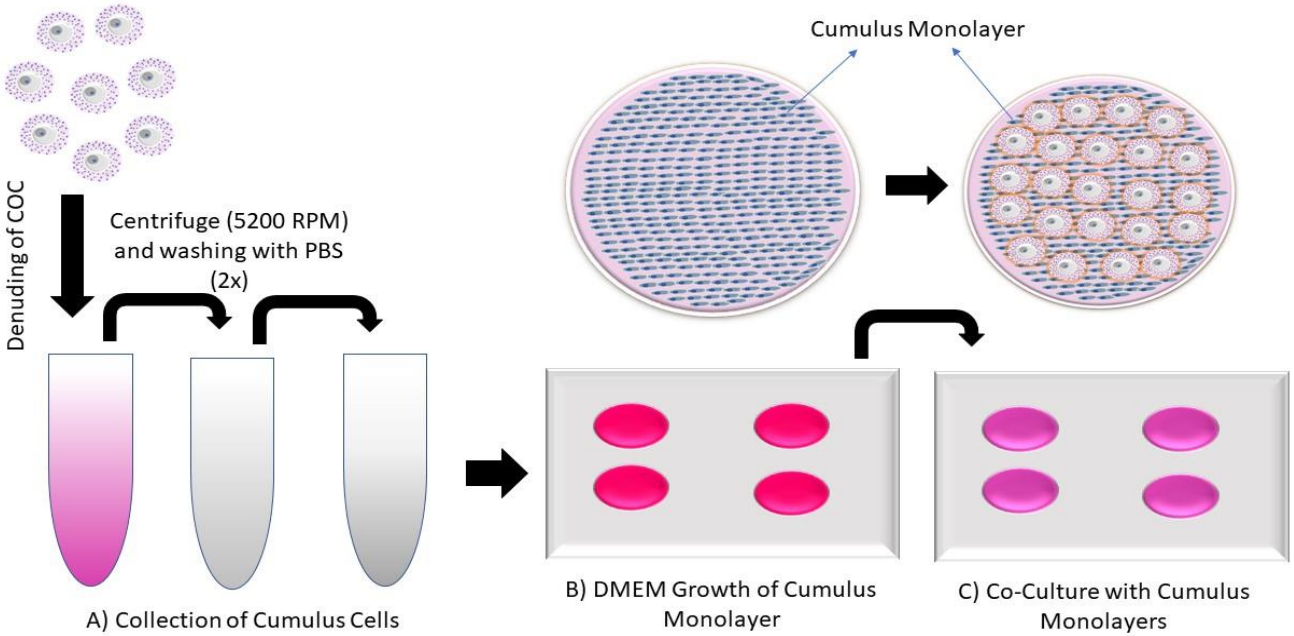


Figure 2. Schematic Diagram of Co-Culture Protocol

Donor	Method of IVM	Average Number of Oocytes[1]	Number of 2-4 cell (%) [1]	Number of 8 cells (%) [1]
SD Female Rat 3-4 weeks old	Simple culture	65±12	53.8 ± 7%*	14.04 ± 7%*
	Co Culture	52±19	59% ± 4*	23% ± 2*

Table 1. Comparison between IVM methods Simple culture vs Co-culture.

[1]Values of results were expressed as the means ± SD.

***Values in the same column with different superscripts are significantly different (P < 0.05).**

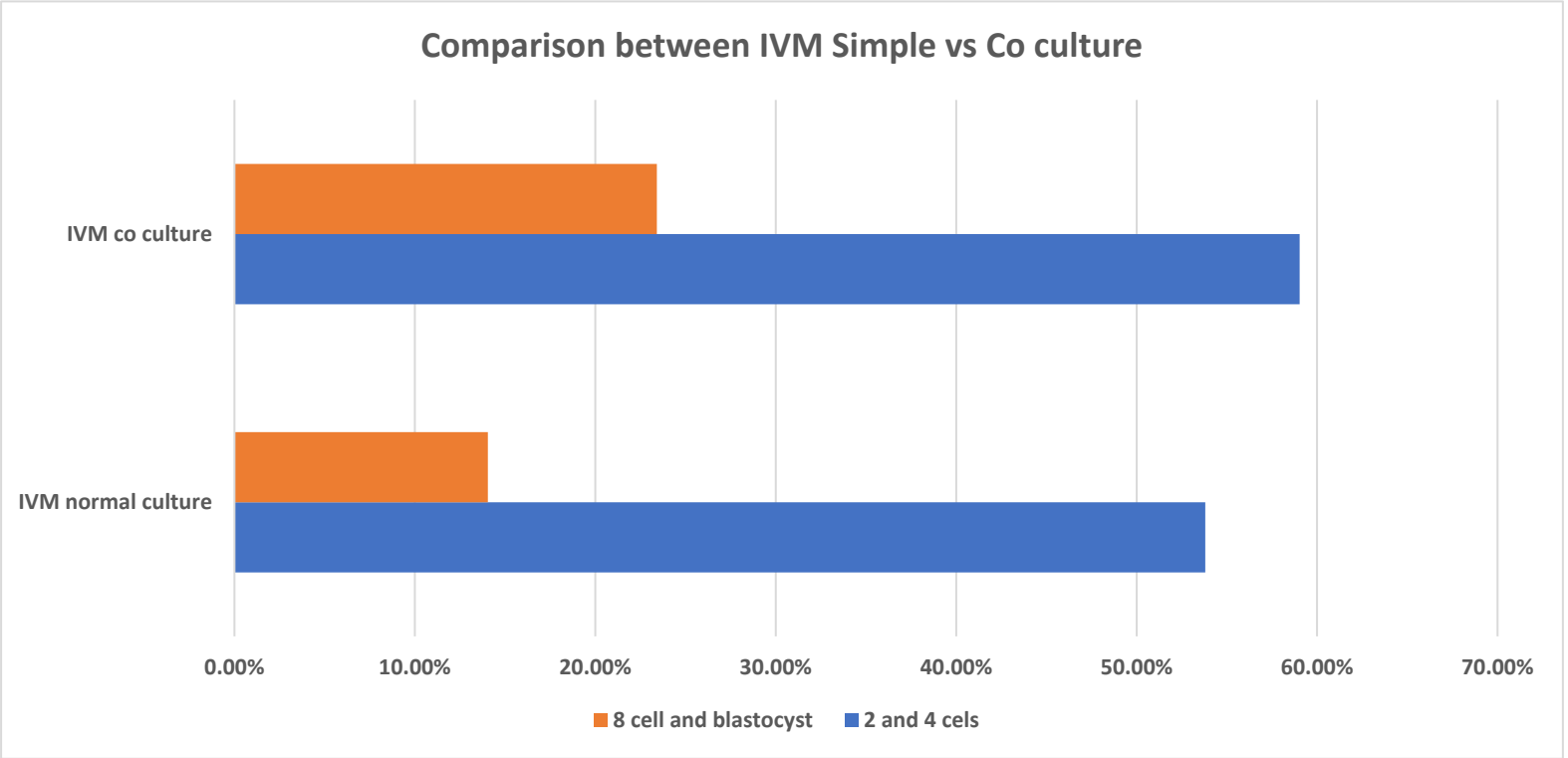


Figure 3 Comparison between In vitro maturation under simple and co-culture conditions. Showing significantly better development in co-culture compared to simple culture.

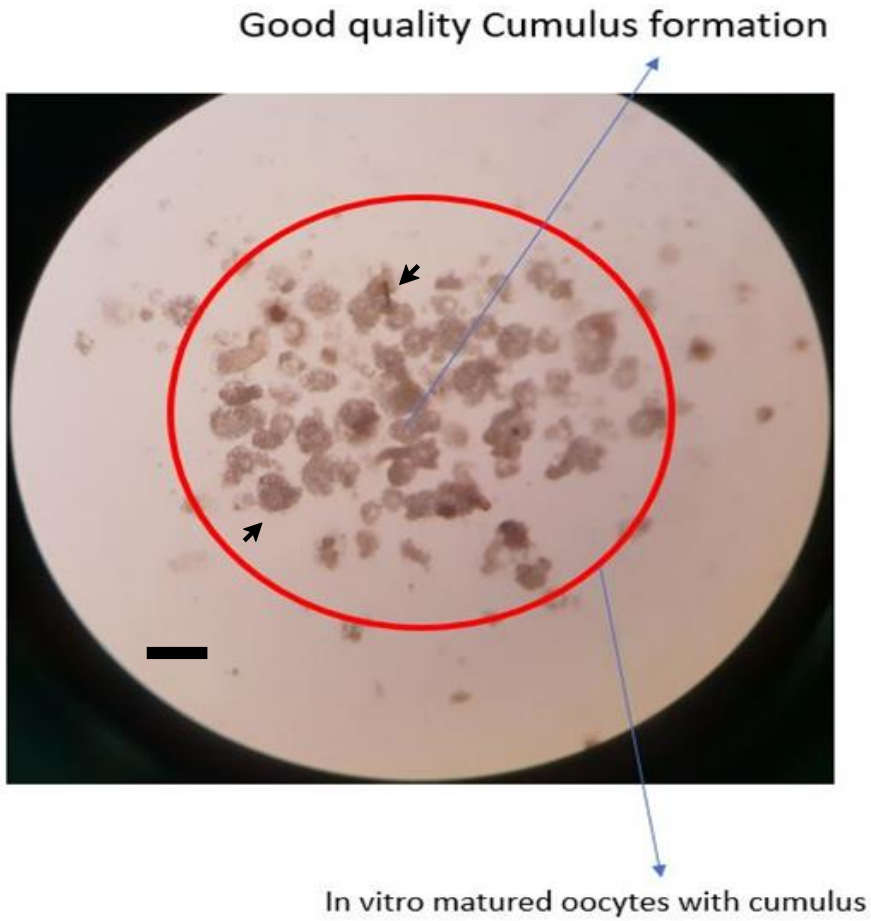
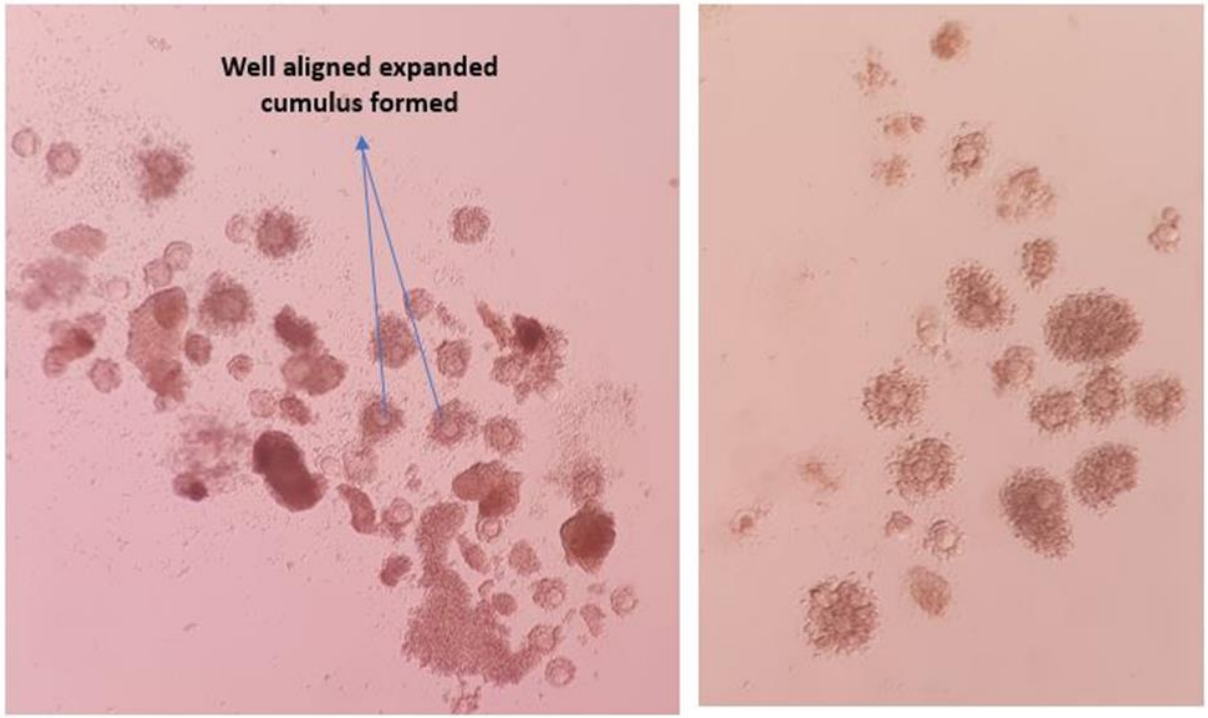


Figure 4 An example of good quality In vitro maturation, well-expanded cumulus cell layers with proper cytoplasmic equilibrium can be seen.

Arrows = scale of 100 µm is kept.

Figure 5 Comparison between oocytes matured in the presence of cumulus cell monolayer co-culture on the left-right vs oocytes matured in simple culture.



(A) Oocytes matured in cumulus co culture (B) Oocytes Matured in Simple Culture

4. Discussion

In this study, I tried to evaluate and develop the in vitro maturation protocol in rat oocytes. I tried to evaluate oocyte quality based on different markers i.e. expansion of the cumulus layer, the formation of polar bodies, cytoplasmic equilibrium, and post parthenogenetic activation development. Our studies showed that those oocytes grown in co-culture with cumulus cell clumps showed better development than simple culture. Many studies show that cytoplasmic maturation is crucial for the development of oocytes [37, 38] One of the most important markers of cytoplasmic maturation is meiotic progression, redox state, and post-fertilization or activation development[22].

Previous studies have shown the metabolic and protective role of cumulus cells, for example, cumulus cells can reduce cystine to cysteine, enhance the uptake of cysteine cumulus cells can reduce cystine to cysteine [39], and increase the intra-oocyte glutathione level and protect the oocytes against oxidative stress [40] Cumulus cells can also metabolize glucose to pyruvate that can be used by the oocyte to improve cytoplasmic maturation [41]. Thus, based on previous studies I tried to develop an IVM co-culture protocol that may provide a key for the future development of transgenic rats in the in vitro environment.

A lot of improvements can be made to the protocol with upcoming research work, for example, glutathione interaction and involvement in maturation of oocytes can be studied in rat oocytes. Also, we can explore different physiological aspects of in vivo maturation and try to mimic and study them using in vitro culture. This protocol can work as a base platform to start with the rat in vitro oocytes studies.

Chapter II. Parthenogenetic Activation

1. Introduction

Parthenogenesis is a naturally occurring procedure in lower species of reptiles, amphibians.[27] In mammals, parthenogenesis does not occur naturally but can be induced artificially, to the date it been induced and transferred in many animals such as mice [2], sheep [42], cows [43], pigs[44], rabbits [45] and monkeys [46]. Parthenogenetic activation is artificially activating oocyte to develop further it done by mimicking the same physiological and chemical changes oocyte goes through during fertilization.

Parthenogenesis can be proposed as a possible embryological model since parthenote development is virtually identical to embryo development during early stages of development as observed in several animal models[47] Also, It can be used to analyze and asses maternal genes expression can be used to track many silenced genes. Moreover, there is low or no ethical concern as embryo formed as result of parthenogenetic activation which is considered artificial[48], combined with in-vitro matured oocytes parthenogenesis can help produce Embryonic stem cells (ESC) because of low Spontaneous activation of IVM oocytes. Also, Artificial activation is a major tool to activate embryo after somatic cell nuclear transfer (SCNT). Further, Parthenogenetic activation can be used as a marker for the successful maturation of oocytes, as they are artificially activated thus ethically more viable than IVF as a maturation marker.

Thus, in this study, I tried to check out in vitro Matured oocytes' developmental capacity using the parthenogenetic activation method. I used chemical agents ethanol and ionomycin for this purpose.

2. Materials

All reagents were purchased from Sigma-Aldrich Co. LLC. (Missouri, USA) unless otherwise specified.

2.1 Care and use of animals

SD rats used in this study were purchased by Orient-bio (Republic of Korea). All-female rats aged 3-4 weeks were maintained in 24 ± 2 ºC, 50% humidity, and 12:12 h light-dark cycle. All animal care and experiments were approved by the IACUC (No.160719a-2-7) and performed under the guideline of SNU.

2.2 Collection of oocytes

Matured oocytes were retrieved and transferred to a 4 well dish containing 500 ul/well 0.1% Hyaluronidase. Almost 50 ± 2 COCs per well are maintained, after incubation for 3 min COC were denuded by gentle pipetting. All denuded oocytes were then analyzed and oocytes with the first polar body are collected for parthenogenetic activation. While cumulus cells were used for further monolayer co-culture production.

2.3 Parthenogenetic activation

Denuded oocytes washed using M2 media 3 times than transferred the activation well. Activation well was of two groups. Group A contained ionomycin while Group B was activated using 7% ethanol. 35 ± 2 oocytes were maintained per well. For ionomycin 4 min dark incubation was done. While for ethanol 3 min of incubation treatment is done.

Activated oocytes were then washed with M2 media 3x times again and transferred to a 35 ul drop of 2mM 6 DMAP. An incubation time of 4 h is provided.

2.4 In vitro culture of embryos

Incubated oocytes were then washed again using M2 media and transferred to 200 ul drop of KSOM covered with oil layer, where they incubated for further 20 h. After 20 h activated oocytes were observed and those with the second polar body or 2 cells were transferred to 100 um MR1ECM media. 25 activated oocytes were kept per drop. Observed for further development, within 2-3 days oocytes usually reach 8 cells and blastocyst stage respectively.

3. Results

The initial development of embryos was almost similar. Most embryos from both culture groups have shown two cell divisions 20 hrs. post-activation (Figure 7). In the case of ethanol, 7% fragmentation was observed after 8 cell stages, with no further development. While in the case of ionomycin development was steadier and more proper. Most of the IVM oocytes reached till blastocyst stage.

On average 116.42±66.13 oocytes were activated using both methods 57% ± 0.05% Shown active cleavage after 20 h of incubation as can be seen in figure 8. But upon further development, a lot of fragmentation was observed in embryos activated using ethanol 7% compared to ionomycin counterparts. Thus, we devised that ionomycin is a much better artificial activation agent compared to ethanol 7%.

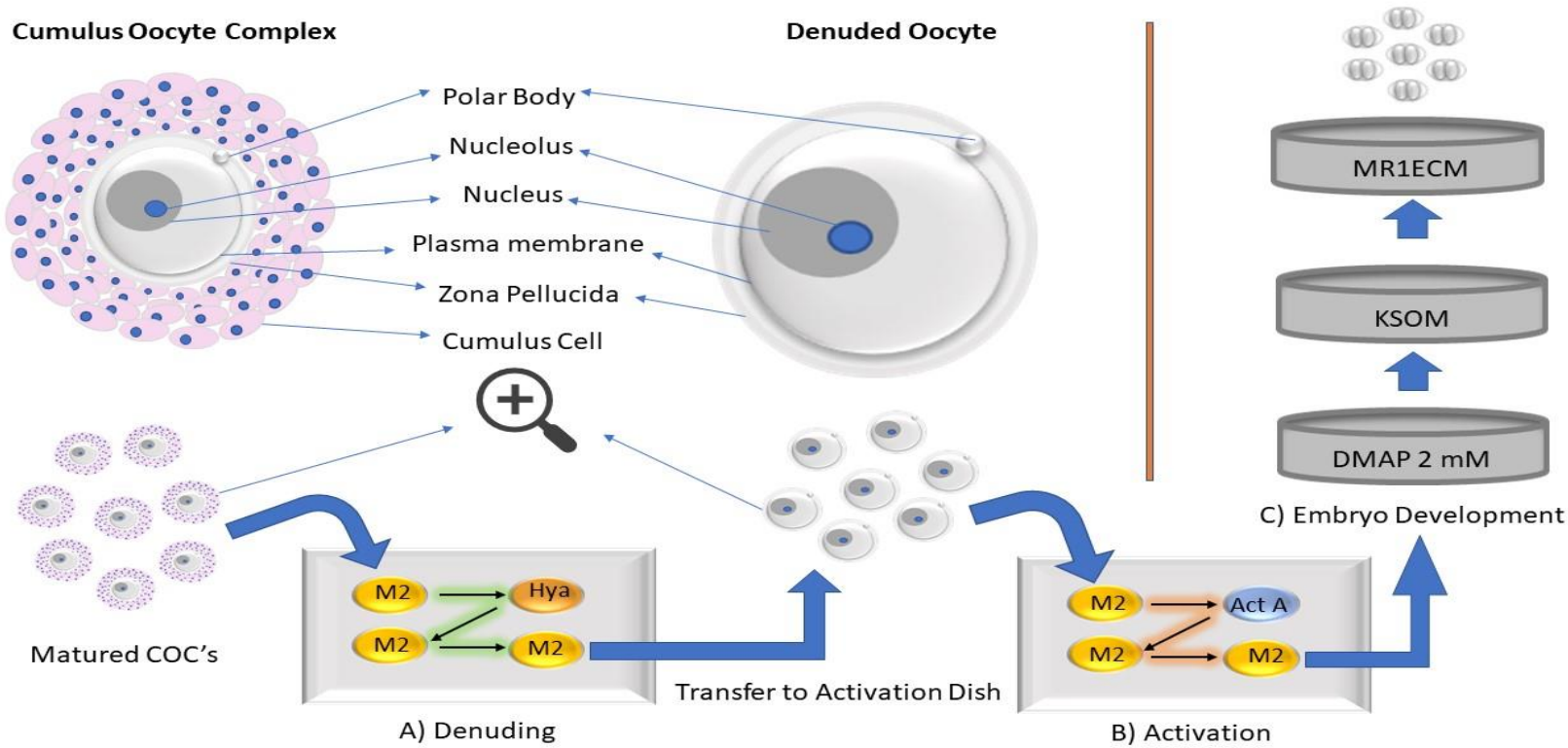


Figure 6. Schematic diagram of Parthenogenetic Activation Protocol

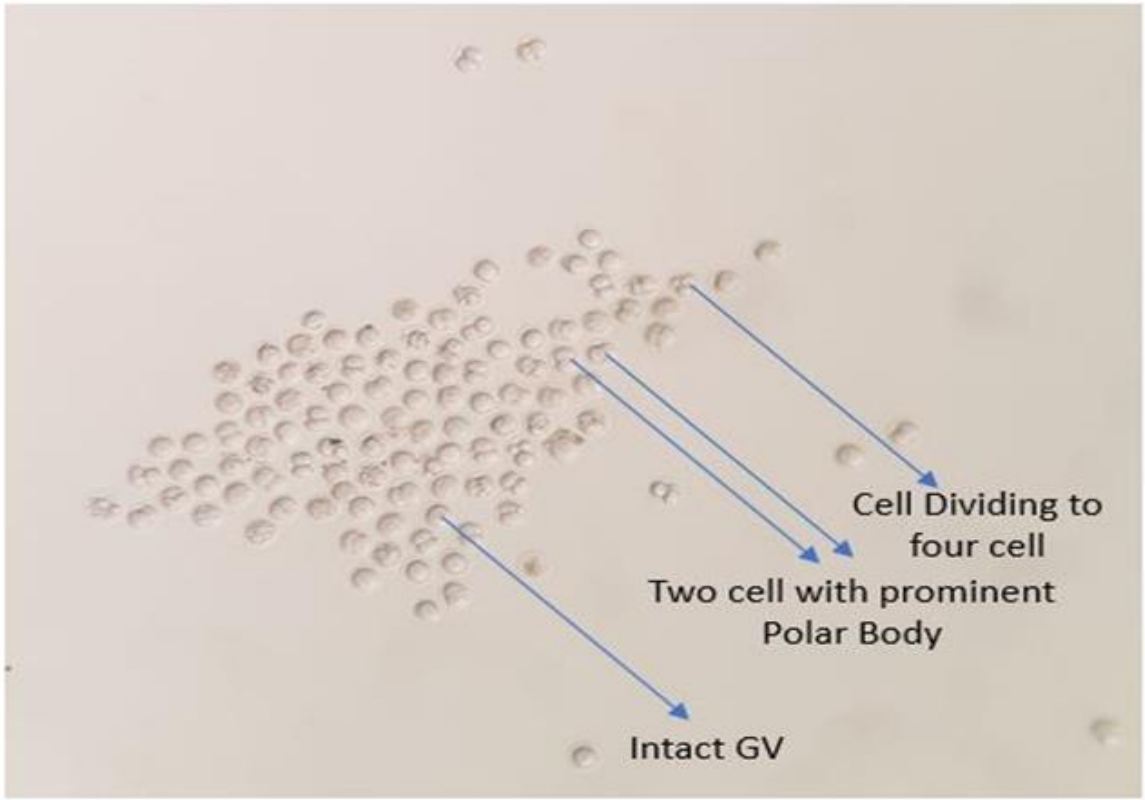


Figure 7 Activated oocytes post 20-hour incubation, one can see dividing oocytes, 2 cells, and 4 cells.

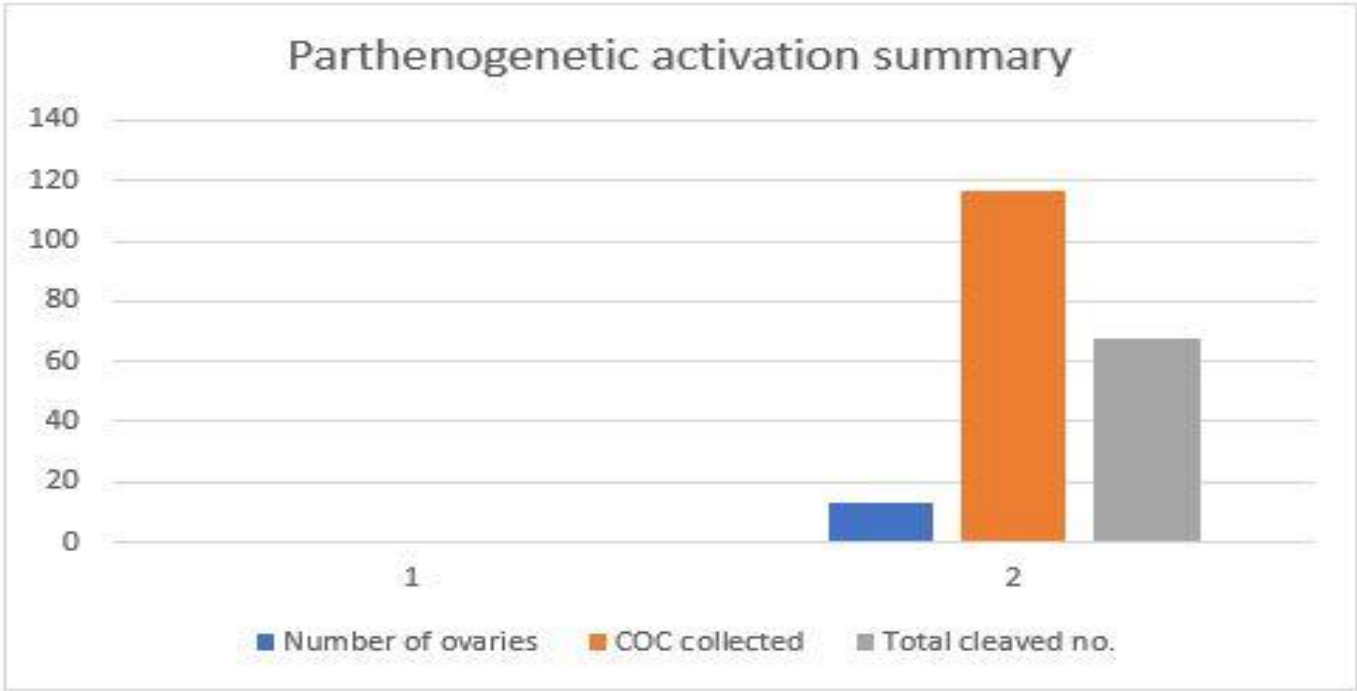


Figure 8 Parthenogenetic activation summary a total of an average of 116.42±66.13 oocytes were activated using both methods 57% ± 0.05% Shown active cleavage after 20 h of incubation.

While the average number of oocytes were calculated as mean ± SD.

4. Discussion

In this experiment, I tried to figure out the development potential of In-vitro matured oocyte post parthenogenetic activation. For successful activation of both nuclear and cytoplasmic maturation is necessary[37], An increased amount of glutathione, proper cytoplasmic equilibrium, and oocyte redox state is one the precursor for in-vitro maturation of oocytes[22]. Thus, I used a parthenogenetic activation tool to maximize our understanding of development in IVM oocytes.

I analyzed two chemical agents to activate IVM oocytes, activated oocytes were matured and developed under similar conditions, later I checked their developmental capacity and analyzed results based on meiotic progression, growth, cytoplasmic maturation, and blastocyst formation.

Our results showed statistically significant data correlating to the proper maturation of rat oocytes as well as that parthenogenetic activation method can be used as a developmental marker for in vitro oocyte maturation. I used two different activation agents and compared their activation capabilities. results have shown that ionomycin with co-culture matured oocytes gives the most optimum results.

Activation via ethanol 7% is comparatively easier as its cheap option is not sensitive to light so no dark incubation is required also an easily available chemical agent. While ionomycin activation required special skills, dosage, also ionomycin is sensitive to light so the procedure must be performed in the darkroom as well as it’s a comparatively expensive option compared to ethanol 7%. Thus, both chemical agents have their pros and cons.

So, based on our results we find out that ionomycin can be a much better option to study in vitro maturation process in rat oocytes. Furthermore, with time and research, many other activation agents can be explored like TPEN, Electric stimuli, etc. Also, the current procedure can be further optimized based on more data and studies by mimicking and studying more in vivo maturation physiology in-depth

and providing the immature oocytes right environment and nutrition required for their development.

Conclusion

This thesis was aimed to generate a basic guideline to develop a protocol for in vitro maturation in rat oocytes. in vitro maturation is one of the important areas that still need to be explored by researchers. In this study, I tried to develop an optimal protocol for rat oocytes.

Firstly, I superovulated 3 to 4 week old female SD rats using PMSG and hCG IU 48 and 16 h respectively. Then I surgically collected ovaries from superovulated female rats, and I collected GI oocytes via ovaries, then cultured them under simple and co-culture methods.

Secondly, I tried to find out their developmental potential using parthenogenetic activation. As parthenogenetic activation is better alternative and ethically more convenient than in vitro fertilization (IVF). For that purpose, I used a chemical activation method. So, I used two major chemicals i.e. ionomycin and ethanol 7%.

I concluded that in vitro maturation under co-culture is better than simple culture, upon more research and advancement it can produce results most close to in vivo developed oocytes. Also, ionomycin is one of the best activation agents available to activate in vitro matured oocytes. Finally, I summarize with the statement that IVM of rat oocytes can be of multipurpose either to study the physiological properties of development, development of embryonic stem cells, gene editing, Transgenic embryo production, or Maternal genetic studies. It is a vast area still pending to be discovered.

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국문초록

래트 난모세포에 대한 시험 관내 성숙 프로토콜의 개발

무하마드조안

(지도교수: 장 구)

서울대학교 대학원

수의학과 수의산과·생물공학 전공

실험실 동물은 의학 연구의 필수 부분이며 새로운 의약품, 백신, 실험실 동물에 대한 의료 제품의 필요성이 크게 증가함에 따라 새로운 필요성이 대두되고 있다. 초기에는 인간의 생리학과 유사하며 크기가 작아 관리가 용이하기 때문에 마우스 모델이 개발되었다. 따라서 지난 30 년에서 40 년 동안 마우스 모델에 대한 매우 다양하고 잘 개발 된 연구를 볼 수 있다. 그러나 마우스 모델은 크기가 작고 수명이 짧기 때문에 한계가 있다. 이는 생물 학자들로 하여금 오랫동안 간과되었던 래트 모델에 대해 재고하도록 하였다. 래트는 마우스에 비해 크기가 커서 우수한 모델이므로 샘플링, 처리 및 절차 성능이

용이하다. 따라서 래트는 심혈관, 신경 생물학, 면역학, 독성학, 생리학, 약리학, 영양학, 행동학 등의 연구에 광범위하게 사용되고 있다. 래트 모델에 대한 수요가 증가함에 따라 래트의 보조 생식 기술 연구에 더 많은 초점을 두게 되면서 많은 연구자들이 생산량을 늘리는 새로운 방법을 찾고 있다. 지금까지 유전자 변형 된 래트 생산의 주요 원천은 생체 내 COC (cumulus-oocyte complex)와 배아이다. 본 연구에서는 쥐 COC 를 위한 체외 성숙 프로토콜을 개발하여 행하였다. (과배란, 체외 성숙, 처녀생식 활성화, 염색).

첫째, 과배란은 최고 수준의 고품질 COC 를 얻을 수 있도록 하는 필수 요소이다. 과배란 유도의 가장 보편적인 방법은 호르몬 말초 성선 자극 호르몬 및 인간 융모 성 성선 자극 호르몬을 사용하는 것이다.

둘째, 시험 관내 성숙 래트를 마취시키고, 난소 샘플을 외과적으로 수집 하였다. 그런 다음 먼저 PBS 로 세척하고 COC 는 바늘 또는 메스 블레이드를 사용하여 난소를 분쇄하여 얻었다. 최소 3 층의 적운 세포를 갖는 COC 를 M2 배지에서 선택하여 세척하고, 미리 만들어진 성숙 배지 TCM 199 (그룹 A), TCM 199 적운 세포 공동 배양 (그룹 B)에서 24 시간 동안 배양 하였다. 그리고 24 시간 후 COC 를 분석하고 현미경으로 관찰하였다.

또한, 시험 관내 성숙 COC 의 품질을 평가하기 위해, 히알루로니다제를 사용하여 제거하고 M2 배지로 세척하였다. 이어서, 상이한 활성화제 이오노마이신, 에탄올, 아연 등을 사용하여 활성화시켰다. 활성화 된 난모세포를 2 mmol DMAP 에서 4 시간 동안 인큐베이션 한 다음, KSOM 배지에서 24 시간 동안 배양하였다. 이어서, 2 개의 세포의 난모세포를 MR1ECM 배지에서 배반포가 형성 될 때까지 추가로 배양하였다.

마지막으로, 배반포 및 활성화 된 난모세포의 질을 확인하기 위해 Hoechst 염색이 수행된다. 배반포를 세척, 염색 및 슬라이드 상에 고정시키고 현미경으로 관찰 한 후 세포의 수를 세었다.

결론적으로, 본 연구에서는 최대의 효능과 성장 잠재력을 가진 시험관 성숙 프로토콜을 개발하려고 노력했으며, 난모세포 적운 덩어리의 공동 배양이 단순 배양보다 훨씬 낫다는 것을 증명했다. 세포 커뮤니케이션, 산화 환원 상태 및 세포질 성숙은 난모세포의 성공적인 성숙에 중요하다.

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키워드: 난소, 난모세포, 시험관 성숙, 처녀생식

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