Doctoral Dissertation

Optimization of Single Embryo Culture and Assay Systems for *In vitro* Fertilization (IVF) Technology

Department of Biomedical Engineering,
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February 2023

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I. Literature Review

Infertility is an inability of a person, both male and female, to conceive a baby even after 1 year or more of unprotected sexual intercourse (1). There can be two types of infertility: primary infertility and secondary infertility. Primary infertility is an inability of a person to have any kind of pregnancy whereas, the secondary infertility is the inability to have a pregnancy after previously successful conception. It is a universal problem and there are around 48 million couples and 186 million individuals around the world with infertility (2, 3). Males contribute to 20-30% of overall infertility cases. Even though there is misconception about the male fertility that they are fertile all around their life, it is not true. Male fertility also decreases with age and semen quantity and quality are best at the age of 30-35 (4). Similarly, the chances of getting pregnant in women decrease with the increase in age. Infertility in women aged above 35 is defined when they cannot conceive a baby even after having unprotected intercourse for 6 months. There are 50% chances of getting pregnant in women aged 19 to 26, <20% chances of conception in women aged above 30 years and <5% in women in their 40s and above. The issues of the infertility in both males and females can be present at birth but in some cases one can develop the problem later in life. Some of the causes of male infertility include abnormal sperm production and function, problems with the sperm delivery, overexposure to certain environmental factors and damage related to cancer and its treatment like radiation and chemotherapy. Cancer treatment procedure can impair the sperm production in males severely. Similarly, the causes

of female infertility may include ovulation disorders, uterine or cervical abnormalities, fallopian tube damage or blockage, endometriosis, primary ovarian insufficiency or early menopause, pelvic adhesions and cancer and treatments involved to treat cancer. Specially the reproductive cancers impair the female infertility. The use of radiation and chemotherapy can be harmful to the female fertility as well. Ovulation disorder in female can be due to polycystic ovary syndrome (PCOS), thyroid problems- overactivation and under activation of thyroid gland can prevent ovulation (5). Sometime the problems of infertility are difficult to diagnose in both male and female. The general reasons for pregnancy failure are shown in Figure 1.

To increase the chances of pregnancy, couples should have regular intercourse several times around the time of ovulation. The chances of infertility are increasing over the years with increase in an unhealthy lifestyle. The lack of exercise, consumption of unhealthy meals, and the environment we are living in contribute to the increase in infertility in both males and females. The other risk factors include use of tobacco, use of alcohol, being overweight, being underweight etc. Although most of the infertility are not preventable one can chose to live a healthy life by exercising, limiting the consumption of alcohol, by limiting the medications that might affect the fertility. As well as in male by avoiding the high temperatures found in hot tubs and hot baths. Also, in female the use of excessive amount of caffeine while trying to get pregnant can affect the end result.

In vitro fertilization (IVF) is one of a common technique developed to treat infertility. The IVF procedure was invented to overcome these short comings and shower the love of a child

to individuals or couples who wish to have them. While the intention of IVF was to solve the problem of infertility, a problem arose when couples with faulty sperm quality and quantity could not undergo the process of IVF.

To solve this problem; a subfertility problem of male partner, a new process has been introduced in which a single spermatozoon is injected directly through the zona pellucida of the oocytes. The process is termed intracytoplasmic sperm injection (ICSI). This process was first introduced in 1987 (6). The development of the ICSI process has made fertilization possible even in severe cases of compromised sperm properties (7). The success rate of ICSI depends on the motility of fresh retrieved or thawed sperm and on the maturity of the sperm selected (8). The chances of pregnancy were better while using fresh motile sperm according to the study conducted by Park et.al. and Shibahara et.al. (8, 9).

The advancement made in IVF and ICSI are the most exciting scientific development for infertility treatment. It has been a boon to humankind, especially for couples who could not have offspring on their own. The first successful case of IVF was observed in the Royal Oldham Hospital, London, UK in 1978 with a successful birth of a baby girl (10). Since then, the interest in IVF has increased drastically over the year. IVF fertilizes both male and female gametes outside of a body in a high-end laboratory with thorough selection of both male and female gametes. The process needs to go through several steps for it to be complete and the success of obtaining healthy embryos and offspring depends on the efficiency of each step of the process. After the patient's health condition has been verified, the oocytes are retrieved and hormonally stimulated. After the collection and classification of the eggs,

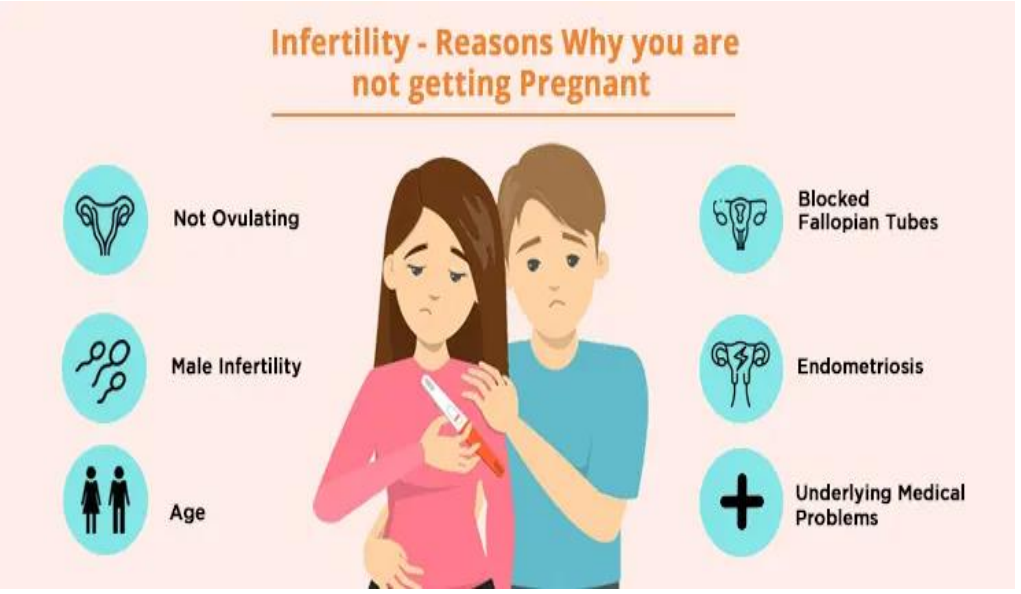

Figure 1: Reasons of pregnancy failure. Figure from (https://aveya.in/infertility-can-be-dreadful/)

insemination takes place for which the semen sample is provided by the male partner. Inseminated oocytes are placed in an incubator overnight at an ideal temperature and pH. Fertilization is assessed the next day. Embryos are generally transferred 3 days after insemination, while some are transferred 5–6 days after insemination, at the blastocyst stage. The number of embryos to be transferred is determined by the age of the patient, the condition of the embryos, and other elated factors. In some cases, when embryos have a thick membrane, assisted hatching is also performed. The pregnancy is tested after 2 weeks by evaluating beta human chorionic gonadotropin (b-hCG) or by other factors. In the developed world, over 4 million IVF babies have been born since 1978, which happens to be largest in the U.S. with 1 in 75 new births followed by Japan and France (11). The schematic diagram to explain the *in vitro* fertilization process is summarized in Figure 2.

This process is extremely sensitive and requires highly experienced technicians. As both male and female gametes are sensitive, it requires extremely careful processes to make the fertilization procedure successful. Therefore, this procedure becomes expensive and mentally draining for both the technicians and individual who wish to have babies using this procedure (12). It becomes even more challenging as there is no 100% guarantee that a baby will be conceived even after this procedure. Also, there occurs the chances of multiple pregnancy like conception and birth of twins, triplets, quadruplets and even more than that sometimes as technicians transfers more than 1 embryo to a body for the successful conception rate as well as birth rate. Women conceiving via IVF are 17% more likely to have chances of giving births to multiple babies. The conception of more than one baby or than required baby is not acceptable as it puts financial to many more burden to the family.

There are lots of gaps to fill in process of IVF to make this technique cost-effective and successful. This process as a whole is out of reach especially for the couples and individuals who belongs to the developing countries as they cannot afford to have a baby with this technology. If we can make this procedure burden free by making it cost-effective with more success rate and as efficient as possible, it will save lots of financial burden and emotional damage, and mental stress to people from both developed to developing countries.

Care in each step of IVF should be given for the successful conception of the baby, every step should be handled carefully and should give extra care from the type of media used to handle the embryos starting from its composition to the type of culture system used for the process. Even the slightest detail can ruin the whole process of IVF leading to the financial burden of people with unsuccessful procedures of IVF as a whole. The use of other assisted technology like microfluidics in each step of this IVF process reduces the chances of error made by human themselves. Microfluidics refers to a manipulation of fluids in channels in micrometer scale. Incorporation of these technologies in this process might also help to make this whole procedure cost effective, easy to use, analyze, and dispose. Over the year, continuous efforts have seen the achievement of milestones in culture systems improvements with notable evolution occurring in an animal as well as clinical IVF (13).

My research study includes the procedure taken to improve the quality of IVF by optimizing the embryo culture media with different media components like EDTA, as well as incorporating different cell types like cumulus cells, oviductal epithelial cells (OEC), uterine epithelial cells (UEC) to enhance the quality of embryos in a mouse model. My research also includes the possible selection procedure of embryo by analyzing their morphology,

time taken by an embryo to develop to certain stage i.e., their morpho kinetics, and by analyzing their metabolic activity like glucose consumption, lactate production to measure glycolytic activity for better implantation after transfer. As well as the miniature culture system for single embryo with introduction of miniature culture system a concave structure microfluidic system was also developed for single embryo culture *in vitro* for better development and easy selection of embryo to transfer for implantation.

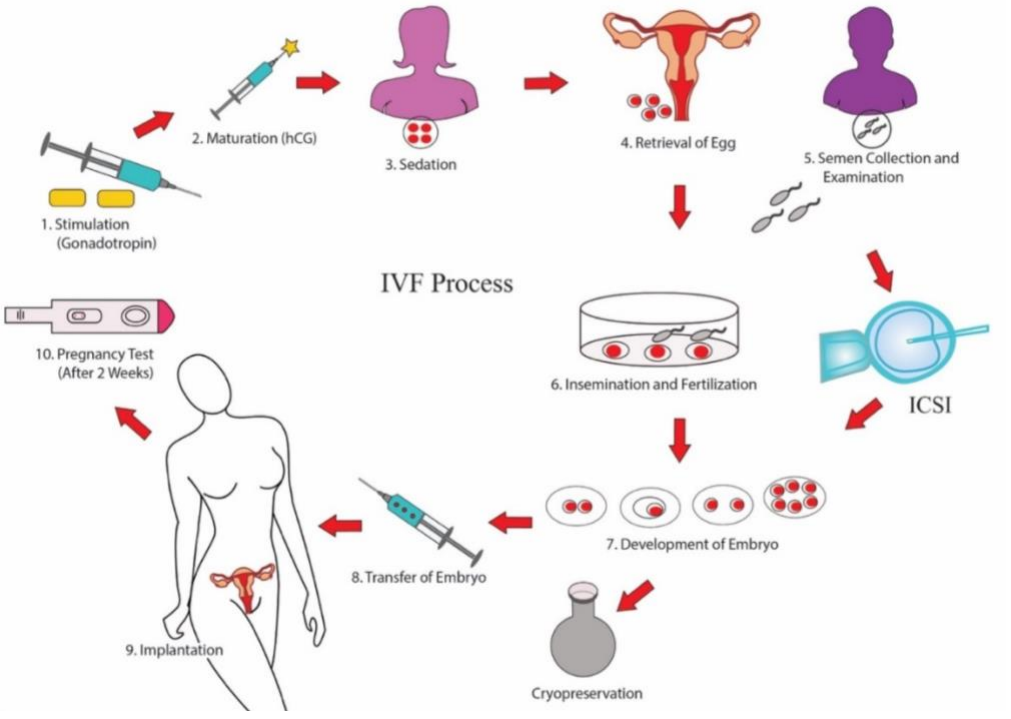

Figure 2: General process of *in vitro* fertilization (IVF) in human (14).

This section of the thesis is summarized in review paper (14).

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Chapter 1: Optimization of IVF culture media using EDTA

1. Introduction

In this chapter, the improvement of IVF procedure was examined by optimizing culture system with ethylenediaminetetraacetic acid (EDTA) along with examining the best culture system for better development of embryo *in vitro*. For decades, different mouse species have been used as an experimental model to develop and modify culture media that later helped to develop culture systems suitable for human embryos (1, 2). However, the most widely employed and well-understood inbred mouse strains in research fail to appropriately account for genetic variation, providing inadequate representation of the genetically diverse human population. Outbred stock mice, a less employed strain, are believed to have a genetic diversity similar to that of humans. The use of outbred mice has greatly increased as they are considered better subjects for biomedical research (3). The Institute of Cancer Research (ICR) mouse was used as a model animal as it is well-developed, well-behaved, and easy to handle. It is also healthy and highly reproducible, and has a wide range of uses for various research purposes (4) as well as toxicity, medicine pharmacy, pharmacology, endocrine and nutritional test (5). From the year 1961 to 2014, the papers using the ICR mouse has gradually increased (6). However, the studies related to *in vitro* culture of such mice embryos are few because *in vitro* developmental block occurs when one cell embryos are cultured in conventional media. If the embryo culture system for ICR mouse is well developed, ICR

mouse can be used as a good model for the assisted reproductive technology (ART). Zygote differentiation in outbred stock mice (ICR mice) gets arrested at 2-cell stage, a phenomenon termed '2-cell block' block" (7). This phenomenon of development ceasing at 2-cell stage of embryo development is usually seen during *in vitro* culture of outbred mice oocytes (8). Sometimes, morula block occurs where the embryos in the morula stage cannot reach blastocyst stage, an important developmental stage. This problem rarely occurs during the culture of inbred and F1 strains of mice *in vitro* (9). 2- cell block can be facilitated by several mechanisms like oviductal transfer of embryos, either *in situ* or as organ culture (10), transfer of cytoplasm by microinjection from a 2- cell embryo of a non- blocking strain of mice to that of a blocking strain (11). Different culture media used for *in vitro* embryo culture contain transitional metal ions like zinc, copper and iron, which produce harmful oxygen radical during chemical reactions (12). The addition of EDTA in culture media helps to chelate such metal ions and reduces the 2-cell block (13). Previous studies indicated that EDTA added at lower concentration, typically 0.005-0.01 mM, also overcame the 2- cell block in some mouse strains (14-16). EDTA helps in this issue as it functions as divalent cation chelator. Transition metal ions like zinc, copper and iron (12) present in tissue culture media is chelated by EDTA which prevent the production of harmful oxygen radicals by preventing ions from taking part in chemical reactions (13, 17, 18). EDTA has been used by adding with other culture media such as CZB(17), G1(19), x-basal HTF (20), P-1 medium (21) and so on to enhance the embryo development *in vitro*. The presence of EDTA in different culture media is shown in Table 1. Embryo cultured without EDTA in culture media shows reduced development rates due to abnormal increase in glycolytic activity (22).

Presently, there are ongoing efforts to overcome these blocks in outbred mice strains (23). The study in this chapter reports the optimized culture condition for *in vitro* development of ICR mice embryos using EDTA.

1.1. Materials and Method

1.1.1. Animal preparation

The animals used in this experiment were ICR stock mice distributed from Hana corporation (South Korea). The female mice were 5-7 weeks old weighing from 21-25g whereas the male mice were 7-12 weeks old weighing 30-40g. Lightening conditions were adjusted to 12 hours light/dark cycle (6:00 am - 6:00 pm) and ad libitum food and drinking water were provided. All the experiments performed were approved by the Institutional Animal Care and Use Committee of the School of Medicine, Keimyung University (Mice IRB no. KM-2017-44R1) and were performed in accordance with the relevant guidelines and regulations.

1.1.2. EDTA preparation

Five hundred millimolar EDTA (Irvine Scientific) was made into different concentrations of EDTA (100,200, 300, 400 and 500μM) and mixed in 1 ml culture media. For droplet culture, EDTA was prepared using media itself. All media were filtered through a 0.22μm filter.

1.1.3. Superovulation and zygote collection

Female mice were superovulated by intraperitoneal injection of 5 IU pregnant mare serum gonadotropin (PMSG; Sigma-Aldrich, G-4877) followed by 5 IU human chorionic gonadotropin (hCG; Life Sciences,367222–1000IU) 48 h later. They were subsequently mated with male mice of the same strain and examined the following morning for vaginal

plugs to confirm that mating had occurred. Female mice were euthanized by cervical dislocation approximately after 16–18h of post hCG injection. Oviducts were dissected and pooled into HEPES-buffered F-10 media (Gibco, 11550-043) with 10% serum synthetic substitute (SSS; Irvine Scientific, CA 92705. USA). Zygotes were isolated by tearing the ampulla of oviducts, and cumulus cells were removed using hyaluronidase from bovine testes (Sigma-Aldrich). Collected embryos were cultured in respective media. General representation is shown in Figure 3.

1.1.4. Embryo culture

Ten percent of SSS and HEPES were added to F-10 media and were used as handling media for the experiment. The same F-10 media were also used as culture media for embryos. The primary culture medium used in this study was a potassium simplex optimized medium; KSOM medium (Merck, MR-121-D).

1.1.5. Experiment 1: conventional large volume culture system (1 ml)

Collected zygotes were distributed randomly in 10–15 groups and were cultured in Falcon IVF one well dish (Irvine Scientific, 353653) containing (i) F-10 media, (ii) 1ml KSOM culture medium, and (iii)different concentration of EDTA mixed in KSOM culture media.

1.1.6. Experiment 2: micro-droplet culture system

The different droplet sizes of media volumes (6.25, 10, 12.5 and 25µl) were prepared for the culture of embryos. Cultures were performed in both KSOM and EDTA optimized KSOM media. Two droplets of media were made in each Falcon IVF one well dish to culture zygotes in six to nine groups (Figure 4). All culture systems were overlaid with oil for embryo culture

(Irvine Scientific, 9305) and were equilibrated overnight before culturing began. Harvested embryos were cultured in the same incubator with a humidified environment of 5% CO_2 at 37°C. Embryo development was assessed every day from 1-cell stage to the formation of blastocyst.

1.1.7. Statistical analysis

Pictures used in this paper were taken using Zeiss Zen 2.5 microscope imaging software. Graphs and percentage of blastocyst development were prepared using Excel 2016 and Origin pro-2019. Percentage data were arcsine transformed before analysis but are presented as non-transformed mean±SEM unless otherwise mentioned. Data were analysed using ANOVA followed by Tukey honestly significant difference test. A value of $p \leq 0.05$ was considered statistically significant.

1.2. Results

1.2.1. Experiment 1: conventional large volume culture system (1 ml)

1.2.1.1. Comparison between different concentrations of EDTA

A plot of EDTA concentrations against the embryonic development rate showed two distinctive peaks, where the development rate increased steadily from 0–200µM concentration but decreased drastically at a concentration of 300µM. However, a slight increase in development rate was seen with increasing EDTA concentration (400–500µM) at a lower rate than the development rate of 200µM EDTA. The 57.6% of 92 zygotes were developed to blastocysts in KSOM media with 200µM of EDTA, whereas, among 508 zygotes, only 16.7% were developed to blastocysts in KSOM media alone Figure 5a. Even

though most EDTA concentrations supported proper development and survival of embryos up to the morula stage, most of them showed ‘morula block’ leading to a low blastocyst development rate. Two hundred micromolar proved to be the best EDTA concentration for media optimization.

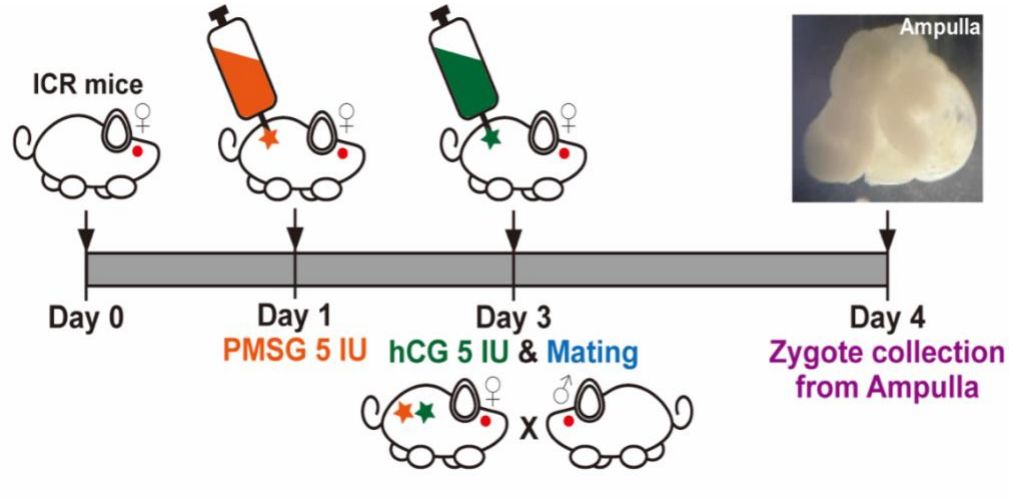

Figure 3: General animal preparation and collection of embryos from mice.

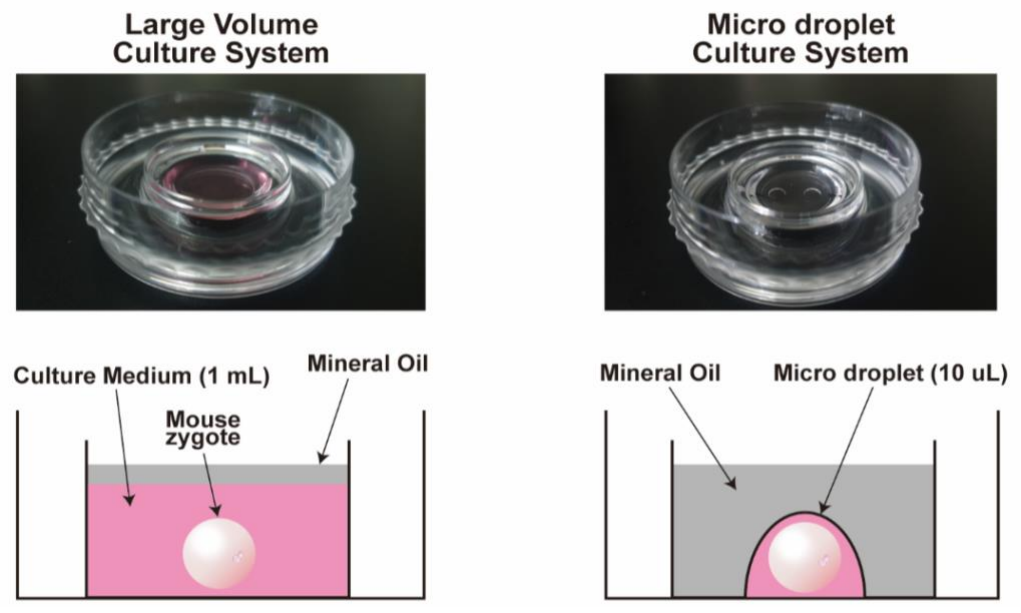

Figure 4: Two types of culture system; large volume culture system and micro-droplet culture system.

1.2.1.2. Comparison between culture media

Among three media used to culture embryos in conventional large volume culture system (F-10, KSOM, KSOM + 200μM EDTA), KSOM with 200μM EDTA showed the highest blastocyst development. Among92 zygotes, 57.6% developed into a blastocyst in this media. Whereas F-10 and KSOM media alone showed '2-cell block' and 'morula block', respectively. Only 0.4% of the 501 zygotes in F-10 media developed to blastocysts. Similarly, in KSOM media, only 16.7% of 508 zygotes developed to blastocysts which were 40.9% less than the blastocysts development rate in KSOM media with 200μM EDTA ($p < 0.01$).

1.2.2. Experiment 2: micro-droplet culture system

1.2.2.1. Micro-droplet culture system optimization

Among the different micro-droplet culture volumes (6.25, 10, 12.5 and 25μl) used for embryo culture (six embryos/droplet-triplicate readings), the largest culture volume (25μl) of KSOM media showed the lowest blastocyst development rate. Despite the fact that a quarter (25.2%) of embryos survived up to the morula stage, only 8.3% survived to the blastocyst stage. Similarly, the lowest culture volume (6.25μl) also weakly supported the development of embryos to blastocysts (18.3%). 10μl and 12.5μl showed the highest blastocyst development rates (25.0%) as shown in Figure 5b. Therefore, we chose a 10μl culture volume for EDTA optimization for further studies. A total number of 18 embryos were used in each case. Like the conventional large volume culture system, different concentrations of EDTA were added to KSOM media to culture mouse embryo in 10μl micro-droplet and a similar growth pattern was seen in stages preceding the morula stage. Most of the 10μl micro-droplets made of the KSOM with different EDTA concentrations

showed decreased ‘morula block’ and increased blastocyst development rates as compared with the 10µl micro-droplets of KSOM media alone. Among these EDTA concentrations, 73.9% of 45 zygotes developed blastocysts in culture media with 200µM EDTA making it optimum culture concentration. KSOM media alone developed 29.6% of 40 zygotes to blastocysts which were 44.3% ($p<0.01$) less than KSOM+200µM EDTA medium as shown in Figure 5c. In Figure 5b, after the optimization of EDTA concentration, the effect of different droplet volumes (6.25, 10, 12.5 and 25µl) was further evaluated with KSOM + EDTA (200µM) simultaneously (n = 18 each). In four different volumes of culture media, the pattern of growth was similar to that of KSOM media alone, however, with a higher rate of blastocyst development. Blastocyst development rates in the lowest and highest culture volume (6.25, 25µl) in KSOM+EDTA (200µM) media were 41.6%. The difference in the blastocyst development rate of the two media (KSOM with 200µM EDTA versus KSOM media alone) in 10µl micro-droplet was 48% (73.9% versus 25.9%), making it a suitable volume size for embryo development ($p<0.01$).

1.2.3. Comparison between culture systems

As shown in Figure 5d, the rate of blastocyst developments in the micro-droplet culture system was seen to be higher than in the conventional large volume culture system after 96 h of culture. The blastocyst development rate in EDTA optimized media was improved by 16.3% (73.9% versus 57.6%) in 10µl micro-droplet culture system ($p<0.05$). However, the optimum concentration of EDTA was the same (200µM) in both culture systems. Blastocyst development rate in KSOM media alone was also higher in the micro-droplet culture system compared with conventional large volume culture system by12.9% ($p < 0.05$).

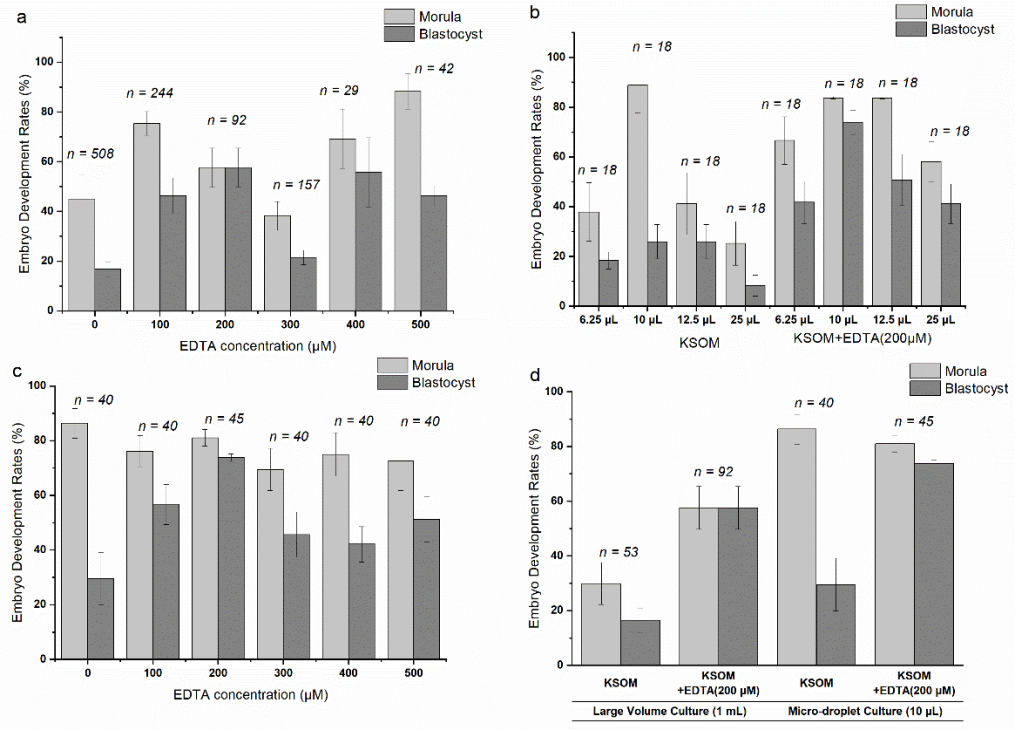

Figure 5: Charts of morula and blastocyst development rates (a) Morula and blastocyst development rate of embryos cultured in KSOM (0) and different concentrations of EDTA (0-500μM) in large volume culture system (1ml), (b) development rate of morula and blastocyst in different droplet media volume in KSOM alone and EDTA optimized KSOM media, (c) development rate of embryos in KSOM and EDTA optimized KSOM media in 10μl micro-droplet culture system, and (d) comparison of morula block and blastocyst development rate in two culture systems with KSOM media alone and KSOM media with 200μM EDTA.

1.3. Discussion

Optimal media condition for the highest blastocyst differentiation rate and its viability still has a gap to be filled. The culture process and the media involved in the culture affect a lot for the development and viability of embryo in *in vitro* culture system. This is why the optimization of *in vitro* culture is considered a fundamental requirement in artificial reproductive technology (ART). For these purposes, before directly implying in human specimens, murine models have been used widely in IVF and culture for the detailed study of physiology and molecular events. Especially, murine embryos have been the number one choice for the research and monitoring of culture conditions as they exhibit similarities in nutritional requirements and developmental milestone with human embryos. The survivability and development rate of the embryos depends on the availability and also the composition of the media involved in each stage of embryo development. Present study experimented with F-10 media, KSOM media, and KSOM with addition of EDTA. As already known, EDTA in culture media acts as chelating agent and helps for better development and differentiation of zygotes to blastocyst. The optimum concentration of EDTA used varies with the study as shown in Table 3. Some of the study has shown the optimum condition to be 10μM whereas some has suggested as 100μM (24-28). The concentration of EDTA used might even differ according to the culture media used. Therefore, the efficient range of EDTA concentration is huge. However, our experiment tried to find out the optimum range of EDTA in KSOM media using ICR mice embryos. Unlike Matsukawa *et.al.*(26), we studied the EDTA concentrations ranging from 0 -700μM in detail which showed optimum range as 200 μM when cultured in both large volume and micro-

droplet culture system.

The result of the present study showed the better development of zygote to blastocyst when cultured in KSOM media along with EDTA. Whereas the embryos cultured in F-10 and KSOM media alone showed the 2-cell and morula block respectively. Significant increase in cleavage rate were observed in EDTA optimized media. Embryos were cultured in the presence of EDTA from day 0 to day 5. The decrease in glycolytic activity of 2- cell stage in mice embryo explains the beneficial effect of EDTA used (29). Some of the previous studies have shown the continuous supply of EDTA in culture media for more than 72 hours is detrimental to the bovine embryo due to the less glycolytic activity (30). Some of the experiment also showed the suppressing effect of EDTA on one cell cleavage but showed higher proportion of 2-cell stage cleavage to blastocyst (18, 31). However, improvement of blastocyst formation and hatching rates in the presence EDTA are also explained in some paper (24, 32). We identified similar beneficial effect of EDTA in the present study despite the longer incubation hours and cleavage stage. Two distinctive peaks were observed when embryonic development rate was plotted from 0-700μM concentrations of EDTA. The second peak at 500μM EDTA concentration showed appropriate amount of embryo development up to morula stage but decreased the number of blastocyst development. However, first peak in comparison with the second one resulted into higher number of blastocyst development where EDTA concentration was equal to 200μM. Nonetheless, reason for fall and rise in embryonic development were not studied. However, the rate of blastocyst formation was seen higher in micro-droplet culture than in large volume culture system by 16.0 %.

Table 1: Different concentrations of EDTA used in different media for embryonic development.

S.N.	Mice Strain	Media	EDTA concentration (µM)	Media volume (µl)	Blastocyst % (hatching &hatched %)	Embryos number in a group	References
1	CD1	EMM	No EDTA	50	88.2 (81.9)	9	(33)
2	Bovine embryo	SOFaa	100	500	43.2	50	(30)
3	CBA/Ca x C57Bl$_6$ F1 hybrid	Amino acid and glutamine free KSOM	10	20	43.8 (hatching) (123 hrs. post hCG)	10	(24)
4	Outbred mice (OF1)	M16	20		0		
		WM	20		10.2	20	(27)
		CZB	110	12	40.32		
		KSOM	10		45.5		
5	B6CBF1	KSOM	10	50	65.8	15-20	(25)
		CZB	100		91.7		
6	Outbred CF1	KSOM	10	50	88.1	5 or10	(34)
		CZB	100		52.5		
7	ICR	WM	10.8	2000	70	NA	(14)
8	ICR	P-1	0.1*		78 (33)	14-10	(35)

				5			
9	ICR	P-1	100	5	63.8±5.3	15-20	(36)
10	ICR	KSOM	EDTA in media	100	87.5 (hatching)	80	(37)
11	ICR	KSOM	10		82		
			100	50	85	NA	(26)
			1000		3		

Our study found 10 µl droplet media with 200 µM EDTA in KSOM as the perfect culture condition than in higher media volume (1 ml, 25 µl) and minimum media volume as 6.25 µl for ICR mice embryos. It can be explained by the fact that embryo quality improves when the embryos are cultured in reduced volume or increased embryo density(33, 38-43). It has been proposed that the production of growth factors like autocrine and paracrine mutually benefit embryos in group culture(44, 45). However, minimal volume of culture media can also be detrimental to the embryos due to toxic metabolite production. Although, EDTA has beneficial effects, its effects are constrained to certain stage of embryo cleavage (15, 30, 32). Culture of embryo in morula and blastocyst stage with EDTA both reduces the development of inner cell mass(30) and development of foetal after transfer to uterus(15). It has also been reported to alter mouse metabolism (29). No studies have been reported on the effects of wide range of concentration of EDTA on KSOM media.

This study optimized the culture media for outbred mice embryo using EDTA in both higher media volume and micro-droplet culture system. However, optimized micro-droplet culture system facilitates higher blastocyst development. Zygotes were cultured in F-10, KSOM and KSOM with different concentrations of EDTA in large volume culture system (1 ml). Among the various media used, the one with KSOM and EDTA concentration equal to 200 µM were found to be effective with highest blastocysts development rate. However, in micro-droplet culture system embryos were cultured only in KSOM or KSOM with different concentrations of EDTA ranging from 0-500 µM. Optimum concentration of EDTA in both culture systems were found to be 200 µM. This result suggests KSOM media with additional EDTA can be used to overcome the 2- cell block as well as morula block more effectively

in outbred mice like ICR. Nevertheless, even in between two culture systems, micro-droplet culture system of volume 10 µl with 200 µM EDTA concentration were found to be more productive with higher number of blastocyst development. In summary, 1. Commercially purchased KSOM medium was optimized 2. using wide range of EDTA from 0-700 µM concentration 3. in different culture volumes (1 ml & different sized micro-droplets) 4. for preimplantation of ICR mice embryos.

Thus, the mice embryo culture media, KSOM, was optimized by using chelating agent EDTA in minimal culture volume of 10 µl to enhance the blastocyst development rate in ICR mice for the first time. This study might help in the use of ICR mice not only in fields like toxicology and pharmacology but also in embryology which might also improve the human embryo culture technique as ICR mice are supposed to relate to the genetic diversity of human as well.

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Chapter 2: Optimization of IVF culture media using somatic cells

2. Introduction

Culture media have vastly improved since the first successful IVF for both human and animal models. However, with new technologies and viability assessments, there remains the possibility for further refined media formulations which benefit embryo development *in vitro* improving the existing clinical outcomes. The modifications of embryo culture media to increase the in vivo-like contact between the developing embryo and the female reproductive tract can go a long way in the field of artificial reproductive technology (ART). Numerous studies have revealed that several species including human itself benefits from embryo-somatic cell co-culture (1-4). The attempt to improve the development and viability of mammalian preimplantation embryos with somatic cells and many other conditioned media *in vitro* is still in practice. However, mimicking *in vivo* culture conditions to develop a perfect *in vitro* culture condition is a never-ending process. Embryo-somatic cell co-culture is believed to manifest the *in vitro* fertilization (IVF) procedure with a faster cleavage rate, overcoming blocks; “2-cell block” and “morula block”, increasing blastocyst cell numbers, improving morphological appearance, expanding blastomeres, and increasing viability of

embryo following escalating transfer rate (5-9). Different somatic cells like endometrial epithelial cells, endometrial stromal cells, embryonic stem cells, embryonic fibroblast cells, granulosa cells, ampullary cells, cumulus cells, etc. (10-13) are in use to co-culture early embryos. Such helper/feeder cells secret an embryo trophic factors like transforming growth factors, insulin-like growth factors (IGF), epidermal growth factors eventually enhancing the quality of the overall embryo (14). It has also been postulated that the helper/feeder cells metabolize the glucose present in culture media which allows embryos to be exposed to tolerable levels of glucose, thus overcoming the cell blocks (15). In addition, the co-culture system is supposed to have a detoxification mechanism that removes the reactive oxygen species (ROS) from the culture media leading to overcoming developmental blocks (16). Among all the helper cells, cumulus cells are routinely available during an IVF cycle which makes it convenient for co-culture. Cumulus cells (CCs) are the group of closely associated granulosa cells that surround the oocytes to form the cumulus-oocyte complexes (COCs). CCs have close physical contact with the oocyte and thereby are exposed to high levels of oocyte-derived regulatory factors that control their function. During the expansion of COCs, CCs get embedded within the expanded extracellular matrix and produce various cytokines and inflammatory factors that get released from the ovary along with oocytes during ovulation (17). CCs also actively take part in the process of oocyte maturation and fertilization (18-20). Therefore, cumulus co-cultured embryos pose the ability to increase implantation rates and increase pregnancy rates as well (15). Cumulus cells protect the oocyte during ovulation and direct it into the oviduct. It also prevents unfavorable changes that affect the fertilization in oocytes in vivo (21, 22). The embryo further benefits from

cumulus cells co-culture *in vitro* because cumulus cells secrete embryotropic factors such as insulin-like growth factors I and II (23), epidermal growth factors (EGF) (24), leukemia inhibitory factors (LIF), and interleukins 1&6 (25), among other factors. Cumulus cells also secret vascular endothelial growth factor (VEGF) which increases uterine receptivity for implantation (26). Thus, it makes a good contender for co-culture embryos *in vitro*.

On the other hand, the ability to select the perfect embryo with the highest implantation potential remains a major challenge among IVF patients. The advancement in this specific area could minimize the risk of multiple pregnancies along with the associated maternal and fetal complications (27). Since the beginning of IVF, methods to evaluate embryo viability have continuously evolved. This is important now more than ever as the worldwide adaptation of elective single embryo transfer (eSET) is increasing (28). eSET has significantly reduced twin pregnancy (29) but also has decreased the number of IVF infants over the years (30, 31). Assessing the embryo quality before the transfer can lead to the rise in successful IVF again. Embryo quality has long been considered an important determinant of successful implantation and pregnancy. There are currently invasive and non-invasive types of technologies to select viable embryos. Embryo grading according to its morphology is well known and common non-invasive technology. Blastocyst grade is closely associated with the success of blastocyst transfer. Blastocysts graded as good show higher implantation rates compared with embryos graded as poor quality (32, 33). Morphological embryo assessment has been enhanced with the introduction of morphokinetics analysis using the Timelapse monitoring (TLM) system in recent years. It is one of the exciting and potential non-invasive technology that can be used for effective embryo selection (34, 35). Whereas,

cleavage and blastocyst stage embryo biopsy is invasive techniques which is useful for genetic testing (36) but have high chances to harm the embryo during the process. The use of morphokinetics technology to assess embryos has enabled us to note the embryo differentiation time leading to the selection of fast-growing embryos. Faster-grown embryos during *in vitro* fertilization increase the chances of pregnancy with the greatest implantation potential (37, 38). The uninterrupted culture with a time-lapse system also produces better blastocyst formation rates with lower embryo arrest rates (39). In addition, time-lapse incubators are considered better as higher pregnancy rates were achieved after the embryo transfer cultured in time-lapse incubators than standard incubators (40).

This chapter of thesis aimed to optimize the culture media by modifying the culture media components using EDTA as well as co-culturing with cumulus cells in one setting for outbred mice embryos. Different concentrations of EDTA were mixed in culture media and 2-cell embryos were co-cultured with monolayers of cumulus cells until the development of blastocysts. Embryo developments were recorded in a culture condition undisturbed by a time-lapse monitoring system. Blastocyst development time was compared in different co-culture conditions and the quality of the embryo was also analyzed to select a viable embryo.

2.2. Materials and Methods

2.2.1. Animals

Outbred ICR mice from Hana Sangsa Corporation (Republic of Korea) were used in this experiment. Mice were housed in 12h light/dark cycle with an unlimited supply of food and water. All procedures for mouse care and use were conducted in accordance with the

guidelines and approved by the Institutional Animal Care and Use Committee of the School of Medicine, Keimyung University (Mice IRB no. KM-2020-01R1).

Female mice (age 7-9 weeks) were superovulated with 7.5IU of pregnant mare's serum gonadotropin (PMSG; Sigma- Aldrich, G-4877) followed by 7.5IU human chorionic gonadotropin (hCG; Life Sciences, 367222-1000IU) 48 h later. Females were mated with the same strain of male mice (age 10-12 weeks) on the same day of hCG injection. Super ovulated females with vaginal plugs were sacrificed after 16-18h and oviducts were collected in M2 media (Merck, M7167) with a 10% serum substitute supplement (SSS; Irvine Scientific, 99193). COCs were then isolated from oviducts.

2.2.2. Preparation of CCs

CCs clumps and oocytes were separated manually from COCs using two 0.5 ml insulin syringes (BD Insulin syringe, Republic of Korea) in regular M2 media. Harvested lump of CCs was dissociated using hyaluronidase from bovine testes (Sigma Aldrich, H3506) and approximately 1.43×10^3cells/ml were seeded in DMEM/F-12 Ham's media (Merck, D6434) with 10% FBS. The culture media was changed to KSOM with 10% SSS (Merck, MR-121-D) after 3-4h when cells were attached to the culture dish surface. The co-culture with embryos proceeded the following day.

2.2.3. Oocyte recovery

After the separation of CCs and oocytes, oocytes surrounded by remaining CCs were denuded by pipetting with a thin pipette in M2 media containing hyaluronidase. Denuded oocytes were collected and cultured in KSOM media with 10% SSS until the next day up to

2 – cell division. All the media were equilibrated overnight before culture began. Embryos with 2 – cells were then collected and co-cultured in respective media conditions.

2.2.4. Embryo co-culture with CCs

For *in vitro* co-culture of oocytes and CCs, KSOM media with 10% SSS were used with and without different concentrations of EDTA (Irvine scientific). 10μM, 50μM, 100μM, and 200μM EDTA concentrations were prepared for culture. The rate of CC development in different EDTA concentrations was examined. The EDTA concentration which supported the CCs growth more favorably was used for further experiments. Four to six droplets of 10μl media volume were made in a 6 - well culture plate (Thermo Fisher Scientific; 140675) covered with Ovoil (Vitrolife, 10029) for culture. 2 – cell embryos were cultured in various culture conditions; only KSOM, KSOM+EDTA, KSOM+CCs, and KSOM+EDTA+CCs. The culture was carried out in a humidified environment of 5% CO_2 at 37°C for 5 days continuously until the embryos developed into blastocysts. After the development of 2-cells staged embryos, all embryos were gathered in one collection droplet and were equally and randomly divided into 10 embryos per group for culture.

2.2.5. Embryo morpho kinetics and blastocyst grading

Non-invasive time-lapse imaging was taken using Lionheart FX automated microscope every 2h using a camera system that utilized bright-field microscopy (Bio Tek Instruments Korea Ltd.). The time of blastocyst formation from the two-cell stage was recorded. 23h time-lapse imaging was taken separately to determine the development of 2-cells from the zygote. The number of embryos reaching the blastocyst stage after 120 hours of culture was

examined. Blastocysts were classified into 3 grades simply based on the degree of expansion and hatching status (41). Blastocysts with blastocoel equal to or less than half (1/3) of the embryo were graded as Grade I. Similarly, a large blastocyst with a blastocoel greater than half of the embryo volume and hatching embryos were considered Grade II and Grade III blastocysts respectively, grade III blastocysts being the best.

2.2.6. Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) staining

TUNEL assay was performed using an In Situ Cell Death Detection Kit, TMR red, according to the manufacturer's protocol (Roche Diagnostics Corp.). Fixed embryos were washed with polyvinyl alcohol (PVA) -PBS and permeabilized with Tyrode's solution. Permeabilized embryos were washed again and incubated with fluorescently conjugated TUNEL at 37°C in dark for 15-20 min. TUNEL stained embryos were washed again and incubated with 10μg/ml Hoechst 33258 for 10 min. Embryos were washed twice again and were mounted on a glass slide in a drop of vectashield plus. Leica Stellaris confocal microscope (Mannheim, Germany) was used to determine the number of cells per blastocyst by counting Hoechst-stained cells (blue) and apoptotic cells (TUNEL positive-red cells).

2.2.7. Statistical analysis

For descriptive statistics, mean ± standard error of the mean (SEM) was used. A two-tailed t-test was performed to compare the embryo development in different culture conditions. A value of $p \leq 0.05$ was considered statistically significant.

2.3. Results

2.3.1. Development of 2-cell staged embryo

The zygotes in our settings started to develop into 2-cell staged embryos from 12h which is similar to the previously reported timeline (42). The development process is shown in the video attached (Supplementary). The co-culture with cumulus cells was initiated the next day after all the embryos were developed into 2 cell stages and cumulus cells reached confluency.

2.3.2. CC culture and its viability in different culture conditions

Upon the examination of different concentrations of EDTA on CC growth, a lower concentration of EDTA (10μM EDTA) supported the growth for a longer period as compared to others. The CC growth increased up to the third day of culture and gradually decreased as the culture day increased. Previously, without the co-culture of CC, embryo development was seen higher in 200μM EDTA (43). However, this concentration along with 100μM and 50μM concentrations of EDTA supported the CCs growth little to none (Figure 6). The CCs survivability was higher in descending order of EDTA concentrations, making the lowest concentration of EDTA (10μM) the most suitable for co-culture. Nonetheless, the KSOM media without EDTA showed the highest CCs confluency. Further experiments were done with CCs in KSOM media with and without the presence of EDTA.

2.3.3. Blastocyst development is favoured by CCs

Among different culture conditions, the blastocyst development was more favourable in

presence of CCs and even more in the combination of CCs and EDTA. Even though the KSOM media alone supported the viability of CCs, the co-culture of embryos was not as higher as the KSOM media with 10µM EDTA. The blastocyst development in KSOM media with EDTA and CCs was 85±2.88 whereas, in KSOM and CC media without EDTA was 75±2.89 (p<0.04). However, the development in KSOM media with CC was higher than in both KSOM media alone and KSOM media with 10µM EDTA by 42.5% (p<0.003) and 5% (p-value- not significant 0.3) respectively. Similarly, the blastocyst development in KSOM media with CCs and 10µM EDTA was higher than in KSOM media alone by 52.5% (p<0.001) and by 15% (p<0.02) in KSOM with 10µM EDTA. Also, the 10µM EDTA in KSOM media improved the blastocyst development by 37.5% (p<0.003) than KSOM media alone (Figure 7 a). However, there was no significant difference between the co-culture with CCs alone and with CCs and 10µM EDTA.

2.3.4. Embryo kinetic

The time taken by embryos to develop from each stage to the next stage was calculated according to the video taken during the period of co-culture. Embryo culture with CCs promoted the early development of the embryo to blastocysts.

The embryo development was observed for 96 hours starting from 2-cells in a co-culture. The time taken varied from developmental stages to culture media used. There was a significant difference between the development time of KSOM with CCs and KSOM+CCs+10µM EDTA in early cleavage stages from 3-cells to 4-cells (3.5h) (p≤0.03). Similarly, in the same developmental stage, KSOM+CCs also showed a significant

difference with the developmental time of KSOM media alone (2.1h) (p<0.04). From 4-cells to 8 cells development, only KSOM+CCs+10μM EDTA showed a significant difference of 5.45h with KSOM media alone (p<0.03) (Table 2). Besides these, the other developmental stages in all culture media did not show any significant time differences.

Similarly, in the later stages of development, embryos in KSOM media with EDTA and CCs developed faster to blastocyst stages as compared to embryos in other groups. Blastocysts in this co-culture system developed 16.25h (88h vs. 71.75h) faster than in KSOM media alone (p<0.01). Similarly, the blastocyst development rate in KSOM media with only CCs was 13h (88h vs. 75h) faster than in the KSOM media alone (p<0.03).

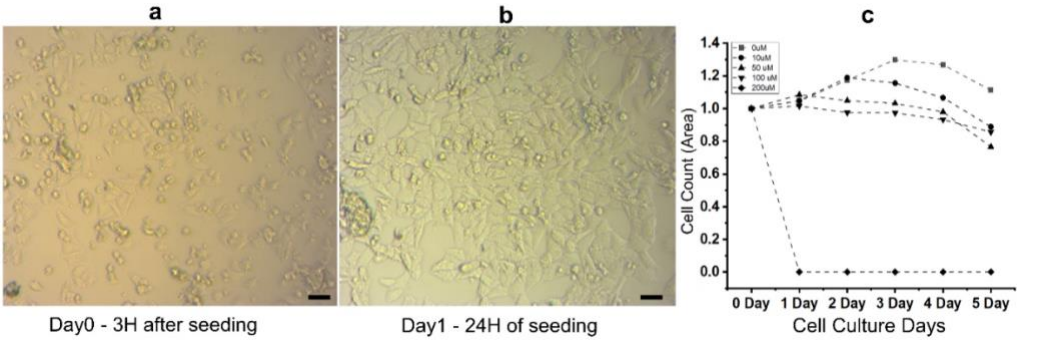

Figure 6:Cumulus cells viability a. Media change and starting of attachment of cells after 3h of seeding. b. Confluent cells after 24h of cell seedings and c. The survivability of cumulus cells in different concentrations of EDTA. Scale bar = 20µm.

However, between other groups, there were no significant differences in the development time and blastocyst formation (Figure 7c). The time taken for hatching embryo to get hatched was less in KSOM media alone (6.667h) as compared to other media, however, no significance was found. The longest time taken to get hatched was by hatching embryos in KSOM with CCs and EDTA (15h) co-culture media. The time to develop certain developmental stages was higher in co-culture media with EDTA and CC, however, the average time to develop blastocyst was still less compared to other culture conditions.

2.3.5. Blastocyst grading

Mostly Grade 2 embryos were seen in all conditions of embryo culture conditions. Among 164 total blastocysts examined, 89 of them were of grade 2 quality whereas, 38 of them were grade 3 qualities followed by 31 blastocysts in grade 1 (Figure 8). Embryos cultured in KSOM with CC and EDTA (n=41) showed highest number of grade 3 blastocysts (34.146%, n=14) followed by embryos cultured in KSOM+10µM EDTA (n=40) without CCs (22.5%, n= 9). The highest number of grade 2 blastocysts were also seen in KSOM+CC+10µM EDTA (60.976%, n=25) followed by KSOM only (n=41) (56.098%, n=23). Also, the highest number of grade 1 blastocysts were seen in KSOM media (24.390%, n=10) followed by blastocysts developed in KSOM with CC (23.809%, n=10). The lowest number of grade 1 blastocysts were observed in KSOM+CC+10µM EDTA culture media (4.878%, n=2).

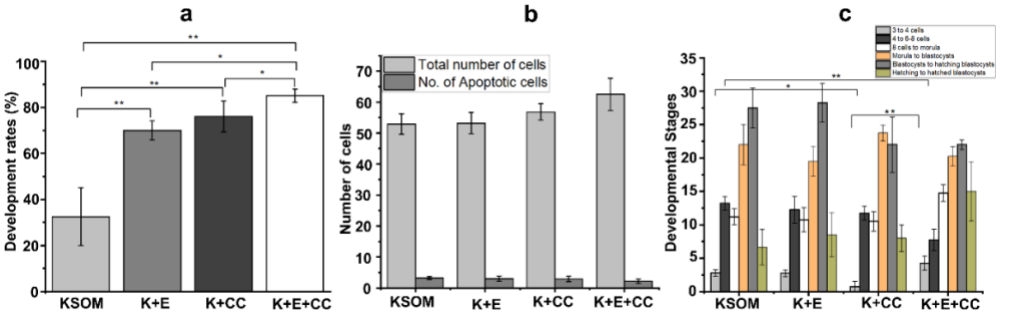

Figure 7: (a) Blastocyst development rates (%), (b) Total number of cells and apoptotic cells, and (c) morphokinetics of different stages embryo. ** $p \leq 0.03$, * $p \leq 0.05$.

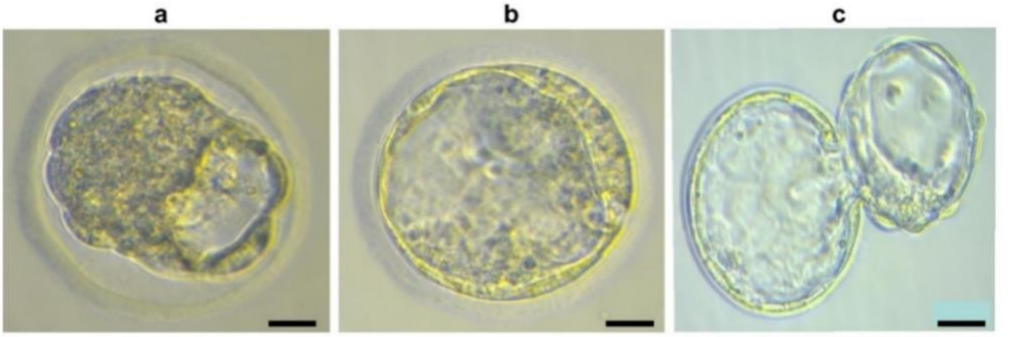

Figure 8: Classifications of blastocysts. a. Grade 1 blastocyst with blastocoel equal to or less than half of embryo volume, b. Grade 2 blastocyst with blastocoel greater than half of the embryo volume or blastocyst with a blastocoel completely filling the embryo and, c. Grade 3 embryo, hatching or hatched embryos. Scale bar= 10μm.

2.3.6. Determination of the total number of total cells and apoptotic cells per blastocyst

The total number of cells and apoptotic cells were counted using TUNEL assay. Lowest number of apoptotic cells (2.2±0.712) with highest number of total cells (62.5±5.235) were seen in co-culture system with CCs and EDTA followed by co-culture system without EDTA. The highest apoptotic cells were seen in control group (3.2±0.489) followed by KSOM with EDTA (3±0.84327). TUNEL assay showing apoptotic cells in different groups are shown in Figure 9.

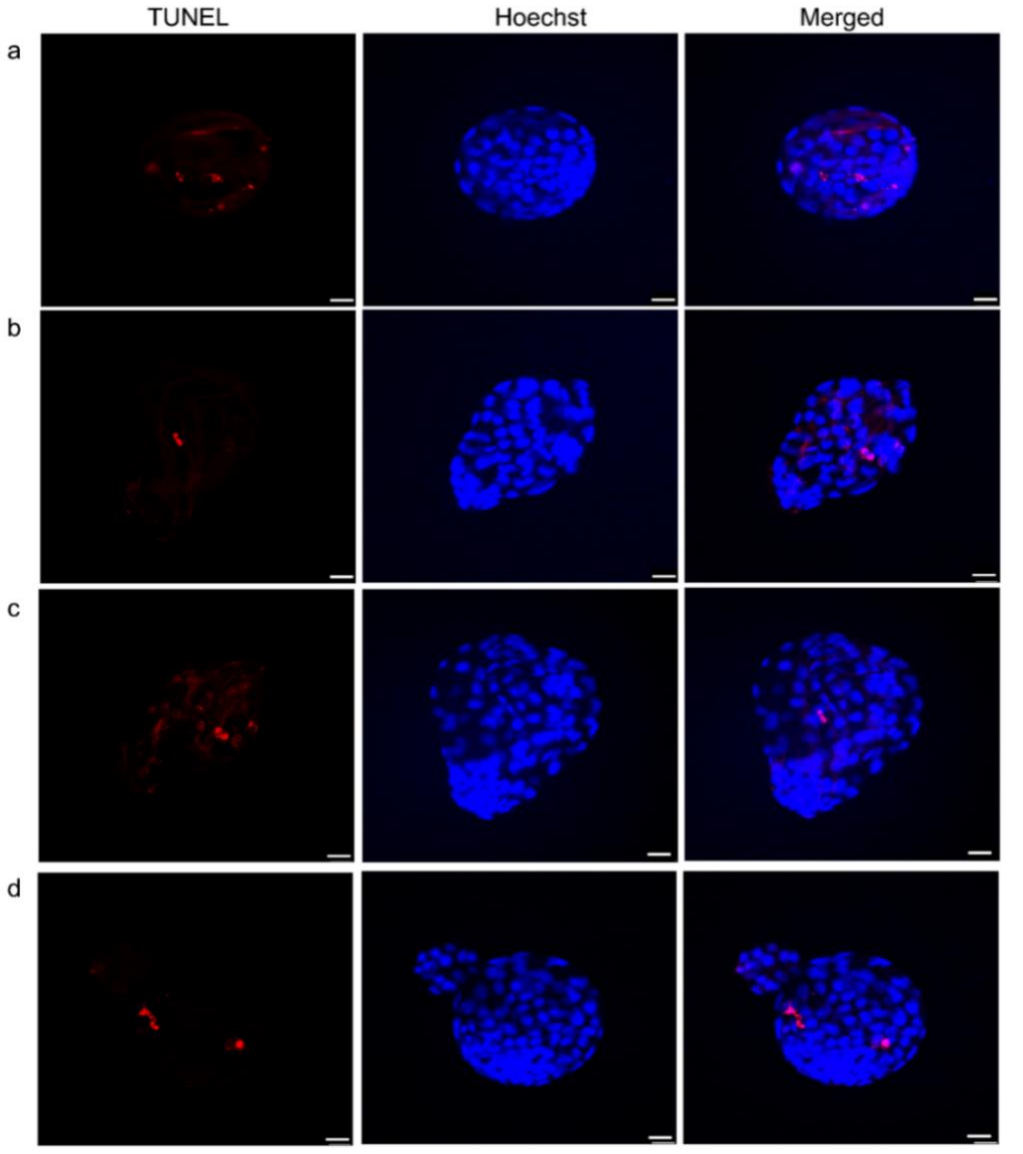

Figure 9: TUNEL assay showing apoptotic cells in control (KSOM) and other EDTA and CC treated blastocysts. Scale bar = 20µm. a. KSOM, b. KSOM+10µM EDTA, c. KSOM+CC, d. KSOM+CC+10µM EDTA.

2.4. Discussion

Somatic cells take a crucial part in embryo development from maturation to fertilization and development *in vivo*. However, it is still a struggle to mimic the same environment while developing an embryo *in vitro.* In our study, we tried to mimic the *in vivo* like development by co-culturing the embryos with cumulus cells that surround the embryo throughout the development. The role of mammalian cumulus cells during oocyte growth and maturation is long proven. In the present study, the co-culture of embryos with CCs showed a significant increase in blastocyst development rate than in embryo culture media, KSOM alone (p<0.003). The higher development rate in the co-culture system might be due to the embryotropic factors secreted by cumulus cells during the development. The blastocyst development in our co-culture study was also higher (76% vs. 47%) than in previously done experiments by Farouk *et al* (44). However, the blastocyst development in KSOM culture media is lower (54.6% vs 32.5%) to the study done by Mancini *et al* (45)**.**

In the present study, the chelating agent; EDTA, was also added to the co-culture media to examine the blastocyst development as the 200µM EDTA has shown significant growth in blastocyst development in a previous study (43). However, the 200µM EDTA was not used for the co-culture procedure as CCs didn't survive in that concentration. The other concentrations, lower than 200µM of EDTA (100µM, 50µM & 10µM) were tested on CCs which lead to 10µM to be an optimal EDTA concentration for co-culture. (Define why cc died but not embryo in 200 µM EDTA concentrations). The EDTA concentration below 10µM was not used for the experiment as the blastocyst development below that

concentration without CCs was less. An experiment done with 5μM EDTA (n=30) with KSOM only supported blastocyst development to 50±0.57735% whereas, the blastocyst development rate in 10μM EDTA was 70±4.082%. Therefore, 10μM EDTA was used for the embryo co-culture with cumulus cells. The co-culture of an embryo with cumulus cells and 10μM EDTA improved the blastocyst development rate (85±2.887%) with a higher number of hatched and hatching embryos (Grade 3). This co-culture system increased the blastocyst development rate by 52.5% (p<0.001) than in KSOM media alone (85% vs 32.5%). The highest number of good-quality embryos based on their morphology were also seen in a co-culture system with EDTA and CCs. Expanded blastocysts (Grade 2), as well as hatching and hatched blastocysts (Grade 3), were seen in the highest number in this co-culture system (60.975% & 34.146% respectively). The high number of blastocyst development and good quality embryos in the co-culture system might be because of the beneficial effect of both EDTA and cumulus cells in overcoming cell blocks that occurs especially in outbred strain mice. The CCs have the factors that help to improve fertilization as well as accelerate cleavage divisions along with 4-cell and 8-cell stages of the development (6, 46). The expression of some growth factors and cytokines by CCs are also known to improve embryo morphology with reduced cellular apoptosis. Many experiments regarding the co-culture of CCs are performed in humans too where, the beneficial effects of CCs co-culture in embryo development are explained (6, 47). The implantation rate in presence of CCs is said to be high as they nurture the embryos during *in vitro* development (22, 48). However, in humans, it is recommended to use the autologous CCs because the protein expression profiles of cumulus cells from different patients are found to be different

in both groups of patients with the same and different stimulation protocols (49).

Further, the embryo development time was analyzed in all four culture groups. The embryos were cultured in a group of 10 embryos per 10µl droplet (1µl/embryo). Zygotes were cultured in KSOM media for 24 h which started to develop into a 2-cell stage after 12 hours of culture. 2-cell staged embryos were then randomly divided into different culture conditions and the embryo morphokinetics were recorded using Lionheart FX automated microscope every 2 h. The morphokinetics of the embryo in this study were recorded for 120 h which showed the development of blastocysts earlier in co-culture media with CCs and 10µM EDTA as compared to the other culture conditions, especially KSOM media alone (p<0.01). Also, the embryos co-cultured in KSOM and CCs without EDTA showed a significant difference in development time as compared to embryos developed in KSOM media alone (p<0.04).

The use of a morphokinetics system can be used in the predictive ability to determine embryonic aneuploidy. This further helps to select the embryos that are less prone to carry chromosomal abnormalities and generally improve the *in vitro* fertilization and birth success rate. The embryos that reach the blastocyst stage are less prone to be aneuploid and have higher implantation rates after transfer (50). Morphokinetics also help to select the earlier cleaving embryos which are more likely to cleave uniformly, which is correlated with a low incidence of chromosomal errors like aneuploidy and have a better chance to develop into blastocysts and eventually implant (51). The embryos with direct cleavage from 1 to 3 cells or very short time as 2- a cell stage embryo, uneven blastomeres at 2-cell stage, and

multinucleation at the 4-cell stage are considered bad embryos with minimal chances of implantation and maximum chances of chromosomal abnormality (38, 52). In our study co-culture with CCs only showed very little time for the development of 3 cells to 4 cells, however, we did not examine the possibility of implantation. Also, the unevenly sized blastomeres are prone to implantation and pregnancy rates with a high possibility of chromosomal defects (53). The multinucleated embryo in our study as well didn't develop into blastocysts (data not shown). Some of the studies have hypothesized that healthy euploid embryos develop faster than unhealthy or aneuploid embryos (54). Also, the same research group hypothesized that the blastomeres with XY chromosomes develop fasters than the blastomeres with XX chromosomes because the X chromosome is larger than the Y chromosome. Time-lapse kinetic parameters can be used to predict the blastocyst formation and can further assist in the morphological assessment of viable embryos ultimately leading to successful IVF birth rates (55). Detection of early cleavage embryos is also beneficial to improve the pregnancy rate in IVF patients (56). However, there is a controversy regarding fast and slow-growing embryos. Some consider fast-growing embryos to resemble the in vivo like timeline, whereas some consider slow-growing embryos are less prone to the loss of genomic imprinting. The paper by Velker et. al. compared the development rate of the embryo obtained from naturally mated females at a two-cell stage which upon comparing many factors found out that the slow-growing embryos are better (57).

Even though the 2-cell staged embryos were randomly distributed in different culture conditions regardless of the cleavage time, the rate of blastocyst formation time in EDTA

and CCs co-culture was high which suggests the high probability of beneficial effect of CCs and EDTA in combination. It might also be because the EDTA is also supposed to have antioxidant properties which eliminate the formation of reactive oxygen species (ROS) which influence the development of early mammalian embryos *in vitro* (58). EDTA is also supposed to induce antioxidant and anti-inflammatory activities in many clinical settings (59). Olson *et al* showed the beneficial effect of EDTA even in as low concentrations as 3μM (60). It is also suggested that the EDTA can be cytotoxic in a dose-dependent manner but is not genotoxic (61) which can be the explanation for the death of cumulus cells in higher concentrations but the improvement in blastocyst formation. According to Basile et al., the types of culture media used do not affect the morphokinetics parameters that are used to select an embryo for transfer (62). The study was done in sequential and single culture media. However, according to our study, the co-culture system affected the time taken by the embryo to develop into a blastocyst. However, there were no significant changes in developmental time of different stages like 4-cells to 8-cells in different culture conditions, it might be because of the insufficient media for both CCs and embryos and may also be due to the toxicity caused by the CCs death as the media used throughout the experiment is single media which was not changed from 2-cell embryo culture to the formation of blastocysts. However, the overall blastocyst formation time was higher in the co-culture system with 10μM EDTA and CCs. A further experiment regarding such a hypothesis was not done in this study. Also, the implantation rate of success in the birth rate was not analyzed in this study.

The co-culture system has slowed down in the last few years as the use of non-autologous

cells was considered unsafe in 2002. However, some of the studies still show that the co-culture system should be reintroduced to mimic the *in vivo* culture condition (63) as CCs co-culture and its aided embryo transfer increase the pregnancy rates in patients undergoing *in vitro* fertilization (64). This study showed the highest blastocyst development in a co-culture system with CCs and 10μM EDTA. The morphokinetics of the embryo also showed the faster development of blastocyst in the co-culture system than in the control, KSOM media alone. In addition, the morphological grades of the embryo were also higher in a co-culture system. This information can be useful in clinical settings as well to increase the successful implantation rate.

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Chapter 3: Metabolic analysis of single embryo for quality assessment

3. Introduction

Along with the optimization of culture media to culture embryo *in vitro*, it is also important to identify the quality of embryo. In this chapter of thesis, an assay system to identify the quality of embryo is optimized. Optimization of specific and sensitive enzyme assays is crucial in all areas of basic and clinical research. One may need to quantify cellular levels of proteins, determine metabolites in serum, and compare normal and diseased tissues by measuring catalytic activities of enzymes, i.e., the extent to which they increase the rate of a given chemical reaction. As enzyme activities depend on the conditions under which they are determined, it is necessary to develop specific assays considering multiple factors such as pH, ionic strength of the reaction mixture, buffer compositions, concentrations of enzyme and substrate, and reaction time and temperature. Here, we optimized an assay for simultaneous measurement of glucose and lactate in important biological samples.

During the preimplantation development of an embryo, lactate, and glucose along with pyruvate act as important sources of energy. Early mouse preimplantation embryo development relies on pyruvate and lactate to feed the tricarboxylic acid (TCA) cycle to produce adenosine triphosphate (ATP). However, during the late stage of development, pyruvate uptake declines and glucose becomes the preferred nutrient above all to increase

the glycolytic activity (1). More than 50% of glucose consumed by the embryo is converted to lactic acid in the presence of oxygen (2). The conversion of lactic acid helps form an acidic environment, which assists embryo implantation during early pregnancy (3). Lactic acid has also been proposed to play a role as a signaling molecule in early pregnancy (3, 4). The selection of a single embryo for implantation is still a big challenge. Multiple embryo transfers during the process of an *in vitro* fertilization (IVF) are still being used, which leads to a high probability of multiple pregnancies. Although morphologic markers and grading systems to check embryo viability have been developed, they are insufficient to assess the physiological status of an embryo. Over the years, analysis of embryo metabolism has been proven to be a valuable marker to check embryo viability. Non-invasive measurements of glucose consumed, and lactate produced (glycolytic activity) by a single embryo during its development can be used to select a single viable embryo for implantation (3, 5). Several reports have already proposed a connection between metabolic activity and embryo viability (6-9). For instance, embryos that develop inside the uterus after implantation have the highest amount of glucose uptake during the stage of blastocyst development. Non-invasive measurement of nutrient uptake to select a viable embryo is the best method as it has a low chance of causing embryo damage.

Clinical applications are of particular importance in cases of rapid changes of glucose and lactate and the association of their levels in many disease conditions. For example, the measurement of metabolites such as glucose and lactate can be used to predict the possible outcome of in-hospital mortality in emergency medicine (10-12). Hyperglycemia and hyperlactatemia are both associated with increased mortality rate of patients (13, 14). Like

glucose, plasma lactate is also profoundly associated with type 2 diabetes as well as obesity in older adults (15-17). Diabetic patients with obesity show higher fasting plasma lactate levels than non-diabetic individuals with obesity (18).

Due to the inseparable relationship between glucose and lactate, these clinical demands have led to an increase in efforts to develop new and effective analytical tools for their simultaneous measurement. Most assays can determine a single compound, either glucose or lactate, in beverages or clinical samples (19, 20). Here, we optimized an enzymatic assay using the same substrate, Amplex red, to measure both D-glucose and L-lactate simultaneously in spent single embryo culture medium to choose a viable embryo for transfer as well as in normal diet (ND) and high-fat diet (HFD) induced obese (DIO) mice blood plasma and human serum samples as a reference in a clinical setting.

3.1. Materials and methods

3.1.1. Reagents and assay protocols for the measurement

Amplex Red Glucose/Glucose Oxidase Assay Kit (A22189) and Lactate oxidase (LOX) for lactate assay (LCO-301) were purchased from Thermo Fisher Scientific (Massachusetts, USA) and Toyobo (Osaka, Japan), respectively. For the lactate assay, reactions were carried out using 50mM potassium phosphate buffer prepared by mixing monobasic and dibasic potassium phosphates using HPLC grade water. All assays were carried out in 96-well black assay microplates (Neo Science, 30496) using an equal volume of freshly prepared reaction cocktail and sample. The reaction cocktail was a mixture of Amplex red (100μM), Horseradish Peroxidase (HRP, 0.2U/ml), its respective oxidases (2U/ml), and buffers. The

concentration of each component became half in the final reaction condition. Fluorescence readings were measured with a Synergy 2 Gen 5 microplate reader (Bio Tek Instruments, Vermont, USA). All readings were taken using an excitation filter of 540/35 and an emission filter of 600/40.

3.1.2. Animal preparation for embryos and blood plasma samples

Two strains of mice were obtained from the Hana Corporation (South Korea) and used for this experiment. Outbred strain ICR mice (IRB no. KM-2020-01R1) were used to measure metabolic activities of single embryos. Inbred male C57BL/6J mice (IRB no. KM-2019-18R2) were used to analyze metabolites in blood plasma. Both mouse strains were housed in a controlled environment with a 12h light/dark cycle (21-23°C) with *ad libitum* access to food and water. All experiments were performed after obtaining approval from the Institutional Animal Care and Use Committee of the School of Medicine, Keimyung University. They were performed following relevant guidelines and regulations.

3.1.3. Embryo collection and culture for metabolic analysis

Female ICR mice (6-9 weeks) were superovulated and mated with the same strain of male mice (10-12weeks). Collected embryos were cultured *in vitro* in commercially purchased KSOM media (Merck, MR-121-D) with 10% serum substitute supplement (SSS; Irvine Scientific, 99193) until the development of morula and early blastocysts. The morula and early blastocysts were incubated individually in 60µl droplets for 24h under Ovoil (Vitrolife, 10029) in a humidified environment at 37°C with 5% CO_2. Control droplets were incubated adjacent to droplets with embryos to check for nonspecific metabolite degradation. The

morphological status of blastocysts was recorded and compared to the status recorded at the beginning of the culture. Measurements of individual embryos were carried out by analyzing differences in substrate levels between incubation droplets and controls. Glycolytic activities of individual embryos were calculated based on the assumption that two moles of lactate were formed from one mole of glucose. Results are expressed in pmol/embryo/h.

3.1.4. Obese mice preparation and blood plasma sampling for fluorescence assay

Male C57BL/6J inbred mice (6 weeks old) were divided into two groups. One group was fed with ND (10% fat) and the other group was given a high-fat diet (HFD-60%) chow to induce obesity. Blood samples were collected from 7-8 weeks old ND-fed mice and up to 20 weeks old HFD fed DIO mice after 15-16h of overnight fasting mice. Blood was collected from the submandibular vein into heparinized Eppendorf tubes. These samples were centrifuged directly after collection to separate the plasma. The separated plasma was then deproteinized using metaphosphoric acid (MPA) and potassium carbonate and then diluted 100-300 times to measure glucose and lactate. Unknown concentrations were evaluated using the equation of a straight line (y = mx+c) obtained from plotted standard curves. Background was corrected by subtracting the no-glucose/no-lactate value from all sample readings. All samples for both assays were analyzed in duplicate on the same assay plate. Lactate measurements in mouse blood plasma were further validated by comparing measured concentrations with a commercially purchased Cayman L-lactate assay kit (Item no. 700510).

3.1.5. Human serum sample

Overnight fasted human serum samples were provided by Keimyung University Dongsan Hospital (Daegu, Korea). Blood serum samples from patients prone to/or with diabetes were collected into heparin-treated tubes according to the IRB-approved procedure (IRB no. 2021-02-053). These samples were centrifuged at 2,000×g for 10 minutes at 4ºC and then diluted 500 times before performing the assay.

3.1.6. Statistical analysis

Data are expressed as mean±SEM (standard error of the mean) for embryo culture media samples and human serum samples or mean±SD (standard deviations) for duplicate measurements of mice plasma samples unless indicated otherwise. To determine the repeatability of measurement, means and SD of each set of repeated measurements were determined to calculate the coefficient of variation [CV=(SD/mean) *100]. Average CVs are expressed as mean±SD. Graphs were made and analyzed using Excel 2016 and Origin pro-2019. Pictures were taken using Zeiss Zen 2.5 microscope imaging software. Paired sample t-tests were performed wherever necessary. Statistical significance was considered when *p*-value was less than 0.05 ($p < 0.05$).

3.2. Results and discussion

3.2.1. Assay optimization

In this study, the development of enzymatic lactate assay was based on LOX & HRP catalyzed oxidation of L-lactate to pyruvate. The choice of the substrate was based on a previously established glucose assay using Amplex red catalyzed by GOX & HRP to

produce D-gluconolactone (21). The amplex red is an extremely versatile peroxidase substrate. The enzyme reaction catalyzed by HRP is highly efficient compared to others. It can increase the yield of resorufin. Therefore, an assay using amplex red and HRP can be used to efficiently measure the extracellular formation of H_2O_2 (22). The glucose assay using the substrate Amplex red and the lactate assay using lactate dehydrogenase (LDH) are well known. However, a protocol for simultaneous measurement of both glucose and lactate using a single substrate is lacking. Here, we established a protocol for both assays using the same substrate (Amplex red) and reaction catalyst (HRP), which simplifies assay procedures. The assay procedure and principle are demonstrated in Figure 10. However, when the same conditions used for glucose assay were applied for lactate assay, the background fluorescent signal fluctuated, unlike that for glucose assay (Figure 11a).

We stabilized background fluorescence signal by replacing the buffer ion from Na^+ in the sodium phosphate buffer with K^+ in the potassium phosphate buffer. It has been reported that activities of enzymes and their stability in an aqueous solution depend on the buffer (23, 24). Different proteins might have different stabilities depending on specific ions such as Na^+ and K^+. As shown in Figure 11a, signal fluctuation was reduced with the K^+ ion of potassium phosphate buffer compared to the sodium phosphate buffer in the lactate assay. Thus, all experiments for lactate assay were carried out with potassium phosphate buffer while sodium phosphate buffer was used for the glucose assay. We further evaluated the effect of each component (pH, temperature, light, HRP, LOX, and Amplex red) on the analytical method of lactate assay with potassium phosphate buffer (Figures 11b-e). The solution pH is an important factor that affects enzyme activity. Fig. 2b shows that the

reaction is much faster in near-neutral solutions than in acidic or basic solutions. Figure 11c illustrates the influence of HRP concentration on the fluorescence intensity of the reaction solution. The fluorescent intensity increased up to a concentration of 0.2U/ml. It then kept decreasing beyond 0.2 U/ml. Figures 11d and 11e show the influence of LOX and amplex red concentrations. Similarly, the fluorescence increased up to 2 U/ml and remained almost constant thereafter for lactate oxidase. The highest fluorescence intensity for amplex red was seen in the 100µM reaction solution. A proper concentration of amplex red is needed to guarantee an appropriate level of H_2O_2 production because an abundant H_2O_2 can further oxidize the desired product resorufin into non-fluorescent resazurin, giving false-positive results (25). Similarly, the temperature is known to affect the fluorescence intensity (Värkonyi and Kabók, 1975).

The effect of reaction temperature on the fluorescence intensity was studied at different temperatures (3°C, 25°C, 37°C, 47°C, and 55°C). The fluorescent intensity slightly increased with increasing reaction temperature up to 37°C. It then started to decrease when the temperature was above 37°C (47°C and 55°C). The fluorescent intensity is also highly affected by exposure to light. When the reaction was exposed to light, the fluorescent intensity increased compared to that without light exposure. All evaluations used 50 µM of lactate. Thus, for the experiment, we chose optimal conditions for each component, which showed the highest value because the highest signal had more advantages in terms of resolution, selectivity, and so on. The optimal conditions in the reaction mixture were pH 7.3, HRP 0.2U/ml, LOX 2U/ml, and Amplex red 100µM at room temperature with minimal light exposure.

3.2.2. Assay validation

Drop-out experiments were carried out to analyze the reaction specificity in which each reagent was independently removed from the reaction cocktail/mixture. The complete reaction mixture showed the highest level of fluorescence, whereas HRP (acted as a catalyst in this experiment) and lactate upon removal showed low signal intensity. The omission of the substrate (Amplex red) and enzyme (LOX), as key factors in the reaction showed no signals (Figure 11f). These results demonstrate that only the complete reaction mixture can give relevant signals.

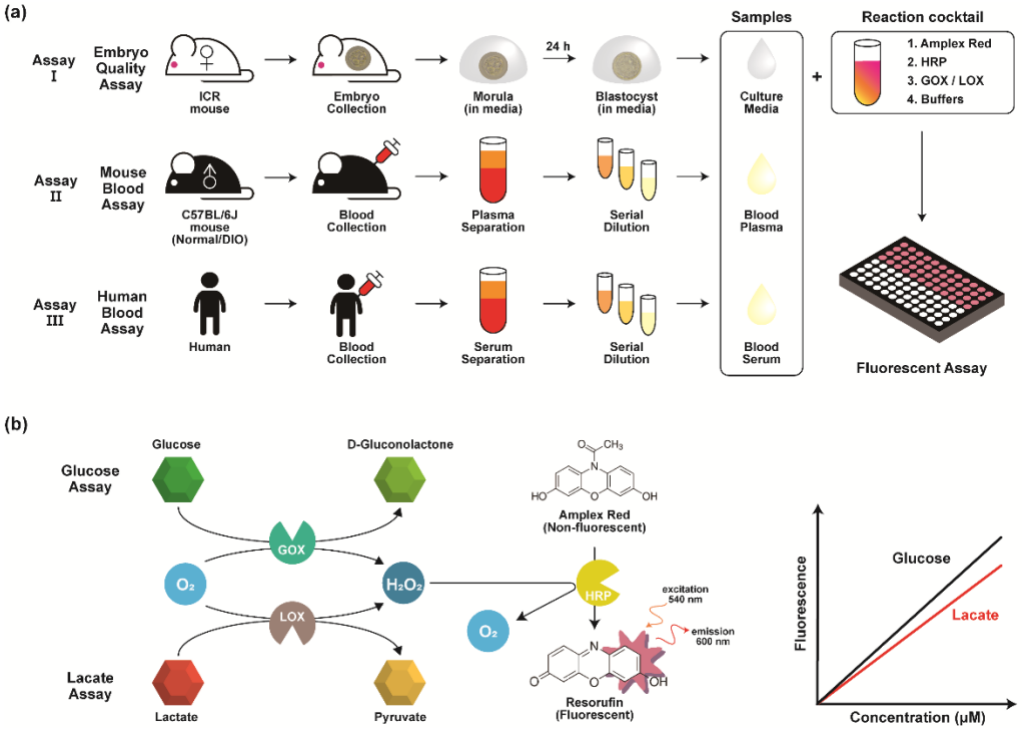

Figure 10: Schematic diagram of the overall experimental setup. (a) Three types of samples; Assay I for embryo quality assay, Assay II for mouse blood plasma assay and Assay III for human blood serum assay measured using optimized assay protocol in 96 well black plate, (b) General representation of glucose and lactate assay principle and supposed standard plot for glucose and lactate.

3.2.3. Assay kinetics

The kinetics of the enzymatic reaction was evaluated by time trace measurements as shown in Figures 12a-12d. In Figures 12a and 12b, fluorescent signals of glucose were continuously increased with an increase in time, whereas fluorescent signals of lactate reached maximal values at 5 minutes followed by a gradual decrease afterward. Figures 12c and 12d are replots of Figure 12a and 12b with time increments in terms of glucose and lactate concentrations, respectively. Both plots and replots demonstrated that the reaction time for the highest signal was 30 minutes for glucose (the commercial product recommendation) and 5 minutes for lactate (our recommendation). In this study, these times were used for glucose and lactate, respectively.

3.2.4. Assay sensitivity

We evaluated the assay sensitivity by analyzing fluorescent signals in a wide range (~up to 500μM) of glucose and lactate concentrations as shown in Figure 12e. Fluorescent signals increased in a hyperbolic fashion and showed a plateau in curves at concentrations over 100μM because all substrates and H_2O_2 were already utilized. However, fluorescent signals ranged from 1μM to 100μM for glucose and lactate, demonstrating a linearity of the assay with coefficients of regression (R^2) of 0.94 and 0.98 and average intra-assay coefficients of variations (CV) 1.34±0.85% and 2.62±2.34% (mean±SD), respectively (Figure 12f). Here, these data points represent mean values. All data were measured twice in duplicate. This linearity in the range up to 100μM allowed us to measure the glycolytic activity of a single mouse embryo based on its glucose consumption and lactate production measurement. We

also analyzed glucose and lactate concentrations in plasma samples of a normal and DIO mouse model along with measurements in human serum samples to determine their metabolic health condition, although an additional dilution procedure was necessary.

3.2.5. Assay Selectivity

The selectivity of the respective assay was evaluated by independently measuring concentrations of glucose and lactate in the mixture of five additional biological samples to show that assays were not affected by non-specific reactions with other compounds. With 50μM glucose (G), 50μM lactate (L), 10μg/L albumin (A), 50μM pyruvate (P), 10μg/ml cholesterol (C), and 10μU/ml insulin (I), five mixture combinations were prepared for glucose and lactate each and measured as shown in Figures 12g and 12h. All concentrations were based on their physiological levels in bodily fluids. The fluorescence signal produced by 50μM glucose or 50μM lactate was taken as the control. The range of glucose recovery rate was from 98.30% to 99.50% with intra-assay CV range of 0.06% to 1.80% (average CV: 0.89±0.64%). The range of lactate recovery rate was from 94.80% to 99.60% with intra-assay CV range of 0.09% to 2.36% (average CV: 1.03±0.84%). The high recovery rate with small CV demonstrates that the assay has a high selectivity for its targeted biomarkers (glucose and lactate) present in different biological samples.

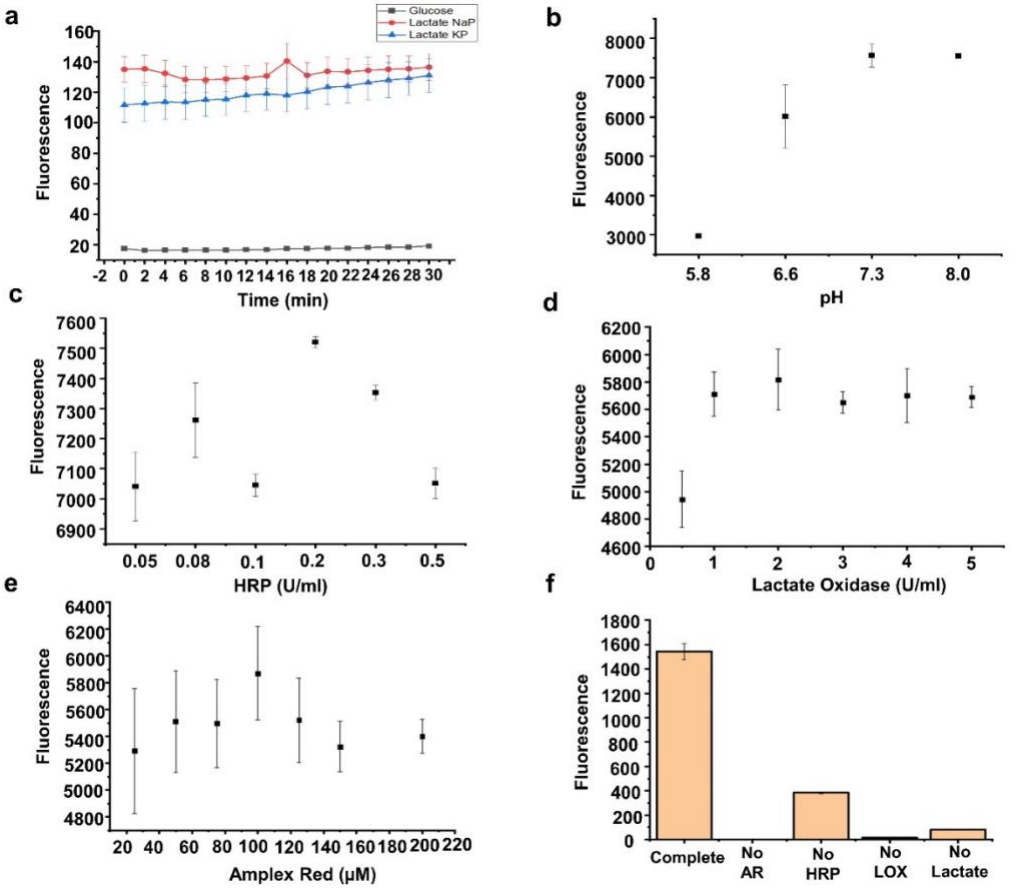

Figure 11: Lactate assay optimization. (a) Stabilization of background signal for lactate assay using sodium phosphate and potassium phosphate buffer in comparison with the glucose assay using sodium phosphate buffer, (b-e) Effects of each component on lactate assay with potassium phosphate buffer: (b) pH, (c) HRP, (d) Lactate oxidase, (e) Amplex Red, and (f) Drop-out experiments with a complete reaction mixture, no Amplex red (AR), no HRP, no lactate oxidase (LOX), and no lactate.

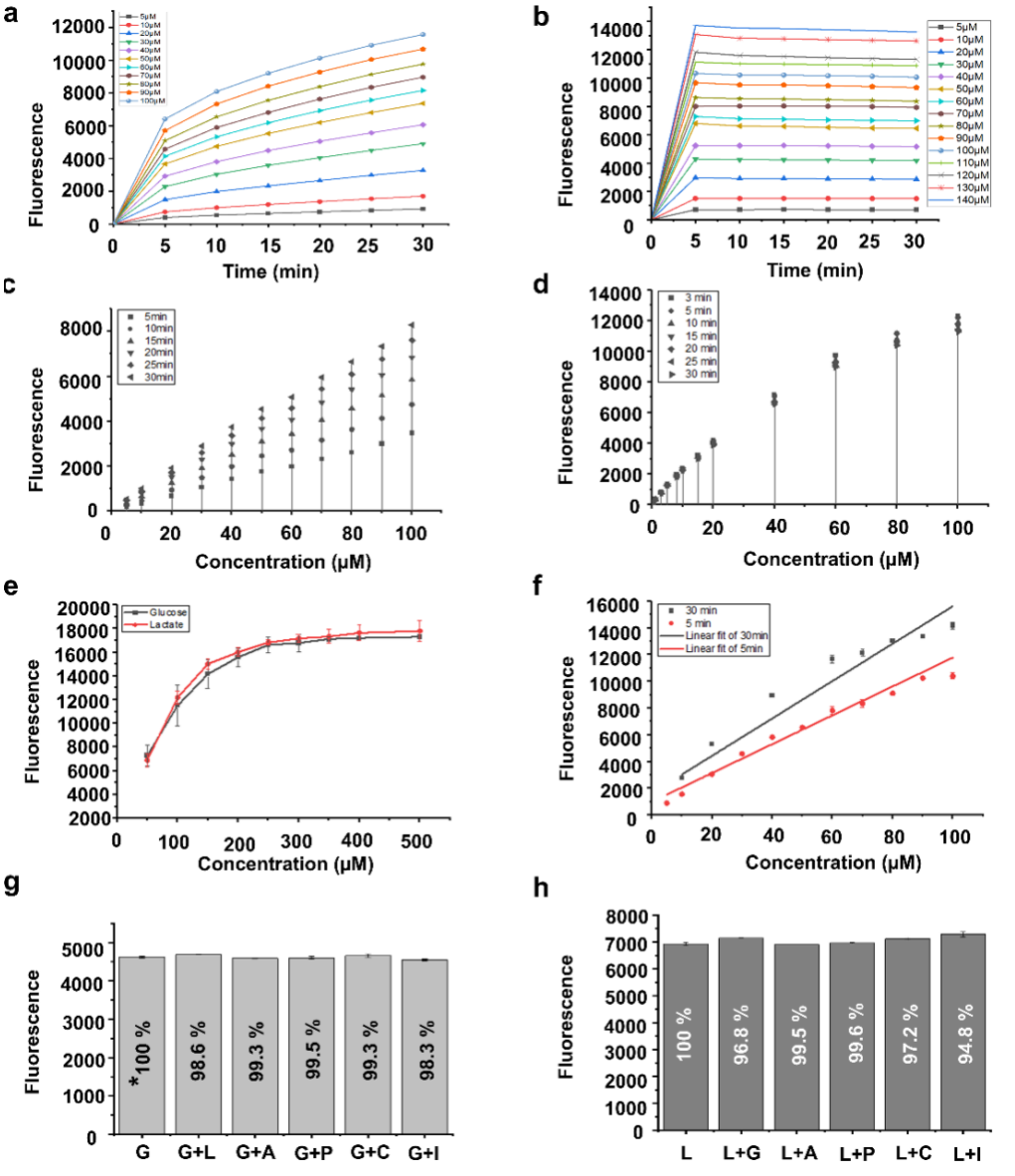

Figure 12: Assay kinetics. (a-b) Increments in fluorescent signals were observed for different glucose (a) and lactate (b) concentrations for 30 minutes with 5-minute intervals. (c-d) Increments in fluorescent signals were observed at different time with 5-minute intervals at

different glucose (c) and lactate (d) concentrations. (e) Fluorescent signals of wide ranges (~up to 500μM) of glucose and lactate concentrations. Data points represent mean values. All data were measured twice in duplicate. (f) Typical glucose standard curve with a coefficient of regression (R^2) of 0.94. The lactate standard curve had a coefficient of regression (R^2) of 0.98. Average CVs of glucose and lactates were 1.34 ± 0.85% and 2.62 ± 2.34%, respectively. (g) Selectivity test of glucose (G = 50μM) under competitive interactions between glucose and each interfering molecule: 50 μM lactate (L), 10 μg/L albumin (A), 50 μM pyruvate (P), 10 μg/ml cholesterol (C), and 10 μU/ml insulin (I). (h) Selectivity test of lactate (L=50 μM) with its interfering molecules: 50 μM glucose (G), 10 μg/L albumin (A), 50 μM pyruvate (P), 10 μg/ml cholesterol (C), and 10 μU/ml insulin (I). The CV (intra-assay) for selectivity test in case of glucose ranged from 0.06 % to 1.8% (average: 0.89 ± 0.64%). *, Recovery percentages.

3.2.7. Metabolic and glycolytic activity analysis of a single embryo

A total of 58 embryos with three different initial morphological stages (morula n=20, early blastocysts n=20, and expanded blastocysts n=18) were cultured for 24h. In general, an embryo develops in the order of zygote (presumed fertilized egg), two-cells, four-cells, eight-cells, morula, early blastocyst, expanded blastocyst, hatching, and hatched stage. Here, because of different development abilities of each embryo, the arrival stages did not correspond to their starting stages. All cultured embryos were in one of three different stages: expanded, hatching, and hatched blastocysts (Figure 13c). To find the efficacy of the proposed assay as well as the correlation between the developmental stages/rates and embryo abilities (represented by metabolic and glycolytic activities), we investigated the amount of glucose consumption and lactate production of each embryo during a 24 h period. Results showed that the glucose consumption rate by every single embryo increased in the later stages of its development. As shown in Fig. 4a, the glucose consumption rate by morula staged embryos was 5.36±0.92pmol/embryo/h. The consumption rate increased gradually in early (6.88±0.89pmol/embryo/h) and expanded blastocysts (8.01±0.87pmol/embryo/h) by 1.53 pmol/embryo/h (p<0.4) and 2.65pmol/embryo/h, respectively (p<0.04). Average CVs (inter-assay) of glucose measurement for morula, early, and expanded blastocysts were 8.48±10.69%, 6.22±12.32%, and 2.02±1.45%, respectively. On the other hand, the lactate production rate of the embryo decreased with embryo development. Lactate production rates in morula, early blastocysts, and expanded blastocysts were 8.84±0.88pmol/embryo/h, 8.31±1.05pmol/embryo/h, and 5.62±0.75pmol/embryo/h with average CVs (inter-assay) of 1.44±1.28%, 2.90±2.93%, and 2.44±3.78%, respectively (Figure 13a). There was no

significant difference in lactate production level between morula and early blastocysts. However, a significant difference in lactate production was found between morula and expanded blastocysts (p<0.009) and between early and expanded blastocysts (p<0.04).

Glycolytic activities of the embryos measured based on glucose consumption and lactate production gradually decreased from morula staged embryos to expanded blastocysts. Early blastocysts cultured in the same condition showed less glycolytic activities than the morula (76.55±16.58% vs. 169.76±38.53%, p<0.03). Similarly, the glycolytic activity of expanded blastocysts (44.02±8.36%) was lower than that of morula staged embryos (p<0.004) or early blastocysts (76.55±16.58%) (p<0.09) (Figure 13b). Based on our results, it seems later stages of embryos need more energy resources (such as glucose) with more metabolic activities to move on to the next stage with increasing numbers of cells. The observed increase in glucose uptake of mouse embryos in this study as the development progresses to later stages is consistent with previous findings in mouse (26, 27) and other species (28, 29). The mean value of glucose in the present study was similar to that reported for mice (9, 30). Similarly, in cases of lactate produced by mouse embryos, the mean value showed a range of lactate production similar to those reported by previous studies (30-32).

The lactate level was lower in the later stages but higher in the earlier stages. In a study conducted by (33), embryos with lower lactate levels showed more successful conception after transfer than those with higher levels of lactate production. Embryos exposed to low lactate levels during post-compaction also showed high viability (34). Furthermore, the glycolytic activity of the embryo was calculated based on glucose consumed and lactate

produced by different stages of embryos. Blastocysts with low glycolytic activities are supposed to be more viable than blastocysts with high levels of glycolytic activities (9). In the present study, the glycolytic level decreased as development proceeded. The highest glycolytic level was found in the morula stage, whereas the lowest level was seen in expanded blastocysts. A study performed previously in human embryos with successful conception showed an elevated glucose uptake but a lower glycolytic rate (33). In that study, morula staged embryos showed higher glycolytic activity than early, expanded, and hatching blastocysts, consistent with our finding. According to the study conducted by Gardner *et. al.*, the glycolytic activity of embryos *in vivo* was ~30% (9, 32). Among 58 embryos, 28 showed similar glycolytic activity (27.81±1.70%) like *in vivo* embryos. Twelve embryos from this group also showed a fast development rate (morula to expanded: 25%, early to hatching/hatched: 10.71%, and expanded to hatched: 7.14%) which highly contributed to the *in vivo* like glycolytic activity. Embryos with fast development and glycolytic activity similar to *in vivo* embryos are said to be highly viable.

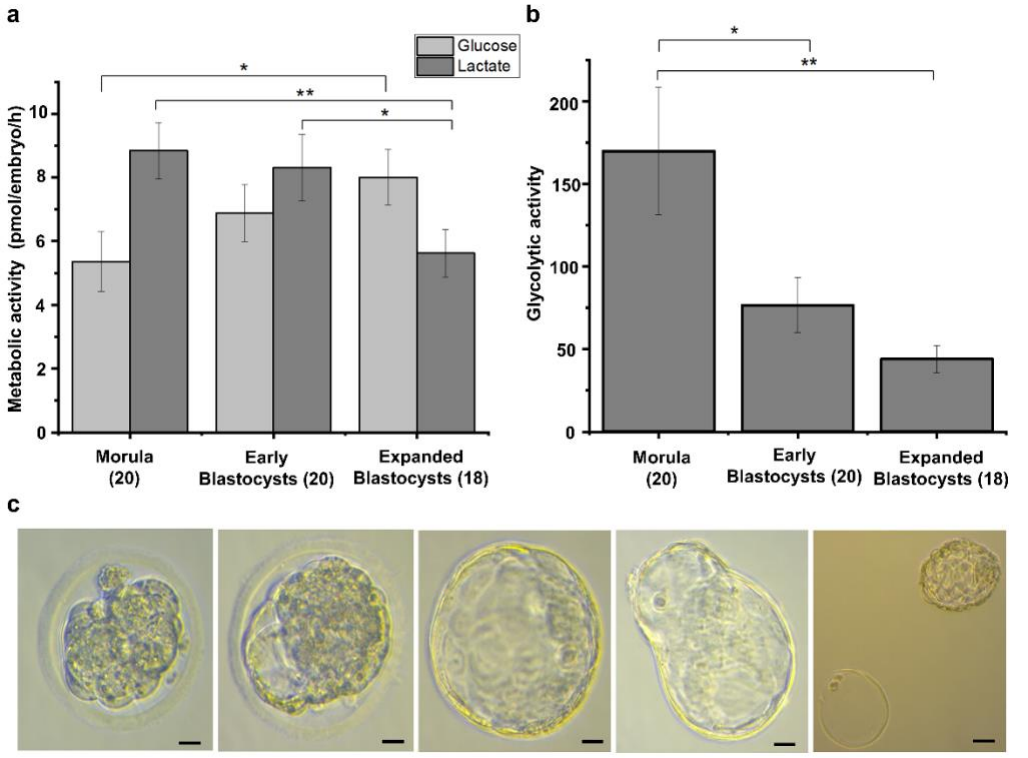

Figure 13: Metabolic analysis of single mouse embryo after 24h of culture. (a) Metabolic activity (glucose consumption and lactate production) and (b) glycolytic activity in the three different stages of embryos: morula, early blastocysts, and expanded blastocysts. Average CVs (mean ± SD) of glucose and lactate measured in embryos were 5.69 ± 9.82% and 2.25 ± 2.84%, respectively. (c) Morphologies of morula, early blastocysts, expanded blastocysts, hatching, and hatched blastocysts (Bar = 20 μm). *, $p < 0.05$; **, $p < 0.01$.

3.2.8. Analytical performance for blood samples

3.2.8.1. Mice blood plasma samples

An HFD or ND diet was introduced to six-week-old C57BL/6J mice with an initial body weight of 19.18±0.6g. Changes in body weight were recorded until 20 weeks of age. After one week of HFD introduction, mouse weight was significantly increased by 1.32±1.10g. For ND fed mice, the weight was increased by 0.91±0.60g, showing higher and faster weight gain for HFD fed mice. Average weight gains of ND-fed mice and HFD fed mice were 0.59±0.64g and 1.04±1.10g, respectively. Mouse weight data are shown in Figure 14a.

Glucose levels in fasted DIO male mice (n=10) were higher than those in ND-fed mice (n=10) by 2.15mmol/l (6.64±0.18mmol/l vs. 4.49±0.16mmol/l) (p<0.001). Likewise, lactate levels in HFD fed mice were increased by 3.80mmol/l (6.42±0.28mmol/l vs. 2.61±0.11mmol/l) than in ND-fed mice (p<0.001) (Figure 14b). Types of diet fed to mice affect their blood glucose and lactate levels. The DIO C57BL/6J mouse group in our study showed an increased level of glucose relative to ND-fed mice, similar to that in a study conducted by (35). Glucose levels of ND-fed mice in this study were also similar to those measured by (36) for 14-16h overnight fasted C57BL/6J ND fed mice.

We also demonstrated that our optimized assay could be used to study blood glucose and lactate levels in a different strain of mice. Blood glucose and lactate levels in mice can also vary depending on different factors such as gender, strain of the mice, sample collection site, stress level, and so on (37). Here, comparisons were made between four ND-fed ICR mice and four ND-fed C57BL/6J mice, each with approximately the same body weight and the

same fasting time (hours). Mean glucose concentrations for ICR mice and C57BL/6J mice were 4.84±0.17 mmol/l and 2.85±0.19mmol/l, respectively. Similarly, a difference of 1.75mmol/l was seen for lactate levels of two mice (3.78±0.10mmol/l for ICR mice and 2.02±1.16mmol/l for C57BL/6J mice, $p<0.03$) (Fig. 5c).

Possible factors such as stress, handling time, storage condition, and sample treatments to minimize the measurement error were taken into consideration. Blood was collected from the submandibular vein of each mouse to reduce stress as it is less strenuous compared to other collection sites (38). The plasma was separated from the collected blood as soon as possible. It was placed on ice. This is because it has been suggested that the plasma should be immediately separated from the blood and placed on ice or should be measured immediately after collection as storage of blood plasma at room temperature even for a few hours can decrease the concentration of glucose (39). Although plasma samples are said to be stable for one month when stored at -80°C, both glucose and lactate concentrations decreased in our samples after storing for just one week. Among the five plasma samples stored, mean glucose value decreased by 1.02mmol/l ($p<0.03$) and the mean lactate value decreased by 2.94mmol/l ($p<0.03$). The best results were obtained using fresh plasma samples with fresh reaction cocktails. Collected plasma samples were also deproteinized using metaphosphoric acid and potassium carbonate to inactivate endogenous enzymes. The presence of glycolytic enzymes such as dehydrogenases reduced NAD(P)H dinucleotides. Other factors in the sample can also affect the assay signal and the background (40). Blood plasma samples examined using a buffer made with Q-water without deproteinization showed a higher concentration of lactate (up to 15mmol/l, data not shown).

In addition to the drop-out experiment and the linearity of the standard curve, the effectivity of the lactate assay was further validated by comparing it with a commercial product. The commercial kit was used to compare lactate levels in diluted mouse blood plasma samples. Quantification differences or percentage relative deviations between commercially measured and those measured with proposed assay procedures were less than 10% for samples from both ND and HFD mice. Average inter-assay CVs for lactates measured using the kit and the present method in six samples done in duplicates were 8.46% and 1.63%, respectively (Table 2).

Table 2: Comparison between lactate concentration measured using a commercial kit and the current protocol.

Mice	Lactate(mmol/l) from kit (Mean±SD)	% CV	Lactate (mmol/l) Proposed Assay (Mean±SD)	% CV	Relative deviation %	Bodyweight (g)
ND 1	1.99±0.02	0.81	1.79±0.06	3.46	9.65	27.71
ND 2	5.61±1.83	32.55	5.30±0.01	0.21	5.49	29.59
ND 3	2.84±0.10	3.66	2.58±0.01	0.21	9.03	30.67
HFD 1	9.07±0.48	5.27	8.96±0.03	0.33	1.20	46.46
HFD 2	9.15±0.29	3.14	8.29±0.41	4.97	9.41	50.73
HFD 3	10.16±0.55	5.37	9.89±0.06	0.61	2.63	51.29

3.2.8.2. Human blood serum samples

We analyzed 20 human serum samples provided by the hospital without patient's personal or physical health information. Results showed that overall glucose concentrations ranged from 3.64mmol/l to 14.19mmol/l with a mean value of 7.99±0.84mmol/l, whereas the overall lactate concentrations ranged from 0.53mmol/l to 11.8 8mmol/l with a mean value of 5.00±0.68mmol/l. Based on the measured glucose concentrations, we were able to categorize 20 samples into three groups: normal, prediabetic, and diabetic groups. Fasting blood glucose concentration <99mg/dl (5.5mmol/l) is considered normal whereas glucose concentration from 100 mg/dl to 125 mg/dl (5.6-6.9mmol/l) is considered the prediabetic group. The diabetic group of people has a fasting glucose concentration >126 mg/dl (>7.0mmol/l) (41). In our case, 5 of them were in the normal group with glucose concentration of 4.73±0.29mmol/l and lactate concentration of 3.53±1.19mmol/l. The other 7 samples were prediabetic groups with a glucose concentration of 6.49±0.13mmol/l and a lactate concentration of 3.79±1.07mmol/l. Similarly, eight people in the diabetic group showed glucose concentrations of 11.34±1.36mmol/l and lactate levels of 6.21±1.17mmol/l. Both glucose and lactate concentrations increased as conditions worsened from normal to diabetic, although lactate concentrations between normal and prediabetic groups were not significantly different. As shown in Figure 14d, glucose concentrations increased in the prediabetic groups relative to the normal groups (p<0.05). Likewise, the diabetic group had higher glucose and lactate concentrations than both the prediabetic group (p<0.05) and the normal group (p<0.05). Previous studies have shown that fasting plasma lactate increases in both types of diabetic patients (17, 42). Lactate is also related to obesity in older adults (17).

Higher lactate levels in diabetic patients than in non-diabetic patients with obesity have been observed (18). Lactate production is also higher in patients with uncontrolled diabetes. A study conducted by (10) on patients at the time of hospital admission revealed that a combination of very high glucose (>11.1mmol/l) and very high lactate (>4mmol/l) showed the highest mortality rate (39.15%), followed by a combination of high glucose (7.8-11.1mmol/l) and very high lactate (34.04%). A study conducted by (43) has suggested that people with diabetes and other health conditions like kidney diseases and heart problems have a high incidence of having high lactate concentrations. In our study, lactate concentration in the normal group with a normal glucose concentration was higher than the normal lactate range of 0.5mmol/l to 2.2mmol/l, which did not indicate diabetes but might indicate other health problems. A study conducted by (44) has suggested that this kind of condition can indicate cases of organ failure in critically ill patients along with an increase in mortality. Sotello et al. (2019) have also suggested that a combination of normal glucose (3.3-7.8mmol/l) but very high lactate can increase the mortality rate. However, in this study, as the patient's information was not disclosed to us, it was difficult to know the patient's medical history and other conditions of the patient.

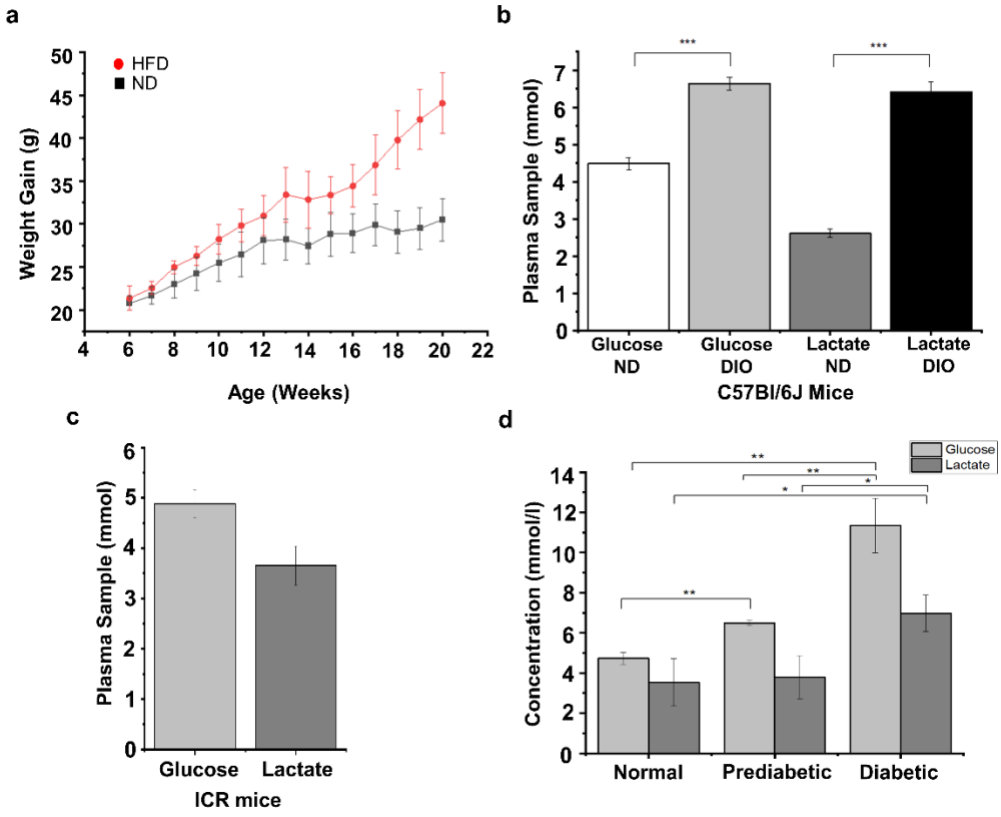

Figure 14: (a) Average weights of male C57BL/6J DIO mice fed with 60 kcal% fat (HFD) and normal mice fed with 10 kcal% diets (ND) between ages of 6 and 20 weeks. Values represent the mean and standard deviation done for 10 mice measured on the same day twice a week, (b) Glucose and lactate levels measured for both ND and DIO mice blood plasma. ***, $p < 0.001$ with average CVs of 3.15±2.76% and 4.73±4.47%, respectively. (c) Glucose and lactate levels in ICR strain mice, and (d) Glucose and lactate levels in human serum samples of normal (5), prediabetic (7), and diabetic patients (8). Average CVs (mean±SD) of glucose and lactate in human serum samples were 3.39±6.45% and 6.50±4.59%, respectively (intra-assay). *, $p<0.05$; **, $p<0.01$.

3.3. Conclusions

In this study, a highly sensitive and selective fluorometric assay for simultaneous measurement of glucose and lactate using the same substrate, Amplex red, was developed with a CV of less than 3%. The lactate assay was stabilized using similar conditions of already established glucose assay to measure glucose and lactate. The optimized assay system was successfully applied for the detection of glucose and lactate in mice embryo culture media as well in plasma samples of mice and human serum samples. This assay system was able to measure samples in a wide range (pmol-mM) either with dilution or without dilution. Measurements of metabolites in embryo culture media (pmol) were used to calculate glycolytic activities of embryos which showed a low glycolytic activity (similar to *in vivo* developed embryos) in fast developing embryos. It can be used as a tool to select viable embryos in further studies. Furthermore, glucose and lactate measured in mice blood plasma (mM) and human serum samples (mM) showed metabolic health conditions that could be further used to analyze health complications that might occur in the future.

This part of the thesis is published in Analytical chemistry (45).

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Chapter 4: Optimization of culture conditions in a microfluidic technology for miniature culture condition

4. Introduction

Since 2010, various approaches have been taken to improve IVF. The microfluidic technology is integrated in each step of the IVF process because the success rate of IVF depends on each step of the process. Microfluidic technology has been one of the best technologies developed for the advancement of this field so far. The technique miniaturizes and simplifies the long hectic procedure of assisted reproductive technology (ART) in a simple chip. The ART field has certainly been enhanced with its introduction. Microfluidic technology was first introduced in 1990s at Stanford University and is defined as the study of the behavior, precise control, and manipulation of fluid in microenvironments. Since then, this technology has been used in different fields such as chemistry [18], molecular biology, and developmental biology [19], although it is most widely used in the biomedical field for the control of fluid transport in the cell analysis system, drug delivery system, and for assisted reproductive technology [19–22]. It is an emerging field used for the miniaturization and simplification of laboratory techniques based on chips with fabricated microchannels and chambers [23]. Beside these fields, it is also used in forensic science [24]. Its use in these areas has provided numerous benefits overall due to its decreased cost in the manufacture, use, analysis, disposal, etc.

In this chapter of thesis, we tried to mimic the mice oviduct by making a concave shaped

microfluidic chip for culture of ICR mice embryos.

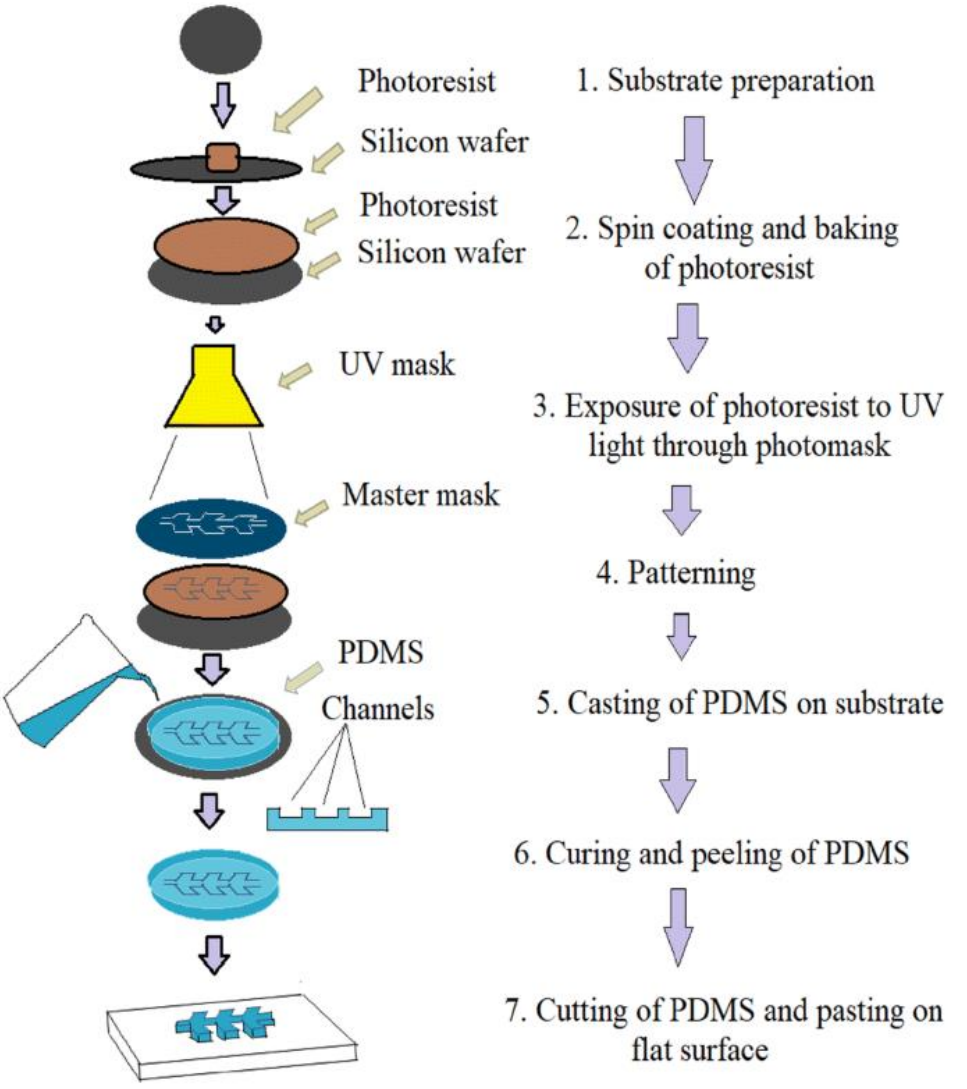

Figure 15: Schematic diagram of the fabrication process of the PDMS microchip in a microfluidic system.

This part of the figure and literature is already published in JMST Advances (1).

4.1. Materials and Methods

4.1.1. Animal preparation for embryos

Outbred ICR mice from Hana Sangsa Corporation (Republic of Korea) were used in this experiment. Mice were housed in 12h light/dark cycle with an unlimited supply of food and water. All procedures for mouse care and use were conducted in accordance with the guidelines and approved by the Institutional Animal Care and Use Committee of the School of Medicine, Keimyung University (Mice IRB no. KM-2022-11).

Female mice (age 7-9 weeks) were superovulated with 7.5IU of pregnant mare's serum gonadotropin (PMSG; Sigma- Aldrich, G-4877) followed by 7.5IU human chorionic gonadotropin (hCG; Life Sciences, 367222-1000IU) 48 h later. Females were mated with the same strain of male mice (age 10-12 weeks) on the same day of hCG injection. Super ovulated females with vaginal plugs were sacrificed after 16-18h and oviducts were collected in M2 media (Merck, M7167) with a 10% serum substitute supplement (SSS; Irvine Scientific, 99193). COCs were then isolated from oviducts

4.1.2. Preparation of CCs

CCs clumps and oocytes were separated manually from COCs using two 0.5 ml insulin syringes (BD Insulin syringe, Republic of Korea) in regular M2 media. Harvested lump of CCs was dissociated using hyaluronidase from bovine testes (Sigma Aldrich, H3506). Approximately 1.43×10^3cells/ml were seeded in DMEM/F-12 Ham's media (Merck, D6434) with 10% FBS in microfluidic chip. The culture media was changed to KSOM with 10% SSS (Merck, MR-121-D) after 24h when cells were attached to the culture dish surface. The co-culture with embryos proceeded the following day.

4.1.3. Oocyte recovery

After the separation of CCs and oocytes, oocytes surrounded by remaining CCs were denuded by pipetting with a thin pipette in M2 media containing hyaluronidase. Denuded oocytes were collected and cultured in KSOM media with 10% SSS until the next day up to 2 – cell division. All the media were equilibrated overnight before culture began. Embryos with 2 – cells were then collected and co-cultured in KSOM media in concave well made in PDMS chip. Single 2 cell embryo were co-cultured with cumulus cells in one well. For control, single embryo was cultured in concave well without cumulus cells.

4.1.4. Microfluidic Fabrication

Different well sized wafers were prepared for the optimization of culture well. Different wells of variable diameters were prepared as 150um, 200um, 250um, and 500um. However, the height of well were same for all as 200um. Briefly, for fabrication process, after the preparation of wafer as such, PDMS prepared using 1:10 ratio of base and curing agent were poured in the wafer and was vacuum treated to remove the bubbles. It was then incubated for 1 hour at 80-90ºC. After solidification of the PDMS, again the PDMS mixture was poured in the structure to make the concave design using friction by raking method. The process of incubation was repeated again for 10-15 minutes and was left to cool down. The PDMS so prepared were plasma treated for hydrophilic property. The design was then used to culture and co-culture the embryos.

4.1.5. Statistical analysis

The design of microfluidics was prepared by using Solid works 2019 and pictures were taken using Zeiss Zen 2.5 microscope imaging software.

4.2. Results and Discussion

4.2.1. Selection of well size

Among different concave well sizes of microfluidics chip prepared, the media content for continuous culture of single embryo was insufficient in smaller well sizes as 150um, 200um, and 250um. The embryo was cultured and co-cultured in biggest well size with diameter 500um. It was very crucial to surround the culture chip with PBS to reduce the chances of media evaporation and also osmolality in media. The occurrence of osmolality shifts through PDMS membranes prevents the mouse embryo growth and also the different type of cell growth (2). By considering this, the PDMS chip prepared was first dipped into distilled water for 24 hours and later, the media was placed over wells along with mineral oils surrounded by PBS buffer in outer wells of IVF dish. The picture of microfluidic design is shown in Figure 16.

4.2.2. Cumulus cell seeding in microfluidic concave well and droplets

The cumulus cells obtained from hyaluronidase treating was seeded in concave well with DMEM/F12 media that was equilibrated in incubator beforehand. The cumulus cells were confluent after 24 hours and the media was changed to warm KSOM media with 10% serum substitute supplements for co-culture purposes.

For control group, cumulus cells were cultured in 10μl droplets of media. Similar as in concave well, cumulus cells were cultured first in DMEM/F12 media with 10% FBS and was later changed to KSOM media to support the development of embryo during co-culture. The embryos were transferred on second day after the cells reached confluency for co-culture.

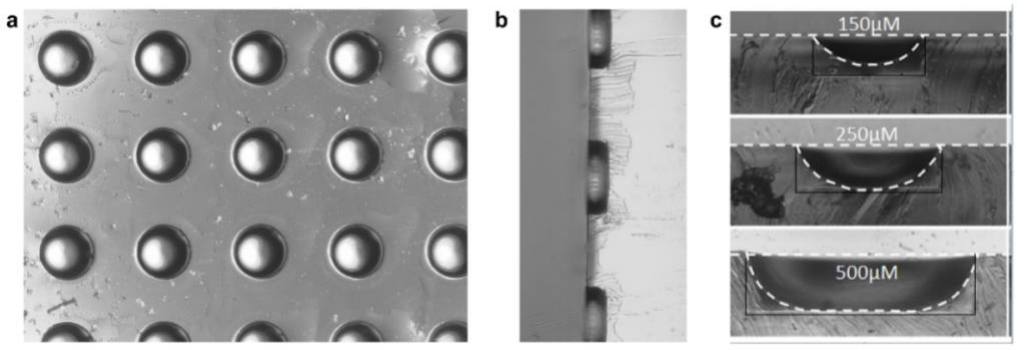

Figure 16: (a) Surface view of microfluidic chip, (b) Cross-section of PDMS before concave fabrication, and (c) Cross-section of PMDS chip after concave design fabrication.

4.2.3. Co-culture in microfluidic concave well

After 24h of cumulus cells, single embryo that is developed to 2-cells was co-cultured carefully in each well where cumulus cells were cultured. It was then cultured for 4 days more at 37ºC with 5% CO_2. The development rate was observed, and co-culture system showed better results than control system that is with only KSOM. However, more experiment is going on regarding this experiment. The figure for culture and co-culture is shown in Figure 17.

Previously, many oviducts mimicking microfluidic chips were prepared. In research done by Wang et. al. human oviduct were mimicked using microfluidic chip which showed that the culture in chips showed decrease in ROS concentration in *in vitro* fertilized embryos of CD-1 mice (3). The design was more focused on mimicking human oviduct which was straight in shape rather concave or wave. The concave microwell designs were also fabricated in past. However, the design was different from ours and the use of cells were also different. The research conducted by Xu et. al. was more focused on fabrication process of device than in biological aspect (4). The fabrication process of different concave microwells for different applications are summarized in a review by Guo et. al. (5). For the preparation of concave wells, different processes are being used, one of them is by using beads. In research article by Lee et. al., they used a bead and magnet to make microwells with variable concave geometries (6).

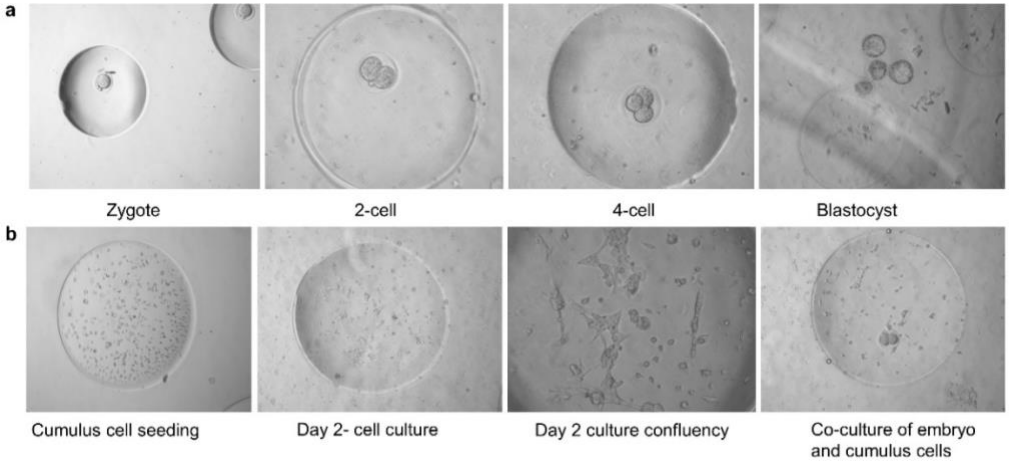

Figure 17: (a) Single embryo culture in microfluidic concave design from zygote stage to blastocyst design, (b) Cumulus cell seeding in concave microfluidic chip and co-culture of two-cell embryo with cumulus cells.

4.3. Conclusion

A miniature culture condition for single embryo was developed using PDMS concave wells. The culture of single embryo is very important for selection of single embryo to transfer for the successful conception of a baby reducing the chances of multiple births. Here, the cumulus cells were cultured successfully and also the embryos were cultured successfully. The co-culture of embryo and cumulus cells were also performed successfully. However, the further study is also required regarding this experiment. In future, oviductal cells can be cultured in the same well to see the developmental rate of embryos.

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Optimization of single embryo culture and assay systems for *in vitro* fertilization (IVF) technology

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(Abstract)

In vitro fertilization (IVF) is still an expensive process that involves lots of complicated procedures. It is an extremely difficult job to handle an embryo outside of a body which makes the work tedious and expensive. The survival rate and development of the embryo *in vitro* depend on the type of culture system, availability, and the composition of the media in each stage of embryo development. Therefore, the optimization of *in vitro* culture systems is a fundamental requirement in artificial reproductive technology (ART). The work is constantly going on to improve the culture conditions and procedures of the embryo to make it easier for embryologists as well as to improve the viability of the embryos for transfer. The main purpose of our study is to optimize the embryo culture condition by altering the nutrients as well as co-culturing with different cells for better development of the embryos. Also, to analyze the metabolic activity of a single embryo to select a viable embryo for successful transfer and conception minimizing the chances of multiple pregnancies.

체외수정(IVF) 기술을 위한 단일 배아 배양 및 분석 시스템의 최적화

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의용공학과

(지도교수 허윤석)

(**초록**)

체외 수정은 여전히 많은 복잡한 절차를 수반하는 비싼 과정입니다. 몸 밖에서 배아를 다루는 것은 매우 어려운 일이어서 작업이 지루하고 비용이 많이 듭니다. 체외 배아의 생존율과 발달은 배아 발달의 각 단계에서 배양 시스템의 종류, 가용성, 배지의 구성에 따라 달라집니다. 따라서 인공 생식 기술(ART)에서 체외 배양 시스템의 최적화는 기본적인 요구 사항입니다. 배아의 배양 조건과 절차를 개선하여 배아학자들이 이식을 위한 배아의 생존 가능성을 향상시키는 작업이 지속적으로 진행되고 있습니다. 우리 연구의 주요 목적은 배아의 더 나은 발달을 위해 다른 세포와 공동 배양 뿐만 아니라 영양분을 변화시킴으로써 배아 배양 조건을 최적화하는 것입니다. 또한, 단일 배아의 대사 활동을 분석하여 성공적인 전이와 임신을 위한 실행 가능한 배아를 선택하여 다수의 임신 가능성을 최소화합니다.