The Seoul Journal of Medicine
Vol. 26, No. 4:361-368, December 1985

Study on *in vitro* Fertilization of Mouse

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= Abstract =The success of human *in vitro* fertilization (IVF) and embryo transfer (ET) has focused attention on the culture conditions that can provide optimal development of the preimplantation embryo. Studies of *in vitro* fertilization using mouse have direct implications to human IVF, since similar conditions are used for both species. Mouse IVF was studied with special reference to the condition of culture media and vessels since April 1984.

The results were summarized as follows:

1. Egg retrieval after superovulation in mice was 17.14 ± 4.79 eggs per mouse.
2. About fifty percent of eggs retrieved showed good quality which has no evidence of fragmentation of the ooplasm or globules of unequal size.
3. In comparison with two media and two culture vessels after 72 hours *in vitro* culture, cleavage rates (six to eight-cell blastocyst) were 9.7% (Ham's F-10 in dish), 10.9% (Ham's F-10 in tube), 21.0% (m-KRB in dish), and 36.6% (m-KRB in tube) respectively.
4. Am-KRB medium was superior to Ham's F-10 in developing mouse embryo.

It was suggested that mouse IVF system has a valuable place in a quality control system of human IVF and a research model.

Key Words: ***Mouse, In Vitro Fertilization, Culture Media***

INTRODUCTION

Since the pioneering work of Brinster and Biggers (1965), Whittingham (1968) and Iwamatsu and Chang (1969), many researchers have investigated the biological and experimental events of *in vitro* fertilization of mouse eggs. Because the study on mouse *in vitro* fertilization and on the growth of preimplantation embryo is very easy way to observe the precess of embryo from early stage of formation to morula and blastocyst, it is a good model to study the reproductive physiology and embryology of mammal. There has been definite progress in research of reproductive physiology due to birth of test tube baby by *in vitro* fertilization and embryo transfer (Edward and Steptoe 1983). The study on *in vitro* fertilization is simple concept, but in reality there always exist technical problems. Several factors such as sperm source, capacitation conditions, insemination conditions and egg source are critical to the success of *in vitro* fertilization of mouse eggs. Studies of *in vitro* fertilization using mice have direct implications to human *in vitro* fertilization, since similar conditions are used for both species (Lopata *et al.* 1978). This paper reports upon various techniques under investigation in our laboratory to maximize mouse *in vitro* fertilization and cleavage rate so we may apply obtained informations to human *in vitro* fertilization. Specifically, in this study we compared cleavage rates using two types of plastic culture ware (organ culture dishes *versus* culture tubes) and a modified Krebs-Ringer Bicarbonate (m-KRB) culture medium routinely used for mice *versus* Ham's F-10 culture medium used for humans.

MATERIALS AND METHODS

1. Experimental animal

Female mice were 6-8 weeks old F-1 (ICR$\times$ Balb/c) bred at Seoul National University farm, and male mice were 3-10 months old ICR strain with proven fertility. All mice were maintained on a lighting regime of 14 hr light: 10 hr dark at room temperature and had free access to food and

water.

2. Culture media

It was manufactured with fifth distillation water utilizing Ham's F-10 (Gibco #430-1200), then added 75 mg of penicillin G (Sigma), 75 mg of streptomycin sulfate (Calbiochem) and after sterilization by millipore filter it was stored in 4°C refrigerator. In this media (4×) 75 cc of fifth distillation water, 24.25 mg of calcium lactate (Sigma) and 210.6 mg of $NaHCO_3$ (Sigma) were added, then pH and osmolarity were adjusted to 7.4 and 280-285 mOsm respectively, and after sterilization by millipore filter just before each experiment, fetal cord serum was added to the insemination culture media and growth culture media to be its concentration 7.5% and 15% respectively. m-KRB culture media was manufactured by the method of Toyoda and Chang (1974) as follows. After manufacturing 250 ml of culture media (4×) (99.6 mM, NaCl:4.78 mM, KCl:1.71 mM, $CaCl_2$:1.19 mM, KH_2PO_4:1.19 mM, $MgSO_4$:25.07 mM, $NaHCO_3$:21.58 mM, Na lactate: 0.5 mM, Na pyruvate: 5.56 mM, glucose: 50 I.U. penicillin/ml: 50 mcg streptomycin/ml), 25cc of m-KRB culture media (4×) was diluted with 75cc of distilled water, then bovine serum albumin (BSA) was added to be its concentration 4 mg/ml, and pH and osmolarity were adjusted to 7.4 and 280-285 mOsm respectively. Only m-KRB culture media was used to wash oviduct and to collect ova in this experiment. Ham's F-10 and m-KRB culture media were taken in Falcon tissue culture dish (Falcon #3016) and Nunc tissue culture tube respectively, and incubated at 37°C in 5% CO_2 in air for 24 hours prior to use.

3. Fetal cord serum

Cord bloods were collected aseptically with 50 ml syringe immediately after vaginal delivery or cesarean section at Seoul National University Hospital delivery room, and stored in 4°C refrigerator, then centrifuged at 1000 rpm. After the serum specimens were inactivated for 45 minutes, and sterilized by millipore filter, they were stored in -20°C refrigerator (Jones *et al.* 1982).

4. Superovulation

Female mice selected at random with regard to their estrus were induced to superovulate by the method of Ackerman *et al.* (1983) who injected 5 I.U./0.1 ml of PMSG (pregnant mare serum gonadotropin) and 5 I.U./0.1 ml of HCG into peritoneal cavity.

5. Retrieval of ova

At 16-18 hours after HCG injection, mouse was sacrificed by cervical dislocation and peritoneum was opened aseptically. The ovary, oviduct and a small segment of uterus were excised and placed in sterile culture dish containing 2 ml of m-KRB culture media. Using microdissecting tools and a dissecting microscope the oviduct was separated from the uterus. Ova were flushed from the oviduct by inserting into the ampulla a 30 gauge needle attached to a 1-cc syringe filled with medium.

6. Preparation of spermatozoa

Two mice were sacrificed by cervical dislocation and testes and cauda epididymis were exposed by same process as female. Cauda epididymis were extirpated from 2 mice and gathered in 35 mm culture dish containing 2 ml of m-KRB culture media. After mincing cauda epididymis with minute operation scissor, the minced tissue and fluid were transferred to 12×75 mm test tube and left at 37°C in 5% CO_2 incubator for 1 hour. After transfer of the supernate containing motile sperm to other test tube, a sperm count and motility determination were performed on the supernate. The concentration of motile sperm was adjusted to $1\text{-}10\times10^6$/ml.

7. Insemination

One oocyte-cumulus mass was put in culture dish containing 3 ml of insemination culture media, and concentration of motile sperm was adjusted to 1×10^5/ml. After insemination it was incubated at 37°C in 5% CO_2 in air for 4 hours, then transferred to growth culture media containing Ham's F-10 culture media with 15% fetal cord serum. In case of m-KRB culture media, growth culture media was same as insemination culture media. After excluding those ova which were degenerated or reached two-cell stage, five to ten of normal ova were put randomly into growth culture media.

8. Observation of embryo

For proper observation of experiment, developing embryo was observed every 24 hours for 3 days.

9. Standardization of culture media

To measure the relative quality of Ham's F-10 being used in human IVF, fertilization ratio was calculated by numbers of embryo which reached six to eight-cell stage, morula and blastocyst after 3 days of culture divided by numbers of one-cell stage fertilized eggs used for culture.

RESULTS

1. Retrieval of ova

Fifteen to twenty of female mice were used in each experiment, and ovulation was confirmed in 70% of them. Numbers of acquired ova per mouse in every experiment were 14.7±2.58~21.2±6.73, and their mean value was 17.14±4.79 (Table 1). It was impossible to count exactly the numbers of ova surrounded by oocyte-comulus mass after washing the ova with m-KRB culture media from the oviduct. Numbers of ova were confirmed by observation after removal of oocyte-cumulus mass after removal of oocyte-cumulus mass after insemination culture. Normal ova with the exception of degenerative or transformed ova were observed in 38.3-64.0% (Table 1).

2. Cleavage rate

2.1 Observation after 24 hours:

After 24 hours it was revealed that cleavage rate to one-cell stage was 57.8%, two-cell stage 8.6%, three to four-cell stage was 6.0%, six to eight-cell stage 7.9%, disproportioned cleavage stage 16.5%, and degenerated embryo 3.2% in first group (Ham's F-10 in dish) (Table 2). In second group (Ham's F-10 in tube), cleavage rate to one-cell stage was 54.3%, two-cell stage 7.3%, three to four-cell stage 5.9%, six to eight-cell stage 11.8%, disproportioned cleavage 17.8% and degenerated embryo 2.9% (Table 3). In third group (m-KRB in dish), 32.4% of them were cleavaged to one-cell stage, 25.1% to two-cell stage, 16.3% to three to four-cell stage, 5.3% to six to eight-cell stage, 16.3% to disproportioned cleavage and 4.6% to degenerated embryo (Table 4). In fourth group (m-KRB in tube), 24.4% cleavaged to one-cell stage, 3.8% to two-cell stage, 6.6% to three to four-cell stage, 6.6% to six to eight-cell stage, 15.6% to disproportioned embryo and 2.1% to degenerated embryo (Table 5). After 24 hours the

Table 1. Ovum retrieval after induction of superovulation*

Exp. No.	No. of amimals**	Total No. of eggs	Mean No. of eggs per mouse	No. of 1-cell (%)	No. of degenerative cell(%)
1	10/15	211	21.1±6.23	135(64.0)	76(36.0)
2	10/20	172	17.2±5.55	73(42.4)	99(57.6)
3	10/17	212	21.2±6.73	121(57.1)	91(42.9)
4	10/15	204	20.4±4.60	96(47.1)	108(52.9)
5	10/17	147	14.7±2.58	72(49.0)	75(51.0)
6	10/15	186	18.6±5.23	85(45.7)	101(54.3)
7	10/15	164	16.4±3.69	87(53.0)	77(47.0)
8	10/16	157	15.7±3.27	89(56.7)	68(43.3)
9	10/15	168	16.8±3.33	72(42.9)	96(57.1)
10	10/17	166	16.6±3.41	98(59.0)	68(41.0)
11	10/16	167	16.7±3.33	64(38.3)	103(61.7)
12	10/16	151	15.1±3.54	70(46.3)	81(53.7)
Total	120/194	2,105	17.14±4.79	1,062(50.5)	1,053(49.5)

* 5 I.U. PMSG & 5 I.U. HCG

** No. of mice of which oviducts were flushed/No. of mice which were induced superovulation

Table 2. Development of embryos from one cell stage during culture with Ham's F-10 in dish

Stage / Hours	1-Cell	2-Cell	3-4 Cell	6-8 Cell	Morula	Blastocyst	Fragmented	Degenerative
24	57.8	8.6	6.0	7.9	—	—	16.5	3.2
48	48.0	0.4	0.8	6.5	6.2	2.3	14.6	21.2
72	12.1	—	—	3.1	1.4	5.2	18.0	60.2

* No. of total embryos: 1,000

Table 3. Development of embryos from one cell stage during culture with Ham's F-10 in tube

Stage / Hours	1-Cell	2-Cell	3-4 Cell	6-8 Cell	Morula	Blastocyst	Fragmented	Degenerative
24	54.3	7.3	5.9	11.8	—	—	17.8	2.9
48	49.6	1.7	2.3	2.4	9.2	1.6	18.3	14.9
72	11.8	0.6	0.4	1.6	2.6	6.3	18.9	57.8

No. of total embryos: 1,000

Table 4. Development of embryos from one cell stage during culture with m-KRB in dish

Stage / Hours	1-Cell	2-Cell	3-4 Cell	6-8 Cell	Morula	Blastocyst	Fragmented	Degenerative
24	32.4	25.1	16.3	5.3	—	—	16.3	4.6
48	28.6	3.2	2.4	8.8	19.2	1.2	18.2	18.4
72	20.1	0.1	0.4	1.8	4.9	14.3	20.8	37.6

No. of total embryos: 1,000

Table 5. Development of embryos from one cell stage during culture with m-KRB in tube

Stage / Hours	1-Cell	2-Cell	3-4 Cell	6-8 Cell	Morula	Blastocyst	Fragmented	Degenerative
24	24.4	31.8	19.5	6.6	—	—	15.6	2.1
48	21.1	4.1	3.4	12.3	25.2	4.6	16.1	13.2
72	17.3	—	—	4.1	8.9	23.6	17.4	28.7

No. of total embryos: 1,000

cleavage rates to six to eight-cell stage were 7.9% in first group, 11.8% in second group, 5.3% in third group and 6.6% in fourth group.

2.2 Observation after 48 hours:

After 48 hours, cleavage rate to one-cell stage was 48.0%, two-cell stage 0.4%, three to four-cell stage 0.8%, six to eight-cell stage 6.5%, morula stage 6.2%, blastocyst 2.3%, disproportioned cleavage 14.6% and degenerated embryo 2.12% in first group (Table 2). In second group, cleavage rate to one-cell stage was 49.6%, two-ell stage 1.7%, three to four-cell stage 2.3%, six to eight-cell stage 2.4%, morula 9.2%, blastocyst 1.6%, disproportioned embryo 18.3%, and degenerated 14.9% (Table 3). In third group, 28.6% of them were cleavaged to one-cell stage, 3.2% to two-cell stage, 2.4% to three to four-cell stage, 8.8% to six to eight-cell stage, 19.2% to morula, 1.2% to blastocyst, 18.2% to disproportioned embryo and 18.4% to degenerated embryo (Table 4). In fourth group, 21.2% of them were cleavaged to one-cell stage, 4.2% to two-cell stage, 3.4% to three to four-cell stage, 12.3% to six to eight-cell stage, 21.2% to morula, 4.6% to blastocyst, 16.1% to disproportioned embryo, and 13.2% to degenerated embryo (Table 5). After 48 hours cleavage rate to six to eight-cell stage was 15.0% in first group, 13.2% in second group. 29.2% in thrid group and 42.1% in fourth group.

2.3. Observation after 72 hours:

After 72 hours cleavage rate of one-cell stage was 12.1% two-cell stage and three to four-cell stage were not observed, six to eight-cell stage 3.1%, morula 1.4%, blastocyst 5.2%, disproportioned embryo 18.0% and degenerated embryo 60.2% in first group (Table 2). In second group, cleavage rate to one-cell stage was 11.8%, two-cell stage 0.6%, three to four-cell stage 0.4%, six to eight-cell stage 1.6%, morula 2.6%, blastocyst 6.3%,disproportioned embryo 18.9%, and degenerated embryo 57.8% (Table 3). In third group, 20.1% of them were cleavaged to one-cell stage, 0.1% to two-cell stage, 0.4% to three to four-cell stage, 1.8% to six to eight-cell stage, 4.9% to morula, 14.3% to blastocyst, 20.8% to disproportioned embryo, and 37.6% degenerated embryo

Table 6. Development of embryos between 6~8 cell and blastocyst after 72 hours

Media in Container	%
Ham's F-10 in dish	9.7
Ham's F-10 in tube	10.5
m-KRB in dish	21.0
m-KRB in tube	36.6

(Table 4). In fourth group, 17.3% of them were cleavaged to one-cell stage, no cleavage to two-cell stage and three to four-cell stage, 4.1% to six to eight-cell stage, 8.9% to morula, 23.6% to blastocyst, 17.4% to disproportioned embryo, and 28.7% to degenerated embryo (Table 5). After 72 hours, cleavage rates to six to eight-cell stage were 9.7% in first group, 10.5% in second group, 21.0% in third group, and 36.6% in fourth group (Table 6). After 72 hours of culture, the results of cleavage from six to eight-cell stage to blastocyst were compared mutually and examined its significance. There was no significant difference between culture vessels in case of using Ham's F-10 culture media, but there was significant difference between culture vessels using m-KRB culture media ($p<0.05$). There was also significant difference between culture media in case of using same culture vessels, so cleavage rate was higher with m-KRB than with Ham's F-10 ($p<0.05$).

DISCUSSION

Since direct observation of early stage fertilization of mammal is possible by IVF and it is not only a research method which can examine systematically the condition of fertilization but can be used for treatment of human infertility and improvement of productivity of livestock, there has been achieved many studies, about it.

Success rate of IVF differs greatly according to investigators. Whittingham (1968) and Kaleta (1979) reported below 25%, but Cross and Brinster (1970), Hoppe and Pitts (1972), Miyamoto and Chang (1973), Fraser and Drury (1975), Wolf and Inoue (1976), and Niwa *et al.* (1980) reported above 90%. The reason of this difference is the discrepancy in experimental methods and success criteria of fertilization of each investigator.

Gonadotropin (PMSG/HCG) is used to induce superovulation in study of mouse IVF. Superovulation rate induced by gonadotropin is influenced by various factors. Even within one mouse strain injected on the same day with the same batch and dose of hormones, the number of eggs ovulated per mouse have ranged from 0 to over 90. Ackerman *et al.* (1983) reported differences in numbers of ova retrieved between mouse strains after induction of superovulation by gonadotropin, that is 24.2 ± 5.1 in CD-1, 33.0 ± 5.8 in CB_6F_1 and 16.3 ± 6.6 in B_6CBAF_1. Numbers of ova retrieved per mouse were $14.7\pm2.58\sim21.26\pm6.73$ (average 17.14 ± 4.19) in this study, lower than 26.4 ± 4.1 of Laufer *et al.* (1983) and $26.7\pm2.1\sim45.8\pm5.5$ of Collins *et al.* (1980).

Because of difficulty in calculating the exact numbers of ova surrounded by cumulus cell, there are some errors (Ackerman *et al.* 1983). Ovulated ova were counted after removal of cumulus cell after fertilization in this study, and it was sometimes impossible to count ova surrounded by cumulus cell. And the *in vitro* cleavage rate reaching two-cell stage after fertilization is influenced by age of experimental animal, method of ovulation induction, site of egg retrieval; method of sperm acquisition and capacitation condition, whether cumulus cell and epididymal fluid exists or not, sort of culture media during fertilization, concentration and amount of drops of sperm, and exposure time of ova to pserm and so on, so it is impossible to compare the cleavage rate uniformly. Also this comparison is influenced by genetic factor, pretreatment for experiment before culture of embryo and biological changeable factor and so on. Sperm capacity of fertilization, sperm-egg interaction and fertilizing ability of ovum are influenced especially by mouse strain, and also by time of entrance of sperm into ovum *in vitro.* Niwa *et al.* (1980) found that the shorter the time required for cleavage, the faster it reached blastocyst stage.

The success of IVF is decided by the methods of comparative observation of developing process from fertilized egg to morula and blastocyst with normal embryo developing in oviduct after fertilization, to see whether the ova are developed into symmetrical cleavage globules, and of observation of developmental normality after transfer to foster-mother of embryo developed by IVF. In this study, growth of fertilized egg to morula and blastocyst 72 hours after fertilization was observed. Observation of two pronuclei and polar bodies was possible but not in all experimental processes.

In this study the cleavage rate of *in vitro* fertilized mice ova, to six to eight-cell stage after 24 hours of observation was 7.9% in first group (Ham's F-10 in

dish), 11.8% in second group (Ham's F-10 in tube), 5.3% in third group (m-KRB in dish) and 6.6% in fourth group (m-KRB in tube). After 48 hours of observation, cleavage rate to six to eight-cell stage was 12.7% in first group, 13.1% in second group, 29.2% in third group, and 42.1% in fourth group. This result was lower than the findings of Ackermann *et al.* (1983) which were 68.4% and 66.9% using m-KRB culture media, and 26.0% and 36.1% using Ham's F-10 culture media. This was also lower than 92% of Hoope and Pitts (1972), 70% of Quinn *et al.* (1984). 62.5% of Mettler *et al.* (1980), and 83±17% of Laufer *et al.* (1983). After 72 hours of observation, results reaching six to eight-cell stage and blastocyst were 9.7% in first group, 10.5% in second group, 21.0% in third group and 36.6% in fourth group, which were lower than those of other studies. In comparison of IVF cleavage rate according to vessel and media of culture by Ackerman *et al.* (1983), it was lowest 24.6% in group using m-KRB in dish, 28.4% in group using Ham's F-10 in dish which was higher than 21.0% and 9.7% of this study. They reported that cleavage rate was best 44.8% in group using m-KRB in tube, also it was best 36.6% in this study.

Quinn *et al.* (1984) pointed out the reason why mouse IVF system being not used as a quality control method in executing human IVF and ET, that were no recognition of its importance, difficulty in acquiring good cleavage rate from mice ova and no establishment of proper experimental method. They reported that they could not find correlation between pregnancy rate of human IVF and ET and use of culture media by which cleavge rate of mouse ova reach blastocyst over 70% although they improved the factors which decreased viability of mice ova during culture. But they insisted on examination of whole process if pregnancy rate decrease or they can't conceive after five consecutive embryo transfer.

Interaction between energy sources and pH is important for the two-cell stage mice ova to reach blastocyst and embryo, for instance, a fall in pH leads to decrease of proper concentration of pyruvate which is energy source necessary to ovum development (Hoppe and Pitts 1972). It is emphasized on composition of culture meida to culture early stage embryo *in vitro.* Because there is a change of surrounding environment according to developmental process of ova *in vivo,* it is reasonable to change the composition of culture media accordint to stage. But it is difficult to control perfect culture composition because secretion in genital tract changes according to sstate of endocrine and method of extraction. Examination about inorganic salts required to develop embryo is depressed compared to that of insemination culture media of IVF, it accords with the concentration in serum as a rule. It is known that Ca^{++} and Mg^{++} influence cleavage of two-cell stage, especially Ca^{++} is known to participate in development of blastocyst. Development is maintained with only pyruvate during one to two-cell stage, lactate is added during two to eight-cell stage, and glucose, frucotose or citrate are used after eight-cell stage, therefore pyruvate, lactate and glucose are added essentially. Protein amount of early mouse embryo decreases between one-cell stage and morula stage, increases during blastocyst stage. pH is determined according to bicarbonate and CO_2 concentration. It is desirable to maintain pH between 7.3-7.4 in CO_2 incubator. Proper osmolarity of culture media for mouse embryo is 277-307 mOsm. It is better using culture media of lower osmolarity than that of mouse serum (310 mOsm). Besides, temperature and humidity of incubator for mouse embryo should be maintained at 37°C and near 100% respectively to prevent the change of pH and osmolarity.

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= 국문초록 =

생쥐난자의 체외수정에 관한 연구

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인간난자의 체외수정 및 자궁내 이식의 성공으로 착상전 태아의 적절한 성장을 유지할 수 있는 배양조건에 관심이 집중되고 있다. 생쥐난자의 체외수정에 관한 연구는 인간난자의 체외수정에 직접 응용될 수 있다. 그 이유는 두 종의 난자의 체외수정에서 유사한 조건이 사용되기 때문이다. 인간난자의 체외수정 및 자궁내 이식에 대한 정도관리의 방법으로 1984년 4월부터 서울대학교 의과대학 산부인과학 교실에서 생쥐난자의 체외수정에 관한 실험을 실시하여 다음과 같은 결과를 얻었다.

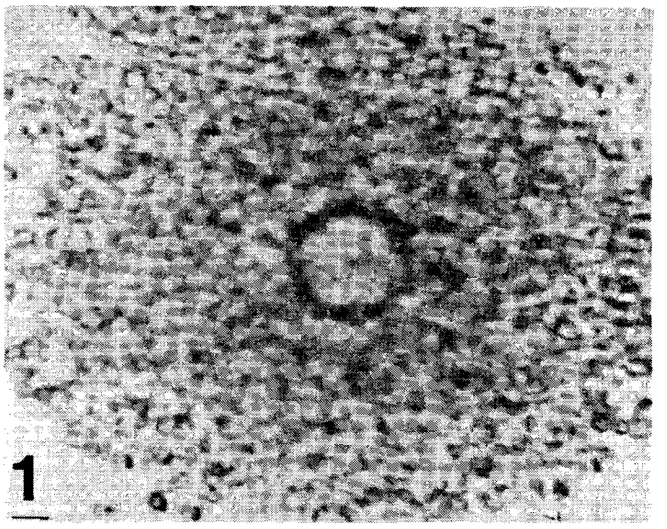
1. 생쥐당 평균 난자 획득수는 17.14±4.79 개이었다.

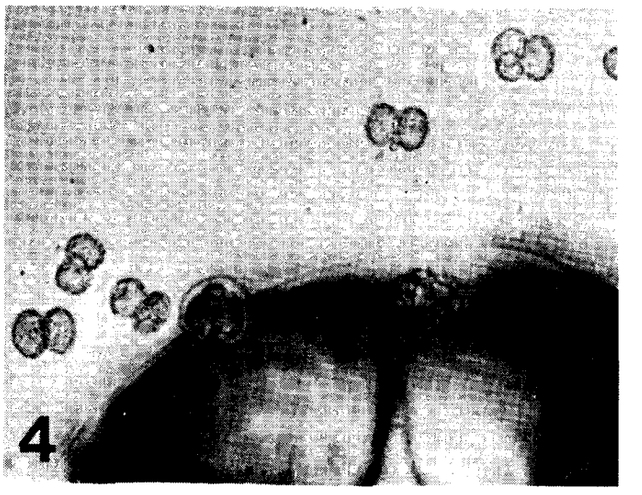
2. 퇴화되었거나 변형된 것을 제외하고 정상적으로 보이는 난자는 38.3-64.0% (평균 50.5)에서 관찰되었다.

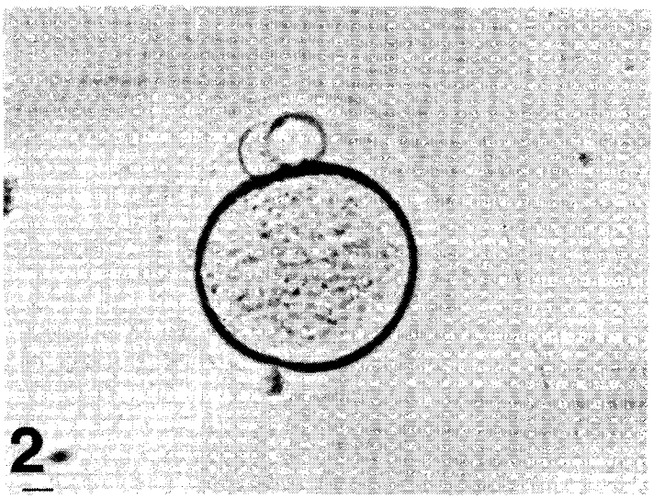
3. 두가지 배양액과 두가지 배양용기를 비교할 때 6-8세포기에서 포배까지 도달한 난활성적을 상호 비교하면 Ham's F-10 in tube 군은 10.9%, m-KRB in dish 군은 21.0%, m-KRB in tube 군은 36.6%이었다.

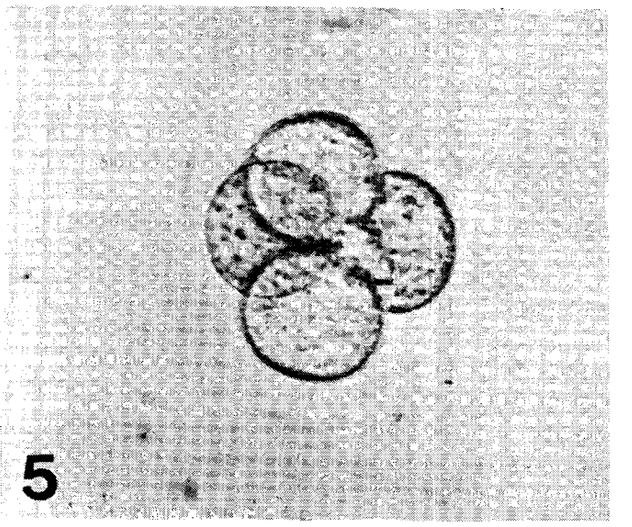
4. 생쥐태아의 성장에 있어서 m-KRB 배양액이 Ham's F-10 배양액에 비해서 난활성적이 높았다.

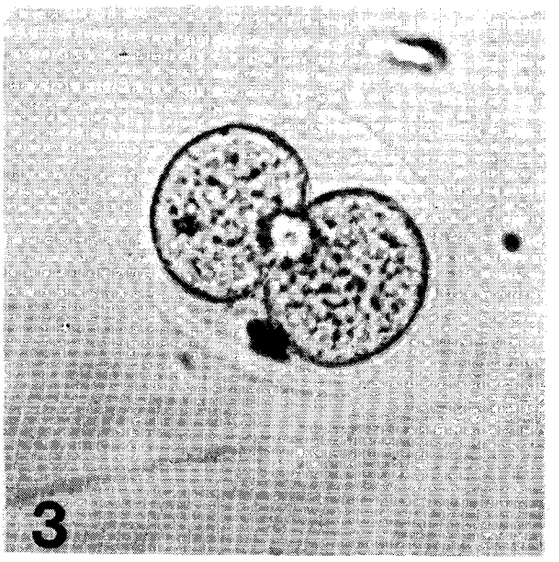
생쥐난자의 체외수정에 대한 연구는 인간난자의 체외수정에 대한 정도관리 및 연구모형으로서 가치있는 방법으로 판단되었다.











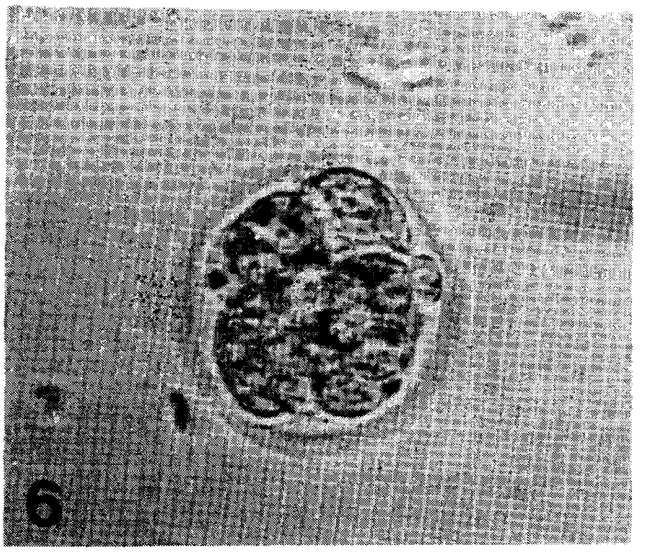


Fig. 1. Mouse oocyte immediately after flushing oviduct (X400).
Fig. 2. 1-cell stage mouse embryo 24 hours after insemination (X400).
Fig. 3. 2-cell stage mouse embryo 24 hours after insemination (X400).
Fig. 4. 2-3 cell stage and degenerative mouse embryo 24 hours after insemination (X40).
Fig. 5. 4-cell stage mouse embryo 48 hours after insemination (X400).
Fig. 6. Morula stage mouse embryo 72 hours after insemination (X400).