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THE EFFECT OF VITRIFICATION OF OOCYTES FOR MICE AND EXAMINING SURVIVED OOCYTE PERCENTAGE

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ABSTRACT

In recent years a lot of efforts have been done in the area of vitrification in order to maintain oocytes because it is necessary to preserve and maintain oocytes in assisted reproductive technology (ART) for example it is necessary to keep oocytes for treatment of infertility among cancerous young women who are subjected to chemotherapy and radiotherapy. Most efforts have been for increasing efficacy and survival rate and oocytes fecundation after fusing and evolving of obtained embryo. In this research the effect of vitrification with cryotop method on the survival, fecundation, and subsequent evolution to two cells stage for mature mouse oocytes is examined. After ovulation stimulation by injecting HMG and HCG hormones to NMRI female mice the oocytes were isolated by the method of flushing uterus corns. Then the granulosa around the oocytes have been isolated by hyaluronidase. After equalization in vitrification solution containing ethylene glycol, di methyl sulfoxide, and sucrose the oocytes are transferred to cryotop and are frozen and maintained in liquid nitrogen. After fusing the rate of oocytes survivals are examined. Survived oocytes are fecundated by IVF method and are cultured in Ham's F10 environment containing BSA 4 mg/ml and certain number of oocytes was fecundated as control too and subsequent evolution of embryos is evaluated by Chi-square test and the open Epi software. The results shows that the high rate of oocytes (84.5%) are survived after cryotop vitrification. And in comparing the fecundation level and evolution to two cells stage with control group no significant

difference was observed (p value =0.21%). Regarding obtained results it can be concluded that the cryotop vitrification method is an appropriate method for oocytes vitrification.

Keyword: Oocytes, mouse, vitrification, cryotop

INTRODUCTION

Cryopreservation is a process in which the cells or tissues are frozen and preserved by cooling in temperature bellow -130^0 C. freezing in the temperature bellow -130^{0}C makes cells and tissues preservation and protection possible for prolonged and unlimited time [1]. In such low temperature all biological activities of the cell including biochemical reactions that lead in cellular death is ceased and the cells after fusing again the biological activity resumes normally. This finding firstly was applied on simple creatures such as bacteria and single-celled and then was operational for multicellular and even organs [2].

Generally smaller samplers with simpler structure have more success change for survival after freezing. since middle of twentieth century along with progress in reproduction technology such as in vitro, some progresses have taken place in cryopreservation of sperm, embryo and obtained oocytes [3].

Success rate in oocytes vitrification was relatively low comparing with embryo vitrification due to oocytes special physiology (big size of oocytes, high level of cytoplasm, high water content) and sensitivity to thermal and cold and chemical shocks [4]. But in recent ten years the process of oocytes vitrification has brought about great change in treatment of infertility and has helped tremendously to success and increase of fertility efficacy, oocyte vitrification and using vitrified oocytes after fusion and pregnancy with embryos obtained from these oocytes has been considered as an important solution in treatment of infertility. Oocytes vitrification has more advantages and less legal and ethical obstacles rather than zygote vitrification. With vitrification of young cancerous patients their gametes is preserved before sustaining the treatment such as chemotherapy or radiotherapy that can cause sterility. Also in some European countries such as Italy that embryo vitrification is forbidden, the egg cells vitrification is the only alternative. Also with oocyte vitrification and producing oocyte bank it is possible to donate oocyte to women who lacks ovary or for any reason they cannot use their oocyte [3]. This reseach examines on the recent findings obtained from details of cryotop vitrification that ensue most level of live delivery in the world by vitrification of human oocytes [3].

Regarding necessity of preservation and vitrification of oocytes and considering ease of use and appropriate result and high efficacy of cryotop vitrification with high vitrification speed and use of low volume of vitrification solutions (less than 0.1 µL) as two important factor in increasing success and efficacy of vitrification , in this research it is attempted to vitrify the adult mouse oocyte in metaphase II stage by this method and evaluate its effects on rate of survival, fecundation and obtained embryos.

RELATED RESEARCHES

[3] Have reported high level of survival after egg cell vitrification and fusions in this method, they stated that almost all vitrified oocytes by cryotop were survived after fusion process. After fecundation by ICSI method, embryos progress till cleavage stage as well as pregnancy rate had no significant difference comparing with control group. After transferring blastocysts 45% of vitrified oocytes have been evolved and turned to healthy infant. [3] Has reported birth of more than 50 healthy infants by cryotop vitrification of oocytes. In such manner that in any infants no evolutional disorder has been observed. This method was adopted by other embryologists in response to patients need all around the world. For example [5] has presented 97% survival after cryotop vitrification of oocyte. The first infant birthed in USA from oocyte donation from oocyte vitrification by [5] group. [6] Have obtained 89.2% survival after oocyte cryotop vitrification and totally 56.5 % pregnancy (13 infants from 23 patients) have been obtained. A Colombian group has obtained first healthy infant after oocyte vitrification in south America [6]. [7] has achieved 90.1% survival (among 445 oocytes, 401 oocytes have been survived) and 34.1 pregnancy after oocyte vitrification [7]. [8]have vitrified 225 oocytes in metaphase II stage and 217 oocytes have survived (96.5%). 165 (76%) oocytes have been fecundated by ICSF method that have no significant difference with control group (fresh or non-vitrified oocytes) 94% of zygotes progressed to cleavage stage and 22.4 % progress to blastocyst stage and no significant difference was observed with control group. Among 21 embryos transferred to uterus 13 pregnancy (61.9%) was reported [8].

It is premise that there are three essential principle for increasing efficiency and success rate of vitrification process including 1) high cooling rate 2) low volume of solutions containing biologic samples for vitrification <1µ 3) using appropriate CPAs for appropriate density, therefore one of important and effective

factors of increasing efficiency in vitrification is CPAs and efforts taken place in this area was for using COAs with minimum toxicity in high density, high solvability in water and high permeability in the cell. Published results from many researches have revealed high success and efficiency of vitrification using types of antifreezes for oocytes of different species.

VS1 was the first vitrification solution that was adopted in 1985 by [9] for vitrification of mouse embryo and lead to good results too, this solution contains di methyl sulfoxide, glycerol, ethylene glycol and 1 and 2 propanediole. Using this solution lead to different results in different studies in terms of survival rate and subsequent evolution [9].

[10]have vitrified mouse oocytes with the same solution [10].

[11] Have vitrified the mouse and humane oocytes comparatively with the same method high amount of oocytes survived and their subsequent evolution was good too. In the reports it is stated that using 2 or more antifreeze reduces particularly toxicity of each one [11].

But CPAs used in vitrification solutions for diluting and equalizing in cryotop vitrification method varies according to species requirement and evolutionary stages [3].

MATERIALS AND METHODS

Super ovulation:

The male mouse (Mus Mua culus) from race of NMRI with the age of 7 to 10 weeks provided from Iran Pastor institute has been maintained within 7 days in animal laboratory of new technology research institute of Avicenna university jihad medical sciences with light-dark cycle (10 hours light-10 hours darkness) and temperature ($23\pm2^{o}C$) and have been nourished with special food of rodent (figure 1-2)

For super ovulation at 18 o'clock hour of first day 100λ prepared solution of HMG containing LH 7.5IU and FSH 7.5 IU was injected to each mouse with intra-peritoneal method by insulin syringe.

48 hours after HMG injection at 18 o'clock equivalent amount of 100λ prepared hormone of HCG is injected due to having similar LH biologic activity leads to steroidogenesis in ovary and as a result intensifying production of estrogen and specially progesterone and then the ovulation takes place.

Tip: injection of prepared hormone for HMG and HCG should be within 48 hours interval and meanwhile the best injection time is within 16-18 o'clock and it is better that these injections take place at a certain time and the injection date and time should be written on label on every cage.

Injection by intra peritoneal

As mentioned above the injection has taken place by intra peritoneal method, in such manner that firstly neck skin (between two ears) should be caught between forefinger and thumb of left hand and the mouse tail should be kept between little finger and the loop of the same hand so that when we pick the mouse up it could not move (figure 2-2).

Then while insulin syringe is in right hand the syringe needle should be inserted between its stomach and thigh with angle 45^{o} in abdominal cavity and the injection would be acomplished.

Figure 2-1: Nourishing mice with rodent special food

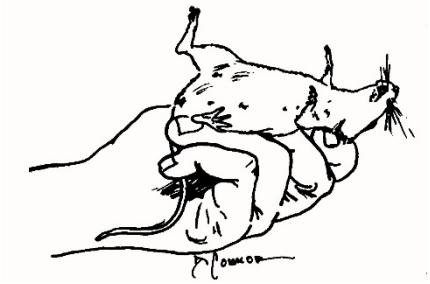


Figure 2-2: the proper manner of catching the mouse in hand for injection

Mice dissection:

Cervical dislocation: first of all for preventing from contamination spread we use glove and mask. We bring out the mouse cage and move it to washing room and then we put the mice on metal cage and we take mouse tail with right hand put the pig forceps on back of mouse neck we drag the mouse tail toward ourselves so that the spinal cord dislocates and so the mouse respiration ceases and the mouse sustain cervical dislocation death (figure 2-3)

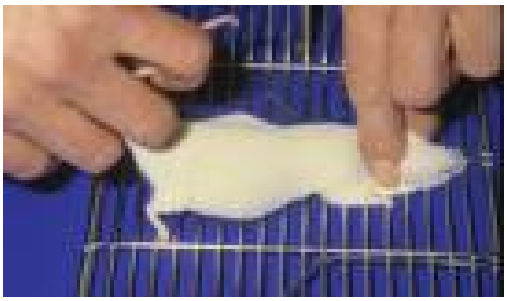
Separating uterine horns:

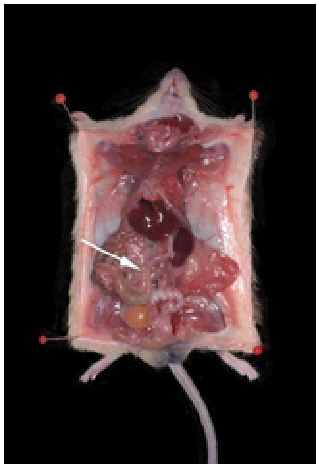
We lay the dislocated mouse supine on a fabric piece and fix the mouse limbs with pin to the fiber and clean its abdominal part with cotton and 70% alcohol. And take up the abdominal skin forceps from lower part (toward legs) and make and cutting and drag two sides of mouse skin to two sides of mouse body (tail and head), we split the

visceral membrane (figure 2-4). We push aside the guts aside so that the reproduction organ of female mouse appears. Then we cut the reproduction organ from fat pod part and distal uterus part.

We transfer the uterus together with ovary, oviduct and fat pod into a plate containing PBS then we move the plate into incubator with temperature 37^0 centigrade and 5% Co2 flow. Dissected mouse is put in freezer bag and transferred to congelation room.

Figure (2-3) a) the way of killing mice by cervical dislocation
b) Disinfection of external skin for dissecting





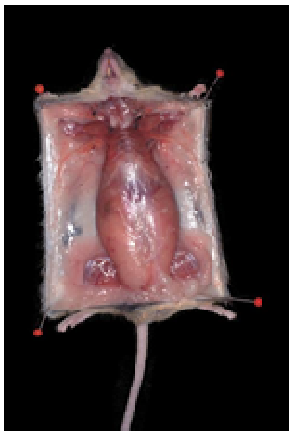


Figure (2-4): a) Pushing aside the external skin of mouse and exposing visceral membrane; b) Splitting and pushing aside the visceral membrane the gut is exposed

The stages of in vitro fertilization: various methods for fertilizing oocytes are used in the laboratory. The oocytes are often placed in great volume of .5-1 ml culture environment and then 50-100 thousands of movable and healthy sperms are added to the environment. In fertilization duct near 2-4 thousand normal sperms are offered to the oocyte. in the cases that the oocytes are fertilized in tiny droplets under oil (0.2 microliter) less number of sperms are needed. Even recently there are some reports that oocytes are fertilized in an environment that is cultured from another cellular layer like to cover cell of oviduct capsule region. For the ease of practice we used the droplet laying under oil that its stages are as follows:

Providing sperm:

Obtaining and selecting semen by standard presented from WHO

- Receiving epididymis
- Capacitation
- Isolation of movable sperm from other cells
- Centrifuge of culture environment containing sperm on puresperm solution (a solution with high volume mass)
- Isolation of obtained sediment and swim up

After killing mail mouse of rave NMRI by cervical disclosure we survey and dissect the mice and cut the reproductive region from upper part of epididymis and under testicles. The detached part is transferred to Ham's f10 environment contained 4 mg/ml BSA and crush completely the part with scalpel blade so that the sperms enter the environment. The culture environment containing sperm is placed into incubator of 37^0 C for 30 minutes for turning the clot phase to liquid. For isolation of movable sperms the puresperm method is applied. In this method the storage solution called

puresperm (manufactured by Nidcon company in Sweden) is used which is produced in 40% and 80% density and respectively at first 2 ml from 40% density is added to recent density so that two separate phases would be provided and then 2 ml from environment containing sperm is gently added to 40% density and is centrifuged in the round 600 g for 5-20 minutes. In this manner the movable sperms are aggregated at the end of the pipe and by isolation of superficial solution the sperm of the end of the pipe are washed with 2 ml of Ham's f10 containing 4 mg/ml BSA and then 0.5 ml from BWW environment containing 5 mg/ml the movable sperm sediment is centrifuged again in round of 1000 g within 1 minute. And then the pipes are brought out gently from centrifuge and placed in incubator of 37^0 C with 45 angle and 5% co2 for 30 minutes so that the movable sperms move upward then by use of pastor pipette the sperms that reached to upper surface of the environment are separated and then after examining movement amount in isolated population it is used under microscope for subsequent stages.

1- Healthy and active sperms are transferred to test group (including vitrified and fused oocytes) and control group (the oocytes that are not subjected to vitrification)

2- after 4-8 hours the oocytes are transferred from culture environment droplet to new droplets of Ham's f10 containing 4 mg/ml BSA.

3- For examining formation of precores and stages of embryo evolution to stage of having two cells after 24 hours the oocytes are evaluated under inverted microscope.

Number of 67 oocytes of experiment group and 80 oocytes of control group are fertilized beside male mouse healthy sperms of race of NMRI and the degree of embryo evolution obtained from two groups to stage of two cells are evaluated.

FINDINGS

Super ovulation:

After super ovulation by injection of 10 IU from prepared hormones of HCG and HMG within 48 hours interval to cycle determined mice (the mice in estrous cycle)(figure 3-1), from total of 15 female mouse of NMRA that have been super ovulated , the number of 170 oocytes are obtained after flushing uterine horns. The oocytes are evaluated by microscope and are placed in incubator of 37^oC.

Evaluating oocytes after vitrification and fusion:

90 mature oocytes containing granulosa bulk placed in Ham's f10 environment containing 4 mg/ml BSA in temperature of

37°C are reevaluated under inverted microscope and all 90 oocytes have good quality. These oocytes after subjecting to hyaluronidase for eliminating the granulosa bulk around the oocyte are reevaluated under microscope once more and among 90 oocytes number of 84 oocytes benefits highly good quality in terms of morphology for vitrification (oocytes with desired morphology, the oocytes with zona pellucida and plasma membrane are healthy the figure (3-3) and (4-3). (Figure 3-2) shows well the granulosa bulk around oocyte and separation of this cellular bulk after care with hyaluronidase.

Number of 84 oocytes without granulosa bulk are transported within 5 times experiment each time 15 to 20 oocyte after two stages of equalizing in vitrification solution (for substituting freezing protectant material or vitrification solution with intercellular water) by cryotop (4 to 5 oocytes transported on cryolife) and they are frozen in liquid nitrogen, after fusion stages (for bringing out vitrified solutions from cell) that include dilution and washing oocytes in Ham's f10 environment containing 4 mg/ml BSA are transferred for evaluating survival rate (figure 3-7). In this stage oocytes survival is evaluated by inverted and loop microscope. Number of 84 mature oocytes are vitrified in 5 times and then fused. Among this number after examination under inverted microscope number of 71 oocytes preserved their natural morphology and survived. High rate of survival (84.5%) suggests appropriateness of this method for oocyte vitrification.

Rate and percentage of oocyte survival after vitrification and fusion is demonstrated in table 1-1.

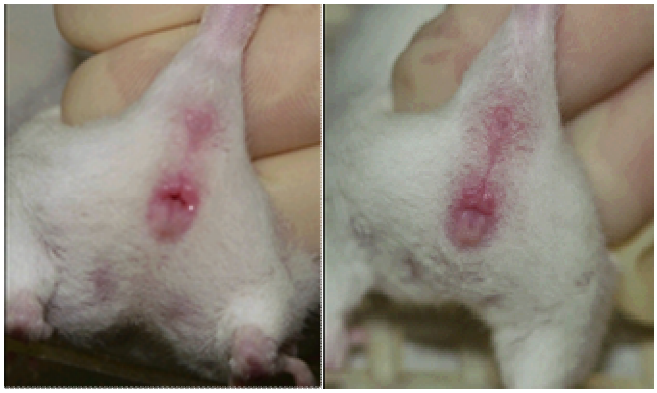


Figure 3-1: when vaginal region in mouse is red, Open and inflated the mouse is in estrous cycle, the right side figure shows that though vaginal region is red and inflated but because this region is closed. The mouse is not in estrous cycle, while in lift side shape, mouse is in estrous cycle

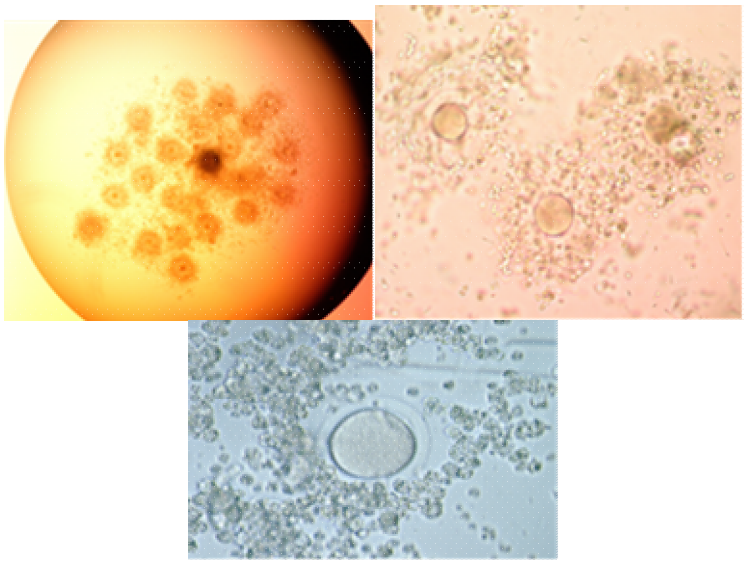


Figure 3-2

a) Accumulation of oocytes together with granulosa cellular bulk
b) Granulosa cellular bulk is detaching after subjecting to hyaluronidase
c) Granulosa cellular bulk is detaching after subjecting to hyaluronidase with higher aggrandizement

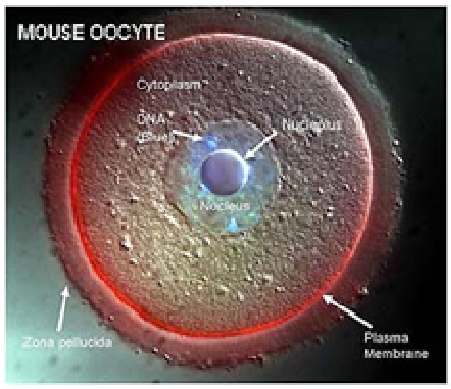


Figure 3-3: An exhibition of zona pellucida oocyte and plasma membrane

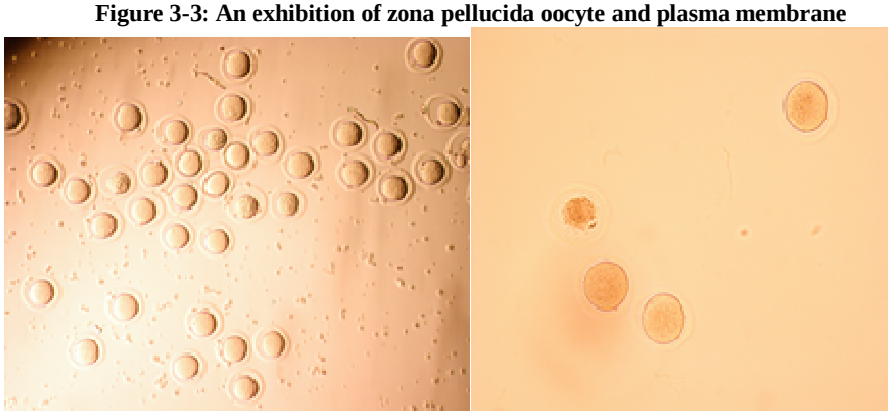


Figure 3-4

a) The oocytes without granulosa cellular bulk before vitrification
b) Oocytes without granulosa cellular bulk before vitrification with higher aggrandizement

The upper left oocytes are not appropriate oocytes for vitrification due to unhealthy plasma membrane

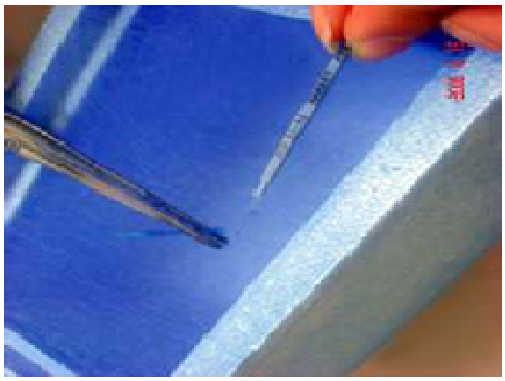


Figure 3-5

Oocytes are vitrifying with cryolife

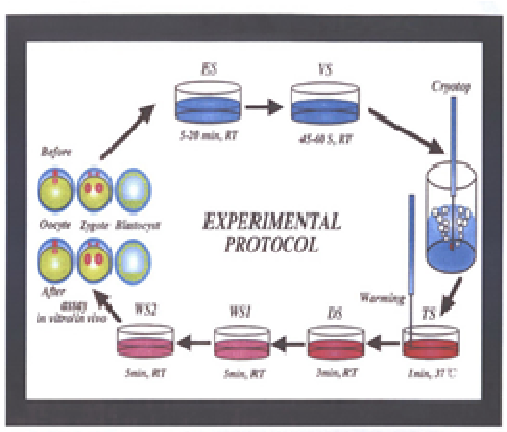


Figure 3-6

Process of vitrification and fusion with cryolife and preserving oocyte morphology after freezing and fusion

Evaluating fertilization and evolution of frozen and fused oocytes:

For evaluating and evolving oocytes necessary condition for fertilization for IVF method is prepared.

Number of 67 mature oocytes that have been frozen by cryotop and then fused in these processes preserved their morphology (experiment group) and number of 80 fresh mature oocytes or non-vitrified oocytes (control group) are placed beside of healthy sperms of the same race of male mouse (NMRI).

Then the degree of fertilization and evolution of oocytes have been evaluate under inverted microscope.

Two cells embryos have been evaluated after 24 hours after adding sperm to the environment. Degree of oocytes evolution to the two cells embryo is demonstrated in table 1-1.

In experiment group among 67 fertilized oocyte member of 49 oocytes turned to two cells embryo.

In control group among 80 fertilized oocytes number of 63 oocyte turned to two cells embryo thus difference between control group and experiment group was not statistically significant (P=0.21).

Statistical analysis: obtained information have been statistically analyzed by statistic test of chi square and open Epi software.

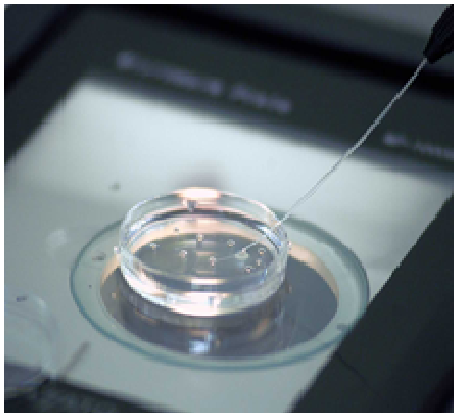


Figure 3-7
Transferring oocytes to culture environment of droplet for evaluating survival rate

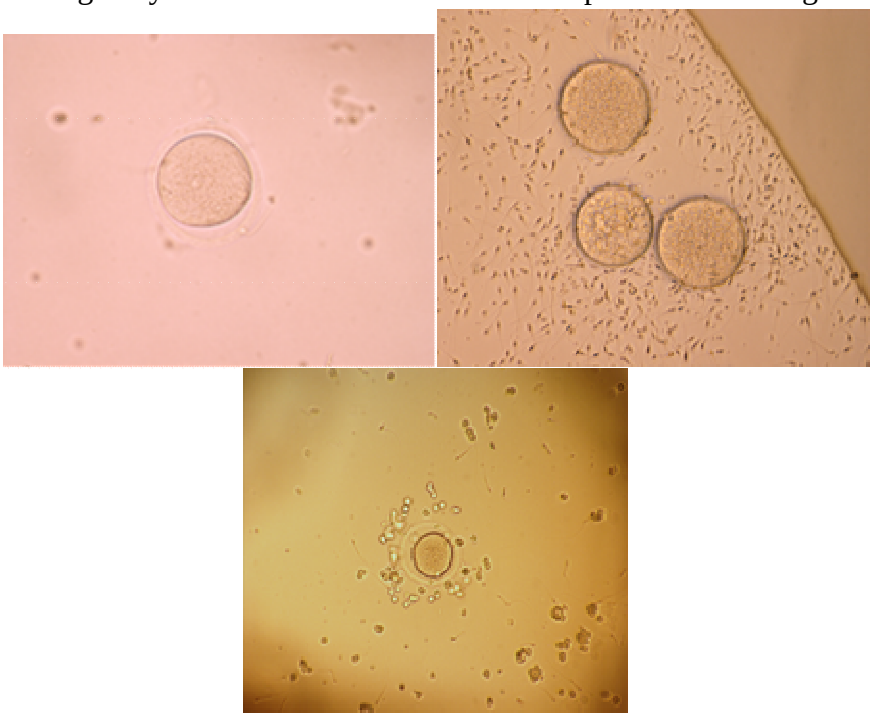


Figure 3-8: Oocyte fertilization by IVF method, a) adding sperm to the environment containing oocyte, b) showing an oocyte confined among sperms c) connecting a sperm to oocyte

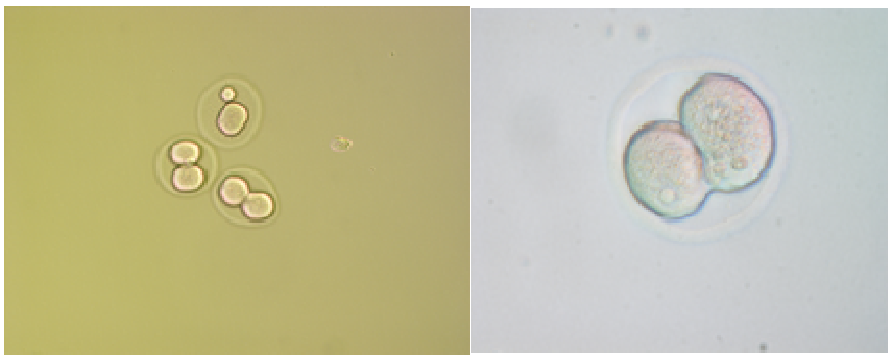
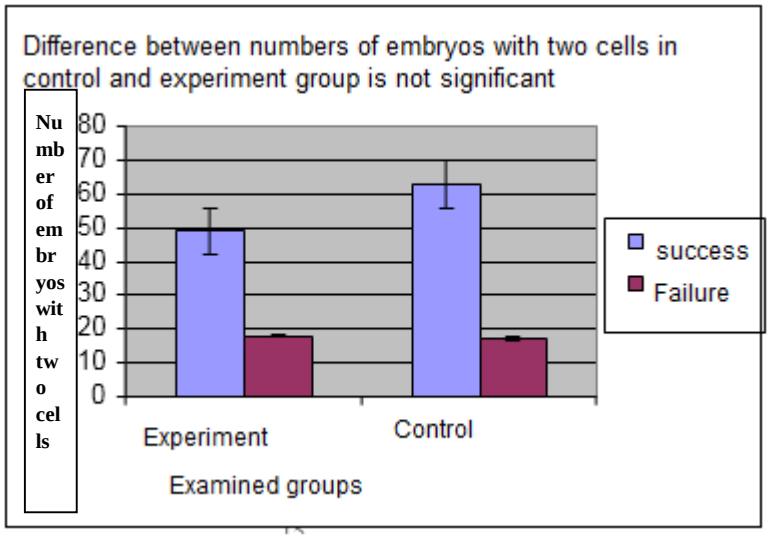


Figure 3-9: A) Two cells embryos 24 hours after fertilization
B) Two cells embryo with higher aggrandizement

Table (3-1) survival rate of oocytes after vitrification by cryotop and comparing embryos evolution obtained from vitrification oocytes after fertilization instill the stage of two cells with control group

grpops / Parameters	Control group	Experiment group
Oocyte numbers	80	84
Healthy oocytes after vitrification and fusion	-	71
Healthy oocytes percentage after vitrification and fusion	-	84.5%
Composed oocytes with sperm	80	67
Number of two cells embryo	63	49
Number of degenerated embryos	17	18
Embryos survival percentage	78.7%	73.1%

X^2=0.63;0.21 The difference with control group is not significant=P-value*



	success	failure
Experiment	49	18
control	63	17

CONCLUSION

The results obtained from this research shows that vitrification in solution containing ethylene glycol and di methyl sulfoxide with cryotop method is an appropriate method for mice oocyte vitrification and consequently for human oocytes because high percentage of oocytes have been survived after vitrification and fusion within cryotop vitrification and after fertilization these oocytes of these oocytes with IVF method the evolution of embryos has progressed to the stage of two cells embryo similar to control group. These results can be compared with the results of other researcher around the world that have used cryotop vitrification for oocytes. For present research we used equal proportions of di methyl sulfoxide and ethylene glycol as most effective compound as well as sucrose in low density for equalizing. Regarding these results of this research it can be said that this solution can be an appropriate solution for oocytes cryotop vitrification.

Ethylene glycol as a CPA with high permeability and low toxicity and low molecular weight is used in most vitrification solution as a standard compound.

One of vitrification assistance devices that is applied in order to provide minimum environmental volume for vitrification is cryotop that is designed by kawayama and kitazito. Regarding very low volume in cryotop method cooling and heating rate reaches higher than 50000 ^{0}C [3].

Cryotop vitrification at present time is the best way of oocytes vitrification for objectives such as creating oocyte bank, or preserving fertility of young cancerous patients after chemotherapy or radiotherapy treatment or for women who whether for disease reasons or any other reason cannot use their own natural oocytes, thus oocyte bank is a solution.

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