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REVIEW ARTICLE

Examining Laboratory Mouse Oocytes and Vitrification

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ABSTRACT
Nowadays in many laboratories around the world cryotop vitrification is used for oocytes vitrification as the best protocol for freezing oocytes. Published reports are an evidence for this important issue. Regarding to the fact that cryotop vitrification is an open system and there is a direct relation between liquid nitrogen and oocytes, for higher biologic safety and preventing disease propagation the vitrification method still can be improved and extended so that a closed system for oocyte vitrification may be evolved so while is biologically safer it has higher efficiency too. This research examines mouse oocytes and vitrification. The results of this research reveals that cryotop vitrification is the best method for oocytes freezing at present time for some objective such as oocyte bank establishment, maintaining fertility of cancerous young patients subjected to chemotherapy or radiotherapy or for the women who for some disease reasons or any other reason cannot use their own natural oocytes, in these cases oocyte bank can be an opportune solution
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INTRODUCTION

In recent years regarding necessity of preservation of oocytes in assisted reproductive technology, for example necessity of preserving oocytes for treatment of infertility in young cancerous women subjected to chemotherapy or radiotherapy great efforts has been done in the area of vitrification for preserving oocytes and most efforts have been carried out in increasing efficiency and survival rate and fertilization of oocytes after melting and evolution of obtained embryos. While it is several years that vitrification is innovated but in many methods of vitrification due to formation of intercellular and extracellular ice crystals , dehydration of cells and osmoses pressure change effect during freezing and melting the success level was fairly low.

Furthermore regarding the fact that the oocytes of different species and races react differently to the same method of vitrification, in spite of abundant effort carried out in this field there is a prominent discussion about the best protocol and method. Vitrification is a new advent event in cellular cryopreservation that prevents formation of ice crystals in the inner and outer space of the cell [1]. Vitrification is a process of cryopreservation that is obtained from high density cryoprotectant agents with high freezing rate.

Recent information indicated that increase in survival rate and fertility level after the course of human oocytes vitrification is increased even in immature vitrified human oocytes [2].

Meta structure of oocytes and its changes during cryopreservation

In mammals the multiplication phase cease in the embryonic phase. Then in fact in embryonic period between second to seventh months of pregnancy oogonia are multiplied dramatically so that there is near 7 million oogonia in human and great number of the oogonia disappears and the remaining turns to initial oocytes that progress to stage diplotene prophase meiosis I and there they cease and remain in this same state until maturity time, in this manner the oocyte is in the form of a small spherical cells with a big spherical core and its colorability is low that is surrounded by a row of follicular cells and this set is termed primary follicle. In primary follicle the golgi organ is seen as individual and propagated and

abundant and the endoplasmic network develops more and is overflowing from ribosome. The free ribosomes are seen in abundant number and mitochondria are propagated between cytoplasm. The oocyte and the surrounding granulosa cells are separated by a thin space from each other where the cell microvilli stand in this space. While oocyte volume increases the transcription and translation take place, the cytoplasm would possess complicated organelles, for example the apparent shape of mitochondria change and the golgi device develops and migrate toward environment, at the end of growth phase the oocyte diameter reaches from 35 micrometer in primary follicle to near 120 micrometer in graph follicle. In this manner it has almost 100 times volume increase and cytoplasmic organelles are increased to great extent and the cortical granules appear as well. The oocyte is located in antral follicle among cumulus cells. Around the transparent membrane a layer of cubic granulosa cell called radial crown appears.

In maturity age the follicle stimulant hormone lead to follicular cells multiplication around the primary oocyte (the growing or secondary follicle) the secondary follicle layer reaches to 7 till 10 layers. Meanwhile a thin layer of glycoprotein with 15 mm thickness emerges around the growing follicle called transparent region. This region emerge from follicular cells. When the number of rows of cellular follicle reaches to 10-11 cellular layer gradually some small and big cavity emerges among follicular cells where follicular liquid accumulate in this cavities and the theca seize around it. The emerged small and big follicular cavities gradually join to each other and make a big follicular cavity that is full of follicular liquid, so that the oocyte with a bulk of follicular cells is pushed aside. Mentioned follicle in this phase is called graph follicle. The primary oocytes together with surrounding follicular cells located in the central cavity are termed cumulus oophorus and the follicular cumulus that has filled around the follicular cavity is named granulosa. Around the granulosa cells it is seized by theca including external and internal theca. The internal theca has more cells and less fibers and external theca has more fibers and less cells. The oocyte that has started its meiosis division on embryonic period and is ceases in prophase I stage when the oocytes is reached to its ultimate growth continue its meiotic division a little before ovulation under influence of hypophyseal gonadotropin and the subsequent stages of meiotic prophase I would be completed. Oocyte meiotic division doesn't lead to equal daughter cells production but brings about a secondary oocyte and a spheroidal cell with a little amount of cytoplasm named first polar mass. The first polar emerges in transparent coating and remains in contact with the oocyte and then the secondary oocyte rapidly enters metaphase II of meiotic division but soon would be ceased and after ovulation and fertility complete it.

OVULATION (RELEASING OOCYTES)

Usually among creatures that takes shelter in mother body and live under its protection, fairly low number of oocytes emerges. In creatures that have external fertilization accidently the growth of the embryo is at hands of destination so that the creatures engender great deal of eggs. For example in oil fish 5000000 eggs and in oyster 50000000 eggs is produced, conversly in some birds and mammals just one egg is released in each period of reproduction. However there is a balance in creatures' number. Cyclic changes in creature behavior and physiology is one of the female (not pregnant) features. In some rodent such as gray mouse and laboratory mouth there is estrous cycle. for example among gray mouse not fertilized eggs is poured out every five days. Such day is called critical sexual day that the female sex accept the male in case of pregnancy till the end of pregnancy period the cycle would not emerge anymore [3]. In fact estrous cycle is a severe sexual activity within a short time that associated with ovulation. The stimulant factor in this case is mostly external factors especially the light. The light amount, intensity, duration and type stimulate hypothalamus. The hypothalamus releases the GnRh in response to this stimulation. The gonadotropins influence on anterior pituitary and leads in secreting FSH and LH and as a result leads in follicular growth and ovulation. Therefore ovulation and estrous take place at the same time and following the estrous stage that is coincident with ovulation the metestrus stages takes place. And afterward the diesterus and prosterus take place that ensue again the estrous stage.

Some creatures have one cycle of estrus per year that is called monostrous and some domestic animals have several estrous cycle within a year called polyestrous [4].

Estrous cycle

Estrous cycle includes alternative physiological changes that take place by sex hormones in most female mammals, however human has menstruation. The mouse reaches to sexual maturity in age of 6-8 weeks and rat in age of 6-10 weeks and the average fertility age within mice is 9 months and rat is 9-12 months.

Estrous cycle begins after sexual maturity in females and ceases by anestrous phase (rest phase). Mostly the estrous cycle continue till the end of life.

The changes and function of mouse sexual tissues (uterus, cervix and vagina) is influenced by ovulatory hormones and estrous cycle. Gonadotropins of pituitary gland control ovulatory hormones and estrogen

and progesterone control cervix and vagina death, growth and performance. Each estrous cycle has 4 phase including proestrus, estrous, metestrus and diestrus.

Proestrus phase: in consequence of increase of FSH secretion, estrogen secretion increases in this phase as well and one or more ovulatory follicle start to grow. The number of follicles is different in various species. Depending to species and animal race this phase continue from 1 to 3 weeks. By induction of estrogen, uterus septum (endometrium) begins to grow and extend, in some animals there is vaginal and sanguinary secretion, but this blood should not confused with menstrual blood. During this phase the number of endometrium superficial epithelial and superbasal cells becomes 2 to 3 times as much. Most of epithelial cells are big and round and sometimes horny and little number of leukocytes are seen among them and the ovary has graph follicle as well. In this phase the female animal is not still acceptant for male animal.

Estrus phase

All epithelium cells have become horny and at the beginning of the phase tends to detach from each other. But in middle or the end of estrus phase they detach in the form of large spangles. At the beginning of the phase no leukocyte is found but at the end some leukocytes are observed. At the beginning of estrus graph follicles ripen in ovary and ovulation takes place 9-10 hours after the cycle. In this phase the females become sexually acceptant. Ovulatory follicles ripen under influence of gonadotropic hormones and estrogen secretions. The animal demonstrates one or more sexual accepting behavior. One of the signals is lordosis reflex. In this reflex the lower part of animal body becomes automatically prominent. In some species the vagina becomes red. The ovulation takes places automatically in some species while in some species the ovulation is induced by mating.

In these cases if the mating does not take place the estrous cycle continues and the next cycles begins again, as long as the mating and ovulation take place.

In female mouse this stage of reproduction takes 4-6 days among mice and the female accepts the male. In female mouse at estrus phase three important sign is visible: 1- the vaginal region opens 2- the vaginal region becomes re. 3- The vaginal region inflates.

Metestrus phase:

This phase is associated with growth of corpus luteim and progesterone secretion. Progesterone secretion leads in preparation of uterus septum for uterus acceptance, in case of failure of pregnancy the corpus luteim would be degenerated. In this phase the signs of estrogen stimulation reduce. The horny cells are piknosis horny cells are together with some amount of leukocytes. Some young epithelial are observed as well. The rapid decrease of uterus epithelial cells and superbasal cells in cervix and vagina may be observed at the beginning of metestrus phase. This short time phase take from 1 to 5 days.

Diestrus phase:

This is the period between metestrus and the beginning of corpus luteim degeneration. With degeneration of corpus luteim the progesterone secretion reduces as well. The numbers of restored cells are low and mostly are leukocyte. In mouse and rat that the mating has been taken place the corpus luteim begins to restore and the future follicles begins at maturing. By failure of pregnancy the diesrtus phase finishes and the uterus septum does not grow anymore and is regulated for next cycle [4].

Ovulation induction:

For follicle ultimate growth and ovulation the LH is necessary. With lack of this hormone the follicle does not enter ovulation stage. Near 2 days before ovulation for unknown reasons LH secretion level increase dramatically from anterior pituitary. The maximum LH secretion takes place near 16 hours before ovulation. (In human) LH is influential in granulosa and theca cell too and leads in higher progesterone and lower estrogen secretion. The ovulation takes place with rapid growth of follicles, decrease of estrogen and start of progesterone secretion. The LH lead in rapid secretion of follicular steroidal hormones that has low progesterone level at the beginning. Within few days two events takes place that their consequence is ovulation. Firstly the external theca begins at releasing proteolytic enzyme from lysosomes that leads in dissolving capsule septum and as a result weakening the septum that results in more inflation of follicle and stigma degeneration. Secondly blood vessels grow rapidly in follicle wall and prostaglandins are secreted in follicular tissues. This leads to more and more inflation of follicles and ultimately follicle inflation leads in ripping follicle by stigma degeneration and oocytes exodus.

Ovary ovulation induction takes place in order to obtaining great number of oocytes in an certain cycle by hypophyseal gonadotropin (LH and FSH). Great number of follicles grows at the same time for this purpose and ultimately the ovulation takes place. Several protocol are reported for ovulation stimulation in different species, in this protocol that we have use for ovulation induction the HMG and HCG are used instead of gonadotropins [4].

For efficiency of ovulation induction in mice some tips are important as follows:

1. An important factor is hormones doze. In this protocol the proposed doze includes HMG 10 IU (for ovulation induction) and HCGT 10 IU (for releasing oocyte) are used in inter peritoneal manner.
   In this ovulation stimulation protocol HCG is used instead of LH that is necessary for releasing oocytes and ripping mature follicles. And before the release of androgens LH takes place the injection must be carried out.
2. Usage time of HMG and HCG depends to each other and to light-dark cycle of the room there mice are kept. In most species the interval between injection of HMG and HCG is 42-48 hours. The ovulation takes place 10-14 hours after HCG injection. [5] have demonstrated that ovulation induction leads in increase of ovulary capillary permeability as well as increase of the gene expression called VEGF [5].
   [6] have showed that the VEGF gene expression level in follicular liquid increases 6 times (6X) after usage of HCG [6]. The research results shows that ovulation stimulation leads in increasing volume of follicular ovary and the corpus luteum.
   Ovulation stimulation effects on ovary performance by changing ovary enzymes activities. Ovulation induction leads in increase of alkaline phosphatase in follicular liquid of mouse ovary. Other findings demonstrate that the alkaline phosphatase activities in cow follicular liquid is increased after ovulation induction, therefore high density of gonadotropins can results in increase of follicular alkaline phosphatase activity.

OOGENESIS

Producing gametes in female sex is termed oogenesis. Due to oogenesis the female gamete or ovum emerges. In creatures with sexual reproduction, dynasty of gametes detach from somatic cells dynasty at early stages of life. These cells are terms as primordial germ cell or PGC. These cells migrate to the emerging sexual gland. Therefore the first stage of gametogenesis (oogenesis) is engendering PGCs and their migration to the primary gonad. Then these cells begin to multiplying through meiotic division. These multiplying cells in female sex are called oogonia and in male sex are spermatogonia. The third stage of oogenesis is meiotic division. Two consequent meiotic division result in decrease of chromosomes to half of initial amount. The fourth stage is maturity and final distinction of gametes. Finally through oogenesis the base cell of 2 N chromosomes reach to the gamete with N chromosome. Oogonic multiplication in mammals is restricted to their life period in the womb. All eggs emerged within maturity period and after it in female sex are obtained from the oocytes of birth time. A while after the birth the oogonia multiplication is almost ceased and the ovary contains all oocytes that may be fertilized throughout of the individual life. Usually the follicles remain at the initial state until maturity period and just before maturity age begins to ripen. From this time on till the end of sexual activity period follicles can be observed in various stages of growths and development in the ovary.

In general all stages cannot be seen in the ovary within oogenesis, based on species of the creature what is released from ovary is different. In some animals such as dog and fox a quite ripe early oocyte is released from ovary that still didn't accomplished its meiotic divisions and after entry of sperm the meiosis I takes place. In most vertebrates a secondary oocyte is released from ovary that progress till the metaphase meiosis II stage and there it ceased. With entry of sperm both mioz are completed and the distinction of the ovum takes place at the same time with its growth and ripening [7].

CRYOPRESERVATION PROTOCOLS

Two prominent methods are proposed for oocyte freezing 1) slow freezing 2) vitrification or ultra-rapid freezing

In both method researchers attempts to decrease formation of ice crystals in and out of the cell during freezing and fusion in order to increase survival level of oocytes. Two important and essential factor contributes in freezing process. Firstly usage of appropriate anti-freezing that leads to rapid exit of intercellular water and secondly rapid freezing that is different in any cells and depend on membrane permeability , the surface/volume ratio and the temperature [7]. Slow freezing method was presented for first time by [8]. [8] Used this method for freezing a two cells embryo of mouse. He managed to obtain successfully great number of healthy two cells embryo of mouse after fusion. The first successful report in mammal oocyte freezing was offered in mice in 1977 by [9].

In slow freezing low density of anti freezes is used (a density between 1-2 moles per liter). Therefore it is necessary to regulate the freezing rate so that to provide necessary time for water to exit from the cell. Cells usually during slow freezing are cooled with near 0.1-0.3 C/min so that water draining takes place slowly and prevent formation of ice crystal within freezing process. Furthermore in slow freezing the warming rate should be to the extent that ice crystals does not form during warming up process , so in general in process of freezing the heating rate is as important as cooling rate [10]. Vitrification is new

event in freezing biologic science. This method was offered for the first time in 1937 by [11]. But its successful usage was reported by the same researcher in freezing and preservation of erythrocytes of blood. Though contradictory reports are published about effect of cellular cumulus on oocyte life but in general oocyte freezing together with intact cellular cumulus is problematic because optimal time for equalizing in oocytes with is different from oocyte without cellular cumulus [9].

VITRIFICATION

Vitrification is new event in cryobiology science that prevents from formation of ice crystals in inter and extra cellular space. Vitrification is a solidifying a solution in low temperature without formation of ice crystal. It is a process through which high density of COA (4-8 mole/lit) and high rate of cooling more than 2000 C/min is obtained [12, 13].

Classic method of slow freezing is based on continuous dehydration during the cooling process and when the sample is placed in liquid nitrogen sufficient time for dehydration is elapsed. Instead for vitrification in fact cells are dehydrated just before beginning of cooling process that it takes place through contact with dense CPAs so that the inter and extra-cellular liquid vitrify simultaneously. Vitrification can be explained as solidifying a solution by increasing density cohesion without producing crystals. It is potential danger of Cryopreservation agents high density vitrification that is fatal for cells. But it is possible that vitrification solution toxicity could be confined by using a different CPA solution. By this measure the relative density of anti-freeze is decreased and the time and temperature to which the cell would encounter can be lowered.

In vitrification the density of CPA is very important. Because firstly the density of COA solution density should be so that the inter- and extra-cellular ice crystal would not be formed, secondly the used anti-freezer density should not be toxic and harmful for the cell. After coming out from freezing state the speed and stages of melting should be in such manner that divitrification would not take place. In other word during melting stages ice crystals should not take place and vitrification environment should turn to liquid phase at once.

Though slow freezing leads successfully in freezing and melting of various cells and it as yet has extensive usage as well, but nowadays attention to advantages of vitrification cause this method to be addressed more than ever.

PRINCIPLES OF VITRIFICATION

For the better vitrification of cells during cells freezing some solution are suggestion. Among them these item can be taken into account:

1. Using minimum solution within freezing and placing samples in very tiny droplets of freezing solution in less than one microliter prevents of heterogeneous nucleation (prerequisite of ice crystallization, and emerging ice cores).
2. Cooling with high rate: freezing rate should be so that the cell would not have the necessary time for crystal formation. As the freezing time is shortened, ice crystal have not the necessary time for growth.
3. Adding antifreeze in high density to vitrification solution is a useful way with respect to ice crystallization and preventing its growth as well as it increases solution viscosity.
4. Increasing hydrostatic pressure during freezing process with adding antifreeze to similar solution, in other word both of them prevent formation or growth of ice crystals.
5. Regarding cell size and its morphologic and biologic properties one of above ways are adopted. For example for freezing thin layers of viruses it can be vitrified by placing it in an hypertonic solution with high cooling rate.

   But for samples that are larger in terms of volume we need adopting two or more of above ways for the better vitrification.

CONCLUSION

Nowadays in many laboratories around the world cryotop vitrification is used for vitrification of oocytes as the best protocol for freezing oocytes. Published reports is an important evident for this issue. The vitrification comparing to slow freezing that is a time consuming method and requires advanced laboratory equipment, leads in improvement of survival, fertilization and evolution of embryos after the fertilization. Analysis of meiotic spindle has proved that the least harm to vitrified oocytes is obtained.

Regarding the fact that the objective of every freezing process is assuring survival and life level of cells after melting and regarding results in this experiment and other tests that are carried out in response to patients need throughout of world, cryotop vitrification with very high cooling rate 25000-50000^{o} C/min

and high efficiency level (near 100 % survival after oocytes freezing and melting as well as oocytes after fertilization with IVF or ICSI method have evolved similar to fresh and unverified oocytes) and also regarding to the fact that it has been reported that the number of birthed infant from cryotop vitrification was higher than all birthed infants obtained from other methods all around the world (it has been said that to date 300 infants are birthed by this method) [14], It can be concluded that cryotop vitrification at present time is the best way for oocytes freezing for objectives such as creating oocytes bank for fertility preservation of young cancerous patient after chemotherapy or radiotherapy treatment or for the women that for some reasons cannot use their own natural oocytes, in such cases oocytes bank can be helpful.

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