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КАФЕДРА БІОТЕХНОЛОГІЇ

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Тема: «Кріоконсервація: спосіб збереження генетичних ресурсів тварин»

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FACULTY ENVIRONMENTAL SAFETY, ENGINEERING AND TECHNOLOGY

DEPARTMENT OF BIOTECHNOLOGY

ALLOWED TO DEFENCE

Head of the graduate department

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«___»_____2021

BACHELOR THESIS

(EXPLANATORY NOTE)

OF APPLICANT OF EDUCATIONAL DEGREE «BACHELOR»

SPECIALTY 162 «BIOTECHNOLOGY AND BIOENGINEERING»

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Theme: «Cryopreservation: a way to preserve the genetic resources of animals»

Executor: student of group 404, FESET Tsimbaliuk A.O.

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Faculty Environmental Safety, Engineering and Technologies

Department of Biotechnology

Speciality: 162 «Biotechnology and bioengineering»

EPP: «Pharmaceutical Biotechnology»

APPROVED

Head of the Department

_____ Baranovsky M.M.

«___» _____2021

TASK to perform a thesis

Tsimbaliuk Anastasiia Oleksiivna

1. Theme of degree work: «Cryopreservation: a way to preserve the genetic resources of animals» approved by the Rector «11» of May 2021 № 715/ст
2. Term of work execution: from «10» of May 2021 to «14» of June 2021.
3. Content of explanatory note: Introduction. Literature review. Materials and Methods. Theoretical and analytical analysis of cryopreservation observation. Conclusions. References.
4. List of necessary graphic (illustrative) material: 12 fig., 2 tables.

НАЦІОНАЛЬНИЙ АВІАЦІЙНИЙ УНІВЕРСИТЕТ

Факультет екологічно безпеки, інженерії та технологій

Кафедра біотехнології

Спеціальність: 162 «Біотехнології та біоінженерія»

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ЗАТВЕРДЖУЮ

Завідувач кафедри

_____Барановський М.М.

«___» _____2021 р.

ЗАВДАННЯ на виконання дипломної роботи

Цимбалюк Анастасії Олексіївни

1. Тема дипломної роботи: «Кріоконсервація: спосіб збереження генетичних ресурсів тварин», затверджена ректором «11» від травня 2021 р. № 715 / ст.
2. Термін виконання роботи: з «10» травня 2021 р. по «14» червня 2021 р.
3. Зміст пояснювальної роботи: Вступ. Літературний огляд. Матеріали і методи. Теоретичний та аналітичний аналіз кріоконсерваційного спостереження. Висновки. Списки використаних джерел.
4. Список необхідного графічного (ілюстративного) матеріалу: 12 рис., 2 таблиці.

5. Schedule

№	Task	Execution term	Signature of the head
1	Coordination of the content of the thesis with the thesis supervisor	10.05.2021 - 12.05.2021	
2	Selection of information sources	12.05.2021 - 17.05.2021	
3	Future work concept development	17.05.2021 - 20.05.2021	
4	Processing information sources	20.05.2021 - 25.05.2021	
5	Registration of the thesis	25.05.2021 - 30.05.2021	
6	Formulation of conclusions	30.05.2021 - 05.06.2021	
7	Thesis defense	14.06.2021	

6. Date of issue of the task: «10» of May 2021

Supervisor of degree work_____ Baranovsky M.M.

Task for execution was taken over by _____Tsimbaliuk A.O.

5. Календарний план–графік

№	Завдання	Термін виконання	Підпис керівника
1	Узгодження змісту дипломної роботи з дипломним керівником	10.05.2021 - 12.05.2021	
2	Підбір джерел інформації	12.05.2021 - 17.05.2021	
3	Розробка концепції майбутньої роботи	17.05.2021 - 20.05.2021	
4	Обробка джерел інформації	20.05.2021 - 25.05.2021	
5	Оформлення дипломної роботи	25.05.2021 - 30.05.2021	
6	Формулювання висновків	30.05.2021 - 05.06.2021	
7	Захист дипломної роботи	14.06.2021	

6. Дата видачі завдання: «10» травня 2021 року

Керівник дипломної роботи _____ Барановський М.М.

Завдання прийняла до виконання_____ Цимбалюк А.О.

ABSTRACT

Explanatory note to the diploma thesis «Cryopreservation: a way to preserve the genetic resources of animals», 59 p., 2 tables, 12 figures, 63 references.

Object of investigation – cryopreservation methods for different types of animal cells.

Subject of investigation – genetic material of animals (semen, oocytes, embryos).

Purpose of the work – to analyze which methods and animal cells will be more suitable for cryopreservation in order to preserve the biodiversity of the animal world.

Methods of investigation – analytical, biochemical, physiological.

The results of the thesis can be used during research and in the practice of specialists – biologists and specialists – biotechnologists.

METHODS OF CRYOPRESERVATION OF ANIMAL CELLS, SPERM, OOCYTES, EMBIOS, VATRIFICATION, SLOW FREEZING, CRYOPROTECTANTS, CRYO□DAMAGE.

РЕФЕРАТ

Пояснювальна записка до дипломної роботи «Кріоконсервація: спосіб збереження генетичних ресурсів тварин», 59 с., 2 таблиці, 12 рисунків, 63 посилання.

Об'єкт дослідження – методи кріоконсервації для різних типів клітин тварин.

Предмет дослідження – генетичний матеріал тварин (сперма, ооцити, ембріони).

Мета роботи – проаналізувати, які методи та типи клітин тварин будуть більш придатними для кріоконсервації з метою збереження біорізноманіття тваринного світу.

Методи дослідження – аналітичні, біохімічні, фізіологічні.

Результати дипломної роботи можуть бути використані під час проведення наукових досліджень та в практичній діяльності фахівців — біологів та фахівців — біотехнологів.

МЕТОДИ КРІОЗБЕРЕЖЕННЯ КЛІТИН ТВАРИН, СПЕРМА, ООЦИТИ, ЕМБІОНИ, ВІТРІФІКАЦІЯ, ПОВІЛЬНА ЗАМОРОЗКА, КРІОПРОТЕКТОРИ, КРІОПОШКОДЖЕНННЯ.

CONTENT

LIST OF SYMBOLS, ABBREVIATIONS, TERMS

AI artificial insemination

AO acridine orange

ATP adenosine triphosphate

ART assisted reproductive technologies

CNPC cryoprotectants that do not penetrate into cells

CPA cryoprotectant

CPCs strong endocellular cryoprotectants

CRs common cryoprotectants

CTC – chlortetracycline

DMAc – dimethylacetamide

DMSO dimethyl sulfoxide

DFI DNA fragmentation index

DNA deoxyribonucleic acid

DNMTs DNA methyltransferases

EG ethylene glycol

FCM flow cytometric

FPT fluorescent polymer thermometer

HBV hepatitis B virus

HCV hepatitis C Virus

HIV the human immunodeficiency viruses

HMBTHEU hexamethylenebistetraoxyethylurea

ICSI intracytoplasmic sperm injection

IIF intracellular ice formation

IMSI intracytoplasmic morphologically selected sperm injection iPS

 induced pluripotent stem

IVF vitro fertilization

LD letal dose

LN liquid nitrogen

MAC mixed-action cryoprotectants

PEG polyethylene glycols

PVP polyvinylpyrrolidone

ROS reactive oxygen species

SCSA sperm chromatin structure assay

UK – the United Kingdom

INTRODUCTION

Actuality of theme. Intense man-made pressure on ecosystems leads to a reduction in biological diversity. At present, deep-freezing of biomaterials – cryopreservation – is widely used to preserve species diversity and duplicate valuable specimens from world collections.

Intense anthropogenic pressure on ecosystems leads to a reduction in the natural capacity for self-extraction of species and limits their reproduction. The number and diversity of valuable and rare, but low-resistant species, is declining; they are replaced by a few inferior or harmful but highly productive and resistant species (gray rat, gray crow, etc.). A significant number of fish, birds, animals (Araripsky Manakin, Red Monkey, Spotted Harlequin, Red–finned Blue-eyed, Java Rhinoceros) were on the verge of extinction. Each species has a unique genotype, and a rapid decline in numbers leads to a reduction in genetic diversity, making it vulnerable to overfishing, environmental pollution and accompanied by depression with consequent extinction.

The steady decline in biodiversity threatens the fact that the degradation processes hidden in natural populations appear suddenly and lead to the complete extinction of the most common species. Such dynamics are growing like a landslide, and the number of such economically and ecologically valuable fish species as the nelma, muksun population in the Siberian sturgeon has been catastrophically reduced. Their ecological niches are occupied by more stable and less in demand carpets, rattan and others.

For sustainable development, modern and future selection, the most important tasks are maintenance of species diversity, conservation of valuable species, development of approaches and organization of long-term storage of the gene pool.

Slow freezing, a common method for cryopreservation of embrions, causes osmotic shock (solution effect) and intracellular ice crystallization leading to cell damage. Vitrification is an alternative method for cryopreservation in which cells are exposed to a

higher concentration of cryoprotectants and frozen with an ultra□rapid freezing velocity, resulting in an ice crystal free, solid glass-like structure. Presently, vitrification is a popular method for cryopreservation of embryos. However, vitrification of oocytes is still challenging due to their complex structure and sensitivity to chilling.

Object of investigation – analysis of cryopreservation methods for different types of animal cells.

Subject of investigation – genetic material of animals (semen, oocytes, embryos).

Purpose of the work – to analyze which methods for which animal cells will be more suitable for cryopreservation in order to preserve the biodiversity of the animal world.

Tasks for execution of the bachelor thesis:

1. Determine the basics of cryopreservation.
2. Analyze two methods of cryopreservation.
3. Analyze which of the cryopreservation methods is more suitable for the two types of cells suitable for freezing.

Methods of investigation – analytical, biochemical, physiological.

ВСТУП

Актуальність теми. Посилений техногенний тиск на екосистеми призводить до зменшення біологічного різноманіття. В даний час глибоке заморожування біоматеріалів □ кріоконсервація □ широко використовується для збереження видового різноманіття та копіювання цінних зразків зі світових колекцій.

Інтенсивний антропогенний тиск на екосистеми призводить до зменшення природної здатності до самовидобування видів та обмежує їх розмноження. Кількість і різноманітність цінних і рідкісних, але низькостійких видів зменшується; їх замінюють кілька неповноцінних або шкідливих, але високопродуктивних і стійких видів (сірий щур, сіра ворона тощо). Значна кількість риб, птахів, тварин

(Араріпський Манакін, Червона Мавпа, Плямистий Арлекін, Червоноплав Синьоокий, Ява□Носоріг) опинилася на межі зникнення. Кожен вид має унікальний генотип, і швидке зменшення чисельності призводить до зменшення генетичного різноманіття, що робить його вразливим до перелову, забруднення навколишнього середовища та супроводжується депресією з подальшим вимиранням.

Постійне зниження біорізноманіття загрожує тим, що процеси деградації, приховані в природних популяціях, з'являються раптово і призводять до повного зникнення найпоширеніших видів. Така динаміка зростає, як зсув, і кількість таких економічно та екологічно цінних видів риб, як нельма, муксун популяція у сибірського осетра катастрофічно зменшилася. Їхні екологічні ніші зайняті більш стабільними і менш затребуваними килимами, ротангом та іншими.

Для сталого розвитку, сучасного та майбутнього відбору найважливішими завданнями є підтримка видового різноманіття, збереження цінних видів, розробка підходів та організація довгострокового зберігання генофонду.

Повільне заморожування, поширений метод кріоконсервації ембріонів, спричинює осмотичний шок (ефект розчину) та внутрішньоклітинну кристалізацію льоду, що призводить до пошкодження клітин. Вітрифікація □ це альтернативний метод кріоконсервації, при якому клітини піддаються підвищеній концентрації кріопротекторів і заморожуються з надшвидкою швидкістю заморожування, в результаті чого утворюється тверда скляна структура, що не містить крижаного льоду. В даний час вітрифікація є популярним методом кріоконсервації ембріонів. Однак склорізація ооцитів все ще є складною через їх складну структуру та чутливість до охолодження.

Об'єкт дослідження □ аналіз методів кріоконсервації для різних типів клітин тварин.

Предмет дослідження □ генетичний матеріал тварин (сперма, ооцити, ембріони).

Мета роботи – проаналізувати, які методи для яких клітин тварин будуть більш придатними для кріоконсервації з метою збереження біорізноманіття тваринного світу.

Завдання на виконання бакалаврської роботи:

1. Визначити основи кріоконсервації.
2. Проаналізувати два методи кріоконсервації.
3. Проаналізувати, який із методів кріоконсервації більше підходить для трьох типів клітин, придатних для заморожування.

Методи дослідження – аналітичні, біохімічні, фізіологічні.

CHAPTER 1 LITERATURE REVIEW

1.1. Cryopreservation

Cryobank is appropriate storage capacity in which sexual products and embryos of valuable organisms are stored in liquid nitrogen using deep‑freezing methods [1]. Such a complex allows to preserve valuable biomaterial and to carry out safe duplication of samples of known world collections in order to ensure maximum saving of the species and minimize

the risk of loss [2; 3]. In it, freezed biological material can be keeped for decades until it is in demand for practical use.

Cryopreservation is a method of converting biological objects into a state of deep cold anabiosis in LN at -196 C; in this case, it becomes possible to store the biomaterial (oocytes, sperms, embryos) for a quite a while without losing their morphological and functional properties. Cryopreservation let to obtain an unlimited amount of genetic material without compromising the natural population, to store it for a long time, as well as easy to transport and, if necessary, relatively quickly restore the species.

The problem of preserving the germ cells of animals and humans had to be solved for the selection of animals and the treatment of infertility. Separate attempts to preserve frozen eggs and sperm of animals were made 200 years ago. However, the scientific foundations of cryobiology were laid in the late nineteenth century. Russian scientist P.I. Bakhmetyev, who studied the phenomenon of hypothermia in insects and anabiosis in bats [4]. In 1899, he established a rule named after the author, according to which a sharp decrease in the temperature of the medium in poikilothermic animals a sudden jump in temperature followed by a drop. The temperature jump is timed to the moment of freezing of the tissue fluid with the release of latent heat of fusion.

At the turn of the XIX□XX centuries the first attempts were made to freeze the gametes of animals. P. Becquerel and G. Ram in the early twentieth century established the ability of various organisms (microorganisms, invertebrates: slugs, rotifers, nematodes), as well as spores and seeds to tolerate deep cooling in the dried state to -

269… -271 C, almost to absolute zero [5].

Cryopreservation of embryos and gametes is commonly used to saving the genetic resources of laboratory and agricultural, uncommon and endangered animal species. Despite the fact that embryos and gametes of several dozen species of mammals have already been successfully frozen, and cryobanks of genetic resources have become an absolutely normal practice for genetic centers, there is yet no single universal protocol for cooling and warming of embryos. In some genetic centers, in which cryobanks of various strains of mice and rats have been created, they prefer to use program freezing, in others, exclusively vitrification.

Meanwhile, over the past several decades, cryobiology has developed very successfully and, as a result, significant advance has been made in understanding the mechanisms of cryo□damage and cryoprotection (Fig.1.1). Recently, new approaches to the study of these issues have appeared, using modern methods of physics. Moreover, with the increasing requirements for experiments on animals and the emergence of a large number of lines of mice and other priority laboratory animals, the organization of cryobanks has become one of the main attributes modern genetic centers [6].

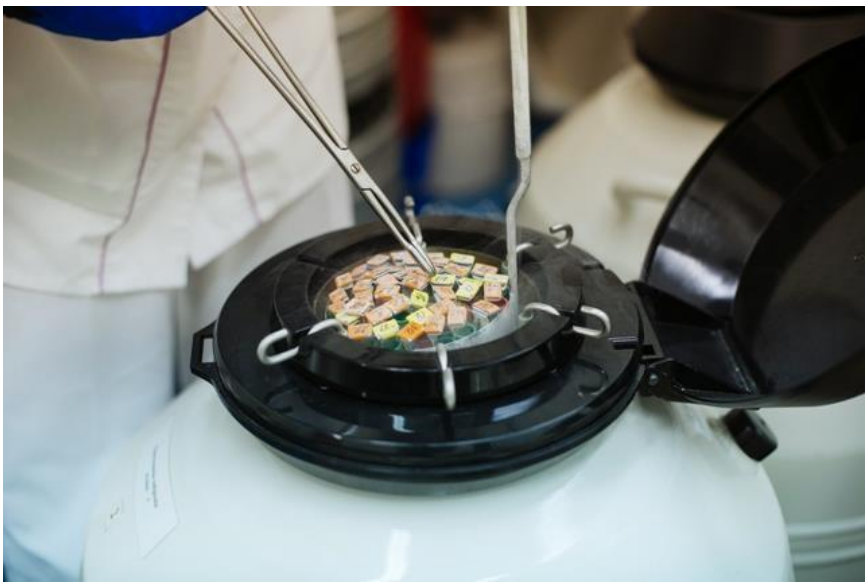

Fig. 1.1. Example of cryopreservation

Creation of a cryobank of gametes and preimplantation embryos and cells of laboratory animals has several main tasks. Very momentous of these is the preservation of the genetic material of animal species, populations and lines. Cryobanks often aim to preserve the biological diversity of a particular mammalian species. If the number of individuals of a particular animal species reaches a certain critical minimum, the probability of the forfeit of this species is high. As the population decreases, the risk of genetic drift, genetic instability and loss of individuals consequently of disease increases. The presence of a cryobank of genetic resources of a particular a different species allows to minimize the risk of loss of the species and its biodiversity.

The use of assisted reproductive technologies (ART) in combination with cellular and genomic tools has led to the creation of many transgenic and knockout animals that make a

significant contribution to biomedical research, ranging from analysis of gene functions to comparative studies of various pathologies in humans [6].

In zootechnology and veterinary medicine, more research is required to preserve existing genetic materials, create valuable, highly productive and disease-resistant lines and types of animals based on modern methods of biotechnology, transplantation and genetic engineering [7].

As a result of advances in the technology of AI, livestock breeders have received a powerful means of improving animals, the possibilities of selecting animals with a high genetic potential for productivity have expanded, and the rate of genetic betterment of entire populations has accelerated.

1.2. Cryoprotectants

Cryoprotectants are substances that have the capacity prevent the development of cryoinjuries biological objects and ensure their viability after thawing [8].

For the first time, the cryoprotective property of glycerin was discovered by N.A. Maximov in 1913. First successful results of rooster semen freezing using cryoprotectant glycerol (fig.1.2.) was obtained by C. Polge, A. Smith, L. Parkes in 1949 [9]. After, for cryopreservation of cells flow of blood and bone marrow in 1959 J. Lovelock suggested using DMSO [10].

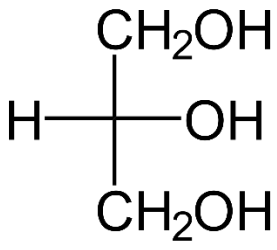

Fig.1.2. Structural formula of glycerol

To date, about 120 organic substances have been identified with cryoprotective properties [9].

Classification of cryprotectors:

 endocellular substances penetrating through the cell membrane with molecular weighing up to 101 g / mol [11];

 exocellular substances that do not penetrate the cell membrane with molecular weight of more than 400 g / mol [10];

 mixed action a substance with a molecular weight of 102 to 400 g / mol [12].

Endocellular cryoprotectants are considered the most effective, however, due to high permeability, they also have the highest toxicity [9]. The properties of the most common cryoprotectants are presented in Table 1.2.

The general principle of action of cryoprotectants during freezing is to bind water, which leads to a decrease in the crystallization rate and a slowdown in the growth of ice crystals, which allows you to protect the cell from osmotic lysis.

Table 1.2

Properties of the most common cryoprotectants [Грицаев С.В., 2015]

Name	Molecular mass, g / mol	Mechanism of action	Half-lethal dose LD50, g / kg	Empirical formula
DMSO	78,13	Endocellular	3.80 ± 0.1	C_2H_6SO
DMAc	87	Endocellular	4.2 (2.5-3.9)	C_4H_9NO
Glycerol	92,1	Endocellular	4.57 ± 0.14	$C_3H_8O_3$
HMBTHEU	378	Mixed	15.5 ± 0.6	-
PEG	400	Mixed	12.5	$HO(CH_2CH_2O)_nH$
PVP	12600	exocellular	non-toxic	$(C_6H_9NO)_n$

The mechanism of action of the endocellular cryoprotectant is realized in the binding of intracell water with rapid penetration through the membrane. Exocellular cryoprotectant binds extracellular water and, when frozen, slows down the growth of extracellular crystals ice, envelops cells and prevents the influence of already formed crystals on them [12].

It should be noted that until now there are no universal principles selection and synthesis of a cryoprotectant. The choice of cryoprotectant is carried out empirically, individually for each cell fraction [13].

Nevertheless, the main general requirements for a cryoprotectant can be formulated:

- keep cells in a viable state;
- provide a cryoprotective effect at a minimum concentration;
- to be low-toxic at the cellular and organism levels; dissolve well in water.

In traditional cold storage technologies, cell viability is ensured by adding special cryoprotectants. Many of them are substances with relatively small molecules that easily penetrate cell membranes. In addition to glycerol, which is widely used for this purpose, other useful cryoprotectants can also be used: dimethyl sulfoxide, sucrose, trehalose, glucose, methanol, proline, glycine, betaine, fructose, galactose, and lactose. The cryoprotectant makes cells completely resistant to the adverse effects of repeated freezethaw cycles and significantly increases the shelf life.

Cryoprotectants are substances that protect a product subjected to freezing from hypothermia and are used in the practice of cryogenic technology because of their potential to stabilize the properties of food or convenience foods during storage.

Cryoprotectants are substances that can prevent the development of damage to biological objects during cooling, freezing and subsequent warming. To date, more than 100 compounds are known as cryoprotectants (alcohols, amines, amino acids and their amides, oxides, carbohydrates, proteins, natural and artificial polymers, inorganic salts, etc.). However, all known cryoprotectants can have a toxic effect on cells, the magnitude of which depends on the temperature and duration of exposure of the cells to the cryoprotectant. Therefore, as a rule, immediately after thawing, the cryoprotectant is removed from the cells

and the medium by successive washing and centrifugation, because many of the cryoprotectants are toxic to the body.

In the cryopreservation of any biological material, adequate conditions must be provided for its survival, and it is there that cryoprotective agents play an important role. Permeable cryoprotectants are low molecular weight substances that can pass through the cell membrane, replacing the volume of intracellular water, avoiding damage caused by the formation of ice crystals and, in turn, maintaining cell volume preventing cell collapse due to excessive dehydration

Among the cryoprotectants, glycerol (GL) has been widely used in the cryopreservation of various types of cells, including mammalian sperm. On the other hand, Dimethyl Sulfoxide (DMSO) is a promising cryoprotective agent for cells, since its low molecular weight (78.13 g / mol) allows it to cross the cell membrane quickly; while ethylene glycol (EG) is a cryoprotectant that has been used universally in the freezing of ovarian tissue and embryos of many species, and with bull semen it exerts a less inhibitory effect on motility than GL or DMSO.

The toxicity of cryoprotectants is characterized by indicators of their general toxicity toxicity at the organismal level, and cytotoxicity toxicity at the cellular level.

The toxicity of substances is assessed by the median lethal dose (LD50, g / kg), when administered, 50% of the experimental animals die [14]. "Strong" endocellular cryoprotectants (CPCs) have significant toxicity. The toxicity of mixed-action cryoprotectants (MAC) is several times less. Cryoprotectants that do not penetrate into cells (CNPC) exhibit very low toxicity. Practice shows that methodological methods for determining general and cytotoxicity require improvement. In cryobiology, it is customary to compare the toxicity of cryoprotectants according to the detected LD50 in white laboratory mice [14, 15]. In the studies of A.M. Kompaniets et al. [16] showed that the toxicity of CPC decreases with an increase in their molecular weight.

1.3. Risks of cryopreservation

The earliest hypothesis about the causes of cell death during freezing was a hypothesis about mechanical damage to cells by growing ice crystals. This assumption seemed obvious due to the known possibility of cannonballs rupture by crystallizing water and the established fact that the volume of formed ice is 10 percent higher than the initial volume of pure water. Another significant cause of cryo□damage is the dehydration of cells and tissues of the body, resulting from the freezing of water and its release from the cell. These processes are associated with a change in the volume of the cell and its deformation, an increase in osmotic pressure and the formation of toxic concentrations of salts, that is, the phenomena known under the generalized name “solution effect”. Cell resistance to the freezing process depends on the degree of dehydration and on the amount of freezing water in the cell, which is determined by the ratio of bound and free water. This state is due to the hydrophobicity and hydrophilicity of macromolecular compounds, viscous protoplasm and is characterized by the reactions of cells of the second stage of the cycle, that is, during freezing. Many researchers believe that damage to biochemical structures mainly occurs not during the freezing of water, but during thawing and recrystallization, when ice crystals grow [17, 18]. With sufficiently slow cooling under a cryomicroscope, it is possible to observe how the cells in the channels between the ice crystals, after compression, begin to swell to a size much larger than the original. They behave in a similar way in hypertensive environments of high osmolarity (Fig.1.3.). The reason for the swelling of cells in a hypertonic environment can be a sharp increase in the permeability of plasma membranes for extracellular solutions, leading to the disappearance of their concentration (and, consequently, osmotic) gradients on the membrane. As a result, a mechanism known as "colloidal osmotic lysis" begins to operate.

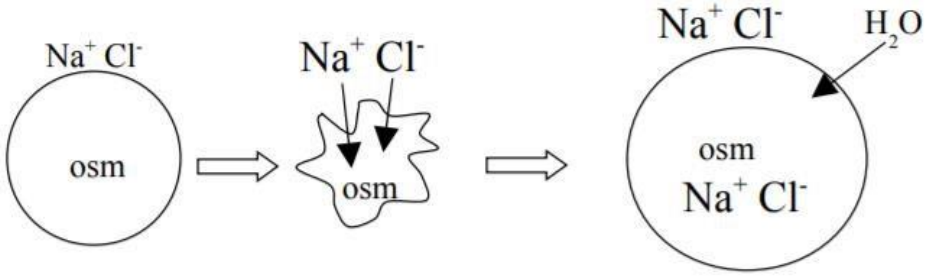

Fig. 1.3. Scheme of hypertensive lysis

Posthypertensive lysis is a consequence of the transfer cells saturated with hypertonic solutions into a medium of less tonicity. Posthypertensive lysis can occur both during thawing of frozen cell suspensions and during their washing from the penetrating cryoprotectant. Cause of posthypertensive lysis when warming up, it is the penetration of extracellular salts concentrated by freezing into the cells. It is still unclear whether this penetration is due to the formation of membrane defects or a normal volume-regulating reaction.

One of the "disadvantages" seen by embryologists who are unfamiliar with nutrition technology is the use of high concentrations of cryoprotectants, which means that refrigeration solutions are more toxic than their counterparts used in conventional refrigeration. However, with a better understanding of the physical and biological principles of rape, this has led to many successful medical applications of this technique for assisted reproduction.

The filter is very simple, does not require expensive equipment to store programs, and, in particular, relies on embedding embryos in very small to medium volume (also known as "minimal method"), which must be refrigerated at very high speeds. The importance of using low volume, also known as the “minimum volume method”. In general, the degree of cooling / heating and concentration of cryoprotectant materials needed to achieve feeding are randomly correlated.

One of the first things we need to learn is to distinguish between their meanings the concept of contamination and the crossing of the sample. The first applies to sample contamination by storing or direct contact with the cooling solution, and the second applies to sample contamination of a common container in contact with all stored samples, some samples may be contaminated or nitrogen liquid may be contaminated, which may to not contaminate. The possibility of disease transmission and survival of diseases caused by liquid nitrogen contamination and evidence of contamination in human patients has been described in a variety of diseases. It should be noted that any post-fertilization in domestic pets cannot be explicitly attributed to the storage and storage process used, but the use of

safety protocols is very important to avoid cell infection animal or cross liquid nitrogen tanks normal.

In the assisted reproduction unit, although no contaminants have been detected in the separation of gametes and embryos, the probability and frequency of occurrence is low, its risk is not zero, so it is recommended to follow the rules in the safety manual and physical hazards and hazardous waste Crucifixion Laboratories.

Subsequent studies have shown that laboratory storage is important. There is evidence that refrigerated samples contained in hermetically sealed fluids are not contaminated, even if they are in contaminated containers contaminated with liquid nitrogen, and liquid nitrogen does not contaminate infectious disease laboratories refrigerated in air-cooled containers. During cryopreservation, biological laboratories go through many processes before being distributed.

Treatment of contaminated biological samples (sperm, follicular fluid, cells, etc.). There looga precautions against contamination from outside the base plate used for protection (cryo□tube, straw, etc.). It is very important to disinfect and sanitize the container before filling it with liquid nitrogen. Therefore, in order to ensure an adequate level of biosafety, it is necessary to study the infectious diseases transmitted by any patient or donor who wants to block any samples.

Use of contaminated cultural media. In these cases, cross-contamination levels reach very high levels, affecting many patients. Regardless of the fact that the embryonic culture media and sperm killer are made up of specific materials, this remains a popular ART practice for animals. However, many substances in the traditional embryonic and spermicide media act as stabilizers for many organisms at high temperatures (milk, whey or whey albumin, sucrose, sorbitol and other sugars). Unfortunately, the most common cryoprotectants (CRs) involved in cryopreservation of oocytes and embryos (glycerin, DMSO, ethylene glycol, propylene glycol, methanol, etc.) are toxic to cells. In addition, bacteria and viruses effectively protect against cryo□damage, for example, the accumulation of DMSO up to 5% of associated viruses protects against cold damage. The fact that the laboratory survives in addition to the plasma of the embryo is important not only in terms

of the possibility of transmitting the disease to the recipient, but also in the form of laboratory storage tests and medical certification of the embryo or international activity. On the other hand, we must also remember that all traditional media including antibiotics prevent or limit biological survival.

Storage of contaminated material or cryo□tube grass is closed or blocked by severe defects, causing the frozen grass to break, causing the contaminated sample to enter directly into the liquid nitrogen tank, the risk of contamination in other laboratories closed systems can be sealed in a variety of ways (heat sealing seal, ultrasonic sealing material, radiofrequency injection molding, polyvinyl alcohol powder and hard hats). Given the locking time and the temperature reached, it does not affect the storage capacity, we must ensure that the seal is sealed and that the device is made of low temperature resistant nitrogen solution (ionomer resins, quartz glass capillary, polyvinyl chloride, polyethylene glycol, etc.).

Poor nitrogen liquid management in our supplier, which contaminates commercial liquid nitrogen that enters our laboratory during the production or transportation and filling of our containers.

Picking up contaminated items in containers. Storage containers should be periodically emptied and cleaned due to the risk of loss of straw or a small portion of contaminated material falling to the bottom of a large container. Most companies with liquid nitrogen containers offer cleaning systems. The main problem is the cleaning of the car lift, which is called "dry", because the liquid nitrogen-absorbing material in these bottles is difficult to clean. Based on the results presented, it was found that sodium hypochlorite and ethylene oxide solutions are equally effective against germs in people carrying dry loads built on hydrophobic absorbent LN. Instead, rariyeyaasha dry without absorption must be hydrophobic, it is advisable to use only sterilized gas sterilization looga stop harmful bacteria disinfection.

The air in the room. If air enters the lab from another source they may be contaminated and there is no good filter. Some laboratories do not analyze or process looga positive pressure to prevent air pollution.

Drivers. If contaminated, this can lead to contact contamination or scratching during sample making or cryogenic tank treatment. Staff must meet certain health and hygiene conditions: severe serotonin HIV, HCV, HBV and hepatitis B vaccine and other viral infections with the vaccine.

Before moving on to cooling, the crop should be maintained in an increasingly active state (growth or growth phase) to ensure optimal health and optimal recovery. Ideally, the practice should be changed 24 hours before cutting. It is also recommended to check the behavior of microbial infections, especially mycoplasmas, and to confirm its identification (cell type) by appropriate methods.

Use a face□to□face microscope to check the general appearance of the culture. Pay attention to the signs of germs. It is also important to examine the culture with the naked eye to find small, isolated colonies of fungi that can float through food and air, so they are not easy to see under a microscope. If antibiotics were used to grow your crops, maintaining non-antibiotic crops for at least one to two weeks before cooling would make it easier to detect anything that could contaminate the cryptocurrency.

1.4. Freezable tissues for cryopreservation

The past decade has seen a rapid and continuous decline in the number of wild mammals, with one in four mammals threatened with extinction [19]. While in situ conservation, such as habitat conservation, is usually the best way to conserve biodiversity, other rescue methods are now needed, such as cryopreservation of germplasm, to help keep natural evolutionary processes going [20]. Unfortunately, this method is useless when the population is declining profusely or when most of the remaining animals are in unprotected areas. As a result, the number of ex situ conservation programs has increased, including both in vivo and in vitro conservation and, naturally, the creation of germplasm banks [21].

The genetic resources of laboratory animals in modern cryobanks are stored mainly in the form of cryopreserved spermatozoa (male gametes) and preimplantation embryos. Freezing of female gametes □ oocytes is used much less frequently.

Cryopreservation of almost all types of female gametes of animals, in contrast to the freezing of spermatozoa, still has certain difficulties. Therefore, for the conservation of animal species, their genetic resources are more often used in the form of frozen embryos or spermatozoa than in the form of oocytes.

Female gametes (eggs) can be obtained by follicle puncture, ovarian tissue biopsy, unilateral or bilateral oophorectomy, or ovarian collection immediately (Fig.1.4.) after the death of the animal, regardless of age [22].

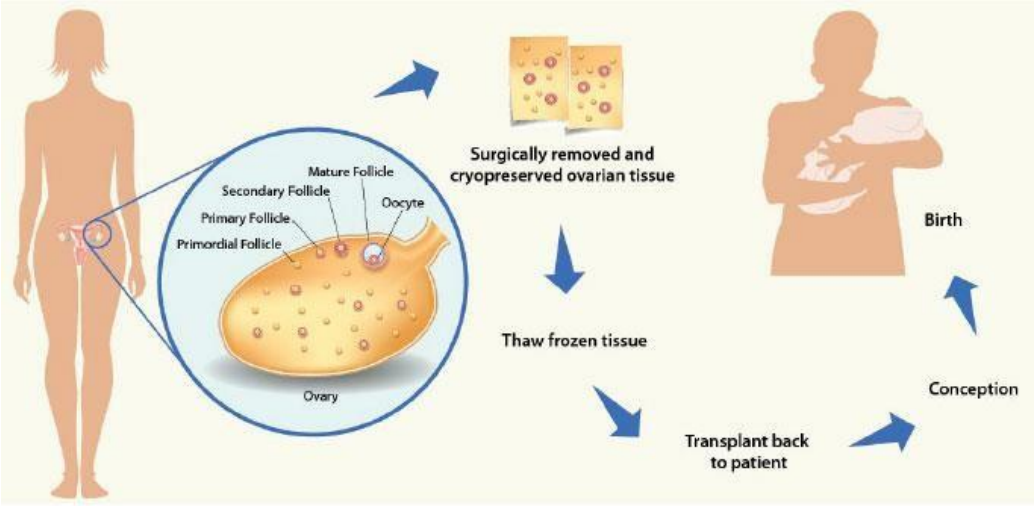

Fig. 1.4. Ovarian tissue transplant

One of the main problems that arise when freezing female reproductive cells is the hardening of the transparent membrane (zona pellucida). This static glycoprotein membrane surrounds the egg, separating it from the environment, and performs a number of vital functions.

Once the sperm enters the oocyte, a cascade of biochemical reactions occurs that causes the contents of the cortical granules to be expelled. These reactions alter the transparent membrane, called the oplo membrane appendage, which prevents multiple sperm from entering the polyspermia of the eggs. Freezing of thawing processes during life leads to spontaneous maintenance of the fertility of cortical granules, which makes it difficult or impossible to further fertilize the formation of such oocytes. Another less serious problem is that the ovulation of most mammals (leaving colic in the ovarian tract) is in a

state of incomplete meiosis, the delicate mechanism of which is often disturbed by cryopreservation, which leads to subsequent fertilization. prevents. For these reasons, egg freezing techniques have not become commonplace, even for laboratory animals such as mice and rats.

Freezing the sperm of male gametes is one of the main methods used to preserve the genetic resources of laboratory animals. Systematic cryopreservation and storage of male gametes from endangered species bypassing the problem of homozygosity in isolated populations by introducing new genetic material between populations, captive sites / zoos / research centers or facilitates genetic exchange between countries [23].

Successful sperm cryopreservation is based on the special structure of sperm. At the beginning of the sperm are lipoproteins and enzymes used to penetrate into the oocyte, which freeze less (Fig. 1.5). In the middle there is a large mitochondrial central filamentous nucleus wrapped to form ATP. The tail or figurine performs anchor movements [24] and has a stronger freezing ability than the head due to their rigid structure and low water content [25].

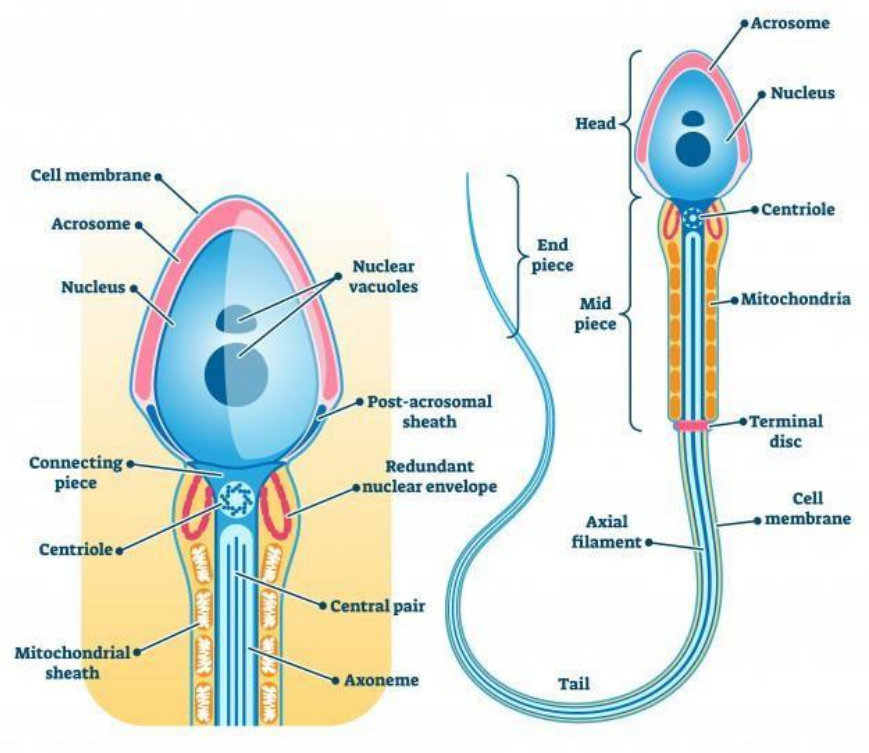

Fig.1.5. Sperm cell anatomy

Cryopreservation of sperm, epididymal and spermatozoa, or spermatogonial stem cells isolated or located in testicular tissue is possible. Each cell type has specific requirements for cryopreservation. Differences in the physical size and shape of sperm can affect their susceptibility to thermal shock and their resistance to osmotic stress during freezing, when membrane stability is compromised (Fig.1.6).

In addition, epididymal sperm are not affected by the complex secretion of accessory gonads, which may alter both sensitivity to cooling and resistance to freezing of the ejaculate [26].

In general, freezing–thawing of mammalian sperm causes cell damage, the degree of damage of which varies, and is highly dependent on the resistance of sperm to cryopreservation procedures [27, 28]. Egg yolk can be replaced by low-density lipoprotein because it can withstand cold shock damage and also maintains the physiological structure of sperm, resulting in increased viability after thawing [29, 30]. He introduced centrifugation to remove seminal plasma, resulting in improved survival after warming and acrosome integrity [25].

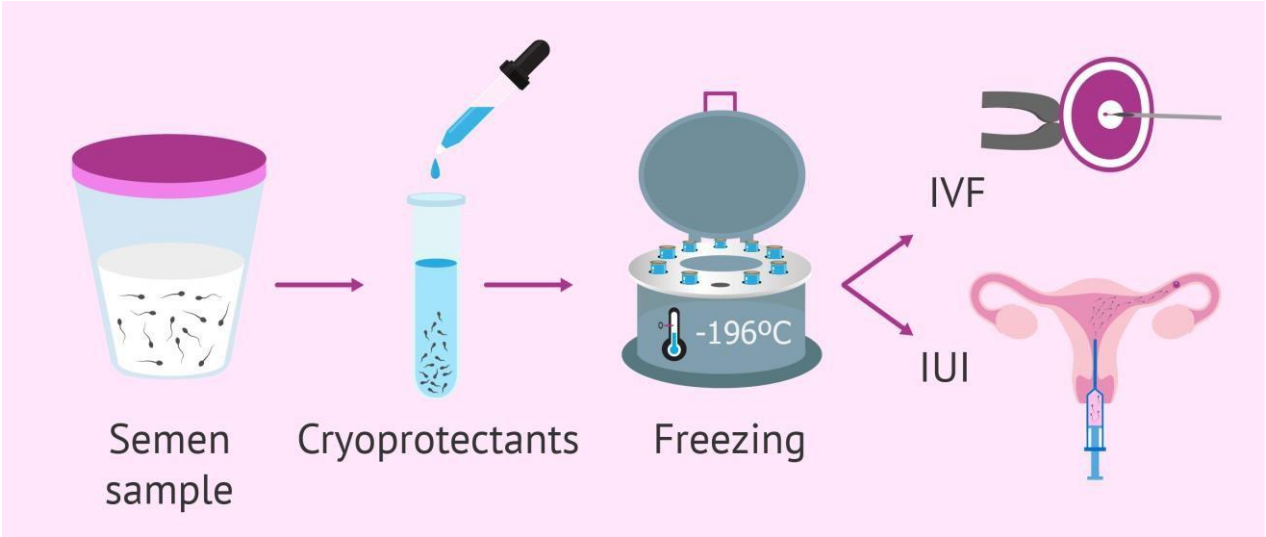

Fig.1.6. A freezing semen

After thawing the frozen seed, it is possible to obtain developing zygotes and embryos using in vitro fertilization (IVF) procedures, with subsequent transplantation of in vitro embryos allowing a particular line of animals to recover.

In particular, sperm cryopreservation, combined with subsequent IVF and transplantation of the resulting embryos into female recipients, is the most appropriate method today with the preservation of the genetic resources of transgenic and knockout animals.

If sperm motility is low after thawing, intracytoplasmic sperm injection or ICSI (intracytoplasmic sperm injection) is used. In this case, the sperm enters the egg using micromanipulators, breaking its transparent membrane. A modern version of this method, which is used in medicine but is not yet widely used in working with laboratory animals, is the selected sperm morphology or intrasytoplasmic injection of IMSI (intrasytoplasmic morphologically selected sperm injection).

Another way to save living people from frozen seeds is through artificial insemination (AI). Embryogenesis is a necessary condition for the economic reproduction of animals used for commercial breeding or reproduction; rearing animals that have been completely altered after mutagenesis or gene transfer.

Embryos are also important for the management and storage of materials obtained in vitro, as they provide significantly higher propagation rates than any other clone distribution system. In addition, embryos originate from a single somatic cell, thereby maintaining the genetic fidelity of the animals from which they are derived. In addition, they promote a potential candidate for transformation because recovery from them is relatively inconvenient, which in many cases becomes a limiting factor in the production of transgenic substances [32]. Embryos contain diploid cells and have a complete set of genetic information of an individual. Once synchronous surrogates are transferred to the uterus, they can develop in individual animals. Therefore, cryopreserved embryos are very suitable for genetic support, transfer, and redirection. Like frozen seeds, pathogens pose a threat to cryopreservation of animal embryos due to their efficacy, ease of storage and transportation, and infectivity [32].

Embryos contain diploid cells and have the complete set of genetic information of an individual (Fig.1.7). They are able to develop into individual animals after being transferred into the uteri of synchronized surrogates. Therefore, cryopreserved embryos are ideal for the maintenance, relocation and rederivation of genetics. Similar to frozen sperm, the

efficiencies, ease of storage and handling, and pathogen transmission are of concern for cryopreservation of animal embryos [32].

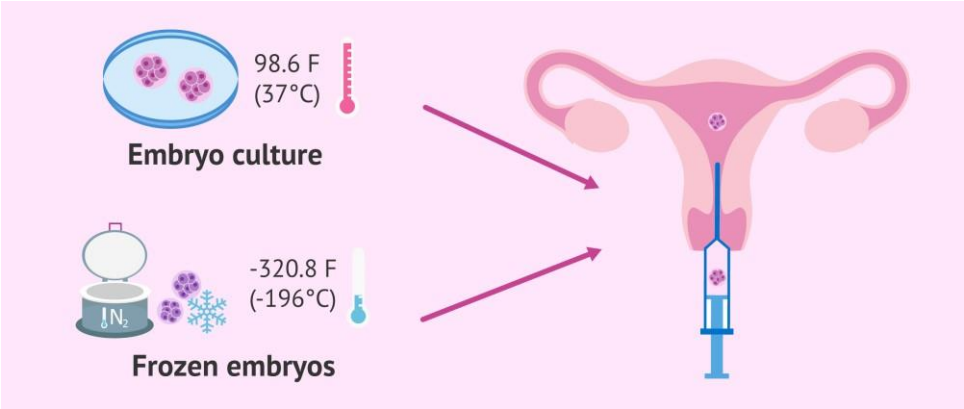

Fig.1.7. Embryo transfer

Cryopreservation of embryos offers several important logistical and economic benefits, including embryo storage, more than the number of recipients available, national and international efforts.

Recent advances in gametes and embryonic biotechnology demonstrate the usefulness of somatic cells for induced pluripotency. Fibroblast□derived pluripotent stem cells (iPS) provide a new approach to the potential formation of germ cells from somatic cells because iPS cells can then differentiate into the desired cell type [32]. The production of iPS cells from fetal fibroblasts was first found in mice [33]. For endangered species, pluripotence occurs in the somatic cells of primates, drills (Mandrillus leucophaeus), almost extinct white rhinos (Ceratotherium simum cottoni), and snow leopards (Panthera uncia) ladi [26].

Cryopreservation of adult cartilage cells, fetal cartilage cells, and lung tissue has shown promising results and is critical for maintaining biodiversity through the biobank. However, wildlife studies are rare and our knowledge is currently limited to domestic animals and marine mammals [26].

1.5. Conclusions to chapter

Cryopreservation is a process in which cells or tissues are frozen at very low temperatures of -196 ° C (boiling point of liquid nitrogen) to reduce the vital functions of the cell or organism. keep him in suspended living conditions for a long time. At these temperatures, any biological activity, including biochemical reactions that could cause cell death, is effectively stopped. Cryopreservation methods strive to achieve low temperatures without causing additional damage caused by ice formation during freezing.

Traditional cryopreservation is based on the coating of a material to be frozen with a class of molecules called cryoprotectants.

There are several types of cells suitable for freezing: sperm, eggs, embryos. The most common are sperm and eggs.

Cryopreservation is very important because it has the ability to preserve animal diversity. Due to various factors, an incredibly large percentage of animal diversity has now been lost, but cryopreservation methods are being studied to prevent this in the future.

CHAPTER 2 RESEARCH MATERIALS AND METHODS

2.1. Diagnosis of freezing material before cryopreservation

A spermogram may include a cryo□test (test for cryotolerance or cryo-resistance). This analysis is performed before semen freezing. The aim of the study is to assess the response of spermatozoa to cryopreservation (Fig.2.1). The cryotest allows you to find out whether they can safely survive the freezing procedure, whether they will retain their fertilizing ability after thawing.

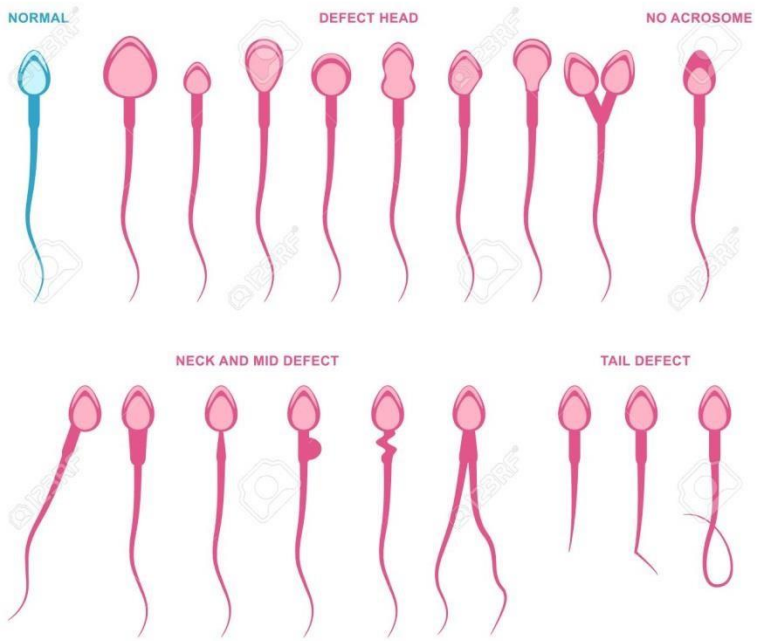

Fig. 2.1. Spermogram and semen parameters for successful cryopreservation

For cryotesting, a portion of sperm is frozen. After a while, some of the sperm cells are thawed and their state "before" and "after" is compared. If the quality of sperm has deteriorated, then this biomaterial is not suitable for cryopreservation and cryo□storage. If 60□75% of surviving cells are found in the sample, then this result is considered normal. In this case, 100% survival is not the goal, as there is usually enough live sperm in the ejaculate sample. Cryopreservation does not have a negative effect on the genetic information and morphology of cells, but sometimes a decrease in their mobility is observed.

It is believed that the success of protocols using gametes that have successfully undergone cryopreservation may be higher, since the strongest spermatozoa survive after freezing.

The use of epididymal sperm cells in ART on endangered animals could be considered a useful source. According to the sperm maturation process, sperm from the cauda epididymis are of good quality and potentially fertile.

These spermatozoa can also be used for IVF procedures even after freezing and thawing. Thus spermatozoa can be collected even from slaughtered male animals if there is no other possibility available. Sperm live/dead ratio can be easily and rapidly assessed by

using Kovács Foote staining procedure. Another approach for semen quality assessment is by the use of combination of several seminal quality attributes. Similarly, Chenoweth reported that choosing semen should reflect different aspects of sperm function (morphology and motility). Morphology or differential count of normal and abnormal cells, the minimum recommended threshold for sperm morphology should be at least 70 percent [34].

For motility, the semen samples are assessed for gross and individual motility with a minimum recommended threshold of fair “generalized oscillation and 30–49 percent” for the gross and individual motility respectively.

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With regard to motility, semen samples are assessed for total and individual motility with the minimum necessary threshold for a fair "generalized fluctuation and 30 49 percent" for gross and individual motility, respectively (Fig.2.2).

Fig. 2.2. Types of sperm motility

It is essential to assess the fertility potentials and guarantee the necessary health qualities of the oocytes since the developmental competence of an embryo is principally dictated by the oocyte [35]. Usually, for in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), oocyte selection is based on morphological parameters related to the cumulus cells, polar body, and cytoplasm.

Graded oocytes into three categories based on the number of cumulus cells and uniformity of the oocytoplasm, these are:

Good: Oocytes with many complete layers of cumulus cells and uniform cytoplasm.

Pair: Oocytes with thin or incomplete layers of cumulus cells and uniform cytoplasm.

Poor: Oocytes with few or no cumulus cells. To achieve good result, only good and pair oocytes should be used.

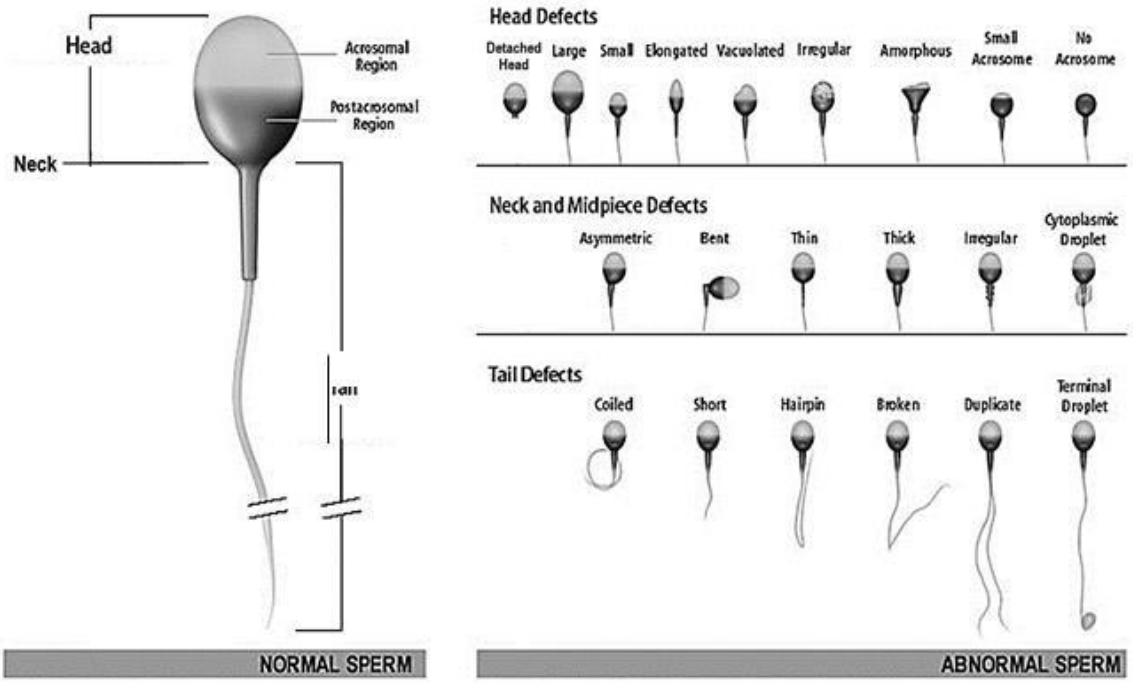

Fig. 2.3. Compares the shape of a normal sperm with common abnormalities

Potentially, oocyte quality can be assessed by using intracellular temperature imaging with the help of fluorescent polymer thermometer (FPT) to evaluate the thermal profile of the oocytes. It can also be determined by assessing molecular markers (microRNAs) and certain morphological factors that are related to oocytes quality like; first polar body morphology, meiotic spindle, cumulus cells and mitochondria [36].

After extraction / recovery of the animal embryo embryo donors, sometimes the total number of embryonic much higher than the number required looga program, that is, there is a need to protect the embryo surplus.

Subsequently, research funding was increased to develop an effective protocol for the protection of residual embryos. This created the idea of mathematical imaging. In sexual regeneration, embryos benefit from sperm because they allow the entire genome to be regenerated (i.e. no regeneration is required), so recovery can be achieved in one generation. Cryopreservation of embryos can be achieved by slow□moving cooling techniques, while maintaining the animal species involved.

Embryos, can be assessed for quality according to their morphological aspect due the time after insemination and the integrity of the pellucid membrane that surround the embryo. In time identified the following criteria for assessing embryo quality;

Developmental rate: embryos must be assessed in culture, during the 1□cell, cleaving and morula/blastocyst stages, and classified according to quality. At day 2, the first cleavage division occurs. After this, embryos double their number of blastomeres each day. Thus embryos have an expected 2–4 cells at day 2, 6–10 cells on day 3, become morula at day 4 and blastocysts at day 5 and 6 [36].

2.2. Slow freezing is a method of cryopreservation

For cryopreservation of cell systems, it is necessary to know and understand the mechanisms that impair the viability and function of cells. At slow freezing, it is not possible to get ride of the formation of ice crystals in the sample. It is therefore necessary to use a work protocol that has an optimal freezing speed that prevents the underlying mechanisms of injury (e.g., IIF, soluble toxicity, and osmotic damage).

Table 2.1

Advantages and drawbacks of the different cryopreservation methods

	Advantages	Drawbacks
Slow freezing	Low concentrations of CPA Simple handling and equipment Possibility to storage large volumes	Ice creation through sample Possible structure/tissue damage

Cryoprotector (CPA) is a term used to describe “any additive that can be given to cells before freezing and provides a degree of survival after warming” [37, 38]. CPA lowers the water content of cells / tissues and increases the total concentration of all dissolved substances. They are added to freezing solutions before cryopreservation to prevent IIF in

the process, often associated with cell damage leading to death [39]. However, in order to obtain a beneficial effect, the CPA concentration is much higher than other solutes in total cell carrier solutions and threatens it with toxicity. If penetrating CPA is used, after the initial compression, the cells swell when the CPA crosses the t cell membrane.

This late exchange of CPAs is due to an increase in cell permeability rather than water [40]. Conversely, if only penetrating CPA is used, this swelling will not occur and the cells will remain dehydrated. Although CPA is required for cryopreservation to prevent IIF, the addition and removal of CPA can cause the aforementioned osmotic stress in cells, which can lead to excessive cell damage or death [41]. Therefore, CPA addition should be carefully controlled to ensure cell performance and viability after storage.

The kinetics of the freezing speed also affect the cryopreservation result. Based on slow-cooling profiles and empirical observation of their good results, Mazur and the colleges proposed a hypothesis to explain the biophysical processes that occur during cryopreservation, i.e., the Mazur two-factor hypothesis (Fig.2.4). During the slow freezing process, the cells must be optimally dehydrated to preserve IIF. At optimal cooling rates, the cells are dehydrated with sensitive molecular and ultrastructural components protected by additional CPA. However, if you use a very fast freezing speed, the cells will not have time to freeze and the intracellular ice residue may form from the water inside the cell. Conversely, if the freezing speed is too slow, the cells will experience irreversible dehydration as a result of prolonged exposure to hypertonic solutions. This can disrupt the biochemical and physical conditions necessary for cell survival, and the mitochondria and endoplasmic reticulum can be systemically disrupted. In this sense, the survival effect of the “twisted U” graphically represented by Mazur is reflected in one of the best collections of cold biology [42]. Even if different cell types are combined in this set and the difference between the good cooling rate of different cell types is large, this “inverse U” living range can be observed in anyone.

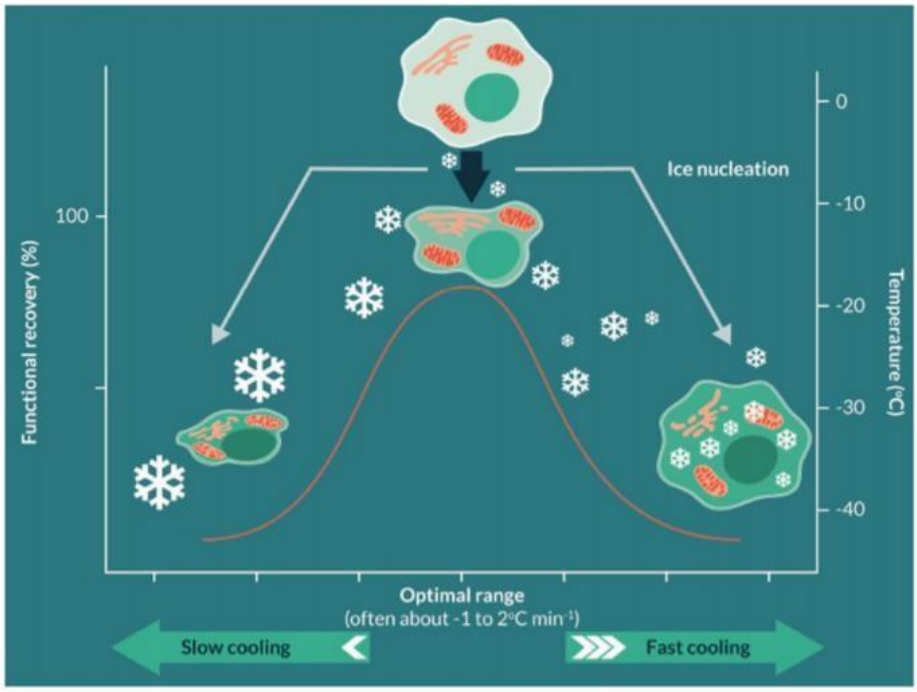

Fig.2.4. Schematic of Mazur´s 2□factor hypothesis

However, this is an oversimplification of the complex biophysical processes that occur during cryopreservation, and many of them are still unclear [43]. However, the twofactor hypothesis has been observed in many other results since its proposition, and its usefulness for studying and optimizing slow freezing protocols of various cellular therapies is undeniable.

Slow freezing involves a programmed gradual decrease in temperature. The process is long and requires the use of expensive tools. The process does not exclude the formation of ice crystals, which can lead to very harmful consequences. Slow freezing is a cryopreservation method based on dehydration. A small amount of cryoprotectant is added to the sample and the mixture is slowly cooled to -196 C, so true dehydration occurs when cooled [44]. At a certain point, ice masses containing pure crystalline water are formed. The unfrozen fraction remains among the growing ice masses, where all the cells and melts are stored. The concentration of sugar, salt, and cryoprotectants increases, while the volume of the unfrozen fraction decreases. An increase in osmotic pressure minimizes the formation of ice inside the cells, leading to water leakage from the cells. With continuous cooling, the viscosity of the unfrozen fraction becomes too high for subsequent crystallization. The

remaining unfrozen fraction eventually becomes an amorphous solid without ice crystals [45].

Slow freezing is the best cryopreservation method for human sperm. Typically, untreated or washed fresh seed samples are used. However, there are various methods for preserving surgically obtained sperm [44]. For example, isolated sperm can be inserted into the hollow zone of a hamster oocyte pelicid and placed between two air bubbles into the straw [46, 47].

Other studies suggest freezing under a layer of paraffin oil with glycerin [48]. A frozen testicular tablet that combines a mixture of sperm and testicular tissue is also possible [49].

2.3. Vitrification is a method of cryopreservation

Vitrification is super□fast method of freezing biological objects. This method is a modified approach developed for cryopreservation of mammalian oocytes and sperms. Vitrification is performed using a high concentration of cryoprotectant, which results in the solidification of the biological object, with the exception of intracellular and extracellular crystallization, which eliminates the underlying cause of cryoprocessing. Vitrification is often used for cryopreservation of sperm of predators, as well as pigs with a large number of lipid granules that are sensitive to cooling in their tissues. This approach requires strict control of the temperature regime and defrost mode of frozen items, which is one of its main drawbacks. It is noted that complex equipment is not necessary for storage and is a cheap cryopreservation method that allows freezing in the field with ultrasound and without the necessary equipment.

Vitrification is a method in which not only the cells but also the whole solution solidifies without ice formation (Fig.2.5). The vitrification method has advantages over the slow freezing method. Vitrification is super□fast cryopreservation method in which the cells travels from 35 to -196 ° C in <1 second and cools very rapidly as a result. The high

concentration of cryoprotectants, along with the rapid cooling rate, are important for cryopreservation of vitreous cell [50].

Vitrification is an unbalanced method that can be considered as a radical approach in which the formation of ice crystals is completely eliminated. However, this requires a very high cooling speed along with a high concentration of cryoprotectants compared to slow freezing [51, 52]. This method does not require expensive equipment and does not take much time.

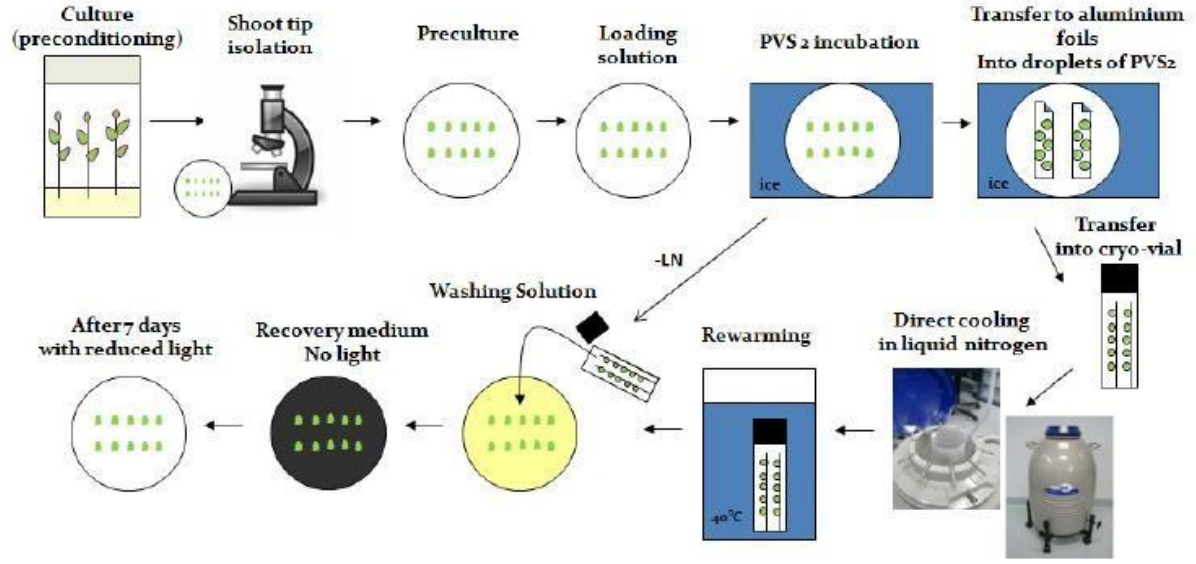

Fig. 2.5. Cryopreservation procedure for the droplet vitrification method

In the eighties of the last century, super-fast method of freezing using a glass transition was introduced, based on stimulating the solidification of the cells and the surrounding glass solution (glass or glassy glass) without the formation of ice crystals. This is possible due to an important combination of the following factors:

 Cryoprotectant concentration needs to be increased: Typically, cryoprotectants are used at very high concentrations for glass rather than slow freezing. Then, to prevent the toxic effects of the cryoprotector, the cells should be balanced in a low-concentration solution before immersion in a high-concentration solution (glass like solution). The equilibrium solution contains 10 15% cryoprotectant, the vitrification solution contains 35 40 percent (approximately 5.5 mol) cryoprotector. Choosing the optimal cryoprotector

is very important. It should have high permeability (i.e., low molecular weight) and be as non-toxic as possible to the cells. Ethylene glycol (EG) meets these requirements as well as DMSO, but its performance is slightly worse.

- Cell dehydration: To achieve this, it is necessary to use a cryoprotector with a high non-penetrating concentration and high molecular weight, such as disaccharide sugar: for example, sucrose or galactose at a concentration of up to 1 mole. The final concentration of this non-penetrating cryoprotector is added to the decomposition solution. The importance of minimizing exposure time to cells in a highly concentrated vitrification solution cannot be overstated; convenient vitrification procedures should limit this effect to a few seconds.

- Extra-fast freezing velocity: The glazing device containing the cells is immersed directly in LN, thus the freezing rate exceeds 20000 C min. The velocity depends on which glass we use to glaze and the amount of freezing. We do not need special equipment to achieve this freezing speed. When LN hits the device, a layer of LN forms around the device, which forms an insulating layer that can reduce the cooling rate. Activating the device by continuously adding lupine to the LN until it is completely solidified easily eliminates this effect.

- Increase the viscosity of the solution to check: the viscosity of the solution increases during the cooling process until the lime hardens. The high viscosity of the vitrification solution is obtained automatically due to the fast freezing process. Viscosity can be increased by using high concentration as well as non-penetrating cryoprotectants, and macro-molecules such as PVP can also be used.

- Creating a minimal number of vitrification. Several devices for vitrification of oocytes and embryos have been developed and used perfectly. When using a ‘plastic ring’, it reduces the amount of vitrification in the liquid film within the cycle, while the ‘open straw’ allows it to receive a very small amount of vitrification, which does not exceed 2 obtainl. The loading of the cells into the cleaning agent and the use of the tool is very high in this form because it is done when the cells are exposed to a high concentration of cleaning solution. The choice of device is an individual decision made depending on the processing

and selection of the individual operator. But velocity and agility are very important because the goal is to reduce the effect of the cells on the high concentration solution before solidification.

- Fast heating rate: Vitreous cells need to be heated quickly to prevent crystallization, as in a fast cooling process. The vitreous solution is immersed in a dilute solution and the cells are usually filled with water in two to three steps.

Advantages of vitrification:

- The vitrification process is simple and fast; this can be done in less than 10 minutes. Avoiding the formation of ice crystals during vitrification successfully eliminates the underlying factors that lead to cell damage during slow freezing.
- Easy to study: Vitrification simply involves moving cells placed in a vitrification device between separated cells and then immersing them in liquid nitrogen.
- One of the advantages of vitrification is that it allows the operator to observe the cells during the vitrification process. No viable zygotes and blastomeres are recognizable because they do not decrease when placed in the first or second vitrification solution. The presence of shrinking cells during heating is a useful sign for cells to survive, except for oocytes, which may survive depression and heating, but show degenerative signs about 1015 minutes after the end of heating.
- Since the vitrification procedure does not require special instrument or investment, it is very economical and offers extraordinary cost effectiveness compared to traditional methods. Vitrification is an extra simple and efficient process and the gains needed to finish it can be achieved in a short period of time due to the training materials that need to be discarded. In several IVF applications, the vitrification method has been used as a uniform procedure for 3 day old fetus and human blastocysts with high velocity of survival and pregnancy. The challenging task here is to find a happy vitrification protocol for human oocytes, for which the slow-freezing method has not yet provided an equal rate of survival and development.

2.4. Conclusions to chapter

Currently, cells can be frozen in two ways: slow freezing or rapid freezing (switching to glass). Prior to the freezing procedure, eggs, sperm, and embryos are mixed with special solutions that exclude the formation of ice crystals in the cells. They are then very cooled and stored in LN. Cells thaw when it's time to use. At slow freezing, the temperature around the cells gradually decreases, allowing more time for ice crystals to form, which can damage or destroy the cells. Not all cells go through a slow freezing process.

Vitrification is a new technique of faster freezing. This improves the survival rate of cells after warming compared to slow freezing.

Clinical studies have shown that the survival velocity of fetus after vitrification is significantly increased compared to slow freezing. At vitrification, the ambient temperature at which the cell fetus is located decreases rapidly. This reduces the risk of ice crystal formation and protects the cells from damage.

Vitrification is a very complex process that requires great training of specialists and great experience in working with this manipulation.

CHAPTER 3 THEORETICAL AND ANALYTICAL ANALYSIS OF CRYOPRESERVATION OBSERVATION

3.1. Disadvantages, problems and consequences of sperm cryopreservation

In frozen and de-frozen sperm there is a decline in mobility, viability and forward progression in the female reproductive tract which causes a reduction in fertility [53]. After cervical insemination, frozen-thawed semen is easier removed from the reproductive tract than fresh semen. Freezing–thawing increases the maturation of sperm membranes and capacitated acrosome reacted spermatozoa. These alterations may not affect mobility but reduces lifespan, ability to interact with the female reproductive tract and sperm fertility [54]. Cooled spermatozoa display chlortetracycline staining and an increase in intracellular free Ca^{2+}, typical of capacitated spermatozoa. Also cryopreservation induces the formation of reactive oxygen species (ROS) that impairs good fertility [55]. The membrane status of spermatozoa (intact, capacitated and acrosome reacted) can be evaluated through functional tests (zona free hamster egg penetration, chlortetracycline (CTC) staining, homologous and heterologous in vitro fertilization).

The freeze–fracture analyses in plasma membrane are not entirely reversible and may have implications for receptor–ligand interactions, namely the interactions between spermatozoa, oocyte and its coating. Studies indicate that frozen and de-frozen sperm can get good fertilization rates because they used hundreds of sperm cells. On the contrary, results of in vivo fertilization are lower because only a few sperm cells reach the site of fertilization.

In many animals, human and ram, cryodamage may change chromatin structure and originate DNA alterations which affect early embryonic development.

Sperm DNA and chromatin abnormalities have been associated with failures in in vitro assisted reproductive techniques. Today there are techniques to evaluate sperm chromatin condensation and stability, namely the flow cytometric (FCM) sperm chromatin

structure assay (SCSA) that is a useful predictor of fertility, either in vivo or in vitro. Also sperm chromatin may be evaluated under a fluorescence microscope after Acridine Orange (AO) staining on slide [56].

These ultrastructural and biochemical cryogenic changes in spermatozoa causes reductions in their functional integrity, survival in vivo and fertilizing capacity [57]. A lower packing quality (chromatin) in morphologically normal and motile spermatozoa is one of the limiting factors for fertilizing capacity. The tertiary and quaternary chromatin structure is essential for protection of genetic information and in early post fertilization events. When DFI (DNA fragmentation index) in semen is de□frozen higher than 30 percent, it decreases in vivo and in VF.

Cryopreservation protocols differ from species to species, as spermatozoa differ in shape, volume, size, and organelle composition. In all species, the differences between individuals are of genetic origin. Differences were found in specific DNA sequences, according to which the quality of de□frozen sperm was classified as good or bad.

Typically, about forty to fifty percent sperm cells do not support cryopreservation even with good protocols, and individual variations have been found.

In mammals, the loss of fertility due to cryopreservation is compensated for by insemination doses containing a greater number of sperm in all domestic species.

In small ruminants, artificial insemination of the cervix with frozen-thawed semen results in low to moderate fertility speed. This may be due to several factors, such as damage to sperm during freeze□thaw, impaired transport, viability and capacity of frozen□thawed sperm, embryonic mortality, and other factors associated with females such as estrus synchronization method, estrus detection, and timing of insemination. [58].

In cervical artificial insemination, a small proportion of sperm reach the oviduct. They are mainly excreted through the vulva or phagocytosed in the reproductive tract. If the total frozen-thawed sperm count falls below the amount required to achieve a good chance of fertilization, fertility will be impaired [59]. In sheep, intrauterine artificial insemination is more effective than posterior cervical fertilization.

In pets, cryopreservation of sperm leads to lower fertility than fresh semen due to loss of sperm viability and decreased function of live sperm.

Laparoscopic frozen‐thawed sperm insemination gave better fertility results than trans‐cervical or cervical artificial insemination. This method requires special expensive equipment and highly qualified specialists. In the UK, conventional laparoscopic insemination of sheep with frozen‐thawed semen is regulated for animal welfare reasons and is not permitted in Norway and Sweden, where the predominant AI technique is based on cervical insemination and is mostly self‐administered [60].

The IVF trials used frozen and de-frozen sperm from goats and sheep, although most IVF studies used freshly ejaculated sperm.

For example, in horses and pigs, artificial insemination with frozen-thawed semen is rarely used. Chilled semen artificial insemination is widely used in pig production with good results. In horses, artificial insemination with chilled semen produces poor to moderate results. Good stud farms sometimes use artificial insemination with frozenthawed semen from excellent horses.

3.2. Disadvantages, problems and consequences of embryo cryopreservation

There is a risk of fracture breakage at cryopreservation, that is more common with slow freezing procedures. In addition, the equilibration phase of any cryopreservation protocol can induce osmotic shock, which can lead to a shriveled embryo. Osmotic breakage can disrupt the cytoskeleton. Depolymerization of microtubules and microfilaments was observed after cryopreservation and traditional vitrification of fetus using straws. Not to do this breakage cytoskeleton stabilizers such as cytochalasin B have been proposed the vitrification process in pigs. But, cytoskeleton stabilizers were not required when ultrafast vitrification procedures were used to cryopreserve porcine morula and blastocysts.

Slow freezing and vitrification cause ultrastructural changes in fetus such as accumulation of cellular debris, enlargement of vesicles, and changes in trophoblastic microvilli.

Cryopreservation also causes abnormal distribution of mitochondria, swelling of mitochondria, reshaping of mitochondria, and rupture of their membranes. Recently, not only morphological changes have been reported, but also about impaired mitochondrial activity in frozen and vitrified fetus. Since mitochondria are required for aerobic metabolism and ATP production in the fetus, it has been proposed to add glycine as a mitochondrial protector to the vitrification medium for overcome the aforementioned deleterious effects [61].

Despite all these morphological changes at the cellular level, even if they are insignificant, the embryo is able to regenerate and eliminate dead cells. In this case, the normal morphology of the embryo can be almost fully recovers after twenty-four hours of cultivation, without affecting the viability of the fetus.

Slow freezing and vitrification affects the integrity of the DNA. Given that the increased DNA fragmentation in cryopreserved fetus is partly caused by excess free radicals, the addition of antioxidants to the medium may reduce this effect. Cryopreserved embryos also showed changed expression of a number of genes compared to fresh embryos. Most of these changes are associated with homeostasis, metabolism, and regulation of cellular and physiological activity, such as cell proliferation, cell cycle, development, biosynthesis, respiration, and expression of stress-related genes [62]. It seems logical that the embryos tried to compensate for osmotic shock and cold by changing their metabolism.

This adaptation and plasticity has implications for the embryo, and little is known about the potential effects on them and the subtle effects on offspring. In contrary, the endometrium, considered as the main sensor of the quality and health of fetus, can distinguish between fresh and vitrified fetus, as evidenced by altered uterine gene expression towards frozen fetus compared to fresh fetus. Moreover, in rabbits, vitrification has been observed to alter the expression pattern of genes and proteins in the placenta after implantation. Research to date shows that vitrification reasons fewer negative changes in the fetus proteome and energy metabolism than slow freezing.

Taken together, altered genes, proteins, and metabolic expression of cryopreserved embryos with different endometrial responses to frozen and fresh embryos may explain

the lower farrowing rate obtained with cryopreserved embryos compared to fresh embryos [63].

There is a growing concern that cryopreservation could initiate epigenetic markings and prolonged changes in the fetus. To date, there are very limited and conflicting studies regarding possible epigenetic effects of the cryopreservation process on the fetus. In contrary, some studies have shown that vitrification does not alter gene methylation patterns in the blastocyst. In contrary, vitrification has been found to increase gene methylation in bi-cellular bovine fetus.

DNA methylation is a clue epigenetic modification that is required for normal embryonic development. Complex patterns of DNA methylation are established and maintained by DNA DNMTs. It has recently been reported that cryopreservation has led to disruption of DNMT expression patterns in preimplantation human fetus. These data warrant further research to assess whether these disrupted patterns of embryonic DNMT expression could have prolonged inference for animal embryonic development.

3.3. Disadvantages, problems and consequences of oocytes cryopreservation

Cryopreservation of oocytes is a crucial step for the dissemination and saving of animal genetic resources. However, oocytes are very sensitive to cooling and cryopreservation, and although new advances have been made in the last few years, the ideal the protocol is not set yet. All oocytes undergo significant morphological and functional damage at cryopreservation, but the degree of breakage as well as the difference in survival and growth rate can vary greatly depending on the species, stage of development and origin.

Oocytes are particularly difficult to good cryopreserve, leading to low blastocyst production after thawing, fertilization, and culture. This problem may be due to the large size of oocytes, which, accordingly, have a low surface□to□volume ratio, which complicates the work of water and cryoprotectants to move across cell plasma membranes. Moreover, oocytes can be cryopreserved after maturation, representing a second meiotic spindle. Another way is cryopreservation of immature oocytes (embryonic bladder), because at this stage the oocyte has a lower microsensitivity to cooling and the absence of

a meiotic spindle. However, several reports have shown that immature oocytes are more sensitive to freezing than mature oocytes.

Due to the known effect of cryopreservation on meiotics spindle oocyte, concerns remain about the potential for chromosomal aneuploidy or other karyotype abnormalities in offspring. Similarly, concerns remain about potential organ malformations and other developmental problems. Despite few promising studies on vitrification, even less is known about the potentially harmful effects of vitrification compared to conventional cryopreservation methods [63].

It was also concerned about the disruption of the meiotic spindle, which is observed when cooling oocytes to a temperature of about 0 □C, which was described as a sign of cooling. At low temperatures, the tubulin is depolymerized, and the support for the structure of the spindle, which supports the chromosomes aligned on the metaphase plate, is disassembled.

There have also been changes in the sensitivity of oocytes to chills in individuals oocytes, as well as in some species of animals, including non□human primates.

But, it has been shown that the spindle reappears in the cytoplasm after heating the oocytes at 37 □C, and chromosomal aberrations do not appear to increase in embryos derived from frozen thawed oocytes compared to fresh embryos.

3.4. Conclusions to chapter

Cryopreservation of oocytes, sperm and embryos is one of the most important procedures for preserving the reproductive capacity of animals.

In order to successfully carry out the cryopreservation procedure, they try to study in advance and analytically predict the shortcomings of cryopreservation for each type of freezing cells.

In any case, each procedure has its disadvantages and advantages, which can be both predicted and identified after the procedure.

CONCLUSIONS

1. Cryopreservation is the process of conservation of tissue and cell viability after defrosting, including freezing and subsequent storage at very low temperatures. Biological

substances can be saved for quite a while at -196 ° C without losing their vitality because the metabolic process fully stops at this temperature.

Antifreezes play an important role in freezing. Traditionally, CPA are divided into several types. The most common and widely used antifreeze agents in the first group are DMSO multivalent alcohols such as glycerol, PEG and EG; monohydric alcohol methanol.

To avoid errors for a successful implementation, it is best to know what issues will occur before and after the cryopreservation process.

2. Today, there are two main ways to freeze biological samples: freezing and bottling applications. The first method consists of cooling at a relatively low speed with a special program consisting of several stages.

Vitrification is usually understood as an ultrafast cooling process in which no ice crystals are formed. Before you like the freezing method, you need to determine if the frozen material is suitable for cryopreservation. Their morphology and survival velosity need to be investigated.

3. The vitrification method is best for oocytes freezing because these cells are very sensitive to low temperatures, and the main problem is the curing of the zona pellucida. Vitrification is also best for sperm freezing because it does not form ice crystals that damage the structure and motility of sperm.

The method of slow freezing is best for embryos, because cryoprotectants are used in vitrification, which are quite toxic and have a negative effect on embryos.

Prosperous application of cryopreservation technology requires investigation of defects in each type of cell for a particular cryopreservation method. Oocytes, fetus, and animal semen have certain morphological features that can avert successful cell freezing. An analytical review of possible problems with the cryopreservation process to avert unsuccessful procedures.

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