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EVALUATION OF DIFFERENT FACTORS AFFECTING THE EFFICIENCY OF OOCYTES CRYOPRESERVATION IN THE BOVINE MODEL

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CHAPTER 1: The Model organisms

INTRODUCTION

Often, in order to understand complex problems like the biological phenomenon, it is helpful to choose and to study a simple "model" in which the phenomenon is present. That is "understand" in the simplify model will be checked in the complex one. This happens when we use a model organism.

A model organism is a non-human species that is diffusely studied to comprehend particular biological phenomena, with the expectancy that discoveries made in the organism model will provide insight into the workings of other organisms (Fields and Johnston, 2005). A more complete definition has been given by Held on the basis of Wessler's original definition (Wessler, 1976): "a living organism in which normative biology or behavior can be studied, or in which a spontaneous or induced pathological process can be investigated, and in which the phenomenon in one or more respects resembles the same phenomenon in humans or other species of animal." What is generally understood by the animal model is modeling humans. In this way, it is not the used animal that is the center of research but the similarity of the physiological behavior of this animal to our own (or another) species. This approach is made possible by the common descent of all living organisms and the conservation, over the

course of evolution, of metabolic and developmental pathways and genetic material (Allen, 1986).

The practice of studying biological phenomena and illnesses in animal models has a long history during which scientists have performed experiments on animals to obtain information of animal and human biological structure and function (Held, 1980). Consequently, most of our present basic knowledge of human biology, physiology, endocrinology, and pharmacology has been resulting from initial studies of mechanisms in animal models (Coffey and Isaacs, 1980). The reason is that ethical and religious concerns as well as social prohibitions have prohibited experimental studies of human biology and pathobiology.

At present, independently of ethical or religious considerations, such studies have not been conducted, and are not possible to be conducted, in humans. Practical, economic, and scientific reasons make initial studies in animals the best solution for studies a biological phenomenon. The use of animal models in science, and in particular, in biomedical research, is accepted by the majority of lay people and scientists. Animals and humans are associated in many real ways and not just on an ideological level. At the heart of it all is the health and safety of human populations. The reason for using animal models in biomedical research is scientific and animal models

are probable to remain necessary until science develops alternative models and systems that are equally complete and healthy.

A SHORT HISTORY OF THE USE OF ANIMAL MODELS IN BIOMEDICAL SCIENCE

The history of model organisms began with the idea that certain organisms can be studied and used to increase knowledge of other organisms or as a control (ideal) for other organisms of the same species.

In the western scientific tradition, the first use of animal models was in experimental surgery, which pre dated all other scientific uses by more than a millennium. In the ancient times, the earliest records of physiology research were carried out on the functions of the heart and respiratory systems in pigs by Erasistratus of Alexandra (302 – 258 BC). Galen (129 – 200) established the discipline of comparative anatomy publishing the first text books founded on dissections not on human cadavers (which was forbidden by religious and legal authorities) but on pigs and apes established, although these observations and their interpretations were frequently erroneous.

During the modern age, William Harvey published his great work on circulation based on studies in animals “Exercitatio anatomica de motu cordis et sanguinis in animalibus” in 1628; the “father” of modern

physiology, Claude Bernard (1813 – 1895) established the basis of the discipline based on animal experimentation and Louis Pasteur (1822 – 1895) used animals in the validation of the experimental method in microbiology. France was the country in which the science of comparing human and animal disease had its birthplace. In fact it was in France, in the later years of the 18th century, that the interest in veterinary science and comparative medicine emerged, but the idea of the model organism first took roots in the middle of the 19th century with the work of men like Charles Darwin and Gregor Mendel and their respective work on natural selection and the genetics of heredity. Some of the first work with what would be considered model organisms started because Gregor Mendel felt that the views of Darwin were unsatisfactory in describing the development of a new species and he began his work with the pea plants. Mendel created a practical guide to breeding and this method has successfully been applied to select for some of the first model organisms of other genus and species such as Guinea pigs, Drosophila (fruit fly), mice and viruses. In the 20th century, cardio-pulmonary resuscitation, the discipline of immunology and translational research on organ transplantation were all primarily developed through the use of animal models. The explosion in molecular biology in the second half of the 20th century increased the importance of animal models. Examples of other

diseases where the use of animal models were crucial to the recent elucidation of pathogenesis include cystic fibrosis, rheumatoid arthritis and spongiform encephalopathies. Increasingly, animal models are now being produced to exhibit specific symptoms and pathology of diseases through selective breeding and genetic modification. The development of in vivo molecular imaging modalities such as the micro-PET and MRI and their application to animal models in the 21st century has brought about a degree of accuracy and sophistication on biomedical research not previously possible.

WHY ANIMALS ARE USED

Animals are used in research when there is a need to find out what happens in the entire, living body, which is far more complex than the sum of its parts. It is difficult, and in most cases simply not yet possible, to replace the use of living animals in research with alternative methods. There are three main reasons why animals are used in research:

1) To advance scientific understanding

Adding to scientific knowledge through basic biological research helps us understand how living things work, and apply that understanding for the benefit of both humans and animals. The study of animals is a vital part of this research process. Many basic cell processes are the same in all animals,

and the bodies of animals are like humans in the way that they perform many vital functions such as breathing, digestion, movement, sight, hearing and reproduction. A great deal of the knowledge of the body's anatomy and functions can be traced to scientific findings from animal research. Comparing different species and studying the differences and similarities between them is one way to gain insights. Even simple animals can be used to study complex biological systems such as the nervous or immune systems, which follow the same basic organization and function in all animals. For example, much has been learnt about the function of neurons from studying the giant squid axon. Information from this sort of work can then be applied to higher animals and humans.

2) As models to study disease

Humans and animals share hundreds of illnesses, and consequently animals can act as models for the study of human illness. While contributing to our understanding of diseases, animal models also enable researchers to explore potential therapies in ways which would be impossible in humans. Studying disease mechanisms in animal models leads directly to development of new technologies and medicines that benefit both humans and animals. These animal models help researchers understand what happens in the body following this type of damage, and have been used in the development of new therapies.

3) To develop and test potential forms of treatment

Once researchers learn more about a particular disease, animals are used to develop and test these potential therapies as part of the applied research process. For example, medicines for Parkinson's disease have been developed using animal models with induced Parkinson's-like symptoms. Models such as these are an essential part of applying biological research to real medical problems, allowing new targets for disease intervention to be identified. Data from animal studies is essential before new therapeutic techniques and surgical procedures can be tested on human patients.

The model must, however, be pertinent to the aims of the study. Biomedical research is a very vast field and there are general and specific competence to consider when a new research starts. These considerations are:

1) Relevance of species

For example, animals are suitable for studies on muscle contraction but data obtained from the whole body has little relevance to humans. In gastrointestinal tract and liver studies, herbivores have highly specialized gastrointestinal parts (e.g. for cellulose digestion) and associated metabolism, which has no counterparts in humans. Omnivores are, thus, most suitable e.g. pigs.

2) Numbers required

In studies where the outcomes between the control and study groups differ only in degree, large numbers of animals are required to achieve statistical significance. Mice and other small mammals are ideal.

3)Transplant and other immunological studies

Inbred or naturally immunosuppressed species may be required.

CLASSIFICATION OF ANIMAL MODELS

What makes a good animal model? Not all animal species are suitable for the purposes of biomedical research and the limitations of the models selected as well as the methodology implicated must be always clear. A good and useful animal model suitable in a research facility should have the following characteristics (adapted from Isselhard, Kushe 1986):

1) The animal model should strictly reproduce the illness or condition under study.

2) The animal model should be easily accessible to many researchers, and for this reason a rare or exclusive animal is not a good model.

3) The animal model, in the case of a vertebrate model, should be large enough for multiple biological sampling (tissue, blood, etc).

4) The animal model should fit into available animal facilities.

5) The animal model should be easily handled by most investigators.

6) The animal model should be available in multiple sub-species.

7) The animal model should survive long enough for results to be significant.

8) The animal model should be sufficiently robust for the purpose of the study.

The selection of an animal model depends on a number of factors relating to the hypothesis to be tested, but often more practical aspects associated with the project and experimental facilities play a significant role. The usefulness of a laboratory animal model should be judged on how well it answers the specific questions it is being used to answer, rather than how well it mimics the human disease. Often a number of different models may advantageously be used in order to scrutinize a biological phenomenon. The appropriateness of any laboratory animal model will eventually be judged by its capacity to explain and predict the observed effects in the target species. For this reasons there are different categories of animal models.

In biomedical research , both invertebrate and vertebrate animals are used as models. Invertebrate models are very useful in the fields of neurobiology, genetics and development and notable examples of invertebrates use for such purposes include the C. Elegans and Drosophila. Vertebrate models are responsible for many advances in biology and medicine and are extremely important in translational research. These

includes the use of both small animal models (e.g. mice, rats, rabbits) and large animal models (e.g. dogs, pigs, monkeys). Broad areas of how vertebrate animal model are used in biomedical research include:

1) Pharmaceutical research including the development of biologics

2) Toxicology testing

3) Development and testing of new medical devices

4) Surgical research

5) Pathophysiological research

6) Study of reproductive aging

7) Vitrification methods for cryopreservation of oocytes

8) Study of ovarian function

9) Cryopreservation of gonads

These models can be in vitro (such as isolated cells, cell culture systems, tissue slice preparations or isolated perfused organs) or in vivo (using live animals).

Biomedical research models can also be either analogues or homologues. Analogous models relate one structure or process to another and are not unique to biomedical research. Homologous models reflect counterpart genetic sequences and are only used in biomedical research. Many animal models are both analogues and true homologues. The ideal model for a

human is another human, which is why randomized controlled clinical trials will always be important in the evaluation of new therapies.

Models can be either spontaneous, where an animal has natural characteristics like those of humans or a human disease-state, or induced where the animal has been altered, for example, through surgery or genetic manipulation. Spontaneous models show how factors, such as diet, genetics, environment and immunity can all contribute to a disease. Induced models are useful for studying the underlying causes of a disease, and are a common research tool for identifying potential drug targets.

The models may be exploratory, aiming to understand a biological mechanism whether this is a mechanism operative in fundamental normal biology or a mechanism associated with an abnormal biological function. Models may also be developed and applied as so-called explanatory models, aiming to understand a more or less complex biological problem. Explanatory models need not necessarily be reliant on the use of animals but may also be physical or mathematical model systems developed to unravel complex mechanisms. Another important group of animal models is predictive models. These models are used to discover and quantify the impact of a treatment, whether this is to cure a disease or to assess toxicity of a chemical compound. The anatomy or morphology of the model

structure of relevance to the studies may be of importance in all three of these model systems.

The validity of the results in experimental research depends on the qualities of the experimental model. For this reason is essential that the model be reliable, reproducible and valid. The model must also be a reasonable representation of the actual situation and the limitations of the model must be identified.

MOST COMMON ANIMAL MODELS

A plethora of animal models has been used and is being used and developed for studies of biological structure and function in humans.

One of the first model systems for molecular biology was the bacterium Escherichia coli, a common constituent of the human digestive system. Several of the bacterial viruses (bacteriophage) that infect Escherichia coli also have been very useful for the study of gene structure and gene regulation (e.g. phages Lambda and T4). However, bacteriophages are not organisms because they lack metabolism and depend on functions of the host cells for propagation.

In eukaryotes, several yeasts, particularly Saccharomyces cerevisiae ("baker's" or "budding" yeast), have been widely used in genetics and cell biology, largely because they are quick and easy to grow. The cell cycle in

a simple yeast is very similar to the cell cycle in humans and is regulated by homologous proteins.

The fruit fly Drosophila melanogaster is studied because it is easy to grow, has various visible congenital traits and has a polytene (giant) chromosome in its salivary glands that can be examined under a light microscope. Within a few years of the rediscovery of Mendel's rules in 1900, Drosophila melanogaster became a favorite model organism for genetics research and today it is one of the most widely used and genetically best-known of all eukaryotic organisms. Its complete genome was sequenced and first published in 2000 (Adams et al., 2000). About 75% of known human disease genes have a recognizable match in the genome of fruit flies, and 50% of fly protein sequences have mammalian homologs. Drosophila is being used as a genetic model for several human diseases including the neurodegenerative disorders Parkinson's, Huntington's, spinocerebellar ataxia and Alzheimer's disease. The fly is also being used to study mechanisms underlying aging and oxidative stress, immunity, diabetes, and cancer, as well as drug abuse.

Caenorhabditis elegans (C. elegans) is a free-living, transparent nematode (roundworm), about 1 mm in length (William, 1988) and extremely fecund. It’s life span is around 2 to 3 weeks under suitable living condition. Compared to the use of other model organisms, such as mice, the short life

cycle of C. elegans reduces the experimental cycle and facilitates biological study (Donald, 1997; Kenyon, 1888; Wood, 1988). These traits make it easy to produce numerous genotypes and phenotypes for genetic research (Donald, 1997; Horvitz, 1997; Wood, 1988). C. elegans is studied as a model organism for a variety of reasons: it is transparent, facilitating the study of cellular differentiation and other developmental processes in the intact organism. In addition, C. elegans is one of the simplest organisms with a nervous system.

Most model organisms have one or several unique attributes that make them ideal for a particular line of research. For instance, zebrafish is therefore a favorite research subject for developmental biologists and they may supplement higher vertebrate models, such as rats and mice. The zebrafish, Danio rerio, is a tropical freshwater fish and is an important vertebrate model organism in scientific research. Danio rerio is commonly useful for studies of development and gene function (Mayden et al., 2007). Research with Danio rerio has allowed advances in the fields of developmental biology, oncology (Xiang et al., 2009), toxicology (Hill et al., 2005), reproductive studies, teratology, genetics, neurobiology, environmental sciences, stem cell and regenerative medicine (Major and Poss, 2007) and evolutionary theory (Parichy, 2006).

The African clawed frog is a species of South African aquatic frog of the genus Xenopus. Although Xenopus laevis does not have the short generation time and genetic simplicity generally desired in genetic model organisms, it is an important model organism in developmental biology. Xenopus oocytes provide an important expression system for molecular biology. By injecting DNA or mRNA into the oocyte or developing embryo, scientists can study the protein products in a controlled system.

The mouse, although the smallest of the common laboratory animals, is in the greatest demand in terms of numbers as an experimental animal. Up to 80% of all animals used in laboratories are mice. The small size, rapid reproduction, and relatively high position on the evolutionary scale provide numerous characteristics useful in all areas of research. The mouse is used in a wide variety of studies including drug toxicity, microbiology, radiobiology, cancer research, behavior research, nutrition, and genetic studies. The mouse has been used in biomedical research since the early 20th century. Several characteristics have made the mouse an appealing research subject. These include the mouse's genetic similarity to humans (at least 80% of DNA in mice is identical to that of humans), small size, short lifespan and reproductive cycle, low maintenance in captivity, and mild manner. For these reasons, house mice constitute the majority of mammals used in research.

FARM ANIMALS AS MODELS FOR BIOMEDICAL RESEARCH

The aim of using animal models in biomedical research is to reconcile biologic phenomena between species. We wish to examine systems existing in one species and extrapolate knowledge to another. Traditional mammalian models include rats, mice, guinea pigs, hamsters, and rabbits and the information about the small-animal models in biomedical research is voluminous. On the contrary, larger domestic animals are proving to be valid research models because of their anatomical and physiological similarity to humans: similar cardiovascular structures, multiparous nature, and omnivorous habits. Also, body and organ sizes are much closer to those of humans and the genome of most domestic species is well characterized. The major limitations to the use of farm animal models for biomedical studies are high vivarium costs, long reproductive cycles and some physiological differences. Researchers continuously identify or develop new animal models to evaluate pathogenic mechanisms, diagnostic and therapeutic procedures, nutrition and metabolic disease, and the efficacy of novel drug development. Animal models for agricultural or food and fiber production research are also contributing data to studies of aging and disease processes of humans and animals. Historically, large animals have made many contributions to greater understanding of human health:

1) The first smallpox vaccine was generated using the cowpox virus (Barquet and Domingo, 1997).

2) Studies on freemartin cattle (females born twin to a male) and allograft tolerance between twins was the basis for the Nobel Prize in 1960 (Anderson et al., 1951).

3) Sperm cells were first frozen in cattle before this technology was applied to humans (Polge et al., 1949).

4) Since 1960, hundreds of thousands of pig and cow heart valves have been placed in humans.

5) The first cloned mammal from a non-reproductive cell was a sheep (Campbell et al., 1996).

6) Pig and sheep hypothalami were used to isolate and sequence thyroid stimulating hormone (TSH) and gonadotropin releasing hormone (GnRH), leading to the Nobel Prize 1977 (Schally, 1977).

7) Prostaglandins were first isolated from sheep, leading to the Nobel Prize 1982 (Flower, 2006).

8) Cow and pigs were used as a source for insulin from the 1920's to the 1980's.

9) Prions first isolated from sheep and goats, leading to the Nobel Prize 1997 (Prusiner, 1982).

What is unique to using large animal models? Size does matter. Large animal research opens up the possibility to take frequent biopsies and samples, obtain many more cells from one sample, as well as catheterize multiple vessels that cannot be done in small animals such as rodents. The similarity in organ size in some animal models allows for better models of surgery, hemodynamics, and possible future xenotransplantation. Large animals can be used for "pharming" to produce large quantities of useful proteins in milk of transgenic cows, pigs, sheep, or goats, such as α1-antitrypsin produced in sheep for patients with pulmonary emphysema that lack this enzyme (Das, 2001) . Besides these general examples, each species has particular attributes which may make it a good candidate for certain types of research.

SWINE

Swine have been useful for biomedical research for the past four decades. The same applies to miniature pigs, which are bred to serve as laboratory animals primarily for biomedical research. Pigs and minipigs are increasingly recognized as useful animals for various areas of biomedical research. Characteristics that make them particularly useful for this purpose are:

1) Convenient body size for most clinical and surgical experiments or trials involving repeated collection of blood samples, biopsies etc.

2) Similarities with the biology of the human, in particular with respect to skin, skeleton, and joints, teeth, gastrointestinal tract, pancreas, liver and kidney, heart and blood vessels, lung, immune mechanism and physiological stage of the newborn.

3) The ease of handling and housing under confined conditions.

4) The relatively low price at which they can be made available.

Pigs and minipigs are already playing a significant role as laboratory animals in the areas of physiology, pharmacology, toxicology, radiology, surgery and organ transplantation, traumatology, pathology, embryology, gastroenterology, nephrology, and pediatrics. Both domestic and miniature swine have been used extensively in cardiovascular research. The pig has been used extensively for studies concerning the metabolism of fats and carbohydrates, such as studies in obesity, diabetes mellitus, atherosclerosis, and hypertension. Swine offer additional advantages over other species by having a renal anatomy and function very similar to human. Swine have also been useful in elucidating the relationship between exercise and cardiopulmonary function. Due to size and anatomic and physiologic similarities, swine have been used extensively in surgical research.

SHEEP

Starting in the later years of the 19th century, medical researchers began to use the sheep (Ovis aries, order Ungulata) as a laboratory animal. Domestic

sheep are placid animals and, because of their size, can easily be handled. Sheep are quite similar in body weight to humans, and sufficiently large to allow serial sampling and multiple experimental procedures (Hubrecht and Kirkwood, 2010). Researchers have successfully studied fetal circulatory system function, anesthesia, catheterization techniques, and steroid radio immunoassays with sheep models. Subsequent to this, pregnant ewes became common subjects in noninvasive studies by ultrasonography, by which the monitoring of fetal physiology could be performed easily and safely. Researchers have studied serology, immunology, and placental transfer of drug and metabolites with sheep. Adult sheep have been models in the development of heart transplantation techniques, and as models for extensive studies of human autoimmune renal disease. Such research included studies on nutrition, reproduction, production, genetics, disease, and wool properties. (Hau and Van Hoosier, 2010).

HORSES

Horses have been used in biomedical work for many purposes over many years. Much of the early use was related to their suitability as sources of blood, in quantity, containing biological factors of medical or experimental value. Very long lifespan, and similar patterns of ovarian follicle development may make this animal useful for studies into fertility in aging women (Carnevale, 2008).

CATTLE

The animal sciences have contributed greatly to the basic understanding of human endocrinology and physiology. Classical endocrinology studies in farm animals led to the current understanding of several reproductive and pituitary hormones. The composition of insulin was first determined for bovine insulin (Sanger et al., 1955; Sanger, 1959) and it was used for several decades to treat human diabetes. Warfarin (coumarin), an anti-coagulant, was first identified in the blood of cattle with sweet clover disease (Stahmann et al., 1941). Parathyroid hormone was first identified in extracts of bovine thyroid/parathyroid (Collip, 1925) and the luteotrophic effect of luteinizing hormone was first demonstrated in cattle (Wiltbank et al., 1961). The growth promoting effects of growth hormone were first identified by administering extracts of bovine pituitaries into the rat (Evans and Long, 1921). Administration of bovine somatotropin (bST) to dairy cattle has provided an abundancy of information on lactational effects of GH.

The bovine model provided the fundamental research platform for developing human reproductive techniques. Current reproductive techniques used in humans such as superovulation, oocyte maturation, in-vitro fertilization, embryo culture, transfer, and freezing, are based upon many years of research with bovine embryos (Brackett et al., 1982; Robl et

al., 1987; Iritani and Niwa, 1977). Research with cattle semen (Polge et al., 1949; Phillips, 1939; Johnson et al., 1987) contributed extensively to using frozen semen for fertilization of human embryos and sexing semen. The most important research lines in the human reproductive techniques are based on the decline in female fertility caused by aging, reproductive diseases or sickness (cancer). To keep alive and then to recover vital gamets is most important in reproductive medicine. From the first experiment of Polge on spermatozoa in 1949 appeared evident the importance to use very low temperature (cryopreservation) in fertility preservation. Even if cryopreservation of embryos and oocytes are considered common technique for this purpose, embryo cryopreservation has many limitations in Italy (Law 40/2004). By prohibiting embryo freezing in humans, the law 40/2004 has restricted the conservation of fertility only to oocytes cryopreservation. Despite many advances in oocytes cryopreservation (ovulated, mature or immature), this technique is still not considered an established procedure and thus its current label as experimental system (Noyes et al., 2010). In the human field, the greatest challenge is oocyte cryopreservation. In fact, oocyte cryopreservation before the start of any kind of therapy would offer a chance to resolve the problem of reduced fertility. The mature oocyte shows a complex cytoplasmic organization as a consequence of the remodelling occurred during the final stages of

maturation, when neogenesis, modification and redistribution of organelles in specific ooplasmic areas did occur. So, oocyte cryopreservation offers a method to preserve fertility but many studies are still necessary to improve this technique. In this scenario the importance of using an animal model is highlighted by the limited availability of human oocytes for research purposes. The choice of bovine oocytes as the model is due to the easy access to experimental material, i.e. oocytes derived from slaughtered animals and to the existence of a well established in vitro embryo production system. The acquisition of more information on cryopreservation-induced oocyte damages is necessary to identify corrective strategies and in the same time many innovative vitrification methods have been proposed that need to be proven effective on animal oocytes before using in the human field.

CHAPTER 2: The Cryopreservation

CRYOPRESERVATION IN MAMMALS

The maintaining of the potential viability of living reproductive cells and tissues of several mammalian species after long-term storage represents a tool of great opportunity for both human and animal reproductive applications (Woods et al., 2004; Godsen, 2005). This is possible with the use of cryopreservation, a process where cells or whole tissues are preserved by cooling to low sub-zero temperatures such as −196 °C (the boiling point of liquid nitrogen). At these low temperatures, any biological activity, including the biochemical reactions that would lead to cell death, is effectively stopped. Cryopreservation occurs naturally. Antifreezing is in fact required for ensuring survival of various organisms.

The concept of preserving life in a suspended animation state with the use of low temperature is not new. Cryobiology history can be traced back to antiquity. As early as in 2500 BC low temperatures were used in Egypt in medicine and the use of cold was recommended by Hippocrates to stop bleeding and swelling. With the emergence of modern science, Robert Boyle studied the effects of low temperatures on animals and in 1949 bull semen was cryopreserved for the first time by a team of scientists led by Christopher Polge. The study on cryopreservation moved into the human world in the 1950s with pregnancies obtained after insemination with frozen sperm. However, the simple immersion in liquid nitrogen for

certain samples, such as embryos and oocytes, did not produce the necessary viability to make them usable after thawing. Increased understanding of the mechanism of freezing injury to cells empathized the importance of controlled or slow cooling to obtain maximum survival on thawing of the living cells. Controlled-rate and slow freezing are well established techniques pioneered in the early 1970s which enabled the first birth from a human frozen embryo in 1984. Since then many studies have developed to preserve human and animal samples from freeze damages until the development of a new technique, that is vitrification, that provides the benefits of cryopreservation without damage due to ice crystal formation.

Different are the advantages to use cryopreservation: the progress in cryopreservation would improve domestic animal breeding by genetic selection programs (Vajta, 2000), to contribute at the maintenance of biodiversity through wildlife species conservation (Pukazhenthi and Wildt, 2004) and preserve the human fertility.

PRINCIPLES OF CRYOPRESERVATION

To maintain long-term viability after long-term storage, living cells must be brought into a state of suspended animation in which they remain for indefinite periods of time, and from which they can be brought back to viability at some point in the future. The temperature that generally is used for storage of mammalian cells −196 °C, the temperature of liquid nitrogen, appears to be adequate for these purposes, although a precise threshold value for this temperature is not known. At these low temperatures, water exists only in a solid state, and no known biological reactions take place. Because oocytes are viable at 37°C and no biological activity takes place at −196 °C, the time of greatest danger during cryopreservation appears to be during the transitions of temperature: during cooling to −196 °C and during subsequent rewarming to 37°C. When water is cooled below its freezing point, it solidifies in a crystalline structure known as ice. Because ice is less dense than liquid water, it necessarily follows that ice crystals occupy a greater volume than does the liquid water from which they were formed. As adjacent volumes of liquid water within a cell solidify, their expansion into ice causes pressure and shearing forces on intracellular organelles, which can suffer considerable damage. Avoidance of ice crystal formation therefore is one of the principal goals of successful cryopreservation. As water transitions from liquid to ice, any solutes in the liquid phase are

excluded from the solid. This lowers the freezing point of the remaining unfrozen solution. As the temperature drops and the solid form proliferates, the concentration of electrolytes and other solutes can reach very high levels (Lovelock, 1954). These concentrations can be quite toxic to intracellular proteins, and thus avoidance of these solution effects is a second major goal of successful cryopreservation. During rewarming, the solid ice melts and releases free water, resulting in decreasing osmolarity of the surrounding solution. When rewarming is slow, there is danger of free water thawing and recrystallizing, thus causing further damage. When rewarming is rapid, sudden drops in extracellular osmotic pressure may lead to rapid shifts of free water across and into the cell, leading to swelling and cell damage (Mazur, 1980). This is called osmotic shock, and its avoidance is a third major goal of successful cryopreservation. Therefore, simply immersing oocytes into liquid nitrogen is not an effective strategy for successful cryopreservation. All successful methods must avoid these three issues: ice crystal formation, solution effects, and osmotic shock. Up to this point in time, all cryopreservation strategies, including all methods of oocyte cryopreservation, have used additional chemicals to avoid cell damage. These chemicals are called cryoprotectants and generally can be divided into two categories, permeating and nonpermeating.

Permeating cryoprotectants

Permeating cryoprotectants are small molecules that readily permeate the membranes of cells. They form hydrogen bonds with water molecules and prevent ice crystallization. At low concentrations in water, they lower the freezing temperature of the resulting mixture. However, at high enough concentrations, they inhibit the formation of the characteristic ice crystal and lead to the development of a solid, glasslike, so-called vitrified state in which water is solidified, but not expanded. In this manner, the permeating cryoprotectants satisfy the first goal of successful cryopreservation, the avoidance of ice crystals. Permeating cryoprotectants play a second important role in cryopreservation, and this is to protect the cell from solution effects. They achieve this goal by remaining in solution and by thus effectively diluting the remaining electrolytes. This effect is described by the phase rule, which states that in a two-phase system, such as liquid water and ice at a given pressure, the total solute concentration in the liquid phase is constant for a given temperature (Friedler et al., 1988). The most commonly used permeating cryoprotectants are: Propylene Glycol (PROH), Ethylene Glycol (ET), Glycerol and Dimethyl sulfoxide (DMSO).

Nonpermeating cryoprotectants

In contrast to the permeating cryoprotectants, nonpermeating cryoprotectants remain extracellular. They act by drawing free water from within the cell, thus dehydrating the intracellular space. As a result, when they are used in combination with a permeating cryoprotectant, the net concentration of the permeating cryoprotectant is increased in the intracellular space. This further assists the permeating cryoprotectant in preventing ice-crystal formation. The nonpermeating cryoprotectants play an important role during thawing. During thawing, the water generated by the melting ice rapidly decreases the extracellular osmotic pressure. Osmotic shock may occur if the intracellular cryoprotectant cannot diffuse out quickly enough to prevent excessive influx of free water and the swelling, or even rupture, of the cell. Therefore, freezing and thawing protocols commonly use a high concentration of nonpermeating cryoprotectants during the thawing phase. The most commonly used nonpermeating cryoprotectant is sucrose, but other disaccharides and other nonpermeating agents also may be used.

DAMAGE AND ITS PREVENTION DURING CRYOPRESERVATION

All oocytes and embryos suffer considerable morphological and functional damage during cryopreservation. The extent of the injury depends on factors including the size and shape of the cells, the permeability of the membranes, and the quality and sensitivity of the oocytes and embryos. All these factors may be highly variable depending on the species, developmental stage and origin (for example, in vitro produced or in vivo derived embryos). However, oocytes and embryos also have a remarkable, sometimes surprising ability to repair this damage fully or partially, and, for optimal cases, to continue normal development. The purpose of cryopreservation procedures is to minimize the damage and help cells to regenerate. Almost all cryopreservation strategies are based on two main factors: cryoprotectants and cooling–warming rates. Since the first successes of mammalian embryo cryopreservation in the sixties of the last century, two major groups of methods can be delineated: traditional slow-rate freezing and vitrification. Storage, warming and rehydration, i.e. removal of cryoprotectants differ only slightly between the two procedures (with some exceptions) and the main difference exists in the addition of cryoprotectants and cooling. Traditional slow-rate freezing was introduced first, and for the majority of domestic animal and human embryologists

this remains the only acceptable approach. Over time, methods have become highly standardized with a considerable industrial and commercial background. Traditional slow-rate freezing can be interpreted as an attempt to create a delicate balance between various factors causing damage including ice crystal formation, fracture, toxic and osmotic damage. The controlled cooling rate allows solution exchange between the extracellular and intracellular fluids without serious osmotic effects and deformation of the cells (this fact is reflected in the other name of the procedure: equilibrium freezing).

The slow-freeze method relies on low initial cryoprotectant concentrations, which are associated with lower toxicity, while the oocyte is still at a temperature at which it is metabolizing. Because cell metabolism is thought to decrease by approximately 50% for every 10°C decrease in temperature, toxicity is limited by having concentrations of cryoprotectants and other solutes increase only after the cell has been cooled to temperatures at which metabolism is quite slow. Cryoprotectants typically are added at room temperature. The temperature then is lowered gradually (about 2°C/min) to the seeding temperature at −6°C. During this time, all of the solution remains liquid. At a temperature of −6°C, ice crystals may be induced in the solution by introducing a seed, a small crystal of ice that allows other water molecules to undergo crystallization. Seeding commonly is

accomplished by touching the outside of the cryopreservation vessel with a very cold instrument, such as a forceps, which then induces a small ice crystal to form in the area at which the cryovessel is touched. The resulting formation of ice releases energy known as the heat of fusion. Therefore, solutions are commonly held at this temperature for some period of time, such as 10 to 30 minutes, to allow equilibration. The growing ice crystals exclude solutes, thus gradually increasing their concentration in the remaining solution. Therefore, seeding is performed in an area distant from the oocyte, so that the ice crystal grows gradually toward the oocyte. After equilibration, the temperature gradually is decreased to a final temperature of −32°C. During this time, the ice crystal gradually propagates in the extracellular medium, thus further increasing the concentration of cryoprotectants, particularly in the intracellular space, which further is dehydrated through the use of nonpermeating cryoprotectants. The very slow rate of cooling (0.33° per minute) allows gradual diffusion into the oocyte of additional permeating cryoprotectant while maintaining equilibrium with the extracellular space. The metabolic rate of the oocyte now is quite slow, further limiting the toxicity of the increasing concentrations of the cryoprotectants. The freezing vessel now is plunged into liquid nitrogen, and the remaining non solidified solution is converted to a solid state. During thawing, a rapid transition of temperature is

preferred to prevent recrystallization of water with the potential for ice-crystal damage. Here caution must be taken to avoid osmotic shock from the permeating cryoprotectant, which now is at a very high concentration in the intracellular space. Therefore, additional nonpermeating cryoprotectant is used. As the permeating cryoprotectant gradually diffuses out of the oocyte, the concentration of the nonpermeating cryoprotectant gradually is decreased, until the oocyte is returned to standard culture medium.

The phenomenon of the solidification of water without ice crystal formation, is also called vitrification. However, the term ‘‘vitrification’’ in cryobiology refers to the other group of cryopreservation methods, where the main purpose is to ensure ice free solidification of the whole solution containing the sample. The process itself can be described as an extreme increase in viscosity of solutions. The benefits are unquestionable: ice crystal formation, one of the most dangerous causes of cryoinjury, is entirely eliminated except for a transitional and very short freezing of the solutions during warming that is generally regarded harmless to the oocytes and embryos. Unfortunately we have to pay the price of this benefit. Vitrification can only be induced in exceptional situations: with dangerously-high concentrations of cryoprotectants and/or with extreme increase of the cooling and warming rates. One characteristic of vitrification is that as the cooling rate chosen increases, the cryoprotectant

concentration can be lowered and vice versa. Extensive research in the past 20 years has resulted in new, sometimes radically-new approaches and created an acceptable compromise between the positive and negative by decreasing cryoprotectant toxicity and increasing cooling rates. As a result, vitrification has become a competitive alternative to slow-rate freezing. Moreover, the high cooling and warming rates applied at vitrification provide an unique benefit compared to the traditional freezing: the possible partial and sometimes total elimination of chilling injury, as the sample passes through the dangerous temperature zone quickly enough to disallow sufficient time for damage to develop. During vitrification, permeating cryoprotectants are added at a high concentration while the cell is at room temperature. Because the toxicity of this high concentration of permeating cryoprotectant is substantial, the oocyte cannot be kept at this temperature for long. Instead, a very short time is allowed for equilibration, after which the oocytes are plunged directly into liquid nitrogen. To further protect against ice-crystal formation, an extremely rapid rate of cooling is used. For this reason, novel cryovessels have been used that allow direct contact between liquid nitrogen and the oocyte-containing solution and that have in common a very high surface-to-volume ratio (Park et al., 2000; Mukaida et al., 2003). This form of ultrarapid freezing must be followed by ultrarapid

thawing to prevent ice recrystallization. To achieve vitrification, three important factors should be considered:

1) Cooling rate, which is achieved with liquid nitrogen or liquid nitrogen slush. When using liquid nitrogen, the sample is plunged into liquid nitrogen resulting in cooling rates of hundreds to tens of thousands degrees Celsius per minute, depending on the container, the volume, the thermal conductivity, the solution composition, etc. (Yavin and Arav, 2007). It was shown for oocytes and embryos that increasing the cooling rate would improve survival rates by up to 37%.

2) Viscosity of the medium in which the embryos are suspended. This is defined by the concentration and behavior of various cryoprotectants and other additives during vitrification. The higher the concentration of cryoprotectants, the higher the glass transition temperature (Tg), thus lowering the chance of ice nucleation and crystallization. The combination of different cryoprotectants is often used to increase viscosity, increase Tg, and reduce the level of toxicity.

3) Volume – the smaller the volume, the higher the probability of vitrification (Arav, 1992; Arav et al., 2002; Yavin and Arav, 2007). Smaller volumes allow better heat transfer, thus facilitating higher cooling

rates. Many techniques have been developed to reduce sample volume with an explosion of methods appearing in the literature during the last decade.

EFFORTS TO IMPROVE VITRIFICATION

IN EMBRYOLOGY: MINUMUM VOLUME COOLING

Vitrification was first introduced in embryology for cryopreservation of mouse embryos (Rall and Fahy, 1985). In the subsequent decade, a moderate number of publications dealing with successes in cryopreservation of oocytes and embryos from several domestic animal species were published. Meanwhile, considerable efforts were made to decrease the toxicity of cryoprotectants by applying less toxic and more permeable chemicals (first of all EG), using two or three cryoprotectants to decrease the specific toxic effect of each, to make stepwise addition, and to cool solutions to temperatures close to 0°C when the final high concentration was applied. Approximately 10 years ago, the development of vitrification seemed to reach a plateau phase. The possibilities for modifying cryoprotectant combinations was almost fully exploited. In most species and developmental phases, vitrification enabled researchers to achieve similar survival and developmental rates to traditional slow rate freezing. However, vitrification did not offer real benefits compared to the

earlier methods. To achieve that, a considerable increase in cooling and warming rates was required. After promising results under experimental conditions (Arav, 1992) the first attempt to use ultrarapid cooling in everyday practice was based on the very simple idea of dropping the embryo containing solution directly into the liquid nitrogen (Riha et al., 1991). Theoretical advantages in maximizing cooling rate by doing so were limited at first by the relatively large volume of the drop as well as the delay before the drop floating on the surface of liquid nitrogen sank. The imagination of researchers both in the domestic animal and human field resulted in a flood of new tools based on the same principle: to increase cooling and warming rates by minimizing the vapor formation around the sample at cooling. The latest development in the practical application of ultrarapid vitrification methods was based on the concept of minimum volume cooling (MVC; Hamawaki et al., 1999). Different carrier tools were applied to minimize the volume and to submerge the sample quickly into the liquid nitrogen. These techniques can generally be divided into two categories, surface techniques and tubing techniques. The surface techniques include:

1) Solid Surface Vitrification (Dinnyes et al., 2000). Solid Surface Vitrification (SSV) method combines the advantages of containerless vitrification in microdrops and the increased heat exchange of a cold metal

surface. A metal cube covered with aluminum foil is partially submerged into liquid nitrogen. Microdrops of vitrification solution, containing the oocytes, are dropped onto the cold upper surface of the metal cube and are instantaneously vitrified. Warming was performed by placing the drop of vitrified solution in a warming solution directly. This approach makes a shortcut to exclude entirely the vapor formation by cooling metal surfaces in liquid nitrogen, and placing the solution containing the biological sample on the surface of metal.

Figure 1. Solid Surface Vitrification

2) Cryoloop (Lane et al., 1999a, 1999b). The Cryoloop method consists in the use of a solution film formed between files of a nylon loop for holding the sample. Due to the absence of solid support and the extremely small volume, the cooling rate upon immersion in liquid nitrogen may be as high as 700,000 C/min (Isachenko et al., 2003) thus also permitting cooling in liquid nitrogen vapor. However, in spite of the unquestionable benefits of

this system from the point of potential disease transmission, one may have concerns regarding the safety of storage, as this very sensitive and fragile system may increase the risk of accidental warming over the safe temperature zone, especially if storage of the container is also performed in the vapor of liquid nitrogen (Fuller and Paynter, 2004).

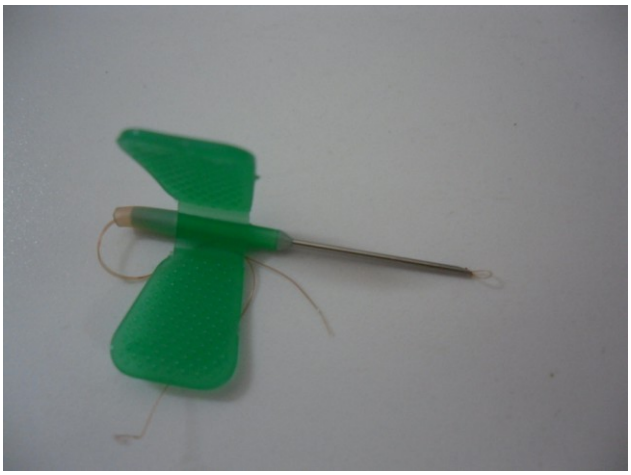


Figure 2. Cryoloop

3) Cryotop (Hamawaki et al., 1999; Kuwayama and Kato, 2000). In the Cryotop procedure (Kuwayama and Kato, 2000), a thin plastic film strip attached to a plastic handle is the carrier tool. Loading is performed with the use of a glass capillary under the control of a stereomicroscope. Before cooling, almost all medium is removed, so the embryos and oocytes are only covered with a very thin solution layer. Submersion of the Cryotop into the liquid nitrogen or into the rehydrating solution after vitrification results in extremely high cooling and warming rates, respectively, permitting further decrease in concentration of cryoprotectants. During

storage, a protective cap is applied to prevent mechanical damage of the film and the vitrified sample. The minimal volume approach of the Cryotop method increases the cooling and especially the warming rates (up to 40,000 8C/min) which may contribute to the improved and consistent survival, and both in vitro and in vivo developmental rates. According to data published by other research groups, the Cryotop technique is at present the most efficient method for cryopreservation of sensitive samples including human oocytes. The Cryotop vitrification method is easy to learn. Anybody with basic experience in embryology can perform it appropriately after a few hours' training period. The method is simple and reliable, provides consistent results and variations between operators are minimal.

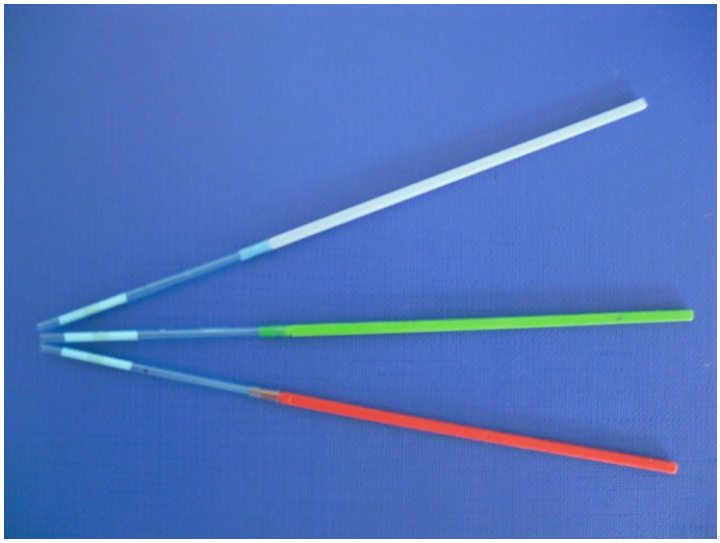


Figure 3. Cryotops

4) Vitri-Inga (Almodin et al., 2010). The Vitri-Inga vitrification strip is an apparatus that consists of a fine, very thin polypropylene strip (0.7 mm thick) with a specially designed round tip, in which there is a minute hole to receive the oocyte; the strip is connected to a hard and thicker plastic handle.

To the tubing techniques belong the:

1) Open Pulled Straw (Vajta et al., 1997, 1998). The Open Pulled Straws (OPS) are French mini-straws (250 µl, IMV, L'Aigle, France) were heat-softened over a hot plate, and pulled manually until the inner diameter and the wall thickness of the central part decreased from 1.7 mm to approximately 0.8 mm, and from 0.15 mm to approximately 0.07 mm, respectively. Loading of sample into these straws is performed using the capillary effect by simply touching a 1- to 2-µl droplet containing the ova or embryos with the narrow end of the straw. This end is then immediately submerged into liquid nitrogen and the liquid column solidified instantaneously. Warming is performed by placing the end of the straw directly into the holding medium. The vitrified medium becomes liquid within 1 to 2 seconds, whereupon the holding medium enters the straw. Immediately afterwards, by means of the sedimentation, the ova or embryos float out of the straw into the holding medium.

2) CryoTip (Kuwayama et al., 2005). The samples are loaded into a plastic capillary with ultrathin walls, both ends are sealed, and the capillary is submerged into the liquid nitrogen. The CryoTip also includes a protective tube preventing mechanical damage during handling and storage. For warming the capillary is submerged into warm water, then decontaminated with ethanol, cut and the sample is expelled into the rehydrating solution.

3) Cryopette (Portmann et al., 2010). A cryopreservation device that is intended to be used to contain, freeze using vitrification procedures. Consists of a polycarbonate Cryo-Straw with a silicone rubber displacement bulb. This device has been optimized as a closed system for cryopreservation procedures.

Each of these two groups has its specific advantages. In the surface methods, if the size of the drop (~ 0.1 ml) can be controlled, high cooling rate can be achieved because these systems are open, and high warming rates are achieved by direct exposure to the warming solution. The tubing systems have the advantage of achieving high cooling rates in closed systems, thus making them safer and easier to handle. Decreasing the vitrified volume and increasing the cooling rate allow a moderate decrease in cryoprotectant concentration so as to minimize its toxic and osmotic hazardous effects (Yavin et al., 2009).

EMBRYO CRIOPRESERVATION

The first mammalian species whose embryos were successfully cryopreserved was the mouse in the early 1970s when Whittingham et al. obtained live mice after the transfer of frozen-thawed morulae (Whittingham et al., 1972). The earliest successes with mouse embryo cryopreservation were achieved with slow cooling, at a constant rate, to deep subzero temperatures (−80°C). Observations of embryos during this cooling procedure demonstrated the extreme dehydration encountered during the process when ice formed from pure water as extracellular ice formed around the cells, leaving the embryos in the small, residual hypertonic unfrozen fraction (Leibo et al., 1974). Conditions that permitted intracellular ice to form (by cooling too fast and leaving water molecules capable of nucleating ice crystal growth) were universally found to be damaging (Mazur, 1970). It was also argued that slow warming was necessary to avoid cell volume stress. In the intervening time, further investigations demonstrated that this low-temperature (and successful embryo cryopreservation) could be achieved if the embryos were slowly cooled only to a relatively high subzero temperature and then transferred directly to –196°C. In this case, warming needed to be rapid to achieve high viability. These findings, developed by Willadsen and Cambridge to freeze sheep embryos (Willadsen, 1977), have also laid the foundations of

cryopreservation techniques widely used in commerce. The first experiment with the technique of vitrification date, however, until 1985, when Rall and Fahy (Rall and Fahy, 1985) applied this technique for the first time in mouse embryos, while the first pregnancy achieved by transfer of vitrified bovine embryos has been reported by Massip et al. in 1986.

Since then, many experiments and significant improvements were achieved in the field of embryo cryopreservation by which, in recent years, have been successfully cryopreserved embryos of most domestic species, especially cattle (Lazar et al., 2000; Zhang et al., 1993), sheep (Ptak et al., 1999; Dattena et al., 2000), goats (Baril et al., 1989; El Gayar and Holtz, 2001; Traldi, 2000), horse (Hochi et al., 1994) and pig (Hayashi et al., 1989; Berthelot et al., 2000). Across all these different species, the protocols applied to embryo cryopreservation tend to fall into one of two broad categories, slow controlled-rate cooling and ultrarapid cooling or vitrification. Throughout these experiences, it became obvious that embryo cryopreservation would too become centrally important in reproductive technologies by allowing optimal utilization of resources in the development of breeding programs in animals (Massip et al., 2004) by permitting genetic banking (for example of rare animals), and by enhancing the clinical approach to infertility treatment.

OOCYTES CRYOPRESERVATION

The history of oocyte cryopreservation in mammals begins in 1958, when the possibility of survival of unfertilized mouse oocytes after freezing and thawing was first demonstrated (Sherman and Lin, 1958). Approximately 20 years later, the fundamental cryobiology of the mouse oocyte began to be systematically studied (Leibo et al., 1975; Parkening et al., 1976), and in 1977 the first successful in vitro fertilization (IVF) and live offspring from cryopreserved mouse oocytes frozen and stored under liquid nitrogen was reported (Whittingham, 1977). Subsequently, relatively successful cryopreservation of oocytes has been achieved for both the mouse (by far the most studied species) and several other species including the hamster, rabbit, pig, cat and cow (Fuku et al., 1992; Parks and Ruffing, 1992; Al-Hasani et al., 1989; Schroeder et al., 1990; Vincent et al., 1989; Sakamoto et al., 2005; Stachecki et al., 2002; Luvoni and Pellizzari, 2000). Overall, however, the procedures developed for oocyte cryopreservation are far less effective than those used for embryos.

The development of a reliable method for the cryopreservation of mammalian oocytes would be an important advance in the field of reproductive biology for the preservation of genetic resources. However, despite significant recent progress, the efficiency of oocyte cryopreservation is still very low. Many of the problems associated with

oocyte cryopreservation relate to the injury due to chilling and to the toxicity of cryoprotectants that cause considerable morphological and functional damage (Zeron et al., 1999; Paynter, 2005). Different strategies have been developed to control these factors and increase oocyte viability and developmental competence, albeit with limited success (Vajta and Kuwayama, 2006; Ledda et al., 2007). At present, oocyte cryopreservation has been performed at two meiotic stages: metaphase II (MII) and germinal vesicle (GV). The cell cycle stage during meiosis appears to influence the survival of mammalian oocytes and affects the results of cryopreservation due to varying sensitivity to cooling procedures. MII oocytes remain the preferred stage for cryostorage owing to better membrane stability during chilling, however, exposure to sub-physiological temperatures induces several form of damage to other structures determining disorganization of the spindles which, in turn, provokes chromosomal aberrations, increasing polyploidy, and fertilization impairment (Chen et al., 2003; Tharasanit et al., 2006). Since one of the main problems in the cryopreservation of mature oocytes arises from the sensitivity of the microtubular spindle to low temperature and cryoprotectants, one way of circumventing these problems would be to preserve oocytes at the immature germinal vescicle stage at which no microtubular spindle is present. Conversely, GV-stage oocytes would not be directly affected by the problem posed by the meiotic

spindle at the MII stage, as the genetic material remains confined within the nucleus. However, the number of experiments using immature oocytes is low, as data obtained to date, indicate that immature oocytes are more susceptible to cryoinjury and that the survival and developmental ability are greatly impaired in comparison to fresh oocytes (Diez et al., 2005; Hochi et al., 1996). Structural modifications of the cytoskeleton, mitochondria, cortical granules and nucleoli have been described (Diez et al., 2005; Hochi et al., 1996). One factor that could affect the success of immature oocyte cryopreservation is the damage occurring to the cumulus cells surrounding the oocytes. The loss of cumulus cells and ultrastructural changes of the cumulus–oocyte complexes following cryopreservation have been reported (Hochi et al., 1996; Ruppert-Lingham et al., 2006). Different lines of evidence clearly indicate that the gap functional coupling between oocytes and cumulus cells fulfills an important role in the maturation process (Gilchrist et al., 2004; Li et al., 2004). It has been demonstrated that GV-stage oocytes which have been stripped of cumulus cells show a lack of coordination between nuclear and cytoplasmic maturation (Goud et al., 1999; Combelles et al., 2002) and undergo a lower development compared to cumulus-enclosed oocytes (Wongsrikeao et al., 2005). The factors contributing to these deficiencies have not yet been fully elucidated. It has been reported that the absence of cumulus cells could

provoke a possible shortcoming in protein synthesis and could reflect the levels of molecules involved in the regulation of meiotic and mitotic cell cycles (Combelles et al., 2005). According to these indications, the success of immature oocyte cryopreservation could depend on the ability to preserve the structural and functional integrity cumulus–oocytes complexes as a whole. However, the need to maintain cumulus cells during cryoconservation of immature oocytes is still a matter for debate because, in that case, cryopreservation protocol would need to be a compromise between the best protocol for oocyte and that for the cumulus cells. Also, another disadvantage of immature oocytes cryopreservation include the fact that an additional maturation procedure is required. Independently from the stage, oocytes are proving to be very difficult cells to cryopreserve for a number of reasons. Development of cryopreservation protocols that allow survival oocyte without damages of internal structures is difficult but the storage of unfertilized oocytes has numerous uses. Applications in animal management include the preservation of precious strains, the preservation of genetically modified strains, thereby reducing the cost of continuous breeding and avoiding problems of genetic drift, and the preservation of endangered species. In animal management, oocyte cryopreservation gives greater flexibility in breeding programs than embryo cryopreservation. The ability to routinely cryopreserve oocytes will have a significant impact on

assisted reproductive technology (ART). Changes in government guidelines in certain countries now restrict the number of oocytes that can be inseminated. This means there is now an increased need for gamete cryopreservation. Furthermore, not only will oocyte cryopreservation circumvent the ethical and legal problems associated with embryo freezing, but it will also benefit numerous patient groups including women at risk of ovarian function loss through premature menopause, surgical treatments or radio/chemotherapy.

OOCYTES COLD DAMAGES

Cryopreservation protocols for gametes, embryos and ovarian tissues have evolved substantially from the first live births from cryopreserved ovulated mammalian oocytes. Researchers have studied many variables that can affect the success of the process including temperature (during cryoprotectant addition, freezing and thawing), type of cryoprotectant, additives, vessels for holding the gametes/embryos, type of machinery and the osmotic properties of solutions.

Oocytes are proving to be very difficult cells to cryopreserve for a number of reasons. The oocyte is a large single cell with low permeability to water. This means that it has a tendency to retain water when cooled and, if this

forms intracellular ice, damage to the cell results. Permeability of oocytes varies between species, strains, and maturational status of the oocyte. The oocyte is also a short-lived cell that must undergo fertilization in order for it to continue to survive and develop. For fertilization to occur naturally, the oocyte must retain integrity of a number of its unique structural features. These include the zona pellucida, the cortical granules, the microtubular spindle and the nucleus.

Oocytes are very different from sperm or embryos with respect to cryopreservation. The volume of the mammalian oocyte is in the range of three to four orders of magnitude larger than that of the spermatozoa, thus substantially decreasing the surface-to-volume ratio and making them very sensitive to chilling and highly susceptible to intracellular ice formation (Toner et al., 1990; Ruffing et al., 1993; Arav et al., 1996; Zeron et al., 1999). During a cryopreservation protocol, cells are cooled and held at sub-ambient temperatures during different procedural steps such as addition and equilibration of cryoprotective agents and seeding. Despite the high survival rates after vitrification, many studies report low rates of developmental competence of vitrified oocytes. It is well documented that the cryopreservation process induces several undesirable effects such as spindle disorganization leading to disrupted chromosomes (Magistrini and Szollosi, 1980; Aman and Parks, 1994; Saunders and Parks, 1999) and

microfilaments (Saunders and Parks, 1999). Partial or complete disassembly of microtubules in the spindle was observed when oocytes of different species were cooled to room or lower temperature (Pickering and Johnson, 1987; Pickering et al., 1990; Parks and Ruffing, 1992; Aman and Parks, 1994; Almeida and Bolton, 1995). Besides spindle damage, several factors might cause the reduced cleavage rates of vitrified oocytes, including their decreased ability to be penetrated by spermatozoa. The reason for the decreased rate of sperm penetration may be a structural change in the zona (zona hardening); changes in the membrane characteristics, believed to be caused by the precocious exocytosis of cortical granules that is triggered by cryopreservation, probably also play a part (Carroll et al., 1990; Fuku et al., 1995; Lim et al., 1991; Matson et al., 1997). A number of studies have speculated that commonly used cryoprotectants and the type of cryopreservation process play a role in the deregulation of cell cycle machinery such as calcium oscillation and calcium signalling pathways (Takahashi et al., 2004; Larman et al., 2006; Larman et al., 2007). Larman et al. (2006) demonstrated that two cryoprotectants commonly used in vitrification protocols (DMSO and EG) cause transient increases in intracellular calcium in mouse MII oocytes causing precocious exocytosis of cortical granules. Oocytes are also more susceptible to damaging effects of reactive oxygen species (ROS). The

production of ROS is the results of normal oxygen metabolism. However, during times of environmental stress ROS levels can increase dramatically. Some reports in porcine oocytes (Gupta et al., 2010; Somfai et al., 2007) have indicated that oocytes that survive cryopreservation may also accumulate ROS (Somfai et al., 2007), which are known to exert harmful effects such as mitochondrial damage, adenosine triphosphate (ATP) depletion, altered calcium oscillation during fertilization, apoptosis, and developmental block (Favetta et al., 2007; Yoneda et al., 2004). Consequently, developmental ability of cryopreserved oocytes may be compromised. Take together all these evidences on damages causes by vitrification suggest that structural and biochemical injuries cooperate to impair the ability of oocytes to achieve the developmental program. Since cryopreservation plays a central role in assisted reproductive technology, further studies are needed to identify the souse and the kind of freeze damages and, possibly, to found a solution for improve the oocyte's vitrification procedure.

THE AIM OF THE WORK

This thesis will be articulated in two parts. In the first part the experiments carried out to investigate several cryopreservation-induced damages in bovine in vitro matured oocytes are reported. The identification of oocyte damages would lead to develop strategies to prevent these problems and hence to improve the cryopreservation efficiency.

In particular, the objectives of the thesis were to evaluate the effects of CTV vitrification on:

1) the chromatin and spindle organization (Experiment 1);

2) the incidence and the causes of parthenogenetic activation (Experiment 2); furthermore, the efficacy of caffeine treatment to prevent the phenomenon of spontaneous activation was investigated (Experiment 3) and

3) the radical oxygen species (ROS) activity (Experiment 4)

Very recently, an intriguing area of research is coming into the limelight, that is the so called stress-induced stress resistance of various cell types. Pivotal studies have been carried out in porcine oocytes pretreated with high hydrostatic pressure (HHP; Du et al., 2008). These pilot studies proved that the sublethal HHP treatment of porcine oocytes results in

improved in vitro developmental competence and cryotolerance, and supports embryonic and fetal development as well as pregnancy establishment and maintenance up to the birth of healthy piglets. Therefore, in the second part of the thesis, that was carried out in parallel, the main objectives were to evaluate whether the exposure of bovine in vitro matured oocytes to a sublethal stress, such as an osmotic stress (Experiment 5) and a thermal stress (Experiment 6) would increase their resistance to a greater stress, such as that of vitrification.

EXPERIMENTAL PART

CHAPTER 3: Studies on cryopreservation-induced damages in bovine in vitro matured oocytes

EXPERIMENT 1: *Structural changes of in vitro matured bovine oocytes following cryopreservation.*

Bovine oocytes are particularly difficult to cryopreserve successfully and only low blastocyst production rates have been achieved after the thawing, in vitro fertilization and in vitro culture (Martino et al., 1996; Vajta et al., 1998; Papis et al., 2000). This problem may be in part due to the large size of bovine oocytes, which consequently have a low surface to volume ratio, making it more difficult for water and cryoprotectants to move across the cell plasma membranes (Leibo, 1981). Undesirable effects of cryopreservation are the altered distribution of cortical granules (CG, Fuku et al., 1995; Hyttel et al., 2000), increased polyploidy at fertilization (Carroll et al., 1989; Bouquet et al., 1992; Eroglu et al., 1998), several ultra-structural (Hyttel et al., 2000) and structural alterations, including damages of the meiotic spindle apparatus in the oocyte of several species (Rho et al., 2002; Rojas et al., 2004; Succu et al., 2008). It is indeed documented that the cryopreservation process may induce spindle and microfilaments disorganization (Saunders and Parks, 1999), leading to

disrupted chromosomes (Magistrini and Szollosi, 1980; Aman and Parks, 1994; Saunders and Parks, 1999).

The meiotic spindle consists of microtubules that are constructed by polymerization of tubulin dimmers of α and β-tubulin (Zhou et al., 2002). Microtubules start from microtubular organizing centers at both poles and anchor chromosomes at the kinetochores, forming a barrel shape (Maro et al., 1986). The chromosomes align at the equatorial plane of the meiotic spindles. The tubulin dimmer would polymerize and depolymerize at various stages of a cell cycle. The meiotic spindles are crucial for the events following fertilization as completion of meiosis, second polar body formation, migration of the pronuclei, and formation of the first mitotic spindle (Schatten et al., 1985). Disorganization of the meiotic spindles could result in chromosomal dispersion, failure of normal fertilization, and termination of development (Eroglu et al., 1998).

It has been reported that meiotic spindle is very sensitive to low temperature. Partial or complete disassembly of microtubules in the spindle was observed when oocytes of different species were cooled to room or lower temperature (Pickering and Johnson, 1987; Pickering et al., 1990; Parks and Ruffing, 1992; Aman and Parks, 1994; Almeida and Bolton, 1995). In cattle it was demonstrated that cooling at either 4 or 25°C

determines spindle anomalies. Furthermore, spindle damages have been reported after slow freezing of bovine oocytes (Aman and Parks, 1994).

One of the most successful ultrarapid vitrification techniques is the Cryotop vitrification (CTV) that has resulted in improved survival and developmental rates with human and bovine Metaphase II (MII) oocytes (Kuwayama et al., 2005). This method has also been successfully used for cryopreservation of ovine (Succu et al., 2008), pig (Fujihira et al., 2005), and buffalo oocytes (Gasparrini et al., 2007).

The aim of this work was to evaluate chromosome and spindle organization of bovine in vitro matured oocytes after vitrification/warming by Cryotop and after their exposure to cryoprotectants (CPs).

MATERIAL AND METHODS

Reagents and media

Unless otherwise stated, reagents were purchased from Sigma-Aldrich® (Milano, Italy).

In vitro maturation (IVM) medium consisted of a commercial medium TCM 199 supplemented with 50 µg/ml of gentamycin, 0.8 mM of L-glutamine, 0.5 µg/ml of FSH, 5 µg/ml LH and with 15% of bovine serum (BS). For the procedure of oocytes washing a Medium TCM199 with 25 mM Hepes, 2 mM sodium bicarbonate , 2 mM pyruvic acid, 1 mM L-

glutammine, 10 μl/ml amphotericin B, and 540 μg/ml heparin (TCM-Aspiration) was used. Vitrification/warming media were made in a base solution consisting of TCM199, buffered with 15mM HEPES and 5mM sodium bicarbonate (H199) supplemented with 20% Fetal Calf Serum (FCS). In particular, the first vitrification solution (VS1) was made of 10% EG and 10% DMSO and the second vitrification solution (VS2) made of 20% EG and 20% DMSO in TCM 199 with 20% FCS + 0.5 M sucrose.

Recovery of the oocytes and in Vitro Maturation

Bovine ovaries were collected from a local abattoir, transported to the lab within 3–4 h after slaughter at approximately 35°C in physiological saline supplemented with 150 mg/L kanamycin. Cumulus–oocyte complexes (COCs) were recovered by aspiration of 2–8mm follicles using an 18-G needle under vacuum (40–50 mmHg). Only COCs with a compact, non-atretic cumulus and a homogeneous cytoplasm were selected for the study, washed twice in TCM-Aspiration and once in the IVM medium. COCs were then put in groups of 25-30 per well with 380 μl of TCM-IVM covered with 400 μl of mineral oil. IVM was carried out at 39°C for 22 h in a controlled gas atmosphere of 5% CO_2 in humidified air.

Vitrification and warming

After IVM, COCs were washed and mechanically stripped of their cumulus cells by vortex in Hepes buffered TCM supplemented with 5% of BS. The denuded oocytes were vitrified by the Cryotop method, previously described (Kuwayama and Kato, 2000). Briefly, oocytes were equilibrated in 200 µl drop of VS1 for 3 minutes and then transferred into 50 µl of VS2. Groups of 4-6 oocytes were loaded with a glass capillary onto the top of the film strip of each Cryotop in <0.1 µl volume. After loading, almost all the solution was removed and the Cryotop was quickly immersed in liquid nitrogen (within 25 seconds), protected with a cap and stored under liquid nitrogen for at least a week. For warming, a procedure of 4 steps with decreasing concentration of sucrose was used. The Cryotop strip was immersed directly into 1 ml of 1.25 M sucrose solution for 1 minute, and the oocytes recovered were subsequently exposed to decreasing concentrations of sucrose (0.62 M, 0.42 M and 0.31 M) for 30 seconds each. After that, the oocytes were washed in 3 ml of H199 + 10 % of FCS for 5 minutes and reallocated in the IVM well for 2 h.

Toxicity test

In order to assess the toxicity of the CPs employed, the oocytes were first exposed to the vitrification solutions and then directly processed through

the warming solutions. Therefore, oocytes were put into the VS1 for 3 minutes, moved to the VS2 for 25seconds and then into the decreasing sucrose warming solutions. After that, the oocytes were washed in 3 ml of H199 + 10 % of FCS for 5 minutes and reallocated in the IVM well for 2 h.

Oocytes fixation and immunostaining

Oocytes were fixed and immunostained for microtubules using a modification of the method described by Messinger and Albertini (1991). Briefly, oocytes were fixed and extracted for 30 minutes at 37°C in a microtubule-stabilizing buffer (0.1 M Pipes [pH 6.9], 5 mM $MgCl_2$·6H2O, and 2.5 mM EGTA) containing 2% formaldehyde, 0.5% Triton X-100, 50% deuterium oxide, and 1 mM of dithiothreitol. Oocytes were then washed three times in a blocking solution of Dulbecco's Phosphate Buffered Saline (PBS) containing 10% normal goat serum (NGS), 0.1% Triton X-100, and 0.02% sodium azide before being stored at 4°C until processing for immunocytochemistry. Oocytes were incubated with fluorescein isothiocyanate-conjugated anti-α-tubulin antibody (final dilution, 1:500; Sigma) in a blocking solution of PBS containing 5% NGS in the dark at 37°C for 1 h. After three washes in the 10% NGS blocking solution, oocytes were incubated for 15 minutes in solution with 10 µg/ml of HOECHST 33342. Finally oocytes were put on a slide into 8 µl drop of

glycerol and PBS in 1:1 ratio. Oocytes observation was made by a fluorescence microscope Nikon Eclipse 90i at 20x magnification.

EXPERIMENTAL DESIGN

A total number of 301 oocytes, over 3 replicates, were selected for IVM.

In order to evaluate the damages to the spindle and chromatin the oocytes were washed and divided into 3 groups: control (fresh oocytes; n=91), toxicity (oocytes exposed to CPs; n=101) and vitrification (n=109). The oocytes of the control group were fixed and stained for microtubules and for nuclei and examined by fluorescence microscopy at 22 h post-maturation. For the vitrification and toxicity groups the oocytes were rinsed after being processed as previously described and allocated into the IVM well for 2 h to allow them to recover. Then they were fixed, stained and evaluated. Oocytes were classified following Saunders and Parks (1999) modified by Tharasanit et al. (2006). With regard to the spindle, it was possible to distinguish, as shown in Figure 4, oocytes with three different typology of meiotic spindle:

- normal (symmetrical barrel-shaped);
- abnormal (disorganized, clumped or dispersed elements, multiple spindle-like structure, i.e. asymmetric and small);
- absent (no visible spindle).

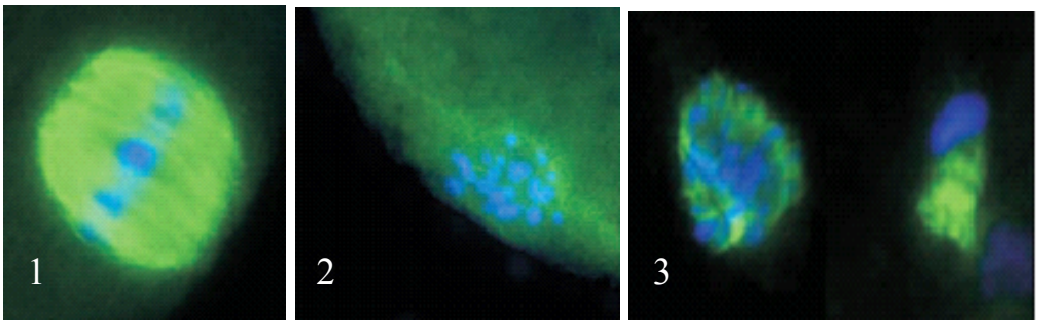


Figure 4. Meiotic spindle observed in cryopreserved oocytes

1) Normal meiotic spindle (green) with compact chromosomes (blu) at the equator of the structure
2) Absent spindle
3) Abnormal spindle

With regard to the chromatin organization, oocytes were divided into two categories:

- normal (two sets of chromatin aligned at the center of the spindle)
- abnormal (clumping or dispersed chromatin from the center of the spindle)

STATISTICAL ANALYSIS

The differences in the percentage of different categories among groups were analyzed by Chi Square Test. The same analysis was used to evaluate the differences among groups in the percentages of activated oocytes.

RESULTS

The results of vitrification and exposure to CPs (toxicity) of bovine IVM oocytes are shown in Table 1.

It is important to specify that no selection of oocytes were made before starting the experiment and for this reason in each experimental group we observed a certain percentage of oocytes at the Germinal Vescicle (GV) stage; surprisingly, this percentage was higher ($P<0.05$) in the vitrification group (26%) than in the other two groups (8% in toxicity group and 13% in control group). In order to eliminate this casual difference, we detracted the GV-stage oocytes from the total when calculating the percentages of MII and TII oocytes.

A very interesting result appears from the analysis of maturation in the three different experimental groups: in particular, the percentage of MII oocytes in the control group was higher than in the toxicity and vitrification groups ($P<0.01$). In addition, the decline of the percentage of MII stage oocytes in the toxicity and vitrification groups corresponded to an increased percentage of oocytes at the TII stage compared to the control group, indicating occurrence of parthenogenetic activation.

The percentages of the different oocyte categories, with regard to the spindle and chromatin organization, were calculated on the total MII-oocytes. As shown in Table 1, the percentage of oocytes with normal

spindle and chromosome organization was greatly reduced ($P<0.01$) in both the vitrification and toxicity groups compared to the control group. However, the percentages of oocytes with a normal spindle configuration and a normal chromosome organization were lower ($P<0.05$) after vitrification than after exposure to CPs. It is interesting to note that, despite similar percentages of abnormal spindle, a higher percentage ($P<0.01$) of oocytes with an absent spindle was found in the vitrified compared to the toxicity group. In addition, it is interesting to report that also the percentage of oocytes with a normal metaphase plate but with an absent or abnormal spindle was lower ($P<0.05$) in the control than in both toxicity and vitrification groups (5, 3 and 18 %, respectively).

Table 1. Effect of vitrification and exposure to CPs (toxicity) on bovine IVM oocytes, spindle and chromosome organization.

		Maturation rate on total number of oocytes			Spindle configuration and chromosome organization in MII oocytes				
		Non matured oocytes	TII n (%)	MII n (%)	Spindle configuration n (%)			Chromosome organization n (%)	
Groups	n				normal	abnormal	absent	normal	abnormal
control	91	12 (13)^{a}	0 (0)^{A}	79 (87)^{A}	75 (95)^{A}	0 (0)^{A}	4 (5)^{a}	79 (100)^{A}	0 (0)^{A}
toxicity	101	8 (8)^{aA}	26 (26)^{Bb}	67 (66)^{B}	49 (73)^{Ba}	16 (24)^{B}	2 (3)^{A}	58 (87)^{Ba}	9 (13)^{Ba}
vitrification	109	29 (26)^{bB}	12 (15)^{Ba}	68 (61)^{B}	38 (56)^{Bb}	18 (26)^{B}	12 (18)^{bB}	47 (69)^{Bb}	21 (31)^{Bb}

^{a,b}Values with different superscripts within columns are significantly different, $P<0.05$

^{A,B}Values with different superscripts within columns are significantly different, $P<0.01$.

DISCUSSION

It is known that vitrification causes several ultrastructural (Hyttel et al., 2000) and structural alterations, including damages of the meiotic spindle apparatus in the oocyte of several species (Rho et al., 2002; Rojas et al., 2004; Succu et al., 2008). These alterations may vary depending on the

species involved and on the cryopreservation procedures (see a review of Chen et al., 2003).

Therefore, we conducted this study aimed to identify spindle and nuclear damages in matured bovine oocytes after Cryotop vitrification. Furthermore, in order to establish whether the damages are due to the chilling injury or to the exposure to CPs a toxicity test was included in the experimental design. The first important result to discuss is the demonstration that the Cryotop vitrification method we used determines damages to the meiotic spindle and to the chromosome organization. Interestingly, also the simple exposure of oocytes to CPs causes these damages but to a lesser extent than the whole vitrification protocol. Indeed, the percentages of oocytes with normal spindle and chromatin organization were significantly reduced after either vitrification or exposure to CPs compared to the control (fresh untreated oocytes).

It has been demonstrated that cryopreservation affects spindle integrity in mature oocytes of different species, such as human (Zenzes et al., 2001), porcine (Liu et al., 2003), bovine (Aman and Parks, 1994) and equine (Tharasanit et al., 2006). It has also been reported that the exposure of mouse oocytes to low temperatures leads to depolymerization of microtubules, disrupting the network of the meiotic spindles (Pickering and Johnson, 1987) and only a small rate of the cooled oocytes is able to

completely recover their spindles after few hour culture at 37°C. Defects in microtubule polymerizations have been attributed to a toxic effect of cryoprotectants (Fahy, 1986) and related both to their concentration and type (Joly et al., 1992; Van der Elst et al., 1992). CPs-induced changes in microtubule organization in mouse (Cooper et al., 1996), human (Sathananthan et al., 1988), bovine (Saunders and Parks, 1999) and equine (Tharasanit et al., 2006) oocytes have been described.

In our study, the percentage of oocytes with an abnormal spindle, i.e. a reduced or asymmetric, were similar in the two treated groups and obviously higher than in the control. Therefore, the difference in the percentage of oocytes with normal spindle configuration between the vitrification and the toxicity groups was mainly related to the significantly higher incidence in the first group of oocytes showing the complete absence of the spindle. This indicates that vitrification induces in a certain percentage of oocytes, in addition to the damages linked to the exposure to CPs, severe microtubular damages leading to the complete disassembly of the spindle structure. These results are in agreement with an earlier study that reported the absence of the meiotic spindle in bovine oocytes following cooling at 4°C or at room temperature (Adam and Parks, 1994). However, it is worth pointing out that, despite the evidence of spindle and chromatin alterations, in our study with cryotop vitrification the extent of the damage

was definitely lower than in another work carried out in cattle with slow freezing (Saunders and Park, 1999). Indeed, with slow freezing only 7-14% of the oocytes showed a normal spindle configuration (vs 56% recorded in this study) and less than 30% of the oocytes had a normal chromatin organization (vs 69% here recorded). It has been demonstrated in the sheep that, besides the influence on viability rates, vitrification procedure causes alterations in tubulin polymerization which in turn affects spindle configuration and chromatin organization depending on the cryodevice. Therefore, the differences we observed compared to the previous work may be due to the different method of cryopreservation employed.

The damages to the meiotic spindle and DNA fragmentation may lead to aneuploidy incompatible with subsequent embryo development and account for the poor embryo development currently recorded. In our preliminary work, in fact we reported low cleavage (approximately 40%) and blastocyst rates (5%) after IVF and IVC of Cryotop vitrified-warmed oocytes (unpublished data). The evidence of these structural alterations provides the basis for developing new strategies to improve the efficiency of cryopreservation in bovine species. It was recently demonstrated, in fact, that taxol treatment of bovine oocytes before cryopreservation by OPS significantly increases the percentage of oocytes showing normal spindle and chromatin (Morato et al., 2008). However, in the control group (no

taxol treatment) the extent of the damages to the spindle and to the chromatin was much higher than in our study. This would suggest that Cryotop determines less damages than OPS and that a pretreatment with taxol and/or other cytoskeleton stabilizers, such as, cytochalasin B, is worth investigating also by using this device, in order to further reduce the incidence of these anomalies.

The most interesting finding of the present study was the occurrence of spontaneous parthenogenetic activation, indicated by the presence of oocytes in TII at 2 h post-warming. In particular, higher rates of activation were recorded in the toxicity group. It has been previously reported that the cryoprotectant DMSO causes zona hardening determined by cortical granule exocytosis that reduced rate of fertilization in mouse oocytes (Vincent et al., 1991). Since DMSO has been shown to cause a transient intracellular calcium rise in various cell lines (Morley and Whitfield, 1993) and fusion of cortical granules to the oocyte plasma membrane is calcium-dependent (Kline and Kline, 1992; Tahara et al., 1996), it is tempting to suggest that DMSO triggers cortical granule release by increasing intracellular calcium. DMSO appears not to be the only cryoprotectant that increases calcium since high concentrations of EG (10–40%) have been recently shown to increase intracellular calcium in mouse oocytes (Takahashi et al., 2004). Larman et al. (2006) showed that the two

cryoprotectants commonly used in vitrification protocols, DMSO and EG, cause large transient increases in intracellular calcium in mouse oocytes that are sufficient to cause zona hardening, which significantly reduces fertilization. The authors also observed that, making the vitrification with calcium-free medium, zona hardening was significantly reduced and hence IVF and embryo development were improved.

The higher rates of spontaneous activation reported in our study on bovine oocytes after vitrification are in agreement with previous reports in the pig (Somfai et al., 2007) and in the sheep (Succu et al., 2007). However, our data on parthenogenetic activation following exposure to CPs are different from the results obtained in the previously cited works. In fact, the higher incidence of activation in the present study was observed in the toxicity group. On the contrary, in the pig high rates of parthenogenetic activation were reported following vitrification but not following exposure to CPs (Somfai et al., 2006). In the sheep parthenogenetic activation also occurred in oocytes exposed to CPs but to a lower extent than in vitrified oocytes (Succu et al., 2008). These authors suggested that the higher parthenogenetic activation recorded with vitrification was due to the cumulative effect of the cryoinjury and the exposure to CPs. It is possible to hypothesize that in bovine oocytes, as demonstrated in the sheep, the exposure to CPs, with the transient intracellular calcium rise, causes an

unexpected fall of the M-phase promoting Factor (MPF) level, after which the meiotic resumption occurs.

An unexpected datum of our study was the evidence of a significantly higher percentage of spontaneously activated oocytes in the toxicity group compared to the vitrification. We speculate that the lower activation observed in the vitrification group may be referred to the slowing down of the metabolic activity subsequent to thermal shock, and hence that activation after vitrification may occur later than 2 h post-warming. To support this hypothesis it is worth pointing out that in the study carried out in the sheep, the oocytes after vitrification or exposure to CPs were cultured for 24 h and then evaluated. Also in the work performed in the pig the assessment of nuclear status was carried out later than in our trial, i.e. at 9 h post-warming.

Therefore, in order to confirm this hypothesis, the same assessment needs to be carried out in bovine oocytes fixed at later times post-warming.

EXPERIMENT 2: *MPF levels and nuclear stage evaluation in bovine mature oocytes after vitrification or CPs exposure*

The most interesting result of Experiment 1 has been the occurrence of spontaneous parthenogenetic activation, indicated by unexpectedly high percentage of oocytes in TII stage at 2 h post-warming. This suggests that vitrification or exposure of MII oocytes to cryoprotectants triggers their activation. It is important to remember that in Experiment 1, in the vitrification and the toxicity groups, oocytes were rinsed and allocated into IVM for 2 h after the procedures. This resting period coincides with the moment where we normally would perform the intervention of IVF because it is believed that during this time the normal morphology of the spindle is restored. It is possible to think that the lower activation rate observed in the vitrification group may be referred to the slowing down of the metabolic activity subsequent to thermal shock, and hence that activation after vitrification may occur later than 2 h post-warming. To confirm this hypothesis, the same assessment needs to be carried out in oocytes fixed at a later time post-warming. Furthermore, in order to hypothesize a corrective strategy it is critical to comprehend the mechanism by which oocytes are activated following vitrification or exposure to the CPs.

Meiotic arrest in mammalian oocytes at metaphase of the second meiotic division is the result of prolonged elevation of kinase activity (Alberts et al., 1994). Both maturation-promoting factor (MPF) and mitogen-activated protein kinase (MAPK) are important in maintaining this arrest. The meiotic progression is regulated by the activity of the MPF, the universal cell cycle regulator of both mitosis and meiosis: in meiosis it induces immature oocytes to undergo meiotic maturation; in mitosis it has a role in the G2/M phase transition. MPF is a heterodimeric protein composed of cyclin B and Cdc2 kinase (Cdc2), and is involved in triggering chromatin condensation, nuclear envelope breakdown (Heald and McKeon, 1990; Peter et al., 1990; Ward and Kirschner, 1990) and meiotic spindle formation (Murray et al., 1989). Active MPF catalyzes the breakdown of the nucleus by directly forcing the lamin molecules to disassemble by phosphorylating them on key serine residues (Alberts et al., 1994). MPF also directly phosphorylates histone H1 and microtubule-associated proteins (Alberts et al., 1994). During activation of MPF, newly synthesized cyclin B associates with Cdc2, and the Cdc2-cyclin B complex is activated by dephosphorylation of Cdc2 by the Cdc25 protein (Minshull et al., 1989). The Cdc25 protein is a phosphatase, which is also activated by phosphorylation (Izumi et al., 1992). Continued protein synthesis, presumably for cyclin B, is needed to maintain meiotic arrest in bovine

oocytes (Verlhac et al., 1994; Levesque and Sirard, 1996; Dekel, 1998). This allows the oocytes to rapidly exit from metaphase arrest simply by terminating protein synthesis. Upon oocyte activation, MPF activity declines dramatically as a result of a decrease in the amount of cyclin B. The maintenance of low MPF activity is further ensured by the phosphorylation of Cdc2. The other kinase known to participate in oocyte maturation is the family of MAPK (reviewed in Thomas, 1992; Ruderman,1993). MAPKs are serine/threonine kinases that are fully activated when dually phosphorylated on both tyrosine and threonine residues (Posada and Cooper,1992). During oocyte maturation, MAPK is likely to exert most of its effects on cytosolic targets including cytoskeletal proteins (Gotoh et al., 1991; Verlhac et al., 1993; Minshull et al., 1994) and phosphorylation of downstream targets such as $p90^{rsk}$ (Jones et al., 1988; Hsiao et al., 1994; Kalab et al., 1996).

We hypothesized that the spontaneous oocyte activation occurring after vitrification and exposure to CPs (Experiment 1) may be due to an interference on the mechanism controlling the meiotic arrest, i.e. a decline of MPF and MAP-kinase.

Therefore, the aim of Experiment 2 was to evaluate the incidence of parthenogenetic activation following vitrification and exposure to the CPs both at 2 h and 4 h post-warming. This would, in part consents to

understand whether giving the oocytes more time in IVM after vitrification, allows to re-establish the metabolic machine and consequently to resume the meiotic maturation to the same rate recorded after simple exposure to CPs.

Furthermore, in order to comprehend the mechanism inducing the spontaneous activation and, hence to hypothesize a corrective strategy, an additional objective was to measure the activity of the MPF and the MAPK in fresh, exposed to CPs and vitrified bovine oocytes.

MATERIAL AND METHODS

Reagents and media

Unless otherwise stated, reagents were purchased from Sigma-Aldrich® (Milano, Italy).

Methods

The oocyte recovery, IVM, vitrification, warming and toxicity test were previously described in Experiment 1. For measuring MPF and MAP kinase activities oocytes were placed in a ice-cold collection buffer made of 1.87 mg/ml of ethylenediaminetetraacetic acid (EDTA), 0.42 mg/ml of sodium fluoride and 1.84 mg/ml of sodium orthovanadate in PBS.

Evaluation of oocyte nuclear status

After each treatment, oocytes were washed in Hepes buffered TCM + 5% of BS and then allocated into 400 µl of the same medium supplemented with 10 µg/ml of HOECHST 33342 at least for 15 minutes. Finally oocytes were put on a slide and the nuclear observation was made by a fluorescence microscope Nikon Eclipse 90i at 20x magnification. After the nuclear evaluation, oocytes were washed 4 times into 1 ml of the collection buffer and then put individually into 2 µl of the same buffer and stored at -80°C until analysis for MPF and MAP kinase assay.

Biochemical determination of MPF and MAP kinase activity

Fresh, exposed and vitrified samples at different time after treatment were processed for MPF and MAPK assay. MPF and MAP kinase activities were measured simultaneously using histone 1 (H1) and myelin basic protein (MPB) as their respective substrate. Samples were recovered from –80°C storage and brought to a final volume of 9µL with a solution containing 45mM β-glycerophosphate, 12mM p-nitrophenylphosphate, 20mM MOPS-KOH, 12mM $MgCl_2$, 12mM EGTA, 0.1mM EDTA, 1mM DTT, 2.3mM $NaVO_4$, 2mM NaF, 0.8mM PMSF, 15µg/mL leupeptin, 30µg/mL aprotinin, 0.1% (w/v) PVA, 1mg/mL histone H1 (type III-S from calf thymus),

myelin basic protein (1mg/mL, MBP), 2.2M protein kinase inhibitor peptide, and 2.5MBq/mL γ-[^{32}P]-ATP. The reaction was started after the addition of γ-[^{32}P]-ATP and performed for 30 min at 37°C. The assay was stopped in 2x concentrated sodium dodecyl sulphate (SDS) sample buffer and boiled for 5 minutes. Proteins were separated on 1D-SDS-PAGE electrophoresis as described by Laemmly (1970) and the phosphorylation of substrates were analysed by autoradiography of the gels. The mean pixel intensity of a preselected set area was measured using Kodak Image Analysis Software 1D 3.6 (Image Analysis Software East Mar Kodak Company, New York). The mean intensity of the bands of fresh controls were assumed to correspond to 100 arbitrary units and the mean band intensities of the mean intensities of the other experimental groups were related to this value (Relative intensity, RI).

EXPERIMENTAL DESIGN

A total number of 151 oocytes was selected for IVM.

After 22 h, all the oocytes were mechanically stripped of their cumulus cells by vortex in Hepes buffered TCM supplemented with 5% BS. The oocytes were washed and divided into 3 groups: control (fresh mature oocytes; n=35), toxicity (oocytes exposed to CPs; n=62) and vitrification

(n=54). The oocytes of each experimental group were stained to evaluate the nuclear status and placed in the collection buffer for the MPF and MAP kinase measurements at 2 h (T2) and 4 h (T4) post-warming.

In particular, in the control group, after the end of IVM (22 h), oocytes were simply left into the IVM well for further 2 h (T2; n=16) and 4 h (T4; n=19).

In order to assess the toxicity of the CPs, oocytes were simply exposed to the vitrification and warming solutions and then divided into the two time-groups. After this procedure, the oocytes were rinsed and allocated into the IVM well for 2 h (T2; n=29) and 4 h (T4; n=33).

For the vitrification group, after warming the oocytes were rinsed and allocated into the IVM well for 2 h (T2; n=25) and 4 h (T4; n=29) respectively.

STATISTICAL ANALYSIS

Data regarding the effect of vitrification and exposure to CPs (toxicity) of bovine IVM oocytes and chromosome organization at different time post-warming were analyzed by Chi Square test. Analysis of variance (ANOVA) was used to assess the significance of differences in MPF and MAPK activity among groups. $P<0.05$ was considered significant.

RESULTS

Results of vitrification and exposure to CPs (toxicity) of bovine IVM oocytes are shown in Table 2.

The nuclear evaluation showed 4 categories of oocytes: immature, mature (oocytes at MII stage) and activated (oocytes at TII and Anaphase II stage). In order to simplify the table, the AII and TII oocytes were unified under the column named activated. As for Experiment 1, no selection of oocytes were made before the experiment and for this reason in treated groups we observed the presence of not matured oocytes even if it was not significant.

The first result that is observed is the high percentage of survival in all the groups. An interesting result appear from the analysis of maturation rate: the percentage of MII oocytes remained elevated in the control group both at T2 and T4 (100 % respectively) compared to the toxicity (T2=76%; T4=64%) and vitrification groups (T2=84%; T4=72%). While no activation was recorded in the control at 2h, this was found in both treated groups. However, no significant differences in the percentage of MII and activated oocytes were observed among groups at 2 h. On the contrary, at T4 the percentage of TII oocytes was lower in the control compared to the toxicity ($P<0.01$) and vitrification groups ($P<0.05$). Furthermore, at the

decline of MII in the toxicity and vitrification groups corresponded an increase of oocytes at TII stage.

Table 2. Effect of vitrification and exposure to cryoprotectants (toxicity) on activation rate of bovine IVM oocytes

				Maturation rate on total number of oocytes					
				Non matured oocytes		MII n (%)		Activated (%)	
Groups	n TOT	n T2	n T4	T2	T4	T2	T4	T2	T4
Control	35	16	19	0 (0)	0 (0)	16 (100)	19 (100)^{Ab}	0 (0)	0 (0)^{Ab}
Toxicity	62	29	33	1 (3)	0 (0)	22 (76)	21 (64)^{B}	6 (21)	12 (36)^{B}
Vitrification	54	25	29	1 (4)	0 (0)	21 (84)	21 (72)^{c}	3 (13)	8 (28)^{c}

^{a,b}Values with different superscripts within columns are significantly different, $P<0.05$

^{A,B}Values with different superscripts within columns are significantly different, $P<0.01$.

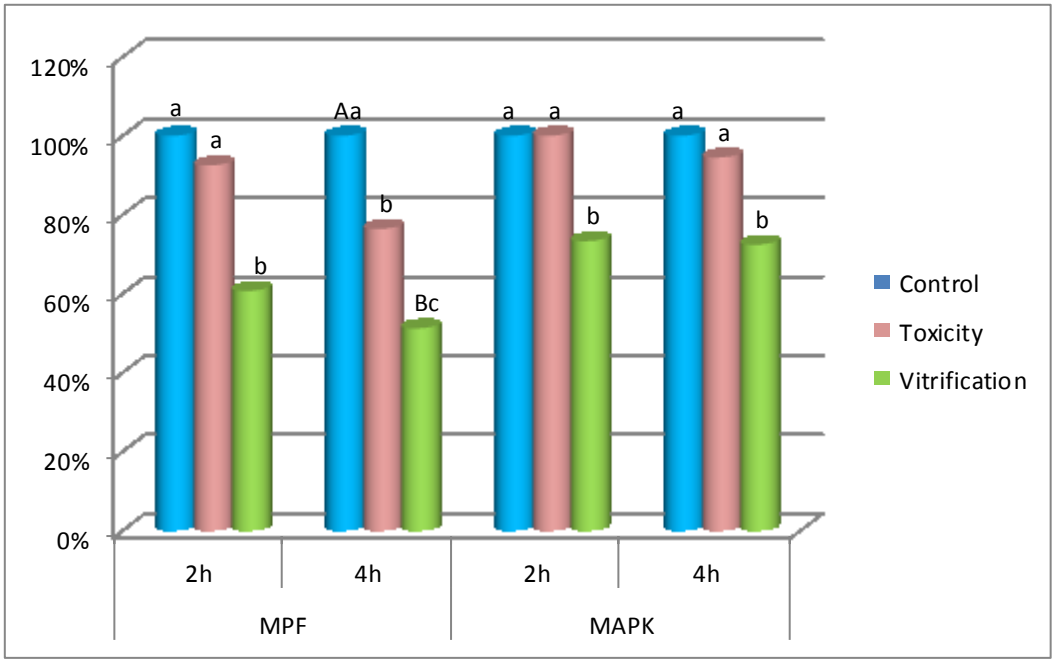
The MPF and MAPK activity in control, toxicity and vitrified oocytes were analyzed for the two time groups and the results are shown in Figure 5. At T2, a significant decrease ($P<0.05$) of MPF activity was observed in the oocytes of vitrification group compared to the control group. Relative intensities (RI) were in fact 60.70 and 100 respectively in vitrification and control group. The MPF activity in the vitrification group was also lower

($P<0.05$) than in the toxicity group (the RI were 60.70 and 92.50, respectively). No differences were observed in MPF levels between the toxicity group and the control. At 2h post-warming, the MAPK activity followed a similar trend of MPF: in fact a significant decrease ($P<0.05$) of MAPK activity was recorded in the oocytes of vitrification group compared to those of the control and toxicity group (RI=100, 100 and 73.30, respectively in control, toxicity and vitrification groups).

At T4, a significant decrease of MPF activity was observed in both treated groups compared to the control. In fact, the MPF levels in the control oocytes were higher than in the toxicity (RI=100 and 76.40, respectively; $P<0.05$), and vitrification groups (RI=100 and 51.20, respectively; $P<0.01$). Furthermore, a significant difference ($P<0.05$) in MPF activity was also found between the toxicity and vitrification groups.

In addition, the MAPK activity followed the same trend observed at T2: in particular, a significant decrease of MAPK activity ($P<0.05$) was recorded in the oocytes of vitrification group compared to the control and toxicity groups (RI=100, 94.50 and 72.40 in control, toxicity and vitrification groups, respectively).

Figure 5: Graphic representation of MPF and MAPK activity measured as relative intensity of histone H1 and myelin basic protein (MPB) electrophoretic bands after kinase assays of Control, Toxicity and vitrified MII oocytes 2 and 4 h after treatment.

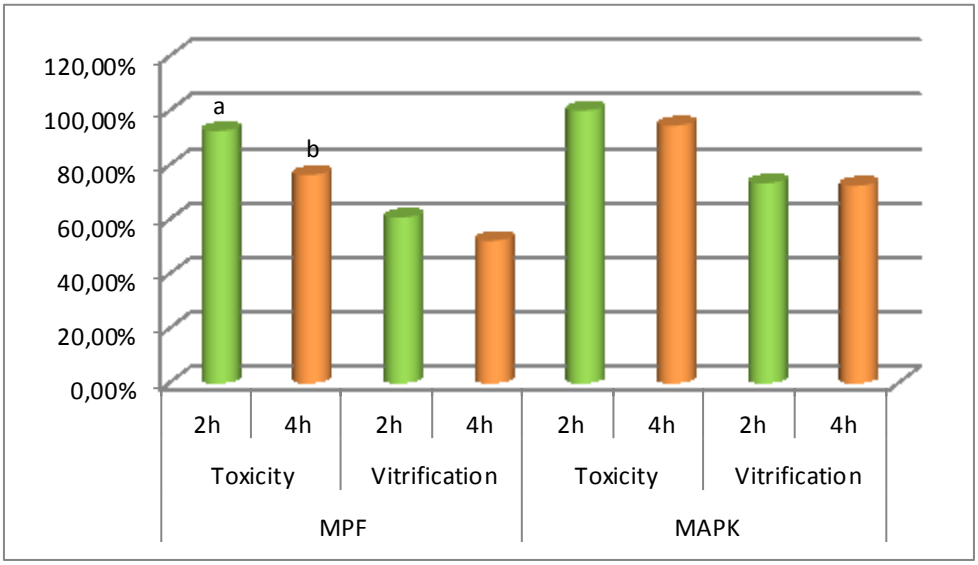


[A,B] Bars with different letters are significantly different; P<0.01

[a,b] Bars with different letters are significantly different; P<0.05

In Figure 6 the MPF and MAPK activities in the toxicity and vitrification groups in relation to the time points are shown. With regard to the MPF values, a significant decrease (P<0.05) was observed at T4 compared to T2 in the toxicity group, whereas no differences were recorded in the vitrification group. No differences were found in MAPK activities between T4 and T2 in both the toxicity and vitrification groups.

Figure 6: Graphic representation of MPF and MAPK activity measured as relative intensity of histone H1 and myelin basic protein (MPB) electrophoretic bands after kinase assays of Toxicity and vitrified MII oocytes 2 and 4 h after treatment.



[a,b] Bars with different letters are significantly different; $P<0.05$

DISCUSSION

After the observation, in Experiment 1, of the occurrence of spontaneous parthenogenetic activation indicated by the presence of oocytes at TII stage in vitrification an toxicity groups, the aim of this work was to evaluate the incidence of activation and the MPF and MAPK activities at 2 and 4 h post-warming, to understand the causes of the phenomenon in order to identify a corrective strategy.

In this Experiment after 2 h of rest period in IVM, we observed the occurrence of spontaneous parthenogenetic activation indicated by the presence of oocytes in TII in both treated groups (21% in toxicity group and 13% in vitrification group), whereas no activation was found in the control. The incidence of activation in the toxicity and vitrification groups enhanced from T2 to T4 (36% and 28% in toxicity and vitrification groups, no statistical difference). However, the phenomenon was more evident at T4. In fact, at T4 the percentage of TII oocytes was lower in the control compared to the toxicity ($P<0.01$) and vitrification groups ($P<0.05$). In this case, no differences were observed between the toxicity and vitrification groups at T4. The result of activation are in accord to Succu et al. (2007) which observed in ovine oocytes an high percentage of parthenogenetic activation in exposed group (18.03%) and in vitrified group (54.54%) after 24 h of culture. These values support our hypothesis referred to the slowing down of the metabolic activity subsequent to thermal shock in oocytes of vitrification group, confirming that activation after vitrification may occur later than 2 h post-warming.

Parthenogenetic activation is caused by a stimulus that can be electrical or chemical that simulates the activation due by sperm penetration. During fertilization, sperm signals induce Ca^{++} mobilization from endoplasmic reticulum that triggers an activation pathways driving to completion of

meiosis by inducing metaphase II-telophase II transition, chromatin decondensation, pronucleus formation and first cleavage (Swann and Ozil, 1994).This transition depends on the kinase activity of MPF, a member of cytostatic factor (Edwards and Brody, 1995), that is the key regulator of meiotic and mitotic cell cycle. Its kinase activity is high at MII stage and decreases after few hours starting from oocytes activation (Bogliolo et al., 2000).

In our study, MPF levels were measured in control and treated oocytes at different time post-warming (T2 and T4). Our results demonstrated that the activity of MPF decreased in oocytes of vitrified and exposed groups compared to the control, which reflected the differences in parthenogenetic activation rates. In particular, we showed that vitrification determined in bovine oocytes a fall in MPF activity, which was evident since 2 h post-warming and was not restored at 4 h post-warming. A similar pattern was found in vitrified oocytes for the MAPK activities. On the contrary, in the toxicity group the MPF levels were similar to the control at 2 h post-warming to significantly drop at 4 h. Furthermore, no differences in MAPK activities were observed in the exposed oocytes either at 2 and 4 h post-warming.

Therefore, our results on MPF and MAPK activities demonstrated that the cumulative effect of CPs toxicity and cryoinjury has a major effect on the

MII-arrest machinery, and this accounts for the high parthenogenetic activation rate that we observed. However, an unexpected result regards the MPF and MAPK activities in the toxicity group. In fact, at 2 h post-warming, although a high percentage of oocytes was in TII stage, the MPF and MAPK levels were similar to the control. However, the exposure to CPs affected the MPF activity only at a later time, when the activation rate increased significantly.

Our results are in accord with data showed in the sheep by Succu et al. (2007). These authors observed that in oocytes vitrified with the Cryotop method the MPF activity fell at warming and was not restored after 2 h culture. On the contrary, in the toxicity group the MPF level dropped at warming but its activity was promptly restored at 2 h post-warming. As we did not measure the MPF activity at warming we cannot rule out that, similar to what demonstrated in sheep, the exposure to CPs caused a transitory drop that would trigger meiotic resumption.

Structural and biochemical damages caused by vitrification cooperate to impair the ability of oocyte to achieve the developmental program. Cryopreserved cell are able to reverse cryoinjuries in different periods of time. In our study, as well as in that by Succu et al. (2007), we showed that MPF activity was not restored after 2 or 4 h. Larman et al. (2006) recently reported that DMSO and EG can induce a large transient increase in

intracellular calcium concentration in mouse MII oocytes, comparable to the initial increase that is seen at fertilization and that the calcium rise was due to both intra cytoplasmic reserve mobilization and extracellular access. Since the transient increase of this ion could be responsible of the cryoprotectant dependent decrease of MPF activity, which induce parthenogenetic activation (Bogliolo et al., 2000), it is possible to hypothesize that a possible strategy to reduce oocytes activation could be the use of calcium-free medium for vitrification. In fact, in the same study, Larman et al. demonstrated that the removal of extracellular calcium from the medium failed to affect the response induced by DMSO but significantly reduced the EG–induced calcium increase. Those investigators suggested that the source of the DMSO-induced calcium increase was from the intracellular calcium pool, whereas EG caused an influx of calcium across the plasma membrane from the external medium. Even Fujiwara et al. (2010) suggested that calcium-free EG-supplemented media were effective for inhibition of cortical granule exocytosis of the vitrified rat oocytes. Taken together, these results clearly demonstrate that vitrifying oocytes in calcium-free media reduced zona hardening, increased subsequent fertilization, and did not adversely affect embryonic development to the blastocyst stage.

An alternative method for reducing the percentage of parthenogenetic activation after oocytes vitrification could be the treatment of the oocytes with substances able to stabilize the action of MPF and MAPK. Theoretically, the stabilization of these molecules could avoid these adverse effects. It has been reported that molecules such as caffeine restore MPF activity in porcine and ovine oocytes by reducing p34 (Cdc2) phosphorylation (Kikuchi et al., 2002; Lee and Campbell, 2006).

On the basis of the results on MPF and MAPK activities obtained in this Experiment, we hypothesize that a pre-treatment of bovine oocytes with a compound as caffeine, known to stabilize MPF, may reduce the incidence of activation and hence increase the vitrification efficiency.

EXPERIMENT 3: *Treatment with caffeine before and after oocyte vitrification*

The most interesting result of Experiment 2 is that MPF and MAPK levels, decreased in oocytes of vitrified and toxicity groups compared to the control, which was reflected by the higher rates of parthenogenetic activation. This confirmed the hypothesis that the parthenogenetic activation recorded in the oocytes following exposure to CPs and vitrification is linked to an action on MPF levels. Since the resumption and completion of meiotic maturation of mammalian oocytes is indeed regulated by MPF, it is critically important to prevent this phenomenon.

As described earlier, elevated levels of MPF and MAP kinase activities maintain the oocytes arrested at metaphase of the second meiotic division. MPF activity is controlled by Cdc2 (Cell Division Cycle 2), also known as CDK1 (Cyclin Dependent Kinase 1), a member of the CDK family of serine/threonine kinases. The Full activation of Cdc2 requires phosphorylation at T161 and association with Cyclin B. In contrast, phosphorylation of Cdc2 at Y15 and T14 during the G2-phase of the cell cycle inhibits activity, and dephosphorylation of Y15 and T14 by CDC25 phosphatase during late G2 stage of meiosis restores activity.

Caffeine, a phosphodiesterase inhibitor, has been reported to artificially increase the activity of MPF by inducing the dephosphorylation of Cdc2 at

T14 and Y15 in pig oocytes (Kikuchi et al., 1999; Kikuchi et al., 2000), cultured mammalian cells (Steinmann et al., 1991) and Xenopus oocytes (Smythe and Newport, 1992). Caffeine can inhibit cAMP phosphodiesterase (Peter et al., 1990) and thus effectively inhibit meiotic resumption of oocytes (Peter et al., 1992; Posada and Cooper, 1992). The employment of substances with stabilizing action on cytoskeleton or MPF may be an helpful strategy to limit the deleterious effects of oocytes cryopreservation. In a number of mammalian oocytes, caffeine is known to increase and maintain the activities of MPF and MAPK in denuded oocytes possibly by inhibition of Myt1/Wee1 kinases (Lee and Campbell, 2006; Kikuchi et al., 2002).

Therefore, the aim of this study was to evaluate whether a treatment with different concentrations of caffeine (0, 10, 20 and 40 mM) of bovine MII oocytes before and after vitrification affects:

1) the MPF and MAPK activity

2) the oocyte spontaneous parthenogenetic activation

3) the embryo development after IVF

MATERIALS AND METHODS

Reagents and media

Unless otherwise stated, reagents were purchased from Sigma-Aldrich® (Milano, Italy).

In vitro fertilization (IVF) medium consisted of a Tyrode's modified medium with 30 mg/mL heparin, 30 mM penicillamine, 15 mM hypotaurine, 0.15 mM epinephrine and 1% BS (IVF-TALP). Medium for oocyte washing and manipulation consisted of Medium 199 with 25 mM Hepes, 2 mM Sodium bicarbonate, 2 mM sodium piruvate, 1 mM L-glutammine (TCM-Hepes) with 5% of BS. For in vitro culture (IVC), Synthetic Oviduct Fluid (SOF; Tervit et al., 1972), modified with the addition of 30 μl/mL essential amino acids, 10 μl/mL non-essential amino acids, 0,34 mM sodium citate, 2,77 mM myo-inositol and 5% BS was used.

Methods

The oocyte recovery, IVM, vitrification and warming procedures were previously described in details in the Material and Methods of the Experiment 1. The biochemical determination of MPF and MAP kinase activity was previously described in Experiment 2.

In Vitro Fertilization (IVF)

After removal from the IVM medium with or without caffeine, the oocytes were washed in IVF-TALP medium, and put (25 for well) in 300 µl of IVF-TALP, covered by mineral oil. Frozen sperm was thawed at 37°C for 40 seconds and the semen was prepared by Percoll density gradient (45 and 80%) through a centrifuge for 25 minutes at 300 x g. After centrifugation, the pellet was reconstituted in 2 ml of modified Tyrode's balanced salt solution (Sperm talp) and centrifuged twice at 160 and 108 x g. The pellet obtained after centrifugation was re-suspended to a final concentration of $1x10^6$ ml^{-1} in the fertilization medium (IVF-TALP) and oocytes were fertilized. IVF was carried out at 39 °C and 5% CO_2 in air.

In Vitro Culture (IVC)

After 20–22 h of gametes co-incubation, presumptive zygotes were washed to remove spermatozoa in buffered medium TCM-Hepes with 5% BS. After this wash, presumptive zygotes were transfered, 30-50 for well, in 400 µl of SOF. IVC was carried out at 39°C under humidified air with 5% CO_2, 7% O_2, and 88% N_2 in air for 7 days, when the percentages of embryos and cleavage were recorded.

Assessment of parthenogenetic activation

After 2 h in IVM supplemented with the 4 different concentration of caffeine (0, 10, 20 and 40 mM) oocytes of each group were placed into well containing 400 µl of SOF. IVC was carried out at 39°C under humidified air with 5% CO_2, 7% O_2, and 88% N_2 in air for 48 h, when the percentages of cleavage was recorded. Then oocytes were fixed and stained (see Experiment 2) and the nuclear observation was made by a fluorescence microscope Nikon Eclipse 90i.

Oocytes fixation and staining

After each treatment, oocytes were washed in Hepes buffered TCM + 5% BS and then allocated into 400 µl of PBS added with 60% of methanol. After 30 minutes, a solution with 10 µg/ml of HOECHST 33342 was added and oocytes were incubated at least for 15 minutes. Finally oocytes were put on a slide into a little drop of 4',6-diamidino-2-phenylindole (DAPI) and the nuclear observation was made by a fluorescence microscope Nikon Eclipse 90i at 20x magnification.

EXPERIMENTAL DESIGN

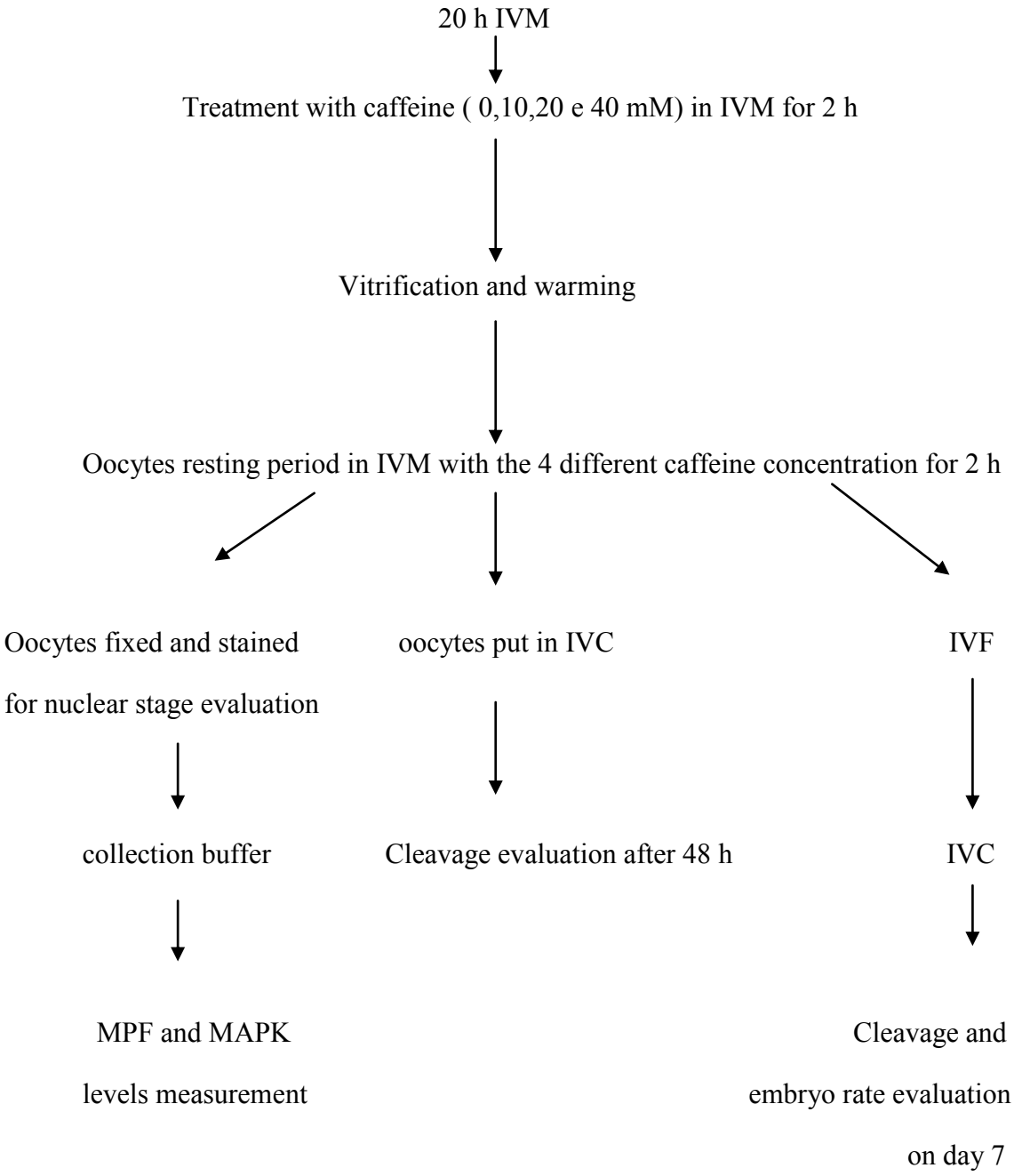
COCs with a compact, non-atretic cumulus and a homogeneous cytoplasm were selected for IVM (n= 1213, over 4 replicates). In order to increase the activity of MPF, three different concentrations of caffeine (0, 10, 20 and 40 mM) were tested.

After 20 h in IVM, oocytes were randomly divided into 4 groups and placed into IVM medium added with four different concentrations of caffeine: 0 mM (n= 314), 10 mM (n= 300), 20 mM (n= 333) and 40 mM caffeine (n= 266). The treatment with caffeine was carried out at 39°C for 2 h in a controlled gas atmosphere of 5% CO_2 in humidified air. After that, oocytes were mechanically stripped of their cumulus cells by vortex in buffered TCM-Hepes supplemented with 5% BS, vitrified and warmed. After a brief wash, oocytes of the 4 different groups were reallocated in IVM + caffeine at the same concentrations for further 2 h in a controlled gas atmosphere of 5% CO_2 in humidified air.

At this point oocytes of each group were further divided: one part was stained for nuclear stage evaluation like previously described (see Experiment 2) and stored in the collection buffer for measuring the MPF and MAP kinase activities (n= 45, 46, 44 and 33 for 0, 10, 20 and 40 mM caffeine, respectively). Another part was destined to the evaluation of

spontaneous activation, i.e. the oocytes were directly cultured in SOF medium, briefly evaluated at 48 h, then fixed and stained (n=123, 115, 114 and 109 for 0, 10, 20 and 40 mM caffeine, respectively). The remaining part of the oocytes was fertilized and cultured in vitro for 7 days, when cleavage and embryo yield were evaluated (n=146, 139, 175 and 124 for 0, 10, 20 and 40 mM caffeine, respectively). The experimental design is also illustrated in a scheme reported in Figure 7.

Figure 7. Scheme of experimental design



STATISTICAL ANALYSIS

Data for nuclear and chromosome organization, parthenogenetic activation and embryo rate after oocyte pretreatment with caffeine were analyzed by Chi Square test. Analysis of variance (ANOVA) was used to assess the significance of differences in MPF and MAPK activity after caffeine pretreatment among groups. The mean intensity of the bands of fresh controls were assumed to correspond to 100 arbitrary units and the mean band intensities of the mean intensities of the other experimental groups were related to this value (Relative intensity, RI). Values of $P<0.05$ were considered statistically significant.

RESULTS

Results of nuclear and chromosome evaluation of bovine oocytes after pretreatment with different caffeine concentrations and vitrification are shown in Table 3. As in Experiment 1and 2, no selection of oocytes were made before the experiment and for this reason in treated groups we observed the presence of not matured oocytes even if it was not significant. A lower ($P<0.05$) percentage of MII stage oocytes was found in the 10 mM caffeine group compared to the control. With regards to the spontaneous activation no statistical difference was highlighted among the 4 groups. The

percentage of oocytes with a normal chromosome organization was high in the control, 10 and 20 mM groups. In particular, in oocytes treated with 40 mM caffeine this percentage was lower ($P<0.05$) than that recorded in the 20 mM caffeine. Obviously, at the decline of normal chromosome organization corresponded an increase ($P<0.05$) of oocytes with an abnormal nuclear organization. An interesting parameter was the higher incidence of condensed nuclei in caffeine-treated groups compared to the control; however, the difference was significant between the control and both 10 and 40 mM caffeine, whereas unexpectedly no difference was found with respect to 20 mM.

Table 3. Effect of vitrification on bovine IVM oocytes, nuclear and chromosome organization after treatment with different concentration of caffeine.

		Maturation rate on total number of oocytes			Chromosome organization in MII oocytes		
Groups	n	Non matured oocytes	MII n (%)	TII n (%)	Condensed nuclei	Normal	Abnormal
0 mM	45	0	38 (84)[a]	5 (11)	2 (4)[aA]	29 (76)	9 (24)
10 mM	46	3 (7)	28 (61)[b]	6 (14)	9 (20)[b]	22 (79)	6 (21)
20 mM	44	1 (2)	34 (77)	4 (9)	5 (11)	28 (82)[a]	6 (18)[a]
40 mM	33	1 (6)	21 (64)	3 (9)	8 (24)[B]	11 (52)[b]	10 (47)[b]

[a,b]Values with different superscripts within columns are significantly different, P<0.05

[A,B]Values with different superscripts within columns are significantly different, P<0.01.

Results of parthenogenetic activation of bovine oocytes after pretreatment with different caffeine concentrations and vitrification are shown in Table 4. Although the percentage of cleavage at 48 h, indicating occurrence of spontaneous activation, showed a tendency to decrease in all treated groups, the difference with the control was significant ($P<0.01$) only when the higher concentration (40 mM) of caffeine was employed.

Table 4. Results of cleavage percentage of bovine oocytes after pretreatment with different caffeine concentration and vitrification

Groups	n	Cleaved	Uncleaved
0 mM	123	37 (30)[A]	86 (70)
10 mM	115	26 (23)[A]	89 (77)
20 mM	114	24 (21)[A]	90 (79)
40 mM	109	8 (7)[B]	101 (93)

[A,B]Values with different superscripts within columns are significantly different, $P<0.01$.

Results of cleavage and blastocyst rates of bovine vitrified-warmed oocytes in different groups are shown in Table 5. No differences were observed in terms of both cleavage and blastocyst rates among groups. However, both parameters showed a tendency to decrease at increasing caffeine concentrations.

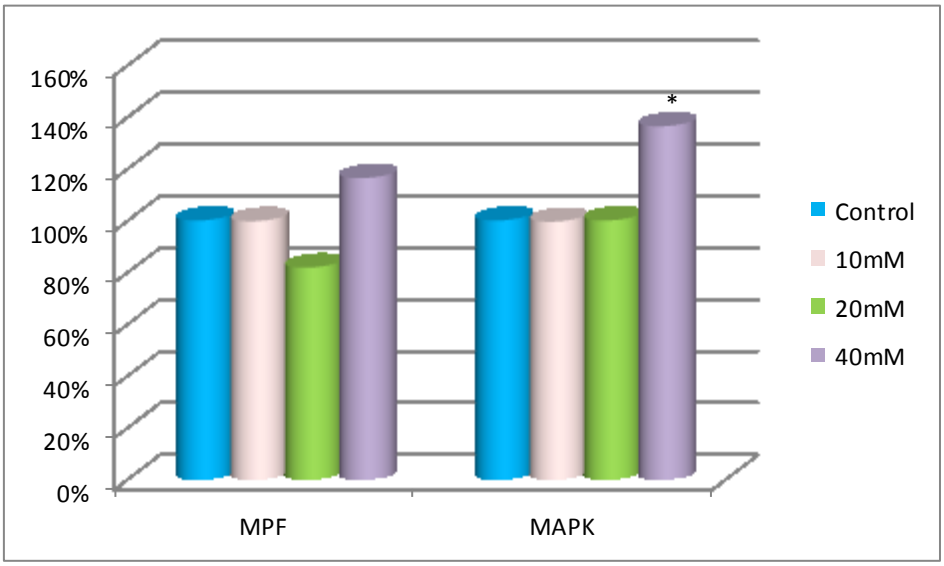
Table 5. Results of cleavage and embryo rates after treatment with caffeine and oocytes vitrification

Groups	n	Cleaved	% Cleavage	TOT TM-BL	% TM-BL/tot	% TM-BL/cl
Control	146	69	51	6	3	6,5
10 mM	139	61	43,6	1	0,9	1,7
20 mM	175	67	41,3	2	0,9	2,4
40 mM	124	47	32,6	1	0,6	1,3

Results of MPF and MAPK activity in vitrified MII oocytes treated with 0 mM (control), 10 mM, 20 mM and 40 mM caffeine are shown in Figure 8. Unexpectedly, no significant differences in MPF activity were observed among groups (RI = 100, 99.70, 81.80 and 116.40, in 0, 10, 20 and 40 mM caffeine groups, respectively). It is worth pointing out that, although not significant, an activity of MPF higher than the control was only found in the 40 mM group. With regard to the MAPK levels, we observed a significant increase ($P<0.05$) in the 40 mM group compared to the other

groups (RI= 100, 99.50, 100 and 136.40 in 0, 10, 20 and 40 mM caffeine, respectively).

Figure 8. Graphic representation of MPF and MAPK activity in vitrified MII oocytes treated with 0 mM (control) 10 mM, 20 mM and 40 mM of caffeine. (*P< 0.05)



DISCUSSION

In the previous experiment the hypothesis that the parthenogenetic activation recorded in the oocytes following exposure to CPs and vitrification is linked to an action on MPF levels was confirmed. The problem of oocytes premature activation can be overcome by caffeine, a phosphodiesterase inhibitor that has been reported to artificially increase the activity of MPF. In fact, it has been reported that molecules such as caffeine could restore MPF activity in porcine and ovine oocytes by

reducing p34 (Cdc2) phosphorylation (Kikuchi et al., 2002; Lee and Campbell, 2006). The use of caffeine in biology is various. First of all, caffeine is frequently used as regulator of aging in oocytes. In fact, recently Maalouf et al. (2009) used a 10 mM concentration of caffeine for the treatment of aged ovine oocytes with rewarding results (caffeine treatment statistically increased development to blastocyst and lowered the frequency of polyspermy in denuded ovine oocytes). In addition, Kikuchi et al. (2000) demonstrated that the treatment of 72-h-cultured aged porcine oocytes with caffeine (5 mM for the last 10 h of culture) decreased the level of pre-MPF and elevated MPF activity and promoted metaphase arrest. The caffeine was tested also to stabilize cytoplast for nuclear transfer (NT) in ovine and porcine oocytes. In particular, treatment of ovine oocytes with 10 mM significantly increased the activities of MPF and MAPK and contributed to an increased reprogramming in NT embryos; treatment with 2.5 mM in porcine oocytes increased not only the rate of chromosome condensation but also the developmental rate to the blastocyst stage of porcine NT embryos.

Furthermore, a previous study on ovine oocytes showed that caffeine can increase MPF and MAPK activity after vitrification (Bogliolo et al., 2007). Taken together, all these evidences represented the basis of our study on caffeine action on kinase activity in bovine oocytes after vitrification.

The results of this study were disappointing as we demonstrated that the caffeine treatments of the oocytes, at the three concentrations tested (10, 20 and 40 mM), fails to improve the vitrification efficiency in cattle. Indeed, caffeine at the lower concentrations, i.e 10 and 20 mM, did not increase the MPF and MAPK activities and hence did not reduce spontaneous activation, as indicated by cleavage rates at 48 h of culture of unfertilized oocytes similar to those obtained in the control (vitrified without caffeine treatment). Subsequently, no improvement was recorded in terms of cleavage and blastocyst rates following IVF and IVC in both groups. Bogliolo et al. also reported similar parthenogenetic activation rates in 20 mM caffeine-treated oocytes and in the control; however, in this study both kinases increased in the caffeine-treated oocytes and the ionomycin-induced activation decreased (Bogliolo et al 2007). When caffeine was used at the highest concentration (40 mM) we recorded a slight increase of the MPF and a significant increase ($P<0.05$) of the MAPK activities, together with a significantly reduced spontaneous activation (7 % vs 30% of the control). These results would suggest that at this concentration caffeine was efficient in stabilizing the kinases and, hence, preventing the problem of spontaneous activation. However, the other results derived from both the nuclear status evaluation and the embryo yields after IVF indicate that the compound also exerted a toxic effect on the oocytes. In fact, in the

40 mM caffeine group, the percentage of oocytes with a regular chromatin configuration in the MII plate was significantly lower ($P<0.05$). In addition, in this group the highest percentage ($P<0.01$) of condensed nuclei was observed. Furthermore, although the statistical analysis did not show significant differences, the lower cleavage rate was recorded in this group. A high incidence of condensed chromatin was also observed by Lee and Campbell (2006) in ovine oocytes treated with caffeine. In their work, the authors observed that treatment with 10 mM caffeine caused a significant increase in the activities of both MPF and MAPK in enucleated oocytes and resulted in a significant increase in the occurrence of nuclear envelope breakdown (NEBD) and premature chromosome condensation (PCC) in the donor nucleus. The high percentage of condensed nuclei that we found particularly at 40 mM in our work may result from the increased kinase activities that we also observed in this group. In fact, it is known that among the effects of MPF and MAPK there is a premature chromosome condensation.

Despite the high cleavage rates after vitrification, low blastocyst rates were reported in all experimental groups. Therefore, we can conclude that, unlike in ovine, the caffeine treatment did not improve the vitrification efficiency. Unfortunately, the only concentration that actually affected the kinases and reduced the spontaneous activation rates was also toxic for the

oocytes, as shown by the high incidence of condensed chromatin, the low percentage of oocytes with a normal MII configuration and low cleavage and blastocyst rates. These results suggest to carry out further studies in order to identify an optimal concentration or to reduce the time of oocyte exposure to 40 mM caffeine.

As our previous experiments highlighted that the vitrification-induced activation of bovine oocytes, consequent to a fall in MPF and MAP kinase levels, is a major factor impairing further development we believe that other strategies should be identified and tested in order to maintain high kinase activities. A possible alternative approach is to evaluate a pre-treatment of the oocytes with other phosphodiesterase-3 inhibitors such as cilostamide and cilostazol, and with the adenylate cyclase activator forskolin.

EXPERIMENT 4: *ROS levels measurement after vitrification and cryoprotectant exposure in bovine MII oocytes*

The low fertilization and development of vitrified-warmed oocytes is suggested to be due to altered meiotic spindle assembly, microtubules, cortical granule distribution, zona pellucida characteristics, and vitrification-induced parthenogenetic activation of oocytes (Wu et al., 2006; Gupta et al., 2007; Somfai et al., 2007; Rojas et al., 2004; Shi et al., 2007). Furthermore, it has been suggested that oocytes and embryos that survive cryopreservation may also accumulate reactive oxygen species (ROS; Somfai et al., 2007; Ahn et al., 2002). ROS such as superoxide (O_2), hydroxyl radical (UOH), hydrogen peroxide (H_2O_2), and hypohalous acids (HOX, where X= Cl^-, Br^-, I^-, Br^-, or SCN^-) are molecules that are highly disruptive to cellular function (Dobrinsky, 2002; Vajta et al., 1997). Therefore, increase in the production of ROS contributes significantly to several diseases including those that may compromise reproduction and fertility (Isachenko et al., 1998; Vajta et al., 1997). Physiological levels of ROS such as H_2O_2 and superoxide are generally produced during the normal metabolism of mammalian embryos (Guerin et al., 2001) and oocytes. Similar to other systems, ROS may be overproduced in the oocyte microenvironment in response to several conditions, such as ongoing acute or chronic infections or inflammation, certain medications, radiation, and

pollutants. Alternatively, a decrease in free radical scavengers may contribute to accumulation of ROS (Szczepanska et al., 2003; Van Langendonckt et al., 2002; Guerin et al., 2001). Likewise, compromise in the oocyte's cellular mechanisms to combat or remove the ROS may also result in accumulation of ROS. In oocytes, as in other cells, ROS are important mediators of intracellular signaling responsible for numerous cellular functions under physiological conditions (Nasr-Esfahani et al., 1991). However, under pathological conditions, the ROS may contribute to oxidative stress, resulting in mutations, inactivation or loss of mitochondrial DNA, and synthesis and accumulation of abnormal or oxidized proteins. To our current knowledge, the few studies that measured ROS in oocytes following vitrification were carried out in the pig (Gupta et al., 2010). Somfai et al. in 2007 demonstrated that the glutathione (GSH) content of vitrified porcine oocytes was significantly reduced whereas the H_2O_2 level increased compared to control oocytes. A more recent work reported an increased level of ROS in vitrified porcine oocytes, that was partially ameliorated using β-mercaptoethanol (βME; Gupta et al., 2010). Indeed, to protect cells from oxidative stress, exogenous antioxidants such as βME, a low molecular weight thiol compound, have been frequently used to increase antioxidant capacity of embryos and oocytes via increasing intracellular levels of ROS scavengers such as GSH. ROS are known to

exert harmful effects such as mitochondrial damage, adenosine triphosphate (ATP) depletion, altered calcium oscillation during fertilization, apoptosis, and developmental block (Favetta et al., 2007; Takahashi et al., 2003; Yoneda et al., 2004). Consequently, developmental ability of cryopreserved oocytes and embryos may be compromised. Despite the evidence of a critical role played by ROS in many early reproductive events, the effect of vitrification on ROS levels has not yet been investigated in bovine oocytes. Therefore, the present study was designed to investigate the effect of vitrification and CPs exposure on ROS activity of bovine in vitro matured oocytes.

MATERIALS AND METHODS

For oocyte recovery, vitrification, warming and toxicity test see Experiment 1.

EXPERIMENTAL DESIGN

A total number of 360 oocytes with a compact, non-atretic cumulus and a homogeneous cytoplasm were selected for IVM, over 6 replicates. After 22 h, all the oocytes were mechanically stripped of their cumulus cells by

vortex in Hepes buffered TCM supplemented with 5% BS, washed in the same medium and divided into 3 groups: control (fresh non treated oocytes; n=120), toxicity (n=120) and vitrification (n=120). After each treatment and a brief wash, oocytes were placed into 500 µl of buffer TRIS-HCL 40 mM pH 7.0 and stored at -80°C until further processing.

ROS levels mesaurement

As previously stated, 120 oocytes per group were examined for ROS production. For this purpose, for each group, 20 denuded oocytes for time were incubated in 500 µl of buffer Tris-HCl 40 mM pH 7.0 for 20 minutes at 37°C into a shaker in the presence of 5 µmol/l 2',7'-dichlorfluorescein-diacetate (DCFH-DA). After the incubation, the extraction was obtained by a syringe and the samples were centrifuged at 3000 rpm for 10 min at 4°C. Fluorescence was monitored in the supernatant using a spectrofluorometer at 495 nm excitation and 525-nm emission. Corrections for autofluorescence were made by blanks measured in each experiment.

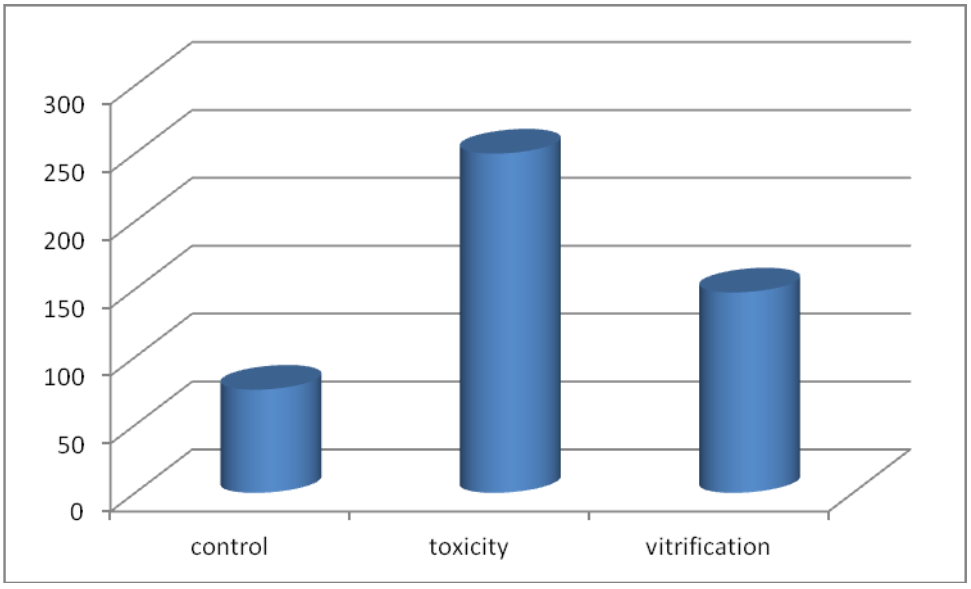
STATISTICAL ANALYSIS

Data were expressed as Arbitrary ROS Units/oocyte/min. The differences among groups were analyzed by ANOVA.

RESULTS

The results of this study showed that in bovine oocytes ROS levels tended to increase in the toxicity and vitrification groups compared to the control (76.0±6.4, 249.9±87.3 and 147.6±42.6 Units/oocyte/min in the control, toxicity and vitrification groups, respectively). However, there were no statistical differences among groups and this was mainly due to the high variability recorded in both treated groups. On the contrary, in the control group of fresh oocytes the variation among replicates was low.

Figure 9. ROS levels (Units/oocyte/min) in fresh, vitrified and exposed to CPs bovine in vitro matured oocytes



DISCUSSION

Little is known about the cooling-related changes in oocytes and embryos that survive cryopreservation. However, one factor that may influence the ability of an oocyte to survive cryopreservation is its intracellular redox status. Free oxygen radicals, which greatly decrease the viability of cells, are formed during cryopreservation of tissues (Whiteley et al., 1992). Mouse embryos cryopreserved at the two-cell stage have elevated levels of intracellular H_2O_2, which is known to induce developmental blockage (Ahn et al., 2002). The production of ROS, such as superoxide anion (O_2^-), hydroxyl radical (OH^-), H_2O_2 and organic peroxides, is a normal process that occurs in the cell when electrons are diverted during their transportation along the mitochondrial respiratory chain and other electron transfer systems. Although the oxidative modification of cell components as a result of the action of ROS is one of the most potentially harmful processes for proper cell function, causing DNA fragmentation, protein oxidation and lipid peroxidation (Yang et al., 1998), biological systems have developed several anti-oxidant mechanisms, both enzymatic (superoxide dismutase, glutathione peroxidase, catalase) and non-enzymatic (α-tocopherol, ascorbic acid, β-carotene and glutathione; Johnson and Nasr-Esfahani, 1994). An enzymatic anti-oxidant system has been also detected in bovine COCs (Cetica et al., 2001). Therefore,

damage to biological systems depends on the balance between ROS production and their removal by the anti-oxidant machinery of the cell (Ho et al., 1996). Oxidative modifications of cell components due to the action of ROS is one of the most potentially damaging processes for proper cell function. However, on the other hand it is well known that ROS participate in many physiological processes.

The objective of our study was to evaluate whether vitrification of bovine mature oocytes determines an increase of ROS accumulation, similar to that described in porcine oocytes (Gupta et al., 2010; Somfai et al., 2007). This was indeed aimed to comprehend if the poor fertilization efficiency recorded following vitrification is due to increased ROS levels. In the present study, ROS production was determined in three different groups of oocytes: control (mature bovine oocytes after 22 h of IVM), vitrification (oocytes vitrified/warmed) and toxicity group (oocytes simply exposed to the vitrification and warming solutions).

The results of the ROS level determination showed an increase of ROS levels following both vitrification and exposure to CPs compared to the control, although the difference was not statistically different. This was mainly due to the high variability among replicates recorded only in both treated groups, suggesting an effect of the treatments on the intracellular redox state. In fact, in the fresh oocytes the variation among replicates was

on the contrary very limited. We may speculate that the increased ROS levels is mainly due to the exposure to CPs, as similar levels of ROS were found in both the treated groups, indicating that the cryoinjury did not exert a cumulative effect on ROS activity.

Previously, it had been demonstrated that the oxygen consumption of COCs increases through IVM (Sutton et al., 2003), with mitochondrial oxidative phosphorylation being the main source of ATP synthesis in the non-fertilized oocyte (Dumollard et al., 2004). The maintenance of ROS production levels during maturation could be due partly to the action of the antioxidant system described in bovine COCs (Cetica et al., 2001; Dalvit et al., 2005). This steady state concentration of ROS could be necessary for the maturation process, as suggested by Blondin et al. (1997), who documented that a certain production of ROS during bovine oocyte in vitro maturation is required to increase blastocyst percentage. Several studies indicate that ROS affects the embryo developmental competence, DNA fragmentation, and fertilization rate of the oocyte (Guerin et al., 2001; Bedaiwy et al., 2004).

The results of this Experiment indicate that one of the damages of bovine oocytes following CTV vitrification is the increased ROS levels, that may be responsible of the poor developmental potentials. To our knowledge this is the first report on ROS levels in bovine oocytes after cryopreservation.

Our results are, however, in agreement with those reported in previous studies in swine (Gupta et al., 2010; Somfai et al., 2007).

Interestingly, Gupta et al. (2010) reported that the vitrification induced increase of ROS activity in porcine oocytes can be partially annihilated by exogenous supplementation of βME to obtain their enhanced post fertilization development in vitro.

In conclusion both the exposure of CPs and vitrification affected the ROS balance in bovine oocytes, increasing the ROS levels. These results suggest, therefore, in future perspective to elaborate a strategy to prevent this phenomenon, likely by the addition of antioxidants to the IVM medium, such as βME, cysteamine, cystine and cysteine etc, providing the oocyte a greater antioxidant capacity prior to vitrification.

CHAPTER 4: Stress induced for stress tolerance: evaluation of the effects of osmotic and heat shock on oocyte cryotolerance

'What does not destroy me makes me stronger'

Friedrich Nietzsche

The dominant approach of applied embryology in mammals in the fifty years' history has been a rather defensive one. The laboratory phase of the work is considered to be potentially dangerous for gametes and embryos (Thompson et al., 2007; Gardner, 2009) and efforts were mostly focused to eliminate these dangers. Even in the cryopreservation field, various strategies have been proposed to increase viability and developmental competence of gametes and embryos trying to satisfy the original purposes of reducing their sensitivity to low temperature. So, different approaches were found to improve cell cryotolerance but, currently, many problems still resist for oocytes cryopreservation: low temperatures substantially disrupts cytoskeletal elements (Aman and Parks, 1994), cortical granules (Pickering et al., 1990), and plasma membranes (Vincent and Johnson, 1992), frequently leading to poor development and cell death. In the first part of this thesis we demonstrated that also using the innovative method of CTV vitrification damages are determined to the bovine oocyte, such as chromatin and spindle anomalies (Experiment 1), spontaneous oocyte

activation (Experiment 2), the latter resulting from a decreased MPF and MAP kinase activity (Experiment 3), poor development into blastocysts and increasing of ROS levels (Experiment 4). A new approach to improve the cryotolerance and developmental competence of gametes and embryos is to expose them to sub lethal stress before manipulation in order to increase the subsequent stress tolerance, morphological intactness and cryopreservation ability. The phenomenon that a sub lethal stress induces a response with a temporary increase in a general, rather non-specific resistance to various further stresses has been observed in almost all levels of life, from bacteria to multicellular organisms including humans. The first reaction for stress in humans or animals is the 'fight-or-flight' or the 'acute stress response' described by Cannon (1915). This response was later recognized as the first stage of a general adaptation syndrome (GAS) that regulates stress responses among vertebrates and other organisms (Selye, 1936). A fully-developed GAS consists of three stages: the alarm reaction, the stage of resistance and the stage of exhaustion. On the cellular level the reaction incorporates sensing, assessing and then counteracting stress-induced damage, consequently temporarily increasing tolerance to such damage (Kultz, 2005). Proteins that are involved in the key functions of the stress response are conserved in all cells, and participate in cellular functions including protein, DNA, chromatin and cytoskeleton stabilization

and repair, cell cycle control, redox regulation, energy metabolism, fatty acid/lipid metabolism and elimination of damaged proteins (Kultz, 2003). Where stress enhances function, it may be considered as a positive impact, also called eustress (Selye, 1975). However, if the stress level is over the limit of tolerance, programmed cell death (apoptosis) or necrosis occur (Hansen, 2007). Everything that surrounds cells could serve as a stressor. Environmental changes such as heat or cold, modifications in the pH, hyper- or hypo-osmosis, presence of oxidative agents, irradiation, light or inappropriate nutrition such as lack of energy, all serve as stress factors and initiate cellular stress reactions. One of the common features of these impacts is that their effect to the cells is gradual and time-dependent, attacking first the cell membrane, then progressing gradually towards the center of the cell. If the intensity of the stress changes, its intracellular consequences are again gradual and time-dependent.

An innovative technique named high hydrostatic pressure (HHP) treatment has recently been reported to improve the cryosurvival of porcine and bovine spermatozoa (Pribenszky et al., 2005b, 2006) and mouse and bovine blastocysts (Pribenszky et al., 2005a, 2005c), as well as to increase the developmental competence of vitrified porcine oocytes after parthenogenetic activation (Du et al., 2008b). In addition, HHP has been reported to improve the developmental competence of porcine IVM

oocytes after hand-made cloning (HMC; Vajta et al., 2001; Kragh et al., 2004), increase the cryotolerance of the blastocysts produced and allow full-term development after the transfer of fresh embryos to recipient sows (Du et al., 2008a).

It is known that during cryopreservation the oocytes are exposed to both an osmotic stress and a thermal stress, that in turn result in mechanical damages to the intracellular organelles.

In the present study, therefore two different sub lethal stress treatments were used to increase the oocyte survival rate and developmental competence following CTV vitrification: osmotic (Experiment 5) and heat (Experiment 6) stress. For clarity, we describe the two experiments separately, whereas the discussion is in common.

EXPERIMENT 5: *Evaluation of osmotic stress on the cryotolerance of in vitro matured oocytes*

Osmotic stress occurs when the concentration of molecules in solution outside the cell is different than that inside the cell. When this happens, water flows either into or out the cell by osmosis, thereby altering the intracellular environment. If the stress impact is over the limit of tolerance, apoptosis or necrosis occurs (Hansen, 2007). Mammalian cells generally demonstrate an ideal osmotic response, characterized by the Boyle–Van't Hoff relationship (i.e., cell equilibrium volume is a linear function of the reciprocal of the extracellular osmolality), and bovine oocytes have been reported to follow this pattern (Ruffing et al., 1983; Myers et al., 1987). Consequences of osmotic stress on unfertilized mouse oocytes, zygotes, cleavage stage embryos (Mazur and Schneider, 1986; McWilliams et al., 1991; Oda et al., 1992; Pedro et al., 1997), unfertilized human oocytes (McWilliams et al., 1995), and 8 cell bovine embryos (Mazur and Schneider, 1986) have been studied using non permeating solutes such as NaCl and sugars (mono and disaccharides). Recently, high osmolarity at early embryonic stage was reported to improve in vitro development, with reduced apoptosis through regulation of Bax-α/Bcl-xl gene expression in porcine nuclear transfer (NT) and IVF embryos (Hwang et al., 2008). In a previous study, Lin et al. (2009) described that pretreatment with elevated

concentrations of NaCl improved the cryotolerance and developmental competence of porcine oocytes.

On the basis of these observations the aim of this study was to investigate the effect of a sublethal transitory osmotic stress, created by using different concentrations of NaCl, on the cryotolerance of bovine oocytes, evaluated in terms of survival and development after IVF. In particular, the oocytes were exposed for1 h to 0, 0.1, 0.15 and 0.2 M of NaCl, given another 1 h to recover prior to vitrification.

MATERIALS AND METHODS

Reagents and media

Unless otherwise stated, reagents were purchased from Sigma-Aldrich® (Milano, Italy). For oocytes osmotic treatment, three different solutions were prepared: 0.1 M, 0.15 M and 0.2 M of NaCl in Hepes buffered TCM medium + 5% BS.

Methods

The oocytes recovery, IVM, vitrification and warming were previously described in Experiment 1. The procedure of IVF and IVC were previously described in Experiment 3.

Osmotic treatment

For osmotic treatment, after 20 h in IVM the oocytes were placed in four-well dishes containing 400 µl of Hepes buffered TCM medium + 5% BS supplemented with three different concentrations of NaCl (0.1 M, 0.15 M and 0.2 M). COCs were then incubated for 1 h at 39°C in a controlled gas atmosphere of 5% CO_2 in humidified air. As controls, COCs were incubated in buffered TCM Hepes medium + 5% BS under the same conditions but without the addition of NaCl. Subsequently, to let them recover from the stress, COCs were washed briefly and then incubated in 400 µl well of IVM medium for 1 h at 39°C in 5% CO_2 in humidified air.

EXPERIMENTAL DESIGN

A total number of 880, oocytes over 4 replicates, was selected for IVM.

After 20 h in IVM, oocytes were divided into 4 groups. Three of these groups were treated with different NaCl concentration as described above and in particular: 0.1 M (n=211), 0.15M (n=228) and 0.2 mM (n=228). The COCs of the fourth group were put into buffered TCM Hepes medium + 5% BS for 1 h and served as the control group: 0 mM (n=213). After 1 h recovery in IVM medium, oocytes were denuded, vitrified and warmed as previously described in the Material and Methods of Experiment 1. After a

brief wash, oocytes of the 4 different groups were located into the well of IVM for 2 h in a controlled gas atmosphere of 5% CO_2 in humidified air. Finally, oocytes were fertilized and cultured in vitro and embryos were evaluated at day 7, as previously described in the Material and Methods of Experiment 3.

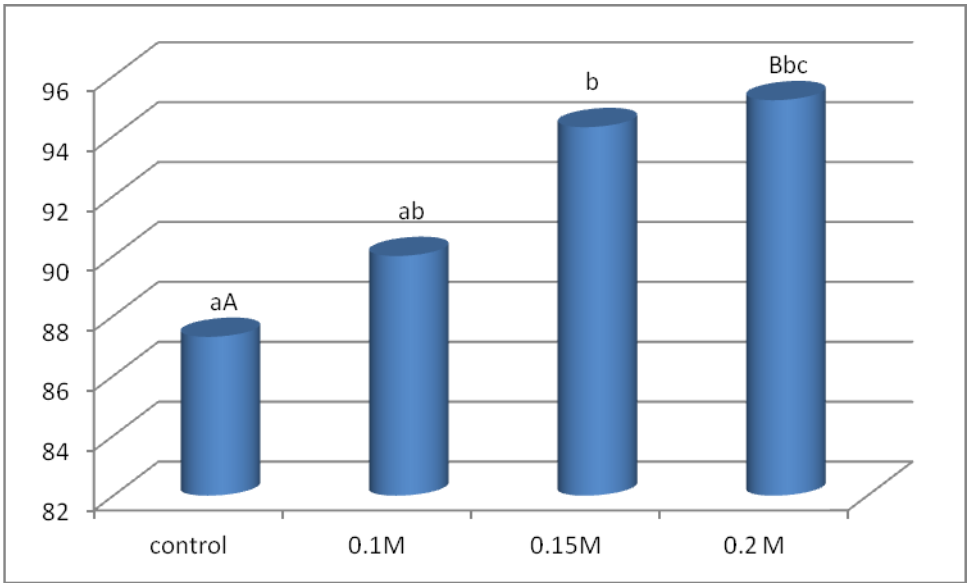
STATISTICAL ANALYSIS

The differences in the percentage of survival, cleavage and blastocyst rates among groups were analyzed by Chi Square Test. When the P value was <0.05 the differences were considered significant.

RESULTS

As shown in Figure 10, the survival rates following vitrification were significantly higher in the 0.15 M group ($P<0.05$) and in the 0.2 M group ($P<0.01$) compared to the control (87.3, 90.0, 94.3 and 95.2 % in the control, 0.1 M, 0.15 M and 0.2 M NaCl, respectively).

Figure 10. Survival rates of bovine oocytes undergone 1 h osmotic treatments prior to vitrification



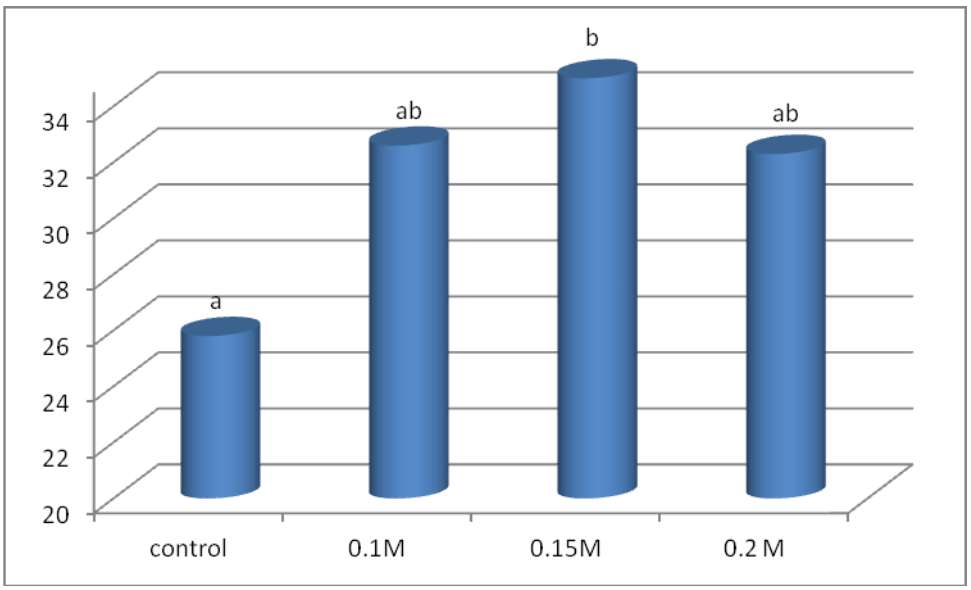
[A,B] Bars with different letters are significantly different; P<0.01

[a,b] Bars with different letters are significantly different; P<0.05

Interestingly, cleavage rate increased (P<0.05) in the oocytes treated with 0.15 M NaCl compared to the control (25.8, 32.6, 35.8, 32.3%, in the control, 0.1 M, 0.15 M and 0.2 M NaCl, respectively), as illustrated in Figure 11. However, no differences were observed in terms of blastocyst rates among groups (0.5, 1.1, 2.3 and 2.3% in the control, 0.1 M, 0.15 M and 0.2 M NaCl groups, respectively). Finally, although the percentage of cleaved oocytes reaching the blastocyst stage tended to increase at higher concentrations of NaCl, the analysis did not show any statistical difference

(2.1, 3.2, 6.5 and 7.1% in the control, 0.1 M, 0.15 M and 0.2 M NaCl groups, respectively).

Figure 11. Cleavage rates of bovine oocytes undergone 1 h osmotic treatments prior to vitrification



[a,b] Bars with different letters are significantly different; P<0.05

EXPERIMENT 6. *Evaluation of thermal shock on the cryotolerance of in vitro matured bovine oocytes*

Cells exposed to heat shock can undergo an array of biochemical responses protection from elevated temperature. One of the main pro survival activities of cells, the heat shock response, was originally described as the biochemical response of cells to mild heat stress (i.e., elevations in temperature of 3–5°C above normal; Lindquist, 1986; Craig, 1985). It has since been recognized that many stimuli can activate this response, including oxidative stress and heavy metals. One of the main cellular consequences of these stresses is protein damage leading to the aggregation of unfolded proteins. In order to counteract this, cells increase the expression of chaperone proteins that help in the refolding of misfolded proteins and alleviate protein aggregation. This confers a transient protection, leading to a state that is known as thermo tolerance, whereby cells become more resistant to various toxic insults, including otherwise lethal temperature elevations or oxidative stress (Samali and Orrenius, 1998; Samali and Cotter, 1996). One group of molecules that play a role in induced thermo tolerance are the heat-shock proteins and the most well characterized of these are proteins of the heat shock protein 70 (HSP70) family. The HSP70 proteins are molecular chaperones that protect cells

from elevated temperature by stabilizing intracellular proteins and organelles and by inhibition of apoptosis (Brodsky and Chiosis, 2006).

It has been demonstrated that exposing porcine parthenogenetic embryos to elevated temperatures immediately after oocyte activation results in a dramatic increase of their developmental potential (Isom et al., 2009). Therefore, in the context of stress-induced stress tolerance, another physical challenge, such as heat shock was here investigated.

The aim of this study was to investigate the effect of exposing bovine in vitro matured oocytes to elevated temperatures (42°C) for different times (3, 6 and 9 h) before vitrification, on their survival and embryo development after IVF.

MATERIALS AND METHODS

Reagents and media

Unless otherwise stated, reagents were purchased from Sigma-Aldrich® (Milano, Italy).

Methods

The oocytes recovery, IVM, vitrification and warming were previously described in Experiment 1. The procedure of IVF and IVC were previously described in Experiment 3.

Heat shock treatment

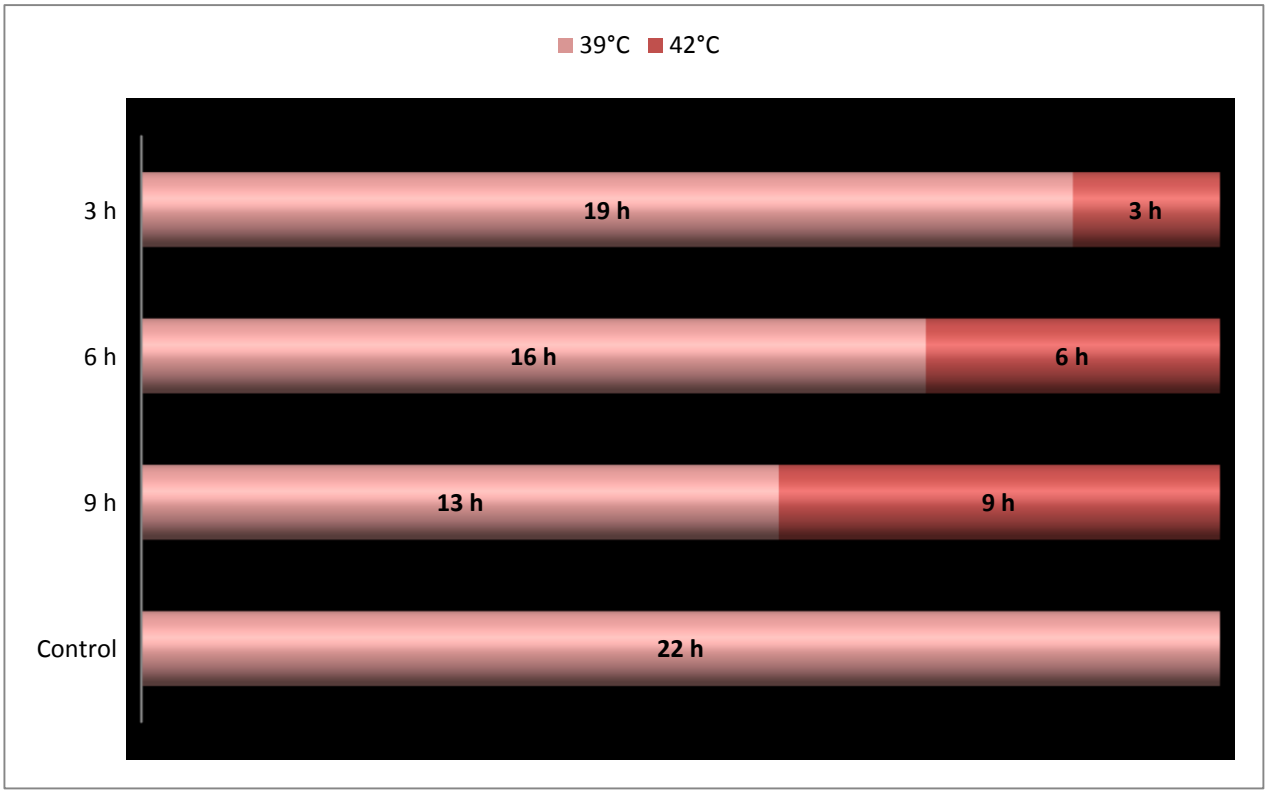
At different maturation times (13, 16 and 19 h of IVM) the oocytes were simply placed at 42°C (3°C above the temperature normally used) in the same well of IVM, in order to test the effect of elevated temperatures for 9, 6 and 3 h prior to vitrification. Another group of oocytes was left for the whole period of IVM (22 h) at 39°C, as a control group. For all groups, the IVM was carried out in a controlled gas atmosphere of 5% CO_2 in humidified air.

EXPERIMENTAL DESIGN

A total number of 532 oocytes, over 2 replicates, was selected for IVM.

As a control, a group of 138 oocytes were matured for 22 h at 39°C. The remaining oocytes were divided into three treatment groups according to the duration of exposure to the heat shock: 3 h (n=132), 6 h (n=128) and 9 h (n=134). In particular, within the treated oocytes, a group was matured for 19h at 39°C and for the last 3h at 42°C; a group was matured for the first 16 h at 39°C and for the last 6 h at 42°C and another group was matured for the first 13h at 39°C and then at 42°C for 9 h, as shown in Figure 12. After the shock treatments and at the end of IVM for the control, oocytes were denuded and vitrified as previously described in the Materials and Methods of Experiment 1. After warming, oocytes were rinsed and allocated into well of IVM medium for 2 h at 39°C and then they were fertilized and cultured in vitro, as previously described in the Materials and Methods of Experiment 3. The cleavage and blastocyst rates were evaluated at day 7.

Figure 12. Scheme of experimental design



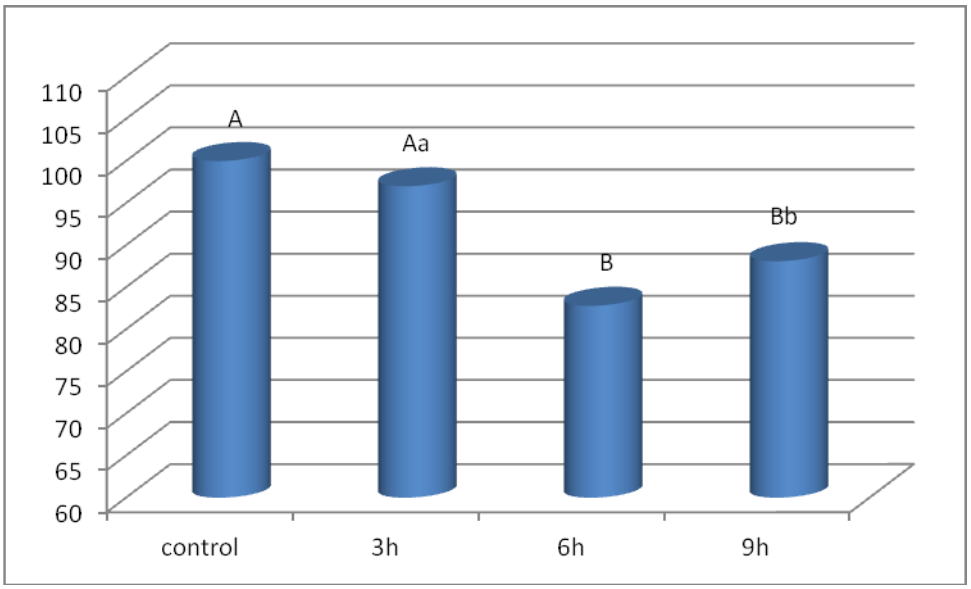
STATISTICAL ANALYSIS

The differences in the percentage of survival, cleavage and blastocyst rates among groups were analyzed by Chi Square Test. When the P value was <0.05 the differences were considered significant.

RESULTS

As shown in Figure 13, a decreased (P<0.01) survival rates was observed when oocytes were exposed to elevated temperatures for 6 and 9 h compared to the control (100.0, 97.0, 82.8 and 88.1, % in the control, 3h, 6h and 9 h groups, respectively). On the contrary, when the heat stress treatment lasted 3 h the survival rates were similar to the control untreated oocytes.

Figure 13. Survival rates of bovine oocytes exposed to elevated temperatures prior to vitrification.

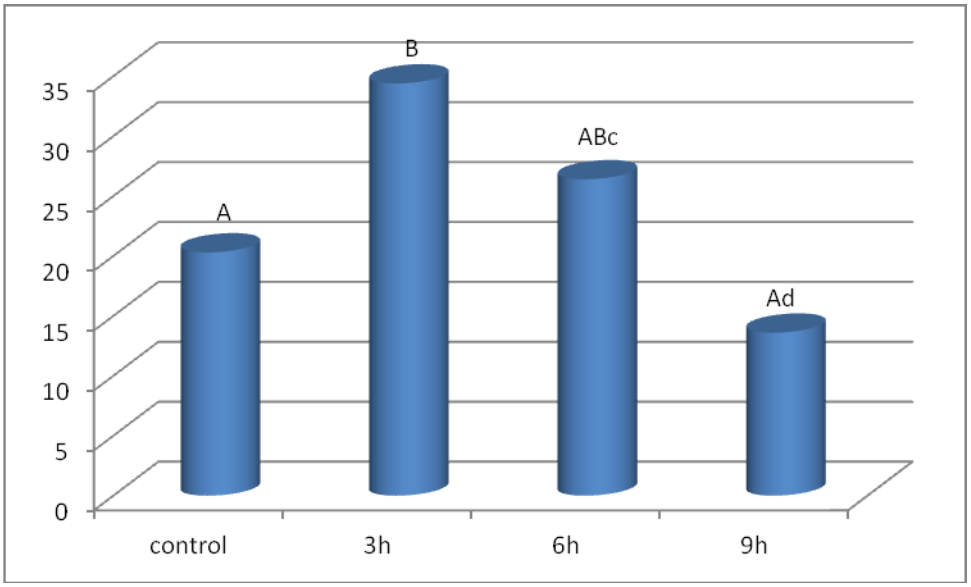


[A,B] Bars with different letters are significantly different; P<0.01

[a,b] Bars with different letters are significantly different; P<0.05

Interestingly, cleavage rate significantly increased (P<0.01) when oocytes were heat-treated for 3 h compared to the control (20.3, 34.4, 26.4 and 13.6 %, in the control, 3h, 6h and 9 h groups, respectively). A 6h treatment gave cleavage rates intermediate between the control and the 3 h group. Differences in cleavage rates were also observed between the 3 h and the 9 h treatment groups. Unfortunately, in this experiment no blastocysts were produced.

Figure 14. Cleavage rates of bovine oocytes exposed to elevated temperatures prior to vitrification.



[A,B] Bars with different letters are significantly different; P<0.01

[a,b] Bars with different letters are significantly different; P<0.05

DISCUSSION (Experiments 5 and 6)

Active training of cells to enhance their viability and developmental competence has recently applied in experiments involving ART procedures. A possible strategy to improve cell's viability and developmental competence consist to expose them to a sub lethal stress before manipulation in order to increase the subsequent stress tolerance and morphological intactness. Cells respond to stress in a variety of ways ranging from activation of pathways that promote survival to elicite programmed cell death that eliminates damaged cells. The cell's initial response to a stressful stimulus is geared towards helping the cell to defend against and recover from the insult. However, if the noxious stimulus is unresolved, then cells activate death signaling pathways. The fact that the cell's survival critically depends on the ability to mount an appropriate response towards environmental or intracellular stress stimuli can explain why this reaction is highly conserved in evolution. There are many different types of stress and the response a cell mounts to deal with these conditions will depend on the type and level of the insult. The adaptive capacity of a cell ultimately determines its fate.

The objectives of this work were to evaluate whether exposing bovine in vitro matured oocytes prior to vitrification to two different sources of stress treatments, i.e. an osmotic (Experiment 5) and thermal (Experiment 6)

shock, would increase their cryotolerance. The results of these experiments demonstrated, indeed, that exposing in vitro matured bovine oocytes to sub lethal stress, such as osmotic and thermal stresses, improves their resistance to vitrification.

Osmotic shock or osmotic stress is a sudden change in the solute concentration around a cell, causing a rapid change in the movement of water across its cell membrane. Under conditions of high concentrations of salts water is drawn out of the cells through osmosis. This also inhibits the transport of substrates and cofactors into the cell thus “shocking” the cell. All organisms have mechanisms to respond to osmotic shock, with sensors and signal transduction networks providing information to the cell about the osmolarity of its surroundings, these signals activate responses to deal with extreme conditions. How cells tolerate changes in osmolality and the associated changes in cell volume plays a major role in the outcome of cell survival during cryopreservation.

Therefore, in order to develop optimal methods for cryopreservation, there is a need for determining the osmotic tolerance excursion limits of bovine oocytes. Agca et al. (2000) studied effects and consequences of osmotic stress on MII and GV bovine COCs exposing them to various anisotonic NaCl solutions. Exposure to anisotonic conditions had a significant effect on the developmental competence of both GV and bovine MII COCs. In

fact, both in GV and MII oocytes a significant decrease of development to blastocyst was observed compared to the isotonic control. Furthermore, Mullen et al. (2004) demonstrated that anisosmotic sucrose solutions lead to disruption of the MII spindle in human oocytes. These data support the hypothesis that osmotic stress is detrimental to bovine oocytes and must be considered when developing optimized cryopreservation procedures for these cells.

The rational of Experiment 5 was to test the hypothesis that exposing the oocytes to a sub lethal (short exposure) osmotic stress would trigger the machinery response to the insult in the cells, so that their defensive mechanism is already activated when they encounter a more severe stress, such as vitrification. In the current experiment NaCl was used as the osmotic solute because during freezing NaCl is most often the primary electrolyte solute responsible for the high osmolality associated with solute damage (Mazur et al., 1984). For this purpose, three different concentrations of NaCl were tested: 0.1M, 0.15 M, 0.2 M compared to a control group, with no addition.

The results of this Experiment demonstrated that a short exposure to an osmotic stress prior to vitrification improves the cryotolerance of in vitro matured bovine oocytes. In particular, within the concentrations of NaCl considered, the most efficient was the intermediate tested, i.e. 0.15 M was.

Indeed, this pretreatment significantly increased ($P<0.05$) both the survival rates and, more importantly, the cleavage rates after IVF of vitrified-warmed oocytes. Furthermore, although the analysis did not show any statistical difference, the percentage of cleaved oocytes reaching the blastocyst stage tended to increase at higher concentrations (0.15 and 0.2 M) of NaCl.

Our results are in agreement with those reported by Lin et al. (2009) who reported that an osmotic pretreatment improved the in vitro developmental competence of vitrified porcine oocytes. In that work, both cleavage and blastocyst rates increased significantly after exposure to 593 mOsmol NaCl that is equivalent approximately to 0.3 M.

A possible mechanism that may in part account for the reduced cryoinjury after osmotic stress is the decrease in the melting temperature (Tm) of the cell membrane. There are several mechanisms and structures involved in chilling injury, including cell membranes. In the cell membrane, chilling injury is related to the lipid phase transition, so the higher the Tm of the cell membrane, the more sensitive cells will be. Methods to reduce chilling injury include the use of lipid stabilizers (Strauss et al., 1986) or passage through the lipid phase transition more rapidly than the kinetics of chilling injury, as in the case of high-speed vitrification methods (Vajta and Nagy, 2006). The use of elevated concentrations of sugars or NaCl would reduce

the Tm of the cell membrane, albeit through different mechanisms (Caffrey and Feigenson, 1984; Roesser and Muller, 2001; Molina-Hoppner et al., 2004).

During cryopreservation cells are obviously exposed to a thermal stress, i.e. to the cold temperatures that are necessary to preserve their viability and function. As a temporary increase of the environmental temperature is more likely efficient in triggering the stress response than a decreased temperature we decided to investigate the effect of a short exposure of in vitro matured bovine oocytes to elevated temperature on their cryotolerance.

Another type of stress that may occur naturally in the cells is the heat shock. Heat shock is the effect of subjecting a cell to a higher temperature than that of the ideal body temperature of the organism from which the cell line was derived. The main cellular consequences of this stress is the heat shock response, a universal response to increased temperature in both prokaryotes and eukaryotes (Craig and Gross, 1991; Georgopoulos and Welch, 1993). Heat shock may enhance the thermo tolerance of, or cause detrimental effects on, a variety of cell types or organisms, depending on the duration and intensity of the thermal challenge.

The objective of Experiment 6 was, therefore, to evaluate whether exposing bovine oocytes during IVM at high temperatures (3°C higher than the physiological temperature) for different times before cryopreservation influences their cryotolerance. The rational of the study was the same as for Experiment 5 but in this case we evaluated the effect of a different source of stress, i.e. that given by elevated temperatures. The hypothesis we tested was that a short heat shock would likely result in activating the cell defensive mechanism so that the oocytes would increase their tolerance to the more severe stress of the cold temperatures they will encounter during vitrification.

However, Roth and Hansen (2004) showed that exposure of bovine oocytes to thermal stress during the first 12 h of maturation reduced cleavage rate and the number of oocytes developed to the blastocyst stage. Furthermore, the same authors in 2005 found that while bovine oocytes matured at 38.5°C were mostly at metaphase II stage, the majority of heat-shocked oocytes (cultured at 40.0 or 41.0 °C for the first 12 h of maturation) were blocked at the first metaphase, first anaphase or first telophase stages.

For this reason, in the present study, we evaluated the effects of heat shock (42°C) after the first 12 h of IVM and precisely after 13 (9 h at 42°C) , 16 (6 h at 42°C) and 19 h (3 h at 42°C).

The first results of this experiment regards the survival rates following vitrification: decreased ($P<0.01$) survival rates were observed when oocytes were exposed to elevated temperatures for 6 and 9 h compared to the control, whereas when the length of heat stress treatment was 3 h the survival rates were similar to the control. The most interesting results, however, was the significant increase ($P<0.01$) of cleavage rate that was recorded when oocytes were heat-treated for 3 h compared to the control. Also this parameter showed a decrease when the treatment was extended for 9 h. Therefore, the observation that survival rates were not affected and the increased cleavage rate indicate that a brief (3 h) exposure of oocytes to elevated temperatures prior to vitrification improves their cryotolerance. On the contrary, a more prolonged exposure to elevated temperatures is detrimental.

A dramatic improvement of development was recently reported by Isom et al. (2009) when porcine parthenogenetic embryos were exposed to elevated temperatures immediately after oocyte activation.

In our study, however, despite the improved cleavage in the 3 h group, the development into blastocysts was not improved. Actually, no blastocysts were produced in any of the groups. This is not surprising considering the poor efficiency of oocyte cryopreservation; however, in previous studies,

although at a low rate, blastocysts were produced. This may in part be accounted for by the oocyte variability.

However, these results clearly indicate that there is a beneficial effect of exposing oocytes for limited time to elevated temperatures, but the positive effect of heat stress before vitrification depends upon the duration of exposure.

With regard to the duration of exposure, Ju et al. (1999) reported that after a treatment of bovine oocytes at 41.5°C the group with the minimum exposure time (30 minutes) showed the highest cleavage (82%) respect to the control (69%) and the other treated group (60 minutes, 68%). In addition, the same authors in 2005 reported that no significant differences were detected in cleavage rates when oocytes were heated for 0, 1, 2, or 4 h but blastocyst development in the 4 h heat shock oocytes was lower than that in the control group. Furthermore, in the same study it was reported that the metaphase spindle became elongated or aberrant and smaller following heat shock, compared to the non-heat shock oocytes.

Our results stand in apparent contrast to results reported by other authors that describe deleterious effects of elevated temperatures on the oocytes.

Recently, more physiologically relevant temperatures of 41–41.5°C have been shown to cause alterations in microtubule and microfilament structure

in matured bovine oocytes (Tseng et al., 2004), porcine oocytes subjected to heat shock during maturation (Ju and Tseng, 2004) and two-cell bovine embryos (Rivera et al., 2004a). Ju et al. (1999) reported that when the temperature increases to 43°C the developmental competence of the treated oocytes is severely reduced following a 45 minute exposure. Edwards and Hansen (1996) found that exposure of bovine cumulus oocyte complexes for 12 or 24 h to 41°C did not alter the number of embryos that cleaved but reduced the number that developed to the blastocyst stage. In contrast, exposure to 42°C for 12 or 24 h reduced both cleavage and developmental rates.

It is also known that heat stress may interfere with the process of oocyte maturation, particularly at the cytoplasmic level, impairing the acquisition of developmental competence (Payton et al., 2004,Edwards and Hansen, 1997; Roth and Hansen, 2004). It has also been suggested that the increased protein turn-over may also lead to premature aging of the oocyte (Seath and Miller, 1946; Monty and Wolff, 1974; Ealy et al., 1993).

We may speculate that the increased capacity to undergo fertilization of the 3h heat stress group recorded in this experiment may be due to the fact that the oocytes of this group had completed the maturation process before the heat stress was applied (the treatment started at 19 h post-IVM) and to the limited exposure time.

However, the contradicting results of literature may suggest caution to draw definite conclusions. Other studies are necessary to understand the right hyperthermic conditions and the exposure time in order to improve developmental potential of vitrified-warmed oocytes.

In conclusion, the results of the present study demonstrated that the induction of sub lethal osmotic (Experiment 5) and thermal stress (Experiment 6) increases the cryotolerance of IVM bovine oocytes. In particular, the pre-treatment of oocytes with 0.15M NaCl increased both the survival and cleavage rates, with a tendency to give more blastocysts. The exposure of oocytes at 42°C for 3 h resulted only in increased cleavage rate after IVF. However, the evidence of a toxic effect of elevated temperatures reported in literature and the failure to obtain blastocysts in Experiment 6 would suggest that the stress tolerance induced by an osmotic stress is preferable in order to improve the vitrification efficiency. Furthermore, a sub lethal osmotic stress is also easier to achieve, as changing the temperature setting is less practical and more risky than using an hypertonic solution.

CONCLUSIONS

Currently, in the context of reproductive technologies in human medicine, particular attention is paid to the decline of female fertility caused by aging, reproductive diseases or sickness (cancer). In our country the law 40/2004 has restricted the preservation of fertility only to oocytes cryopreservation because embryo freezing is prohibited. This has been the subject of many debates because women who undergo repeated hormonal stimulation in ART programs may end up producing more embryos than necessary and these cannot be saved. Therefore, there has been an increasing interest in oocyte cryopreservation because the safe storage of the surplus oocytes may avoid to repeat harmful stimulation in case of pregnancy failure. Furthermore, oocyte cryopreservation is potentially the best tool to preserve the fertility of women that, for health reasons, need to undergo severe cycles of chemotherapy and radiotherapy. The optimization of oocyte cryopreservation efficiency would then allow these patients to save their germinal material before entering therapies.

Despite the success of sperm and embryo cryopreservation, oocyte cryopreservation in most species is still an open challenge, due to the peculiar characteristics of the oocyte. The mammalian oocyte is indeed a complex cell type, with low permeability to water and a low surface to

volume ratio that make it very sensitive to chilling and highly susceptible to intracellular ice formation.

At present, although many improvements have been obtained in oocytes cryopreservation, this technique is still not considered an established procedure and it is currently still an experimental system (Noyes et al., 2010).

In this scenario the importance of using an animal model is highlighted by the limited availability of human oocytes for research purposes. The choice of bovine oocytes as the model is due to the easy access to experimental material, i.e. oocytes derived from slaughtered animals and to the existence of a well established in vitro embryo production system. The acquisition of more information on cryopreservation-induced oocyte damages is necessary to identify corrective strategies and in the same time many innovative vitrification methods have been proposed that need to be proven effective on animal oocytes before using in the human field.

Therefore, in the first part of this work we carried out a series of experiments that were focused on the evaluation of cryopreservation-induced damages using an innovative and promising minimum volume vitrification tool, that recently came into the limelight, i.e. the cryotop (Kuwayama and Kato, 2000). In all the experiments we used in vitro matured bovine oocytes as it has been previously proven that they are more

resistant than the immature counterparts (Lim et al., 1992). The rational of this study was to identify key problems related to cryopreservation in order to develop corrective treatments.

In Experiment 1. it was demonstrated that also using this innovative vitrification tool damages to the meiotic spindle and to the chromosome organization of the oocytes are observed. Furthermore, it was observed that also the simple exposure of oocytes to CPs causes these damages but to a lesser extent than the whole vitrification protocol. However, it is worth pointing out that, despite the evidence of spindle and chromatin alterations, in our study with cryotop vitrification the extent of the damage was definitely lower than in another work carried out in cattle with slow freezing (Saunders and Park, 1999). The damages to the meiotic spindle and DNA fragmentation may lead to aneuploidy incompatible with subsequent embryo development and account for the poor embryo development currently recorded.

The most interesting finding of the present study was the occurrence of spontaneous parthenogenetic activation, indicated by the presence of oocytes in TII at 2 h post-warming. Spontaneous activation has been observed in cryopreserved oocytes of other species where it has been hypothesized that CPs determine an intracellular Ca^{++} increase (Larman et al., 2006) that may trigger the meiotic resumption. An unexpected datum of

our study was the evidence of a significantly higher percentage of spontaneously activated oocytes in the toxicity group compared to the vitrification. We speculated that the lower activation observed in the vitrification group might be referred to the slowing down of the metabolic activity subsequent to thermal shock, and hence that activation after vitrification may occur later than 2 h post-warming.

Therefore, in order to confirm this hypothesis, in Experiment 2 the same assessments were carried out in bovine oocytes fixed also at a later time (4h) post-warming. In the same time we decided to evaluate the mechanism by which activation occurs in order to prevent the phenomenon. Therefore, in addition to the assessment of nuclear status we also measured the MPF and MAP kinase activities.

In particular, we showed that vitrification determined in bovine oocytes a fall in MPF activity, which was evident since 2 h and was not restored at 4 h post-warming. A similar pattern was found in vitrified oocytes for the MAPK activities. On the contrary, in the toxicity group the MPF levels were similar to the control at 2 h post-warming to significantly drop at 4 h. Furthermore, no differences in MAPK activities were observed in the exposed oocytes either at 2 and 4 h post-warming. Therefore, our results on MPF and MAPK activities demonstrated that the cumulative effect of CPs

toxicity and cryoinjury has a major effect on the MII-arrest machinery, and this accounts for the high parthenogenetic activation rate that we observed.

On the basis of the results on MPF and MAPK activities obtained in Experiment 2 we hypothesized that a pre-treatment of bovine oocytes with a compound as caffeine, a phosphodiesterase inhibitor known to stabilize MPF, may reduce the incidence of activation and hence increase the vitrification efficiency (Experiment 3). The results of this study were disappointing as we demonstrated that the caffeine treatments of the oocytes, at the three concentrations tested (10, 20 and 40 mM), fail to improve the vitrification efficiency in cattle. Indeed, caffeine at the lower concentrations, i.e 10 and 20 mM, did not increase the MPF and MAPK activities and hence did not reduce spontaneous activation. Subsequently, no improvement was recorded in terms of cleavage and blastocyst rates following IVF and IVC in both groups.

Furthermore, the only concentration (40 mM) that actually increased the kinases and reduced the spontaneous activation rates was also toxic for the oocytes, as shown by the high incidence of condensed chromatin, the low percentage of oocytes with a normal MII configuration and low cleavage and blastocyst rates.

Despite the evidence of a critical role played by ROS in many early reproductive events, the effect of vitrification on ROS levels has not yet

been investigated in bovine oocytes. Therefore, another objective of the present study was to investigate the effect of vitrification and CPs exposure on ROS activity of bovine in vitro matured oocytes (Experiment 4). The results of Experiment 4 showed an increase of ROS levels following both vitrification and exposure to CPs compared to the control, although the difference was not statistically different. The most evident result was that the ROS levels in fresh control oocytes were stable, whereas a high variability among replicates was recorded in both vitrified and exposed oocytes. These results indicate that one of the damages of bovine oocytes following CTV vitrification is the increased ROS levels, that may be responsible of the poor developmental potentials.

The results of the first part of this thesis demonstrated that even with an innovative vitrification method such as CTV several damages occur in bovine in vitro matured oocytes. The evidence of these alterations provides the basis for developing new strategies to improve the efficiency of cryopreservation in bovine species. In particular, in order to further reduce the spindle and chromatin damages, an interesting approach may be a pretreatment of the oocytes with taxol and/or other cytoskeleton stabilizers, such as, cytochalasin B. Furthermore, in order to prevent the occurrence of spontaneous parthenogenetic activation a possible alternative approach may be a pre-treatment of the oocytes with other phosphodiesterase-3 inhibitors

such as cilostamide and cilostazol, and with the adenylate cyclase activator forskolin. Finally, to prevent ROS accumulation, it may be suggested to add antioxidants to the IVM medium, such as βME, cysteamine, cystine and cysteine etc, providing the oocyte a greater antioxidant capacity prior to vitrification.

In the second part of this thesis we investigated a new intriguing approach to improve the cryotolerance and developmental competence of oocytes, based on the concept that exposing cells to sub lethal stress before manipulation may increase the subsequent stress tolerance. Both an osmotic stress and a thermal stress, that in turn result in mechanical damages to the intracellular organelles, occur during cryopreservation. Therefore, we evaluated whether exposing bovine in vitro matured oocytes to a sub lethal osmotic stress (Experiment 5) and thermal stress (Experiment 6) increases the oocyte survival rate and developmental competence following CTV vitrification.

The rational of Experiment 5 was to test the hypothesis that a short pretreatment of the oocytes with a sub lethal osmotic stress would trigger the cell response to the insult, so that when the cells encounter a major stress, such as vitrification, they can more promptly respond. For this purpose, three different concentrations of NaCl were tested: 0.1M, 0.15 M, 0.2 M compared to a control group, with no addition. The results of

Experiment 5 demonstrated that a short exposure to 0.15 M NaCl prior to vitrification improves the cryotolerance of in vitro matured bovine oocytes, as indicated by increased survival and cleavage rates after IVF of vitrified-warmed oocytes. Furthermore, the percentage of cleaved oocytes reaching the blastocyst stage also tended to increase.

The objective of Experiment 6 was to evaluate whether exposing bovine oocytes during IVM at high temperatures (3°C higher than the physiological temperature) for different times before cryopreservation influences their cryotolerance. The rational of the study was the same as for Experiment 5, with the only difference of the stress source, in this case elevated temperatures. The hypothesis we tested was that a short heat shock would likely result in activating the cell defensive mechanism so that the oocytes would increase their tolerance to the more severe stress of the cold temperatures they will encounter during vitrification. For this reason, in the present study, we evaluated the effects of heat shock (42°C) for 3, 6 and 9 h prior to vitrification. The results of Experiment 6 demonstrated that a brief (3 h) exposure of oocytes to elevated temperatures prior to vitrification improves their cryotolerance, as shown by the increased cleavage rate, whereas a more prolonged exposure to elevated temperatures is detrimental.

In conclusion, these results demonstrated that both an osmotic (Experiment 5) and thermal stress (Experiment 6) prior to cryopreservation improves the cryotolerance of IVM bovine oocytes. However, the contradicting results on the toxic effects of elevated temperatures present in literature, together with the failure to obtain blastocysts in Experiment 6, would suggest that it is more advisable to induce the stress tolerance by an osmotic treatment. Furthermore, a sub lethal osmotic stress is very easy to achieve in the laboratory practice, by simply making an hypertonic solution, whereas changing the temperature setting is not practical and even more risky for both cells and incubators.

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