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Communication in Genomics and Proteomics

Sequence analysis of cDNA encoding follicle-stimulating hormone and luteinizing hormone β-subunits in the Mongolian gerbil (*Meriones unguiculatus*)

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Abstract

To examine the molecular basis for efficient superovulation in the Mongolian gerbil, the cDNA sequences of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) β-subunits were determined and compared with those of other mammals. FSHβ and LHβ cDNAs were 1637 and 507 bp long, respectively, from the 5′-end to putative polyA sites. The deduced sequences of the FSHβ and LHβ precursor proteins were 129 and 141 amino acids in length, respectively. The amino acid sequences of both Mongolian gerbil hormone subunits showed overall similarity to those of other rodents, confirming that the combination of equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) should be effective for induction of superovulation in Mongolian gerbils, as in mice and rats. However, the use of hCG might need to be re-evaluated owing to its low homology to rodent LH.

Keywords: Mongolian gerbil; FSHβ; LHβ; cDNA; Phylogeny

1. Introduction

The Mongolian gerbil (*Meriones unguiculatus*) is frequently used as a rodent model in the study of epilepsy (Loskota et al., 1974) and gastric infection with *Helicobacter pylori* (Hirayama et al., 1996). Embryo manipulation techniques, such as cryopreservation of embryos/gametes and transgenic technologies, need to be employed to improve the usability of laboratory animals, but these techniques are not fully applicable to gerbils yet. For these applications, many embryos/oocytes are required, making induction of superovulation an essential technique. In general, superovulation is induced by injection of gonadotropins. In particular, the combination of equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) is widely used for induction of superovulation in many species. However, this method is not efficient in all species. In rabbits, multiple injections of FSH are more effective than a single injection of eCG (Hirabayashi et al., 2000). In guinea pigs, human menopausal gonadotropin (hMG) is a better inducer of superovulation than are the chorionic gonadotropins (Suzuki et al., 2003). In Mongolian gerbils, the combination of eCG plus hCG is effective for superovulation, but a problem arises in that a considerable number of oocytes remain trapped within the corpora lutea (Fischer and Fisher, 1975). A careful choice of gonadotropins is still needed for practical superovulation in this animal.

In this paper, we describe the cDNA sequences of the FSH and LHβ-subunits in order to develop a molecular basis for efficient superovulation in the Mongolian gerbil. Phylogenetic analyses of the deduced proteins were also performed. The possibility of using this sequence information as a criterion for selection of gonadotropins for superovulation of Mongolian gerbils is discussed.

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Chromosomal Mapping and Zygosity Check of Transgenes Based on Flanking Genome Sequences Determined by Genomic Walking

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Abstract: *Transgenes can affect transgenic mice via transgene expression or via the so-called positional effect. DNA sequences can be localized in chromosomes using recently established mouse genomic databases. In this study, we describe a chromosomal mapping method that uses the genomic walking technique to analyze genomic sequences that flank transgenes, in combination with mouse genome database searches. Genomic DNA was collected from two transgenic mouse lines harboring pCAGGS-based transgenes, and adaptor-ligated, enzyme restricted genomic libraries for each mouse line were constructed. Flanking sequences were determined by sequencing amplicons obtained by PCR amplification of genomic libraries with transgene-specific and adaptor primers. The insertion positions of the transgenes were located by BLAST searches of the Ensembl genome database using the flanking sequences of the transgenes, and the transgenes of the two transgenic mouse lines were mapped onto chromosomes 11 and 3. In addition, flanking sequence information was used to construct flanking primers for a zygosity check. The zygosity (homozygous transgenic, hemizygous transgenic and non-transgenic) of animals could be identified by differential band formation in PCR analyses with the flanking primers. These methods should prove useful for genetic quality control of transgenic animals, even though the mode of transgene integration and the specificity of flanking sequences needs to be taken into account.*

Key words: *chromosomal mapping, flanking primers, genomic walking, zygosity check*

Introduction

Transgenes can exert effects in transgenic animals by transgene expression and by the so-called positional effect. Transgene expression often varies among multiple lines that have been derived from different founders. This variation may result from the position of the transgene integration site as well as the copy

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A Mutation in the Serum and Glucocorticoid-Inducible Kinase-Like Kinase (*Sgkl*) Gene is Associated with Defective Hair Growth in Mice

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Abstract

YPC is a mutant mouse strain with defective hair growth characterized by thin, short hairs and poorly developed hair bulbs and dermal papillae. To identify the gene associated with the phenotype, we performed genome-wide linkage analysis using 1010 backcross progeny and 123 microsatellite markers covering all chromosomes. The mutant locus (*ypc*) was mapped to a 0.2-cM region in the proximal part of mouse chromosome 1. This 0.2-cM region corresponds to a 450-kb region of genome sequence that contains two genes with known functions and five ESTs or predicted genes with unknown functions. Sequence analysis revealed a single C-to-A nucleotide substitution at nucleotide 1382 in the *Sgkl* gene, causing a nonsense mutation at codon 461. *Sgkl* encodes serum and glucocorticoid-inducible kinase-like kinase (SGKL), which belongs to a subfamily of serine/threonine protein kinases and has been suggested to have a role downstream of lipid signals produced by activation of phosphoinositide 3-kinase (PI3K). In the mutant SGKL, a serine residue in the C-terminal end of the protein (Ser486), which is indispensable for activation of SGKL upon phosphorylation, is abolished by premature termination. Specific expression of the *Sgkl* gene in the inner root sheath of growing hair follicles was also identified by *in situ* hybridization. Therefore, we concluded that the nucleotide substitution in the *Sgkl* gene is the causative mutation for defective hair growth in the *ypc* mutant mouse and that the signaling pathway involving SGKL plays an essential role in mammalian hair development.

Key words: Hair follicle; SGKL/SGK3/CISK; WNT signaling; Mutant mouse; IRS

1. Introduction

Hair follicle morphogenesis and the hair growth cycle are complex processes dependent on a series of mesenchymal-epithelial interactions in skin.[1] Reciprocal exchange of signals between dermal and epidermal cells of skin regulates the formation of hair placodes during embryonic development, and it also regulates cyclic transformation of the growth (anagen), regression (catagen), and quiescent (telogen) phases in the hair cycle in adult skin. As these processes show a high degree of organization and self-renewal, hair follicle development and hair cycling are thought to be excellent models for investigating the molecular mechanisms of mesenchymal-epithelial interactions.

Numerous growth factors and cytokines have been shown to be involved in morphogenesis and cycling of hair follicles. WNT,[2,3] TGFα,[4,5] BMPs,[6,7] and FGFs[8] in particular, as well as their signal transduction molecules,[9,10] play essential roles in these processes. Experiments with transgenic mice or those with knockout mutations in these genes have demonstrated a number of abnormalities in morphogenesis and cycling of hair follicles, including a short-hair phenotype and cyclical balding in transgenic mice overexpressing the *Wnt3* gene in skin[3] and abnormally long hair in *Fgf5* knockout mice, which is caused by defective regulation of the hair cycle.[8] On the other hand, spontaneous mutant mouse strains showing abnormalities in hair morphogenesis have also provided useful information on the molecular mechanisms of these processes. For example, the hairless (*hr*) mutant, which

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Skeletal Muscle FOXO1 (FKHR) Transgenic Mice Have Less Skeletal Muscle Mass, Down-regulated Type I (Slow Twitch/Red Muscle) Fiber Genes, and Impaired Glycemic Control*[S]

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FOXO1, a member of the FOXO forkhead type transcription factors, is markedly up-regulated in skeletal muscle in energy-deprived states such as fasting and severe diabetes, but its functions in skeletal muscle have remained poorly understood. In this study, we created transgenic mice specifically overexpressing FOXO1 in skeletal muscle. These mice weighed less than the wild-type control mice, had a reduced skeletal muscle mass, and the muscle was paler in color. Microarray analysis revealed that the expression of many genes related to the structural proteins of type I muscles (slow twitch, red muscle) was decreased. Histological analyses showed a marked decrease in size of both type I and type II fibers and a significant decrease in the number of type I fibers in the skeletal muscle of FOXO1 mice. Enhanced gene expression of a lysosomal proteinase, cathepsin L, which is known to be up-regulated during skeletal muscle atrophy, suggested increased protein degradation in the skeletal muscle of FOXO1 mice. Running wheel activity (spontaneous locomotive activity) was significantly reduced in FOXO1 mice compared with control mice. Moreover, the FOXO1 mice showed impaired glycemic control after oral glucose and intraperitoneal insulin administration. These results suggest that FOXO1 negatively regulates skeletal muscle mass and type I fiber gene expression and leads to impaired skeletal muscle function. Activation of FOXO1 may be involved in the pathogenesis of sarcopenia, the age-related decline in muscle mass in humans, which leads to obesity and diabetes.

Skeletal muscle is the largest organ in the human body, comprising about 40% of the body weight. The mass and composition of skeletal muscle are critical for its functions, such as exercise, energy expenditure, and glucose metabolism (1, 2). Elderly humans are known to undergo a progressive loss of muscle fibers associated with diabetes, obesity, and decreased physical activity (sarcopenia) (3). In human skeletal muscle, there are two major classifications of fiber type: type I (slow-twitch oxidative, so-called red muscle) and type II (fast-twitch glycolytic, so-called white muscle) fibers (2). Mass, fiber size, and fiber composition in adult skeletal muscle are regulated in response to changes in physical activity, environment, or pathological conditions. For example, space flight experiments using rats showed a reduction in total skeletal muscle mass of up to 37% as well as a significant loss of contractile proteins in type I but not type II fibers by 1–2 weeks of microgravity (4). Furthermore, the ratio of type I to type II fibers is associated with obesity and diabetes; the number of type I fibers is reduced in obese subjects and diabetic subjects compared with that in controls (5–7).

Skeletal muscle mass is positively regulated by hormones such as insulin-like growth factors (IGFs)[1] and growth hormone (8). Induction of hypertrophy in adult skeletal muscle by increased load is accompanied by the increased expression of IGF-1 (9). Systemic administration of IGF-1 results in increased skeletal muscle protein and reduced protein degradation (10). In addition, overexpression of IGF-1 blocks the age-related loss of skeletal muscle (11). Supplementation of IGF-1 to muscle cells *in vitro* promotes myotube hypertrophy, suggesting that hypertrophy can be mediated by autocrine- or paracrine-produced IGF-1 (12). Thus, delivery of the *IGF-1* gene specifically into skeletal muscle has been proposed as a genetic therapy for skeletal muscle disorders. A better understanding of the role of IGF-1 in skeletal muscle is therefore of great importance.

Specialized/differentiated myofiber phenotypes, including type I and type II fibers, are plastic and are physiologically

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[1] The abbreviations used are: IGF, insulin-like growth factor; CaMK, calmodulin-dependent kinase; PGC-1α, peroxisome proliferator activated receptor-γ coactivator-1α; STZ, streptozotocin; MLC, myosin light chain; mtCK, mitochondrial creatine kinase; IGFBP, IGF-binding protein; COX, cytochrome *c* oxidase; DEXA, dual energy X-ray absorptiometry; EDL, extensor digitorum longus.

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Communication in Genomics and Proteomics

Sequence analysis of cDNA encoding follicle-stimulating hormone and luteinizing hormone β-subunits in the Mastomys (*Praomys coucha*)

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Abstract

To examine the molecular basis of efficient superovulation in the Mastomys (*Praomys coucha*), the cDNA sequences of the follicle-stimulating hormone (FSH) and luteinizing hormone (LH) β-subunits were determined and compared with those of other mammals. FSHβ and LHβ cDNAs were 1606 and 513 bp long, respectively, from the 5′-ends to the putative polyA sites. The deduced sequences of the FSHβ and LHβ precursor proteins were 130 and 141 amino acids in length, respectively. The amino acid sequences of both mastomys hormone subunits showed overall similarity to those of other rodents. In particular, the N-terminus of the FSHβ precursor protein is of the MM-type, like those of mice and rats, which suggests that the MM-type is characteristic of the subfamily *Murinae*. As we reported earlier for the Mongolian gerbil, the use of hCG for superovulation of mastomys might need to be re-evaluated, due to the low homology between hCG and rodent LH sequences.

Keywords: Mastomys; FSHβ; LHβ; cDNA; Phylogeny

1. Introduction

The mastomys (multimammate mouse, *Praomys coucha*) is an African rodent that is a well-known reservoir of serious diseases, such as *Yersinia pestis*, the rickettsia that causes bubonic plague (Dippenaar et al., 1993). It is used as a laboratory animal for studies of virology, oncology, and reproductive biology (Solleveld, 1987). For instance, *mastomys* are used to study prostate endocrinology, since both males and females have functional prostates (Ohta et al., 1994). Although there has been some research on reproduction technology in mastomys (Nohara et al., 1998; Ogonuki et al., 2003), it is quite difficult to apply standard embryo manipulation techniques, such as transgenic technologies or cryopreservation of embryos and gametes, to mastomys. Many embryos and oocytes are required to establish these techniques, necessitating the induction of superovulation. In mastomys, the combination of equine chorionic gonadotropin (eCG) plus human chorionic gonadotropin (hCG) is effective for superovulation, but a problem arises in that treated females rarely have fertile matings, perhaps due to a perturbation of estrous behavior by the exogenous hormones (Ogura et al., 1997). More information is needed to permit a careful selection of appropriate gonadotropins that will be practical and functional for superovulation in this animal.

This paper describes the cDNA sequences of the follicle-stimulating hormone (FSH) and luteinizing hormone (LH) β-subunits in order to develop a molecular

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マストミス精子の凍結保存法の検討
Development of a method for cryopreservation of mastomys spermatozoa

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Mastomys (*Mastomys coucha*) is a rodent which has been used as an laboratory animal in biomedical researches such as oncology, parasitology and epidemiology. In this study, we attempted to develop a suitable method for the cryopreservation of mastomys sperm. Four sugars (sucrose, lactose, trehalose and raffinose), egg yolk and surface-active agents (Equex Stem, SDS) were examined as cryopotectants for the mastomys sperm cryopreservation. Sparmatozoa from cauda epididymides of mastomys were transferred into different cryoprotectants containing solutions and the sperm suspension was loaded into plastic straw and frozen in LN_2 vapor for 5 mins before being plunged into NL_2. The frozen sperm suspension was thawed in 37℃ water and was diluted with incubation media at 37℃ to evaluate sperm motility. When the spermatozoa frozen in the solution of various

MOLECULAR REPRODUCTION AND DEVELOPMENT 70:464–470 (2005)

Birth of Offspring After Transfer of Mongolian Gerbil (*Meriones Unguiculatus*) Embryos Cryopreserved by Vitrification

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ABSTRACT The Mongolian gerbil (*Meriones unguiculatus*) has been used as a laboratory species in many fields of research, including neurology, oncology, and parasitology. Although the cryopreservation of embryos has become a useful means to protect valuable genetic resources, its application to the Mongolian gerbil has not yet been reported. In this study, we investigated the in vitro and in vivo developmental competence of Mongolian gerbil embryos cryopreserved by vitrification. In vivo-fertilized embryos were vitrified on the day of collection using the ethylene glycol (EG)-based solutions EFS20 and EFS40, which contained 20% and 40% EG, respectively, in PB1 containing 30% (w/v) Ficoll 70 and 0.5M sucrose. First, we compared one-step and two-step vitrification protocols. In the one-step method, the embryos were directly transferred into the vitrification solution (EFS40), whereas in the two-step method, the embryos were exposed serially to EFS20 and EFS40 and then vitrified. After liquefying (thawing), late two-cell embryos (collected on day 3) vitrified by the two-step method showed significantly better rates of in vitro development to the morula stage compared to those vitrified by the one-step method (65% vs. 5%, $P < 0.0001$). We then examined whether the same two-step method could be applied to early two-cell embryos (collected on day 2), four-cell embryos (day 4), morulae (day 5), and blastocysts (day 6). After liquefying, 87%–100% of the embryos were morphologically normal in all groups, and 23% and 96% developed to the compacted morula stage from early two- and four-cell embryos, respectively. After transfer into recipient females, 3% (4/123), 1% (1/102), 5% (4/73), and 10% (15/155) developed to full-term offspring from vitrified and liquefied early two-cell embryos, late two-cell embryos, morulae, and blastocysts, respectively. This demonstrates that Mongolian gerbil embryos can be safely cryopreserved using EG-based vitrification solutions. *Mol. Reprod. Dev. 70: 464–470, 2005.* © 2005 Wiley-Liss, Inc.

Key Words: cryopreservation; ethylene glycol; embryo transfer

INTRODUCTION

The Mongolian gerbil (*Meriones unguiculatus*), also called the "laboratory gerbil", is a myomorph rodent that is native to China and Mongolia. It has been widely used as a laboratory animal in biomedical research, including the study of epilepsy (Jobe et al., 1991), tumor (Meckley and Zwicker, 1979), hypercholesterolemia (Dictenberg et al., 1995), and cerebral ischemia (Levine and Payan, 1966). This species has also been used to develop good animal models for a variety of infectious diseases caused by bacteria, viruses, and parasites; for example, *Helicobacter pylori* (Yokota et al., 1991; Sugiyama et al., 1998), Borna disease virus (Nakamura et al., 1999), *Echinococcus multilocularis* (Williams and Oriol, 1976), *Cryptospordium muris* (Koudela et al., 1998), *Brugia-pahangi* (Klei et al., 1981), *Giardia duodenalis* (Buret et al., 1991), and *Entamoeba histolytica* (Chadee and Meerovitch, 1984). Although gerbils were randomly bred in closed laboratory colonies for the first decades of their use, selective breeding has recently been conducted to establish laboratory strains that are suited for each research purpose. The best-characterized strains include seizure-sensitive and -resistant strains (Loskota et al., 1974; Robbins, 1976; Seto-Ohshima et al., 1997) and mutant strains with different coat colors (Robinson, 1973; Shimizu et al., 1990).

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FULL PAPER *Anatomy*

Decreased Expression of Matrix Metalloproteinases and Tissue Inhibitors of Metalloproteinase in the Kidneys of Hereditary Nephrotic (ICGN) Mice

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ABSTRACT. Matrix metalloporoteinases (MMPs), which are dominantly regulated by tissue inhibitors of metalloproteinase (TIMPs), play important roles in extracellular matrix (ECM) degradation and are involved in the progression of kidney diseases. In glomeruli and tubulointerstitum of hereditary nephrotic (ICR-derived glomerulonephritis: ICGN) mouse kidneys, hyper-accumulation of ECM components occurred, and MMP activity decreased. In the present study, because lower levels of MMP activity may contribute to the progression of renal fibrosis in ICGN mice, Western blotting analysis and immunohistochemical staining for MMPs and TIMPs were performed to verify the expression levels of these proteins. Levels of MMP-2, MMP-9, MT1-MMP, TIMP-1 and TIMP-2 in the kidneys were decreased in ICGN mice in comparison with normal ICR mice. These results indicate that small amounts and low levels of activity of MMPs cause the progression of renal fibrosis in ICGN mice.

KEY WORDS: extracellular matrix (ECM), ICR-derived glomerulonephritis (ICGN) mouse, matrix metalloproteinase (MMP), renal fibrosis, tissue inhibitor of metalloproteinase (TIMP).

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The quality and quantity of extracellular matrix (ECM) components are tightly regulated in normal tissue, cell migration, proliferation, apoptotic cell death and so on. A balance between the production and degradation of the ECM is maintained to achieve tissue homeostasis, but is disrupted under pathological conditions [1]. Hyper-production and/or hypo-degradation of the ECM can cause fibrosis in many organs, ex. kidneys, liver, lungs and so on. ECMs are dominantly degraded by serine proteinase, plasmin, and matrix metalloproteinases (MMPs) [14, 15, 20]. Based on substrate specificity, MMPs are classified as follows: (1) MMP-1 (interstitial collagenase), primarily responsible for the degradation of type I collagen; (2) MMP-2 and MMP-9 (gelatinases), dominantly degrade type IV collagen; (3) MMP-3 (stromelysin), has a broad substrate specificity and degrades type IV and V collagens, proteoglycans and laminin; and (4) membrane type MMP (MT-MMP: membrane associated MMP), degrades not only various ECM components, such as type I collagen, but also processes the precursor MMP. Each MMP is secreted as precursor enzyme (pro-MMP; non-active form) into the extracellular space and binds with tissue inhibitor of metalloproteinase (TIMP), which is a dominant regulator of MMP activation. When the degradation of ECM is required, the appropriate TIMP is removed from the MMP via digestion by a proteolytic enzyme (ex. Plasmin, MT-MMP and so on), and pro-MMP is activated and the against ECM.

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ICR-derived glomerulonephritis (ICGN) mice develop severe proteinuria at an early age which progresses to nephrosis [7, 8]. This strain suffers from hypoalbuminemia, hypercholesterolemia, and anemia. Histological studies show a thickened glomerular basement membrane (GBM) and effacement of podocyte foot processes [6–11]. We previously showed that components of the ECM accumulate in glomeruli and tubulointerstitum of ICGN kidneys [11], and that the accumulation was due to hyper-production and less degradation of the ECM [17, 18]. We biochemically measured the activity of MMP-1, MMP-2 and MMP-9 in ICGN kidneys, and found decreased levels in comparison with normal ICR mice [17]. However, the reason for the decrease was not clear. In the present study, to verify whether lower expression levels of these proteins provoke lower levels of activity or not, we compared the expression levels of MMPs (MMP-2, MMP-9 and MT1-MMP) and TIMPs (TIMP-1 and TIMP-2) in the kidneys of ICGN mice and in of normal ICR mice by Western blotting and immunohistochemical staining.

MATERIALS AND METHODS

Animals and tissue preparation: ICGN mice were prepared by mating homozygous males (*nep/nep*) with heterozygous females (*nep/–*) at the laboratory of the National Institute of Infectious Diseases (NIID) [8–11]. Early stage (8-week-old; slightly renal fibrosis) and terminal stage (15-week-old: progressed stage of renal fibrosis) male homozygous ICGN mice from a specific-pathogen-free colony in NIID [16–18] and age- and sex-matched ICR mice pur-

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Morphological appearance of the cryopreserved mouse blastocyst as a tool to identify the type of cryoinjury

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BACKGROUND: If it were possible to deduce the mechanism of injury in cryopreserved embryos by their appearance, it would help to optimize cryopreservation protocols. METHODS: Mouse blastocysts were treated so that they were damaged by the six types of cryoinjuries listed below, and their appearance was observed at recovery in sucrose solution and a modified phosphate-buffered saline (PB1), and after culture for 1 and 24 h. RESULTS: (i) Intracellular ice: the embryos shrank normally in sucrose solution, but swelled in PB1 and collapsed after culture. (ii) Chemical toxicity of the cryoprotectant: the embryos looked normal in sucrose solution and PB1. After 1 h of culture, however, the blastomeres showed decompaction and degenerated thereafter. If the toxicity was extremely high, embryos looked nearly normal in PB1, but the surface of the cytoplasm was wrinkled as if they were 'fixed'. (iii) Osmotic swelling: the embryos looked normal in PB1, but after culture they shrank. (iv) Osmotic shrinkage: the embryos swelled in PB1, and then collapsed. (v) Fracture damage: the zona pellucida of the embryos was dissected. (vi) Extracellular ice: the zona of the embryos was elongated. CONCLUSIONS: It was often possible to deduce the type of injury that had occurred in cryopreserved embryos from their appearance at recovery and during subsequent culture. This may help to improve cryopreservation protocols for embryos of many species, including man.

Key words: blastocyst/cryopreservation/morphology/mouse/vitrification

Introduction

Embryo cryopreservation technology has been applied to the preservation of genetic variants in laboratory animals, to breeding in livestock, and to assisted reproduction in humans. Since each embryo, having the ability to develop into an individual, is valuable, it is important to minimize the decrease in survival after cryopreservation. In some cases, e.g. for mouse morulae, a reliable method without appreciable loss of viability has been established (Kasai *et al.*, 1990; Shaw and Kasai, 2001). In most cases, however, improvement and refinement of the procedure is necessary, or the development of a reliable method is still underway. An example is the human blastocyst. An improved culture system has made it possible to develop IVF embryos to blastocysts, which seems to be a promising option to raise the pregnancy rate (Gardner *et al.*, 1998). Accordingly, the need to cryopreserve human blastocysts is increasing. Various reports have been made on the successful cryopreservation of human blastocysts (Cohen *et al.*, 1985; Ménézo *et al.*, 1992). However, a reliable method which can reproduce high survival rates has not been established, probably because human blastocysts are much less permeable not only to cryoprotectant but also to water (Mukaida *et al.*, 2001).

For the cryopreservation of various mammalian embryos, vitrification has proven to be the preferred strategy; embryos of mice (Kasai *et al.*, 1990), rabbits (Kasai *et al.*, 1992a) and cattle (Ishimori *et al.*, 1993; Tachikawa *et al.*, 1993) have been successfully vitrified at quite high survival rates by a simple method. As yet, limited application has been made in humans, but vitrification also seems to be a promising strategy in fertility centres, since successful vitrification of IVF embryos has recently been reported using cryostraws (Mukaida *et al.*, 1998; Yokota *et al.*, 2001) or cryoloops (Mukaida *et al.*, 2001). In vitrification, however, a slight difference in the conditions for embryo handling will lead to a great difference in the survival of cryopreserved embryos, because the time and temperature of exposure of embryos to the vitrification solution before cooling is critical. In addition, the concentration of the vitrification solution surrounding each embryo may vary depending on the skill of the handler and even on the instrument (i.e. pipettes) used.

To find optimal conditions for embryo cryopreservation, it is essential to identify the mechanism by which embryos are injured in each protocol or procedure. Embryos are at risk of various types of injuries during cryopreservation (Kasai, 1996, 2002). The main injuries are those from intracellular ice and concentrated solutes. In slow freezing of embryos, intracellular ice is a major cause of injury (Whittingham *et al.*, 1972), whereas in vitrification, the effect of the chemical toxicity of a high concentration of cryoprotectant is a major obstacle (Rall

生殖医療における凍結技術

葛西孫三郎

Summary

細胞を凍結保存するとさまざまな種類の傷害が生じる可能性がある。ウシやヒトの精子は比較的簡単に凍結することができるが，胚や卵子は大型の細胞であり，特に細胞内氷晶による傷害を受けやすい。胚の凍結方法として，緩慢凍結法が確立されているが，より簡便で生存性の向上が期待されるガラス化法も普及してきた。最近では，保存液量を減らした超急速ガラス化法が有効なことも報告されている。未受精卵は，胚とほぼ同様の方法で凍結が可能であるが，生存性の改善が必要である。

Key words

精子●胚●卵子
凍結保存●ガラス化

はじめに

人工授精や体外受精などの生殖工学的技術を用いるためには，精子や胚の凍結保存はきわめて重要な技術である。主要な家畜であるウシでは，古くから凍結精子による人工授精技術が確立されている。また，胚移植技術も普及し，ウシなどの家畜の改良やマウスなどの実験動物の系統保存のために胚の凍結保存技術が利用されている。精子や胚の凍結保存技術は，ヒトの不妊治療にも応用され，人工授精用の精子や体外受精によって得られた余剰胚が凍結保存されている。凍結した細胞の生存性は，動物種や細胞の種類によって異なるが，基本的な生存の原理は同じである。本稿では，低温生物学的な視点から，精子，胚および卵子の凍結保存の概要を述べる。

細胞凍結保存の原理

細胞を長期間保存するためには，液体窒素(−196℃)で保存する必要がある。細胞を生理的溶液のまま凍結すると，ほとんどの場合死滅してしまう。特に胚や卵子は大型の細胞をもっており，全く生存できない。細胞の凍結保存には，保護物質(耐凍剤)が必要である。しかし，凍結融解した細胞の生存性は，耐凍剤の種類・濃度・添加方法，冷却速度，融解速度，耐凍剤除去方法などの条件によって左右される。凍結融解操作の過程で，細胞は以下に示すようなさまざまな種類の傷

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卵細胞と初期胚の凍結保存

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はじめに

哺乳動物の卵子や初期胚は，通常の細胞と比べると巨大な体積を持っており，グリセロールなどの保護物質（耐凍剤）を用いても，培養細胞や精子と同じ条件では凍結保存することができない．現在，マウス，ウシ，ヒトなどの胚の凍結保存に広く用いられている方法は，緩慢凍結法である．しかし，緩慢凍結法は手間と時間を要し，温度制御装置（プログラムフリーザー）を必要とする．さらに，生存性の面からも改善の余地があり，凍結保存法の改善が続けられてきた．凍結手順の革新的な簡便化をもたらしたのはガラス化（Vitrification）法である．ガラス化法はきわめて高濃度の耐凍剤を用いることによって，室温から直接液体窒素で冷却することを可能にした．さらに最近では，ガラス化法の変法である超急速ガラス化法が，従来凍結保存が困難であった卵子や胚の凍結に有効なことが報告されている．

凍結した細胞の生存性は，動物種や細胞の種類，さらに凍結法によって異なるが，基本的な生存の原理は同じである．従来，凍結保存に関する研究は，経験的なアプローチが多かったが，凍結した細胞の生存のメカニズムが明らかになるにつれて，より論理的なアプローチが取り入れられるようになってきた．

本稿では，低温生物学的視点に基づいて，卵子や胚の凍結保存の概要と，最近の超急速ガラス化法について紹介したい．

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1．卵子と胚の低温生物学

1 傷害メカニズム

細胞を長期間保存するためには，細胞を浸透圧の高い濃縮した状態にして，ガラス転移温度（−130°C付近）以下まで冷却しなければならない．濃縮した水溶液や細胞質を−130°C以下まで冷却すると，結晶（氷晶）を形成しないまま固体となる．すなわちガラス化する．細胞の長期保存にはガラス化が必要であり，そのために液体窒素（−196°C）が用いられている．細胞を生理的溶液のまま凍結すると，ほとんどの場合死滅してしまう．特に胚や卵子の細胞は大型であるので，まったく生存できない．卵子や胚の凍結保存には，耐凍剤を透過させ，さらに細胞内を濃縮することが必要である．しかし，凍結融解後の生存性は，

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Artificial Expression of Aquaporin-3 Improves the Survival of Mouse Oocytes after Cryopreservation[1]

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ABSTRACT

Successful cryopreservation of mammalian cells requires rapid transport of water and cryoprotective solutes across the plasma membrane. Aquaporin-3 is known as a water/solute channel that can transport water and neutral solutes such as glycerol. In this study we examined whether artificial expression of aquaporin-3 in mouse oocytes can improve water and glycerol permeability and oocyte survival after cryopreservation. Immature mouse oocytes were injected with aquaporin-3 cRNA and were cultured for 12 h. Then the hydraulic conductivity (L_p) and glycerol permeability (P_{GLY}) of matured oocytes were determined from the relative volume changes in 10% glycerol in PB1 medium at 25°C. Mean ± SD values of L_p and P_{GLY} of cRNA-injected oocytes (3.09 ± 1.22 μm min^{-1} atm^{-1} and 3.69 ± 1.47 × 10^{-3} cm/min, respectively; numbers of oocytes = 25) were significantly higher than those of noninjected oocytes (0.83 ± 0.02 μm min^{-1} atm^{-1} and 0.07 ± 0.02 × 10^{-3} cm/min, respectively; n = 13) and water-injected oocytes (0.87 ± 0.10 μm min^{-1} atm^{-1} and 0.08 ± 0.02 × 10^{-3} cm/min, respectively; n = 20). After cryopreservation in a glycerol-based solution, 74% of cRNA-injected oocytes (n = 27) survived as assessed by their morphological appearance, whereas none of the water-injected oocytes survived (n = 10). When cRNA-injected oocytes that survived cryopreservation were inseminated in vitro, the penetration rate was 40% (n = 48) and the cleavage rate was 31% (n = 70), showing that oocytes retain their ability to be fertilized. This is the first report to show that artificial expression of a water/solute channel in a cell improves its survival after cryopreservation. This approach may enable cryopreservation of cells that have been difficult to cryopreserve.

oocyte development

INTRODUCTION

Cryopreservation of mammalian embryos has been used for various purposes (i.e., for preservation of genetic variants in laboratory animals, for breeding and reproduction of farm animals, and for treatment of infertility in humans) [1]. Successful cryopreservation of oocytes would also greatly assist the application of several reproductive biotechnologies, such as in vitro production of embryos, treatment of infertility, cloning, and gene banking [1]. However, oocyte cryopreservation has been achieved only in a limited number of mammalian species.

For successful cell cryopreservation to occur, it is important that water and cryoprotective solutes be transported across the plasma membrane. There are two mechanisms by which water and cryoprotectants move across the plasma membrane: by simple diffusion across the membrane and by a channel-mediated process. During the last decade, small intrinsic membrane proteins that act as water channels were discovered and have been characterized [2]. These proteins, called aquaporins (AQPs), occur in two types in mammals: one subgroup, including AQP1, AQP2, AQP4, and AQP5, is highly selective for the passage of water, and the other subgroup, including AQP3, AQP7, and AQP9, transports water as well as neutral solutes with a small molecular weight, such as glycerol. Because glycerol is a representative cryoprotectant, expression of the latter subgroup of AQPs might be relevant to the survival of cryopreserved cells. Specifically, if a channel that transports water and neutral solutes such as AQP3 can be artificially expressed in cells, it may be possible to improve the survival of the cells that presently suffer high mortality during cryopreservation.

Vitrification, an innovative, rapid method for cryopreserving mammalian embryos and oocytes, has been extensively studied by many cryobiologists because mammalian embryos and oocytes can be cryopreserved quickly and simply by this method [3]. However, for successful vitrification to occur, high concentrations of cryoprotectants are required, which may damage the cells, probably by cryoprotectant toxicity. On the other hand, insufficient exposure can cause intracellular ice formation. To minimize damaging exposure times to cryoprotectants, rapid movement of water and cryoprotectants through the plasma membranes is essential. Mouse oocytes have been cryopreserved successfully and effectively by slow-freezing methods, mostly using DMSO as the cryoprotectant [4], and also by vitrification using DMSO [5] or ethylene glycol [6]. However, using a glycerol-based solution, mouse oocytes have not been successfully cryopreserved by vitrification, because the permeability of mouse oocytes to glycerol is low [7, 8]. Therefore, vitrification of mouse oocytes coupled with glycerol is a good model for examining changes in the permeability and survival after cryopreservation through artificial expression of water/cryoprotectant channels. In this study we examined whether artificial expression of AQP3 in mouse oocytes can improve their water and glycerol permeability, and their survival after vitrification with glycerol.

MATERIALS AND METHODS

Preparation of AQP3 cRNA

AQP3 cDNA was cloned from rat kidney cDNA by polymerase chain reaction (PCR). The sense strand was 5'-CGGGATCCCATGGGTCGA-

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Vitrification of human blastocysts using cryoloops: clinical outcome of 223 cycles*

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BACKGROUND: The need to cryopreserve human blastocysts is increasing. The successful birth has been reported of a baby from a blastocyst vitrified using the cryoloop technique. The present study expands on this earlier report to confirm the effectiveness of this vitrification procedure. METHODS: In patients undergoing IVF at one of three clinics, supernumerary blastocysts on day 5 or 6 at various stages of development were vitrified using cryoloops. RESULTS: Of 725 vitrified blastocysts, 583 (80.4%) survived. After the transfer of 493 blastocysts in 207 cycles, 76 women (37%) became clinically pregnant. Among these women, 21 pregnancies ended in miscarriage, 23 healthy babies were born in 18 deliveries, and 37 pregnancies are ongoing. The survival rate of day 5 blastocysts (87%) was higher than that of day 6 blastocysts (55%), but implantation rates and pregnancy rates were not statistically significantly different. CONCLUSIONS: Clinical outcomes with 725 blastocysts and 207 transfers showed that vitrification using cryoloops is effective and practical for the cryopreservation of human blastocysts. Early blastocysts on day 5 seem to be the most suitable in terms of stage and age for cryopreservation, but developed and day 6 blastocysts can also be cryopreserved.

Key words: blastocyst/cryoloop vitrification/cryopreservation/embryo/human

Introduction

In assisted reproductive technology, the cryopreservation of embryos has proved important for the best use of supernumerary embryos. In the cryopreservation of embryos, there is a risk of various types of injury (Kasai, 1996; Kasai *et al.*, 2002), among which the formation of intracellular ice appears to be the most damaging. The first strategy to prevent intracellular ice formation was to adopt a lower concentration of cryoprotectant and a long slow-cooling stage. This slow-freezing method has proven effective for embryos of a wide range of mammalian species. Unlike embryos of laboratory animals and domestic animals, in which dimethyl sulphoxide (DMSO), glycerol or ethylene glycol (EG) is commonly used as the cryoprotectant, human embryos at early cleavage stages have most often been frozen in a solution of propanediol supplemented with sucrose (Lassalle *et al.*, 1985), although those at the blastocyst stage have more frequently been frozen with glycerol (Fehilly *et al.*, 1985; Hartshorne *et al.*, 1991; Ménézo *et al.*, 1992). With slow freezing, however, it is difficult to eliminate injuries from ice completely. Furthermore, the slow-freezing method requires a long period of time before the embryos can be stored in liquid nitrogen.

In 1985, the first report was made of an innovative approach called vitrification, in which injuries related to ice are minimized by using very high concentrations of cryoprotectant (Rall and Fahy, 1985). This approach simplifies the cooling process, because embryos can be cooled directly in liquid nitrogen. Although embryos subjected to vitrification are liable to be injured by the toxicity of the high concentration of cryoprotectant, the method has been refined and proven effective for the cryopreservation of embryos at various stages of development in laboratory and domestic species. In 1998, it was shown that vitrification using an EG-based vitrification solution (EFS40) (Kasai *et al.*, 1990) with conventional cryo-straws was effective for human embryos at the 4- to 8-cell stage (Mukaida *et al.*, 1998). The effectiveness of vitrification was confirmed for human embryos at the 8- to 16-cell stage (Saito *et al.*, 2000) and the morula stage (Yokota *et al.*, 2001b), also using EG-based solutions.

Recent advances in culture systems with sequential media have made it possible to develop human IVF embryos into blastocysts quite easily. Because the blastocyst is better suited to the uterine environment, and because blastocyst formation is

*Part of this work (126 transfers) was presented in a symposium in Williamsburg USA in April 2002, and a summary of the presentation is published in RBM online (2003)

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Theriogenology

Vitrification of rat embryos at various developmental stages

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Abstract

The effect of developmental stage on the survival of cryopreserved rat embryos was examined. Wistar rat embryos at various developmental stages were vitrified by a 1-step method with EFS40, an ethylene glycol-based solution, or by a 2-step method with EFS20 and EFS40. After warming, the survival of the embryos was assessed by their morphology, their ability to develop to blastocysts (or expanded blastocysts for blastocysts) in culture, or their ability to develop to term after transfer. Most (91–100%) of the embryos recovered after vitrification were morphologically normal in all developmental stages. However, the developmental ability of 1-cell embryos was quite low; exposing them to EFS40 for just 0.5 min decreased the in vitro survival rate from 76 to 9%. The survival rates of 2-cell embryos and blastocysts, both in vitro and in vivo, were significantly higher with a 2-step vitrification process than with a 1-step vitrification process. Very high in vitro survival rates (94–100%) were obtained in 4- to 8-cell embryos and morulae in the 1-step method. Although survival rates in vivo of 4-cell (40%) and 8-cell (4%) embryos vitrified by the 1-step method were comparatively low, the values were similar to those obtained in non-vitrified fresh embryos. When morulae vitrified by the 1-step method were transferred to recipients, the in vivo survival rate (61%) was high, and not significantly different from that of fresh embryos (70%). These results show that rat embryos at the 2-cell to blastocyst stages can be vitrified with EFS40, and that the morula stage is the most feasible stage for embryo cryopreservation in this species.

Keywords: Developmental stage; EFS; Embryo transfer; Rat; Vitrification

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Articles

Blastocyst cryopreservation: ultrarapid vitrification using cryoloop technique

Dr T Mukaida

Dr Mukaida completed his certified training in Obstetrics and Gynaecology at the Kochi Medical School Hospital, Japan, in 1990. During that training, he studied abroad to the University of Miami School of Medicine, in the Biochemistry and Molecular Biology department. His area of study was the mechanism of ovulation related to serine protease (plasminogen, collagenase). Simultaneously he joined the IVF programme in the Reproductive Endocrinology department at the same institution as an embryologist. A period as lab director in a private fertility clinic, the Diamond Institute for Infertility in New Jersey, USA, enabled him to study assisted reproductive technology. For the last 7 years, he has worked as clinical director of the Hiroshima HART Clinic, where he is responsible for managing assisted reproduction in patients for 500 oocyte retrievals per year. Dr Mukaida's main interest lies in vitrification for gametes and embryos: he is responsible for the two first reports in the world of successful birth from vitrified embryos. Both of these day 2–3 embryos were cryopreserved by vitrification with straw and the blastocysts by ultra-rapid vitrification using a cryoloop technique.

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Abstract

Human embryos have been cryopreserved mainly by slow freezing, but vitrification has also proven effective for embryos at early cleavage stages. However, clinical results on blastocyst cryopreservation have not been consistent. A feasible option appears to be ultrarapid vitrification, in which embryos are vitrified with a reduced amount of solution to achieve extremely high rates of cooling and warming. The cryoloop is a tiny nylon loop connected to the lid by a small metal tube; a metal insert on the lid enables the use of a stainless steel handling rod with a small magnet, and the loop can be stored in the cryovial. In the HART Clinic group, of 444 supernumerary human blastocysts that were vitrified by cryoloops 79% survived after warming, and of 126 recipients 36% became pregnant. The outline of ultrarapid vitrification using cryoloops is described.

Keywords: *blastocyst, cryoloop, cryopreservation, human, vitrification*

Introduction

The cryopreservation of embryos has become a powerful tool in human assisted reproduction. Various methods have been developed for the cryopreservation of mammalian embryos. Each method must prevent injury from intracellular ice forming. To prevent this, cells must be solidified without crystallization, i.e. the cells must be vitrified.

Cryopreservation methods can be broadly classified as slow freezing and vitrification. The slow freezing method makes use of relatively low concentrations of cryoprotectant and a long slow cooling stage, by which cells are concentrated gradually (Whittingham *et al.*, 1972) (**Figure 1**). To alter and program specific cooling rate, the use of a machine is necessary. Vitrification, by contrast, enables rapid cooling of samples by direct plunging into liquid nitrogen from temperatures above 0°C without ice forming even in the extracellular medium, by use of a very high concentration of cryoprotectant (Rall and Fahy, 1985) (**Figure 1**). In vitrification, the chance of intracellular ice forming can be minimized, but toxic effect of cryoprotectant on the cell becomes a great obstacle.

Recently, several modified vitrification methods have been devised which require a reduced volume of concentrated solution and thus enable ultrarapid cooling and warming (Kasai, 2002) (**Figure 1**). Ultrarapid vitrification of human blastocysts has been attempted, and one successful delivery reported (Mukaida *et al.*, 2001). Subsequently, this approach has been continued for the cryopreservation of human blastocysts. This report gives an outline of a ultrarapid vitrification method using cryoloops.

Cryopreservation of human embryos

Human embryos are usually cryopreserved by slow freezing, using propanediol as the cryoprotectant (Lassalle *et al.*, 1985). However, it has been shown that vitrification using an ethylene

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In Vivo Development of Vitrified Rat Embryos: Effects of Timing and Sites of Transfer to Recipient Females[1]

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ABSTRACT

In cryopreserved rat embryos, survival rates obtained in vitro are not always consistent with the rates obtained in vivo. To determine the optimal conditions for in vivo development to term, rat embryos at the 4-cell, 8-cell, and morula stages were vitrified in EFS40 by a one-step method and transferred into oviducts or uterine horns of recipients at various times during pseudopregnancy. Vitrified and fresh 4-cell embryos only developed after transfer into oviducts of asynchronous recipients on Days −1 to −2 of synchrony (i.e., at a point in pseudopregnancy 1–2 days earlier than the embryos). Approximately half the vitrified embryos transferred into oviducts on Day −1 developed to term, but only a minority of embryos, whether vitrified (10%–34%) or fresh (24%–33%), transferred at later times did so, suggesting that this may not be the most suitable stage for cryopreservation. Very few 8-cell embryos, either vitrified or fresh, developed when transferred into oviducts on Day 0 to −0.5. However, when transferred into uterine horns, high proportions of vitrified 8-cell embryos (~63%) developed to term in reasonably synchronous recipients (Day 0 to −0.5) but not in more asynchronous ones (6%; Day −1). A majority of vitrified morulae also developed to term (52%–68%) in a wider range of recipients (Days 0 to −1), the greatest success occurring in recipients on Day −0.5. Similar proportions of vitrified and fresh 4-cell embryos, 8-cell embryos, and morulae developed to term when appropriate synchronization existed between embryo and recipient. Thus, vitrification of preimplantation-stage rat embryos does not appear to impair their developmental potential in vivo.

early development, embryo, oviduct, pregnancy, uterus

INTRODUCTION

Transfer of embryos to recipient females has become a valuable experimental tool, particularly in the fields of embryology and genetics [1]. Since Heape [2] performed the first successful embryo transfer in the rabbit, many studies have been carried out on the transfer of mammalian embryos. In rats, the first transfer experiment constituted an embryo viability test and established the importance of synchrony between donors and recipients [3]. Later studies demonstrated that the development of transferred embryos is dependent on close synchronization between embryonic development and endometrial preparation in a number of mammalian species, such as rabbits [4, 5], mice [6], sheep [7], rats [8], cattle [9], and ferrets [10]. It has also been shown that asynchrony is more tolerated when embryos are at a more progressed stage than the recipient uteri [6, 8, 10].

Rat embryos have been successfully cryopreserved at various developmental stages, such as the 1-cell [11, 12], 2-cell [13–15], 4-cell [13], 8-cell [13, 16–18], morula [19], and late-morula to early blastocyst [14] stages. In many cases, survival of the embryos was assessed by transfer to recipients, probably because the in vitro culture system for rat embryos was not as effective as that for mouse embryos. Consequently, reported survival rates have been variable but generally low.

In a recent study [20], we compared the survival of vitrified rat embryos, ranging from the 1-cell to the blastocyst stage, using an efficient culture system (in vitro) and a successful embryo-transfer technique (in vivo). Because very high proportions (94%–100%) of vitrified embryos developed in vitro, we concluded that the 4-cell, 8-cell, and morula stages are suitable for embryo cryopreservation; furthermore, the good developmental potential in vitro of these embryos led us to expect they would develop equally well in vivo. However, the in vivo survival rate of vitrified 4-cell embryos was relatively low (40%) and that of 8-cell embryos extremely low (4%), although similar poor results were obtained with fresh embryos (29% and 5%, respectively). In contrast, the in vivo survival rate of vitrified morulae (61%) was high and very similar to that of fresh embryos (70%). In that study, both fresh and vitrified 4- and 8-cell embryos were transferred into oviducts of pseudopregnant recipients that were at a point in pseudopregnancy 1 day earlier than the embryos (Day −1 of synchrony), whereas morulae were transferred to uterine horns of synchronous recipients (Day 0).

We hypothesized that it might be possible to improve the in vivo survival of vitrified embryos at all stages by adjusting the synchrony/asynchrony with respect to the recipient females and by altering the site of transfer. The present study was undertaken to test this hypothesis using rat embryos vitrified at the 4-cell, 8-cell, and morula stages.

MATERIALS AND METHODS

All experiments were conducted in accordance with the International Guiding Principles for Biomedical Research Involving Animals as promulgated by the Society for the Study of Reproduction.

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Symposium: Cryopreservation and assisted human conception

Cryopreservation of animal and human embryos by vitrification

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Abstract

Vitrification is a method in which not only cells but also the whole solution is solidified without the crystallization of ice. For embryo cryopreservation, the vitrification method has advantages over the slow freezing method. For example, injuries related to ice is less likely to occur, embryo survival is more likely if the embryo treatment is optimized, and embryos can be cryopreserved by a simple method in a short period without a programmed freezer. However, solutions for vitrification must include a high concentration of permeating cryoprotectants, which may cause injury through the toxicity of the agents. Since the development of the first vitrification solution, which contained dimethylsulphoxide, acetamide, and propylene glycol, numerous solutions have been composed and reported to be effective. However, ethylene glycol is now most widely used as the permeating component. As supplements, a macromolecule and/or a small saccharide are frequently added. Embryos of various species, including humans, can be cryopreserved by conventional vitrification using insemination straws or by ultrarapid vitrification using minute tools such as electron microscopic grids, thin capillaries, minute loops, or minute sticks, or as microdrops. In the ultrarapid method, solutions with a lower concentration of permeating cryoprotectants, thus having a lower toxicity, can be used, because ultrarapid cooling/warming helps to prevent ice formation.

Keywords: *cryopreservation, embryo, ethylene glycol, human, vitrification*

Introduction

In cryopreservation, cells are suspended in a suitable solution, cooled, stored in liquid nitrogen, warmed to room temperature, and returned to a physiological solution. During each step of this process, cells are at risk for various types of injuries. The primary injury is that caused by the formation of intracellular ice during cooling and warming. To prevent this injury, inclusion of a cryoprotectant is essential for large cells like mammalian embryos. However, the cryoprotectant brings other causes of injuries, i.e. chemical toxicity of the agent and osmotic over-swelling of the cells during removal of the permeated cryoprotectant. During the removal, embryos are usually exposed to a hypertonic solution with sucrose, and embryos can be injured by osmotic over-shrinkage in some cases. In addition, embryos can be dissected physically by a fracture plane if such a plane is formed in the medium and traverses the embryos during passage through the glass transition temperature. Furthermore, certain types of embryos are injured just by chilling at 20–0°C. In order for embryos to survive cryopreservation, the effect of each of these injuries must be minimized (Kasai *et al.*, 2002).

Vitrification is a reasonable and effective strategy for preventing the primary cause of injury, that is, intracellular ice formation. Fracture damage and chilling injury may also be minimized in vitrification. Vitrification also has advantages over slow freezing in that survival of embryos is more likely if the embryo treatment is optimized, and embryos can be cryopreserved by a simple method in a short period without a programmed freezer. This study provides a brief outline of cryopreservation of animal and human embryos by vitrification.

2 体外受精・顕微授精　④ 難治性不妊症に対する体外受精の工夫

卵子の凍結保存

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はじめに

ヒト不妊治療のためのARTの進歩とともに，凍結保存技術の果たす役割は増大している．体外受精で得られる余剰胚の凍結保存は，妊娠率の向上，多胎の防止，患者の負担軽減に役立てられている．さらに，受精前の卵子を凍結保存する試みが増加してきた．卵子の凍結保存は，未婚女性が放射線療法や化学療法によって卵巣内卵子に傷害を受ける場合や，卵子採取時に精子が得にくい場合などに利用できる．また，卵子提供が認められる場合にも有効な手段となる．さらに，胚に比べて取り扱いに倫理的制約が少ない利点もある．

1977年に，マウスにおいて，胚と同様の方法を用いて第2成熟分裂中期（MII期）の卵子を凍結保存できることが示された．また，1989年にはウサギで，1992年にはウシで，凍結保存したMII期卵子由来の産子が得られた．ヒトにおいては凍結胚による初の出産例が報告されたわずか2年後の1986年に，凍結保存したMII期卵子を用いた妊娠例が報告されている．しかし動物，ヒトのいずれにおいても，凍結保存した卵子の生存率や受精後の着床・発生率は凍結胚に比べてかなり低く，保存技術は実用的に普及するレベルには達していない．

卵巣中には，減数分裂前期の卵核胞期（GV期）にある未成熟な卵子が多数存在しており，ウシやブタの体外受精には，発育したGV期卵子が用いられている．一方，通常ヒト卵子は受精直前のMII期に回収されるが，GV期で回収されるケースも想定される．GV期卵子の凍結保存も試みられ，マウスにおいては1994年に，ウシにおいては1996年に，そしてヒトでは1998年に，それぞれ凍結保存したGV期卵子由来の産子が得られている．これらの成功を同じ種におけるMII期卵子の最初の成功と比較すると，マウスでは17年，ウシでは4年，ヒトでは12年遅れている．このことは，GV期卵子はMII期卵子よりもさらに凍結保存が困難なことを示している．

卵巣内の未成熟な卵子を凍結保存して利用する手段として，卵巣ごと凍結する手段が考えられる．メン羊，マウス，ラットでは，凍結保存した卵巣組織由来の産子が得られている．ヒトにおいても最近，卵巣組織の凍結保存の試みが多数報告されるようになってきた．卵巣の凍結は，凍結保存技術とともに，卵巣移植や未熟な卵子の体外発育技術が進めば，卵子の有効な保存法として期待できる．

本稿では卵子の凍結保存について，技術的側面から概説する．

1. 卵子の低温生物学的特性

哺乳動物卵子は特別大型の細胞で，細胞質の約85％は水分である．生理的な溶液に浸した卵子をそのまま氷点下まで冷却すると，細胞内の水分も凍結し氷晶が生じる．細胞内の氷晶は細胞の構造を物理的に破壊し，致命的な損傷を与える．卵細胞内に氷晶を形成させないためには，保存液に耐凍剤を加える必要がある．ジメチルスルフォキシド（DMSO），グリセロール，プロピレングリコール，エチレングリコール，アセトアミドなどの耐凍剤は，いずれも分子量100以下の荷電していない物質で，保存液と細胞質の浸透圧を高めることによって細胞内氷晶を防止する働きがある．

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Water- and cryoprotectant-permeability of mature and immature oocytes in the medaka (*Oryzias latipes*)☆

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Abstract

The permeability of the plasma membrane plays a crucial role in the successful cryopreservation of oocytes/embryos. To identify a stage feasible for the cryopreservation of teleost oocytes, we investigated the permeability to water and various cryoprotectants of medaka (*Oryzias latipes*) oocytes at the germinal vesicle (GV) and metaphase II (MII) stages. In sucrose solutions, the volume changes were greater in GV oocytes than MII oocytes. Estimated values for osmotically inactive volume were 0.41 for GV oocytes and 0.74 for MII oocytes. Water-permeability (μm/min/atm) at 25 °C was higher in GV oocytes (0.13 ± 0.01) than MII oocytes (0.06 ± 0.01). The permeability of MII oocytes to various cryoprotectants (glycerol, propylene glycol, ethylene glycol, and DMSO) was quite low because the oocytes remained shrunken during 2 h of exposure in the cryoprotectant solutions at 25 °C. When the chorion of MII oocytes was removed, the volume change was not affected, except in DMSO solution, where dechorionated oocytes shrunk and then regained their volume slowly; the P_{DMSO} value was estimated to be $0.14 \pm 0.01 \times 10^{-3}$ cm/min. On the other hand, the permeability of GV oocytes to cryoprotectants were markedly high, the P_s values ($\times 10^{-3}$ cm/min) for propylene glycol, ethylene glycol, and DMSO being 2.21 ± 0.29, 1.36 ± 0.18, and 1.19 ± 0.01, respectively. However, the permeability to glycerol was too low to be estimated, because GV oocytes remained shrunken after 2 h of exposure in glycerol solution. These results suggest that, during maturation, medaka oocytes become less permeable to water and to small neutral solutes, probably by acquiring resistance to hypotonic conditions before being spawned in fresh water. Since such changes would make it difficult to cryopreserve mature oocytes, immature oocytes would be more suitable for the cryopreservation of teleosts.

Keywords: Medaka; Oocyte; Cryoprotectant; Water; Permeability

Long-term storage of embryos is useful for the management of various stocks of model organisms. Such storage has been used for several mammalian embryos. However, the cryopreservation of

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Successful Cryopreservation of Mouse Ovaries by Vitrification[1]

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ABSTRACT

We developed a new method of cryopreservation of whole ovaries by vitrification using DAP213 (2 M dimethyl sulfoxide, 1 M acetamide, and M propylene glycol) as a cryoprotectant. Four-week-old C57BL/6 mice that underwent partial ovariectomy were orthotopically transplanted with cryopreserved or fresh ovaries (experimental or control group) isolated from 10-day-old green fluorescent protein (GFP)-transgenic mice (+/+). GFP-positive pups were similarly obtained from both groups by natural mating or in vitro fertilization (IVF) followed by embryo transfer, indicating that the cryopreserved ovaries by vitrification retain their fecundity. However, a statistically significant difference ($P < 0.05$) was found between both groups with respect to the following parameters: the number of GFP-positive pups born by natural mating/grafted ovary (0.8 ± 0.3 for the experimental group versus 2.0 ± 0.7 for the control group, mean ± SEM), the number of collected oocytes by superovulation per mouse (7.0 ± 1.7 for the experimental group versus 22.7 ± 3.2 for the control group), the percentage of two-cell embryos obtained from GFP-positive oocytes by IVF (38.5% for the experimental group versus 90.0% for the control group). Histologically, normal development of follicles and formation of corpora lutea were observed in frozen-thawed grafts. However, estimated number of follicles decreased in frozen-thawed ovaries compared with fresh ovaries. Taken together, cryopreservation of the ovary by vitrification seems a promising method to preserve ovarian function, but further studies are required to overcome the possible inhibitory effects of this method on the growth of the ovarian graft.

in vitro fertilization, ovary, ovulation, ovum pick-up/transport, pregnancy

INTRODUCTION

The cryopreservation of ovarian tissue is potentially a useful technology for preservation of genetic resources of experimental, domestic, and wild animals. Moreover, it is employed in clinical medicine to restore the fecundity of young women suffering from infertility and premature menopause due to iatrogenic loss of ovarian function resulting from chemotherapy and/or radiation therapy of malignant neoplasms. Parrot reported the birth of live offspring after orthotopic transplantation of a slice of cryopreserved mouse ovary [1]. Since then, various cryoprotectants have been developed [2–7], and slow freezing methods to control freezing rate have been employed for the cryopreservation of ovaries.

The vitrification method was applied to the field of biology as a method of cryopreservation, and its recent development has greatly simplified the cryopreservation procedures. Vitrification can essentially be defined as the solidification of a liquid brought about not by crystallization but by an extreme elevation in viscosity during cooling [8]. Extensive studies have proven the usefulness of this method for cryopreservation of oocytes and embryos. The survival rates of oocytes and embryos after the freezing-thawing procedure by vitrification are comparable with those by slow freezing methods [9].

Although the technical simplicity of vitrification has revolutionized cryopreservation of oocytes and embryos, its application to whole ovaries has been considered to be difficult. In the present study, we examined whether ovaries retain their fecundity after cryopreservation by vitrification utilizing DAP213 solution [10] (2 M dimethyl sulfoxide, 1 M acetamide, and 3 M propylene glycol) as a cryoprotectant. For this purpose, we investigated the fertility of mice that had undergone orthotopic transplantation of the cryopreserved ovaries by natural mating and in vitro fertilization (IVF) followed by embryo transfer (ET). In order to distinguish graft-derived pups and oocytes and embryos from those derived from residual host ovarian tissue, we employed jellyfish green fluorescent protein (GFP)-transgenic mice [11] that express the GFP transgene in the entire body as sources of ovarian grafts.

MATERIALS AND METHODS

Animals

Female and male C57BL/6 and female ICR mice were purchased from CLEA Japan Inc. (Tokyo, Japan). GFP-transgenic C57BL/6TgN(act-EGFP) OsbY01 mice (+/+) [11], referred to as GFP-transgenic mice in this article, were kindly provided by Dr. Okabe of the Genome Information Research Center at Osaka University and were bred in our animal facilities under specific pathogen-free conditions. Mice were housed under a 12L: 12D regimen at 22°C and 55% humidity. Food and water were freely available at all times. All the experiments using animals were in accordance with the International Guiding Principle for Biomedical Research Involving Animals and the experimental protocol was approved by the Ethics Committee for Experimental Animals of our institute.

Collection of GFP+ Ovaries

In vitro fertilization and embryo transfer were performed to obtain the offspring of GFP-transgenic mice (+/+). Briefly, female homozygous GFP-transgenic mice were superovulated by injections of 5 IU pregnant mare serum gonadotropin (serotropin) and 5 IU hCG (gonatropin), obtained from Teikoku Hormone Mfg. Co., Ltd. (Tokyo, Japan), at 48-h

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