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SYMPOSIUM: OOCYTE CRYOPRESERVATION
REVIEW

Reprint of: Theoretical and experimental basis of slow freezing

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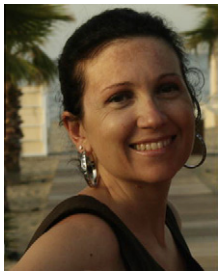

Lucia De Santis graduated in biological sciences at the University of Milan in 1993. During 1989–1994, she worked in the surgical pathology department in the university. In 1995, she established the IVF laboratory of S. Raffaele Hospital, Milan of which she is laboratory director and where she has developed a large IVF programme and introduced basic and more advanced techniques. Lucia has been awarded a Masters in reproductive medicine by University of Padova and is lecturer of the Masters in biotechnology applied to assisted reproduction at Vita-Salute University, Milan. Her current interests concern fundamental and clinical aspects of oocyte cryopreservation.

Abstract In human IVF, cryopreservation of oocytes has become an alternative to embryo storage. It has also shown enormous potential for oocyte donation, fertility preservation and animal biotechnology. Mouse oocytes have represented the elective model to develop oocyte cryopreservation in the human and over several decades their use has made possible the development of theoretical and empirical approaches. Progress in vitrification has overshadowed slow freezing to such an extent that it has been suggested that vitrification could soon become the exclusive cryopreservation choice in human IVF. However, recent studies have clearly indicated that human embryo slow freezing, a practice considered well established for decades, can be significantly improved by a simple empirical approach. Alternatively, recent and more advanced theoretical models can predict oocyte responses to the diverse factors characterizing an entire slow-freezing procedure, offering a global method for the improvement of current protocols. This gives credit to the notion that oocyte slow freezing still has considerable margins for improvement.

KEYWORDS: cryopreservation, cryoprotectants, fertility preservation, oocytes, slow freezing

Introduction

Cryopreservation of oocytes lends itself to numerous applications, some of which have recently become normal practice in many IVF laboratories. In infertility treatments, storage of mature metaphase II (MII) oocytes ensures that the whole reproductive potential of an ovarian stimulation cycle may be conveniently used, at the same time circumventing legal and ethical problems derived from the

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generation of surplus embryos. Oocyte storage has also enormous potential for making oocyte donation safer (by allowing a suitable temporal window for donor screening) and for giving a hope for the preservation of fertility in women at risk of premature loss of ovarian function. In animal biotechnology, oocyte cryopreservation can preserve economically important and genetically modified strains, thereby avoiding the implications of genetic drift.

The first signs of an interest in the low-temperature storage of mammalian oocytes can be traced back as early as the late 1950s, when Sherman and Lin (1958) described the consequences of cooling in mouse oocytes. In 1977, for the first time live births of mice from mature oocytes stored in liquid nitrogen (LN_2) were documented (Whittingham, 1977).

Since these initial achievements, mouse oocytes have represented the elective model to develop oocyte cryopreservation in the human. Experimentally, mouse oocytes are suitable for several reasons. For example, they have plasmalemma permeability characteristics which dictate a rate of shrinkage—swelling response upon exposure to propanediol (PROH) similar to that of human oocytes (Paynter, 2005). Over several decades, their use has made possible the development of theoretical and empirical approaches to oocyte cryopreservation.

In human IVF, in which the first reported pregnancy from cryopreserved oocytes was described in 1986 (Chen, 1986), slow freezing was the method of choice until a few years ago. This approach has generated thousands of births, many of them unreported, and is still being used in numerous laboratories. In recent years, vitrification has gained increasing credit for oocyte cryopreservation, by improving post-storage survival rates and apparently preserving more efficiently oocyte developmental ability (Cobo et al., 2008).

Vitrification has overshadowed slow freezing to such an extent that it has been foreseen that vitrification could soon become the exclusive cryopreservation choice in human IVF (Vajta and Nagy, 2006). However, recent studies have clearly indicated that embryo slow freezing, a practice considered well established for decades, can be significantly improved by a simple empirical approach (Edgar et al., 2009). Alternatively, novel theoretical models have the potential to predict the oocyte response to freezing and thereby indicate protocol modifications which can reduce or eliminate cryoinjury (McGrath, 2009). This gives credit to the notion that slow freezing still includes uncharted territories whose exploration could make oocyte cryopreservation more efficient.

Between empiricism and theory

In the absence of control, freezing is lethal to cells. Because at temperatures well below 0°C, the freezing of water is the prevailing phenomenon and because water constitutes up to 80% of the living matter, it has long been believed that freezing causes cell death exclusively as a consequence of injuries caused by the formation of ice crystals. In reality, the freezing of aqueous solutions involves a number of phenomena that may affect cell viability, such as changes in solute concentrations, osmotic stress and pH alterations. Therefore, successful cryopreservation requires the control of several detrimental factors which interact in a very complex and often poorly understood fashion.

A twist in cryopreservation history occurred when, in fact rather accidentally, Polge, Smith and Park found that solutions containing glycerol were able to preserve the viability of cock spermatozoa for extended periods of time at −80°C (Polge et al., 1949). Other empirical attempts rapidly followed and soon it was realized that cell survival could be improved by cooling samples very slowly. Collectively, these initial experiences led to the definition of conditions generally necessary, and sometimes sufficient, for the slow freezing of different types of cells: (i) permeation with freezing solutions of 10—20% glycerol or molecules with similar properties, thereafter known as cryoprotective agents (CPA); (ii) cooling at rates of −1°C or slower; (iii) storage at −130°C or lower; and (iv) rapid warming.

Protocols applied for freezing mouse oocytes were generated empirically from those previously developed for embryos of the same species (Whittingham, 1971). Generally, mouse oocytes are exposed at low supra-zero temperatures to 1.5 mol/l dimethylsulphoxide (DMSO), cooled slowly (0.3—0.5°C/min) to temperatures between −30°C and −40°C and finally rapidly cooled and stored in LN_2. Thawing is performed slowly (at about 8—20°C/min) or rapidly (200°C/min or faster) before CPA is diluted and samples rehydrated. Further studies have highlighted the importance of other factors, such as chemical identity of CPA (Nash, 1962), temperature and mode of exposure and removal of CPA (Paynter et al., 1997), dehydration (Fabbri et al., 2001), showing how apparently marginal changes in any of the above steps can have important consequences on oocyte survival and development.

Progress has also been achieved in the theoretical understanding of fundamental principles that govern slow freezing. For example, the hypothesis formulated by Mazur (1963) identified excessive intracellular ice formation (IIF) and high solute concentration, derived from the freezing of water, as the major causes of cell injury during slow freezing. Having stood the test of time, the Mazur hypothesis is essentially correct and has inspired several attempts to improve freezing protocols. However, the fact that other principles are not yet fully understood engenders the belief that cryopreservation still has inherent margins for improvement. A recently developed computer model can predict how, depending on specific sets of operating parameters (e.g. CPA concentration) the probability of IIF changes (McGrath, 2009). Therefore, protocols can be designed to establish conditions that limit injury or stress and maximize cell viability.

Basics of slow freezing

Water and aqueous solutions possess a distinct tendency to cool below their melting point before ice begins to form. For example, despite the fact that the melting point of water is 0°C, in the presence of a permeating CPA in excess of 1.0 mol/l an aqueous sample may be cooled to approximately −40°C before ice is formed. At such low temperatures, once started, conversion of liquid water into ice occurs very rapidly if the intracellular compartment remains hydrated (Mazur, 2010; Mazur et al., 2005). To preserve viability, cells need to be dehydrated before ice may propagate intracellularly,