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Effects of reproductive aging and postovulatory aging on the maintenance of biological competence after oocyte vitrification: insights from the mouse model

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Abstract

Cryopreservation of female reproductive cells allows preservation of fertility and provides materials for research. Although freezing protocols have been optimized, and there is a high survival rate after thawing, the *in vitro* fertilization (IVF) pregnancy rate is still lower in cycles with cryopreserved oocytes, thus highlighting the importance of identifying intrinsic limiting factors characterizing the cells at time of freezing. The aim of the present study is to investigate in the mouse model the impact of reproductive aging and postovulatory aging on oocyte biological competence after vitrification. Metaphase II oocytes were vitrified soon after retrieval from young and reproductively old mice. Part of the oocytes from young animals was vitrified after 6 h incubation (*in vitro* aged oocytes). All classes of oocytes showed similar survival rate after vitrification. Moreover, vitrification did not alter chromosomal organization in young cells, whereas *in vitro* aged and old oocytes presented an increase of slightly aberrant metaphase configurations. Compared to fresh young oocytes, *in vitro* aged and old oocytes showed increased ROS levels which remained unchanged after vitrification. By contrast, cryopreservation significantly increased ROS production in young oocytes. Both the aging processes negatively impacted oocyte ability to undergo pronucleus formation and first cleavage after vitrification by stimulating cellular fragmentation. These results could be helpful for establishing the correct time table for cryopreservation in the laboratory routine and improving its application in reproductively old females. Moreover, our observations highlight the importance of oxidative stress protection during vitrification procedures.

Keywords: Reproductive aging; Postovulatory aging; Oocyte vitrification; ROS; Parthenogenetic activation

1. Introduction

Cryopreservation of female reproductive cells is an important goal in modern medicine. It allows for preservation of fertility in healthy women by avoiding repetitive stimulation cycles and the effects of reproductive aging, and in cancer patients who have to undergo sterilizing treatments. Moreover, cryopreservation of mammalian oocytes is an important way to provide a steady source of materials for research on parthenogenetic activation, *in vitro* fertilization, and nuclear transfer.

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In recent years there have been significant improvements in cryopreservation techniques which have led to an increase in clinical applications of these procedures. Nevertheless, in frozen-thawed oocytes employed in IVF, the pregnancy rate is still lower than in fresh cells [1,2]. Moreover, only about 1000 babies have been derived from cryopreserved oocytes as opposed to tens of thousands that developed from frozen embryos [3]. One of the possible reasons for this partial failure is the generalized high chilling sensitivity of the oocyte enhanced by the elevated susceptibility to cooling of the metaphase apparatus [4]. Current studies reveal increasing efforts in the optimization of protocols and understanding of mechanisms responsible for cryodamage [5]; the possible influence of oocyte intrinsic limiting factors has been so far poorly investigated. The positive outcome of ART cycles is strictly linked to the biological properties of the female gamete at the time of fertilization [6]. Thus, it is reasonable to hypothesize that the maintenance of biological competence following cryopreservation is affected by characteristics of the cell at the time of freezing.

The developmental competence of the mature oocyte is related to positive and negative signals targeting the germ cell before and after its recruitment in the growing phase. Negative signals include the aging processes which oocytes have to face in the ovary, in the oviduct, or during *in vitro* culture prior to *vitro* insemination. Prolonged stay of the oocyte in a resting phase as well as its exposure to the aged ovarian microenvironment during growth and final maturation are responsible for a female age-dependent process known as "reproductive aging" [7]. Prolonged stay in the oviduct before fertilization or *in vitro* culture prior to insemination involves a time-dependent aging process, known as "postovulatory aging" [8]. These two aging phenomena induce in the oocyte similar alterations, such as metaphase II aberrations, spontaneous activation, cellular fragmentation, and initiation of an apoptotic pathway. Moreover both reproductive aging and postovulatory aging have been shown to lead to faulty spindle checkpoint predisposing oocytes to premature chromosome separation and aneuploidy [9]. Further alterations shared by the two aging processes involve decline of mitochondrial functions and changes in the redox state [10,11,12]. The regulation of intracellular redox potential is a crucial determinant of oocyte competence [13] and is known to influence the ability of cells to survive cryopreservation [14]. The hypothesis that cryopreservation induces oxidative stress in the female gamete has been recently investigated by Gupta et al. [15], who found that vitrification increases ROS activity in *in vitro* matured porcine oocytes.

Recent studies indicate that it is difficult to cryopreserve human and mouse oocytes, in contrast to numerous cell types including embryonic stem cells, by slow equilibrium freezing, and that it yields low results. This problem can be circumvented by applying ultrarapid vitrification procedure, a simple and fast technique. There is a developing consensus that this method yields better survival, fertilization, and developmental rates as well as a faster spindle recovery after warming, avoiding aging in the whole process, when compared with slow freezing procedures [16,17]. As recently reported in clinical research, vitrification imparts less stress to the cells, although trauma to the female gamete cannot be completely avoided [1].

The aim of the present study is to investigate potential oocyte properties, increasing sensitivity to cryodamage. Since it is well established that aging processes affect cell response to stress conditions [18], we evaluated the impact of reproductive and postovulatory aging on the maintenance of biological competence after vitrification. To this purpose we assessed morphological survival, chromosomal metaphase configuration, intracellular reactive oxygen species (ROS), and activation potential in oocytes obtained from young and reproductively old mice.

2. Materials and methods

2.1. Oocyte collection and treatment

CD-1 mice were obtained from Charles River Italia s.r.l. (Calco, Italy). Animal care and experiments were carried out in accordance with the Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH, 80-23). At the age of 4–8 weeks (young mice) and 48–52 weeks (reproductively old mice), females were superovulated by intraperitoneal injection of 10 IU of PMSG (Folligon; Intervet-International, Boxmeer, Holland) and 10 IU of hCG (Profasi HP 2000; Serono, Roma, Italy) 48 h apart. After 15 h, mice were killed by cervical dislocation and oviducts were removed. Cumulus masses were released into the M2 medium (Sigma, St. Louis, MO) and oocytes arrested at metaphase II stage (MII oocytes) were isolated after a brief exposure to 0.3 mg/ml hyaluronidase (Sigma).

Oocytes from young animals were divided into two experimental groups called "young oocytes" if they were processed immediately after collection, and "*in vitro* aged oocytes" if they were cultured at 37 °C, 5% CO_2 in M16 medium (Sigma) for 6 h before processing.