Reproductive BioMedicine Online (2015) **30**, 493–503

www.sciencedirect.com
www.rbmonline.com

ARTICLE

Kit ligand decreases the incidence of apoptosis in cultured vitrified whole mouse ovaries

Shabnam Abdi [a], **Mojdeh Salehnia** [a,*], **Saman Hosseinkhani** [b]

[a] *Anatomy Department Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran;* [b] *Department of Biochemistry, Faculty of Biological Sciences, Tarbiat Modares University, Tehran, Iran*

* Corresponding author. *E-mail address:* mogdeh@dr.com; salehnim@modares.ac.ir (M Salehnia).

Shabnam Abdi obtained her PhD degree in Anatomy at the Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran in 2013. Her special interest lies in the field of cryopreservation of ovarian tissue and in-vitro maturation of ovaries in two- and three-dimensional culture systems.

Abstract To investigate the development of follicles and incidence of apoptosis in vitrified cultured mouse ovaries in the presence and absence of Kit ligand, 1-week-old mouse ovaries were cultured in the presence or absence of Kit ligand for 7 days. Development and function of ovarian follicles was evaluated by histology and hormonal analysis. Apoptosis assessment was conducted by analysis of DNA laddering, TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end-labelling and caspase-3/7 activity. The proportion of preantral follicles and the level of 17-β oestradiol, progesterone and dehydroepiandrosterone were increased in all cultured groups, and it was significantly higher in Kit ligand treated groups than in the control ($P < 0.001$). The number of apoptotic signals in both vitrified samples is significantly higher than in the non-vitrified control ($P < 0.01$), and these signals are significantly lower in both Kit ligand treated groups than in non-Kit ligand treated groups ($P < 0.001$). The level of caspase-3/7 activity was higher in vitrified cultured ovaries than non-vitrified group ($P < 0.01$). Kit ligand was shown to improve in-vitro development of follicles, and also acted as an anti-apoptotic factor in vitrified ovaries. The developmental potential of follicles in vitrified groups was lower than that in fresh ovaries.

KEYWORDS: caspase- 3/7, in-vitro culture, kit ligand, vitrification

Introduction

Primordial follicles in the ovaries of neonatal mammals and young women are the earliest form of ovarian follicles (Hovatta, 2004, 2012; Kerr et al., 2013). These primordial follicles are considered an important source of immature oocytes for fertility preservation (Adhikari, 2013; Wiedemann et al., 2013).

http://dx.doi.org/10.1016/j.rbmo.2015.01.009
1472-6483/

After cryopreservation of ovarian tissue, the primordial follicles are well preserved with minimal damage (Salehnia et al., 2012; Sheikhi et al., 2013; Van den Hurk and Santos, 2009; Zhang et al., 2011). Some studies, however, have indicated that the development and survival of follicles in vitrified ovarian tissue were lower than those in fresh ovaries (Choi et al., 2007; Milenkovic et al., 2012).

A reduction in the development of follicles followed by ovarian vitrification may be caused by cell death or apoptosis in follicular and theca cells (Choi et al., 2007; Milenkovic et al., 2012; Rolaki et al., 2005). At the molecular level, however, it is not absolutely clear if the expression of apoptosis-related genes is altered in vitrified and warmed ovarian tissue (Abdollahi et al., 2013). Therefore, the analysis of apoptosis may be used to evaluate the safety and outcome of the vitrification procedure.

An alternative technique to improve the development of follicles within ovarian tissue is ovarian organ culture after cryopreservation. Organ culture provides a suitable support for three-dimensional follicular development (Devine et al., 2002; Eppig and O'Brien, 1996). To improve in-vitro development of primordial follicles, growth factors, hormones and peptides are added to the culture medium (Esmaielzadeh et al., 2013; Lima et al., 2011).

Kit ligand known as stem cell factor, steel factor or mast cell growth factor is involved in the activation of primordial follicles, oocyte growth and proliferation of granulosa and theca cells (Roskoski, 2005; Thomas et al., 2008). Kit ligand is secreted by granulosa cells, and its receptors (c-kit) are found on the surface of oocytes and theca cells in mouse, rat and human postnatal ovaries (Jones and Pepling, 2013; Reynaud et al., 2000), and stimulates primordial follicle activation (Hutt et al., 2006; Parrott and Skinner, 1999).

It has been shown that Kit ligand is essential for follicular cell survival and the protection of preantral follicles from apoptosis (Driancourt et al., 2000; Jin et al., 2005a; Reynaud et al., 2001). Kit ligand, however, may also be effective as a survival factor in supporting the viability of ovarian follicles and to decrease the incidence of apoptosis. To the best of our knowledge, there is little information on the effects of Kit ligand on follicular growth and apoptosis after the culture of vitrified whole ovaries. Therefore, the aim of this study was to determine the development and growth of follicles in vitrified mouse whole ovaries in the presence of Kit ligand after 7 days of culture. In addition, the incidence of apoptosis was analysed in long-term cultured fresh and vitrified mouse whole ovaries using complementary techniques, including TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end-labelling (TUNEL) assay, DNA laddering and caspase-3/7 assay.

Materials and methods

All chemicals were purchased from Sigma-Aldrich (St, Louis, USA), unless otherwise noted.

Animals and ovarian tissue

Female National Medical Research Institute mice were bred and cared for in accordance with the Tarbiat Modares University Guide for the Care and Use of Laboratory Animals and housed under a 12-h light/12-h dark regime and temperature-controlled conditions (22 ± 2°C). Animal experiments were carried outo in accordance with the Ethical Committee's approval of Tarbiat Modares University (approved 5 February 2011, reference: 52/99424).

One-week old female mice (n = 95) were killed by cervical dislocation, and their ovaries were removed and washed in alpha minimal essential medium (MEM) (Gibco, UK) supplemented with 5% fetal bovine serum (FBS, Gibco, UK) and were entered into this study.

Experimental design

The collected ovaries from 1-week old mice were divided randomly into non-vitrified (n = 95) and vitrified (n = 95) groups. The ovaries in both groups were cultured for 7 days in alpha-MEM medium in the presence and absence of Kit ligand as subgroups. The apoptosis assessments in all non-cultured and cultured ovaries were carried out using TUNEL assay, DNA laddering technique and Caspase3/7 assay. The six experimental groups were non-vitrified-non-cultured, vitrified-non-cultured, non-vitrified-cultured-Kit ligand$^-$, non-vitrified-cultured-Kit ligand$^+$, vitrified-cultured-Kit ligand$^-$ and vitrified-cultured-Kit ligand$^+$.

Vitrification and warming procedures

The vitrification procedure was based on the previously described method (Salehnia et al., 2002) with some modifications. The ovaries (n = 95) were equilibrated in vitrification medium containing 40% ethylene glycol (v/v), 30% ficoll 70 (w/v), and 1 mol sucrose (EFS40) for 5 min at room temperature. The ovaries were then placed on the inner surface of the CryoLock (Biotech, USA) and immediately plunged into liquid nitrogen and stored for 7 days.

For warming, the CryoLocks were put into 1 ml of descending concentrations of sucrose (1, 0.5 and 0.25 M) for 5 min at room temperature. The warmed ovaries were then equilibrated for 30 min in alpha-MEM media supplemented with10% fetal bovine serum before any assessments.

Ovarian culture

Ovaries (n = 45 for each subgroup) were placed individually on culture inserts (Millicell-CM, 0.4-μm pore size; Millipore Corp, Billerica, MA) in 24-well plates and cultured in basic culture medium consisted of alpha MEM supplemented with 1% insulin, transferrin, and selenium (ITS; Gibco, UK), 10% fetal bovine serum, 100 mIU/ml recombinant FSH (rFSH or Gonal-f; Serono, Switzerland) in a humidified incubator with 5% CO2 at 37°C for 7 days. In groups treated with Kit ligand, 100 ng /mL recombinant mouse kit-ligand was added to the culture media and the group without Kit ligand was considered as the control group.

About 0.4 ml of culture media was added below the membrane insert. The culture media was renewed every 2 days. A total of 150 μl media was changed with fresh culture media