European Journal of Obstetrics & Gynecology and Reproductive Biology 178 (2014) 16–20

Contents lists available at ScienceDirect

European Journal of Obstetrics & Gynecology and Reproductive Biology

journal homepage: www.elsevier.com/locate/ejogrb

Effect of necrostatin on mouse ovarian cryopreservation and transplantation

Jung Ryeol Lee [a,b], Hye Won Youm [a,b], Seul Ki Kim [a], Byung Chul Jee [a,b], Chang Suk Suh [a,b], Seok Hyun Kim [b,*]

[a] Department of Obstetrics and Gynaecology, Seoul National University Bundang Hospital, Seongnam, Republic of Korea
[b] Department of Obstetrics and Gynaecology, Seoul National University College of Medicine, Seoul, Republic of Korea

ARTICLE INFO

Article history:
Received 21 September 2013
Received in revised form 23 January 2014
Accepted 29 April 2014

Keywords:
Fertility preservation
Ovarian tissue cryopreservation
Vitrification
Necrostatin

ABSTRACT

Objective: To investigate the effects of necrostatin-1 (Nec-1) supplementation on vitrification, warming and transplantation of ovarian tissue.
Study design: Ovaries from 4-week-old ICR mice were vitrified using a two-step procedure; ovaries were suspended in equilibration solution for 10 min, and then mixed with vitrification solution for 5 min. Ovaries were divided at random into three groups and 0 (control), 25 or 100 μM Nec-1 was added to the vitrification solution. After warming, follicular morphology and apoptosis were assessed. For each group, a sample of vitrified, warmed ovaries was autotransplanted. The same dose of Nec-1 that was added to the vitrification solution was added to each warming solution and injected intraperitoneally. Follicular morphology and apoptosis of transplanted ovaries were assessed after 2 weeks.
Results: After vitrification and warming, morphological analysis revealed that the intact follicle ratio was significantly higher in the Nec-1-treated groups compared with the control group (control, 45.1%; 25 μM Nec-1, 51.7%; 100 μM Nec-1, 57.9%). The rate of apoptosis was lower in the Nec-1 treated groups compared with the control group (control, 11.2%; 25 μM Nec-1, 8.5%; 100 μM Nec-1, 7.2%). After transplantation of the vitrified, warmed ovaries, morphological analysis revealed that the intact follicle ratio was significantly higher in the Nec-1 treated groups compared with the control group (control, 43.1%; 25 μM Nec-1, 60.6%; 100 μM Nec-1, 70.7%). The rate of apoptosis was lower in the Nec-1 treated groups compared with the control group (control, 5.3%; 25 μM Nec-1, 2.5%; 100 μM Nec-1, 2.0%).
Conclusions: Nec-1 supplementation during vitrification, warming and transplantation has beneficial effects on the survival of ovarian tissue. These results can help to improve ovarian tissue vitrification and transplantation protocols for fertility preservation.

Introduction

Advances in the diagnosis and treatment of cancer have substantially improved the life expectancy of premenopausal women. However, aggressive chemotherapy or radiotherapy for cancer treatment has an adverse effect on gonadal function, and subsequently leads to a loss of fertility and premature ovarian failure. Therefore, the preservation of ovarian function in female cancer survivors has become an important issue. Several options are available for fertility preservation in these women, such as cryopreservation of embryos, oocytes or ovarian tissue [1,2]. Cryopreservation of embryos and oocytes is a clinically established procedure that has been used for infertility treatment. Until recently, these procedures were considered to be the first option for fertility preservation in women. However, these procedures are preceded by controlled ovarian stimulation, which takes 2–3 weeks, resulting in a delay in cancer treatment.

Cryopreservation and transplantation of ovarian tissue is one of the most promising options for fertility preservation of female cancer survivors, and has several advantages compared with other options. Ovarian stimulation is not necessary for ovarian cryopreservation, so there is no need to delay cancer treatment. In addition, this procedure is an option for single women and prepubertal girls because a male partner is not required. To date, more than 20 live births have been reported in humans after transplantation of cryopreserved ovarian tissue [3].

However, cryopreservation of ovarian tissue is currently considered to be an experimental technique because the success rate is not appropriate for routine clinical use. The low success rate is due to damage to ovarian tissue during cryopreservation,

* Corresponding author. Tel.: +82 2 2072 3773; fax: +82 2 762 3599.
E-mail addresses: leejrmd@snu.ac.kr (J.R. Lee), seokhyun@snu.ac.kr (S.H. Kim).

http://dx.doi.org/10.1016/j.ejogrb.2014.04.040
0301-2115/

thawing and transplantation. One of the major barriers to cryopreservation of ovarian tissue is that a large number of primordial follicles are lost when the ovarian tissue is transplanted. Ischaemic injury is a threat to tissue survival, resulting in necrosis, apoptosis and fibrotic changes soon after transplantation [4,5]. Many attempts have been made to reduce this damage, such as the addition of antioxidants [6–9], anti-apoptotic agents [10,11] or angiogenic factors [12,13] during cryopreservation and/or transplantation. However, not all of the agents studied have shown beneficial effects on tissue survival, and there have been conflicting results among reports that have studied the same agent.

Traditionally, three main types of programmed cell death have been classified morphologically or biochemically: (1) apoptotic (caspase mediated or caspase independent); (2) autophagic (without the involvement of chromatin condensation); and (3) necrosis (necroptosis, paraptosis). Necroptosis is a recently discovered, regulated form of programmed necrosis that depends on the serine/threonine kinase activity of receptor-interacting protein kinase 1(RIP1) [14], and participates in the pathogenesis of diseases, including ischemic injury, neurodegeneration and viral infection [15]. The pro-necrotic function of RIP1 has been studied in various disease models using necrostatins; a series of chemical inhibitors against RIP1 activity [16]. Necrostatin-1 (Nec-1) has been reported to suppress necroptotic cell death triggered by an array of stimuli in various cell types. Nec-1-inhibitable non-apoptotic death is an important contributor to ischaemic injury in mouse models of cerebral ischaemia and myocardial infarction [17,18]. Therefore, it was hypothesized that Nec-1 supplementation may help to reduce ischaemic necrosis after ovarian transplantation. The aim of this study was to investigate the effect of Nec-1 supplementation during vitrification and transplantation of ovarian tissue.

Materials and methods

Vitrification and warming of ovarian tissue

The experimental procedures performed were similar to those reported previously [11]. The animals in this study were cared for and used in accordance with the institutional guidelines established by the Animal Care and Use Committee of Seoul National University of Bundang Hospital. ICR mice (Orient Co., Seoul, Korea) were maintained under a 12-h light:12-h dark cycle at 23 °C and fed *ad libitum*. After 1 week of adaptation, both ovaries of 4-week-old mice were resected. Ovaries were vitrified using a two-step method involving exposure to equilibration and vitrification solutions [19]. Equilibration solution contained 20% ethylene glycol (Sigma Chemical Co., St. Louis, MO, USA), and vitrification solution was composed of 40% ethylene glycol, 18% Ficoll (Sigma) and 0.3 M sucrose (Sigma). All solutions were based on Dulbecco's phosphate-buffered saline (DPBS; Gibco BRL, Grand Island, NY, USA) containing 20% fetal bovine serum (Gibco).

Intact ovaries were initially suspended in 1 ml equilibration solution for 10 min and then mixed with 0.5 ml vitrification solution for 5 min. The ovaries were divided randomly into three groups, and 0 (control), 25 or 100 μM Nec-1 (Sigma) was added to the vitrification solution. After exposure to equilibration and vitrification solutions, ovaries were transferred immediately to 1.2-ml cryotubes (Nunc; Fisher Bioblock Scientific, Illkirch, France) and plunged into liquid nitrogen. After 2 weeks, the tissues were warmed by immersing the vials rapidly in a water bath at 37 °C, and suspended serially in 0.5 M sucrose in DPBS containing 20% fetal bovine serum for 5 min, 0.25 M for 5 min, and 0 M for 10 min.

Morphological assessment of follicles

Ovarian samples were fixed in 10% buffered formalin and then embedded in a paraffin block. Paraffin-embedded ovarian sections were cut (thickness 5 μm) for routine histological examination. After deparaffinization and rehydration, the sections were stained with haematoxylin and eosin. The numbers of each type of follicle were counted for the entire cut ovarian surface (magnification ×400). All follicles found in one section of each ovary were scored. Each type of follicle was categorized according to the following classification [20]:

(1) primordial – single layer of flattened pre-granulosa cells;
(2) primary – single layer of granulosa cells; one or more being cuboidal cells;
(3) secondary – two or more layers of cuboidal granulosa cells with antrum absent; and
(4) antral – multiple layers of cuboidal granulosa cells with antrum present.

The integrity of each follicle was evaluated using the following criteria [21]:

(1) Primordial/primary follicle.
G1 – spherical with even distribution of granulosa cells;
G2 – granulosa cells pulled away from the edge of the follicle but oocyte still spherical; and
G3 – pyknotic nuclei, mis-shapen oocyte or vacuolation.
(2) Secondary/antral follicle.
G1 – intact spherical follicle with evenly distributed granulosa and theca cells, small space and spherical oocyte;
G2 – intact theca cell, disruption of granulosa cells and spherical oocyte; and
G3 – disruption and loss of granulosa and theca cells, pyknotic nuclei and missing oocyte.

Detection of apoptotic follicles by TUNEL assay

Paraffin-embedded ovarian sections (thickness 5 μm) were cut and assessed for apoptosis using a commercial TUNEL assay kit (*In Situ* Cell Death Detection Kit, Fluorescein, Roche Applied Science, Germany). After deparaffinization and rehydration, sections were rinsed in PBS (pH 7.2) and digested using proteinase K (20 μg/ml, 37 °C, 30 min) in 10 mM Tris–HCl buffer. After rinsing in DPBS, the specimens were incubated with 50 μl TUNEL reaction mixture at 37 °C for 60 min in a humidified chamber in the dark, and subsequently rinsed with DPBS. Positive control slides were prepared by treating 1500 U/ml DNase I (Roche Applied Science) in 50 mM Tris–HCl (pH 7.5, including 1 mg/ml bovine serum albumin) for 10 min at room temperature to induce DNA strand breaks, prior to labelling procedures. Some ovarian tissue specimens were used as negative controls by substituting terminal deoxynucleotidyl transferase with distilled water in the reaction mixture following the protocol.

The slides were covered with DAPI (Vector Laboratories, Burlingame, CA, USA) to counterstain DNA. TUNEL-stained and DAPI-counterstained slides were examined under an inverted fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Green fluorescence was visualized in TUNEL-positive cells at an excitation wavelength in the range of 450–500 nm and detection in the range of 515–565 nm. DAPI reached excitation at approximately 360 nm and emitted at approximately 460 nm when bound to DNA, producing blue fluorescence in all nuclei. Only follicles with a visible nucleus were counted regardless of their type. Follicles were considered to be apoptotic if >30% of the cells were TUNEL stained (Fig. 1).