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Vitrified Xenograft Survived Human Ovarian Follicles Using Erythropoietin

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Abstract

Xenotransplantation is a useful method for studying ovarian function to improve cryopreservation and grafting protocols. Due to graft rejection, many strategies have been presented to reduce problems like hormone therapy. In present study, erythropoietin (EPO) and human menopausal gonadotropin (HMG) were applied in xenotransplantation of human ovarian tissue into the castrated nude mice. Ovarian fragments provided from Rasool-e- Akram hospital (Tehran, Iran) were divided into 4 equal ($5 \times 5 \times 1$ mm²) Pieces- control: fresh piece of ovary, Fresh-trans-treated (FTT): fresh piece of ovary treated with EPO and HMG during xenotransplantation, Vit-trans-treated (VTT): vitrified-warmed piece of ovary treated with both EPO and HMG during xenotransplantation, and Vit-trans-non-treated (VTnT): vitrified-warmed piece of ovary non-treated with EPO and HMG during xenotransplantation. After 8 weeks of transplantation, morphologically healthy primary follicles were observed in both fresh and vitrified grafts (FTT and VTT groups), but no follicle was observed within vitrified xenotransplanted tissue into nude mice receiving no treatment with EPO & HMG (VTnT group).

Keywords: Xenotransplantation; Vitrification; Human ovary; Follicles

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Introduction

Xenotransplantation is used as a model to examine the ovarian function and follicle development in vivo and optimize cryopreservation and grafting protocols [1]. Reduction of follicular growth could be detected just 48 hours after transplantation attributable to hypoxia and lacking angiogenesis. Also the production of free radicals during revascularization affects follicular development in grafted ovary [2]. Consequently, rapid neovascularization and stimulation of the follicle growth are the main challenges in xenotransplantation. Erythropoietin and human menopausal gonadotropin (HMG) are the factors enjoyed for this purpose to improve angiogenesis. Also the Erythropoietin plays a role in reducing oxidative stress and risk of ischemia during the first days of transplantation [3]. This study aimed to record the folliculogenesis rate during xenotransplantation of human ovarian fragments retrieved from transsexual patients into the nude castrated mice.

Materials and Methods

The human ovarian samples were collected from 22-30 years old transsexual patients. The cortexes of ovarian tissues were cut into $5 \times 5 \times 1$ mm². At first, cryosections were prepared from the fresh ovarian tissues and stained with hematoxylin and eosin (H & E) to approve the presence of follicles within the ovarian pieces. Each ovarian fragment was individually vitrified based on Royan ovarian tissue bank (Royan OTB) protocol: the stripes are vitrified in a two-step process, equilibration and vitrification. In the first step (equilibration), each strip is washed in equilibrium medium composed of HTCMT, ethylene glycol (EG, Sigma, St. Louis, MO, USA), Dimethyl sulphoxide (DMSO, Sigma, USA, each 7.5%) and 20% HSA for 15 minutes at 4°C. In the second step (vitrification), each strip is washed in 15% HTCMT, 15% DMSO and 15% EG, 0.25M sucrose, and 20% HSA for 10 minutes at 4°C. The extra medium is completely removed from the strips, after which they are directly transferred into liquid nitrogen. Warming is performed in 4 steps in descending concentrations (1, 0.5, 0.25, and 0.125) of sucrose. The base medium is comprised of

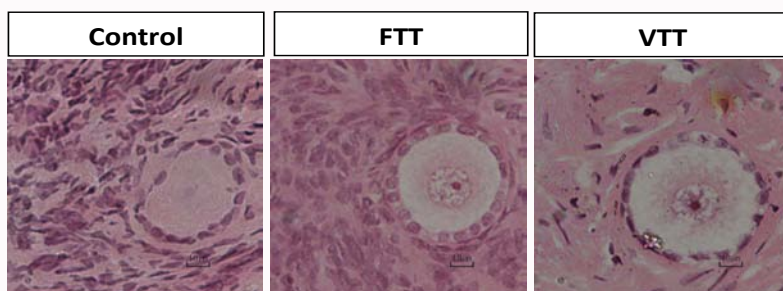


Figure 1: (Control) Primary follicle in cryosection of human ovarian fragment, (FTT) fresh human xenotransplanted ovarian tissue treated with EPO and HMG, (VTT) Primary follicle within vitrified human xenotransplanted ovarian tissue treated with EPO and HMG, (VTnT) vitrified human xenotransplanted ovarian tissue non-treated with EPO and HMG.

HTCM+20% HSA.

Then, fresh and vitrified human ovarian strips were xenotransplanted into the gonadectomized male nude mice which (6- 8-weeks-old) were obtained from the animal house of Royan institute.

The nude mice were anesthetized by intraperitoneal (IP) injection of 50mg/kg ketamine and 5mg/kg xylazine 2%. They were all castrated bilaterally and the grafts were transplanted into the dorsal muscle of neck. 500IU/kg erythropoietin was injected immediately after xenotransplantation until 5 days. Also, during the last 10 days before removing the grafts, 7.5 IU/ml HMG was injected into each recipient every other day. Eight weeks after transplantation the grafts were removed and fixed in Bouin's and formalin solutions. After serial sectioning of the entire grafted ovarian tissues (5 μ m thickness), they were subjected to H&E staining.

Results

Cryosections demonstrated the presence of follicles within ovarian pieces (Figure 1A). Strips with appropriate follicular storage were xenografted into the gonadectomized male nude mice. After two months, the morphologic results showed healthy growing and intact primary follicles within the fresh and vitrified human xenotransplanted ovaries, but no follicle was observed in vitrified human xenotransplanted ovaries into EPO & HMG non-treated nude mice (Figure 1B and 1C).

Discussion

In present study, primary follicles were observed in ovarian grafts as a result of optimizing xenotransplantation outcomes. Due to the difficulty in preparing the human ovarian samples, obtaining ovarian tissues from female-to-male transsexuals could be an appropriate material for research. These samples are suitable for studying the procedures of ovarian cryopreservation, culture and graft.

In this regard, transplantation site has critical effects on follicle survival and growth. Different sites such as kidney capsule [4], ovarian bursa [2] and intraperitoneum [5,6] are noted in many studies. In this research, human ovarian pieces were transplanted into the dorsal muscle of the neck in nude mice. This site is suggested on account of its easier surgery, convenience of monitoring the tissue during the graft time and preparing adequate space for follicular development [2]. Soleimani et al. reported the largest oval-shape antral follicle after ovarian tissue xenotransplantation into back muscle [4]. Likewise, Wang et al, xenotransplanted fresh human ovarian cortex into back muscle of immunosuppressed rabbits and they used VEGF

and bFGF as improving factors to achieve the best transplantation consequences. His study reported to reduce apoptosis and fibrosis and promote follicle survival rate 6 weeks after transplantation [7].

The body environment is a suitable incubator for maturation and growing of ovarian follicles through xenotransplantation procedure; many researchers enjoy this incubator as a live model to evaluate the ovarian cryopreservation protocols and their follicular dynamic activities [2,6,8]. As a result, it is important to improve the condition of xenotransplantation.

There are some limitations problems such as hypoxia, ischemia and free radical production causing the rejection after xenotransplantation. Antioxidants, hormones, cell therapy, growth factors and scaffolds are the strategies applied for reducing the risk of rejection [9]. Owing to these reasons, erythropoietin and HMG could improve vascularization and folliculogenesis. In present study, no follicle was observed in xenotransplantation of cryopreserved-thawed human ovarian tissue in EPO & HMG non-treated nude mice. But EPO & HMG caused survival of the follicle after 2 months of xenotransplantation of human vitrified ovarian tissue.

Mahmoodi et al., studied the effects of EPO on ischemia and follicular survival after transplantation. She and her colleagues presented increase in the proportion of different types of follicles in EPO-treated group. By measuring the plasma concentration of malondialdehyde (MDA), they discussed that EPO could reduce lipid peroxidation by its antioxidant properties. Also they observed significant increase of serum concentration of E2 in EPO-treated groups that indicated to induction ability of the mentioned factors in endocrine function during transplantation [3]. Erythropoietin attenuate oxidative stress by increasing the number of erythrocytes [10], It can also improve angiogenesis by increasing the expression of angiogenic factors and reduce apoptosis rate and ischemia-reperfusion (IR) injury during transplantation [11].

Kolusari et al, reported the survival follicles after using erythropoietin in autotransplanted ovarian tissue into the rat. They discussed a significant decrease of VEGF-C levels expressed in hyperthermia and oxidative stress condition [12]. In a study carried out in Royan institute by Tahaei et al. a better folliculogenesis rate was achieved after applying the erythropoietin and HMG in sheep ovarian tissue xenotransplantation into manually immunodeficient rats [13]. whilst injection of gonadotropin plays a role as an angiogenic factor, providing an appropriate vascular supply and regulating the vascular system during transplantation, it will improve folliculogenesis [13,14]. The secretion of FSH & LH regulates follicular development [15]. Therefore, the injection of combined gonadotropins has the same

effect as those hormones released from pituitary glands [13]. Different gonadotropins have been used to improve follicular development after transplantation [5,8,13]. Maltaris et al, induced primary follicles to grow into antral stage in ovarian tissue xenotransplantation in gonadectomized SCID mice by injecting HMG [16]. Similarly, Amorim and her colleagues showed folliculogenesis improvement by gonadotropin stimulation (FSH & HCG) after xenotransplantation human ovarian cortex into SCID mice [8]. Additionally, Luyckx et al. reported xenotransplantation of cryopreserved prepubertal ovarian tissue into SCID mice for the first time. After twenty-one weeks, they achieved high number of survived and developing follicles by exogenous FSH therapy [5].

In conclusion, it is important that xenotransplantation outcomes will be improved by the means of suitable supportive exogenous factors like EPO and HMG and they can be appropriate elements for improving the condition of folliculogenesis during xenotransplantation.

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