Anim. Reprod., v.5, n.3/4, p.97-102, Jul./Dec. 2008

Negative effect of vitrification of whole rat ovarian tissue and its subsequent autotransplantation into omentum

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Abstract

This study was designed to examine the viability of follicles in whole rat ovary after vitrification-warming and transplantation into omentum. Sixteen 6-8 week old female Wistar rats were randomly divided into four groups, with four rats in each group. Freshly isolated ovaries saved as a control (group 1; fresh ovaries) in formalin-fixed or vitrified immediately after dissection (group 2; vitrified ovaries). In group 3 (fresh grafts), ovarian tissues were dissected out and implanted into the omentum. In group 4 (vitrified grafts), ovarian tissues were dissected out and used for autologous transplantation into omentum after vitrification and warming. Ovaries in the vitrified groups were exposed sequentially to two vitrification solutions: 10% ethylene glycol and 10% DMSO in holding medium [HM; human tubal fluid (HTF) medium with 20% FBS] for 15 min and then 20% ethylene glycol, 20% DMSO and 0.5 M sucrose in HM for 2 min before plunging into liquid nitrogen. After warming at 37°C, cryoprotectants were diluted with 1.0 M sucrose in HTF for 10 min. All of the collected ovaries underwent hematoxylin and eosin-stained paraffin serial sections. Follicles at each developmental stage were counted and divided according to the morphological changes. Among the morphologically changed follicles after vitrification, developing and antral follicles significantly accounted for high proportions compared to the fresh control group. A significant decrease in the percentage of healthy follicles was observed in the vitrified ovarian tissues implanted into the omentum in comparison with the fresh omental graft (8.3% vs. 46.3%, $P < 0.05$). Cryopreservation of whole rat ovarian tissue by the vitrification method used had detrimental effect on follicles. Subsequent autotransplantation of the ovaries into the omentum was not able to support viability of follicles and also insufficient blood supply associated ischemia caused more damage and significant decrease in the number of follicles.

Keywords: omentum, ovarian tissue, transplantation, vitrification.

Introduction

With the significant increase in studies on cryopreservation of ovarian tissue conducted since the 1990's, subsequent transplantation after completed cancer treatment has been suggested as an alternative to restore fertility for women who are at high risk for ovarian failure after chemotherapy or radiotherapy (Torrents *et al.,* 2003). Autotransplantation of frozen-thawed ovarian tissue has proven to be an effective method not only to restore endocrine function (Kim *et al.,* 2004a), but also to restore fertility in humans (Donnez *et al.,* 2004) and animals (Lee *et al.,* 2004). Although the technical simplicity of vitrification has revolutionized cryopreservation of oocytes and embryos, its application to whole ovaries has been considered to be difficult.

To date, there have been several reports about orthotopic (Candy *et al.,* 2000; Kim, 2006) and heterotopic (Kiran *et al.,* 2004; Lee *et al.,* 2005) autotransplantation of ovarian tissue. The site of orthotopic transplantation was in the ovarian cortex (Radford *et al.,* 2001). For heterotopic transplantation, the ovarian tissues were often introduced to the renal capsule (Liu *et al.,* 2001), subcutaneously (Aubard *et al.,* 1999), to the bursal cavity (Shaw *et al.,* 2000a) and to the uterine cavity (Kagabu and Umezu, 2000). However, orthotopic or heterotopic transplantation of cryopreserved ovarian tissue is of little practical value since it makes ovum pick up (OPU) difficult. Subcutaneous transplantation would solve this problem (Lee *et al.,* 2005). Despite this progress, ovarian tissue cryopreservation and transplantation is still in its experimental stage, and further researches are necessary to elucidate an optimal protocol for cryopreservation, development of nontoxic cryoprotectants and prevention of ischemia-induced tissue damage (Kim *et al.,* 2004b).

Tissue ischemia without vascular anastomosis remains a problem for implants because the process of revascularization can take 2 to 7 days to complete, dependent on factors such as the size of implanted tissue (Dissen *et al.,* 1994; Gosden *et al.,* 1994). In theory, immediateness in tissue revascularization could achieve

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Received: March 3, 2008
Accepted: June 19, 2008

a suitable blood supply in vivo to maximize graft survival. However, the role of omentum and its great ability in revascularization of tissues has been well documented (Oloumi *et al.,* 2006). In this study, we implanted whole fresh and vitrified-warmed rat ovarian tissue into the omentum and located it to the subcutaneous to determine the viability of follicles.

Materials and Methods

Animals

The experiments were carried out using Wistar female rats, aged between 6 to 8 weeks old, purchased from Neuroscience Research Center of Kerman, IRAN. All animals were cared for and used in accordance with the International Guiding Principle for Biomedical Research Involving Animals at Kerman University of Medical Science. They were housed under a lighting regimen of 12 h light and 12 h darkness and temperature-controlled conditions (22 ± 2°C). Animals were fed a standard laboratory diet (Javaneh Khorasan Co., Mashhad, Iran) and water ad libitum.

Experimental design

The animals were randomly divided into four groups, with four rats in each group. Only the whole right ovary of each rat was dissected out following superovulation. In group 1 (fresh ovaries, n = 4), freshly isolated ovaries were used as control. In group 2 (vitrified ovaries, n = 4), freshly isolated ovaries were vitrified immediately after dissection and then were preserved in buffered formalin. In group 3 (fresh graft, n = 4), ovarian tissues were dissected out and implanted into the omentum of the same rat. In group 4 (vitrified grafts, n = 4), ovarian tissues were vitrified after oophorectomy and then implanted into the omentum of the same rat.

Surgical procedure

The rats were anesthetized with the injection of 100 mg/kg ketamine (Rotexmedica, Germany) and 10 mg/kg xylazine (Alfasan, Holland) intraperitoneally. All of the following surgical procedures were performed aseptically. A single median longitudinal skin incision was made on the lumbar portion to expose the subcutaneous tissue over the ovary. A small incision was made on the fascia and muscles immediately above the ovary, thereby exteriorizing the reproductive tract, to isolate and resect the ovary. Fresh or vitrified-warmed ovaries isolated from the rats were then autotransplanted into the omentum and immediately implanted subcutaneously in the right sublumbar area (Fig. 1).

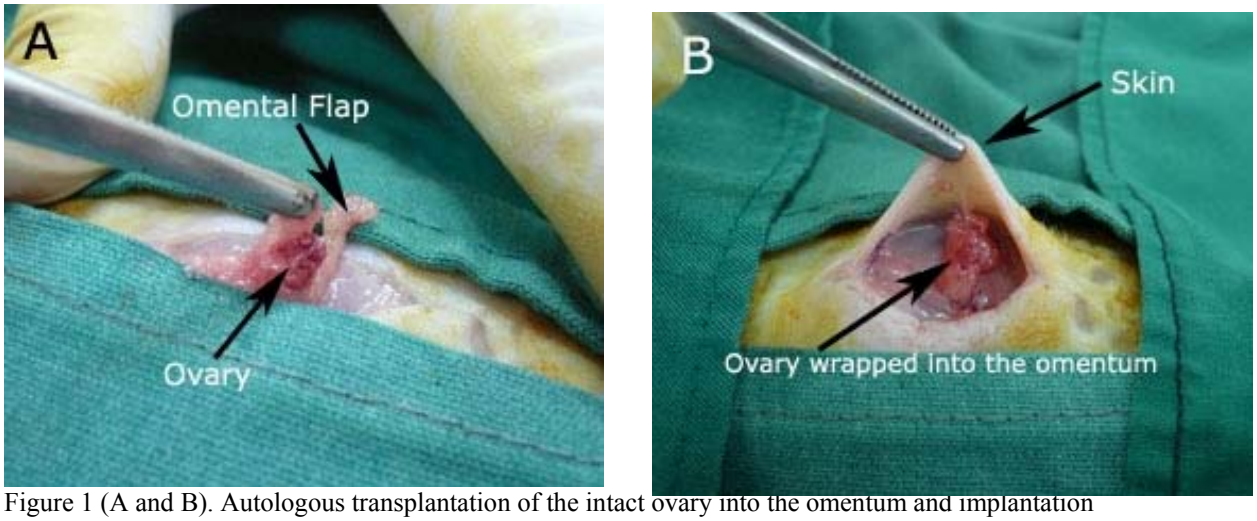

Figure 1 (A and B). Autologous transplantation of the intact ovary into the omentum and implantation subcutaneously.

Vitrification and warming procedure

We adopted the method of Vajta *et al.* (1998) with some modification. Briefly, intact ovaries were immersed in an equilibration solution composed of 10% ethylene glycol (EG, Sigma-Aldrich Company, USA) and 10% DMSO (Sigma-Aldrich Company, USA) in holding medium [HM: human tubal fluid (HTF) medium with 20% FBS (Gibco, UK)] for 15 min at room temperature, and then in a vitrification solution composed of 20% EG, 20% DMSO and 0.5 M sucrose (Sigma-Aldrich Company, USA) in HM for 2 min. After equilibration, the ovaries were loaded into the cryotubes with 1 ml of vitrification medium and were directly plunged in liquid nitrogen.

The vitrified samples were warmed up rapidly by immersing the end of the tubes in a warming solution composed of 1.0 M sucrose in HM for 10 min at room

temperature. The temperature of the media used for warming was held at 37°C.

Microscopic evaluation of ovaries

Twenty-one days after autotransplantation, all the rats in four experimental groups underwent superovulation as follows: equine chorionic gonadotropin (eCG, Intervet, 50 IU) was injected intraperitoneally and 48 h later, the animals were euthanized by intracardiac injection of 20 mg/kg sodium thiopental (Sandoz GmbH, Kundl-Astria). The right ovarian tissues were recovered by a skin and sublumbar muscle incision and fixed in 10% buffered formaldehyde solution (Merck, Germany) for at least 48 h. After routine paraffin embedding, the entire samples were serially sectioned at 5 µm and stained with haematoxylin and eosin and examined microscopically (Smith *et al.,* 1991). Follicles at each developmental stage were counted and classified (Gougeon, 1986) according to the general morphological aspects as follows: (i) morphologically healthy follicles, which have regular oocytes, intact theca, well-arranged follicle cells and regular corona radiata; (ii) morphologically damaged follicles, which have deformed oocytes, eosinophilic ooplasm, disordered follicle cells and clumped chromatin material (see Fig. 2).

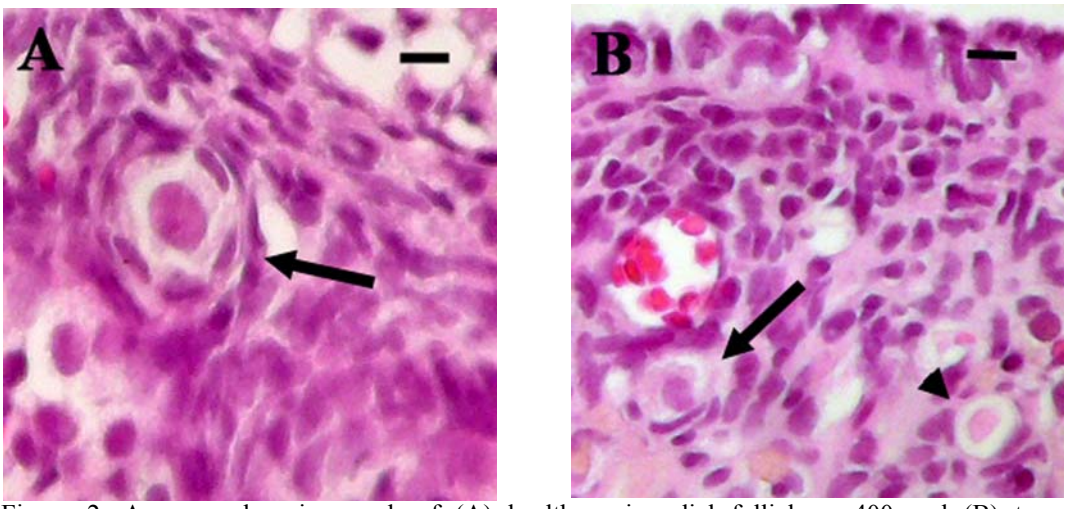

Figure 2. An example micrograph of (A) healthy primordial follicle × 400 and (B) two cryodamaged primordial follicles × 400. H & E staining. Scale bar = 25 µm.

For this study, primordial follicles were defined as those containing one layer of flattened granulosa cells surrounding oocytes, primary follicles as those with one layer of cuboidal granulosa cells and preantral follicles as those with two or more layers of cuboidal granulosa cells, but no antrum and antral follicles those with an antral cavity. We used the term "developing follicles" in this study to describe those that were beyond the primordial stage, i.e., primary and preantral follicles together.

Statistical analyses

The SPSS® program was used for data evaluation. Data were expressed as mean ± SEM and compared using ANOVA. A P-value of < 0.05 was considered statistically significant.

Results

To determine the effect of vitrification of ovarian tissue on morphologic properties of follicles, the number of intact and damaged follicles was counted in ovarian sections. The total number of healthy follicles was significantly decreased in vitrified ovaries compared to the fresh control group ($P < 0.05$). Although the majority of primordial follicles appeared morphologically healthy, the percentage of intact primordial follicles was significantly lower than that of the fresh ovaries (12.7 vs. 23.3%, $P < 0.05$, respectively; Table 1).

In addition, we evaluated whether the decrease in the number of intact follicles after the whole rat fresh and vitrified-warmed ovarian graft was prevented by transplantation into the omentum. Table 2 shows the number of intact and damaged follicles in fresh and vitrified-warmed ovaries 3 weeks after transplantation into the omentum. The number of intact follicles in fresh ovarian grafts was decreased when compared to fresh ovaries (19 ± 1.2 vs. 91 ± 4.5, $P < 0.05$, respectively) and a significant reduction in the percentage of intact follicles was observed in vitrified ovarian tissues implanted into the omentum in comparison with fresh grafts (8.3 vs. 46.3%, $P < 0.05$, respectively).

Table 1. Numbers (mean ± SEM) of healthy and damaged and types of healthy follicles found in fresh and vitrified-warmed ovaries.

	No. of Follicles	Healthy				Total Damaged (%)
		Primordial (%)	Developing (%)	Antral (%)	Total (%)	
Fresh ovaries (n=4)	116	27 ± 1.5[a] (23.3)	33 ± 1.2[a] (28.4)	31 ± 1.8[a] (26.7)	91 ± 4.5[a] (78.4)	25 ± 1.5[a] (21.5)
Vitrified ovaries (n=4)	126	16 ± 1.6[b] (12.7)	7 ± 1.7[b] (5.5)	4 ± 2.1[b] (3.2)	27 ± 1.7[b] (21.4)	99 ± 5.7[b] (78.6)

[a,b]Means within column with different superscripts differ (P <0.05).

Table 2. Numbers (mean ± SEM) of healthy and damaged follicles found in fresh and vitrified-warmed grafts 3 weeks after omental transplantation.

	No. of Follicles	Healthy follicles (%)	Damaged follicles (%)
Fresh omental grafts (n = 4)	41	19 ± 1.2[a] (46.3)	22 ± 1.2[a] (53.6)
Vitrified omental grafts (n = 4)	36	3 ± 0.5[b] (8.3)	33 ± 2.1[b] (91.6)

[a,b]Means within column with different superscripts differ (P <0.05).

Discussion

Recently, in an experimental study we observed that small follicles in mouse ovaries underwent the least damage after vitrification-warming using EG and DMSO as cryoprotectants (Babaei *et al.,* 2007). This is in agreement with our present study in which among the morphologically changed follicles after vitrification-warming, developing and antral follicles accounted for high proportions, whereas primordial follicles underwent less changes (Table 1). Primordial follicles have been reported to have tolerance to vitrification and warming injuries compared to other stages due to their special characteristics: (i) they represent 90% of the entire follicular population in the ovary; (ii) they have a relatively inactive metabolic rate; (iii) their small size (~20 µm diameter); (iv) a low number of granulosa cells around the small oocyte; and (v) an absence of the zona pellucida and peripheral cortical granules (Shaw *et al.,* 2000a; b). In addition, primordial follicles have been reported to have more potential to repair sublethal damage to organelles and other structures during their prolonged growth phase (O'Brien *et al.,* 2003).

Another cause of damage of cryopreserved ovaries is devitrification that may occur during the warming procedure. In order to avoid the effects of devitrification, it is necessary to increase the warming rate to the highest extent by either using small ovaries or cutting the ovaries into smaller pieces (Migishima *et al.,* 2003). The vitrification of whole mouse ovarian tissue using ethylene glycol was reported to be useful without any harmful effects on the morphology of follicles (Salehnia, 2002). In the previous report (Salehnia, 2002), ovaries of mice seemed to be sufficiently small to minimize devitrification effects. In contrast, Sugimoto *et al.* (1999) observed that rat ovarian follicles survived after vitrification and transplantation with a decreased number of small and large follicles. This is in agreement with the present experiment in which after transplantation the number of intact follicles had decreased markedly.

One of the important major challenges for ovarian cryopreservation and transplantation includes prevention of ischemia-induced tissue damage (Chen *et al.,* 2006). Although detrimental effects of freezing-thawing ovarian tissue may decrease by application of optimal protocol for cryopreserving, developing of less toxic cryoprotectants and slicing ovarian tissue into small pieces (Isachenko *et al.,* 2003; Migishima *et al.,* 2003), efficiency is adversely determined by subsequent transplantation with or without immediate vascular anastomosis (Yin *et al.,* 2003). Attempts have been made to overcome ischemia-related damage that exists at transplantation by treating with GnRH (Imthran *et al.,* 2000), antioxidant agents such as alpha-tocopherol (Newton and Illingworth, 2001) or by using microvascular anastomosis (Wang *et al.,* 2002; Bedaiwy *et al.,* 2003; Yin *et al.,* 2003).

Host tissues with potential capacity for providing angiogenesis factors could result in sufficient blood supply. Recently, it was reported that implantation of ovarian grafts into angiogenic granulation tissue improved graft vascularization and follicular survival (Israely *et al.,* 2006). Considering the angiogenic factors present in the omentum, it can be assumed that this tissue could play its angiogenic role at providing sufficient blood supply. A number of

polypeptide growth factors that possess potent angiogenic properties have recently been identified in greater omentum. Zhang *et al.* (1997) analyzed the level of vascular endothelial growth factor (VEGF) in a number of rat tissues. The highest VEGF secretion rate was found in the omentum. They suggested that VEGF is the major angiogenic factor produced by omentum and possibly underlies the mechanism of omentum-induced angiogenesis. In the present study, we found that all the omental graft groups showed significantly decreased proportions of morphologically normal follicles than the fresh control group. This result suggested that both the fresh and vitrified ovarian graft into the omentum could cause morphological changes to some follicles mainly because of temporary ischemia after transplantation. Therefore, in spite of previous reports about angiogenic potential of omentum (Oloumi *et al.*, 2006), our results showed that it is not capable of providing immediate and sufficient blood supply. We do not know the reason of this difference and further researches should be performed to elucidate this discrepancy. Possibly, an intact tunica albuginea that encloses the ovary prevents immediate neovascularization by the omentum and this problem might be facilitated by slicing the ovarian tissue.

In conclusion, our study demonstrated that the vitrification method used for storage of whole rat ovarian tissues induces detrimental effects on follicles. Subsequent autotransplantation of the ovaries into the omentum was not able to support viability of follicles and also insufficient blood supply associated ischemia caused more damage and significant decrease in the number of follicles. The results of this experiment showed that large follicles invariably undergo damage, whereas smaller ones survive well. In agreement with the previous reports, there is a direct relationship between the developmental stages of the follicles and their susceptibility to insufficient blood supply.

Acknowledgments

This work was supported by a research grant from the Shahid Bahonar University of Kerman, Iran.

References

Aubard Y, Pirer P, Cogni Y, Fermeaux V, Poulin N, Driancourt MA. 1999. Orthotopic and heterotopic autografts of frozen-thawed ovarian cortex in sheep. *Hum Reprod,* 14:2149-2154.

Babaei H, Derakhshanfar A, Kheradmand A. 2007. Vitrification of mouse ovaries using ethylene glycol and DMSO as cryoprotectants: histopathological evaluation. *Vet Arh,* 77:19-27.

Bedaiwy MA, Jeremias E, Guranluglu R, Hussein MR, Siemianow M, Biscotti C, Falcone T. 2003. Restoration of ovarian function after autotransplantation of intact frozen-thawed sheep ovaries with microvascular anastomosis. *Fertil Steril,* 79:594-602.

Candy CJ, Wood MJ, Whittingham DG. 2000. Restoration of a normal reproductive lifespan after grafting of cryopreserved mouse ovaries. *Hum Reprod,* 15:1300-1304.

Chen C-H, Chen S-G, Wu G-J, Wang J, Yu C-P, Liu J-Y. 2006. Autologous heterotopic transplantation of intact rabbit ovary after frozen banking at -196°C. *Fertil Steril,* 86:1059-1064.

Dissen GA, Lara HE, Fahrenbach WH, Costa ME, Ojeda SR. 1994. Immature rat ovaries become revascularized rapidly after autotransplantation and show a gonadotrophin-dependent increase in angiogenic factor gene expression. *Endocrinology*, 134:1146-1154.

Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squifflet J. 2004. Live birth after orthotopic transplantation of cryopreserved ovarian tissue. *Lancet,* 364:1405-1410.

Gosden RG, Baird DT, Wade JC, Webb R. 1994. Restoration of fertility to oophorectomized sheep by ovarian autografts stored at -196 ºC. *Hum Reprod,* 9:597-603.

Gougeon A. 1986. Dynamics of follicular growth in the human: a model from preliminary results. *Hum Reprod,* 1:81-87.

Imtharn B, Cox SL, Jenkin G, Trounson AO, Shaw JM. 2000. Gonadotrophin administration can benefit ovarian tissue grafted to the body wall: implications for human ovarian grafting. *Mol Cell Endocrinol,* 163:141-146.

Isachenko E, Isachenko V, Rahimi G, Nawroth F. 2003. Cryopreservation of human ovarian tissue by direct plunging into liquid nitrogen. *Eur J Obstet Gynecol Reprod Biol*, 108:186-193.

Israely T, Nero N, Harmelin A, Neeman M, Tsafriri A. 2006. Reducing ischaemic damage in rodent ovarian xenografts transplanted into granulation tissue. *Hum Reprod,* 21:1368-1379.

Kagabu S, Umezu M. 2000. Transplantation of cryopreserved mouse, Chinese hamster, rabbit, Japanese monkey and rat ovaries into recipients. *Exp Anim,* 49:17-21.

Kim SS, Hwang IT, Lee HC. 2004a. Heterotopic autotransplantation of cryobanked human ovarian tissue as a strategy to restore ovarian function. *Fertil Steril,* 82:930-932.

Kim SS, Yin H, Godsen RG. 2004b. Cryobanking of ovarian and testicular tissue for children and young adults. *In:* Tulandi T, Godsen RG (Eds.). *Preservation of Fertility*. London: Taylor & Francis. pp. 159-163.

Kim SS. 2006. Fertility preservation in female cancer patients: current developments and future directions. *Fertil Steril,* 85:1-11.

Kiran G, Kiran H, Coban YK, Guven AM, Yuksel M. 2004. Fresh autologous transplantation of ovarian cortical strips to the anterior abdominal wall at the pfannenstiel incision site. *Fertil Steril,* 82:954-956.

Lee DM, Yeoman RR, Battaglia DE, Stouffer RL, Zelinski-Wooten MB, Fanton JW. 2004. Live birth after ovarian tissue transplant. *Nature,* 428:137-138.

Lee RK, Ho H-Y, Yu S-L, Lu C-H. 2005. Blastocyst development after cryopreservation and subcutaneous transplantation of mouse ovarian tissue. *J Assist Reprod Genet,* 22:95-101.

Liu J, Van der Elst J, Van den Broecke R, Dhont M. 2001. Live offspring by in vitro fertilization of oocytes from cryopreserved primordial mouse follicles after sequential in vitro transplantation and in vitro maturation. *Biol Reprod,* 64:171-178.

Migishima F, Suzuki-Migishima R, Song S-Y, Kuranochi T, Azuma S, Nishijima M, Yokoyama M. 2003. Successful cryopreservation of mouse ovaries by vitrification. *Biol Reprod,* 68:881-887.

Newton H, Illingworth P. 2001. In vitro growth of murine pre-antral follicles after isolation from cryopreserved ovarian tissue. *Hum Reprod,* 16:423-429.

O'Brien MJ, Pendola JK, Eppig JJ. 2003. A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence. *Biol Reprod,* 68:1682-1686.

Oloumi MM, Derakhshanfar A, Molaei MM, Tayyebi M. 2006. The vasculogenic potential of autogenous free omental graft in experimental tibial defects in rabbit: a short term preliminary histopathological and immunohistochemical studies. *J Exp Anim Sci,* 43:179-187.

Radford JA, Leberman BA, Brison DR, Smith AR, Critchlow JD, Russell SA. 2001. Orthotopic reimplantation of cryopreserved ovarian cortical strips after high dose chemotherapy for Hodgkin's lymphoma. *Lancet,* 357:1172-1175.

Salehnia M. 2002. Autograft of vitrified mouse ovaries using ethylene glycol as cryoprotectant. *Exp Anim,* 5:509-512.

Shaw JM, Cox SL, Trounson AO, Jenkin G. 2000a. Evaluation of the long-term function of cryopreserved ovarian grafts in the mouse, implications for human applications. *Mol Cell Endocrinol,* 161:103-110.

Shaw JM, Oranratnachai A, Trounson AO. 2000b. Fundamental cryobiology of mammalian oocytes and ovarian tissue. *Theriogenology,* 53:59-72.

Smith BJ, Plowchalk DR, Glenn Stpes I, Mattison DR. 1991. Comparison of random and serial sections in assessment of ovarian toxicity. *Reprod Toxicol,* 5:379-383.

Sugimoto M, Maeda S, Manabe N, Miyamoto H. 1999. Development of infantile rat ovaries autotransplanted after cryopreservation by vitrification. *Theriogenology,* 53:1093-1103.

Torrents E, Boiso I, Barri PN. 2003. Applications of ovarian tissue transplantation in experimental biology and medicine. *Hum Reprod Update,* 9:471-481.

Vajta G, Holm P, Kuwayama M, Booth PJ, Jacobsen H, Greve T, Callesen H. 1998. Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos. *Mol Reprod Dev,* 51:53-58.

Wang X, Chen H, Yin H, Kim SS, Lin Tan S, Godsen RG. 2002. Cryopreservation: fertility after intact ovary transplantation. *Nature,* 415:385.

Yin H, Wang X, Kim SS, Chen H, Tan SL, Godsen RG. 2003. Transplantation of intact rat gonads using vascular anastomosis: effect of cryopreservation, ischaemia and genotype. *Hum Reprod,* 18:1165-1172.

Zhang QX, Magovern CJ, Mack CA, Budenhender KT, Ko W, Rosengart TK. 1997. Vascular endothelial growth factor is the major angiogenic factor in omentum: mechanism of the omentum-mediated angiogenesis. *J Surg Res,* 67:147-154.