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Beneficial Effect of Hyperbaric Oxygen Therapy on the Follicular Survival in the Mouse Ovary after Transplantation

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Abstract. A large proportion of follicles are lost during the initial ischemia that occurs after transplantation of ovarian tissues. Thus, the effect of hyperbaric oxygen therapy (HBO) on the follicular loss of ovarian tissues after transplantation was examined in mice. Ovarian slices from ICR mice were transplanted under the kidney capsule in ovariectomized ICR. Hyperbaric oxygen with 100% oxygen was initiated for 30 min at 2.5 atmospheres absolute immediately after transplantation, and this treatment was repeated at 48-h intervals for 2 weeks. The number of follicles was dramatically reduced at 2 weeks post transplantation. However, HBO was significantly effective in enhancing the survival of transplanted ovarian follicles. The survival rates of primordial and primary follicles in ovarian tissues of mice with HBO were significantly higher than those without HBO. These results indicate HBO can be effectively used for the enhancement of survival of transplanted ovarian tissues.

Key words: Hyperbaric oxygen, Mice, Ovary, Transplantation

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Several options such as cryopreservation of embryos, oocytes or ovarian tissue are currently available to preserve fertility in cancer patients. However, cryopreservation of ovarian tissue is the only option available for prepubertal girls and women in need of immediate chemotherapy [1, 2]. Additionally, the cryopreservation of ovarian tissues is a potentially significant technology for the preservation of the genetic resources of working dogs as well as other laboratory and domestic animals [3, 4]. Several reports have indicated that follicular loss in the mammalian ovary is not drastically extended by the cryopreservation procedure itself [3–5]. It is believed that the reason the primordial follicle is observably resistant to cryoinjury is because the oocyte it contains has a relatively inactive metabolism and lacks a meiotic spindle, zona pellucida and cortical granules [6]. In fact, a high percentage of oocytes as well as granulosa cells survive the cryopreservation and thawing procedures [3, 7–9]. Despite being able to survive freezing, it is well established that a large proportion of follicles are lost during the initial ischemia that occurs after transplantation of ovaries of mouse [10, 11], sheep [12, 13], dog [4, 5] and human [14, 15]. Most follicles that survive cryopreservation undergo ischemic loss during neovascularisation [16]. Several attempts have been made to prevent the follicular loss of cryopreserved ovarian tissues after transplantation. Treatment of ovarian tissues with a water soluble antioxidant (ascorbic acid) is known to reduce apoptosis in the ovarian cortex *in vitro* [17]. However, local application of sphingosine-1-phosphate, an apoptosis inhibitor, does not prevent follicular loss after transplantation in sheep [18]. It has been reported that treatment with vitamin E, a lipid soluble antioxidant, improves the survival of follicles in ovarian grafts by reducing ischemic injury [19]. More recently, we have shown that both local [9] and systemic administration (our unpublished observation) of desialylated erythropoietin effectively increase in the survival of follicles in cryopreserved canine ovarian tissues after xenotransplantation. However, a fully effective solution has not been found to date.

Application of HBO is well established in the treatment of decompression sickness and generally accepted for the treatment of carbon monoxide poisoning, gas embolism and radionecrosis [20]. Recently, effects of HBO on transplantation have been reported in several organs and tissues such as the liver, bone, thyroid and pancreatic islet cells [21]. However, the effect of HBO on follicular survival after ovarian tissue transplantation has not been examined to date. Thus, we examined the effect of HBO on follicular survival in the mouse ovary after transplantation as a model, since a large proportion of follicles are lost during the initial ischemia that occurs after ovarian transplantation in mammals including humans [4, 5, 10–15].

Eighty percent (20/25) and 65% (17/26) of transplanted ovarian tissues were recovered from recipients with and without HBO treatment at 2 weeks after transplantation, respectively. As shown in Table 1, the average numbers of primordial, primary, secondary and antral follicles in ovarian tissues of mice decreased from 3.4 ± 0.69, 3.1 ± 0.65, 5.7 ± 1.07 and 2.1± 0.67/mm^2 to 0.3 ± 0.08, 0.2 ± 0.09, 0.2 ± 0.08 and 0/mm^2 at 2 weeks after transplantation, respectively. However, higher numbers of each stage of follicles were retained when recipient mice were treated with hyperbaric oxygen. The mean numbers of primordial, primary and secondary follicles per square millimeter in ovarian sections were 1.7 ± 0.39, 1.4 ± 0.34 and 0.9 ± 0.29 in the HBO group. These values were significantly higher than those of the control (without hyperbaric

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Table 1. Effect of hyperbaric oxygen therapy on the follicular reserve in mouse ovarian tissues

Exp. group	No. grafts examined	Number of follicles /mm^2 (Mean ± SE)				
		Primordial	Primary	Secondary	Antral	Total
Untransplanted	21	3.4 ± 0.69^a	3.1 ± 0.65^a	5.7 ± 1.07^a	2.1 ± 0.67^a	14.3 ± 1.58^a
Control	17	0.3 ± 0.08^b	0.2 ± 0.09^b	0.2 ± 0.08^b	0 ^{b}	0.7 ± 0.16^b
HBO*	13	1.7 ± 0.39^a	1.4 ± 0.34^a	0.9 ± 0.29^b	0.1 ± 0.07^b	4.1 ± 0.82^c

The different superscript letters within a column indicate significantly different values (P<0.05). *: hyperbaric oxygen therapy.

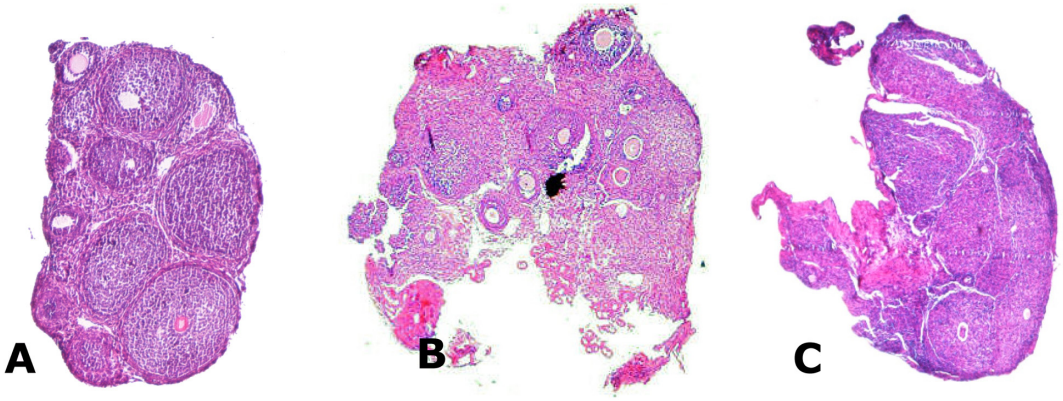

Fig. 1. Hematoxylin and eosin staining of transplanted mouse ovarian tissues with HBO. ICR mouse ovarian tissues were transplanted under the kidney capsule in ovariectomized ICR mice. The recipients were treated with hyperbaric oxygen with 100% oxygen for 30 min at 2.5 ATA, and the treatment was repeated at 48-h intervals for 2 weeks. Larger numbers of follicles are seen in mouse fresh (A) and HBO-treated (B) ovarian tissues; however, there are fewer follicles in transplanted tissue without HBO treatment (C).

oxygen) in each follicular developmental stage (P<0.05). The survival rates of primordial (49.6%) and primary (45.5%) follicles in ovarian tissues of mice with HBO were higher than those without HBO (8.7 and 7.3%, respectively). HBO dramatically enhanced the follicular survival of transplanted mouse ovarian tissues (Table 1 and Fig. 1). The survival rates of primordial, primary and secondary follicles in ovarian tissues with HBO were approximately fivefold higher than those without HBO. These results indicate that HBO is applicable for transplantation of ovarian tissues as well as other organs and tissues.

It has been shown that HBO reduces the severity of ischemia, preservation and reperfusion injury (IPRI) in liver transplantation [21]. In addition, HBO has a beneficial therapeutic effect in several models of IPRI including those for the myocardium, skeletal muscle and small intestine [22]. The mechanisms underlying the effectiveness of HBO appear to be related to a reduction in tissue hypoxia, decreased levels of cytokines such as TNF-α or IL-1 and inhibition of apoptosis [21]. A number of *in vitro* and *in vivo* studies have shown that HBO selectively induces endothelial nitric oxide synthase (eNOS) while inhibiting inducible NOS (iNOS) production [23]. NO produced by iNOS activation results in increased vascular leakage and tissue injury, unlike the beneficial effects of eNOS [21]. Naturally, eNOS is constitutively expressed in endothelial cells and plays a role in vasodilation of the microcirculation in response to hypoxia [21]. In addition, the effect of HBO may be through the inhibition of apoptosis. A study of the cerebral ischemic model in rats showed the ability of HBO to inhibit apoptosis and promote neurologic functional recovery [24]. It has been proposed that the effects of HBO for fetal spinal transplantation were due to a combination of reduction in tissue hypoxia and tissue edema and an increase in microvascular proliferation at the graft interface leading to better graft survival [25]. HBO also improves islet function after transplantation due to a significant decrease in hypoxia, apoptosis and vascular endothelial growth factor (VEGF) expression [26]. It is well known that hypoxia inducible factor (HIF)-1α upregulates VEGF under ischemic conditions [27]. Thus, HBO-related HIF-1α reduction is followed by a strong reduction in VEGF-1 expression. As increased VEGF production delays vessel maturation [27], decrements in hypoxia and subsequent decreases in VEGF may accelerate the development of mature vessels. Although precise mechanisms of the effect of HBO on ovarian transplantation were not examined in this study, those factors and/or the mechanisms described above could account for the higher follicular survival after ovarian transplantation in mice (Table 1 and Fig. 1). Since it has been reported that most follicles are lost during the time required for neovascularization after ovarian tissue transplantation [16], the initial treatment within 72 hr post transplantation might contribute to follicular survival.

In addition to the combination of HBO and hormone treatment [4], provision of pretransplantation HBO to donors [28] might also

be effective for follicular survival after ovarian transplantation.

Methods

Both female and male ICR mice were purchased from a commercial supplier (CLEA Japan, Tokyo, Japan) and were bred in our animal facility. All animals were housed in polycarbonate cages, and maintained in a specific pathogen-free environment in light-controlled (lights-on from 0700 to 1900 h) and air-conditioned rooms (temperature of 24 ± 1 C, humidity of 50 ± 10%). They had free access to standard laboratory chow (CA-1; CLEA Japan, Tokyo, Japan) and water *ad libitum*.

Female mice (8-week-old, n=16) were anesthetized by inhalation of isoflurane (ISOFLU, Dainippon Sumitomo Pharma, Osaka, Japan), and then the dorsal skin was incised to draw out the ovaries and kidneys. An incision was made on the lateral side of each ovary to remove the mouse ovary from the ovarian bursa. After ovariectomy, one to three pieces of ovarian tissues (1.0–1.5 mm cubes) obtained from 3-week-old female mice were transplanted under the kidney capsule of both sides of the kidneys. The skin incision was closed with a clip (9-mm auto clip, 427631, Becton, Dickinson and Company). The operated mice were placed on a warm plate until sufficient recovery occurred to allow movement. Hyperbaric oxygen with 100% oxygen (purity: >99.5 vol %, Air Water Techno Supply, Obihiro, Japan) was initiated for 30 min at 2.5 atmospheres absolute (ATA) immediately after transplantation. The mice were placed in a hyperbaric chamber (Model TM5R, Unicontrols, Tokyo, Japan) with a compression rate of 0.1 ATA/min (1 ATA=14.233 pound-force per square inch). Air exhaled by the mice was expelled from the chamber by ventilating the chamber with 100% oxygen every 15 min during HBO. HBO was repeated at 48-h intervals for 2 weeks. In the control group, the operated mice were not treated with HBO. At 2 weeks after transplantation, the transplanted ovaries were removed. The removed transplanted ovaries and pretransplant ovarian tissues were fixed with 10% formalin and then subjected to hematoxylin and eosin staining. To evaluate the effects of transplantation subsequent to hyperbaric oxygen therapy, follicles that visibly contained an ovum (oocyte) with a nucleus were counted according to the classification of Myers *et al.* [29] as follows: Primordial follicles were defined as oocytes surrounded by either a partial or complete layer of squamous granulosa cells. Primary follicles showed a single layer of cuboidal granulosa cells. Secondary follicles were surrounded by more than one layer of cuboidal granulosa cells, with no visible antrum. Antral follicles possessed a clearly defined antral space. For ovarian tissue, about ten sections (5 µm in thickness) were sequentially prepared for a tissue specimen (a block). A total of 13–21 graft samples were examined for each experimental group including the untransplanted, without HBO (control) and HBO groups. The distance between sections was 100 µm. The number of follicles in each section was counted, and the area of each section was calculated by using the ImageJ 1.41 software bundled with Java 1.6.0-10. The number of follicles in each stage in each section was expressed per square millimeter. The data were expressed as means ± standard error of the mean. The survival rates of follicles were calculated as the number of follicles in transplanted ovarian tissues / number of follicles in pretransplant ovarian tissue samples × 100. Statistical analysis was performed by using the Wilcoxon signed-rank test. P values less than 0.05 were considered to be significant.

The tissues and animals used in this study were treated under the Guiding Principles for the Care and Use of Research Animals established by Obihiro University of Agriculture and Veterinary Medicine.

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