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Protective role of adrenomedullin in heterotopic ovarian transplant

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Summary

Purpose of investigation: To study adrenomedullin (ADM) in preventing ischemia and morphological changes in heterotopically transplanted ovary. *Materials and Methods:* Forty female Sprague-Dawley rats were divided into four groups. In groups 1 and 2 each ovary was transplanted to the corresponding inguinal region by heterotopic transplantation. In groups 3 and 4, ovaries were left intact without transplantation. Treatment was injected to left inguinal region. ADM was given in groups 1 and 3 and placebo was given in groups 2 and 4. Left ovaries showing local treatment effect in heterotopic transplantation was designated as A (1A, 2A, 3A, and 4A), right ovaries showing systemic treatment effect were designated as (1B, 2B,3B, and 4B). Main outcome measures were ischemia, follicle count, and CD 31 expression. *Results:* Ovaries treated with local ADM (group 1A) were in consonance with normal rat ovaries. The ovaries of rats in group 1B and placebo treated transplant group (groups 2A, 2B) exhibited varying effects of ischemia. The mean follicle numbers in groups 1A, 1B, 2A, 2B were 28 ± 3.3, 16.8 ± 2.5, 17.7 ± 2.1, 16.4 ± 2.9, respectively. The mean follicle number in groups 3A, 3B, 4A, and 4B were 27.7 ± 2.0, 28.3 ± 2.2, 28.3 ± 2.2, 27.8 ± 1.9, respectively. Corpus luteum number and CD31 expression was found to be significantly higher in group 1A. *Conclusion:* Subcutaneous injection of ADM to heterotopic ovarian graft site causes vasodilatation and increases angiogenesis and may protect ovarian graft against hypoxic damage.

Key words: Heterotopic transplantation; Adrenomedullin; Ovary; Ischemia; CD 31.

Introduction

Population of oocytes is fixed before birth and oocytes cannot be renewed in mammalians after birth [1]. Depletion of oocytes and cessation of ovarian function result in menopause. Pelvic radiotherapy in young patients with cervical cancer can lead to premature ovarian failure. Approximately 30,000 cases of cancer are diagnosed annually in women aged between 25–49 years old, and cervical cancer comprises 2% of female cancers in reproductive period [2]. Forty-one percent of cervical cancer patients are less than 45 years of age [3]. Young survivors of cancer experience somatic symptoms of menopause and are at increased risk of osteoporosis, bone fracture, cardiovascular diseases, hot flushes, stroke, anxiety, and sexual dysfunction [4, 5]. Therefore, preservation of gonadal function is of great concern in young patients with cancer. Risk of ovarian metastasis in cervical cancer is very low if the ovaries are macroscopically normal [6]. Ovarian transposition is the choice of surgical procedure to preserve ovarian function by transposing the ovaries out of the field of radiotherapy, however the effectiveness of ovarian transposition is debatable in the literature [7, 8].

Fresh transplantation of ovaries far away from the field of pelvic irradiation (heterotopic transplantation) may be an alternative to young patients with cancer to preserve ovarian function [9]. Transplantation of ovary under the skin is the preferred grafting site, because monitoring of ovary is simple [10]. Vascularization of the ovarian graft is the major factor limiting transplantation, particularly in heterotopic transplantation. Ischemic damage results in apoptosis of ovarian follicles. Majority of the follicles die due to ischemia during transplantation, and finding ways to accelerate graft vascularization is essential for development of reproducible and reliable procedures for ovarian transplantation [11].

Adrenomedullin (ADM) is a 52-amino acid peptide, structurally and functionally related to calcitonin, calcitonin gene-related peptide [12]. ADM has been shown to mediate multifunctional responses in cell culture and animal systems, particularly regulation of growth and apoptosis [13]. ADM is a potent vasodilator [14]. ADM regulates vascular permeability and plays a major role in forming blood vessels in physiological and pathological conditions [14-16]. Immunostaining by CD31 revealed

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that ADM increased capillary formation in ischemia, hypoxia, neoplasia, and tumor angiogenesis [16, 17]. Inflammation and hypoxia increases ADM expression in tumors [17]. Elevated levels of ADM is shown to be associated with tumor neovascularization in xenografted endometrial tumors and renal cell carcinoma [17, 18]. ADM also acts as a cell survivor factor; it plays a potent protective role against apoptosis and maintains cellular integrity [19]. Protective effect of ADM by preventing apoptosis and inducing angiogenesis is reported in ischemia and hypoxic conditions such as stroke, age-related macular dysfunction, intrauterine growth restriction, myocardial infarct, and carcinomas of various organs [19-22]. However, protective role of ADM in transplantation has not been studied before in the literature. Aim of the present study was to investigate whether ADM can prevent ischemia and morphological changes in heterotopically transplanted whole ovary.

Materials and Methods

An animal research was designed to study the effect of ADM in heterotopic whole ovary transplantation. The study was approved by institutional review board and all procedures were approved by the Animal Care and Use Committee of the University.

Forty female Sprague-Dawley rats, weighing 160-210 grams, were allowed to acclimatize for seven days. Rats were housed in a controlled environment at 23 ± 2°C on an illumination schedule of 12 hours of light and 12 hours of darkness each day. Rats were fed standard food and tap water ad libitum. The animals were maintained in accordance with Animal Care and Use Committee regulations. After acclimatization, rats were divided into four groups and the surgical procedures were undertaken. The protocols in this study followed guiding ethics for research involving animals as recommended by the Declaration of Helsinki and the Guiding Principles in the Care and use of Animals [23].

All rats were operated with ketamine (80 mg/kg body weight) and xylazine (ten mg/kg body weight) for anesthesia. Abdominal and inguinal skin was shaved and sterilized with iodine. A two-cm skin incision was made and the subcutaneous transplantation site was prepared. Ovaries were harvested by midline laparotomy. After being freed from any foreign tissue, the ovary which was to be transplanted was washed in physiological solution (0.9% NaCl), just before transplantation. After removal, cleaning and rinsing, the entire ovary was immediately placed into previously prepared subcutaneous transplantation site without vascular anastomosis. The incision was sutured using 4–0 sutures.

Rats were divided into four groups. In groups 1 and 2, heterotopic transplantation was performed; in groups 3 and 4 ovaries were left intact without transplantation. In groups 1 and 2, both ovaries were harvested and each ovary was transplanted to right and left inguinal region. ADM treatment was given to group 1 rats and placebo was given in group 2 rats for control. Groups 3 and 4 were control groups without transplantation. In groups 3 and 4, only midline laparotomy was made. Treatment by ADM was administered to rats in group 3 and placebo was administered to rats in group 4.

ADM was administered ten μg/kg, daily for 21 days. ADM was administered in group 1 (transplantation and ADM treated group) and group 3 (no transplantation, ADM treated group). 0.9% NaCl was used for placebo in group 2 (transplantation, no treatment group) and group 4 (no transplantation, no treatment). Injections were made to left inguinal operation site.

Left heterotopic ovaries of the rats from groups 1 and 2 showed the local treatment effect, while right heterotopic ovaries showed the systemic treatment effect after subcutaneous injection. Left ovaries were showing local treatment effect in heterotopic transplantation was designated as A (1A, 2A, 3A, and 4A), right ovaries showing systemic treatment effect were designated as (1B, 2B, 3B, and 4B). Rats were sacrificed at the end of 21-day treatment, ovaries were removed for macroscopic gross examination and microscopic examination.

Macroscopic examination for viability was assessed and then tissue samples were fixed in a 10% formalin solution, dehydrated through a graded ethanol series, cleared in xylene, and processed for embedding in paraffin wax, according to routine protocols. Five-μm-thick sections were cut by microtome and stained with hematoxylin and eosin (H&E) according to the standard method. Sections were evaluated to detect the follicle and corpus luteum number by using a CX41 bright-field microscope.

Immunohistochemical analysis for CD31 (rabbit polyclonal, 1:200) was performed on formalin fixed, paraffin-embedded tissue blocks using the streptavidin-biotin-peroxidase technique.Following antigen retrieval, four-μm-thick sections were washed gently in deionized water, then treated with 2% trypsin in Tris buffer (50 mMTris base and 150 mMNaCl dissolved in deionized H_2O) at 37°C for 15 minutes. Endogenous peroxidase was blocked with 3% hydrogen peroxide for ten minutes. Slides were incubated with avidin and biotin blocking solutions for 15 minutes each, and 3% normal goat serum for 20 minutes in order to prevent nonspecific staining in the background. All slides underwent overnight incubation at 4°C. Negative controls for immunostaining were provided by omitting the primary antibody step. After washing with TBST, biotinylated goat anti-rabbit IgG (1:1000) were applied to the sections for 30 minutes at room temperature. Then all of the sections were incubated with strepavidin-HRP for 30 minutes at room temperature. Finally, 3-amino-9-ethylcarbazole was used as the chromagen and hematoxylin as the counterstain.

Depending on the size of the H&E section, three to five high power areas within the slide were selected randomly for evaluation. Image-analyzing software was used to lock on these preselected areas for each histological section of the same paraffin block. The microvessel density (MVD) measurements for CD31 were performed within each area at a ×100 magnification. The MVD was measured based on Weidner's method [24]. Each positive endothelial cell cluster of immunoreactivity in contact with the selected field was counted as an individual vessel in addition to the morphologically identifiable vessels with a lumen.

Results

Macroscopic and microscopic examination of ovaries revealed obvious tissue modifications between heterotopic transplant group rats treated with ADM and placebo. Ovaries from control group rats (groups 3 and 4) were normal and they had follicles in different stages of development and a cellular stroma. Ovaries treated with local ADM (group 1A) were in consonance with normal rat ovaries as described before [20, 21] and control groups. The ovaries of rats in group 1B (systemic effect of ADM) and placebo treated transplant group (groups 2A and 2B) were exhibiting varying effects. There were changes in

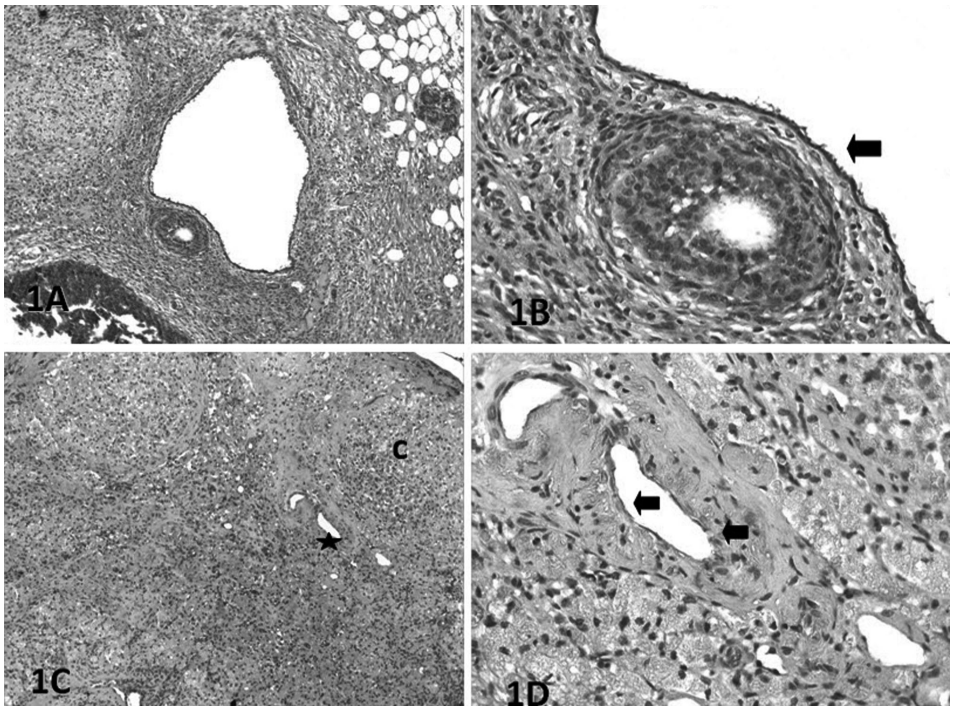

Figure 1. — There is marked vasodilation by ADM local injection in heterotopically transplanted ovary (CD31, ×100). B) CD31 expression (arrow) in the vascular endothelium in group 1A (CD31, ×400). C) Corpus luteum (C) number was significantly higher along with the vascularization (*) in group 1A (CD31, ×100). D) Remarkable lipid accumulation in the CL of ADM treated rats and increased vascularization (arrows). This might be due to the increased steroid hormone synthesis by ADM (H&E, ×400).

Table 1. — *A: follicule counts of transplantation and control groups. B: corpus luteum counts of transplantation and control groups.*

Group	Follicle count	CL count
1A	28.0±3.3	26.4±2.4
2A	17.7±2.1	15.4±2.0
3A	27.7±2.0	23.8±2.2
4A	28.3±2.2	20.6±2.5
1B	16.8±2.5	15.7±1.8
2B	16.42.9	15.4±2.0
3B	28.3±2.2	22.0±2.1
4B	27.8±1.9	21.2±2.0

Table 2. — *CD31 expressions of transplantation and control groups.*

Transplantation	Group 1A	Group 1B	Group 2A	Group 2B
	283±8.9	180±7.2	180±7.3	178±9.6
Controls	Group 3A	Group 3B	Group 4A	Group 4B
	110±7.5	104±3.4	100.6±3.7	101±4.5

the color, size, and macroscopic viability of ovaries.

Microscopic examination was in concordance with macroscopic evaluation. Morphology of follicles was protected in locally ADM treated group 1A rats. There were significantly more corpus luteum structures and lipid accumulation in the corpus luteum was remarkable. There was aberrant vascular dilatation in this group (Figure 1); however, the follicles were remarkably scant in groups 1B, 2A, and 2B. There was no significant vascular dilatation in groups 1B, 2A, and 2B.

The mean follicle number in groups 1A, 1B, 2A, 2B were 28 ± 3.3, 16.8 ± 2.5, 17.7 ± 2.1, 16.4 ± 2.9, respectively. The mean follicle number in groups 3A, 3B, 4A, and 4B were 27.7 ± 2.0, 28.3 ± 2.2, 28.3 ± 2.2, 27.8 ± 1.9,respectively (Table 1). The mean number of follicles in group 1A was similar to control groups 3 and 4, whereas it was significantly higher than the mean follicle number in groups 1B and group 2. This finding showed that ADM was useful locally to preserve viability of ovary. The corpus luteum number was also found to be significantly higher in group 1A. CD31 expression was increased in heterotopic transplant groups (groups 1 and 2). CD31 expression was highest in group 1A. CD31 expression was similar in groups 1B, 2A, and 2B (Table 2).

Discussion

Hypoxic-ischemic damage is the major challenge for large grafts in heterotopic transplantation. Ischemia causes depletion of follicles during the first days after transplantation and this continues for one week [22, 25]. The present authors have studied local and systemic effect of ADM to salvage transplanted ovaries from ischemia. This is the first study investigating the novel, angiogenetic, anti-apoptotic factor (ADM) in heterotopic transplantation. Subcutaneous injection of ADM to transplantation field was

found to protect the ovarian follicles from hypoxia and increased the vascularization of the graft after seven days. Subcutaneous injection of ADM to another site did not protect the transplanted ovary.

One of the limitations of the present study was that the authors were not able to detect hormones such as FSH, LH, estradiol, inhibin, or anti-Müllerian hormone (AMH) with a sensitive analysis. It is reported that these markers may not be useful as a marker of ovarian reserve after transplantation, although they reflect ovarian function in other clinical situations [26, 27]. Ovarian function may also return late after transplantation [27]. Longer follow-up after transplantation is needed to evaluate hormonal function of the ovary. The present aim was to study the viability, loss of follicles, and vasculogenesis of the ovary by ADM injection after heterotopic transplantation. ADM may also effect the secretion of hormones, which may cause a bias for hormonal status [28, 29]. Hormonal function, development of follicles, and fertility may further be investigated in future studies with longer duration. Another limitation of the present study may be the follow-up after transplantation. The decision to sacrifice animals on the eighth day of transplantation is based on previous studies. Experimental studies showed that the fall in number of follicles in grafted tissue due to hypoxia and delay re-vascularization occurs in two to four days [26]. The critical time for the full recovery of ovarian function after transplant happens during the first 24 hours [30]. Dissen *et al.* showed profuse re-vascularization 48 hours after transplantation [31]. Neovascularization is completed by seven to ten days after transplantation [32,33].

CD31 is a reliable marker to evaluate angiogenesis in tranplants [16, 34]. The present authors have found the microvessel density (expression of CD31) to be significantly higher in locally ADM treated transplanted ovaries. ADM is released under hypoxic conditions and ADM is a potent vasodilator and induces angiogenesis [15-17]. ADM also regulates vascular permeability [15], which may contribute to the graft nourishing and survival [33]. ADM was protective against hypoxia; number of follicles and corpus luteum was significantly higher in locally treated ovarian grafts. Chaung *et al.* reported that peripheral administration of ADM may reduce stroke-induced brain ischemic injury [35]. In the present study, the authors found that ADM was protective only in local injection to the site of transplantation.

Damous *et al.* reported that corpus luteum appeared seven days after transplantation [32]. Presence of corpus luteum indicates immature follicles can grow and mature to the preovulatory stage, eventually ovulating and forming corpora lutea. These findings suggested that heterotopic ovarian transplantation into subcutaneous tissue can resume normal function and estrous cycle. The number of corpus luteum was higher in group 1A. Corpus Luteum is a well vascularized structure and formation of CL may be associated with ADM. ADM and its mRNA were reported in the follicles and the corpora lutea (CL) of rat and human ovaries [28,36]. In human ovary, ADM levels were low in the mature follicle but increased in the CL of the mid-luteal phase and remained high in CL of early pregnancy [36]. The present authors have observed remarkable lipid accumulation in the CL of ADM treated rats. This might be due to the increased steroid hormone synthesis by ADM.

Conclusion

Subcutaneous injection of ADM to heterotopic ovarian graft site causes vasodilatation and increases angiogenesis. These benefical effects of ADM may protect the ovarian graft against hypoxic damage, and depletion of follicles. The heterotopic graft treated by ADM is viable, can resume normal function, and the follicles may develop to CL. Further studies on ADM and ovarian transplantation is necessary for a better understanding of interaction of ADM, ischemia, and grafts.

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