Original Article

Anti-aging Effect on Skin of Autologous Transplantation of Tissue Fragments from Thawed Cryopreserved Ovaries

Hiroharu Imanishi¹, Suguru Igarashi¹, Yoko Yamaguchi², and Nao Suzuki¹

(Received for Publication: August 21, 2017)

Abstract

Transplanting the ovaries of young mice into menopausal mice has been shown to extend their lifespan, suggesting that the reproductive organs may play an important role in combating aging. Preventing skin aging is an extremely important matter with respect to maintaining quality of life, but little basic research has been carried out on this issue. The effects of treatment with cryopreserved ovarian tissue, tissue hormone therapy (THT), and hormone replacement therapy on inhibiting skin aging in experimental animals were investigated. The effects on skin elasticity and body weight changes in 6-week-old mice resulting from the transplantation of cryopreserved ovarian tissue were evaluated, as were the effects on skin of estrogen administration after bilateral oophorectomy or transplantation. Estrogen was secreted by mouse ovaries that had been frozen, thawed, and transplanted, and the estrus cycle was restored, but this was insufficient to have any effect on skin elasticity. After oophorectomy, the body weight of the mice increased, and their skin elasticity decreased. Estrogen administration after these changes had occurred neither restored skin elasticity nor suppressed body weight gain. However, when estrogen was continuously administered from immediately after oophorectomy, skin elasticity decreased transiently and then improved. If techniques for THT using cryopreserved ovaries to maintain the blood estrogen concentration above a specific level can be established, this might help to prevent or improve the deterioration of skin appearance in young women who require oophorectomy due to gynecological disease and also in childhood, adolescent, and young adult cancer patients.

Key words

Tissue hormone therapy, cryopreservation of ovarian tissue, mouse, ovarian transplantation, skin

Introduction

Women who have had an ovary removed at a young age are at increased risk of cognitive impairment or dementia, Parkinson’s disease, and death from a neuropsychiatric disorder¹. Conversely, transplanting the ovaries of young mice into menopausal mice has extended their lifespan², suggesting that the reproductive organs may play an important role in the appearance of disorders, control of the lifespan of living organisms, and combating aging.

Hormone replacement therapy (HRT) is an effective treatment for a range of symptoms that appear during the menopausal period, and it has also been found to have a cosmetic effect by improving skin elasticity and increasing collagen³⁴.

Preventing skin aging by suppressing the wrinkling and bagginess of the skin that appear with increasing age is an extremely important matter with respect to maintaining quality of life not only for menopausal women, but also for cancer survivors suffering from primary ovarian insufficiency after chemotherapy or radiotherapy. However, little basic research has been carried out on this issue.

Therefore, the anti-aging effects on the skin of experimental animals treated with cryopreserved ovarian tissue, tissue hormone therapy (THT), and HRT were investigated.

¹ Departments of Obstetrics and Gynecology, St. Marianna University School of Medicine, Kawasaki, Japan
² NANOEGG Research Laboratories, Inc., Tokyo, Japan
Experimental Procedures

Experimental Animals
Four-week-old and 6-week-old female Hos:HR-1 mice (Hoshino Laboratory Animals, Inc., Bando, Japan) were used. The mice were housed in a room of the St. Marianna University School of Medicine animal care unit under conditions of constant temperature (23 ± 1°C), constant humidity (55% ± 5%), and daylight (6:00–18:00). They were fed commercial chow and given tap water to drink. Oophorectomy and transplantation were carried out under inhalation anesthesia. All experiments were approved by the Animal Care and Use Committee of the Animal Care Unit of St. Marianna University School of Medicine (Approval number: 1510009).

Freezing, thawing, and transplantation of ovaries
Ovaries were frozen and thawed using the vitrification technique\(^5\). The harvested ovaries were then finely sliced into eight pieces and placed in phosphate buffer I (PBI) medium containing 1M dimethyl sulfoxide (room temperature)\(^6\), after which they were placed into cryotubes (Nalge Nunc International KK, Yokohama, Japan). These were placed on ice for 5 min, after which 95 μL of DAP 213 solution (2M dimethyl sulfoxide, 1M acetamide, 3M propylene glycol in PBI) maintained at 0°C was added to each cryotube. The tubes were again placed on ice for 5 minutes and then placed in liquid nitrogen for cryopreservation. Thawing was carried out by removing the cryotubes from the liquid nitrogen and allowing them to thaw at room temperature for 30 seconds, after which they were placed in dilution fluid consisting of PBI medium containing 0.25M sucrose (37°C). After washing with PBI medium, they were stored in Whitten medium at 0°C until transplantation. This method refers to the experimental method reported in 2003 that succeeded in obtaining cryopreservation of ovarian tissue offspring with reproducible results by cryopreservation of ovaries using DAP 213 preservation solution, followed by transplantation to recipients after thawing\(^7\).

Anti-aging effect on skin of the transplantation of cryopreserved ovarian tissue
Six-week-old mice were divided into Group A (sham surgery, n=5), Group B (oophorectomy, n=5), and Group C (cryopreserved ovarian tissue transplantation, n=5).

Body weight; skin elasticity as assessed by R2 (the restoration ratio after extension), R6 (the ratio of viscoelasticity to elastic deformation), and R7 (the ratio of the elastic portion at retraction); and blood estrogen concentrations were measured every week after oophorectomy was performed on Groups B and C. The skin of all three groups was subjected to histological analysis (hematoxylin-eosin [H-E] staining and picrosirius red staining) 13 weeks after the start of the experiment.

In Group C, the cryopreserved ovarian tissue was thawed, and subcutaneous autologous transplantation was performed 9 weeks after oophorectomy. The return of the estrus cycle was confirmed 1 week after the slices were transplanted.

Effect of postmenopausal HRT on skin
Ovaries were removed from 6-week-old mice, and 17β-estradiol (E2) was administered subcutaneously at 0 pg, 250 pg, 2500 pg, and 25,000 pg (each, n=2) and intraperitoneally at 0 pg and 25,000 pg (each, n=2) after 13 weeks\(^8\). Body weight and skin elasticity (R2, R6, and R7) were measured at weekly intervals.

Effect of perimenopausal HRT on skin
Six-week-old mice were allocated to a bilateral oophorectomy group, a sham surgery group, and a control group, an estrogen administration group, a progesterone administration group, an estrogen plus progesterone administration group, and a cryopreserved ovarian tissue transplantation group (each group n=5). Estrogen 25,000 pg and/or progesterone 1 mg (Wako Pure Chemical Industries, Osaka, Japan) was administered intraperitoneally every 3–4 days, and body weight and skin elasticity were measured over time. Both estrogen and progesterone were dissolved in olive oil (Wako Pure Chemical Industries, Osaka, Japan) before administering to the mice, and only the olive oil was administered to the control group.

The skin of the mice of all groups was subjected to histological evaluation (H-E staining and picrosirius red staining) 3 weeks after the start of the experiment. Thawing and transplantation of cryopreserved ovarian tissue were performed the day after oophorectomy in the transplantation group.

Statistical analysis was performed using the Student t-test, with P<0.05 considered the level of significance. Data analysis was conducted with Microsoft Excel for Mac 2011.
Results

Skin elasticity, measured in terms of the restoration ratio after extension (Figure 1a), the ratio of viscoelasticity to elastic deformation (Figure 1b), and the ratio of elastic portion at retraction (Figure 1c), was significantly decreased in Groups B (oophorectomy group) and C (cryopreserved ovarian tissue transplantation group), both of which had undergone oophorectomy, compared with those in Group A, which did not undergo oophorectomy. Mean body weight was also significantly greater in Groups B and C than in Group A (Table 1) (Figure 2a, b). Autopsies showed the accumulation of visceral fat in the lower abdomen, particularly around the uterus, and a pronounced increase in fat near the rump.

These parameters were compared at 4 weeks after the thawing and transplantation of cryopreserved ovaries, but no significant differences were observed between Group C and Group A or B (Table 2). Histological evaluation also found no significant differences between any of the three groups in terms of the thickness of the dermal or muscle layers and collagen content (Figures 2c, d). The mean blood E2 concentration was 5 pg/mL in Group A, much higher than that in Groups B and C (Figure 3). The mean blood E2 concentration in groups B and C was 2 pg/mL or less, which is lower than the detection sensitivity of the kit; therefore, the average E2 concentration was actually unknown.

Observations were carried out for two weeks after E2 administration. There were no significant differences between any of the three groups in body weight or skin elasticity measured in terms of R2 (Figure 4a), R6 (Figure 4b), and R7 (Figure 4c), and estrogen administration had no effect on improving the decrease in skin elasticity.

Body weight and elasticity were evaluated 3 weeks after the start of the experiment. Body weight increased significantly in the animals that had undergone transplantation after oophorectomy (Group C) compared with those that had undergone sham surgery (Group A) (Figure 5a). The R2 (Figure 5b) and R7 (Figure 5c) values were significantly lower in the animals that had received E2 administration than in those that still possessed ovaries.

Discussion

HRT is an effective treatment for a range of symptoms that appear during the menopausal period as a result of diminished ovarian function and is also effective against osteoporosis, fat metabolism, arteriosclerosis, diminished cognitive function, and depressed mood. However, it is known to increase the risk of several disorders including breast cancer9).
Figure 2. (a) Photo of a control (group A) mouse. (b) Photo of an oophorectomy group mouse (groups B and C). The oophorectomy group mouse had a body weight of about 5 g, and obesity centered on the lower body is present. (c) and (d) show hematoxylin-eosin staining for evaluation of the thickness of the dermal layer and muscle layer.

Table 2. Average weight change after cryopreserved ovarian tissue transplantation

<table>
<thead>
<tr>
<th>Weeks/Groups</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>6w Group A</td>
<td>28.1±0.48 g</td>
<td>28.7±0.527 g</td>
<td>28.5±0.562 g</td>
<td>29.4±0.466 g</td>
</tr>
<tr>
<td>6w Group B</td>
<td>32.3±0.464 g</td>
<td>33.0±0.642 g</td>
<td>33.5±0.566 g</td>
<td>34.2±0.512 g</td>
</tr>
<tr>
<td>6w Group C</td>
<td>32.2±0.422 g</td>
<td>32.7±0.498 g</td>
<td>33.3±0.502 g</td>
<td>39.9±0.724 g</td>
</tr>
</tbody>
</table>

Figure 3. Results of measurements of estrogen blood concentration in each group of 6-week-old mice. In groups B (oophorectomy) and C (ovarian tissue transplantation), the estrogen level was below the detection sensitivity of the kit used for measurement.
heart disease due to arteriovenous thromboembolism\textsuperscript{10}, cerebrovascular disease\textsuperscript{11}, and exacerbation of liver dysfunction\textsuperscript{12}.

In the field of reproductive medicine, the technology for cryopreservation of ovarian tissue with the aim of preserving fertility has made rapid strides since the beginning of the 21st century, and there have been many reported cases of young cancer patients becoming pregnant and giving birth following treatment with anticancer drugs\textsuperscript{13,14}. Women who underwent ovarian tissue transplantation became pregnant with a success rate of 35\%\textsuperscript{15}. In one recent case, a 9-year-old girl diagnosed as having Ewing’s sarcoma had her ovaries cryopreserved prior to the start of chemotherapy and radiotherapy and transplanted after treatment at age 13 years. Subsequently, female hormones were secreted by the transplanted ovaries and menarche occurred, but she became amenorrheic after approximately 6 months\textsuperscript{16}.

THT, comprising treatment with female hormones secreted by the patient’s own transplanted ovaries for purposes other than of becoming pregnant, is a promising new method of treatment\textsuperscript{17}. Whether the transplantation of cryopreserved ovarian tissue has a cosmetic effect on skin equivalent to that of HRT was investigated using the ovarian tissue cryopreservation techniques developed via continuing basic studies in \textit{Macaca fascicularis} and rats\textsuperscript{18–20}.

Studies have shown that visceral fat increases after oophorectomy due to the lack of female hormones\textsuperscript{21}, and in the present study, the amount of estrogen secreted by transplanted ovarian tissue was insufficient to improve the loss of skin elasticity induced by oophorectomy. In reproductive medicine, the objective of ovarian tissue transplants is the growth and development of ovarian follicles after transplantation, and follicle-stimulating hormone (FSH) is administered extracorporeally\textsuperscript{18}. Follicle growth stimulates estrogen secretion, but as pregnancy was not the objective in the present study, FSH was not administered.

Given that future improvement of freezing and thawing techniques for the ovary and implantation sites will likely lead to the clinical use of THT using ovarian tissue, an additional experiment was performed to investigate the effect of postmenopausal HRT on skin. When ovaries were removed from mice, the elasticity of their skin diminished and their

Figure 4. Ovaries were removed from 6-week-old mice, and E2 was administered subcutaneously (a.s) (0 pg, 250 pg, 2500 pg, 25,000 pg; each, n=2) and intraperitoneally (a.i) (0 pg, 25,000 pg; each, n=2) after 13 weeks\textsuperscript{7}. Skin elasticity were measured at weekly intervals. (a) R2: restoration ratio after extension, (b) R6: ratio of viscoelasticity to elastic deformation, and (c) R7: ratio of elastic portion at retraction.
Six-week-old mice were allocated to a bilateral oophorectomy group (OVX), sham surgery group (Sham), control group (Vehicle), estrogen subcutaneous administration group (E), progesterone subcutaneous administration group (P), estrogen plus progesterone subcutaneous administration group (EP), and cryopreserved ovarian tissue transplantation group (Transplant) (each group, n=5). Body weight increases significantly in the mice in the Transplant group compared with those in the Sham group (Figure 5a). Two skin elasticity parameters, R2 (restoration ratio after extension) (Figure 5b) and R7 (ratio of elastic portion at retraction) (Figure 5c), were significantly lower in the mice in the OVX group than in those in the Sham group.

Body weight increased, and the administration of E2 after these changes had occurred neither improved the decrease in skin elasticity nor suppressed body weight gain (Figure 4).

A further experiment was then performed to investigate the effect of perimenopausal HRT on skin when E2 was administered immediately after oophorectomy, before changes in skin elasticity became apparent. When mice were given E2 continuously from immediately after oophorectomy, skin elasticity showed a transient decrease but improved again 3 weeks after ovary removal. If HRT is started within 10 years after menopause, the risk of cardiovascular impairment is lower than if it is started more than 20 years after menopause, which suggests that if HRT is started at a comparatively early stage of estrogen deficiency, it may prevent the decrease in skin elasticity.

This study has two limitations. First, it is not clear why E2 was not secreted from the transplanted ovaries, and whether the problem relates to the freezing method or the transplant site requires further investigation. Second, rodents were used in the experiments. It is difficult to draw comparisons between studies in rodents, which have a short menstrual cycle, and humans. Ideally, primates (monkeys) should have been used because their menstrual cycle is similar to that of humans. However, primates are expensive, and few institutions are equipped to look after them; thus, it was not possible to use them in the present study.

In this study, the amount of estrogen secreted from thawed and transplanted mouse ovaries was insufficient to improve skin elasticity. In addition, skin elasticity was not improved by E2 administration after elasticity had decreased following the removal of
ovaries from mice. However, when mice were given E2 continuously from immediately after oophorectomy, skin elasticity showed a transient decrease, but then improved again. If techniques for THT using cryopreserved ovaries to maintain the blood estrogen concentration above a specific level can be established, this might help to prevent or improve the deterioration of skin appearance in young women who require oophorectomy due to gynecological disease and also in childhood, adolescent, and young adult cancer patients.

Acknowledgments

We thank Teruaki Nagasawa, Yoshiki Kubota, and Nanako Shimura of NANOEGG Research Laboratories, Inc. for helping with the research.

Author’s contributions

Hiroharu Imanishi conducted the experiments, performed the analysis, and wrote the manuscript; Yoko Yamaguchi contributed intellectually to the experimental design and edited the manuscript; Suguru Igarashi contributed to data interpretation and manuscript writing; Nao Suzuki conceived the project, designed the experiments, and wrote the manuscript.

References

18) Igarashi S, Suzuki N, Hashimoto S et al. Hetero-
topic autotransplantation of ovarian cortex in
cynomolgus monkeys. Hum Cell 2010; 23: 26–
34.

19) Igarashi S, Suzuki N, Osada M, Takae S, Tarumi
W, Ishizuka B. Cryopreservation of ovarian tis-
sue after pretreatment with a gonadotropin-re-
leasing hormone agonist. Reprod Med Biol

20) Suzuki N, Hashimoto S, Igarashi S et al. Assess-
ment of long-term function of heterotopic trans-
plants of vitrified ovarian tissue in cynomolgus

21) Zoth N, Weigt C, Laudenbach-Leschowski U,
Diel P. Physical activity and estrogen treatment
reduce visceral body fat and serum levels of lep-
tin in an additive manner in a diet induced ani-
mal model of obesity. J Steroid Biochem Mol

22) Rossouw JE, Prentice RL, Manson JF et al.
Postmenopausal hormone therapy and risk of
cardiovascular disease by age and years since