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Isolation of Viable Ovarian Cells From Cryopreserved Ovarian Tissues of Women Undergoing Chemotherapy Induced Premature Ovarian Failure (Chemo-POF) and Their Qualification by Flow Cytometry

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Abstract

Objectives: Female cancer patients undergoing chemotherapy have an elevated risk of developing premature ovarian failure (POF) and infertility. Therefore, this study aimed to isolate the ovarian cortex cells from the cryo-banked ovarian tissues undergoing chemotherapy or radiotherapy for in vitro culture or seeding into the artificial ovary.

Materials and Methods: Ovarian tissues were obtained from five chemotherapy induced premature ovarian failure (Chemo-POF) women. The ovarian medullas were carefully removed. The cortex was finely minced and enzymatically digested, and the isolated cells were fixed. As for cell characterization, flow cytometry for vimentin (stromal cells), FSH-R (granulosa cells), and OCT-4 (oogonial stem cells) were performed. The evaluation was carried out in order to isolate the ovarian cortex cells from Chemo-POF women and qualify them by flow cytometry.

Results: Flow cytometry showed that 90% of isolated cells were vimentin-positive. Out of this pool, 4%-5% were granulosa cells and 2%-3% were oogonial stem cells. Consequently, the population of ovarian stromal cells was 90%. Moreover, the stromal cells represented the larger population of cells in the human ovarian cortex.

Conclusions: It was concluded that alive cells, including stromal cells, granulosa and oogonial stem cells (OSCs) , may have been isolated from the ovaries of Chemo-POF patients undergoing chemotherapy.

Keywords: Cryo-banked ovarian cells, Chemo-POF patients, Flow cytometry

Introduction

Cancer is still one of the leading causes of death; however, the survival of children and adolescents with cancer has recently improved by an average of five years with a pediatric cancer survival rate of more than 80% (1-4). Common treatments for this group of patients, including chemotherapy and radiotherapy for dealing with the pelvis and/or central nervous system (CNS), can adversely affect hormonal regulation, puberty, fertility and, consequently, quality of life (1). Severe gonadotoxic effects have been reported for some radiotherapy and/or chemotherapy procedures, which may subsequently develop premature ovarian failure (POF) (2,5-7).

Improving survival rates for children and adolescents with cancer have created a great interest among growing number of survivors in having their own offspring. Available therapeutic strategies to preserve fertility have given many young patients hope to continue these procedures.

There are several treatment options for women with cancer related to fertility preservation, including ovarian transplantation (pelvic irradiation only), cryopreservation of embryo or oocyte, and ovarian tissue cryopreservation (OTC) (7-9), which can be integrated with immature oocytes collection. Some factors including the type of malignancy, type of treatment, patient's condition, as well as partner's status and age influence the selection of the alternatives.

Despite the availability of several options in this regard, cryopreservation and transplantation of ovarian tissue can usually be helpful in preserving fertility for prepubertal girls and patients who are unable to delay gonadotoxic therapy. Ovarian tissue cryopreservation has been introduced as one of the candidates for fertility preservation due to numerous advantages such as needlessness for ovarian stimulation as well as suitability for prepubertal girls or patients with delayed gonadotoxic therapy (8,9). However, there are also some disadvantages for this approach,

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Key Messages

- Artificial ovary prepared a new niche for ovarian cells and follicular reconstruction.
- Ovarian follicular reconstruction is possible in the case of ovarian insufficiency by engineering artificial ovary.

including ischemic tissue damage following ovarian tissue transplantation, resulting in follicle loss, and the risk of re-introduction of malignant cells for malignancies with the ability for metastasis to the ovary (9-11). To date, no substitute has been developed for restoring fertility after cancer recovery for these patients.

Various research groups around the world have recently attempted to develop a transplantable artificial ovary to restore fertility and prevent the risk of malignant cells re-introduction to the body. Two requirements must be met in this regard: a three-dimensional physical structure for extracted follicles (12,13) and/or cell lines (14) like ovarian cells for angiogenesis, extracellular matrix synthesis, and follicular structure production (14).

Structural support from ovarian cells may be created in a transplanted artificial ovary. Their presence is essential due to the presentation of a complex bidirectional paracrine signals with follicles and their role in vascularization in ovarian tissue, as essential parameter for the survival and development of follicles (15,16).

Ovarian cells like granulosa, stromal, and oogonial stem cells (OSCs) may also provide structural support in an artificial ovary. Additionally, oogonial stem cells are necessary for differentiating into in vitro derived oocytes when cultured on artificial ovary. Also, it is not known whether chemotherapy can adversely affect ovarian cells like granulosa, stromal, and OSCs. Therefore, this study aimed to extract ovarian cells by applying biopsy from the cryo-banked human ovarian tissues that underwent chemo- or radiotherapy-induced premature ovarian failure (Chemo-POF) in the human ovary. Flow cytometry was employed to analyze the ovarian suspension as well as to detect viable ovarian cells and cell viability rate post chemotherapy.

Materials and Methods

Ovarian Tissue Biopsy and Freezing Process

The biopsy was performed for all female volunteers with Chemo-POF and having had laparoscopic surgery for benign gynecologic condition in order to obtain human ovarian tissues (Table 1). The tissue samples obtained from Mahdieh hospital (Tehran, Iran) were instantly transferred to the Ovarian Tissue Bank (OTB) of Royan Institute (Tehran, Iran) in DMEM/F12+GlutaMAX TM (Dulbecco's Modified Eagle Medium, Gibco, Paisley, UK) on ice in less than one hour. A surgical scissor was used to detach the ovarian medullas from the cortical part. The incisions made from the extracted cortexes were strips measuring 5×5×5 mm^3, which were then slowly frozen according to standard protocol of OTB (17) until thawing for next *in vivo* and *in vitro* experiments. The cortical tissues had been collected from five Chemo-POF women with the mean age of 33 years (ranging from 22 to 44).

Ovarian Cells Isolation Procedure

The protocols of Shahri et al (2) with slight modifications were followed to extract human ovarian cortex cells (HOCCs) from women with Chemo-POF. This approach was developed in our laboratory with a focus on enzymatic dissociation. For isolating ovarian cell, two sections of frozen-thawed human ovarian cortex measuring 5×5×5 mm^3 were cut to smaller pieces by a bistoury in Hanks' Balanced Salt Solution (HBSS) free of magnesium or calcium (BOSTER; Wuhan, China) with 1.5 mg/mL of collagenase IA (Sigma- Aldrich, UK). The resultant mix was incubated for 40 minutes at 37°C while slowly agitating and pipetting every 15 minutes. Enzymatic digestion was completed using equal volumes of HBSS blended with heat-inactivated fetal bovine serum (15% FBS, Gibco, USA) and penicillin-streptomycin (1%, Gibco, USA). A cell strainer (70 µm, 40 µm, Dutscher SAS, Brumath, France) was utilized to filter the cell suspension for discarding remaining connective tissue fibers, followed by centrifugation of the cell suspension at 4°C with 300 g for 5 minutes. The pellet was suspended again in a certain amount of Dulbecco's Modified Eagle Medium (DMEM-F12, Gibco, 11330057). Finally, the cells were incubated in T75 culture flask in the absence of a feeder layer. The medium required for the cell cultivation at 37°C contained 15% FBS, DMEM/F-12, epidermal growth factor (10 ng/mL EGF; Invitrogen, MA, USA), basic fibroblast growth factor (1ng/ml bFGF; BD Biosciences, USA), 1 mM non-essential amino acids (Gibco, USA), 1 mM Glutamax (Gibco, USA), penicillin/streptomycin (Thermofisher, USA) 1× concentrated and incubated in the presence of 97% humidity, 5% CO_2, and 21% O_2. High amount of the medium was renewed by fresh medium every 4 days.

Table 1. Type of patient

Patient	Cancer type	Age of patient	Number of Chemotherapy	Type of Chemotherapy
1	Breast cancer	25	6	Cyclophosphamide
2	Breast cancer	39	5	Cyclophosphamide
3	Breast cancer	28	5	Cyclophosphamide
4	Breast cancer	44	5	Cyclophosphamide
5	Breast cancer	23	6	Cyclophosphamide

Analysis of Ovarian Isolated Cells by Flow Cytometry

To this end, cisplatin-treated or untreated cells were gathered and washed with PBS two times. Thus, 10^6 cells were exposed to 100 µL of 2-4% cold paraformaldehyde for 20 minutes at 4°C, then rinsed with PBS-/Tween 0.05%, permeabilized by appending Triton X-100 for 10 minutes at RT, and rinsed again with PBS-/tween 0.05% twice. The cells were exposed to primary antibody (vimentin, OCT4, and FSH-R) at 4°C for one hour; then unbound antibodies were discarded by rinsing with PBS-/Tween 0.05% twice. Cell staining was conducted by using dye-conjugated secondary antibody (anti-mouse, anti-rabbit) in dark place at 4°C for 45 minutes, then rinsing with PBS-/Tween 0.05% was performed twice and thus re-suspension was completed in 0.5 mL PBS before flow cytometry analysis. In each assay, an antibody-specific IgG isotype was utilized to detect background staining.

Statistical Analysis

Cell Quest software (Becton-Dickinson, Bedford, MA, USA) was used for statistically analysis of all data (18). Results were expressed as mean intensity of fluorescence.

Results

Through sufficient physical cutting and collagenase digestion of five ovaries from Chemo-POF women, a disperse cell solution was obtained (Figure 1), which contained stromal cells, granulose cells, and a small number of OSCs (Figure 2A-C). After culturing for seven days, some small round cells with small colonies were formed, which resembled OSCs in terms of morphology (Figure 2D-E). After culturing for 14 days, the density of the above cells reached confluence and larger cells, typical of oocyte-like structures, were observed (Figure 2F).

Characterization of the Cells After Isolation

The isolated cells from Chemo-POF women were identified adopting flow cytometry (Figure 3) according to their expression of stromal, granulosa, and oogonial stem cells markers as vimentin, FSH-R, and OCT4, representatively. The results showed that most of the cell population isolated from ovarian cortex tissue expressed high level of vimentin, while low expression levels of FSH-R and OCT4 (4.8% and 2.5%, respectively) were evident (Figure 3). Consequently, the proportion of ovarian stromal cells was estimated at ~93%. On the other hand, the proportion of ovarian stromal cells was higher than the proportions of granulosa and oogonial stem cells. Moreover, oogonial stem cell population was lower than that of granulosa cells.

Discussion

In this study, a simple method was adopted to characterize three different populations of cells from the cryo-banked ovarian tissues from patients with chemotherapy induced POF. To the best of our knowledge, this study was the first to extract viable human ovarian cortical cells from women with Chemo-POF. In earlier investigations, human ovarian cortical cells had been isolated from women under fertility preservation (healthy ovaries) (2). In our study, no attempt was made to obtain ovarian cortex cells from healthy ovaries, but an attempt was made to separate ovarian cells from ovaries left over from radiotherapy or chemotherapy. Since the ovaries of these patients are atrophic and without follicles after treatment for cancer, similar to the ovaries of postmenopausal women (2,19,20), the ovaries of these patients were used to determine if the ovarian cells were still alive.

Since chemotherapy reduces the number of follicles in the ovarian tissue, moreover, a prediction was made in this study regarding the isolation of a low number of granulosa cells (21). Therefore, flow cytometry technique was employed to characterize ovarian cell suspension.

Our findings revealed that viable ovarian cells – granulosa, in particular – stromal and oogonial stem cells were achieved following the dissociation of ovarian

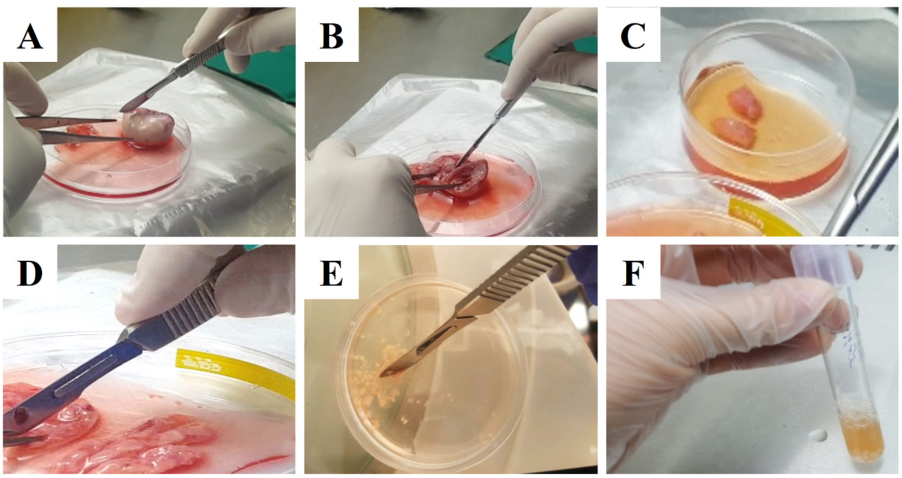

Figure 1. Cells Separation Procedure From Human Ovarian Cortex by Enzymatic Digestion (Collagenase I) (A-F).

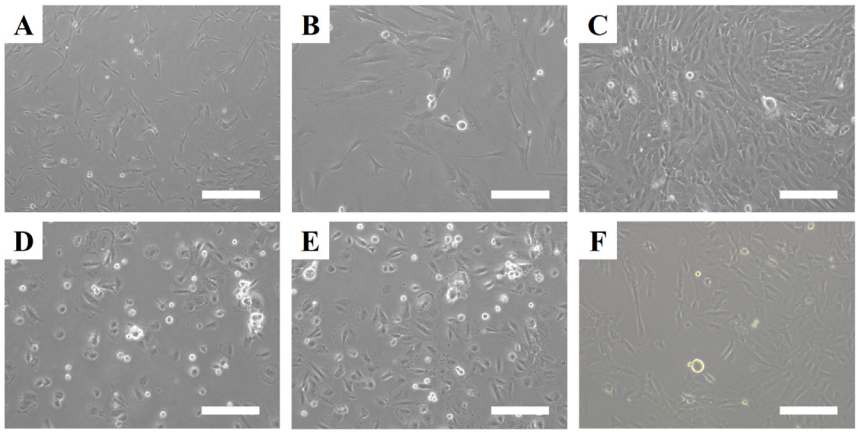

Figure 2. Morphology of HOCCs Isolated From Chemo-POF Ovaries. After ovarian digestion by collagenase I, dispersed ovarian cells were cultured. These figures illustrate the morphology of all the dispersed cells from Chemo-POF (passage 1: A-C, passage 2: D-F). Scale bars: 400 µm. HOCCs: human ovarian cortex cells; Chemo-POF: chemotherapy induced premature ovarian failure.

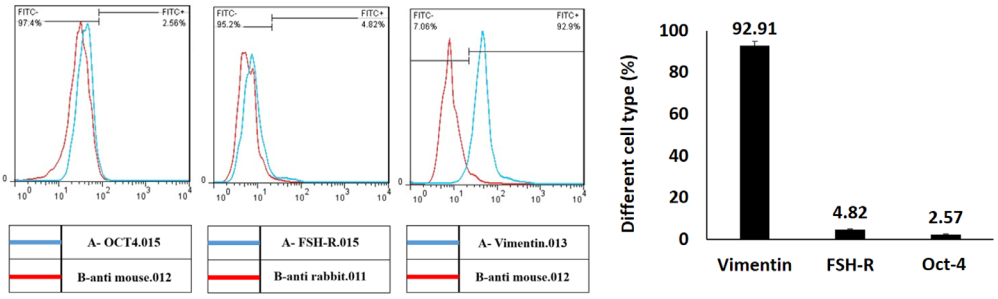

Figure 3. Expression of Surface Markers of HOCCs by Flow Cytometry. Purified cells from Chemo-POF ovaries were incubated with vimentin, FSH-R, and OCT4 primary antibodies against the respective antigens followed by secondary antibody. Results were representative of (n=3) independent samples. HOCCs: human ovarian cortex cells; Chemo-POF: Chemo-POF: chemotherapy induced premature ovarian failure.

tissue. However, our results showed a proportion of approximately 5% and 3% for FSH-R and OCT4-positive cells, respectively, soon after the isolation while a higher proportion (93%) was observed for stromal cells.

According to several studies, stromal cells account for the majority of cells in the ovarian cortex in humans (2). There were findings about OSCs and granulosa cells, which were in line with our study results. The low proportions of OSCs and granulosa cells after ovarian tissue dissociation could negatively affect follicle survival and growth. In this case, an alternative could be increasing the number of these cells through isolation and culturing.

Hence, chemotherapy can have a limited effect on the rate and viability of cells isolated from ovarian tissue (22). Assisted reproductive technologies can be employed for women with cryopreserved embryos or oocytes before gonadotoxic therapies (23-26). Embryos or eggs can be preserved initially by hormonally stimulating the patient to gather mature oocytes. It takes two weeks or more for controlling ovarian stimulation, and there is no viable option for patients reaching reproductive maturity or unable to delay treatment (26-29). For these women, the only way to normally restore endocrine activity and fertility is cryopreservation of intact ovarian tissues before the treatment. Despite promising advances in ovarian tissue transplantation, there is a potential risk of malignant cells returning to the body (26,30). Multiple preclinical experimental studies have introduced artificial ovaries as an approach to decrease the risk of malignant cells. Unwanted contamination of malignant cells can be prevented by separating the ovarian cells from the remaining ovaries undergoing radiotherapy or chemotherapy. After separation of the cell suspension from remaining ovaries undergoing chemotherapy, moreover, the population of OSCs and granulosa cells can be increased by culturing the cells. This enhances the chance of follicle formation after seeding on artificial ovary.

More evidence was found in our study for the incidence of OSCs in the ovarian cortex of women with Chemo-POF and the potential of such cells to differentiate the generate oocyte-like cells under *in vitro* conditions. Thus, there was a possibility for *in vitro* production of oocyte-like cells by even using the OSCs from the women with Chemo-POF. Taking into account our study results, a more effective technique may have been developed to treat female

infertility through a neo-oogenesis when more accurate understanding of the artificial ovary was achieved by isolating ovarian cells (in particular OSCs) coupling with implantable biomimetic scaffolds (Unpublished data).

Conclusions

In sum, separation and detection of Chemo-POF female ovarian cells highlighted the presence of various cell types such as granulosa, stromal, and oogonial stem cells. Stromal cells were found to account for the majority of cells present in the human ovarian cortex. A review of the literature revealed that our study was the first report addressing the characterization of OSCs from HOCCs from women with Chemo-POF.

Authors' Contribution

Rouhollah Fathi and Hamid Nazarian planned and designed the experiments. Sara Khaleghi performed the experiments, analyzed the data and wrote the manuscript. Farideh Eivazkhani contributed to perform the experiments. Ashraf Moini and Marefat Ghaffari Novin reviewed and discussed the data.

Conflict of Interests

Authors declare that they have no conflict of interests.

Ethical Issues

All participants were informed of the study protocol and then asked to sign the informed written consent before using the ovarian specimens and performing the related tests. This study was approved by the Ethical Committee of the Royan Institute (IR.ACECR.ROYAN.REC.1396.174).

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