FERTILITY PRESERVATION IN ASIA

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FERTILITY PRESERVATION IN ASIA

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EDITORIAL
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Editorial: Fertility Preservation in Asia

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Keywords: fertility preservation, Asia, oncofertility, cancer, Asian countries

Editorial on the Research Topic

Fertility Preservation in Asia

In recent years, fertility preservation (FP) has become an important concern for cancer patients, and also for women seeking protection against future infertility due to aging or other causes. The Asian Society for Fertility Preservation (ASFP) was founded in 2015 by experts from 14 countries (Australia, China, Hong Kong, India, Indonesia, Japan, Korea, Pakistan, Philippines, Singapore, Taiwan, Thailand, Turkey, and Vietnam) to promote FP science and practice. In Asia, awareness of the importance of FP has gradually increased with new developments in clinical practice and research. The purpose of this editorial is to introduce a Research Topic consisting of articles published in a Research Topic of the journal *Frontiers in Endocrinology*, entitled "Fertility Preservation in Asia".

The ASFP is the first initiative by experts across Asia to promote FP research and clinical application. Its mission is to raise awareness of FP among healthcare professionals and the public, improve technical skills, and keep healthcare practitioners informed about the latest developments in the field and research environment (Harzif et al.). The ASFP strives to develop FP programs in Asian countries through exchanges between countries, conferences, and educational programs such as hands-on workshops. Future goals include development of a strategy to promote multidisciplinary approaches between practitioners, policy creation, and advancement of a referral system to benefit patients (Harzif et al.).

In the following section, we introduce two review papers addressing FP practices in Asian countries. Takae et al. investigated currently available FP options in Asian countries for child and adolescent (CAYA) cancer patients. In November 2018, a questionnaire survey of founding members of the ASFP was conducted to identify FP barriers for CAYA cancer patients, and the results indicated that most Asian countries could provide FP treatment. Among the 11 Asian countries responding to the survey, five had organizations or academic societies promoting FP, and Australia, Japan, and Korea had organizations specializing in FP. In contrast, China and Indonesia maintained committees or branches of large academic societies focused on reproductive or maternal-child health medicine. Furthermore, Hong Kong and the Philippines are planning to establish organizations or academic societies specializing in FP. However, the lack of experience and an established framework for FP promotion are factors that may hinder its implementation, and more constructive discussion is needed to support FP for CAYA cancer patients in Asian countries. Harzif et al. suggested that the particular situations existing in each country should be addressed to provide optimal FP development and fulfill the needs of patients and physicians. Necessary conditions for implementing FP in various Asian countries were described by the authors of the abovementioned studies.

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Cryopreservation programs for oocytes or tissues from cancer patients are available in most countries that have joined the ASFP. In Korea, a live birth resulting from vitrified-warmed oocytes harvested from a chronic myeloid leukemia patient was reported (1). In Japan, reports showed that auto-transplantation of ovarian tissue after cryopreservation successfully resulted in live births (2). And in 2013, cases of pregnancies and live births were reported in Singapore after ovarian cancer patients underwent oophorectomy and IVM oocyte cryopreservation (Harzif et al.). Since FP programs have only recently been initiated, additional reports of pregnancies and live births in similar cases are likely to occur. We anticipate that the review articles discussed above will become useful resources to help overcome barriers and encourage future development of FP programs in Asian countries.

Recent developments in "omics" have accelerated research on genes and RNAs related to FP. Tu et al. revealed that microRNAs of granulosa cells (GCs) are essential regulators of GC function under physiological and pathological conditions. The authors suggested that specific microRNAs could be targeted in the future for treatment of ovarian-related diseases such as polycystic ovary syndrome (PCOS), premature ovarian failure (POF), and granulosa cell tumors (GCT). Choi et al. found that expression levels of various genes related to DNA double-strand break (DSB) repair decreased in women with endometriosis, and consequently FP should be considered in these cases since impaired DSB repair gene expression may reduce ovarian reserve.

This special issue introduces various FP-related diagnostic, treatment, and prognostic studies performed by Asian research groups. Son K-A et al. analyzed the association between BRCA mutations and anti-Mullerian hormone (AMH) levels in young breast cancer patients using linear and logistic regression analysis. As a result of the analysis, breast cancer patients with BRCA mutations showed significantly lower serum AMH levels, and the authors suggested that young breast cancer patients should consider preserving fertility more actively. Son W-Y et al., in a review paper on *in vitro* maturation (IVM) of human oocytes, suggested the possibility of successful IVM for FP in women at risk of losing ovarian function. The authors also explained the benefits of IVM associated with oocyte vitrification. Kim et al. reported that using autologous platelet-rich plasma (PRP) in patients with refractory thin endometrium improved implantation, pregnancy, and live birth rates, introducing a possible new treatment for patients with infertility due to endometrial factors.

Two articles on infertility are also covered in this issue. Che et al. reported that cardiovascular disease and recurrent miscarriage shared risk factors, and some cardiovascular disease-related candidate genes were associated with recurrent miscarriage. Cai et al. showed that higher thyroid-stimulating hormone (TSH) levels were related to hyperandrogenism in women with euthyroid PCOS independent of age, BMI, and thyroid autoimmunity. Although it is widely known that thyroid dysfunction can lead to infertility, this report highlights the relationship between TSH and a specific disease.

These articles collectively cover current developments and predict future directions for FP in Asia. Patient demand for FP is expected to increase in the future since it is a key area of reproductive medicine. We expect that the articles in this Research Topic will serve as an important cornerstone to mark the beginning of future FP development, and also to stimulate FP research and practice in Asia and worldwide.

AUTHOR CONTRIBUTIONS

JL: draft, revision, and final approval of the manuscript. ST: draft, revision, and final approval of the manuscript. NS: draft, revision, and final approval of the manuscript. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Association Between Impairment of DNA Double Strand Break Repair and Decreased Ovarian Reserve in Patients With Endometriosis

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Background: Repair of DNA double strand break (DSB) is an important mechanism for maintaining genetic stability during a DNA damage event. Although, a growing body of recent evidence suggests that DNA DSBs and related repair mechanisms may be important in ovarian aging and in various cancers, there are few reports in endometriosis. We, therefore, examined expression levels of genes pertaining to DNA DSB repair in patients with endometriosis to assess the potential effects on ovarian reserves.

Materials and methods: A total of 69 women undergoing laparoscopic surgery for endometriosis and other benign conditions was included; endometriosis group (n = 38) vs. controls (n = 31). DNA DSBs in endometrial and ovarian tissues of both groups were compared via immunohistochemistry, aimed at γ-H2AX expression. To gauge genotoxin-induced DNA DSBs in endometrial stromal cells, γ-H2AX expression was determined by western blot after H_2O_2 treatment of cultured endometrial stromal cells (endometriosis group and controls) and Ishikawa cell-line cultures. Endometrial and ovarian tissue levels of BRCA1, BRCA2, Rad51, and ATM (ataxia-telangiectasia mutated) mRNA expression were also compared. Correlations between expression levels of genes of interest and serum anti-müllerian hormone (AMH) levels were assessed as well.

Results: Expression of γ-H2AX in immunostained endometrial and ovarian tissue preparations was greater in the endometriosis group, compared with controls. After H_2O_2 treatment, γ-H2AX expression levels were also significantly greater in cultured stromal cells of the endometriosis group and in the Ishikawa cell line than in controls. Endometrial expression of *BRCA1* and *Rad51* mRNA proved significantly lower in the endometriosis group (vs. controls), as did ovarian expression of *BRCA1* and *BRCA2* mRNA. Serum AMH concentration showed a significant correlation with ovarian *BRCA1* mRNA expression in women with endometriosis (p = 0.03).

Conclusions: In women with endometriosis, expression levels of various genes implicated in DSB repair are decreased and ovarian *BRCA1* expression correlates with

ovarian reserves. These findings indicate that impaired DSB repair may contribute to diminished ovarian reserves in this setting.

Keywords: endometriosis, ovarian reserve, double stranded DNA break, BRCA1 gene, gamma-H2AX

INTRODUCTION

Endometriosis is characterized by the presence of endometrium-like epithelium and stroma outside the endometrium and myometrium (1), and not necessarily restricted to the pelvic compartment. This disease is one of the most common gynecologic disorders affecting ∼10% of all reproductive-age women and 20–50% of women with chronic pelvic pain and/or infertility (2).

The biologic mechanisms that may link endometriosis and subfertility have not been fully explained. However, several mechanisms (e.g., pelvic adhesions, altered peritoneal, hormonal, or cell-mediated function, abnormal cytokine release, endocrine and ovulatory abnormalities, and impaired implantation) have been proposed (3). Studies from oocyte donation–*in vitro* fertilization and embryo transfer, which may exclude the impact of endometrial receptivity, suggest that subfertility in endometriosis is attributed to quality of oocytes rather than endometrial receptivity (4). In addition to the qualitative issues of oocytes, quantitative declines in ovarian reserve are also of concern for women with endometriosis (5). The impact of minimal-to-mild endometriosis on ovarian reserves has seemed inconsistent, but it is speculated that advanced-stage endometriosis may be detrimental in this regard, regardless of any surgical damage. As serum anti-Müllerian hormone (AMH) concentrations and antral follicle counts (AFCs) confirm, endometriomas *per se* are associated with diminished ovarian reserves (6, 7); and endometriosis or endometriomas may impact responsiveness to controlled ovarian stimulation, reflected by numbers of oocytes collected (8). Thus, declining ovarian reserves appear directly related to endometriosis. Such deterioration due to both pathologic underpinnings of endometriosis and any surgical damage incurred is the source of mounting attention on fertility preservation in women with endometriosis (5, 9).

At present, however, the pathophysiologic mechanisms of diminished ovarian reserves in endometriosis remain unclear. Tissues bordering endometriomas often show morphologic alterations (i.e., substantial loss of cortical stroma, fibrosis, and considerably less follicular density) that are not present near other benign cysts (10, 11). Similar pathologic changes, including focal fibrosis and vascular deficiency, have likewise been observed in ovarian cortex exposed to chemotherapy (12). The proportion of primordial follicles present in ovaries with endometriomas is distinctly lower by comparison, and there are notable increases in proportions of non-resting growing follicles (13). This "burn-out" effect on follicular reservoirs is also suggested as a major mechanism leading to chemotherapy-induced loss of ovarian reserves (14, 15). Hence, patients with endometriosis may follow a path similar to chemotherapy-induced ovarian damage.

Cells sustain DNA damage through both external and internal means. Among the various types of DNA damage due to environmental genotoxins, DNA double-strand breaks (DSBs) are capable of substantially altering genetic integrity and thus are the most deleterious. The ataxia-telangiectasia mutated (ATM)–mediated DNA damage signaling (DDS) pathway regulates repair of DNA DSBs via a homologous recombination mechanism. In instances of irreparable DNA damage, cells are eliminated by apoptotic cell death or undergo senescence (complete cell-cycle withdrawal) to avoid severe mutagenic consequences (16). Impaired DNA DSB repair may be associated with loss of ovarian follicular reserves, *BRCA1*, and other key genes in the ATM pathway that decline with age in human oocytes (17). Furthermore, chemotherapy causes massive DNA DSBs in primordial follicles, oocytes, and granulosa cells. Such damage is associated with apoptotic oocyte death, which then triggers the DNA repair response by activating the ATM-medicated DDS pathway (18, 19).

It has been suggested that endometriosis *per se* affects ovarian reserve. Although evidence of an association between DNA DSBs and ovarian reserves has emerged, there has been no pertinent data on women with endometriosis. Considering that it was reported that morphologic changes in ovary of women with endometriosis were similar to those after chemotherapy-induced ovarian damage, we hypothesized that diminished ovarian reserve in women with endometriosis may have similar pathway to chemotherapy-induced ovarian damage (e.g., increased DNA damage and impaired repair mechanism). In the present study, we assessed the extent of genotoxin-induced DNA damage to *in vitro* cultures of endometrial stromal cells. We also used endometrial and ovarian tissues of women with endometriosis to investigate DNA damage and expression levels of genes implicated in DNA DSB repair in an effort to determine the ramifications for ovarian reserves.

MATERIALS AND METHODS

Study Population and Sample Collection

Among candidates undergoing laparoscopic surgery for various indications (e.g., pelvic masses or pain, endometriosis, infertility, and diagnostic evaluations of benign gynecologic diseases) from January 2015 to September 2017, only those aged 25–40 years with a body mass index (BMI) of 18.5–29.9 kg/m^2 were included in this prospective case-control study after granting written informed consent. The present study was performed in university research center and approved by the Institutional Review Board of Gangnam Severance Hospital (IRB number 3-2015-0250). Postmenopausal status, previous use of a hormone or a gonadotropin-releasing hormone (GnRH) agonist within 6 months, prior ovarian surgery, or other medical disorders (including adenomyosis; endometrial

hyperplasia, polyps, or cancer; infectious diseases; chronic or acute inflammatory diseases; malignancies; autoimmune diseases; or cardiovascular diseases) were grounds for study exclusion.

At the time of surgery, all possible endometriotic lesions were excised and sent for pathologic examination to confirm the diagnosis. Patients were assigned to the endometriosis group only after pathologic confirmation of the excised tissue. The extent of endometriosis was determined using the American Society of Reproductive Medicine (ASRM) revised classification (20). Overall, 38 patients displayed moderate-to-severe endometriosis (stages III and IV), histologically confirmed. Another 31 patients were confirmed that they had no endometriosis by laparoscopy and served as controls. They were diagnosed with the following ovarian neoplasms: dermoid cyst ($n = 20$), serous cystadenoma ($n = 6$), and mucinous cystadenoma ($n = 5$). Endometrial samples were collected from the patients by Pipelle sampler (Cooper Surgical, Trumbull, CT, USA) during surgery irrespective of menstrual phase. Tweleve out of 38 patients (31.5%) in the endometriosis group and 9 out of 31 patients (29.0%) in the controls were in proliferative phase and others in secretory phase.

Main outcomes were γ-H2AX protein expression, mRNA expression of BRCA1, BRCA2, and Rad51, and ATM in eutopic endometrium and ovarian tissue, γ-H2AX protein expression of cultured endometrial stomal cells after H2O2 treatment, and correlation between DNA DSB repair genes of interest and AMH.

Immunohistochemistry

Immunohistochemical staining was performed in five participants of each group. γ-H2AX protein expression was assessed by ready-to-use immunostain application (Bond Polymer Intense Detection System; Vision BioSystems, Wetzlar, Germany) following the manufacturer's instructions. Surgically resected tissues were first fixed in 10% neutral buffered formalin for 12–24 h. Samples were then selected for routine processing (in automated system), embedding in paraffin, and slide preparation, sectioning at 4 μm by rotary microtome. Once deparaffinized (Bond Dewax Solution; Vision BioSystems), antigen retrieval proceeded (Bond Epitope Retrieval Solution; Vision BioSystems) by treating sections for 30 min at 100°C. Endogenous peroxidases were quenched by a 5-min hydrogen peroxide pretreatment. Sections were then incubated for 15 min at ambient temperature with rabbit polyclonal γ-H2AX antibody (1:500 dilution; Bethyl Laboratories, Montgomery, TX, USA). A biotin-free polymeric horseradish peroxidase-linked antibody conjugate system was finally applied (BOND-MAX automatic slide stainer; Vision BioSystems), and sections were developed using 1 mM 3,3′-diaminobenzidine as the chromogen, 50 mM Tris-hydrogen chloride buffer (pH 7.6), and 0.006% hydrogen peroxide, with hematoxylin as counterstain. Positive (breast cancer tissue), and negative control slides were generated for each reaction to minimize inter-assay variation. For the negative controls, the primary antibody was replaced by non-immune serum to yield no detectable γ-H2AX staining.

Culture of Primary Endometrial Stromal Cells and Ishikawa Cell Lines

Since Ishikawa cell line, a well-differentiated human endometrial adenocarcinoma cell line bears estrogen and progesterone receptors, the cells have been used numerous basic research areas such as reproductive biology and molecular science including endometriosis researches (21). Since it was also suggested that genotoxic exposure such as chemotherapeutic agent induced substantial increase in gamma-H2AX in Ishikawa cell lines (22), these cell lines were used as a positive control to quantify DNA damage in *in vitro* cell culture study. Eutopic endometrium of women with endometriosis shows fundamental differences compared with that of healthy control (23). Since, therefore, eutopic endometrial stromal cells of women with endometriosis may have altered responses to genotoxic exposure, *in vitro* cell culture study was performed to compare the extent of DNA damage following genotoxic stimuli between endometrial stromal cells obtained from endometriosis group and controls.

We utilized a previously published method to culture endometrial stromal cells (24). Endometrium was finely minced, and the cells were dispersed by incubation at 37°C for 60 min with agitation while adding (pipetting) Hanks balanced salt solution (HBSS) containing 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; 2 mmol/mL), penicillin/streptomycin (1%), and collagenase (1 mg/mL, 15 U/mg). The cells were pelleted, washed, suspended in Ham's F12:Dulbecco's Modified Eagle Medium (DMEM) in a 1:1 ratio containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, passed through a 40-μm cell strainer (Falcon, Corning, NY, USA), and plated onto 75-cm^2 Falcon tissue culture flasks (BD Biosciences, Bedford, MA, USA). Cultured primary human endometrial stromal cells (HESCs) at passages 3–5 were used for analysis. Ishikawa cells were maintained in MEM (Invitrogen, Carlsbad, CA, USA) containing 2.0 mmol/L l-glutamine and Earl's Salts, supplemented with 10% FBS, 1% sodium pyruvate, and 1% penicillin/streptomycin. HESCs from patients with endometriosis and Ishikawa cells were harvested from culture flasks using trypsin/EDTA (0.05%). The cells were then counted (5×10^6) for plating in six-well plates at 37°C in a 5% CO_2 humidified environment and grown as previously described. At 80% confluency, the cells were treated with 250 μmol/L H_2O_2 concentrations, extracting proteins 4 h later. *In vitro* cell culture experiments were triplicated.

RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

Endometrial samples of all participants were analyzed by quantitative RT-PCR. Total RNA was extracted using a kit (RNeasy Mini; Qiagen, Valencia, CA, USA). A total of 2 μg RNA from each sample was reverse transcribed into cDNA (SuperScript III First-Strand Synthesis System; Invitrogen), all according to manufacturer protocols. Expression of candidate gene mRNA was measured by SYBR RT PCR on an ABI 7300 instrument (Applied Biosystems, Forster, CA, USA). We designed specific primers for BRCA1 (forward primer 5′-AG CTGTGTGGTGCTTCTGTGGT-3′, reverse primer 5′-TGG

CTGCACAACCACAATTGGG-3′), BRCA2 (forward primer 5′-CTTGCTTTCAAATTGGCACTGA-3′, reverse primer 5′-GTTTAAAAGGGCATAGGCT CTG-3′), Rad51 (forward primer 5′-TAGC AAAGGGAATGGGT CTGC-3′, reverse primer 5′-GCACAAGACTCCATAACCAAAC-3′), and ATM (forward primer 5′-GCTCAGTGTTGGTGGACAGGT-3′, reverse primer 5′-TCCATCCTGGGAAAAGTCG GCT-3′). The PCR reaction was performed in 20 μL buffer containing 2 μL of cDNA, 5 pM each primer, and power SYBR green PCR master mix (Applied Biosystems). The thermal cycling conditions were pre-incubated for 2 min at 50°C, then denatured for 10 min at 95°C, followed by 40 cycles of denaturation for 15 s at 95°C, and annealing and extension for 1 min at 60°C. To normalize the amount of total RNA present in each reaction, we amplified the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GADPH). Sequences for GAPDH were as follows: forward primer 5′-GAAGGT GAAGGTCGGAGTC-3′ and reverse primer 5′-GAAGATGGTGATGGGATTTC-3′. The amount of target, which was normalized to the endogenous reference (GAPDH) and was compared to the calibrator, was defined by the ΔΔCT method, as previously described (25). Endometrial tissue contributed by a normal patient was used as the calibrator in the following normalization formula: target amount $= 2^{-\Delta\Delta Ct}$, where ΔΔCt = [Ct (target gene sample) – Ct (GAPDH sample)] – [Ct (target gene calibrator) – Ct (GAPDH calibrator)]. The latter was calculated by Light Cycler v4.0 software.

Protein Extraction and Western Blot Analysis

Protein extracts were prepared using RIPA buffer (Thermo Scientific, Rockford, IL, USA) containing freshly added protease and phosphatase inhibitor cocktail (Thermo Scientific). Concentrations of total cell lysates were measured using BCA protein assay kit (Thermo Scientific). Altogether, 30 μg of total protein were mixed with 5 × sample buffer and heated at 95°C for 5 min. The samples were loaded onto 12% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and electrotransferred to polyvinylidene fluoride membranes (Millipore Corp, Billerica, MA, USA) using a Trans-Blot apparatus (Bio-Rad, Hercules, CA, USA). The membranes were blocked using 5% non-fat skim milk in Tris-buffered saline solution [10 mmol/L Tris-HCl (pH 7.4) and 0.5 mol/L NaCl), adding Tween-20 (0.1% vol/vol]. Blots were probed using primary antibodies to γ-H2AX (1:2,000; Bethyl Laboratories) and GAPDH (1:5,000; Santa Cruz Biotechnology, Dallas, TX, USA), followed by horseradish peroxidase-conjugated secondary anti-mouse (1:6,000; Thermo Scientific) or anti-rabbit antibody (1:5,000; Thermo Scientific). Protein detection was achieved by enhanced chemiluminescence (Santa Cruz Biotechnology). The experiment was performed in triplicate for analyses, all data shown being representative.

Immunofluorescence Staining

Immunofluorescence staining of BRCA1 was performed in five participants of each group. Selected cells on glass coverslips were fixed in cold acetone for 10 min, incubated with 1% BSA/PBS for 10 min, and then stained using mouse anti-BRCA1 monoclonal antibody as primary antibody (sc-135732; Santa Cruz Biotechnology) and goat anti-mouse secondary antibody conjugated with Cy3 (Abcam, Cambridge, UK). Fluorescence images from the slides were viewed and captured using a LSM510 microscope (ZEISS, Jena, Germany) and processed using proprietary LSM image software.

TABLE 1 | Clinical characteristics of study participants with and without endometriosis.

	EMS ($n = 38$)	Control ($n = 31$)	p-value
Age (years)	35.02 ± 1.22	36.64 ± 1.18	0.353
Gravidity	1.13 ± 0.23	2.16 ± 0.31	0.009
Parity	0.64 ± 0.15	1.35 ± 0.17	0.003
BMI (kg/m^2)	20.47 ± 0.30	22.39 ± 0.44	<0.001
CA-125 (U/mL)	65.27 ± 8.28	19.03 ± 2.59	<0.001
EMS STAGE			
III	18 (47.4%)	N/A	
IV	20 (52.6%)		
rAFS scores	49.22 ± 25.57	N/A	
Serum AMH (ng/mL)	2.05 ± 0.40	4.97 ± 1.18	0.039

Data expressed as mean ± standard error mean (SEM) BMI, body mass index; EMS, endometriosis; rAFS, revised American Fertility Society; AMH, anti-Müllerian hormone.

Statistical Analysis

The sample size was calculated to compare mRNA expression of genes related to DNA DSB repair between two groups. A power calculation was performed using PS Power and Sample Size Calculations (Version 3.1.2). Power analysis showed that at least 30 patients would be needed to detect a 30% difference with a significance level of 0.05 and 80% power. Considering 10% drop-out rate, the sample size required per group was 33.

All data (expressed as mean ± SEM) were subjected to Kolmogorov-Smirnov or Shapiro-Wilk test to check for normal distribution and compared using Student's *t*-test or Mann-Whitney *U*-test, as appropriate. Correlations between ovarian *BRCA* mRNA expression and serum AMH levels were assessed using Spearman's correlation coefficient. All computations relied on standard software (SPSS v16.0; SPSS Inc, Chicago, IL, USA), setting statistical significance at $p < 0.05$.

RESULTS

Clinical Characteristics

The endometriosis group and controls did not differ significantly in terms of age. However, controls significantly surpassed endometriosis group members in gravidity (2.16 ± 0.31 vs. 1.13 ± 0.23; $p = 0.009$) and parity (1.35 ± 0.17 vs. 0.63 ± 0.15; $p = 0.003$); and serum CA 125 levels were significantly higher in the endometriosis group than in controls (19.03 ± 2.59 vs. 65.27 ± 8.28; $p < 0.001$). BMI was significantly lower in the endometriosis group than in controls (20.47 ± 0.30 vs. 22.39 ± 0.44; $p < 0.001$). All patients in the endometriosis group had advanced-stage endometriosis, showing a mean revised AFS score of 49.22 ± 25.57. Serum AMH levels were significantly

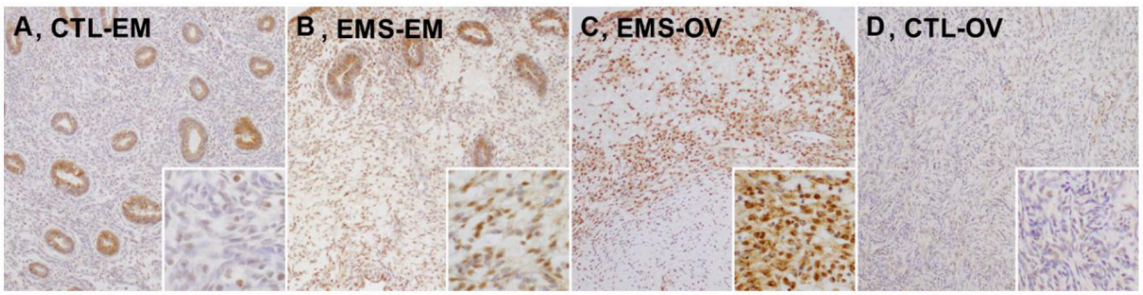

FIGURE 1 | Expression levels of γ-H2AX protein in tissue samples of eutopic and ectopic endometrium of patients with and without endometriosis. **(A)** CTL-EM, eutopic endometrium of control group: γ-H2AX expression absent, or very faint in stromal cells; **(B)** EMS-EM, ectopic endometrium of endometriosis group: patchy γ-H2AX expression of variable staining intensity, most stromal cells showing moderate nuclear γ-H2AX immunoreactivity; **(C)** EMS-OV, ectopic endometrium of endometriosis group: stromal cells demonstrating uniform and strong γ-H2AX immunoreactivity (γ-H2AX expression by glandular epithelium similar in control and in endometriosis groups); and **(D)** CTL-OV, normal ovarian stroma: absence of γ-H2AX expression (original magnification: **A–D**, × 100; inset, × 400).

lower in the endometriosis group, compared with controls (2.05 ± 0.40 vs. 4.97 ± 1.18; $p = 0.039$) (**Table 1**).

Immunohistochemical Staining of γ-H2AX

We evaluated **γ**-H2AX protein expression in eutopic and ectopic endometrial tissues of patients with or without endometriosis in immunostained tissue sections. Representative images **γ**-H2AX immunoreactivities are shown in **Figure 1**. In eutopic endometrial tissue of the control group, **γ**-H2AX expression was absent or faint in nuclei of stromal cells (**Figure 1A**); whereas most stromal cells in eutopic endometrium of patients with endometriosis showed moderate nuclear **γ**-H2AX expression (**Figure 1B**), and some stromal cells showed faint cytoplasmic **γ**-H2AX positivity. In a sample of ovarian endometrioma, **γ**-H2AX expression was clearly increased, compared with eutopic endometrial tissues. Uniform and strong nuclear **γ**-H2AX expression was also observed in ectopic endometrium of patients with endometriosis (**Figure 1C**). In normal ovarian stroma, no **γ**-H2AX expression was evident (**Figure 1D**). No between-group difference in glandular expression of **γ**-H2AX was observed.

Western Blot Analysis of γ-H2AX Expression After H_2O_2 Treatment of Cultured Endometrial Stromal Cells and Ishikawa Cell Lines

After H_2O_2 treatment of cultured Ishikawa cell lines and endometrial stromal cells from patients with endometriosis and controls, **γ**-H2AX expression as a marker of DNA DSB was measured by western blot (**Figure 2**). Post-treatment **γ**-H2AX expression levels in Ishikawa cells and endometrial stromal cells of patients with endometriosis patients were significantly elevated, compared with pretreatment levels [relative **γ**-H2AX expression/GAPDH: 0.88 ± 0.25 vs. 5.03 ± 1.02 ($p = 0.049$) and 1.00 ± 0.15 vs. 2.02 ± 0.26 ($p = 0.016$), respectively]. However, no **γ**-H2AX expression was evident in endometrial stromal cells of controls, despite observed H_2O_2 treatment (relative **γ**-H2AX expression/GAPDH: 1.07 ± 0.15 vs. 0.86 ± 0.11; $p = 0.32$).

Expression Levels of *BRCA1, BRCA2, Rad51,* and *ATM* mRNA in Endometriosis Group and Controls

Endometrial and ovarian expression levels of *BRCA1*, *BRCA2*, *Rad51,* and *ATM* mRNA in the endometriosis group and in controls are shown in **Figures 3**, **4**. Although endometrial *BRCA2* mRNA expression was comparable in both groups (2.19 ± 0.59 vs. 9.86 ± 3.02; $p = 0.051$), levels of *BRCA1*, *Rad51*, and *ATM* were significantly lower in endometriosis group, compared with controls [0.25 ± 0.03 vs. 0.54 ± 0.11 ($p = 0.014$); 4.95 ± 1.11 vs. 11.77 ± 3.05 ($p = 0.024$); and 0.25 ± 0.03 vs. 0.545 ± 0.11 ($p = 0.016$), respectively]. Ovarian expression of *BRCA1* and *BRCA2* was significantly lower in the endometriosis group, compared with controls [0.12 ± 0.06 vs. 0.22 ± 0.09 ($p = 0.045$) and 0.16 ± 0.08 vs. 1.26 ± 0.52 ($p = 0.001$), respectively]; but *Rad51* and *ATM* expression levels were comparable in the two groups [0.38 ± 0.11 vs. 0.51 ± 0.16 ($p = 0.621$) and 0.79 ± 0.13 vs. 0.76 ± 0.26 ($p = 0.526$), respectively].

Serum AMH concentration and ovarian *BRCA1* mRNA expression correlated significantly in the endometriosis group (correlation coefficient, 0.541; $p = 0.03$) (**Figure 5**).

Immunofluorescence Staining of *BRCA1*

Decreased expression of BRCA1 protein in endometrial and ovarian tissues of the endometriosis group was confirmed by immunofluorescence staining (**Figure 6**).

DISCUSSION

In the present study, we have demonstrated that mRNA expression levels for key genes related to DSB repair are reduced in women with endometriosis. Indeed, expression levels of *BRCA1*, *Rad51*, and *ATM* in endometrial tissue proved to be lower in the endometriosis group than in control subjects, as were expression levels of *BRCA1* and *BRCA2* in ovarian tissue. Ovarian BRCA1 expression in particular correlated with serum AMH levels as a marker of ovarian reserves. To our knowledge, this is the first study to evaluate the role of impaired DNA DSB

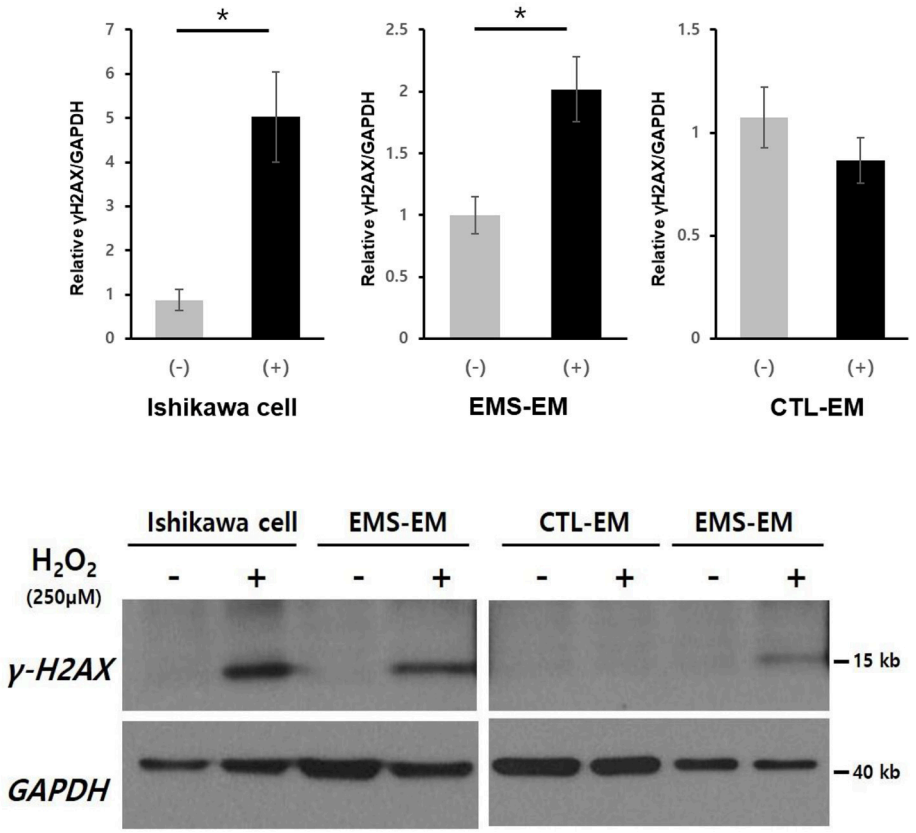

FIGURE 2 | Western blot analysis of γ-H2AX in cultured Ishikawa cell lines and in primary endometrial stromal cells of patients with and without endometriosis after H_2O_2 treatment. *$p < 0.05$. Data are expressed as mean ± SEM values. +, with H_2O_2 treatment; –, without H_2O_2 treatment; CTL-EM, eutopic endometrium of patients without endometriosis; EMS-EM, eutopic endometrium of patients with endometriosis.

repair in the pathogenic declines of ovarian reserves experienced by women with endometriosis.

Above findings are aligned with previous studies of diminished ovarian reserves in women with *BRCA* mutations. Women with germline mutations of the *BRCA1* gene showed low responses to controlled ovarian stimulation for fertility preservation by oocyte or embryo cryopreservation (26) and had lower age- and BMI- adjusted serum AMH levels, indicating that *BRCA1* mutations may be associated with decreased ovarian reserves. Furthermore, women with *BRCA1* mutations may also be associated with earlier menopause (27, 28). In a recent comparative study of ovarian tissue obtained from unaffected *BRCA* mutation carriers and age-matched cadaveric organ donors, *BRCA* mutations were associated with reduced ovarian reserves as well as accelerated loss of primordial follicle and oocyte DNA damage (29). These observations support that DNA DSB repair plays an important role in maintaining ovarian reserve and suggest that such reserves are prematurely depleted in women with *BRCA1* mutations.

Apart from *BRCA1/2* germline mutations, other BRCA defects (including methylation of the *BRCA1* promoter, low-level expression, and copy-number deletions) in some sporadic cancers share phenotypic traits of tumors that carry BRCA1/2 mutations (30). *BRCA1/2* mRNA expression levels have been previously investigated in women with breast and ovarian cancers (31, 32), suggesting that they correspond with survival rates or chemotherapeutic sensitivity. Although the present study was aimed at mRNA expression levels of genes related to DNA DSB repair, faltering mRNA expression of these genes may affect ovarian reserves in a manner similar to women with *BRCA* germline mutations. It is believed that *BRCA1*-related DNA DSB repair efficiency may be an important determinant of oocyte aging in women (17). Researchers have found that *BRCA1* gene expression showed a significant age-related decline in oocytes and that oocyte-specific knockdown of *BRCA1* expression increases DSBs.

Endometriosis is a disease related to elevated oxidative stress in the follicular environment, paracrine environment, and systemically (33). Oxidative stress causes DNA damage (34). Histone H2AX, one of several variants of the nucleosome core histone H2A, becomes phosphorylated on Ser139 in response to DSBs (γ-H2AX). Within seconds of DSB occurrence, γ-H2AX foci appear at sites of DNA damage, which are detectable by confocal microscopy, or immunohistochemistry in quantifying DNA damage. Foci of γ-H2AX represent DSBs in a 1:1 ratio, enabling sensitive quantitation of DSBs (35).

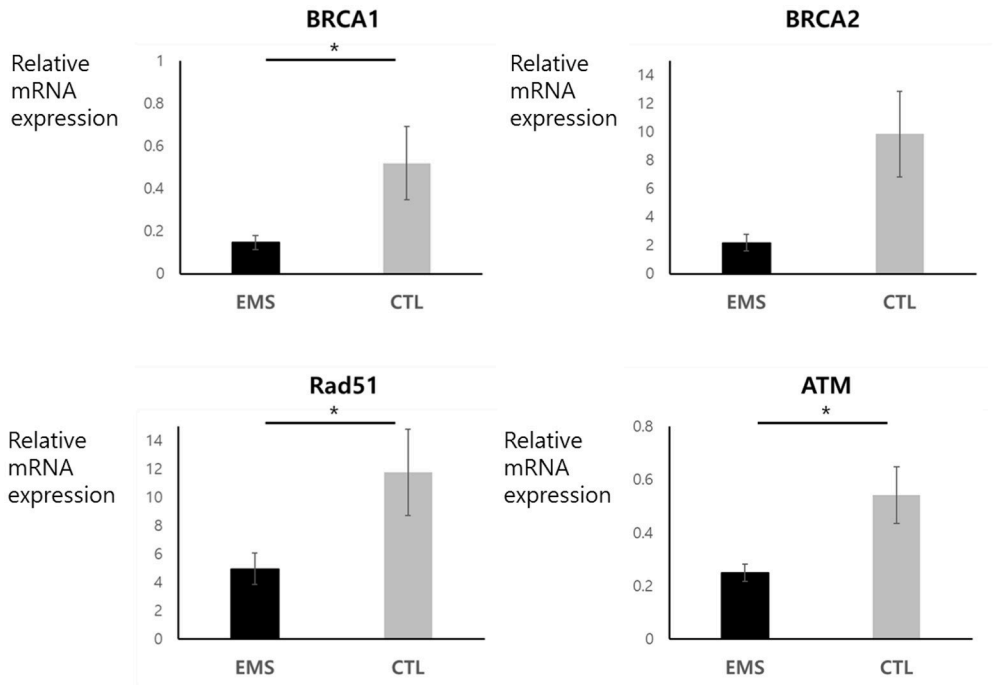

FIGURE 3 | Endometrial mRNA expression of *BRCA1*, *BRCA2*, *Rad51*, and *ATM* in endometriosis group and in controls. $^{*}p < 0.05$. Data are expressed as mean ± SEM.

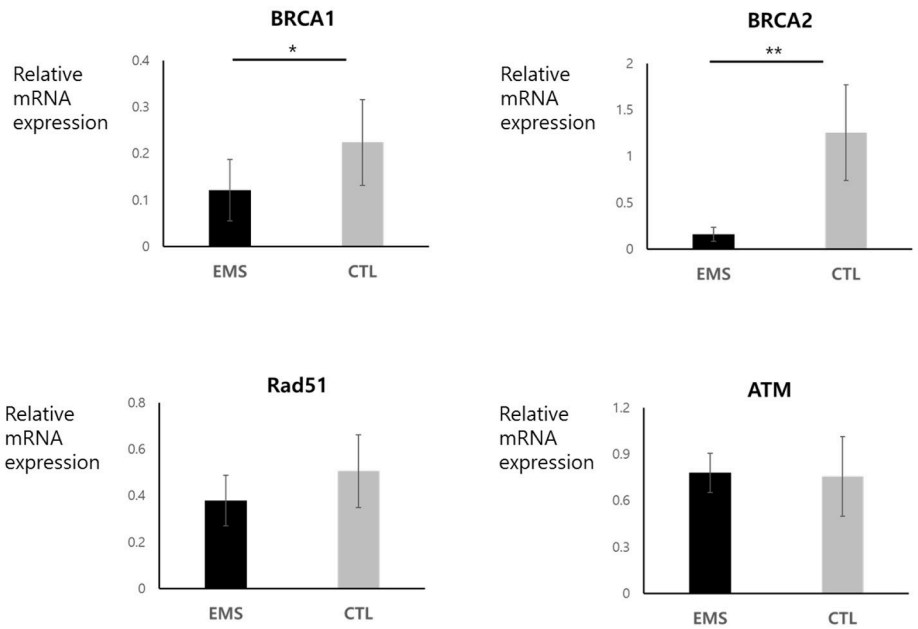

FIGURE 4 | Ovarian mRNA expression of *BRCA1*, *BRCA2*, *Rad51*, and *ATM* in endometriosis group and in controls. $^{*}p < 0.05$, $^{**}p < 0.01$. Data are expressed as mean ± SEM.

In the present study, we found that occurrences of DNA DSBs, as represented by γ-H2AX protein expression, increased in endometrial, and ovarian endometrioma samples of patients with endometriosis. However, two earlier immunohistochemical studies have reported results contradictory to ours. In women with endometriosis, persistence of proliferative markers in eutopic endometrial cells seemed to be associated with virtually complete loss of γ-H2AX (36); and endometrial cells

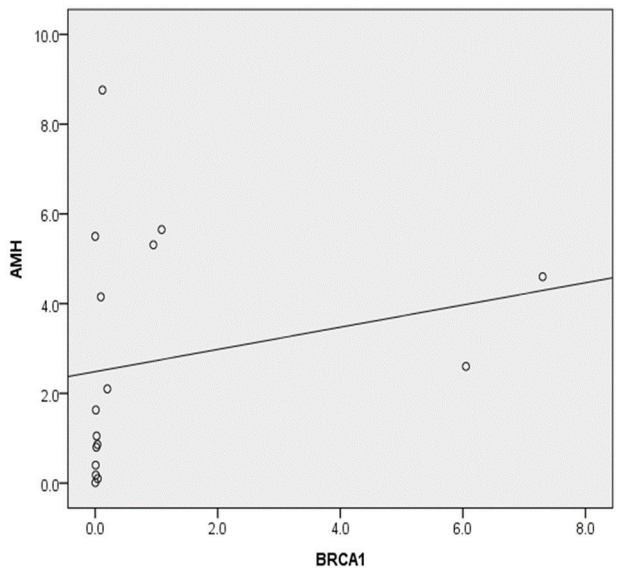

FIGURE 5 | Correlation between serum anti-Müllerian hormone levels (AMH) and ovarian *BRCA1* expression in patients with endometriosis (Spearman's rho, 0.541; $p = 0.030$).

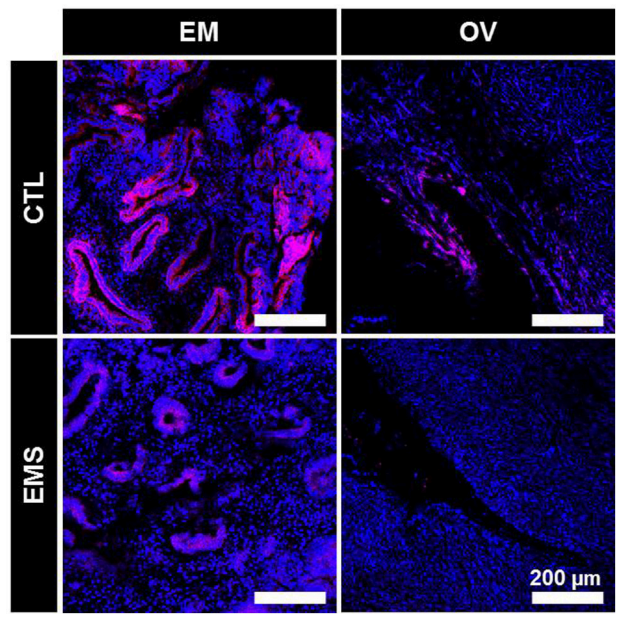

FIGURE 6 | Immunofluorescence staining of BRCA1 in eutopic endometrium and ovarian tissue of patients with and without endometriosis. CTL-EM, eutopic endometrium of patients without endometriosis; EMS-EM, eutopic endometrium of patients with endometriosis; CTL-OV, Non-endometriotic ovarian tissue; EMS-OV, ovarian endometriotic cyst.

from ectopic sites dysplayed immune-staining for proliferative markers, with concomitant loss of the γ-H2AX staining in ectopic endometriotic lesions of both human and baboon endometriosis model (37). These differences may be due to the differing characteristics of subjects selected for study. Although all of our patients had advanced-stage endometriosis with endometrioma(s), all stages of peritoneal endometriosis (I-IV) were included. Moreover, immunnostains performed in the latter study were confined to peritoneal endometriotic lesions. Immunodetection of γ-H2AX is used to quantify DNA damage in cells and tissues, and has diagnostic and prognostic value in cancer. High phosphorylation levels are indicative of defective DNA repair and genomic instability in premailignant lesions and in tumors; and they are associated with higher-grade malignancy and poor prognoses in various cancers, including breast, colorectal, lung, and ovarian cancers, and melanoma (38) as well as endometrial cancer (39).

In our experiments with *in vitro* cell cultures, we also demonstrated that endometrial and ovarian tissues of patients with endometriosis are more vulnerable to genotoxic stimuli, showing an increased propensity for DNA damage compared with controls. Following genotoxic exposure with H_2O_2, γ-H2AX expression by endometrial stromal cells, and Ishkawa cell line increased in tandem, whereas endometrial stromal cells of control subjects were devoid of γ-H2AX expression, even after H_2O_2 treatment. These findings may certainly stem from impaired DNA DSB repair.

Although we did not directly demonstrate attenuated DNA DSB repair or DSB repair mechanisms in ovarian follicles or oocytes, we did establish that *BRCA1* expression in ovarian endometriotic tissue and serum AMH level (a marker of ovarian reserve) correlated significantly. Diminished expression of *BRCA* in ovarian endometrotic tissue is then indicative of impaired DNA DSB repair and ultimately is attributable to follicular damage. Given that endometriosis is associated with oxidative stress locally and systemically and incite chronic inflammation within the peritoneal cavity, heightened DNA damages, and impairment of DNA repair in ectopic endometrial tissue may mirror the status of ovarian follicles, both oocytes and surrounding follicular cells. Finally, pathologic findings akin to chemotherapy-induced ovarian damage (i.e., focal fibrosis, vascular deficiency, and concomitant lowering of follicular density) in women with endometriosis may attest to microvascular and stromal damage. As documented in the mouse ovary, doxorubicin insult initially induces DNA damage in stroma, theca, and granulosa cells, followed by oocytic DNA damage (40). Thus, DNA damage in adjacent somatic cells and stroma also plays a role in decreased ovarian reserves.

Although gene expression patterns of endometrium might be different according menstrual phase, we compared gene expression patterns irrespective of menstrual phase. However, their distribution in the present study was comparable between the two groups. When we also compared mRNA expression of BRCA1, BRCA2, Rad 51, and ATM between proliferative phase and secretory phase endometrium using quantitative RT-PCR, there were no significant differences in mRNA expressions of BRCA1, BRCA2, Rad 51, and ATM between proliferative and secretory phase endometrium (data were not shown).

Eutopic endometrium of women with endometriosis shows fundamental differences compared with that of healthy control (23). The eutopic and ectopic endometrium of women with

endometriosis shares alterations that are not found in the eutopic endometrium of women without endometriosis, corroborating the idea that this altered endometrium in the peritoneal cavity has the initial potential to develop endometriosis (41). In the present study, we observed increased DNA damages and impaired DNA DSB repair mechanism in eutopic endometrium as well as ectopic endometrium. However, there were no significant correlations between genes related to DNA DSB repair in eutopic endometrium and AMH. Although it could not be clearly explained, it may due to small sample size. On the other hand, it may be explained as a direct consequence of the different endocrine microenvironments such as the peritoneal fluid and the intraovarian microenvironment of ectopic endometrium and the intrauterine environment in eutopic endometrium. Nonetheless, considering that autocrine and paracrine effects following DNA damage, findings of ectopic endometrium may be more important than those of eutopic endometrium with respect to ovarian reserve.

In the clinical characteristics, BMI was significantly lower in endometriosis group compared with controls. Oxidative stress in obesity poses a significant threat to DNA stability and integrity as indicated by the growing number of investigations reporting a positive correlation between markers of oxidative DNA damage and increased adiposity (42). Obesity induces DNA damage in hematopoietic stem cell transplant recipients that have been treated by cyclophosphamide (43). Since there were significant increases in DNA damages and impairment of genes related DNA DSB repair in spite of protective effect of low BMI in endometriosis group, the difference of BMI could not affect our results.

In conclusion, we have determined that expression levels of various genes implicated in DSB repair are diminished in patients with endometriosis. In particular, *BRCA1* expression is decreased in both ovarian and endometrial tissues of such patients and appears to correlate with AMH, a marker of ovarian reserves. These findings indicate that impaired DSB repair is a likely contributor to diminished ovarian reserves in women with endometriosis. However, further studies will be necessary to confirm the association between impairment of DNA DSB repair and decreased ovarian reserve in this setting.

AUTHOR CONTRIBUTIONS

YC, SC, and H-SK contributed to study conception and design, acquisition, analysis, and interpretation of data and drafting of the manuscript. JiP, JL, J-KY, BY, JoP, SS, and H-JS aided in acquisition and analysis of data for the work. BL contributed to conception and design. All authors participated substantially in these research efforts and then critically appraised, revised, and approved the manuscript.

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Effect of Autologous Platelet-Rich Plasma Treatment on Refractory Thin Endometrium During the Frozen Embryo Transfer Cycle: A Pilot Study

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Objective: Thin or damaged endometrium remains to be an unsolved problem in the treatment of patients with infertility. The empirical preference for endometrial thickness (EMT) among clinicians is >7 mm, and the refractory thin endometrium, which doesn't respond to standard medical therapies, can be the etiology of recurrent implantation failure (RIF). Autologous platelet-rich plasma (PRP) is known to help tissue regeneration and is widely used in various fields. In the present study, we conducted PRP treatment and investigated its effect on the refractory thin endometrium.

Design: Prospective interventional study (https://cris.nih.go.kr/cris, clinical trial registration number: KCT0003375).

Methods: Women who had a history of two or more failed IVF cycles and refractory thin endometrium were enrolled in this study. The main inclusion criteria were EMT of <7 mm after more than 2 cycles of previous medical therapy for increasing the EMT. Twenty-four women were enrolled in this study. The subjects were treated with intrauterine infusion of autologous PRP 2 or 3 times from menstrual cycle day 10 of their frozen-thawed embryo transfer (FET) cycle, and ET was performed 3 days after the final autologous PRP infusion. 22 patients underwent FET, and 2 patients were lost to follow up.

Results: The ongoing pregnancy rate and LBR were both 20%. The implantation and clinical pregnancy rates were 12.7 and 30%, respectively, and the difference was statistically significant. The average increase in the EMT was 0.6 mm compared with the EMT of their previous cycle. However, this difference was not statistically significant. Further, EMT of 12 patients increased (mean difference: 1.3 mm), while that of seven patients decreased (mean difference: 0.7 mm); the EMT of one patient did not change. There were no adverse effects reported by the patients who were treated with autologous PRP.

Conclusions: The use of autologous PRP improved the implantation, pregnancy, and live birth rates (LBR) of the patients with refractory thin endometrium. We assume that the ability of autologous PRP to restore the endometrial receptivity of damaged endometrium has some aspects other than increasing the EMT. The molecular basis of the treatment needs to be revealed in future studies.

Keywords: refractory thin endometrium, platelet-rich plasma, recurrent implantation failure, frozen embryo transfer, endometrial receptivity

INTRODUCTION

Since the first introduction of *in vitro* fertilization and embryo transfer (IVF-ET), the technology has evolved rapidly, and the pregnancy rate with IVF-ET has significantly increased. However, thin or damaged endometrium remains to be an unsolved problem in the treatment of patients with infertility. Several treatments to restore endometrial receptivity have been attempted, including administration of exogenous estrogen, vitamin E, vaginal sildenafil citrate, and pentoxifylline (1–3). Patients with refractory thin endometrium who do not respond to the abovementioned treatment do not have many options, and an endometrium with a thickness below 7 mm is assumed as non-optimal for embryo implantation and is associated with a low pregnancy rate (4, 5). Recently, some progress in treating damaged or thin endometria has been made with the use of the cell proliferation method, including stem cell therapy (6, 7). However, there are still unsolved issues concerning the safety and usability of bone marrow-derived stem cells (8, 9).

Autologous platelet-rich plasma (PRP) is one alternative that is well-known for its safety. Such platelet products have been used since the 1970s, and they have become more popular since the 1990s (10). Platelets are known as the blood component that plays a crucial role in hemostasis. During the healing process, growth factors, cytokines, and chemokines are secreted from the α-granules inside platelets. The various secreted proteins have paracrine effects on myocytes (11), tendon cells (12), mesenchymal stem cells from different origins (13, 14), chondrocytes (15), osteoblasts (11, 16), fibroblasts (17), and endothelial cells (18), stimulating cell migration, cell proliferation, and angiogenesis and consequently inducing tissue regeneration (19). A study on a murine model was performed, which reported that intrauterine infusion of autologous PRP accelerated and enhanced regeneration of damaged endometria and that the fibrosis within decreased (20).

The first study on PRP for treating human thin endometrium *in vivo* was published in 2015 (21). Four studies followed and concluded that PRP is a potent treatment for thin endometri um (22–25). They stated that autologous PRP promotes endometrial growth and improves pregnancy outcomes. However, the number of patients was small, and they did not provide sufficient information on the type or concentration of PRP they used. It is known that the efficacy of PRP can vary according to the platelet concentration and cell component (19, 26). In the present study, we defined the platelet concentration and type of PRP that we used and investigated its effect on refractory thin endometrium regarding the pregnancy and live birth rates.

MATERIALS AND METHODS

Study Population and Inclusion Criteria

We conducted an interventional prospective cohort study. Patients were recruited from December 2015 to June 2017 in a fertility center of a university hospital. Women who had a history of two or more failed IVF cycles and refractory thin endometrium were enrolled in this study. The inclusion criteria were as follows: (a) age of 20–45 years at the time of enrollment, (b) endometrial thickness (EMT) of <7 mm on the human chorionic gonadotropin (hCG) administration day in fresh ET cycles or on the end of estrogen priming day in frozen ET cycles in all of the previous cycles, (c) two or more failed IVF cycles, (d) more than two cycles of previous therapy for increasing the EMT, such as, hysteroscopic adhesiolysis following hormone replacement therapy, high dose estradiol valerate, transvaginal sildenafil administration, or pentoxifyilline combination with vitamin E, (f) frozen embryo available for ET, and (g) informed consent form signed. The exclusion criteria were as follows: (a) hematologic disorders, hemoglobin level of <9.0 g/dL or platelet count of $<100{,}000/\mu$L, (b) auto-immune disease, (c) chromosomal abnormality in the patient or spouse, (d) peripheral NK cell proportion of $\geq 12\%$, (e) body mass index (BMI) of ≥ 30 kg/m^2, and (f) uncontrolled endocrine or other medical conditions, such as prolactinemia or thyroid diseases.

Autologous PRP Preparation

On each PRP administration day, 18 mL of venous blood was drawn from the patients using 30 mL syringes coated with 2 cc of acid citrate A, anticoagulant solution (ACD-A; Arya Mabna Tashkhis, Iran). The blood samples were then moved into an aseptic PRP centrifuge kit (PROSYS PRP; Prodizen, Korea) and centrifuged at 1017 G for 3 min. The buffy coat and plasma just above the buffy coat were collected, and 0.7–1.0 mL of PRP was produced and infused into uterine cavity. Based on the data provided by the manufacturer, the platelet concentration of PRP ranged from 717×10^3 to $1565 \times 10^3/\mu$L, and the WBC concentration varied from 24,000 to 37,000/μL.

Autologous PRP Administration and ET

Intrauterine autologous PRP administration was performed at the estrogen-primed FET cycle. The patients started to take a

daily dose of 4–6 mg of estradiol valerate (Progynova; Bayer Schering Pharma, France) from menstrual cycle day (MCD) 2 to prepare the endometrium. The first autologous PRP infusion was performed on MCD 10 and was repeated at 3 day intervals until the EMT reached 7 mm. PRP was administered into the uterine cavity using an ET catheter within 1 h from completion of PRP preparation. The syringe containing the PRP was connected to ET catheter and the PRP was infused. Then the syringed filled with the air was used to push in the remaining PRP. Then the air bubble was confirmed in ultrasonography. Thereafter, the patients were prescribed with second-generation cephalosporin for 2 days as prophylaxis for infection. The maximum number of autologous infusions was limited to three.

Ultrasonography was performed to measure the EMT on MCD 2 and every autologous PRP administration day until ET. ET was conducted 3 days after the final autologous PRP administration. Luteal phase support was performed using either 90 mg of vaginal progesterone (Crinone gel 8%; Merck, Germany) or 50 mg of progesterone (Sugest Inj. 50 mg; Uni-Sankyo, India) administered via intramuscular injection daily from 3 days before the ET day. The serum β-hCG level was measured from peripheral blood 2 weeks after ET. Those with positive β-hCG results underwent ultrasonography another 2 weeks later to confirm clinical pregnancy. Clinical pregnancy was defined as the presence of intrauterine gestational sac. The luteal phase support was continued until 9 weeks of pregnancy. The obstetric progress of the pregnant patients was followed up via a timely chart review.

Comparison of the Outcomes Between the Treatment and Previous Cycles

The variables of the most recent ET cycles were compared with those of the treatment cycle. The primary outcomes were the ongoing pregnancy rate and LBR. The secondary outcomes were the implantation rate, clinical pregnancy rate, and EMT increment compared with those on the previous cycle.

Data Analysis

The statistical analysis was performed using the IBM SPSS® software, version 24 (IBM Corporation, Armonk, NY, USA). Wilcoxon signed-rank test was used to compare the differences between the pre-PRP and post-PRP EMT. A P value of < 0.05 was considered statistically significant. The implantation rate, clinical pregnancy rate, and live birth rate were analyzed using Fisher's exact test.

Ethics Approval

This study was approved by the Institutional Review Board committee of Bundang CHA Medical Center.

RESULTS

Study Population and Baseline Characteristics

A total of 24 women were recruited, and 22 of them underwent ET. One patient underwent preimplantation genetic screening, and all embryos were abnormal. Another patient had withdrawn owing to personal reasons. Among the 22 patients who underwent ET, two patients were lost to follow-up, and the data of the 20 remaining women were collected.

The average age of the patients was 38.4 years. The mean duration of infertility in the 20 women was 5.7 years, and the mean number of dilatation and evacuation performed was 1.3. The mean number of failed IVF cycle was 2.7. The mean EMT on the previous-cycle hCG administration or the final estrogen priming day was 5.4 mm. Sixteen of them were diagnosed with endometrial sclerosis or adhesion via hysteroscopy; the cause was radiation therapy for treating colon cancer in one patient and pelvic tuberculosis in another patient (**Table 1**).

Treatment Outcome

The number of embryos transferred in each patient was 2 or 3. The cleavage stage embryo grading was performed using the qualification scale by Veeck (27). The blastocysts were graded using the Gardner grading system (28). A good-grade embryo was defined as a grade I or II cleavage stage embryo with six or more cells and blastocyst score of 3BB or higher. The morula was considered as a good-grade embryo. Seventeen patients had at least one good-grade embryo; however, three patients had only poor-grade cleavage embryos.

The gestational sac was confirmed in 30% ($n = 6$) of the patients. One patient had missed abortion at 8+2 weeks of gestational age. Another patient had heterotopic pregnancy, and the intrauterine fetus was aborted at 6 weeks soon after laparoscopic removal of the ectopic conceptus. The live birth rate was 20% ($n = 4$). All the ongoing pregnancies resulted in live births without obstetric complications. The mean EMT after the PRP treatment was 6.0 mm. The average increment in the EMT was 0.6 mm. However, this difference was not statistically significant. Individually, the EMT of 12 patients increased (mean difference: 1.3 mm), while that of seven patients decreased (mean difference: 0.7 mm); however, the EMT of one patient did not change. Among the six clinical pregnancy cases, two were increased and four were decreased in EMT (**Figure 1**). There were no adverse effects reported by the patients. The outcomes of the treatment are summarized in **Table 2**.

Comparison of the Outcomes Between the Treatment and the Previous Cycles

The treatment cycle outcomes were compared with the most recent ET cycle outcomes of each patient; the latter cycle was considered as the control cycle. The implantation, clinical pregnancy, and live birth rates in the treatment cycle were 12.7, 30, and 20%, respectively. The implantation, clinical pregnancy, and live birth rates in the control cycle were all 0%. The implantation and clinical pregnancy rates were significantly higher in the treatment cycle than in the control cycle. The age, BMI, number of transferred embryos, and number of good-grade embryos transferred were not significantly different. The comparison results are summarized in **Table 3**.

TABLE 1 | The baseline characteristics of the patients.

Parameters / Patient no.	Age	BMI (kg/m^2)	Infertility factor	No. of D&E	Failed IVF cycles	Infertility duration (years)	Parity[#]				Hystero-scopic finding	Medical history	EMT (mm), On previous cycle hCG day or final priming day
							T	P	A	L			
1	31	20.1	Tubal, IUA	0	2	3.1	0	0	0	0	IUA	Past Tuberculosis	5.8
2	35	28.4	Tubal, IUA	4	2	6	0	0	4	0	Synechia		4.8
3	39	20.7	IUA	2	2	7.1	0	0	2	0	Central IUA		6.7
4	40	23.6	Tubal DOR IUA	3	2	10.5	0	0	3	0	Erythematous EM		6.0
5	30	17.7	MF, IUA	1	2	1.9	0	0	1	0	No specific	Past PID	6.4
6	34	22.3	Tubal, IUA	0	3	6	0	0	0	0	Severe IUA		4.9
7	45	25.3	DOR,	1	3	8	0	0	1	0	No specific		5.2
8	33	22.4	IUA	1	2	3.7	0	0	1	0	Synechia		5.5
9	35	22.3	Tubal, IUA	0	4	6.5	0	0	1	0	Severe IUA		4.9
10	36	20.8	IUA	1	5	8.5	0	0	1	0	Synechia		5.5
11	37	21.3	POI, IUA	0	2	3.3	0	0	0	0	Severe IUA	Past RT (colon ca.)	4.0
12	38	25.4	unexplained	0	2	10	0	0	1	0	No specific		4.8
13	39	26.0	unexplained	1	4	4	0	0	1	0	Sclerotic EM		5.8
14	39	28.6	SM myoma, PGD	0	3	5	1	0	0	1	Synechia		6.8
15	41	24.0	IUA, MF	0	4	4.3	0	0	0	0	Severe IUA		4.3
16	41	20.6	IUA	3	2	1.5	0	0	3	0	Synechia		5.3
17	43	28.6	DOR IUA	5	2	6.6	0	0	5	0	Septum c fistula		5.7
18	43	19.2	IUA	1	2	5.7	1	1	2	1	Sclerotic fundus		4.5
19	44	22.4	MF, IUA	2	3	9	0	0	2	0	Sclerotic walls		6.5
20	44	25.7	DOR	0	2	2.4	2	0	0	2	Synechia		5.4
Mean ± SD or explanation	38.4 ± 4.3	23.3 ± 3.1		1.3 ± 1.5	2.7 ± 0.9	5.7 ± 2.6	17 primary 3 secondary				16 patients with endometrial pathology	–	5.4 ± 0.8

EMT, endometrial thickness; hCG, human chorionic gonadotropin; RT, radiation therapy; IUA, intrauterine adhesion; DOR, diminished ovarian reserve; POI, primary ovarian insufficiency; MF, male factor; SM, submucosal; D&E, dilatation and evacuation; T, term birth; P, preterm birth; A, Abortion; L, living birth. [#]The abortion count of parity includes chemical abortion.

DISCUSSION

The purpose of the present study was to determine whether intrauterine administration of PRP would improve the pregnancy outcomes of patients with refractory thin endometrium. A total of 20 women were enrolled, and a clinical pregnancy rate of 30% and a live birth rate of 20% were achieved in these patients with poor prognosis. However, contrary to the expectation, even the mean EMT increased after treatment, and there was no association between the EMT changes and the ET outcomes.

Since the first study on *in vivo* autologous PRP on the human endometrium in 2015, five studies have been published (21–25). The inclusion criteria differed to some extent; however, all studies showed that autologous PRP is effective in repairing the damaged endometrium and improving the pregnancy outcomes. The LBRs reported by three studies were all above 25%. The autologous PRP preparation method and cell contents were not reported in three of the five studies. **Table 4** summarizes the five previous studies on PRP for treating patients with repeated implantation failure owing to endometrial factors.

Although PRP is widely applied in different clinical areas, the procedure in preparing PRP is not yet standardized. Therefore, the platelet quantification and growth factor contents are not defined (19). The previous studies did not present critical information on the PRP used, such as cell contents, platelet concentration, and activation. We attempted to provide information on PRP and its preparation method and searched for the best-known evidence to improve the effectiveness of PRP. The optimal biological effect seems to occur when PRP with a platelet concentration of approximately 1,000,000/μL (503,000–1,729,000/μL) is used. At lower concentrations, the effect is suboptimal, while higher concentrations might have a paradoxically inhibitory effect (29). We employed a PRP preparation method using an aseptic PRP preparation kit that

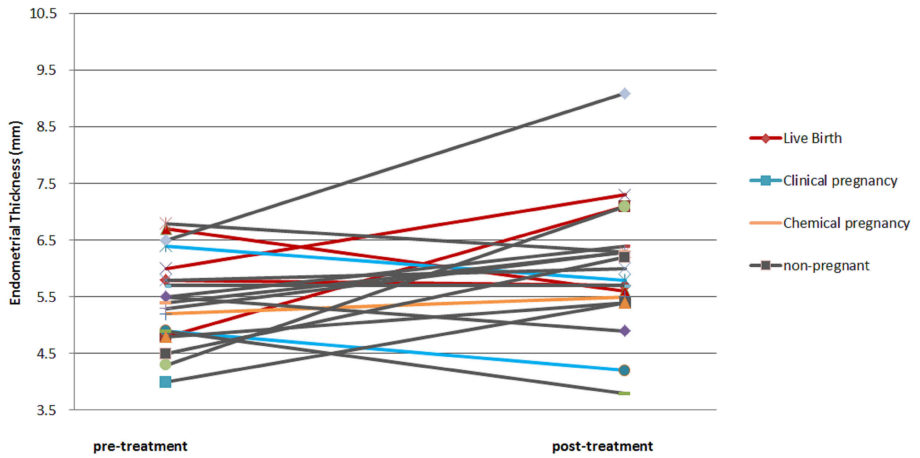

FIGURE 1 | Pre- and post- endometrial thickness of each patient. The color of the line indicates the obstetric result of the patient.

TABLE 2 | The results of autologous platelet-rich plasma treatment.

Pat. No.	Obstetric result		EMT Final (mm)		Embryo grade and number	β-hCG¥	No. of G sac
	Week	Result		AVG			
1	37+5	Live birth	5.7	6.4	10C GIII, 8C GI	+	1
2	38+6		7.1		Mor, 12C GII x 2.	+	1
3	38+3		5.6		Mor, 12C GI, 8C GI	+	2*
4	37+2		7.3		Mor, 12C GI, 10C GIII	+	1
5	8+2	Abortion	5.8	5.0	Mor, 10C GIII	+	1
6	6		4.2		Mor, 12C GII, 12C GIII	+	1‡
7	5	Chemical pregnancy	5.5	5.5	12C GII, 10C GI, 6C GIII	+	0
8	Not-pregnant	Not-pregnant	6.4	6.1	12C GI, 10C GI	–	N/A
9			3.8		Mor x 2, 12C GII	–	
10			4.9		12C GIV, 10C GIII, 10C GIV	–	
11			5.4		8C GII, 6C GIII, 4C GIII	–	
12			5.4		6C GIV x 2	–	
13			6.0		Mor, 12C GII, 10C GIII	–	
14			6.3		Mor x 2, 8C GIV	–	
15			7.1		8C GIII, 7C GIII, 6C GIII	–	
16			6.3		Mor x 2, 10C GIII	–	
17			5.7		12C GII, 12C GIII, 8C GI	–	
18			6.3		Mor, 8C GII, 4C GI	–	
19			9.1		Mor, 10C GII, 6C GIII	–	
20			6.2		Mor x 2, 10C GIII	–	
Average/ counts	Full term: 4 patients (20%)	LBR: 20% ABR: 15%	6.0 ± 1.6			7 patients (35%) (35%)	6 patients (30%)

*EMT, endometrial thickness; Mor, Morula; hCG, human chorionic gonadotropin; G sac, gestational sac; LBR, live birth rate; ABR, abortion rate; ¥ β-hCG cut off: 35 mIU/mL, * Vanishing twin, ‡ Missed abortion after laparoscopy for heterotopic pregnancy.*

had manufacturer's information on the platelet count of the final product as 717,000 to 1,565,000/μL and the WBC concentration as 24,000 to 37,000/μL.

There are four categories of platelet concentrate preparations: leukocyte-poor or pure PRP (P-PRP), leukocyte PRP (L-PRP), pure platelet-rich fibrin clot, and leukocyte platelet-rich fibrin

TABLE 3 | Comparison of outcomes between the treatment and the previous cycles.

Parameters / cycle	Previous Cycle	Treatment Cycle	*P*-value
Age	37.6 ± 4.4	38.4 ± 4.3	0.547
BMI (kg/m^2)	22.89 ± 3.2	23.3 ± 3.1	0.640
EMT on hCG triggering or final preparation day* (mm)	5.4 ± 0.8	6.0 ± 1.1	0.070
Cycle types (fresh/frozen)	10/10	0/20	
Number of transferred embryos	2.6 ± 0.7	2.8 ± 0.4	0.640
Number of good quality embryos transferred	1.7 ± 0.8	1.7 ± 0.9	0.967
Implantation rate (%)	0 (0/52)	12.7 (7/55)	0.015
Clinical pregnancy rate (%)	0 (0/20)	30 (6/20)	0.020
Ongoing pregnancy rate (%)	0 (0/20)	20 (4/20)	0.106
Live birth rate (%)	0 (0/20)	20 (4/20)	0.106

*EMT, endometrial thickness; hCG, human chorionic gonadotropin. *EMT on hCG triggering day in fresh cycles and on final preparation day in frozen-thawed cycle.*

clot. Among them, two families contain a significant number of leukocytes. P-PRP and pure platelet-rich fibrin clot are made without the buffy coat and considered to contain a minimal amount of leukocytes (30). The variety of PRP preparations currently available on the market has led to considerable confusion in the evaluation of the potential clinical benefits of PRP in different applications (26). The advantage of each type of PRP in specific tissues has not been defined yet.

Two of the previous studies (21, 25) provided information that they used the buffy coat of the centrifuge, and this implies that they employed L-PRP. There are conflicting opinions on the leukocyte content in PRP. One view is that leukocytes increase inflammation and reduce tissue regeneration (31). Another view is that inflammation is an essential step in the healing process (32), especially for protection against infection and clearance of tissue debris (33). There was also a recent study by Cousins et al. that provided evidence that mononuclear phagocytes have roles in scar-less endometrial healing in menstrual cycles (34). We also used the buffy coat of the centrifuge, and thus, L-PRP was employed. The leukocytes in PRP could have increased inflammation; however, the implantation and pregnancy rates improved. Since no studies have stated the use of P-PRP, its effectiveness needs to be explored in the future.

LBR was reported in two of the previous studies (23, 24). The first study reported 26.3% of live birth after PRP treatment and the LBR of the second study was 38.2%. The difference of LBR between the previous and the present studies may be caused by the difference in patient characteristics. The inclusion criteria of the first study was "aged between 33 and 45 years with a previous history of refractory endometrium and at least one failed IVF attempt" and the second study criteria was "between 22 and 40 years of age with a suboptimal endometrial pattern, as identified by ET <7 mm despite standard dose of estradiol valerate, or suboptimal endometrial vascularity, defined as <5 vascular signals reaching the central zone (zones 3 and 4 as per Applebaum grading) of the endometrium." The patients of our study had at least 2 failed previous IVF cycles and had no improvement in endometrial thickness after two or more cycles of medical therapies. The average of infertile period was 5.7 years and more than 2/3 of them had intrauterine adhesion from hysteroscopic findings.

The EMT was reported to have increased after PRP treatment in the previous studies. In the present study, the average increase in the EMT was 0.6 mm. However, this difference was not statistically significant. Furthermore, there was no correlation between the EMT increase and pregnancy outcomes. Among the six clinical pregnancy cases, two were increased, and four were decreased in EMT. A study examining the pregnancy outcomes of euploid ET (35) and a systemic review with meta-analysis on the EMT as a prognostic factor of pregnancy (36) reported that the EMT was not significantly associated with the pregnancy outcomes. Accordingly, we assumed that autologous PRP intrauterine administration improved the endometrial receptivity of the patients with refractory endometrium through the way that cannot be checked by EMT.

There was no difference in other clinical characteristics including age, infertility duration, number of failed IVF cycles, and transferred embryo number and grade according to pregnancy outcomes. Therefore, there is no prognostic factors expecting successful results in PRP treatment. However, this result might be due to small number of cases and further study with larger number of subjects is necessary to confirm this finding.

Endometrial receptivity is controlled by dynamic and precise molecular and cellular events of cytokines, homeobox transcription factors, and genes (37). Of the cytokines, leukemia inhibitory factor (LIF) has been found to have a role in uterine preparation and embryo attachment (38, 39). *Lif* -deficient female mice showed an implantation failure and were rescued with LIF supplementation (40, 41). PRP treatment upregulates LIF expression in endometrial stromal cells (42), and upregulated LIF expression could enhance endometrial receptivity. It is also suggested that PRP may exert some effect to enhance the placentation of trophoblasts. Amable et al. showed that the levels of 12 proteins increased in activated PRP in comparison with whole blood plasma or platelet-poor plasma. Six growth factors (i.e., PDGF-AA, PDGF-AB, PDGF-BB, TGF-β1, TGF-β2, and EGF), three anti-inflammatory cytokines (i.e., IL-4, IL-13, and IFN-α), and three pro-inflammatory cytokines (i.e., IL-8, IL-17, and TNF-α) were included (19). These cytokines and growth factors may increase endometrial receptivity. The vascularity of the endometrium increased in the study by Tandulwadkar et al. The endometrial vascularity measured using power doppler after PRP treatment significantly increased, especially in the group that achieved pregnancy after PRP treatment (24). More studies on the molecular basis of PRP treatment are required to reveal the exact mechanism and to specify which group of patients would benefit the most from the autologous PRP treatment of the endometrium.

Prevention of intrauterine adhesion after curettage or hysteroscopic operation of myoma or endometrial polyp is a good candidate for endometrial PRP treatment. However,

TABLE 4 | Previous studies on autologous PRP treatment of human endometrium.

References	Patients			PRP preparation	Result and Conclusion				
	No	HRT on FET	PRP repeat		EMT	β- hCG	On-going pregnancy	Live birth	Missed Ab
Chang et al. (21)	5	Yes	2	L-PRP No information on platelet concentration	>7 mm (100%)	5 (100%)	4 (80%)	Not reported	1 (20%)
	EMT < 7 mm on previous hCG day despite HRT				PRP promote endometrial growth and improve pregnancy outcome				
Zadehmodarres et al. (25)	10	Yes	2	L-PRP No information on platelet concentration	>7 mm (100%)	5 (50%)	4 (40%)	Not reported	–
	EMT < 7 mm 4 patients were diagnosed as intrauterine adhesion by HSC				PRP is effective for endometrium growth				
Molina et al. (23)	19	Yes	2	No information on PRP preparation method, platelet concentration or WBC's in PRP	>9 mm (100%)	15 (73.7%)	5 (26.3%)	5 (26.3%)	1 (.26%)
	history of the refractory endometrium with at least 1 failed previous IVF cycle				PRP seems beneficial for endometrial microvasculature and endometrial receptivity of the refractory endometrium				
Colombo et al. (22)	8	–	–	No information	>6.5 mm (88%)	6 (85.7%)	4 (57%)	2 (28.5%)	1 (14.3%)
	more than 3 canceled FET d/t EMT < 6 mm HSC: no EM pathology				Inefficient expression of adhesion molecules can be replaced by PRP				
Tandulwadkar et al. (24)	68	Yes	2	No information on platelet concentration or WBC's in PRP	Average 7.22 mm	39 (60.9%)	31 (45.3%)	26 (38.2%)	5 (7.35%)
	suboptimal endometrial growth; thickness<7 mm or <5 vascular signals reaching central zone				Endometrial vascularity measured with power Doppler was increased				

EMT, endometrial thickness; hCG, human chorionic gonadotropin; MCD, menstrual cycle day; HRT, hormonal replacement therapy; FET, frozen-thawed Embryo Transfer; HSC, hysteroscopy; L-PRP, Leucocyte-platelet rich plasma.

concerns have been raised regarding PRP use for regeneration or reconstruction on cancer tissue removal site because PRP contains and induces various growth factors and cytokines to promote cell proliferation and regeneration. There have been a few clinical studies reporting favorable outcomes of using PRP in breast reconstruction after mastectomy in breast cancer patients (43, 44). However, there is no study on endometrial PRP treatment after curettage in endometrial cancer patients. Although *in vitro* studies reported that the growth factors and VEGF of PRP could promote cancer recurrence (45, 46), the role of PRP in tumor proliferation and recurrence in cancer patients yet needs further investigation.

In the present study, the PRP treatment was performed during the FET cycle; however, half of the most recent cycles that were used as control cycles were conducted during the fresh cycle. It is still controversial whether FET increases the pregnancy rate in IVF-ET. In a recent large-scale prospective randomized clinical trial (RCT), Shi et al. reported that there is no significant difference in the pregnancy outcomes between fresh and frozen embryos when transferred to ovulatory women (47). Further, a meta-analysis including four RCTs also showed that there is no clear evidence on the difference in the cumulative pregnancy rates between fresh and frozen-thawed ET cycles (48). In the present study, 14 of the 20 patients have undergone FET in the previous cycles, and all the cycles failed to achieve pregnancy. Among the six pregnant cases after the PRP treatment, three underwent fresh ET, and the other three underwent FET as the control cycle. Therefore, we assumed that the difference in the transfer cycle characteristics (fresh vs. frozen) would not affect the outcomes significantly in our study.

There are limitations in this study. First, the study population was small to show a statistically significant result on live birth

rate. The live birth rate was 20% in the treatment cycles, but was not significantly increased compared with that in the control cycles showing no pregnancy. A follow-up study consisting of larger number of patients is necessary and is actually currently being performed. Second, this study was not an RCT; thus, the effectiveness of the PRP treatment was shown only by comparison with the most recent previous cycle of each patient.

The present study was conducted as a pilot study to determine the effects of autologous PRP treatment on refractory thin endometrium. The implantation, clinical pregnancy and live birth rates reached up to 12.7, 30, and 20%, respectively. This result is a noticeable improvement considering the patients' history. Further studies on the molecular basis of this PRP treatment and well-designed RCTs are necessary to reveal the exact mechanism and to obtain more solid evidence on the beneficial effect of PRP on the endometrium of various pathophysiology.

AUTHOR CONTRIBUTIONS

HKi: collection, analysis, and interpretation of data, drafting, and revision of the manuscript; JS, HKo, HKw, and DC: conception and design, data interpretation, and revision of the manuscript; JK: conception and design, data analysis, data interpretation, revision and final approval of the manuscript.

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Investigation of Each Society for Fertility Preservation in Asia

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Fertility preservation (FP) remains a future direction for reproductive medicine. FP development is needed to keep up with advancements in other areas of medicine, such as cancer research that has successfully prolonged patients' survival. The demand for optimum FP is sought by both patients and practitioners alike. The American Society of Clinical Oncology has published and updated several FP guidelines. However, these guidelines need to be optimized for each country due to the nature of FP that interacts with the local policy, social decorum, and economic factors. Furthermore, the availability and success rate for each procedure might differ since there is a requirement for advanced and innovative technologies involved in FP. These guidelines should ideally be supported by the FP society to overcome unique barriers that may arise in each country. Here we try to illustrate the most recent update on the condition of FP practice in several countries in Asia. This hopefully will encourage future FP development and might become a useful reference for other countries, especially in Asia.

Keywords: fertility preservation, cancer, Asian countries, reproductive technology, cancer therapy

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INTRODUCTION

Cancer remains highly prevalent despite discoveries of its risk factors. In 2012, there were 14.1 million new cases and around 8.2 million deaths were caused by cancer. In Indonesia, the prevalence of cancer of all ages in 2013 was 1.4‰ or estimated at 347,792 people (1). Breast cancer, lymphoma, skin cancer (excluding basal and squamous types), and leukemia are the most prevalent cancers in young adults aged 20–44 years (1). The adverse outcome of infertility due to premature gonadal failure is prominent, despite the overall 5-year relative survival rate improvement to 82.7% for individuals younger than 45 years (2). In 2017, an estimated 15,270 children and adolescents ages 0–19 were diagnosed with cancer (2).

Patients with cancer can now live longer owing to advances in diagnosis and treatment. Their lives, however, are handicapped by long-term effects of cancer and its treatments, in terms of being psychological, economic, social, sexual, and biological. The commonly prescribed cancer treatment can negatively impact major female reproductive systems and may lead to the loss of reproductive organs, premature ovarian failure, or an inability to produce mature eggs (3). Women also face the risk of immediate or premature menopause (4). In addition to the biological consequences, loss of fertility can be devastating for younger adults with respect to its severe and long-lasting emotional impact (3).

Fertility preservation (FP) is a developing field that envelops an assortment of fertility treatments for patients envisioning restorative treatment that could influence future conceptive results (5). The first widely-known guideline produced was by American Society of Clinical Oncology (ASCO) in 2006 for patients facing cancer therapy (6). FP is often linked with cancer treatment, however, it has also been applied to patients who require fertility affecting treatments, such as lupus, glomerulonephritis, and myelodysplasia. Furthermore, FP can also be applied for adolescent females with Turner mosaicism or other conditions that cause premature ovarian failure. The 2006 ASCO Recommendations on FP in Cancer Patients advises that all oncologists should address potential treatment-associated infertility with patients of childbearing age. This is based on the fact that cancer survivors believed that they will be a better parent upon surviving cancer which drove their intention toward pregnancy (7).

FERTILITY PRESERVATION OPTIONS

A recent ASCO guideline published in 2018 did not differ significantly from the 2013 guideline. Notwithstanding, the 2013 ASCO is more focused on promoting a holistic approach to include all providers to discuss FP to cancer patients (8). More importantly, it emphasizes the use of Gonadotropin-Releasing Hormones agonist (GnRHa) as an option for FP. In contrast, ASCO 2018 indicates that GnRHa should not be used to replace other means of more reliable FP options whenever possible despite a lower likelihood of chemotherapy-induced ovarian failure is noted with the use of GnRHa. ASCO 2018 added recommendations that all patients in reproductive age with cancer are routinely counseled about the effect of cancer treatment on fertility and all options available.

ETHICAL CONSIDERATION

Several arguments exist to support the use of fertility preservation. One would argue that everyone has the right to reproduce as a basic human right (4). Several studies concluded that given the right support system and counseling, patients undergoing fertility preservation procedures are satisfied (9–11). Fertility preservation has given hope to some patients and give a stronger reason to live (12). Without proper counseling, these benefits may instead cause anxiety and depression among the cancer patients (13, 14). Consideration should also be taken when the right to reproduce is outweighed by others' right. This concerns the welfare of the children born with a higher chance of losing the parent in addition to the possibility of inheriting the disease (4).

Another argument would be a non-maleficence aspect of healthcare providers, as it is our duty to do no harm, or at least reverse the harm done by treatment whenever possible (15). This can also be debated with the possibility of delay in curative treatment from the fertility preservation procedures. Despite the expected longer delay in the cancer treatment, mortality, and recurrence rate does not differ between women who underwent fertility preservation and not (16).

Although ethical aspects often change over the course of history, it can be inferred from recent studies that cancer survival rate is higher than ever. Thus, it is ethical to provide FP options when it is accompanied with appropriate counseling.

ASIAN SOCIETY FOR FERTILITY PRESERVATION

The Asian Society for Fertility Preservation (ASFP) already established and there are many countries that have joined the society, which are Japan, Hong Kong, India, Singapore, Korea, Taiwan, Thailand, Indonesia, Vietnam, Philippines, China, and Pakistan. ASFP is the first initiative of experts from all over Asia to promote FP science and practice. Being the first, ASFP sparks countries in Asia to establish their own FP society. ASFP's mission is to raise awareness of FP among medical professionals and the public, to improve technical skills, and to keep health practitioners informed about the latest developments in the field and in a research setting (17).

JAPAN

Japan Society for Fertility Preservation (JSFP) is a non-profit organization found in 2012 aiming to appropriately organize, implement, and understand the healthcare system for oncofertility therapy. While ASCO is readily accessible, it is not necessarily applicable to Japanese Cancer patients (18). They also serve to provide patients with issues regarding oncofertility treatments among healthcare professionals from multiple specialties. The society states that "treatment of the patient's malignancy must receive the highest priority," thus FP should be completed within a limited period. It then becomes the treating physicians' responsibility to inform the patient whether to abandon FP and suspending anticancer treatment unnecessarily (19). Written in a native language, JSFP website provides information for patients and healthcare professionals.

Japan has published several guidelines from Japanese Society for Reproductive Medicine (JSRM) and Japanese Society of Obstetrics and Gynecology (JSOG). These guidelines, however, are not comprehensive enough to address FP for various cancer types (18). In 2017, the guideline on FP for young cancer patients has been published by the Japan Society of Clinical Oncology that can be applied by healthcare providers. Options available for female patients in Japan are oocyte, embryo, and ovarian tissue cryopreservation. Oocyte cryopreservation has been the first option as a method for FP in Japan. However, in the case when the delay for cancer treatment is not tolerable or increased estradiol due to ovarian stimulation may cause adverse effects on cancer, ovarian tissue cryopreservation is preferred.

As a safeguard, all frozen oocyte or embryo should be reported by the physician to JSOG (20). Oocyte cryopreservation is considered as a medical practice to counter the adverse effects of cancer therapy and patients should be informed of this option regardless of their desire for childbirth at the time.

Patients should also be informed about the procedures impacts on the disease prognosis, the possibility and safety of pregnancy in the future, so that they can make their own independent decision (18).

Among patients who are eligible for oocyte cryopreservation, ovarian tissue cryopreservation can still be offered. The only contraindication is when the cancer cells might be present in the ovarian tissue. The method for ovarian tissue cryopreservation in Japan favors the use of closed method vitrification, which is different from the recommended slow-freezing technique by ASRM 2013 (21). They have conducted a systematic review and meta-analysis and concluded that the vitrification technique is superior in terms of DNA strand breaks and stromal cells preservations (22). This technique is expected to be used as standard method of ovarian tissue cryopreservation in the near future. After the tissue cryopreservation, auto-transplantation can be done with a successful birth rate of 25% (23). Alternatively, the immature oocytes can be collected from antral follicles and cultured for further growth in a specific medium for oocytes *in vitro* maturation.

FP option for male patients is restricted to sperm cryopreservation. When facing difficulty in extracting sperm, Penile Vibratory Stimulator, Electroejaculation under general anesthesia, or Microsurgical Testicular Sperm Extraction (TESE) can also be an option. Testicular tissue cryopreservation remains restricted to experimental research as it is not possible to mature spermatogonial stem cells *in vitro*. However, since it is the only option for pre-pubertal boys it can still be done with the hope that auto-transplantation may resume sperm production (18).

Japanese Government also enforced Law regarding FP. With regards to cost, Japan has not established coverage for the patients. Insurance also does not cover cryopreservation procedures thus patients are asked to fund the procedures themselves including consultation fee. One survey around the globe reports Japan has one of the highest cost of FP procedures with the cost ranging from 150 to 8,000 USD$ (24).

REPUBLIC OF KOREA

In 2013, Korean Society for Fertility Preservation (KSFP) was established to ease teamwork between medical doctors and researchers specializing in reproductive medicine and oncology. The aim of KSFP is to help the patients who undergo treatments that may affect fertility. The society also has established standard protocols and policies including referral system for optimum FP application through an annual conference and postgraduate courses. As a result, Korea has a well-established network for their hospitals including the regional ones. This ensures the availability of high-quality FP treatments in each institution. As a global partner of Oncofertility Consortium, together with JSFP, they share information and experience for improvement of oncofertility research and clinical programs in Asia (25). KSFP does not limit the application of FP to oncologic patients, instead all patients facing treatment-related infertility such as lupus, rheumatoid arthritis, or Crohn disease, and those planning to undergo bone marrow or stem cell transplantation for hematologic diseases are also candidates (26).

KSFP guidelines promote a multidisciplinary team approach, involving physicians, nurses, mental health professionals, office staff, and laboratory personnel. This is done to understand patients' unique situation and develop the flow system to build successful FP program (26). Several guidelines are also published regarding FP for breast cancer (27), hematologic malignancies (28), and gynecologic malignancies (29). These guidelines provide considerations between FP techniques for specific cases. In addition to similar recommendations with 2018 ASCO guideline, gonadal shielding and ovarian transposition is encouraged for gynecological malignancies (29).

KSFP acknowledges that the time pressure for cancer treatment causes patients having difficulties in processing the information regarding FP. Therefore, oral, printed materials, and web-based resources need to be prepared (30), as well as encouraging patients to ask and additional FP consultations with fertility specialist (31) to ensure patients understand the risk of the chosen FP and cancer treatments. It is also stated in the guideline that despite clinical judgement may be used whether FP techniques are appropriate, early and prompt referral should be made to minimize time delay to begin the cancer treatment (26).

Despite the effort, a review stated that Korea faces barriers for FP in terms of issues with referrals, a financial burden for patients, and an inability to secure funding for research. Since there is no insurance coverage, patients must pay roughly $US 2,000–3,000. For ovarian tissue cryopreservation, only operation costs may be partially covered by insurance, which results to the same costs for ovarian tissue cryopreservation and oocyte or embryo cryopreservation (24).

INDIA

Fertility Preservation Society of India (FPSI) is the first organization in India to promote the science and practice of fertility preservation mainly for cancer treatment but also for some diseases that can cause premature infertility (Premature Ovarian Failure, Autoimmune diseases, and Fragile X Syndrome). They vision to promote science and practice of FP in India and active interaction between medical specialties to accomplish patient's reproductive health expectations and quality of life regarding FP. They claim that all patients should receive reproductive information, referrals, and decision making supports about FP from the healthcare professionals (32).

India offers embryo cryopreservation, oocyte cryopreservation, gonadal shielding, and ovarian transposition surgery as standard FP options for post-pubertal to pre-menopausal female patients aged 15–45 years old. The ovarian transposition option, however, is reserved for patients who are not eligible for embryo or oocyte cryopreservation. Following the cancer treatment regimen, the ovary can then be relocated into the pelvis via minimally invasive laparoscopic surgery for traditional IVF oocyte retrieval (32). Ovarian shielding and transposition might become a beneficial routine in the future, since the risk of miscarriage is not increased when ovaries were shielded (RR = 0.90, $p = 0.88$) (33). Alternatively, ovarian tissue cryopreservation and gonadal suppression using GnRHa remain an experimental option.

The standard FP options for post-pubertal males are sperm cryopreservation and gonadal shielding. The options for obtaining sperm cells are masturbation, TESE, or electroejaculation. Gonadal shielding, in this case for the testes, has been shown to reduce 3–10 fold reduction in radiation dose, which leads to <1% of the patient's prescript dose (34). The procedure may reduce the radiation dose to 0.5Gy in most radiotherapy treatments, which only leads to temporary oligospermia (35). This non-invasive procedure may be useful to be applied as a routine. In addition, nerve-sparing surgery as to not damage the nerves involved in ejaculation is also offered for patients in India (32).

India also offers third option parenting, allowing oocytes donation for infertile females. A 42-year-old woman with bilateral ductal carcinoma of the breast has been able to conceive with full term baby with no congenital anomalies by IVF performed with donor oocyte (36). The male counterpart of third-party sperm donation has never been reported, although this does not seem to be restricted by the Law (37).

FP in India are not covered by insurance. Fortunately, in some cases, tissue storage costs are, covered by in-house funding or grants (24).

CHINA

FP practice in China had been happening prior to the establishment of Chinese Fertility Preservation Society (CFPS) in 2017. CFPS was then founded with the aim of advancing research and clinical practice of FP, as well as enhance public awareness of FP especially oncofertility in China. However, CFPS President admits that there have not been any specific clinical regulations nor guidelines regarding FP in China, which may translate to overlooked FP options when cancer patients are treated. Opportunely, CFPS continues to move forward with its collaboration with international society, namely Oncofertility Professional Engagement Network (38). In addition, a survey among reproductive health professionals in China reveals a positive attitude toward interdisciplinary collaboration despite the lack of knowledge for standard (39). This study shows the continuous effort to provide information about the need and expectation for FP development in China.

In the research field, China has shown several novel findings for FP. The biotechnology advancement has allowed 3D *in vitro* follicle growth and organ-on-a-chip to be applied for ovarian tissue cryopreservation (38). Potentially, an ovary-on-a-chip will be able to further demonstrate the intricate reproductive physiological process as well as the potential toxicological effects of medical treatments (40). Also, Israel collaborates with China as the first to have achieved the initial step with regards to *in-vitro* maturation of spermatocytes. The chemotherapy (busulfan) treated spermatogonial cells shows proliferation and development into sperm-like cells using methylcellulose as a 3D *in-vitro* culture system (41). This will hopefully be a solution for FP in pre-pubertal male patients.

SINGAPORE

Singapore does not have a society for Fertility Preservation. However, the Singapore Hospital (SGH) and several private practices offer FP to the patients. SGH is the national referral center and the oldest and largest tertiary hospital in Singapore. The Center for Assisted Reproduction (CARE) by SGH is a one-stop center offering a full range of assessment services and proven assisted reproduction procedures, to help couples address infertility issues when starting a family including FP options. CARE provides Oocyte, sperm, embryo, and ovarian tissue cryopreservation including gamete, sperm, and embryo donation (42). SingHealth group offers an informative website available for the public regarding medical interventions including FP, but there is no specific section regarding the options (43).

SGH reported that in 2012 a method of FP through cryopreserved ovary has been successful. The 40-year-old woman patient had breast cancer thus the ovary was frozen for 3 years. Three months after the re-transplantation, menstruation resumes indicating resumed ovulation (44).

Singapore is one of the leading developers for novel FP methods. The first pregnancy and live birth resulting from cryopreserved embryos obtained from IVM oocytes after oophorectomy in an ovarian cancer patient achieved in 2013 in Singapore (45). In 2017, one experimental case for FP has also been reported on a pre-pubertal 13-year-old boy with beta-thalassemia major. Nine months following hCG and FSH administration to induce puberty and spermatogenesis, three adequate samples of sperm were obtained and cryopreserved (46). This is the first method that can be offered as an alternative since IVM of sperm from spermatogonial cell lines is not well-established.

With regards to funding, Singaporean government covers 35–75% of the cost of IVF procedure with the cost resides within a range of $6,000–$13,000 (47). However, FP in Singapore is restricted by the law such that it is only allowed based on medical reason (48).

INDONESIA

Indonesia has yet to establish the society for FP regardless of its ongoing practice. Initiatives to create FERTI-protect team in Indonesia has been sounded and is an on-going process. The referral system has also not been well-established. Nonetheless, human tissue from fertility centers can be referred to the FERTI-protect team for further applications. Information toward the patients is mostly managed by private practice that offers FP techniques. Regardless, the FP being offered in one of the centers are all well-established technique (49). It is generally agreed that multi-disciplinary team approach to FP is encouraged. Seminars regarding FP awareness for the experts and health practitioners have also been promoted by the Indonesian Obstetrics and Gynecology Association (POGI).

Researchers and clinicians have published several articles regarding FP in Indonesia. A study regarding the attitudes and knowledge of medical doctors specializing in obstetrics and gynecology found that there is a lack of knowledge about FP

TABLE 1 | Summary of fertility preservation status in Asian countries.

Country	Established FP society	Local guideline	Information for patients*	Referral system	Legal involvement	Insurance coverage
Japan	Yes	Yes	Yes	Yes	Yes	Partial
Korea	Yes	Yes	Yes	No	No	Partial
India	Yes	No	Yes	No	No	No
China	Yes	No	No	No	Yes	No
Singapore	Yes	No	No	No	No	No
Indonesia	No	No	No	No	Yes	No

**Considers the availability of official website that openly provides FP information for patients.*

options, leading to sub-optimal provision of information to patients (50). Most participants only felt knowledgeable about pre-treatment with GnRH Agonists. The practitioners seem to be willing to provide FP with 92.5% agreed that FP is a high priority to discuss with newly diagnosed cancer patients, and 80% suggested FP for their patients, although 45% agreed that treating the primary cancer was more important than FP. With regards to referring the patients, only 35% reported having referred patients to a fertility specialist, and 15.1% provided patients with written information.

Among the patients with Turner Syndrome, fertility issue is more concerning than health and social issues. There seem to be a very high expectation for preserving fertility among these patients (85%) and they would like to undergo fertility treatment (97.5%) (51). This expectation, however, is challenged by their lack of information, fear of complication, and the cost of fertility treatment.

As a religious country, third party parenting is not allowed in Indonesia. The universal health coverage offered by the government does not include FP options and insurance companies also do not cover the cost. The main barriers to for offering discussions regarding FP in Indonesia were poor success rates (97.5%), affordability (93.8%), poor prognosis (92.6%), lack of obstetrician and gynecologists' knowledge (91.3%), and lack of fertility services in their area (81.3%) (50).

OTHER ASIAN COUNTRIES

FP status in some Asian countries has been discussed (**Table 1**). Several other countries in Asia, for instance, Malaysia, Vietnam, and Iran just to name a few, face similar condition as in Indonesia. The practice of FP has been done by the experts although there is no society of FP in the country. As expected, a survey in Iran found that only 15% of all parents reported that they are aware of the danger of cancer treatment on fertility and only one-third of these patients received the knowledge from the treating physicians. Furthermore, the survey also found that despite a small percentage of success, many parents would still prefer to try FP for their sons (52). This situation that is surely detrimental for the patients' quality of life might also be applicable to other Asian countries.

Practitioners in these countries might also face legal and ethical challenges. With no guideline that addresses the legal and cultural aspect, FP practice is bound to face some issues. In Israel, where there is no direct policy in the issue, existing guidelines are often vague and ignored by the physicians. Furthermore, roughly half of the physicians are willing to perform more innovative procedures if backed by official guidelines (53). The lack of a support system for practitioners in several Asian countries will ultimately lead to poor conduct of FP and might be detrimental for the patients.

CONCLUSION

FP is a highly demanded field and may improve the quality of life among patients. Further research should be conducted to explore new methods. Ideally, FP society should be established to promote a multi-disciplinary approach between practitioners, produce policies, and promote referral system at the very least for the benefit of the patients.

AUTHOR CONTRIBUTIONS

AH and VS write and prepare the manuscript. MM and BW supervised manuscript writing.

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REVIEW
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The Role of microRNAs in Ovarian Granulosa Cells in Health and Disease

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The granulosa cell (GC) is a critical somatic component of the ovary. It is essential for follicle development by supporting the developing oocyte, proliferating and producing sex steroids and disparate growth factors. Knowledge of the GC's function in normal ovarian development and function, and reproductive disorders, such as polycystic ovary syndrome (PCOS) and premature ovarian failure (POF), is largely acquired through clinical studies and preclinical animal models. Recently, microRNAs have been recognized to play important regulatory roles in GC pathophysiology. Here, we examine the recent findings on the role of miRNAs in the GC, including four related signaling pathways (Transforming growth factor-β pathway, Follicle-stimulating hormones pathway, hormone-related miRNAs, Apoptosis-related pathways) and relevant diseases. Therefore, miRNAs appear to be important regulators of GC function in both physiological and pathological conditions. We suggest that targeting specific microRNAs is a potential therapeutic option for treating ovary-related diseases, such as PCOS, POF, and GCT.

Keywords: miRNA, granulosa cells, PCOS (polycystic ovary syndrome), POF, GCT

INTRODUCTION

The granulosa cell (GC) is a type of somatic cell arising from the sex cord in the ovary (1). During folliculogenesis, GCs develop from a thin, one-cell thick, layer around the oocyte in the primordial follicle to the multilayered cumulus oophorus surrounding the oocyte in the dominant follicle, that also has huge numbers of synthetically active mural GCs cells on its inner wall. The transition from primordial follicles to mature follicles involves differentiation and functional transformation of GCs. The interaction between GCs and oocytes is critical for coordinated oocyte maturation (2). Therefore, investigating the molecular pathways involved in proliferation, differentiation and functional transformation of GCs will lay a solid foundation for the mechanistic understanding of folliculogenesis (3).

GCs not only play a critical role in normal folliculogenesis, but also in pathological folliculogenesis in both benign disorders, such as polycystic ovary syndrome (PCOS) (4), premature ovarian failure (POF), which is also referred to as premature ovarian insufficiency, (POI) (5) and malignant disease such as ovarian GC tumors (GCT) (6). PCOS is a complex, multifactorial endocrine disorder affecting ∼10% of all women of reproductive age (7). It is associated with multiple small antral follicles within the ovary that fail to develop into larger dominant follicles. GC function is different in polycystic ovaries. Increased follicle number and GC proliferation are observed in murine PCOS models (6). In addition, upregulated GC proliferation in smaller follicles is also observed in the ovaries of PCOS women (7, 8). Therefore, it is likely that abnormal ovarian GC proliferation has a role in the pathogenesis of PCOS. However, the underlying mechanism remains largely unclear. POF, with increased gonadotropin concentrations and hypoestrogenism, occurs in up to 4% of women under the age of 45 and it is associated with anovulation and infertility. The apoptosis of GC causes ovarian atresia and might eventually leads to POF. Therefore, the GC dysfunction is a main pathological feature of POF (8–12). The GC is the cell of origin in the pathogenesis of GCT (6), which is a clinically and molecularly unique subtype of ovarian cancer. GCT stems from the sex cord stromal cells of the ovary and represents ∼5% of all ovarian cancers. While the etiologies of PCOS, POF, and GCT are unclear it is likely that both genetic and epigenetic factors may contribute to their pathogenesis (13–15), suggesting a role for differential gene function in these conditions. Genetic factors including, for example, critical genes for steroidogenesis and hormonal regulation and action, have been well-studied in these conditions. Additionally, in recent years, many noncoding portions of the genome have been recognized as being able to influence the epigenome with potential effects on the phenotypes of PCOS, POF, and GCT.

MicroRNA (miRNA) is a class of small noncoding RNAs (16). They are estimated to regulate mRNA translation of more than 70% of the protein-coding genes and are widely involved in both the normal and diseased states (17). Biogenesis of miRNAs is divided into several steps. Firstly, miRNAs genes are transcribed to primary miRNA (pri-miRNA) in the nucleus. Next, Drosha (a RNAase III enzyme) processes pri-miRNA to miRNA precursor (pre-miRNA). Following that, exportin-5 mediates the transport of pre-miRNA from nucleus to cytoplasm. Lastly, pre-miRNA is modified by Dicer to form mature miRNA, which forms the RNA-induced silencing complex (RISC) with Argonaute proteins. The complex searches for specific sequences of mRNA via the "seed sequence," thus suppressing the translation or changing the stability of the target mRNAs (18). The role of miRNAs in normal development (19–22) and follicle pathogenesis (23, 24) has been summarized in previous reviews. Here, we focus on the recent findings regarding the roles of miRNAs in normal GCs and in the GCs of pathological conditions (PCOS, POF, and GCT). An in-depth understanding of miRNAs will provide valuable insights into the mechanism of normal GC development and the etiology and pathophysiology of these diseases with different GC phenotypes. In addition, the limitations and persistent issues that necessitate further investigation in the related areas are also discussed.

THE ROLE OF DICER IN GC

As mentioned above, Dicer is an evolutionarily conserved ribonuclease III that is necessary for miRNA biogenesis. However, the specific functions of Dicer in the GC of the female reproductive system are unknown. Therefore, investigating the loss-of-function of Dicer in GC provides insight into the role of miRNAs in GC.

Otsuka et al. (25) firstly demonstrated that reduction of Dicer expression by a hypomorphic mutation causes infertility due to defects in ovarian angiogenesis and corpus luteum insufficiency. The terminal differentiation of GCs into luteinized GCs is known to regulate ovarian angiogenesis and normal corpus luteum function. In addition, *in vivo* ectopic expression of two microRNAs (miR-17-5p and let-7b) allows partial vascular recovery in the corpus luteum of these Dicer hypomorphic mice, suggesting that these effects are indeed miRNA-dependent. Other ovarian functions, including folliculogenesis, oocyte maturation, and ovulation, are not affected (25). However, this mouse model is not a GC specific knockout of Dicer, just a global effect of Dicer deficiency.

A possible role for miRNAs in the ovary is demonstrated by conditional knockout (cKO) of Dicer 1 in GC using the anti-Müllerian hormone (AMH) receptor type 2 promoter-driven expression of Cre recombinase (26). Therefore, two groups used this GC specific knockout of Dicer model and both showed that Dicer knockout in GC lead to female sterility. Nagaraja et al. (27) found that Dicer knockout in GC not only cause female sterility, but also induce multiple reproductive defects including decreased ovulation rates, compromised oocyte and embryo integrity, prominent bilateral paratubal cysts, and shorter uterine horns. MiRNA sequencing revealed differential expression of specific miRNAs in Dicer cKO mice. The majority of these miRNAs are predicted to regulate genes important for Mullerian duct differentiation and mesenchyme-derived structures, and several of these putative target genes were greatly affected upon Dicer cKO (27).

Second group also showed that adult female GC-specific Dicer KO mice display female sterility. Morphological and histological assessments of the reproductive tracts of immature and adult mice indicated that the uterus and oviduct were hypotrophic, and the oviduct was highly disorganized. Oviductal transport was disrupted in the GC-specific Dicer KO mice as evidenced by the failure of embryos to enter the uterus. These studies implicate Dicer/miRNA mediated posttranscriptional gene regulation in reproductive somatic tissues as critical for the normal development and function of these tissues and for female fertility (28).

Another group also used this GC specific knockout of Dicer mouse, but they focused on the regulatory role of Dicer in folliculogenesis. Lei et al. (29) demonstrated that the specific deletion of Dicer in GC led to an increased primordial follicle pool endowment, accelerated early follicle

recruitment and resulted in more degenerate follicles. In addition, significant differences were observed in the expression of some follicle development related genes between cKO and WT mouse ovaries, such as Amh, Inhba, Cyp17a1, Cyp19a1, Zps, Gdf9, and Bmp15. With the Dicer inactivation, miR-503, a miRNA that is more abundant in ovary than in other tissues, was significantly decreased. Meanwhile, the expression of miR-503 decreased notably with follicle development in the gonadotropin-primed mouse ovary. Overexpression of miR-503 in primary GC resulted in the decreased expression of genes that related to GC proliferation and luteinization. Therefore, Dicer plays essential roles in follicular cell development through the differential regulation of expression of miRNA and target genes (29).

These studies vary but overall show that Dicer in GC is fundamental for normal ovarian function and female fertility. Since Dicer is responsible for the synthesis of mature miRNAs, it is important to identify the key miRNAs that are essential for GC function (**Figure 1**).

PHYSIOLOGICAL CONDITIONS

Transforming Growth Factor (TGF)-β (TGFB) Pathway

TGFB signaling plays an important role in reproduction. TGFB receptor 1 and 2 (types I and II) are membrane-bound serine/threonine kinase receptors that upon complexing with TGFB, activate SMAD 2/3 intracellular signaling by phosphorylation. Phosphorylated SMAD2/3 binds SMAD4 and translocates to the nucleus to regulate transcription of many downstream genes. Up-regulation of TGFB signaling represses GC apoptosis. In contrast, repressed TGFB signaling induces GC apoptosis, suggesting that TGFB signaling regulates GC survival (30, 31). However, the mechanism underlying TGFB signaling for GC survival/apoptosis has not been fully elucidated. TGFB pathway regulates a bunch of miRNAs in GC (32). In addition, emerging evidence shows that miRNAs might play a role in the regulation of the TGFB signaling pathway (**Figure 2**). The interaction of miRNA-TGFB pathway in GC seems to mainly dysregulate the normal growth of GCs.

Du et al. (33) show that the TGFB pathway controls ovarian porcine GC apoptosis through the SMAD4/miR-143 axis. Specifically, miR-143 enhances porcine GC apoptosis by regulating two direct targets, the FSH receptor (FSHR) and SMAD4. SMAD4 binds to the miR-143 promoter to form a feedback loop between the TGFB pathway and miR-143. Activated TGFB signaling rescues miR-143-reduced FSHR and intracellular signaling molecules, and miR-143-induced GC apoptosis. MiRNA-424/503 cluster members are involved in modulating bovine GC proliferation and cell cycle progression through targeting SMAD7 (a blocker of TGFB pathway) and consequently inducing phosphorylation of SMAD2/3 in GC (34). TGFB1 induces lysyl oxidase expression via repressing miR-29a, and lysyl oxidase is proven to be a direct target of miR-29a in human GCs (35). MiRNA let-7g promotes porcine GC apoptosis by targeting the TGFB1 receptor and repressing phospho-SMAD3. Therefore, let-7g blocks the TGFB pathway in GC (31). In addition, TGFB1 represses miR-383, which consequently inhibits another miRNA (miR-320) in murine GCs (36). The same research group further validated miR-383 as a downstream target of the TGFB pathway via direct repression of RNA-binding morif single-stranded-interacting protein (RBMS) 1 (37). The relationship between TGFB and miR-224 has also been investigated. Yao et al. (38) demonstrate that ectopic expression of miR-224 stimulates TGFB1-induced mouse GC proliferation through targeting SMAD4. This is the first demonstration that miRNAs could control reproductive functions by promoting TGFB1-induced GC proliferation. The same group further demonstrated that transcriptional cooperation between p53, p65, and TGFB regulates miRNA-224 transcription in murine GCs (39). Taken together, these studies support and further expands the knowledge of the powerful interaction between miRNAs and TGFB pathways in GC. It is reasonable to conclude that these miRNAs potentially regulate some essential cellular feature of GC, such as proliferation and apoptosis, via communication with the TGFB signaling pathway.

Follicle-Stimulating Hormone (FSH) Pathway

In ovaries, Follicle-stimulating hormone receptor (FSHR) is exclusively expressed in GCs (40). It is essential for their proliferation, growth, function, and differentiation (41). The ovarian follicle is characterized by two layers of GCs with different features; the cumulus GCs surrounding the oocyte and the mural GCs peripheral to the antrum (42). Treatment with FSH can induce the expression of more than 100 genes in GCs (43–46). FSH-induced regulation of the TGFB pathway has been demonstrated in GCs of different mammalian species, such as humans (47), mice (48), rats (49), and cows (50). On the other hand, TGFB signaling cooperates with Forkhead box L2 (FOXL2) to regulate FSHR and consequently affects GC functions, such as normal proliferation and differentiation, in pre-hierarchical follicles. During folliculogenesis FOXL2 plays a bidirectional modulating role involving intracellular FSHR transcription and GC proliferation via an autocrine regulatory mechanism, in a positive or negative manner, in cooperation with members of the TGFB superfamily; activin A (and its binding protein, follistatin) and GDF9 (51). MiRNAs can regulate GC proliferation, angiogenesis, hormone secretion and differentiation via interacting with the FSH pathway in GCs (**Figure 3**).

Shukla et al. (52) showed that FSH induced miR-210 expression in GC, and miRNA-210 consequently regulates pre-ovulatory GC proliferation and angiogenesis by repressing H-Ras and ephrin-A3. Zhang et al. (53) demonstrated that the FSH/miR-143/KRAS regulatory axis pathway plays an important role in regulating proliferation and estradiol secretion in murine GCs. In addition, the TGFB and FSH pathways interact via the miR-143/FSHR axis (33), highlighting the central role of miR-143 in GCs. Our group has found that two members in miR-10 family, miR-10a and miR-10b, are repressed by FSH in human, mouse,

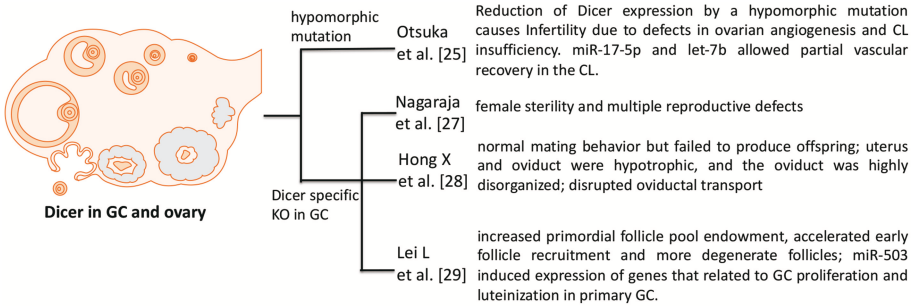


FIGURE 1 | Phenotypes in Dicer KO ovary. Four studies investigate the role of Dicer in GC and ovary. Otsuka et al. (25) use a Dicer hypomorphic mutation mouse. Other three papers [Nagaraja et al. (27), Hong et al. (28), and Lei et al. (29)] show that specific deletion of Dicer in GC hampers the normal function and development of folliculogenesis in ovary.

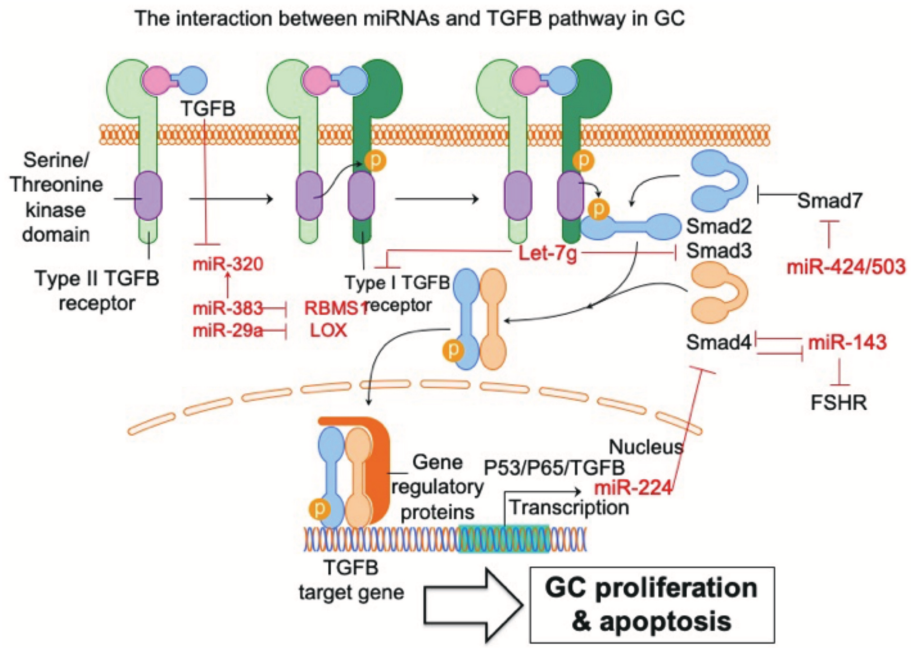


FIGURE 2 | The interaction between miRNAs and TGFB pathway in GC. The interaction of miRNA-TGFB pathway in GC mainly affects the growth and survival of GC in ovary. FSHR, follicle-stimulating hormone receptor; SMAD3, SMAD family member 3; SMAD4, SMAD family member 4; SMAD7, SMAD family member 7; LOX, lysyl oxidase; RBMS1, RNA-binding morif single-stranded-interacting protein 1. Arrowhead line, promotion; Flat-end line, repression.

and rat GCs (54), suggesting a conserved role of miR-10 family in GC function. Indeed, we showed that both miR-10a and miR-10b could disturb normal development of GCs and folliculogenesis in different species. Overexpression of androgen receptors (AR) in GCs increased miR-126* (miR-126* is an old designation, it is designated as miR-125-5p now) and decreased FSHR expression. The FSHR gene is a direct target of miR-126*, which inhibits FSHR expression and increases the rate of AR-induced apoptosis. Therefore, AR and miR-126* may cooperate to inhibit FSHR expression and induce apoptosis in GC (55). As GCs differentiate, they also express the luteinizing hormone/chorionic gonadotropin receptor (LHCGR). The regulatory role of miR-122 in FSH-induced LHCGR expression during follicle development was examined by Menon et al. (56). MiR-122 could completely abrogate FSH-mediated upregulation of LHCGR. MiR-122 also blocks the FSH-induced decrease in luteinizing hormone receptor mRNA-binding protein (LRBP) expression and increases the binding of LHCGR mRNA to LRBP. A transcription factor, Sterol Regulatory Element Binding Protein (SREBP-1a and SREBP-2 isoforms), is an intermediate in miR-122-mediated LHCGR mRNA regulation (57, 58). Therefore, miR-122 plays a regulatory role in LH/hCG-induced LHCGR mRNA downregulation by increasing LRBP expression through the activation of SREBP pathway.

FSH pathway has been implicated in modulating several key cellular functions of GC, such as proliferation, differentiation, and steroidogenesis, which are essential for the female reproductive system. Related studies have proven that these miRNAs are under control of FSH pathway in GC, including miR-10a/b, miR-143, miR-126*, miR-122, and miR-210. The

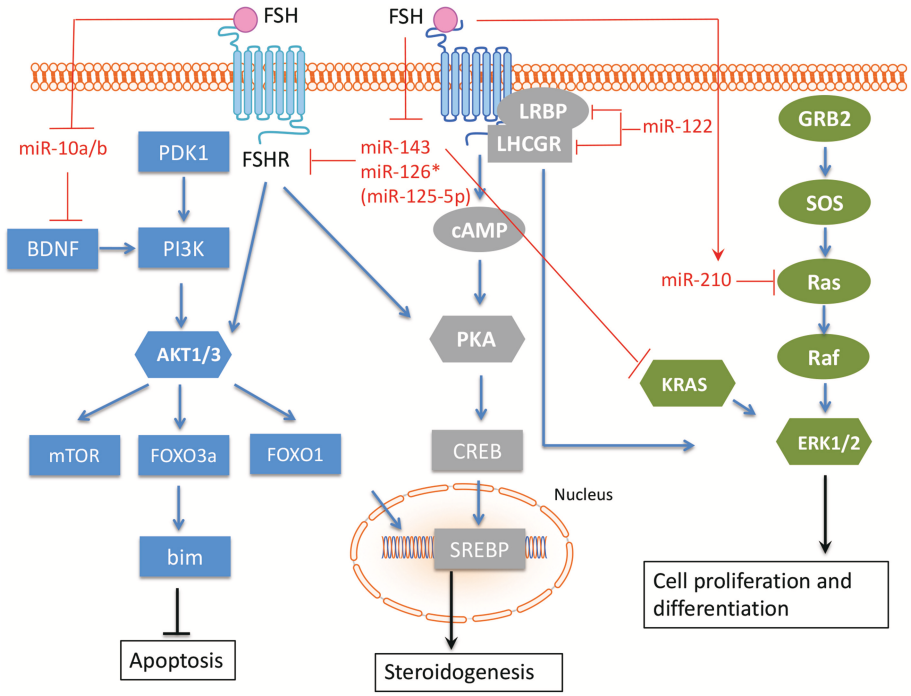


FIGURE 3 | The interaction between miRNAs and FSH pathway in GC. The related miRNAs regulate GC proliferation, angiogenesis, hormone secretion and differentiation via interacting with FSH pathway in GC. FSH, Follicle-stimulating hormone; FSHR, Follicle-stimulating hormone receptor; BDNF, Brain-derived neurotrophic factor; KRAS, V-Ki-Ras2 Kirsten Rat Sarcoma 2 Viral Oncogene Homolog; Ras, rat sarcoma viral oncogene homolog; LHCGR, luteinizing hormone/chorionic gonadotropin receptor; LRBP, luteinizing hormone receptor mRNA-binding protein. Arrowhead line, promotion; Flat-end line, repression.

miRNA-FSH pathway network in GC, especially the interaction among different miRNAs, should be further studied in the future. The interaction between above-mentioned miRNAs and the FSH pathway in GC is summarized in **Figure 3**.

Hormone-Related Pathways

The differentiation process from primordial germ cells to fertilizable oocytes takes place within follicles (59). During this process, the key proteins required for oocyte maturation are progressively synthesized (60). This complicated follicular development is precisely modulated by different signals from different cells, including oocyte and the surrounding somatic GCs (61), in addition to the complex interactions between gonadotropin hormones, sex steroids, and other diverse growth factors (62). During folliculogenesis, these hormones, steroids, and growth factors produced by GCs also affect the process of follicular growth and GC development and function (63). Their differential effects on folliculogenesis is likely to be mediated by GCs and involve changes in GC proliferation, apoptosis, and hormone secretion (64). Here, we summarize the miRNAs that affect hormone secretion in GCs (**Table 1**).

MiR-144 represses prostaglandin E2, an important intrafollicular paracrine regulator with particular roles during ovulation, by inhibiting cyclooxygenase-2 expression (65). The corticotropin-releasing hormone signaling system is involved in numerous stress-related physiological and pathological responses, including inhibiting estradiol synthesis and follicular development in the ovary. MiR-375 mediates the corticotropin-releasing hormone signaling pathway and inhibits follicular estradiol synthesis (66). MiR-96 targets Forkhead box protein O1 (FOXO1) to induce steroid production in human GCs (67). MiR-764-3p regulates 17β-estradiol synthesis by repressing an essential transcriptional factor, steroidogenic factor-1 (SF-1), in mouse GCs (68). MiR-378 regulates ovarian estradiol production by targeting aromatase in porcine GCs (69). MiR-320 regulates steroid production by targeting E2F1 and SF-1 during follicular development (36). MiRNA-133b stimulates ovarian estradiol

TABLE 1 | GC secretes hormones related miRNAs.

miRNA	Hormones	Regulation	Target	Species	References
miR-144	Prostaglandin E2 (PGE2)	Suppression	COX-2	Mouse	(65)
miR-375	Estradiol	Suppression	CRH	Porcine	(66)
miR-96	Steroid	Promotion	FOXO1	Human	(67)
miR-764	Estradiol	Suppression	SF-1	Mouse	(68)
miR-378	Estradiol	Suppression	Aromatase	Porcine	(69)
miR-320	Steroid	Promotion	E2F1 and SF-1	Mouse	(36)
miR-133b	Estradiol	Promotion	FOXL2	Mouse/human	(70)
miR-224	Estradiol	Suppression	SMAD4	Mouse	(39)
miR-383	Estradiol	Promotion	RBMS1	Mouse	(37)

synthesis by targeting Foxl2 (70). MiR-224 is involved in TGFB1-mediated GC growth and estradiol production by targeting SMAD4 (39). Transactivation of miRNA-383 by SF-1 promotes estradiol release from mouse GCs by targeting RBMS1 (37). As mentioned before, several GC related hormones are essential for normal folliculogenesis and other associated diseases. Therefore, these miRNAs require more of our attention as they could regulate the secretion of related hormones.

GC is the main resource for synthesis and production of related hormones, such as prostaglandin E2, estradiol, steroid, for local use and providing endocrine signaling to other cells within ovary. Therefore, to investigate the role of those miRNAs that could regulate hormones synthesis in GC is essential for a lot of important functions in ovary, including follicular development, cumulus cell expansion, luteinization, ultimately oocyte maturation, and ovulation.

Apoptosis-Related Pathways

Ovarian follicle atresia is mediated via apoptosis in higher vertebrates (71, 72). In most instances, GC are the first group of cells to show the features of apoptosis. Although follicle atresia is often considered as a normal physiological function to ensure the highest chance for ovulation of an appropriate number of healthy fertilizable oocytes, exceptional atresia leads to chronic infertility or menopause (73). Most research to investigate the molecular mechanism of GC apoptosis have been conducted in preclinical mammalian model systems (74). A series of studies have been shown that several miRNAs play essential roles in GC apoptosis. TGFB pathway, known to be regulated by miRNAs, affects apoptosis in GCs and the associated miRNAs have been summarized previously. Apart from the TGFB pathway, miRNAs can regulate GC apoptosis by targeting several other genes (75). Evidence for miRNAs that regulate GC apoptosis is shown in **Figure 4** and discussed below.

The function of the miRNAs on apoptosis of GC can be divided into two groups: positively regulated apoptotic miRNAs and negatively regulated apoptotic miRNAs. On one hand, several miRNAs have been proven as apoptosis promoter of GC. MiR-4110 induces GC apoptosis via targeting SMAD2, and the ratio of an apoptosis index, Bcl-2-associated X protein (BAX)/B-cell CLL/Lyphoma 2 (BCL2), is also up-regulated in miR-4110 overexpressing GCs. Thus, MiR-4110 promotes GC apoptosis by targeting SMAD2 in the ovary (76). MiR-23a and miR-27a promotes apoptosis in human GC by targeting SMAD5 in the Fas ligand (FasL)-Fas signaling pathway (77). Anti-apoptotic genes BCL-2 and myeloid cell leukemia sequence (MCL) 1 are repressed by let-7g in GCs. Let-7g induces the expression of FOXO1 in GCs and leads to nuclear accumulation of dephosphorylated FoxO1, as a consequence of repression of the expression of Mitogen-activated protein kinase 1 (MAP3K1) in GC. Let-7g has been shown to induce GC apoptosis by targeting MAP3K1 in the porcine ovary (78). Inducers of oxidative stress, hydrogen peroxide (H2O2) and 3-nitropropionic acid (NP), induced miR-181a expression in GC *in vitro* and *in vivo*. Ectopic and knockdown of miR-181a promoted and repressed GC apoptosis, respectively. SIRT1 was also identified as a direct target of miR-181a and mediated the effects of miR-181a on GC apoptosis (79).

On the other hand, there are also some miRNAs that protect GC from apoptosis. Some cytokines and growth factors, such as Tumor necrosis factor-α (TNF-α), Fas ligand (FasL) and nerve growth factor (NGF) are specific inducers of apoptosis by affecting oxidative stress in GC (80). GC-specific miR-145 over-expression attenuates apoptosis in the *in vivo* ovarian oxidative stress model promoted by targeting Kruppel-like factor 4 (KLF4). Therefore, MiR-145 protects GC against oxidative stress-induced apoptosis via repressing KLF4 (81). In addition, miR-22 increases during follicular atresia and suppresses GC apoptosis. Further investigation showed that miR-22 inhibits mouse ovarian GC apoptosis by targeting NAD-dependent deacetylase sirtuin-1 (SIRT1) (82).

In addition, preantral and antral follicles involve several cell death pathways. Antral follicular degeneration stem from GC apoptosis, while preantral follicular atresia is mainly initiated by upregulated GC autophagy (24, 83). A series of publications reported the essential role of miRNAs in modulating autophagy (84). However, there is only one publication about miRNA on GC autophagy until now (85). This recent report showed that let-7g induced autophagy facilitate mouse GC apoptosis by targeting insulin like growth factor 1 (IGF-1). Therefore, further investigation definitively should focus on the effect of miRNAs on GC autophagy.

Taken together, these miRNA-mRNA interactions modulate GC apoptosis during the process of follicle atresia. Specifically, more attention should be paid to the two groups of miRNAs (promoter or suppressor of GC apoptosis) in GC apoptotic related disorders, such as POF and PCOS.

PATHOLOGICAL CONDITIONS

PCOS

The definition of PCOS is based on criteria from three different organizations: the National Institutes of Health (NIH) (86), the European Society of Human Reproduction and Embryology/American Society for Reproductive Medicine (Rotterdam) (87), and the Androgen Excess Society (88). The prevalence of PCOS in women of reproductive age varies from ∼7% (NIH criteria) to up to 20% (the Rotterdam criteria) (7, 89). Polycystic ovaries, anovulation, and hyperandrogenism are the main features of PCOS according to all three criteria. In addition, PCOS is closely associated with many other pathological conditions, such as infertility, obesity, type 2 diabetes, dyslipidemia, insulin resistance and hypertension. There is evidence for differential GC function in PCOS and it is useful to summarize the differentially expressed functional miRNAs in GCs to determine potential therapeutic targets (**Figure 5**).

MiR-93 and miR-21 are increased in GCs from hyperandrogenic (HA) PCOS patients compared to Normo-androgenic (NA) patients. Free testosterone and free androgen index are positively correlated with of miR-93 and miR-21 in PCOS GCs. Androgens are fundamental in the pathophysiology of PCOS and androgens have effect on follicle growth, health and survival. As miR-93 and miR-21 have been highlighted as androgen responsive factors they may play a role in the follicular dysfunction involved in the pathogenesis of PCOS in

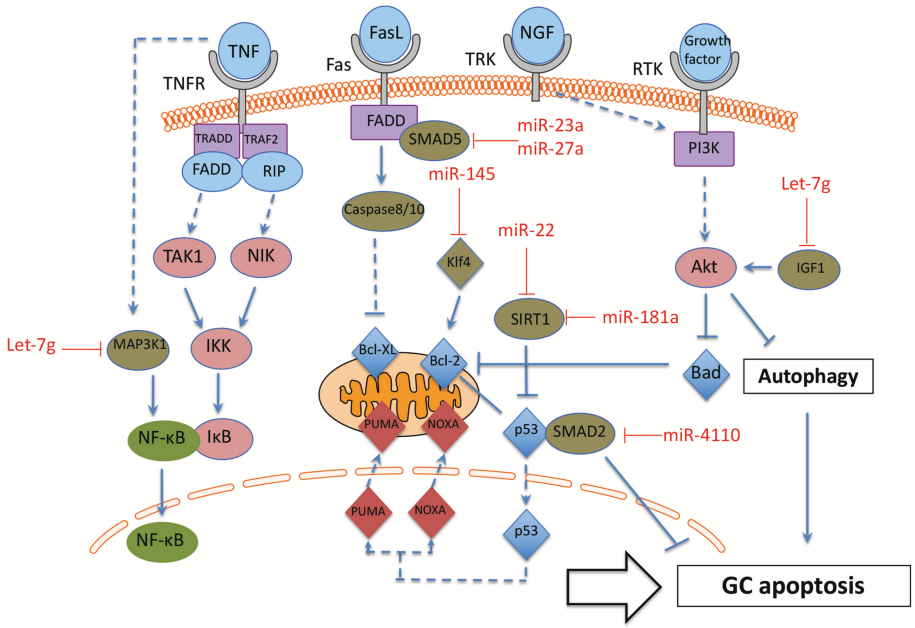


FIGURE 4 | The interaction between miRNAs and apoptosis pathway in GC. These miRNA-mRNA communications could greatly modulate GC apoptosis during the process of follicle atresia. MAP3K1, Mitogen-activated protein kinase 1; Klf4, Kruppel-like factor 4; SMAD2, SMAD family member 2; SMAD5, SMAD family member 5; SIRT1, NAD-dependent deacetylase sirtuin-1; IGF1, insulin like growth factor 1. Arrowhead line, promotion; Flat-end line, repression.

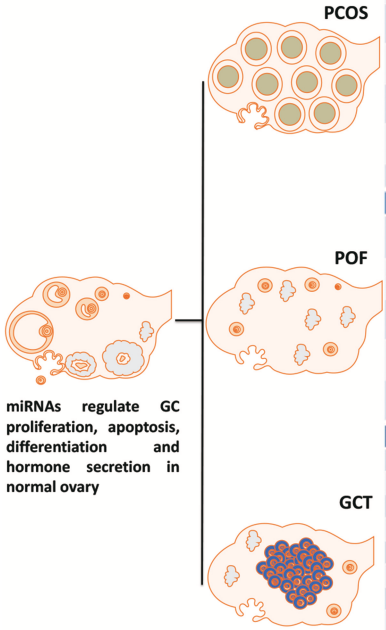
hyperandrogenic condition (90). MiR-320a decreases in GCs from PCOS patients and this down-regulation is thought to cause relative estrogen deficiency. IGF1 regulated miR-320a in GCs, and miR-320a potentiates the steroidogenesis in CCs through modulation of cytochrome P450, family 11, subfamily a polypeptide 1 (CYP11A1) and cytochrome P450, family 19, subfamily a polypeptide 1 (CYP19A1), by directly targeting the osteogenic transcription factor Runt-related transcription factor 2 (RUNX2) (91).

Another miRNA, miR-27a-3p, also represses CYP19A1 via targeting cyclic AMP response element (CRE)-binding protein 1 (Creb1) in mouse GC from PCOS mice model. MiR-27a-3p induces apoptosis and decreases expressions of estradiol, aromatase and testosterone in mouse GC. The effects of miRN-27a-3p on the dysfunction of related hormones and apoptosis of GC could be involved in the PCOS pathophysiology (92). MiR-145 negatively regulates cell proliferation through targeting Insulin receptor substrate 1 (IRS1) in isolated ovarian GCs from PCOS patients. MiR-145 represses the activation of p38 mitogen-activated protein kinase (p38 MAPK) and extracellular signal-regulated kinase (ERK) and IRS1 rescues the suppressive effect of miR-145 on MAPK/ERK signaling pathways (93). "MiRNAome" and transcriptome in GCs from five PCOS and five control patients have been determined by a miRNA and cDNA microarray. The differentially expressed miRNA-509-3p and its potential target gene Mitogen-activated protein kinase 8 (MAP3K8) were identified from the miRNA and cDNA microarrays, respectively. MiRNA-509-3p promotes estradiol secretion by targeting MAP3K8 in GCs from PCOS patients (94). Repressed miR-483 expression is observed in the ovarian cortex from PCOS patients. MiR-483 is down-regulated in PCOS and inhibits GC proliferation via targeting IGF1 (95). MiR-483–5p and miR-486–5p are down-regulated in the cumulus GCs surrounding metaphase II oocytes taken from women with PCOS. MiR-486–5p promotes the proliferation of PCOS GCs via inducing PI3K/Akt pathway (96). MiR-93 expression is up-regulated in PCOS GCs and its predicted target, cyclin dependent kinase inhibitor 1A (CDKN1A), is repressed in PCOS GCs. MiR-93 induces GC proliferation and G1 to S transition. Inhibition of CDKN1A shows the similar cellular phenotype in GC. Therefore, miR-93 promotes the proliferation of ovarian GC through targeting CDKN1A in PCOS (97).

In summary, there is evidence for dysregulation of miRNAs in the GCs of women with PCOS with plausible actions that can be associated with the GC dysfunction in PCOS. That suggests that miRNA manipulation may have a plausible role in improving follicular health in women with PCOS. The above-mentioned miRNAs in GC may provide new insights into pathophysiology of PCOS.

POF

POF is a reproductive disorder with significant health complications (98), including disorders of the genital tract, cognitive dysfunction, and cardiovascular related diseases (99). It is associated with a markedly reduced number of follicles in the ovary, which when present show some features consistent with GC dysfunction and it is likely that GC dysfunction can increase the likelihood of POF [6]. Therefore, summarizing miRNAs that could regulate GC dysfunction in POF may allow novel



miRNA	Function in PCOS GC	target	Ref
miR-21 & miR-93	Increase in GC from hyperandrogenic PCOS patients	Not mentioned	80
miR-320a	miR-320a/RUNX2/CYP11A1 cascade plays an important role in the development of estrogen deficiency in CC	RUNX2	81
miR-145	MiR-145 Negatively Regulates Cell Proliferation in GC From PCOS Patients	IRS1	82
miR-509-3p	promotes oestradiol in GC from PCOS patients	MAP3K8	83
miR-483	miR-483 is Down-Regulated in PCOS and Inhibits GC Proliferation	IGF1	84
miR-483–5p and miR-486–5p	down-regulated in GC of metaphase II oocytes from women with PCOS	Not mentioned	85
miR-93	promotes ovarian GC proliferation in PCOS	CDKN1A	86

miRNA	Function in POF GC	target	Ref
miR-210	regulate pre-ovulatory during follicular-luteal transition	*HRas* and *EFNA3*	49
miR-146a/ 196a2 SNP	associate with some primary ovarian insufficiency related-gene regulation in GC	Not mentioned	89
miR-938 G>A Polymorphisms	associate with primary ovarian insufficiency-related gene expression in GC	Not mentioned	90
miR-146a	promote GC apoptosis in POF via the caspase cascade pathway	IRAK1 and TRAF6	91
miR-139	Hyaluronic acid rescues GC apoptosis via regulating miR-139 in POF.	PGRMC1	92

miRNA	Normal GC	JGCT	AGCT
miR-138	low	low	high
miR-184	low	low	high
miR-203	low	low	high
miR-29c	low	low	high
miR-328	low	low	high
miR-501	low	low	high
miR-15	low	middle	high
miR-16	low	middle	high
miR-21	low	middle	high
miR-21*	low	middle	high
miR-184	Low miR-184 is correlated with tumorrecurrence in early stage AGCT patients.		

FIGURE 5 | The effect of miRNAs on GC from PCOS, POF and GCT. These miRNAs are potential markers or targets for diagnostic, therapeutic, or prognostic treatment of PCOS, POF, or GCT.

paradigms to improve GC health and slow the loss of follicles in POF patients (**Figure 5**).

The differential expression of miRNA-210 during the follicular-luteal transition regulates pre-ovulatory functions by targeting HRas and Ephrin A3 (EFNA3) (52). Single nucleotide polymorphisms of miR-146a/196a2, and their POI-related target genes, regulate GCs (100). In addition, there is an association of miR-938 G>A polymorphisms in GC with POI-related gene expression (101). MiR-146a has an important promoting effect on GC apoptosis by targeting interleukin-1 receptor associated kinase 1 (IRAK1) and tumor necrosis factor receptor-associated factor 6 (TRAF6) via the caspase cascade pathway (102). Hyaluronic acid is involved in promoting progesterone receptor membrane component 1 (PGRMC1) expression by regulating miR-139, which may be dysregulated in POF (103). Further studies are required for the potential application of these miRNAs as diagnostic, therapeutic, or prognostic markers of POF or even therapeutic targeting in the management of POF.

GCT

Current histopathological and genetic markers, such as FOXL2 mutations, to distinguish between the two major subtypes, adult GCT (AGCT) and juvenile GCT (JGCT), are not accurate. The potential clinical utility of miRNAs as markers of GCT for tumor diagnosis and prognosis was evaluated by Cheng et al. (15). MiRNA-array results demonstrated that 37 miRNAs are differentially expressed between AGCT and JGCT. Six miRNAs, including miRs-138-5p,-184,-204-5p,-29c-3p,-328-3p, and -501-3p, were validated by RT-qPCR. Moreover, miR-184 was a potential predictor of tumor recurrence in AGCT, specifically for patients diagnosed with stage I and II and stage I only disease. It is the first report to profile miRNAome of human GCT. The role of these miRNAs in granulosa cell tumors is not yet understood and further studies are required to validate the clinical use of these miRNAs as diagnostic and recurrence markers as well as targets for manipulation.

CONCLUSION AND PROSPECTS

This review describes miRNAs as a group of key posttranscriptional regulators in GCs of both physiological and pathological conditions. A single miRNA could repress hundreds, even thousands of genes, and a single gene could be modulated by multiple miRNAs. Many miRNAs are expressed in GC and directly regulate normal development and function of ovarian follicles (16), including atresia, ovulation, and ovarian

steroidogenesis by targeting specific molecules and modulating various signaling pathways, such as TGFB-, FSH-, hormone-, and apoptosis-related pathways. In addition, miRNAs also play important roles by affecting GC in female reproductive diseases, such as PCOS, POF, and GCT. Systematically identifying miRNAs specific to GC will help researchers to better understand the underlying mechanisms relevant to ovarian disorders. For example, miR-320 affects steroidogenesis in both physiological (folliculogenesis) and pathological (PCOS) conditions of GC via targeting different targets. On one hand, during folliculogenesis, E2F1/SF-1 mediated miR-320-induced suppression of GC proliferation and of GC steroidogenesis; On the other hand, in PCOS patients, miR-320 decreases in GC and this down-regulation is thought to cause relative estrogen deficiency via targeting RUNX2, implying the potential therapeutic role of this miRNA in PCOS by regulating steroidogenesis. It may shed light on the essential regulatory roles of specific miRNAs in the development and function of the GCs, and follicles, paving the foundation for novel therapeutic strategies. Therefore, a deeper understanding of the regulation of GC function by miRNAs for treating ovarian diseases is definitely worth further investigation.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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The lncRNA MALAT1 rs619586 G Variant Confers Decreased Susceptibility to Recurrent Miscarriage

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Cardiovascula disease and recurrent miscarriage have shared risk factors, and some cardiovascular disease-related candidate genes have been confirmed to be associated with recurrent miscarriage. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is a long non-coding RNA (lncRNA) that is considered to be associated with susceptibility to cardiovascular disease. However, whether lncRNA MALAT1 polymorphisms are related to recurrent miscarriage susceptibility is unclear. We genotyped three lncRNA MALAT1 polymorphisms (rs591291, rs619586, and rs3200401) in 284 patients and 392 controls using TaqMan methods. Logistic regression was used to evaluate the odds ratios (ORs) and 95% confidence intervals (CIs) adjusted for age. Our results showed that the rs619586 G variant had protective effects against recurrent miscarriage (AG vs. AA: adjusted OR = 0.670, 95% CI = 0.457–0.982, $p = 0.040$; GG vs. AA: adjusted OR = 0.278, 95% CI = 0.079–0.975, $p = 0.046$; GG/AG vs. AA adjusted OR = 0.621, 95% CI = 0.429–0.900, $p = 0.012$). In a combined analyses of protective genotypes, with regard to the three single nucleotide polymorphisms (SNPs), we found that individuals with two or three protective genotypes exhibited a significantly lower risk of recurrent miscarriage than those with no or only one protective genotype (adjusted OR = 0.369, 95% CI = 0.199–0.684, $p = 0.002$). Moreover, the decrease in recurrent miscarriage risk with two or three protective genotypes was most pronounced in women less than 35 years of age (OR = 0.290, 95% CI = 0.142–0.589, $p < 0.001$) and in women with 2–3 miscarriages (adjusted OR = 0.270, 95% CI = 0.126–0.580, $p < 0.001$). In conclusion, our study suggests that the rs619586 G variant may have potential protective effects conferring a decreased risk of recurrent miscarriage in the southern Chinese population.

Keywords: recurrent miscarriage, MALAT1, susceptibility, rs619586, single nucleotide polymorphism

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INTRODUCTION

Recurrent miscarriage is defined as the loss of two or more consecutive pregnancies before 20 weeks of gestation (Jaslow et al., 2010; Diejomaoh, 2015). The occurrence of recurrent miscarriage is associated with many factors, including genetic factors, immunological dysfunction, endocrine disorders, unhealthy lifestyles and defects of the reproductive organs (Saravelos and Regan, 2014; Sen et al., 2014; Garrido-Gimenez and Alijotas-Reig, 2015; Kaur and Gupta, 2016; Pereza et al., 2017; Shi et al., 2017). In recent years, many studies have revealed a relationship between miscarriage and cardiovascular disease. Cardiovascular disease and recurrent miscarriage share risk factors, and women who experience miscarriages may have an increased risk of cardiovascular disease (Kharazmi et al., 2010; El Achi et al., 2018). For example, women with a history of miscarriage appear to have an increased risk of ischemic heart disease (Wagner et al., 2015). Smith et al. (2011) reported that the parents of women who have experienced recurrent miscarriage also have an increased risk of ischemic heart disease, and Kharazmi et al. (2010) suggests that women who experience spontaneous pregnancy loss may have an increased risk of myocardial infarction (Zhu et al., 2018). Moreover, studies have found that genetic polymorphisms may be involved in the pathogenesis of recurrent miscarriage (Hyde and Schust, 2015), and some cardiovascular disease-related candidate genes have been confirmed to be associated with recurrent miscarriage, such as MTHFR (C677T), APO-E3, and Factor II (G20210A) (El Achi et al., 2018).

Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is a long non-coding RNA (lncRNA) that participates in splicing and epigenetic regulation (Tripathi et al., 2010; Yang et al., 2011). Recent studies have found that the MALAT1 gene polymorphism is associated with a susceptibility to various diseases, such as cancer risk and congenital heart disease (Huang et al., 2018; Li et al., 2018). Moreover, MALAT1 is involved in angiogenesis and inflammation, where inflammation is associated with the occurrence of recurrent miscarriage, and recurrent miscarriage may increase the risk of cardiovascular disease (Thum and Fiedler, 2014; Vitagliano et al., 2017). Studies have also confirmed that the expression level of MALAT1 was reduced in the villus samples of recurrent miscarriage patients and the regulation of MALAT1 is one of the factors that contributes to the pathogenesis of recurrent miscarriage (Wang Y. et al., 2018). These studies suggest that the MALAT1 gene polymorphism may be associated with recurrent miscarriage. However, reports have not focused on whether the MALAT1 gene polymorphism is associated with miscarriage susceptibility. Research has confirmed that the rs619586 G allele of the MALAT1 gene is associated with a decreased risk of coronary atherosclerotic heart disease (Wang G. et al., 2018), and genetic variations in MALAT1 (rs591291) showed a significantly better hepatocellular cancer prognoses in female patients (Wang B.G. et al., 2018). Furthermore, the lncRNA MALAT1 rs619586 and rs3200401 variants are associated with a decreased susceptibility to breast cancer (Peng et al., 2018). Therefore, we investigated whether recurrent miscarriage susceptibility is related to specific MALAT1 gene polymorphisms (rs591291, rs619586, and rs3200401) in a case-control study that included 248 cases and 392 controls from a southern Chinese population.

MATERIALS AND METHODS

Study Population

In the current study, a total of 248 recurrent miscarriage patients and 392 healthy controls were recruited at the Gynecology Department of Guangzhou Women and Children's Medical Center, between June 2017 and July 2018. Recurrent miscarriage was diagnosed as the occurrence of two or more spontaneous miscarriages of unknown etiology (5–24 weeks of gestation) with the same husband, and the control women had had at least two normal pregnancies and no history of a miscarriage. None of the patients with recurrent spontaneous miscarriage or the control women had a history of metabolic disorders, autoimmune conditions, hypertension, endocrine disorders, arterial or venous thrombosis, uterine anomalies, liver or kidney dysfunction, or embryo chromosomal abnormalities. Chromosomal abnormalities were excluded in all couples in the recurrent miscarriage group.

This study was approved by the Ethics Committee of Guangzhou Women and Children's Medical Center (201802202, Guangzhou, China). Written informed consent was obtained from each recurrent miscarriage patient and control subject before participation in the study. The clinical data, personal information, and demographic information were collected with a medical record system.

SNP Genotyping and DNA Extraction

Genomic DNA was extracted from 200 μL samples of peripheral blood leukocytes from all participants by using a Blood DNA Isolation Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The specific fluorescent probes for single nucleotide polymorphism (SNP) (rs591291, rs619586, and rs3200401) genotyping were purchased from ABI (Thermo Fisher Scientific, United States). Genotyping of the three SNPs was performed in a 384-well plate on an ABI Q6 instrument (Thermo Fisher Scientific, United States) according to the TaqMan real-time polymerase chain reaction protocol. A random selection of 10% of the samples was repeated for detection, and the results showed 100% concordance.

Statistical Analysis

The data were analyzed using SAS statistical analysis software (version 9.4; SAS Institute, Cary, NC, United States). The tests were two-tailed, and *P*-values less than 0.05 were considered statistically significant. Hardy-Weinberg equilibrium (HWE) for the control group was calculated using the goodness-of-fit χ^2 test. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were used to estimate the associations between the MALAT1 gene polymorphisms (rs591291, rs619586, and rs3200401) and recurrent miscarriage susceptibility, based

TABLE 1 | Frequency distribution of selected characteristics of the recurrent miscarriage and control groups.

Variables	Patients (n = 248)		Controls (n = 392)		P^a
	No.	%	No.	%	
Age range, years	20–44		22–44		0.722
Mean ± SD	31.00 ± 4.83		31.44 ± 4.39		
<35	187	75.4	288	73.47	
35–40	52	20.97	92	23.47	
>40	9	3.63	12	3.06	
No. of abortions/%					
2–3	169	68.15			
≥4	79	31.85			

[a] *Two-sided χ^2 test for distributions of recurrent miscarriage patients and controls.*

on extracted genotype data. Adjusted ORs were calculated using multiple-variable unconditional logistic regression after adjustment for age. In addition, analyses stratified by age and the number of miscarriages were performed. We divided the patients into two groups according to the number of abortion occurrences (two to three miscarriages or four or more miscarriages).

RESULTS

Population Characteristics and SNP Selection

In total, we recruited 248 recurrent miscarriage patients and 392 healthy controls with ages ranging from 20 to 44 (**Table 1**). There was no significant difference between the recurrent miscarriage patients and the controls in terms of age (31.00 ± 4.83 vs. 31.44 ± 4.39 years, p = 0.722). Approximately 68.15% of the recurrent miscarriage patients had undergone two or three spontaneous miscarriages, and more than 31.85% had suffered four or more spontaneous miscarriages.

Association Between MALAT1 Gene Polymorphisms and Recurrent Miscarriage Susceptibility

The genotype frequency distribution of the three SNPs was analyzed with the goodness-of-fit χ^2 test. As shown in **Table 2**, upon analysis of the genotypic and allelic frequencies of SNPs between the recurrent miscarriage patients and the healthy controls, the P-values for HWE in the control group were above 0.05 (p = 0.227 for rs591291, p = 0.123 for rs3200401, and

TABLE 2 | Genotype and allele frequencies of MALAT1 in recurrent miscarriage patients and controls.

Genotype/allele	RM (N = 248)	Controls (N = 392)	P-value[a]	OR (95% CI)	P-value	Adjusted OR (95% CI)	P-value[b]
MALAT1/rs591291 C > T (HWE = 0.227)							
CC	84 (33.87)	149 (38.01)	0.531	1.00	/	1.00	/
CT	116 (46.77)	176 (44.90)	/	1.169 (0.819–1.668)	0.389	1.163 (0.815–1.661)	0.405
TT	48 (19.35)	67 (17.09)	/	1.271 (0.805–2.007)	0.304	1.271 (0.805–2.009)	0.306
Dominant	164 (66.13)	243 (61.99)	0.288	1.197 (0.858–1.670)	0.289	1.193 (0.855–1.665)	0.299
Recessive	200 (80.65)	325 (82.91)	0.469	1.164 (0.772–1.755)	0.468	1.168 (0.774–1.761)	0.459
C	284 (57.26)	474 (60.46)	0.257	1.00	/	1.00	/
T	212 (42.74)	310 (39.54)		1.141 (0.908–1.434)	0.256	1.141 (0.908–1.434)	0.257
MALAT1/rs3200401 C > T (HWE = 0.123)							
CC	180 (72.58)	277 (70.66)	0.412	1.00	/	1.00	/
CT	63 (25.40)	100 (25.51)	/	0.969 (0.672–1.399)	0.869	0.963 (0.667–1.390)	0.840
TT	5 (2.02)	15 (3.83)	/	0.513 (0.183–1.437)	0.204	0.507 (0.181–1.420)	0.196
Dominant	68 (27.42)	115 (29.34)	0.601	0.910 (0.639–1.296)	0.601	0.903 (0.634–1.287)	0.573
Recessive	243 (97.98)	377 (96.17)	0.187	0.518 (0.186–1.442)	0.208	0.512 (0.184–1.428)	0.201
C	423 (85.28)	654 (83.42)	0.372	1.00	/	1.00	/
T	73 (14.72)	130 (16.58)		0.868 (0.636–1.186)	0.374	0.863 (0.632–1.179)	0.354
MALAT1/rs619586 A > G (HWE = 0.259)							
AA	194 (78.23)	271 (69.13)	**0.014**	1.00	/	1.00	/
AG	51 (20.56)	106 (27.04)	/	**0.672 (0.459–0.984)**	**0.041**	**0.670 (0.457–0.982)**	**0.040**
GG	3 (1.21)	15 (3.83)	/	**0.279 (0.080–0.978)**	**0.046**	**0.278 (0.079–0.975)**	**0.046**
Dominant	54 (21.77)	121 (30.87)	**0.011**	**0.623 (0.431–0.903)**	**0.012**	**0.621 (0.429–0.900)**	**0.012**
Recessive	245 (98.79)	377 (96.17)	**0.039**	0.308 (0.088–1.074)	0.065	0.307 (0.088–1.072)	0.064
A	439 (88.51)	648 (82.65)	**0.004**	1.00	/	1.00	/
G	57 (11.49)	136 (17.53)		**0.619 (0.444–0.862)**	**0.005**	**0.617 (0.442–0.860)**	**0.004**
Combined protective effect of genotypes*							
0–1	233 (93.95)	332 (84.69)	**<0.001**	1.00	/	1.00	/
2–3	15 (6.05)	60 (15.31)		**0.373 (0.201–0.690)**	**0.002**	**0.369 (0.199–0.684)**	**0.002**

**The protective genotypes used for the calculation were rs591291CC+rs3200401 CT/TT+rs619586 AG/GG.* [a] *χ^2 test for genotype distributions between recurrent miscarriage patients and controls.* [b] *Adjusted for age. RM: recurrent miscarriage patients. Statistically significant values are shown in bold ($P < 0.05$).*

$p = 0.259$ for rs619586), suggesting that the genotype frequencies for those SNPs conformed to HWE. Single-locus analysis suggested that the rs619586 G variant in lncRNA MALAT1 was associated with decreased recurrent miscarriage susceptibility (AG vs. AA: adjusted OR = 0.670, 95% CI = 0.457–0.982, $p = 0.040$; GG vs. AA: adjusted OR = 0.278, 95% CI = 0.079–0.975, $p = 0.046$; GG/AG vs. AA adjusted OR = 0.621, 95% CI = 0.429–0.900, $p = 0.012$). However, we found no significant relationship between rs591291 or rs3200401 in lncRNA MALAT1 and recurrent miscarriage risk. Upon combined analysis of the protective genotypes with regard to the three SNPs, we found that individuals with two or three protective genotypes exhibited significantly lower recurrent miscarriage risk than those with no or only one protective genotype (adjusted OR = 0.369, 95% CI = 0.199–0.684, $p = 0.002$).

Stratified Analysis of Selected Polymorphisms and Recurrent Miscarriage Susceptibility

We further explored the associations between lncRNA MALAT1 gene polymorphisms (rs619586) and combined effects of protective genotypes and recurrent miscarriage susceptibility in analyses stratified by age and number of miscarriages (as shown in **Table 3**). Compared with the rs619586 AA variant, the AG/GG variant was more protective in women less than 35 years of age (OR = 0.534, 95% CI = 0.345–0.827, $p = 0.005$) and in women who had undergone 2–3 miscarriages (adjusted OR = 0.577, 95% CI = 0.375–0.888, $p = 0.012$). Moreover, the combined analysis suggested that the presence of two or three protective genotypes decreased the recurrent miscarriage risk in women less than 35 years of age (OR = 0.290, 95% CI = 0.142–0.589, $p < 0.001$) and in women who had undergone 2–3 miscarriages (adjusted OR = 0.270, 95% CI = 0.126–0.580, $p < 0.001$) compared with the presence of no or only one protective variant.

FPRP Values for All Significant Associations

In **Table 4**, the false-positive report probability (FPRP) values of the positive results of the MALAT1 gene discovery are shown. The predicted value of the false positive report was 0.2, and the prior probability was 0.1. Compared with the rs619586 A genotype carrier, the probability that the rs619586 G genotype can reduce the risk of recurrent abortion is still credible (FPRP = 0.115). In the FPRP analysis, most of the meaningful findings are not noteworthy, which is likely due to the limited sample size in the current study. Therefore, important findings from current research need to be further validated with large sample sizes.

DISCUSSION

To the best of our knowledge, this study is the first to investigate the associations between lncRNA MALAT1 gene polymorphisms (rs619586, rs3200401, and rs591291) and recurrent miscarriage susceptibility. Included in our study were 248 recurrent

TABLE 3 | Stratification analysis of associations between MALAT1 polymorphisms and recurrent miscarriage risk in a southern Chinese population.

Variable	rs619586 (cases/controls)		*p*	OR (95% CI)	*p*	Adjusted OR (95% CI)	p^a	Combined (cases/controls)*		*p*	OR (95% CI)	*p*	Adjusted OR (95% CI)	p^a
	AA	AG/GG						0–1	2–3					
Age, years														
<35	**150/197**	**37/91**	**0.004**	**0.534 (0.345–0.827)**	**0.005**	/	/	**177/241**	**10/47**	**<0.001**	**0.290 (0.142–0.589)**	**<0.001**	/	/
35–40	38/65	14/27	0.756	0.887 (0.415–1.896)	0.757	/	/	48/80	4/12	0.314	0.556 (0.170–1.821)	0.332	/	/
>40	6/9	3/3	0.677	1.500 (0.223–10.077)	0.677	/	/	8/11	1/1	0.831	1.375 (0.074–25.433)	0.831	/	/
No. of abortions/%														
2–3	134/271	35/121	**0.012**	**0.585 (0.381–0.381)**	**0.014**	**0.577 (0.375–0.888)**	**0.012**	161/332	8/60	**<0.001**	**0.275 (0.128–0.589)**	**<0.001**	**0.270 (0.126–0.580)**	**<0.001**
4≥	60/271	19/121	0.219	0.709 (0.406–1.240)	0.228	0.723 (0.413–1.266)	0.256	72/332	7/60	0.116	0.538 (0.236–1.225)	0.140	0.549 (0.241–1.253)	0.155

**The combination of protective genotypes used for the calculation were MALAT1: rs591291CC+rs3200401 CT/TT+rs619586 AG/GG. [a]Adjusted for age. RM, recurrent miscarriage patients. Statistically significant values are shown in bold (P < 0.05).*

TABLE 4 | False-positive report probability values for associations between recurrent miscarriage risk and genotypes of MALAT1 polymorphisms.

Genotype/allele	OR (95% CI)	*P*-value[a]	Statistical power[b]	Prior probability				
				0.25	0.1	0.01	0.001	0.0001
MALAT1/rs619586 A > G								
AG vs. AA	0.672 (0.459–0.984)	0.041	0.505	**0.196**	0.422	0.889	0.988	0.999
GG vs. AA	0.279 (0.080–0.978)	0.046	0.115	0.546	0.783	0.975	0.998	1.000
AG/GG vs. AA	0.623 (0.431–0.903)	0.012	0.358	**0.091**	0.232	0.769	0.971	0.997
G vs. A	0.619 (0.444–0.862)	0.005	0.346	**0.042**	**0.115**	0.589	0.935	0.993
AG/GG vs. AA								
<35 years	0.534 (0.345–0.827)	0.005	0.171	**0.081**	0.208	0.743	0.967	0.997
2–3 abortions	0.585 (0.381–0.381)	0.014	0.277	**0.132**	0.313	0.834	0.981	0.998
Protective genotypes								
0–1 vs. 2–3	0.373 (0.201–0.690)	0.002	0.064	**0.086**	0.219	0.755	0.969	0.997
<35 years	0.290 (0.142–0.589)	0.001	0.028	**0.096**	0.243	0.779	0.973	0.997
2–3 abortions	0.275 (0.128–0.589)	0.001	0.023	**0.115**	0.281	0.811	0.977	0.998

[a]*Calculated the genotype frequency distributions using the omnibus χ^2 test in **Tables 2, 3**.* [b]*Calculated the statistical power using the number of observations and the OR and P-values in **Tables 2, 3**. Statistically significant values are shown in bold ($P < 0.05$).*

miscarriage patients and 392 healthy controls. Our results suggested that the lncRNA MALAT1 rs619586 G allele was associated with a decreased risk of recurrent miscarriage, and the protective effect was most pronounced in women less than 35 years of age, and in the subgroup of women with two to three prior miscarriages. In contrast, other lncRNA MALAT1 SNPs (rs3200401 and rs591291) were not associated with recurrent miscarriage susceptibility.

MALAT1 is one of the lncRNAs that has been proven to be associated with disease, and a growing number of studies have indicated that MALAT1 also participates in various pathological processes (Wu et al., 2015). Several studies have revealed that MALAT1 gene polymorphisms are associated with disease susceptibility. For example, the MALAT1 rs619586 G variant was associated with a decreased risk of hepatocellular carcinoma and colorectal cancer (Liu et al., 2012; Zhao et al., 2018). In addition, Wang G. et al. (2018) found that rs619586 AG and GG genotypes in MALAT1 are associated with reduced risk of coronary atherosclerotic heart disease in a Chinese population and play protective roles in preventing the occurrence of coronary atherosclerotic heart disease. A study by Peng et al. (2018) found that the lncRNA MALAT1 rs619586 AG genotype and the rs3200401 CT genotype are associated with a decreased susceptibility to breast cancer, and compared to the rs619586AA genotype, carriers with the rs619586 G variant have lower expression of MALAT1 in the Chinese Han population. Similarly, in our case-control study, the results suggested that the MALAT1 gene rs619586 G variant decreased the risk of recurrent miscarriage in a southern Chinese population and that it was likewise a protective factor against recurrent miscarriage susceptibility. Although we did not detect the expression of MALAT1 in miscarriage patients, we speculate that the rs619586 G variant may reduce the risk of miscarriage by regulating the expression of MALAT1. In future research, we will detect the expression level of MALAT1 and further verify our speculation with larger sample sizes. MALAT1 rs591291 showed significantly better hepatocellular cancer prognoses in female patients (Wang B.G. et al., 2018). Another study by Zhu et al. (2018) found that MALAT1 gene polymorphisms (rs619586 and rs3200401) were not significantly associated with ischemic stroke susceptibility in a northern Chinese Han population. However, these studies suggest that MALAT1 gene polymorphisms may play different roles in different diseases. In our case-control study, two SNPs of the MALAT1 gene (rs3200401 and rs591291) were not related to recurrent miscarriage susceptibility. These results suggest that MALAT1 gene variants may play similar roles in the pathological processes of recurrent miscarriage and cardiovascular diseases. To the best of our knowledge, this case-control study is the first to validate the association between genetic variants of lncRNA MALAT1 (rs619586, rs3200401, and rs591291) and recurrent miscarriage susceptibility. We propose that the rs619586 G variant may play a significant role in the pathogenesis of recurrent miscarriage. Dysregulation of MALAT1 contributes to various human diseases. MALAT1 is upregulated in many types of cancer, myocardial infarction, diabetes mellitus, and diabetic retinopathy. MALAT1 mainly regulates inflammation, cell proliferation, migration, and metastasis and affects endothelial function (Zhang et al., 2017; Masoumi et al., 2018). Cardiovascular diseases and diabetes mellitus are high risk factors for miscarriage. The rs619586 G variant may reduce the risk of miscarriage by regulating the expression of MALAT1. Currently, the molecular mechanism of MALAT1 in miscarriage patients is still not clear. Therefore, further studies of the functional role of MALAT1 in miscarriage are needed.

Numerous studies have demonstrated that advanced age is a risk factor for miscarriage; beyond the age of 40 years, the risk of miscarriage in women is five times greater than that in 31- to 35-year-old women (van Kooij et al., 1996; Nybo Andersen et al., 2000; Agenor and Bhattacharya, 2015). This study also confirmed that the number of prior miscarriages is strongly associated with the risk of miscarriage, consistent with previous reports that miscarriage rates increase with the number of previous miscarriages (Ogasawara et al., 2000). Kharazmi et al. (2010)

found that women with a history of miscarriage have increased risk of myocardial infarction. However, some previous research findings have suggested that the rs619586 G variant is associated with decreased risk of coronary atherosclerotic heart disease and congenital heart disease (Li et al., 2018; Wang G. et al., 2018). Similarly, we found that the rs619586 AG/GG variant was more protective in women less than 35 years of age and in women with two to three miscarriages than the rs619586 AA variant. Moreover, combined analysis suggested that the presence of two or three protective genotypes decreased the recurrent miscarriage risk in women less than 35 years of age and in women who had undergone 2–3 miscarriages, which may be one reason that the incidence of recurrent miscarriage was relatively low in women less than 35 years of age. The molecular mechanism underlying this phenomenon deserves further exploration. In addition, further studies with larger sample sizes are needed to confirm these results.

This case-control study was the first to evaluate the association between lncRNA MALAT1 polymorphisms and recurrent miscarriage susceptibility. However, several limitations of our research should be noted. First, we only studied the relationships between lncRNA MALAT1 gene polymorphisms and susceptibility to recurrent miscarriage; MALAT1 gene expression in patients was not determined. Second, only three SNPs (rs619586, rs3200401, and rs591291) were analyzed in our study. Other SNPs, such as rs11227209, should be included in future research. Third, in the stratified analysis, we only analyzed the association of age and number of miscarriages with lncRNA MALAT1 gene polymorphisms. Because our study is retrospective, we were not able to collect and control for other factors, such as smoking, drinking status, eating habits, and these are important factors in miscarriage. Fourth, the sample size of this study, with 640 participants, was still not sufficiently large, which may have limited the statistical power. Future studies with larger sample sizes and inclusion of other factors that are important for miscarriage are needed to validate our findings regarding the roles of the lncRNA MALAT1 gene in recurrent miscarriage susceptibility.

In summary, our study confirmed the significant protective effect of the MALAT1 rs619586 G variant in recurrent miscarriage in a Chinese population. Moreover, the protective effect was more pronounced in women less than 35 years of age, than in women of other age groups; in addition, the protective effect was stronger in subgroups of women who had undergone two to three miscarriages, than in other subgroups. Thus, the rs619586 G allele may be involved in decreasing the number of miscarriages. However, future studies with larger sample sizes and practical experiments should be performed to further validate the roles of MALAT1 gene variants in recurrent miscarriage susceptibility.

ETHICS STATEMENT

This study was approved by the Ethics Committee of Guangzhou Women and Children's Medical Center (Guangzhou, China). Written informed consent was obtained from each recurrent miscarriage patient and control subject before participation in the study.

AUTHOR CONTRIBUTIONS

All authors contributed significantly to this work and supported the publication of the manuscript. DC, LL, and ZF devised the research plan. ZF and QL analyzed the data. DC wrote the manuscript. YT, YY, ZL, and HZ performed the experiments. LP and LF designed the experimental methods. XG and QX modified and polished the manuscript.

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High Thyroid Stimulating Hormone Level Is Associated With Hyperandrogenism in Euthyroid Polycystic Ovary Syndrome (PCOS) Women, Independent of Age, BMI, and Thyroid Autoimmunity: A Cross-Sectional Analysis

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Background: Infertility and dyslipidemia are frequently present in both women with polycystic ovary syndrome (PCOS) and subjects with thyroid dysfunction. Limited study regarding the association between thyroid stimulating hormone (TSH) level and phenotypes in euthyroid PCOS women. We aimed to determine whether the variation of TSH level associates with phenotypes in euthyroid PCOS patients.

Methods: Cross-sectional study including 600 PCOS and 200 age, body mass index (BMI), and thyroid autoimmunity-matched Chinese women from Renji hospital, Shanghai Jiaotong university during January 2010 and August 2018. The anthropometric and serum biochemical parameters related to TSH, thyroid autoimmunity, lipid profiles, and sex steroids were detected.

Results: The TSH level is higher in (2.29 ± 1.24 vs. 1.86 ± 0.90 mu/L, $p < 0.001$) in PCOS than controls. In euthyroid PCOS patients, TSH, TG, TC, LDL-c, and apoB level increased from non-hyperandrogenism (nonHA) to HA group (all $p < 0.05$). TSH level is positively associated with TG, apoB, free T, FAI, and negatively associated with apoA (all $p < 0.05$). The percentage of HA increased from TSH level (57.93% in TSH $<= 2.5$ group vs. 69.46% in TSH > 2.5 mU/L group, $p = 0.006$). HA phenotype is increased with TSH level independently of age, BMI, WC, LDL-C. Besides, in multivariate logistic regression analysis TSH and TG significantly associated with HA phenotype.

Conclusions: Higher TSH level is associated with increased prevalence of HA phenotype independent of age, BMI and thyroid autoimmunity in euthyroid PCOS.

Keywords: thyroid stimulating hormone, polycystic ovary syndrome, dyslipidemia, hyperandrogenism, euthyroid

INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder with systemic metabolic manifestations and neuroendocrine-immunity disturbance in women of reproductive age (1). It is characterized by hyperandrogenism (HA)/hirsutism, oligo- or amenorrhea, and polycystic ovaries (PCO). PCOS is also a heterogeneous disorder that affects many body functions, resulting in several health complications, including infertility, menstrual dysfunction, hirsutism, acne, obesity, metabolic syndrome as well as autoimmune disease.

Thyroid hormone disorders or thyroid autoimmunity is associated with increased risk of infertile, spontaneous miscarriage, preterm delivery, and metabolic dysfunctions, which is also commonly observed in PCOS (2, 3). In PCOS population, the prevalence of subclinical hypothyroidism (SCH) and thyroid autoimmunity are reported higher than that for women in general (4–6). Emerging studies have investigated the association between thyroid function/thyroid autoimmunity and metabolic parameters in PCOS, especially in dyslipidemia and insulin resistance. SCH is observed in PCOS women and has been found to be associated with hyperlipidemia and affect pregnancy rate in both PCOS and general population (7–11). Bakker et al. reported serum thyroid stimulating hormone (TSH) value is associated with a higher risk for dyslipidemia and severe cardiovascular risk factors (12). Our previous study analyzed TSH level and lipid profile in PCOS population, suggesting the cut-off point of TSH is 4.07 mU/L for elevated LDL-c risk (11). Thyroid hormones may also act as insulin agonists in muscle and as antagonists in the liver, so deficiency of thyroid hormones may lead to a decrease in glucose production and utilization (13–15). So some authors have considered insulin resistance (IR), which has been considered to be the principal factor in the pathogenesis of PCOS, as a consequence of hypothyroidism (1, 16).

Thyroid hormones not only plays an important role in regulating metabolism but also in reproductive health. Both thyroid receptor and TSH receptor are expressed in ovary, uterus and widely expressed in the feto-maternal unit during implantation (1). Deficiency of thyroid hormones may affect gonadal function and fertility, leading to delayed puberty onset and anovulatory cycles (17). SCH is associated with body weight gain, sex hormone-binding globulin (SHBG) increase, androstenedione to testosterone conversion increase, and aromatization to estradiol (18). TSH has been described as the most sensitive parameters for detecting minor degrees of primary thyroid hormone deficiency (18). But there is a controversy on treatment threshold based on TSH value in infertile women. According to 2017 American thyroid association (ATA) guideline, evidences suggesting TSH 4.0 mU/L instead of 2.5 mU/L as treatment threshold of levothyroxine (L-T4) in women before or in pregnancy (18). While in those undergoing *in-vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) ATA guideline suggest they treated with L-T4 and goal of treatment is to achieve TSH concentration <2.5 mU/L. That indicating in infertile women, there might has underlying subtle hypothyroidism even in those with normal thyroid function. Although PCOS is the most common cause in women with infertility, rare studies investigate the association of TSH and HA in euthyroid PCOS population.

In this present study, we hypothesized that underlying subtle hypothyroidism in PCOS would be associated with HA in women with PCOS. The aim of the present study was to investigate the relationship of TSH and HA phenotypes between PCOS and age, body mass index (BMI), and thyroid autoimmunity matched euthyroid controls.

MATERIALS AND METHODS

Subjects

We consecutively recruited participants who attended outpatient endocrine clinics of Renji hospital for investigation of oligo- or amenorrhea, fertility problems, hirsutism, or acne during January 2010 and August 2018. All the subjects were non-smokers from eastern China. Age, BMI and thyroid-autoimmunity matched control and PCOS subjects were included by a ratio of 1:3. In brief, PCOS and control women were stratified by age, BMI and the presence of autoimmune thyroiditis (AIT). Totally 600 PCOS (age: 27.67 ± 5.21 years, range: 14–50 years, BMI: 26.44 ± 5.69 kg/m^2, range: 14.89–50.59 kg/m^2) and 200 healthy control women (age: 28.26 ± 5.29 years, range: 14–49 years; BMI: 26.72 ± 5.37 kg/m^2, range: 15.02–50.09 kg/m^2) are analyzed. All subjects were received thyroid autoimmune antibody measurement and thyroid USG evaluation. Those with TSH level out of reference range (0.25–5.0 mIU/L), took thyroid treatment in last 6 weeks, or had thyroid, pituitary surgery history were excluded. The presence of either thyroid peroxidase (TPO) or thyroglobulin antibodies is defined as AIT. Euthyroidism is defined as TSH level within the reference range. The PCOS diagnosis was based on the revised Rotterdam 2003 criteria. These include the following: (i) clinical and/or biochemical signs of hyperandrogenemia; (ii) oligomenorrhoea or anovulation; and (iii) findings of polycystic ovaries by ultrasound (19). All patients of other related disorders should be excluded prior to examination, including non-classical 21-hydroxylase-deficient adrenal hyperplasia, hyperprolactinemia, thyroid dysfunction, Cushing's syndrome, or androgen-producing tumors (20). A free androgen index (FAI) >7 was considered diagnostic of hyperandrogenemia (HA) (21). PCOS was divided into four phenotypes: oligo/amenorrhea + HA (O + HA), polycystic ovaries + HA (PCO + HA), O + PCO, and O + PCO + HA. Subjects in the control group were nonHA healthy women and serum total testosterone values ≤0.6 ng/ml. Moreover, all of them had normal ovulation cycles and ovarian appearance in ultrasound examinations. All the study evaluations and procedures were conducted in accordance with the guidelines of the Helsinki Declaration on human experimentation. Written informed consent was obtained from all subjects and this study is approved by the Ethical Committees of Renji Hospital.

Anthropometric Measurements

We used a digital scale and a stadiometer to measure the height and weight of each subject to the nearest 0.1 cm and 0.1 kg, respectively. The BMI was calculated as the body weight (kg) divided by the height (m) squared. The waist circumference

(WC) was measured to the nearest 0.1 cm by placing measuring tape around the body in a horizontal position at a level midway between the lower rib margin and the iliac crest.

Laboratory Assays

All the laboratory evaluations were performed on subjects in the fasting state between 7:00 a.m. and 8:00 a.m. during day 2 to 5 of the spontaneous menstrual cycle. If the patient had amenorrhea for more than 3 months, the examination was performed during a bleeding episode after progestin withdrawal. The blood samples were stored at 4°C on the day of the collection. All measurements, including total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-c), and low-density lipoprotein cholesterol (LDL-c) were performed with Roche reagents (D 2400 and E 170 Modular Analytics modules with Roche/Hitachi analyzers; Roche Diagnostics).Analysis of thyrotropin (TSH), luteinizing hormone (LH), follicle stimulating hormone (FSH), total testosterone (T),

TABLE 1 | Clinical and biochemical characteristics in PCOS patients and controls.

	PCOS ($n = 600$)					
Characteristics[a]	**nonHA ($n = 229$)**	**HA ($n = 371$)**	P^c	**Total**	**Control ($n = 200$)**	P^d
Age (years)	27.73 ± 4.90	27.63 ± 5.40	0.822	27.67 ± 5.21	28.26 ± 5.29	0.170
BMI (kg/m^2)[b]	23.44 ± 4.70	28.26 ± 5.47	<0.001	26.44 ± 5.69	26.72 ± 5.37	0.537
Normal BMI	150	104	<0.001	252 (42.00)	77 (38.50)	0.516
Overweight	56	138		196 (32.67)	74 (37.00)	
Obese	23	129		152 (25.33)	49 (24.50)	
WC (cm)	79.99 ± 12.51	91.89 ± 14.16	<0.001	87.35 ± 14.73	86.52 (13.90)	0.188
WHR	0.85 ± 0.09	0.89 ± 0.07	<0.001	0.88 ± 0.08	0.87 ± 0.08	0.249
TSH (mIU/L)	2.16 ± 1.01	2.37 ± 1.37	0.045	2.29 ± 1.24	1.86 ± 0.90	<0.001
Phenotype			<0.001			
HA-O-PCO	0	190		190 (31.67)	NA	
O-HA	0	86		86 (14.33)	NA	
PCO-HA	0	95		96 (16)	NA	
PCO-O	229	0		228 (38)	NA	
HA phenotype	0	371	<0.001	371 (61.83)	NA	
AIT	53 (23.14)	66 (11.18)	0.11	119 (19.83)	34 (17.00)	0.378
TGAB positive	47 (20.32)	60 (16.11)	0.176	107 (17.83)	29 (14.50)	0.277
TPOAB positive	27 (11.13)	30 (8.09)	0.133	57 (9.50)	19 (9.50)	1.000
TG (mmol/L)	0.90 (0.67–1.37)	1.37 (1.01–2.02)	<0.001	1.19 (0.82–1.81)	1.01 (0.69–1.57)	0.002
Tch (mmol/L)	4.61 ± 0.96	4.88 ± 0.99	0.001	4.78 ± 0.99	4.63 ± 0.91	0.070
HDL (mmol/L)	1.51 ± 0.40	1.23 ± 0.32	<0.001	1.34 ± 0.38	1.43 ± 0.40	0.006
LDL (mmol/L)	2.57 ± 0.72	2.95 ± 0.81	<0.001	2.8 ± 0.80	2.63 ± 0.76	0.007
apoA (mg/L)	125.00 (41–252)	108.00 (48.95–259.00)	0.68	112.9 (46.23–253.45)	107.45 (41.00–259.50)	0.302
apoB (g/L)	0.85 ± 0.23	1.00 ± 0.28	<0.001	0.95 ± 0.27	0.88 ± 0.27	0.007
LH (IU/L)	8.72 ± 7.03	8.86 ± 6.01	0.787	8.81 ± 6.41	8.07 ± 7.31	0.201
FSH (IU/L)	6.40 ± 2.56	6.12 ± 3.50	0.205	6.23 ± 2.50	7.31 ± 5.48	0.012
LH/FSH	1.47 ± 1.15	1.50 ± 0.98	0.759	1.49 ± 1.05	1.21 ± 0.90	0.001
PRL (ug/L)	12.59 (8.96–19.36)	11.55 (8.76–16.02)	0.095	11.95 (8.96–16.92)	12.68 (9.40–16.62)	0.726
E_2 (pmol/L)	183.00 (110.75–300.50)	168.00 (125.00–243.00)	0.338	174.00 (116.50–259.00)	172 (121.50–276.50)	0.956
T (nmol/L)	1.79 ± 0.75	2.53 ± 0.92	<0.001	2.25 ± 0.93	1.73 ± 0.75	<0.001
FT	25.35 ± 9.68	61.21 ± 21.89	<0.001	47.52 ± 25.21	27.05 ± 13.73	<0.001
FAI	3.73 (2.76–5.23)	12.46 (9.43–17.73)	<0.001	8.68 (4.66–14.17)	4.32 (2.74–5.68)	<0.001
mean ovary volume (ml^3)	50.53 ± 24.12	19.62 ± 9.16	<0.001	10.41 ± 4.54	5.69 ± 1.26	<0.001
SHBG (nmol/L)	202.50 (149.25–258.75)	257.00 (183.75–321.25)	<0.001	24.2 (15.93–42.28)	39 (28.60–58.10)	<0.001
DHEAs (ng/mL)	3.78 ± 2.16	4.58 ± 2.26	<0.001	243.33 ± 104.59	198.31 ± 93.20	<0.001
A_2 (ug/dL)	10.88 ± 4.92	10.10 ± 4.26	0.061	4.28 ± 2.26	2.94 ± 1.66	<0.001

WC, waist circumference; WHR, waist to hip ratio; HA, hyperandrogenism; AIT, autoimmune-thyroiditis; FT, free testosterone; FAI, free androgen index; SHBG, sex hormone binding globulin; DHEAs, dehydroepiandrosterones.

[a] *Continuous data are shown as mean ± sd or medians (IQR) and categorical variables as n (%).*

[b] *Body mass index was calculated as weight (kg)/height (m)^2.*

[c] *P-value for nonHA vs. HA PCOS.*

[d] *P-value for PCOS vs. control.*

progesterone (P), estradiol (E2), prolactin (PRL), sex hormone binding globulin (SHBG), and sulfated dehydroepiandrosterone (DHEA-S) were detected by chemiluminescence (Elecsys Auto analyzer, Roche Diagnostics). The FAI level was calculated as [(total testosterone × 100)/SHBG] (21).

Statistical Analysis

All statistical analyses were performed using SPSS software version 17.0 (SPSS, Chicago, IL, USA). The PCOS patients were further divided into two groups on the basis of TSH level: Group 1: 0.25 ≤ TSH ≤ 2.5 mIU/L, Group 2: 2.5 < TSH ≤ 5.0 mIU/L. The data were presented as the mean ± SD, except for skewed variables, which were presented as the median with the interquartile range (IQR) given in parentheses. Ln transformation were conducted to achieve normal distribution for skewed variables. Student's *t*-test or Mann–Whitney *U*-test was used for the comparisons of continuous data among groups, whereas the Chi-squared test was used for the comparisons of categorical variables. Spearman correlation analysis were used to detect the associations between TSH level, lipid profiles and the present of HA. Multiple logistic regression analysis was used to assess the odds ratio (OR) for the presence of HA in PCOS subjects. Factors that were significantly associated with HA in the univariate analyses were entered in the multivariate regression model. A two-tailed test was applied, and a *P*-value of <0.05 was considered statistically significant.

RESULTS

Clinical and Biochemical Characteristics of PCOS and Controls

General demographic and laboratory characteristics of the study cohort are summarized in **Table 1**. The mean age of our population was 27.67 ± 5.21 years in PCOS women and 28.26 ± 5.29 years in control. Among all participants, 153 were autoimmune-thyroiditis (AIT), 119 (19.83%) in PCOS women and 34 (17.00%) in control ($p = 0.378$). Compared to the age, BMI and thyroid autoimmunity matched control group, TSH level of PCOS women is higher (2.29 ± 1.24 mU/L vs. 1.86 ± 0.90 mU/L, $p < 0.001$). Lipid profiles showed higher TG (1.19 (0.82–1.81) mmol/L vs. 1.01 (0.69–1.57) mmol/L, $p = 0.002$), TC (4.78 ± 0.99 mmol/L vs. 4.63 ± 0.91 mmol/L, $p = 0.07$), LDL-c (2.80 ± 0.80 mmol/L vs. 2.63 ± 0.76 mmol/L, $p = 0.007$) and apo B (0.95 ± 0.27 mmol/L vs. 0.88 ± 0.27 mmol/L, $p = 0.007$) were observed in PCOS population than controls. Furthermore, FSH ($p = 0.012$), ratio of LH/FSH, total T, free T, FAI, A_2, and DHEAs were all significantly elevated in PCOS while SHBG was lower compared with controls ($p < 0.001$).

Clinical and Biochemical Characteristics of PCOS With or Without HA

The clinical and biochemical characteristics of PCOS with or without HA were summarized in **Table 1**. We found that HA subjects of PCOS have significantly higher BMI, WC, and WHR (all $p < 0.001$) compared to nonHA subjects. The prevalence of AIT is comparable in nonHA and HA subjects (23.14% vs. 11.18, $p = 0.110$). While TSH, TG, TC, LDL-c and apoB level increased from nonHA to HA group (all $p < 0.05$). As for the sex steroids, lower SHBG, higher total T, FAI, free T, DHEA-S, and A_2 were observed, as expected.

Clinical and Biochemical Characteristics of PCOS Women Grouped by TSH Level

As the controversy on treatment threshold mainly based on whether TSH value above 2.5 or 4.0 mIU/L, we further subdivided PCOS patient by TSH 2.5 mU/L (**Table 2**, Group 1, 0.25 ≤ TSH ≤ 2.5 mU/L; Group 2, 2.5 < TSH ≤ 5.0 mU/L) and TSH 4.0 mU/L (**Table S1**, Group S1, 0.25 ≤

TABLE 2 | Clinical and biochemical characteristics in PCOS patients grouped by TSH level.

Characteristics[a]	Group 1[b] ($n = 397$)	Group 2 ($n = 203$)	*P*
Age (years)	27.76 ± 5.35	27.48 ± 4.94	0.533
BMI (kg/m^2)[b]	26.06 ± 5.51	27.12 ± 5.98	0.032
Normal BMI	179 (45.09)	75 (36.95)	0.111
Overweight	126 (31.74)	68 (33.50)	
Obese	92 (23.17)	60 (29.56)	
WC (cm)	86.15 ± 14.67	89.69 ± 14.59	0.005
WHR	0.87 ± 0.08	0.88 ± 0.07	0.136
TSH (mU/L)	1.64 ± 0.50	3.57 ± 1.28	<0.001
PHENOTYPE			
HA-O-PCO	116 (21.17)	74 (40.22)	0.053
O-HA	56 (10.22)	30 (16.30)	
PCO-HA	59 (10.77)	37 (20.11)	
PCO-O	166 (30.29)	62 (33.70)	
HA phenotype	230 (57.93)	141 (69.46)	0.006
AIT	72 (18.14)	47 (23.15)	0.145
TGAB positive	65 (16.37)	42 (20.69)	0.191
TPOAB positive	31 (7.81)	26 (12.81)	0.048
TG (mmol/L)	1.15 (0.82–1.71)	1.34 (0.83–2.02)	0.048
Tch (mmol/L)	4.73 ± 0.97	4.88 ± 1.01	0.084
HDL (mmol/L)	1.33 ± 0.37	1.35 ± 0.39	0.569
LDL (mmol/L)	2.76 ± 0.79	2.9 ± 0.82	0.043
apoA	105.35 (44.00–244.25)	124 (50.75–266.25)	0.236
apoB	0.92 ± 0.26	0.98 ± 0.29	0.027
LH (IU/L)	8.94 ± 6.71	8.55 ± 5.79	0.462
FSH (IU/L)	6.38 ± 2.75	5.93 ± 1.89	0.021
LH/FSH	1.49 ± 1.06	1.49 ± 1.03	0.957
PRL (ug/L)	11.69 (8.96–16.99)	12.27 (8.96–16.78)	0.548
E_2 (pmol/L)	173 (113.5–272.00)	178 (127.00–243.00)	0.985
T (nmol/L)	2.23 ± 0.93	2.29 ± 0.93	0.431
FT	45.7 ± 24.24	51.09 ± 26.71	0.016
FAI	8.18 (4.39–12.92)	10 (5.47–16.44)	0.001
A_2 (ug/dL)	4.32 ± 2.26	4.20 ± 2.25	0.517
SHBG (nmol/L)	25.8 (16.95–43.75)	20.9 (13.90–35.60)	0.001
DHEAs (ng/mL)	237.64 ± 100.70	254.58 ± 111.27	0.061
mean ovary volume (ml^3)	10.59 ± 4.78	10.10 ± 4.08	0.247

WC, waist circumference; WHR, waist to hip ratio; HA, hyperandrogenism; AIT, autoimmune-thyroiditis; FT, free testosterone; FAI, free androgen index; SHBG, sex hormone binding globulin; DHEAs, dehydroepiandrosterones.

[a] *Continuous data are shown as mean ± sd or medians (IQR) and categorical variables as n (%).*

[b] *Group 1, 0.25 ≤ TSH ≤ 2.5 mU/L; Group 2, 2.5 < TSH ≤ 5.0 mU/L.*

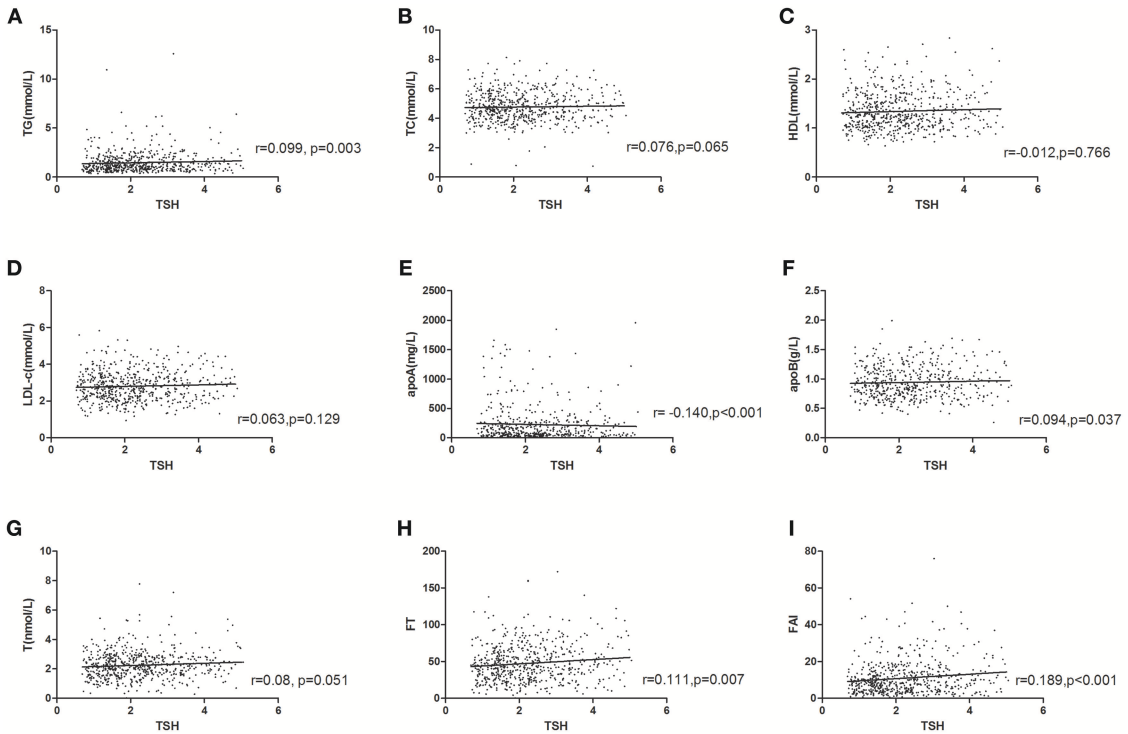

FIGURE 1 | Relationship of TSH level with lipid and androgen parameters in PCOS. **(A)** TG ($r = 0.099$, $p = 0.003$); **(B)** TC ($r = 0.076$, $p = 0.065$); **(C)** HDL-c ($r = -0.012$, $P = 0.766$); **(D)** LDL-c ($r = 0.063$, $p = 0.129$); **(E)** apoA ($r = -0.140$, $p < 0.001$); **(F)** apoB ($r = 0.094$, $p = 0.037$); **(G)** total T ($r = 0.08$, $P = 0.051$); **(H)** free T ($r = 0.111$, $p = 0.007$); **(I)** FAI ($r = 0.189$, $P < 0.001$). p-value for Pearson (normal distribution)/Spearman's (skewed distribution) correlation tests.

TSH $\leq$ 4.0 mU/L; Group S2, 4.0 $<$ TSH $\leq$ 5.0 mU/L). As shown in **Table 2**, when divided by TSH 2.5 mU/L we found women with relatively higher TSH level (Group 2) had significantly higher BMI, WC,TG, apoB, LDL-c, FT, FAI, and lower SHBG compared with Group 1 (all $p < 0.05$). Besides, higher prevalence of HA phenotype were observed in Group 2 (69.46% vs. 57.93%, $p = 0.006$). Furthermore, we analyzed those with AIT (**Table S2**), lipid profiles and HA prevalent were not different between Group 1 and Group 2 (all $p > 0.05$). We then subdivided PCOS patients by TSH level 4.0 mU/L, Group S2 had comparable HA phenotype, lipid profiles and sex steroids except lower SHBG compared to Group S1 (**Table S1**).

Relationships Between TSH Level, Lipid Profiles, and Androgen Related Parameters

We further analyze the relationships of TSH level with lipid and androgen related parameters in PCOS women. As shown in **Figure 1**, the level of TSH was positively associated with TG, ($r = 0.099$, $p = 0.003$),TC ($r = 0.076$, $p = 0.065$), LDL-c ($r = 0.063$, $p = 0.129$), apoB ($r = 0.094$, $p = 0.037$), total T ($r = 0.08$, $P = 0.051$), free T ($r = 0.111$, $p = 0.007$), FAI ($r = 0.189$, $P < 0.001$), and negatively associated with HDL-c ($r = -0.012$, $p = 0.766$), apoA ($r = -0.140$, $P < 0.001$).

TABLE 3 | Associations of TSH level with HA in PCOS patients.

	TSH level			
	Group 1	Group 2	95% CI	*P* for trend
HA	1	1.632	1.111–2.433	0.004
adjusted for age, BMI, WC	1	1.521	1.103–2.410	0.019
Multivariate-adjusted*	1	1.143	1.033–2.016	0.038

Data are OR (95%CI) unless otherwise indicated.
**OR with corresponding 95% CI has been adjusted for age, BMI, WC, LDL-c.*

Analyses of Factors Associated With HA in Women With PCOS

Multinomial logistic regression analyses show that the risk for prevalent HA increased across TSH level after adjustment for age, BMI, WC, and LDL-C (**Table 3**). In multivariate-adjusted models, the ORs (95% CIs) for HA in the higher TSH level compared with the TSH below 2.5 mIU/L were 1.143 (95%CI: 1.033–2.016, $p = 0.038$). Furthermore, we conducted univariate binary logistic regression to evaluate the risk factors associated with HA in PCOS women (**Table 4**). We found HA was significantly associated with TSH, TG, TC, apo B, and LDL-c. In multivariate logistic regression analysis TSH $>$ 2.5 mIU/L and TG

TABLE 4 | Binary logistic regression with HA as output variable.

	Univariate				Multivariate			
Variables	**OR**	**95% CI**		***P***	**OR**	**95% CI**		***P***
TSH	1.815	1.216	2.887	0.004	1.573	1.024	2.416	0.038
TG	2.001	1.554	2.578	<0.001	1.343	1.01	1.785	0.043
TC	1.342	1.125	1.6	0.001	0.654	0.399	1.071	0.092
apoB	9.884	4.537	21.531	<0.001	2.327	0.511	10.601	0.275
LDL-c	1.485	1.148	1.923	0.003	1.595	0.832	3.056	0.159

Data are OR (95%CI) unless otherwise indicated.

remained significantly associated with the presence of HA (all $p < 0.05$).

DISCUSSION

In the current study, we show evidence to confirm PCOS women with relatively higher TSH level is associated with increased risk of HA phenotype from a large population. The elevated HA risk is evident after statistical correction for differences in age, BMI, thyroid autoimmunity, and increased across TSH level divided by 2.5 mU/L, suggesting the important role of TSH level and HA in PCOS women. To the best of our knowledge, this is the first study to investigate the association between TSH levels and HA risk in a large PCOS population with normal thyroid function in a single center.

In our cohort, serum TSH level is significant higher in PCOS population than age, BMI, and thyroid autoimmunity matched counterparties with normal thyroid function. TSH level is increased from nonHA to HA group ($p = 0.04$). Previous study revealed SCH and PCOS occur in conjunction had higher risk of metabolic disorders especially in lipid profiles. Total cholesterol (TC), triglyceride (TG) and LDL-c were higher in PCOS with SCH (11, 22, 23). Dittrich et al. (24) reported in a 103 PCOS cohort that women with TSH $\geq$ 2.5 mIU/L had a significantly higher BMI, fasting insulin, HOMA-IR, TC, FAI levels, and decreased SHBG level in comparison with those with TSH $<$ 2.5 mIU/ L (24–26). This is accordance with our result, as in our euthyroid PCOS population, FT and FAI were significant higher in TSH $\geq$ 2.5 mIU/L group than their age-matched counterparts.

Among women of reproductive age, the prevalence of thyroid autoimmunity is 8–14% worldwide (3, 5). While in women from eastern China the prevalence is 21.4% (27), which is comparable with our cohort (20.13%). In addition, we find no difference between PCOS and controls (119/600, 19.83% vs. 34/200, 17.00%; $p = 0.378$). Women with positive thyroid antibodies have been reported to be at 2–3-fold higher risk of spontaneous miscarriage than those who test negative (28). The 2011 American Thyroid Association (ATA) and the 2012 Endocrine Society guidelines for the diagnosis and treatment of thyroid disease during pregnancy both declared that there was insufficient evidence to recommend for or against treating women with normal thyroid function experiencing sporadic or recurrent miscarriage or undergoing IVF-ET with levothyroxine (28, 29). Furthermore, ATA took a similar position in its 2017 guideline (18). Conflicting results were reported involving the relationship between TSH level and conception rates or time to pregnancy in infertility: Plowden et al. reported TSH $\geq$ 2.5 mIU/L has not been associated with increased time to pregnancy (30). Karmon et al. found preconceptional TSH $\geq$ 2.5 mIU/L is not associated with adverse intrauterine insemination outcomes in 1,477 euthyroid women (31). While in a large population including 11,254 Danish women, higher TSH level is associated with higher risk of not having children and not getting pregnant in age-adjusted and multi-adjusted models (32). In euthyroid women with unexplained infertility from Orouji et al. TSH $\geq$ 2.5 mIU/L has a higher risk of infertility (33). Whether levothyroxine treatment target for infertile women within 2.5mU/L is suitable for PCOS women was also still uncertain. Wang et al. (34) conducted a randomized clinical trial recently. Their result showed among women undergoing IVF-ET in China who had intact thyroid function and positive antithyroperoxidase antibodies, treatment with L-T4, compared with no levothyroxine treatment, did not reduce miscarriage rates or increase live-birth rates (34). So further clinical trials will be needed to investigate whether L-T4 treatment is suitable in PCOS population.

PCOS is the most common cause of female infertile in women during reproductive age. In our study, TSH value above 2.5 mU/L was associated with HA phenotype, which plays a key role in pathogenesis of PCOS. Gonadotropin-releasing hormone (GnRH) treatment has been shown to moderately increase TSH secretion in amphibians (35), suggesting that GnRH can modulate thyroid hormons at the pituitary level. As GnRH regulates the biosynthesis and secretion of both LH and FSH which were observed elevated in PCOS. Thyroid hormones modulate androgen biosynthesis through direct and indirect regulation of the expression and activity of steroidogenic enzymes associated synthesis (35). Recently, Flood et al. performed an *in silico* analysis of the promoter of several receptors and enzymes involved in both the androgen and hypothalamic-pituitary-thyroid axes (36). It was found that several putative androgen responsive elements (AREs) and thyroid responsive elements (TREs) were present in all of the androgen and thyroid hormone-related genes (36). This may give indication for there might be relatively subtle hypothyroidism in even normal PCOS population. If this were the case,

supplementation of L-T4 before pregnancy in those PCOS women with HA phenotype may improve pregnancy outcomes. Further studies will be needed to confirm this hypothesis.

Several limitations of our study should be considered. These data come from cross-sectional analyses and thus have substantial limitations for drawing causal inferences. Prolonged follow-up was needed as a prospective study to evaluate the pregnant outcome in PCOS patients with different TSH level can provide better evidence on the relationship of TSH level and pregnancy.

CONCLUSIONS

In conclusion, our results lend support to the postulation that the TSH level is associated with HA phenotype in PCOS women. We analyzed the TSH level in different PCOS phenotypes, we found TSH level is associated with HA phenotype in a well-characterized, large number of PCOS cohort. Our results show that TSH level, independently of age, BMI and thyroid autoimmunity, is associated with a higher HA prevalence in PCOS. Further studies are warranted to elucidate the role of TSH and HA phenotype in PCOS patients.

AUTHOR CONTRIBUTIONS

WL and TT designed the study. JC, YZ, YW, SL, JZ, LW, YJ, YD, HZ, YH, JM, and TT collected the data. JC, YZ, WL, and TT analyzed the data. JC and TT wrote the first draft of this report. All authors made critical revisions of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00222/full#supplementary-material

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Association of *BRCA* Mutations and Anti-müllerian Hormone Level in Young Breast Cancer Patients

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Background: Several preclinical and clinical studies have suggested that *BRCA*-mutation carriers may have decreased ovarian reserve. However, data in this area are limited and inconsistent, especially in young breast cancer patients.

Objective: This study evaluated the association between *BRCA* mutation status and serum anti-Müllerian hormone (AMH) level in young, reproductive-aged patients with breast cancer.

Materials and Methods: Patients ≤ 40 years of age with breast cancer and who had known *BRCA* status and baseline serum AMH level at Samsung Medical Center, Seoul, Korea, were considered for inclusion. A total of 52 *BRCA* mutation carriers (27 *BRCA1* and 25 *BRCA2*) and 264 non-carriers were selected for analyses. The serum level of AMH was compared according to presence of a *BRCA* mutation, and linear and logistic regression analyses were performed to evaluate the association between *BRCA* mutation and serum AMH level.

Results: No difference was found in clinical characteristics between *BRCA*-mutation carriers and non-carriers. Subjects with any *BRCA* mutation had a significantly lower median AMH than those without a mutation (2.60 vs. 3.85 ng/mL, 32% reduction, $P = 0.004$). Linear regression analysis showed a significant negative association between *BRCA* mutation and AMH level. In addition, logistic regression demonstrated non-significantly increased odds of mutation carriers having AMH < 1.2 ng/mL. However, no difference was found between *BRCA1/2* mutations.

Conclusions: Breast cancer patients with *BRCA* mutation have significantly lower serum AMH level. Fertility preservation should be considered more aggressively in young breast cancer patients with *BRCA* mutation.

Keywords: breast cancer, anti-Müllerian hormone, *BRCA1*, *BRCA2*, ovarian reserve

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INTRODUCTION

BRCA mutations are associated with high risk of breast and ovarian cancer in reproductive-aged women (1, 2). The lifetime risks of breast and ovarian cancer are 65 and 39%, respectively, in *BRCA1* mutation carriers and 45 and 11% in *BRCA2* mutation carriers (3).

In addition to cancer risk, it has been suggested that *BRCA* mutation may be related to decreased ovarian reserve, due to BRCA's function in repairing double-strand

DNA breaks (4). Several studies have demonstrated significantly decreased serum anti-Müllerian hormone (AMH) level, a biomarker representing ovarian reserves (5), in *BRCA* mutation carriers (6–9). Moreover, in breast cancer patients who underwent ovarian stimulation for fertility-preservation, there was a higher rate of poor ovarian response (POR) in *BRCA*-mutation carriers compared to non-carriers (10, 11). However, some studies have found no difference in serum AMH level according to *BRCA* mutation status (12–15). Therefore, the association between *BRCA* mutation status and decreased ovarian reserve is not conclusive. In addition, only a few studies have shown a significant association between *BRCA* mutation and decreased ovarian reserve in young breast cancer patients (16, 17).

Considering that it is currently recommended for *BRCA*-mutation carriers to complete childbearing by age 40 and to undergo a risk-reducing salpingo-oophorectomy, and that breast cancer patients with *BRCA* mutation are at increased risk of infertility as a result of anticancer treatment (18), issues of fertility preservation should be a priority for young patients.

Therefore, this study aimed to clarify the relationship between *BRCA* mutation and the level of ovarian reserve by comparing serum AMH level between *BRCA*-mutation carriers and non-carriers in breast cancer patients.

MATERIALS AND METHODS

This retrospective study included all premenopausal patients ≤40 years of age who were diagnosed with breast cancer and had a known baseline status regarding *BRCA* mutation and serum AMH level at Samsung Medical Center, Seoul, Korea, from December 2011 to May 2018. We excluded patients who (1) no longer had spontaneous menstruation at the time of tests, (2) had a history of any cancer treatment for breast cancer (i.e., chemotherapy or endocrine therapy), (3) had a history of another malignancy, (4) had a history of any ovarian surgery, (5) were pregnant, (6) had been diagnosed with any gynecologic problem that might affect AMH level (i.e., polycystic ovarian syndrome or endometriosis), and (7) had *BRCA* mutation of undetermined significance. Among the 316 patients included in this study, 264 were *BRCA*-negative and 52 were *BRCA*-positive (27 *BRCA1*-positive and 25 *BRCA2*-positive). The study was approved by the Institutional Review Board of Samsung Medical Center and exempted from informed consent requirements.

Measurements

Serum AMH level was measured using AMH ELISA kits (Beckman Coulter, Fullerton, CA, USA) following the manufacturer's directions. The minimum detectable concentration was 0.16 ng/mL, and the inter- and intra-assay coefficients of variation were 5.6 and 5.4%, respectively.

BRCA testing was conducted on peripheral blood using direct sequencing. When pathogenic variants were identified in the genetic tests, all mutations were interpreted utilizing the Human Gene Mutation Database (HGMD; http://www.hgmd.cf.ac.uk/), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar), and Korea ONCOgene Research and Diagnosis (KONCORD; http://koncord.kr). Mutation nomenclatures from the Breast Cancer Information Core (BIC; http://research.nhgri.nih.gov/bic/) were used for the genetic test reports.

Statistical Analysis

Statistical analysis was executed using Statistical Analysis System software, version 9.4 (SAS Institute Inc., Cary, NC, USA).

Clinical characteristics and serum AMH level were compared based on the presence of *BRCA* mutation. Data are presented as median (interquartile range) or number (percentage). Differences between the groups were analyzed using Chi-square test or Fisher's exact tests for categorical variables and Student's *t*-test or Mann–Whitney *U*-test for continuous variables. A $P < 0.05$ was considered statistically significant.

Regression analyses were performed to reveal the relationships between serum AMH level and *BRCA* mutation status after adjusting for age, body mass index, and history of smoking and oral contraceptive use. Linear regression analysis was performed on log-transformed serum AMH levels due to the non-normal distribution of the AMH values. In addition, logistic regression analysis was conducted to examine the association between *BRCA* mutation status and low AMH level, which represents poor ovarian reserve. For analysis, AMH <1.2 ng/mL was considered as poor ovarian reserve based on a previous report (19).

RESULTS

Table 1 shows the clinical characteristics of the study subjects. The median age was 34 years for both the *BRCA*-positive and *BRCA*-negative groups. No differences were found in reproductive or menstrual history, and smoking and alcohol intake did not differ between the two groups. However, the proportions of patients who had progesterone receptor- or human epidermal growth factor receptor 2-positive cancer were significantly higher in the *BRCA*-positive than in the *BRCA*-negative group.

Figure 1 shows the median serum AMH level according to *BRCA*-mutation status. Patients with any *BRCA* mutation had a significantly lower median AMH than those without a mutation (2.60 vs. 3.85 ng/mL, 32% decrease, $P = 0.004$). Serum AMH levels of the *BRCA1* (2.56 ng/mL, $P = 0.001$) and *BRCA2* groups (2.64 ng/mL, $P = 0.036$) were significantly lower than that of *BRCA*-negative group, but no difference was found between the *BRCA1* and *BRCA2* groups.

Table 2 shows the results of linear regression analysis. Log-transformed AMH was negatively associated with age ($P < 0.001$). After adjusting for age, body mass index, and history of smoking and oral contraceptive use, serum AMH level was still significantly lower in the *BRCA*-positive group than in the *BRCA*-negative group ($P = 0.043$). **Table 3** shows the results of the logistic regression model evaluating the association between risk of POR and *BRCA* mutation status. Thirty-five (13.3%) and 9 (17.3%) patients had AMH level <1.2 ng/mL in the *BRCA*-negative and *BRCA*-positive groups, respectively, presenting no statistical difference. After adjusting for age, body mass index, and history of smoking and oral contraceptive use, there was no increased

TABLE 1 | Clinical characteristics of the study subjects.

	BRCA-negative ($n = 264$)	*BRCA*-positive ($n = 52$)	*P*-value
Age, years	34.0 (30.0–36.0)	34.0 (30.5–36.0)	0.802
Body mass index, kg/m^2	21.0 (19.5–23.0)	20.9 (19.3–24.0)	0.820
Age at menarche, years	14.0 (13.0–15.0)	14.0 (13.0–15.0)	0.863
Parity			0.635
0	148 (56.1%)	25 (48.1%)	
≥1	116 (43.9%)	27 (51.9%)	
Menstruation			
Regularity	199 (75.4%)	45 (86.5%)	0.064
Duration (days)	5.0 (5.0–6.5)	5.0 (4.0–6.5)	0.449
Infertility treatment history	7 (2.7%)	1 (1.9%)	0.760
Receptor status			
ER (+)	161 (61.0%)	27 (51.9%)	0.224
PR (+)	133 (50.4%)	18 (34.6%)	**0.038**
HER2 (+)	45 (17.1%)	0 (0.0%)	**0.001**
Smoking use	1 (0.38%)	0 (0.0%)	0.657
Alcohol use	2 (0.76%)	1 (1.9%)	0.428

Data are presented as median (IQR) or number (%). Statistically significant differences are in bold. ER, estrogen receptor. PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

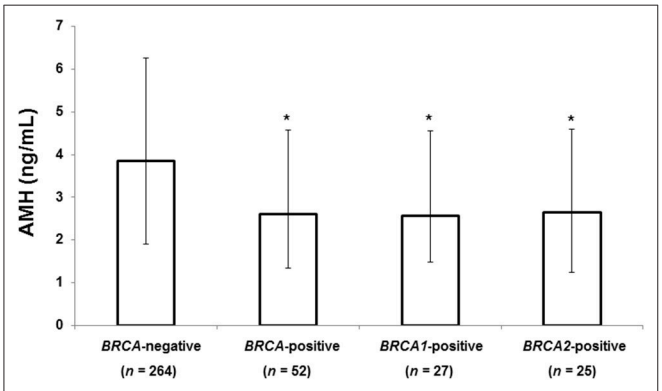

FIGURE 1 | Median AMH level according to *BRCA* mutation status. The error bars indicate interquartile ranges. *$P < 0.05$ vs. *BRCA*-negative patients. AMH, anti-Müllerian hormone.

likelihood of POR in the *BRCA*-positive group. In addition, no differences were found between the *BRCA1*- and *BRCA2*-positive groups in either the linear or logistic regression analysis (data now shown).

DISCUSSION

This study evaluated the association between *BRCA* mutation and serum AMH level in breast cancer patients aged ≤40 years. Median AMH was significantly lower in *BRCA*-positive breast cancer patients compared to *BRCA*-negative patients, but there was no difference in AMH level between the *BRCA1*-positive and *BRCA2*-positive groups.

TABLE 2 | Results of linear regression modeling of AMH level.

	Parameter estimate	Standard error	*P*-value
Intercept	4.843	0.605	**<0.001**
BRCA 1/2 carrier	−0.309	0.152	0.043
BRCA non-carrier	(ref)		
ADJUSTED VARIABLES			
Age	−0.092	0.014	**<0.001**
Body mass index	−0.017	0.017	0.336
Smoking	0.517	1.004	0.607
Oral contraceptive use	0.067	0.359	0.853

Regression of log transformed AMH level. Statistically significant differences are in bold. AMH, anti-Müllerian hormone.

Our results are similar to those of a previous study demonstrating a trend of lower AMH level (1.8 vs. 2.6 μg/L, $P = 0.109$) in 29 *BRCA*-positive breast cancer patients compared to 72 *BRCA*-negative breast cancer patients (17). Since age *per se* is an important factor determining serum AMH level, and patients with *BRCA* mutation show accelerated loss of ovarian follicular reserve and an earlier menopausal age (20), the differences in statistical significance in the studies might have resulted from inclusion of younger patients compared to our study (median age 31 vs. 34 years). Indeed, another study on patients with a median age of 34–36 years reported that AMH level was significantly lower in *BRCA*-positive breast cancer patients (1.22 vs. 2.23 ng/mL; $P < 0.001$) (16).

Several studies have shown that, in non-cancer, healthy subjects, serum AMH level was also significantly lower and ovarian follicles are fewer in *BRCA*-positive groups than in *BRCA*-negative groups (7–9). However, in one study, AMH levels were similar between 41 healthy *BRCA*-positive subjects and 324 healthy *BRCA*-negative subjects (12). Overall, the relationship between serum AMH level and *BRCA* mutation status remains contested.

When we analyzed the *BRCA1*-positive and *BRCA2*-positive groups separately in the present study, both had significantly lower AMH level than *BRCA*-negative patients, but no significant difference was found between the *BRCA*-positive groups. This finding is in accordance with previous studies presenting no significant difference in serum AMH level between *BRCA1*-positive and *BRCA2*-positive subjects (16, 17). In other studies, however, the serum AMH level was only significantly lower in either the *BRCA1*-positive (7) or *BRCA2*-positive (11) group compared to *BRCA*-negative subjects. Further studies are needed to evaluate the associations between each *BRCA* mutation and ovarian reserve.

In the current study, the prevalence of patients expected to exhibit POR, defined as AMH <1.2 ng/mL according to POSEIDON criteria (19), was not different according to *BRCA* mutation status, and the odds of AMH <1.2 ng/mL did not significantly increase after adjustment for age, body mass index, and history of smoking and oral contraceptive use. The results were the same when POR was assessed using an AMH level

TABLE 3 | The prevalence of poor ovarian reserve and the results of logistic regression model.

	***BRCA*-negative ($n = 264$)**	***BRCA*-positive ($n = 52$)**	***P*-value**
AMH level, number of patients (%)			0.441
<1.2 ng/mL	35 (13.3%)	9 (17.3%)	
≥1.2 ng/mL	229 (86.7%)	43 (82.7%)	
Odds ratio	(reference)	1.40	0.415
95% CI	(reference)	0.62–3.17	

Adjusted for age, body mass index, and history of smoking and oral contraceptive use. AMH, anti-Müllerian hormone; CI, confidence interval.

of <1.1 ng/mL (Bologna criteria) (21) or <1.0 ng/mL (17). Although the mean AMH of 2.64 ng/mL was lower in *BRCA*-mutation carriers than in non-carriers, the level should be sufficient for pregnancy due to the young age (34 years) of the patients in the current study.

The association between serum AMH level and *BRCA* mutation may be due to repair of double-strand DNA breaks and maintenance of chromosomal telomeres by BRCA (4, 22, 23). During reproduction, the telomere is shortened after every cycle of DNA replication, and telomere shortening is related to ovarian aging and reproductive lifespan (24). Furthermore, *BRCA1* gene expression decreases significantly with age in human oocytes. In a previous study, *BRCA1*-mutant mice had fewer oocytes after ovarian stimulation compared to wild-type mice and showed a tendency for DNA damage as a consequence of a deficiency in DNA double-strand break repair (16). Although BRCA2 also repairs DNA double-strand breaks, decreased *BRCA2* gene expression typically occurs at the end of the reproductive window, and the proportion of *BRCA2* gene expression among all DNA repair genes is small (25).

Our findings have substantial clinical importance in decision-making for young patients with breast cancer and *BRCA* mutation. From our results, *BRCA* mutation is an important factor associated with AMH level. Since fertility is attenuated with age, and a risk-reducing salpingo-oophorectomy should not be delayed over the long-term based on current recommendations, comprehensive, and individualized counseling for fertility preservation, such as oocyte or embryo cryopreservation, should be stressed in this population (26, 27).

This study has several strengths. First, the study population was relatively large ($n = 316$), and this is the largest reported study focusing on breast cancer patients. With the current sample size and difference in serum AMH level, a power of the current study is 98% with an alpha of 0.05. Second, our study evaluated the associations between *BRCA* mutations and decreased ovarian reserve in young breast cancer patients. Associations might differ between those who developed disease and those who were simply mutation-carriers, but most studies have only assessed healthy, non-cancer subjects. In addition, this is the first study of this kind in an Asian population. Genetic background differs across ethnicities; therefore, studies on various ethnicities are clinically important. For example, some studies (7, 12) have been performed on patients who carried at least 1 Ashkenazi Jewish founder mutation that is associated with a higher risk of breast and ovarian cancer (28, 29), and the results were different from ours. Finally, we analyzed the prevalence of POR according to *BRCA* mutation.

However, there are some limitations to our study. First, this was a retrospective study performed in one center. Second, we only analyzed serum AMH level to evaluate ovarian reserve. Although serum AMH level may be a reliable marker for ovarian reserve, addition of antral follicle count or serum follicle-stimulating level would be useful. Third, AMH is generally considered as the best ovarian reserve test, but it does not directly measure the primordial follicle pool. Fourth, although we addressed several factors that could affect serum AMH level, not all the potential confounders affecting serum AMH level were considered for analysis. Moreover, although we measured AMH and estimated POR, long-term fertility outcomes were not assessed in the present study.

In conclusion, young breast cancer patients with *BRCA* mutation have significantly lower AMH value, which is indicative of decreased ovarian reserve, compared to *BRCA*-negative patients. With further studies, our finding can support decision-making for fertility preservation.

ETHICS STATEMENT

The study was approved by the Institutional Review Board of Samsung Medical Center and exempted from informed consent requirements.

AUTHOR CONTRIBUTIONS

K-AS, DC, and D-YL were responsible for the concept and design of the study, searching for and analyzing data, and the writing of the manuscript.

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REVIEW
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Immature Oocyte for Fertility Preservation

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In vitro maturation (IVM) of human immature oocytes has been offered to women who are at risk of developing ovarian hyperstimulation syndrome (OHSS) caused by gonadotropin stimulation, such as PCO(S) patients or who have poor ovarian reserve. Cryopreservation of oocytes matured *in vivo* obtained in IVF cycles has improved after implementing the vitrification method and many successful results have been reported. Now, this procedure can be successfully offered to fertility preservation programs for patients who are in danger of losing their ovarian function due to medical or social reasons, and to oocyte donation programs. This vitrification technique has also been applied to cryopreserve oocytes obtained from IVM program. Some advantages of oocytes vitrification related with IVM are: (1) eliminating costly drugs and frequent monitoring; (2) completing treatment within 2 to 10 days (3) avoiding the use of hormones in cancer patients with hormone-sensitive tumors; and (4) retrieving oocytes at any point in menstrual cycle, even in the luteal phase. In addition, immature oocytes can also be collected from extracorporeal ovarian biopsy specimens or ovaries during caesarian section. Theoretically, there are two possible approaches for preserving immature oocytes: oocyte cryopreservation at the mature stage (after IVM) and oocyte cryopreservation at the Germinal Vesicle (GV)-stage (before IVM). Both vitrification of immature oocyte before/after IVM is not currently satisfactory. Nevertheless, many IVF centers worldwide are doing IVM oocyte cryopreservation as one of the options to preserve fertility for female cancer. Therefore, more studies are urgently required to improve IVM- and vitrification method to successfully preserve oocytes collected from cancer patients. In this review, present oocyte maturation mechanisms and recent progress of human IVM cycles will be discussed first, followed by some studies of the vitrification of human IVM oocyte.

Keywords: cancer, fertility preservation, immature oocyte, *in vitro* maturation, vitrification

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INTRODUCTION

Fertility preservation is a technique which may prolong the ability to conceive, either for medical or social reasons. Cancer is a major health concern, though survival rates have increased as treatment methods are improving, especially in young people (1). However, treatment of these cancers is commonly detrimental to reproductive function, with a high risk of losing one's fertility even after recovery, especially for young cancer survivors (2, 3).

Today, it is possible to preserve reproductive potential for these cancer patients using cryopreservation technic of either embryos, oocytes, or ovarian tissue (4). Embryo cryopreservation is a well-established routine procedure in IVF clinics worldwide and is the option with the best chances of reproductive success in the future that is offered to female cancer patients (5). However, since it requires a sperm source, many young single women are not able to choose this option unless they want to use donor sperm. In addition, there may be other various constraints to produce embryos and subsequently store them due to ethical, religious and social reasons (6).

In these cases, oocyte cryopreservation is an alternative option to embryo freezing (6). Although the first live birth using slow-cooling method was reported in 1986 (7), it remains technically challenging and has yet to become a routine procedure in IVF laboratories until oocyte vitrification method is properly established (8, 9).

Oocyte vitrification now offers increased success rates in comparison to slow freezing worldwide. Several IVF centers in the world have reported similar pregnancy rates between fresh- and vitrified-oocytes matured *in vivo* obtained in IVF cycles (10–13). The American Society of Reproductive Medicine (ASRM) and American Society of Clinical Oncology (ASCO) have endorsed oocyte cryopreservation as a "fertility preservation strategy for women with cancer and other illnesses requiring treatments that pose a serious threat to their future fertility" (14, 15). Nevertheless, oocyte cryopreservation requires ovarian stimulation, thus potentially resulting in a delay in cancer treatment.

Other fertility preservation methods include ovarian tissue cryopreservation and *in-vitro* maturation (IVM). Both methods can be performed without a delay in cancer treatment even for prepubertal girls. Ovarian tissue cryopreservation method requires two surgical procedures such as harvesting and orthotopic transplantation of the tissues after thawing (16). There is also the risk of reintroducing malignant cells at transplantation (16, 17).

IVM oocyte cryopreservation involves the retrieval of immature oocytes from ovaries after minimal or no gonadotropin priming and then either cryopreservation at immature stage or at matured stage after IVM. There are several advantages of IVM such as simplified treatment, reduced cost and avoidance of potential side effects such as ovarian hyperstimulation syndrome (OHSS). IVM program has already been offered to women with polycystic ovary syndrome (PCOS) to avoid the risk of developing OHSS caused by exogenous gonadotrophin stimulation. Although the clinical outcome is still suboptimal, improved pregnancy rates in IVM cycles of PCO(S) patients have recently been reported by some centers to be 32.4 and 46.7% clinical pregnancy per embryo transfer (18, 19). Based on these results, currently, IVM technique has also been applied for women with poor response to ovarian stimulation (20, 21) and for women who need fertility preservation urgently (22–24). However, IVM procedure is still considered as experimental (25) and small number of IVF centers are doing this procedure worldwide since antagonist cycle, GnRH agonist triggering, and elective cryopreservation strategies have been improved.

Although vitrification of oocytes retrieved from IVF cycles has been used successfully in the oocyte donation and fertility preservation programs, controlled ovarian stimulation for IVF is contraindicated for patients with certain forms of cancer. In addition, many cancer patients do not have enough time to do an IVF cycle before beginning chemo- or radiation-therapy. In these cases, immature oocyte collection can be an alternative (**Figure 1**) (26). However, very few live births have been reported after IVM oocyte cryopreservation. The first live birth was reported after oocyte cryopreservation using the slow-cooling method at the immature Germinal Vesicle (GV)-stage oocytes retrieved from conventional IVF cycles (27). Following this, the McGill Reproductive Center reported 5 pregnancies with live births after vitrification at MII-stage after IVM of immature oocytes collected from hCG-primed IVM cycles (28). However, as of yet, there have been no reports of successful pregnancies or live births after cryopreservation of IVM oocytes using either slow-cooling or vitrification method for cancer patients.

Therefore, it is important to understand oocyte maturation mechanisms and the current status of the human IVM program in order to make improvements in this program and in IVM oocyte vitrification procedures. In this manuscript, we will review our understanding of oocyte maturation mechanisms and recent advances in the field of human IVM cycles. Following this, some studies of the vitrification of human IVM oocytes will be discussed.

METHODS

This review is based on material published found via an electronic search of PUBMED between January 1996 and January 2019. We included articles that were published in English language for studies regarding fresh and cryopreserved oocytes produced from IVM program.

Oocyte Maturation

It is important to understand the mechanism of oocyte maturation *in vivo* in order to obtain "high quality oocytes" *in vitro*. Oocyte maturation consists of nuclear maturation and cytoplasmic maturation (29). Oocyte nuclear maturation implies the re-initiation of the first meiotic division and progression to metaphase two. This process can be divided into several parts including meiotic resumption/germinal vesicle breakdown (GVBD), chromatin condensation, formation of the meiotic spindle, separation of chromosome with extrusion of first polar body, and meiotic re-arrest before fertilization (29). Cytoplasmic maturation involves metabolic structural changes in the organelle that will lead to successful fertilization and early embryo development (29).

Oocyte maturation *in vivo* is a complex process regulated through hormonal signals, interactions with surrounding somatic cells, and involvement of transcription factors regulating gene expression (30). It has been established that meiotic arrest until luteinizing hormone (LH) surge is regulated by cyclic adenosine 3', 5'-monophosphate (cAMP) levels within the oocyte. The high level of cAMP is mainly controlled by gap

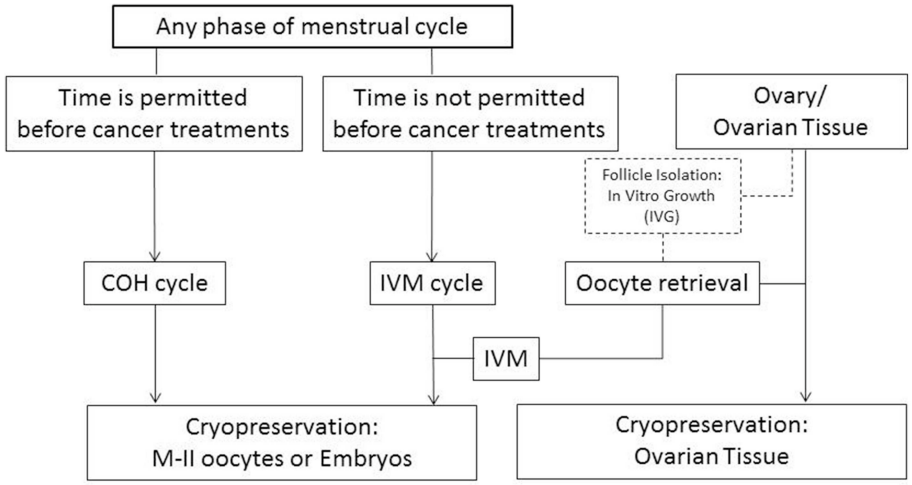

FIGURE 1 | Fertility preservation strategies for female cancer patients depending on their clinical situations. COH, controlled ovarian hyperstimulation; IVM, *in vitro* maturation. Discontinuous lines/box: experimental procedure.

junction connection between oocytes and cumulus cells (CC), and between CC themselves (30). Each gap junction is composed of connexin (Cx) proteins such as Cx37 and Cx43 (30). *In vivo*, there are three mechanisms to maintain the high cAMP level within the oocyte before the LH surge. (1) cAMP enters the egg from CC through the gap junction (31). (2) cAMP is produced by the oocyte itself via G-protein coupled receptors in the oocyte membrane (32–34). (3) Guanosine 3′,5′-cyclic monophosphate (cGMP), which is produced in the mural and cumulus granulosa cells via the activity of the guanylate cyclase natriuretic peptide receptor 2 (NPR2) by C-type natriuretic peptide (CNP), passes through gap junctions into the oocyte (35–39), where it inhibits hydrolysis of cAMP by the phosphodiesterase 3A (PDE3A:oocyte specific phosphodiesterase) (40). The high intra-oocyte cAMP concentration inactivates meiosis promoting factor and thus blocks meiotic progression.

After the LH surge, oocyte maturation is induced by cascade signaling pathways as well as physiological changes in preovulatory follicles *in vivo*. Mural granulosa layers express LH-receptors (LH-R) in much higher numbers than those in CC. Therefore, LH activation of mural granulosa cells induces the expression of the EGF-like growth factors such as betacellulin, amphiregulin, and epiregulin, as second signals (41, 42). The EGFs bind to their receptors in cumulus cell and mitogen-activated protein kinase (MAPK) in CC is activated immediately. The increased activation of MAPK may achieve meiosis resumption by inducing synthesis of downstream meiosis resumption-inducing factor(s) as well as blocking the gap junction via phosphorylation of gap junction proteins. In addition, the LH surge inactivates NPR2 and activates cGMP phosphodiesterase PDE5, and induces a rapid drop in follicular and oocyte cGMP levels resulting in decreasing cGMP supply to the oocyte (37, 38, 43, 44). Physiologically, a Graafian follicle rapidly increases in volume by inflow and accumulation of follicular fluid. Concomitantly, the cumulus oocyte complex (COC) synthesizes a muco-elastic extracellular matrix (ECM). This brings about volumetric enlargement called cumulus expansion which is important to oocyte. Hyaluronan (HA) is mainly involved in the cumulus cell expansion after the LH surge *in vivo* and HA is synthesized by HAS2 (Hyaluronan synthase) in the plasma membrane and directly extends into the ECM (45–47). The gap junctions in the COCs are disrupted by the cumulus cell expansion which stops the transport of cAMP through the gap junction, and leads to activation of MPF and meiotic resumption of oocytes (48, 49). Oocyte itself is also actively involved in HA synthesis related with cumulus expansion while secreting soluble factors. Growth differentiation factor-9 (GDF-9), bone morphogenetic protein 15 (BMP-15), and BMP-6 are likely candidate molecules for oocyte-secreted factors (50, 51). These growth factors induce HAS2 gene expression and cumulus expansion in the presence of FSH (50, 52).

Clinical Application of Human IVM Program

There are differences in the process of oocyte maturation *in vivo* and *in vitro*. *In vivo*, although a follicle gets dominance and grows to Graafian follicle, the fully grown oocyte inside of the follicle remains arrested in GV-stage (prophase I) until LH surge (53). However, the immature oocytes retrieved from small antral follicles start nuclear maturation spontaneously *in vitro* (53). This spontaneous maturation causes a premature breakdown of oocyte-cumulus cell gap junctions, leading to a loss of beneficial cumulus cell metabolites, such as mRNA, proteins, substrates, and nutrients that are required to achieve successful fertilization and embryo developmental competence (53). Researchers think that is the main reason why IVM oocytes have lower reproductive potential than those of IVF oocytes in general (53). Therefore, some studies have tried to mimic *in vivo* systems by the approach of delaying or temporarily preventing spontaneous IVM with CNP (54) or with chemicals such as cAMP analog, kinase or PDE inhibitors (53, 55–58). However, the effect is still unknown and more research is required.

In the human IVM program, *in vivo* stimulation with gonadotropins has been commonly used to improve the quantity and quality of oocytes such as FSH-, hCG-, and combined FSH-hCG-primed IVM cycles (59). In IVM cycles at McGill, hCG-priming is performed prior to egg collection in IVM cycles.

The hCG-primed IVM program has improved following several studies related to both clinical and embryological aspects over the last several years (60).

Clinical Protocol

In the routine IVM procedure, a baseline ultrasound scan (US) is performed for all patients between Days 2 and 4 of the menstrual cycle to confirm that no ovarian cysts are present and to assess the antral follicle count (AFC). A transvaginal US is repeated on Days 8–12 of the cycle until the dominant follicle reaches ≤12 mm, and the endometrial thickness is at least 6 mm, after which 10,000 IU hCG is administered.

In the case of fertility preservation for cancer patients, immature oocyte retrieval is performed at any point in the menstrual cycle depending on timing of chemotherapy, either in the early-, late-follicular, or luteal stage when hCG priming was performed (22).

Oocytes retrieval is performed 38 h after hCG administration with a specially designed 19-gauge single-lumen aspiration needle (K-OPS-7035-RWH-ET; Cook, Australia). The aspiration pressure is 85 mmHg.

Laboratory Procedures

Laboratory process of IVM cycles is more time consuming and technically demanding than IVF cycles (60). Therefore, the embryologists should obtain adequate training from an individual skilled in this technique. It is important also to define the best conditions for the laboratory procedures in IVM cycles in order to increase clinical outcomes.

At the time of collection, the follicular aspirate is first examined under a stereomicroscope to identify COC. After, the follicular aspirate is filtered through a cell strainer composed of nylon mesh with 70-μm pores to identify more oocyte with few CC.

In the IVM cycles treated with hCG priming, *in vivo* mature oocytes may be retrieved at oocyte collection (60). In the IVM cycles without hCG priming, no *in vivo* mature oocytes can be retrieved at collection or on the day of retrieval.

The complex culture media as the basic IVM media have been used in research or in clinical purposes of human immature oocyte culture for IVM (60). Recently, commercialized IVM media such as SAGE (Coopersurgical) IVM medium and Medi-Cult IVM medium have been used in several IVF centers as they have the advantage of being certified as IVF quality controlled (61–63). However, no IVM medium is superior to other media. You could choose an IVF media as a convenient basic media such as blastocyst media (61, 63). Serum albumin and gonadotropins are typically supplemented to the IVM medium. After Day 1 (24–30 h) to Day 2 (48 h) culture, matured oocytes are cryopreserved using vitrification method or fertilized with partner sperm.

Recently, efforts have been made to try and improve human IVM culture system in order to mimic *in vivo* maturation process such as the introduction of 3-D culture systems (57, 58, 64), using C-Type Natriuretic Peptide (CNP) to keep gap junction for a certain time before starting oocyte maturation *in vitro* (54), and adding EGF-like growth factors (amphiregulin and epiregulin) (65) or oocyte secreting factors (GDF-9 and BMP-15) (66, 67) to culture medium.

In cases of cryopreservation of IVM embryos, historically, intracytoplasmic sperm injection (ICSI) has been used to inseminate the oocytes matured *in vitro* in order to increase the chances of fertilization due to potential zona pellucida hardening (68, 69). ICSI is performed more than 1 h later after the first polar body extrusion based on a report (70). Culture conditions for fertilized embryos generated from IVM oocytes are the same as those in IVF cycles.

Cryopreservation of Oocytes

Cryo-injuries may occur at all phases of the cryopreservation process such as chilling injury (+15°C ∼ −5°C), ice crystal formation (−5°C ∼ −80°C) and facture damage (−50°C ∼ −150°C) depending on the temperature (71). Therefore, it is important to know the causes and mechanisms of cryo-damage in order to develop the optimal cryopreservation method.

There are two general approaches to prevent these cryo-injuries: slow-cooling and vitrification. The major difference is initial concentration of cryoprotectants and variation in cooling procedures (71). In the slow cooling method, they use a low concentration of cryoprotectants and very slow cooling rate using specialized equipment which can control the temperatures to avoid ice crystallization formation inside the cells (71). Compared to the slow-cooling method, in the vitrification, high concentration of cryoprotectants and ultrarapid cooling are used to prevent the ice crystal formation in and out of the oocyte/embryos (71, 72). Therefore, vitrification takes only a few seconds to cool oocytes or embryos after exposing them to cryoprotectants, and does not require expensive specialized equipment. Recently, many cryo-devices as a carrier have been used to increase cooling and warming rates resulting in significant increases in the success rate of human oocyte vitrification (71, 72).

Since the slow-freezing method is inefficient and inconstant, vitrification has gradually replaced slow-freezing during the past decade as a main cryopreservation method based on comparable success rates with fresh oocytes in IVF program (12, 73, 74). In addition, the vitrification method has also been applied to cryopreserve oocytes or embryos generated from IVM program (75).

IVM Program for Fertility Preservation

Some advantages to cryopreserve oocytes generated from IVM program for fertility preservation of cancer patients are as follows;

1) Eliminating costly drugs and frequent monitoring.
2) Since IVM treatment does not need high gonadotropin stimulation, it takes no more than 48 h from the decision to perform oocyte retrieval (76). However, a stimulated IVF

cycle requires more days until oocyte retrieval even though stimulation starts any phase of menstrual cycles. Therefore, when patients cannot delay chemotherapy IVM treatment may be a good option (**Figure 1**).

3) Avoiding the use of hormones in cancer patients with hormone-sensitive tumors. As mentioned previously, *in vivo* matured oocytes obtained from IVF cycles can be easily cryopreserved using vitrification methods with high success rates for fertility preservation. However, in some patients who have some special medical conditions or certain cancers, such as hormone-sensitive cancers, the high levels of estrogen during gonadotropin hyperstimulation are worrisome to both physicians and patients, even though several modified ovarian stimulation protocols with antiestrogen therapy have been developed (77–80). At McGill, around 70% of women who had frozen oocytes/embryos using IVM program for fertility preservation were patients with breast cancer (81).
4) Retrieving oocytes at any phase of the menstrual cycle, even in the luteal phase, without affecting the quantity and quality of the immature oocytes (22, 82). In cancer patients who are subject to time limits, immature oocyte retrieval in the luteal phase can be considered before cancer treatment in order to maximize the possibility of fertility preservation. According to our data, we did not find any significant differences in the number of oocytes retrieved, maturation and fertilization rates, or total number of available oocytes and embryos to vitrify when immature oocyte retrieval was performed during the early-, late- follicular phase compared with the luteal phase of the cycle for fertility preservation (22). There is evidence that pregnancy has occurred after donation of immature oocytes, which had been exposed to high progesterone level, retrieved from ovaries during cesarean section (83). In IVF programs, it has already been demonstrated that there is no difference of the number of oocytes collected, fertilization, their embryo developmental potential and clinical outcomes between oocytes generated from stimulations started during any phase of the menstrual cycle (84, 85).
5) The ability to harvest immature oocytes from ovarian biopsy specimens (86). Sometimes, several visible antral follicles are present on ovarian tissue biopsied for cryopreservation for fertility preservation. In this case, retrieving immature oocytes from the follicles using a syringe is an additional benefit to maximize fertility preservation (24, 86, 87). After applying this strategy, actually, a few successful live birth cases have been reported after cryopreservation of IVM embryos obtained from the ovarian tissue of cancer patients (88, 89). In addition, immature oocytes can be aspirated from the ovaries during caesarian section for the women who have cancer during pregnancy for fertility preservation (90, 91).
6) Combined *in vitro* growth (IVG) of isolated small follicles and *in vitro* maturation of the immature oocytes.

As mentioned previously, there is the risk of reintroducing malignant cells at transplantation of cryopreserved tissue (16, 17). In these cases, as an alternative, follicle isolation, *in vitro* growth (IVG) of the follicles and *in vitro* maturation of the immature oocytes would be another strategy to minimize the risk. Ideally, it is important to culture from primordial follicle stages. However, it is still challenging to establish the IVG culture system from the primordial follicle stages with no success in humans yet (92). On the other hand, M-II oocyte production has successfully been reported after combined IVG of secondary follicles isolated from fresh ovarian tissues and IVM of the immature oocytes. According to literature, human GV-stage oocytes have 3 different stages (93) and early antral follicle stage of GV-oocytes have lower maturation rate *in vitro*. Therefore, another option to preserve more mature oocytes would be *in vitro* growth (IVG) of secondary /early antral follicles isolated from ovarian tissue until meiotically competent GV oocytes are achieved, removed from the follicle and *in vitro* matured (92).

Theoretically, there are two approaches for preserving immature oocytes: oocyte cryopreservation at the mature stage (after IVM) and oocyte cryopreservation at the GV- stage (before IVM). The first successful pregnancy and live birth using immature human oocytes was after freezing at GV-stage oocytes (27), but further successful cases were from cryopreservation at MII-stage oocytes after IVM (28).

Some advantages and disadvantages in both approaches have been reported.

Table 1 shows characteristics of GV-stage and MII- stage oocytes. MII-stage oocyte has chromosomes attached with meiotic spindles. These microtubules are considered prone to damage at low temperature (chilling injuries), and the spindle dysfunction increases the risk of aneuploidy caused by chromosome misalignment. There is therefore a potential risk of affecting the meiotic spindle during vitrification and warming procedures in the MII-stage oocytes. It is possible that GV-stage oocytes are more stable when cryopreserved than MII-stage of oocytes, since they does not have temperature-sensitive meiotic spindle and their nuclear membrane could protect prophase-I chromatin genetic material during cryopreservation. In addition, the first successful live birth with immature oocytes was after freezing at GV-stage oocytes (27). Paradoxically, however, increasing chromosome and spindle abnormalities were observed in MII-stage oocytes matured *in vitro* after cryopreservation at GV-stage oocytes using slow-cooling method (8). This is probably due to lower cell membrane stability in immature oocytes associated with membrane lipid phase transition temperature (94). In addition, some data showed that aneuploidy rate was not increased after vitrification of human oocytes matured *in vivo* or *in vitro* (95, 96).

TABLE 1 | Different characteristics of oocyte between GV- and MII- stage.

Characteristics	GV-stage	MII-stage
Meiotic spindle	−	+
Nuclear membrane	+	−
Cell membrane lipid stability	Lower	Higher
Cumulus cells - cell size - gap junction	+	−

While MII-stage oocytes do not require CC to be attached to the oocyte at cryopreservation, GV stage oocytes need CC in order to get nutrients and regulatory molecules through gap junctions after thawing/warming, which are needed for oocyte maturation and further embryo development (97). However, there are two problems when trying to preserve these physiological interactions. The first, since there is a difference in the surface/volume ratio between oocyte and CC, the optimal exposure times and concentrations of cryoprotectants for equilibration are likely to be different and so it is difficult to make optimal cryopreservation conditions for both cell types. The other problem is that immersion GV-stage oocytes with CC in hypertonic cryoprotectants cause cell shrinkage of both oocyte and CC leading to disruptions in the gap junction between the oocyte and CC during cryopreservation (98).

Studies Concerning Human IVM Oocyte Vitrification

Several studies have been published in the field of IVM oocyte vitrification.

Chung et al. (95) compared survival and embryo development after vitrification of different stages of oocytes retrieved from unstimulated or stimulated ovaries. The immature oocytes collected from unstimulated ovaries were vitrified either at GV- or MII-stages after IVM. The immature oocytes retrieved from stimulated ovaries (conventional IVF cycles) were vitrified at GV-, GVBD- (MI-), or MII-stages after IVM for different time periods. After warming, they compared survival, maturation and embryo developmental competence in the oocytes vitrified at different stages. Although the number of samples was too small to get statistical significance in both sources of immature oocytes, in the immature oocytes collected from unstimulated ovaries, there were no differences in the rates of IVM, survival and blastocyst development between oocytes vitrified at GV- (before) (63.2, 63, 43%) and MII- (after) stage (69.6, 56, 40%), respectively. However, in the immature oocytes collected from conventional IVF cycles, the rates of survival, fertilization and blastocysts development were better in the group where oocytes were vitrified at M-II stage (100, 83.3, 40%) compared to GV- (65, 66.7, 33.3%) or GVBD-stage (64.2, 55.6, 20%), respectively. In addition, IVM was affected after vitrifying the oocytes at GV- or GVBD-stage. The blastocysts produced from each group had normal chromosome competency. This study showed different embryological results depending on the source of immature oocytes obtained from either stimulated or unstimulated ovaries.

Cao et al. (99) compared embryological aspects between oocytes before and after IVM of immature oocytes obtained in IVM cycles. This study included PCOS patients who were given clomiphene and hMG for 5 days and primed with hCG when leading follicles reached 8 to 10 mm. Egg collection was performed 36 h after administration of 10,000 IU hCG. The retrieved oocytes were divided into three groups; control fresh *in vitro* matured M-II, before IVM (GV stage) and after IVM (M-II stage) groups. They did not observe any difference in the survival rate between oocytes vitrified at GV- and MII-stages (85.4% vs. 86.1%). However, there was a significant difference in the IVM rates between immature oocytes with and without vitrification. Higher IVM was obtained in the oocyte without vitrification (85.4%) than that of vitrified immature oocytes (50.8%). Better quality embryos at the cleavage stage were produced from the group where oocytes were vitrified at M-II stage (33.3%) than the group where oocytes were vitrified at GV-stage (12.2%). In both groups, the embryo developmental competence was lower than that of fresh IVM oocytes (49.3% of good quality and 46.3% of blastocysts developed). Therefore, from this study it would appear that vitrifying oocytes at the M-II stage, after IVM, improves the chance of success compared with freezing them at the GV stage and *in vitro* maturing them after warming. However, they did not assess the oocyte maturity at the time of collection. As mentioned before, IVM cycles related with hCG priming can induce oocyte maturation *in vivo* and it would be possible to collect some M-II- and GVBD-stage oocytes even from follicles <10 mm in diameter. According to the published literature, over 50% of oocytes are retrieved from follicles sized 10 mm diameter at oocyte retrieval, even in conventional IVF (100). Therefore, this study needs to be confirmed.

Conversely, Wu et al. (101) reported embryo developmental competence as being similar between oocytes with and without vitrification in GV-stage oocytes collected from unstimulated ovaries, even though survival rate was low after vitrification (59.0%). Similarly, Al-khtib et al. (102) observed that the IVM rate of GV-stage oocytes retrieved from stimulated ovaries was similar with and without vitrification (75.5% vs. 70.8%).

Chang et al. (103) compared survival and embryo development after vitrification between oocytes matured *in vivo* and *in vitro* in oocyte donation (OD) IVF cycles and reported that there was no difference in survival rates. However, embryo development was better in embryos generated from oocytes matured *in vivo* than *in vitro*. Nevertheless, it is difficult to know whether the lower embryo development of the oocytes matured *in vitro* was because of oocyte innate characteristics or because of the vitrification and warming process. In general, the embryos generated from immature oocytes in conventional IVF cycles have less developmental potential than sibling embryos produced from *in vivo* matured oocytes.

Fasano et al. (104) used GV- and GVBD (MI)-stage oocytes obtained from ICSI cycles in order to compare oocyte survival, maturation and embryo developmental competence between oocytes vitrified before and after IVM of immature oocytes. Although the survival rate of oocytes after warming was similar (86.9% vs. 84.5%), maturation rate was significantly higher in the oocytes matured *in vitro* before vitrification (46%) than that of oocytes vitrified at immature stages before IVM (23.8%) ($P < 0.01$). After insemination, the fertilization and embryo developmental rates were not significantly different, but no blastocysts were produced in both groups. Accordingly, vitrifying oocytes after IVM was more efficient than that of IVM after vitrification to get more MII oocytes to inseminate. This is because vitrifying oocytes at GV-stage affect their IVM capability negatively after warming.

However, Molina et al. (105) presented different results showing higher IVM rate after vitrifying oocytes at GV-stage than that of control fresh IVM oocytes obtained from stimulated IVF cycles. Although activation rate was lower, activated oocytes from the group where oocytes were vitrification at GV-stage developed more embryos compared to the group where oocytes

were vitrified at MII after IVM. Therefore, vitrifying GV-stage oocytes seemed to be better than that of MII-stage in terms of IVM rate and embryo development.

Song et al. (106) were also using GV- and GVBD (MI)-stage oocytes generated from IVF cycles. Control was oocyte matured *in vivo* without vitrification and vitrification groups were where vitrification was performed before or after IVM of immature oocytes. After warming, cleavage rate was significantly higher in the oocytes vitrified at MII-stage after IVM than the group where oocytes vitrified at immature stage before IVM ($P < 0.05$). However, there were no statistical differences in fertilization, embryo developmental competence and aneuploidy rate in the three groups. This study indicates that oocytes at MII-stage after IVM were more suitable to vitrify than oocytes at immature stages.

Kasapi et al. (107) used GV oocytes collected from stimulated donation cycles. Oocytes were either vitrified at the GV stage or at MII-stage after IVM. Control group was oocytes matured after IVM without vitrification. There were no significant differences in the survival rate or incidence of normal spindle/chromosome configurations in oocytes matured *in vitro* before or after vitrification. A higher incidence of normal spindle/chromosome configurations existed in the fresh IVM oocytes. However, a significantly higher maturation rate was obtained in the group where oocytes were vitrified after IVM (82.9%) compared to oocytes vitrified at GV stage (51%). Their study also demonstrated that vitrification of *in vitro* matured MII-oocytes obtained from stimulated cycles was more efficient than GV oocytes vitrification.

Table 2 summarizes these 9 studies. There were differences among the groups in terms of devices, solution, source of immature oocytes, and IVM culture system. In addition, sample sizes in most of studies were not enough. Therefore, it appears difficult to draw general conclusions from these studies. However, in stimulated cycles, it seems that vitrifying oocytes at MII-stage after IVM is better than at GV-stage in terms of IVM rate after warming, even though Molina et al. (105) reported a reverse result. This needs to be verified in oocytes obtained from unstimulated cycles since two studies in **Table 2** showed no difference of IVM rate with and without vitrification at the GV stage. Nevertheless, they were overall similar in the rates of survival, fertilization and embryo developmental potential. However, the studies show that vitrification/warming of oocytes either at GV- or MII-stages severely affect their fertilization and embryo developmental potential compared to fresh oocytes. This is evidence that oocytes obtained from IVM

TABLE 2 | Summary of IVM oocyte vitrification studies.

References	Carrier	Cryo-solution	Source of immature oocytes	IVM media	Affect on IVM after vitrifying at immature stage	Survival rate	2 PN	Embryo development
Chung et al. (95)	EM-grid	EG	Unstimulated IVF	TCM-199 + PMSG + HCG	No Yes	GV≈MII GV< MII	GV≈MII GV < MII	GV≈MII GV < MII
Cao et al. (99)	Cryoleaf	EG +PROH	CC+HMG	TCM-199 + FSH + HCG	Yes	GV≈MII	GV≈MII	GV < MII
Wu et al. (101)	EM-grid	EG	Unstimulated	Ham's F10 + HMG	No	59%	Frozen ≈ Fresh	Frozen ≈ Fresh
Al-khtib et al. (102)	High-security Straw	EG +PROH	IVF	Medicult IVM + FSH + HCG	No	55.4%	NA	NA
Chang et al. (103)	Cryotop	EG +DMSO	IVF	Fertilization Medium (SAGE)	–	*In vivo* MII ≈ *in vitro* MII	*In vivo* MII > *in vitro* MII	*In vivo* MII > *in vitro* MII
Fasano et al. (104)	High-security Straw	EG +DMSO	IVF	SAGE IVM medium + FSH + LH	Yes	GV/MI < MII	GV/MI ≈ MII	GV/MI ≈ MII
Molina et al. (105)	Cryo-tip	EG +DMSO	IVF	Medicult IVF medium + FSH + LH	No (Improve)	GV≈MII	GV < MII (by activation)	GV > MII (by activation)
Song et al. (106)	Cryoleaf	EG +PROH	IVF	SAGE IVM medium + FSH + LH	Yes	GV/MI≈MII	GV/MI < MII	GV/MI > MII
Kasapi et al. (107)	Closed carrier system (VitriSafe, VitriMed, Austria)	EG +DMSO	IVF	SAGE IVM medium + FSH + LH	Yes	GV≈MII	–	–

are more fragile for the cryopreservation compared to *in vivo* matured oocytes.

Again, there were different characteristics of immature oocytes depending on their source of origin and the method of vitrification. As shown in the Cao et al. (99) study, different results could be obtained in immature oocytes retrieved from different sources of oocytes with and without gonadotropin stimulation. Most of IVM cases for cancer patients are from unstimulated ovaries. Therefore, further investigations with immature oocytes retrieved from unstimulated ovaries are needed to improve fertility preservation options of IVM.

Based on overall results from these studies, we vitrified MII-stage oocytes after IVM in unstimulated IVM cycles at McGill (108). We recruited patients for IVF and IVM oocyte vitrification studies and compared the two groups. There were no differences in patient characteristics between two groups. Compared with *in vivo* MII-stage oocytes generated from IVF cycles, MII-stage oocytes obtained after IVM of immature oocytes in unstimulated regular IVM cycles had significantly lower survival (81.4% vs. 67.5%) and fertilization (75.6% vs. 64.2%) rates after vitrification ($P < 0.05$). In addition, implantation (19.1% vs. 9.6%), clinical pregnancy (44.7% vs. 20.0%), and live birth (39.5% vs. 20%) rates were lower in IVM-oocyte vitrification groups, but it was not statistically significant since the number of samples was not sufficient. From this study, it is difficult to determine whether the lower embryological and clinical outcomes in the oocytes from our IVM program is due to the low quality of IVM oocytes, our vitrification procedure, or both since there was no fresh IVM oocytes control group included in the study. In addition, no reproductive potential has been reported to compare fresh and vitrified IVM oocytes. Therefore, we analyzed embryological and clinical outcome of IVM oocyte vitrification with that of fresh IVM cycles performed during the same period (28).

We included 267 patients for the study, 56 patients were for IVM oocyte vitrification group and 219 patients were fresh IVM group during the same period. Oocyte maturation rates were similar between the groups. Survival rates of IVM oocytes after performing vitrification/warming was 59.8%. The rates of fertilization and embryo cleavage in the vitrified IVM oocytes (58%, 72%) were significantly lower compared to fresh IVM oocytes (72%, 90%) ($P < 0.01$). Clinical pregnancy (10.7% vs. 36.1%) and live birth rates (8.9% vs. 25.9%) were also significantly lower in the group where IVM oocytes were vitrified than those in the group where IVM oocytes were fresh ($P = 0.005$ and $P < 0.001$, respectively). Therefore, reproductive potential was negatively affected after vitrification of IVM oocytes. This implies that vitrification/warming itself could also induce some detrimental effects on IVM oocytes.

Given *in vitro* matured oocytes may never be equivalent to *in vivo* matured oocytes, it is necessary to modify vitrification methods/process appropriately for oocytes retrieved from the IVM programs, to improve survival and embryo developmental rates of IVM oocytes. There is evidence that *in vivo* matured oocytes collected from advanced aged women (>35 years old) also had lower survival rates (82.4%) after vitrification than those of oocytes collected from younger women (≤35 years old) (94.6%) (109). It indicates that oocyte quality is one of the main factors related with survival rates as well as clinical outcomes.

Actually, present vitrification methods have been adapted to use good quality *in vivo* matured oocytes from young women. Therefore, studies to improve survival and further embryological developmental competence of the oocytes retrieved from IVM program are urgently required in order to successfully apply them to IVM fertility preservation program for cancer patients.

CONCLUSIONS

Immature oocytes may be the answer for fertility preservation in the long term. Healthy live births can be achieved from the combination of IVM oocytes and vitrification even though no live births have been reported using cryopreserved oocytes generated from IVM program of cancer patients. The efficiency of IVM oocyte cryopreservation is still low and requires further improvements in IVM- or/and Cryo-technology. Different strategies may be implemented in order to improve the results such as improving oocyte quality by optimizing the *in vitro* culture conditions of immature oocytes and/or establish a more refined vitrification/warming procedure which can adjust to cellular properties of oocytes before/after IVM. Improved understanding of mechanisms regulating IVM and developing subsequent optimal IVM medium may give rise to more refined vitrification methods for oocytes before/after IVM. This will in turn help maintain the same oocyte quality before and after vitrification, resulting in improved quality of embryos and pregnancy rates.

AUTHOR CONTRIBUTIONS

W-YS's role included study design, data collection, statistical analysis, and manuscript writing. SH was involved in data collection and manuscript writing and review. YC's role was data collection and statistical analysis. MD was involved in study design and manuscript writing. WB's role included study design, manuscript writing, and review.

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Fertility Preservation for Child and Adolescent Cancer Patients in Asian Countries

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Background: At present, fertility is one of the main concerns of young cancer patients. Following this trend, "fertility preservation (FP)" has been established and has become a new field of reproductive medicine. However, FP for child and adolescent (C-A) cancer patients is still developing, even in advanced countries. The aim of the present study was to assess the barriers to FP for C-A patients by investigating the current status of FP for C-A patients in Asian countries, which just have started FP activities.

Method: A questionnaire survey of founding members of the Asian Society for Fertility Preservation (ASFP) was conducted in November 2018.

Main findings: Of the 14 countries, 11 country representatives replied to this survey. FP for C-A patients is still developing in Asian countries, even in Australia, Japan, and Korea, which have organizations or academic societies specialized for FP. In all countries that replied to the present survey, the patients can receive embryo cryopreservation (EC), oocyte cryopreservation (OC), and sperm cryopreservation (SC) as FP. Compared with ovarian tissue cryopreservation (OTC), testicular tissue cryopreservation (TTC) is an uncommon FP treatment because of its still extremely experimental status (7 of 11 countries provide it). Most Asian countries can provide FP for C-A patients in terms of medical technology, but most have factors inhibiting to promote FP for C-A patients, due to lack of sufficient experience and an established system promoting FP for C-A patients. "Don't know how to provide FP treatment for C-A" is a major barrier. Also, low recognition in society and among medical staff is still a particularly major issue. There

is also a problem with cooperative frameworks with pediatric departments. To achieve high-quality FP for C-A patients, a multidisciplinary approach is vital, but, according to the present study, few paramedical staff can participate in FP for C-A patients in Asia. Only Australia and Korea provide FP information by video and specific resources.

Conclusion: The present study demonstrated the developing status of FP for C-A patients in Asian countries. More intensive consideration and discussion are needed to provide FP in Asian societies based on the local cultural and religious needs of patients.

Keywords: fertility preservation, child cancer patients, ovarian tissue cryopreservation, oncofertility, Asia

INTRODUCTION

Based on the Global Burden of Disease study, cancer incidence rate continues to increase in the world including Asian countries (1). Also, incidence of childhood cancer is increasing (2). Development of cancer therapy has resulted in increasing numbers of cancer survivors. In particular, more than 70% of child cancer patients will be cancer survivors (3). Unfortunately, one in 10 cancer patients experience fertility due to impairment in ovarian or testis function, as a result of the gonadotoxic treatments (chemotherapy and radiation therapy) as cancer therapy. Recently, several reviews of fertility preservation (FP) which based on assured clinical study have indicated the risk of infertility associated with specific diseases and therapies among different age groups. Especially, high-dose Alkylating agent represented by Cyclophosphamide may cause serious damage to gonads (4, 5). In addition, cancer itself and cancer treatment could cause the sexual dysfunction due to physical and psychological problems for cancer survivors (both men and women) including survivors of childhood cancer. To begin with, couple infertility and sexual dysfunction are highly prevalent in general population. Therefore, cancer and cancer treatment have possibilities getting worse this contemporary condition (6–8).

For adult patients with cancer, fertility preservation treatments have been established to improve quality of life for cancer survivors. In 2006, the "Oncofertility consortium" and "FertiPROTEKT," which are representative associations to promote FP for young cancer patients, were established (9). The "International Society for Fertility Preservation (ISFP)" was established in 2009 as the first academic society specialized in FP treatments. Additionally, the "Japan Society for Fertility Preservation (JSFP)" and the "Fertility Preservation Society of India (FPSI)," and the "Asian Society for Fertility Preservation (ASFP)" were founded in 2012 and 2014, and 2015, respectively. Also, Australasian Oncofertility Consortium started 2015. As a consequence of efforts or actions to promote FP by these organizations, FP is now becoming a new field of reproductive medicine.

Based on the latest guideline that was updated by the American Society of Clinical Oncology (ASCO), only oocyte and embryo cryopreservation is endorsed as an "established method" for fertility preservation for female patients who face a threat to their own fertility due to cancer treatment (10). Meanwhile, ovarian tissue cryopreservation (OTC) is still an "experimental method" according to this guideline, although many experts believe that OTC fulfills the criteria for an "established method" (11, 12). The indications for OTC are specifically FP for child and adolescent patients and adult patients who do not have enough time to receive another fertility preservation treatment (5, 10, 11). Based on the literature, around 1,000 cases per year of oocyte cryopreservation (OC) for serious medical reasons and until now, more than 4,500 cases of OTC are performed in Europe (13), and more than 1,000 cases of OC and 200 cases of OTC are performed in Japan as FP (2006-2016, unpublished data). For male cancer patients, sperm cryopreservation before receiving chemotherapy is strongly recommended as the sole effective FP treatment. Hormonal therapy is not recommended as FP treatment for men. Testicular tissue cryopreservation (TTC) with later re-implantation is considered a highly experimental method (10). To determine the FP procedure for child and adolescent (C-A) patients, sexual maturity as we say "puberty" is one of important factors. It is menarche for female and spermarche for male. Generally, OC is the FP procedure which method is the most likely to result in subsequent pregnancy, but this is only for post-menarchal females (those who have begun to menstruate) since it would require developing follicles. Therefore, for pre-pubertal females, OTC is the only FP option. As a FP options for males, sperm cryopreservation is the most established option and should be offered to all peri- and post-pubertal male adolescents with a fertility-threatening situation. Although the age at which to offer sperm cryopreservation is unclear, an adequate semen specimen can be obtained in adolescents as young as 11 years of age. For pre-pubertal boys with lack of mature sperm, TTC is solely option as FP treatment (14, 15). At present, there are only two live birth cases from transplanted ovarian tissues that were cryopreserved before menarche, and there are no live birth cases from patients who underwent TTC (15, 16). In addition, there are few reports of OC for C-A patients. Even OC for late teenagers is still challenging because it needs ovarian stimulation with multiple hormonal injections and follicle monitoring using ultrasound, with subsequent oocyte retrieval under sedation or

Abbreviations: FP, fertility preservation; C-A, child and adolescent; ISFP, International Society for Fertility Preservation; ASFP, Asian Society for Fertility Preservation; JSFP, Japan Society for Fertility Preservation; FPSI, Fertility Preservation Society of India; ASCO, American Society of Clinical Oncology; JSCO, Japan Society of Clinical Oncology; EC, embryo cryopreservation; OC, oocyte cryopreservation; OTC, ovarian tissue cryopreservation; OTT, ovarian tissue transplantation; GnRHa, gonadotropin releasing hormone agonist; SC, sperm cryopreservation; TTC, testicular tissue cryopreservation; GDP, gross domestic product; ART, assisted reproductive technology.

anesthesia (these procedures need a transvaginal approach) (15, 17). For these reasons, FP for C-A patients is still uncommon compared with FP for adult patients, even in advanced countries performing FP although they have OTC and TTC cases for infant (18, 19). The aim of the present study was to assess the barriers to FP for C-A patients by investigating the current status of FP for C-A patients in Asian countries whom are members of the Asian Society of Fertility Preservation (ASFP).

MATERIALS AND METHODS

Survey Design

On November 2018, a survey was sent to country representatives of ASFP (Australia, China, Hong Kong, India, Indonesia, Japan, Korea, Philippines, Taiwan, Thailand, Vietnam, Pakistan, Singapore, Turkey) to collect information about the current status of FP services for child patients and the barriers that inhibit promoting this treatment. The participating countries gross national income per capita is very different (five high income countries, three upper-middle income countries, five lower income countries, and one with no data). The survey was approved by the institutional review board of our institution with revisions in keeping with the Declaration of Helsinki. The final version was sent by email to 14 contacts of the ASFP.

Potential survey participants were identified from existing members of the ASFP and international experts in the field. Potential participants received an email with an invitation to participate in the survey. Following the initial email, each participant received two reminders, one on November 1, 2018 and one on November 15, 2018, in order to maximize the number of responses.

Survey Inclusion/Exclusion

Surveys were excluded from the analysis if participants failed to provide contact or identification information, if the survey was left blank, or if duplicate responses were submitted.

Survey Questions

Survey participants were asked a total of 12 questions about the following areas: organization to promote FP treatment, patient access to medical professionals, current status of FP for adult and child patients, barriers that inhibit promotion of FP for C-A patients, and systems for providing information about FP for child patients. Three questions were dichotomous scaled questions (yes/no) with space for providing open-ended comments. Three questions were multiple-choice format, where only one answer could be selected. Four questions were multiple response questions, where participants could select one or more answers. One question was for free descriptive answer, and one was defining the priority order.

Analysis of Survey Results

Survey responses were exported to Microsoft Excel. The dichotomous and multiple response questions were coded with numerical values to facilitate statistical analysis.

Ethics Approval and Informed Consent

The present study was approved by the IRB of St. Marianna University (approval No. 4191, UMIN000035723). This survey is questionnaire survey targeted to medical professionals (representatives of society). On the explanation of this survey, we had written about consent to participate this survey at the front of questionnaires. We told them to reply when they could agree with participating this survey as participants.

RESULTS

Organizations to Promote FP, Patient Access to Medical Professionals, and Current Status of FP for Adult Patients (Table 1)

From the 14 countries, 11 country representatives replied to the survey. Of the 11 countries, five had organizations or academic societies to promote FP, and three countries (Australia, Japan, and Korea) had organizations or academic societies that are specialized for FP in the true sense, whereas two (China and Indonesia) had a committee or branch society of a large academic society in the area of reproductive medicine or maternal-child health medicine. Two countries (Hong Kong and Philippines) are planning to establish organizations or academic societies specialized for FP. Although most countries do not have aid funds or insurance for FP, only Australia has a registration system for FP which requires individual patient consent and partial financial assistance or insurance system (Medicare) covering extensive FP treatment [embryo cryopreservation (EC), OC, consultation, ovarian transposition, sperm cryopreservation (SC)]. Also, Korea has partial funds for FP treatment (EC only).

In all countries that replied to the survey, the patients can receive EC, OC, and SC as FP. Compared with OTC, TTC is uncommon FP treatment because of its still extremely experimental status. Therefore, even Australia, which is an advanced country for FP, has only one institution that has ethics approval for TTC although TESE can be done in post-pubertal patients in a number of centers if required.

Current status of FP for C-A Patients (Table 2)

All of Asian countries have experience of FP for C-A patients. However, in most countries, the opportunities for FP for C-A patients are limited compared with FP for adult patients, because all participants (except for Indonesia) chose "not so often" regarding opportunities for FP for C-A patients. The main reasons were "not enough information for physicians, oncologists, patients and family" and "lack of public awareness." Also, the numbers of facilities that can provide FP treatment for C-A patients are limited. Especially, in Australia, the facilities that can do OTC and TTC are strictly consolidated.

Barriers That Inhibit Promotion of FP for C-A Patients

To investigate the barriers that inhibit promotion of FP for C-A patients, multiple-choice questionnaires were prepared (**Table 3**).

TABLE 1 | Organizations to promote FP, patient access to medical professionals, and current status of FP for adult patients in Asian countries.

		Australia	China	Hong Kong	India	Indonesia	Japan	Korea	Philippines	Taiwan	Thailand	Vietnam
Specialized organization for FP		Yes	Yes	No (in planning)	No	Yes	Yes	Yes	No (in planning)	No	No	No
Name of the organization		FUTuRE Fertility	Chinese Maternal and Child Health Association	(Hong Kong Society of Reproductive Medicine)	FPSI (Fertility Preservation Society of India)	Indonesian Association for IVF	JSFP (Japan Society for Fertility Preservation)	KSFP (Korea Society for Fertility Preservation)	PSFP (Philippine Society of Fertility Preservation)	–	–	–
Aid fund or insurance for FP		Yes (several)	No	No (in planning)	No	No	No (in planning)	Yes (EC only)	No	No	No	No
FP for female	EC	Yes (100–199)	Yes (>200)	Yes (1–49)	Yes (>200)	Yes (1–49)	Yes (100–199)	Yes (1–49)	Yes (6)	Yes (1–49)	Yes (1–49)	Yes (1–49)
	OC	Yes (100–199)	Yes (>200)	Yes (1–49)	Yes (>200)	Yes (1–49)	Yes (100–199)	Yes (1–49)	Yes (6)	Yes (1–49)	Yes (1–49)	Yes (1–49)
	OTC	Yes (10)	Yes (1–49)	No	Yes (3)	Yes (1–49)	Yes (38)	Yes (1–49)	Yes (1)	Yes (1–49)	Yes (1–49)	No
	GnRHa	Yes (unknown)	Yes (>200)	Yes (rare) (1–49)	Yes (>200)	Yes (1–49)	Yes (not standard)	Yes (1–49)	Yes (6)	Yes (1–49)	Yes (1–49)	No
FP for male	SC	Yes (>200)	Yes (1–49)	Yes (1–49)	Yes (>200)	Yes (1–49)	Yes[a] (around 100)	Yes (1–49)	Yes (6)	Yes (1–49)	Yes (1–49)	Yes (1–49)
	TTC	Yes (1)	Yes (1–49)	No	Yes (>200)	Yes (1–49)	Yes (rare)	No	No	Yes (1–49)	No	No

FP, fertility preservation; EC, embryo cryopreservation; OC, oocyte cryopreservation; OTC, ovarian tissue cryopreservation; GnRHa, gonadotropin releasing hormone agonist; SC, sperm cryopreservation; TTC, testicular tissue cryopreservation. [a]*Based on literature (49). Adult patients can be provided EC, OC, and SC as FP in all countries. Compared with OTC, TTC is an uncommon FP treatment. Only Australia and Korea provide funds for FP for patients.*

Although there was variation in ranking, 9 of 11 participants identified "b: Low recognition among medical staff" as one of the major issues. Also, "f: Information is insufficient," "a: Low recognition in society," and "g: There is a problem with the cooperative system with the pediatrics department" were major reasons for inhibiting the promotion of FP for C-A patients (**Table 3**). Three of the 11 selected "e: There is technology, but we don't know how to provide it" and "j: Economically impossible." Only one participant from Thailand chose "k: It is not necessary because the adoption system is popular." As other comments, participants from Australia mentioned "weakness of evidence for FP for C-A patients." To improve the level of FP awareness, 3 of 11 participants (India, Japan, Korea) are providing opportunities for lecture presentations, oral presentations at scientific conferences, and education for parents or patients.

Framework for Providing FP Treatment for C-A Patients

To improve FP treatment for C-A patients, the kinds of specialists that provided FP for C-A patients were investigated, and 10 of 11 participants replied. In half of the countries (5 of 10), only a medical doctor could provide FP treatment for C-A patients. On the other hand, in four of five countries, nurses and/or psychologists could collaborate with the medical team in FP treatment for C-A patients. Although, patient navigators as independent position and child life specialists are not involved in FP for C-A patients, in Australia, nurses and psychologist are involved as patient navigators aiming to assist decision-making and psychological support. In addition, peer supporters including cancer survivors are not involved in FP treatment for individual cases (**Table 4**). However, as described below, patient consumer organization and Consumer Charter are collaborating with FP organization to develop FP in Australia (20). Also, JSFP have peer supporter group to promote FP.

Resources for Providing Information About FP for C-A Patients

All of the participants selected "Oral explanation" for informed assent, and "article" is used for informed assent as supplementary material (China, Japan, Philippines, Vietnam). To improve the quality of informed assent, Korea has animations about FP treatment, including sexual education. Only Australia has an "online or printed resource" and a "video a peer supporter has done" as "other" means (**Table 5**).

DISCUSSION

Improvement of the survival rate following childhood cancer has led to an increased focus on the late effects of cancer treatment (3, 21) and "fertility" is a prime concern for both female and male cancer survivors (3, 22) which can result in psychological distress (23). Although Asia consists of 48 countries that have various backgrounds in terms of culture, economic status, religion, and status of medical care, FP is becoming increasingly common as medical care. In particular, countries that participate in the

TABLE 2 | Current status of FP for C-A patients in Asian countries.

			Australia	China	Hong Kong	India	Indonesia	Japan	Korea	Philippines	Taiwan	Thailand	Vietnam
Experience with FP for C-A patients			Not very often	Not very often	Not very often	Not very often	Most of the time	Some of the time	Some of the time	Not very often	Not very often	Not very often	Not very often
Reason or comments			Routinely only two centers done	Not enough information	Not enough information, lack of oncology support	Oncologist and parents are reluctant to provide FP	Two centers can provide FP	Not enough information, patient's disease	Lack of information to physicians, parents, patients	Fertility-sparing surgery and radiation shielding are done	Lack of public awareness	Parents concerned about cancer treatment more than FP	Lack of information, FP for C-A patients have not been established
FP for female	Children (0–14 y.o)	OC	No	No	No	Yes (>200)	Yes (1–49)	Yes (rare)	Yes (1–49)	No	Yes (1–49)	Yes (1)	No
		OTC	Yes (4)	Yes (1–49)	No	Yes (3)	Yes (1–49)	Yes (less than 38)	Yes (1–49)	Yes (1)	Yes (1–49)	Yes (1–49)	No
		GnRHa	No	Yes (>200)	Yes (1–49)	Yes (>200)	Yes (1–49)	Yes (not standard)	Yes (1–49)	No	Yes (1–49)	–[b]	No
	Adolescents (≥15 y.o)	OC	Yes (100–199)	Yes (1–49)	Yes (1–49)	Yes (>200)	Yes (1–49)	Yes (not so many)	Yes (1–49)	Yes (6)	Yes (1–49)	–[b]	Yes (1–49)
		OTC	Yes (10)	Yes (1–49)	No	Yes (3)	Yes (1–49)	Yes (less than 38)	Yes (1–49)	Yes (1)	Yes (1–49)	–[b]	only for research
		GnRHa	Unknown	Yes (>200)	Yes (1–49)	Yes (>200)	Yes (1–49)	Yes (not standard)	Yes (1–49)	Yes (6)	Yes (1–49)	–[b]	No
FP for male	Children (0–14 y.o)	SC	Yes (4, 5)	No	Yes (1–49)	Yes (>200)	Yes (1–49)	Yes (rare)	Yes (1–49)	Yes (6)	Yes (1–49)	No	No
		TTC	Yes (1)	Yes (1–49)	No	No	Yes (1–49)	Yes (rare)	No	No	Yes (1–49)	No	No
	Adolescents (≥15 y.o)	SC	Yes (>200)	Yes (1–49)	Yes (1–49)	Yes (>200)	Yes (1–49)	Yes[a] (less than 100)	Yes (1–49)	Yes (6)	Yes (1–49)	Yes (1–49)	Yes (1–49)
		TTC	Yes (50–99)	Yes (1–49)	No	Mature tetsis only	Yes (1–49)	Yes (rare)	No	No	Yes (1–49)	No	No

FP, fertility preservation; EC, embryo cryopreservation; OC, oocyte cryopreservation; OTC, ovarian tissue cryopreservation; GnRHa, gonadotropin releasing hormone agonist; SC, sperm cryopreservation; TTC, testicular tissue cryopreservation.

[a] *Based on literature (49).*

[b] *Detailed number is unknown.*

The opportunities of FP for C-A patients are limited compared with FP for adult patients, because all participants (except for Indonesia) chose "not so often" for opportunities for FP for C-A patients. Also, the numbers of institutions that can provide FP treatment for C-A patients are limited.

TABLE 3 | Barriers to FP for C-A patients in Asian countries.

	Australia	China	Hong Kong	India	Indonesia	Japan	Korea	Philippines	Taiwan	Thailand	Vietnam
a		1		1		1	3		1	1*	1
b	2			4	1*	2	1	2	2	1*	2
c											4
d				3							
e			1*	4			4				
f		2	1*	4	1*	3	2	1			3
g		3	1*	2	1*		4		3		
h											4
i											
j	1							3		1*	
k										1*	
l											
m											
Other	3										

[a] *Low recognition in society.*
[b] *Low recognition among medical staff.*
[c] *Medical technology is behind.*
[d] *Family doctor does not agree with fertility preservation.*
[e] *There is technology, but we do not know how to provide it.*
[f] *Information is insufficient.*
[g] *There is a problem with the cooperative system with the pediatric department.*
[h] *Even the prevalence of fertility preservation for adults is still low.*
[i] *Prohibited/limited by law or academic society.*
[j] *Economically impossible.*
[k] *It is not necessary because the adoption system is popular.*
[l] *Regional disparity of medical technology is large.*
[m] *Religious reason.*
Other: Evidence for pediatrics is still limited (Australia).
[*] *The participants did not specify the priority order.*
Numbers are defined in order of critical factors as "Barrier." According to this multiple response question, "Low recognition in society and medical staff" is a major issue. Cooperative system with pediatrics department is also a big issue. Most countries have issues related to system barriers rather than technology.

TABLE 4 | Framework for providing FP treatment for C-A patients in Asian countries.

		Australia	China	India	Indonesia	Japan	Korea	Philippines	Taiwan	Thailand	Vietnam
Medical doctor	Oncologist and/or reproductive medicine specialist	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
	Pediatrician (Oncologist)	✓	✓	✓	✓	✓	✓			✓	✓
	Pediatrician (Other)		✓			✓					
	Pediatric surgeon	✓	✓			✓					✓
	Hematologist	✓	✓	✓	✓		✓			✓	✓
Paramedical staff	Nurse	✓				✓	✓				✓
	Social worker	✓					✓				
	Psychologist	✓		✓		✓					✓
	Patient navigator	✓									
	Child-life specialist	✓									
Others	Peer supporter	★				★					

★ *Australia and Japan have organizations which are consisted peer supporters and cancer survivors. However, it is difficult to attend FP treatment for individual cases.*
In half of the countries (5 of 10), only a medical doctor could provide FP treatment for C-A patients. On the other hand, 4 of 5 countries achieved a multidisciplinary approach.

ASFP and have specialized organizations for FP can provide contemporary FP treatment. Indeed, Australia is one of the advanced countries in the FP area, which has already established its own registration system and partial public funding for patients receiving FP treatment. Japan is also one of advanced country which has guideline of FP treatment collaborate JSFP with JSCO (Japan Society of Clinical Oncology) (24). JSFP may start a registration system for FP treatment within 1 year to understand

TABLE 5 | Resources for providing information about FP for C-A patients in Asian countries.

	Australia	China	Hong Kong	India	Indonesia	Japan	Korea	Philippines	Taiwan	Thailand	Vietnam
Oral explanation	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Illustrated book											
Article		✓				✓		✓			✓
Anime or movie							✓				
Other	✓										

In most countries, only "Oral explanation" is the main procedure for informed assent. "Article" is used for informed assent as supplementary material (China, Japan, Philippines, Vietnam). Korea has animation about FP treatment including sexual education. Only Australia has "online or printed resource" and "video peer supporter has done.

the present status of FP in Japan based on national survey for FP (25, 26). However, FP for C-A patients is not as common as FP for adult cancer patients (27). The present study data have shown that the numbers of hospital or institutions that can provide FP for C-A patients are much fewer than for adult patients. The reason for lower number of FP in C-A patients are multi-factorial (28).

Barriers to promoting FP treatment for C-A patients may be divided into "medical factors" and others. For female C-A patients, OC and OTC are options as FP treatments, with SC and TTC for male C-A patients. In general, the selection of FP treatments depends on the patient's pubertal status. For post-pubertal female patients, EC with OC is one of the options for FP treatment (15). Although OC has been the standard FP treatment for young or unmarried female patients since 2013 as per ASCO (5), it is uncertain whether will be acceptable OC for teenagers. In fact, reports of OC for post-pubertal female patients as FP are very few, and its status is challenging, as mentioned above. Some reports and clinical data already demonstrated that OC is a practical technology for children (17), but there are issues to be resolved before pediatric fertility preservation programs can be universally available (ovarian stimulation, transvaginal procedure, sedation) (14). Furthermore, concern about delays in therapy is one of the greatest barriers to offering OC for patients (15), especially C-A patients who often require the urgent initiation of treatment due to hematological or systemic disease. In addition, OC for pre-pubertal female patients is also challenging. Although there is a report of a pre-pubertal OC patient (29), in general, only OC as a combined procedure (oocyte retrieved from ovarian cortex which extracted OTC) is available for pre-pubertal female patients (30, 31). Based on the literature, OC as a combined procedure can be available to around 40% of under 15-year-old child patients (minimum 3.5 months) (30). However, the effectiveness of the combined procedure is still very limited (32), and it has been demonstrated that the percentage of degenerated oocytes was significantly higher in girls than in adult patients (33).

OTC is the only FP treatment for pre-pubertal females and for post-pubertal patients who are unable to delay the initiation of chemotherapy, although its status is still experimental. It has been completed in patients of all ages and has been demonstrated to be safe and effective, with a low complication rate with minimal delay (15) allowing cancer treatment to commence very soon after laparoscopic surgery for OTC (34). In promoting OTC for C-A patients, the primary disease is one of the major issues. For C-A patients, leukemia is a representative primary disease. Although there is a live birth case with leukemia who received Ovarian tissue transplantation (OTT) after treatment (35), OTT following OTC in leukemia patients is challenging and requires further investigation to avoid re-introducing minimum residual disease (MRD) (10, 36). According to the European Society for Blood and Marrow Transplantation (EBMT), both pre-pubertal and post-pubertal OTC from patients with leukemia can be considered, in view of future developments, for *in vitro* maturation and subsequent *in vitro* fertilization (37). Already, as future developments, an artificial ovary and multiple-step primordial follicle culture system has demonstrated encouraging results (38). Currently, OTC has been becoming an established treatment in some countries (10); there have already been more than 130 live birth cases (11). In general, the hospital or institution that provides OTC treatment for adult patients can perform OTC for C-A patients, because both are technically the same procedure. Indeed, based on the present study, most countries that can provide OTC for adult patients replied that "it is possible to do OTC for C-A patients." However, there are few countries that can provide OTC for C-A patients at the same level as for adult patients (although the actual numbers of OTC cases for C-A patients are unknown), due to several child-specific barriers.

For post-pubertal male patients, SC with patient assent and parent or guardian consent is an actual established method for FP (10, 34). Although the minimum age for SC is unclear (15), the success rate of SC has been reported to be up to 64.5% for adolescents aged 11–14 years (15, 39). At least Tanner stage 3 pubertal development is needed for successful SC (15, 39, 40). In general, ejaculated sperm is collected by masturbation, but penile vibratory or electro ejaculation under general anesthesia is used for patients who cannot perform masturbation (15). Also, surgical sperm extraction called "ONCO-TESE" (TESE: Testicular sperm extraction) is one of the effective procedures for patients who show cancer-induced azoospermia with a testicular tumor or lymphoma (41–43). Based on the literature, patients who underwent "ONCO-TESE" can be started on chemotherapy the same day as sperm retrieval (43). For pre-pubertal male patients, TTC is the sole treatment for FP, even though its status is still extremely experimental (10). Until now, there have been no retrievals of mature sperm or achievement of pregnancy using this treatment (15). These current situations are congruent with the findings of the present study. In conclusion, based on

present survey, almost of female child cancer patients can receive OTC (except Hong Kong and Vietnam), and female adolescent patients can receive OC in Asian countries which participate this survey, although OC is uncertain whether will be acceptable for teenagers. And all adolescent male patients can receive sperm cryopreservation, also almost child male patients can receive sperm cryopreservation according to their sexual maturation (except China, Thailand, Vietnam). However, TTC for male child and adolescent cancer patients is still uncommon procedure as described above in Asia. As a limitation, age restriction was still unclear on this survey (almost participants did not clearly state). There are some possibilities that these differences to select the procedure of FP is ascribed to the developing and economical status of country.

According to the present study, there are several factors based on "medical aspects" and "social aspects" that impede the progress of FP for C-A patients. Importantly, "How to provide FP treatment for C-A" is a major issue, more so than "medical technology" as a medical factor. When we provide FP treatment for C-A patients, there are some difficulties in explaining FP treatment and obtaining informed assent/consent from children/parents. For discussion about FP with C-A patients, "Knowledge about FP (guidelines, costs, facilities and specialist, informed assent/consent process)," "low referrals," "low priority," "Sense of comfort for health care professionals (they feel embarrassed to discuss FP)," "Patient factors (prognosis, cost, age, feel discomfort)," "Parent factors (contradictory opinions, feel discomfort)," and "Educational resources for patients and families" (44, 45) are issues (28). Also, "provider bias" is identified as a potential barrier. Providers feel difficulties giving information about FP to patients who have low potential for fertility and/or cure, and who have a lower socioeconomic status. Furthermore, if the hospital does not have the capability to perform experimental FP treatments, it is difficult to discuss FP with patients (15, 46). These situations are among the reasons for "low referrals." Until now, we had only five studies about decision-making for C-A patients, and all of them were performed in Western European countries (47). Therefore, we should perform surveys in Asian countries based on the different and varied cultures, including many different religions. In addition, for investigating this survey accurately, we need to consider economic status (GDP: gross domestic product) of countries, developing status of fertility treatment (especially ART: assisted reproductive technology), cost issue, distance between centers which provide FP treatment currently. And as a social aspect, difference of sanitary system is one of important factor. In Japan, the government had stated the policy for supporting young cancer patients to promote FP in 2018. Also, leading society for cancer treatment in Australia and Japan had published the guidance for FP. To promote the FP, academic societies are established in each Asian country. These societies hold opportunities of scientific meeting and symposium for advertising, dissemination in the territory.

The present study demonstrated the variety of frameworks for FP treatment among countries and the need to implement consistent oncofertility models of care in Asian countries (28). In most countries, pediatricians and pediatric oncologist/hematologist can participate in FP, but participation of pediatric surgeons is still not common. Based on the reports investigating the safety of OTC for pediatric patients by pediatric surgeons, there are no cases of delay, and they concluded that OCT is safe procedure (18). They considered port placement according to the size of the patient's body. We strongly agree with them that collaboration with pediatric surgeons is needed for OTC. The participation of paramedical staff (multidisciplinary approach) is also vital to improve FP treatment (28). According to the present study, nursing staff, social workers, and psychologists participated in FP in a few countries. Based on the national guidelines of FP for C-A patients in Sweden, involvement of a psychologist and/or counselor to give information about FP is recommended as part of a multidisciplinary approach (48). Not only medical staffs, but peer supporter and cancer survivor are important for developing FP treatment. In Australia, The FUTuRE Fertility Research Group led a collaborative consultation process with the Australasian Oncofertility Consumer group and oncofertility specialists to explore consumers' experiences of oncofertility care (20). The importance of resources (brochures and videos) for decision-making has also been emphasized (48). Although only Australia and Korea can provide video information about FP for C-A patients, most countries provide information by oral explanations. Unfortunately, there are no Asian countries in which child-life specialists and patient navigators can participate in FP treatment, likely because there are still very few child-life specialists and patient navigators in Asian countries. On the other hand, some child-life specialists are already participating in FP in the USA. As a future task, establishment of system to follow-up the reproductive issue of C-A patients after cancer treatment. In almost of Asian countries don't have system and network to follow-up C-A patients focused on reproductive issues, although some countries have guideline of long-term follow-up C-A patients.

As limitations, we investigated current status of FP for C-A patients in Asian countries, however it is difficult to compare them simply. Because they have various backgrounds of priority, culture, religion, and economical situation among them. Also, our survey had covered mainly developed countries in Asia. To assess the current status more accurately, we need to investigate remaining 34 of Asian countries which didn't participate this study.

CONCLUSION

The present study demonstrated the developing status of FP for C-A patients in Asian countries. The problem that needs to be resolved is how to establish a system providing FP for C-A patients while being part of the research strategy to improve the current FP options. Asian countries hold a high value on family and so it is important that we develop an oncofertility model of care which will support the implementation of local, national and international guidelines and include healthcare providers and patients. In addition, greater consideration and more discussion needs to occur about "How to apply FP to our own society"

are needed based on the various cultures and religions in the region.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

ST drafted the manuscript. NSuz and AA revised manuscript. ST and NSuz designed the research and contributed to the critical discussion. JL, NM, BW, NSuk, VN, AA, DG, C-RT, AD, CL, WL, WD, R-CC, and SK contributed to collecting and analyzing data.

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Corrigendum: Fertility Preservation for Child and Adolescent Cancer Patients in Asian Countries

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In the original article, the affiliations of all, but the corresponding author, were missing. These have now been added.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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