Research Article

Effect of Rat Medicated Serum Containing Zuo Gui Wan and/or You Gui Wan on the Differentiation of Stem Cells Derived from Human First Trimester Umbilical Cord into Oocyte-Like Cells In Vitro

Xiang Hu, Hua Lu, Yan-li Deng, Qian Wan, and Shang-mian Yie

Department of Gynecology and Obstetrics, The Second Medical College/Teaching Hospital of Chengdu University of Traditional Chinese Medicine, Remin Nanlu Road, Chengdu, Sichuan 610041, China

Correspondence should be addressed to Hua Lu; kjclh@126.com and Shang-mian Yie; yieshangmian@yahoo.ca

Received 17 June 2014; Revised 16 September 2014; Accepted 27 October 2014

Academic Editor: Min Li

Copyright © 2015 Xiang Hu et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Zuo Gui Wan (ZGW) and You Gui Wan (YGW) are two classic formulas used in clinical treatment of infertility in traditional Chinese medicine (TCM). However, the actions of the formulas remain to be proven at the cellular and molecular levels. In this study, we investigate whether the two formulas have any effect on germ cell formation and differentiation by culturing rat medicated serums containing YGW or ZGW with stem cells derived from human first trimester umbilical cord. Our results showed that while the normal rat serums had no significant effects, the rat medicated serums had significant effects on the differentiation of the stem cells into oocyte-like cells (OLCs) based on (1) cell morphological changes that resembled purative cumulus-oocyte complexes (COCs); (2) expressions of specific markers that were indicative of germ cell formation and oocyte development; and (3) estradiol production by the COC-like cells. Furthermore, ZGW medicated serums exhibited more obvious effects on specific gene expressions of germ cells, whereas YGW medicated serums showed stronger effects on estradiol production. Accordingly, our study provides evidence demonstrating for the first time that one of molecular and cellular actions of YGW or ZGW in treating human reproductive dysfunctions may be through an enhancement of neooogenesis.

1. Introduction

It is well known that TCM therapies were successfully used in reproductive medicine over thousands of years in China. Major theories of TCM including theories of Yin-Yang, Five Elements, Qi and Blood, and Zang-fu organs are completely different from Western medicine [1, 2]. For example, the “Kidney,” as one of the Zang-fu organs, has the ability to store congenital essence from one’s parents, as well as the ability to control reproduction, growth, menstruation, conception, and aging in women [3].

Moreover, according to TCM theories, disease is generally viewed as a result of disharmony or imbalance between two opposite yet complementary forces: Yin and Yang [4]. As such, a “Kidney” Yin or Yang deficiency is the root of many reproductive disorders in both males and females. Thus, a major purpose of TCM therapies is to reinforce the Yin or Yang deficiency [4, 5].

There are a number of herbal formulas that have been successfully used to reinforce the “Kidney” Yin or Yang deficiency. Of these, ZGW and YGW are two classic formulas that have been successfully used in the clinical treatment of infertility. ZGW is usually prescribed for female patients with infertility when fertility issues arise that show signs of “Kidney” Yin deficiency. On the other hand, YGW is widely used as an herbal remedy for impotence and male infertility. For example, it has been reported that ZGW could restore ovarian functions effectively and promptly in patients with premature ovarian failure (POF) after 8 months of failed treatment with clomiphene citrate [6]. It has also been reported that YGW was useful in improving all sperm parameters, as well as facilitating the metabolism of toxins...
and improving general immune status [7]. Despite these studies, the actions of the two formulas remain to be proven at the cellular and molecular levels.

Germ cells are biological cells that can differentiate into mature gametes, either as eggs or sperm, of an organism that reproduces sexually. From this point of view, germ cells may correspond to the congenital essence found in TCM theories [8], and the actions of herbal formulas that reinforce the Ying or Yang deficiency may be presumed to influence germ cell fertilization abilities as well as the formation and/or differentiation of germ cells. This hypothesis has been supported by our recent studies which demonstrate that YGW decoctions can enhance the fertilizing abilities of mouse sperm in vivo and in vitro [9] and that rat medicated serums containing YGW can increase mouse oocytes in vitro maturation and subsequent in vitro fertilization abilities [10].

Stem cells are undifferentiated biological cells that can differentiate into specialized cells and can self-renew to produce more stem cells. Interestingly, a number of studies published in the past decade indicate that stem cells derived from different sources are able to differentiate into germ cells in vitro [11–14]. Thus, the generation of germ cells from embryonic or somatic stem cells in vitro may provide a valuable model for identifying factors involved in germ cell formation and differentiation.

In one of our recent reports, we demonstrated that stem cells derived from human first trimester umbilical cord (hFTUC) have an intrinsic ability to differentiate into oocyte-like cells (OLCs) in vitro by culturing the cells with human follicular fluid (hFF), FSH/LH, and estradiol [15]. This method provides a novel model for studying the effects of Chinese herbal medicine on the process of germ cell formation and oocyte differentiation. Accordingly, in the present study, we investigate whether or not ZGW or YGW has any effect on germ cell formation and differentiation by using this novel model that we developed.

In addition, it has been reported that serum pharmacology is more scientific and more befitting to use in TCM research than traditional pharmacology [16, 17]. In traditional pharmacology, crude drugs are directly added into a culture system of cells or organs in vitro. By contrast, in serum pharmacology, drugs or drug compounds are first administered to animals orally. After a period of time, the blood of the animals is then collected to separate the serum. The end result is a medicated serum that has undergone a series of biotransformation after digestion and absorption in the gastrointestinal tract. Subsequently, the medicated serum is added into a culture system of cells or organs in vitro. This approach is much more similar to the way in which drugs work in vivo [18].

Accordingly, in the present study, we first cultured hFTUC-derived stem cells in the presence of rat medicated serums containing YGW or ZGW or common herbal ingredients of the two formulas. We then compared the rat medicated serums to normal rat serums based on cell morphological changes, gene expressions indicative of germ cell formation and oocyte development, and estradiol production.

2. Materials and Methods

2.1. Isolation and Culture of hFTUC-Derived Stem Cells. Isolation and expansion of hFTUC-derived stem cells were based on a method used in our recent reports [15, 19]. Briefly, first trimester umbilical cords were collected following therapeutic pregnancy interruptions that were conducted with the written informed consent of the patients and approval from the Second Medical College/Teaching Hospital Institutional Review Board (see Supplementary Materials 1–3 available online at http://dx.doi.org/10.1155/2014/825805). The cords were rinsed several times with sterile saline and cut into pieces followed by an immersion in a 1% collagenase type I (Sigma-Aldrich, St. Louis, USA) solution for 1 hour at 37°C to isolate the stem cells. The denuded tissues were discarded after removal of the cells during digestion. The cells were pelleted by a low-speed centrifugation (250 g for 5 min) and suspended in a fresh medium. Next, the cells were plated onto a 25 cm culture flask with an expansion medium (Minimum Essential Medium Eagle, Alpha Modification; α-MEM, Sigma-Aldrich), which was supplemented with a 10% fetal bovine serum (Gibco-BRL, Gaithersburg, USA), 2 mmol/L L-glutamine, 50 IU/mL penicillin, and 50 mg/mL streptomycin (Gibco-BRL) in 5% CO2 at 37°C. The culture medium was replaced every 3 days. Subconfluent (70%–80%) cells were detached with 0.05% trypsin-0.01% EDTA (Gibco-BRL) and plated at a density of 2.4 × 10^5 cells/cm^2.

2.2. Preparation of Rat Medicated Serums. The Animal Care and Use Committee at the Chengdu University of Chinese Traditional Medicine approved this study and all procedures as protocol number 30472225.

Three herbal decoctions were prepared by using standard methods [20]. Of the three decoctions, one decoction included YGW ingredients such as Radix rehmanniae preparata, Eucommia ulmoides, Radix angelicae sinensis, Cinnamomum, Radix aconitae lateralis preparata, Colla cornus cervi, Rhizoma Dioscoreae, Fructus corni officinalis, Cuscuta chinensis lam, and Fructus lycii and another decoction included ZGW such as Radix rehmanniae preparata, Fructus corni officinalis, Rhizoma Dioscoreae, Fructus lycii, Radix cyathulae, Cuscuta chinensis lam, Colla cornus cervi, and Colla carapacis et plastri testudinis, while the third decoction included common ingredients found in ZGW and YGW (e.g., Radix rehmanniae preparata, Fructus corni officinalis, Rhizoma Dioscoreae, Fructus lycii, Colla cornus cervi, and Cuscuta chinensis lam). The three decoctions were diluted with saline (preparation: saline, 1:1 v/v) and stored at 4°C prior to use.

Rat medicated serums were prepared according to published protocols [21, 22]. Briefly, 160 Sprague Dawley (SD) female rats, aged 6–8 weeks and weighing 220–250 grams, were supplied by the Laboratory Animal Service Center of the Sichuan Academy of Medical Sciences and maintained in an air-conditioned room with a controlled temperature of 22 ± 2°C and a humidity level of 45–65% in a 12/12 hour light/dark cycle. The rats were randomly divided into four groups (40 in each group). The first, second, and third groups were orally administered with decoctions of YGW,
ZGW, and common ingredients of ZGW/YGW, respectively. The dosage of the medicated decoctions was 20 mL/kg body weight, which was equal to 5.0 grams of formula per kg body weight. The forth group was administered with normal saline (NS) with the same volume as the medicated decoctions. Before the preparation of the serums, the rats were fasted for 16 hours and then singly administered with either the medicated decoctions or the NS for 5 days. One hour after the last administration, blood from the rats was collected and centrifuged (3000 g for 10 min) to obtain the serums. Finally, the serums were sterilized by filtration and stored at −20°C prior to use.

2.3. Rat Plasma Samples Preparation for HPLC Analysis. The rat medicated serums and the normal rat serums were subjected to a high-performance liquid chromatography (HPLC) analysis by using a Shimadzu LC2010C HPLC gradient system. To perform the analysis, 1 mL of each of the serums was mixed with 5 mL of methanol and ultrasonicated for 20 min. The mixture was centrifuged at 3000 rpm for 10 min at room temperature, and the obtained supernatant was evaporated to dryness in vacuo. The residue was then reconstituted in 500 μL of methanol solution and filtered through a 0.45 μm membrane filter (Millipore, Tokyo, Japan). Aliquots (20 μL) of the filtered solution were used in the HPLC analysis.

2.4. Immunofluorescent Staining. Cell culturewares, which were treated for 24 days with the rat medicated serums or the normal rat serums, were washed with PBS and fixed with 4% ice-cold paraformaldehyde for 10 min. After three washes with PBS, the fixed cells were incubated for 1 hour at room temperature with one of the primary antibodies in an antibody dilution solution (Dako, Denmark). Antibodies against SP1 (1:100 dilution), SCP3 (1:100 dilution), GDF9 (1:100 dilution), and ZP1 or ZP2 (1:200 dilution) were obtained from either Santa Cruz Biotechnology (USA) or Abcam (UK). Subsequently, the cells were incubated with FITC-conjugated goat anti-rabbit antibodies (Molecular Probes, Invitrogen, Carlsbad, CA). The membranes were washed three times again and antigen-antibody complexes were visualized with tetramethylbenzidine (TMB) (Sigma).

2.6. Western Blot Analysis. To examine protein expression, cells were collected with 200 μL cell lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 1% Triton X-100, and 1% SDS) and centrifuged at 12,000 rpm for 10 min at 4°C. Membrane protein concentrations were determined using a BCA assay kit (Pierce, Rockford, IL). Equal amounts of membrane lysates were separated using 10% SDS-PAGE and electroblotted to polyvinylidene fluoride (PVDF) membranes (Invitrogen, Carlsbad, CA). The membranes were blocked in PBS containing 0.5% Tween-20 (PBS-Tween) and 5% skim milk for 2 hours at room temperature and then incubated with polyclonal rabbit anti-human OCT4, Stella, GDF9, ZP1, and anti-beta-actin (Abcom UK; 1:500 to 1:1000) overnight at 4°C. Membranes were washed three times (10 min/wash) with PBS-Tween and incubated with a horseradish peroxidase-conjugated goat anti-rabbit antibody (Sigma; 1:2000) for 1 hr at room temperature. Membranes were washed three times again and antigen-antibody complexes were visualized with tetramethylbenzidine (TMB) (Sigma).

2.8. Data Analysis. Statistical analysis was performed using the SPSS software package (SPSS Inc., Chicago, USA). Because the data did not violate assumptions of homogeneity of variance and normal distribution, the one-way analysis of variance was used to compare the differences in the medium estradiol and medium estradiol concentrations among different days of the culture and among the different experimental groups. If significance was found in the one-way analysis of variance, the data underwent post hoc comparisons. P < 0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. Treatment of hFTUC-Derived Stem Cells with the Rat Medicated Serums or Normal Rat Serums. To test the effects of the rat medicated serums on the differentiation of hFTUC-derived stem cells into OLCs, the hFTUC-derived stem cells were cultured with α-MEM medium containing 2% rat medicated serums (containing either ZGW or YGW or common
The rat medicated serum containing common ingredients of ZGW/YGW was used to culture the hFTUC-derived stem cells. The cells were grown in 24-well plates at a density of 1000 cells per well and were maintained in a humidified incubator at 37°C with 5% CO2. The culture medium was replaced with fresh medium every 3 days and maintained for a total of 24 days. During this period, the cells changed their morphology and gradually differentiated into OLCs. Morphology of the cells was examined by using an inverted microscope (Leica DMI 3000B, Leica, Wetzlar Germany) before the medium changes. At the end of differentiation, the OLCs were examined by using immunofluorescent staining to identify whether the OLCs expressed oocyte specific markers. Meanwhile, the cells were subjected to real-time RT-PCR analysis as well as Western blot analysis at various time points to investigate gene expressions related to germ cell formation and oocyte differentiation. Further, supernatant fractions were collected from the cell cultures to determine estradiol production.

3.2. Effect of Rat Medicated Serum Containing Common Ingredients of ZGW/YGW on the Differentiation of hFTUC-Derived Stem Cells into OLCs. Figure 1(a) shows a representative HPLC profile for the rat medicated serum containing common ingredients of ZGW/YGW. Five distinct peaks at 2.2, 3.2, 10.8, 15.9, and 16.4 min retention times were detected in the rat medicated serum but not in the normal rat serum. This indicates that the rat medicated sera may contain certain active ingredients and/or metabolic components.
To characterize the OLCs, oocyte specific markers in these cells were tested by using immunofluorescent staining on day 24 of the culture. Figure 3(a) shows that the OLCs expressed oocyte specific markers such as SP1, SCP3, GDF9, and ZP1. Notably, the zona pellucida-like structure surrounding the OLCs could be detected in some of the cells by using anti-ZP antibodies, which was very similar to what was described in a recent report [27].

A real-time RT-PCR was performed to further verify whether the differentiated cells can be identified based on morphological changes that expressed specific gene markers related to primordial germ cells (PGC) transition and oocyte development. In the real-time RT-PCR analysis, the specificity of each amplified product in the control and differentiated cells was verified by a bidirectional sequence analysis (Supplemental Material 6). Once the specificity of the products was established, temporal changes in relative mRNA levels for each of the markers were evaluated by using GAPDH as an inner control. The Ct values for all products were below 35 and the efficiencies of targets and reference were approximately equal.

Transcripts of all the markers could be detected in the differentiated cells as well as the undifferentiated cells. However, fold changes of the differentiated versus undifferentiated cells for all the markers showed certain dynamic changes during the differentiation process. On day 7 of the differentiation,
Figure 3: Continued.
Figure 3: Detection of oocyte markers in OLCs by immunofluorescent analysis. (a), (b), and (c) show immunofluorescent stains of SP1, SCP3, GDF9, and ZP1 or ZP2 in OLCs on day 24 after treatment with the rat medicated serums containing common ingredients of ZGW/YGW, YGW, and ZGW, respectively. (d) shows the immunofluorescent analysis of expressions of SP1, SCP3, GDF9, and ZP1 in stems cells treated with normal rat serum. (e) shows negative controls obtained by incubating the stems cells with secondary anti-rabbit or anti-mouse antibody alone after staining with DAPI. Scale bar is set to 50 μm.

Oct4, SSEA1, and Blimp1 mRNA levels decreased by 20% and 31%, respectively, while mRNA levels of Prdm14, Ttap2c, SSEA1, and Stella increased by approximately 4.8-fold, 5.1-fold, 1.2-fold, and 9.3-fold, respectively (P = 0.037, 0.035, 0.118, and 0.001, resp.) when compared to levels obtained on day 0 (Figure 4(a)). On day 24 of the differentiation, in which OLCs developed, the mRNA levels of SCP3, GDF9, ZPI, and ZP2 increased by approximately 1.4-fold (P = 0.004), 4.8-fold (P = 0.033), 6.7-fold (P = 0.031), and 2.2-fold (P = 0.046), respectively, while the mRNA levels in ZP3 showed no changes when compared to levels obtained on day 0 (Figure 4(a)).
Figure 4: Continued.
Figure 4: Detection of germ cell and oocyte markers in germ cell-like cells and OLCs by real-time RT-PCR. Real-time RT-PCR analysis of expressions of germ cell markers (OCT4, SSEA-1, Stella, Blimp1, Prdm14, and Tfap2c) in the PGC-like cells after 7 days of differentiation and oocyte markers (SCP3, GDF9, ZP1, ZP2, and ZP3) after 24 days of treatment with the rat medicated sera containing common ingredients of ZGW/YGW (a), YGW (b), ZGW (c), and normal rat serum (d). Relative mRNA levels are normalized for the GAPDH housekeeper. The results are presented relative to the control cells \(2^{-\Delta\Delta Ct}\). Data represents the mean \(\pm\) SEM of three independent experiments. * indicates statistical significance when compared to day 0 of the culture.
In the western blot analysis, protein expressions of Stella on day 7 and GDF-9 and ZP1 on day 24 after the treatment were obviously increased while Oct4 protein expression had a slight decrease from day 0 to day 24 (Figure 5(a)). These changes in protein expressions were similar to those of the mRNA expressions.

Estradiol concentrations in the cultured medium on day 0 was 3.7 ± 0.18 pg/mL with the levels of estradiol in cultured medium gradually increasing from 14.6 ± 0.23 to 26.0 ± 0.30 pg/mL during days 15 to 21 of the treatment ($F = 39.3$, $P = 0.0001$; Figure 6(a)).

### 3.3. Effect of Rat Medicated Serum Containing YGW on the Differentiation of hFTUC-Derived Stem Cells into OLCs.

Figure 1(b) shows a representative HPLC profile for the rat medicated serum containing YGW. Seven distinct peaks at 3.5, 3.8, 8.8, 12.3, 16.7, 17.2, and 21.6 min retention times were detected in the rat medicated serum but not in the normal rat serum (Figure 1(d)).

Similar to the rat medicated serum containing common ingredients of YGW/ZGW, Figure 2(b) shows that some subpopulations of the cells became morphologically distinct from the starting cultures after 7 days of culture with 2% of the rat medicated serum containing YGW. On day 24 of the differentiation, OLCs surrounded by smaller cells also appeared.

Figure 3(b) shows that the OLCs expressed SPI1, SCP3, GDF9, and ZP1 as detected by using immunofluorescent staining. In the real-time RT-PCR analysis, fold changes of differentiated versus undifferentiated cells for all of the markers showed certain dynamic changes during the differentiation process. On day 7 of the differentiation, Blimp1 mRNA levels decreased by 75% ($P = 0.005$) and SSEA1 mRNA levels remained unchanged (96%), while mRNA levels of Oct4, Prdm14, Tfap2c, and Stella increased by approximately 1.38-fold, 10.16-fold, 4.97-fold, and 8.1-fold, respectively ($P = 0.114, 0.02, 0.009, 0.001$, resp.) when compared to levels obtained on day 0 (Figure 4(b)). On day 24 of the differentiation, in which OLCs developed, the mRNA levels of SCP3, GDF9, ZP1, and ZP3 increased by approximately 1.62-fold ($P = 0.018$), 6.05-fold ($P = 0.043$), 27.9-fold ($P = 0.036$), and 2.49-fold ($P = 0.048$), respectively, while the mRNA levels of ZP3 decreased by 51% ($P = 0.035$) when compared to levels obtained on day 0 (Figure 4(b)). Figure 5(b) showed that protein expressions of Stella on day 7, GDF-9 on day 24, and ZP1 on day 7 and day 24 after the
Figure 6: Determination of changes of estradiol production. Estradiol production during the treatment with the rat medicated serums containing common ingredients of ZGW/YGW (a), YGW (b), ZGW (c), and normal rat serum (d). Data represents the mean ± SEM of three independent experiments. During 24 days of treatment, estradiol productions of OLCs were gradually increased in the three rat medicated serum groups (all $P$ values $= 0.0001$, one-way analysis of variance) while no significant difference was detected in cultures treated with normal rat serum ($P = 0.092$). * indicates statistical significance when compared to day 0 of the treatment.

3.4. Effect of Rat Medicated Serum Containing ZGW on the Differentiation of hFTUC-Derived Stem Cells into OLCs.

Figure 1(c) shows a representative HPLC profile for the rat medicated serum containing ZGW. Six distinct peaks at 2.2, 3.2, 11.3, 16.6, 17.0, and 22.6 min retention times were detected in the rat medicated serum but not in the normal rat serum (Figure 1(d)).

Similar to rat medicated serums containing common ingredients of YGW/ZGW and YGW, after 7 days of culture with 2% of the medicated serum containing ZGW, some subpopulations of the cells also became morphologically distinct from the starting cultures, and OLCs developed from days 14 to 24 (Figure 2(c)).

The OLCs expressed proteins of SP1, SCP3, GDF9, and ZP1 as detected by immunofluorescent staining (Figure 3(c)). The expressions of levels of mRNA in Oct4, Blimp1, Prdm14, Tfap2c, SSEA1, and Stella increased by approximately 3.77-fold ($P = 0.009$), 1.42-fold ($P = 0.027$), 2.80-fold ($P = 0.006$), 14.1-fold ($P = 0.001$), 1.17-fold ($P = 0.016$), and 36.7-fold ($P = 0.001$) on day 7 when compared to levels obtained on day 0 (Figure 4(c)). Meanwhile, after 24 days, the mRNA levels of SCP3, GDF9, ZP1, ZP2, and ZP3 increased by approximately 2.8-fold ($P = 0.019$), 15.5-fold ($P = 0.016$), 2.7-fold ($P = 0.018$), 3.89-fold ($P = 0.01$), and 1.88-fold ($P = 0.007$) when compared to levels obtained on day 0 (Figure 4(c)). Similarly, the western blot analysis showed that the protein expressions of OCT4 and Stella on day 7 and GDF-9 and ZP1 on day 24 after treatment were obviously increased (Figure 5(c)).

differentiated were increased while no clear changes in Oct4 protein expression during the differentiation were observed.

Estradiol concentrations in the cultured medium on day 0 was $6.0 \pm 0.60$ pg/mL with the levels of estradiol in cultured medium gradually increasing and reaching $28.3 \pm 1.41$ pg/mL by day 9, which remained at this high level until day 21 of the treatment ($F = 62.6, P = 0.0001$; Figure 6(b)).

The OLCs expressed proteins of SP1, SCP3, GDF9, and ZP1 as detected by immunofluorescent staining (Figure 3(c)). The expressions of levels of mRNA in Oct4, Blimp1, Prdm14, Tfap2c, SSEA1, and Stella increased by approximately 3.77-fold ($P = 0.009$), 1.42-fold ($P = 0.027$), 2.80-fold ($P = 0.006$), 14.1-fold ($P = 0.001$), 1.17-fold ($P = 0.016$), and 36.7-fold ($P = 0.001$) on day 7 when compared to levels obtained on day 0 (Figure 4(c)). Meanwhile, after 24 days, the mRNA levels of SCP3, GDF9, ZP1, ZP2, and ZP3 increased by approximately 2.8-fold ($P = 0.019$), 15.5-fold ($P = 0.016$), 2.7-fold ($P = 0.018$), 3.89-fold ($P = 0.01$), and 1.88-fold ($P = 0.007$) when compared to levels obtained on day 0 (Figure 4(c)). Similarly, the western blot analysis showed that the protein expressions of OCT4 and Stella on day 7 and GDF-9 and ZP1 on day 24 after treatment were obviously increased (Figure 5(c)).
Estradiol concentrations in the cultured medium on day 0 were $4.0 \pm 0.2$ pg/mL with the levels of estradiol in cultured medium gradually increasing and reaching a peak value of $30.0 \pm 1.52$ pg/mL by day 9. Afterward, the estradiol levels decreased but were still higher than day 0 until day 21 of the treatment ($F = 36.0, P = 0.0001$; Figure 6(c)).

3.5. Effect of Normal Rat Serum on the Differentiation of hFTUC-Derived Stem Cells. Normal rat serum was used as a control to demonstrate the effects of the rat medicated serums on the differentiation of hFTUC-derived stem cell into OLCs. Figure 1(d) shows a representative HPLC profile for the normal rat serum. Only one peak at 2.75 min retention times was detected.

During the 24 days of culture with 2% of the normal rat serum, the morphology of cells remained the same as the morphologic features of undifferentiated fibroblast-like cells (Figure 2(d)). Immunofluorescent staining showed no positive reaction with anti-SP1, -SCP3, -GDF9, and -ZP1 antibodies in during the 24 days of culture (Figure 3(d)). In the real-time RT-PCR analysis, there were no significant fold changes in the mRNA levels of Oct4, Blimp1, Tafap2c, SSEA1, and Stella while the mRNA level of Prdm14 increased by 1.3-fold ($P = 0.045$) after 7 days when compared to levels obtained on day 0 (Figure 4(d)). On day 24, there were no significant changes in the mRNA levels of SCP3 and GDF9, while the mRNA levels of ZP1 and ZP3 decreased by 85% ($P = 0.01$) and 70% ($P = 0.02$), respectively, when compared to levels obtained on day 0 (Figure 4(d)). The unchanged mRNA levels of Oct4, Stella, GDF9, and ZPI were also confirmed by their protein expression assayed with the western blotting (Figure 5(d)).

Estradiol concentrations in the cultured medium on day 0 was $3.8 \pm 0.20$ pg/mL with the levels of estradiol in cultured medium increasing slightly to $8.42 \pm 0.42$ and $7.6 \pm 0.39$ pg/mL by days 12 and 15 of the treatment, respectively ($F = 2.15, P = 0.096$; Figure 6(d)).

3.6. Comparison of the Effect of the Three Types of Rat Medicated Serums on Gene Expressions Related to Germ Cell Formation, Oocyte Development, and Estradiol Production. Although the three types of rat medicated serums contained different active herbal ingredients and/or biotransformed components based on the HPLC analysis, no significant differences in cell morphologic changes and protein expression of oocyte markers, such as SP1, SCP3, GDF9, and ZPI, were observed among the rat medicated serums. However, the effects of the three types of rat medicated serums on gene transcripts related to germ cell formation and oocyte development as well as estradiol production were observed to be different. In particular, as shown in Figure 7, mRNA levels of Oct4, Blimp1, Prdm14, Tafap2c, and Stella in the rat medicated serum containing ZGW were significantly higher than those in the other two types of rat medicated serums after 7 days of treatment. The mRNA levels of SCP3 and GDF-9 in the rat medicated serum containing ZGW were also significantly higher than those in the other two types of rat medicated serums after 24 days of treatment. However, no significant differences in the mRNA levels of SSEA1, ZP-1, ZP-2, and ZP-3 were detected among the rat medicated serums. Interestingly, COC-like cells induced by the rat medicated serum containing YGW produced more estradiol when compared to the rat medicated serum containing ZGW or the rat medicated serum containing common herbal ingredients of ZGW/YGW ($P = 0.042$ and 0.048, resp., Table 1). Note that no significant difference was found in the average numbers of OLCs among the three treatment groups ($4.0 \pm 0.36$ versus $3.8 \pm 0.40$ versus $4.1 \pm 0.40$ (mean $\pm$ SE) for common herbal ingredients of ZGW/YGW versus YGW versus ZGW). Accordingly, the differences in the estradiol production could be attributed to the different effects of the three types of medicated serums.

4. Discussion

Many studies have shown that OLCs can be generated from embryonic stem cells (ESCs), induced from pluripotent stem cells (iPSc) and adult stem cells in vitro [11, 26–32]. In these previous studies, the induction of ESCs or somatic stem cells into OLCs was generally performed by cultivating the cells with growth factors [28, 30], estrogenic stimuli [12], conditional medium from testicular cell cultures [33], follicular fluid, and gonadotrophins [15, 26, 27] or with ovarian granulose cells [29]. In the present study, we showed that hFTUC-derived stem cells are also able to differentiate into OLCs by the treatment of rat medicated serums containing ZGW or YGW or common ingredients of ZGW/YGW. The germ cell precursors in our study showed close resemblance to oocytes, which were based on (1) morphologic changes; (2) marker expression profiles at the mRNA and/or protein levels; and (3) production of estradiol from COC-like structures. By contrast, no significant morphological changes, germ cell marker expressions, or estradiol productions were found in the cells treated with normal rat serum.

Germ cell development requires a series of multiple well-orchestrated steps, which involve the concurrent up- and downregulation of specific determinant gene expressions [34]. It has been demonstrated that Blimp1, Prdm14, and Tafap2c are key germ cell determinants for regulating PGC specification [35], and Oct4 is proposed to be required for the survival of primordial germ cells [36]. In the present study, the rat medicated serums caused an increase in the mRNA levels of Oct4, Blimp1, Prdm14, and Tafap2c after 7 days of development as well as estradiol production were observed among the rat medicated serums. However, contrasts, no significant morphological changes, germ cell marker expressions, or estradiol productions were found in the cells treated with normal rat serum.

Table 1: Comparison of estradiol production from the differentiated cells treated with rat medicated serums containing common components of YGW and ZGW, YGW, or ZGW.

| Comparison     | F    | P    |
|----------------|------|------|
| NS versus YGW/ZGW | 33.88| 0.006|
| NS versus YGW   | 60.42| 0.0005|
| NS versus ZGW   | 35.98| 0.0054|
| YGW/ZGW versus YGW | 2.47| 0.042|
| YGW/ZGW versus ZGW | 1.53| 0.167|
| YGW versus ZGW  | 2.45 | 0.048|
Figure 7: Continued.
Figure 7: Comparison of the effect of the three types of rat medicated serums on gene expressions. Significant differences in the mRNA levels of Oct4, SSEA-1, Stella, Blimp1, Prdm14, and Tfap2c can be seen on day 7, with the highest expression seen in the stem cells treated with the rat medicated serum containing ZGW. No significant difference in any of the gene expressions was found on day 24, except for Tfap2c. On day 24, the mRNA levels of SCP3 and GDF-9 in the rat medicated serum containing ZGW were higher than the mRNA levels of SCP3 and GDF-9 in the other two types of rat medicated serums. No significant differences in mRNA levels of ZP-1, ZP-2, and ZP-3 were detected among the three types of rat medicated serums.
treatment, although the effects were different among the rat medicated serums.

Moreover, the PGC-like cells also expressed SSEA1 and Stella, which are markers indicative of germ cell formation. It has been reported that human germ cells express SSEA1 [37], and Stella plays a role in facilitating germline and endodermal differentiation of human ES cells [38]. A lack of Stella expression at an earlier stage could indicate a transition of cells into the germ lineage [33].

Further, the rat medicated serums caused an increase in the levels of mRNA of oocyte specific markers such as SP1, SCP3, GDF-9, ZP1, ZP2, and ZP3 during the differentiation process. SCP3 is a meiosis-specific protein [39], while GDF-9 is required for normal folliculogenesis [40], and zona pellucida glycoproteins are expressed only in oocytes [41]. The SP1 protein, which could promote rapid germ-cell differentiation [42], was also detected in the COC-like cells/OLCs. Accordingly, our results indicate that the rat medicated serums have the potential to induce the differentiation of hFTUC-derived stem cells into OLCs.

Moreover, the detection of estradiol production in our cultures provides evidence for the functional activity of somatic cells around the OLCs, since the 4–8-fold increase in estradiol concentrations over the controls on day 21 should be due to the COC-like cells. However, further investigation is needed to confirm the existence and function of granulosa cell-like cells in the COC-like cells.

The HPLC profiles revealed that the rat medicated serum containing ZGW had six distinct peaks (at 2.2, 3.2, 11.3, 16.6, 17.0, and 22.6 min retention times), the rat medicated serum containing YGW had seven distinct peaks (at 3.5, 3.8, 8.8, 12.3, 16.7, 17.2, and 21.6 min retention times), and the rat medicated serum containing common ingredients of ZGW/YGW had five distinct peaks (at 2.2, 3.2, 10.8, 15.9, and 16.4 min retention times). At least 3 peaks, at 2.2, 3.2, and 16 min retention times, were similar in all three types of rat medicated serums. Since the three types of rat medicated serums have similar effects on the differentiation of OLCs, the common distinct peaks may represent active herbal ingredients and/or biotransformed components in the rat medicated serums that are responsible for the induction of cell specific differentiation in terms of cell morphological changes and expressions of SCP3, GDF9, and ZPI or ZP2 proteins. However, the rat medicated serum containing ZGW had stronger effects on mRNA expressions of genes related to germ cell formation and oocyte development, while the rat medicated serum containing YGW showed more obvious influence on estradiol production. The findings were also confirmed by the western blot analysis since protein expressions of Oct4, Stella, GDF9, and ZPI from day 0 to day 24 of the differentiation have a similar changing pattern to that of mRNA levels. This suggests that other distinct peaks in these two types of rat medicated serums may be responsible for the differences. Our findings are consistent with clinical observations that ZGW could restore ovarian functions effectively and promptly [5, 6, 8] and with a previous report that showed that YGW can enhance estradiol secretion by mice granulose cells in vitro [43].

Moreover, one of our recent studies showed that rat medicated serums containing YGW had a significant effect on mouse oocyte maturation in vitro via molecular signal pathways similar to those induced by FSH [10]. The results suggest that some active or biotransformed herbal ingredients in the medicated serums may have gonadotrophin functions to stimulate the production of estradiol from differentiated granulosa cells.

Following the development of tissue engineering and stem cell therapy, stem cell transplantations are emerging as an important research topic due to their therapeutic potentials in a variety of diseases. For example, investigations into the application of MSCs in improving liver fibrosis have been carried out in animal models and clinical trials [44]. Because stem cells are able to differentiate into germ cell-like cells in vitro [11–14], stem cell therapy may also become an important research topic in the future due to their therapeutic potentials in reproductive diseases such as POF. Interestingly, it has been reported that the differentiation, proliferation, and migration of MSCs can be regulated by a combination of TCM treatments [44], which may be of great benefit for infertility therapy in the future.

According to TCM theories, the “Kidney” houses the reproductive essence (jing) and governs reproduction [45]. In modern terminology, the essence refers to eggs and sperm, the most primordial building blocks of reproduction [8]. While the “Kidney” essence correlates with eggs and sperm, the “Kidney” Yin and “Kidney” Yang can be thought of as the hormonal underpinnings of the reproductive system [8]. Because YGW and ZGW are used in patterns with “Kidney” Yang and Yin deficiency, respectively, they may regulate the endocrine function to promote germ cell transition, although other important underlying mechanisms may be also involved. However, this hypothesis needs to be experimentally demonstrated in future studies by using isolated herbal ingredients. Additionally, future studies are needed to identify the chemical compositions and properties of these herbal ingredients.

5. Conclusion

In summary, we have demonstrated that rat medicated serums containing YGW or ZGW or common ingredients of ZGW/YGW can induce the differentiation of hFTUC-derived stem cells into OLCs in vitro. We also showed that the rat medicated serum containing ZGW has more obvious effects on gene transcript expressions related to germ cell transition determination and oocyte development than the other two types of rat medicated serums. Further, the rat medicated serum containing YGW showed stronger effects on estradiol production by COC-like cells. Our findings suggest that one of actions of the ZGW and YGW formulas is an enhancement of neooogenesis. This should play an important role in the understanding of the pharmacological mechanisms of ZGW or YGW at the cellular and molecular levels in treating human reproductive dysfunctions such as premature ovarian failure. Consequently, this can lead to better clinical practices in the future which combine both TCM and modern reproductive medicine. Nevertheless, further studies are necessary in order to identify which
active ingredients and/or biotransformed components are responsible for the underlying mechanisms of the herbal formulas.

Conflict of Interests

The authors declare no conflict of interests.

Authors’ Contribution

Shang-mian Yie, Hua Lu, and Xiang Hu conceived and designed the experiments. Xiang Hu and Shang-mian Yie performed the experiments. Xiang Hu and Shang-mian Yie analyzed the data. Xiang Hu, Yan-li Deng, and Qian Wan contributed to reagents/materials/analysis tools. Shang-mian Yie, Hua Lu, and Xiang Hu wrote the paper.

Acknowledgment

This study was supported by grants from the National Basic Research Program of China (973 Plan, Grants nos. 2010CB530403 and 2010CB530400).

References

[1] Z. G. Li, “Comparative study on WHO western pacific region and world federation of Chinese medicine societies international standard terminologies on traditional medicine: diseases of gynecology and obstetrics (part 1),” Journal of Integrative Medicine, vol. 12, no. 2, pp. 127–130, 2014.
[2] H. Satoh, “Pharmacological characteristics of Kampo medicine as a mixture of constituents and ingredients,” Journal of Chinese Integrative Medicine, vol. 11, no. 1, pp. 11–16, 2013.
[3] S.-T. Huang and A. P.-C. Chen, “Traditional Chinese medicine and infertility,” Current Opinion in Obstetrics and Gynecology, vol. 20, no. 3, pp. 211–215, 2008.
[4] J.-H. Wang, “Traditional Chinese medicine and the positive correlation with homeostatic evolution of human being: based on medical perspective,” Chinese Journal of Integrative Medicine, vol. 18, no. 8, pp. 629–634, 2012.
[5] J. Zhou and F. Qu, “Treating gynaecological disorders with traditional Chinese medicine: a review,” African Journal of Traditional, Complementary and Alternative Medicines, vol. 6, no. 4, pp. 494–517, 2009.
[6] S.-L. Chao, L.-W. Huang, and H.-R. Yen, “Pregnancy in premature ovarian failure after therapy using Chinese herbal medicine,” Chang Gung Medical Journal, vol. 26, no. 6, pp. 449–452, 2003.
[7] A. S. Crimmel, C. S. Conner, and M. Monga, “Withered Yang: a review of traditional Chinese medical treatment of male infertility and erectile dysfunction,” Journal of Andrology, vol. 22, no. 2, pp. 173–182, 2001.
[8] L. Rosenthal and B. Anderson, “Acupuncture and in vitro fertilisation: recent research and clinical guidelines,” Journal of Chinese Medicine, no. 84, pp. 28–35, 2007.
[9] X.-H. Jiang, S.-M. Yie, X. Zhen et al., “Effect of You Gui Wan on mouse sperm fertilising ability in vivo and in vitro,” Andrologia, vol. 46, no. 3, pp. 283–289, 2014.
[10] X. H. Jiang, Y. L. Deng, H. Lu et al., “Effect of rat medicated serum containing You Gui Wan on mouse oocyte in vitro maturation and subsequent fertilization competence,” Evidence-Based Complementary and Alternative Medicine, vol. 2014, Article ID 152010, 11 pages, 2014.
[11] A. I. Marques-Mari, O. Lacham-Kaplan, J. V. Medrano, A. Pellicer, and C. Simón, “Differentiation of germ cells and gametes from stem cells,” Human Reproduction Update, vol. 15, no. 3, pp. 379–390, 2009.
[12] A. T. Clark, M. S. Bodnar, M. Fox et al., “Spontaneous differentiation of germ cells from human embryonic stem cells in vitro,” Human Molecular Genetics, vol. 13, no. 7, pp. 727–739, 2004.
[13] H.-F. Chen, H.-C. Kuo, C.-L. Chien et al., “Derivation, characterization and differentiation of human embryonic stem cells: comparing serum-containing versus serum-free media and evidence of germ cell differentiation,” Human Reproduction, vol. 22, no. 2, pp. 567–577, 2007.
[14] A. Bukovsky, M. Svetlikova, and M. R. Caudle, “Oogenesis in cultures derived from adult human ovaries,” Reproductive Biology and Endocrinology, vol. 3, article 17, 2005.
[15] X. Hu, H. Lu, S. Cao et al., “Stem cells derived from human first-trimester umbilical cord have the potential to differentiate into oocyte-like cells in vitro,” Submitted to International Journal of Molecular Medicine.
[16] W. Bochu, Z. Liancai, and C. Qi, “Primary study on the application of Serum Pharmacology in Chinese traditional medicine,” Colloids and Surfaces B: Biointerfaces, vol. 43, no. 3–4, pp. 194–197, 2005.
[17] Z.-G. Peng, H.-S. Chen, Z.-M. Guo, B. Dong, G.-Y. Tian, and G.-Q. Wang, “Anti-HIV activities of Achyranthes bidentata polysaccharide sulfate in vitro and in vivo,” Yaoxue Xuebao, vol. 43, no. 7, pp. 702–706, 2008.
[18] S. Tashino, “Serum pharmacology and ‘serum pharmaceutical chemistry’ from pharmacology of Chinese traditional medicines to start a new measurement of drug concentration in blood,” Therapeutic Drug Monitoring Research, vol. 5, pp. 54–64, 1988.
[19] S.-H. Hong, L. Maghen, S. Kenigsberg et al., “Ontogeny of human umbilical cord perivascular cells: molecular and fate potential changes during gestation,” Stem Cells and Development, vol. 22, no. 17, pp. 2425–2439, 2013.
[20] S. C. Sze, Y. Tong, Y. B. Zhang et al., “A novel mechanism: Erxian Decoction, a Chinese medicine formula, for relieving menopausal syndrome,” Journal of Ethnopharmacology, vol. 123, no. 1, pp. 27–33, 2009.
[21] J. Li, B. Li, W.-J. Zhou, F.-G. Zhao, D.-J. Li, and W.-J. Wang, “Medicated rat serum containing Gengnianchun decoction reduces apoptosis of pheochromocytoma cells insulted by amyloid beta protein,” Journal of Chinese Integrative Medicine, vol. 8, no. 5, pp. 472–479, 2010.
[22] D. K. Yin, Z. Q. Liu, D. Y. Peng et al., “Serum containing tao-hong-si-wu decoction induces human endothelial cell VEGF production via PI3K/Akt-eNOS signaling,” Evidence-Based Complementary and Alternative Medicine, vol. 2013, Article ID 195158, 9 pages, 2013.
[23] S.-M. Yie, L.-H. Li, G.-M. Li, R. Xiao, and C. L. Librach, “Progesterone enhances HLA-G gene expression in JEG-3 choriocarcinoma cells and human cytotrophoblasts in vitro,” Human Reproduction, vol. 21, no. 1, pp. 46–51, 2006.
[24] K. J. Livak and T. D. Schmittgen, “Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method,” Methods, vol. 25, no. 4, pp. 402–408, 2001.
[25] P. Qiu, Y. Bai, S. Pan, W. Li, W. Liu, and J. Hua, “Gender depended potentiality of differentiation of human umbilical
cord mesenchymal stem cells into oocyte-Like cells in vitro,” *Cell Biochemistry and Function*, vol. 31, no. 5, pp. 365–373, 2013.

[26] P. W. Dyce, L. Wen, and J. Li, “In vitro germline potential of stem cells derived from fetal porcine skin,” *Nature Cell Biology*, vol. 8, no. 4, pp. 384–390, 2006.

[27] X. L. Yu, N. Wang, R. Qiang et al., “Human amniotic fluid stem cells possess the potential to differentiate into primordial follicle oocytes in vitro,” *Biology of Reproduction*, vol. 90, no. 4, p. 74, 2014.

[28] K. Hubner, G. Fuhrmann, L. K. Christenson et al., “Derivation of oocytes from mouse embryonic stem cells,” *Science*, vol. 300, no. 5623, pp. 1251–1256, 2003.

[29] T. Qing, Y. Shi, H. Qin et al., “Induction of oocyte-like cells from mouse embryonic stem cells by co-culture with ovarian granulosa cells,” *Differentiation*, vol. 75, no. 10, pp. 902–911, 2007.

[30] W. Wei, T. Qing, X. Ye et al., “Primordial germ cell specification from embryonic stem cells,” *PLoS ONE*, vol. 3, no. 12, Article ID e4013, 2008.

[31] K. Kee, J. M. Gonsalves, A. T. Clark, and R. A. Reijo Pera, “Bone morphogenetic proteins induce germ cell differentiation from human embryonic stem cells,” *Science*, vol. 15, no. 6, pp. 831–837, 2006.

[32] S. Panula, J. V. Medrano, K. Kee et al., “Human germ cell differentiation from fetal- and adult-derived induced pluripotent stem cells,” *Human Molecular Genetics*, vol. 20, no. 4, Article ID ddq520, pp. 752–762, 2011.

[33] O. Lacham-Kaplan, H. Chy, and A. Trounson, “Testicular cell conditioned medium supports differentiation of embryonic stem cells into ovarian structures containing oocytes,” *Stem Cells*, vol. 24, no. 2, pp. 266–273, 2006.

[34] K. Linher, P. Dyce, and J. Li, "Primordial germ cell-like cells differentiated in vitro from skin-derived stem cells," *PLoS ONE*, vol. 4, no. 12, Article ID e8263, 2009.

[35] E. Magnúsdóttir, S. Dietmann, K. Murakami et al., “A tripartite transcription factor network regulates primordial germ cell specification in mice,” *Nature Cell Biology*, vol. 15, no. 8, pp. 905–915, 2013.

[36] J. Kehler, E. Tolkunova, B. Koschorz et al., “Oct4 is required for primordial germ cell survival,” *EMBO Reports*, vol. 5, no. 11, pp. 1078–1083, 2004.

[37] C. L. Kerr, C. M. Hill, P. D. Blumenthal, and J. D. Gearhart, “Expression of pluripotent stem cell markers in the human fetal testis,” *Stem Cells*, vol. 26, no. 2, pp. 412–421, 2008.

[38] P. Wongtrakoongate, M. Jones, P. J. Gokhale, and P. W. Andrews, “STELLA facilitates differentiation of germ cell and endodermal lineages of human embryonic stem cells,” *PLoS ONE*, vol. 8, no. 2, Article ID e56893, 2013.

[39] L. Yuan, J.-G. Liu, M.-R. Hoja, J. Wilbertz, K. Nordqvist, and C. Höög, "Female germ cell aneuploidy and embryo death in mice lacking the meiosis-specific protein SCP3," *Science*, vol. 296, no. 5570, pp. 1115–1118, 2002.

[40] J. Aaltonen, M. P. Laitinen, K. Vuojolainen et al., “Human growth differentiation factor 9 (GDF-9) and its novel homolog GDF-9B are expressed in oocytes during early folliculogenesis,” *The Journal of Clinical Endocrinology & Metabolism*, vol. 84, no. 8, pp. 2744–2750, 1999.

[41] L. Lefèvre, S. J. Conner, A. Salpekar et al., “Four zona pellucida glycoproteins are expressed in the human,” *Human Reproduction*, vol. 19, no. 7, pp. 1580–1586, 2004.

[42] S. Toyoda, T. Yoshimura, M. Mizuta, and J. Miyazaki, “Auto-regulation of the SOHLH gene by the SOHLH2/SOHLH1/SP1 complex: implications for early spermatogenesis and oogenesis,” *PLoS ONE*, vol. 9, no. 7, Article ID e101681, 2014.

[43] X. J. Xu, S. R. Jin, and X. H. Qin, “Effect and mechanism of water extraction of Yougui Bulus on mice ovary granule cells secreting estrogenic and progesterational hormones,” *Journal of Sichuan Traditional Chinese Medicine*, no. 5, p. 24, 2006.

[44] S. Dong and S. B. Su, “Advances in mesenchymal stem cells combined with traditional Chinese medicine therapy for liver fibrosis,” *Journal of Integrative Medicine*, vol. 12, no. 3, pp. 147–155, 2014.

[45] C. Q. Ling, L. N. Wang, Y. Wang et al., “The roles of traditional Chinese medicine in gene therapy,” *Journal of Integrative Medicine*, vol. 12, no. 2, pp. 67–75, 2014.