Decreased levels of sRAGE in follicular fluid from patients with PCOS

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Abstract

This study aimed to explore the association between soluble receptor for advanced glycation end products (sRAGE) levels in follicular fluid and the number of oocytes retrieved and to evaluate the effect of sRAGE on vascular endothelial growth factor (VEGF) in granulosa cells in patients with polycystic ovarian syndrome (PCOS). Two sets of experiments were performed in this study. In part one, sRAGE and VEGF protein levels in follicular fluid samples from 39 patients with PCOS and 35 non-PCOS patients were measured by ELISA. In part two, ovarian granulosa cells were isolated from an additional 10 patients with PCOS and cultured. VEGF and SP1 mRNA and protein levels, as well as pAKT levels, were detected by real-time PCR and Western blotting after cultured cells were treated with different concentrations of sRAGE. Compared with the non-PCOS patients, patients with PCOS had lower sRAGE levels in follicular fluid. Multi-adjusted regression analysis showed that high sRAGE levels in follicular fluid predicted a lower Gn dose, more oocytes retrieved, and a better IVF outcome in the non-PCOS group. Logistic regression analysis showed that higher sRAGE levels predicted favorably IVF outcomes in the non-PCOS group. Multi-adjusted regression analysis also showed that high sRAGE levels in follicular fluid predicted a lower Gn dose in the PCOS group. Treating granulosa cells isolated from patients with PCOS with recombinant sRAGE decreased VEGF and SP1 mRNA and protein expression and pAKT levels in a dose-dependent manner.

Introduction

Polycystic ovarian syndrome (PCOS) is the most common endocrine disorder, occurring in 5–12% of reproductive women, and is one of the main causes of female infertility (Hart & Doherty 2015). Interactions of insulin resistance and hyperandrogenism have been implicated in the pathogenesis of PCOS. Recent studies have demonstrated that chronic inflammation is involved in the occurrence of PCOS, but a clinical cure requires elucidation of the pathogenesis of PCOS (Pertynska-Marczewska et al. 2015).

Vascular endothelial growth factor (VEGF) is a 46-kDa dimeric protein whose expression level gradually increases with the development of follicles in the normal menstrual cycle of women. Babitha et al. (2013) suggested that VEGF promotes capillary proliferation related to increases in the diameter, selection, and maturation of the pre-ovulatory follicle. VEGF may contribute to the occurrence of PCOS by promoting angiogenesis (Toulis et al. 2011); reducing VEGF expression and secretion can improve ovarian function in women with PCOS (Abramovich et al. 2012). Thus, our aim was to evaluate the effect of sRAGE on VEGF in granulosa cells from patients with PCOS.

Advanced glycation end products (AGEs) are a heterogeneous group of non-enzymatically modified proteins that form slowly under normal physiological conditions. Obesity, aging, hyperglycemia, oxidative stress, and insulin resistance accelerate the generation of AGEs (Piperi et al. 2015). The receptor of AGEs (RAGE) is a member of the immunoglobulin superfamily. The upregulation and accumulation of AGEs, which activate MAP kinase; NF-kB; and other major cellular signaling pathways leads to cellular dysfunction and have been implicated in many pathological processes and diseases (Prasad 2014, Palimeri et al. 2015).

Soluble RAGE (sRAGE) is an extracellular form of RAGE. sRAGE lacks the cytosolic and transmembrane domains of RAGE and thus acts as a decoy that prevents the adverse intracellular effects of the AGE–RAGE axis. sRAGE consequently serves as not only a biological marker that reflects pathological changes within the body but also a protective factor that delays
the occurrence of diabetes, atherosclerosis, and other diseases (Basta et al. 2006).

sRAGE can also be used as a marker of an acceptable development environment and reproductive potential (Merhi 2014). However, some scholars believe that a high concentration of sRAGE is negatively related to embryo quality (Bonetti et al. 2013). sRAGE is closely related to inflammatory factors such as insulin resistance and ovarian reserve, while the inflammatory response, insulin resistance, and ovulatory dysfunction are clinical and pathological characteristics of PCOS (Spritzer et al. 2015). In many cells and tissues, sRAGE can inhibit NF-κB (Tang et al. 2015) and reduce reactive oxygen species (ROS) formation (Shubbar et al. 2012). NF-κB and ROS are involved in promoting the expression of VEGF. Additionally, elevated sRAGE concentrations can reduce VEGF expression and then inhibit the growth of cultured human breast cancer cells (Shubbar et al. 2012). However, Goova et al. 2001 found that sRAGE enhances VEGF expression and accelerates skin wound healing. Nevertheless, the regulation of VEGF by sRAGE in granulosa cells of patients with PCOS remains unclear. The present study investigated the effect of sRAGE on VEGF expression to reveal the potential role of sRAGE in the development of PCOS and to determine the potential underlying mechanism by which sRAGE regulates VEGF expression.

Materials and methods

All protocols were approved by the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. All subjects that were enrolled in the study gave written formal consent to participate.

Subjects

The subjects in this study were recruited from patients who underwent in vitro fertilization–embryo transfer (IVF-ET) at our reproductive center. Two sets of experiments were performed in this study. The first part of our study included the control and PCOS groups. The control group included 35 patients with infertility caused only by tubal factors, and the PCOS group included 39 patients with PCOS; follicular fluid (FF) was extracted from both groups. The second part of our study included 10 additional patients with PCOS from whom granulosa cells were isolated for in vitro culture. The inclusion criteria for the control group were as follows: (1) no hormonal drug use within the last 3 months and normal levels of follicle-stimulating hormone (FSH; <10 IU/L), luteinizing hormone (LH; <10 IU/L), and estradiol (E2; <50 pg/mL); (2) no polycystic morphology; and (3) regular menstruation and normal ovulation. PCOS was diagnosed according to the Rotterdam criteria (Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group 2004). All subjects in the study had no history of genetic disease, immune infertility, ovarian surgery, uterine malformation, or endocrine disease.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Follicular fluid sRAGE and VEGF levels and clinical characteristics of the patients in the PCOS and control groups.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical characteristics</strong></td>
<td><strong>PCOS group (n = 39)</strong></td>
</tr>
<tr>
<td>Age (years)</td>
<td>27.600 ± 3.236</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.049 ± 2.169</td>
</tr>
<tr>
<td>FSH (pg/mL)</td>
<td>5.877 ± 1.110</td>
</tr>
<tr>
<td>LH/FSH</td>
<td>1.739 ± 0.671</td>
</tr>
<tr>
<td>T (ng/mL)</td>
<td>0.217 ± 1.293</td>
</tr>
<tr>
<td>E₂ (pg/mL)</td>
<td>27.836 ± 23.10</td>
</tr>
<tr>
<td>Antral follicle count</td>
<td>25 ± 4.771</td>
</tr>
<tr>
<td>Total dose of gonadotropin</td>
<td>1221.54 ± 278.612</td>
</tr>
<tr>
<td>Number of oocytes retrieved</td>
<td>17.770 ± 5.234</td>
</tr>
<tr>
<td>Number of mature oocytes</td>
<td>11.82 ± 5.321</td>
</tr>
<tr>
<td>High-quality embryos</td>
<td>5.87 ± 2.839</td>
</tr>
<tr>
<td>VEGF (pg/mL)</td>
<td>1609.024 ± 17.912</td>
</tr>
<tr>
<td>sRAGE (pg/mL)</td>
<td>666.753 ± 233.153</td>
</tr>
</tbody>
</table>

Values are presented as the mean ± s.d. The PCOS group compared with the control.

*P < 0.05.

All patients had normal levels of thyroid stimulating hormone (TSH) and prolactin (PRL). sRAGE levels are lower in women older than 35 years and in those with a body mass index (BMI) greater than 25 (He et al. 2014). Additionally, sRAGE has been related to diabetes, chronic kidney disease and related complications, chronic metabolic diseases, and endometriosis (Kalousová et al. 2007, Fujii et al. 2008, Stewart et al. 2008); therefore, patients with these diseases were excluded. All patients were younger than 35 years of age and had a BMI between 18 and 25. Two high-quality embryos were transferred into each patient because of China’s family planning policy. General data for the two groups are shown in Table 1.

Experimental sample collection and determination

Pituitary desensitization was initiated in midluteal phase by subcutaneous injection of a gonadotropin-releasing hormone agonist (GnRH-a, Decapeptyl, Ferring GmbH, Germany). Gonadotropin (Gn) stimulation was initiated after standard downregulation with human menopausal gonadotropin (hMG, Livzon, Guangdon, China) and recombinant FSH (Gonal-F; Merck, Darmstadt, Germany), followed by human chorionic gonadotropin (hCG, Livzon, Guangdon, China) administration based on follicular size. Oocytes were collected by transvaginal ultrasound-guided puncture 34–36 h later. The fluid from the first large aspirated follicle without blood was subjected to enzyme-linked immunosorbent assay (ELISA) to quantify sRAGE and VEGF (R&D Systems, Inc.). The lower limit of sensitivity was 4.12 pg/mL for sRAGE and 15.6 pg/mL for VEGF. The intra- and inter-assay coefficients of variation for both sRAGE and VEGF were each less than 5%.

Collection and cultivation of granulosa cells

Blood-free FF was centrifuged at 400 g for 10 min. Phosphate-buffered saline (PBS, 12 mL from HyClone, USA)
was added to the cells to prepare a single-cell suspension. The suspension was added to a centrifuge tube containing hydroxypropylmethyl cellulose (Hao Yang Biological Formulation Company, Tianjin, China) in a 1:1 ratio, followed by centrifugation at 2000 r/min for 30 min. PBS was added to the white cell layer in a 1:1 ratio, followed by centrifugation at 200 g for 10 min. The cells were placed in red cell lysis buffer and centrifuged at 1000 r/min at room temperature for one minute. After the supernatant was removed, the sample was immediately stored at −80°C until use in RT-PCR and Western blotting experiments. To cultivate granulosa cells, 10 additional reproductive-age women with PCOS were prospectively enrolled. The granulosa cells were cultured in a humidified atmosphere containing 95% air and 5% CO2 at 37°C in DMEM/F-12 medium supplemented with 10% charcoal/dextran-treated FBS, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, and 1X GlutaMAX. Cells were plated in 12-well plates at a density of 5 × 104 cells/cm² with 1 mL of culture medium. After 5 days, the medium was changed to medium containing 0.5% charcoal/dextran-treated FBS. After another 48 h, sRAGE was added at a concentration of 0, 0.6, 0.9, or 1.2 µg/mL (Liu et al. 2016a). Granulosa cells cultured without sRAGE stimulation were used as the control group. After another 48 h, each group of granulosa cells was divided into two equal portions. One portion was used for RT-PCR, and the other portion was used for Western blotting.

**Determination of VEGF and SP1 mRNA in granulosa cells**

Total RNA was isolated from the collected primary granulosa cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA quality was verified using a NanoDrop spectrophotometer (samples with a minimum concentration of 10 ng/µL and an OD 260:280 ratio of 1.8–2.0). First-strand cDNA was synthesized from 2 µg total RNA using reverse transcriptase (Fermentas, Canada). The following primers were used for RT-PCR: VEGF, 5'-TTGCTGCTACTCCACAGC-3' (sense) and 5'-GGTTTATCCGCAATACCT-3' (antisense); GAPDH, 5'-AATCCCCATCACCTTCC-3' (sense) and 5'-CCTGCTTACCACCTTCTT-3' (antisense); specificity protein 1 (SP1), 5'-GATGAGCTACAGAGGCACAAACG-3' (sense) and 5'-GGTTGAAATAGGCGCTAAGGAGG-3' (antisense); and β-actin, 5'-CAGCATGGGAGGGCAGACTAC-3' (sense) and 5'-TAAAGACCTTACAGCGCAACT-3' (antisense).

VEGF and SP1 mRNA levels were determined by RT-PCR (EDC-810, EASTWIN, China). The cDNA was then amplified in triplicate using SYBR Green/FluoresceinPCR master mix (Fermentas, Canada) and detected on an ABI Prism 7900 Sequence PCR machine (Illumina, USA). All RT-PCR experiments were performed in triplicate, and the mean value was used to determine the mRNA levels. Water and mRNA without reverse transcriptase were used as negative controls.

The levels of mRNA relative to GAPDH or β-actin were calculated using the 2-ΔΔCt threshold cycle method. All primers were provided by GenScript Company (China).

**Statistical analysis**

The mean and standard deviations (mean ± s.d.) were used to describe variables that were all normally distributed. Student's t-test followed by an F test was applied for single comparisons between the PCOS group and the control group. For data that exhibited homogeneity of variance, Pearson correlation analysis was used for comparisons between FF sRAGE and clinical parameters such as age, BMI, days of stimulation, total dose of Gn, number of oocytes retrieved, number of matured oocytes, and number of high-quality embryos.

Multiple regression models were used to analyze the effect of the independent variable FF sRAGE on the total dose of Gn, and the potential explanatory variables were age, BMI, and day 3 FSH. Multiple regression models were also used to analyze the effect of the independent variable FF sRAGE on the number of oocytes retrieved, and the potential explanatory variables were age, BMI, day 3 FSH, and the dose of Gn. Furthermore, multiple logistic regression models were used to analyze the effect of the independent variable FF sRAGE on pregnancy, and potential explanatory variables were age and BMI.

VEGF and SP1 mRNA and protein expression and pAkt levels in cells treated with different concentrations of sRAGE were compared using Student's t-test or the Mann–Whitney U test followed by an F test. All statistical analyses were performed using SPSS software (version 12.0 for Windows; SPSS Inc.). We considered P < 0.05 to indicate statistical significance.

**Results**

**FF sRAGE levels positively correlate with the number of oocytes retrieved in the control group**

sRAGE concentrations (mean, 1518.608 ± 872.130 pg/mL) were measured in FF samples from 35 participants in the control group. Correlation analysis revealed no correlation between FF sRAGE protein levels and age, BMI, days of stimulation, the number of mature oocytes,
Table 2 Correlation between the follicular fluid (FF) sRAGE protein concentration and other clinical parameters.

<table>
<thead>
<tr>
<th>Variable</th>
<th>( r )</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>−0.012</td>
<td>0.946</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>−0.247</td>
<td>0.153</td>
</tr>
<tr>
<td>Days of stimulation</td>
<td>0.168</td>
<td>0.329</td>
</tr>
<tr>
<td>Total dose of Gn (IU)</td>
<td>−0.328</td>
<td>0.041</td>
</tr>
<tr>
<td>Oocytes retrieved</td>
<td>0.488</td>
<td>0.005</td>
</tr>
<tr>
<td>Mature oocytes</td>
<td>0.376</td>
<td>0.171</td>
</tr>
<tr>
<td>High-quality embryos</td>
<td>0.207</td>
<td>0.232</td>
</tr>
<tr>
<td>PCOS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.134</td>
<td>0.417</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>−0.192</td>
<td>0.242</td>
</tr>
<tr>
<td>Days of stimulation</td>
<td>−0.104</td>
<td>0.523</td>
</tr>
<tr>
<td>Total dose of Gn (IU)</td>
<td>−0.451</td>
<td>0.004</td>
</tr>
<tr>
<td>Oocytes retrieved</td>
<td>−0.037</td>
<td>0.831</td>
</tr>
<tr>
<td>Mature oocytes</td>
<td>−0.228</td>
<td>0.067</td>
</tr>
<tr>
<td>High-quality embryos</td>
<td>0.119</td>
<td>0.407</td>
</tr>
</tbody>
</table>

Statistical significance using the Pearson product–moment correlation coefficient. Significance at \( P < 0.05 \).

or the number of high-quality embryos (Table 2). We observed an inverse relationship between FF sRAGE protein levels and the total dose of Gn (expressed as international units used per cycle) \( (r = −0.328, P = 0.041) \) (Table 2); this relationship was independent of age, BMI, or day 3 FSH (\( \beta = −0.319, P = 0.010 \)) (Table 3). Correlation analysis showed a correlation between FF sRAGE protein levels and the total number of oocytes retrieved \( (r = 0.488, P = 0.005) \) (Table 2). After the analysis was adjusted for age, BMI, day 3 FSH, and the dose of Gn used, FF sRAGE protein levels could be used to predict the total number of oocytes retrieved \( (\beta = 0.005, P = 0.008) \) (Table 3). Women who successfully conceived after IVF transplantation had significantly higher sRAGE levels than women who failed to conceive \( (1906.201 ± 1160.407 \text{ vs } 1145.147 ± 383.716 \text{ pg/mL}, P = 0.048) \) (Fig. 1A). After the analysis was adjusted for age, BMI, day 3 FSH, and the dose of Gn used, FF sRAGE protein levels could be used to predict the pregnancy outcome (odds ratio = 1.014, \( P = 0.039 \)) (Table 4).

Table 3 Multinomial regression analysis of follicular fluid (FF) sRAGE concentrations in both groups.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Beta</th>
<th>( P ) value</th>
<th>( R^2 )</th>
<th>Overall model ( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total dose of Gn (IU)</td>
<td></td>
<td>0.184</td>
<td>0.010</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.270</td>
<td>0.086</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>0.259</td>
<td>0.112</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3 FSH (IU)</td>
<td>−0.226</td>
<td>0.157</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FF sRAGE (pg/mL)</td>
<td>−0.319</td>
<td>0.010</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of oocytes retrieved</td>
<td></td>
<td></td>
<td>0.311</td>
<td>0.045</td>
</tr>
<tr>
<td>Age (years)</td>
<td>−0.145</td>
<td>0.764</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>0.121</td>
<td>0.888</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3 FSH (IU)</td>
<td>0.877</td>
<td>0.493</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total dose of Gn (IU)</td>
<td>−0.001</td>
<td>0.680</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FF sRAGE (pg/mL)</td>
<td>0.005</td>
<td>0.008</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCOS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total dose of Gn (IU)</td>
<td></td>
<td>0.203</td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.192</td>
<td>0.198</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>−0.055</td>
<td>0.716</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 3 FSH (IU)</td>
<td>0.172</td>
<td>0.248</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FF sRAGE (pg/mL)</td>
<td>−0.539</td>
<td>0.004</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Statistical significance using multinomial regression analysis. Significance at \( P < 0.05 \).

The FF sRAGE concentration is higher in patients with PCOS and negatively correlates with the total dose of Gn (expressed as international units used per cycle)

The FF sRAGE concentration was significantly lower in the PCOS group than in the control group \( (666.753 ± 233.153 \text{ vs } 1518.608 ± 872.130 \text{ pg/mL}, P < 0.001) \) (Fig. 1B). Correlation analysis revealed no correlation between FF sRAGE protein levels and age, BMI, or day 3 FSH (\( \beta = −0.328, P = 0.041 \)) (Table 2). After the analysis was adjusted for age, BMI, day 3 FSH, and the dose of Gn used, FF sRAGE protein levels could be used to predict the pregnancy outcome (odds ratio = 1.014, \( P = 0.039 \)) (Table 4).
BMI, days of stimulation, the number of mature oocytes, or the number of high-quality embryos (Table 2). FF sRAGE protein levels in patients with PCOS were negatively related to the total dose of Gn (expressed as international units used per cycle) after adjusting for age, BMI, and day 3 FSH (beta = −0.539, P = 0.004) (Table 3). After the analysis was adjusted for age, BMI, day 3 FSH, and the dose of Gn (expressed as international units used per cycle), there was no relationship between FF sRAGE levels and the number of oocytes retrieved (r = −0.037, P = 0.831) (Table 2), in contrast to the results obtained for the control group. There was no significant difference in sRAGE levels between women who successfully conceived and women who failed to conceive after two embryos were transferred (772.527 ± 344.248 vs 687.026 ± 158.561 pg/mL, P = 0.524) (Fig. 1C).

Exogenous sRAGE decreases VEGF mRNA and protein expression levels in ovarian granulosa cells

VEGF protein levels in FF aspirated without blood at the time of egg collection were measured by ELISA (n = 39). The VEGF concentration in the FF was significantly higher in the PCOS group than in the control group (1609.024 ± 17.912 vs 1109.360 ± 12.021 pg/mL, P < 0.001) (Fig. 2A). An additional 10 patients with PCOS were enrolled and used as sources of granulosa cells for culture. VEGF mRNA and protein levels were detected by RT-PCR and Western blotting, respectively, after cells were treated with different concentrations of sRAGE (0, 0.6, 0.9, or 1.2 µg/mL). VEGF mRNA and protein levels were both lower in granulosa cells treated with sRAGE compared to untreated granulosa cells, and the effects of sRAGE on VEGF expression were dose-dependent (Fig. 2B and C).

Exogenous sRAGE decreases pAKT levels and SP1 mRNA and protein levels in ovarian granulosa cells

pAKT levels and SP1 protein and mRNA expression were detected by Western blotting and RT-PCR after cells were treated with different concentrations of sRAGE (0, 0.6, 0.9, or 1.2 µg/mL). The levels of pAKT, SP1 protein,
and SP1 mRNA were all lower in granulosa cells treated with sRAGE compared to untreated granulosa cells, and these effects of sRAGE were dose-dependent (Fig. 3A, B and C).

Discussion

A consensus has been reached regarding the effect of the AGE–RAGE axis on ovarian dysfunction. Jinno et al. (2011) revealed that AGEs have negative effects on the growth and development of the follicles, resulting in ovarian dysfunction and unfavorable IVF outcomes. However, Jinno et al. (2011) did not address the association between ovarian dysfunction and sRAGE, and the role of sRAGE in the follicular microenvironment remains unclear. Recent evidence links the AGE–RAGE axis to ovarian dysfunction. However, the function and mechanism of sRAGE have been largely neglected. This study aimed to compare the differences in FF sRAGE levels between non-PCOS and PCOS patients and to explore the potential role of sRAGE in PCOS.

We measured sRAGE concentrations in FF samples from 35 women of childbearing age in the control group and determined that there was no correlation between FF sRAGE protein levels and age, BMI, or days of stimulation. These results are consistent with the findings of Merhi et al. (2014). Serum sRAGE concentrations are negatively related to the BMI. However, one study showed that FF sRAGE levels negatively correlate with BMI (Bonetti et al., 2013), in contrast to the findings of the present study. A possible explanation for this difference could be that Bonetti et al. (2013) used a BMI of less than 35 kg/m² as a cutoff for the inclusion criteria, whereas we used a BMI of less than 25 kg/m² because FF sRAGE protein levels decreased in the participants with a BMI of 25 kg/m² or greater.

In the control group, sRAGE protein levels were negatively related to the total dose of Gn (expressed as international units used per cycle), independent of age, BMI, or day 3 FSH, which is in accordance with the previous results reported by Merhi; however, it is unclear whether the gonadotropin dose truly influences the synthesis of sRAGE in FF during the period of ovulation induction. To characterize the relation between sRAGE concentration and gonadotropin dose, it would be more clinically significant to measure the serum sRAGE concentration before gonadotropin administration.

The FF sRAGE protein levels could be used to predict the total number of oocytes retrieved after adjusting for age, BMI, day 3 FSH, and the dose of Gn used. Merhi (2014) demonstrated that sRAGE positively correlates with AMH protein levels in FF, whereas AMH is a recognized marker of ovarian reserve capacity in women of childbearing age (Brodin et al., 2015). These results suggest that sRAGE may also be a marker of reproductive environment and fertility. Correlation analysis revealed no correlation between FF sRAGE protein levels and the number of high-quality embryos. A recent study by Bonetti et al. (2013) showed that high FF sRAGE levels predicted poor-quality embryos. A possible explanation for this difference could be the differences in the inclusion criteria. The authors used a BMI of less than 35 kg/m² as the cutoff for the inclusion criteria, whereas we used a BMI of less than 25 kg/m². The authors also used a basal FSH level of less than 14 IU/L as the cutoff for the inclusion criteria, whereas we used a basal FSH level of 10 IU/L.

We also compared the concentrations of sRAGE in FF of women who successfully conceived and in that of women who failed to conceive during a fresh embryo transfer cycle and determined that sRAGE was higher in the pregnancy group, further illustrating that sRAGE concentrations in FF can be used to predict favorable IVF outcomes. Ota et al. (2014) reported that elevated serum sRAGE levels are associated with recurrent pregnancy loss (RPL) through a reduction in uterine blood flow, which causes ischemia in the fetus. However, Rzepka et al. (2015) observed a negative trend for sRAGE and CRP plasma levels in a group of women with preterm premature rupture of membranes, and these authors determined that a high sRAGE concentration could be a favorable prognostic factor in the presence of symptoms of threatened premature labor.

In recent years, there has been increasing evidence indicating that the AGE–RAGE axis contributes to inflammation and increases oxidative stress, processes that play a major role in many chronic diseases (Uribarri et al., 2015). However, sRAGE exerts protective effects in a wide range of diseases through mechanisms other than the sequestration of RAGE ligands (Schmidt, 2015). In the present study, mean sRAGE concentrations in the PCOS group were decreased compared with the concentrations in patients without PCOS. In previous studies, lower levels of sRAGE were significantly associated with an increased risk of heart failure (Lazo et al., 2015). The effect of sRAGE on the genesis of PCOS remains unknown. Multiple analyses revealed that the FF sRAGE protein level in patients with PCOS was negatively related to the total dose of Gn. However, the relationship between FF sRAGE and the total number of oocytes retrieved is not statistically significant. The effects of reduced sRAGE concentration in patients with PCOS remain unclear, and the association between lower intrafollicular sRAGE levels and the genesis and evolution of PCOS requires further exploration.

AGE–RAGE interactions stimulate VEGF production and cause changes in the follicular environment, leading to reproductive dysfunction (Merhi, 2014). VEGF may be involved in the pathogenesis of PCOS by influencing angiogenesis (Guruvaiah et al., 2014). However, research on the direct impact of sRAGE on VEGF in granulosa cells is lacking.
FF VEGF concentrations were significantly higher in the PCOS group than in the control group. The cultured ovarian granulosa cells isolated from PCOS patients were treated *in vitro* with different concentrations of sRAGE, and the expression and secretion of VEGF were detected. VEGF mRNA and protein levels decreased in these cells compared with ovarian granulosa cells that were not treated with sRAGE, and the effects of sRAGE were dose dependent. As reducing the expression and secretion of VEGF can improve ovarian function in patients with PCOS (Abramovich et al. 2012), and elevated FF VEGF levels reduce the pregnancy rate in assisted reproductive technology (Manau et al. 2000), we thus inferred that sRAGE may play a protective role in patients with PCOS by inhibiting VEGF. Determining whether sRAGE can prevent the progression of PCOS by inhibiting VEGF requires further study.

The phosphatidylinositol 3-kinase (PI3K)/AKT pathway is involved in the regulation of cellular metabolism in various types of cancer, and this pathway is a common therapeutic target of anticancer drugs. One of the outcomes of this signaling pathway is the regulation of VEGF production (Papломата & O’Regan 2014). A previous study by Zong et al. (2016) showed that in U937-derived foam cells, VEGF may be upregulated by CD147 through the PI3K/AKT pathway. The PI3K/AKT pathway can activate many transcription factors, including SP transcription factors (Archer 2011). SP1 is an SP transcription factor that can bind specific sites within the VEGF promoter to play an important role in regulating VEGF production (Liu et al. 2016b). Research by Pang (2013) indicated that the activated PI3K/AKT pathway increases the interaction between VEGF and SP1, resulting in the upregulation of VEGF expression. Curry et al. (2008) confirmed that artificial mutation of the SP1 binding sites decreases the activity of the VEGF promoter. In our study, pAKT, SP1 protein, and SP1 mRNA levels were all lower in sRAGE-treated granulosa cells compared to untreated granulosa cells, and the effects of sRAGE were dose-dependent, perhaps indicating that sRAGE regulates VEGF expression via the PI3K/AKT/SP1 signaling pathway.

In summary, the findings of the present study suggest an association between FF sRAGE levels and the number of oocytes retrieved in women of childbearing age. Although the role of sRAGE in PCOS is unknown, the present results provide additional evidence of an association between FF sRAGE and VEGF concentrations in patients with PCOS.

### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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