INTRODUCTION

Vascular endothelial growth factor (VEGF) is the most important angiogenine discovered so far and is a growth factor yet known to have both pro-angiogenesis and vascular permeability enhancing functions in all proangiogenic factors.\(^1,2\) Previous studies have shown that, in the ovulation cycle, with the follicle growth and maturation, VEGF levels in serum and follicular fluid were significantly higher, suggesting that VEGF may participate in the development of follicles.\(^3,4\) However, there are few reports on the related research of VEGF in human embryonic development. VEGF gene polymorphisms are associated with recurrent miscarriage, preeclampsia and premature birth.\(^5,6\) However, few studies have been reported on the association between VEGF gene polymorphisms and IVF-ET/ICSI clinical outcomes. Relationship between vascular endothelial growth factor (VEGF) 936 C/T gene polymorphisms and IVF-ET/ICSI treatment outcomes can be studied by detection of serum VEGF genotypes in the pregnant and non-pregnant women, thus providing reference for IVF - ET/ICSI therapy. The aim of this study was to analyse the association between VEGF levels in embryo culture mediums and clinical outcomes after embryo transplantation.

METHODOLOGY

This study was done in The Reproductive Medical Center, Yantaishan Hospital, Yantai, Shandong Province, China, from January to December 2016. All patients met
the IVF-ET/ICSI indications and were Chinese Han women. This study was approved by the Hospital Ethics Committee and the patients signed informed consent. Using the unit's conventional ovulation-promoting programme to promote ovulation. Oocyte pickup was carried out under transvaginal B ultrasound guidance. Conventional in-vitro fertilisation was performed after seminal fluid treatment. Fertilisation was observed at 19-hour after addition of sperm. After fertilisation, the fertilised ovums were transferred to embryonic culture droplets, with one culture droplet for one embryo. The embryos were incubated at 37°C in a 6% CO₂ incubator and observed after 48 hours for embryo scoring. The best two embryos were chosen for transplantation. Embryo culture medium was loaded as per 2030 μL/drop after transfer of embryos and stored at -80°C ultra-low temperature refrigerator for testing.

On the 3rd day of culture, the embryos were scored. According to the number of blastomeres, whether the cells were homogenous and whether there were debris, embryos were graded in line with Wetzels embryo scoring method. Grade I embryos (4 points) had uniform cells, regular shape, intact zona pellucida, even and clear cytoplasm, with cell debris at 5%. Grade II embryos (3 points) had non-uniform cell size, slightly irregular shape, granule in cytoplasm, with cell debris between 10-20%. Grade III embryos (2 points) had obviously uneven cell size, obviously irregular shape, granule in cytoplasm, with cell debris between 21-50%. Grade IV embryos (1 point): extremely uneven cell, severe granule in cytoplasm, with cell debris above 50%. Grade V embryos (0 point) were degenerated, dead embryos. The score for each embryo was obtained by multiplying embryonic morphological score with number of embryonic cells, and cumulative embryo score (CES) of each patient's transfer cycle was obtained by totaling the transplanted embryos' score in the transfer cycle.

According to clinical outcomes, all the 98 patients were divided into clinical pregnant group and non-pregnant group. The diagnosis of clinical pregnancy is ultrasound examination at 4 weeks after embryo transfer, seeing gestational sac and fetal buds, original heart tube or seeing villus at uterine curettage.

VEGF concentration in the culture medium was measured by ELISA. The reagent used for the assay was a VEGF kit and a pre-test was conducted to adjust the dilution ratio.

For all subjects, 5 mL of fresh peripheral venous whole blood was collected under aseptic conditions and anticoagulated with ethylenediaminetetraacetic acid. The specific operation of genomic DNA extraction was performed in strict accordance with the kit instructions. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technology was used to perform PCR amplification with white blood cell genomic DNA in peripheral blood as a template. The upstream primer of VEGF 936 C/T was 5'-AAGGAAGA GGAGACTTGCGCAGAGC-3', and the downstream primer was 5'-TAATTGATGTGGTGTCAGG-3'. The 50 μL total reaction system contained one μL of upstream and downstream primers respectively, and approximately one μL of genomic DNA, and 25 μL 2xHiFi-PCR Master (3 mmol/L MgCl₂, 0.2 mmol/L dNTP, 0.1 U/μL Taq DNA polymerase, 2x PCR buffer), which was complemented by double distilled water. Amplification was performed on a PCR thermocycler: pre-denaturation at 94°C for 8 minutes, denaturation at 94°C for 30 seconds, annealing at 65°C for 30 seconds, extension at 72°C for 30 seconds, 30 cycles and final extension at 72°C for 10 minutes. The 15 μL of the PCR reaction product was digested with restriction enzyme NiaIII 1L. The digested products were electrophoresed on 1.2% agarose, stained with ethidium bromide, observed under a UV lamp and photographed. The 208 bp 1 band was observed for TT genotype enzyme-free cutting site; the NiaIII restriction enzyme cutting site of CC genotype was cut into122 bp and 86 bp bands. The CT genotype part was cut, and 208, 122 and 86 bp bands were observed.

SPSS 21.0 statistical software package was used for data processing. Measurement data are expressed as mean ± standard deviation, count data are expressed as frequencies with percentages. Measurement data are tested with independent-sample t-test, and count data are tested with Chi-square test. P<0.05 indicates statistically significant differences.

**RESULTS**

Ninety-eight patients undergoing IVF-ET/ICSI treatment were selected, including 46 (46.94%) cases of primary infertility and 52 (53.06%) cases of secondary infertility.

<table>
<thead>
<tr>
<th>Index</th>
<th>Pregnant group (n=58)</th>
<th>Non-pregnant group (n=40)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female age (year)</td>
<td>34.37 ±3.69</td>
<td>34.84 ±4.96</td>
<td>0.601</td>
</tr>
<tr>
<td>Infertility duration (year)</td>
<td>4.57 ±0.50</td>
<td>4.43 ±0.51</td>
<td>0.180</td>
</tr>
<tr>
<td>Body mass index</td>
<td>20.98 ±1.26</td>
<td>20.87 ±2.03</td>
<td>0.741</td>
</tr>
<tr>
<td>Number of previous grafts</td>
<td>1.14 ±0.25</td>
<td>1.21 ±0.15</td>
<td>0.119</td>
</tr>
<tr>
<td>Basic FSH (mIU/mL)</td>
<td>7.09 ±0.38</td>
<td>7.06 ±0.35</td>
<td>0.693</td>
</tr>
<tr>
<td>Basic LH (mIU/mL)</td>
<td>4.69 ±0.39</td>
<td>4.82 ±0.76</td>
<td>0.282</td>
</tr>
<tr>
<td>Basic E2 (pg/mL)</td>
<td>46.07 ±1.29</td>
<td>46.91 ±3.04</td>
<td>0.064</td>
</tr>
<tr>
<td>Chorionic gonadotropin (HCG) intimal thickness (cm)</td>
<td>7.43 ±0.84</td>
<td>7.27 ±1.52</td>
<td>0.505</td>
</tr>
<tr>
<td>Estrogen (E2) level, (pg/mL)</td>
<td>3693.35 ±167.54</td>
<td>3681.06 ±151.91</td>
<td>0.712</td>
</tr>
<tr>
<td>Progesterone (P) level (ng/mL)</td>
<td>13.33 ±1.41</td>
<td>13.1 ±0.46</td>
<td>0.822</td>
</tr>
<tr>
<td>Number of retrieved oocytes</td>
<td>19.46 ±4.19</td>
<td>19.07 ±4.56</td>
<td>0.663</td>
</tr>
<tr>
<td>Embryo number</td>
<td>10.57 ±2.09</td>
<td>9.88 ±2.53</td>
<td>0.150</td>
</tr>
<tr>
<td>Quality embryo number</td>
<td>7.15 ±0.84</td>
<td>6.75 ±1.32</td>
<td>0.070</td>
</tr>
<tr>
<td>Quality embryo rate (%)</td>
<td>0.75 ±0.06</td>
<td>0.73 ±0.10</td>
<td>0.204</td>
</tr>
<tr>
<td>Cumulative embryo score (Score)</td>
<td>43.36 ±6.61</td>
<td>42.25 ±5.06</td>
<td>0.263</td>
</tr>
<tr>
<td>VEGF (pg/mL)</td>
<td>11.35 ±1.88</td>
<td>14.08 ±2.63</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Ranging from 24 to 48 years, the patients had an average age of 34.56 ±2.41 years. Of the 98 cases of embryo transplanted, 58 (59.18%) cases got pregnant, making 58 (59.18%) cases in the pregnant group and 40 (40.82%) cases in the non-pregnant group. There were no significant differences in average age, infertility duration, body mass index, number of previous grafts, basic follicle-stimulating hormone (FSH) levels, luteinizing hormone (LH) levels, chorionic gonadotropin (HCG) intimal thickness, estrogen (E2) level, progesterone (P) level, number of retrieved oocytes, embryo number, quality embryo number, quality embryo rate, and cumulative embryo score (CES) between the two groups. VEGF level in embryo culture medium of pregnant group was lower than that of non-pregnant group (p<0.001, Table I). VEGF level in the culture medium of grade I embryos (4 points) was 8.03 ±0.76 pg/mL, and that of grade II embryos (3 points) was 17.12 ±3.04 pg/mL. VEGF level in the culture medium of grade I embryos with better implantation potential was lower than that of slightly deficient grade II embryos, the difference is statistically significant (p<0.001).

Both pregnant and non-pregnant groups’ VEGF genes have C and T alleles and CC, CT and TT genotypes. The distribution was consistent with Hardy-Weinberg equilibrium method (Figure 1). The frequencies of CC genotypes of VEGF 936 in non-pregnant and pregnant group were 90.0% (n=36) and 63.79% (n=37), respectively. C allele frequencies were 95.00% (n=76) and 81.03% (n=94), respectively. The frequencies of CC genotype and C allele in the non-pregnant group are higher than those in the pregnant group (p=0.003, and 0.005, respectively, Table II).

**DISCUSSION**

Embryo quality is a key factor in the success of pregnancy. In the current human assisted reproductive technology, morphological parameters of embryos are still the main non-invasive indicators for embryo selection. Although morphological assessment provides a simple and effective method for selection of embryos for transplantation, many quality embryos as assessed by morphological methods, failed to implant after transplantation. At present, there are many problems in embryo morphological assessment, such as the lack of quantifiable indicators and subjective factors of laboratory technicians. Therefore, how to increase prediction method and improve the ability to predict the potential of early embryo development has become a top priority. The assessment of embryonic developmental potential by measuring embryonic endogenous factors in embryonic metabolites may become an effective method for assessing embryo quality due to its non-invasive nature and low risk. Relevant data show that after removal of VEGF in the process of mouse embryo culture in-vitro, embryo growth retarded or even stopped. But returned to normal and showed cardiovascular formation after VEGF was added. However, there are few reports on the related research of VEGF in human embryonic development.

Previous studies have suggested that clinical pregnancy of in-vitro fertilisation (IVF) is related to embryo quality and endometrial receptivity. However, the results of this study show that there was no difference in clinical characteristics (average age, infertility duration, gonadotropin dosage, number of gonadotropins, and HCG intimal thickness) and embryonic development (number of received oocytes, number of embryos, number of quality embryos, and the cumulative embryo score of transplanted embryos) between the two groups. At the same time, expression levels of VEGF in embryo culture medium of pregnant group and non-pregnant group were initially compared. The result showed that VEGF level in embryo culture medium of pregnant group was lower than that of non-pregnant group and was significantly lower than that of non-pregnant group (p<0.001).
slightly deficient grade II embryos. The difference is statistically significant (p<0.001). All these results suggest that VEGF levels in embryo culture medium may predict clinical outcomes after embryo transfer earlier and more sensitively than embryo morphology and may serve as a reference indicator for predicting pregnancy outcomes.

Gene polymorphism refers to differences in nucleotide sequences between individuals. Polymorphisms occur at gene level mutations. There may be more than two genotypes in the same gene locus, which is prevalent in areas with noncoding protein or without important adjustment functions in gene sequences. The human VEGF gene is located on long arm of chromosome 6p21.3. The gene encoding VEGF is about 14 Kb in size and is composed of 8 exons and 7 introns alternately. The 3' region, 5' region and intron of human VEGF gene are all highly polymorphic. VEGF gene has at least 30 single nucleotide polymorphism sites. Some single nucleotide polymorphisms have higher frequencies, with 10 polymorphisms just in its promoter region. Where, +936 C/T at the 3'-end of the promoter is associated with RNA splicing. Studies have shown that distribution of VEGF 936 C/T polymorphism is significantly different in patients with endometriosis and normal controls. There was statistically significant difference in VEGF 936 C/T allele frequency between III-IV grade endometriosis patients and normal controls. There has been no report on the relationship between VEGF 936 C/T gene polymorphism and IVF-ET/ICSI treatment outcome. Our results showed that frequencies of CC genotype and C allele in the non-pregnant group were higher than those in the pregnant group. The difference was statistically significant. This suggests that VEGF 936 C/T polymorphism may be associated with IVF-ET/ICSI treatment outcomes. Thus, using the combination of VEGF levels, embryo morphological parameters and VEGF 936 C/T gene polymorphisms to select embryos may obtain embryos with optimal growth potential for transplantation and improve the success rate of pregnancy.

There are some limitations and shortages in this research; for example, this study is limited to only Chinese women. No comparison between the Chinese women and the Western population in VEGF 936 C/T gene polymorphisms. So further relevant researches need to continue.

CONCLUSION

VEGF level in the embryo culture medium can be used as one reference indicator for predicting embryo quality and pregnancy outcome. The VEGF936C/T gene polymorphism may be related to IVF-ET/ICSI treatment outcome, and C allele may be a susceptibility gene for IVF-ET/ICSI failure.

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REFERENCES


