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The correlation analysis of human embryonic MMP-9 secretion and embryo quality

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Abstract. – OBJECTIVE: The correlation of the embryo matrix metalloproteinase-9 (MMP-9) secretion, embryonic development and clinical pregnancy was investigated.

MATERIALS AND METHODS: The embryo culture from in vitro fertilization-embryo transfer (IVF-ET) patients were collected in the Xuzhou Central Hospital from January 2013 to September 2014. At the same time, the embryo grade was recorded. The secretion of the MMP-9 in the embryo culture was detected through hybridization (Dot-blot) and enzyme-linked immunosorbent assay (ELISA). The clinical pregnancy outcome was followed after one month of the embryo transfer.

RESULTS: With the embryonic development from 2-cell to 8-cell, embryonic MMP-9 secretion increased gradually ($p < 0.05$). Though 8/I embryo, the secretion of MMP-9 are not identical. The quantitative detection of the MMP-9 secretion of the 8-cell embryo by ELISA is higher as is the embryo score, but the low secretion of embryo score is lower ($p < 0.001$). As 8/I embryos, the clinical pregnancy rate (77.3%) of the MMP-9 high secretion embryos is higher than the low secretion embryos (16.7%) ($p < 0.001$).

CONCLUSIONS: The secretion of the embryonic MMP-9 is closely related to the quality of the embryo and embryo implantation. It is speculated that MMP-9 may become one of the criteria to evaluate the quality of the embryos.

Key Words:
Embryo, MMP-9, IVF-ET, Embryo quality.

Introduction

With the rapid development of reproductive medical technology in recent years, the success rate of *in vitro* fertilization-embryo transfer (IVF-ET) increased from 30-40% to 50%. However, there are still nearly half of the patients that are not satisfied. The factors that influence the success rate of IVF-ET are mainly the quality of the embryo, endometrium receptivity, and the establishment of the correct "dialogue" between embryo and endometrium. The traditionally used method of morphologic embryo selection is not predictive enough to allow routine single embryo transfer. The low implantation rate often results in the transfer of several embryos and leads to multiple gestations. Therefore, new screening tools are needed. The various methods have been studied that could help embryo selection[1,2]. Conaghan et al[3] tested an automated time-lapse analysis system to determine whether it can predict blastocyst formation. However, large prospective trials are needed to study before their introduction into daily practice can be recommended. This paper mainly focuses on the MMP-9 that is related to the embryo development and implantation in the hope of finding a method of judging the quality of the embryo without destroying it.

Materials and Methods

Embryo culture medium samples were provided by the Reproductive Center Laboratory of Xuzhou Central Hospital. Embryo culture medium samples of the IVF-ET patients from January, 2013 to September, 2014 in the Xuzhou Central Hospital were collected. The storage temperature is -20°C.

Ovulation drugs were used in infertile patients to stimulate multiple ovarian follicular maturations simultaneously in the IVF-ET pre-clinical period. Transvaginal ultrasound monitored follicular development. When the follicles mature, the oocyte was extracted using transvaginal ultrasound.

The egg and the sperm were cultivated in the embryonic laboratory of 37°C, 5% CO_2. Embryo after fertilization will be cultivated singly. Then, the fertilized embryo was transferred into the patient's uterus to continue to develop three days after fertilization. The fertilized egg will develop

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into 2-cell, 4-cell, 6- cell, 8-cell embryo. The culture medium was changed in different developmental stages of the embryo and collected.

The various embryo development stage and rating standards were provided by the reproductive center embryonic laboratory and annotated for embryo transfer.

The embryo quality is divided into four levels judging from the morphological index and includes the number and size of the blastomere, symmetry between blastomeres, the number of pieces in the blastomere and the thickness of the zona pellucida[4]. Grade I: Blastomeres are the same in size that are homogeneous and transparent with no debris. Grade II: Blastomeres are not the same in size that are homogeneous with no debris. Grade III: Blastomeres are the same in size that are homogeneous with few of debris. Grade IV: Blastomeres are not the same in size and turn into black with uneven granules. Embryos of grade I to III are transplantable of which grade I and II embryos are with high quality.

Detection of MMP-9 Secretion in Embryo Culture Medium by Dot-blot Method

The specific protocol is as follows: (1) Take a piece of nitrocellulose membrane (NC membrane) and a piece of filter paper and cut its size in accordance with Dot-blot apparatus (MBI Company, Denver, CO, USA). Incubate in tris-buffered saline (TBS) for ten minutes. (2) The filter paper is tiled in the Dot-blot apparatus, and the NC membrane is tiled in filter paper. Tighten the locking screw of the dot hybridization instrument. Dry the water on the membrane using the negative pressure suction machine. Stop suction when the NC film is semi-dry. Add 25 μl of the embryo culture medium to NC film by the hole of Dot-blot apparatus and keep suctioning for 15 min to make the protein adsorb to the NC film. Take out the NC membrane to dry. Incubate the membrane in 5% bovine serum albumin (BSA) at 37°C for 2 hrs. (3) Wash the membrane with TBS for three times and ten mins for each time. Incubate the membrane in anti-human matrix metalloproteinase-9 (MMP-9) antibody (1:200, pH7.4, Diluted with TBS (Santa Cruz Biotech, Santa Cruz, CA, USA) 37°C for two hrs. (4) Wash the membrane with TBS for three times and ten mins for each time. Incubate the membrane in alkaline phosphatase labeled anti-human IgM (1:1500 pH8.0, dilated with TBS, Santa Cruz Biotech Company) at 37°C for 40 minutes. (5) Wash the membrane with TBS for three times and ten minutes for each time. The membrane was colorized by alkaline phosphatase chromogenic substrate NBT/BCIP (Gibco/BRL, Grand Island, NY, USA) for 5-10 minutes avoiding light. (6) Wash with distilled water to terminate the reaction. Pat dry on the filter paper. Store in dark and analyze by a photograph.

Detection

The secretion of the MMP-9 of grade I embryo in different developmental stages was detected by Dot-blot method. The procedure was as follows: collect the culture medium of 2-cell, 4-cell, 6-cell, 8-cell embryo respectively. All embryo scores were grade I. The Dot-blot method was used to detect the secretion of MMP-9 in the culture medium.

The secretion of the embryo MMP-9 with the same cell period was detected and scored by Dot-blot method. The procedure was as follows: 15 cases of the medium of the embryos with single embryo transfer whose embryo scores were 8-cell grade I after the embryo transfer were collected. The Dot-blot method was used to detect the secretion of MMP-9 in the medium.

The secretion of MMP-9 in 8-cell embryo with different scores was detected by the ELISA. The procedure was as follows: analyze the relationship between the secretion level of MMP-9 and embryos development quality.

The kit was purchased from ZSJB-Bio Beijing Co Ltd. The procedure of ELISA was performed according to the protocols of the experiment. All 98 cases of IVF-ET patients are single embryo transfer with a total 98 8-cell embryo, 55 cases of 8/I embryo transplantation, 25 cases of 8/II embryo transplantation and 18 cases of 8/III embryos transplantation. Embryos of Grade I and II are of high quality. Embryos of Grade I, II and III are transplantable. The culture medium after the embryo transfer was collected for the detection of the pro-MMP-9 secretions by ELISA. The different secretions of MMP-9 were compared in the different 8-cell embryos with the same score and analyzed the correlation between the secretion level of the MMP-9 and embryo quality.

The correlation analysis of the secretion level of the embryonic MMP-9 and clinical pregnancy was as follows: 8/I embryos were detected by the above 1.4 steps and transferred into patients.

95% confidence interval of pregnancy was calculated based on the concentration of MMP-9 in the corresponding 8/I embryo culture medium. Patients were divided into high secretion and low

secretion group according to this critical value. Telephone follow-up was conducted one month after the embryo transfer. The ultrasound of the intrauterine gestational sac confirmed clinical pregnancy.

Statistical Analysis

The statistical processing of the results is expressed by ± SD. Mean comparison of the groups were compared using the *t*-test. The enumeration data were compared with the χ^2 test. The results of the experiment were repeated three times. The results of Dot-blot hybridization were analyzed using Scan Image software to mark the relatively gray value of each point. Compared to the blank control for the reference of the relative gray value of each sample. The application of SPSS11.5 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. $p < 0.05$ indicates that there was a significant difference.

Results

The secretion of the embryonic MMP-9 grade I for the different developmental stages was detected by Dot-blot method. Figure 1 shows that as the same grade I embryos, the MMP-9 secretion in the embryonic medium enhanced by the development of the embryo during the development from two cell to eight cells. The results were analyzed by Scan Image. The relative gray levels in the different period were statistically analyzed. The difference was statistically significant ($p < 0.05$).

The MMP-9 secretion for embryos with the same cell stages and scores was detected by Dot-blot method. Figure 3 shows that as 8-cell stage-I embryos, the secretion of MMP-9 in human embryo culture medium had significant differences. This difference is visible by Dot-blot hybridization method.

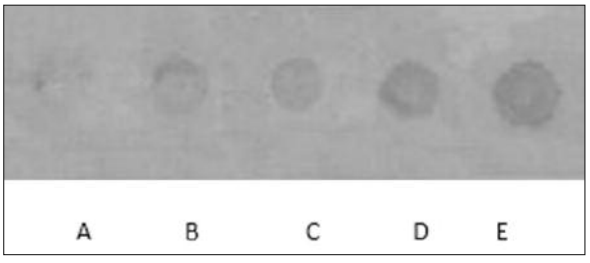

Figure 1. ***A,*** Control group. ***B,*** 2-cell. ***C,*** 4-cell. ***D,*** 6-cell. ***E,*** 8-cell.

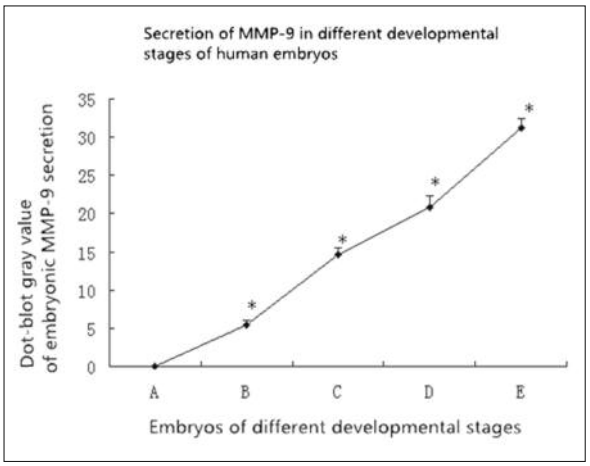

Figure 2. Body weight loss and survival rate in DSS-induced acute IBD mice. Treatment with anti-IL-6 neutralizing antibodies led to more severe symptom of IBD (n = 20).

The correlation of the embryo quality with MMP-9 secretion in 8-cell embryos with different embryo scores was detected by ELISA method**.** As is shown in Table I, for the same 8-cell embryos, the secretion of MMP-9 gradually decreased with the relegation of the embryo scores. The difference was statistically significant ($p < 0.001$).

The Correlation of Clinical Pregnancy Rate With the Secretion of MMP-9 in Embryo Culture Medium

As shown in Table II, the clinical pregnancy rate for MMP-9 high secreted group (77.3%) was significantly higher than that of that of low secreted group (16.7%). The difference was statistically significant ($p < 0.005$).

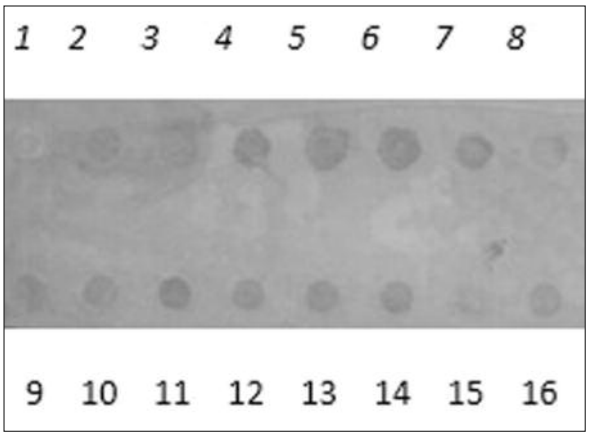

Figure 3. 1 for the blank control.

Table I. The secretion of 8-cell embryo MMP-9 with different embryo scores.

	8/I (n = 55)	8/II (n = 25)	8/III (n = 18)
Concentration of Pro-MMP-9 (ng/ml)	0.615 ± 0.062	0.530 ± 0.055	0.415 ± 0.051
t	5.880	6.970	
p	$p < 0.001$	$p < 0.001$	

Discussion

The study on the mechanism of mammalian embryo implantation showed that many factors such as matrix metalloproteinases (MMPs), leukemia inhibitory factor (LIF), growth factors, extracellular matrix metalloproteinase are necessary for regulating embryo development and implantation[5]. In the present study, we focused on MMPs as candidate biomarkers of embryo development. MMP-9 is one of the most important proteolytic enzymes that hydrolyze the extracellular matrix in the mammalian reproductive implantation process[6]. MMPs are crucial for the normal physiology of the reproductive system[7-9]. However, expression of MMP-2 remains consistent throughout the reproductive cycle. MMP-9 also expresses in the endometrium throughout the cycle, though, its expression increases in the glandular cells particularly during midsecretory phase[10]. The expression of MMP-9 on embryo rarely is studied. Cyclosporin A promotes the embryonic adhesion and invasion, up-regulating integrinβ3 and MMP-9 expression[11]. We also tend to study the influence of the MMP-9 change in this phase compared to the steady expression of MMP-2 in the embryonic stage of development.

Animal studies have shown that mice embryos can be detected for the expression of many related genes in 2-cell stage[12] and the human embryo can be detected for the expression of related genes in 4-cell stage[13]. All these genes show high or low expression in some specific embryonic development stage so that it can be used as an evaluation index of the embryo development. Morphology has been used as the only index of the clinical score of the embryo at present[4]. Although more than 90% of IVF treated patients were successfully fertilized, the implantation rate of the fertilization-embryo into the uterus *in vitro* and the fertility rate is low, indicating that morphological observation failed to judge the embryo quality[14]. Detection of the implantation factor secretion in the embryo culture medium helps judge the embryo quality[15]. We detected the secretion of the MMP-9 in the 2-cell stage by the Dot-blot method in this study. The expression of the MMP-9 increased gradually with further development of the embryo indicating that MMP-9 is an important factor in the process of embryo development. The 8/I stage embryos transplantation are conducted in the clinic with pregnancy for some patients and failure of implantation in some other patients after multiple 8/I embryo transplantation. It shows that in Figure 3, the secretion of the MMP-9 had a significant difference for the different 8-cell embryos. Further detection of 8-cell stage embryos by ELISA method found that secretion of MMP-9 in grade I embryo was significantly higher than those of grade II and III embryos. The difference was significant. The clinical pregnancy rate for higher secretion of embryonic MMP-9 (77.3%) was significantly higher than that of the low expression group (16.7%) for the same 8/I stage embryos. All results further show that embryonic MMP-9 secretion level are closely related to the quality

Table II. The correlation of embryo MMP-9 secretion with clinical pregnancy.

	Concentration of Pro-MMP9 (ng/ml)	The clinical pregnancy rate (%)
High secreted group (n = 22)	0.698 ± 0.022	77.3 (17/22)
Low secreted group (n = 18)	0.486 ± 0.031	16.7 (3/18)
c^2		14.55
p		$p < 0.005$

of the embryo and embryo implantation, which suggests that MMP-9 may become one of the hallmarks for evaluating embryo quality. The detection of the MMP-9 is for the zymogen form (pro-MMP-9) because the antibodies only specify binding protein N-terminal while detecting the 8-cell embryo by ELISA.

Conclusions

We investigate factors related to the embryo development in a single embryo level in this study and observe the collection of the embryo culture growth, development, and implantation of early embryos.

The culture medium was collected as the research object to ensure that the normal embryo transplantation was successful in order to obtain clinical pregnancy. It also has practical guiding significance for assisting reproductive technology and may develop a theoretical basis for reproductive medicine. However, the mechanism of MMP-9 in the process of embryonic development and implantation still needs further research.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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