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Association between follicular fluid levels of HMGB1 protein and outcomes in patients undergoing *in vitro* fertilization/intracytoplasmic sperm injection cycles

SAIJIAO LI*, TAILANG YIN*, WEI LI, JING YANG, WANGMING XU and DANNI ZHOU

Reproductive Medicine Center, Renmin Hospital of Wuhan University, Wuhan, Hubei 430060, P.R. China

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Abstract. The aim of the present study was to evaluate the association between follicular fluid (FF) levels of high-mobility group box 1 (HMGB1) protein and the reproductive outcome in patients undergoing *in vitro* fertilization (IVF) with intracytoplasmic sperm injection (ICSI). FF samples were collected from the ovarian follicles (≥14 mm) of 143 infertile patients that had undergone IVF/ICSI, and the HMGB1 expression levels were determined using ELISA. Spearman's correlation and receiver operating characteristic (ROC) curve analysis were applied to analyze the results. Significantly increased levels of HMGB1 protein (7.38±2.02 vs. 6.14±2.52 ng/ml; P<0.01), endometrial thickness on the day of human chorionic gonadotropin (hCG) administration (10.3±1.3 vs. 9.7±1.7 mm; P<0.01) and retrieved oocyte counts (11.68±6.51 vs. 11.00±6.34; P<0.01) were observed in the pregnant group when compared with the non-pregnant group. Conversely, the level of luteinizing hormone on the day of hCG administration was significantly reduced in the pregnant group compared with the non-pregnant group (0.92±1.78 vs. 1.78±2.03 pmol/l, P<0.01). The ROC curve indicated a significant association between the FF level of HMGB1 protein and the pregnancy rate, with an area under the ROC curve of 0.673 (0.581-0.765; P<0.01). In addition, the HMGB1 protein level was shown to have a significant positive correlation with the endometrial thickness (r=0.170; P<0.05). Therefore, the present study indicated that the FF levels of HMGB1 protein are increased in pregnant patients and are positively correlated with endometrial thickness. Thus, FF levels of HMGB1 may be a useful factor for predicting the outcome of IVF/ICSI treatments.

Correspondence to: Professor Jing Yang, Reproductive Medicine Center, Renmin Hospital of Wuhan University, 238 Jie Fang Road, Wuhan, Hubei 430060, P.R. China
E-mail: dryangqing@hotmail.com

*Contributed equally

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Introduction

An increasing number of studies have indicated that the follicular microenvironment of a human oocyte is a crucial factor for its developmental competence (1). Follicular fluid (FF) is the medium that ensures oocyte maturation, fertilization and transport, and facilitates *in vitro* sperm capacitation (2,3). Thus, FF serves a function in early embryo development and implantation (4). Immune cells, including leukocytes and cytokines, are involved in the physiology of follicular development, ovulation and corpus luteum formation (5-7). As modulators of the immune system, cytokines influence the physiology and pathology of the female reproductive system.

To date, associations between particular cytokines and the outcome of *in vitro* fertilization (IVF) have been extensively studied (8-11). Thus, ovulation is a well-recognized inflammatory process that is putatively modulated by cytokines. Numerous studies have demonstrated that a variety of proinflammatory cytokines, including interleukin (IL)-6, IL-8, IL-11, tumor necrosis factor (TNF)-α, leukemia inhibiting factor and the IL-1 system, are crucially involved in folliculogenesis (12), implantation (13) and follicular maturation (14-16).

High-mobility group box 1 (HMGB1; also known as amphoterin or HMG1) is a highly conserved nuclear protein, which exhibits DNA-binding properties and participates in DNA transcription, replication and repair (17). Certain studies have demonstrated that HMGB1 is a necessary and sufficient mediator of lethal inflammation and functions as a novel proinflammatory cytokine (18-20). Various cell types, such as activated macrophages/monocytes, natural killer cells, mature dendritic cells, pituicytes and erythroleukemic cells, are known to secrete HMGB1 into the extracellular milieu. Following release from necrotic cells and macrophages, HMGB1 functions as an inflammatory stimulator to upregulate the expression levels of IL-1, IL-6, TNF-α, C-reactive protein and macrophage inflammatory proteins-1α and -1β (21,22).

Since HMGB1 is a crucial cytokine that mediates the response to infection and inflammation (23), the FF levels of HMGB1 protein were hypothesized to correlate with follicular development and IVF/ intracytoplasmic sperm injection (ICSI) outcome. To date, there have been a limited number of studies examining the correlation between HMGB1 protein and IVF/ICSI outcome. Thus, the aim of the present study was to investigate the presence of HMGB1 protein in human FF.

In addition, the study investigated the possible association between FF concentrations of HMGB1 protein and reproductive outcomes in normal ovarian responders, patients with an antral follicle count between 5-15, undergoing IVF/ICSI.

Materials and methods

Ethical approval and patient consent. The study was approved by the Ethical Committee of Wuhan University (Wuhan, China) and was conducted in accordance with the institutional guidelines. Informed consent was obtained from each patient.

Subjects. In total, 143 cases of infertile couples who had undergone IVF/ICSI at Renmin Hospital of Wuhan University were included in the study. The study protocol was approved by the Institutional Review Board. Infertile couples with male and female etiologies were included, such as sperm abnormalities and tubal factors, respectively. Patients with endometriosis, ovulatory disorders, polycystic ovary syndrome or a history of ovarian surgery and a poor response to IVF were excluded from the study. The 143 cases of modified natural IVF/ICSI cycles that were examined were all first attempts. Informed consent was provided by each of the couples.

Ovarian stimulation, FF sampling and oocyte collection. Patients underwent a long treatment protocol with gonadotropin-releasing hormone agonist administration in the midluteal phase, which was followed by ovarian stimulation with recombinant follicle-stimulating hormone (FSH; Gonal-f®, Merck Serono, Geneva, Switzerland; or Puregon®; Schering-Plough Corporation, Kenilworth, NJ, USA) or human menopausal gonadotropin (Livzon Pharmaceutical Group Inc., Zhuhai, China). Following confirmation of ovarian suppression and when at least three follicles had reached a mean diameter of 18 mm under transvaginal ultrasound examination (GE Logiq 400 Pro, GE Healthcare Life Sciences, Shanghai, China), 10,000 IU human chorionic gonadotropin (hCG; Livzon Pharmaceutical Group Inc.) was administered subcutaneously. After 34-36 h, oocytes were retrieved using an Aloka ProSound SSD-3500 ultrasound-guided transvaginal puncture (Hitachi-Aloka Medical, Ltd., Guangzhou, China). An individual aspiration was used to collect the oocytes and each follicle was recovered in a separate tube. FF was collected from ovarian follicles that were ≥14 mm, and was pooled for each patient. FF samples were centrifuged at 2,000 x g for 10 min, and the supernatants were stored at -80˚C for further analysis.

Assessment of oocyte morphology and maturation. Oocytes isolated from FF samples were evaluated. The cumulus oophorus and corona radiata were removed from the oocytes by mechanical pipetting in SynVitro Flush containing 300 IU/ml hyaluronidase (Sigma-Aldrich) for up to 1-2 min depending on the extent of cumulus investment. Nuclear maturation of the oocytes was determined by the identification of the first polar body. On day 2 or 3, the oocytes were sorted into four categories, based on their morphologic appearance, zonal thickness, cytoplasmic fragmentation and blastomere size. The categories were as follows: Grade I (high quality, embryos with equal blastomeres and no observed cytoplasmic fragmentation; grade II (good quality), embryos with equal blastomeres and <20% fragmentation of the cytoplasm; grade III (fair quality), embryos with unequal blastomeres and 20-50% fragmentation of the cytoplasm; and grade IV (poor quality), embryos with unequal blastomeres and >50% fragmentation of the cytoplasm (24).

Assessment of fertilization, cleavage and embryo quality. Fertilization results were assessed 18 h following the ICSI treatment for the appearance of two distinct pronuclei and two polar bodies. Cleavage was evaluated 24 h after fertilization. Embryo quality was assessed on the second day following insemination and was graded using the aforementioned protocol (24).

Determination of HMGB1 protein levels in the FF. HMGB1 protein levels in the FF were determined using a commercially available ELISA kit (HMGB1 ELISA kit II; Shino-Test Corporation, Tokyo, Japan), according to the manufacturer's instructions. Sensitivity was <1.0 ng/ml, while the intra-assay and inter-assay coefficients of variation were <10.0%.

Determination of estradiol (E2), progesterone (P) and luteinizing hormone (LH) concentrations in the FF. Serum levels of E2, P, LH and FSH were measured using an Immulite® 2500 immunoassay analyzer (Siemens, Munich, Germany). Prior to each test, the Immulite® 2500 was calibrated with three control samples containing low, medium and high concentrations of the appropriate hormones. Dilutions were performed prior to the measurement of E2 (1:1,000) and P (1:500), depending on the calibration range.

Statistical analysis. SPSS software, version 13.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Results are expressed as the mean ± standard deviation. Between-group differences were evaluated using the Student's t-test, while Spearman's correlation was applied for correlation analysis between the concentrations of HMGB1 protein in the FF and other variables. Receiver operating characteristic (ROC) curve analysis was used to determine the respective performances and sensitivity/specificity values. The following thresholds were used to classify the area under the ROC curve (AUCROC) data: 0.9-1, Perfect separation; 0.8-0.9, excellent discrimination; 0.7-0.8, acceptable discrimination; 0.6-0.7, poor discrimination; and 0.5-0.6, no discrimination. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Clinical characteristics of the patients. Following the first round of IVF/ICSI treatment, 64 patients were pregnant and 70 patients remained non-gravid. Standard IVF was used for 101 oocytes and ICSI was applied for 41 oocytes. In eight cases, an oocyte was collected but unsuccessfully fertilized (one ICSI and seven IVF), and in one case no oocyte was retrieved. Infertility was due to sperm abnormalities in 32/143 cases and tubal abnormalities in 111/143 cases. Table I details the characteristics of the patients in the three groups, namely the pregnant, non-pregnant and oocyte unfertilized groups. Statistically significant differences were observed

Table I. Clinical and IVF/ICSI patient characteristics.

Characteristic	Pregnant	Non-pregnant	Oocyte unfertilized
Patients (n)	64	70	8
Age (years)	30.30±4.19	31.67±4.42	30.62±4.66
HMGB1 protein in FF (ng/ml)	7.38±2.02[a]	6.14±2.52	7.49±1.79
Duration of infertility (years)	5.47±3.67	4.86±3.13	5.28±2.31
BMI (kg/m^2)	21.69±2.68	21.55±2.45	20.72±1.62
Basal level			
E2 (pg/ml)	46.60±21.89	50.70±29.42	52.19±22.58
LH (pmol/l)	4.41±5.64	4.08±2.34	3.77±1.49
FSH (mIU/ml)	6.05±2.03	6.01±2.09	5.83±2.78
Antral follicle count (n)	12.73±3.66	12.09±3.09	9.98±2.91
Day of hCG administration			
E2 (pg/ml)	5,169.09±2,264.4	4,507.56±2,467.98	3,616.55±1,937.16
LH (pmol/l)	0.92±1.78[ab]	1.78±2.03	1.64±2.29
P (pmol/l)	1.50±0.96	1.35±0.52	1.46±0.48
Endometrial thickness (mm)	10.3±1.3[c]	9.7±1.7	10.3±0.09
Retrieved oocytes (n)	11.00±6.34[c]	11.68±6.51	11.02±5.09
Fertilization rate (%)	71.79±28.00	67.14±23.26	-
Grade I/II embryos (n)	4.65±4.44	3.99±3.45	-
Transferred embryos (n)	2.48±1.14	2.64±1.12	-

Data are presented as the mean ± standard deviation. [a]P<0.01, vs. non-pregnant group; [b]P<0.01, vs. oocyte unfertilized group; [c]P<0.05 vs. non-pregnant group. IVF/ICSI, *in vitro* fertilization/intracytoplasmic sperm injection; HMGB1, high-mobility group box 1; FF, follicular fluid; BMI, body mass index; E2, estradiol; LH, luteinizing hormone; FSH, follicle-stimulating hormone; hCG, human chorionic gonadotropin; P, progesterone.

between the pregnant and non-pregnant groups with regard to the FF levels of HMGB1 protein, the LH levels on the day of hCG administration, endometrial thickness and the number of retrieved oocytes. The oocyte unfertilized group exhibited higher levels of LH on the day of hCG administration when compared with the pregnant group (Table I).

FF levels of HMGB1 protein and the prediction of cycle outcome and oocyte fertilization. A ROC curve was produced for the prediction of pregnancy outcomes for patients undergoing IVF/ICSI, based on the HMGB1 protein level in the FF. The AUCROC was 0.673 (0.581-0.765; P<0.01), indicating a poor discrimination. The optimal threshold according to the ROC curve for HMGB1 protein was 5.13 ng/ml, with a sensitivity of 89.1% and a specificity of 62.9% (Fig. 1). In eight cases, no oocyte was fertilized. Statistical analyses were conducted to identify any associations between the measured parameters and the failure of oocyte fertilization. A ROC curve was produced for predicting the fertilization outcome of patients undergoing IVF/ICSI, based on the HMGB1 protein level in the FF. The AUCROC was 0.616 (0.433-0.798; P=0.273), indicating no statistically significant association.

Correlations between HMGB1 protein levels and IVF/ICSI data. Spearman's correlation analyses of the HMGB1 protein level with pregnancy and numerous associated factors are presented in Table II. Statistically significant correlations were identified when comparing the HMGB1 protein level in the FF with the pregnancy rate and endometrial thickness on the day of hCG administration (r=0.30; P<0.01 and r=0.170; P<0.05,

Table II. Spearman correlations of the HMGB1 protein level with associated factors.

Variable	r_s	P-value
Pregnancy	-0.300	<0.01
BMI	-0.017	-
Day of hCG administration		
E2	0.070	-
LH	-0.115	-
P	0.081	-
Endometrial thickness	0.170	<0.05
Retrieved oocytes	0.009	-
Fertilization rate	-0.067	-
Grade I/II embryos	-0.031	-

HMGB1, high-mobility group box 1; BMI, body mass index; hCG, human chorionic gonadotropin; E2, estradiol; LH, luteinizing hormone; P, progesterone.

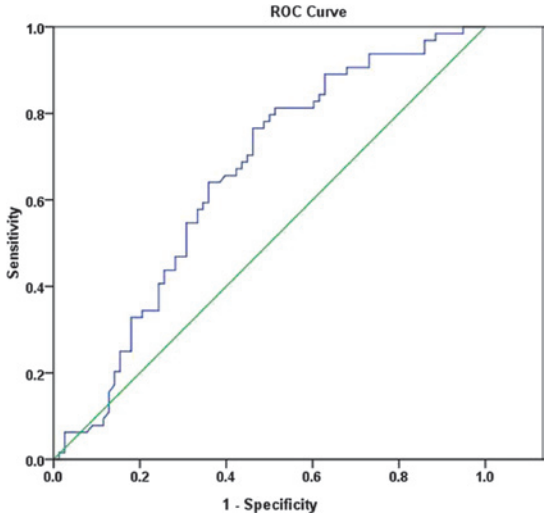

Figure 1. ROC curve predicting the pregnancy rate of patients undergoing *in vitro* fertilization/intracytoplasmic sperm injection, based on the high-mobility group box 1 protein level in the follicular fluid. Area under ROC curve = 0.673 (0.581-0.765; P<0.01). ROC, receiver operating characteristic.

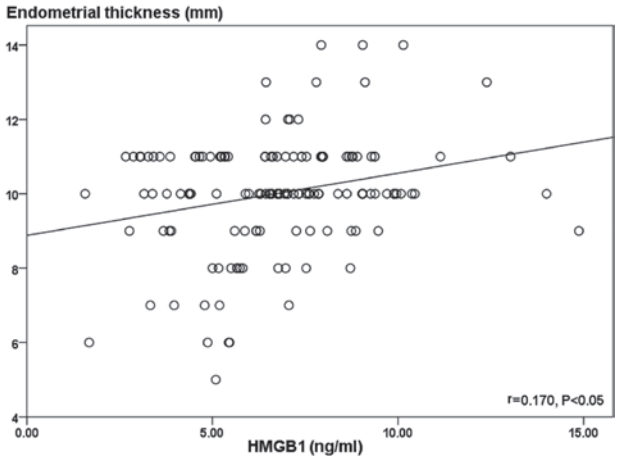

Figure 2. Positive correlation between the HMGB1 protein level in the follicular fluid and endometrial thickness (r=0.170; P<0.05). HMGB1, high-mobility group box 1.

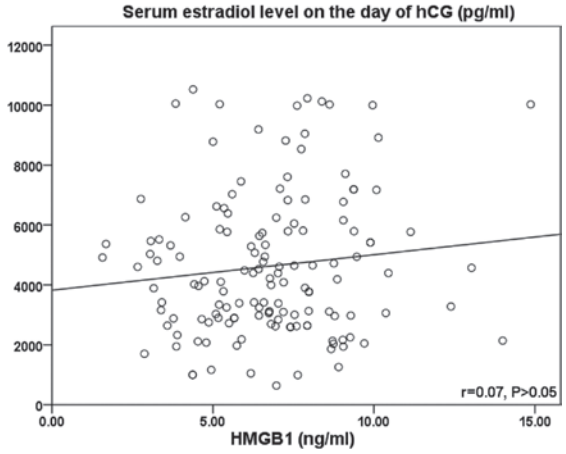

Figure 3. Association between the follicular fluid HMGB1 protein level and the serum estradiol level on the day of hCG administration in patients undergoing *in vitro* fertilization/intracytoplasmic sperm injection (r=0.07, P>0.05). hCG, human chorionic gonadotropin; HMGB1, high-mobility group box 1.

respectively; Fig. 2). However, no significant correlation was observed between the HMGB1 protein level and the serum E2 level on the day of hCG administration (r=0.07; P>0.05) (Table II and Fig. 3).

Discussion

The present study investigated the association between HMGB1 protein levels and the treatment outcomes in infertile patients undergoing induction of superovulation. The results demonstrated that there were statistically significant differences in the level of HMGB1 protein in the FF, LH levels on the day of hCG administration, endometrial thickness and the number of retrieved oocytes when comparing the pregnant and non-pregnant groups. To date, there are limited data concerning the correlation between HMGB1 protein levels and IVF/ICSI outcome.

The present study indicated that LH levels on the day of hCG administration were higher in the non-pregnant group compared with the pregnant group (1.78±2.03 vs. 0.92±1.78 pmol/l; P<0.01). Previous studies have reported elevated LH levels in patients undergoing IVF/ICSI treatment; however, the observed effect of these increases on pregnancy rates has differed between studies. Cantineau and Cohlen (25) observed an incidence of spontaneously elevated LH levels of 35% without a significant alteration in pregnancy rates. However, Huirne *et al* (26) observed reduced pregnancy rates in the presence of elevated LH levels at the time of hCG administration in patients undergoing IVF/ICSI treatment. Furthermore, no successful pregnancies were observed in patients with a combined elevation in LH and P levels, which was consistent with the present study. Elevated LH levels appear to affect pregnancy rates via mechanisms other than ovulation alone. LH induces the synthesis of growth factors, cytokines and chemokines, which exert a variety of effects, including influencing specific features of the endometrium (27).

HMGB1 protein is a crucial cytokine that mediates the immune response to infection and inflammation (21). HMGB1 serves a key function in numerous chronic inflammatory diseases, including rheumatoid arthritis, systemic lupus erythematosus and inflammatory myositis (28). To the best of our knowledge, the present study is the first to report the presence of HMGB1 protein in human FF, demonstrating that FF levels of HMGB1 protein are significantly increased in pregnant patients. Furthermore, the present study performed ROC curve analyses to determine if a threshold concentration of HMGB1 protein in the FF was able to indicate pregnancy outcomes. The ROC curve indicated that FF levels of HMGB1 protein were significantly associated with pregnancy rates, with an AUCROC of 0.673 (0.581-0.765; P<0.01; Fig. 1). In addition, the ROC curve exhibited discriminating capacity and the optimal threshold, according to the ROC curve, for HMGB1 protein was 5.13 ng/ml. These results suggested that FF levels of HMGB1 protein may be a useful factor to predict the outcome of IVF/ICSI treatment.

A number of studies have evaluated the association between endometrial thickness and pregnancy rates in patients undergoing assisted reproductive technology; however, the results remain controversial (29-38). A number of studies have indicated a higher pregnancy rate at a partic-

ular endometrial thickness (29-33), while alternative studies indicated no significant correlation between endometrial thickness and pregnancy rates in IVF/ICSI patients (34-39). The present study indicated that clinical pregnancy rates following embryo transfer are positively correlated with endometrial thickness, which is consistent with previous studies (39-41).

Growing ovarian follicles produce increasing quantities of E2, which induces proliferative endometrial alterations. He *et al* (42) reported an estrogen-induced increase in the transcription of HMGB1 in breast cancer cells. This observation suggested that endometrial HMGB1 expression, and possibly secretion, is steroid-dependent. The presence of estrogen response elements in the HMGB1 gene supports this hypothesis (43). This explanation may account for the current observation that levels of HMGB1 protein in the FF exhibited a significant positive correlation with endometrial thickness ($r=0.170$; $P<0.05$). However, no association was observed between the FF levels of HMGB1 protein and serum E2 levels on the day of hCG administration in the IVF cycle patients ($r=0.07$; $P>0.05$). The underlying mechanism remains unknown and further preliminary and clinical studies are required.

In the present study, the number of retrieved oocytes was significantly reduced in the non-pregnant group compared with the pregnant group on the day of hCG administration (11.00±6.34 vs. 11.68±6.51; $P<0.01$). However, the majority of existing studies reported no association between the number of oocytes retrieved and the pregnancy rate.

However, there were limitations to the present study. The study population included a small number of Chinese patients; thus, future studies with larger cohorts are required. Furthermore, the precise mechanisms underlying the observations and their clinical relevance remain unclear and require elucidation.

In conclusion, the current study reported the presence of HMGB1 protein in the FF, and indicated elevated levels of HMGB1 protein in the FF of pregnant patients. In addition, the level of HMGB1 protein in the FF was shown to positively correlate with endometrial thickness. Therefore, the FF levels of HMGB1 protein may be a useful factor for predicting the outcome of IVF/ICSI treatment. Future studies are required to investigate the precise mechanisms underlying the possible influence of HMGB1 protein on the outcome of IVF/ICSI.

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