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ARTICLE

Follicular fluid humanin concentration is related to ovarian reserve markers and clinical pregnancy after IVF–ICSI: a pilot study

BIOGRAPHY

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KEY MESSAGE

Humanin is present in the human ovary and can be secreted to the follicular fluid. The humanin concentration in follicular fluid was positively associated with ovarian reserve markers, fertilization rate and clinical pregnancy.

ABSTRACT

Research question: Is humanin present in the human ovary and follicular fluid? What relationship exists between humanin concentration in the follicular fluid and ovarian reserve and clinical outcomes after IVF and intracytoplasmic sperm injection (ICSI)?

Design: Follicular fluid samples were collected from 179 patients undergoing their first IVF or ICSI cycle during oocyte retrieval. Ovarian tissues were collected from two patients undergoing surgery for ovarian cysts. Ovarian humanin localization was analysed using immunofluorescence staining. Expression of humanin in granulosa cells was confirmed by reverse transcription polymerase chain reaction (RT-PCR) analysis. Follicular fluid humanin levels were evaluated with enzyme-linked immunosorbent assay. Relationships between follicular fluid humanin levels and ovarian reserve markers and clinical outcomes were analysed.

Results: Strong humanin expression was found in the granulosa cells, oocytes and stromal cells of the ovary. Agarose gel electrophoresis of RT-PCR products showed rich humanin mRNA expression in human granulosa cells (119 bp). Follicular fluid humanin concentrations ranged from 86.40 to 417.60 pg/ml. They significantly correlated with FSH ($r = -0.21$; $P < 0.01$), LH ($r = -0.18$; $P = 0.02$), antral follicle count ($r = 0.27$; $P < 0.01$), anti-Müllerian hormone ($r = 0.24$; $P = 0.03$) and inhibin B ($r = 0.46$; $P < 0.01$) levels. Patients were subdivided into four groups according to follicular fluid humanin concentration quartiles (Q1–Q4). Patients in Q4 were more likely to achieve a pregnancy than Q1 (OR = 3.60; 95% CI 1.09 to 11.84).

Conclusions: Humanin concentration in the follicular fluid was positively associated with ovarian reserve and clinical pregnancy rate.

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KEYWORDS

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INTRODUCTION

Humanin is a mitochondrially derived 24-amino-acid peptide encoded in the mitochondrial genome by the 16S ribosomal RNA gene *MT-RNR2* (*Hashimoto et al., 2001*). Published evidence suggests that humanin is secreted and expressed in many tissues, including the brain (*Hashimoto et al., 2001; Matsuoka, 2009*), retinal pigment epithelium (*Minasyan et al., 2017; Nashine et al., 2017*), blood vessels (*Bachar et al., 2010; Muzumdar et al., 2010*), pancreatic beta cells (*Hoang et al., 2010*), tumours (*Gottardo et al., 2017; Omar et al., 2017*) and testes (*Lue et al., 2010; Moretti et al., 2010*). Studies have shown that humanin interacts extracellularly with a tripartite receptor composed of gp130, WSX1 and CNTFR, as well as with the formyl peptide receptor 2 (formylpeptide-like-1 receptor) (*Ying et al., 2004; Hashimoto et al., 2005; 2009*). Intracellular interactions with BAX (*Guo et al., 2003; Luciano et al., 2005; Zhai et al., 2005*) and insulin-like growth factor binding protein 3 (IGFBP3) (*Lue et al., 2010; Njomen et al., 2015*) have also been reported as prerequisites for the cytoprotective effect against apoptosis and oxidative stress on exposure to chemotherapy, ischaemia–reperfusion and hyperthermia (*Muzumdar et al., 2010; Jia et al., 2013; 2015*).

Humanin is richly expressed in the testes of a variety of species, including the mouse (*Jia et al., 2015*), rat (in which it is also known as rattin) (*Surampudi et al., 2015*) and humans (*Moretti et al., 2010*). It is mainly localized in the cytoplasm of Leydig cells, as well as in the cytoplasm and nucleus of spermatocytes and spermatids and in the chromatin and mitochondria of mature ejaculated sperm (*Moretti et al., 2010; Surampudi et al., 2015*). Administration of humanin has been shown to rescue germ cells from apoptosis secondary to testicular stress, caused by hormonal deprivation induced by gonadotrophin-releasing hormone (GnRH) antagonist (*Jia et al., 2013*). Humanin has also been reported to ameliorate chemotherapy-induced male germ cell apoptosis, via the suppression of mitochondrial-mediated apoptosis (*Jia et al., 2015*). In addition, humanin localization differs between normal and abnormal sperm, indicating an effect on sperm quality (*Moretti et al., 2010*). *Yadav et al. (2004)* reported humanin expression in the ovaries of monkeys. More specifically, they found that humanin expression was increased during the late luteal phase and after GnRH antagonist treatment; however, the expression was decreased during simulated early pregnancy. The results of humanin expression in that study suggested that it played an important role in the survival or demise of the corpus luteum in monkeys. Nevertheless, no study has described the presence of humanin in the human ovary or the role of humanin in follicular development. In this present study, we sought to investigate the distribution or localization pattern of humanin in the human ovary and follicular fluid, and to analyse the relationships between humanin concentration in the follicular fluid of women undergoing ovarian stimulation for IVF–ICSI and ovarian reserve. We therefore evaluated the correlations between humanin concentration and clinical outcomes after IVF–ICSI.

MATERIALS AND METHODS

Ethical approval

This study was approved by the Ethics Committee of Reproductive Medicine Centre, Tongji Medical College, Huazhong University of Science and Technology (number 201605) on 29 October 2016. This approval included the collection of blood and follicular fluid samples (including granulosa cells) for humanin and steroid hormone level measurements, and the collection of ovarian tissues for humanin expression analysis. Written informed consent was obtained from every participant enrolled in this study.

Study participants

Blood and follicular fluid samples were collected from women who underwent oocyte retrieval for IVF–ICSI at the reproductive medical centre at Tongji Medical College of Huazhong University of Science and Technology, from January to December 2016. Participants were required to meet the following eligibility requirements: age 20–45 years; unable to conceive naturally for at least 1 year, regardless of male, female, both, or uncertain factors; primary or secondary infertility; and IVF or ICSI cycles fertilized by husband or donor sperm. Patients were excluded if they were diagnosed with polycystic ovary syndrome, endometriosis or premature ovarian failure; women with previous trauma in ovaries caused by surgery or women with secondary diseases; and uterine abnormality. A total of 179 women (aged 21–43 years) were eligible and enrolled into the study. Seventy-eight of the 179 women also participated in another study (not yet published) in our research group, of which the follicular fluid samples were subjected to ostradiol and progesterone concentration analysis, and the data were also analysed in this present study. To evaluate the distribution or localization pattern of humanin in different types of cells in the ovary, a small piece of ovarian tissue was obtained from another two women (aged 28 years and 32 years, respectively) who underwent surgical removal of ovarian cysts. The inclusion criteria of women for ovarian tissue collection included age between 20 and 35 years; the cyst pathologically diagnosed to be benign; and normal reproductive hormone levels. Patients were excluded if they were diagnosed with endometriosis or polycystic ovary syndrome. Granulosa cells were collected from the follicular fluid of another five patients (aged 24–35 years) who underwent oocyte retrieval for IVF–ICSI in the same reproductive centre. These patients received IVF–ICSI treatment owing to male infertility factor. The isolated granulosa cells were used for both humanin fluorescence staining and humanin expression with reverse transcription polymerase chain reaction (RT-PCR).

Ovarian stimulation and IVF–ICSI

Women were treated with different ovarian stimulation protocols, including standard long GnRH agonist (long protocol, $n = 99$), prolonged pituitary down-regulation with GnRH agonist (prolonged protocol, $n = 25$), GnRH antagonist (antagonist protocol, $n = 46$) and short agonist protocol ($n = 9$), according to patients' conditions, as detailed in previously published studies (*Li et al., 2008; Xu et al., 2012; Duan et al., 2017*). A standard full dose (3.75 mg) of triptorelin depot (Decapeptyl®; Ipsen Pharma Biotech, Paris, France) and 0.1 mg/day triptorelin acetate (Decapeptyl®; Ferring, Saint-Prex, Switzerland) were used for the long and prolonged protocols, respectively. The GnRH antagonist (Cetrotide®, Serono, Geneva, Switzerland) (0.25 mg/day) was used for the antagonist protocol. The results of B-ultrasound imaging and serum hormone levels guided the clinicians' decisions on the timing and

dosage of gonadotrophin (Gonal-F@, Merk Serono, Geneva, Switzerland). Recombinant HCG (250 mg; Ovidrel; Serono) was administered when two leading follicles reached a mean diameter of 18 mm.

Oocytes were retrieved transvaginally 34–36 h after HCG administration, and the follicles were aspirated using a single lumen needle attached to a syringe under transvaginal ultrasound guidance. The oocytes were identified in a culture dish using a stereomicroscope. Semen from the participant's husband or a sperm bank was prepared using the swim-up technique, and insemination was carried out by conventional IVF or ICSI. Oocyte fertilization was assessed 18–20 h after insemination by confirmation of the presence and location of two pronuclei. IVF fertilization rate (two pronuclei/number of oocytes inseminated) and ICSI fertilization rate (two pronuclei/number of oocytes injected) were calculated. Up to three embryos were transferred on day 2 or 3 after oocyte retrieval. The antral follicle count (AFC) in both ovaries was measured by transvaginal ultrasonography scans on day 2 or 3 of the menstrual cycle as described in a previously published study (*Du et al., 2016*). Clinical pregnancy was diagnosed by ultrasound visualization of fetal cardiac activity 4 weeks after embryo transfer. The miscarriage rate was defined as the number of miscarriages in the first 20 weeks of gestation per clinical pregnancy; and the live birth rate was defined as the number of deliveries that resulted in at least one live-born baby per initiated cycle, as described in our previous study (*Rao et al., 2018*).

Follicular fluid and granulosa cell collection

During oocyte retrieval, follicular fluid from the first punctured follicle from either ovary was obtained. One millimetre of fluid from each patient was collected in a frozen tube and centrifuged at 1000 g for 10 min at 4°C, and the supernatant was immediately stored at −80°C until assay for humanin using enzyme-linked immunosorbent assay (ELISA). We also collected 60 follicular fluid samples from smaller follicles (<18 mm) to compare the difference in humanin concentration with larger follicles (≥18 mm). Granulosa cells were collected from the follicular fluid of another five patients as described in *Grondahl et al. (2012)*. After isolation, granulosa cells were seeded and cultured on coverslips. After a 48-h culture, the cells were fixed in 4% paraformaldehyde for 15 min for immunofluorescence of humanin. The FSH receptor (FSHR) was also stained to test the purity of the granulosa cells.

Ovarian tissue collection

Ovarian tissue samples obtained from two women were fixed with 4% paraformaldehyde and embedded in paraffin. Then 4-μm sections were cut for the immunofluorescence staining of humanin.

ELISA assay of humanin in the follicular fluid

The concentration of humanin in the follicular fluid was examined using a commercial immunoassay (Cusabio, Wuhan, China) according to the manufacturer's protocol, as described in another study (*Nikolakopoulos et al., 2017*). The intra- and inter-assay precision were less than 8% and 10%, respectively. The sensitivity was 7 pg/ml.

Immunofluorescence staining

Both sections of the ovary and slides of granulosa cells were stained for humanin with immunofluorescence. Additionally, slides of granulosa cells were stained for FSHR to confirm the purity of isolated granulosa cells. Briefly, after immersion in 0.5% Triton x-100 for 20 min, the slides were treated with blocking serum for 30 min, and incubated with polyclonal rabbit FSHR antibody (Proteintech, 1:50) and polyclonal rabbit humanin antibody (Thermofisher, 1:100) at 4°C overnight. After washing with phosphate buffered saline with Tween 20 for 5 min three times, the slides were incubated with goat anti-rabbit fluorescence conjugated secondary antibody (KPL, 1:400). 4′,6-Diamidino-2-phenylindole (DAPI) was then used for counterstaining. In this assay, we conducted negative controls for both humanin and FSHR staining, by using non-immune rabbit serum as a substitute for the primary antibodies. The other steps, reagents as well as dilutions were all the same.

RT-PCR analysis of humanin in the granulosa cells

RT-PCR analysis was carried out to further confirm the expression of humanin in granulosa cells. Total RNA was isolated from granulosa cells collected from five patients, using Trizol reagent (Invitrogen, USA). cDNA was synthesized from mRNA via reverse transcription using a commercial kit from Takara (Japan). The cDNA samples were subjected to PCR amplification on a Light Cycler® 96 System (Roche, Switzerland) by the following specific primers (5′–3′): Humanin sense, CACTCCACCTTACTACCAG and anti-sense, ATAATTTTTCATCTTTCCC; β-actin sense, CCTTCCTGGGCATGGAGTC, and anti-sense, TGATCTTCATTGTGCTGGGTG. The PCR products were examined by 1.5% agarose gel electrophoresis, stained with ethidium bromide (10 μg/ml), then visualized and photographed on a Bio-Rad Universal Hood II machine (Bio-Rad, USA).

Steroid hormone, anti-Müllerian hormone and inhibin B assays

Serum collected on day 2 or 3 of the menstrual cycle was assayed for FSH, LH, oestradiol, prolactin, progesterone and testosterone using a chemiluminescent immunoassay method on a UniCel DxI 800 analyser and commercial kits (Beckman Coulter, Brea, USA), as described previously (*Rao et al., 2015*). Both serum anti-Müllerian hormone (AMH) and Inhibin B concentrations were assayed using commercially available ELISA kits (Beckman Coulter, Brea, USA), and were assayed only in 83 and 46 participants, respectively, as these two indices were measured only when the clinicians deemed it necessary. Follicular fluid oestradiol and progesterone concentrations were assayed using a commercially available ELISA kit (R&D, USA) according to the manufacturer's instructions. All of the assays were carried out by the same experienced technician, to minimize the effect of between-assay variability.

Statistical analysis

Humanin concentrations in follicular fluid, expressed as means ± SD, were compared with analysis of variance (ANOVA) across different age ranges (21–29, 30–35 and 36–43 years) and different body mass indices (<18.5, 18.5–24.9, 25–27.9 and ≥28). Humanin concentrations in follicular fluid collected from small (<18 mm) and large follicles (≥18 mm) were compared using a paired t-test. To evaluate the correlation between humanin concentration and ovarian reserve related indices (hormones, AFC, AMH and inhibin B), we first subdivided all 179 samples according to their

follicular fluid humanin concentration quartile, then the serum and follicular fluid reproductive hormones, AMH, AFC and inhibin B levels of the four patient groups (Qs 1–4) were expressed as means and a 95% confidence interval and compared by ANOVA or Kruskal–Wallis test (if the data were not normally distributed). Spearman correlation analyses were then conducted to evaluate the correlations between humanin concentration and ovarian reserve related indices involving serum FSH (n = 179), LH (n = 179), oestradiol (n = 179), AMH (n = 83) and inhibin B (n = 46) levels and AFC (n = 179).

Fertilization rates were compared across different patient groups with a chi-squared test. Multi-variable logistic regression models were used to analyse the relationship between follicular fluid humanin concentration and the clinical outcomes of clinical pregnancy, live birth and miscarriage, adjusting for age (21–29, 30–35 and 36–43 years), BMI (<18.5, 18.5–24.9, 25–27.9 and ≥28), ovarian stimulation protocol (long, prolonged, antagonist and short protocols), cause of infertility (male, female, both male and female, and uncertain), type of infertility (primary or secondary), insemination (IVF by husband's sperm, IVF by donor sperm, ICSI by husband's sperm and ICSI by donor sperm), duration of infertility (≤2, 3–5 and >5 years) and number of transferred embryos (one, two or three). All of the statistical tests were two-sided, and $P < 0.05$ was considered statistically significant. SPSS 17.0 (SPSS Inc., Chicago, USA) was used for data analysis.

RESULTS

Characteristics of participants

The patients' demographic and baseline characteristics are shown in TABLE 1. The mean age and body mass index (BMI) were 30.64 years and 22.44, respectively. The duration of infertility was 5.03 ± 3.97 (mean ± SD) years. Primary and secondary infertility were reported in 124 (69.27%) and 54 (30.73%) women, respectively. One hundred and twelve patients underwent IVF treatment, 61 of whom received their husband's sperm and 51 of whom received donor sperm from a sperm bank. Fifty-two women underwent ICSI with their husband's sperm and 15 did so with donor sperm. The mean baseline levels of FSH, LH and oestradiol were 7.60 IU/l, 4.70 IU/l and 37.72 pg/ml, respectively and peak oestradiol concentration was 5.23 ng/ml. Mean AMH and inhibin B levels were 4.20 ng/ml and 109.55 pg/ml, respectively. An average of 16.65 AFC was detected in all patients.

Immunostaining of humanin in the ovary and granulosa cells

To determine the distribution or localization pattern of humanin, two normal healthy ovarian tissue samples were immunostained with anti-humanin antibody. As shown in FIGURE 1, humanin was detected in the cytoplasm of a variety of cells in the ovary, including stromal cells, granulosa cells in the follicle, granulosa cells in the corpus luteum and oocytes. Oocytes in both primordial and primary follicles were stained with the humanin-specific antibody. To confirm the expression of humanin in granulsoa cells, granulosa cells were isolated from follicular fluid and also examined by immunostaining. Staining specific to FSHR was observed on the membrane of all isolated cells, indicating the purity of the isolated granulosa cells. We also detected a strong humanin-specific signal in the cytoplasm of isolated granulosa cells. None of the negative controls showed positive staining (FIGURE 1).

RT-PCR analysis of humanin in the granulosa cells

Agarose gel electrophoresis of RT-PCR products showed rich humanin mRNA expression in human granulosa cells. The lengths of the amplified products of humanin and beta-actin were 119 and 189 bp, respectively (FIGURE 2). This result further confirmed humanin expression in human granulosa cells.

Humanin concentration in the follicular fluid

Humanin was detected in every follicular fluid sample, with a concentration ranging from 86.40 to 417.60 ng/ml. The concentrations were not found to be related to the patients' age or BMI, but smaller follicles had a significantly higher concentration of humanin than larger follicles (P = 0.004) (FIGURE 3). The patients were subdivided into four groups according to the humanin concentration quartile: Q1: 86.40–142.82 pg/ml, n = 45; Q2: 143.07–173.46 pg/ml, n = 45; Q3: 174.28–221.54 pg/ml, n = 45; and Q4: 221.98–417.60 pg/ml, n = 44. The FSH and LH levels decreased with the increase of humanin concentration ($P < 0.01$ and 0.03, respectively), except that FSH in Q4 was higher than in Q3, and LH in Q2 was higher than in Q1. The AFC in different humanin concentration intervals was significantly different (P = 0.01), with a much lower value in Q1 than in quartiles 2–4. Other serum and follicular fluid hormone levels were similar across the different humanin groups (TABLE 2).

Correlations between humanin concentration and ovarian reserve

Humanin concentrations in the follicular fluid were found to be negatively correlated to FSH (r = –0.21; $P < 0.01$) and LH (r = –0.18; P = 0.02) levels. Positive correlations were observed between humanin and AFC (r = 0.27; $P < 0.01$), AMH (r = 0.24; P = 0.03) and inhibin B (r = 0.46; $P < 0.01$) levels. Serum oestradiol level was not statistically related to humanin concentration (FIGURE 4).

Correlation of humanin concentration and assisted reproductive technology outcomes

For IVF cycles inseminated with husband or donor sperm, ICSI cycles inseminated with donor sperm, the fertilization rates across different patient groups were significantly different (all $P < 0.01$), with higher levels in the Q4 intervals than in quartiles 1–3 (except for IVF cycles inseminated with husband sperm in the Q2 interval); whereas the rates in ICSI cycles inseminated with husband sperm across different patient groups were similar, as shown in TABLE 3. Multiple regression analysis showed that patients in the Q4 group experienced an increased chance of clinical pregnancy when taking patients in Q1 group as a reference (OR = 3.60, 95% CI 1.09 to 11.84; P = 0.03). Although patients in the Q4 group seemed to have an increased chance of live birth compared with patients in the Q1 group, the difference was not statistically significant (OR = 2.74, 95% CI 0.91 to 8.23). No obvious correlation was observed between humanin concentration and miscarriage (TABLE 4).

DISCUSSION

In this study, we demonstrated the localization and distribution of humanin in human ovarian cells. We found strong humanin expression in the ovary in multiple types of cells, including granulosa cells, oocytes and stromal cells. Further study revealed that follicular fluid humanin levels were correlated with ovarian reserve markers, and were

TABLE 1 DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF ENROLLED PARTICIPANTS

Variable	Mean	SD	*n* (%)
Age	30.64	5.39	
Age, years			
21–29			78 (43.58)
29–35			62 (34.64)
36–43			39 (21.79)
BMI, mean	22.44	3.61	
BMI			
<18.5			20 (11.17)
18.5–24.9			115 (64.25)
25–27.9			32 (17.88)
≥28			12 (6.70)
Duration of infertility (years)	5.03	3.97	
Primary infertility			124 (69.27)
Secondary infertility			55 (30.73)
Female baseline levels			
FSH (IU/l)	7.60	2.32	
LH (IU/l)	4.70	2.22	
Oestradiol (pg/ml)	37.72	19.99	
AMH (ng/ml)[a]	4.20	3.57	
Inhibin B (pg/ml)[b]	109.55	63.95	
AFC	16.65	9.81	
Peak oestradiol level (ng/ml)	5.23	2.84	
Ovarian stimulation protocol			
Long protocol			99 (55.31)
Prolonged protocol			25 (13.97)
Antagonist protocol			46 (25.70)
Short protocol			9 (5.03)
IVF with husband sperm			61 (34.08)
IVF with donor sperm			51 (28.49)
ICSI with husband sperm			52 (29.05)
ICSI with donor sperm			15 (8.38)

[a] AMH was assayed only in 83 participants.

[b] Inhibin B was assayed only in 46 participants.

AFC, antral follicle count; AMH, anti-Mullerian hormone; ICSI, intracytoplasmic sperm injection; SD, standard deviation.

associated with clinical pregnancy for women undergoing IVF–ICSI.

Humanin locates in the cytoplasm of the ovarian cells, as it is a mitochondrially derived peptide. This is consistent with other studies, which have also reported the cytoplasmic localization of humanin in human retinal pigment epithelial cells (*Sreekumar et al., 2016*), rat germ cells and Leydig cells (*Jia et al., 2013*), and monkey corpus luteum cells (*Yadav et al., 2004*). It has been demonstrated that humanin exerted the cytoprotective effect via an intracellular, autocrine, or paracrine mechanism (*Hashimoto et al., 2001; Yamagishi et al., 2003; Muzumdar et al., 2010*). We therefore aimed to investigate the presence of humanin in the follicular fluid, as follicular fluid is an important medium for the interaction of granulosa cells and oocytes, both of which were shown to have strong humanin expression as indicated by our immunofluorescence results. RT-PCR analysis further confirmed the expression of humanin in granulosa cells. Humanin was detected in every follicular fluid sample, with a large range of concentration (88.4–417.6 pg/ml) across different samples, indicating its potential role in follicular development.

Considering that follicular fluid is mainly produced by granulosa cells (*Rodgers and Irving-Rodgers, 2010*), we predicted that granulosa cells were the cells most likely to be secreting humanin into the follicular fluid Additionally, our immunostaining showed a strong signal of humanin in the cytoplasm of isolated human granulosa cells. As no direct evidence regarding the origin of follicular fluid humanin was obtained in this study, this issue needs further investigation.

Our further analysis showed that humanin concentration in the follicular fluid was negatively associated with

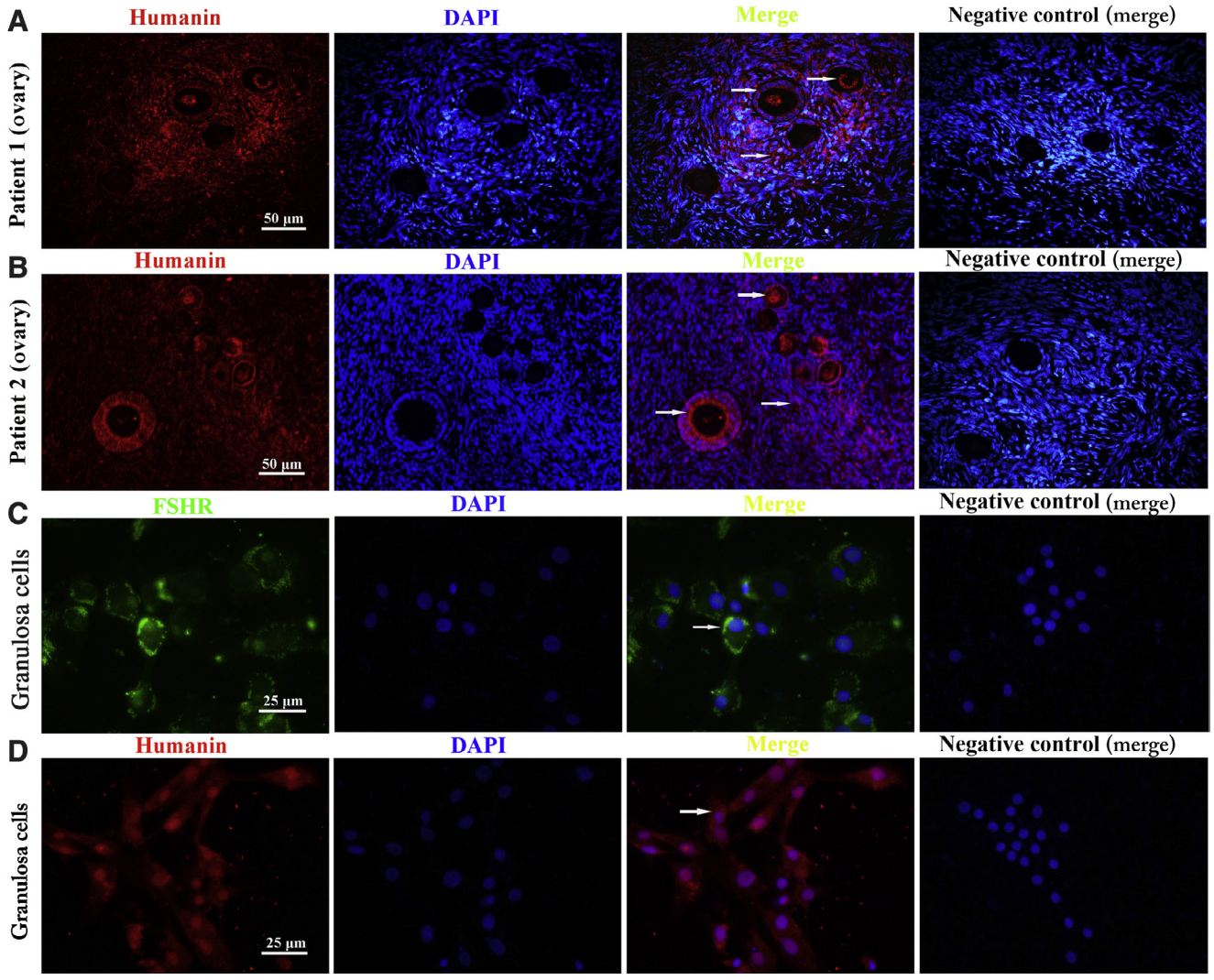

FIGURE 1 Localization of humanin in human ovary and isolated human granulosa cells. Humanin (red fluorescence) was strongly expressed in human ovary tissues collected from (A) patient 1 (aged 28 years) and (B) patient 2 (aged 32 years). It was detected in the oocytes and granulosa cells of the primordial and primary follicles (secondary and antral follicles were not found in our samples) and also stromal cells. Granulosa cells were isolated and stained to confirm humanin presence. FSH receptor staining was used to test the purity of isolated granulosa cells (C); humanin was strongly expressed in the granulosa cells and located in the cytoplasm (D) (the arrows show positive staining). Immunofluorescence staining with non-immune rabbit serum as a substitute for the primary antibody was employed as the negative control. DAPI, 4′,6-Diamidino-2-phenylindole.

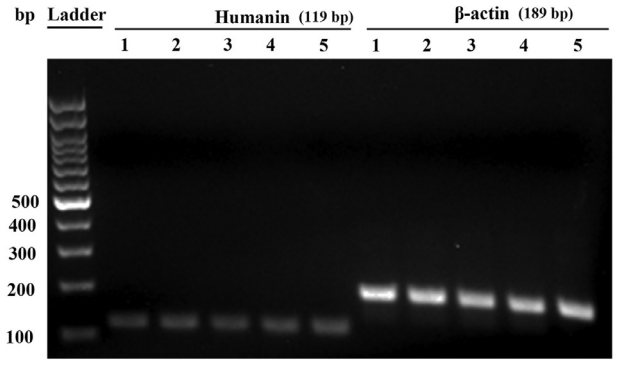

FIGURE 2 Humanin expression in human granulosa cells analysed using reverse transcription polymerase chain reaction. The lengths of the amplified products of humanin and beta-actin were 119 and 189 bp, respectively.

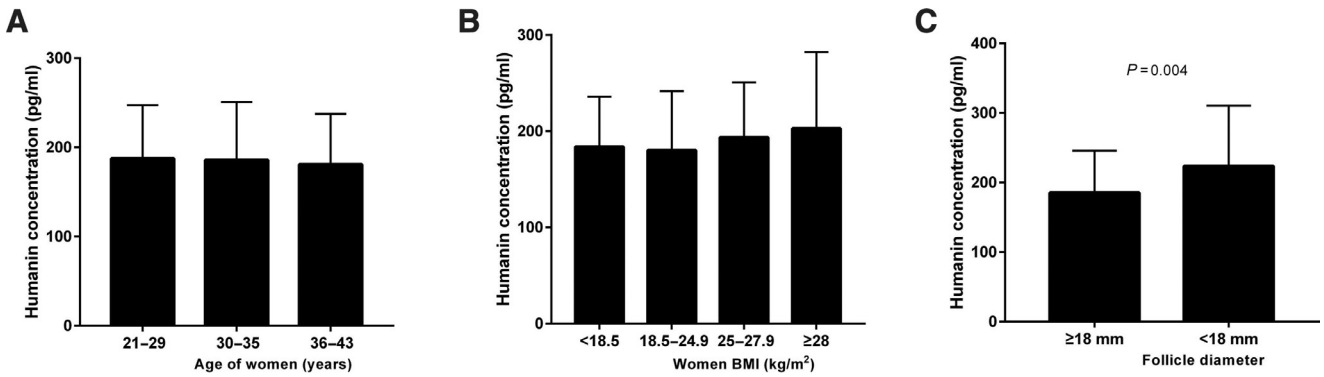

FIGURE 3 Follicular fluid humanin levels in 179 women stratified according to age and body mass index, and in 60 women with paired samples from a small and large follicle. BMI, body mass index.

FSH and LH, and positively associated with AMH, inhibin B and AFC, all of which are useful predictors of ovarian reserve (*Jamil et al., 2016; Tal and Seifer, 2017*), indicating an association between humanin and ovarian function and fertility. No obvious relationship, however, was observed between follicular fluid humanin levels and patient's age or BMI, which have also been reported to affect ovarian function (*May-Panloup et al., 2016*). These contradictory results may be due to multiple confounders involving the cause of infertility and ovarian stimulation protocol. Interestingly, a higher concentration of humanin was detected in smaller follicles (<18 mm) than in larger ones (≥18 mm), suggesting an association with the functional and dynamic state of individual follicles. In fact, follicular fluid composition changes according to the follicle size in mammals, including humans (*Malizia et al., 2010; Nishigaki et al., 2011*). Moreover, porcine and bovine granulosa cells from small antral follicles were more susceptible to apoptosis than those from large follicles (*Yang and Rajamahendran, 2000; Lin and Rui, 2010*). Considering that anti-apoptosis is a major molecular function of humanin as previously shown (*Jia et al., 2013; Jia et al., 2015*), higher levels of humanin in small follicles may be an important molecular response to granulosa cell apoptosis.

Follicular fluid composition strongly influences oocyte quality, developmental competence and the quality of the subsequent embryo (*Borowiecka et al., 2012; Scalici et al., 2014; Buyuk et al., 2017; Cavallo et al., 2017*). For this reason, many studies have highlighted follicular fluid as an important source of potential non-invasive biomarkers for oocyte and embryo quality, as well as for clinical outcome prediction (*Scalici et al., 2014; Buyuk et al., 2017; Cavallo et al., 2017*). In this study, we observed a significantly different fertilization rate across quartiles of follicular fluid humanin concentrations in IVF cycles using husband and donor sperm (both $P < 0.01$), and in ICSI cycles using donor sperm ($P < 0.01$), but not husband sperm, with the highest rates mostly at the fourth quantile. These observations suggest a positive effect of humanin on oocyte

TABLE 2 ASSOCIATIONS BETWEEN FOLLICULAR FLUID HUMANIN CONCENTRATION AND REPRODUCTIVE HORMONE, ANTRAL FOLLICLE COUNT, ANTI-MÜLLERIAN HORMONE AND INHIBIN B LEVELS[a]

Variables	Q1	Q2	Q3	Q4	*P*-value
	86.40–142.82 ng/ml	**143.07–173.46 ng/ml**	**174.28–221.54 ng/ml**	**221.98–417.60 ng/ml**	
	***n* = 45**	***n* = 45**	***n* = 45**	***n* = 44**	
FSH (IU/L)	8.37 (7.66–9.09)	7.93 (7.10–8.75)	6.80 (6.13–7.46)	7.27 (6.75–7.79)	<0.01
LH (IU/L)	4.81 (4.10–5.52)	5.23 (4.48–5.98)	4.91 (4.34–5.49)	3.86 (3.25–4.47)	0.03
Oestradiol (pg/ml)	37.10 (31.19–43.01)	34.08 (29.99–38.18)	40.99 (32.78–49.21)	38.89 (33.07–44.73)	NS
Prolactin (mIU/l)	291.11 (244.59–337.63)	274.28 (234.48–314.07)	300.43 (253.43–347.44)	297.08 (257.12–337.05)	NS
Progesterone (ng/ml)	0.93 (0.61–1.24)	0.91 (0.68–1.13)	0.84 (0.47–1.20)	0.80 (0.59–1.00)	NS
Testosterone (nmol/l)	1.26 (1.02–1.49)	1.16 (0.98–1.35)	1.25 (1.02–1.49)	1.22 (0.96–1.49)	NS
Follicular fluid progesterone (μg/ml)	18.02 (13.54–22.50)	17.29 (14.19–20.40)	16.72 (11.87–21.58)	16.08 (11.44–20.73)	NS
Follicular fluid oestradiol (μg/ml)	0.73 (0.39–1.07)	1.21 (0.55–1.86)	1.16 (0.64–1.67)	0.83 (0.43–1.24)	NS
AFC	12.73 (10.62–14.84)	17.20 (14.66–19.74)	18.83 (14.92–22.75)	18.48 (15.61–21.35)	0.01
AMH (ng/ml)[b]	3.40 (2.45–4.35)	5.07 (2.88–7.25)	5.89 (3.63–8.15)	11.81 (0.59–24.21)	NS
Inhibin B (pg/ml)[c]	85.26 (71.24–99.28)	114.33 (82.31–146.34)	105.2 (79.39–131.18)	140.16 (73.14–207.18)	NS

[a] Data are given as mean (95% CI).

[b] AMH was assayed only in 83 participants.

[c] Inhibin B was assayed only in 46 participants.AMH, anti-Mullerian hormone; AFC, antral follicle count; NS, not statistically significant, Q, quartile.

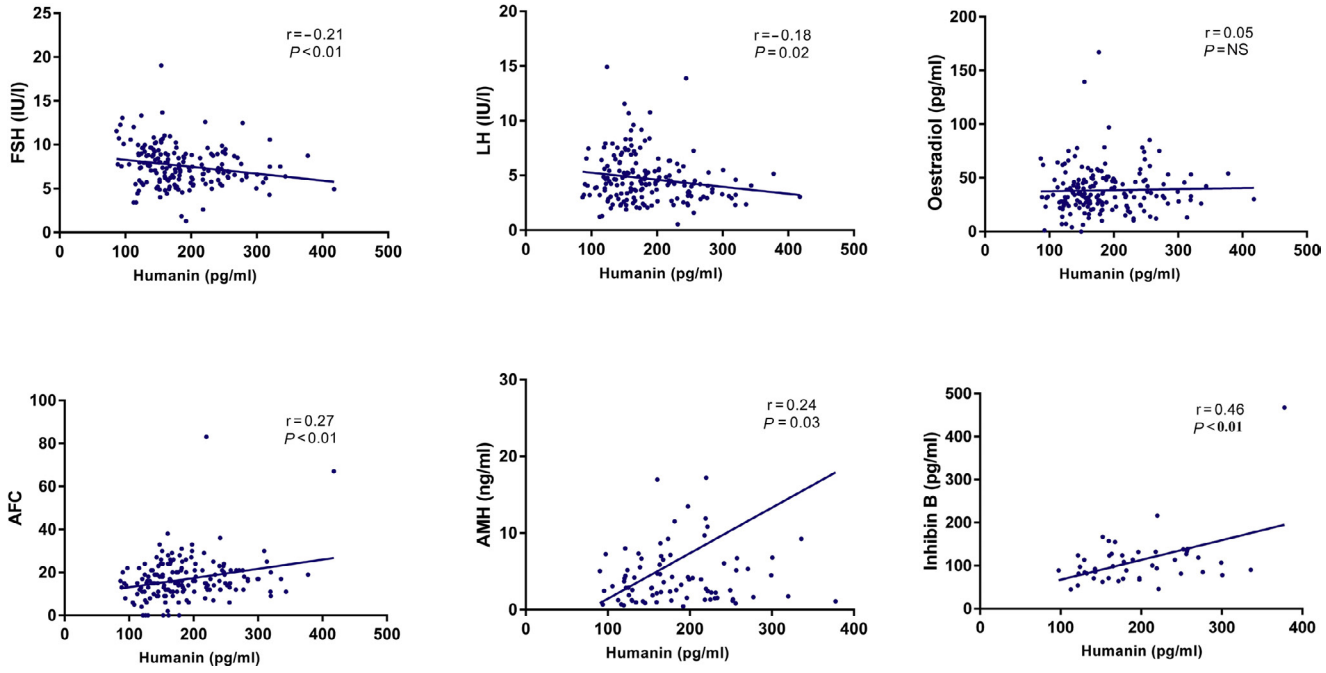

FIGURE 4 Correlations between follicular fluid humanin concentration levels and reproductive hormones, antral follicle count, anti-Müllerian hormone (AMH) and inhibin B levels. Serum FSH, LH, oestradiol levels and antral follicle count (AFC) were tested in all women (n = 179). AMH and inhibin B levels were assayed in 83 and 46 women, respectively. NS, not statistically significant.

TABLE 3 ASSOCIATIONS BETWEEN FOLLICULAR FLUID HUMANIN CONCENTRATION AND FERTILIZATION RATE

Parameters	Quartile of follicular fluid humanin concentration								*P*-value
	Q1		Q2		Q3[a]		Q4		
	Number of 2PN/ number of oocytes inseminated	%	Number of 2PN/number of oocytes inseminated	%	Number of 2PN/number of oocytes inseminated	%	Number of 2PN/number of oocytes inseminated	%	
IVF cycles									
With husband sperm	137/237	57.8	187/264	70.8	141/272	51.8	93/136	68.4	<0.01
With donor sperm	111/201	55.2	100/167	59.9	107/173	61.8	74/94	78.7	<0.01
ICSI cycles									
With husband sperm	105/164	64.0	113/175	64.6	114/189	60.3	140/210	66.7	NS
With donor sperm	26/56	46.4	52/107	48.6	–	–	47/64	73.4	<0.01

[a] No patients were treated with intracytoplasmic sperm injection using donor sperm.
NS, not statistically significant; 2PN, two pronuclei; Q, quartile.

maturation and function. Our further regression analysis showed that patients with higher humanin levels (only Q4) experienced an increased chance of clinical pregnancy after adjusting for a series of confounders. As noted above, humanin plays an important role in maintaining the structural and functional homeostasis of mitochondria, and is involved in mitochondria-dependent apoptosis, oxidative stress and energy metabolism (*Kariya et al., 2005; Muzumdar et al., 2010; Jia et al., 2013; Klein et al., 2013; Jia et al., 2015*). As all of these processes have been shown to affect oocyte development and maturation clearly (*Ruder et al., 2008; Pandey et al., 2010; Tripathi et al., 2013; Sugiyama et al., 2016; Dadarwal et al., 2017; Yang et al., 2017*), humanin in the follicles may also act to protect granulosa cells from apoptosis, balance oxidative stress in the microenvironment of follicular fluid, and improve the metabolic status of oocytes via mechanisms similar to those in other organs. Consequently, higher levels of humanin may improve the fertilization rate and promote subsequent clinical pregnancy. On the basis of this result, future study of the therapeutic effect of supplemental humanin on developmental dysfunction of follicles is needed to better clarify its protective role and evaluate its clinical application.

The most important strength of this study is that, to the best of our knowledge,

TABLE 4 MULTI-VARIABLE LOGISTIC REGRESSION ANALYSIS[a] OF THE ASSOCIATIONS BETWEEN FOLLICULAR FLUID HUMANIN CONCENTRATION AND CLINICAL OUTCOMES AFTER IVF AND INTRACYTOPLASMIC SPERM INJECTION (OR 95% CI)

	Clinical pregnancy	Live birth	Miscarriage
Q1	Reference	Reference	Reference
Q2	1.82 (0.62 to 5.45)	1.07 (0.37 to 3.05)	1.05 (0.13 to 8.53)
Q3	2.22 (0.70 to 7.00)	1.51 (0.51 to 4.52)	0.61 (0.07 to 5.43)
Q4	3.60 (1.09 to 11.84)[b]	2.74 (0.91 to 8.23)	1.34 (0.34 to 13.00)

[a] Adjusted for age, body mass index, ovarian stimulation protocol, cause of infertility, type of infertility, type of insemination, duration of infertility and number of transferred embryos.

[b] $P = 0.03$.

Q, quartile.

the presence of humanin in the follicular fluid was evaluated for the first time, as was the relationship between follicular fluid humanin levels and ovarian function. Additionally, our results were based on a moderate population, and follicular fluid samples were collected from the first punctured follicles which were similar in follicle size. The study, however, is not without limitations. First, the enrolled patients were heterogeneous in the cause of infertility and treatment protocols (IVF or ICSI, with husband or donor sperm), which may affect clinical outcomes although these factors were adjusted in multi-variable logistic regression analysis. Second, only two human ovarian samples were used for immunofluorescence staining. Third, no direct evidence on the origin and regulation of follicular fluid humanin was obtained in this study, and further study is needed to clarify this issue.

In conclusion, humanin is present in the human ovary and can be secreted to the follicular fluid. The concentration levels of humanin in the follicular fluid were found to be positively related to the ovarian reserve markers and fertilization rate of women undergoing IVF–ICSI. Patients with higher levels of humanin in the follicular fluid were more likely to achieve clinical pregnancy. These results indicate that humanin may be an important molecule affecting follicular development and ovarian function. Further studies of the origin of follicular fluid humanin, and its regulative factors and the detailed role of humanin in follicle development and maturation are needed.

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