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Original Article

Correlation analysis between C natriuretic peptide and pregnancy outcome

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Abstract: C-type natriuretic peptide (CNP) plays a key role in female reproduction and is related to oocyte quality. This study analyzed the relationship of CNP with pregnancy outcome to provide a new indicator of pregnancy outcome. Follicular fluids were collected from 158 patients undergoing the IVF/ICSI procedure at the Center for Reproductive Medicine, Tongji Medical College, Huazhong University of Science and Technology. CNP and cGMP levels in human follicular fluids were tested by ELISA. Then, the distribution patterns of CNP and NPR-B from GV oocytes to blastocysts in mice were tested by confocal microcopy. Finally, CNP was added to the fertility or embryo development medium to observe fertility rate and the development of the embryo. CNP levels in follicular fluids from nonpregnant women were significantly higher than those in follicular fluids from pregnant women. A strong positive correlation between CNP and cGMP concentrations in human follicular fluids was observed. Both CNP and NPR-B were expressed in the plasma of cells at different stages from GV to blastocyst. CNP could increase the 2-cell rate of embryos and the rate of blastocysts when added to either fertility culture medium or embryo culture medium. In a word, CNP in human follicular fluid could predict the pregnancy outcome of IVF patients, and the mechanism of CNP in follicular fluid is related to the quality of oocyte or embryo competence and could promote the development of embryos.

Keywords: CNP, NPR-B, pregnancy outcomes, IVF, follicular fluid

Introduction

Natriuretic peptides (NPs) are a family of peptides that have similar sequences and conformations and include atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), C-type natriuretic peptide (CNP) and dendroaspis natriuretic peptide (DNP). C-type natriuretic peptide (CNP), encoded by the NPPC (Natriuretic Peptide Precursor C) gene, was found in porcine brain extracts in 1990 [1]. Both ANP and BNP are cardiac hormones involved in the regulation of the water-electrolyte balance and blood pressure [2]. In contrast, CNP is a paracrine and endocrine factor expressed in a variety of tissues [3]. The biological effect of CNP is mediated by intracellular cyclic guanosine monophosphate (cGMP) accumulation specifically through membrane-bound natriuretic peptide receptor B (NPR-B). CNP is involved in many kinds of physiological processes, such as maintaining cartilage and bone function [4], regulating cardiac and vascular function [5], modulating blood-brain barrier permeability [6], protecting kidney function [7] and maintaining meiosis [8].

Recent studies have shown that CNP plays a key role in female reproduction. *Npr-B*$^{-/-}$ female mice are all sterile, and studies have indicated that the uterus horn is string-like, with a very thin endometrium and myometrium relative to WT animals [9]. In addition, ovarian size was smaller in *Npr-B*$^{-/-}$ females, whereas only primordial through secondary antral follicles existed in the ovaries [9]. Geister et al. discovered a new spontaneous mutant allele of Npr2 named peewee (pwe), which exhibits severe disproportionate dwarfism and female infertility, and reported that the primary cause of Npr2 (pwe/pwe) female infertility was premature oocyte meiotic resumption [10]. Zhang M et al. found that the granulose cell ligand CNP and its receptor NPR-B in cumulus cells prevent precocious meiotic maturation, which is critical for maturation and ovulation synchrony and for normal female fertility [8]. Zhang et al. suggested that CNP can be used to delay meiotic resumption

and enhance the developmental competence of goat oocytes matured in vitro [11]. Moreover, CNP could simulate preantral and antral follicle growth [12]. In vitro, stimulating goat granulosa cells with FSH led to an increase in the expression of NPPC in a dose- and time-dependent manner, and a rapid decline was induced by LH stimulation, but the expression of NPPA (natriuretic peptide precursor A) and NPPB (natriuretic peptide precursor B) did not change after FSH or LH treatment [13]. Therefore, CNP treatment, as a substitute for follicle stimulating hormone, could provide an alternative therapy for female infertility [12-14].

Follicular fluid, surrounding oocytes, provides an important microenvironment that influences oocyte maturation and embryo development [15]. Follicular fluid is generated mainly through two pathways: one part is derived from the plasma elements that penetrate the blood-egg barrier, while the other part comes from granular and follicular cell secretions. Karen et al. reported that follicular somatic cells sustain meiotic arrest via the natriuretic peptide C/natriuretic peptide receptor 2 (NPPC/NPR2) system and possibly also via high levels of the purine hypoxanthine in follicular fluid [16]. Zhang et al. reported that CNP existed in porcine follicular fluid, and its concentration increased with follicular size [17]. CNP is also found in the human ollicular fluid [18]. Although a few reports have confirmed the presence of CNP in follicular fluid [16-18], its relationship with IVF outcomes remains unknown.

In this study, we performed CNP quantification in individual follicular fluids (n=158) from 158 patients undergoing IVF to investigate the relationships between CNP levels in follicular fluids and IVF outcomes. In addition, we successfully characterized the CNP and NPR-B expression patterns during mouse oocyte maturation and embryo development after conventional IVF by scanning confocal microscopy. Moreover, CNP was used to incubate oocyte and embryos for the improvement of IVF outcomes, such as better fertilization rates and blastocyst formation rates.

Methods

This study was approved by the Ethical Committee of Tongji Medical College, Huazhong University of Science and Technology (No. S1188). Informed consent was obtained from all participants. The 158 patients in this study were undergoing the IVF/ICSI procedure at the Center for Reproductive Medicine, Tongji Medical College, Huazhong University of Science and Technology.

Follicular fluid collection and CNP/cGMP quantification by ELISA

On the day of oocyte retrieval, follicular fluid from large follicles (≥18 mm) was collected, and the follicles were aspirated without flushing. Only blood-free follicular fluids (n=158) were collected and centrifuged at 2000 g for 10 min. The supernatants were removed and stored at -80°C for CNP or cGMP quantification. CNP and cGMP concentrations in each follicular fluids were quantified using a Human CNP/cGMP ELISA kit (no. 3715-1HP-2 Mabtech AB, Sophia Antipolis, France) following the manufacturer's instructions.

Immunofluorescence and confocal microscopy

Oocytes or embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 30 min at room temperature. After permeabilization with 0.5% Triton X-100 at room temperature for 30 min, the oocytes or embryos were blocked in 1% bovine serum albumin (BSA)-supplemented PBS for 1 h and incubated overnight at 4°C in the appropriate primary antibodies diluted in 1% BSA-supplemented PBS. The antibodies and the dilutions used in the study were as follows: CNP (1:300); NPR-B (1:300); and α-tubulin (1:200). After three washes with PBS containing 0.1% Tween 20 and 0.01% Triton X-100 (PBST) for 5 min each, the oocytes or embryos were labeled with 1:64 tetramethyl rhodamine isothiocyanate conjugate (TRITC)-conjugated goat anti-rabbit IgG or rabbit anti-goat IgG for 1 h at 37°C. After three washes with PBST, the oocytes or embryos were blocked in 1% BSA for 1 h and then labeled with anti-α-tubulin antibody (1:200), tetramethyl rhodamine isothiocyanate conjugate (FITC)-conjugated donkey anti-mouse IgG for 1 h at 37°C. The nuclear status of the oocytes or embryos was evaluated by staining with 10 μg/L Hoechst for 10 min. Finally, the oocytes or embryos were mounted on glass slides and examined with a confocal laser scanning microscope (Zeiss LSM 510 META, Jena, Germany). Immunofluorescent staining of CNP and NPR-B in mouse oocytes and embryos was carried out according to the method described above. Each experiment was repeated three times. The instrument settings were kept constant not only for each replicate but also between different groups.

Table 1. Basic characteristics of IVF patients

Parameter	Pregnant (n=73)	No pregnant (n=85)	P
Age (year)	30.19±4.71	31.31±5.03	0.157
BMI (kg/m^2)	22.69±3.10	22.02±3.16	0.206
Infertility time (year)	5.24±3.85	5.34±4.05	0.876
AFC (n)	19.37±10.55	16.51±6.70	0.048
Basic FSH (IU/L)	7.46±2.33	7.56±2.05	0.768
Basic E2 (pmol/L)	71.73±68.74	79.11±65.54	0.505
Total amount of Gn (IU)	2143.06±847.07	2351.07±798.81	0.119
Oocytes obtained	14.68±8.03	13.58±6.84	0.358
Egg maturation rate (%)	0.93±0.13	0.93±0.13	0.798
Optimal embryo rate (%)	0.62±0.32	0.60±0.33	0.724
Fertilization rate (%)	0.77±0.20	0.76±0.21	0.864

Isolation of GV and GVBD oocytes

Female KM mice (5-8 wk old; Charles River, St. Constant, PQ, Canada) were superovulated by the intraperitoneal injection of 10 IU pregnant mare serum gonadotropin (PMSG) (catalog no. G4877; Sigma, St. Louis, MO). After 48 h, the ovaries were isolated, punctured to release the cumulus oocyte complexes (COCs). After isolation, the COCs were washed in the final incubation medium and cultured in a modular incubation chamber (Billups Rothenberg, Del Mar, CA) with an atmosphere of 5% O_2, 5% CO_2, and 90% N_2 at 37°C. Denuded oocytes (DOs) were obtained by repeated drawing in and out of glass pipets with a diameter slightly smaller than that of the oocytes and dispersing the cumulus cells. At the end of the 4 h culture period, oocytes with germinal vesicles (GV; meiotic arrest) and germinal vesicle breakdown (GVBD; meiotic resumption) were counted under a stereomicroscope.

In vitro fertilization (IVF)

Female NRG mice (4-10 weeks of age) were superovulated by intraperitoneal injection with 10 IU pregnant mare serum gonadotropin, followed by an injection with 10 IU human chorionic gonadotropin (hCG, National Hormone & Peptide Program, NIDDK) at 48 h later. The animals were sacrificed at 16 h following hCG administration, and the oviducts were collected. COCs were released from the oviducts and placed in pre-equilibrated human tubal fluid (HTF; Millipore catalog no. MR-070-D) drops, and subsequently incubated for insemination at 37°C in 5% CO_2.

Spermatozoa from young adult male KM mice were squeezed out of freshly isolated cauda epididymides in HTF using sterile forceps and allowed to "swim out" for 10 min at 37°C. The spermatozoa were counted using a hemocytometer and incubated in an HTF droplet for 60 min to allow capacitation before insemination.

Insemination and the subsequent washing steps occurred in 50 µl HTF drops overlaid by sterile mineral oil in a culture dish. COCs and denuded oocytes were inseminated at 106 sperm/ml in HTF for fertility. After incubation for 6 h (37°C, 5% CO_2), presumptive zygotes were washed twice and transferred to KSOM-AA (Millipore catalog no. MR-121-D) droplets for embryo development. Successful fertilization was assessed at 24 h after insemination, indicated by the presence of a second polar body or cleavage. Embryo development was recorded at 96 h after IVF. The trials for each group were repeated at least three times. In this experiment, different concentrations of CNP were added to HTF or KSOM-AA to assess the effect of CNP on embryo development at different stages.

Statistical analyses

All data were statistically analyzed using SPSS 23.0 Software/Stata 21.0 software. The means ± standard deviations (SD) and 95% confidence intervals (CI) were reported for quantitative data and percentages were reported for categorical data. A level of $P<0.05$ was considered statistically significant.

Results

Basic characteristics of IVF patients

A total of 158 female patients between 21 and 45 years of age (mean ± SD: 30.79±4.90 years) and with a BMI between 15.63 and 30.82 kg/m^2 (mean ± SD: 22.33±3.14 kg/m^2) were included in our study. The mean infertility period was 5.29±3.94 years. A total of 101 couples had primary infertility (63.92%) and 57 couples had secondary infertility (36.08%). The patient clinical characteristics are summarized in **Table 1**.

Table 2. Relationships between CNP concentrations in human follicular fluids and pregnancy outcome

	Nonpregnant	Clinical pregnancy			
		Total	Normal pregnancy	Abortion	Ectopic pregnancy
N (%)	85 (53.80)	73 (46.20)	60 (37.97)	5 (3.16)	8 (5.06)
CNP (ng/L)	17.41±13.15	13.61±5.31*	13.31±5.25*	14.33±4.66	15.42±6.32

*Comparison to nonpregnant patients, P<0.05.

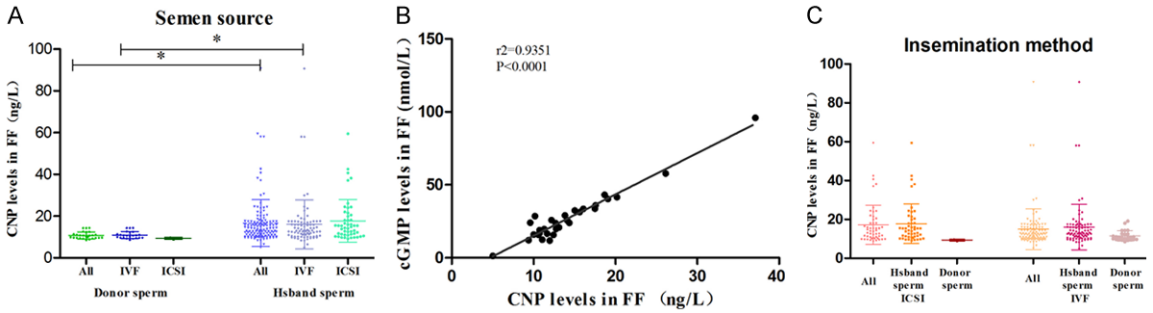

Figure 1. Relationships between CNP concentrations with different types of IVF and with cGMP in human follicular fluids. (A) The relationships between CNP levels with different sources of semen or different IVF insemination methods were analyzed, the results showed the CNP concentrations in patients undergoing IVF using husband sperm were significantly lower than those in patients using donor sperm (*P<0.05), but there were no differences in CNP concentrations in follicular fluids between patients undergoing IVF and ICSI (B). (C) There was a strong positive correlation between CNP and cGMP concentrations in human follicular fluids.

Table 3. Relationships between CNP and cGMP in human follicular fluids

	N	Concentration		R^2	Regression coefficient β
		Mean±SD	95% CI		
CNP (ng/L)	30	14.35±5.92	[12.14; 16.57]		
cGMP (nmol/L)	30	27.47±17.33	[21.00; 33.94]	0.935	2.83

R^2 determination coefficient, β regression coefficient.

Relationships between CNP concentrations in follicular fluids with pregnancy outcomes

As shown in **Table 2**, the CNP levels in follicular fluids from nonpregnant patients were significantly higher than those in follicular fluids from pregnant patients. The pregnant patients were divided into three groups: normal pregnancy, abortion, and ectopic pregnancy. Although CNP concentrations in abortion and ectopic patients were higher than those in normal pregnancy patients, there were no differences. Only CNP in normal pregnancy patients was significantly lower than that in non-pregnant patients.

Relationships between CNP concentrations in follicular fluid with different types of IVF

In this study, the CNP concentrations in different types of IVF were analyzed, and the results showed that the CNP concentrations in patients undergoing IVF using husband sperm were significantly lower than those in patients using donor sperm (**Figure 1A**). However, there were no differences in CNP concentrations in follicular fluids between patients undergoing IVF and ICSI (**Figure 1B**).

Relationships between CNP and cGMP in human follicular fluid

As shown in **Table 3** and **Figure 1C**, we found a strong positive correlation between CNP and cGMP concentrations in human follicular fluids (R^2=0.935, β=2.83).

Distribution patterns of CNP and NPR-B from GV oocytes to blastocysts in mice

Confocal microcopy showed immunostaining for CNP and NPR-B in oocytes at GV, GVBD, and MII stages or in embryos at the 2-cell, 4-cell, 8-cell, and blastocyst stages (**Figures 2**, **3**). The results showed that both CNP and NPR-B were

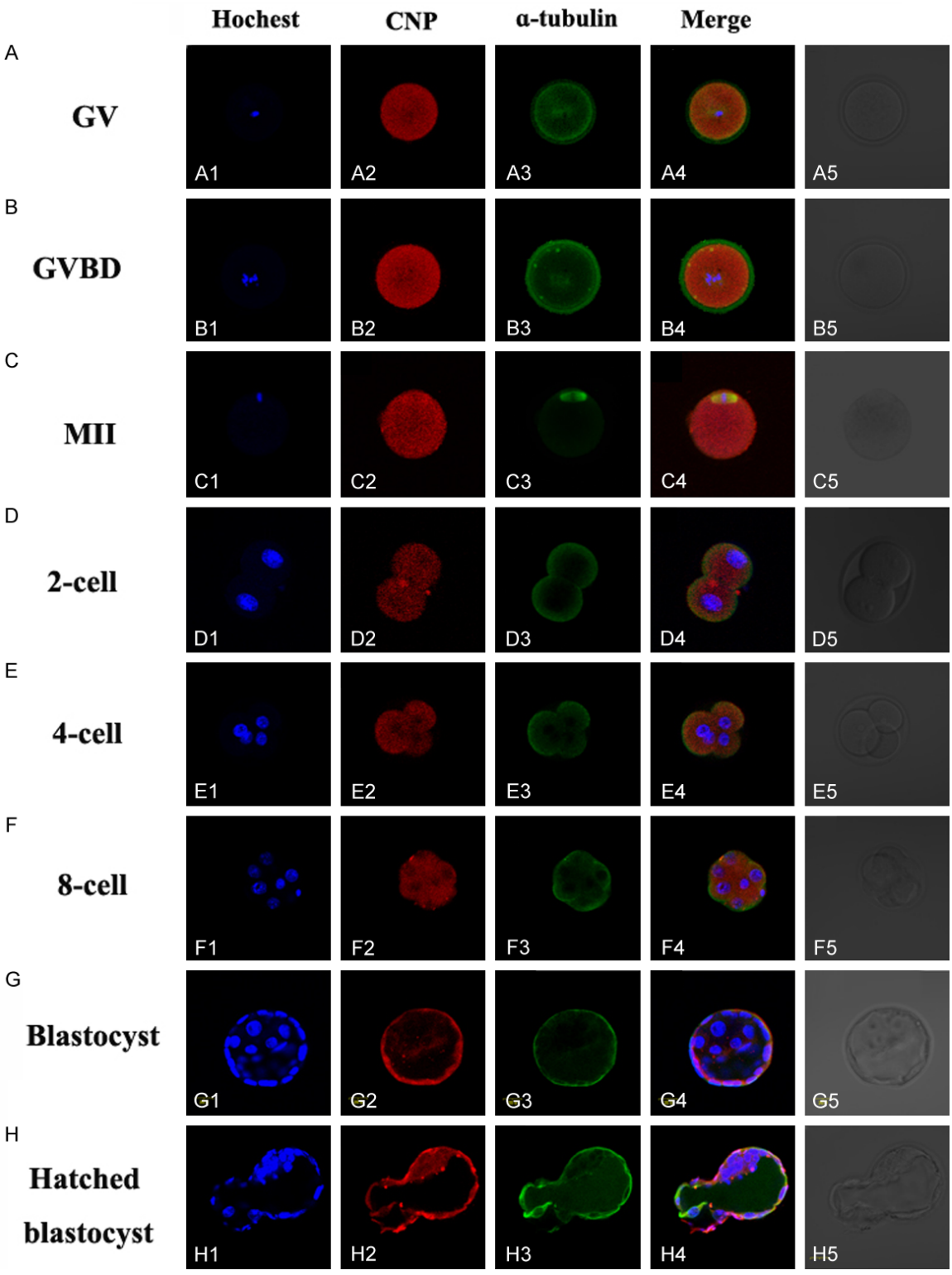

Figure 2. Confocal microcopy showed CNP was expressed in the plasma of cells at different stages from GV oocyte to blastocyst. Red shows the expression of CNP, green shows the expression of α-tubulin, blue shows the expression of DNA, scale bar =20 μm. (A-H) represent CNP expression in GV, GVBD, MII, two-cell, four-cell, eight-cell, blastocysts, and hatched embryos, respectively.

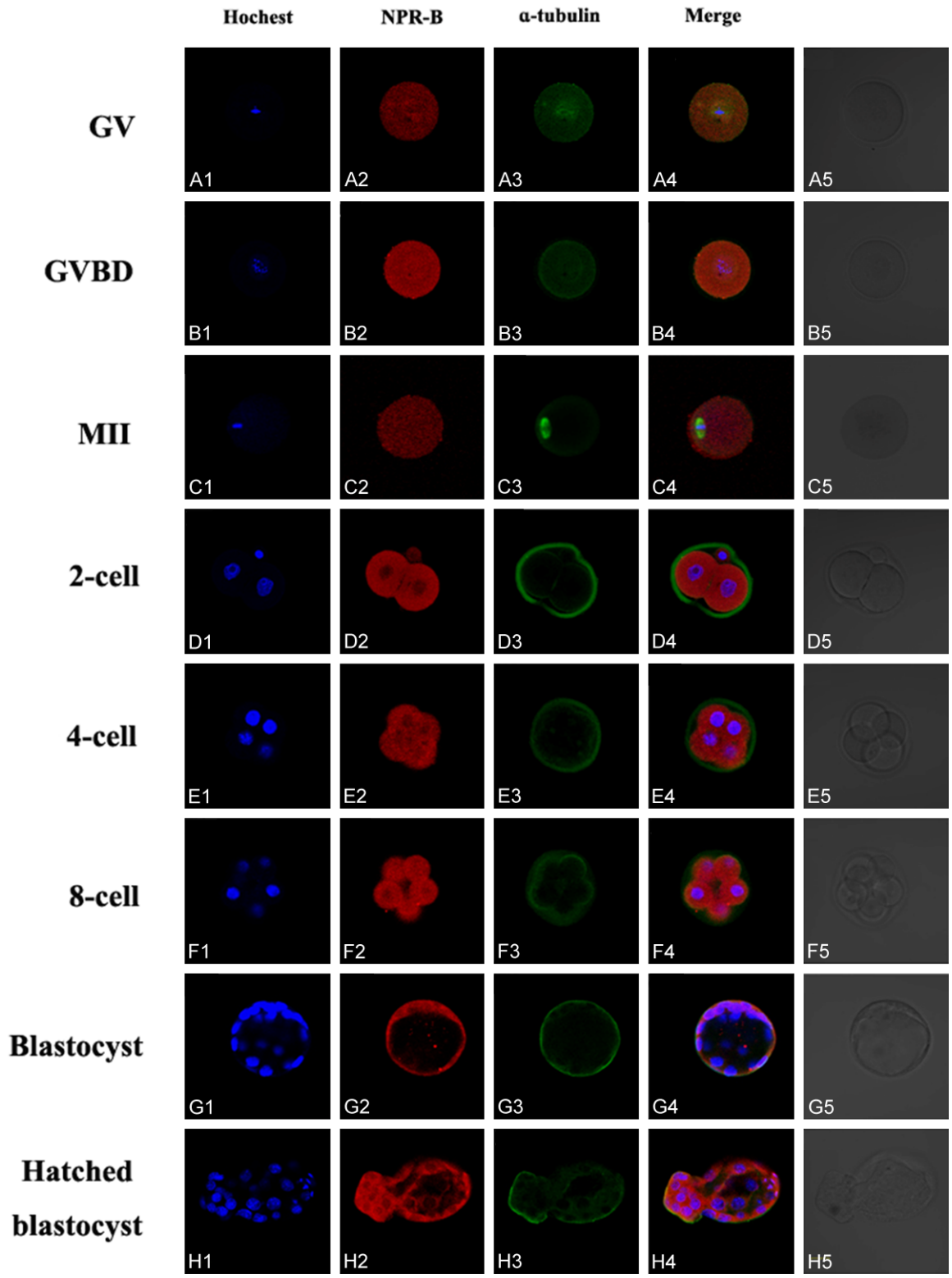

Figure 3. Confocal microcopy showed NPR-B was expressed in the plasma of cells at different stages from GV oocyte to blastocyst. Red shows the expression of NPR-B, green shows the expression of α-tubulin, and blue shows the expression of DNA (scale bar =20 μm). (A-H) represent CNP expression in GV, GVBD, MII, two-cell, four-cell, eight-cell, blastocysts, and hatched embryos, respectively.

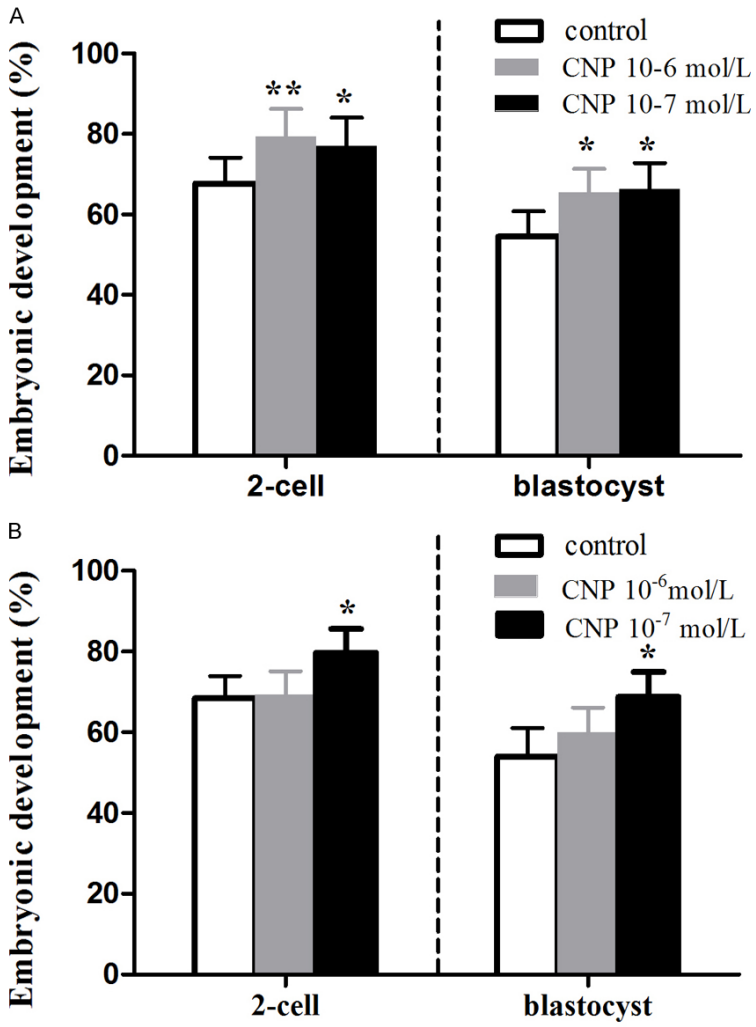

Figure 4. CNP could increase the fertilization rate and promote early embryonic development. Different concentrations of CNP were added to the fertility culture medium (HTF) or embryonic development medium (KSOM-AA). A. Both 10^{-7} mol/L and 10^{-6} mol/L CNP could increase the 2-cell and blastocyst formation rates when added to HTF. B. 10^{-7} mol/L CNP could improve the 2-cell and blastocyst formation rates when added to the KSOM-AA. Data presented was representative of multiple experiments (mean ± SD, *P<0.05, **P<0.01).

expressed in the plasma of cells at different stages from GV to blastocyst (GV= oocytes at the germinal vesicle stage; GVBD= oocytes undergoing germinal vesicle breakdown; MII= oocytes at the metaphase of second meiosis; day 2 embryos usually at 2 cell stage; day 3 embryos at 4-8 cell stage; day 5 embryos at blastocyst stage; days 6-7 embryos at the hatched blastocyst stage).

Effects of CNP on fertilization and early embryonic development in mice

To detect the effects of CNP on fertilization and early embryonic development, different concentrations of CNP were added to the fertility culture medium (HTF) or embryonic development medium (KSOM-AA) separately. The results showed that the 2-cell and blastocyst formation rates cultured in CNP were higher than those in the control groups. Both 10^{-6} mol/L and 10^{-7} mol/L CNP improved the 2-cell and blastocyst formation rates when added to HTF. Furthermore, the 2-cell rate increased as the concentration of CNP increased. In addition, 10^{-7} mol/L CNP improve the 2-cell and blastocyst formation rates when added to KSOM-AA (**Figure 4**).

Discussion

Mammalian oocytes first undergo meiotic progression during embryonic development, and at the time of birth, these cells become arrested in the diplotene stage of prophase I. Meiosis arrest provides a relatively longer time for cytoplasmic maturation, optimizes the synchronization of nuclear and cytoplasmic maturation and improves the developmental capacity of immature mammalian oocytes [10, 19]. This meiotic arrest of oocytes is maintained until shortly before ovulation. During each reproductive cycle, the preovulatory LH surge triggers the resumption of meiosis and its progression to metaphase II (MII), a process commonly termed oocyte maturation [20]. Oocyte maturation is the first step affecting successful fertilization and preimplantational embryo development. In humans, incomplete oocyte maturation results in infertility that is attributed to a poorly defined phenomenon known as “oocyte factor” [21, 22]. CNP has been demonstrated to function as an oocyte maturation inhibitor (OMI) in many species [12, 13, 17]. The mechanism is that CNP stimulates the production of cGMP by binding to its receptor NPR2 on cumulus cells; then, cGMP is trans-

ferred to the oocyte though gap junctions and inhibits phosphodiesterase (PDE) 3A, resulting in the inhibition of cAMP degradation and the maintenance of meiotic arrest [10, 23]. Ovulatory LH surge can suppress NPPC expression and significantly decrease CNP levels in murine ovaries and human follicular fluid [18]. The decline in CNP levels lowers intraoocyte cGMP levels, which play a central role in maintaining meiotic arrest, and triggers meiotic resumption [24]. Egbert et al. showed that LH signaling causes dephosphorylation and inactivation of NPR2 within 10 min, accompanied by a rapid phosphorylation of the cGMP phosphodiesterase PDE5; later, levels of the NPR2 agonist C-type natriuretic peptide decrease in the follicle, and these sequential events contribute to the decrease in cGMP that causes meiosis to resume in the oocyte [25]. In our study, there was a strong correlation between the level of CNP and cGMP in follicular fluid, which suggested that the level of CNP in follicular fluid reflected the level of cGMP. Therefore, the detection of CNP content in follicular fluid can reflect egg nuclear maturation to a certain extent.

Oocyte maturation is one of the major steps for the oocyte to attain competence for successful fertilization and subsequent embryonic development. Although IVF technology has recently made significant progress, the successful live birth rate is no more than 40% [25, 26]. So, a critical step in IVF treatment is the assessment of oocyte maturation and embryo development competence to determine the most viable embryo(s) to be transferred [27]. Currently, oocyte maturation is mainly determined by morphology. However, morphological parameters do not accurately reflect the maturity of eggs. Therefore, the development of an objective and accurate test to assess oocyte and embryo viability remains one of the most significant contemporary goals of reproductive medicine [28]. Oocyte quality can be evaluated through the biological functions of granulosa cells and molecular components of follicular fluid within the ovarian follicle [29]. Human follicular fluid is a superfluous, rich and readily available substance that can be used as a noninvasive tool to assess the quality of oocytes and the outcome of pregnancy [30]. As described above, CNP plays a key role in oocyte maturation and exists in the follicular fluid. Therefore, the CNP level in the follicular fluid may be a new marker for oocyte maturation. Our results showed that the concentrations of CNP in the follicular fluids of nonpregnant women were significantly higher than those in the follicular fluids of clinical pregnant women. In addition, there is no significant difference in the level of CNP between nonpregnant women and abnormal pregnant women (such as abortion and ectopic pregnancy) who experienced embryonic termination. The results indicated that the high expression of CNP after LH surge could not trigger meiotic resumption efficiently, resulting in the insufficient maturity of the egg nucleus and a decrease in the quality of the egg, which is not easily conceived.

There are different methods of IVF to treat different kinds of infertility. Usually, if the cause of infertility is mainly in females (such as tubal disease, PCOS, poor ovarian function), then the patients will undergo IVF using husband sperm; if the cause of infertility is mainly in males (such as severe oligozoospermia, azoospermia), the patients will adopt IVF using donor sperm or intracytoplasmic sperm injection (ICSI) [31, 32]. In this study, we further analyzed CNP in follicular fluid between different types of IVF and found that the concentration of CNP in women using husband sperm was significantly higher than that in women using donor sperm,. We presumed the high CNP level may be related to poor ovarian function and decreased egg quality in IVF women who use their husband's sperm. While women undergoing IVF with donor sperm are usually normal and have better ovary function [33]. Furthermore, the results indicated that there were no obvious differences in CNP concentrations in follicular fluids between IVF and ICSI women. ICSI is used to solve not only male infertility (such as severe oligozoospermia, teratozoospermia), but also female infertility (abnormal zona pellucida, fertilization failure, poor ovarian response) [34, 35]. Therefore, the patients undergoing ICSI are often accompanied by female ovarian dysfunction. This may be the reason why there is no difference between conventional IVF and ICSI in this experiment.

In adult female mice, the uterus and ovaries are the organs with the highest CNP mRNA concentrations, exceeding the CNP levels in other organs, such as the brain or kidneys [36]. Uterine CNP-mRNA concentrations increase

during pregnancy up to a seven-fold concentration, whereas in the ovaries, these levels decrease to 10% compared to those in non-pregnant controls. Moreover, the concentrations of CNP in placental tissues greatly exceed those in intercaruncular uterine tissue throughout pregnancy [37, 38]. In this study, immunofluorescence showed that CNP and NPR-B were expressed from immature oocytes to mature oocytes and later at the 2-cell and blastocyst stages. The results indicated that CNP and NPR-B might participate not only in oocyte maturation but also in the early development of embryos. Recently, several studies have confirmed that CNP can increase the in vitro maturation rate of immature oocytes and significantly improve the fertilization rate and blastocyst rate of in vitro matured oocytes [39]. The two-step IVM system in which oocytes were pretreated with 200 nM CNP for 6 h and then cultured IVM for 28 h yielded a significantly ($P<0.05$) increased blastocyst rate and cell number after in vitro fertilization (IVF) compared to the conventional one-step IVM method [40]. A strategy involving prematuration culture in the presence of CNP followed by IVM using FSH + amphiregulin increases oocyte maturation potential, leading to a higher availability of day 3 embryos and good-quality blastocysts for single embryo transfer [41]. CNP treatment significantly increased the oocyte maturation rate from less than half to more than 80%. After IVF, temporary meiotic arrest mediated by 10 and 50 nM CNP significantly improved the fertilization and blastocyst rate of oocytes matured in vitro up to approximately 55% and 30%, respectively [42]. Romero S et al. [43] reported for the first time a long-term precocious culture system with CNP as a key factor that can effectively obtain oocytes from premature meiotic mice without meiosis and promote their development. In this study, the fertilization rate and blastocyst rate were significantly improved after adding the appropriate concentration of CNP to the culture medium. Both 10^{-6} mol/L and 10^{-7} mol/L CNP could promote the 2-cell and blastocyst formation rates when added to fertility culture medium. But only 10^{-7} mol/L CNP could increase the 2-cell and blastocyst formation rates when added to embryonic development medium. This may be due to the facts that oocyte fertilization and embryo development need different environments in vivo [44]. The fallopian tubes are the natural environment for human oocyte fertilization and the stage where early embryonic development unfolds, while the endometrial cavity provide the optimal environment for blastocyst formation and implantation [45]. Our previous study showed that CNP was expressed in the genital tract of female rats, especially in the mucosa epithelium cell of the oviduct; the CNP level in the rat oviduct was higher than that in the uterus [46].

In summary, CNP not only plays a key role in inhibiting the meiosis of eggs but also improves fertilization and subsequent embryonic development, which is important for a successful pregnancy outcome. CNP in follicular fluid may be a potential biomarker for evaluating the quality of oocytes or embryo competence during IVF and for predicting the outcome of IVF pregnancy.

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Disclosure of conflict of interest

None.

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