Supplementation of c-type natriuretic peptide during the whole in vitro growth period benefits the development of murine secondary follicles

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Research

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Abstract

Background Supplementation of c-type natriuretic peptide (CNP) in the culture medium shortly before in vitro maturation (IVM) has been reported to be effective in delaying meiotic resumption of murine oocyte. The present study investigated the effect of CNP supplementation during the whole period of in vitro growth (IVG) on the development of murine secondary ovarian follicles.

Methods Late secondary ovarian follicles isolated from ovaries of Kunming mice were cultured in vitro with and without supplementation of CNP. In experiment 1, CNP was supplemented at the early stage and the follicle development was evaluated. In experiment 2 and 3, CNP was supplemented during the whole period of IVG. In experiment 2, follicle development and oocyte maturity were evaluated. In group 3, follicle development and rate of cleaved embryos after in vitro fertilization (IVF) was assessed.

Results In control group in all 3 experiments, granulosa cells migrated from within follicle and adhered to the plate at different degrees. The follicles flattened and could not reach antral stage. About 39.8% (39/98) of the oocytes ovulated nakedly. As no antral follicle was obtained, IVF was not performed in control group in experiment 3. In experiment group in all 3 experiments, no migration of granulosa cells was observed and the follicles grew three-dimensionally. Ovulation of naked oocyte decreased substantially. The rate of antral stage follicle were 45% (18/40) in experiment 1. This parameter was 75.9% (44/58) in experiment 2 and 3 combined. In experiment 2, in preovulatory follicles without ovulation induction, oocytes at germinal vesicle (GV) stage and germinal vesicle breakdown (GVBD) stage were 87.5% (14/16) and 12.5% (2/16), respectively. In preovulatory follicles with ovulation induction, no GV stage oocyte was retrieved, oocytes at GVBD and metaphase II (MII) stage were 50% (8/16), respectively. In experiment 3, among 18 follicles cultured, 12 cumulus-oocyte complexes (COC) ovulated automatically after ovulation induction. Eleven oocytes were fertilized and cleaved. Compared with control groups, the follicle development assessed by naked oocyte ovulation and follicle stage (preantral follicle and antral follicle) in experiment groups were significantly superior ($p < 0.0001$). CNP effectively maintained oocytes' meiotic arrest and enhanced fertilization competency.

Conclusions The supplementation of CNP in culture system of murine late secondary follicle during the whole period of IVG could sustain the 3-dimensional structure of follicle, increase the antral formation rate. As a result, the oocyte's competency to be fertilized was greatly improved.

Plain English Summary

The supplementation of CNP in the culture system of murine late secondary follicle during the whole period of IVG could sustain the 3-dimensional structure of follicle, increase the antral formation rate and maintain oocytes' meiotic arrest. As the oocyte's competency to be fertilized was greatly improved, this protocol might benefit the further research in the field of ovarian follicle culture.

Background

Oocyte development takes place in ovarian follicles. During folliculogenesis, oocytes grow and reach metaphase of meiosis II (MII). To be developmentally competent, it is very important that the cytoplasm matures at the same time when oocyte's meiosis resumed. This include accumulation of proteins and energy substrates, organelles reallocation and changes in the structure of chromatin etc. If the meiosis resumed precociously before cytoplasmic maturation, the developmental competence of oocyte, e.g. the ability to be fertilized and the ability to develop to blastocyst, will be negatively affected.

The importance of cytoplasmic maturation was confirmed by a test reported by Cheng et al. When the cytoplasm at GV stage was used as recipient and karyoplast at metaphase in meiosis I (MI) or MII stage as donor, the constructed oocyte extruded a polar body after electrofusion and culture. While both the cytoplasm and the polar body had a metaphase spindle in the MI-GV pair, only a clutch of condensed chromatin was observed in the cytoplasm and polar body of the MII-GV pair. When MI cytoplasm was used as recipient and GV or MII karyoplast as donor, the reconstructed oocyte also extruded a polar body. Each had one spindle and a group of metaphase chromosomes in the cytoplasm and polar body, respectively. When MII cytoplasm was used as recipient and GV or MI karyoplast as donor, the reconstructed oocytes were activated, became parthenogenetic embryos and even developed to hatching blastocysts after electrofusion. Immunoblotting showed that mitogen-activated protein kinase (MAPK) activity was high in MI and MII cytoplasts but not detected in GV cytoplast. The results demonstrated that the cytoplasmic environment determines the behavior of asynchronous donors [1].

In ovary, follicular cells communicate via paracrine and juxtacrine mechanism. CNP is encoded by natriuretic peptide precursor C (Nppc) gene expressed mainly in mural granulosa cells. It stimulates natriuretic peptide receptor B (NPRB) on the membrane of cumulus cells to produce cyclic guanosine monophosphate (cGMP). cGMP of cumulus origin diffuses into oocytes to suppress phosphodiesterase 3 (PDE3) activity, leading to elevation of cyclic adenosine 3',5'-monophosphate (cAMP) in oocyte. cAMP binds to protein kinase A (PKA) which in turn activates WEE 1 homolog 2 (WEE1B) and myelin transcription factor 1 (MYT1) kinase. WEE1B and MYT1 are known to block cyclin-dependent kinase 1 (CDK1). Thus, cAMP-dependent activation of PKA results in CDK1 inhibition leading to meiotic arrest in oocyte [2,3,4]. This effect of CNP was confirmed in mouse[5,6], porcine [7], bovine [8], cat[9], goat [10] and sheep [11] etc.

In most Graafian follicles of Nppc or Npr2 mutant mice, meiotic arrest was not sustained and meiosis resumed precociously[5]. In in vitro condition, the physiological condition including the cAMP level within oocyte is changed. This will probably result in spontaneous resumption of meiosis. As the maturation of cytoplasm does not catch up with that of nuclear, the developmental competence of such oocytes might decrease. Zhang et al reported that approximately 50%-70% of fertilized oocytes cultured in vitro fail to develop into preimplantation embryos[12,13].

To solve this issue, CNP was supplemented in the in vitro culture system to retard nuclear maturation, which in turn allows time for ooplasmic maturation to catch up and results in better synchronization of nuclear and cytoplasmic compartments [3,6,7,8,9,10,11,13]. But, it was reported that although CNP alone

could enhance the concentration of cGMP during in vitro culture of COC from porcine antral follicle, follicle stimulating hormone (FSH) suppressed the effect if it was added simultaneously with CNP. However, if CNP was added into culture medium 1 h before FSH supplementation, the high level of cGMP induced by pretreatment of CNP could be maintained for up to 8-12 hours[14]. As the oocytes for in vitro culture were isolated from follicles close to ovulation, if the pretreatment of CNP lasted longer, the oocytes cultured in vitro demonstrated a significant decrease in meiotic arrest [13]. So, to prevent premature meiotic resumption, some scholars pretreated COC from antral follicle with CNP for only several hours before conventional in vitro maturation (IVM) [11,12,13]. This protocol was called two-step in vitro culture method.

Besides maintaining meiotic arrest, CNP could also stimulate ovarian follicle growth and increase ovarian follicle viability. In mice, Nppc and Npr2 begin expression in early preantral follicles and increase during early to late preantral follicle development. In cultured somatic cells from infantile ovaries and granulosa cells from prepubertal animals, CNP stimulated cGMP production. Also, treatment of cultured preantral follicles with CNP stimulated follicle growth, whereas treatment of cultured ovarian explants from infantile mice with CNP promoted the development of primary and early secondary follicles to the late secondary stage. In vivo studies indicated that in infantile mice, daily injection of CNP for 4 d promoted ovarian growth and the follicles ovulated after ovulation induction. In prepubertal mice, CNP treatment alone also promoted early antral follicle growth to the preovulatory stage resulting in efficient ovulation by gonadotropin. Mature oocytes retrieved after CNP treatment could be fertilized in vitro and developed into blastocysts. After embryo transfer (ET), viable offspring was delivered [15,16]. It was reported that CNP increased the expression of paracrine or autocrine factors such as Wingless-type mouse mammary tumor integration site family 2b (Wnt2b), Wnt5a, cytochrome P 450 11 a 1 (Cyp11a1) and repressed the expression of estrogen metabolic enzymes cytochrome P 1a1 (Cyp1a1) resulting in follicle growth [16]. This beneficial effect of CNP is more prominent in small follicles compared with large follicles in in vitro culture [17].

As FSH receptor was expressed in preantral follicle onwards, the development of preantral ovarian follicles were jointly regulated by CNP and FSH physiologically. To mimic the in vivo condition, the present study cultured murine late secondary follicles with supplementation of CNP and FSH during the whole period of IVG. Follicle development, oocyte's maturity and fertilization competency were evaluated. The objective is to investigate if supplementation of CNP in combination with FSH during the whole IVG period could stimulate preantral follicle growth and at the same time effectively maintain oocyte meiotic arrest.

Methods

Chemicals

The purchasing information of chemicals were as follows: DMEM/F12 from HyClone Company (USA); recombinant FSH from Merck Serono Company (Italy); human chorionic gonadotropin (HCG) from

Sansheng Pharmaceutical Company (China); insulin from Wanbang Pharmaceutical Company (China); transferrin, epidermal growth factor (EGF) and sodium selenium from Sigma Company (USA); CNP from TOCRIS Company (Britain); serum protein substitute (SPS) from SAGE Company (USA); and human tubal fluid (HTF) from Merck Company (USA).

Animals, follicle culture and in vitro fertilization

Female Kunming mice aged 3 weeks were obtained from Laboratory Animal Center of Shanxi Medical University. Late secondary follicles with multilayer granulosa cells (130-180µm in diameter) were isolated from ovary with tuberculin syringe needle. Each follicle was cultured in 30 µl of droplet under 37°C in a humidified atmosphere of 5% CO₂. The basic culture medium was DMEM/F12 and it was supplemented with 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenium, 10% SPS, 100nM/ml CNP, 25 mIU/ml FSH. The medium was changed half volume every other day. Follicle development was assessed on the day of medium change.

For follicle ovulation, 1.5 IU/ml HCG and 5 µg/ml EGF were added into medium and incubated for 17 hours to induce ovulation when the follicle size reached 350-400µm and part of the follicle bulged out which indicates the follicle is about to ovulate.

For oocyte maturity assessment, the granulosa cells in COC ovulated from antral follicles were stripped off by incubating COC with 80 IU/ml hyaluronadase for 30 seconds and repeated aspiration with capillary glass pipette. The preovulatory follicles not ovulated were pierced with tuberculin syringe needle to retrieve COC. The denuded oocytes were classified into stages of GV, GVBD and MII.

For the assay of fertilization, the COC ovulated was transferred into 100 µl of HTF medium supplemented with 10% SPS. 1×10^5 of motile sperm retrieved from the epididymis of male mice aged 13 weeks were added to the droplet containing COC for IVF. Twenty hours later, fertilization and embryo cleavage were checked.

Experiment design

Experiment 1. The objective of experiment was to investigate the effect of early CNP supplementation on follicle development. In experiment group, CNP was supplemented into medium for only 48 h and omitted in the later culture period. In control group, the only difference from the experiment group was the absence of CNP in the culture medium. The culture was concluded when the dominant antral follicles reached 350-400 µm in diameter. Follicle stage in experiment group and control group were compared.

Experiment 2. The objective of experiment was to investigate the effect of CNP on oocyte maturity. In experiment group, CNP was supplemented into medium for the whole period of IVG. In control group, the only difference from the experiment group was the absence of CNP in the culture medium. As oocyte is matured only in antral stage follicle, in 50% of antral stage follicles, EGF and HCG were added to induce ovulation. In the other antral stage follicles, EGF and HCG were not added. The oocytes in the COC from

antral stage follicle were denuded. Follicle stage in experiment group and control group were compared. In addition, the oocyte maturity was compared between groups with and without ovulation induction in experiment group.

Experiment 3. The objective of experiment was to investigate the fertilization potential of oocytes cultured in vitro. The culture media in experiment and control group during IVG were the same as in experiment 2, respectively. After ovulation induced by EGF and HCG, sperm were added to COC for in vitro fertilization. The rate of 2-cell stage embryo after IVF were evaluated. In addition, follicle stage in experiment group and control group were compared.

In all 3 experiments, the follicles were cultured for at least 7 days based on the preliminary tests. If the follicles have not reached antral stage on the 7th day of culture, or the color darkened, they have the least potential to develop into antral follicle. For antral follicle, when the follicle size reached 350-400µm and part of the follicle bulged out, ovulation was induced with EGF and HCG. If the size increment of antral follicle in 2 days was less than 10µm and the size was more than 300 µm, the oocyte inside the follicle might be competent and perhaps degenerate in the later period. For such follicles, ovulation was also induced with EGF and HCG as a salvage measures.

Statistical analysis

Statistical analysis was performed with software GraphPad Prism 6.0 (La Jolla, CA,USA). Data were analyzed with contingency table (Chi-square test for table 1, 2 and Fisher's exact test for table 3). A level of $p < 0.05$ was considered significant.

Results

Supplementation of CNP improved follicle development

In all control groups in 3 experiments, the granulosa cells in all follicles migrated out from within follicle with different degree and adhered to the plate. As a result, originally ball-shaped follicles flattened (figure 1).

In all experiment groups in 3 experiments, this phenomenon was not observed and all follicles grew in 3-dimension fashion (figure 2).

In all control groups in 3 experiments, 39.8% (39/98) follicles ovulate prematurely before the follicle reached antral stage and the oocyte ovulated was not accompanied by granulosa cells (figure 3).

In all experiment groups in 3 experiments, the abnormal ovulation (oocyte ovulated without granulosa cells) was 3.1% (3/98). In the rest follicles, oocytes ovulated with granulosa cells (figure 4).

In addition, in all control groups in 3 experiments, no follicle reached antral stage. In all experiment groups in 3 experiments, 63.3% (62/98) follicles reached antral stage. All three experiments

demonstrated significant improvement upon follicle development through CNP supplementation (table 1,table 2). In experiment 1, even CNP was only added at the early stage of IVG in experiment group, the migration of guanulosa cells from within follicle was not observed and the follicles grew 3-dimensionally. Besides, the rate of antral follicle was 0% (0/40) in control group and 45% (18/40) in experiment group. The culture period lasted 10 days, 7-9 days and 8 days in experiment 1, 2 and 3, respectively.

Supplementation of CNP effectively maintained meiotic arrest of oocytes

In experiment 2, 32 and 0 antral follicles were formed in the experiment group and control group, respectively. EGF and HCG were added into 16 antral follicles randomly allocated to induce ovulation but not to the other 16 antral follicles. The result demonstrated that 87.5% (14/16) of the oocytes in the follicles without ovulation induction retained at the GV stage. But 0% (0/16) of the oocytes in the follicles with ovulation induction maintained meiotic arrest (table 3).

Supplementation of CNP resulted in cleaved embryos after IVF

In experiment 3, 12 and 0 antral follicles were formed in the experiment group and control group, respectively. In 12 oocytes retrieved from antral follicles in experiment group, 11 were fertilized and cleaved into 2-cell stage embryo 20 h after IVF.

Table 1. The development of ovarian follicles in experiment 1

Groups	naked oocyte	ovulation	preantral follicle	antral follicle
Without CNP (n=40)	15	25	0	
CNP supplementation during the first 48 hours (n=40)	2	20	18	

The development of ovarian follicles between experiment group and control group was significant different (p<0.0001).

Table 2. The development of ovarian follicles in experiment 2 and 3

Groups	naked oocyte	ovulation	preantral follicle	antral follicle
Without CNP (n=58)	24	34	0	
CNP supplementation during the whole period of IVG (n=58)	1	13	44	

The development of ovarian follicles between experiment group and control group was significantly different (p<0.0001).

Table 3. The maturity of oocytes in experiment 2

Groups	GV	MIand MII
Without ovulation induction (n=16)	14	2
With ovulation by HCG and EGF (n=16)	0	16

The maturity of oocytes between group with ovulation induction and group without ovulation induction was significantly different (p<0.0001).

Discussion

Ovarian follicle cryopreservation and in vitro culture after warming is a potential strategy for fertility preservation. In addition, ovarian follicle culture might be helpful for recognizing the mechanism of oogenesis. In 1977, Epigg successfully cultured isolated ovarian follicles in vitro [18]. In 1989, Epigg et al reported the first live offspring after in vitro culture of mouse ovarian follicle and IVF-ET [19]. Then, the live offspring born with modified culture protocol of ovarian follicle were reported successively [20,21,22,23,24].

Although live offspring have been born after ovarian follicle culture, the fertility competency of oocyte cultured in vitro was much lower than that in vivo. Even the oocytes attained MII stage, the fertilization rate especially blastocyst formation rate were significantly decreased [20,21].

Ovarian follicles are grown 3-dimensionally in vivo. Maintenance of intricate 3-dimension architecture and granulosa-oocyte interaction may be critical for successful in vitro maturation of follicles. In conventional 2-dimensional tissue culture systems, granulosa cells surrounding the oocytes tend to migrate out from within follicle and the follicles become flattened. This will change the follicle structure and the communication between oocyte and granulosa cells leading to decreased fertility competence of oocyte. To keep 3-dimensional structure of follicle and enhance the fertility competency of oocyte, v-shaped microwell plates [25], inverted hanging droplet [26,27], alginate encapsulation [28,29], collagen encapsulation [30], fibrin-alginate interpenetrating encapsulation [31,32] and encapsulation with alginate plus amino acids [33] have been used. Although these modifications maintained follicles' 3-dimensional structure, it was reported that compared with oocytes grown in vivo, spindle formation and chromosome alignment of oocytes cultured with some of the protocols mentioned above was abnormal and the developmental competence was compromised[29].

Contrary to the conventional protocol in which CNP was added into culture system several hours before IVM, the present study supplemented CNP in combination with FSH during the whole period of IVG of murine late secondary follicles. By this strategy, oocytes' meiotic arrest was efficiently maintained. In addition, follicles grew 3-dimensionally although ovarian follicles were not encapsulated by alginate or collagen etc. As a result, follicle development and fertilization competency of oocyte were greatly improved. It indicated that besides the effect of increasing cGMP production in cumulus cells, CNP might play some other roles in the development of ovarian follicle. Sergio et al reported that CNP-treated COC showed a higher ($p<0.05$) density of transzonal projections (TZP) between granulosa cells and oocyte [34]. Franciosi reported that the percentage of COCs with functionally open gap junctions in experiment group with CNP supplementation for 6-8 h was similar to that at the time of COCs collection and significantly higher than that in control COCs as measured by Lucifer yellow injection assay [8]. Only on the premise of functional gap junction, could factors such as cGMP etc. flow into oocyte. High density TZP and functionally open gap junctions will both strengthen the communication between granulosa cells and oocyte and help to maintain the 3-dimensional structure of follicle. In addition, CNP could stimulate follicle growth and decrease reactive oxygen species (ROS) [15,16,35]. The improvement of

COC's developmental competency in the present study might be at least partly explained by the mechanism mentioned above in this paragraph.

Several chemical besides CNP such as forskolin, 6-dimethylaminopurine (6-DMAP) etc. have also been reported as substances which have the potency to improve the nuclear-cytoplasmic synchronization of oocyte after IVM. But it was reported that some of these chemicals may have prolonged inhibitory effect due to a longer half-life or have detrimental effects by inducing cellular apoptosis [36,37,38,39,40]. In contrast, CNP is a natural substance in vivo, and could orchestrate meiotic progress in cooperation with involved growth factors and hormones. More importantly, the inhibitory effect of CNP-NPR2 signaling is reversible and will be inactivated soon after luteinizing hormone (LH) surge. This make CNP a promising agent to improve the developmental competency of ovarian follicles cultured in vitro.

Conclusions

The supplementation of CNP in the culture system of murine late secondary follicle during the whole period of IVG could sustain the 3-dimensional structure of follicle, increase the antral formation rate and maintain oocytes' meiotic arrest. As the oocyte's competency to be fertilized were greatly improved, this protocol might benefit the further research in the field of ovarian follicle culture.

Abbreviations

cAMP: cyclic adenosine 3',5'-monophosphate; CDK1: cyclin-dependent kinase 1; cGMP: cyclic guanosine monophosphate; CNP: c-type natriuretic peptide; Cyp1a1: cytochrome P 4501a1; Cyp11a1: cytochrome P 450 11 a1; 6-DMAP: 6-dimethylaminopurine; DMEM/F12: Dulbecco modified eagle's medium/Ham's F12 nutrient medium; EGF: epidermal growth factor; FSH: follicle stimulating hormone; HCG: human chorionic gonadotropin; GV: germinal vesicle; GVBD: germinal vesicle breakdown; IVF: in vitro fertilization; IVG: in vitro growth; IVM: in vitro maturation; LH: luteinizing hormone; MI: metaphase in meiosis I; MII: metaphase in meiosis II; MAPK: mitogen-activated protein kinase; MYT1: myelin transcription factor 1; NPPC: natriuretic peptide precursor; NPRB: natriuretic peptide receptor B; COC: cumulus-oocyte complexes; PKA: protein kinase A; SPS: serum protein substitute; TZP: transzonal projections; WEE1B: WEE 1 homolog 2; Wnt2b: Wingless-type mouse mammary tumor integration site family 2b; Wnt5a: Wingless-type mouse mammary tumor integration site family 5a.

Declarations

Ethical approval and consent to participate

The experiment was performed in accordance with the guidelines of animal experiment in Shanxi Provincial Key Laboratory of Cell Regeneration and Birth Defects. The study protocol was approved by the ethics committee of Shanxi Provincial Key Laboratory of Cell Regeneration and Birth Defects.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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None.

Authors' contributions

HW and X G jointly designed the experiment. HW wrote the manuscript. AL and HC did the experiment. HL and RL worked as assistants in the experiment. All authors read and approved the final manuscript.

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Figures

Figure 1

The follicular development without CNP supplementation (The granulosa cells migrated from within follicle and the follicle flattened)

Figure 2

The follicular development with CNP supplementation. (The follicle grew 3-dimensionally.)

Figure 3

The ovulation without CNP supplementation. (Oocyte ovulated nakedly from preantral follicle)

Figure 4

The ovulation with CNP supplementation. (COC ovulated from antral follicle.)