UNIVERSIDADE FEDERAL DE MINAS GERAIS

Faculdade de Medicina

Programa de Pós-Graduação Medicina Molecular

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Peptídeo natriurético tipo C no líquido folicular humano: relação com a maturação oocitária

Belo Horizonte

2019

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Dissertação apresentada ao Programa de Pós-Graduação em Medicina Molecular da Universidade Federal de Minas Gerais como requisito para obtenção do título de Mestre.

Orientador: Prof. Dr. Fernando M. Reis
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Belo Horizonte
2019

T274p

Telles, Maíra Casalechi Badin.

Peptídeo Natriurético tipo C no líquido folicular humano [manuscrito]: relação com a maturação oocitária. / Maíra Casalechi Badin Telles. - - Belo Horizonte: 2019.

44f.: il.

Orientador (a): Fernando M. Reis.

Coorientador (a): Cynthia Dela Cruz.

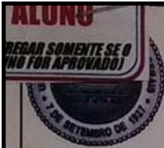
Área de concentração: Reprodução Humana.

Dissertação (mestrado): Universidade Federal de Minas Gerais, Faculdade de Medicina.

1. Peptídeo Natriurético Tipo C. 2. Técnicas de Maturação in Vitro de Oócitos. 3. Fertilização In Vitro. 4. Dissertações Acadêmicas. I. Reis, Fernando M. II. Cruz, Cynthia Dela. III. Universidade Federal de Minas Gerais, Faculdade de Medicina. IV. Título.

NLM: QU 68

Bibliotecário responsável: Fabian Rodrigo dos Santos CRB-6/2697



UNIVERSIDADE FEDERAL DE MINAS GERAIS

PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA MOLECULAR

FOLHA DE APROVAÇÃO

PEPTÍDEO NATRIURÉTICO TIPO C NO LÍQUIDO FOLICULAR HUMANO: RELAÇÃO COM A MATURAÇÃO OOCITÁRIA

MAÍRA CASALECHI BADIN TELLES

Dissertação submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em MEDICINA MOLECULAR, como requisito para obtenção do grau de Mestre em MEDICINA MOLECULAR, área de concentração MEDICINA MOLECULAR.

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Belo Horizonte, 15 de fevereiro de 2019.

“Ninguém ignora tudo. Ninguém sabe tudo.

Todos nós sabemos alguma coisa. Todos nós ignoramos alguma coisa.

Por isso *aprendemos sempre*.”

Paulo Freire

AGRADECIMENTOS

Ao meu orientador **Professor Fernando Marcos dos Reis**, por dividir seu conhecimento comigo, por toda confiança e disponibilidade e por tanto colaborar com a minha formação profissional;

À minha co-orientadora **Cynthia Dela Cruz,** pela amizade, por todos os ensinamentos e pelo suporte em todas as etapas do trabalho e na vida;

Aos **amigos do Laboratório de Reprodução Humana do Hospital das Clínicas da UFMG**, pela deliciosa convivência e pela disponibilidade em ajudar sempre que necessário, em especial **Dr Francisco de Assis, Dra Ines Katerina, Dra Júlia Dias, Lorena Velozo, Maria Thereza Veloso** e **Verônica Lobach**, por tanta dedicação em ajudar na obtenção das amostras;

Ao meu esposo **Leonardo,** por todo o amor, por muita compreensão e pelo apoio incondicional ao longo desses anos;

Aos meus **amigos e familiares,** por estarem sempre por perto nos momentos mais importantes da minha vida, em especial à minha mãe, **Delise**, e ao meu pai, **Fabiano**;

Às **pacientes**, por toda disposição em colaborar com o estudo.

Ao **Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq),** pelo apoio financeiro por meio do **Instituto Nacional de Ciência e Tecnologia** em **Hormônios e Saúde da Mulher (INCT-HSM)**.

RESUMO

O peptídeo natriurético tipo-C (CNP) é um produto das células da granulosa (GC) que se liga a seu receptor transmembrana (receptor de peptídeos natriuréticos tipo 2, NPR2) e sinaliza através do segundo mensageiro monofosfato de guanidina cíclico (cGMP). Estudos em camundongos demonstraram que a sinalização do CNP contribui para o crescimento folicular ovariano e para a parada meiótica oocitária até o momento do pico pré-ovulatório do hormônio luteinizante (LH). Em humanos, por outro lado, a relação entre os níveis de CNP folicular e a retomada meiótica dos oócitos ainda é desconhecida. O objetivo deste estudo foi investigar se os níveis do CNP e do seu receptor no ovário variam de acordo com o estágio de maturação dos oócitos humanos. Coletamos fluido folicular (FF) e GC murais em pool de diversos folículos de 47 pacientes, e também FF, GC murais e CC de 96 folículos pré-ovulatórios individuais de outras 39 pacientes submetidas a estimulação ovariana controlada (COS) para tratamento de fertilização in vitro (IVF). O estágio de maturação de cada oócito coletado oriundo de folículo puncionado individualmente foi avaliado após denudação para injeção intracitoplasmática de espermatozoides (ICSI). Os níveis de CNP foram avaliados no FF por ensaio imunoenzimático (EIA). Os mRNAs codificadores do precursos do CNP (*NPPC*) e do seu receptor NPR2 foram quantificados por reverse-transcriptase real-time PCR (RT-PCR). Foi encontrada uma correlação positiva entre os níveis de CNP no pool de FF com a contagem de folículos antrais (AFC, r_s=0,458 p=0,002), de folículos pré-ovulatórios >16mm (r_s=0,361; p=0,016) e de oócitos coletados (r_s=0,378; p=0,01) e uma correlação negativa entre os níveis de CNP no pool

de FF e a porcentagem de oócitos maduros metáfase II, MII) coletados (r_s=-0,39; p=0,033). Os níveis de CNP no FF de folículos com oócitos MII foram consideravelmente menores que de folículos contendo oócitos imaturos (metáfase I, MI) (mediana=0,44 vs 0,57 ng/mL, $p<0,05$). Em concordância, a expressão gênica de *NPPC* foi diminuída em 50% nas GC de folículos contendo oócitos MII quando comparada aos folículos contendo oócitos MI ($p<0.01$). A expressão gênica de NPR2 é diminuída nas CC oriundas de oócitos MII quando comparada à expressão em CC oriundas de oócitos MI (redução de 60%, $p<0.01$). Foi detectado mRNA do receptor de LH (LHR) tanto em CC quanto em GC de folículos contendo oócitos MII e contendo oócitos MI, sendo quatro vezes mais abundante nas CC que nas GC. Concluímos que a sinalização do CNP é diminuída nos folículos ovarianos contendo oócitos maduros. Entretanto, mais estudos in vitro são necessários para esclarecermos se a sinalização do CNP é essencial na manutenção da parada meiótica oocitária em humanos.

Palavras-chave: CNP, NPR2, maturação oocitária, fertilização in vitro

ABSTRACT

C-type natriuretic peptide (CNP) is a product of granulosa cells (GC) that binds to a transmembrane receptor (natriuretic peptide receptor type 2, NPR2) and signals through the second messenger cyclic guanosine monophosphate (cGMP). Studies in mice have shown that CNP signaling contributes to ovarian follicle growth and oocyte meiotic arrest until the preovulatory luteinizing hormone (LH) surge. In humans, however, the relationship between follicular CNP levels and oocyte meiotic resumption is still unknown. The aim of this study was to investigate whether ovarian CNP levels and NPR2 expression change according to the meiotic phase of human oocytes. We collected follicular fluid (FF) and mural GC in pool from several follicles (n=47 participants), and FF, mural GC and CC from 96 preovulatory follicles (n=39 participants) of women undergoing controlled ovarian stimulation (COS) for in vitro fertilization (IVFI). We kept track of the maturation stage of each oocyte from follicles assessed individually at the time of oocyte denudation. CNP levels were measured in the FF by enzyme immunoassay. The mRNAs encoding for the CNP precursor *NPPC* and its receptor NPR2 were quantified by reverse-transcription real-time PCR (RT-PCR). There was a positive linear correlation between CNP levels in FF pools and basal antral follicle counting (r_s=0,458; p=0,002), number of preovulatory follicles >16 mm (r_s=0,361; p=0,016) and number of oocytes retrieved (r_s=0,378; p=0,011) and a negative correlation between CNP levels in FF pools and the percentage of mature (metaphase II, MII) oocytes retrieved (r_s=-0,39; p=0,033). FF CNP levels in follicles containing MII oocytes were considerably lower than in follicles containing immature (metaphase I, MI)

oocytes (median = 0.44 vs. 0.57 ng/ml, $p < 0.05$). Accordingly, *NPPC* gene expression was reduced by 50% in the GC from follicles containing MII oocytes when compared to GC from follicles containing MI oocytes ($p<0.01$). In addition, NPR2 mRNA was down-regulated in CC surrounding MII oocytes compared to CC from MI oocytes (60% reduction, $p<0.01$). LH receptor mRNA expression was detected in both CC and GC of follicles containing MII or MI oocytes, being four-fold more abundant in CC than in GC. CNP signaling is downregulated in human ovarian follicles containing mature oocytes. Further in vitro studies should clarify whether CNP signaling is essential to keep oocyte meiotic arrest in humans.

Key-words: CNP, NPR2, oocyte maturation, in vitro fertilization

LISTA DE ABREVIATURAS E SIGLAS

AFC: antral follicle count

ANP: atrial natriuretic peptide

BNP: brain natriuretic peptide

cAMP: cyclic adenosine monophosphate

CC: cumulus cells

cDNA: complementary deoxyribonucleic acid

cGMP: cyclic guanosine monophosphate

CNP: c-type natriuretic peptide

COC: cumulus-oocyte complex

COS: controlled ovarian stimulation

Ct: threshold cycle

DPBS: Dulbecco’s Phosphate Buffered Saline

EIA: enzyme immunoassay

FF: follicular fluid

FSH: follicle-stimulation hormone

GC: granulosa cells

GC-A: guanylyl cyclase-A

GC-B: guanylyl cyclase-B

GnRH: gonadotropin releasing hormone

GV: germinal vesicle

GVBD: germinal vesicle breaking down

hCG: human chorionic gonadotropin

hMG: human menopausal gonadotropin

ICSI: intracitoplasmatic sperm injection

IVF: in vitro fertilization

IVM: in vitro maturation

LH: luteinizing hormone

LHR: luteinizing hormone receptor

LGC: luteal granulosa cell

MI: metaphase I

MII: metaphase II

mRNA: messenger ribonucleic acid

NPPC: natriuretic peptide precursor C

NPR: natriuretic peptide receptor

NPR1: natriuretic peptide receptor 1

NPR2: natriuretic peptide receptor 2

PCR: polymerase chain reaction

RT-PCR: reverse-transcription real time polymerase chain reaction

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Figure 5: Lack of correlation between CNP levels in the follicular fluid (FF) and *NPPC* mRNA expression in luteinized granulosa cells (LGC). r_s = Spearman's rank correlation coefficient.

Figure 6: CNP levels in follicular fluid (FF) and gene expression analyses in luteinized granulosa cells (LGC) and cumulus cells (CC) of individual follicles containing oocytes at germinal vesicle (GV), metaphase I (MI) or metaphase II (MII) stage. Relative mRNA expression is reported as fold change using as reference (Ref) LGC from follicles where no oocyte was retrieved. *p<0.01 vs. GV and MII groups. #p<0.05 vs. MI group.

Figure 7: Extrapolation of the measures shown in Figure 6, performed in single FF samples collected at the time of oocyte pickup (dotted black line and syringe), to

hypothetical scenarios of changes over time. Follicles containing GV oocytes are represented in orange; MI oocytes in blue; and MII oocytes in green. After hCG injection for ovulation trigger, LHR is activated and meiosis resumed with germinal vesicle breakdown (GVBD). Oocytes that were retrieved in the MI stage may have responded later to the LHR activation and had a delayed meiosis resumption. LHR activation may be followed by its own downregulation, which would explain the lower LHR mRNA levels in follicles containing MII oocytes. Based on previous animal experiments, we hypothesize that LRH activation by hCG inhibited CNP release by granulosa cells into the FF. The higher FF CNP levels in follicles containing MI oocytes compared to those containing MII oocytes may suggest two possibilities: that follicles with MI oocytes had already decreased their CNP signal as part of the mechanism evoking GVBD (filled blue line), or there was no decrease of CNP levels and nevertheless GVBD occurred (dotted blue line). Follicles containing oocytes arrested at germinal vesicle (GV) phase had deficient LHR expression, which could be the cause of nonresponse to hCG and arrest at GV stage.

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1. Introduction

The hormonal profile of human oocyte maturation has been extensively studied in recent years. However, further elucidation regarding the mechanisms interfering in follicular development and oocyte maturation is still required.

The natural history of the human oocyte begins with the onset of meiosis and formation of the primordial follicle still in fetal life. At the time of birth, the oocytes into meiosis are arrested in the prophase of the first meiotic division, known as germinal vesicle (GV) stage, and there is the establishment of relative metabolic quiescence (1). In humans, GV arrest lasts for several years until the onset of puberty (2-4), when some activated primordial follicles begin to growth and develop into primary, secondary and antral follicle stages (5). Most early antral follicles undergo physiological atretic degeneration (6), but some of them reach the preovulatory stage (7, 8), which is when the oocyte acquires the ability to resume meiosis.

The fate of each antral follicle is controlled by endocrine and paracrine factors that act in a not fully understood manner (8, 9). In response to the preovulatory luteinizing hormone (LH) surge at each menstrual cycle, oocytes from preovulatory follicles resume meiosis, undergo ovulation and become fully competent to be fertilized (10, 11).

Morphologically, meiotic resumption from diplotene arrest (GV) is characterized by germinal vesicle breakdown (GVBD). Once the GVBD occurs, there is a proper organization of the meiotic spindle that makes the first meiotic division progress through metaphase I, followed by the extrusion of the first polar body (telophase I) and then the second meiotic division, which is arrested at metaphase II until fertilization (2, 12).

Failed oocyte meiotic arrest, resulting in precocious meiotic resumption, compromises oocyte developmental competence, and, consequently, the quality of the embryos to be generated (12).

Since 1987, it has been known that a high level of cyclic adenosine monophosphate (cAMP) in oocytes is essential for the maintenance of oocyte meiotic prophase arrest of preovulatory ovarian follicles (1, 13-16). The intracellular cAMP concentration in oocytes reflects a balance between its synthesis and degradation, catalyzed by adenylyl cyclase and phosphodiesterase 3A (PDE3A), respectively (17).

The natriuretic peptides comprise a family of three active ligands: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) (18, 19). ANP and BNP act as endocrine hormones whereas CNP acts in an autocrine/paracrine fashion (20). The CNP precursor gene, *NPPC,* is expressed in several cell types and once cleaved it gives rise to the biologically active CNP, a peptide of 22 amino acids (21, 22). The natriuretic-peptides perform their function once bound to a specific receptor, a guanylyl cyclase enzyme anchored to the plasma membrane, known as natriuretic peptide receptor (NPR) (20). ANP and BNP activate, mainly, the natriuretic peptide receptor subtype 1 (NPR1), also known as NPRA or guanylyl cyclase-A (GC-A), regulating blood volume homeostasis through their natriuretic and diuretic kidney effects (23, 24). CNP, in its turn, binds to the natriuretic peptide receptor subtype 2 (NPR2), also known as NPRB or guanylyl cyclase-B (GC-B) (25). Once the NPR2 is activated by CNP, the signaling occurs via the intracellular production of cyclic guanosine monophosphate (cGMP) (20).

The CNP/NPR2/cGMP axis has been shown to be crucial in local regulatory

pathways in reproductive tissues (26). Studies have reported ovarian gene expression of *NPPC* and NPR2 and their regulation by gonadotropins in female rats (27, 28). Gene transcription for NPR2 has also been found in mouse and rat ovarian granulosa and cumulus cells (CC) from antral and preovulatory follicles (9, 17, 28-30). Moreover, CNP and its receptor have been described in the human reproductive system (31) (Figure 1).

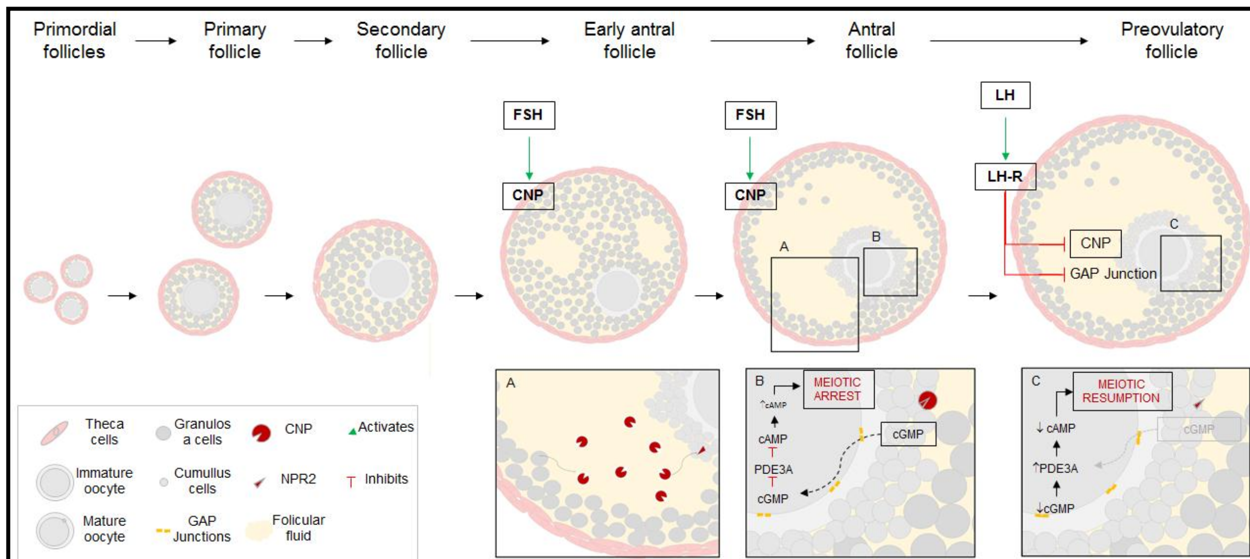

Figure 1: CNP signaling in the follicular environment

Recently, CNP has been described as an important factor in the maintenance of intra-oocyte cAMP concentration, which is essential to enable the meiotic arrest until ovulation (17). Studies in mouse cumulus-oocyte complexes (COC) have shown that CNP synthesized by granulosa cells (GC) binds and activates NPR2 in cells surrounding the oocytes, the cumulus cells. This activation generates intracellular cGMP in the CC, and the presence of gap junctions between the oocyte and the CC allows cGMP to diffuse into the oocyte. Within the oocyte, cGMP inhibits PDE3A activity and cAMP hydrolysis, and the resulting high cAMP levels maintain meiotic arrest (32, 33). Studies have documented this role of CNP in several mammal species, including

mouse (33, 34), pig (35), bovine (36, 37), cat (38), goat (39) and, more recently, sheep (40). The preovulatory LH surge decreases CNP release and cGMP production in somatic cells (41), thereby reducing oocyte cAMP levels and prompting meiosis resumption (16, 40).

Despite the compelling evidence that CNP is a key paracrine regulator of follicle growth (19, 42), oocyte maturation, nuclear and cytoplasmic synchronization, and ovulation (42) in several mammals species, its correlation with ovulation induction in humans is unknown. Therefore, this study was designed to characterize CNP levels in human FF after controlled ovarian stimulation (COS) for IVF and to investigate whether ovarian CNP levels and NPR2 expression change according to the meiotic phase of human oocytes.

2. Materials and Methods

2.1. Ethical approval

The study design, protocol and informed consent form were approved by the local Institutional Review Board (registration number 53504516.8.0000.5149) and all participants freely signed the informed consent upon enrollment in the study.

2.2. Study design and participants

This was a prospective cross-sectional study including 86 women scheduled to in vitro fertilization (IVF) treatment at the Division of Human Reproduction of Federal University of Minas Gerais, in Belo Horizonte, Brazil (Figure 2). The first participants (n = 47) were enrolled between May and July 2016 and provided samples of FF and luteinized GC suspended in the FF (LGC) collected in pool from several follicles. A second series of participants (n=39) was enrolled from February to September 2017 and provided 96 samples of FF, LGC and CC obtained from individual follicles, whose oocytes were immediately inspected after denudation and classified according to the

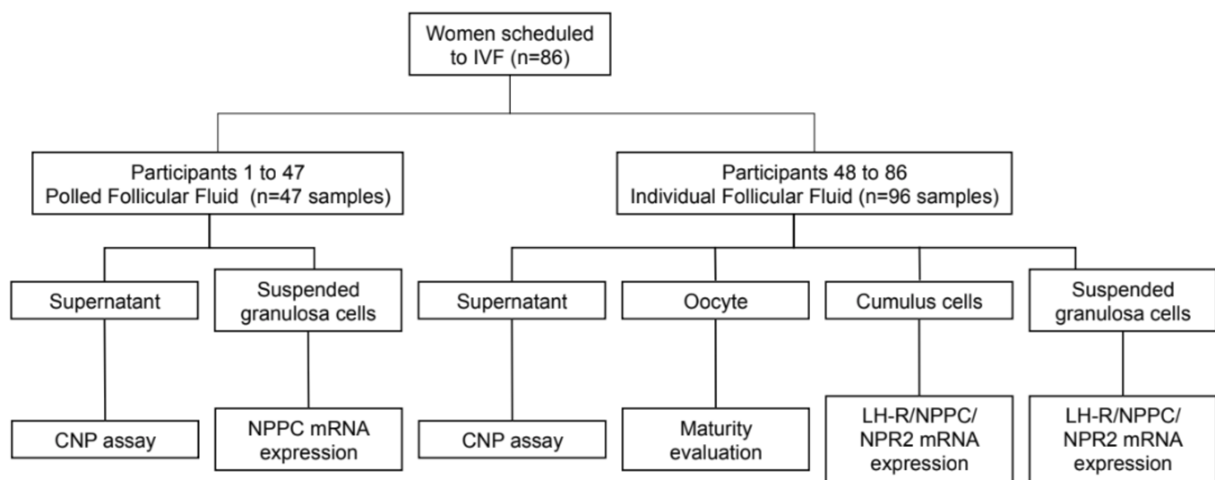

Figure 2: Study flow diagram.

maturity stage (Figure 2). The baseline characteristics of the study participants are summarized in Table 1.

Table 1: Baseline clinical characteristics of the study participants.

	All (n=86)	Participants 1 to 47	Participants 48 to 86
Age (years)	37 (34-39)	36 (35-39)	37 (34-39)
Duration of infertility (years)	7 (5-10)	6 (4-10)	8 (5-11)
BMI (kg/m²)	25 (23-28)	24 (22-28)	25 (23-27)
FSH (U/L)	7.4 (5.9-9.0)	7.1 (5.5-8.4)	7.9 (6.6-10.5)
Antral follicle count	11 (8-16)	12 (7-17)	10 (8-14)
Dose of gonadotropins used (IU)	1950 (1575-2100)	1950 (1650-2100)	1950 (1500-2100)
Cause(s) of infertility			
Male factor	58 (67%)	29 (62%)	29 (74%)
Ovulatory factor	5 (6%)	4 (9%)	1 (3%)
Tubal factor	18 (21%)	12 (26%)	6 (15%)
Endometriosis	9 (10%)	7 (15%)	8 (21%)
Unexplained	10 (12%)	6 (13%)	4 (10%)

Quantitative variables are expressed as medians and interquartile intervals.

2.3. Controlled Ovarian Stimulation and oocyte retrieval

COS and collection of follicular aspirates were performed as detailed elsewhere (43). Briefly, the patients were submitted to a COS protocol chosen according to the cause of infertility, age, follicle-stimulating hormone (FSH) levels and antral follicle count (AFC). Human menopausal gonadotropin (hMG) or recombinant FSH were used subcutaneously. Ovarian blockade was performed with gonadotropin releasing hormone (GnRH) agonist (leuprolide acetate) or antagonist (cetrorelix or ganirelix), according to

the individualized protocol. Serial transvaginal ultrasonography was performed from the sixth day of COS. When one or more follicles had a mean diameter greater than 18 mm, 250 μg of recombinant human chorionic gonadotropin (hCG) was administered and follicle aspiration for oocyte retrieval was performed ~35 hours later.

Follicle aspiration was guided by transvaginal ultrasonography using a 17-gauge follicular aspiration needle connected to a transvaginal probe and a craft suction unit with a negative pressure of 100–200 mmHg. The first 47 patients had the FF collected in pool from several follicles. In the second series (patients 48 to 86) up to five follicles from each patient were aspirated individually and the FF collection tube was changed and identified after each follicle aspiration.

Semen was prepared using simple washing, swim-up, or discontinuous density gradient technique – according to clinical criteria for each patient – and insemination was performed by ICSI. Oocyte fertilization was assessed at 18–20 hours after insemination by confirmation of the presence and location of two pronuclei. Pregnancy was assessed with quantitative serum βhCG two weeks after embryo transfer (44).

2.4. Cumulus cells sample collection

Each oocyte retrieved from the FF of individual follicles had part of its CC mechanically removed with microdissection needles (16-gauge needle). Each sample of CC was transferred into a microfuge tube and 1 mL of TRIzol® Reagente (Invitrogen Life Technologies, Carlsbad, CA, USA) was added to posterior mRNA mRNA isolation. It was stored in freezer at -80 °C until analysis. All the procedures occurred in sterile and nuclease-free conditions.

2.5. Follicular fluid and luteinized granulosa cells sample collection

Immediately after the collection of the oocyte by the embryologist, FF samples were chilled on ice and then centrifuged at 4 ^{o}C for 10 minutes at 1000 g. One mL of the supernatant was transferred to a microfuge tube and was stored at -80 ^{0}C until analysis.

The remaining supernatant was discarded, and the resulting pellet was resuspended with 1 mL of Dulbecco's Phosphate Buffered Saline (DPBS). One mL of Histopaque 1077 (Sigma-Aldrich, Saint Louis, USA) was added to a 15 mL tube and carefully overlayed with the 1 mL DPBS cell suspension. Further centrifugation at 1000 g was performed for an additional 20 minutes. After this process, the LGC were suspended in a cloud between the DPBS and the Histopaque 1077 layers. The cell cloud was aspirated, transferred to a fresh microfuge tube and again centrifuged at 4 ^{o}C for 15 minutes, at 1200 g. After discarding the supernatant, the cells were stored in a freezer at -80 ^{o}C (43, 45). This method yields approximately 97% of viable cell recovery with up to 19% of contaminating macrophages (46).

2.6. CNP Enzyme Immunoassay

CNP levels were measured by enzyme immunoassay (EIA) using an extraction-free EIA kit purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA; catalog number EKE-012-03). The assay sensitivity is 0.07 ng/mL with a linear range 0.07-1.23 ng/mL and there is no cross-reaction with other natriuretic peptides. The assay was performed according to manufacturer's instructions. Absorbance was read in an automatic microplate reader at 450 nm. The results were converted to CNP concentration (ng/mL) by linear regression using the assay standard curve. All samples

from the same series (pool or individual follicles) was run in a single plate, with a coefficient of variation of 8%.

2.7. RNA extraction, complementary DNA synthesis and quantitative PCR

Expression of the messenger ribonucleic acids (mRNA) encoding the CNP precursor *NPPC*, the CNP receptor NPR2 and LH receptor (LHR) was evaluated in CC and LGC by quantitative reverse-transcription real time polymerase chain reaction (RT-PCR).

For processing, CC and LGC samples stored in TRIzol® Reagent were removed from the freezer at -80 °C and maintained at room temperature until complete thawing followed by vigorous vortex homogenization. RNA was extracted using the TRIzol® protocol, unmodified. Total RNA was extracted, and it was quantified by light absorbance at 260 nm (NanoDrop - Thermo Fisher Scientific, Wilmington, Delaware, USA). First-strand complementary deoxyribonucleic acid (cDNA) was synthesized from 1 µg of total RNA using Superscript IV first-strand synthesis system (Invitrogen, Carlsbad, CA, USA). Real-time PCR was carried out as described previously (43, 47) in a ABI-Prism 7500 Sequence Detection System, with use of the fluorescent dye Power SYBR Green Master Mix Kit (Invitrogen Life Technologies, Carlsbad, CA, USA). The PCR parameters were: [stage 1] a cycle of 95 °C / 10min; [stage 2] 40 cycles of 95 °C / 15 seconds, 60 °C / 15 seconds and 72 °C / 20 seconds; [Stage 3] 95 °C / 15 seconds, 54 °C / 15 seconds and 95 °C / 15 seconds. The gene encoding the ribosomal protein S26 was used as the internal control (48). The synthesized primer sequences used for PCR amplification are shown in Table 2. Primers were designed to span intron–exon

Table 2: Oligonucleotide primers designed for real-time PCR

Gene	Sense (5' to 3')	Antisense (5'to 3')
s26	CCGCCATCCGGCTAAATAGT	GGGTGGAAATGCGTTCCTAGT
LHR	GCCGGTCTCACTCGACTATC	TGCTCCGGGCTCAATGTATC
NPPC	CTCCGTTGTGCTGAGGTCAT	CAGCTGGTGTTGTGTATTCCC
NPR2	GATGCTGGAGAAGGAGCTGG	CATACTGTTCCATGCGCAGC

borders and thus anneal only to cDNA. The specificity of PCR products was confirmed by single peak dissociation melting curves. No amplification of fragments occurred in negative control samples prepared without reverse transcriptase. Threshold cycle (Ct) values were normalized to S26 (ΔCT) and the differential gene expression between groups was expressed as fold change, calculated by the formula $2^{-\Delta\Delta CT}$ (49) where ΔΔCT is the difference between the mean ΔCT of the group and the mean ΔCT of LGC from FF samples where no oocyte was found (reference group).

2.8. Statistical Analysis

Variables were tested for normality with use of D’Agostino-Pearson test. CNP levels departed from normal distribution and therefore were summarized as medians with interquartile ranges and analyzed with Wilcoxon's signed rank test. Linear correlations were tested with Spearman's rank correlation coefficient. The relative mRNA expression of target genes was summarized as fold change ± standard deviation and submitted to analysis of variance with Tukey's post test. The analyses were conducted using Graphpad Prism version 6 and IBM SPSS Statistics version 22.

3. Results

3.1. CNP levels in FF pools and their correlation with clinical data

We evaluated the possible correlations between FF CNP levels and some clinical characteristics of the patients. CNP was measurable in all samples analyzed, with concentration ranging from 0.07 to 0.47 ng/ml. As shown in Figure 3, there was a positive linear correlation between CNP levels in FF pools and basal AFC (r_s= 0.458, p = 0.002), number of preovulatory follicles larger than 16mm at the day of oocyte pickup (r_s = 0.361, p = 0.016) and number of oocytes retrieved (r_s = 0.378, p = 0.011).

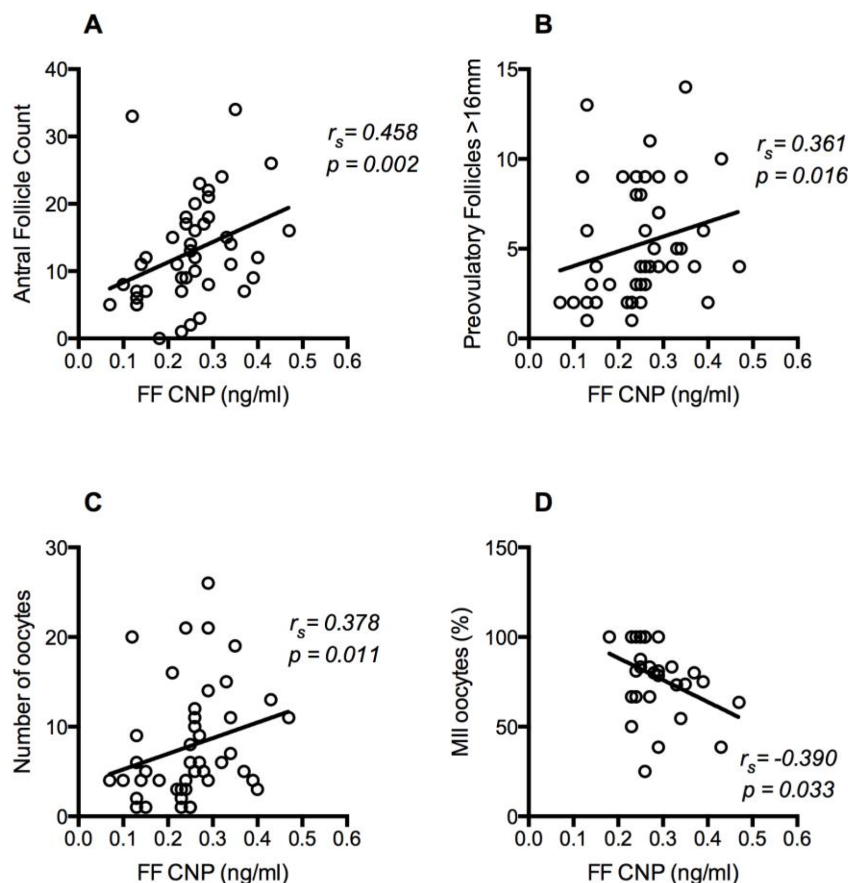

Figure 3: Linear correlation between CNP levels in follicular fluid pools and antral follicle count (A), number of preovulatory follicles larger than 16mm (B), number of oocytes retrieved (C) and proportion of mature oocytes (D). r_s = Spearman's rank correlation coefficient.

Conversely, a negative linear correlation was observed between the percentage of MII oocytes retrieved and the intrafollicular CNP levels (r_s = -0.390, p = 0.033). FF CNP had no correlation with the woman's age, duration of infertility, basal serum FSH or total dose of gonadotropins used in COS. In addition, there was no difference in FF CNP levels between patients who used recombinant FSH for COS and those who received hMG, a mixed formulation containing both FSH and LH (Figure 4).

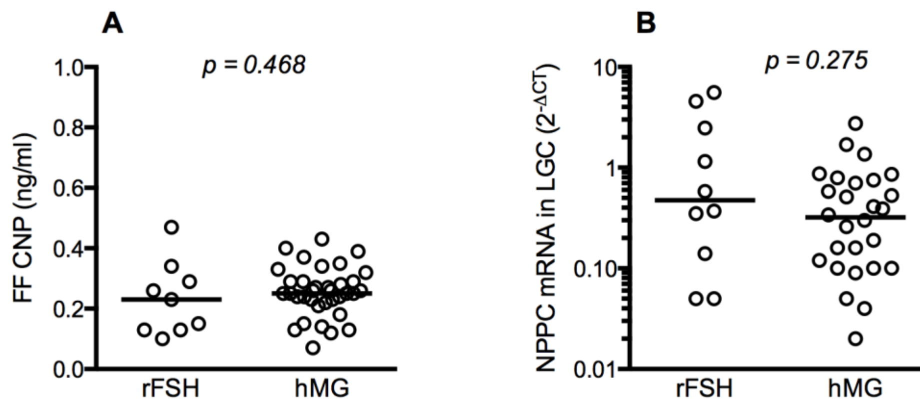

Figure 4: CNP levels (A) and *NPPC* mRNA expression in luteinized granulosa cells (B) in ovarian aspirates from patients stimulated with recombinant FSH (rFSH) or human menopausal gonadotropin (hMG). The two groups were compared with Mann-Whitney test.

3.2. NPPC and NPR2 mRNA expression in LGC from FF pools

LGC suspended in FF pools expressed both *NPPC* and *NPR2* mRNAs. The RT-PCR results were homogeneous for amplification of the s26 reference gene. The samples duplicates were concordant and the melting curves showed a single peak for each target gene, demonstrating the reliability of the results.

There was no correlation between *NPPC* mRNA expression in LGC and CNP levels in the same FF pool (Figure 5). *NPPC* mRNA expression in LGC did not differ between patients treated with recombinant FSH and those who used hMG (Figure 4).

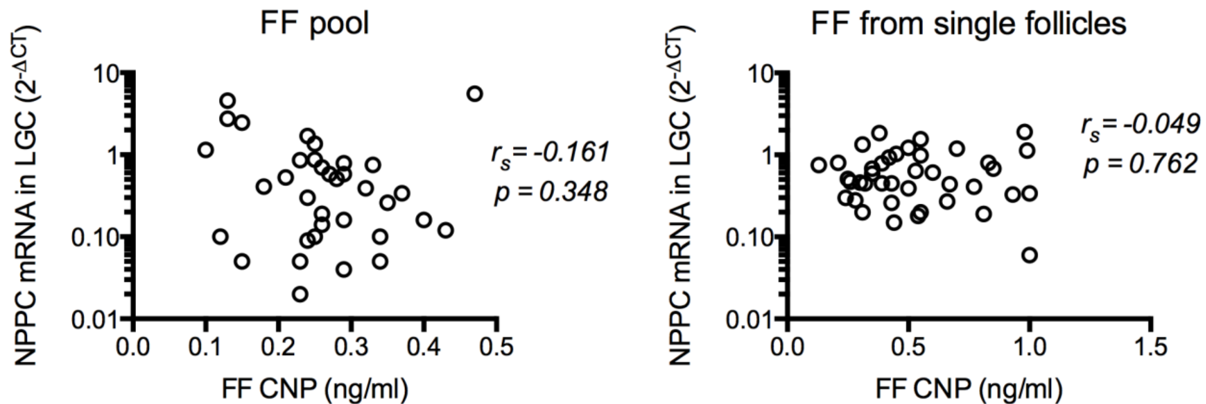

Figure 5: Lack of correlation between CNP levels in the follicular fluid and NPPC mRNA expression in luteinized granulosa cells (LGC). r_s = Spearman's rank correlation coefficient.

3.3. CNP protein and mRNA levels according to the oocyte maturity stage

As shown in Figure 6, FF CNP levels were lower in individual follicles containing MII oocytes than in follicles containing MI oocytes (median = 0.44 vs. 0.57 ng/ml ; p<0.05). Moreover, *NPPC* mRNA expression in LGC and CC differed significantly between follicles containing MI oocytes and follicles harboring GV or MII oocytes, being maximal in LGC associated with MI oocytes (p<0.01, Figure 6).

3.4. NPR2 and LHR mRNA expression according to the oocyte maturity stage

NPR2 gene expression was significantly higher in CC surrounding MI oocytes when compared to CC surrounding MII or GV oocytes (p<0.01, Figure 6).

Because oocyte meiotic resumption is driven by the LH surge through paracrine messages passed by GCs, we tested the expression of LRH in LGC and CC according to the oocyte maturity stage. LHR mRNA expression was detected in both CC and LGC of follicles containing MII or MI oocytes, being four-fold more abundant in CC than in

GC. The relative abundance of LHR mRNA in LGC and CC was significantly lower in follicles containing MII oocytes than in follicles containing MI oocytes ($p<0.01$, Figure 6). Importantly, LHR mRNA was nearly undetectable in both CC (fold change 0.52, $p<0.01$) and LGC (fold change 0.13, $p<0.01$) from follicles containing GV oocytes.

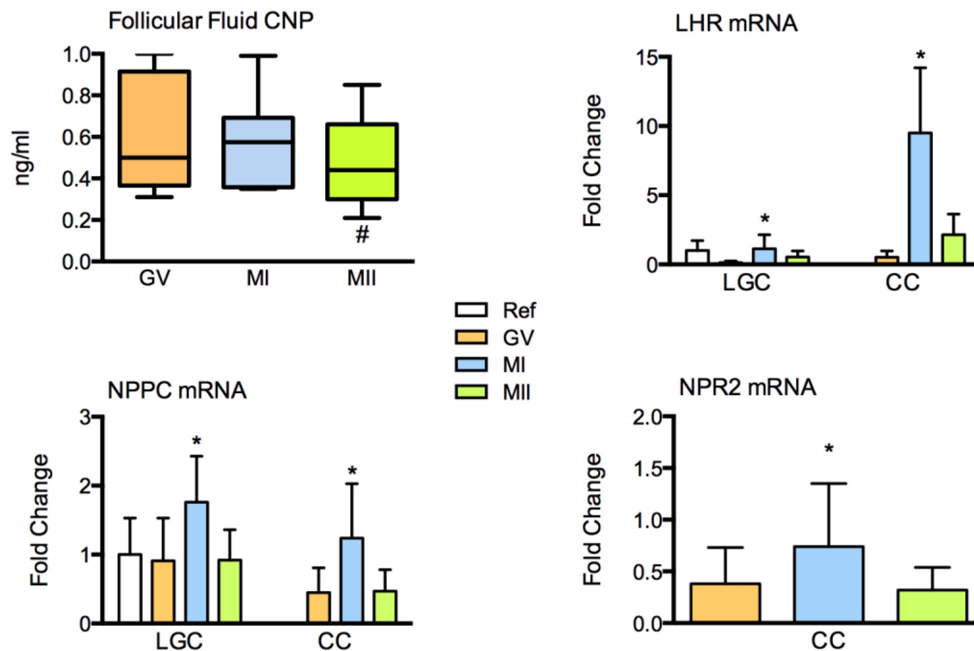

Figure 6: CNP levels in follicular fluid and gene expression analyses in luteinized granulosa cells (LGC) and cumulus cells (CC) of individual follicles containing oocytes at germinal vesicle (GV), metaphase I (MI) or metaphase II (MII) stage. Relative mRNA expression is reported as fold change using as reference (Ref) LGC from follicles where no oocyte was retrieved. *$p<0.01$ vs. GV and MII groups. #$p<0.05$ vs. MI group.

4. Discussion

In this study, we evaluated whether intraovarian CNP levels and *NPR2* expression change according to the meiotic phase of human oocytes retrieved for IVF. Previous studies have demonstrated that CNP maintains meiotic arrest in many mammal species (35, 37, 40), but the association of CNP with human oocyte maturation had not been reported yet. In the present study, intrafollicular CNP levels were evaluated in women submitted to IVF. We demonstrated that CNP is present in human FF along with gene expression of its precursor *NPPC*, as well as its receptor NPR2, in human LGC and CC. Moreover, we observed that follicles containing mature oocytes retrieved for IVF had lower FF CNP levels and lower *NPPC* and *NPR2* mRNA expression compared to follicles containing MI oocytes. Coherently with these findings in individual follicles, CNP levels in FF pools correlated negatively with the proportion of MII oocytes retrieved for IVF.

In order to determine factors that might influence the intraovarian levels of CNP, we correlated FF CNP levels with some clinical characteristics of the participants. An interesting finding was the lack of difference in FF CNP levels between women stimulated only with recombinant FSH and those treated with menopausal gonadotropins containing LH. This was unexpected because hCG inhibits CNP secretion in mouse ovary (41). However, we measured FF CNP levels only after hCG injection to emulate the preovulatory LH surge, therefore we cannot rule out the possibility that patients stimulated with hMG had lower FF CNP levels than patients stimulated with recombinant FSH before ovulation triggering. The mechanisms that ultimately control the production and release of CNP in the human ovary remain

unknown, and we have not been able to measure intrafollicular CNP levels before hCG injection, which would require an invasive procedure without benefit to the patient. In a previous human study, high levels of CNP were found in FF samples aspirated before the spontaneous mid-cycle LH peak from women undergoing laparotomy for surgical treatment of uterine myomatosis, when compared to another group of women who had been stimulated with gonadotropins for IVF treatment (41). In the present study, paired FF samples before and after the LH peak or hCG injection were unachievable due to ethical constrains.

Animal studies have shown that cGMP signaling promotes pre-antral follicle growth in vitro (50), while CNP treatment stimulates early antral follicle growth in vivo (42). CNP is an endogenous stimulator of murine oocyte growth that acts in the same direction as FSH but through an independent mechanism (33, 51). Evaluating the samples of pooled FF in our study we found out that patients with higher number of oocytes retrieved also had higher intrafollicular CNP levels. These results are in agreement with a previous study in mice showing that CNP is present in the FF and correlates with the number of oocytes and with the response to gonadotropin stimulation (41).

On the other hand, patients with higher proportion of mature oocytes retrieved had lower intrafollicular CNP levels. This finding is in consonance with murine studies, suggesting that a decrease in CNP may induce oocyte meiotic resumption and thereby increase the rate of mature oocytes, a cause-effect mechanism that has been experimentally demonstrated (17, 41). Despite of the inherent limitations of in vivo human studies, which do not allow us to experimentally manipulate intrafollicular CNP

levels, the inverse relationship between CNP and the number of immature oocytes found in the present study reinforces the hypothesis that this peptide may have a relevant function in human oocyte maturation.

In the follicles containing GV oocytes there was minimal expression of LHR, suggesting that the maturation did not occur because of deficient LHR activation. The mechanisms behind LHR deficiency in some follicles deserve further investigation. Case reports have documented patients whose repeated IVF attempts yielded only GV oocytes despite the correct use of gonadotropins for COS and ovulation triggering (52). Moreover, in typical IVF cycles about 9% of the retrieved oocytes are arrested in GV stage (53). Until now, there is no clear explanation to the lack of oocyte maturation in response to COS. Our finding reveals a potential mechanism for this condition and suggests that novel drugs acting beyond LHR should be developed to treat IVF patients with a high proportion of GV oocytes.

The lower LHR expression in CC from follicles with mature oocytes, in contrast to the follicles with MI oocytes, suggests that this decreased expression may be due to the completion of the metaphase II. Once the oocyte is mature, there is active secretion of several factors, such as hyaluronic acid (54, 55), which change the microenvironment within the COC and might inhibit the mRNA expression of LHR. In IVF treatments, the natural LH surge is replaced by an injection of hCG, a peptide with more affinity to LHR and that lasts longer in vivo. Therefore, it is unknown whether the same pattern in LHR expression occurs in follicles from natural cycles.

Our findings show that CC surrounding MI oocytes had more *NPR2* gene expression than CC linked to MII oocytes, suggesting that gamete meiosis resumption

was associated with downregulation of NPR2 in CC. A decreased expression of NPR2 together with the decrease in CNP production likely contributes to inhibit the CNP/cGMP pathway in the COC. In mouse ovarian follicles, LH exposure further inhibits NPR2 activity by mechanisms that do not reduce NPR2 protein levels (32).

Studies have suggested that pre-incubation with CNP would improve the quality of the oocytes submitted to in vitro maturation (IVM), mainly because CNP has the potential to synchronize nuclear and cytoplasmic oocyte maturation, generating blastocysts with a reduced proportion of DNA-fragmented nuclei (40). Pre-treatment with CNP in a precise concentration range would keep the oocyte arrested for a certain amount of time. However, the studies were performed in animals that had not been exposed to COS, and the oocytes were only pre-incubated with CNP before IVM (40, 56). In human, the higher expression of CNP and NPR2 in follicles containing MI oocytes means that these oocytes were exposed to CNP signal too long, which may overdo the synchronicity between nuclear and cytoplasmic maturation and concur to the poorer quality of the these oocytes.

It is important to consider that human oocytes arrest only in GV and MII stages (52), and that the MI is just transitional, a way through both arrests. Considering that, the oocytes retrieved in MI stage are not arrested but just delayed, being able to reach the MII stage in a few hours of incubation after denudation, despite their weakened quality (57). In the present study, oocytes that were retrieved in the MI stage may have responded later to the LHR activation and had a delayed meiosis resumption. As depicted in Figure 7, the higher CNP levels, *NPPC* and NPR2 expression in follicles containing MI oocytes compared to those containing MII oocytes may suggest two

possibilities: that follicles with MI oocytes had already decreased their CNP signal as part of the mechanism evoking GVBD, or there was no decrease of CNP levels and nevertheless GVBD occurred.

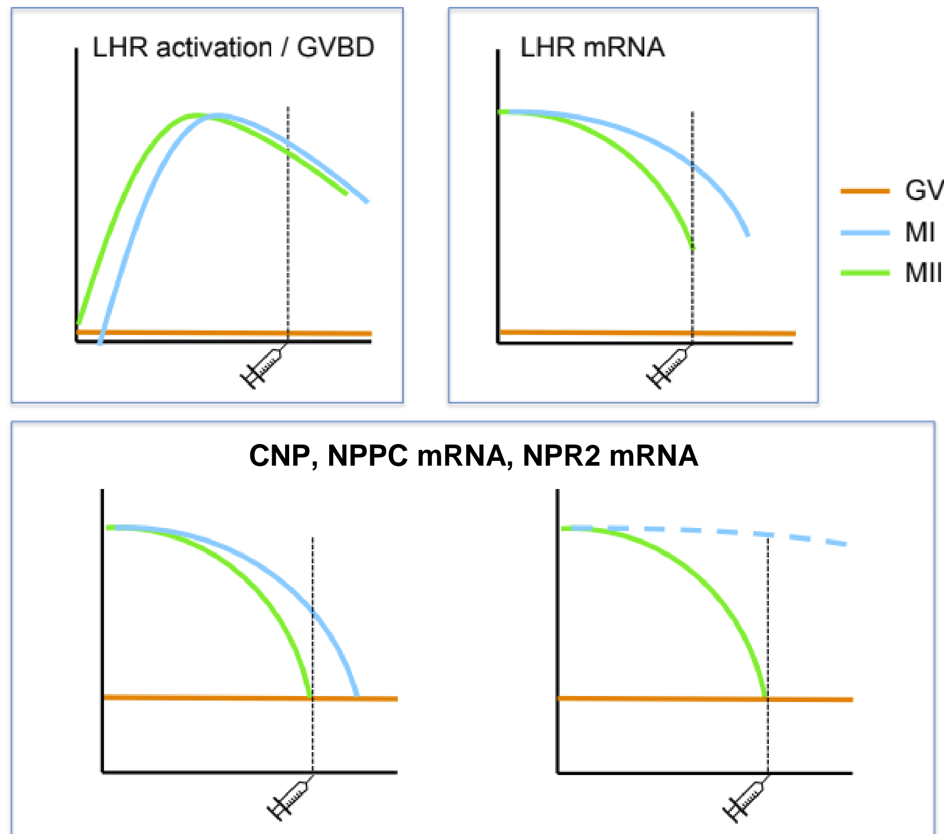

Figure 7: Extrapolation of the measures shown in Figure 6, performed in single FF samples collected at the time of oocyte pickup (dotted black line and syringe), to hypothetical scenarios of changes over time. Follicles containing GV oocytes are represented in orange; MI oocytes in blue; and MII oocytes in green. After hCG injection for ovulation trigger, LHR is activated and meiosis resumed with germinal vesicle breakdown (GVBD). Oocytes that were retrieved in the MI stage may have responded later to the LHR activation and had a delayed meiosis resumption. LHR activation may be followed by its own downregulation, which would explain the lower LHR mRNA levels in follicles containing MII oocytes. Based on previous animal experiments, we hypothesize that LRH activation by hCG inhibited CNP release by granulosa cells into the FF. The higher FF CNP levels in follicles containing MI oocytes compared to those containing MII oocytes may suggest two possibilities: that follicles with MI oocytes had already decreased their CNP signal as part of the mechanism evoking GVBD (filled blue line), or there was no decrease of CNP levels and nevertheless GVBD occurred (dotted blue line). Follicles containing oocytes arrested at germinal vesicle (GV) phase had deficient LHR expression, which could be the cause of nonresponse to hCG and arrest at GV stage.

In summary, the present data provide the first evidence that CNP levels in human FF correlate with follicle growth and oocyte maturation during COS for IVF, but we should not extrapolate these findings to the natural cycle at this time. The expansion of knowledge about the interruption of meiosis in oocytes opens up a number of research avenues, such as the possibility of developing alternatives for ovarian stimulation, new contraceptives based on oocyte maturation arrest and a novel tool for the fine-tuning of in vitro oocyte maturation.

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6. Anexo

UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM PESQUISA - COEP

Projeto: CAAE – 53504516.8.0000.5149

Interessado(a): Prof. Fernando Marcos dos Reis
Departamento de Ginecologia e Obstetrícia
Faculdade de Medicina - UFMG

DECISÃO

O Comitê de Ética em Pesquisa da UFMG – COEP aprovou, no dia 16 de maio de 2016, o projeto de pesquisa intitulado **"Quantificação do peptídeo natriutético atrial no líquido folicular de mulheres com síndrome dos ovários policísticos",** bem como o Termo de Consentimento Livre e Esclarecido.

O relatório final ou parcial deverá ser encaminhado ao COEP um ano após o início do projeto.

Telma Campos Medeiros Lorentz

Profa. Dra. Telma Campos Medeiros Lorentz
Coordenadora do COEP-UFMG