UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU

EFEITOS DA ASSOCIAÇÃO DO SISTEMA PRÉ-MIV COM NPPC COM MIV SUPLEMENTADA DE MODO SEQUENCIAL COM LH SOBRE A PRODUÇÃO *IN VITRO* DE EMBRIÕES BOVINOS

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"A mente que se abre a uma nova ideia, jamais voltará ao seu tamanho original."

Albert Einstein

RESUMO

In vivo, a maturação oocitária é gonadotrofina-dependente (FSH e LH) e, até haver o devido estímulo, o oócito é mantido em parada meiótica (prófase I) devido à manutenção dos níveis intraoocitários de cAMP. O FSH é o responsável pelo crescimento inicial do folículo antral enquanto o LH promove a maturação final oocitária (no estágio pós-desvio), sinalizando a produção de peptídeos EGF-*like* (fator de crescimento semelhante ao epidermal) nas células da granulosa. Estes se ligam ao seu receptor (EGFR) nas células do *cumulus* ativando a via da proteína cinase ativadora de mitógeno (MAPK), causando o fechamento das junções comunicantes do tipo GAP e culminando na retomada meiótica. Os peptídeos AREG (Ampirregulina)e EREG (Epirregulina) são utilizados na maturação *in vitro* em diferentes espécies e em substituição ou associação com o FSH. Alguns estudos demonstram que, quando maturados com esses peptídeos, os oócitos desenvolvem embriões de melhor qualidade, com maior número de células da massa celular interna e mais parecidos com aqueles maturados *in vivo*. No entanto, o oócito retirado do microambiente folicular retoma a meiose espontaneamente, de forma precoce e não sincronizada entre o núcleo e o citoplasma. Para melhorar os resultados da maturação *in vitro,* é importante retardar a retomada espontânea meiótica. A literatura descreve a eficácia do precursor do peptídeo natriurético tipo C (NPPC) em manter *in vitro* a concentração de cAMP oocitária similar à fisiológica. Diante disso, neste estudo foi observado o efeito do AREG sobre a abundância de transcritos alvo de oócitos e células do *cumulus*, além do seu efeito em adição às gonadotrofinas (LH ou FSH) quanto à progressão meiótica e a produção de embriões, quando precedida pela pré-MIV com NPPC. Nossos resultados corroboram que o AREG é eficiente tanto quanto o FSH na maturação de complexos *cumulus*-oócito e não altera as taxas de quebra da vesícula germinativa (GVBD) ou oócitos que chegaram a meiose II (MII). No entanto, o perfil dos transcritos alvo estudado apresentou diferença entre ambos, indicando distintas ações celulares do AREG e do FSH. Demonstramos ainda que o LH, sozinho, é ineficiente na maturação e produziu uma alta taxa de GV mesmo após 24 horas. No entanto, quando a maturação foi precedida por pré-MIV utilizando NPPC, a taxa de blastocisto e a abundância dos transcritos alvo, o LH não diferiu dos demais grupos. Em suma, como já descrito na literatura, o AREG foi eficaz para a MIV e não estimulou a expressão de receptores de LH nas células do *cumulus*. Adicionalmente, o uso do NPPC na pré-MIV

foi capaz de retardar a retomada da meiose, por tempo suficiente para que o oócito adquirisse competência para suportar o desenvolvimento embrionário inicial.

ABSTRACT

In vivo, the oocyte maturation is gonadotropin-dependent (LH and FSH) and, until there is a due stimulation, the oocyte is maintained in meiotic arrest (prophase I) due to the maintenance of intra-oocyte levels of cAMP. The FSH is responsible for initial growth of the antral follicle while the LH promotes final oocyte maturation (post-deviation), signaling the production of EGF-*like* (Epidermal Growth Factor-*like*) peptides in the granulosa cells. They bind to their receptor (EGFR) on *cumulus* cells activating the mitogen-activating protein kinase (MAPK) pathway causing closure of Gap Junctions culminating in meiosis resumption. The AREG (amphiregulin) and EREG (epiregulin) peptides are used *in vitro* maturation in different species and in substitution or association with FSH. Same studies shown that when mature with these peptide, oocytes develop better-quality embryos, with more cells in the inner cell mass, and more similar to those *in vivo*. However, the oocyte removed from the follicular microenvironment, resumes meiosis spontaneously, early and not synchronized between the cytoplasm and nucleus. To improve the *in vitro* maturation results, it is important delay spontaneous meiotic resumption. The literature describes the efficacy of the C-type natriuretic peptide (NPPC) precursor in maintaining the physiological-like oocyte cAMP concentration *in vitro*. In this study, the effect of AREG on the abundance of oocyte and *cumulus* cell transcript was observed, as well as its effect in addition to gonadotrophins (LH or FSH) on meiotic progression and embryo production, when preceded by pre-IVM with NPPC. Our results corroborate that the AREG is efficient as well as FSH in maturation of *cumulus*-oocyte complex (COC) and does not alter the rates of germinal vesicle breakdown (GVBD) or oocytes that reached meiosis II (MII) However, the target transcript profile showed a difference between the two, indicating distinct cellular actions of the AREG and FSH. We also demonstrate that LH, alone, is inefficient in maturation and produced a high GV rate even after 24 hours. However, when the maturation was preceded by pre-IVM using NPPC, blastocyst rate and abundance of target transcripts in the LH group did not differ from the other groups. In summary as describe in the literature, AREG was effective for IVM and did not stimulate the expression of LH receptor in *cumulus* cells. In addition, the use of NPPC in pre-IVM can postpone the meiosis resumption, long enough for the oocyte to acquire competence to support early embryonic development.

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LISTA DE ABREVIATURAS

ADAM	Desintegrina e Metaloproteinase A
AREG	Ampirregulina
BTC	Betacelulina
cAMP	3’-5’ Adenosina Monofosfato Cíclica
CDK	Cinase dependente de Ciclina
cGMP	Guanosina 3’, 5’-Monofosfato Cíclica
CNP	Peptídeo Natriurético tipo C
COC	Complexo *cumulus*-oócito
COX2	Cicloxigenase 2
Cx	Conexina
EGF-*like*	Fator de Crescimento semelhante ao Epidermal
EGFR	Receptor do Fator de Crescimento Epidermal
EREG	Epirregulina
ERK1/2	Cinase Regulada por sinal extracelular 1/2
FSH	Hormônio Folículo Estimulante
GDP	Guanosina difosfato
GJ	GAP Junctions (junções comunicantes do tipo GAP)
GnRH	Hormônio liberador de gonadotrofinas
GPCR	Receptor Acoplado à Proteína G
Grb2	Proteína ligada ao receptor do fator de crescimento 2
GTP	Guanosina trifosfato
GV	Vesícula Germinativa (estágio dictiado da prófase I)
GVBD	Quebra da Vesícula Germinativa
LH	Hormônio Luteinizante
LHR	Receptor do Hormônio Luteinizante (ou oficialmente denominado o gene LHCGR)
MAPK	Proteína Cinase Ativadora de Mitógeno
MAS	Esterol Ativado de Meiose
MEK	Família de cinases MAP/ERK
MII	Meiose II

MIV	Maturação *in vitro*
MPF	Fator Promotor da Maturação, da Meiose ou da Mitose
NPPA	Precursor do Peptídeo Natriurético tipo A (Atrial)
NPPB	Precursor do Peptídeo Natriurético tipo B (Brain)
NPPC	Precursor do Peptídeo Natriurético do tipo C
NPR2	Receptor do Peptídeo Natriurético tipo 2
P4	Progesterona
PDE	Fosfodiesterase
PGF2α	Prostaglandina F2α
PIVE	Produção *in vitro* de embriões
PKA	Proteína cinase tipo A
PKC	Proteína cinase tipo C
PTGER2	Receptor de prostaglandina E2
PTX3	Pentraxina 3
SH2	Domínio de homologia 2 à Src
TACE	Enzima de Conversão do Fator de Necrose Tumoral Alfa

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2018-IRRS- International Ruminants Reproduction Symposium; setembro/2018- Foz do Iguaçu-PR

2018- II CURSO DE INVERNO DE FARMACOLOGIA E BIOTECNOLOGIA; Julho2018- Botucatu-SP (Comissão Organizadora)

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2018- PIOLTINE, E.M.; SAWA, A.G.; ALMEIDA, A.M.; **PRONUNCIATE, M.**; FRANCHI, F.F.; NOGUEIRA, M.F.G. Gene expression related to the meiosis resumption after associated use of NPPC and rhFSH during *in vitro* prematuration of bovine *cumulus*-oocyte complexes. In IRRS (International Ruminants Reproduction Symposium), Setembro/2018; Foz do Iguaçu/PR

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2018- Artificial Intelligence-based grading quality of bovine blastocyst digital images: Direct capture with juxtaposed lenses of SmartPhone camera and Steromicroscope ocular lens. Sensors, 2018.

INTRODUÇÃO

O Brasil detém o maior rebanho comercial bovino do mundo com mais de 218 milhões de cabeças em todo seu território (IBGE, 2017), número que supera a própria população do país (207 milhões em 2017) sendo ainda o maior exportador de carne bovina (IBGE, 2017). Além disso, é líder mundial na produção *in vitro* de embriões bovinos, responsável por 70% da produção mundial (Ereno, 2015). O programa de produção *in vitro* de embriões (PIVE) bovinos começou em 1990, mas somente após 4 anos ocorreram os primeiros nascimentos (Mello, 2016). A PIVE é uma biotecnologia na qual se mimetiza um ou mais processos reprodutivos, tais como maturação e fertilização do oócito e a cultura de embriões por aproximadamente sete dias. Um dos principais processos limitantes para o sucesso dessa técnica é a maturação *in vitro* (MIV).

A maturação oocitária é definida como o processo pelo qual o oócito adquire a capacidade de suportar o desenvolvimento gradual até que o genoma embrionário seja ativado (Ferreira *et al*., 2009). Assim, quando a maturação é insatisfatória há alterações no desenvolvimento, sobrevivência e implantação embrionária. Embora tenha havido avanços recentes na PIVE, os resultados desta técnica ainda são inferiores quando comparados com o processo *in vivo* (Farin *et al.,* 2007).

Durante a gestação de vacas, por volta do 72º a 82º dias, as oogônias iniciam a meiose I até a primeira parada da meiose no estágio de diplóteno ou vesícula germinativa da prófase I (GV-*germinal vesicle*; Richards 1980; van den Hurk R. Zhao 2005). Ao nascimento, todos os oócitos estão em prófase da primeira divisão meiótica e permanecem no estágio de GV até a retomada da meiose e sua progressão até a meiose II, o que caracteriza a maturação oocitária (Sun *et al.,* 2009). A retomada da meiose ocorre em resposta ao pico pré-ovulatório de LH (Edwards 1965). O estímulo hormonal ativa a via da proteína cinase ativadora de mitógeno –MAPK (Dekel *et al*., 1981) e de outras como o fator promotor da maturação (MPF, conhecido como complexo proteico CDK/ciclina B; Wu *et al*., 1997). Logo após, ocorre a quebra da vesícula germinativa (GVBD, *germinal vesicle break down*; Gordon 1994). Estudos sugerem que o LH, através das vias cAMP e proteínas cinases (PKA e PKC), induz a síntese de substâncias parácrinas tais como os fatores semelhantes ao de crescimento epidermal (EGF-*like*) e esterol ativador de meiose (MAS) para regular a GVBD dos oócitos (Sun *et al.* 2009).

In vitro, o isolamento do oócito do seu ambiente folicular desencadeia a retomada espontânea da meiose sem o estímulo gonadotrófico (Pincus e Enzmann, 1935; Franciosi

et al., 2014). Este fato é baseado na incapacidade do oócito em manter a concentração de cAMP alta, o que previne a retomada da meiose (Dekel *et al.,* 1981; Vivarelli *et al.*, 1983; Aktas *et al.,* 1995; Mamo, 2011). O cAMP tem a função de segundo mensageiro e é sintetizado pelo oócito mediante um receptor acoplado à proteína-G e também é fornecido ao ooplasma por meio das comunicações tipo gap. A concentração intraoocitária de cAMP é regulada por fosfodiesterases (PDE; Conti e Beavo, 2007; Franciosi, 2014), enzimas que degradam o cAMP. A principal PDE no oócito é a PDE3A, cuja atividade é inibida pela guanosina 3', 5'-monofosfato cíclico (cGMP; Tornell *et al.*, 1991; Norris *et al.,* 2009; Franciosi *et al.,* 2014).

Dados da literatura demonstram que essa limitação pode ser contornada com a regulação artificial da retomada da meiose através do aumento dos níveis de cAMP intra-oocitário (Luciano *et al.,* 2004; Zeng *et al.,* 2013). Existem duas abordagens: utilizar na MIV inibidores específicos de PDE para evitar a degradação e consequente queda das concentrações presentes de cAMP ou o cultivo com fármacos que aumentam a concentração de cAMP em COCs, tais como o forskolin ou dibutiril-cAMP (dbcAMP; Richani *et al.,* 2014).

Dentre os inibidores indiretos de PDE, podemos citar o precursor do peptídeo natriurético do tipo C (NPPC), sintetizado pelas células da granulosa (Zhang *et al.*, 2010; Zhang *et al.,* 2011). Quando ligado ao seu receptor nas células do cumulus (NPR2) estimula a síntese de cGMP. Este, quando transportado para o oócito pelas TZP e GJ inibe a PDE3A, dessa forma mantendo a parada meiótica (Richard *et al.,* 2014; Franciosi *et al.,* 2014).

Apesar da retomada espontânea da meiose, o processo *in vitro* necessita de fatores (hormônios, peptídeos, entre outros) que estimulem a maturação citoplasmática e nuclear de forma sincronizada. Assim, o uso de gonadotrofinas como o FSH se tornou comum, já que na maturação *in vitro*, as células da granulosa não estão presentes e as células do cumulus não respondem diretamente ao LH. Recentemente, têm-se aceito a ideia do uso de peptídeos EGF-*like* em substituição às gonadotrofinas, principalmente o AREG. Ele faz parte da cascata de sinalização *in vivo* promovida pelo pico pré-ovulatório de LH e quando ele se liga com seu receptor, presente nas células do cumulus, há o estímulo da ativação de genes de remodelagem da matriz extracelular, melhorando a expansão.

O ambiente folicular, garante ao oócito todo o suprimento e regulação endócrina para que este se desenvolva e adquira competência para os processos reprodutivos. Competência oocitária é definida como a capacidade do oócito em suportar os processos

de maturação, fertilização, o desenvolvimento -desde as primeiras clivagens até o estágio de blastocisto – e, consequentemente, a manutenção da gestação (Sirard *et al.*, 2006).

Este trabalho buscou elucidar os efeitos do uso de AREG em associação ou não com gonadotrofinas sobre a maturação *in vitro* de complexos cumulus-oócito. Além disso, foram observados os resultados sobre a produção de embriões quando foi utilizado uma MIV em duas fases (pré-MIV com NPPC e a MIV com AREG/gonadotrofinas).

CAPITULO 1

1. MATURAÇÃO OOCITÁRIA

1.1. O papel das gonadotrofinas na maturação oocitária: do crescimento do folículo antral à ovulação

A fêmea bovina é uma espécie mono ovulatória e tem ciclo estral com duração entre 18 e 24 dias. Estro é o período de receptividade sexual, em que a vaca aceita a monta de um touro ou de outras vacas (Gordon, 1994; Forde *et al.*, 2010). Esse período do ciclo estral é regulado por hormônios provenientes do hipotálamo (GnRH), da hipófise anterior (FSH e LH), dos ovários (progesterona, estradiol e inibinas) e do útero (prostaglandina F2α-PGF2α; Forde *et al.*, 2011). O GnRH é liberado de forma pulsátil e em níveis basais, do centro tônico do hipotálamo, prevenindo a dessensibilização dos seus receptores presentes nas células gonadotróficas na adenohipófise. Durante a fase folicular do ciclo, um ambiente hormonal com níveis basais de progesterona e crescente concentrações de estradiol, induz um pico de GnRH (Forde *et al.*, 2010). Após esse evento, o GnRH é transportado para a hipófise via sistema sanguíneo portal-hipofisário (Moenter *et al.*, 1992) e se liga a receptores acoplados a proteína G desencadeando a liberação de cálcio intracelular e a ativação de vias MAPK. Com isso há a concomitante liberação de estoques adenohipofisários de FSH e de LH e o estímulo para a expressão destes genes e a síntese proteica das gonadotrofinas (Weck *et al.*, 1998).

Os hormônios FSH e LH, apesar de serem sintetizados na mesma glândula, são secretados de formas diferentes de acordo com a frequência (pulsátil ou fásica) e amplitude do sinal do GnRH. (Farnworth, 1995). A crescente concentração de FSH leva ao aumento da atividade enzimática da aromatase (P450; CYP19) nas células da granulosa e convertem andrógenos em 17 β estradiol. Conforme o folículo dominante cresce, a concentração de estradiol e de inibinas – que são secretadas por ele - também aumentam e fazem com que os níveis de FSH voltem ao basal por meio de *feedback* negativo (Hillier, 1994). O LH é liberado de forma pulsátil, mas de maneira específica para cada fase do ciclo. Na fase luteal, o pulso de LH tem alta amplitude e baixa frequência (aproximadamente ocorrendo um pulso a cada 4 horas), já na fase pré-ovulatória esse pulso tem baixa amplitude e alta frequência (um ou mais pulsos por hora; Figura 1; Gordon, 1994). A função do LH é estimular a produção as células da teca para produzir andrógenos que serão usados como substrato à aromatase nas células da granulosa, em folículos dominantes (pós-desvio folicular). Nesse momento, as células da

granulosa também adquirem a capacidade de responder ao LH Em folículos dominantes (pós-desvio folicular) as células da granulosa adquirem a capacidade de responder ao LH e as células da teca sintetizam andrógenos para servir de substrato à aromatase nas células da granulosa (Richards *et al.,* 2002). Um desses substratos é a testosterona, sintetizada a partir do colesterol nas células da teca e difundida para as células da granulosa, onde será transformada em estradiol (Dorrington *et al.,* 1975).

O desenvolvimento folicular, ocorre em padrões de duas (Rhodes *et al.*, 1995) ou três (Figueiredo *et al.,* 1997) ondas por ciclo (Figura 1). Nelas, um pequeno grupo de folículos é recrutado para iniciar uma fase de desenvolvimento por, normalmente, três dias (Ginther *et al.,* 2003). Conforme os folículos crescem, as células da granulosa produzem e liberam estradiol e inibinas que, em sinergia, suprimem o FSH, fazendo com que seus níveis retornem ao basal (Ginther *et al.,* 2000). Dentre os folículos recrutados, apenas um continua o desenvolvimento (folículo dominante) e os demais folículos (chamados subordinados) regridem devido à queda na concentração circulante de FSH, agora insuficiente para manter o desenvolvimento de todos os demais folículos que não o dominante (fenômeno conhecido como desvio folicular; Ginther *et al.,* 1996; Gimenes *et al.,* 2008). O folículo dominante é refratário à concentração decrescente de FSH devido à aquisição de LHR pelas células da granulosa. (Nogueira *et al.* 2007; Barros *et al.,* 2010). Evidências sugerem que ocorre um aumento transitório de LH circulante, próximo do desvio folicular (Ginther *et al.,* 2003), permitindo que a produção de estradiol continue em um ambiente com baixa concentração de FSH (Ireland e Roche, 1983). Após a dominância, o LH é a gonadotrofina responsável pelo desenvolvimento e maturação folicular final *in vivo* até o estágio pré-ovulatório (Ginther *et al.,* 1996). Somente quando os pulsos de LH ocorrem a cada 40 a 70 minutos, e em um período de 2 a 3 dias, é que o folículo dominante é ovulado (Roche *et al.,* 1996). Isso ocorre, em média24 (Borges et al., 2004) ou 26 (Pinheiro et al., 1998) horas após o início do estro em vacas da raça Nelore

Logo após a ovulação, começa a fase luteal (metaestro) que tem duração de 3 a 4 dias e é caracterizada pela formação do corpo lúteo a partir do folículo ovulado. As células da granulosa e da teca são luteinizadas e começam a produzir progesterona, com a finalidade de manter a provável prenhez ou de reiniciar o ciclo estral. Ainda com a concentração de progesterona alta, as ondas de crescimento folicular voltam a ocorrer sob estímulo da liberação do FSH (período chamado de diestro), mas os folículos dominantes

decorrentes dessa fase não são ovulados devido à alta concentração de progesterona. Por fim, em um período nomeado próestro, o corpo lúteo regride em resposta a prostaglandina secretada pelo endométrio e as concentrações de progesterona declinam (Figura 1; Hansel e Convey, 1983; revisado por Forde *et al.*, 2010).

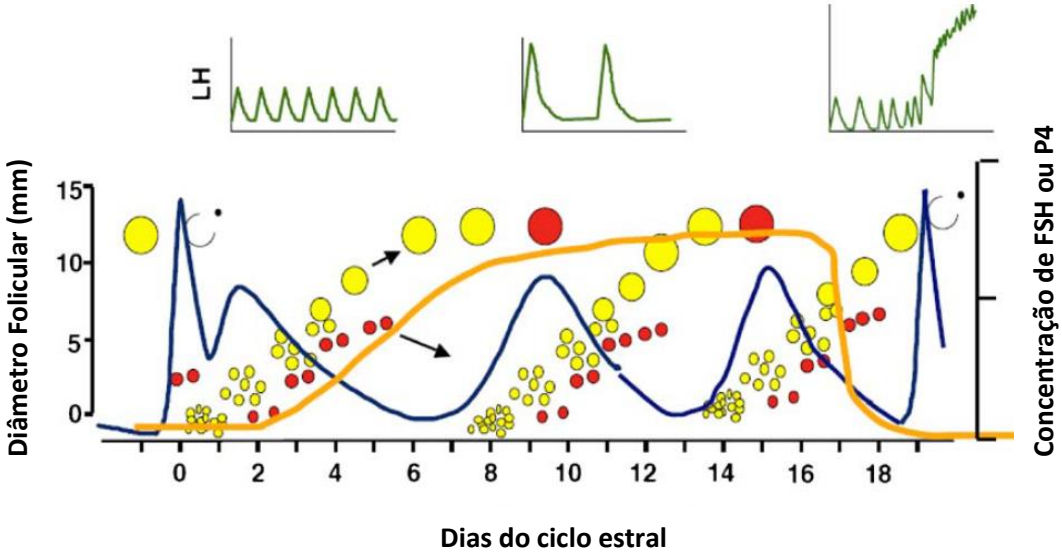

Figura 1 - Esquema do padrão de secreção de FSH, LH e P4 e o padrão de crescimento de folículos durante o ciclo estral na espécie bovina. Adaptado de Forde *et al.*, 2010. *Animal Reproduction Science, 124 (2011) 164-169.*

1.2. O uso dos peptídeos EGF-like na maturação in vitro

A superfamília EGF-*like* é composta por onze ligantes (EGF, HB-EGF, AREG, EREG, BTC, EPIGEN, NRG1, 2, 3 e 4 e TGF-α) e quatro receptores (EGFR/ERBB1, ERBB2, ERBB3 e ERBB4). Juntos, eles participam de vários processos e, dentre os quais, os relativos a reprodução, ou seja, maturação, ovulação e fertilização do oócito e/ou a implantação de embriões (Schneider & Wolf, 2008; Celestino *et al.*, 2012). Os três peptídeos que participam diretamente do processo de maturação são: AREG (ampirregulina), EREG (epirregulina) e BTC (betacelulina). Eles são sintetizados nas células da granulosa e regulados pela concentração de cAMP, estando a jusante (*downstream*) da via estimulatória do LH (Shimada *et al.*, 2016).

O LH induz rapidamente à produção de cAMP que regula a produção de peptídeos EGF-*like* para induzir a sinalização da via do receptor EGF-ERK1/2 (Park *et al.*, 2004; Ashkenazi *et al.*, 2005; Shimada *et al.*, 2006; Fan *et al.*, 2009; Gilchrist *et al.*, 2016).

Esses peptídeos, após clivados nas células da granulosa, se ligam ao receptor EGF (ERBB1) expresso pelas mesmas e pelas células do cumulus (Hisieh *et al.*, 2007). Essa ligação induz a dimerização do receptor e auto-fosforilação em múltiplos resíduos de tirosina que, entre outras funções, ativa o ERK1/2 (Yamashita *et al.*, 2007; Fan & Richards, 2010). Desse modo, o ERK1/2 promove a produção de prostaglandina E2 que se liga ao receptor (PTGER2) para produzir peptídeos EGF-*like*. Essas moléculas são importantes mediadores intraovarianos que estimulam a expressão de genes críticos para a remodelagem da matriz extracelular tais como a cicloxigenase 2 (COX2), fator estimulador de necrose tumoral 6 (TSG6), hialurona sintase 2 (HAS2) e pentraxina 3 (PTX3), modulando assim a expansão das células do cumulus (Park *et al.*, 2004; Richards *et al*., 2002). Além disso, os fatores EGF-*like* mimetizam os efeitos do LH sobre as células do cumulus - uma vez que estas não são responsivas ao LH (Shimada *et al.*, 2006; Gilchrist *et al.*, 2016) - e promovem a maturação oocitária nuclear e citoplasmática *in vitro* (Downs *et al.*, 1988; Downs, 1989; Smitz *et al.*, 1998; De La Fuente *et al.*, 1999).

Os peptídeos EGF-*like* são sintetizados como um pró-peptídeo ancorados a membrana e que, após ser clivado por uma protease, se tornam solúveis podendo induzir a ativação parácrina ou autócrina do seu receptor (EGFR ou ERBB1; Levano & Kenny, 2012; Berasain & Avila, 2014). A protease responsável pela clivagem do peptídeo é a enzima ancorada a membrana de conversão do fator alfa da necrose tumoral (TACE, do inglês *tumor necrosis factor-alpha converting enzyme*), membro da família de desintegrinas e metaloproteinases, e também conhecida como ADAM17 (para os peptídeos AREG e EREG; Sahin *et al.*, 2004) ou a ADAM10, que cliva a BTC (Ceresa e Peterson, 2014).

A forma solúvel da AREG, quando ligada ao receptor que é do tipo tirosina quinase, estimula a dimerização do mesmo e a fosforilação de resíduos de tirosina na cauda C-terminal (Berasein e Ávila, 2014). A fosforilação recruta moléculas adaptadoras como a SH2 e a Grb2 que vão ativar a proteína Ras, trocando um GDP (guanosina difosfato) por um GTP (guanosina trifosfato). A forma ativa da Ras, fosforila e ativa a proteína Raf provocando a fosforilação da MEK e das MAPK (ERK1/2; Pearson et al., 2001). A ativação da ERK1/2, fosforila as conexinas 43 (Cx43), levando a diminuição da permeabilidade das junções tipo GAP, fazendo cessar a passagem de cGMP para dentro do oócito o que culmina na retomada da meiose (Sun *et al.*, 2009).

Nos últimos anos, dados da literatura demonstram a efetividade do peptídeo AREG na maturação *in vitro* na presença ou não de gonadotrofinas. Em camundongos, Richani *et al.* (2013) observaram que oócitos maturados na presença de AREG ou EREG resultavam em blastocistos (no 6º dia do cultivo) com maior quantidade de massa celular interna em comparação com aqueles maturados com FSH ou EGF. Em suínos, foi demonstrado que folículos antrais médios são responsíveis ao AREG em uma concentração de 100 ng/mL e quase 80% dos complexos cumulus-oócito (COCs) chegavam a fase de metáfase II - taxa muito similar àquela de COCs provenientes de maturação com gonadotrofinas no mesmo experimento (Sugimura *et al.,* 2015). Na mesma espécie, Procházka *et al.* (2017) relataram que COCs maturados na presença de AREG, EREG ou AREG/EREG tiveram expansão do cumulus completa assim como os maturados com FSH. No entanto, o curso de maturação foi diferente entre os grupos FSH e AREG. Enquanto o grupo maturado com FSH apresentou expansão coesa e gradual, chegando ao máximo apenas após as 42h completas de maturação, os que foram maturados com AREG, apresentavam expansão completa mais rápido (20-24 horas de maturação), mas de uma forma mais desorganizada do que no primeiro grupo. Em primatas a taxa de oócitos que chegam a fase de metáfase II foi aumentada quando esses foram maturados em meio suplementado com AREG na presença ou não de gonadotrofinas (Peluffo *et al.,* 2012). Por fim, um trabalho com mulheres portadoras da síndrome de ovário policístico, o meio suplementado com FSH e AREG, precedido de uma fase de pré-maturação com CNP, aumentou a taxa de metáfase II e de embriões (com 3 dias de cultivo) com o grau bom de qualidade em comparação com o protocolo padrão da instituição (Sánchez *et al.,* 2017).

Na espécie bovina Sugimura *et al.* (2014), testaram o uso de AREG no meio de maturação na presença de fatores secretados por oócito como o GDF9 e BMP15. Os resultados desse trabalho demonstraram que, em cooperação com o BMP15, o uso de AREG melhorou a formação e a qualidade de blastocisto além deles apresentarem maior consumo de glicose e produção de lactato.

1.3. *Regulação da retomada da meiose: O Sistema NPPC-NPR2*

Até que haja a sinalização gonadotrófica, ou o estímulo que a mimetize, o oócito fica em parada meiótica na prófase da meiose I. A chave para que o bloqueio meiótico

permaneça por longos períodos está na concentração de nucleotídeos cíclicos como o cGMP e o cAMP.

O cAMP é sintetizado nas células da granulosa e do cumulus, e suprem o oócito através das junções GAP, além de ser também sintetizado pelo próprio oócito (Downs *et al.*, 2010). A síntese do nucleotídeo cíclico é iniciada pela ativação do receptor acoplado à proteína G (GPCR3/GPCR12; Zhang *et al.*, 2010) que promove a conversão de guanosina difosfato (GDP) em guanosina trifosfato (GTP), estimulando a adenilato ciclase a produzir cAMP através da ligação da GTP com a subunidade α da proteína G (Saraiva *et al.*, 2010). O cAMP tem um papel ambíguo na maturação oocitária, podendo promove-la ou inibi-la. Variações em sua concentração intraoocitária podem modular a retomada da meiose. As concentrações ótimas mantêm a PKA ativa, o que inibe os fatores promotores da maturação e mantem o oócito em parada meiótica (Sirard *et al.*, 1998; revisado por Botigelli *et al.*, 2017).

O cGMP é sintetizado pelas células da granulosa e do cumulus e transportado para o oócito via junções GAP, inibindo competitivamente a hidrólise de cAMP pela PDE3A (Shuhaibar *et al.*, 2018). O estímulo necessário para a síntese vem de várias vias, tais como as do óxido nítrico (NO), do bicarbonato e dos peptídeos natriuréticos (Sasseville *et al.*, 2008; Potter *et al.*, 2011).

O sistema dos peptídeos natriuréticos é composto por três peptídeos endógenos com alta homologia entre si: precursor natriurético tipo A (NPPA), tipo B (NPPB) e tipo C (NPPC), e também três receptores, NPR1, NPR2 e NPR3. NPPA e NPPB, tem alta afinidade pelo receptor NPR1, enquanto que o NPPC tem afinidade pelo NPR2; ambos possuem atividade guanilato ciclase e, quando estimulado, aumenta a síntese de cGMP (Cesaro *et al.*, 2015). O NPR3 não possui atividade guanilato ciclase e é responsável pela internalização e degradação dos três peptídeos, ou seja, promove o controle da biodisponibilidade dos ligantes.

Neste capítulo, vamos enfatizar somente a via que envolve o precursor do peptídeo natriurético do tipo C. O NPPC é essencial para o bloqueio meiótico em suínos (Zhang *et al.*, 2014) e em camundongos (Zhang *et al.*, 2010). Sua síntese ocorre nas células da granulosa e seu receptor (NPR2) está apresente nas mesmas e nas células do cumulus (Zhang *et al.*, 2010). Em ratos, Kawamura *et al.* (2011), observaram que tanto o FSH quanto o eCG aumentam a síntese de NPPC nas células da granulosa enquanto o estradiol

e os fatores derivados do oócito são capazes de aumentar a expressão de NPR2 e manter a sua funcionalidade (Zhang *et al.*, 2011). De modo contraditório e mediante a ativação do EGFR, o LH/hCG diminui a expressão de NPPC nas células da granulosa (Kawamura *et al.*, 2011) e da atividade de NPR2 nas células do cumulus de roedores (Wang *et al.*, 2013).

Um recente trabalho demonstrou, em suínos, que a concentração de NPPC dentro do folículo (com 3 a 8 mm de diâmetro) é diretamente proporcional ao seu diâmetro, mas a expressão do NPR2 não se altera. Além disso, a adição de NPPC no meio de cultura contribuiu para a elevação da taxa de clivagem e de blastocistos provenientes de diferentes tamanhos de folículos (3-4; 4-6; 6-8 mm; Zhang *et al.*, 2017). Soares *et al.* (2017) demonstraram que o NPPC é capaz de aumentar o número de oócitos em estado de vesícula germinativa em vacas da raça Holstein (*Bos t. taurus*) e também a porcentagem de oócitos que mantêm abertas as junções comunicantes do tipo GAP. Já na raça Nelore (*Bos t. indicus*), o NPPC aumentou a expressão de NPR2 após 9 horas de cultura (Soares *et al*., 2017).

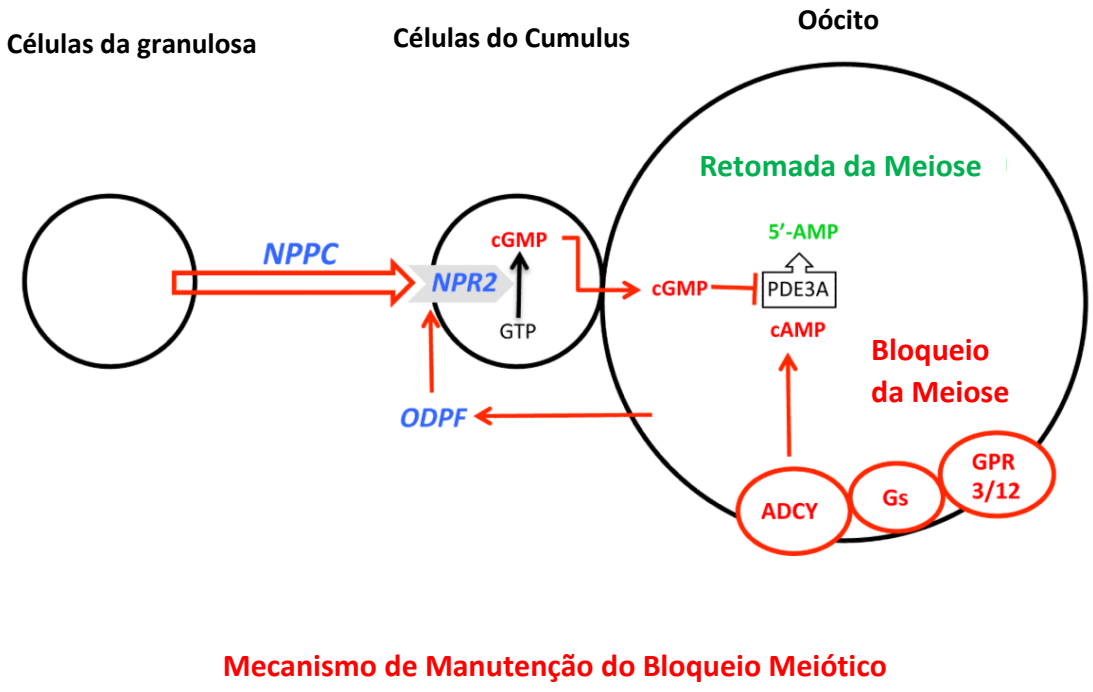

Figura 2. Modelo descritivo do papel do NPPC e seu receptor (NPR2), sobre a manutenção do bloqueio meiótico. ADCY (adenilato ciclase); GPR3/12 (receptores acoplados à proteína Gs 3 e 12); ODPF (do inglês Oocyte-derived paracrine factor; fatores parácrinos derivados do oócito). Adaptado de Zhang *et al.*, 2010. *Science 330(6002): 366–369.*

2. HIPÓTESES

As células do *cumulus oophorus* possuem a capacidade de expressar o gene do LHR e a utilização de AREG, no meio de cultivo, é capaz de estimular sua expressão no decorrer da MIV. Adicionalmente, a cinética de expressão gênica será distinta quando adicionado FSH ou AREG ao meio de cultura. Além disso, a ação de AREG, em sinergia com o LH, melhora a taxa de blastocistos produzidos. O NPPC retém o oócito em parada meiótica e irá melhorar a sincronia da maturação entre citoplasma e núcleo tornando a produção de blastocistos semelhantes para LH, AREG e FSH ou associações.

3. OBJETIVOS

1) Caracterizar a cinética da abundância de transcritos alvo (genes dos receptores de LH, FSH e EGF) nas células do *cumulus* e nos momentos de 0, 9, 15 e 24 horas da MIV, quando utilizado o rhFSH ou a AREG;

2) Investigar a ação de ligantes (LH, rhFSH e AREG), quando adicionados à MIV, sobre a progressão meiótica;

3) Investigar a ação dos mesmos ligantes sobre a abundância de transcritos alvo de blastocistos produzidos em sistema de pré-maturação *in vitro* com NPPC;

4) Avaliar a taxa de produção *in vitro* de blastocistos com o uso do sistema de pré-maturação com NPPC e de ligantes (rhLH, rhFSH, AREG ou combinações) na MIV.

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CAPÍTULO 2

EFFECTS OF PRE-MATURATION WITH NPPC AND *IN VITRO* MATURATION WITH DIFFERENT LIGANDS ON *CUMULUS* CELLS TRANSCRIPT ABUNDANCE AND BOVIVE BLASTOCYST PRODUCTION

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Abstract

Background: EGF-*like* peptide, such AREG, propagates the LH stimulus *in vivo* to meiosis resumption and *in vitro*, has been related as equal or superior than FSH to oocyte

maturation in various species. Whereas NPPC maintains high cAMP levels in *cumulus* cells and temporarily sustain *in vitro* the oocyte in meiotic arrest.

Methods: We evaluated the germinal vesicle breakdown (GVBD) and metaphase II stage (MII) rates in time course way using four time points (0, 9, 15 and 24 hours) comparing FSH and AREG ligands in the maturation medium. Also, we observed the transcript abundance of target genes by qRT-PCR BioMark platform in the same conditions. The MII rate also was assessed comparing the same groups plus LH addition. Finally, we observed the effectiveness of this association when IVM was preceded of pre-IVM with NPPC in the embryo production and the expression of targeted genes. The rates were arcsine-transformed, the value generate by qRT-PCR log-transformed (when necessary) and analyzed by ANOVA. The general differentially expressed gene were tested by correlation test and the results gave in correlation coefficient (R). To all analyses, we considered statically significance when $p<0.05$.

Results: Our data did not show difference (GVBD or MII rates) when we compared just AREG and FSH in time course experiment. It was maintained even with LH addition, although LH alone produced the lowest rate of MII. When we produced embryos in this condition plus pre-IVM with NPPC, it was observed a difference on cleavage (D3) and blastocyst (D7) rates, but not in the hatched blastocyst (D9).

Conclusions: We conclude that AREG induced a different gene expression profile in *cumulus* cells when compared to the FSH stimulus, maybe by activation of different pathways. The embryo production by LH was acceptable when associated with pre-IVM system with NPPC. Furthermore, is necessary more tested to elucidate if that is an effect just LH or NPPC had a crucial participation.

Keywords: Oocyte, *cumulus* cells, AREG, NPPC, LH, transcript abundance, IVM, cattle.

Background

In vivo, the luteinizing Hormone (LH) surge is responsible to stimulate the oocyte maturation and ovulation. The first intracellular signaling after the LH binding at Luteinizing Hormone Receptor (LHR) is a transitory increase in cAMP levels that stimulate the synthesis of EGF-like peptides, amphiregulin, epiregulin and betacellulin (AREG, EREG and BTC, respectively) from the granulosa cells (GC; Park *et al.*, 2004; Ashkenazi *et al.*, 2005; Shimada *et al.*, 2006; Fan *et al.*, 2009; Gilchrist *et al.*, 2016). After their cleavage from cellular membrane, they interact with Epidermal Growth Factor-*like* Receptor (EGFR; present on cumulus and granulosa cells) and active the MAPK pathway (ERK1/2; Shimada *et al.*, 2006; Richani *et al.*, 2014). This activation, leads to conexin 43 (Cx43) phosphorylation and decrease the traffic of cyclic nucleotides, as cGMP, from GC to CC. This block is a stimulus to the phosphodiesterase activity in oocyte and hydrolyses cAMP. Low concentration of cAMP inside oocyte, causes GVBD and meiosis resumption (Norris *et al.*, 2008). Besides that, the EGF-like peptides, promotes the expression of crucial gene to CC expansion.

AREG is used as alternative to supra physiological doses of Follicular Stimulating Hormone (FSH) or in association with gonadotropins in maturation medium (Richani e*t al.*, 2014). It increases the rate of oocytes in meiosis II (MII) stage closer the levels of maturation with gonadotropins in porcine (Sugimura *et al.*, 2015; Prochàzka *et al.*, 2011) and primates (Pellufo *et al.*, 2012). In mice, embryos-derived from AREG maturation medium showed a great number of inner cell mass (Richani *et al.*, 2013) and increase the

embryo quality (trophectoderm and total cell number, comparable to *in vivo*-derived embryo; Richani *et al.*, 2014).

The mechanical removal of oocyte from follicle causes a decrease in the cGMP levels and leads to spontaneous meiosis resumption. The Natriuretic Peptide Precursor type C (NPPC) is a candidate to delay this process. Its receptor (NPR2) has a guanylyl cyclase activity and is expressed on CC (Potter and Hunter, 1998). When actived by NPPC, there is an increase of the cGMP concentration thus preventing the meiosis resumption (Zhang *et al*., 2010).

The NPPC was reported such great to prevent meiosis resumption in mouse enclosed oocyte and elevate the cGMP levels (Zhang *et al.,* 2010) and in porcine (Hiradate *et al.,* 2013). *In vitro*, NPPC also affect the intercellular communication between CC and oocyte. It prolongs the time that gap junction is open and promote the transport of nutrients and metabolites in bovine COCs (Soares et al., 2017). The use of NPPC during the bovine *in vitro* maturation (IVM), causes an upregulation of PTX3 (Botigelli *et al.,* 2018), a marker gene of quality and oocyte competence and involved with a CC extracellular matrix formation (Assidi *et al.*, 2008; Uyar *et al.,* 2013).

The present study observed the effect of AREG on meiosis progression and gene expression compared with FSH. Also, we compared the meiosis progression among the gonadotropins (LH or FSH), AREG or associations. Additionally, we tested the same associations on bovine embryo production after a pre-IVM with NPPC. Currently, we observed if AREG can induce the LHR expression in any time through of maturation.

Methods

Chemicals

Unless specified, all chemicals were purchased from Sigma-Aldrich (St Louis MO, USA). Rh-FSH (Gonal 75IU; Folitropin Alfa) and rh-LH (Luveris 75IU; Lutropin Alfa) were obtained from MERCK.

Experimental Design

Experiment 1- Genetic expression and meiosis resumption through the maturation (Time Course Test)

We performed 5 replicates with 300 COCs per routine. The structures were separated in 3 groups (25 COCs/group): CTRL group (TCM 199, FBS and rh-FSH); AREG group (TCM 199, BSA and AREG); and FSH group (TCM 199, BSA and rh-FSH). The concentrations were used as described below. The COCs were denuded and stained by Hoechst and analyzed under epifluorescence microscopy at 0, 9, 15 and 24 hours of maturation. Furthermore, the *cumulus* cells and oocytes were preserved at -80° C until the RNA extraction.

Experiment 2- Alternative IVMs

We compared the effectiveness of AREG and FSH in combination or not with LH. To this aim, we separated the COCs in 6 groups (25 COCs/group): Control group (just TCM and BSA); AREG group (100ng/mL); FSH (0.1IU/mL) group; LH (0.01IU/mL) group; AREG/LH (100ng/mL; 0.01IU/mL) group and FSH/LH group (0.1UI/mL; 0.01IU/mL). After 24 hours of maturation, they also were colored by Hoechst and analyzed under epifluorescence microscopy.

Experiment 3- Embryo Production

Lastly, we tested the alternative IVMs above preceded by a Pre-IVM with NPPC (100ng/mL) on the embryo production. After fertilization, the presumptive zygotes were

transferred to a CIV medium and cultivated until the Day 9 (post-fertilization). The cleavage rate was observed on Day 3 and the blastocyst rate was observed on Days 7, 8 and 9. At Day 9, hatched blastocysts were collected and stored at -80° C until the RNA extraction.

Oocyte Collection and In Vitro Maturation

To all experiments, Nellore or cow bread ovaries were obtained from a local abattoir in Assis, São Paulo state. They were transported to the laboratory in saline solution (0.9%) at 36.5°. Follicles (2-8mm) were aspirated with an 18-gauge needle and pooled in a 15mL conical tube. After the sedimentation, we recovered and select *cumulus*-oocyte complexes (COCs) using a stereomicroscope. Only COCs with homogenous cytoplasm and compact multilayer of *cumulus,* grade 1 and 2, according Bó & Mapletoft (2013) were selected to maturation.

After the selection, we washed the COCs in the TCM 199 Hepes media supplemented with 4 mg/mL fatty acid free BSA (Bovine Serum Albumin; Sigma-Aldrich), amikacin sulfate 16.67 µL/mL and 11 µL/mL sodium pyruvate (Sigma-Aldrich). Then, they were cultured in four well board in 250 µL of TCM 199 containing Earle's salts media with 4mg/mL fatty acid free BSA (Bovine Serum Albumin; Sigma-Aldrich) or 10% fetal bovine serum, amikacin sulfate 16.67 µL/mL and 11 µL/mL sodium pyruvate (Sigma-Aldrich). According the experimental design, the IVM media were supplemented with 0.1 IU/mL rh-FSH (Gonal 75IU; Folitropin Alfa, Merck), 100 ng/mL AREG (rhAR; RD System), 0.01 IU/mL rh-LH (Luveris 75 IU; Lutropin Alfa, Merck) or the combination of AREG and LH or FSH and LH and covered with mineral oil and incubated for up to 24 h at 38.5° C in humidified air containing 5% CO_2. To 0 hour we used immature COCs. The matured COCs were denuded by pipetting and the oocyte were

stained by Hoechst or both *cumulus* cells and oocyte were maintained at -80 for molecular techniques.

Pre-IVM

Following the selection, we placed the structures in 250 µL of pre-IVM media covered with mineral oil and incubated for 6 hours. The media were composed for TCM 199 Earle's salt with 4mg/mL fatty acid free BSA (Bovine Serum Albumin; Sigma-Aldrich), amikacin sulfate 16.67 µL/mL and 11 µL/mL sodium pyruvate (Sigma-Aldrich) supplemented with 100 ng/mL NPPC.

In Vitro Fertilization

After 24 h of maturation the mature COCs were fertilized with a semen from one bull. The COCs were washed in TCM 199 Hepes three times and transferred to 90 µL drops of fertilization media covered with mineral oil. The semen was thawed at 37°C for 30s and spermatozoa were washed in commercial media Select Sperm (Botupharma, Brazil). The semen sample were added on the top of 800 µL Select Sperm and centrifuged at 4.500×g for 5 min (MiniSpin® Eppendorf). After, supernatant was removed and the spermatozoa pellet was added on top in 1 mL of IVF medium and centrifuged at 1.500×g for 3 min. The spermatozoa pellet was resuspended in IVF medium, counted in a Neubauer chamber. Sperm (7 µl of a final concentration=1×10^6 cells/mL) was added into each drop. Incubation was carried out at 38.5 °C in a saturated humidity atmosphere containing 5% CO_2 for 18h.

RNA extraction and qPCR

The total RNA was extracted from both *cumulus* cells and oocyte, by the RNeasy Micro Kit® (Qiagen, Mississauga, ON, CA) following the manufacturer's protocol. After purification, the samples were eluted in 14 µL of RNAse water free. Then, we measure

the *cumulus* cells RNA concentration using the spectrophotometry method by NanoDrop ND 1000 (Thermo Scientific, Wilmington, DE, USA). The obtained RNA was transcribed by incubation in 25° to 10 minutes, 37° to 2 hours and 85° to 5 minutes using Oligo-d(T) primer and the enzyme High Capacity (Life Technologies). The target transcripts evaluation from *cumulus* cells and oocytes was performed using Biomark® HD 96.96 (Fluidigm, South San Francisco, CA, USA) with TaqMan assays (ThermFisher®) specific to *Bos taurus* (Table 1).

Table 1. Definition and functions of genes from the selected assays used to perform transcript array by BioMark platform.

GENE SYMBOL	DEFINITION	FUNCTION
ACAT1	acetyl-CoA acetyltransferase 1	LIPIDIC METABOLISM
ACSL1	acyl-CoA synthetase long-chain family member 1	LIPIDIC METABOLISM
ACSL3	Acyl-CoA Synthetase 3	LIPIDIC METABOLISM
ACSL6	Acyl-CoA Synthetase 6	LIPIDIC METABOLISM
ACTB	actin, beta	HOUSEKEEPING
ADCY3	adenylate cyclase 3	MEIOTIC ARREST
ADCY6	adenylate cyclase 6	MEIOTIC ARREST
ADCY9	adenylate cyclase 9	MEIOTIC ARREST
AGPAT1	1-acylglycerol-3-phosphate O-acyltransferase 1	QUALITY PREDICTION
AGPAT9	1-acylglycerol-3-phosphate O-acyltransferase 9	QUALITY PREDICTION
AQP3	aquaporin 3	WATER CHANNEL
AREG/LOC538751	Amphiregulin	MEIOTIC ARREST
ARO	Aromatase	HORMONAL FUNCTION
ATF4	Activating transcription factor 4	OXIDATIVE STRESS
ATP5L	ATP synthase, H+ transporting, mitochondrial Fo complex subunit E	QUALITY PREDICTION
B2M		HOUSEKEEPING

BAX	BCL2-Associated X Protein	APOPTOSIS
BCL2	B-cell CLL/lymphoma 2	APOPTOSIS
BDNF	Brain-derived neurotrophic factor	MEIOSIS RESUMPTION
BID	BH3 interacting domain death agonist	APOPTOSIS
BMP15	Bone Morphogenetic Protein 15	OOCYTE SECRETED FACTOR
CASP3	caspase 3, apoptosis-related cysteine peptidase	APOPTOSIS
CASP9	Caspase 9, apoptosis-related cysteine peptidase	APOPTOSIS
CAT	Catalase	ANTIOXIDANT DEFENSE
CCND2	cyclin D2	CELL CYCLE
CD40	CD40 molecule	APOPTOSIS
CDCA8	Cell division cycle associated 8	CELL CYCLE
CDH1	cadherin 1	CELL ADHESION
CDK6	Cyclin-Dependent Kinase 6	CELL CYCLE
DICER1	Dicer 1, Ribonuclease III	EPIGENETIC
Dnmt1	DNA (Cytosine-5-)-Methyltransferase 1	EPIGENETIC
Dnmt3A	DNA (Cytosine-5-)-Methyltransferase 3[a]	EPIGENETIC
Dnmt3B	DNA (Cytosine-5-)-Methyltransferase 3B	EPIGENETIC
EGFR	Epidermal growth factor – receptor	EGF RECEPTOR
ELOVL1	ELOVL fatty acid elongase 1	LIPIDIC METABOLISM
ELOVL2	ELOVL fatty acid elongase 2	LIPIDIC METABOLISM
ELOVL3	ELOVL fatty acid elongase 3	LIPIDIC METABOLISM
ELOVL4	ELOVL fatty acid elongase 4	LIPIDIC METABOLISM
ELOVL5	ELOVL fatty acid elongase 5	LIPIDIC METABOLISM
ELOVL6	ELOVL fatty acid elongase 6	LIPIDIC METABOLISM
FOXO3	forkhead box O3	APOPTOSIS
FSHR	Follicle-stimulating hormone receptor	FSH RECEPTOR
GADD45A	growth arrest and DNA-damage-inducible, alpha	CELL CYCLE
GATM	Glycine amidinotransferase	QUALITY PREDICTION

GDF9	growth differentiation factor 9	OOCYTE SECRETED FACTOR
GFPT2	glutamine-fructose-6-phosphate transaminase 2	OXIDATIVE STRESS
GLRX2	glutaredoxin 2	OXIDATIVE STRESS
GREM1		QUALITY PREDICTION
GUCY1B3	Guanylate cyclase 1, soluble, beta 3	GMPc
H1FOO	H1 histone family, member O, oocyte-specific	EPIGENETIC
H2AFZ	H2A histone family, member Z	EPIGENETIC
HAS2	Hyaluronan synthase 2	*CUMULUS* CELL EXPANSION
HDAC2	histone deacetylase 2	EPIGENETIC
HSF1	Heat Shock Transcription Factor 1	HEAT SHOCK
HSP90AA1	Heat Shock Protein 90kDa Alpha	HEAT SHOCK
HSPA1A	heat shock 70kDa protein 1A	HEAT SHOCK
MORF4L2	Mortality Factor 4-like protein 2	EPIGENETIC
IGFBP2	Insulin-Like Growth Factor Binding Protein 2	
IGFBP4	Insulin-Like Growth Factor Binding Protein 4	
LHCGR	Luteinizing hormone/choriogonadotropin receptor	LH RECEPTOR
LUM	Lumican	QUALITY PREDICTION
MAPK1	Mitogen-Activated Protein Kinase	CELL CYCLE
MTIF3	mitochondrial translational initiation factor 3	VIABILITY
NLRP5	NLR Family, Pyrin Domain Containing 5	VIABILITY
NOS2	Nitric oxide synthase 2, inducible	MEIOTIC ARREST
NOS3	nitric oxide synthase 3	MEIOTIC ARREST
NPPA	Natriuretic peptide A (ANP)	GMPc/PKG
NPPB	Natriuretic peptide B (BNP)	GMPc/PKG
NPPC	Natriuretic peptide C (CNP)	GMPc/PKG
NPR1	Natriuretic peptide receptor 1	GMPc/PKG
NPR2	Natriuretic peptide receptor 2	GMPc/PKG
NPR3	Natriuretic peptide receptor 3	GMPc/PKG

OOSP1	oocyte-secreted protein 1	OOCYTE SECRETED FACTOR
PDE5A	Phosphodiesterase 5A, cGMP-specific	MEIOTIC ARREST
PFKP	Phosphofructokinase	QUALITY PREDICTION
PGK1	Phosphoglycerate Kinase 1	METABOLISM
PPIA	Peptidylprolyl Isomerase A	HOUSEKEEPING
PRDX1	Peroxiredoxin-1	QUALITY PREDICTION
PRDX3	peroxiredoxin 3	QUALITY PREDICTION
PTGS2/COX2	Prostaglandin-endoperoxide synthase 2	*CUMULUS* CELL EXPANSION
PTX3	Pentraxin 3, long	*CUMULUS* CELL EXPANSION
RPL30		HOUSEKEEPING
SLC2A1	solute carrier family 2 (facilitated glucose transporter), member 1	GLUCOSE TRANSPORTER
SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3	GLUCOSE TRANSPORTER
SLC2A4	solute carrier family 2 (facilitated glucose transporter), member 4	GLUCOSE TRANSPORTER
SLC2A5	solute carrier family 2 (facilitated glucose/fructose transporter), member 5	GLUCOSE TRANSPORTER
SOD1	superoxide dismutase 1, soluble	ANTIOXIDATIVE DEFENSE
SOD2	superoxide dismutase 2, mitochondrial	ANTIOXIDATIVE DEFENSE
STAT3	Signal Transducer and Activator of Transcription 3	VIABILITY
TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	*CUMULUS* CELL EXPANSION
TNFRSF21	tumor necrosis factor receptor superfamily member 21	APOPTOSIS
TP53	tumor protein p53	APOPTOSIS
TXNRD1	thioredoxin reductase 1	OXIDATIVE STRESS
VCAN	Versican	*CUMULUS* CELL EXPANSION
VEGFA	Vascular Endothelial Growth Factor A	CELL CYCLE
VNN1	vanin 1	OXIDATIVE STRESS

The relative expression values for each gene were calculated with the ΔCt method and data were normalized by geometric means of most stable reference genes for each

cellular type (NormFinder software tested). The mRNA abundance of ACTB, B2M, PPIA and RPL30 was used to normalize oocytes and *cumulus* cells gene expression.

To embryos, the RNA was extracted using the Pico Pure RNA isolation Kit (Thermo Fisher) following the manufacturer's protocol. We used 4 pools of 3 embryos to each group. The cDNA was obtained by RT-PCR using High Capacity (Life Technologies) as described above and stored at -20° until the qPCR. The qPCR was performed by StepOnePlus™ (Applied Biosystems) using the Power SYBR Green PCR Master Mix (Applied Biosystem, São Paulo, Brazil). The analyzed genes, primers sequences and annealing temperatures for each target gene are shown in Table 2. The final volume of mix was 25 µl and PCR cycling conditions were: 95°C for 10 min (1 cycle), denaturing at 95°C for 10 seconds followed by annealing and extension for 1 min at 60ºC (40 cycles). Reactions were optimized to provide maximum amplification efficiency for each gene. Each sample was run in duplicates. The relative expression values for each gene were calculated using the ΔΔCt method with efficiency correction and using one control sample as calibrator (Pfaffl, 2001). Mean efficiency values for each gene were calculated from the amplification profile of individual samples with LinRegPCR software (Ramakers *et al*., 2003).

Table 2. Sequence of primers used in the qPCR analyze. F: forward; R: reverse.

GENE SYMBOL	PRIMER SEQUENCE	Annealing temperature (°C)
PPIA	F 5'-GCCATGGAGCGCTTTGG- 3' R 5'-CCACAGTCAGCAATGGTGATCT-3'	60°
RPL30	F 5'-TGGTGTCCATCACTACAGTGGCAA-3' R 5'-ACCAGTCTGTTCTGGCATGCTTCT-3'	60°
BAX	F 5'TGTTTTCTGACGGCAACTTCA-3' R 5'-CGAAGGAAGTCCAATGTCCAG-3'	60°
BCL2	F 5'-TTCGCCGAGATGTCCAGTCAGC-3' R 5'-TTGACGCTCTCCACACACA-3'	60°
OCT4	F 5'-TGGAGGAAGCTGACAACAACGAGA-3' R 5'-ATGTGGCTAATTTGCTGCAGGGTG-3'	60°
PLAC8	F 5'- GACTGGCAGACTGGCATCTT-3' R 5'-CTCATGGCGACACTTGATCC-3'	60°
SOD2	F 5'-AGCACGAGCAGGAGACTGGT-3' R 5'-GACGTTGAGGTTGTTCACGTAG-3'	60°

Statistical Analyze

The cleavage, meiosis progression and blastocyst rates were calculated as mean of the percentages and arcsine-transformed. The mRNA abundance data were transformed to logarithms in order to reach normal distribution, when necessary. If the data demonstrated normal distribution it was analyzed by ANOVA and the different means compared used Tukey-Kramer HSD post-test. If the data was no parametric, the Kruskal-Wallis test was used. The analyses were performed with JMP® program (SAS Inst., Inc., Cary, NC) and the results are presented as mean ± SEM. Differences were considered significant when $p \leq 0.05$.

The network genes were created using the STRING platform (https://string-db.org/) with the genes that were different and significantly expressed. All interactions among the genes given by this program are based on database sites such as PubMed and others.

Results

Experiment 1- Genetic expression and meiosis resumption through the maturation (Time Course Test)

We observed the meiotic resumption throughout the time course way, using the Hoechst staining at 0, 9, 15 and 24 hours of the maturation. The result shows no difference on the meiosis resumption among the groups (Table 3) in each time point analyzed.

Table 3. Percentagen of GV, GVBD and MII at 9, 15 or 24 hours after maturation with FSH or AREG media.

	CTRL			AREG			FSH		
Time (h)	GV (% ± S.E.M.)	GVBD (% ± S.E.M.)	MII (% ± S.E.M.)	GV (% ± S.E.M.)	GVBD (% ± S.E.M.)	MII (% ± S.E.M.)	GV (% ± S.E.M.)	GVBD (% ± S.E.M.)	MII (% ± S.E.M.)
9	50.4 ± 0.14	49.6 ±0.22		28 ±0.14	72 ±0.12		39 ±0.14	61 ±0.10	
15	37.25 ±0.30	62.75 ±0.42		36.4 ±0.32	63.6 ±0.53		30.3 ±0.13	67.3 ±0.13	
24	4.25±0.19	32.25±0.12	63.5±0.43	5.5±0.15	25±0.12	69.5±0.61	2.8±0.14	19.4±0.12	78±0.12

The results were obtained from five replicates.

To evaluate the kinetics of gene expression, we analyzed 96 genes in time course form. These genes were relative to cellular circle, apoptosis, oxidative stress, epigenetic regulation, meiosis progression and quality prediction (Table 1). Contrary to our expectations, none of treatments were capable to enhance the mRNA abundance of LHR (data not shown). Despite this, in the *cumulus* cells a total of 57genes (12 at 9 hours; 37 at 15 hours and 39 genes at 24 hours; Table 4) were differentially expressed (some genes, showed difference in more than one time point). A general analyze of parametric correlation (Pearson), demonstrated that had a positive correlation among all genes of the three tested groups. It means that when the abundance mRNA of genes increased in one group, it also occurred in the others and vice versa. The dispersion graphic on the Figure 1 depicts this.

Table 4. Genes differently expressed in the time course test at 9, 15 and 24 hours after.

	9 HOURS			*15 HOURS*			*24HOURS*		
GENE	**CTRL (Means ± S.E.M.)**	**FSH (Means ± S.E.M.)**	**AREG (Means ± S.E.M.)**	**CTRL (Means ± S.E.M.)**	**FSH (Means ± S.E.M.)**	**AREG (Means ± S.E.M.)**	**CTRL (Means ± S.E.M.)**	**FSH (Means ± S.E.M.)**	**AREG (Means ± S.E.M.)**
ACAT1	-	-	-	0.037 ± 0.003 (ab)	0.049 ± 0.003 (a)	0.029 ± 0.003 (b)	0.061 ± 0.003 (a)	0.051 ± 0.003 (a)	0.037 ± 0.003 (b)
ACSL1	-	-	-	0.011 ± 0.0007 (a)	0.013 ± 0.0007 (a)	0.006 ± 0.0007 (b)	0.0089 ± 0.0010 (b)	0.0136 ± 0.0009 (a)	0.0073 ± 0.0009 (b)
ADCY6	-	-	-	0.110 ± 0.007 (a)	0.102 ± 0.007 (a)	0.055 ± 0.007 (b)	0.174 ± 0.014 (a)	0.147 ± 0.012 (a)	0.081 ± 0.012 (b)
AGPAT1	-	-	-	0.261 ± 0.023 (a)	0.227 ± 0.0023 (ab)	0.142 ± 0.023 (b)	-	-	-
AGPAT9	3.372 ± 0.399 (a)	2.609 ± 0.39 (ab)	1.268 ± 0.39 (b)	0.386 ± 0.047 (a)	0.551 ± 0.047 (a)	0.056 ± 0.047 (b)	0.120 ± 0.014 (b)	0.181 ± 0.012 (a)	0.043 ± 0.012 (c)
ARO	0.017 ± 0.003 (ab)	0.023 ± 0.003 (a)	0.005 ± 0.003 (b)	0.002 ± 0.0005 (b)	0.005 ± 0.0005 (a)	0.0006 ± 0.0005 (b)	-	-	-
ATP5L	-	-	-	0.443 ± 0.033 (b)	0.571 ± 0.033 (a)	0.334 ± 0.033 (b)	0.540 ± 0.040 (ab)	0.569 ± 0.036 (a)	0.406 ± 0.036 (b)
BCL2	-	-	-	0.029 ± 0.004 (a)	0.040 ± 0.004 (a)	0.006 ± 0.004 (b)	0.098 ± 0.014 (a)	0.091 ± 0.013 (a)	0.016 ± 0.013 (b)
BDNF	-	-	-	0.001 ± 0.0006 (b)	0.003 ± 0.0006 (ab)	0.005 ± 0.0006 (a)	0.0013 ± 0.0008 (b)	0.0047 ± 0.0008 (a)	0.0043 ± 0.0007 (ab)
BID	-	-	-	0.014 ± 0.001 (b)	0.019 ± 0.001 (a)	0.012 ± 0.001 (b)	0.013 ± 0.001 (ab)	0.018 ± 0.001 (a)	0.010 ± 0.001 (b)
CASP3	-	-	-	-	-	-	0.0071 ± 0.0007 (b)	0.0129 ± 0.0006 (a)	0.0060 ± 0.0006 (b)
CASP9	-	-	-	0.037 ± 0.002 (a)	0.044 ± 0.002 (a)	0.024 ± 0.002 (b)	0.038 ± 0.002 (a)	0.040 ± 0.002 (a)	0.028 ± 0.002 (b)
CAT	-	-	-	0.033 ± 0.003 (ab)	0.041 ± 0.003 (a)	0.023 ± 0.003 (b)	0.056 ± 0.004 (a)	0.058 ± 0.004 (a)	0.025 ± 0.004 (b)
CCND2	0.997 ± 0.123 (a)	0.936 ± 0.123 (a)	0.381 ± 0.123 (b)	-	-	-	0.406 ±0.035 (b)	0.725 ± 0.031 (a)	0.208 ± 0.035 (c)
CDCA8	-	-	-	0.015 ± 0.001 (a)	0.014 ± 0.001 (a)	0.007 ± 0.001 (b)	-	-	-
CDK6	-	-	-	-	-	-	0.021 ± 0.002 (ab)	0.028 ± 0.002 (a)	0.011 ± 0.002 (b)
DICER1	0.030 ± 0.002 (a)	0.022 ± 0.002 (ab)	0.017 ± 0.002 (b)	0.033 ± 0.002 (a)	0.030 ± 0.002 (a)	0.014 ± 0.002 (b)	0.024 ± 0.002 (ab)	0.028 ± 0.002 (a)	0.015 ± 0.002 (b)
DNMT3B	-	-	-	0.004 ± 0.0007 (a)	0.003 ± 0.0007(ab)	0.001 ± 0.0007 (b)	-	-	-
EGFR	-	-	-	0.050 ± 0.010 (b)	0.112 ± 0.010 (a)	0.075 ± 0.010 (ab)	-	-	-
ELOVL1	0.019 ± 0.004 (b)	0.028 ± 0.004 (ab)	0.038 ± 0.004 (a)	-	-	-	-	-	-
ELOVL4	-	-	-	0.014 ± 0.002 (ab)	0.020 ± 0.002 (a)	0.009 ± 0.002 (b)	0.012 ± 0.001 (b)	0.019 ± 0.001 (a)	0.010 ± 0.001 (b)
FOXO3	-	-	-	0.011 ± 0.001 (a)	0.015 ± 0.001 (a)	0.006 ± 0.001 (b)	0.020 ± 0.001 (a)	0.019 ± 0.001 (a)	0.006 ± 0.001 (b)
FSHR	-	-	-	0.001 ± 0.0003 (b)	0.003 ± 0.0003 (a)	0.002 ± 0.0003 (ab)	-	-	-
GADD45A	0.072 ± 0.012 (ab)	0.114 ± 0.012 (a)	0.036 ± 0.012 (b)	0.020 ± 0.005 (b)	0.051 ± 0.005 (a)	0.023 ± 0.005 (b)	0.015 ± 0.005 (b)	0.038 ± 0.004 (a)	0.010 ± 0.004 (b)
GATM	-	-	-	0.199 ± 0.038 (a)	0.177 ± 0.025 (a)	0.077 ± 0.057 (b)	0.012 ± 0.001 (ab)	0.014 ± 0.001 (a)	0.007 ± 0.001 (b)
GFPT2	0.128 ± 0.012 (a)	0.078 ± 0.012 (b)	0.032 ± 0.012 (b)	-	-	-	0.332 ± 0.019 (a)	0.307 ± 0.017 (a)	0.133 ± 0.017 (b)
GLRX2	-	-	-	-	-	-	0.084 ± 0.007 (a)	0.087 ± 0.006 (a)	0.044 ± 0.006 (b)
GUCY1B3	-	-	-	0.003 ± 0.0002 (b)	0.004 ± 0.0002 (a)	0.003 ± 0.0002 (b)	-	-	-
HAS2	-	-	-	0.281 ± 0.036 (b)	0.477 ± 0.036 (a)	0.168 ± 0.036 (b)	0.291 ± 0.033 (b)	0.517 ± 0.029 (a)	0.191 ± 0.029 (b)
HDAC2	0.090 ± 0.006 (a)	0.083 ± 0.006 (ab)	0.060 ± 0.006 (b)	0.080 ± 0.006 (ab)	0.087 ± 0.006 (a)	0.058 ± 0.006 (b)	-	-	-
HSF1	-	-	-	0.149 ± 0.11 (a)	0.178 ± 0.011 (a)	0.085 ± 0.011 (b)	0.204 ± 0.018 (a)	0.213 ± 0.016 (a)	0.084 ± 0.016 (b)
HSP90AA1	-	-	-	0.759 ± 0.055 (a)	0.894 ± 0.055 (a)	0.520 ± 0.055 (b)	-	-	-
IGFBP2	-	-	-	-	-	-	0.019 ± 0.004 (ab)	0.022 ± 0.004 (a)	0.007 ± 0.004 (b)
IGFBP4	0.026 ± 0.002 (a)	0.016 ± 0.002 (b)	0.008 ± 0.002 (b)	-	-	-	0.022 ± 0.002 (a)	0.015 ± 0.002 (a)	0.004 ± 0.004 (b)
LUM	-	-	-	-	-	-	0.004 ± 0.001 (ab)	0.007 ± 0.001 (a)	0.003 ± 0.001 (b)
MAPK1	-	-	-	0.041 ± 0.008 (b)	0.078 ± 0.008 (a)	0.079 ± 0.008 (a)	0.107 ± 0.011 (a)	0.105 ± 0.010 (a)	0.047 ± 0.010 (b)
MORF4L2	0.199 ± 0.024 (ab)	0.243 ± 0.024 (a)	0.143 ± 0.024 (b)	0.244 ± 0.012 (a)	0.290 ± 0.012 (a)	0.167 ± 0.012 (b)	0.237 ± 0.016 (a)	0.283 ± 0.014 (a)	0.172 ± 0.014 (b)
MTIF3	-	-	-	0.004 ± 0.0005 (ab)	0.005 ± 0.0005 (a)	0.002 ± 0.0005 (b)	0.0047 ± 0.0006 (ab)	0.0062 ± 0.0005 (a)	0.0028 ± 0.0005 (b)
NOS2	-	-	-	-	-	-	0.009 ± 0.001 (b)	0.014 ± 0.001 (a)	0.003 ± 0.001 (c)
NOS3	-	-	-	-	-	-	0.009 ± 0.001 (a)	0.011 ± 0.001 (a)	0.004 ± 0.001 (b)
NPPC	-	-	-	-	-	-	-	-	-
NPR1	-	-	-	-	-	-	-	-	-
NPR3	-	-	-	0.0011 ±0.0002 (ab)	0.0016 ± 0.0002 (a)	0.0005 ± 0.0002 (b)	-	-	-
PDE5A	-	-	-	0.055 ± 0.007 (b)	0.073 ± 0.007 (b)	0.103 ± 0.007 (a)	-	-	-
PGK1	0.414 ± 0.031 (a)	0.352 ± 0.031 (ab)	0.243 ± 0.031 (b)	-	-	-	0.269 ± 0.032 (ab)	0.307 ± 0.029 (a)	0.192 ± 0.029 (b)
PRDX1	-	-	-	0.324 ± 0.031 (a)	0.313 ± 0.031 (a)	0.189 ± 0.031 (b)	0.375 ± 0.029 (a)	0.263 ± 0.026 (b)	0.160 ± 0.026 (c)
PRDX3	-	-	-	-	-	-	0.040 ± 0.004 (ab)	0.048 ± 0.003 (a)	0.025 ± 0.003 (b)
SLC2A1	0.986 ± 0.102 (a)	0.709 ± 0.102 (ab)	0.592 ± 0.102 (b)	-	-	-	-	-	-
SLC2A4	-	-	-	0.012 ± 0.001 (a)	0.007 ± 0.001 (ab)	0.004 ± 0.001 (b)	-	-	-
SOD1	-	-	-	-	-	-	0.181 ± 0.018 (ab)	0.225 ± 0.016 (a)	0.147 ± 0.016 (b)
STAT3	-	-	-	-	-	-	0.098 ± 0.011 (ab)	0.132 ± 0.010 (a)	0.072 ± 0.010 (b)
TNFAIP6	-	-	-	-	-	-	1.439 ± 0.241 (a)	1.817 ± 0.216 (a)	0.301 ± 0.216 (b)
TP53	-	-	-	0.035 ± 0.003 (a)	0.038 ± 0.003 (a)	0.021 ± 0.003 (b)	0.042 ± 0.004 (a)	0.040 ± 0.003 (a)	0.025 ± 0.003 (b)
TXNRD1	-	-	-	0.024 ± 0.002 (ab)	0.030 ± 0.002 (a)	0.018 ± 0.002 (b)	-	-	-
VCAN	-	-	-	-	-	-	5.205 ± 0.527 (b)	7.965 ± 0.472 (a)	4.254 ± 0.472 (b)
VEGFA	-	-	-	0.159 ± 0.020 (a)	0.183 ± 0.020 (a)	0.080 ± 0.020 (b)	-	-	-
VNN1	-	-	-	-	-	-	0.0018 ± 0.0005 (ab)	0.0035 ± 0.0005 (a)	0.0006 ± 0.0005 (b)

All the data are showed as Mean ± S.E.M. Different letters in the same row means statistical difference in the same time point. All results were from five replicates.

Although all correlations were significant ($p<0.05$) the strongest correlation was found between AREG and FSH groups (R=0.891), whereas it was intermediary between FSH and CTRL (0.849) and the lowest value was found between CTRL and AREG (R=0.801) groups.

Almost all genes differentially expressed, showed a decreased profile in the treatment with AREG in comparation to CTRL or FSH. Despite this, four gene (BDNF,

PDE5A, NPR1 and ELOVL1) were discrepant with that profile. In those cases, a major mRNA abundance was observed in the AREG group as showed in the Figure 2.

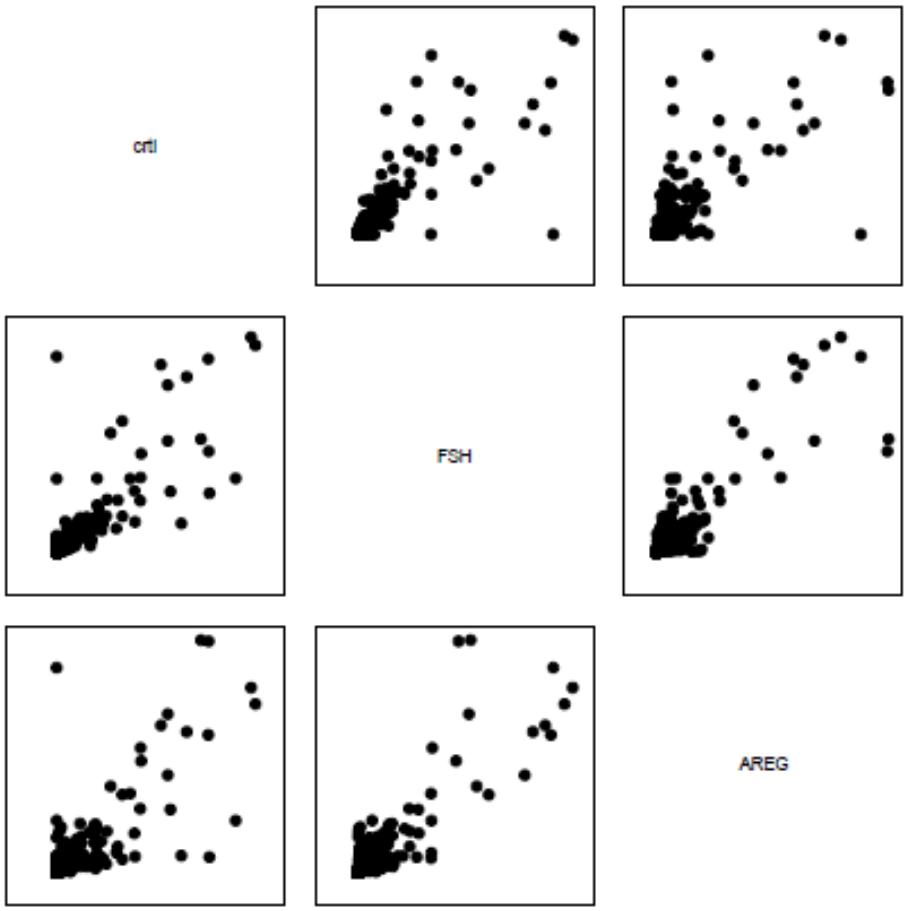

Figure 1. Dispersion graph with the illustrative global gene expression profile of the groups analyzed. Each column represents a group, and each row compare the groups on pairs. The results were obtained from five replicates.

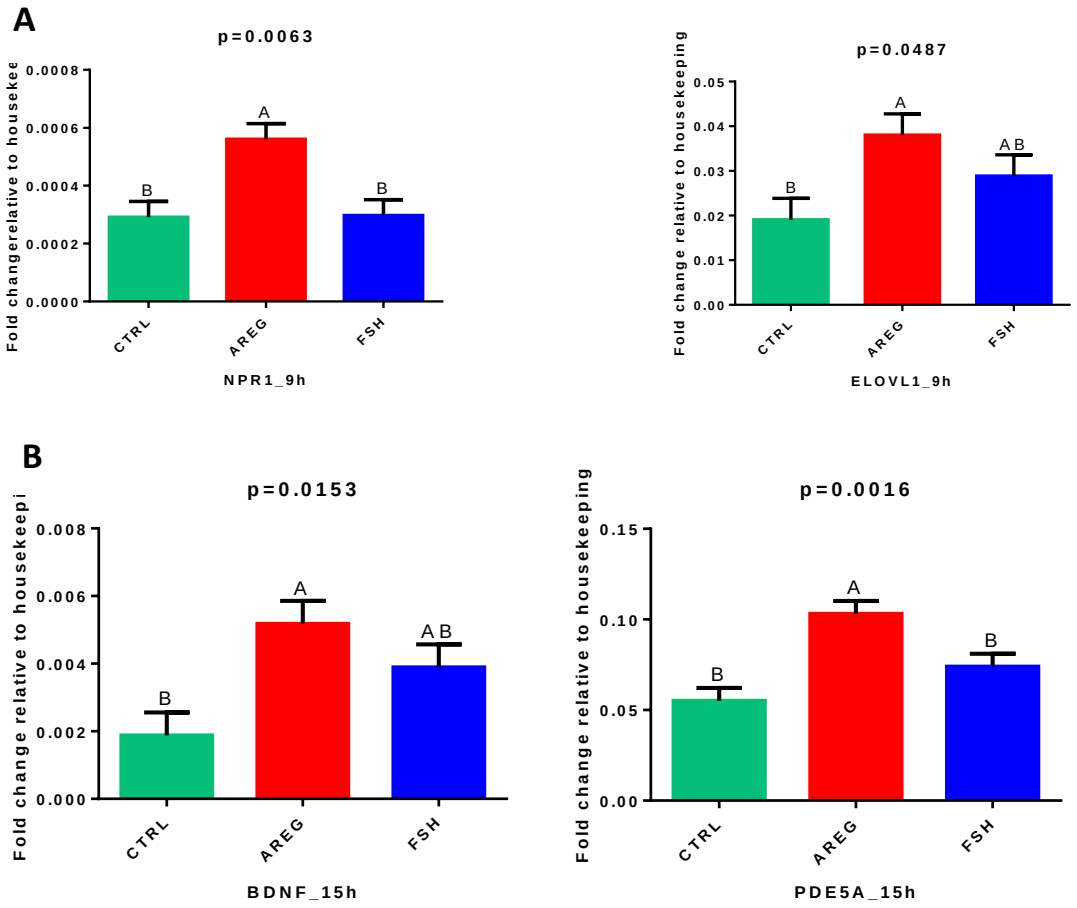

Figure 2. *Cumulus* cell abundance of target transcripts. Bar charts showing the mRNA abundance in the different treatments. A) target genes NPR1 and ELOVL1 at 9 hours of maturation. B) target genes BDNF and PDE5A at 15 hours of maturation. The results were obtained from five replicates. Different letters mean statistical difference.

Contradictory of *cumulus* cells, the oocyte appeared not underwent changes regards on gene expression, independently which medium was used. In all times point evaluated, only two gene were differently expressed. The gene H1FOO, a member of H1 histone family and oocyte-specific, showed major transcript abundance on FSH media at 9 hours, following the profile of the majority of genes. The second gene was the SLC2A4, a member of solute carrier member (facilitated glucose transporter, Figure 3). The

SLC2A4 abundance was increased with AREG at the 15 hours. At the same time point, this gene was downregulated in *cumulus* cells by AREG (data not show).

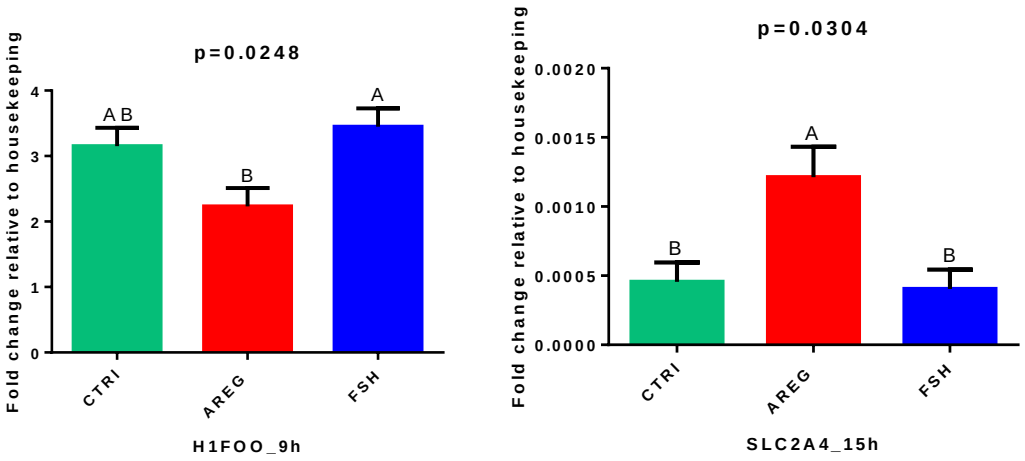

Figure 3. Oocyte abundance of target transcripts. Bar charts showing the mRNA abundance difference among the groups to the genes H1FOO and SLC2A4 from oocyte after 9 and 15 hours, respectively. Different letter means statistical difference among the groups. The results were obtained from five replicates.

From data analysis by ANOVA, a network was constructed with the all 57-target gene with a differently expression. A smaller network was designed (Figure 4) including only the genes with the greatest number of interactions (nodes) among them (confidence score of 0.400). There were 13 genes, and all had some relationship with TP53. Among them, two genes (GADD45A and DICER1) were differently expressed from 9 until 24 hours; three genes (EGFR, HSP90AA1and VEGFA) just at 15 hours of maturation; five genes (BCL2, CASP9, FOXO3, MAPK1 and TP53) were differently expressed from 15 hours until 24 hours. At 24 hours, three genes (CASP3, STAT3 and CDK6) showed differential expression. The transcript abundance of DICER1 was equal in both AREG and FSH media at 9 hours (after this, the abundance was lower in AREG

media than FSH). To the genes EGFR and FSHR at 15 hours, the expression was similar too. The other genes showed a downregulation profile in AREG media compared with FSH. The transcripts abundance is showing in the Table 4.

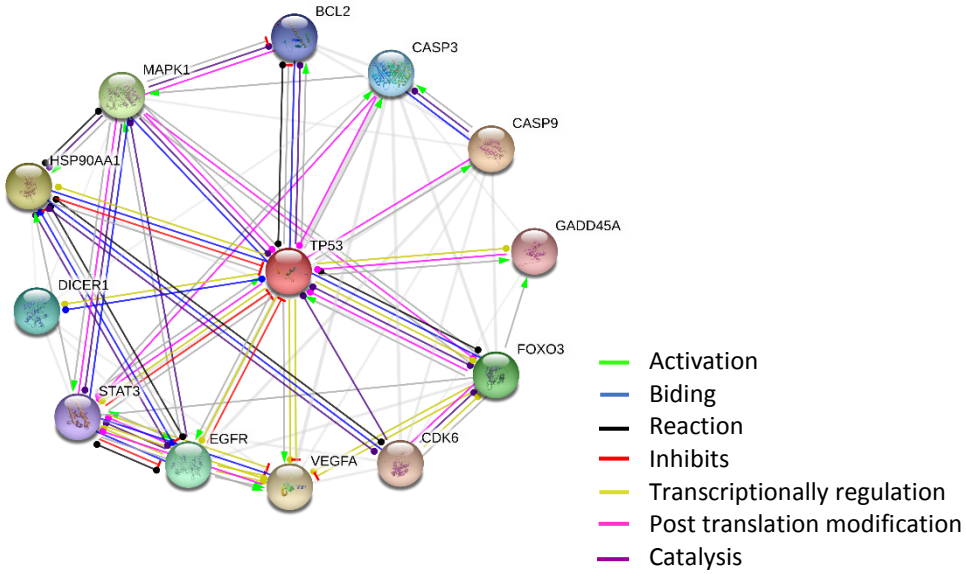

Figure 4. Gene network designed with genes that showed the r interactions among them. The genes in the network was differentially expressed at 9, 15 or 24 hours as indicated in the text. The results were obtained from *cumulus* cells in five replicates. Legend:(→) positive action; (●) unspecific action; (⊦) negative action.

Experiment 2- Alternative IVMs - Meiotic resumption

We tested AREG, FSH or LH alone or in association (AREG/LH and FSH/LH) above the MII rate after 24 hours of maturation treatment. At the end of this time, we found oocytes that were still in the GV stage. The highest GV rate was found in the LH and CTRL groups and, due to an inversely and proportional relationship, the lowest MII rate was found in the same groups. There was no difference in the GVBD rate (Figure 5).

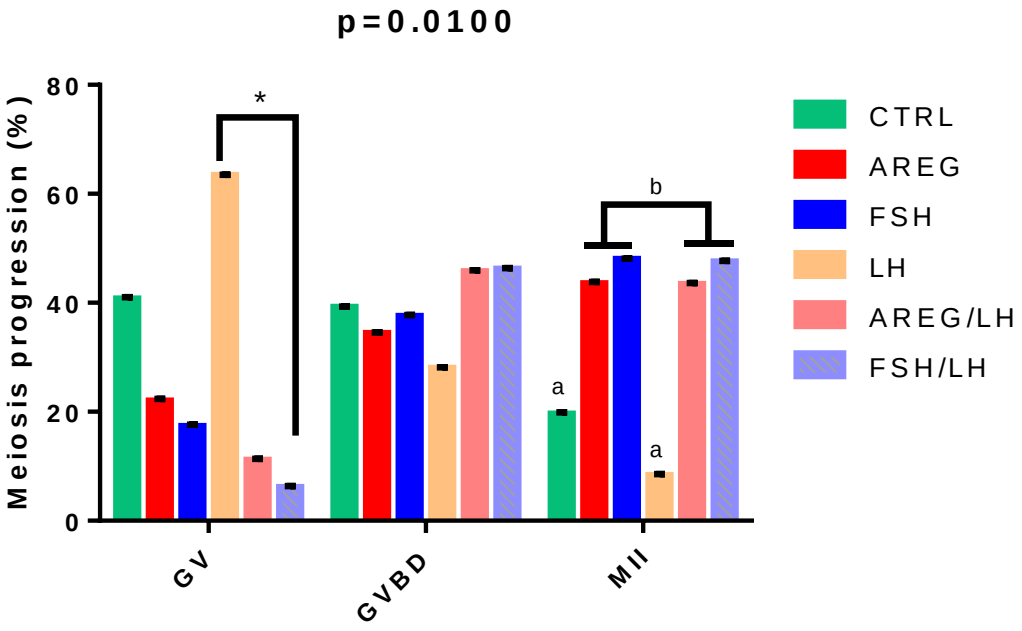

Figure 5. Meiosis resumption. Bars chart showing the rates of GV (germinal vesicle), GVBD (germinal vesicle breakdown) and MII (metaphase II) after 24 hours of maturation. Bars with asterisk (*) are different (GV) or bars with different letters indicate a statistical difference among the groups (MII). CTRL: control group (basic media); FSH group (basic media plus rhFSH); AREG group (basic media plus AREG); LH group (basic media plus rhLH); AREG/LH (basic media plus AREG and LH); FSH/LH (basic media plus FSH and LH). The concentrations used were described in the text above.

Experiment 3 - Embryo Production

On Day 3, we observed the cleavage rate (Figure 6a) and found a difference among the groups. The higher rate was observed in the CTRL and AREG groups, while the LH group produced a low rate in comparison to CTRL group (Figure 6a). When the hatching kinetics were evaluated, was observed a difference in blastocyst development on Day 7 (Figure 6b). There was no difference in the hatching rate on Day 9 (Figure 6c). To gene expression analysis there was no difference when all groups were compared.

However, when just compared the groups CTRL, FSH/LH and LH, there was a difference in the mRNA abundance of PLAC8 gene (Figure 6d).

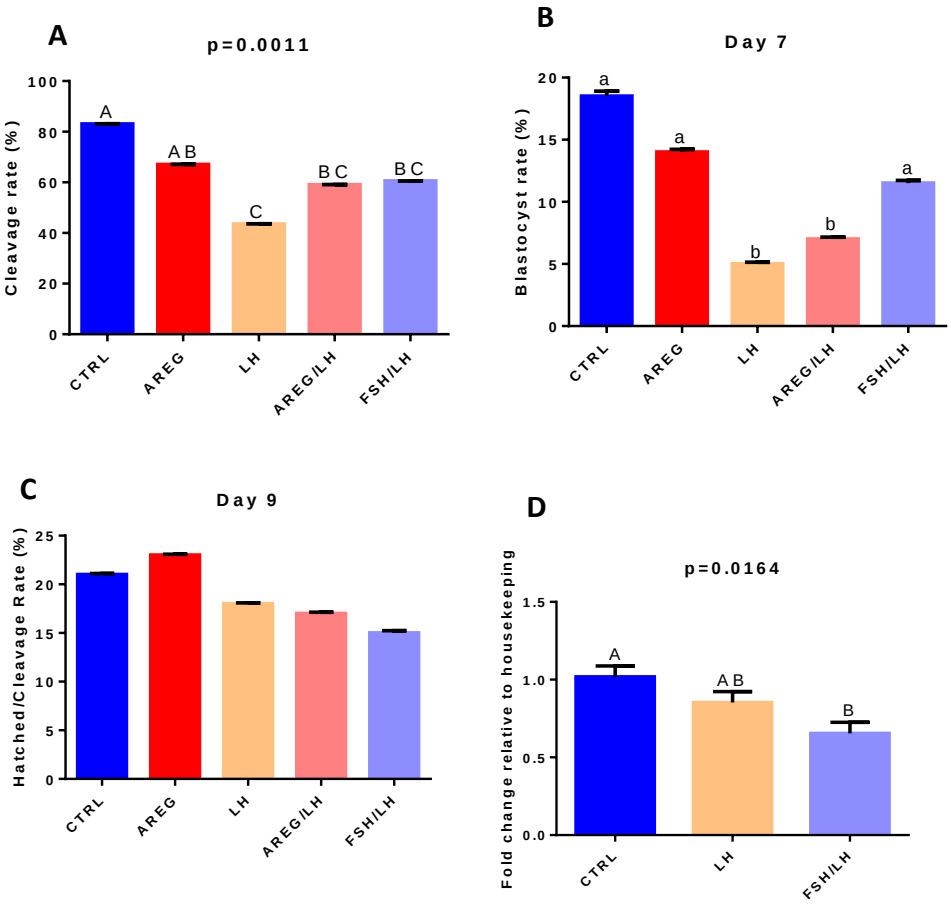

Figure 6. Cleavage and blastocyst rates and PLAC8 transcript abundance. A) The cleavage rate on Day 3 of *in vitro* culture; B) The blastocyst rate on day 7 (p=0.0431); C) The hatching rate on Day 9; D) mRNA abundance of PLAC8 gene. The results were obtained from four replicates. Different letters above the bars indicate statistical difference.

Discussion

In the present study, we demonstrated the maturation with AREG on bovine *cumulus*-oocyte complex and its effects in the gene expression. Furthermore, we tested it

in association or compared with gonadotropins (FSH and LH) in a pre-IVM system using NPPC on embryo production.

As related in the literature, AREG is efficient as much as or superior to FSH in *in vitro* maturation and embryo production. Our results did not support any statistical difference in rates of GVBD or MII as described in porcine (Sugimura *et al*., 2015) or in primates (Peluffo *et al*., 2012). But, numerically, the GVBD rate at 9 h was the highest in the AREG media as the MII rate at 24 h.

The embryo production showed that there was no statistical difference in cleavage or blastocyst formation rates - although the hatching rate was numerical the highest in the AREG media. This, supporting the hypothesis that AREG is an alternative to the high doses of FSH in *in vitro* maturation of bovine oocytes. However, the use of LH (in the tested dose here) or association with AREG or FSH seems to be not efficient on bovine oocyte maturation. The MII rate was low when the oocytes were matured by LH, because it seems to postpone the meiotic resumption by delaying the time that GVBD occurs and prolonging the cytoplasm maturation (Li *et al*., 2011). However, data from embryos gene expression, showed that LH or association with FSH but not AREG, can reduce the transcription of PLAC8 (placental-specific 8), a marker gene of embryo competence and essential to embryo survival. It is expressed on trophectoderm and it is involved in placental development. an upregulation is observed in embryos successfully implanted and that can establish a pregnancy (El-Sayed *et al*., 2006). While potentially deleterious, this observation should be confirmed with the PLAC8 protein evaluation before a final conclusion. Explaining the hatching rate from LH, in our view it is perhaps the NPPC stimulate the meiosis resumption (de Cesaro *et al.*, 2015). In this way, to confirm if LH has no or negative effect in embryo production, we need more data.

Regarding the gene expression, our results are the first to bring some clues on how the AREG affects the general gene expression in bovine *cumulus*-oocyte complex. To oocyte, just two genes were differentially expressed. The first (SLC2A4), is a glucose transporter with high affinity with glucose, insulin-dependent and is related as expressed just on *cumulus* cells and in macaque Rhesus oocytes (Zheng *et al*., 2007), but not in bovine oocytes, contradictory to our results. Here, the increased expression on oocyte maturation with AREG may be correlated with the fact that AREG can increase the glycolysis by reducing the glucose flux through the hexosamine pathway (Sugimura *et al*., 2014). The hexosamine pathway is known to affect the oocyte developmental competence in cattle and pigs (Sutton-McDowall *et al*., 2006). Prochàzka *et al*. (2011) believed that the increase in glycolysis can offer more ATP and is one mechanism for that AREG can promotes the oocyte developmental competence.

The second gene (H1FOO), is a histone 1 oocyte-specific involved in gene expression control during the oogenesis and initial embryogenesis. It is responsible to DNA compaction in eukaryotes cells, that is essential to cell division. It is localized on nucleus of the GV stage, at the chromatin during the metaphase II and at the second polar body (Furuya *et al*., 2007). The expression of H1FOO decrease constantly through oocyte maturation until embryonic genome activation (Tanaka *et al*., 2003; McGraw *et al*., 2006). Considering those data from the literature, the fact of the low expression of this gene by AREG at 9 h and the numerically high rate of GVBD at the same time, we agree that AREG can to conduce the maturation faster than FSH, as shown Prochàzka et al., (2011) in the porcine species.

About cumulus cells gene expression, we observed an upregulation on four genes in the AREG group (NPR1 and ELOVL1 at 9 hours and BDNF and PDE5A at 15 hours). The BDNF (Brain-derived neurotrophic factor) is expressed in granulosa cell and

play a role in the follicle development (Dissen *et al*., 1995; Ojeda *et al.,* 2000). In the bovine, Yi *et al*. (2008) showed that BDNF also promote the development and growth in *cumulus* cell. In humans, it showed to be secreted by *cumulus* cells (Seifer *et al.,* 2002). A recent study reported that BDNF was able to activate both Akt and the ERK1/2 (Chen *et al.,* 2018) and active MAPK pathway via Ras beyond extend the PKB phosphorylation (Zhang *et al.,* 2010). *In vivo*, its expression is increased in response to the pre-ovulatory LH surge and may acts with EGF-*like* peptide to promote the extrusion of the first polar body (Kawamura *et al.,* 2005). We know that AREG propagates the LH stimulus and the difference on expression between the FSH and AREG suggest that they act in different way. Maybe the AREG containing medium is closer related to the physiological via, although the action of FSH (as presented as well *in vivo* during the pre-ovulatory LH surge) could be not ruled out.

The ELOVL1 (Fatty acid elongase 1) is member of the family that are responsible for elongation of fatty acids producing a very long chain of fatty acids either saturated, monounsaturated or polyunsaturated (Wang *et al.,* 2006). ELOVL1 is specifically involved in elongation of saturated and monounsaturated fatty acids (SFAs and MUFAs, respectively; Jakobsson *et al.,* 2006; revised by Kihara, 2011). In HeLa cells, a knockdown of ELOV1 promotes change in cells membrane and increase the cellular susceptibility to apoptosis (Sassa *et al.,* 2012).

The NPR1 (Natriuretic Peptide Receptor 1) makes part of natriuretic system and is expressed in the cell surface. It has an effect in the steroidogenesis and corpus luteum formation (Kobayashi *et al.,* 2002; de Cesaro *et al.,* 2018). The NPPA and NPPB have high affinities with this receptor (de Cesaro *et.al.,* 2018).

The PDE5A is member of phosphodiesterase family that hydrolyze cyclic nucleotides. It is specifically from granulosa cells and hydrolyze cGMP. We know that

PDE5A can be to activate directly by the LH (Gupta *et al.*, 2017) or indirectly through the activation of EGFR (Park *et al.*, 2004; Hsieh *et al.*, 2007). In the second way, maybe AREG has a participation. Vaccari *et al.* (2009) using the same concentration of AREG that induces maturation in oocyte, noted that the concentration of cGMP was reduced shortly after 30 minutes of culture. In those facts, we believe that AREG could potentially increase the PDE5A production what in turns could decrease in cGMP levels and, at the end, speed up the meiosis resumption as seen in porcine species (Procházka *et al*., 2011).

Except for the genes cited above, we observed a downregulation on the major number of genes in AREG group compared with FSH on the time course experiment. We believe that this is due to the AREG and FSH cannot active the same pathways. For example, both of than are able to active the MAPK and PI3K/Akt pathway, but AREG cannot active the cAMP-PKA, whereas FSH can. That pathway is responsible to stimulate the HAS2 expression, that is essential to cumulus expansion and is reduced in the maturation by AREG. But, it is not prejudicial to oocyte maturation, it leads just a lower cumulus expansion, because the hyaluronic acid is not stable in this condition. Another explanation is because FSH can induce the progesterone synthesis whereas AREG not (Prochàzka *et al*., 2011). Progesterone helps in the induction and manutention of TACE/ADAM17 expression. It is a metalloproteinase member family responsible to cleave AREG from the cellular membrane (Yamashita *et al*., 2010).

Using the STRING platform, we designed a network with all genes differentially expressed (Figure 7) with a high score confidence (>0.700).

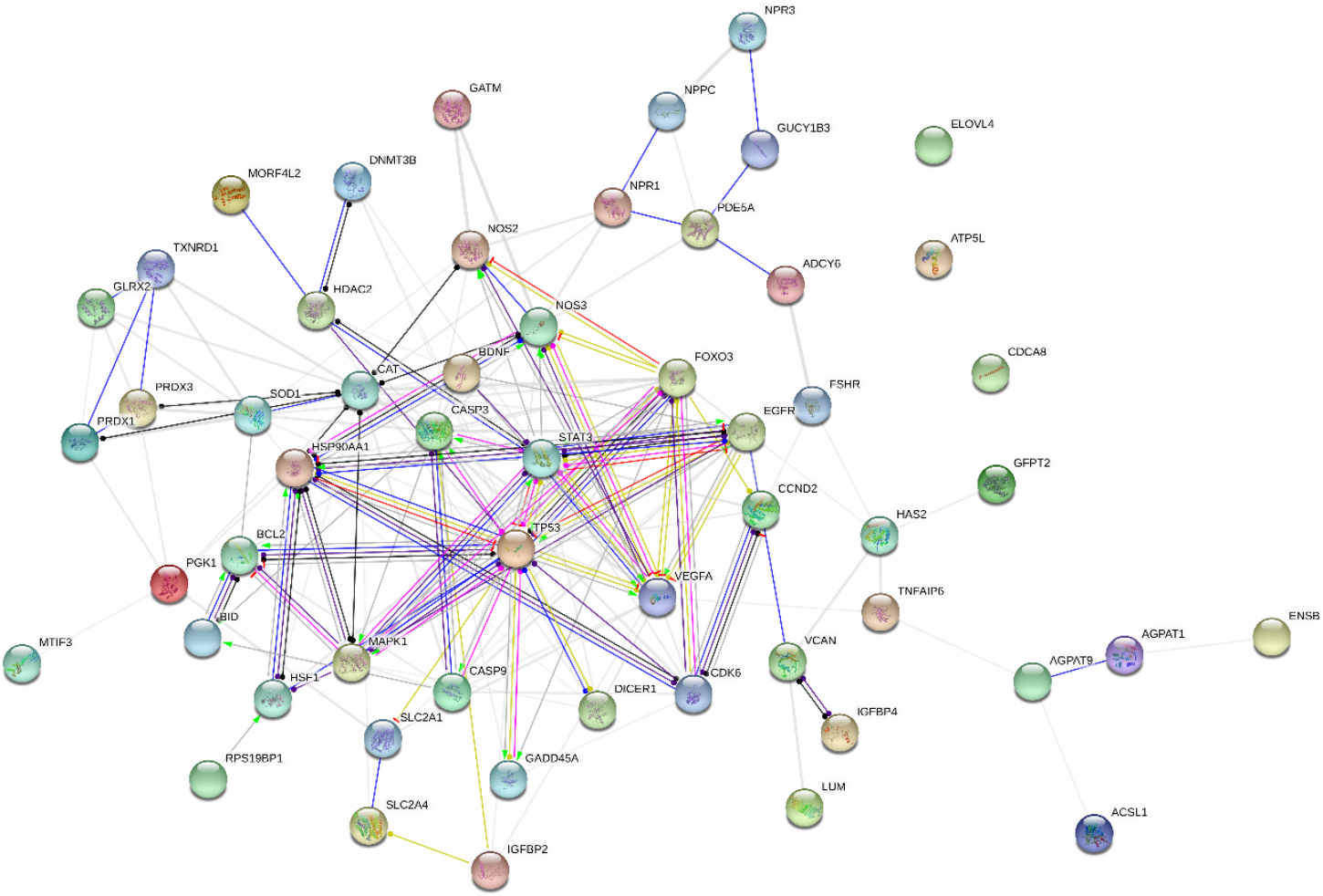

Figure 7. The entire network formed with all genes differentially expressed in this work. The results were obtained from 5 replicates of *cumulus* cells samples.

The TP53 gene induces growth arrest or apoptosis depending on the physiological circumstances and cell type. It is also involved in cell cycle regulation as a trans-activator that acts to negatively regulate cell division by controlling a set of genes required for this process. In the follicular environment, TP53 is related to apoptosis, atresia and follicular stress and play a role in the oocyte competence acquisition. Is highly expressed in granulosa cells from those follicles (Landry and Sirard *et al.*, 2018). In the presence of FSH during in vitro maturation, the TP53 levels decrease, suggesting that FSH can reduce the apoptosis (Khan *et al.*, 2015). So, to our knowledge, the smallest transcript abundance of TP53 and the faster maturation with AREG, promoted an environment under less stress than that maturated in FSH or CTRL group.

Conclusion

We concluded that AREG can support the full maturation of bovine COCs but in a faster way than FSH - that could be the reason in a general downregulation expression. The LH associated with a pre-IVM system with NPPC can support the production of blastocyst and they retained their hatching potential. In this way, there was an evidence of the LH capacity to the oocyte maturation but in a slower way than control and poorly effective to produce blastocyst when compared to the control, AREG and FSH/LH groups.

Abbreviations

Not applicable

Additional files

Selected target genes list

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Not applicable

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Availability of data and materials

All data are available in the text

Authors' Contributions

MP conduced all experiments and wrote the initial draft of manuscript. EMP performed the qRT-PCR by BioMark HD. FFF helped with the statistical analyze and all steps of molecular biology tests. PHS helped in all embryo production process and in time course and maturation experiments. MFGN designed the experiments, obtained founds to this work execution and review the manuscript. All authors read and approved the final manuscript.

Competing interest

The authors declare there is no conflict of interest.

Consent for publication

Not applicable

Ethics approval and consent to participate

Not applicable

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MP conduced all experiments and wrote the initial draft of manuscript. EMP performed the qRT-PCR by BioMark. FFF helped with the statistical analyze and all steps of molecular biology tests. MFGN designed the experiments, obtained founds to this work execution and manuscript editing. All authors read and approved the final manuscript.

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CONSIDERAÇÕES FINAIS

Em seu conceito inicial, esse trabalho foi delineado para esclarecer a informação não consensual da presença de receptores de LH (LHR) nas células do *cumulus* e que sua expressão poderia ser induzida em algum momento da maturação *in vitro* utilizando o peptídeo ampirregulina (AREG), o que foi refutado pelos dados de expressão gênica. Esperávamos ainda que o perfil de expressão de genes relacionados a apoptose, metabolismo lipídico, retomada meiótica, entre outros fosse distinto entre AREG e FSH sendo isto corroborado pelos dados deste trabalho.

Como não houve expressão que indicasse em que horário o LHR é expresso nas células do *cumulus*, testamos sua eficácia na maturação e em associação com AREG ou FSH. As taxas de maturação para o LH sozinho, foram baixas e reforçam os dados da literatura. No entanto, quando utilizamos um sistema de pré-maturação (NPPC), o meio contenho apenas LH foi capaz de gerar embriões que eclodiram até o 9º dia de cultivo. Esses dados nos dão indício de que o LH tem capacidade de fazer com que o oócito conclua a maturação, ainda que de um modo mais lento e menos efetivo do que os demais ligantes testados.