Universidade Estadual Paulista “Júlio de Mesquita Filho”
Instituto de Biociências de Botucatu
PPG – Farmacologia e Biotecnologia

Desenvolvimento embrionário e efeitos da criopreservação após a suplementação deCNP no cultivo *in vitro* de embriões bovinos

Camila Bortoliero Costa Giovannetti

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“A satisfação está no esforço e não apenas na realização final”.

Mahatma Gandhi

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Preâmbulo

RESUMO

A produção *in vitro* de embriões bovinos avança tecnologicamente a cada ano. No entanto, a criopreservação desses embriões não é condizente com as melhorias gerais da técnica. Um dos principais fatores relacionados com as baixas taxas de sobrevivência pós-criopreservação é o conteúdo e o perfil lipídico diferentes nos embriões produzidos *in vitro* (PIV). Estes estão presentes em forma de gotículas lipídicas no citoplasma e na membrana celular dos embriões. Dessa forma, algumas tentativas de adição e remoção de moléculas, no meio de cultivo *in vitro* (CIV), já foram testadas com o objetivo de reduzir ou alterar o conteúdo lipídico, o que resultou em melhorias após o aquecimento dos embriões submetidos à criopreservação. Embora a utilização do peptídeo natriurético do tipo-C (CNP) – um modulador das concentrações de AMPc e GMPc intracelular – já tenha sido descrita na maturação ou na pré-maturação *in vitro*, o seu uso na modulação do perfil lipídico das células embrionárias no CIV de embriões bovinos ainda é incipiente. Desta forma, os objetivos gerais desta tese foram: Capitulo 1- detectar a presença do receptor do peptídeo natriurético NPR2 em embriões pré-implantação, encontrar uma concentração não embriotóxica para o cultivo do embrião bovino e avaliar possíveis modulações que o cultivo com CNP pode causar no desenvolvimento embrionário; e Capítulo 2- avaliar se a adição do CNP no cultivo *in vitro* de embriões bovinos altera sua criotolerabilidade através da modulação do teor e perfil lipídico, assim como com a modulação de transcritos vinculados ao metabolismo embrionário. Foi detectado e quantificado o receptor NPR2 (pela primeira vez e até onde tenhamos conhecimento) em estágios pré-implantação do embrião bovino produzido *in vitro*. Além disso, foi determinado que o uso de CNP em concentração superior, àquela descrita na literatura, pode alterar o metabolismo embrionário com base na abundância de transcritos e na análise do perfil lipídico. Entretanto, não houve modificação no teor lipídico de embriões bovinos cultivados com CNP. Ainda, foi observada uma manutenção nas taxas de produção após a vitrificação dos embriões cultivados com CNP. Provas funcionais *in vivo* poderão comprovar, ou não, se essas alterações são traduzidas em uma melhor performance reprodutiva por parte desses embriões tratados com o CNP no CIV.

Palavras-chave: Blastocisto; CNP; Cultivo; Criotolerabilidade; Perfil lipídico.

ABSTRACT

The *in vitro* production of bovine embryos advances technologically every year. However, the cryopreservation of these embryos is not consistent with the general improvements in the technique. One of the main factors related to low post-cryopreservation survival rates is the different lipid content and profile in *in vitro* produced (IVP) embryos. These are present in the form of lipid droplets in the cytoplasm and cell membrane of the embryos. Thus, some attempts to add and remove molecules in the *in vitro* culture (IVC) medium have already been tested with the aim of reducing or altering the lipid content, which resulted in improvements after heating the embryos submitted to cryopreservation. Although the use of C-type natriuretic peptide (CNP) – a modulator of intracellular cAMP and cGMP concentrations – has already been described in *in vitro* maturation or pre-maturation, its use in the modulation of the lipid profile of embryonic cells in the IVC from bovine embryos is still incipient. Therefore, the general objectives of this Thesis were: Chapter 1- detect the presence of the natriuretic peptide receptor NPR2 in pre-implantation embryos, find a non-embryotoxic concentration for bovine embryo culture and evaluate possible modulations that culture with CNP can cause in embryonic development; and Chapter 2 - evaluate whether the addition of CNP in the *in vitro* culture of bovine embryos modificate its cryotolerability through the modulation of the lipid content and profile, as well as with the modulation of transcripts linked to embryonic metabolism. The NPR2 receptor was detected and quantified (for the first time and as far as we know) in pre-implantation stages of bovine embryo produced *in vitro*. Furthermore, it was determined that the use of CNP at a concentration higher than that described in the literature may alter embryonic metabolism based on the abundance of transcripts and analysis of the lipid profile. However, there was no change in the lipid content of bovine embryos cultured with CNP. Furthermore, maintenance of production rates was observed after vitrification of embryos cultured with CNP. Functional tests *in vivo* will be able to confirm, or not if these alterations are translated into a better reproductive performance on the part of these embryos treated with CNP in the IVC.

Keywords: Blastocyst; CNP; Cultivation; Cryotolerability; Lipid profile.

PREÂMBULO

Esta Tese para o programa de Pós-graduação em Farmacologia e Biotecnologia se encontra dividida em quatro sessões principais. A primeira, denominada **"*Introdução"***, descreve o contexto e a problemática relacionada com este trabalho de maneira mais profunda e detalhada. As duas partes subsequentes, Capítulo 1 - ***"Developmental and molecular effects of CNP supplementation in in vitro culture of bovine embryos"*** *e* Capítulo 2 - ***"Can the use of C-Type Natriuretic Peptide in in vitro culture of bovine embryos alter the characteristics of their cryotolerance?",*** correspondem a duas propostas de manuscritos a serem submetidos como artigos resultantes do trabalho efetuado. Finalizando, a sessão ***"Considerações Finais"*** compreende as conclusões gerais (descrevendo de forma sucinta os resultados obtidos).

Conforme normas do programa de Pós-graduação, o presente exemplar consiste na versão definitiva da tese a ser encaminhada à biblioteca e que comporá o conjunto de publicações do Instituto de Biociências de Botucatu da UNESP. Entretanto, os artigos científicos aqui apresentados estão no prelo, mas não comprometidos com a publicação em quaisquer periódicos, estando, portanto, passíveis de correções e modificações de amplo espectro, especialmente por parte da banca de defesa.

INTRODUÇÃO

As biotécnicas da reprodução contribuem decisivamente para a cadeia produtiva da pecuária de corte e de leite. Dentre as biotécnicas disponíveis, a produção *in vitro* (PIV) de embriões destaca-se no cenário mundial. Em 2020 um total de 1.156,422 mil embriões foram produzidos *in vitro* no mundo, cerca de 12% a mais que em 2019 (1.156,422 *vs.* 1.031,567, respectivamente; IETS, 2021). No entanto, apesar dos recentes avanços relacionados com a PIV, a baixa eficiência nos processos de criopreservação ainda se mostra um gargalo no avanço comercial da técnica. Existem hoje inúmeros métodos de criopreservação, sendo eles classificados basicamente em criopreservação com a curva lenta de resfriamento e vitrificação (Sudano *et al*., 2016).

Embriões produzidos *in vitro* possuem alta sensibilidade à criopreservação a qual, aparentemente, está associada com o maior conteúdo lipídico presente nas células desses embriões quando comparados aos produzidos *in vivo* (Camargo *et al*., 2011). Apesar dos avanços nas biotécnicas *in vitro*, a técnica em si, ainda proporciona estresse para os oócitos e embriões afetando o desenvolvimento (Melo-Sterza *et al*., 2021).

Sabe-se que a técnica *in vitro* altera os embriões, devido a inúmeros fatores, como manipulação excessiva, estresse oxidativo, consequentemente o surgimento de espécies reativas a oxigênio, meios utilizados e principalmente a inclusão do (SFB; Sudano *et al*., 2016).

Assim, embriões PIV, possuem um elevado depósito lipídico, sendo a adição do SFB no cultivo desses embriões uma das possíveis explicações (Paschoal *et al*., 2017). Apesar dos lipídios serem um obstáculo na PIV, eles possuem extrema importância nos processos metabólicos, diferenciação celular, quimiotaxia e ajudam a coordenar eventos fundamentais durante o desenvolvimento embrionário inicial (Sudano *et al*., 2016).

Lipídios como os triacilglicerídeos (TAG), a principal classe lipídica encontrada no citoplasma das células de mamíferos, são armazenados como gotículas lipídicas (Sudano *et al*., 2016) e, nos processos de criopreservação, a sua presença é um fator possivelmente comprometedor devido às injúrias celulares que causa. Em situações de alta demanda energética, pode ocorrer a hidrólise dos TAG no organismo, gerando ácidos graxos e glicerol em um processo denominado lipólise (Ravnskjaer *et al*., 2015; Razza *et al*., 2018). Sendo que a detecção e caracterização desses lipídeos em oócitos e embriões mamíferos, somente foi possível com o avanço de técnicas que utilizam como instrumento principal a espectrometria de massas (MS; Ferreira *et al*., 2012; Pirro *et al*., 2014; Laskowski *et al*., 2018).

Além da detecção dos lipídios, a alteração do perfil ou diminuição dos lipídios para as biotécnicas é altamente utilizado. Sabe-se que a adenosina monofosfato cíclica (AMPc) está envolvida em inúmeras atividades celulares (Razza *et al*., 2018). Sua modulação nas células embrionárias já foi associada com a redução do depósito lipídico intracitoplasmático (Paschoal *et al*., 2017), juntamente com as modulações de guanosina monofosfato cíclica (GMPc) por meio da inibição da enzima fosfodiesterase 5 (PDE5; Schwarz *et al*., 2018). Em um estudo recente conduzido por nosso grupo de pesquisa, foi identificada a alteração do conteúdo lipídico em embriões bovinos com a utilização do forskolin (um indutor inespecífico da adenilato ciclase) e da 3-isobutil-1-metilxantina (um inibidor da fosfodiesterase não específica PDE3), fármacos estes que são moduladores da concentração de AMPc intracelular (Razza *et al*., 2018).

O precursor do peptídeo natriurético do tipo C (C-type natriuretic peptide - CNP) é um potencial modulador das concentrações de AMPc intracelulares e já foi utilizado para a manutenção do bloqueio meiótico em oócitos de diversas espécies (Dubeibe *et al*., 2017). Em bovinos, sabe-se que o CNP é produzido fisiologicamente pelas células da

granulosa, *cumulus* e oócitos (Xi *et al*., 2018), diferentemente, em camundongos o CNP é produzido apenas pelas células da granulosa e se liga ao seu receptor de eleição CNP tipo 2 (NPR2) presente nas células do *cumulus*, oócitos e embriões pré-implantação (Xi *et al*., 2019). A ativação do NPR2 induz a síntese de GMPc, que é transferido – das células do cumulus para o oócito – por meio de projeções transzonais as quais terminam em junções do tipo gap com a membrana citoplasmática do oócito. Nas células do cumulus, o GMPc tem ação inibitória sobre a fosfodiesterase 3A (PDE3A), mantendo altas as concentrações de AMPc no oócito e sustentando o bloqueio da meiose. A interação do CNP com as células do *cumulus* bem como a identificação das fosfodiesterase 3 e 5 em oócitos já é conhecida. No entanto, a relação entre o CNP e o embrião bovino possui escassos relatos na literatura (Botigelli *et al*., 2018). Do mesmo modo, a identificação das enzimas PDE-3 e 5 em embriões bovinos e sua relação com a concentração de AMPc e GMPc ainda necessita de mais estudos (Schwarz *et al*., 2018).

Como o CNP modula as concentrações de AMPc – fato este já demonstrado em oócitos bovinos – e está relacionado com o metabolismo lipídico em embriões bovinos o objetivo, com este projeto de pesquisa, foi detectar a presença de receptor do tipo NPR2 (receptor de eleição CNP tipo 2) em embriões pré-implantação, determinar os efeitos *in vitro* que a suplementação de CNP no cultivo *in vitro* teria sobre a taxa de produção embrionária, o conteúdo e o perfil lipídico, a abundância de alguns transcritos alvo (genes relacionados com o próprio sistema CNP-NPR2, e sua criotolerabilidade após a exposição dos embriões ao CNP.

REVISÃO DE LITERATURA

Alterações do metabolismo embrionário *in vitro*

Embriões produzidos *in vitro* apresentam menor rendimento de blastocisto por oócitos e menor qualidade dos embriões, contribuindo para a maior sensibilidade a criopreservação, comparados ao produzidos *in vivo*. A qualidade intrínseca do oócito no início da maturação *in vitro* é relacionada a falha de até 60% do avanço para o estágio de blastocisto. No entanto, entre os estágios de zigoto e blastocisto, ocorrem eventos determinantes para a qualidade embrionária (Lonergan *et al*., 2002). Estudos demostraram que o sistema de cultivo influencia na expressão de alguns genes (Lonergan *et al*., 1999; Khosla *et al*., 2001; Lee *et al*., 2001;) e no metabolismo do embrião, sendo o principal determinante na qualidade do blastocisto (Kiijn *et al*., 2001).

Apesar dos avanços nas biotécnicas *in vitro*, o processamento *in vitro* ainda proporciona estresse para os oócitos e embriões afetando seu desenvolvimento (Melo-Sterza *et al*., 2021). Características como, citoplasma mais escuro (Pollard *et al*., 1994), maior proporção de lipídeos da classe dos TAGs (Abd El Karek *et al*., 2000), vacúolos nas células trofoblásticas, alterações nas conexões intercelulares (Fair *et al*., 2001) e maior fragilidade da zona pelúcida (Duby *et al*., 1997) são relatadas como diferenças entre os embriões de produção *in vitro* e *in vivo*.

Em embriões bovinos, o consumo de glicose aumenta gradativamente com o desenvolvimento. Antes da compactação das mórulas, o requerimento de energia é baixo e sua produção baseia-se na fosforilação oxidativa para gerar adenosina trifosfato (ATP) (Gardner, Harvey, 2015). Com a ativação do genoma, ocorre o aumento do requerimento de energia e ativação do metabolismo oxidativo (Gardner, Harvey, 2015; Rollo *et al*., 2017). Nas etapas de compactação e formação de blastocele o consumo de glicose e o metabolismo aumentam. Simultaneamente ocorre maior síntese de proteína e atividade

de íons envolvidos na formação da blastocele (Collinet e Lecuit, 2021). Após a formação do blastocisto, as células sofrem diferenciação e modificação no perfil metabólico, com a massa celular interna consumindo maior quantidade de glicose e o trofectoderme consumindo alto nível de piruvato e produzindo lactato (Gopichandran e Leese, 2003). Devido as características fisiológicas do blastocisto é necessário encontrar o equilíbrio entre a produção e a velocidade de consumo de energia necessário para o desenvolvimento embrionário (Dias *et al*., 2017).

Diversos problemas estão associados ao metabolismo embrionário, e alguns danos aos embriões, chegam a ser irreversíveis (Dias *et al*., 2017). A tensão de oxigênio empregada durante o cultivo *in vitro* é um dos fatores que tem influenciado no desenvolvimento embrionário. Embriões bovinos, produzidos em sistemas *in vitro* de alta tensão de oxigênio, apresentam maior proporção de genes relacionados com a oxidação (*SOD1, GPX1* e *PRDX1*), estresse do retículo endoplasmático (*HSF1* e *HSP90*) e mecanismos de morte celular (*BAX, CASP9* e *DDIT3*), além de quantidades aumentadas de radicais livres. Evidenciando que, a tensão de oxigênio está altamente relacionada a modulação do metabolismo embrionário (De Lima *et al*., 2020).

Embriões produzidos em sistemas *in vitro* de baixa tensão de oxigênio apresentam benéfica regulação de genes envolvidos no transporte de glicose (*GLUT3*), intermediários/reguladores da glicólise (*PFK1* e *GAPDH*), conversão de piruvato em lactato (*LDHA*) e elevada expressão de proteínas envolvidas na síntese de colesterol (*HMGCS1* e *HMGCR*) e degradação de ácidos graxos (*ACAT2* e ACSL4) (Shahzad *et al*., 2020). A melhor qualidade e criotolerância de embriões produzidos *in vitro* sob baixa tensão de oxigênio tem sido explicadas pelo Efeito Warburg (internalização da glicose e sua conversão em lactato) e suas reações, que desempenham um papel essencial no desenvolvimento embrionário (De lima *et al*., 2020; Melo-Sterza, Poehland, 2021).

A glicose e os ácidos graxos são as fontes mais comuns de energia para as células de mamíferos e seus substratos passam por oxidação nas mitocôndrias. Apesar dos processos para produção de energia ser rigorosamente controlados, estes acabam se adaptando as condições ambientais, em que, as células são submetidas (Warzych e Lipinska, 2020). O excesso de glicose em embriões produzidos *in vitro*, favorece a formação de lipídeos intracitoplasmáticos e desequilíbrio no estado de oxido-redução, ocasionando alterações na função mitocondrial, promovendo um acumulo de lipídeos (De La Torresanchez *et al*., 2006; Barcelo-Fimbres, Seidel Júnior, 2007).

Embriões bovinos cultivados em ambientes *in vitro* com baixa concentração de oxigênio e de glicose (0,6 mM) respondem o mais próximo, de um eficaz metabolismo. Apresentando mitocôndrias funcionais com capacidade de alta produção de ATP e baixa quantidade de espécies reativas de oxigênio (EROs) (De Lima *et al*., 2020). O adequado funcionamento do metabolismo energético é importante para a capacidade de metabolização de substratos para produção de energia (Dias *et al*., 2017).

Os lipídeos atuam como fonte de energia para oócitos e embriões (Dias *et al*., 2017), sendo potencialmente um reservatório de energia para o desenvolvimento embrionário inicial antes da ativação do genoma embrionário e estão envolvidos na biossíntese da membrana plasmática (Melo-Sterza, Poehland, 2021). Ainda, participam nos processos metabólicos (Lehner, Quiroga, 2016) e quimiotaxia (Sudano *et al*., 2016). Apesar de sua extrema importância para o desenvolvimento embrionário, em sistemas *in vitro*, ocorre um acumulo considerável de lipídios intracitoplasmáticos em oócitos e embriões bovinos (Dias *et al*., 2017) e o seu armazenamento como gotículas lipídicas (Sudano *et al*., 2016; McKeegan *et al*., 2011). Esse acumulo é consequência do estresse durante o processo de desenvolvimento embrionário *in vitro* (Melo-Sterza, Poehland, 2021).

A presença de gotículas lipídicas é um fator comprometedor no processo de criopreservação e aumenta os riscos de lesões celulares (Costa *et al*., 2020; Melo-Sterza, Poehland, 2021). A formação abundante de gotículas lipídicas está relacionada a alterações na função mitocondrial, sendo que as classes de triglicerídeos, ácidos graxos livres, colesterol e fosfolipídios tem sido associada a baixa sobrevivência embrionária pós-criopreservação (Dias *et al*., 2017; Melo-Sterza, Poehland, 2021). Uma possível explicação para o maior teor lipídico dos embriões produzidos *in vitro,* está relacionada à suplementação de SFB no meio de cultura *in vitro* (Moore *et al*., 2007; Sudano *et al*., 2011; Paschoal *et al*., 2016).

O desenvolvimento embrionário *in vivo* ocorre sob baixa tensão de oxigênio, com equilíbrio na produção de EROs e de substâncias antioxidantes. Fisiologicamente, os radicais livres tem pronunciado efeitos no DNA, RNA e na síntese de proteínas, porém, podem alterar a membrana celular, aumentar o pH intracelular e interferir na função mitocondrial (Dias *et al*., 2017). Embriões produzidos *in vitro*, principalmente em sistemas de alta tensão de oxigênio (20%), sofrem desequilíbrio na produção e/ou acumulo de EROs, caracterizando o estresse oxidativo (EO). Esse desequilíbrio pode produzir efeitos deletérios no desenvolvimento embrionário como, alterações metabólicas, redução dos níveis de ATP, peroxidação lipídica e alterações na síntese proteica, na permeabilidade da membrana e nas funções mitocondriais e do reticulo endoplasmático (Takahachi *et al*., 2000; Guerin *et al*., 2001; Cagnone e Sirad, 2013; Yoon *et al*., 2014).

Perfil lipídico do embrião produzido *in vitro*

Os perfis lipídicos dos embriões durante o primeiro estágio de clivagem exibem várias semelhanças. No entanto, no estágio de 8 a 16 células, quando o genoma embrionário bovino é ativado, o perfil lipídico do embrião é altamente alterado, o que pode estar correlacionado com a ativação de mecanismos que regulam o metabolismo lipídico para preparar sua transição para a mórula e estágios de blastocisto (Melo-Sterza, Poehland, 2021). Já foi demonstrado uma redução significativa de gotas lipídicas da fase de mórula para o estágio de blastocisto (Sudano *et al*., 2016). Além disso, sabemos da diferença de conteúdo e perfil lipídico de embriões PIV comparados aos embriões *in vivo*.

Diversos estudos tem demostrado que o acúmulo excessivo de gotículas lipídicas intracelulares durante a desenvolvimento embrionário *in vitro* está associado a efeitos negativos na qualidade dos embriões, aumentando sua sensibilidade ao estresse oxidativo e criopreservação (Risos *et al*., 2002; Pereira *et al*., 2008; Sudano *et al*., 2016). Diante deste cenário, tentativas de modificações nos meios de cultivo são feitas, com o objetivo de reduzir o acúmulo ou modificar o perfil de lipídios em embriões PIV. Ainda, almeja-se atingir condições *in vitro* que, reduzam ou neutralizem os fatores de estresse, e consequentemente favoreçam o metabolismo embrionário. Abe *et al*. (2001) mostraram que o meio contendo SFB, em contraste com o meio sem a presença do soro, levou ao acúmulo anormal de gotículas lipídicas em blastocistos iniciais, consequentemente, problemas após a criopreservação. Além disso, também demonstraram que após o CIV com o SFB houve gotículas lipídicas gigantes e (>6 µm) e altos níveis de mitocôndrias imaturas (Abe *et al*., 2001). Em estudo conduzido por Del Collado *et al*. (2016) foi demonstrado que a utilização de SFB em comparação a albumina sérica em meio de maturação, pode elevar até 18 vezes as concentrações lipídicas. No entanto, os autores acima, não encontraram alterações no blastocisto ou nas taxas de prenhez após o embrião transferido (Choi *et al*., 2019). Além disso, embriões cultivados em alta tensão de

oxigênio (20%) também apresentaram metabolismo lipídico diferente daqueles cultivados em baixa tensão de oxigênio (5%). Houve modulação na expressão de genes responsáveis pela síntese de ácidos graxos, também quantidades relativas aumentadas de ácidos graxos livres totais, e particularmente de ácidos esteárico e oleico em embriões cultivados em 20% de O2. (De Lima *et al*., 2020). O excesso de ácidos graxos livres acumulados intracelularmente pode causar lipotoxicidade (Mylonis, Simos, Paraskeva, 2019), desta forma, com o objetivo de evitar essa alteração, as células podem converter os ácidos graxos livres em TAGs neutros que são armazenados em gotículas lipídicas e servem como depósitos de energia (Wang *et al*., 2017). Previne a formação de radicais livres, com as gotículas lipídicas servindo como blocos para a produção de ésteres de esteróis essenciais e fosfolipídios necessários em células altamente proliferativas para a biogênese de novas membranas (Ufer, Wang, 2011).

Além da interferência própria da técnica de produção *in vitro,* cada etapa de desenvolvimento embrionário possui lipídios de diferentes classes em maiores ou menos abundâncias. Em oócitos bovinos foi possível observar uma grande variação entre oócitos imaturos e maturados. As fosfatidilcolinas se apresentaram em maior abundância em oócitos imaturos, já os ácidos graxos livres se apresentaram em maior quantidade nos oócitos maturados (De Lima *et al*., 2018). Fato este, pode estar relacionado nos oócitos maturados, pois os ácidos graxos livres são presentes tanto no fluido folicular quanto nos estoques intracelulares do complexo cumulus oócito (Li, Butka, Wang, 2014). O oócito maduro é rapidamente capaz de incorporar os ácidos graxos que estão no seu microambiente, como foi demonstrado *in vitro* para ácidos oleico e linoleico insaturados (Aardema *et al*., 2011; Carro *et al*., 2013). Assim, os ácidos graxos livres podem ser usados para produzir energia através da via de beta-oxidação, potencializando a progressão da maturação e a competência de desenvolvimento do provável embrião em

bovinos (Sturmey, Leese, 2003). Além disso, De Lima *et al*. (2019) mostrou que a abundância relativa dos ácidos graxos como palmítico (16:0), esteárico (18:0) e oleico (18:1), foram maiores em oocitos maduros, como já sido relatado na literatura anteriormente (Aardema *et al*., 2011; Carro *et al*., 2013).

Em oócitos bovinos maturados, foi observada uma maior concentração de lipídicos da classe dos TAGs, fosfotidilcolina, fosfatidiletolamina, esfingomielina, fosfatidilinositol, fosfatidilserina, fosfatidilglicerol, quando comparado aos embriões de 2 células. Já em blastocistos há uma maior predominância de lipídios da classe dos TAGs, colesterol ésteres e fosfatidiletolamina (De Lima *et al*., 2018). O acúmulo aumentado de lipídios durante a fase de pré-maturação (oócito imaturo) já foi descrito pois há o enriquecimento em fosfolipídios e ésteres de colesterol (na forma de gotículas lipídicas) por causa de seus papéis cruciais na formação de membranas e na produção de energia durante as divisões celulares após a fertilização do oócito (Prates, Nunes, Pereira, 2014). No entanto, no estágio de blastocisto, o genoma embrionário é totalmente ativado e o primeiro evento de diferenciação celular ocorre (formação do trofoblasto e da massa celular interna) (De Lima *et al*., 2018).

Modulação do conteúdo e perfil lipídico embrionário em bovinos

A tensão de oxigênio no cultivo *in vitro*, a adição de SFB ou substituição de soro como fonte de substrato e as potenciais substâncias reguladoras, que se destinam a influenciar o equilíbrio lipídico, são aspectos de interesse ao referir-se ao metabolismo energético ou lipídico embrionário (Melo-Sterza e Poehland, 2021). Essas alternativas de mudança do meio de cultura, substituição e/ou retirada do SFB ou o uso de substâncias delipidantes, visam reduzir o conteúdo lipídico e melhorar a qualidade do embrião,

ocasionando aumento na sobrevivência embrionária pós-criopreservação (Dias *et al*., 2017).

Sudano *et al*., (2016) sugeriram que em oócitos imaturos e em embriões pré-implantação, as gotículas lipídicas citoplasmáticas e os níveis de transcrição de mRNA de *ACSL3, ELOVL5* e *ELOVL6* estão associados à estrutura química de seus lipídios de membrana. A utilização de SFB em meios de cultivo é uma das questões mais relevantes a esse respeito (Abe *et al*., 2002; Sudano *et al*., 2012). Com o objetivo de reduzir o teor de lipídios, várias moléculas já foram testadas, seja adicionando ou removendo-as do meio CIV (Sanches *et al*., 2013; Paschoal *et al*., 2016; Razza *et al*., 2018; Costa *et al*., 2019; Costa *et al*., 2020).

Botigelli *et al*. (2018) relataram que os genes envolvidos no metabolismo lipídico e acúmulo de lipídios (*DGAT1, PLIN2* e PLIN3) de embriões resultante de oócitos tratados com CNP não foram afetados. Embora, ocorreu o aumento na expressão de *PTX3* (biomarcador de expansão de células *cumulus*) e maior taxa de eclosão, sugerindo que a suplementação com CNP poderia melhorar o desenvolvimento e a qualidade dos blastocistos bovinos. Recentemente Costa *et al*. (2020) observaram que a adição do CNP no cultivo modificou o perfil lipídico de embriões *in vitro*, com redução na quantidade relativa da maioria dos lipídios avaliados pertencentes a derivados do colesterol e subclasses dos TAGs. Além disso, apresentaram alteração na expressão na abundância de transcritos de genes relacionados ao metabolismo lipídico e um efeito positivo nas taxas de eclosão de embriões. Além disso, suspeitaram que o CNP possa estimular o cGMP, que exerce uma ação semelhante ao AMPc, estimulando a lipólise e reduzindo a quantidade de lipídeos (Costa *et al*., 2020).

As condições a que os oócitos e embriões são expostos *in vitro*, elevam os níveis de espécies reativas ao oxigênio (EROs), potencializando as perdas embrionárias, devido

à parada do desenvolvimento, disfunção mitocondrial, dano ao DNA, indução de apoptose e peroxidação lipídica (Santos *et al*., 2018). A suplementação das condições de cultura com ácido tauroursodesoxicólico (TUDCA), um potente ácido biliar, anteriormente usado para aliviar o estresse da membrana endoplasmática do retículo (Song *et al*., 2011; Zhao *et al*., 2015; Mochizuki *et al*., 2018), foi associada com diminuição da produção de EROS e aumento da abundância de transcritos antioxidantes em oócitos e embriões (Pioltine *et al*., 2021).

Apesar de inúmeros experimentos investigando estratégias para refinar a PIV de embriões bovinos, incluindo a suplementação de meios de cultivo com inúmeras moléculas, a introdução de novos tipos de incubadoras e a redução da tensão de oxigênio, a taxa de desenvolvimento de blastocisto por COC não ultrapassou 40-50% desde o início dos anos 1990 (Lonergan e Fair, 2008). Desta forma, além das reconhecidas limitações dos protocolos de PIV, a qualidade do oócito continua sendo o outro fator primordial (Sirard, 2018).

As estratégias destinadas a aumentar a competência do oócito incluem o bloqueio temporário da meiose para permitir a conclusão da maturação citoplasmática do oócito. Entre os sistemas desenvolvidos, os mais promissores são aqueles que inibem ou retardam farmacologicamente a retomada meiótica (Franciosi *et al*., 2014).

O uso de Peptídeo Natriurético do Tipo C na produção *in vitro* de embriões

Em 1981, De Bold *et al*. Propuseram a existência de fatores atriais diuréticos e sua atividade. Entretanto, em 1984 ocorreu o isolamento e a purificação destes fatores revelando sua sequência de aminoácidos em ratos e humanos (Kishimoto; Rossi; Garbers, 2001). O primeiro fator a ser descoberto foi o peptídeo natriurético atrial ou fator

natriurético atrial (Atrial Natriuretic Peptide- ANP), distribuído, principalmente, pelos átrios cardíacos dentro dos grânulos de miócitos. Sua função, basicamente, é normalizar a volemia sanguínea e a pressão arterial quando a musculatura cardíaca é excessivamente distendida, sendo o mais conhecido e estudado dos peptídeos natriuréticos encontrados.

Em 1988, Sudoh *et al*. descobriram um homólogo com atividade biológica semelhante ao ANP, o peptídeo natriurético de tipo B (BNP; Sudoh *et al*., 1988) produzido principalmente nos ventrículos. Em 1990, um terceiro tipo de peptídeo foi isolado e denominado peptídeo natriurético tipo C (CNP). Inicialmente, foi proposto que ele interagisse somente com o cérebro (Misono *et al*., 2011), porém, mais tarde foi evidenciada sua produção em tecidos periféricos, como o endotélio vascular (Suga *et al*., 1998), as células musculares lisas e os macrófagos (Naruko *et al*., 1996), as células do *cumulus* oócitos (Kawamura *et al*., 2011). Hoje sabemos que o efeito do CNP na reprodução de mamíferos, inclui a regulação da espermatogênese (Sogawa *et al*., 2014; Codognoto *et al*., 2019), atração do espermatozoide ao oócito (Kong *et al*., 2017), desenvolvimento folicular (Sato *et al*., 2012) e manutenção do bloqueio temporário da meiose em oócitos de várias espécies de mamíferos (Zhang *et al*., 2010; Franciosi *et al*., 2014; Zhang *et al*., 2014; Zhang *et al*., 2015; Dubeibe *et al*., 2017, Botigelli *et al*., 2018).

Estes três peptídeos (ANP, BNP e CNP) possuem elevada homologia, sendo que seus respectivos receptores (NPR 1 e 2) estão localizados na superfície da célula alvo (Misono *et al*., 2011; De Cesaro *et al*., 2015). Estes peptídeos apresentam efeitos variados em diferentes tecidos em uma mesma espécie. Desta forma, os ANP e BNP utilizam como comunicação celular o receptor NPR-1, enquanto o CNP, utiliza como receptor o NPR-2 (Potter *et al*., 2009).

O bloqueio da meiose em oócitos ocorre pelo acúmulo de AMPc (adenosina monofosfato cíclica) intracelular até o momento da maturação, sendo a queda desta

concentração necessário para a retomada da meiose, consequentemente a maturação oocitária (Franciosi *et al*., 2014). As células do *cumulus* são responsáveis pela síntese do AMPc que passa para o oócito pelas junções comunicantes (gap junctions; Dubeibe *et al*., 2017). Similarmente ao que ocorre *in vivo*, o CNP já foi utilizado em alguns estudos com o objetivo de elevar os níveis de cAMP intracelular de oócitos na fase de maturação *in vitro* (Franciosi *et al*., 2014; De Cesaro *et al*., 2015).

Em bovinos, foi detectado que o CNP é produzido fisiologicamente pelas células da granulosa, *cumulus* e oócitos (Xi *et al*., 2018), e sua adição na maturação *in vitro* pode afetar o metabolismo lipídico em oócitos e embriões (Botigelli *et al*., 2018). Em contraste, em camundongos o CNP é produzido apenas pelas células da granulosa e se liga ao seu receptor de eleição CNP tipo 2 (NPR2) presente nas células do cumulus, oócitos e embriões pré-implantação (Xi *et al*., 2019). A presença de NPR2 em oócitos bovinos já foi relatada, mas não há relatos de sua presença em estruturas após a fertilização e desenvolvimento embrionário. Recentemente, a suplementação de CNP em cultivo in vitro (Dia 5) de embriões bovinos modulou o perfil lipídico, bem como a abundância de alguns transcritos relacionados ao metabolismo lipídico embrionário (Costa *et al*., 2020).

Além disso, para que ocorra ação do CNP sobre as células é necessário a presença de seu receptor, o NPR2. Em bovinos, a presença de NPR2 foi observada em oócitos no estágio de vesícula germinativa na membrana e, após a retomada da meiose, nos quais houve uma diminuição da detecção do receptor em oócitos maturados em metáfase II (Xi *et al*., 2018). Já em camundongos, foi detectado e quantificado a presença de receptores NPR2 em todas as estruturas em desenvolvimento, tais como oócito imaturo, oócito maturado, provável zigoto, mórula e blastocistos (Xi *et al*., 2019).

Criopreservação de embriões produzidos *in vitro* e seu efeito no metabolismo embrionário

A criopreservação de embriões é extremamente importante e essencial para a indústria de transferência de embriões bovinos, especialmente em nossa atual escala comercial (Hasler, 2014). O primeiro relato sobre a criopreservação foi na utilização em embriões de camundongos, por Whittingham *et al.* (1971). Posteriormente, outros estudos surgiram e em diversas espécies (Arav *et al*., 1987; Vajta *et al*., 1998; Kuwayama *et al*., 2005), sendo uma delas especialmente estudada, a bovina. A vitrificação se apresenta como uma das técnicas de vitrificação, a qual pode ser executada utilizando altas temperaturas de resfriamento e alta concentração de soluções crioprotetoras (Sudano *et al*., 2016a).

Os embriões obtidos pela produção *in vitro* (PIV) possuem baixa resistência à criopreservação (isto é, baixa crio tolerância) a qual, aparentemente, está associada com os lipídios (sua composição na membrana celular, conteúdo e o tamanho das gotículas citoplasmáticas), bem como a composição e a morfologia das organelas presentes nas células embrionárias (Leibo *et al*., 1995; Abe *et al*., 2002; Paschoal *et al*., 2017; López-Damián *et al*., 2018). Uma das possíveis explicações para esse elevado depósito lipídico está relacionada com a suplementação de SFB nos meios de cultivo *in vitro* (Paschoal *et al.*, 2017). Lipídios como os TAGs, a principal classe lipídica encontrada no citoplasma celular de mamíferos, são armazenados em forma de gotículas lipídicas (Sudano *et al*., 2016) e, nos processos de criopreservação, a sua presença é um fator possivelmente comprometedor devido às injúrias celulares por elas causadas. Geralmente, as maiores concentrações de ácidos graxos correspondem a uma baixa resistência ao resfriamento (Zeron, 2002). Os ácidos graxos saturados modificam o metabolismo celular, podendo inibir a proliferação celular (Gordon, 1977) e induzir a apoptose (Beeharry *et al*., 2003).

Em estudo conduzido por López-Damián *et al*. (2019), foi demostrado que embriões das duas subespécies (*Bos t. taurus* e *Bos t. indicus*) produzidos *in vivo* sofreram

maior peroxidação lipídica, número de células apoptóticas e ROS, comparados a embriões que não passaram pela criopreservação. Sabendo-se que uma das consequências da peroxidação lipídica é aumento do conteúdo lipídico intracitoplasmático, impactando diretamente na sobrevivência após aquecimento, deduz-se que após a criopreservação, os embriões de PIV ficam mais vulneráveis aos danos oxidativos, por motivo de sua estrutura ser delicada e não carecer de mecanismos de defesa desenvolvidos (Nedambale *et al*., 2006).

Em situações de alta demanda energética, pode ocorrer a hidrólise dos TAG no organismo, gerando ácidos graxos e glicerol em um processo denominado lipólise (Ravnskjaer *et al*., 2015; Razza *et al*., 2018). Sendo assim, todos os efeitos gerados pela técnica e manipulação dos oócitos e embriões podem interferir no metabolismo celular. Assim, a utilização de antioxidantes no meio de cultivo e nos meios pós aquecimento pode viabilizar o potencial de desenvolvimento dos embriões, como inibidores do estresse do RE, como o ácido tauroursodesoxicólico (TUDCA; Pioltine *et al*., 2021), ácido fenilbutírico (PBA) e salubrinal (Song *et al*., 2012; Zhang *et al*., 2012; Sutton-McDowall *et al*., 2015). Além do que a utilização de agentes delipidantes como forskolin (Sanches *et al*., 2013; Costa *et al*., 2019) 3-isobutil-1-metilxantina (um inibidor da fosfodiesterase não específica PDE3; Razza *et al*., 2018) e o CNP (Costa *et al*., 2020) podem reduzir ou modificar o teor e perfil lipídico desses embriões.

HIPÓTESES

A suplementação de CNP, no cultivo *in vitro* de embriões bovinos:

i) altera o metabolismo celular e, consequentemente, o conteúdo e perfil lipídico dos blastocistos derivados dessa suplementação;

ii) melhora a qualidade embrionária e, independentemente de sua criopreservação, as taxas de eclosão *in vitro*.

OBJETIVOS

Objetivo geral

Investigar os efeitos nas taxas de produção, perfil lipídico e metabolismo embrionário da suplementação do CNP no cultivo *in vitro* de embriões bovinos, em especial com relação à sua criotolerância.

Objetivos específicos

i) Detectar a proteína do receptor para o CNP (NPR2) em embriões pré- e pós-compactação (D5 e D7 do cultivo *in vitro*) e avaliar o efeito da suplementação de CNP, em diferentes concentrações, nas taxas de clivagem, de blastocisto e de eclosão;

ii) Quantificar e caracterizar o conteúdo lipídico de blastocisto submetidos ao CNP durante o cultivo embrionário;

iii) Comparar a abundância de transcritos de genes relacionados com o metabolismo energético e lipídico, e a qualidade dos embriões submetidos ao CNP no cultivo;

iv) Comparar a criotolerância *in vitro* dos blastocistos, derivados da suplementação com o CNP, por meio da re-expansão e da eclosão após a criopreservação.

CAPÍTULO 1

DEVELOPMENTAL AND MOLECULAR EFFECTS OF CNP SUPPLEMENTATION IN *IN VITRO* CULTURE OF BOVINE EMBRYOS

Developmental and molecular effects of CNP supplementation in *in vitro* culture of bovine embryos

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ABSTRACT

The use of C-type natriuretic peptide (CNP) in the interaction with the oocyte and in the temporary blockade of meiosis has already been very well described. However, its action in pre-implantation embryos is still little known. Thus, the aim of our study was to detect the presence of the CNP receptor of choice (natriuretic peptide receptor NPR2) in germinal vesicle (GV), metaphase II (MII), presumptive zygotes (PZ), morula (MO), and blastocyst (BL) and later to observe possible modulations that culture with CNP can cause in embryonic development. First, in Experiment I, we detected and quantified the NPR2. From there, we carried out Experiment II, with the objective of standardizing a higher concentration of CNP in *in vitro* culture that was not embryotoxic, we tested different concentrations (100, 200 or 400 nM of CNP) at different times of inclusion in the IVC (inclusion from the beginning, i.e., Day 1 of the IVC, or from day 5). After the results, we observed that the concentration of 400 nM of CNP on day 1 (day 1) in the IVC did not show embryotoxicity results, in addition numerically, it showed more promising results in terms of production rates. Thus, we analyzed the embryonic development rates of bovine embryos (D7) and hatching kinetics (D7, D8, and D9). Subsequently, morula and blastocyst were collected and evaluated for transcript abundance in relation to competence and quality (apoptosis, oxidative stress, proliferation, differentiation) and lipid metabolism in a microfluidic platform. Differences with probabilities less than $P < 0.05$, and/or Foldchange >1.5 were considered significant. We observed the presence of NPR2 until the blastocyst development phase, which was the structure in which there was

a significant decrease in membrane receptors. We observed no difference in production rates after cultivation with 400 nM CNP. However, when we evaluated the abundance of morula transcripts, they showed upregulated transcription *ADCY6* (*P=0.0113*), *IMPDH1* (*P=0.0401*); and downregulated transcripts, *BMP15* (*P= 0.007*), *ACAT1* (*P= 0.040*), and, *CASP3* (*P=0.034*). In addition, when evaluated from the Fold change (FC), a variation was observed in 12 targets (*FC >1.5*). In blastocysts, treatment with CNP downregulated transcription *AGPAT9 (P*= 0.0097), and *BDNF* (*P=0.0322*). However, it was upregulated in the CNP group for *BID, CASP3, SOX2, HSPA5* transcripts, and downregulated for *NLRP5, ELOVL1, ELOVL4, IGFBP4*, and *FDX1* transcripts (FC >1.5). Thus, our study identified and quantified for the first time the presence of NPR2 in bovine pre-implantation embryos. Furthermore, a concentration of 400 nM of CNP not previously described in the literature was used in *in vitro* culture, observing a modulation of some transcripts, related to embryonic metabolism.

Keywords: C-type natriuretic peptide, embryonic metabolism, NPR2, transcript abundance, embryotoxicity, cattle.

INTRODUCTION

The C-type natriuretic peptide (CNP) molecule plays a central role in regulating the meiotic progress of the oocyte into growing follicles in mammals (Zhang *et al*. 2010, Hiradate *et al*. 2014, Franciosi *et al*., 2014; Dubeibe *et al*., 2017, Botigelli *et al*., 2018, Xi *et al*., 2019). However, the relation between CNP and the embryo has few reports in the literature (Botigelli *et al*., 2018; Xi *et al*., 2019; Costa *et al*., 2020).

In cattle, it has been detected that CNP is physiologically produced by granulosa cells, *cumulus*, oocytes (Xi *et al*., 2018), in extracellular vesicles (EV; Pioltine *et al*., 2020) and its addition in *in vitro* maturation may affect lipid metabolism in oocytes and embryos (Botigelli *et al*., 2018) and its inclusion in the *in vitro* culture can alter some target genes and the lipid profile (Costa *et al*., 2020). In contrast, in mice CNP is produced only by granulosa cells and binds to its election receptor CNP type 2 (NPR2) present in *cumulus* cells, oocytes, and pre-implantation embryos (Xi *et al*., 2019). The presence of NPR2 in bovine oocytes has been reported, but there are no reports of its presence in structures after fertilization and embryonic development. Recently CNP supplementation

in *in vitro* culture (Day 5) of bovine embryos modulated the lipid profile, as well as the abundance of some transcripts related to embryonic lipid metabolism (Costa *et al*., 2020). However, there were not studies about possible receptors related to the action of CNP on the bovine embryo, as already described in oocytes. Thus, initially, the objective of our study was to confirm and quantify the NPR2 receptor in bovine oocytes and pre-implantation embryos.

After that, our objective was initially to investigate the presence of the CNP receptor of choice -NPR2- at different stages of embryonic development in bovines. Furthermore, predict a non-embryotoxic concentration of CNP applicable during IVC and lastly, evaluate the action of CNP on *in vitro* production rates, as well as its action on the abundance of transcripts in bovine morula and blastocysts.

MATERIAL AND METHODS

All animal procedures were approved by the Ethics and Animal Handling Committee of the São Paulo State University (UNESP), Botucatu, São Paulo, Brazil, certificate #1180. The experimental design performed in our study is illustrated below (Figure 1).

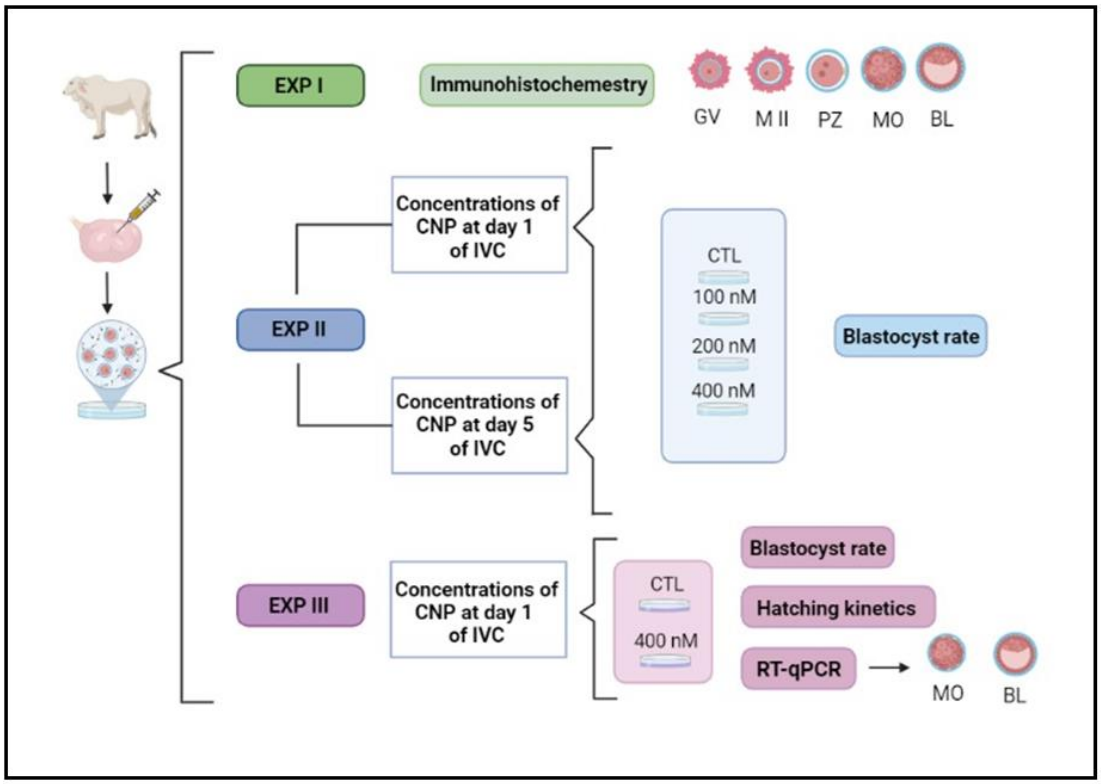

Figure 1 - Illustrative experimental design. IVC, *in vitro* culture; EXP, experiment; GV, germinal vesicle; MII, metaphase II; PZ, presumptive zygotes; MO, morula; and BL, blastocyst; CTL, control group; CNP, group treated with C-type natriuretic peptide.

In vitro production of embryos

Ovaries from a commercial slaughterhouse, from bovine females with a predominantly *Bos taurus indicus* phenotype of the Nellore breed were collected, packaged and transported to the laboratory in 0.9% saline solution between 30 and 35ºC. Follicles with a diameter of 2–8 mm were aspirated with hypodermic needles (30x8; 21G) attached to 10 mL syringes for the recovery of *cumulus-oocyte* complexes (COCs), with a maximum interval - between the arrival of the ovaries from the slaughterhouse and the end of aspiration – of four hours. Only COCs of qualities I and II were used, and the classification was performed as described by Seneda *et al*. (2001).

In vitro maturation

Previously to *in vitro* maturation (IVM), COCs were washed three times in TCM-HEPES 199 supplemented with 10% (v/v) fetal bovine serum, (FBS), 0.20 Nm sodium pyruvate and 83.4 mg /mL of gentamicin (ABS Global Brasil®, Mogi Mirim, São Paulo, Brazil). The COCs were matured in drops of 100 Ml of TCM-199 medium bicarbonate supplemented with 10% (v/v) Fetal Bovine Serum (FBS), and 50 μg of gentamycin/mL (ABS Global Brasil®, Mogi Mirim, São Paulo, Brazil) and incubated for 24 h in an environment with maximum humidity, 38.5°C and 20% O_2.

In vitro fertilization

After maturation, the COCs were washed in HEPES-buffered TCM-199 medium and transferred to 100-Ml droplets of the fertilization medium that consisted of Tris-buffered medium (TBM) supplemented with 8 mg/Ml fatty acid-free bovine serum albumin (BSA) and 1 Mm glutamine (ABS Global Brasil®, Mogi Mirim, São Paulo, Brazil). For fertilization in drops of 100 μL, semen from a single Nellore bull was used (Adamo Fiv Kubera; Código 011NE03127, registro ACF 3522, Alta Genetics), which was previously validated in our laboratory. The cryopreserved semen was heated at 36°C for 30 seconds. Sperm selection was performed by Percoll gradient (ABS Global Brasil®; Percoll 45% in the upper part and 90% in the lower) by centrifugation (12,100 g, for 5 minutes), the supernatant (600 μL) was discarded and the sperm pellet resuspended in 300 μL of fertilization medium and homogenized. The semen was centrifuged again (8,127 g, for 2 minutes) and, after discarding the supernatant, the sperm concentration was adjusted to obtain a final concentration of $1x10^6$ live spermatozoa in each drop containing 20 COCs. They were co-incubated for 20 to 22 hours in an environment with

maximum humidity, 20% O_2, and 38.5° C. The day of insemination was considered day zero (Day 0).

In vitro culture

For *in vitro* culture, presumptive zygotes were subjected to the removal of *cumulus* cells by successive pipetting and then incubated in SOF (Synthetic Oviduct Fluid) medium supplemented with 8 mg/Ml fatty acid-free BSA (ABS Global Brasil®, Mogi Mirim, São Paulo, Brazil) under mineral oil at sob the same temperature and gaseous atmospheric condition used in the previous steps. On the first day of cultivation (D1) or four days later (D5) the structures were divided into experimental groups: control (without the addition of CNP) and CNP groups (C-type natriuretic peptide, Sigma–Aldrich /St. Louis, MO, United States). On D3, 50% of the culture media volume was replaced by fresh media (1st feeding) and the same occurred on D5 (50% of the culture media volume was replaced with fresh SOF medium supplemented with glucose; 2nd feeding). During culture, blastocyst (D7) and hatching (D7, D8 and D9) rates were evaluated.

Experiment I – Detection and quantification of NPR2 receptors

Cell membrane detection of the receptor NPR2 was germinal vesicle (GV), metaphase II (MII), presumptive zygotes (PZ), morula (MO), and blastocyst (BL) through the technique of immunocytochemistry. The collected oocytes and embryos (N=10/cell type) were washed three times with phosphate-buffered saline (PBS; Sigma-Aldrich, St. Louis, MO) and fixed immediately in 4% paraformaldehyde for 20 min. After fixation, the oocytes and embryos were incubated with the primary antibody against NPR2

(HPA011977 Sigma-Aldrich; Xi *et al*., 2019) diluted 1:100 overnight at 4 °C. Subsequently, oocytes and embryos were washed with PBS, incubated with anti-rabbit secondary antibody (Invitrogen) diluted 1:200 for 1 h at 37 °C and nuclei were stained with 4′, 6-diamidino- 2-phenylindole (DAPI; Sigma-Aldrich) for 5 min. The fluorescent signals were examined using a Leica fluorescence optical microscope (Leica – THUNDER, Pirassununga, Brazil) and analyzed by densitometric analysis using ImageJ (Version 1.53). The intensity of DAPI and NPR2 is presented as the mean of the fluorescence intensity in arbitrary units.

Experiment II – Evaluation of production rates on the dose-response effect with different CNP concentrations and culture time

Initially, two experiments were carried out with the objective of testing some concentrations (control, 100 nM of CNP; 200 nM of CNP and 400 nM of CNP groups) on the embryotoxicity; and, to test the inclusion of CNP in the IVC, at the beginning of culture (Day 1) or on Day 5. The blastocyst rate of the treated groups compared to the control were evaluated.

Experiment III – Blastocyst production and abundance of target-transcripts in morula and blastocyst

After Experiment II data, we observed that the highest concentration used (400 nM) was not morphologically embryotoxic. Therefore, we used the concentration of 400 nM of CNP from the first day of the IVC (Day 1) and evaluated the impact on blastocyst production and hatching rate. Furthermore, we evaluated the abundance of transcripts in

morula (Day 5) and hatched blastocyst (Day 7 and 8) from the control group (without CNP) compared to those treated with 400 nM CNP.

Reverse Transcription and Quantitative Polymerase Chain Reaction (RT-qPCR)

RNA Isolation and Reverse Transcription

Total RNA from morula (5 morulas/group in 4 replicates) and hatched blastocysts (3 embryos/group in 4 replicates) was extracted using the PicoPure RNA Isolation kit (Life Technologies, Foster City, CA, United States) following the manufacturer's protocol. Extracted RNA was stored at -80°C until further analysis by qPCR. RNA concentration was quantified using a spectrophotometer (Nanodrop, Thermo Fisher Scientific, Waltham, MA, United States). For each sample, it was used a pool of five morulae and a pool of three blastocysts for reverse transcription. cDNA synthesis was performed using a High-Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA, United States), following the manufacturer's instructions. All samples were treated with DNase according to the manufacturer's instructions before reverse transcription.

Pre-amplification and qPCR

Gene expression analyses of bovine morulas and blastocysts were performed independently using Applied BiosystemsTM TaqMan©R Assays specific for *B. taurus* and based on Fontes *et al*. (2020). We analyzed the abundance of 95 transcripts using a panel of genes formatted to investigate embryonic competence and quality (apoptosis, oxidative stress, proliferation, and differentiation) and lipid metabolism in a microfluidic platform (Supplementary Table S1 describing all the genes and their signaling pathways). Prior to qPCR thermal cycling, each sample was subjected to a sequence specific preamplification process as follows: 1.25 mL assay mix (TaqMan©R Assay was pooled

to a final concentration of 0.2× for each of the 96 assays), 2.5 mL TaqMan PreAmp Master Mix (Applied Biosystems, #4391128), and 1.25 mL cDNA (5 ng/mL). The reactions were activated at 95°C for 10 min, followed by denaturation at 95°C for 15 s, annealing, and amplification at 0°C for 4 min for 14 cycles. These preamplified products were diluted fivefold (morula and blastocysts) prior to RT-qPCR analysis. Assays and preamplified samples were transferred to an integrated fluidic circuit plate. For gene expression analysis, the sample solution preparation consisted of 2.25 mL cDNA (preamplified products), 2.5 mL of TaqMan Universal PCR Master Mix (2×, Applied Biosystems), and 0.25 mL of 20× GE Sample Loading Reagent (Fluidigm, South San Francisco, CA, United States); the assay solution included 2.5 mL 20× TaqMan Gene Expression Assay (Applied Biosystems) and 2.5 mL of 2× Assay Loading Reagent (Fluidigm). The 96.96 Dynamic Array^{TM} Integrated Fluidic Circuits (Fluidigm) chip was used for data collection. After priming, the chip was loaded with 5 mL each of the assay solution and each sample solution and loaded into an automated controller that prepares the nanoliter-scale reactions. The qPCR thermal cycling was performed in the Biomark HD System (Fluidigm) using the protocol TaqMan GE 96 × 96 Standard, which involved one stage of Thermal Mix (50°C for 2 min, 70°C for 20 min, and 25°C for 10 min) followed by a hot start stage (50°C for 2 min and 95°C for 10 min), 40 cycles of denaturation (95°C for 15 s), primer annealing, and extension (both at 60°C for 60 s).

Statistical Analysis

To estimate the fluorescence intensity (Experiment I), the results were evaluated regarding the data distribution, being non-parametric data, thus the Kruskal-Wallis test was used followed by the *post hoc* Dunn test. Blastocyst rate was evaluated and the hatching kinetic were tested for normal distribution. Data normality was assessed using

the Shapiro-Wilk tests. If the data had a normal distribution, Tukey's test or one-way ANOVA was applied. If they were non-parametric, data transformation (Log10) was applied or the non-parametric Mann-Whitney test was applied. Differences with probabilities less than 0.05 ($P < 0.05$) were considered significant. Data are presented as mean values and standard error of the mean (SEM) or median, 1sd and 3rd interquartiles. In Experiment II, five replicates/group were performed and Experiment III was performed with eight replicates/group (for blastocyst rate). All of the above analyzes were performed with SigmaStat 4.0 software.

Quantitative PCR data were assessed using the ΔCq values relative to the geometric mean of the best reference genes among the 95-gene set, i.e., *GAPDH*, and *ACTB*. Fold-changes (FC) were calculated using the $2^{-\Delta Cq}$ method (Livak *et al*., 2001). All analyses were performed using SigmaStat 4.0 and MetaboAnalyst 5.0. The evaluation of the transcripts was initially performed with the univariate statistical analysis method, using FC, T-test, Volcano Plot. In a second moment, we analyzed the data by multivariate methods, considering Principal Component Analysis (PCA) and Partial Least Squares-Discriminant Analysis (PLS-DA) and their variations. Differences with probabilities less than $P < 0.05$, and/or FC >1.5 were considered significant.

RESULTS

Experiment I – Localization of NPR2 in bovine oocyte and pre implantation stage embryos

Based on fluorescent, we demonstrate the localization of NPR2 in different stages of embryonic development. NPR2 is located primarily on oocyte membranes at the germinal vesicle stage, germinal vesicle breakdown, and presumptive zygote. However,

263 the morula stage showed the highest concentrations and the blastocyst stage the lowest
264 (Figure 2 and Figure 3; $P \leq 0.05$).

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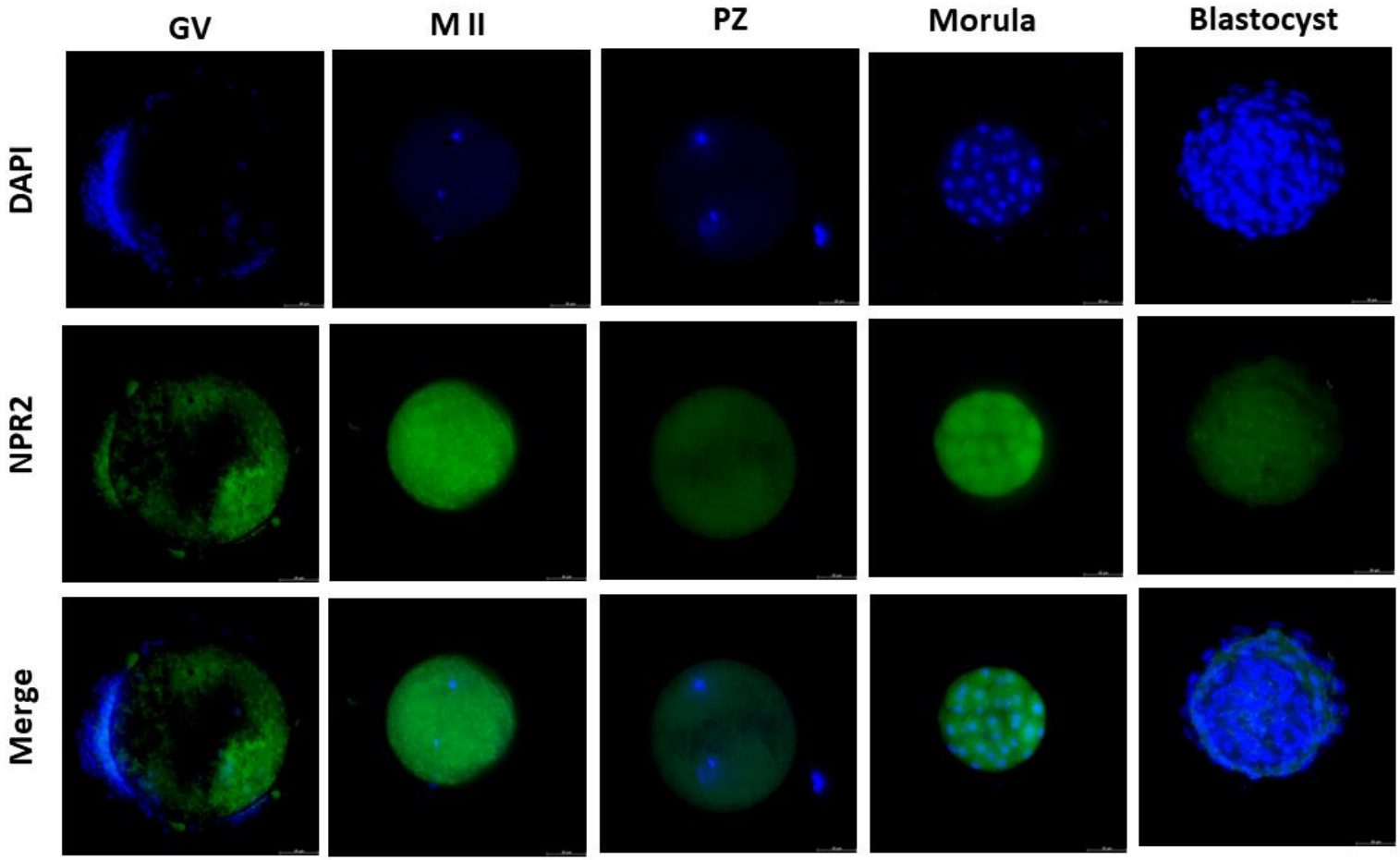

Figure 2- NPR2 localization in bovine oocytes and pre-implantation stage embryos. The green color indicates NPR2 staining and the blue color indicates nuclear staining DAPI. NPR2 protein was expressed in bovine oocytes and embryos at all stages. Bar = 50 µm.

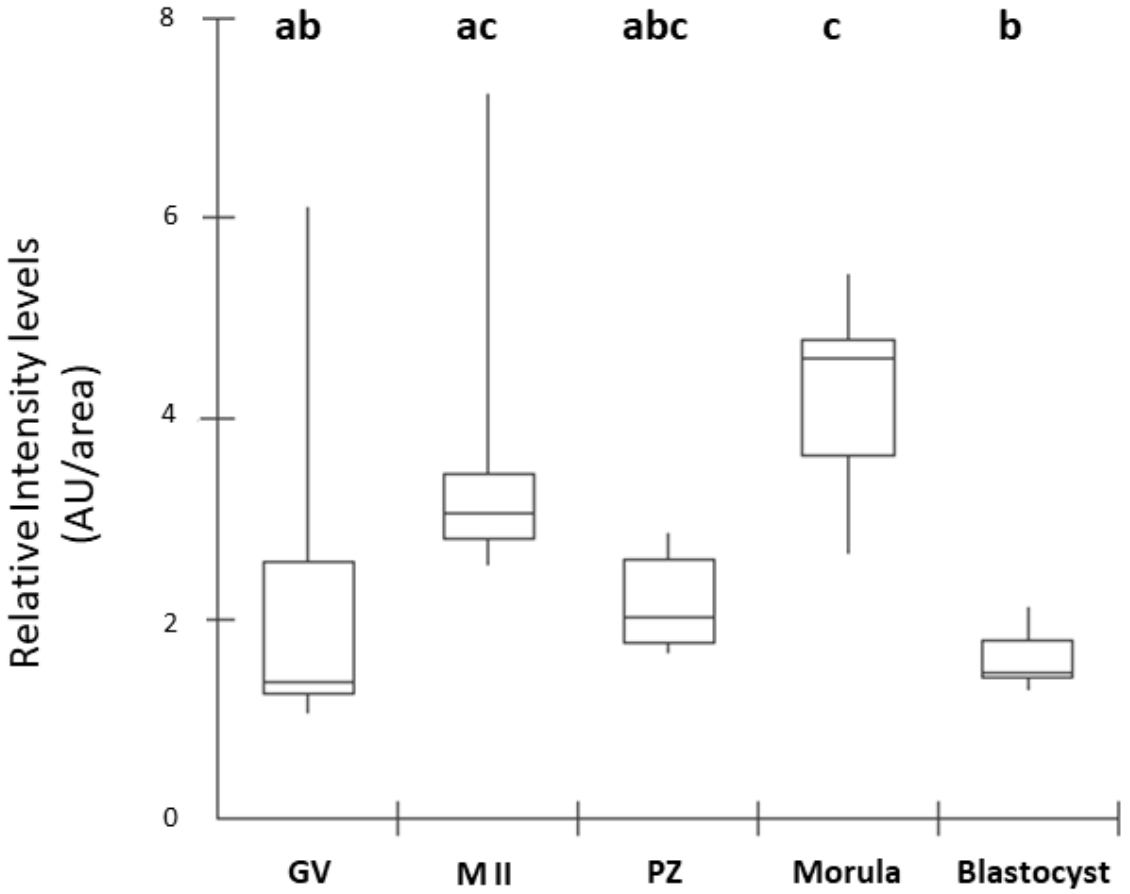

Figure 3- Fluorescence intensity of NPR2 in oocytes and preimplantation stage embryos. Results are presented as the median and 1º and 3º interquartile intervals of five replicates/stage and using 8 structures in total. Different letters above each box represent significant differences ($P \leq 0.05$). GV, germinal vesicle; M II, metaphase II; PZ, presumptive zygotes; Morula, morula stage; Blastocyst, blastocyst stage. AU, arbitrary units.

Experiment II - Effect on production rates of concentration-response with CNP and its exposure time

No significant difference was observed between the groups treated with CNP (100, 200 and 400 nM) compared to the control group on Day1 (P=0.7432) and Day 5 (P=0.082) of IVC. The evaluated embryos did not show signs of toxicity (there was no reduction in blastocyst rates and morphological features), so the highest concentration of CNP (400 Nm) was chosen to be used together with the Control group. Likewise, the use of CNP from the beginning of culture (D1) proved to be useful to maintain regular blastocyst rate (Tables 1 and 2). Therefore, we chose to keep the embryos exposed to CNP from the beginning of the IVC (D1) considering the development and activation of the embryonic genome in this period.

Table 1- Blastocyst rate in *in vitro* produced bovine embryos supplemented with different concentrations of CNP from day 1 (D1) of IVC.

Group	*Cumulus*-oocyte complexes N	P.Z. N	Blastocysts N (% mean ± SEM)
Control	91	90	29 (32.81 ± 14.24)
C-100	87	87	30 (34.14 ± 5.61)
C-200	94	93	33 (35.45 ± 5.06)
C-400	90	88	41 (46.09 ± 7.76)
P-value	-	-	0.7432

C-100: 100 nM of CNP; C-200: 200 nM of CNP; C-400: 400 nM of CNP.
PZ = Presumptive zygotes.
Data are the mean ± standard error of the mean (SEM) of 4 replicates.

Table 2-Blastocyst rate in *in vitro* produced bovine embryos supplemented with different concentrations of CNP from day 5 (D5) of IVC.

Group	*Cumulus*-oocyte complexes N	P.Z. N	Blastocysts N (% mean ± SEM)
Control	182	180	60 (32.41 ± 5.45)
C-100	184	181	52 (28.62 ± 12.52)
C-200	186	183	48 (26.33 ± 5.64)
C-400	184	183	55 (29.17 ± 8.94)
P-value	-	-	0.082

C-100: 100 nM of CNP; C-200: 200 nM of CNP; C-400: 400 nM of CNP.
PZ = Presumptive zygotes.
Data are the mean ± standard error of the mean (SEM) of 5 replicates.

Experiment III- Effect of CNP on blastocyst and hatching kinetic rates, and on the abundance of target-transcripts in morula and blastocyst

A total of 2,005 presumptive zygotes were cultured, obtaining blastocyst rate (D7) of 32.6% and 34.1% in the C-400 (400 nM of CNP) and Control groups, respectively ($P < 0.05$). There was no significant difference in the observed hatching rate between groups ($P < 0.05$; Table 3).

Table 3 – Evaluation on embryonic development rate of bovine embryos (D7) and hatching kinetic (D7, D8, and D9) cultured in the presence or absence of CNP (when added on the first day of culture).

Grupo	PZ N	Blastocyst N (% mean ± SEM)	Hatched blastocyst D7 N [% median (1st, 3rd)]*	Hatched blastocyst D8 N (% mean ± SEM)	Hatched blastocyst D9 N (% mean ± SEM)	Hatched blastocysts TOTAL N (% mean ± SEM)
Control	979	332 (34.13 ± 2.11)	4 [0.98 (0.00, 2.11)]	84 (25.76 ± 4.96)	62 (17.88 ± 2.75)	150 (44.80 ± 5.51)
C-400	1026	331 (32.55 ± 1.14)	3 [0.00 (0.00, 1.06)]	71 (20.58 ± 3.79)	68 (20.00 ± 2.09)	142 (41.34 ± 5.34)
P-value	-	0.52	0.57	0.42	0.55	0.66

Data are presented as median and 1st and 3rd interquartiles.
CNP-400: 400 Nm de CNP.
PZ = Presumptive zygotes.
Data are the mean ± standard error of the mean (SEM) of 8 replicates except for *.

Transcript abundance in morula showed differences in *BMP15* (*P* = 0.013), *ACAT1 (P* = 0.040), *ADCY6 (P* = 0.057) and, tended in *CASP3* (*P*=0.074) (Figure 4). Fold change analysis evidenced a variation in 12 targets (FC >1.5; Table 4 and Table 5). In Principal Component Analysis (PCA) and Partial Least Squares – Discriminant Analysis (PLS-DA) it was possible to observe an overlap in the PCA plot, and in PLS-DA a separation between the control and CNP-treated group (Figure 5A and 5B). In the heatmap, there was discriminant clustering between samples from both groups (Figure 5C).

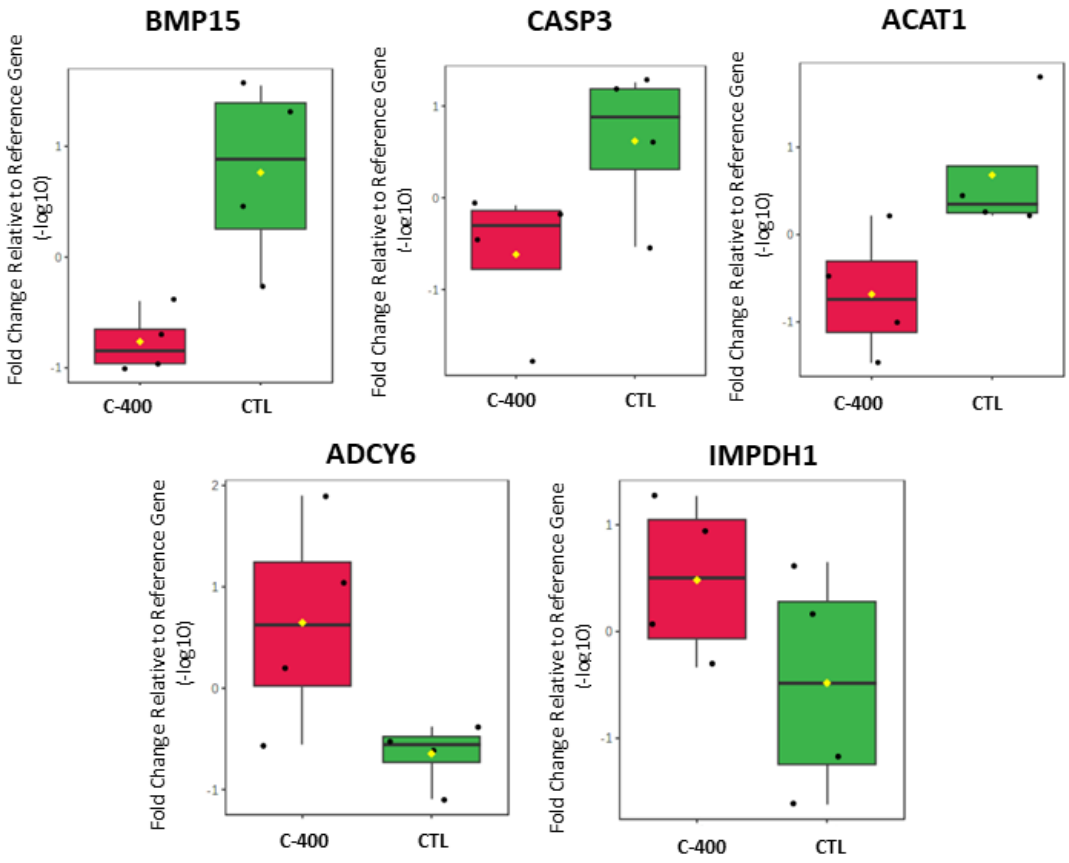

Figure 4 -Effect of CNP treatment in IVC on differential gene expression in morula. Data represent the fold change of relative abundance related to the reference gene in the -log10 scale. Upregulated transcription *ADCY6* (*P= 0.0113*) *IMPDH1* (*P=0.0401*); and downregulated transcription *BMP15* (*P*= 0.007); *CASP3* (*P*=0.034); *ACAT1* (*P*= 0.040). Control (no treatment) and C-400 (400 nM of CNP).

Table 4 – Upregulated transcription observed in bovine morulas and blastocyst after treatment with CNP. The relative abundance of transcripts was selected by the Fold change analysis (with magnitude greater than 1.5 times, that is, with the threshold > 1.5). The values shown were calculated as the ratio of the control group to the treated group (treated /control).

Structure	Gene symbol	Place of action and metabolic pathways	Fold Change	*P-value*
Morula	ADCY6	cAMP / Meiotic arrest	3.298	0.0113
	IMPDH1	GTP / cGTP	1.508	0.0401
	NPR2	cGMP / Meiotic arrest	5.256	0.0914
	ELF5	Cell differentiation / trofectoderm	5.020	0.5268
	CD40	Apoptosis	1.596	0.6726
Blatocyst	HSPA5	Folding and assembly of proteins in the endoplasmatic reticulum / degradation of misfolded proteins	1.571	0.4751
	SOX2	Pluripotency/chromatin binding/DNA methilation	1.752	0.4754
	CASP3	Apoptosis	1.950	0.5926
	BID	Apoptosis/Pro-Apoptotic	2.445	0.721

Table 5- Downregulated transcription observed in bovine morulas and blastocyst after treatment with CNP. The relative abundance of transcripts was selected based on the Fold change analysis (with magnitude greater than 1.5 times, ie, with the threshold < 0.667). The values shown were calculated as the ratio of the control group to the treated group (treated /control).

Structure	Gene symbol	Place of action and metabolic pathways	Fold Change	*P-value*
Morula	BMP15	Oocyte maturation / follicular development	0.143	0.0007
	CASP3	Apoptosis	0.545	0.0347
	GFPT2	Oxidative stress	0.536	0.0914
	HSPA1A	Cell survival / facilitates DNA repair	0.633	0.2049
	FSHR	Follicle stimulating hormone receptor / gonad development	0.308	0.2059
	NRP2	Cell survival / follicular development	0.524	0.6728
	NANOG	Pluripotency (ICM/TE) / when overexpressed, promotes cells to enter the S phase and proliferation	0.533	1.000
Blatocyst	AGPAT9	Predict embryo quality/Lipid metabolism	0.557	0.0097
	BDNF	Supporting meiotic progression	0.508	0.0322
	FDX1	Synthesis steroid hormones/ catalyzes cholesterol cleavage	0.652	0.0742
	IGFBP4	Either inhibit or stimulate the growth promoting effects of the IGFs	0.588	0.1588
	ELOVL4	Fatty acid biosynthesis, elongation, endoplasmic reticulum	0.590	0.2841

	ELOVL1	Fatty acid biosynthesis, elongation, endoplasmic reticulum	0.600	0.2843
	NLRP5	maternal oocyte protein / required for normal early embryogenesis	0.535	0.3725

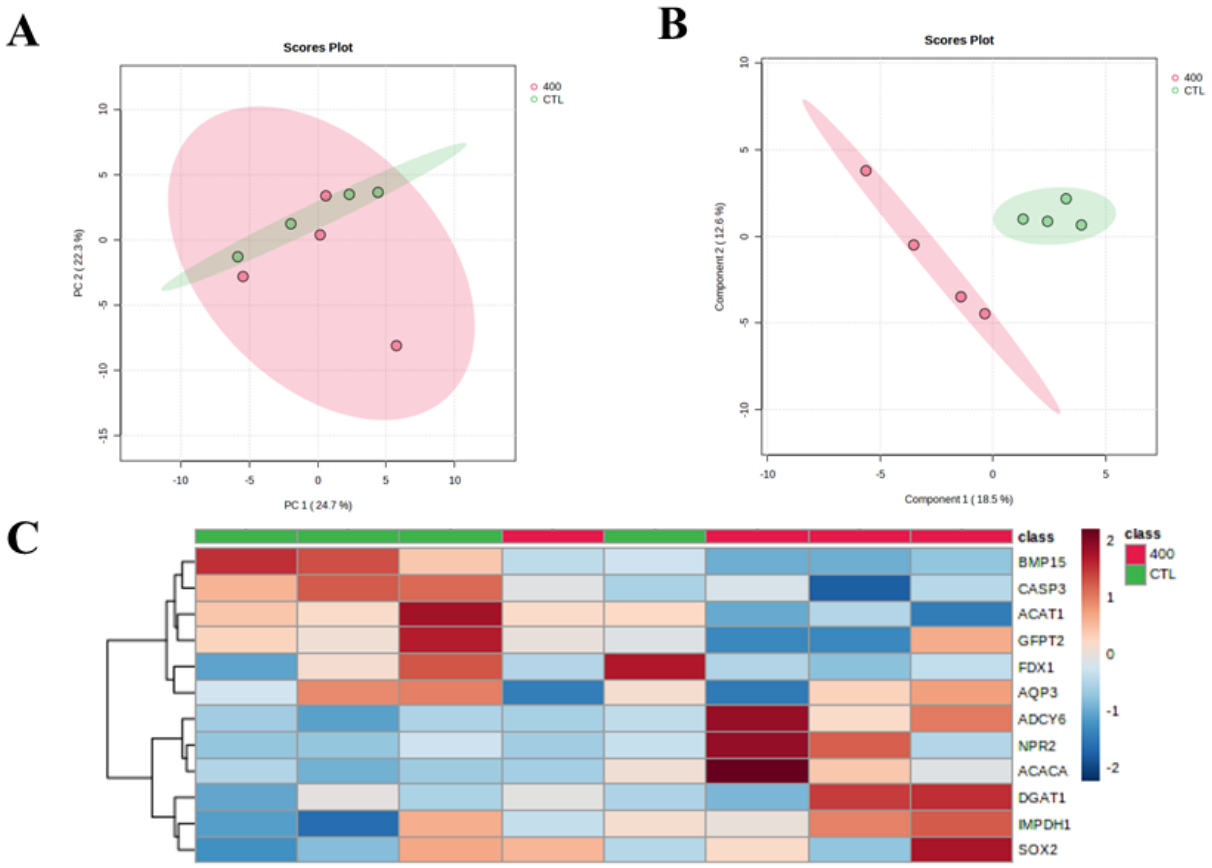

Figure 5- Multivariate analysis plots of the abundance of transcripts derived from untreated (control) and CNP-treated morula. **(A)** PCA plot of morula from control and treated groups; **(B)** 2D PLS-DA discrimination score plot between groups. **(C)** Heatmap showing transcriptional profiles abundance of morula treated with 400 nM CNP and the control group (n = 4 / group).

Transcript abundance of blastocyst to be reduced in the CNP group for *AGPAT9* (*P = 0.094*), BDNF (*P=0.0322*). However, it was upregulated in CNP group for *BID*, *CASP3*, *SOX2*, *HSPA5* transcripts, and downregulated for *BDNF*, *NLRP5*, *AGPAT9*, *ELOVL1*, *ELOVL4*, *IGFBP4* and *FDX1* transcripts (Table 4 and Table 5). All the others

target transcripts analyzed in morula and blastocyst did not differ in a statistically significant way (Supplementary Table S1). In addition, it was possible to observe a modest separation of the groups when 2D PLS-DA was applied (Figure 6).

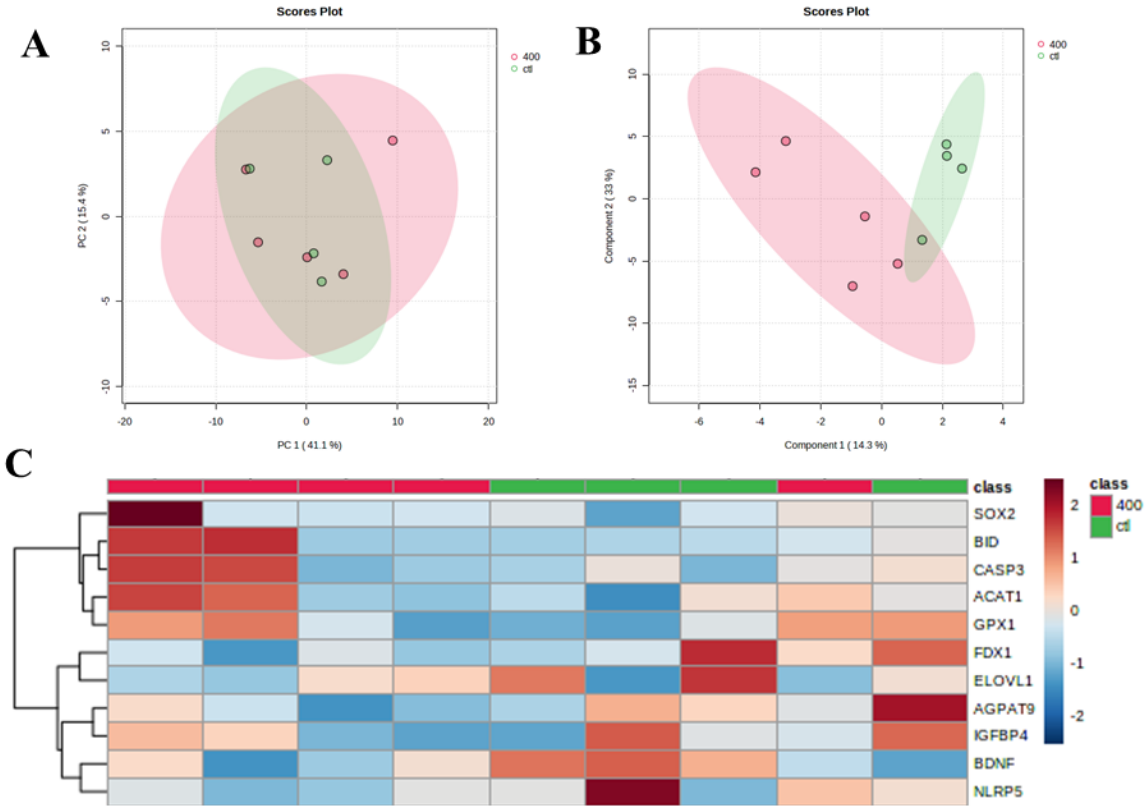

Figure 6 – Multivariate analysis plots of the abundance of transcripts derived from untreated (control) and CNP-treated blastocysts. **(A)** PCA plot of blastocysts from control and treated groups; **(B)** 2D PLS-DA discrimination score plot between groups. **(C)** Heatmap showing transcriptional profiles abundance of *in vitro* produced bovine embryos with 400 nM CNP and control group (n = 4/group).

DISCUSSION

To our knowledge, this study is the first to detect and quantify the NPR-2 receptor in bovine pre-implantation embryos. The results supported the hypothesis that the use of CNP in the culture of bovine embryos would alter the embryonic metabolism – sustained by data from our group that observed changed transcript abundance in embryos submitted to 100 nM of CNP from day 5 of the IVC (Costa *et al*., 2020). In the present study, when testing the use of 400 nM of CNP, no morphological embryotoxicity was observed (ie, morphological characteristics and rate of blastocyst production), although no benefit was also noted. However, when some transcripts were evaluated, related to lipid metabolism, embryonic development, and oxidative stress, a change in their abundance was observed in morulas and blastocysts treated with CNP.

The effect of CNP on regulation includes spermatogenesis (Sogawa *et al*., 2014; Codognoto *et al*., 2019), sperm attraction (Kong *et al*., 2017), follicular development (Sato *et al*., 2012) and maintenance of blockade meiosis in oocytes of several species of mammals (Zhang *et al*., 2010; Franciosi *et al*., 2014; Zhang *et al.*, 2014; Zhang *et al.*, 2015; Dubeibe *et al*., 2017, Botigelli *et al.*, 2018) and is well established.

The use of exogenous CNP in pre-IVM and IVM has been used for years, with different concentrations and in different species: 100 nM in cattle (Franciosi *et al*., 2014; Soares *et al*., 2017; Botigelli *et al*., 2018) 100 nM, 500 nM in murine (Xi *et al*., 2019; Ang *et al*., 2021), 100 nM in cats (Zhong *et al*., 2016), 150 ng/mL in goats (Zhang *et al*., 2015). However, the relationship between CNP and the embryo has few reports in the literature (Costa *et al*., 2020; Botigelli *et al*., 2018; Xi *et al*., 2019). Most importantly, when its use in *in vitro* culture step is searched, there is only one study (Costa *et al*., 2020).

The NPR2 receptor when activated by CNP binding triggers a guanylyl cyclase domain, which generates cGMP. This process causes the elevation of cGMP, and transferred through gap junctions from *cumulus* cells to oocytes, cGMP has an inhibitory action on phosphodiesterase 3A (PDE3A), maintaining high cAMP concentrations in oocytes, and blocking meiosis resumption.

In addition, for CNP to action cells, the presence of its receptor, NPR2, is required. In our study, the presence of NPR2 receptors was observed in all stages of embryonic development analyzed. The detection of NPR2 in oocytes (GV and MII stages) and presumptive zygotes was similarly expressed and quantified. In blastocysts, there was a pronounced decrease in the number of receptors in relation to the morula, something already described by Xi *et al*. (2019) in mouse embryos. In cattle, the presence of NPR2 was observed in oocytes at the germinal vesicle stage in the membrane and, after the resumption of meiosis, there was a decrease in receptor detection in matured oocytes in metaphase II (Xi *et al*., 2018). Thus, our results corroborate the findings by Xi *et al*., 2019 in mice.

Adenylyl cyclases (AC) are a group of enzymes that convert adenosine-5'-triphosphate (ATP) into 3',5'-adenosine monophosphate (cyclic AMP/cAMP) and pyrophosphate. Furthermore, AC is reported to presence in 13- to 16-day-old bovine embryos has been reported to modulate cAMP and cGMP concentrations - a fact that determines the rapid proliferation of embryonic cells or even signaling to the endometrium (Grealy, Sreenan, 1999). The action of CNP described in oocytes aims to increase cAMP and cGMP concentrations through the granulosa cell and transferred via GAP junctions to the oocyte. The detection, in the present study, of the presence of the NPR2 receptor - and its potential activity, in view of the differences found when the CNP ligand is added to the IVC medium - in a location other than the *cumulus* cells, assumes

462 a natural function for the NPR2 complex -CNP also in pre-implantation embryos (at least
463 in bovine and murine species). We presume that the elevation of *ADCY6* levels occurred
464 due to exposure of morulas to CNP, ultimately triggering greater conversion of ATP to
465 cAMP.

466 A reduction in the abundance of *ACAT1* gene transcripts (acetyl-CoA
467 acetyltransferase 1) was observed in CNP-treated morulas. *ACAT1* converts free
468 intracellular proteins which promote free cholesterol to be esterified into cholesterol
469 esters (Tian *et al*., 2012). In that regard, treatment with Acyl-CoA synthetase (GW3965
470 hydrochloride) caused an increase in transcripts related to lipid metabolism (*ACSL3,*
471 *ACAT1,* and *AUH;* Valente *et al*., 2019). In our study, however, we inferred that the
472 reduction of *ACAT1* transcripts may have been caused by the action of CNP, which
473 modulated the metabolism of some lipid classes, reducing some cholesterol esters.

474 Bone morphogenic protein 15 (*BMP15*) was elevated in morula that did not
475 receive CNP treatment. *BMP15*, in mammals, is related to oocyte maturation and also in
476 cholesterol biosynthesis, improving oocyte competence and embryonic development in
477 cattle (Caixeta *et al*., 2013; Cajas *et al*., 2020). Several studies have reported an increase
478 in the *BMP15* transcript during maturation in oocytes from buffaloes, dogs, and cows
479 (Kathirvel *et al*., 2013; Lee *et al*., 2017; Cajas *et al*., 2020). Furthermore, the metabolic
480 pathways of *cumulus* cells, particularly glycolysis and cholesterol biosynthesis, are highly
481 affected when there is a mutation in the *BMP15* gene (Su *et al*., 2008). A possible
482 inference to the finding in our results would be that CNP could be reducing *BMP15*
483 expression by modulating cholesterol biosynthesis through cGMP elevations. This
484 hypothesis is based on the fact that there is no decrease in blastocyst production and
485 hatching rates, there is no increase in transcripts negatively related to embryonic quality
486 (with CNP treatment), and that oocyte maturation (where the role of *BMP15* is more

associated) has not been tested and therefore the role of *BMP15* in the IVC of embryos is the point of this finding.

Another important point was that CNP-treated morulas tended to lower concentrations of Caspase-3 gene transcripts (*CASP3*). This gene is directly linked with the cell death program, apoptosis (Xie *et al*., 2002). *CASP3*, in oocytes, is associated with low competence and death of the oocyte (Pang *et al*., 2018; Pioltine *et al*., 2021). Initial studies suggest that mitochondria play a central role in apoptosis (Berridge, 2002), and after their injury, there is a loss of mitochondrial membrane potential and release of factors such as the apoptosis initiation factor and cytochrome C. Cytochrome released C activates caspase-9, which then activates effector caspases such as caspase-3 (Xie *et al*., 2002). Thus, when we observed the reduction in the abundance of *CAPS3* transcripts, we inferred that the morulae possibly had fewer lesions associated with cell death.

In blastocysts treated with CNP, it was possible to observe a reduction, when evaluating the fold change, of related targets predict embryo quality, lipid metabolism, fatty acid biosynthesis, elongation, endoplasmic reticulum action, synthesis steroid hormones, and catalyzes cholesterol cleavage *(BDNF, NLRP5, AGPAT9, ELOVL1, ELOVL4, IGFBP4* and *FDX1* transcripts). An indication of the decrease in the abundance of the *AGPAT9* transcript was observed in blastocysts treated with CNP. *AGPAT9* has been identified as a key regulator of lipid accumulation in adipocytes (Hosoi *et al*., 2010), suggesting that this may be a biomarker for lipid droplet content in the embryo. In theory, with the reduction in the abundance of *AGPAT9* and other targets, blastocysts cultured with CNP should have a lower lipid content, however, based on the results presented we were unable to measure its content or lipid profile. Thus, further studies are needed regarding embryonic metabolism, in order to understand what are the real changes caused by the inclusion of CNP.

512 The family of elongases of very long-chain fatty acids (*ELOVL*) are enzymes
513 responsible for the condensation reaction necessary for the biosynthesis of long-chain
514 fatty acids (FA). Increased *ELOVL1* expression is directly involved in the elongation of
515 saturated and monounsaturated FA (Monroig *et al*., 2010). The *ELOVL4* is an elongase
516 responsible for the biosynthesis of very long chain (VLC, ≥C28) saturated (VLC-SFA)
517 and polyunsaturated (VLC-PUFA) fatty acids (Hopiavuori *et al*., 2019). It is known that
518 FA are mainly stored as triacylglycerides (TAGs; main lipid class found in the cytoplasm
519 of mammalian cells), are stored as lipid droplets (Sudano *et al*., 2016), and their presence
520 may be a compromising factor in cryopreservation processes, increasing risks of cellular
521 injuries (Costa *et al*., 2020). In our results, we showed that *ELOVL4* was upregulated -
522 considering the fold change - therefore, CNP treatment may have potentiated the
523 biosynthesis of long-chain acids. However, we did not observe a reduction in the
524 production rates of CNP-treated embryos. Concomitantly, it was observed that control
525 blastocysts (without CNP treatment) had a greater abundance of pro-apoptotic and
526 apoptosis-related transcripts (*BID* and *CASP3*) than those that received CNP. This fact
527 may suggest a potential protective effect of CNP on cell metabolism in bovine embryos
528 during IVC.

529 Brain-derived neurotrophic factor (*BDNF*) is a member of the growth factors and
530 neurotrophin family. This factor is found in the brain and periphery, helping neuronal
531 survival and neurogenesis (Scheidt *et al*., 2015). Some studies suggest the importance of
532 *BDNF* - together with its high-affinity receptor, neurotrophin receptor kinase-2 (*NTRK2*)
533 - in the development and implantation of human embryos (Yu *et al*., 2012; Chow,
534 Wessels, Foster, 2020) and placental function (Garces *et al*. 2014; Sahay *et al*., 2017).
535 Thus, what we observed in the results may be an indication of a better competence in
536 embryos cultured with CNP, as they presented a significant downregulated *BDNF* -

although as it would be, this theoretical benefit was not evident in relation to the observed production rates.

In summary, this study detected and quantified the NPR2 receptor (for the first time to the best of our knowledge) in pre-implantation stages of the *in vitro* produced bovine embryo. Furthermore, it was determined that the use of CNP at a higher concentration than that described in the literature may alter embryonic metabolism based on the abundance of transcripts. Furthermore, maintenance was observed in the production rates of embryos cultured with CNP. *In vivo* functional tests will be able to prove, or not, whether these alterations are translated into better reproductive performance by these embryos treated with CNP in the IVC.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

CBC contributed to the conception and design of the study, collected and analyzed data, and wrote the manuscript. NCS performed most of the experiments. ANS assisted in performing the immunocytochemistry analysis. EMP and TD performed RNA extraction and reverse transcription for cDNA of samples subjected to gene expression analysis. ANS contributed to immunochemistry analysis. AAA, MMS and FVM provided technical support. MFGN contributed to the conceptualization and design of the entire study and supervised and contributed to critical revision and intellectual input to the manuscript. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

Supplementary Material

Table S1: Gene symbol, functions and primers assay ID used for microfluidic expression analyses (Biomark HD System - Fluidigm).

Gene Symbol	Function	Assay ID*
GAPDH	Reference gene	Bt03210912_g1
ACTB	Reference gene	PA5-16914
CASP9	Apoptosis	Bt04282453_m1
CD40	Apoptosis	Bt03817804_g1
TNFRSF21	Apoptosis	Bt03250597_m1
TP53	Apoptosis	Bt03223213_m1
MORF4L2	Apoptosis	Bt03270996_m1
BID	Apoptosis	Bt03241255_m1
BAX	Apoptosis	Bt03211777_g1
CASP3	Apoptosis	Bt03250954_g1
ADCY3	Cell development	Bt04289077_m1
NPPA	Cell development	Bt03223175_g1
NPPB	Cell development	Bt04301375_g1
NPR1	Cell development	Bt04297034_g1
TNFAIP6	Cell development	Bt03210223_m1
VCAN	Cell development	Bt03217633_m1
IGFBP4	Cell development	Bt03259500_m1
AQP3	Cell development	Bt03253663_m1
NLRP5	Cell development	Bt03218031_m1
VEGFA	Cell development	Bt03213282_m1
IMPDH1	Cell development	Bt00995384_m1
IMPDH2	Cell development	Bt03226238_g1
ATP5L	Cell development	Bt03210836_g1
DSC2	Cell development	Bt03649202_m1
DSC3	Cell development	Bt04301926_m1
MAPK1	Cell development	Bt03216718_g1
LHCGR	Cell development	Bt03213974_m1
LEP	Cell development	Bt03211909_m1
BMP15	Cell development	Bt03286494_u1
GDF9	Cell development	Bt03223996_m1
NANOG	Cell development	Bt03220541_m1
SOX2	Cell development	Bt03278318_s1
G6PD	Cell development	Bt03649181_m1
HSPD1	Cell development	Bt04301470_g1
NRP1	Cell development	Bt04284287_m1
NRP2	Cell development	Bt04284287_m1
BDNF	Cell development	Bt04316732_m1

ACSL5	Cell development	Bt03241747_m1
FSHR	Cell development	Bt03212674_m1
IGF2	Cell development	Bt03259224_m1
RPL15	Cell development	Bt03288449_g1
ADCY6	Cell development	Bt03816767_m1
ADCY9	Cell development	Bt04287024_m1
EGFR	Cell development	AJT96D7
HSPA1A	Cell development	Bt03292670_g1
NPPC	Cell development	Bt03212844_m1
NPR2	Cell development	Bt04316732_m1
GADD45A	Cell development	Bt03225650_m1
POU5F1	Cell development	Bt03223846_g1
CCND2	Cell development	Bt03249250_m1
ELF5	Cell development	Bt03220307_m1
FASN	Cell development	Bt03210485_m1
NOS3	Cell development	Bt03217679_m1
HSPA5	Oxidative stress	Bt03244880_m1
GFPT2	Oxidative stress	Bt03250351_m1
GLRX2	Oxidative stress	Bt03229700_m1
TXNRD1	Oxidative stress	Bt03215471_m1
VNN1	Oxidative stress	Bt03220248_m1
SOD1	Oxidative stress	Bt03215423_g1
GPX1	Oxidative stress	Bt03259217_g1
FDX1	Oxidative stress	Bt03217449_m1
ATF4	Oxidative stress	Bt03221057_m1
CAT	Oxidative stress	Bt03228713_m1
KEAP1	Oxidative stress	Bt03817661_m1
SLC2A3	Glucose transport facilitator	Bt03259514_g1
SLC2A1	Glucose transport facilitator	Bt03215314_m1
SLC2A5	Glucose transport facilitator	Bt03258296_m1
AKR1B1	Glucose transport facilitator	Bt03218049_g1
SLC2A4	Lipid metabolism	Bt03215316_m1
FADS2	Lipid metabolism	Bt03256255_g1
SCD	Lipid metabolism	Bt04307476_m1
DGAT1	Lipid metabolism	Bt03251719_g1
ACSL1	Lipid metabolism	Bt03248469_m1
ACAT1	Lipid metabolism	Bt03238649_g1
LPL	Lipid metabolism	Bt03240493_m1
CD36	Lipid metabolism	Bt03212335_mH
PNLIPRP2	Lipid metabolism	Bt03267914_m1
PNPLA2	Lipid metabolism	Bt03234129_g1
LIPE	Lipid metabolism	Bt03253691_m1
PLIN2	Lipid metabolism	Bt03212182_m1
PLIN3	Lipid metabolism	Bt03230537_m1
AGPAT9	Lipid metabolism	Bt04292093_m1
AGPAT1	Lipid metabolism	Bt03224587_g1
SREBF1	Lipid metabolism	Bt03276370_m1

ACACA	Lipid metabolism	Bt03213389_m1
AUH	Lipid metabolism	Bt03275798_m1
ACSL3	Lipid metabolism	Bt04282138_m1
ACSL6	Lipid metabolism	Bt03231692_m1
HMGCS1	Lipid metabolism	Bt04296095_g1
HMGCS2	Lipid metabolism	Bt03233809_m1
ELOVL1	Lipid metabolism	Bt03286627_s1
ELOVL2	Lipid metabolism	Bt03256849_m1
ELOVL4	Lipid metabolism	Bt03270721_m1
ELOVL5	Lipid metabolism	Bt03235956_m1
ELOVL6	Lipid metabolism	Bt00907566_m1

* ThermoFischer Scientific

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CAPÍTULO 2

CAN THE USE OF C-TYPE NATRIURETIC PEPTIDE IN *IN VITRO* CULTURE OF BOVINE EMBRYOS ALTER THE CHARACTERISTICS OF THEIR CRYOTOLERANCE?

Can the use of C-Type Natriuretic Peptide in *in vitro* culture of bovine embryos alter the characteristics of their cryotolerance?

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ABSTRACT

Despite the wide spread of *in vitro* production bovine embryos around the world, there is still a bottleneck when it comes to the distribution of these embryos. Cryopreservation is extremely important, but the results are not satisfactory. Thus, the objective of this study was to evaluate whether the addition of CNP in the *in vitro* culture of bovine embryos alters its cryotolerability through the modulation of the lipid content and profile, as well as the modulation of transcripts linked to embryonic metabolism. Initially, a concentration of 400 nM of CNP was used throughout the *in vitro* culture (inclusion from the beginning, i.e., Day 1 of the IVC) and blastocysts were collected for lipid content analysis by Sudan Black B. In addition, blastocysts from the control and CNP-400 groups were selected by morphological quality (excellent or good) and developmental stage (blastocysts to expanded blastocysts), and the collected were analyzed using *Multiple Reaction Monitoring* (MRM)-*profiling* technique. After that, blastocysts were vitrified using OPS. After 12 hours of warming, the rate of re-expansion and hatching was evaluated; and 24 and 48 hours the hatching rate. Subsequently the warming, hatched blastocysts from the two experimental groups were collected and evaluated for transcript abundance in relation to competence and quality (apoptosis, oxidative stress, proliferation, differentiation) and lipid metabolism in a microfluidic platform. Differences with probabilities less than $P < 0.05$, and/or Foldchange >1.5 were considered significant. We observed a reduction in the relative concentrations of palmitic (16:0), stearic (18:0) and cervonyl carnitine (22:6) in blastocysts cultured with CNP. A modulation of blastocyst transcripts was also observed, they showed upregulated transcription *ATF4* (*P=0.0004*), *BMP15* (*P=0.0043*); GFPT2 (*P=0.0043*), *PNPLA2* (*P=0.0322*), and *SOX* (*P=0.0302*); and downregulated transcript, *ELF5* (*P= 0.0124*). No difference was in production rates after warming with 400 nM CNP, and the embryonic lipid content also did not change. Thus, our study

identified possible modulations in the embryonic lipid profile, as well as in the abundance of some transcripts related to lipid metabolism. However, no change was observed in embryonic survival rates after vitrification. Thus, further studies are needed to evidence and prove a possible positive modification after cultivation with CNP.

Keywords: C-type natriuretic peptide, lipid metabolism, Multiple Reaction Monitoring profiling, targets, vitrification.

INTRODUCTION

Reproduction biotechniques make a decisive contribution to the beef and dairy production chain (Lopes *et al*., 2012). Among the available biotechniques, *in vitro* production (IVP) embryo stands out on the world stage. About 1,156,422 embryos were produced *in vitro* worldwide, about 12% more than in 2019 (1,156,422 *vs.* 1,031,567, respectively; IETS, 2021). Despite recent advances related to IVP, low efficiency in cryopreservation processes is still an obstacle. Since the first successful cryopreservation, several procedures have been developed. These methods can basically be classified into two main strategies: cryopreservation with a slow cooling curve and vitrification (Sudano *et al.*, 2016). Embryos produced *in vitro* have a high sensitivity to cryopreservation, which is apparently associated with the higher lipid content present in the cells of these embryos when compared to those produced *in vivo* (Camargo *et al*., 2011).

Thus, IVP embryos have a high lipid deposit, and the addition of fetal bovine serum (FBS) in the culture of these embryos is one of the possible explanations (Paschoal *et al.*, 2017). Lipids such as triacylglycerides (TAG), the main lipid class found in the cytoplasm of mammalian cells, are stored as lipid droplets (Sudano *et al.*, 2016) and, in cryopreservation processes, their presence is a possibly compromising factor due to the cellular damage it causes. In this way, numerous substances have already been tested with the aim of reducing or modulating the lipid profile (Sanches *et al.*, 2013; Paschoal *et al.*,

2016; Razza *et al.*, 2018; Costa *et al.*, 2019; Costa *et al*., 2020). Recently, the inclusion of the C-type natriuretic peptide (CNP) proved to be a possible modulator of the embryonic lipid profile (Costa *et al.*, 2020). Thus, the objective of our study was to evaluate whether the addition of CNP in the *in vitro* culture of bovine embryos alters its cryotolerability through the modulation of the lipid content and profile, as well as the modulation of transcripts linked to embryonic metabolism.

MATERIAL AND METHODS

All animal procedures were approved by the Ethics and Animal Handling Committee of the São Paulo State University (UNESP), Botucatu, São Paulo, Brazil, certificate #1180. All medium for the production of bovine embryos and solutions used in vitrification and devitrification were provided by ABS Global Brasil®, Mogi Mirim, São Paulo, Brazil.

In vitro production of embryos

Sample collection

Ovaries from a commercial slaughterhouse and from phenotypically seemed Nellore breed bovine females (that is, with the phenotype mostly from *Bos taurus indicus* animals of the Nellore breed) were collected, packaged, and transported to the laboratory in 0.9% saline solution between 30 and 35ºC. Follicles with a diameter of 2–8 mm were aspirated with hypodermic needles (30x8; 21G) attached to 10 mL syringes for the recovery of *cumulus-oocyte* complexes (COCs), with a maximum interval between the arrival of the ovaries at the slaughterhouse and the end of aspiration for one hour. Their classification was performed as described by Seneda *et al*. (2001). Only quality grade I and II CCOs were used.

937

938 *In vitro maturation*

939 Previously to *in vitro* maturation (IVM), COCs were washed three times in TCM-
940 HEPES 199 supplemented with 10% (v/v) fetal bovine serum, (FBS), 0.20 nM sodium
941 pyruvate and 83.4 mg /mL of gentamicin. The COCs were matured in drops of 100 µL of
942 TCM-199 medium bicarbonate supplemented with 10% (v/v) FBS, 5 µg of luteinizing
943 hormone, 0.5 µg of follicle-stimulating hormone, 1 µg of estradiol, 2.2 µg of pyruvate and
944 50 µg of gentamycin/mL and incubated for 24 h in an environment with maximum
945 humidity, 38.5°C and 20% CO_2.

946

947 *In vitro fertilization*

948 After maturation, the COCs were washed in HEPES-buffered TCM-199 medium
949 and transferred to 100-µL droplets of the fertilization medium that consisted of Tris-
950 buffered medium (TBM) supplemented with 8 mg/mL fatty acid-free bovine serum
951 albumin (BSA) and 1 mM glutamine. For fertilization in drops of 100 µL, semen from a
952 single Nellore bull was used (Adamo Fiv Kubera; Código 011NE03127, registro ACF
953 3522, Alta Genetics), which was previously validated in our laboratory. The
954 cryopreserved semen was heated at 36°C for 30 seconds. Sperm selection was performed
955 by Percoll gradient (Percoll 45% in the upper part and 90% in the lower part) by
956 centrifugation (12,100 g, for 2 minutes), the supernatant (600 µL) was discarded and the
957 pellet sperm resuspended in 300 µL of fertilization medium and homogenized. The semen
958 was centrifuged again (8,127 g, for 45 seconds) and, after discarding the supernatant, the
959 sperm concentration was adjusted to obtain a final concentration of $1x10^6$ live
960 spermatozoa in each drop containing 20 COCs. They were co-incubated for 20 to 22 hours

961 in an environment with maximum humidity, 20% CO_2, and 38.5° C. The day of
962 insemination was considered day zero (Day 0).

963 *In vitro culture*

964 For *in vitro* culture, presumptive zygotes were subjected to the removal of
965 cumulus cells by successive pipetting and then incubated in SOF (synthetic oviduct fluid)
966 medium supplemented with 8 mg/mL fatty acid-free BSA under mineral oil at sob the
967 same temperature and gaseous atmospheric condition used in the previous steps. On the
968 first day of cultivation (D1) the probable zygotes were divided into experimental groups:
969 control (without the addition of CNP) and C-400 groups (400 nM of CNP, Sigma–Aldrich
970 /St. Louis, MO, United States). After day three (D3), 50% of the culture media volume
971 was replaced by fresh media (1st feeding). At day 5 (D5), 50% of the culture media
972 volume was replaced with fresh SOF medium but this time was supplemented with
973 glucose (2nd feeding). During culture, blastocyst (D7) and hatching rates (D8 and D9)
974 were determined.

975

976 ***Lipid Content by Sudan Black B***

977 Expanded blastocysts from the control and CNP-400 groups (D7; 6 replicates)
978 were randomly selected during the experimental replicates and had their lipid content
979 measured as described by Sudano *et al.* (2012). Briefly, the embryos were fixed in a 10%
980 formaldehyde solution (diluted in PBS- pH 7.4) for 2 hours at room temperature. After
981 that, they were washed in distilled water with 0.05% polyvinyl alcohol (PVA) and
982 transferred to drops with 50% ethanol. After 2 minutes, the embryos were transferred to
983 drops with a 1% Sudan Black B solution (w/v) diluted in 70% ethanol for 1 to 2 minutes.
984 Subsequently, they were washed in three 50% ethanol baths for 5 minutes each and then
985 with distilled water containing 0.05% PVA. For mounting the slides, 10 µL of glycerol

was used. The evaluation was performed using light microscopy. To estimate lipid content, digital images were captured and processed using Image J 1.41 Software (Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) based on the methodology described by Sudano *et al.* (2012), and Costa *et al.* (2019).

Analysis of the lipid profile by MRM–profiling

Blastocysts from the control and CNP-400 groups (D7; n=10/group, 5 replicates) were selected by morphological quality (excellent or good) and developmental stage (blastocysts to expanded blastocysts). Thus, the collected structures were analyzed using *Multiple Reaction Monitoring* (MRM)-*profiling* technique. Briefly, the embryos were collected, washed in methanol:water (1:3 v/v) to remove the culture medium and stored at -80° C. Lipid extraction was performed according to Bligh and Dyer (1959) and adapted for small volume samples. Briefly, 40 µL of ultrapure water was added to a microtube containing the embryos, and the mixture was to promote cell lysis. Then, 50 µL of chloroform and 90 µL of methanol were added and mixed by pipetting for 15 seconds (one-phase solution). After, another 50 µL of CHCl3 and 50 µL of ultrapure H_2O were added and the samples were incubated for 5min at room temperature. Samples were centrifuged to enhance polar from nonpolar phase separation (two-phase solution). This procedure is intended to leave precipitated protein behind. The combined organic and water phases were dried in a centrifugal evaporator and samples were vacuum-sealed and stored at -80oC until MS analysis (De Lima *et al*., 2018).

Vitrification of embryos

On D7 and D8 (n=50 blastocysts/group, 5 replicates), only expanded blastocysts and IETS grade I blastocysts (excellent or good quality; Bó and Mapletoft, 2013) were subjected to vitrification using the OPS technique (Open Pulled Straw) developed by Vajta *et al.* (1997). The blastocysts were washed in three drops of H-SOF solution and submitted to a maintenance solution until the beginning of vitrification. Groups of no more than 5 blastocysts at a time were transferred to one well of a four-well plate containing 400 µL of maintenance solution, 50 µL of ethylene glycol, and 50 µL of dimethylsulfoxide (DMSO). After 1 minute, the structures were transferred to a drop of 10 µL of S1M solution plus 100 µL of ethylene glycol and 100 µL of DMSO. They remained in this drop for 20 seconds and then were placed at the end of the OPS with as little medium as possible. Immediately after loading, the OPS was immersed in liquid nitrogen, where it was stored until warming.

For warming, the OPSs was removed from the N2 and one OPS was devitrified at a time. The tip of the OPS, where the blastocysts were located, was immersed in a well of a four-well plate containing 800 µL of maintenance solution and 400 µL of S1M. Then, the blastocysts were transferred to the second well containing 400 µL of maintenance solution and 200 µL of S1M. The blastocysts spent time between well 1 and 2 did not exceed 5 minutes. The blastocysts were transferred to well 3 containing 400 µL of maintenance solution and 100 µL of S1M for 5 minutes and finally to well 4 in which there was only maintenance solution. After that, the blastocysts were washed in three drops of SOF medium and transferred to the culture plate. After 12 hours of warming, the rate of re-expansion and hatching was evaluated; and 24 and 48 hours the hatching rate.

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA from blastocysts after vitrification (3 embryos/group in 4 replicates) was extracted using the PicoPure RNA Isolation kit (Life Technologies, Foster City, CA, United States) following the manufacturer's protocol. Extracted RNA was stored at −80◦C until further analysis by qPCR. RNA concentration was quantified using a spectrophotometer (Nanodrop, Thermo Fisher Scientific, Waltham, MA, United States). For each sample, we used a pool of three blastocysts for reverse transcription. cDNA synthesis was performed using a High-Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA, United States), following the manufacturer's instructions. All samples were treated with DNase according to the manufacturer's instructions before reverse transcription.

Pre-amplification and qPCR

Gene expression analyses of bovine blastocysts were performed independently using Applied BiosystemsTM TaqMan©R Assays specific for B. taurus and based on Fontes *et al.* (2020). We analyzed the abundance of 95 transcripts using a panel of genes formatted to investigate embryonic competence and quality (apoptosis, oxidative stress, proliferation, differentiation), and lipid metabolism in a microfluidic platform (Supplementary Table S1 describing all the genes and their signaling pathways). Prior to qPCR thermal cycling, each sample was subjected to a sequence specific preamplification process as follows: 1.25 mL assay mix (TaqMan©R Assay was pooled to a final concentration of 0.2× for each of the 96 assays), 2.5 mL TaqMan PreAmp Master Mix (Applied Biosystems, #4391128), and 1.25 mL cDNA (5 ng/mL). The reactions were activated at 95◦C for 10 min, followed by denaturation at 95◦C for 15 s, annealing, and amplification at 0◦C for 4 min for 14 cycles. These preamplified products were diluted

fivefold (morula and embryos) prior to RT-qPCR analysis. Assays and preamplified samples were transferred to an integrated fluidic circuit plate. For gene expression analysis, the sample solution preparation consisted of 2.25 mL cDNA (preamplified products), 2.5 mL of TaqMan Universal PCR Master Mix (2×, Applied Biosystems), and 0.25 mL of 20× GE Sample Loading Reagent (Fluidigm, South San Francisco, CA, United States); the assay solution included 2.5 mL 20× TaqMan Gene Expression Assay (Applied Biosystems) and 2.5 mL of 2× Assay Loading Reagent (Fluidigm). The 96.96 Dynamic ArrayTM Integrated Fluidic Circuits (Fluidigm) chip was used for data collection. After priming, the chip was loaded with 5 mL each of the assay solution and each sample solution and loaded into an automated controller that prepares the nanoliter-scale reactions. The qPCR thermal cycling was performed in the Biomark HD System (Fluidigm) using the protocol TaqMan GE 96 × 96 Standard, which involved one stage of Thermal Mix (50◦C for 2 min, 70◦C for 20 min, and 25◦C for 10 min) followed by a hot start stage (50◦C for 2 min and 95◦C for 10 min), 40 cycles of denaturation (95◦C for 15 s), primer annealing, and extension (both at 60◦C for 60 s).

Statistical Analysis

To estimate the lipid content, the results of the analysis were evaluated of data distribution, being non-parametric data, the Kruskal-Wallis test was used followed by the *post hoc* Student-Newman-Keuls test. The data are present in the form of median, 1sd and 3rd interquartiles, and analyzes were performed with SigmaStat 4.0 software

To evaluated blastocyst rates and hatching kinetics after warming, data were tested for normality using the Shapiro–Wilk and Bartlett tests. After then, Tukey's test was applied. Differences with probabilities less than 0.05 ($P < 0.05$) were considered

significant. The data is presented as mean values and standard error of the mean (SEM), and analyzes were performed with SigmaStat 4.0 software.

The lipid profile data by MRM-profiling, data analysis was performed, with the first step (discovery) applying a list of MRMs generated by combining the *m/z* of the analysis in relation to the ions based on the online platform LipidMAPS (http://www.lipidmaps.org/) with expected product ion resulting from precursor scan (Prec) and neutral loss scans (NL; Supplementary Table S1). In the case of data analysis in the second stage (screening), instead of simply using all MRMs above the blank to create the lipid profile, a noise reduction filter was applied, which consists of eliminating the MRMs that did not produce an ion signal above 30% of the blank sample in at least one of the analyzed samples. Subsequently, the ionic intensity value of each MRM was normalized by the total ion count (TIC) to obtain the relative amounts, that is, the lipid profile. MetaboAnalyst 4.0 (http://www.metaboanalyst.ca) was used for multivariate statistics by Principal Component Analysis (PCA). The two experimental groups, control and C-400 group (400 nM of CNP) were evaluated with Student's t test. Fold-change values were also calculated and considered significant when Fold-changes (FC) > ±1.5 e $P<0.05$.

Quantitative PCR data were assessed using the ΔCq values relative to the geometric mean of the best reference genes among the 95-gene set, i.e., GAPDH, and ACTB. Fold-changes (FC) were calculated using the $2^{-\Delta Cq}$ method. All analyses were performed using SigmaStat 4.0 and MetaboAnalyst 5.0. The evaluation of the transcripts was initially performed with the univariate statistical analysis method, using FC, T-test, Volcano Plot. In a second moment, we analyzed the data by multivariate methods, considering Principal Component Analysis (PCA) and Partial Least Squares- Discriminant Analysis (PLS-DA)

and their variations. Differences with probabilities less than $P < 0.05$, and FC >1.5. were considered significant.

RESULTS

Lipid content by Sudan Black B

There was no difference in lipid content analyzed between embryos without CNP supplementation (control) compared to embryos supplemented with 400nM CNP (Table 1; Figure 1).

Table 1- Intracytoplasmic lipid content of IVP bovine embryos supplemented or not with CNP (when added on the first day of culture).

Parameter	**Control**	**C-400**	***P*-value**
Gray intensity (X 10^{-6})	2.29 ± 6.66	2.12 ± 5.11	0.109
Gray intensity per area (X 10^{-12}/ μm^2)	5.17 [3.35, 7.03]*	4.77 [2.84, 6.04]*	0.230
Gray intensity per volume (X 10^{-15}/ μm^3)	10.04 [6.02, 1.52]*	9.37 [4.90, 1.26]*	0.266

* The data are present in the form of median, 1sd and 3rd interquartiles.
CNP-400: 400 nM de CNP.
Data are the mean ± standard error of the mean (SEM) of 6 replicates. Control, n=69; C-400, n= 66.

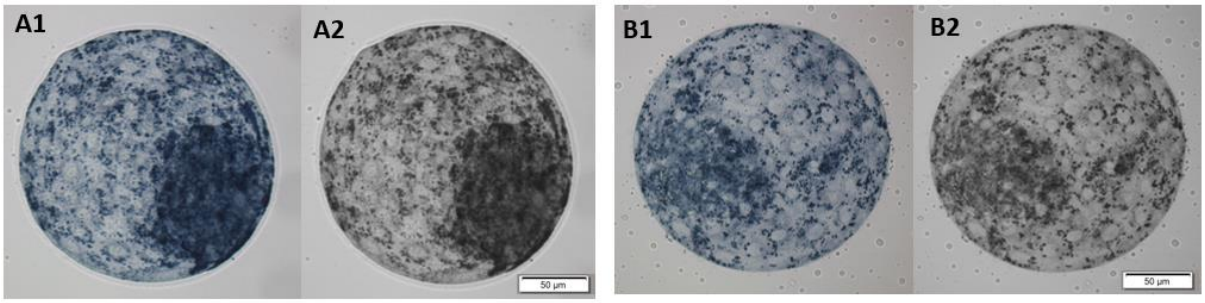

Figure 1- Illustrative light microscopy images of IVP bovine embryos submitted to culture supplemented with CNP. **(A)** Bovine embryo without CNP supplementation (control); **(B)** Bovine embryo submitted to 400 nM CNP supplementation. (1) Images captured by light microscopy; (2) Images converted to grayscale using Image J 1.41 Software. Original magnification x 500

Lipid profile of embryos cultured with CNP

In the analysis of the lipid profile of bovine embryos, an alteration in different lipid subclasses was observed after cultivation with CNP (Table 2). However, there was no discriminating grouping between embryos cultured or not with CNP (Figure 2).

Table 2- List of compounds that showed difference in relative intensity between blastocysts from groups not cultured with CNP (control) and treated with 400 nM CNP (C-400) (*P < 0.09*).

Lipid Subclass	**Possible Compound**[a]	***P-value***
Acyl-carnitines	2-ethylacryloylcarnitine, Tiglylcarnitine	0.02276
Triacylglycerol	TAG(52:3)_FA 16:1	0.03288
	TAG(56:3)_FA 18:1	0.04092
	TAG(52:2)_FA 18:2	0.05211
	TAG(52:2)_FA 16:1	0.07285
Phosphatidylcholine	PC(32:0)	0.05471
	PC(36:4)	0.07445
	PC(34:1)	0.07618
	PC(34:2)	0.08099
	PC(36:0), PCp(38:6)	0.08592
Sphingomyelin	SM(d18:0/18:0)	0.03806
	SM(d18:0/20:0)	0.05367
Cholesteryl esters	20:0 Cholesteryl ester	0.01974
	18:0 Cholesteryl ester	0.04315
	22:5 Cholesteryl ester	0.06706
	16:0 Cholesteryl ester	0.08723
Free Fatty Acids	C16:0	0.01552
	C18:0	0.01645
	C22:6	0.01841
	C22:0	0.05237
	C20:0	0.06161
	C20:1	0.06984
	C24:5	0.08354
Glycerophospholipids	PS(28:0)	0.01083
	PG(36:0), PGp(38:6)	0.05024
	PG(34:1)	0.05467
	PCo(32:3)	0.06729
	PS(32:2)	0.08094

[a] Tentative assignment does not guarantee the actual structures of corresponding ions. The names were acquired from LIPID MAPS Structure Database (http://www.lipidmaps.org/). TAG = triacylglycerides; FA = fatty acids; PC = phosphatidylcholine; PCp = phosphatidylcholinephosphate; SM = sphingomyelin; PS = phosphatidylserine; PG = phosphatidylglycerol; PGp = glycerophosphoglycerophosphate; PCo = phosphocholine.

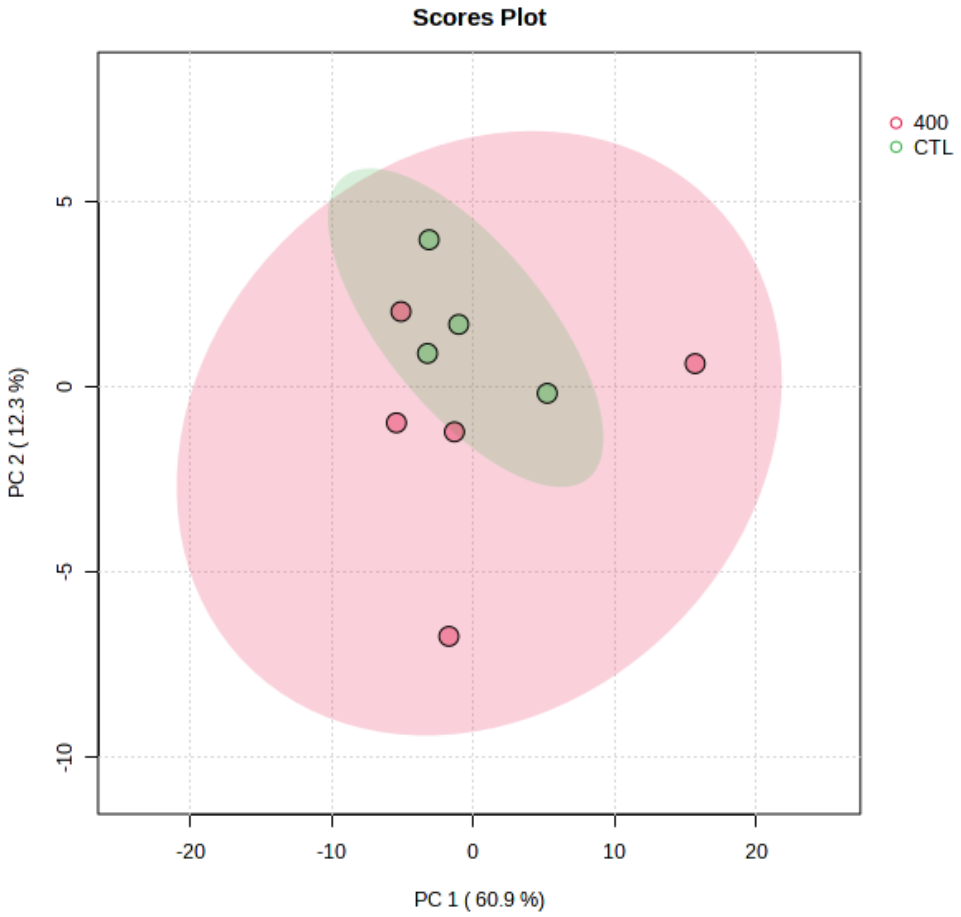

Figure 2- Multivariate analysis of the difference in relative intensity of lipid ions between blastocysts from the groups treated with CNP (400 nM of CNP; 400) and control (CTL). PLS-DA shows the 2D score plot between groups.

Embryos cultured with CNP showed a reduction in the relative abundance of some ions belonging to different lipid classes, such as acylcarnitines (Figure 3), fatty acids (Figure 4), glycerophospholipids (Figure 5) and sphingomyelins (Figure 6).

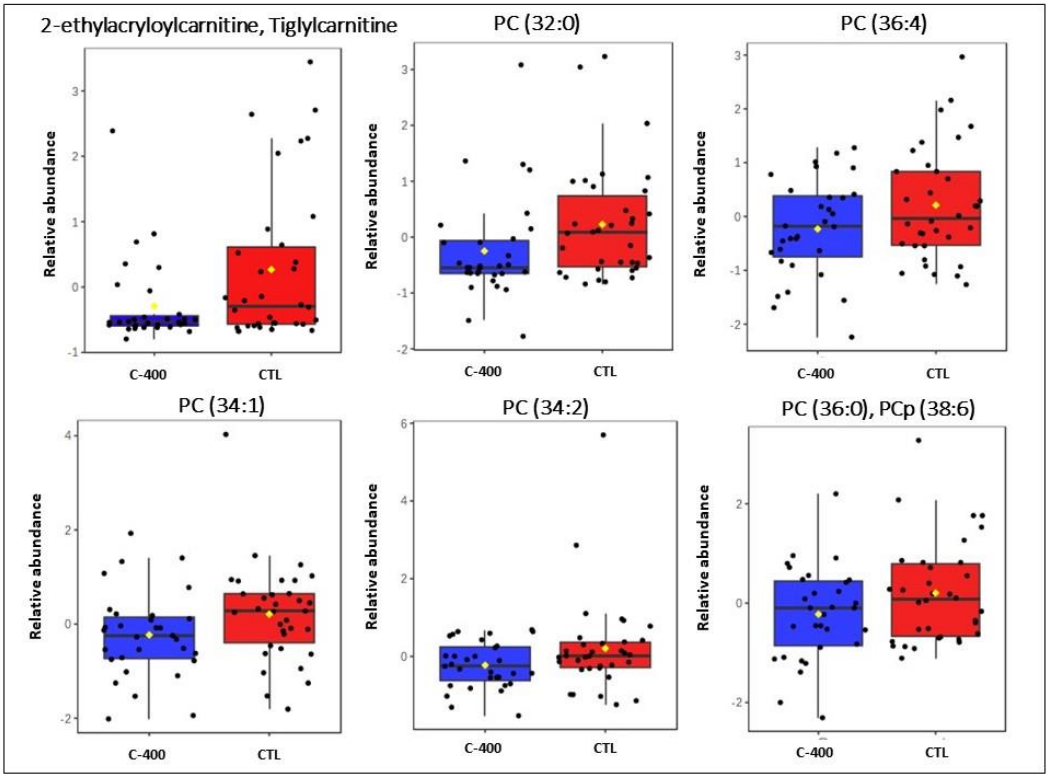

Figure 3 - Boxplot of the lipid classes of acylcarnitines and phosphatidylcholines and their relative abundances of lipid ions in bovine embryos, modulated after culture with 400 nM of CNP (C-400). PC=phosphatidylcholine; PCp= phosphatidylcholinephosphate.

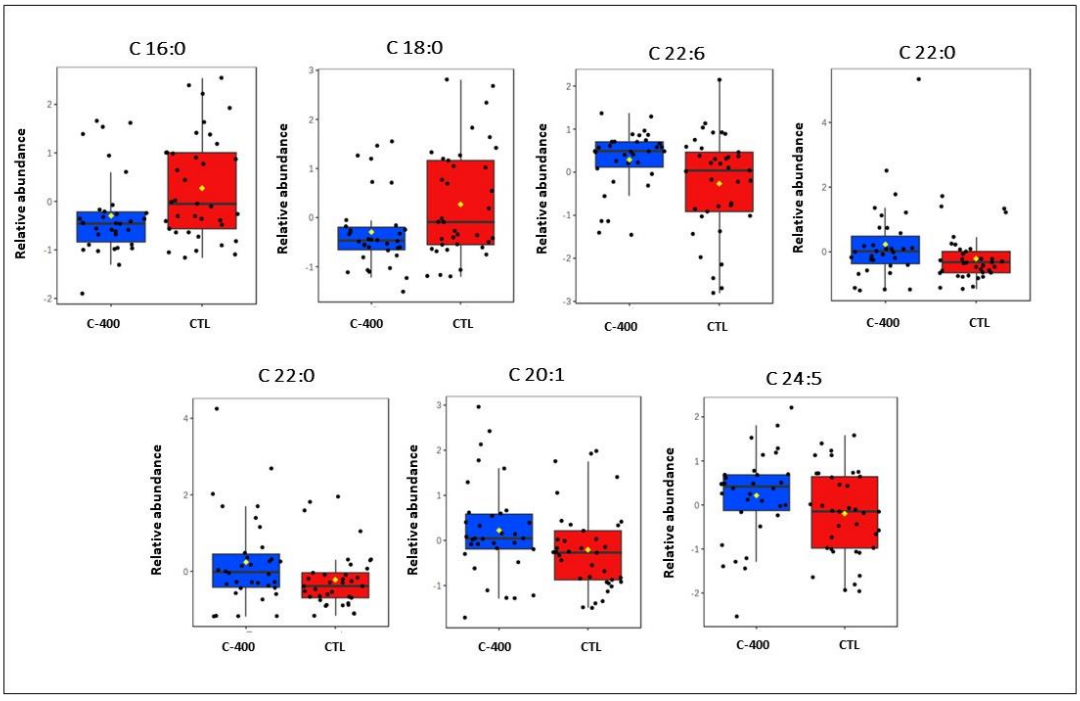

Figure 4 - Boxplot of the lipid class of fatty acids and their relative abundances of lipid ions in bovine embryos, modulated after culture with 400 nM of CNP (C-400).

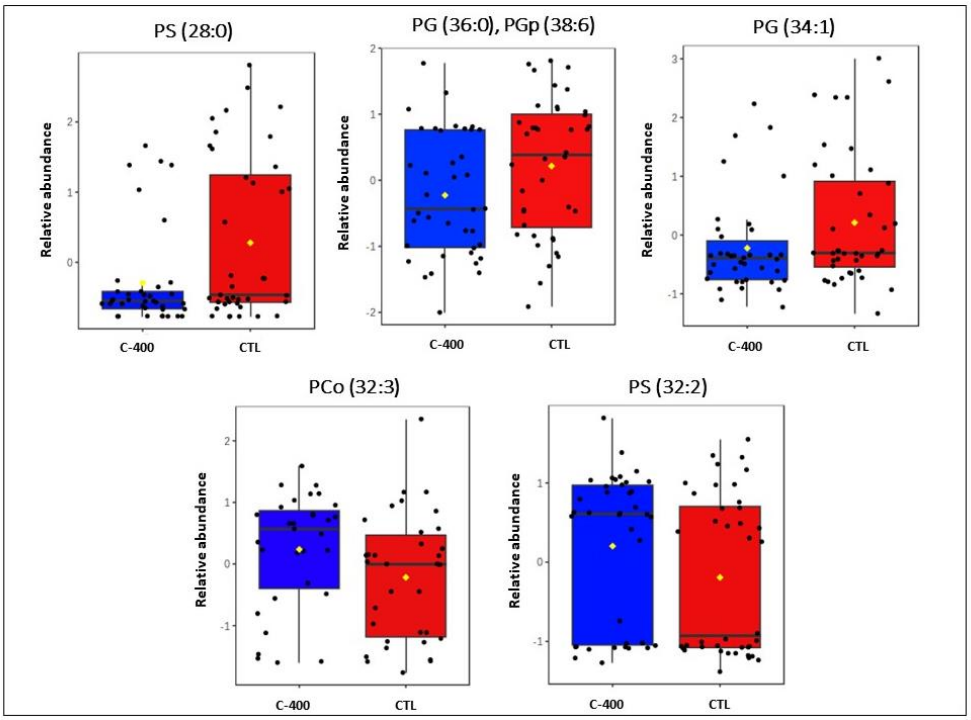

Figure 5 - Boxplot of the class of glycerophospholipids and their relative abundances of lipid ions in bovine embryos, modulated after culture with 400 nM of CNP (C-400). PS= phosphatidylserine; PG=phosphatidylglycerol; PGp= glycerophosphoglycerophosphate; PCo= phosphocholine.

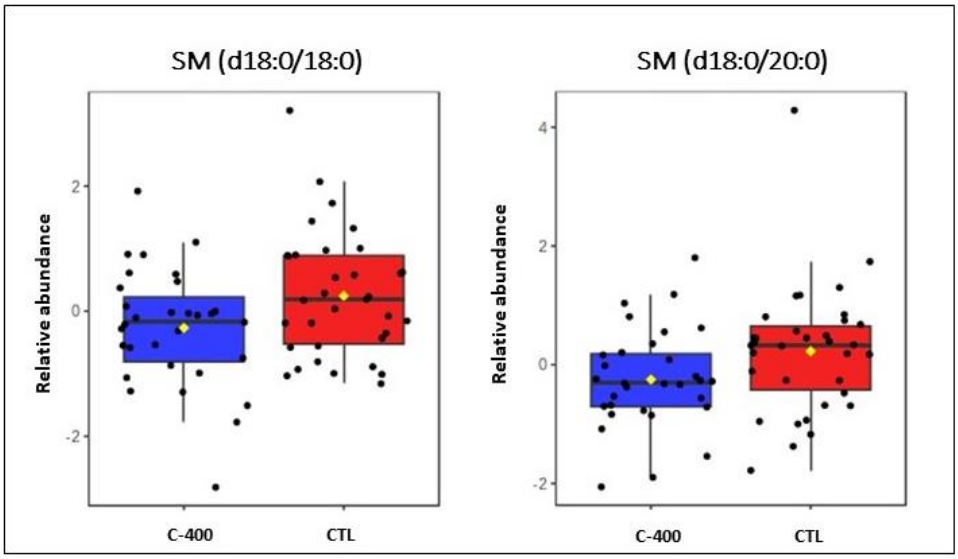

Figure 6 - Boxplot of the sphingomyelin (SM) class and its relative abundances of lipid ions in bovine embryos, modulated after culture with 400 nM of CNP (C-400).

Warming, Re-expansion and Hatching rates of blastocysts

Regarding the rate of re-expanded and hatched blastocysts, there was no difference between the rates of re-expansion and hatching at 12, 24, and 48 hours after warming between the evaluated groups (Tables 3 and 4).

Table 3- Evaluation on embryonic development rate of bovine embryos (D7) cultured in the presence or absence of CNP (supplementation with 400 nM of CNP) which underwent vitrification.

Group	PZ N	Blastocyst N (% mean ± SEM)	Hatched Blastocysts N (% mean)*
Fresh control	104	29 (27.75 ± 5.37)	22 (75.86)
Control	1714	476 (27.88 ± 3.81)	
C-400	1740	504 (28.92 ± 4.93)	
P-value	-	0.21	

CNP-400: 400 nM de CNP.
PZ = Presumptive zygotes.
Data are the mean ± standard error of the mean (SEM) of 8 replicates except for *.
* Statistical comparisons were only applied to vitrified blastocyst groups. The fresh CTL group was our laboratory control.

Table 4- Re-expansion and hatching rate of embryos after vitrification on Days 7 and 8 (only expanded blastocysts) from groups Control or C-400 (supplementation with 400 nM of CNP).

Group	Vitrified Embryos (N)	Re-exp. rate12h (%) [N (%média ±SEM)]	Hatch. rate12 h (%) [N (%média ± SEM)]	Hatch. rate24 h (%) [N (%média ± SEM)]	Hatch. rate48 h (%) [N (%média ± SEM)]	Hatch. rate [N (%média ± SEM)]
Control	88	47 (51.22 ± 1.60)	4 (9.52 ± 0.46)	14 (39.10 ± 0.76)	17 (28. 57 ± 0.71)	35 (76.19 ± 0.93)
C-400	92	50 (45.45 ± 2.12)	2 (4.00 ± 0.35)	14 (32.00 ± 0.93)	29 (40.00 ± 1.15)	45 (76.00 ± 1.69)
P-value		0.37	0.59	0.36	0.48	0.26

CNP-400: 400 nM de CNP.
Abbreviations: Re-exp.: re-expansion; Hatch.: hatching.
Data are the mean ± standard error of the mean (SEM) of 5 replicates.

The Effect of CNP after cryopreservation on the Abundance of Target-Transcripts in embryos during IVC

Transcription abundance on embryos of differences in *ATF4* (*P*= 0.0004), *BMP15* (*P*=0.0043), *GFPT2* (*P*=0.0043), *PNPLA2* (*P*=0.0322), and *SOX* (*P*=0.0302). In the analysis of the Fold change, a variation was observed in 10 targets (FC >1.5; Table 5). In Principal Component Analysis (PCA; Figure S1) and Partial Least Squares - Discriminant Analysis (PLS-DA) it was possible to observe an overlap in PCA plot, and in PLS-DA a slight separation between the control and CNP-treated groups (Figure 7A). In the Heatmap, there was a slight clustering between the samples (Figure 7B). All the others target transcripts analyzed in blastocysts did not differ in a statistically significant way (Supplementary Table S2).

Table 5- Upregulated and downregulated transcription observed in bovine blastocyst after cryopreservation. The relative abundance of transcripts was selected by the Fold change analysis (upregulated with magnitude greater than 1.5 times, that is, with the threshold > 1.5; and downregulated with magnitude greater than 1.5 times, ie, with the threshold < 0.667). The values shown were calculated as the ratio of the control group to the treated group (treated /control).

	Gene symbol	**Site of action and metabolic pathways**	**Fold Change**	***P-value***
Upregulated	ATF4	ER stress	1.6179	0.0004
	BMP15	Oocyte maturation / follicular development	2.6287	0.0043
	GFPT2	Oxidative stress	2.3075	0.0043
	PNPLA2	Lipid metabolism/Glycerolipid/Degradation	1.5038	0.0322
	SOX2	Pluripotency/chromatin binding/DNA methilation	1.577	0.0302
	ELOVL4	Fatty acid biosynthesis, elongation, endoplasmic reticulum	1.5046	0.0779
	IGFBP4	Either inhibit or stimulate the growth promoting effects of the IGFs	1.8001	0.4751
Downregulated	ELF5	Cell differentiation/trofectoderm	0.59532	0.0124
	PDE5A	cGMP/Meiotic arrest	0.58397	0.1532
	VCAN	Cumulus expansion/Matrix	0.63453	0.7212

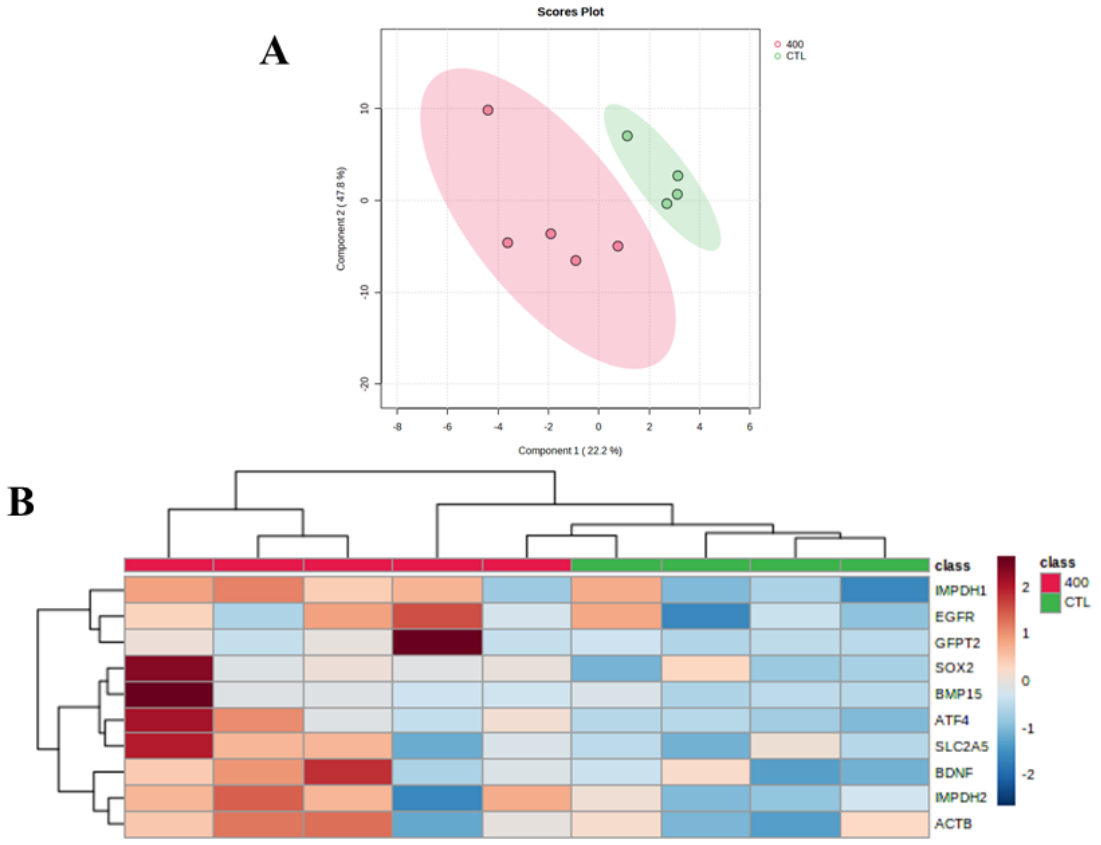

Figure 7- Plots derived from multivariate analysis the abundance of transcripts derived from untreated (control) and CNP-treated blastocyst after cryopreservation**. (A)** PLS-DA shows the 2D score plot between groups. **(B)** Heatmap showing transcriptional profiles abundance of *in vitro*–produced bovine embryos with 400 nM CNP and control group. N = 4 per group.

DISCUSSION

Our study is the first to present the use of CNP in the *in vitro* culture of bovine embryos with the aim of modulating the profile and lipid content, making it more cryotolerable. With regard to transcript analysis - and these related to lipid metabolism, embryonic development and oxidative stress - changes were observed in blastocysts treated with CNP. Furthermore, when analyzing the embryonic lipid profile, it was possible to observe changes in some lipid classes in their relative concentration. However, there was no change in the hatching kinetics and rate of re-expansion and hatching after

warming blastocysts cultured with CNP, together with no change in the embryonic lipid content.

The use of exogenous CNP in pre-IVM and IVM has been used for years, with different concentrations and in different species (Franciosi *et al.*, 2014; Soares *et al*., 2017; Botigelli *et al*., 2018; Xi *et al*., 2019; Ang *et al*., 2021). However, the relationship between CNP and the embryo has few reports in the literature (Botigelli *et al*., 2018; Xi *et al.*, 2019; Costa *et al*., 2020). It is known that CNP binds to its receptor NPR2 and modulates intracellular cAMP concentrations in oocytes in the *in vitro* maturation phase (Franciosi *et al.*, 2014). In addition, it is suspected that CNP can stimulate cGMP, which exerts an action similar to cAMP, stimulating lipolysis and reducing the amount of lipids. (Costa *et al.*, 2020). However, our study did not show alteration in the relative lipid content of embryos cultured with a concentration of 400 nM of CNP, similarly to what occurred in a study previously carried out by our research group (Costa *et al*., 2020). Despite the use of 4 times the concentration of CNP - we used 400 nM of CNP in our study, unlike the one used by Costa *et al.* (2020), who used 100 nM of CNP - together with a longer exposure time, we did not observe differences in content.

Unlike the results with the semiquantitative analysis by Sudan Black B, we observed that the culture of embryos with CNP altered the lipid profile. Some lipid ions from the classes of triacylglycerides, cholesterol esters, glycerophospholipids and free fatty acids, in addition to acylcarnitine, underwent changes in their relative concentration. Phospholipids and cholesterol esters (in the form of lipid droplets) play a crucial role in membrane formation and energy production during rapid cell divisions after oocyte fertilization, and fatty acids such as palmitic (16:0), stearic (18:0) and oleic (18:1) have already been mentioned as of great importance and in the supplementation of the *in vitro* production of embryos (Prates *et al*., 2014; De Lima *et al*., 2018). In the present study, a

reduction in the relative concentrations of palmitic (16:0), stearic (18:0) and cervonyl carnitine (22:6) was observed, with the inference that the embryos had a potential increase in metabolism and, consequently, there was a breakdown of these fatty acids - or there was no incorporation of fatty acids derived from the means of production due to CNP supplementation in the IVC. Comparatively, the excess of fatty acids and triacylglycerides in rat spermatozoa was negatively associated with movement alteration, resulting in infertility of these animals (Kuang *et al*., 2021).

Bovine embryos produced *in vitro* already appear to have an overabundance of glycerophospholipids and triacylglycerides (González-Serrano *et al*., 2013). However, after using CNP there was a reduction in some ions of this lipid class. An overabundance of phospholipids, particularly phosphatidylcholine, appears to be regarded as a positive biomarker for successful cryopreservation (Wu *et al*., 2010; González-Serrano *et al*., 2013). The results obtained in this study detected a reduction in the abundance of phosphatidylcholine [PS(28:0)] and phosphatidylglycerol [PG(36:0), PGp(38:6) and PG(34:1)]. However, there was no reduction in survival after vitrification and warming of these blastocysts. Vitrified (and CNP-cultured) embryos showed no improvement in re-expansion or hatching rates. However, the results of *in vivo* embryo transfer are still needed, whether cryopreserved or freshly transferred, so that there is greater clarity about possible effects of CNP on the IVC of these embryos.

The transcript of bone morphogenetic protein 15 (*BMP15*) was upregulated in blastocysts that received CNP in the IVC and evaluated after cryopreservation. *BMP15*, in mammals, is related to oocyte maturation and cholesterol biosynthesis, with the aim of improving oocyte competence and consequently the development of the bovine embryo (Caixeta *et al*., 2013; Cajas *et al*., 2020). It has already been observed that mutation in the *BMP15* gene affects glycolysis and cholesterol biosynthesis in *cumulus* cells (Su *et al*.,

2008). A possible inference for the finding in the present study would be that CNP could be intensifying the expression of *BMP15* by modulating cholesterol biosynthesis through a potential increase in cGMP, in addition to the stress that the cryopreservation process can cause in cells.

In the blastocysts evaluated after vitrification and warming, it was possible to observe a greater abundance of the *ATF4* gene transcript. Activating transcription factor 4 (*ATF4)* is a transcription factor that upregulates genes involved in amino acid import, glutathione biosynthesis, and antioxidant stress response (Harding *et al*., 2000; 2003). In addition, *ATF4 i*s related to the prevention of endoplasmic reticulum stress (Botigelli *et al*., 2018). Thus, we infer that culture, supplemented with CNP, may have a greater potential for preventing damage resulting from oxidative stress, mainly caused by cryopreservation processes.

Furthermore, there was an increase in the abundance of the *GFPT2* gene (Glutamine-Fructose-6-Phosphate Transaminase 2). *GFPT2* is related to the metabolism in the insulin and EGF network, and its expression was regulated by reduced glutathione (GSH) and suppressed by the oxidative stress regulator GSK3-β (Wang *et al.*, 2022). This fact, in our study, may be related to the lack of protection perhaps by CNP. However, we did not observe any decrease in rates after warming, but further studies are needed to prove the viability of this embryo after supplemented culture and vitrification.

The *PNPLA2* gene (Patatin Like phospholipase domain containing 2) encodes an enzyme which catalyzes the first step in the hydrolysis of triglycerides in adipose tissue and where triacylglycerols are hydrolyzed to diacylglycerols (Lehner and Quiroga, 2016). O *PNPLA2* works in cells, along with other lipases, degrading TAGs, resulting in the release of FAs and glycerol structure (Yang and Mottillo, 2020). However, this lipolysis may occur non-linearly, and may result in a re-esterification and recycling of FAs (Zhang

et al., 2019). Thus, with our results, we did not observe an expressive reduction of TAGs after cultivation with CNP, but there was a modulation in some lipid classes. We also did not observe an increase in the relative concentrations of FAs, so with the results obtained, we cannot infer a precise action of *PNPLA2*.

In summary, this study showed changes in embryonic metabolism based on the abundance of transcripts and analysis of the lipid profile. Furthermore, maintenance of production rates was observed after vitrification of embryos cultured with CNP, in addition to no change in embryonic lipid content. More studies are needed to really prove the action on embryonic metabolism, consequently, these changes will bring better results *in vivo*.

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SUPPLEMENTARY MATERIAL

Supplementary Material

Table S1: Complete method containing the 348 MRMs that were used for the screening step.

Compound Name	MRM
2-ethylacryloylcarnitine, Tiglylcarnitine	244.1 -> 83.1
(2E)-hexenedioylcarnitine, O-octanoylcarnitine	288.1 -> 85.1
Decanoylcarnitine	316.2 -> 85.1
O-(11-carboxyundecanoyl)carnitine	374.2 -> 60.1
2-Hydroxymyristoylcarnitine, 3-hydroxytetradecanoylcarnitine	388.3 -> 60.1
(7Z,10Z)-hexadecadienoylcarnitine	396.3 -> 85.1
Palmitoylcarnitine, (5Z)-13-carboxytridec-5-enoylcarnitine	400.3 -> 60.1
O-(13-carboxytridecanoyl)carnitine	402.3 -> 60.1
3-hydroxypalmitoleoylcarnitine, Heptadecanoyl carnitine	414.3 -> 60.1
Stearidonyl carnitine	420.3 -> 60.1
Linoelaidyl carnitine, O-linoleoylcarnitine, 9,12-Hexadecadienylcarnitine	424.3 -> 85.1
O-oleoylcarnitine, Elaidic carnitine	426.4 -> 60.1
Stearoylcarnitine, hexadecanedioic acid mono-L-carnitine ester	428.4 -> 60.1
(9Z,12Z,15Z)-3-hydroxyoctadecatrienoylcarnitine	438.3 -> 85.1
3-hydroxylinoleoylcarnitine,	440.3 -> 85.1
(9Z)-3-hydroxyoctadecenoylcarnitine	442.3 -> 60.1
12-Hydroxy-12-octadecanoylcarnitine, 3-hydroxyoctadecanoylcarnitine	444.4 -> 60.1
O-arachidonoylcarnitine	448.3 -> 85.1
(11Z)-eicoseneoylcarnitine	454.4 -> 85.1
Arachidyl carnitine, O-[(9Z)-17-carboxyheptadec-9-enoyl]carnitine	456.4 -> 60.1
O-(17-carboxyheptadecanoyl)carnitine	458.3 -> 60.1
3-hydroxyarachidonoylcarnitine	464.3 -> 60.1
Docosa-4,7,10,13,16-pentaenoyl carnitine, Clupanodonyl carnitine	474.4 -> 60.1
(7Z,10Z,13Z,16Z)-docosatetraenoylcarnitine	476.4 -> 60.1
O-behenoylcarnitine	484.4 -> 85.1
STD 18:1 (d7) Lyso PC	529.6 -> 184.1
SM(d18:1/14:0)	675.5 -> 184.1
SM(d16:1/18:1)	701.6 -> 184.1
PC(30:2)	702.5 -> 184.1
SM(d18:1/16:0)	703.6 -> 184.1
PC(30:1)	704.5 -> 184.1
SM(d18:0/16:0)	705.6 -> 184.1
PC(30:0)	706.5 -> 184.1
PCo(32:3)	714.5 -> 184.1
PCo(32:2)	716.6 -> 184.1
PCo(32:1)	718.6 -> 184.1
PC(32:3)	728.5 -> 184.1
SM(d18:1/18:1)9Z))	729.6 -> 184.1

PC(32:2)	730.5 -> 184.1
SM(d18:1/18:0)	731.6 -> 184.1
SM(d18:0/18:0)	733.6 -> 184.1
PC(32:0)	734.6 -> 184.1
STD_d18:1-18:1(d9) SM	738.64 -> 184.1
PCo(34:3)	742.6 -> 184.1
PCo(34:2)	744.6 -> 184.1
PCo(34:1)	746.6 -> 184.1
PCo(34:0)	748.6 -> 184.1
PC(34:5)	752.5 -> 184.1
STD_15:0-18:1(d7) PC	753.5 -> 184.1
PC(34:4)	754.5 -> 184.1
PC(34:3)	756.6 -> 184.1
SM(d16:1/22:1)	757.6 -> 184.1
PC(34:2)	758.6 -> 184.1
SM(d18:1/20:0)	759.6 -> 184.1
PC(34:1)	760.6 -> 184.1
SM(d18:0/20:0)	761.6 -> 184.1
PC(34:0)	762.6 -> 184.1
PCp(36:5)	764.6 -> 184.1
PCo(36:5)	766.6 -> 184.1
PCo(36:4)	768.6 -> 184.1
PCo(36:3)	770.6 -> 184.1
PCo(36:2)	772.6 -> 184.1
PC(36:8), PCo(36:1)	774.5 -> 184.1
PC(36:7), PCo(36:0)	776.5 -> 184.1
PC(36:5)	780.6 -> 184.1
PC(36:4)	782.6 -> 184.1
SM(d18:2/22:1)	783.6 -> 184.1
PC(36:3)	784.6 -> 184.1
SM(d16:1/24:1)	785.6 -> 184.1
PC(36:2)	786.6 -> 184.1
SM(d16:1/24:0)	787.7 -> 184.1
PC(36:1)	788.6 -> 184.1
SM(d18:0/22:0)	789.7 -> 184.1
PC(36:0), PCp(38:6)	790.6 -> 184.1
PCo(38:6)	792.6 -> 184.1
PCo(38:5)	794.6 -> 184.1
PCo(38:4)	796.6 -> 184.1
PC(38:7), PCo(38:0)	804.6 -> 184.1
PC(38:6)	806.6 -> 184.1
PC(38:5)	808.6 -> 184.1
PC(38:4)	810.6 -> 184.1
SM(d18:2/24:1)	811.7 -> 184.1
PC(38:3)	812.6 -> 184.1
SM(d18:1/24:1)15Z))	813.7 -> 184.1

PC(38:2)	814.6 -> 184.1
SM(d18:1/24:0)	815.7 -> 184.1
PC(38:1)	816.6 -> 184.1
SM(d18:0/24:0)	817.7 -> 184.1
PCp(40:6), PC(38:0)	818.6 -> 184.1
PCo(40:6)	820.6 -> 184.1
TAG(48:2)_FA 18:1	820.8 -> 521.8
TAG(48:2)_FA 18:2; TG 18:2_30:0	820.8 -> 523.8
TAG(48:2)_FA 16:0	820.8 -> 547.8
TAG(48:2)_FA 16:1	820.8 -> 549.8
TAG(48:1)_FA 18:1	822.8 -> 523.8
TAG(48:1)_FA 16:0	822.8 -> 549.8
TAG(48:1)_FA 16:1	822.8 -> 551.8
TAG(48:0)_FA 18:0	824.8 -> 523.8
TAG(48:0)_FA 16:0	824.8 -> 551.8
STD_15:0-18:1(d7)-15:0 TAG	829.8 -> 570.4
PC(40:5)	836.6 -> 184.1
PC(40:0), PCp(42:6)	846.7 -> 184.1
TAG(50:4)_FA 18:1	846.8 -> 547.8
TAG(50:4)_FA 18:2	846.8 -> 549.8
TAG(50:4)_FA 16:0	846.8 -> 573.8
TAG(50:4)_FA 16:1	846.8 -> 575.8
PCo(42:6)	848.6 -> 184.1
TAG(50:3)_FA 18:1	848.8 -> 549.8
TAG(50:3)_FA 18:2	848.8 -> 551.8
TAG(50:3)_FA 16:0	848.8 -> 575.8
TAG(50:3)_FA 16:1	848.8 -> 577.8
TAG(50:2)_FA 18:0	850.8 -> 549.8
TAG(50:2)_FA 18:1	850.8 -> 551.8
TAG(50:2)_FA 16:0	850.8 -> 577.8
TAG(50:2)_FA 16:1	850.8 -> 579.8
TAG(50:1)_FA 18:0	852.8 -> 551.8
TAG(50:1)_FA 18:1	852.8 -> 553.8
TAG(50:0)_FA 16:0	852.8 -> 579.8
TAG(52:5)_FA 20:4	870.8 -> 549.796
TAG(52:4)_FA 20:4	872.8 -> 551.8
TAG(52:4)_FA 18:1	872.8 -> 573.8
TAG(52:4)_FA 18:2	872.8 -> 575.8
TAG(52:4)_FA 16:0	872.8 -> 599.8
TAG(52:3)_FA 18:1	874.8 -> 575.8
TAG(52:3)_FA 18:2	874.8 -> 577.8
TAG(52:3)_FA 16:0	874.8 -> 601.8
TAG(52:3)_FA 16:1	874.8 -> 603.8
TAG(52:2)_FA 18:0	876.8 -> 575.8
TAG(52:2)_FA 18:1	876.8 -> 577.8
TAG(52:2)_FA 18:2	876.8 -> 579.8

TAG(52:2)_FA 16:0	876.8 -> 603.8
TAG(52:2)_FA 16:1	876.8 -> 605.8
TAG(52:1)_FA 18:0	878.8 -> 577.8
TAG(52:1)_FA 18:1	878.8 -> 579.8
TAG(52:1)_FA 16:0	878.8 -> 605.8
TAG(52:0)_FA 18:0	880.8 -> 579.8
TAG(52:0)_FA 16:0	880.8 -> 607.8
TAG(54:8)_FA 18:0	892.7 -> 591.696
TAG(54:8)_FA 18:1	892.7 -> 593.7
TAG(54:5)_FA 20:4	898.8 -> 577.8
TAG(54:5)_FA 18:1	898.8 -> 599.8
TAG(54:5)_FA 18:2	898.8 -> 601.8
TAG(54:5)_FA 16:0	898.8 -> 625.8
TAG(54:4)_FA 20:4	900.8 -> 579.8
TAG(54:4)_FA 18:1	900.8 -> 601.8
TAG(54:4)_FA 18:2	900.8 -> 603.8
TAG(54:4)_FA 16:0	900.8 -> 627.8
TAG(54:3)_FA 18:0	902.8 -> 601.8
TAG(54:3)_FA 18:1	902.8 -> 603.8
TAG(54:3)_FA 18:2	902.8 -> 605.8
TAG(54:3)_FA 16:0	902.8 -> 629.8
TAG(54:2)_FA 18:0	904.8 -> 603.8
TAG(54:2)_FA 18:1	904.8 -> 605.8
TAG(54:2)_FA 18:2	904.8 -> 607.8
TAG(54:2)_FA 16:0	904.8 -> 631.8
TAG(54:1)_FA 18:0	906.8 -> 605.8
TAG(54:1)_FA 18:1	906.8 -> 607.8
TAG(54:1)_FA 16:0	906.8 -> 633.8
TAG(54:0)_FA 18:0	908.9 -> 607.896
TAG(56:8)_FA 18:1	920.8 -> 621.8
TAG(56:8)_FA 16:1	920.8 -> 649.796
TAG(56:6)_FA 20:4	924.8 -> 603.8
TAG(56:6)_FA 18:1	924.8 -> 625.8
TAG(56:5)_FA 18:1	926.8 -> 627.796
TAG(56:5)_FA 18:2	926.8 -> 629.8
TAG(56:5)_FA 16:0	926.8 -> 653.8
TAG(56:4)_FA 16:0	928.8 -> 655.8
TAG(56:3)_FA 18:1	930.8 -> 631.796
TAG(56:3)_FA 18:2	930.8 -> 633.8
TAG(58:7)_FA 20:0	950.8 -> 621.8
TAG(58:6)_FA 18:1	952.8 -> 653.8
TAG(58:5)_FA 18:1	954.8 -> 655.8
TAG(58:3)_FA 18:1	958.9 -> 659.896
TAG(58:2)_FA 18:1	960.9 -> 661.9
TAG(58:2)_FA 18:2	960.9 -> 663.9
TAG(58:1)_FA 18:1	962.9 -> 663.9

TAG(60:9)_FA 18:1	974.9 -> 675.896
TAG(60:7)_FA 18:1	978.9 -> 679.896
TAG(60:4)_FA 20:4	984.9 -> 663.9
TAG(60:4)_FA 18:1	984.9 -> 685.896
TAG(60:2)_FA 18:1	988.9 -> 689.896
PS(14:1)	468.2358 -> 283.2358
PE(18:1), Lyso PE(18:1)	480.3086 -> 339.3086
PSp(16:0)	482.2879 -> 297.2879
PE(18:0), Lyso PE(18:0)	482.3242 -> 341.3242
PSo(16:0)	484.3035 -> 299.3035
STD 18:1 (d7) Lyso PE	487.5 -> 346.3
PE(20:4), Lyso PE(20:4)	502.2929 -> 361.2929
PE(20:1), Lyso PE(20:1)	508.3399 -> 367.3399
PSo(18:0)	512.262 -> 327.262
PG(16:0), PGo(18:0), Lyso PG(17:0)	516.2668 -> 327.2668
PG(18:0), Lyso PG(18:0)	530.3188 -> 341.3188
PE(22:4), Lyso PE(22:4)	530.3242 -> 389.3242
STD C15 ceramide-D7	531.5 -> 271.3
PSo(20:0)	540.3661 -> 355.3661
PGp(20:0), Lyso PG(19:1)	542.3552 -> 353.3552
PGo(20:0), Lyso PG(19:0)	544.3709 -> 355.3709
Lyso PG(20:0)	558.3427 -> 369.3427
PI(14:1)	560.2566 -> 283.2566
PG(20:0), Lyso PG(21:0)	572.3294 -> 383.3294
PG(22:6), Lyso PG(22:6)	574.2875 -> 385.2875
PG(22:0), Lyso PG(22:0)	586.3814 -> 397.3814
12:0 Cholesteryl ester	586.5219 -> 369.1
STD 15:0-18:1-d7 DG	605.6 -> 346.1
14:1 Cholesteryl ester	612.5376 -> 369.1
14:0 Cholesteryl ester	614.5532 -> 369.1
PG(24:0)	628.392 -> 439.392
PI(20:5)	636.2879 -> 359.2879
16:3 Cholesteryl ester	636.5376 -> 369.1
16:2 Cholesteryl ester	638.5532 -> 369.1
16:1 Cholesteryl ester	640.5689 -> 369.1
16:0 Cholesteryl ester	642.5845 -> 369.1
PG(26:0)	656.4233 -> 467.4233
18:3 Cholesteryl ester	664.5689 -> 369.1
18:2 Cholesteryl ester	666.5845 -> 369.1
18:1 Cholesteryl ester	668.6002 -> 369.1
18:0 Cholesteryl ester	670.6158 -> 369.1
STD_18:1(d7) Chol Ester	675.64 -> 369.1
PS(28:1)	678.4342 -> 493.4342
PS(28:0)	680.4498 -> 495.4498
20:5 Cholesteryl ester	688.5689 -> 369.1
PE(32:1)	690.507 -> 549.507

20:4 Cholesteryl ester	690.5845 -> 369.1
20:3 Cholesteryl ester	692.6002 -> 369.1
20:2 Cholesteryl ester	694.6158 -> 369.1
20:1 Cholesteryl ester	696.6315 -> 369.1
20:0 Cholesteryl ester	698.6471 -> 369.1
PEo(34:2)	702.5433 -> 561.5433
PS(30:2)	704.4498 -> 519.4498
PEo(34:1)	704.559 -> 563.559
STD_15:0-18:1(d7) PE	711.56 -> 570.4
22:6 Cholesteryl ester	714.5845 -> 369.1
PE(34:2)	716.5226 -> 575.5226
22:5 Cholesteryl ester	716.6002 -> 369.1
PE(34:1)	718.5383 -> 577.5383
22:4 Cholesteryl ester	718.6158 -> 369.1
22:3 Cholesteryl ester	720.5435 -> 369.1
PE(34:0)	720.5539 -> 579.5539
22:2 Cholesteryl ester	722.5744 -> 369.1
PEo(36:5)	724.5277 -> 583.5277
22:1 Cholesteryl ester	724.6628 -> 369.1
22:0 Cholesteryl ester	726.6784 -> 369.1
PEo(36:3)	728.559 -> 587.559
PEo(36:2)	730.5746 -> 589.5746
PE(36:8), PEo(36:1)	732.46 -> 591.46
PS(32:2)	732.4811 -> 547.4811
PE(36:5)	738.507 -> 597.507
PG(32:0)	740.5172 -> 551.5172
PE(36:4)	740.5226 -> 599.5226
PE(36:3)	742.5383 -> 601.5383
PE(36:2)	744.5539 -> 603.5539
PE(36:1)	746.5696 -> 605.5696
PE(36:0), PEp(36:6)	748.5852 -> 607.5852
PEo(38:6)	750.5433 -> 609.5433
PEo(38:5)	752.559 -> 611.559
24:1 Cholesteryl ester	752.6941 -> 369.1
PEo(38:4)	754.5746 -> 613.5746
STD_15:0-18:1(d7) PS (Na Salt)	755.53 -> 570.4
PE(38:9), PEo(38:2)	758.4757 -> 617.4757
STD_15:0-18:1(d7) PG (Na Salt)	759.54 -> 570.4
PG(34:2)	764.5172 -> 575.5172
PE(38:6)	764.5226 -> 623.5226
PG(34:1)	766.5328 -> 577.5328
PE(38:5)	766.5383 -> 625.5383
PG(34:0)	768.5485 -> 579.5485
PE(38:4)	768.5539 -> 627.5539
PE(38:3)	770.5696 -> 629.5696
PE(40:2)	772.5852 -> 631.5852

PE(40:1), PEp(40:7)	774.6009 -> 633.6009
PS(36:8)	776.4498 -> 591.4498
PG(36:8), PGo(36:1)	780.4546 -> 591.4546
PEo(40:5)	780.5903 -> 639.5903
PG(36:4)	788.5172 -> 599.5172
PS(36:2)	788.5437 -> 603.5437
PG(36:3)	790.5328 -> 601.5328
PE(40:7), PEo(40:0)	790.5383 -> 649.5383
PS(36:1)	790.5594 -> 605.5594
PG(36:2)	792.5485 -> 603.5485
PE(40:6)	792.5539 -> 651.5539
PG(36:1)	794.5641 -> 605.5641
PE(40:5)	794.5696 -> 653.5696
PG(36:0), PGp(38:6)	796.5798 -> 607.5798
PE(40:4)	796.5852 -> 655.5852
PS(38:7), PSo(38:0)	806.4968 -> 621.4968
PS(38:4)	812.5437 -> 627.5437
PG(38:4)	816.5485 -> 627.5485
PS(38:2)	816.575 -> 631.575
PG(38:3)	818.5641 -> 629.5641
PS(38:1)	818.5907 -> 633.5907
PSp(40:6), PS(38:0)	820.5488 -> 635.5488
PS(40:4)	840.575 -> 655.575
STD_15:0-18:1(d7) PI (NH4 Salt)	847.6 -> 570.4
PSp(42:6), PS(40:0)	848.5801 -> 663.5801
PI(34:2)	852.5332 -> 575.5332
PG(40:0), PGp(42:6)	852.6424 -> 663.6424
PI(34:1)	854.5489 -> 577.5489
PI(36:4)	876.5332 -> 599.5332
PI(36:3)	878.5489 -> 601.5489
PI(36:2)	880.5645 -> 603.5645
PI(36:1)	882.5802 -> 605.5802
PI(38:9), PIo(38:2), PIp(38:1)	894.4863 -> 617.4863
PI(38:7)	898.5176 -> 621.5176
PI(38:5)	902.5489 -> 625.5489
PI(38:4)	904.5645 -> 627.5645
PI(38:3)	906.5802 -> 629.5802
PI(38:2)	908.5958 -> 631.5958
PIo(40:4), PIp(40:3)	918.6166 -> 641.6166
PI(40:10), PIo(40:3), PIp(40:2)	920.5019 -> 643.5019
PI(40:6)	928.5645 -> 651.5645
PI(40:5)	930.5802 -> 653.5802
PI(40:4)	932.5958 -> 655.5958
PI(40:0), PIp(42:6)	940.6584 -> 663.6584
C12:1	197.2 -> 197.2
C12:0	199.2 -> 199.2

C14:1	225.2 -> 225.2
C14:0	227.3 -> 227.3
C15:1	239.3 -> 239.3
C15:0	241.3 -> 241.3
C16:1	253.3 -> 253.3
C16:0	255.3 -> 255.3
C17:1	267.3 -> 267.3
C17:0	269.3 -> 269.3
C18:4	275.3 -> 275.3
C18:3	277.3 -> 277.3
C18:2	279.3 -> 279.3
C18:1	281.3 -> 281.3
C18:0	283.3 -> 283.3
C19:0	297.3 -> 297.3
C20:5	301.3 -> 301.3
C20:4	303.3 -> 303.3
C20:3	305.3 -> 305.3
C20:1	309.3 -> 309.3
C20:0	311.3 -> 311.3
C22:6	327.3 -> 327.3
C22:5	329.3 -> 329.3
C22:4	331.3 -> 331.3
C22:1	337.3 -> 337.3
C22:0	339.3 -> 339.3
C24:5	353.3 -> 353.3
C24:6	355.3 -> 355.3
C24:1	365.3 -> 365.3
C24:0	367.3 -> 367.3
C26:1	393.3 -> 393.3
C26:0	395.3 -> 395.3
C28:0	423.4 -> 423.4
C30:0	451.4 -> 451.4
C32:0	479.4 -> 479.4
C34:0	507.5 -> 507.5

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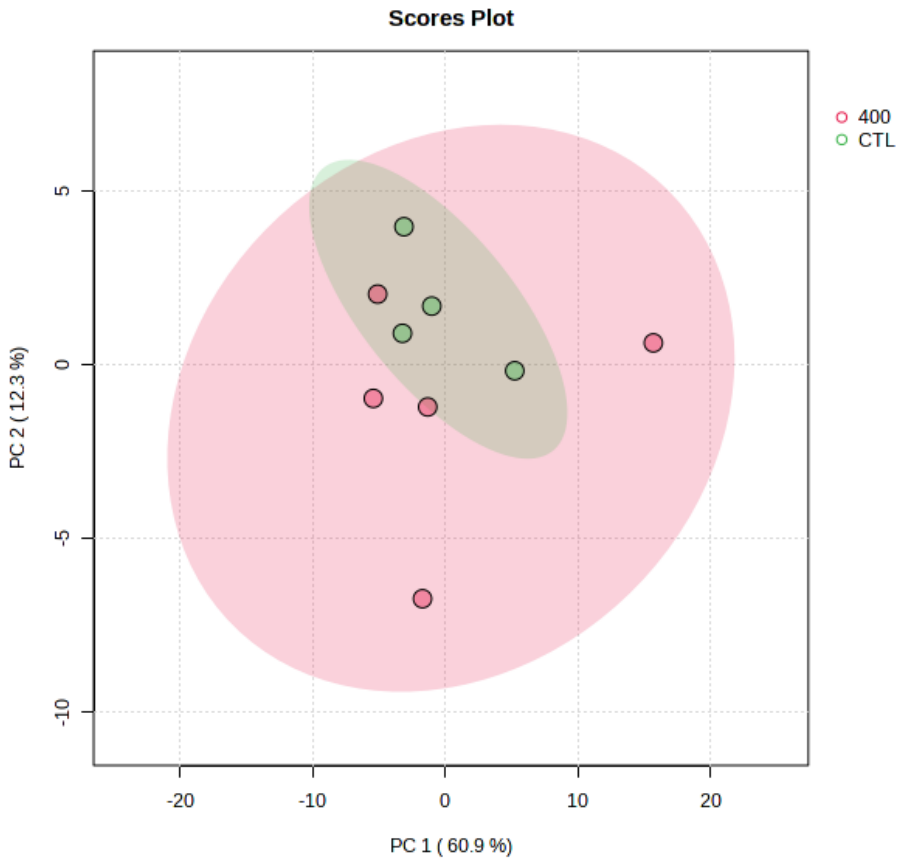

Figure S1- PCA plot from multivariate analysis the abundance of transcripts derived from untreated (control) and CNP-treated blastocyst after cryopreservation. N = 4 per group.

Table S2: Gene symbol, functions and primers assay ID used for microfluidic expression analyses (Biomark HD System - Fluidigm).

Gene Symbol	Function	Assay ID*
GAPDH	Reference gene	Bt03210912_g1
ACTB	Reference gene	PA5-16914
CASP9	Apoptosis	Bt04282453_m1
CD40	Apoptosis	Bt03817804_g1
TNFRSF21	Apoptosis	Bt03250597_m1
TP53	Apoptosis	Bt03223213_m1
MORF4L2	Apoptosis	Bt03270996_m1
BID	Apoptosis	Bt03241255_m1
BAX	Apoptosis	Bt03211777_g1
CASP3	Apoptosis	Bt03250954_g1
ADCY3	Cell development	Bt04289077_m1
NPPA	Cell development	Bt03223175_g1
NPPB	Cell development	Bt04301375_g1
NPR1	Cell development	Bt04297034_g1
TNFAIP6	Cell development	Bt03210223_m1
VCAN	Cell development	Bt03217633_m1
IGFBP4	Cell development	Bt03259500_m1
AQP3	Cell development	Bt03253663_m1
NLRP5	Cell development	Bt03218031_m1
VEGFA	Cell development	Bt03213282_m1
IMPDH1	Cell development	Bt00995384_m1
IMPDH2	Cell development	Bt03226238_g1
ATP5L	Cell development	Bt03210836_g1
DSC2	Cell development	Bt03649202_m1
DSC3	Cell development	Bt04301926_m1
MAPK1	Cell development	Bt03216718_g1
LHCGR	Cell development	Bt03213974_m1
LEP	Cell development	Bt03211909_m1
BMP15	Cell development	Bt03286494_u1
GDF9	Cell development	Bt03223996_m1
NANOG	Cell development	Bt03220541_m1
SOX2	Cell development	Bt03278318_s1
G6PD	Cell development	Bt03649181_m1
HSPD1	Cell development	Bt04301470_g1
NRP1	Cell development	Bt04284287_m1
NRP2	Cell development	Bt04284287_m1
BDNF	Cell development	Bt04316732_m1
ACSL5	Cell development	Bt03241747_m1
FSHR	Cell development	Bt03212674_m1
IGF2	Cell development	Bt03259224_m1
RPL15	Cell development	Bt03288449_g1
ADCY6	Cell development	Bt03816767_m1

ADCY9	Cell development	Bt04287024_m1
EGFR	Cell development	AJT96D7
HSPA1A	Cell development	Bt03292670_g1
NPPC	Cell development	Bt03212844_m1
NPR2	Cell development	Bt04316732_m1
GADD45A	Cell development	Bt03225650_m1
POU5F1	Cell development	Bt03223846_g1
CCND2	Cell development	Bt03249250_m1
ELF5	Cell development	Bt03220307_m1
FASN	Cell development	Bt03210485_m1
NOS3	Cell development	Bt03217679_m1
HSPA5	Oxidative stress	Bt03244880_m1
GFPT2	Oxidative stress	Bt03250351_m1
GLRX2	Oxidative stress	Bt03229700_m1
TXNRD1	Oxidative stress	Bt03215471_m1
VNN1	Oxidative stress	Bt03220248_m1
SOD1	Oxidative stress	Bt03215423_g1
GPX1	Oxidative stress	Bt03259217_g1
FDX1	Oxidative stress	Bt03217449_m1
ATF4	Oxidative stress	Bt03221057_m1
CAT	Oxidative stress	Bt03228713_m1
KEAP1	Oxidative stress	Bt03817661_m1
SLC2A3	Glucose transport facilitator	Bt03259514_g1
SLC2A1	Glucose transport facilitator	Bt03215314_m1
SLC2A5	Glucose transport facilitator	Bt03258296_m1
AKR1B1	Glucose transport facilitator	Bt03218049_g1
SLC2A4	Lipid metabolism	Bt03215316_m1
FADS2	Lipid metabolism	Bt03256255_g1
SCD	Lipid metabolism	Bt04307476_m1
DGAT1	Lipid metabolism	Bt03251719_g1
ACSL1	Lipid metabolism	Bt03248469_m1
ACAT1	Lipid metabolism	Bt03238649_g1
LPL	Lipid metabolism	Bt03240493_m1
CD36	Lipid metabolism	Bt03212335_mH
PNLIPRP2	Lipid metabolism	Bt03267914_m1
PNPLA2	Lipid metabolism	Bt03234129_g1
LIPE	Lipid metabolism	Bt03253691_m1
PLIN2	Lipid metabolism	Bt03212182_m1
PLIN3	Lipid metabolism	Bt03230537_m1
AGPAT9	Lipid metabolism	Bt04292093_m1
AGPAT1	Lipid metabolism	Bt03224587_g1
SREBF1	Lipid metabolism	Bt03276370_m1
ACACA	Lipid metabolism	Bt03213389_m1
AUH	Lipid metabolism	Bt03275798_m1
ACSL3	Lipid metabolism	Bt04282138_m1
ACSL6	Lipid metabolism	Bt03231692_m1
HMGCS1	Lipid metabolism	Bt04296095_g1
HMGCS2	Lipid metabolism	Bt03233809_m1

ELOVL1	Lipid metabolism	Bt03286627_s1
ELOVL2	Lipid metabolism	Bt03256849_m1
ELOVL4	Lipid metabolism	Bt03270721_m1
ELOVL5	Lipid metabolism	Bt03235956_m1
ELOVL6	Lipid metabolism	Bt00907566_m1

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CONSIDERAÇÕES FINAIS

Conclusões Gerais

A partir dos resultados apresentados nos Capítulos 1 e 2, as hipóteses propostas nesta Tese foram parcialmente aceitas. Foi observado alteração no metabolismo celular e no perfil lipídico dos blastocistos derivados dessa suplementação com CNP no CIV. No entanto, não foram observadas modificações no teor lipídico embrionário. Com relação a melhora na qualidade embrionária, não foi observada alteração nas taxas de produção e também sem alteração nas taxas após a criopreservação. Os principais resultados observados com este trabalho foram:

- A adição de CNP (100, 200 e 400 nM) durante o cultivo *in vitro* (CIV) não alterou taxas de produção *in vitro*.

- A adição de 400 nM de CNP, durante o CIV, não foi embriotóxica, sendo assim não modificou as taxas de produção (taxa de blastocisto e cinética de eclosão).

- A adição de 400 nM de CNP, durante o CIV, modulou a abundância de transcritos relacionados com o metabolismo energético, competência e qualidade embrionária (apoptose, estresse oxidativo, proliferação celular, diferenciação) e metabolismo lipídico, em mórulas (dia 5) blastocistos (dia 7 e 8) e blastocistos após a vitrificação (dia 8 e 9).

- A adição de 400 nM de CNP, durante o CIV, modulou a abundância relativa de lipídicos em blastocisto, modificando o perfil lipídico embrionário.

- A adição de 400 nM de CNP, não alterou o teor de lipídios presentes em blastocistos cultivados.

- A adição de 400 nM de CNP, não alterou a criotolerabilidade dos embriões submetidos a técnica de vitrificação.

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