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INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU

Efeito do IGF-1 e da PAPP-A sobre aspectos do metabolismo lipídico e estresse oxidativo de oócitos e embriões bovinos produzidos *in vitro*

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Tese apresentada ao Programa de Pós Graduação do Instituto de Biociências de Botucatu, Universidade Estadual Paulista – UNESP, para a obtenção do título de Doutora em Farmacologia e Biotecnologia

Orientador: Anthony César de Souza Castilho

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Presidente e Orientador

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Prof. Dra. Gisele Zoccal Mingoti

Membro

Departamento de Produção e Saúde Animal (FMVA)

Unesp – Araçatuba – São Paulo

Prof. Dr. Mateus José Sudano

Membro

Centro de Ciências Naturais e Humanas

UFABC – Santo André – SP

Fabiana Ferreira de Souza

Membro

Departamento de Cirurgia Veterinária e Reprodução Animal

Unesp – Botucatu – SP

Dra. Raquel Zaneti Puelker

Membro

Progest Biotecnologia em Reprodução Animal – Botucatu – SP

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Dedico esse trabalho aos meus pais, Mario e Eliana,
E aos meus tios, Augusto e Regina.

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RESUMO

A proteína plasmática A associada à gravidez (PAPP-A) é uma metaloprotease, que tem como função aumentar a biodisponibilidade do fator de crescimento semelhante a insulina tipo 1 (IGF-1), um peptídeo com diversas funções em prol do bom desenvolvimento oocitário e embrionário. Assim, a temática principal dessa tese foi utilizar a PAPP-A como um potencializador dos efeitos atribuídos ao IGF-1 durante a PIVE de bovinos. No primeiro artigo, investigamos o efeito da adição de PAPP-A durante a MIV suplementada com soro fetal bovino (SFB). Os complexos cumulus-oócitos (CCOs) foram maturados *in vitro* por 24 horas na presença ou ausência de SFB e, posteriormente, foram fertilizados e cultivados *in vitro*. Analisamos o conteúdo lipídico dos oócitos e blastocistos usando o corante Sudan-Black B, a taxa de blastocistos produzidos e a expressão gênica de 96 genes por RTqPCR. Não houve diferença no conteúdo lipídico dos oócitos e blastocistos avaliados, nem na taxa de blastocistos produzidos. No entanto, a presença de PAPP-A durante a MIV diminuiu a expressão de 22 genes relacionados à síntese de lipídeos, apoptose e estresse celular e qualidade embrionária nos blastocistos produzidos. No segundo artigo, os CCOs foram maturados (tratamento na MIV) ou os embriões cultivados (tratamento no CIV) por 24h com adição de IGF-1, IGF-1/PAPP-A, ou na ausência de ambos, em um ambiente estresse-induzido pela presença de menadiona. Avaliamos nos oócitos a progressão da meiose, concentração de espécies reativas de oxigênio (EROs) e o potencial de membrana mitocondrial por técnicas de coloração utilizando sondas específicas. Também analisamos as concentrações de glutationa total (GSH) nos CCOs, blastocistos e meios de MIV e CIV, além da taxa de blastocistos produzidos e a expressão gênica dos embriões por RTqPCR. A combinação IGF-1/PAPP-A resultou nas menores concentrações de EROs em oócitos e blastocistos e também modulou positivamente nos

embriões genes envolvidos na defesa antioxidativa, metabolismo lipídico e qualidade embrionária, além de aumentar a taxa de blastocistos produzidos quando utilizadas na CIV. Assim, concluímos que o uso da PAPP-A, durante a PIVE de bovinos, foi efetivo como uma estratégia para maximizar as ações mediadas por IGF-1. Especificamente, reiteramos que a combinação IGF-1/PAPP-A teve efeitos mais marcantes, potencializando a proteção antioxidativa e a qualidade embrionária, além de aumentar quantitativamente o número de blastocistos produzidos em um ambiente estresse-induzido.

Palavras-chave: Pappalisina, fator de crescimento, EROs, IGFPB, IGF-1 livre, biodisponibilidade, PAPP-A.

ABSTRACT

Pregnancy-associated plasma protein-A (PAPP-A) is a metalloprotease which role is to increase insulin-like growth factor type 1 (IGF-1), a peptide with several beneficial functions for oocyte and embryo development. Thus, the thesis main subject was using the PAPP-A as an enhancer of IGF-1 attributed-effects during bovine in vitro production. In the first article, we investigated the effects of PAPP-A addition during IVM supplemented with fetal bovine serum (FBS). The cumulus-oocyte complexes (COCs) were in vitro matured for 24 hours in the presence or absence of FBS, and after, in vitro fertilized and cultured. We analyzed the oocytes and blastocysts lipid content by Sudan-Black B staining, the blastocysts yield, and the expression of 96 genes by RTqPCR. There was no difference in oocytes and blastocysts lipid content or in the blastocysts yield. However, the PAPP-A addition during IVM downregulated the expression of 22 genes related to lipid synthesis, apoptosis and cell stress, and embryonic quality in the blastocysts. In the second article, the COCs were matured (IVM treatment) or the embryos were cultured (IVC treatment) for 24 hours with IGF-1, IGF-1/PAPP-A addition, or in absence of both at a stress environment-induced by the menadione addition. We assessed the meiosis progression, reactive oxygen species (ROS) concentration and mitochondrial membrane potential levels in the oocytes using staining techniques. We also analyzed the concentrations of total glutathione (GSH) in COCs, blastocysts, and IVM and IVC media, besides the blastocysts yield and the expression of 96 genes by RTqPCR. The IGF-1/PAPP-A combination showed the lower ROS concentrations in oocytes and blastocysts and also positively modulated genes involved in antioxidative defense, lipid metabolism, and embryonic quality, besides increasing the blastocysts yield when used in IVC. Thus, we conclude the PAPP-A uses during bovine IVP was effective to improve the IGF-1 mediated-actions. Specifically,

the IGF-1/PAPP-A shows the highlight effects, increasing antioxidative protection and embryonic quality, besides the number of blastocysts in a stress-induced environment.

Keywords: pappalysin, growth factor, ROS, IGFPB, free IGF-1, bioavailability, PAPP-A.

SUMÁRIO

PREFÁCIO

A presente tese a ser defendida pelo o programa de Pós-graduação em Farmacologia e Biotecnologia se encontra dividida em quatro sessões. A primeira, denominada **Capítulo I**, compreende a introdução e revisão da literatura, que descrevem o contexto e a problemática relacionada com este trabalho de maneira mais profunda e detalhada. As duas partes subsequentes, **Capítulo II - "Molecular phenotypes of bovine blastocyst derived from *in vitro*-matured oocyte supplemented with recombinant pappalysin-1"** e **Capítulo III - "New approach using IGF-1 combined with PAPP-A during bovine *in vitro* embryo production: could this system enhance IGF-1 antioxidative role?"**, correspondem a duas propostas de manuscritos a serem submetidos como artigos resultantes do trabalho realizado nos periódicos "Theriogenology" e "Biology of Reproduction", respectivamente. Por fim, 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 fará parte do conjunto de publicações do Instituto de Biociências de Botucatu da UNESP. No entanto, ambos os manuscritos aqui apresentados estão no prelo, mas não comprometidos com a publicação em quaisquer periódicos, estando, assim, passíveis de correções e modificações por parte da banca de defesa.

CAPÍTULO I

1. INTRODUÇÃO

A proteína plasmática A associada à gravidez (PAPP-A) é uma metaloprotease, cuja função é a modulação da biodisponibilidade dos fatores de crescimento semelhantes à insulina (IGFs). Essa proteína, por meio da reação de hidrólise, cliva as proteínas ligadoras de IGFs (IGFBPs), aumentando as concentrações desse peptídeo livre, sua forma bioativa (Laursen, et al. 2001, Lawrence, et al. 1999, Mazerbourg, et al. 2003, Monget, et al. 2003, Rivera and Fortune 2003). Essas proteínas, e a dinâmica dos mecanismos envolvidos em seu funcionamento, constituem o sistema IGF.

Esse sistema está presente durante o desenvolvimento oocitário e embrionário bovino. O receptor IGF-1R está presente em oócitos e embriões, e o ligante, IGF-1, é produzido pelas células da granulosa (Bao, et al. 1995, Sirotkin, et al. 1998) e encontrado no soro, no fluido folicular, e nas células ovidutais e uterinas (Echternkamp, et al. 1990, Spicer, et al. 1992, Spicer, et al. 1988, Yaseen, et al. 2001). No entanto, durante a produção *in vitro* de embriões (PIVE) há perda de contato dos oócitos e embriões com esses ambientes, que parecem ser as fontes de IGF-1 durante o desenvolvimento oocitário e embrionário.

Outra desvantagem é que os processos *in vitro* também geram maiores concentrações de espécies reativas de oxigênio (EROs; Nasr-Esfahani, et al. 1990) do que *in vivo*. A alta concentração de EROs pode levar a indução da peroxidação lipídica ligada ao bloqueio da divisão celular (Nasr-Esfahani, et al. 1990, Noda, et al. 1991), danos na atividade mitocondrial (Richter, et al. 1995, Roth 2018) e modificações no DNA, como a fragmentação do material nuclear (Birben, et al. 2012), podendo ser prejudicial ao desenvolvimento de oócitos e embriões (Kala, et al. 2017). Assim, a

adição de substâncias antioxidantes durante a PIVE pode ser uma estratégia para diminuir os danos causados pelas concentrações excessivas de EROs.

Em roedores e bovinos, a adição de substâncias oxidativas, como o H_2O_2, ou que induzem a produção de EROs, como a menadiona (Monks, et al. 1992, Nutter, et al. 1992) durante a CIV, diminuíram a taxa de blastocistos produzidos e aumentaram o número de células apoptóticas nesses embriões, mas a adição de IGF-1 restaurou as taxas de embriões produzidos e reduziu apoptose celular, apesar de não reduzir diretamente a concentração de EROs nesses embriões (Kurzawa, et al. 2004, Moss, et al. 2009).

Outro processo importante para o desenvolvimento dos oócitos e embriões que parece ser modulado pelo IGF-1 é o metabolismo de lipídeos. Alguns estudos mostraram que concentrações de triglicerídeos em oócitos bovinos podem diminuir após a MIV (Cetica, et al. 2002, Ferguson and Leese 1999, Sturmey and Leese 2003), sugerindo que esses lipídeos podem estar sendo utilizados para geração de energia durante a maturação oocitária via β-oxidação (Paczkowski et al. 2013; Dunning et al. 2014). De fato, estudos em roedores mostraram que a terapia com IGF-1 reduziu os níveis de triglicerídeos, aumentando significativamente as concentrações de ácidos graxos livres sistêmicas (García-Fernández et al. 2008), que é a forma de lipídeo que pode ser metabolizada via β-oxidação.

Lipídeos presentes no microambiente podem ser incorporados pelos CCOs e embriões (Dunning et al. 2014) e estudos tem demonstrado que a utilização de soro fetal bovino (SFB) durante a PIVE aumenta a concentração de triglicerídeos, tanto em CCOs quanto em blastocisto (Kim et al. 2001; Del Collado et al, 2015; Sudano et al. 2011; Abe et al. 2002). Esse componente é amplamente utilizado na PIVE convencional de

bovinos, principalmente por resultar nas melhores taxas de produção de blastocistos (Sudano et al. 2011; Del Collado et al. 2015), porém o aumento do conteúdo lipídico dos embriões produzidos na presença de SFB, também resulta na diminuição da taxa de re-expansão embrionária após a criopreservação (Sudano et al. 2011). O SFB possui diversas substâncias em sua composição, dentre elas, altas concentrações de lipídeos e IGF-1 (Abe, et al. 2002, Del Collado, et al. 2015, Honegger and Humbel 1986, Kim, et al. 2001, Tu, et al. 2018). Assim, a indução do metabolismo lipídico pela ação do IGF-1 poderia aumentar a utilização de lipídeos como fonte de energia e, ainda, diminuir o conteúdo lipídico nos blastocistos produzidos, o que favoreceria protocolos de criopreservação, por exemplo.

Um estudo recente em bovinos mostrou que, a adição de PAPP-A durante a MIV aumentou a concentração de IGF-1 livre no meio de maturação, e ainda, modulou genes envolvidos com o metabolismo lipídico e estresse oxidativo nos embriões produzidos a partir dos complexos cumulus-oócito (CCOs) que foram maturados com adição de PAPP-A (Giroto, et al. 2019). No entanto, nenhum estudo investigou se a PAPP-A, devido sua função no aumento da biodisponibilidade de IGF-1, poderia funcionar como um potencializador dos efeitos atribuídos a esse peptídeo. Assim, a utilização de PAPP-A durante protocolos de PIVE, como estratégia para maximizar a indução do metabolismo lipídico em culturas que levam soro fetal bovino (SFB), quanto no papel antioxidativo em ambientes com altas concentrações de EROs, pode resultar em um aumento do sucesso obtido com o uso de biotécnicas *in vitro*.

2. REVISÃO DE LITERATURA

2.1 Sistema IGF: Ações do IGF-1 durante o desenvolvimento oocitário e embrionário

O sistema IGF é composto por dois peptídeos ligantes (o IGF-1 e IGF-2), seus receptores (IGF-1R e IGF-2R) e proteínas de ligação de IGFs denominadas IGFBPs (IGF-binding proteins), cuja função é se ligar a esses peptídeos nos mesmos sítios de ligação destes aos seus receptores, os tornando indisponíveis para utilização celular. Dessa forma, a ligação das IGFBPs aos IGFs modula a biodisponibilidade desses pequenos peptídeos, visto que, para que os IGFs sejam bioativos, eles devem estar em sua forma livre, não complexados as IGFBPs (Froesch, et al. 1985, Jones and Clemmons 1995, LeRoith, et al. 1992, Mazerbourg and Monget 2018). O sistema IGF está presente durante o desenvolvimento folicular, oocitário e embrionário (Armstrong, et al. 2000, Yaseen, et al. 2001) e está envolvido em diversos processos como a estimulação da proliferação, migração e diferenciação celular (Duan, et al. 2005) e até mesmo no mecanismo de dominância folicular (Rivera and Fortune 2003). Oócitos e embriões bovinos expressam os receptores *IGF-1R* e *IGF-2R*, o ligante *IGF-2* (Wang, et al. 2009) e também as *IGFBP-2, IGFBP-3, IGFBP-4* e *IGFBP-5* (Armstrong, et al. 2002, Satrapa, et al. 2013, Sawai, et al. 2005, Winger, et al. 1997) enquanto, a expressão do *IGF-1* é controversa (Armstrong, et al. 2002, Bertolini, et al. 2002, Lopes, et al. 2017, Nuttinck, et al. 2004, Satrapa, et al. 2013, Wang, et al. 2009, Winger, et al. 1997, Yoshida, et al. 1998).

As células da granulosa bovina produzem IGF-1 (Bao, et al. 1995, Sirotkin, et al. 1998), e ele também é encontrado no soro, no fluido folicular, e nas células ovidutais e uterinas (Echternkamp, et al. 1990, Spicer, et al. 1992, Spicer, et al. 1988, Yaseen, et al. 2001), podendo então, essas serem as fontes desse peptídeo para oócitos e embriões

durante o desenvolvimento oocitário e embrionário, respectivamente. No entanto, para a realização da PIVE ocorre a perda de contato tanto dos CCOs com as células da granulosa e fluido folicular, quanto dos embriões com as células ovidutais e uterinas e, consequentemente, com o IGF-1 presente nesses ambientes. Já se sabe que a diminuição das concentrações desse peptídeo, glicose e insulina no soro de bovinos leva produção de oócitos com baixa qualidade (De Rensis and Scaramuzzi 2003), assim, o IGF-1 se tornou alvo de interesse de diversos estudos, que têm investigado os efeitos de sua adição durante a MIV e CIV de CCOs e embriões.

Os resultados desses trabalhos têm mostrado que a adição IGF-1 durante a MIV, diminui a porcentagem de oócitos com fragmentação de DNA e a atividade da proteína pró-apoptótica *CASP3* (Wasielak and Bogacki 2007), e também, os efeitos deletérios causados pela exposição de CCOs ao estresse térmico (Rodrigues, et al. 2016). Além disso, pode aumentar o número de oócitos que atingem a metáfase II, estágio requerido para que a fertilização ocorra, apesar disso não ser consenso entre os estudos (Lorenzo, et al. 1994, Meiyu, et al. 2015, Rieger, et al. 1998, Sirotkin, et al. 2000). Durante a CIV, a adição desse peptídeo também mostrou diminuir a presença de células apoptóticas em embriões, protegê-los contra estresse térmico (Jousan and Hansen 2004, 2007, Jousan, et al. 2008), aumentar a proliferação celular (Byrne, et al. 2002, Jousan and Hansen 2007, Jousan, et al. 2008, Makarevich and Markkula 2002) e também a taxa de blastocistos produzidos (Palma, et al. 1997, Prelle, et al. 2001, Sirisathien, et al. 2003), embora alguns estudos não tenham observado tais efeitos benéficos (Block, et al. 2008, Hernandez-Fonseca, et al. 2002).

Assim, da mesma forma que expressão e presença do IGF-1 em oócitos em embriões bovinos não é consenso, os resultados dos estudos *in vitro* que utilizaram a adição desse peptídeo, também são contraditórios. Não se sabe exatamente o porquê

desses resultados ambíguos, no entanto, uma das possibilidades é que pode haver uma variação na biodisponibilidade do IGF-1 e que isso interfira nas ações desse peptídeo.

2.2 PAPP-A: função no sistema IGF

A PAPP-A é uma metaloprotease membro da superfamília de metzincinas e foi identificada, pela primeira vez, há 46 anos em altas concentrações no plasma de mulheres grávidas e, por isso, recebeu esse nome. As metaloproteases são enzimas hidrolíticas que requerem a ligação de um metal (zinco, no caso da PAPP-A) em seu sítio catalítico para exercerem suas atividades. Essa superfamília é um grupo diversificado de peptidases de zinco: as actacinas, reprolisinas, serralisinas, metaloproteases de matriz (MMPs) e pappalisinas, onde se encontra a PAPP-A (Boldt, et al. 2001, Fialova and Malbohan 2002, Lin, et al. 1974).

Sua função no sistema IGF, é a clivagem proteolítica de IGFBPs (Conover, et al. 1999, Lawrence, et al. 1999) tornando assim os IGFs livres, que é sua forma bioativa. A PAPP-A é capaz clivar as IGFPB-2, 4 e 5 em muito animais, inclusive em bovinos aumentando, assim, a biodisponibilidade de IGF-1 (Laursen, et al. 2007, Laursen, et al. 2001, Lawrence, et al. 1999, Mazerbourg, et al. 2003, Monget, et al. 2003, Rivera and Fortune 2003). A PAPP-A se liga as superfícies celulares que possuem glicosaminoglicanos, tornado-se assim ativa, deste modo, acredita-se que nos tecidos, a maior parte dessas metaloproteases está ligada a superfícies. Nesse sistema, a clivagem das IGFBPs ocorre próximo ao IGF-1R, aumentando a chance de que o IGF liberado resulte na estimulação de receptor (Laursen, et al. 2007, Laursen, et al. 2001, Oxvig 2015; Figure1).

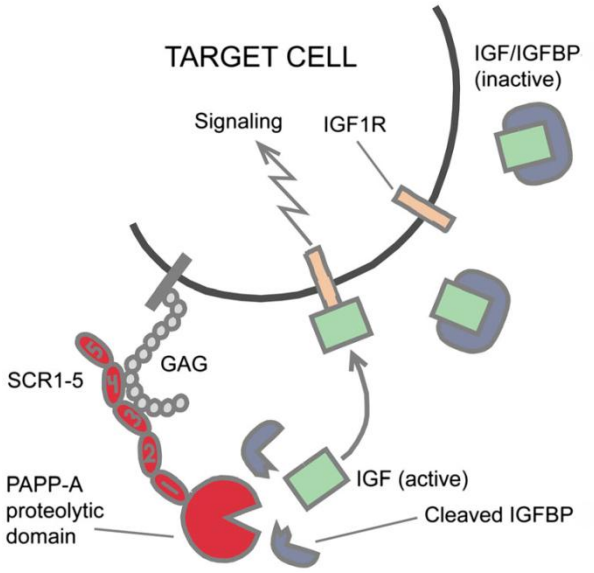

Figura 1. O papel da PAPP-A e IGFBPs no sistema IGF (Oxvig 2015; adaptado).

No ovário, a PAPP-A é secretada pelas células da granulosa e foi encontrada no fluido folicular (Conover, et al. 2001, Conover, et al. 1999). Ela também é expressa em oócitos de vacas de diferentes subespécies (Nelore e Holandesa) sugerindo que essa metaloprotease pode ter uma considerável ação durante o desenvolvimento oocitário (Satrapa, et al. 2013). Um estudo com roedores PAPP-A "*knockout*" encontrou que a ausência dessa proteína causou redução da fertilidade dessas fêmeas que, além de serem menores, apresentaram menor número de CCOs ovulados e menor concentração tanto de progesterona, quanto de estradiol, durante ciclos de superovulação (Nyegaard, et al. 2010). Além disso, a PAPP-A é produzida por células uterinas decíduas e pelo trofoblasto em resposta ao estímulo da progesterona, onde promove assim, a proliferação e adesão das células trofoblásticas (Bischof, et al. 1984a). Ela também foi encontrada no fluido uterino (Bischof, et al. 1984b, Wang, et al. 2014b) e é altamente expressa em células ovidutais em fase luteal (Hess, et al. 2013) sugerindo que a PAPP-A possui um papel importante no desenvolvimento embrionário tanto inicial, quanto em estágios mais tardios como a fase implantacional.

Recentemente, um estudo analisou os efeitos da adição de PAPP-A durante a MIV de CCOs bovinos sobre a biodisponibilidade de IGF-1, e foi observado que a presença dessa proteína aumentou a concentração de IGF-1 livre no meio de maturação após 24 horas. Além disso, a presença de PAPP-A durante a MIV modulou genes envolvidos com o metabolismo lipídico e estresse oxidativo nos embriões produzidos a partir desses CCOs tratados (Giroto, et al. 2019). Esses resultados indicam que o aumento na concentração de IGF-1 livre, mesmo que durante a MIV, foi capaz de modular genes no embrião produzido e que talvez a biodisponibilidade de IGF-1 seja um fator chave para alcançar o potencial desse peptídeo em protocolos *in vitro*.

2.3 Estresse oxidativo: implicações na produção in vitro de embriões

O estresse oxidativo é causado quando há um desbalanço entre espécies reativas de oxigênio (EROs) e substâncias antioxidantes nos organismos. EROs são formas reduzidas de oxigênio energeticamente mais reativas que o oxigênio molecular, e portanto, têm maior facilidade em reagir com outras substâncias podendo gerar uma cascata de reações celulares (Birben, et al. 2012).

As EROs são produzidas pelos organismos aeróbicos como resultantes comuns do metabolismo celular, principalmente, pela fosforilação oxidativa, que é uma reação de geração de energia no organismo através do ATP na mitocôndria e, adicionalmente, podem ser produzidas como resposta a fatores ambientais. Elas são formadas durante a etapa intermediária da redução de O_2, e podem ser radicais livres, apresentando um elétron desemparelhado na sua última camada, como o superóxido (O^{2-}) e a hidroxila ($OH\cdot$), ou não, como o peróxido de hidrogênio (H_2O_2) (Birben, et al. 2012, Guerin, et al. 2001, Lambert and Brand 2009). EROs causam danos ao DNA, RNA, às proteínas, lipídeos e membranas celulares, assim, células que sobrevivem sob condições aeróbicas

possuem um sistema onde enzimas e substâncias antioxidantes presentes tanto no citosol, quanto nas mitocôndrias, atuam defendendo as células contra as ações prejudiciais desses radicais livres (Sugino 2005).

Os sistemas antioxidantes podem divididos em enzimáticos, onde enzimas catalizam reações que convertem EROs em substâncias menos tóxicas ao organismo, ou não enzimáticos, como a glutathiona (GSH) que doa elétrons para reduzir as EROs (Birben, et al. 2012, Guerin, et al. 2001), e este último, parece ser o principal antioxidante em oócitos em embriões (Gardiner and Reed 1994, Guerin, et al. 2001, Luberda 2005, Takahashi, et al. 1993). Oócitos e embriões são sensíveis ao estresse oxidativo e a presença de EROs reduz significativamente o desenvolvimento de ambos, portanto, a manutenção do balanço entre a geração de compostos oxidantes e a atuação dos sistemas antioxidantes é essencial para a maturação e desenvolvimento oocitário e embrionário (Kala, et al. 2017).

No ambiente materno, em condições fisiológicas naturais, esse sistema antioxidante é capaz de manter o equilíbrio necessário. No entanto, em um sistema de produção *in vitro* onde há maior geração de EROs (Goto, et al. 1993, Nasr-Esfahani, et al. 1990), a alta concentração dessas substâncias pode ser um empecilho para o desenvolvimento do oócito e do futuro embrião (Kala, et al. 2017). Existem indícios de que a presença de EROs durante o desenvolvimento embrionário pode ser responsável por diversos danos ao embrião, como a indução da peroxidação lipídica ligada ao bloqueio da divisão celular (Nasr-Esfahani, et al. 1990, Noda, et al. 1991), danos na função mitocondrial (Richter, et al. 1995, Roth 2018) e modificações no DNA, sejam elas, perda de bases ou fragmentação do material nuclear (Birben, et al. 2012). Assim, a utilização de antioxidantes em sistema de cultivo *in vitro* pode ser uma alternativa para minimizar os danos causados pela presença das EROs (Cavallari, et al. 2019, Chiou, et

al. 2003, de Matos, et al. 2002, Marques, et al. 2018, Sovernigo, et al. 2017, Tamura, et al. 2008).

2.4 IGF-1: ação antioxidante

Além das ações já conhecidas do IGF-1, ele ainda parece ser um dos aliados do sistema antioxidante. Em humanos, pacientes com menor concentração de IGF-1 no sangue apresentaram maior concentração de EROs, além disso, o tratamento com GH (do inglês: g*rowth hormone*) promoveu restauração das concentrações sanguíneas de IGF-1, o que resultou na diminuição desses compostos reativos (Evans, et al. 2000). Em estudos conduzidos em camundongos, o aumento do IGF-1 na corrente sanguínea reduziu o estresse oxidativo, tanto sistêmico quanto vascular (Higashi, et al. 2010, Sukhanov, et al. 2007) e, a administração exógena de IGF-1 em animais que apresentavam baixa concentração sanguínea desse peptídeo devido à idade avançada, resultou na diminuição do dano oxidativo no cérebro e fígado associado à normalização das atividades de enzimas antioxidantes e função mitocondrial (Garcia-Fernandez, et al. 2008). Além disso, a peroxidação lipídica, que é um biomarcador do estresse oxidativo ou disfunção mitocondrial, foi reduzida em camundongos com deficiência de IGF-1 quando estes foram tratados com esse peptídeo exógeno (Puche, et al. 2016).

Para avaliar os efeitos do IGF-1 sob o estresse oxidativo na PIVE, estudos têm utilizado durante a CIV a adição de substâncias oxidativas, como o H_2O_2, ou ainda, substâncias que induzem a produção de EROs, como a menadiona, que na presença de O_2, gera O^{-2}, H_2O_2 e outros radicais livres (Kurzawa, et al. 2004, Monks, et al. 1992, Moss, et al. 2009, Nutter, et al. 1992). Em roedores, a adição H_2O_2 na CIV reduziu a porcentagem de embriões produzidos, e a adição de IGF-1 restaurou as taxas alcançadas no grupo controle, onde não houve adição de H_2O_2 (Kurzawa, et al. 2004). Em bovinos,

a presença de menadiona, nessa mesma etapa da PIVE, reduziu as taxas de blastocistos produzidas e aumentou a porcentagem de células apoptóticas nos embriões resultantes. No entanto, a adição do IGF-1 amenizou os efeitos desse estressor, diminuindo a queda das taxas de blastocistos e a presença de células apoptóticas nesses embriões (Moss, et al. 2009).

Outro fator que induz a produção de EROs é o estresse térmico. Em bovinos, embriões de oito células que foram expostos a esse tipo de estresse durante a CIV, resultaram em menor taxa de blastocistos produzidos e com maior concentração de EROs intracelular, sugerindo que o aumento dessas espécies reativas seja a causa primária da mortalidade embrionária durante o estresse térmico (Cavallari, et al. 2019, Sakatani, et al. 2004, Sakatani, et al. 2008). No entanto, a adição de IGF-1 durante essa etapa da PIVE preveniu a queda na taxa de blastocistos bovinos produzidos quando foram submetidos ao estresse térmico e também reduziu a apoptose celular nesses embriões (Bonilla, et al. 2011, Jousan and Hansen 2004, 2007). Além disso, os principais genes modulados nesses blastocistos pela presença desse peptídeo foram relatados com a apoptose celular e proteção contra EROs (Bonilla, et al. 2011). Assim o IGF-1 mostrado um papel na proteção contra o estresse oxidativo, reduzindo as concentrações de EROs e também a presença de células apoptóticas em embriões, o que consequentemente, reflete no aumento da quantidade e qualidade embrionária.

2.5 IGF-1 e metabolismo lipídico

Tanto oócitos quanto embriões de espécies de mamíferos como o suíno, bovino e ovino contêm altas quantidades de gotículas lipídicas no citoplasma (Aardema, et al. 2011, Abe, et al. 2002, Cran 1985, Sturmey, et al. 2009). Essas gotículas são formadas

por ácidos graxos armazenados na forma de triacilglicerídeos no citoplasma e representam um estoque de energia metabólica (Dunning, et al. 2014, McKeegan and Sturmey 2011), onde os ácidos graxos livres podem através da β-oxidação, resultar na formação de adenosina trifosfato (ATP) na matriz mitocondrial (Dunning, et al. 2010, Sadaba, et al. 2016, Smitz, et al. 2011).

O IGF-1 é um dos fatores que está relacionado à regulação do metabolismo lipídico principalmente modulando a adiposidade celular (Christians, et al. 2015, Conover, et al. 2013, Hausman, et al. 2001, Kim 2013, Ruan and Lai 2010, Siddals, et al. 2002, Smith, et al. 1988). Smith et al. (1988) detectou 13 mil locais de ligações de alta afinidade com esse peptídeo em pré-adipócitos de ratos, e em células hepáticas de roedores, a expressão de genes envolvidos no metabolismo de glicose e lipídeos foi normalizada pela terapia com IGF-1 (De Ita, et al. 2015). Adicionalmente, estudos têm mostrado que o tratamento com IGF-1 foi capaz de reverter a resistência à insulina e reduzir os níveis de colesterol e triglicerídeos, aumentando significativamente as concentrações de ácidos graxos livres (Garcia-Fernandez, et al. 2008).

Nos CCOs, o metabolismo de ácidos graxos através da β-oxidação é importante para a aquisição da competência oocitária e subsequente desenvolvimento embrionário em várias espécies (Dunning, et al. 2014, Paczkowski, et al. 2013), e sua inibição durante a maturação oocitária resulta na redução do desenvolvimento de embriões (Dunning, et al. 2010, Ferguson and Leese 2006, Hillman and Flynn 1980, Sturmey, et al. 2009). Alguns estudos mostraram que as concentrações de triglicerídeos em oócitos podem diminuir após a MIV (Ferguson and Leese 1999, Sturmey and Leese 2003), e que a atividade lipase aumenta (Cetica, et al. 2002), além de oócitos com maior conteúdo lipídico serem mais competentes resultando em maiores taxas de embriões produzidos (Jeong, et al. 2009). Em embriões, estudos também têm sugerido que

lipídeos podem ser metabolizados e usados como fonte de energia durante o desenvolvimento embrionário inicial, por meio de mitocôndrias que podem aumentar a produção de ATP necessária para a formação de blastocele ou diferenciação de linhagens celulares (Jeong, et al. 2009, Tarazona, et al. 2006). Juntos, esses resultados sugerem que esses lipídeos podem ser uma fonte energética durante o desenvolvimento oocitário e embrionário inicial.

Os meios de cultivo utilizados em protocolos de PIVE também podem interferir no metabolismo lipídico devido à difusão dos componentes desses meios para o interior das células (Dunning, et al. 2014, Kim, et al. 2001). Oócitos maturados na presença de SFB possuem mais triglicerídeos do que aqueles cultivados na ausência de ácidos graxos (Abe, et al. 2002, Del Collado, et al. 2015, Kim, et al. 2001), e estudos mostraram que oócitos com maior teor lipídico, apresentaram maior capacidade de desenvolvimento, e isso pode ser devido apresentarem também, maior quantidade de ATP disponível (Jeong, et al. 2009, Nagano, et al. 2006a, b). Além disso, há relatos de que alterações da composição de ácidos graxos em oócitos bovinos podem melhorar a maturação e a criopreservação destes (Aardema, et al. 2011, Kim, et al. 2001), uma vez que a maturação de oócitos na presença de SFB promoveu maiores taxas de β-oxidação (Dunning, et al. 2010) e aumentou a taxa de blastocistos (Del Collado, et al. 2015).

O SFB possui também alta quantidade de IGF-1 (Honegger and Humbel 1986, Tu, et al. 2018), e esse peptídeo parece estar envolvido na modulação da β-oxidação através da normalização da expressão de enzimas dessa via (De Ita, et al. 2015). Assim, o SFB pode ser uma fonte energética, tanto para oócitos, quanto para embriões, e a utilização de substâncias que induzem o metabolismo lipídico visando gerar maior produção de energia, pode ser uma estratégia para melhorar as taxas de blastocistos produzidas durante procedimentos *in vitro.*

Objetivo

Investigar os efeitos da PAPP-A como potencializadora dos efeitos atribuídos ao IGF-1 durante a PIVE de bovinos.

Hipóteses

1- A adição de PAPP-A durante a MIV convencional de CCOs bovinos irá diminuir o conteúdo lipídico, tanto de oócitos, quanto dos blastocistos, modulará positivamente genes relacionados ao metabolismo lipídico e qualidade embrionária nos embriões;

2- A adição de PAPP-A, tanto na MIV quanto na CIV de bovinos, potencializará a ação antioxidante do IGF-1 em um ambiente estresse-induzido.

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CAPÍTULO II

MOLECULAR PHENOTYPES OF BOVINE BLASTOCYST DERIVED FROM *IN VITRO*-MATURED OOCYTE SUPPLEMENTED WITH RECOMBINANT PAPPALYSIN-1

MOLECULAR PHENOTYPES OF BOVINE BLASTOCYST DERIVED FROM *IN VITRO*-MATURED OOCYTE SUPPLEMENTED WITH RECOMBINANT PAPPALYSIN-1

Fernanda Fagali Franchi[1], Thaisy Tino Dellaqua[1], Priscila Helena dos Santos[1], Alan Brunholi Giroto[2], Victor Augusto Ferreira de Lima[2], Vitória Bertogna Guilherme[3], Patricia Kubo Fontes[1], Mateus José Sudano[4], Anthony César de Souza Castilho[2].

[1] Departament of Pharmacology, Institute of Biosciences, Universidade Estadual Paulista (UNESP), Botucatu, São Paulo, Brazil.
[2] Departament of Animal Science, University of Western São Paulo (UNOESTE), Presidente Prudente, São Paulo, Brazil.
[3] Departament of Biology, São Paulo State University (UNESP), Assis, São Paulo, Brazil.
[4] Center of Natural and Human Science, Federal University of ABC, Santo André, São Paulo, Brazil.

Corresponding Author:
Anthony César de Souza Castilho; Universidade do Oeste Paulista (UNOESTE), Pró-Reitoria de Pesquisa e Pós-Graduação – Campus II Rodovia Raposo Tavares, km 572 – Bairro Limoeiro, 19067-175, Presidente Prudente, São Paulo, Brasil.
Email: castilho.anthony@gmail.com

Abstract

Oocyte and embryo development requires a lot amount of energy, and lipids are a good source of it. During the oocyte maturation, the lipid content decreased indicating these lipids could be a good source of energy production. The IGF-1 (insulin-like growth factor-1) is a possible regulator of this process and PAPP-A (Pregnancy-Associated Plasma Protein-A) is a positive regulator of IGF-1 bioavailability. Then, this study aimed to evaluate oocytes and blastocysts lipid content and embryo transcriptional profile produced from cumulus-oocyte (COCs) *in vitro* matured with fetal bovine serum

(FBS) and PAPP-A presence (+PAPP-A) or not (-PAPP-A). The PAPP-A and FBS combination (+PAPP-A group) did not affect oocyte (-PAPP-A: 8.807 ±0.583 and +PAPP-A: 9.134±0.747; P=0.785) or blastocysts (-PAPP-A: 3.590±0.244 and +PAPP-A: 3.568±0.146; P=0.822) content. However these combination impact the embryos transcriptional profile by downregulation (P > 0.05) of *ACACA*, *ACSL1*, *ACSL3*, *ELOVL1*, *ELOVL5*, *SREBF2*, and *FASN* related to lipid metabolism; *CASP3*, *CASP9*, and *BAX* pro-apoptotic genes *HSPA1A*, *HSPA5*, *HIF1α*, and *NFkB2* involved in cellular damage processes; and *AKR1B1*, *S100A14*, *SLC2A5*, *CDX2*, IFN-τ, *GATM*, *NANOG* and *REST* markers of embryo quality. Taken together, these results suggest even though PAPP-A/FBS combination did not affect oocytes or blastocysts lipid content, the transcriptional modulation could contribute to maintaining the balance in lipid metabolism of the embryos, and further controlling key intracellular pathways such as apoptosis, cellular damage and embryo quality.

Keywords: *in vitro* maturation, lipolysis, lipid metabolism, IGF-1, PAPP-A, FBS, gene expression, Sudan-Black B.

1. Introduction

In mammals, *in vitro* maturation (IVM) of cumulus-oocytes complex (COCs) is an important stage for success *in vitro* fertilization and early embryo development [1]. The efficiency of IVM is limited by the oocyte's intrinsic developmental competence which refers to the biochemical and molecular state that allows the meiotic progression of immature oocyte for that posteriorly to be fertilized and develop to an embryo [2,3]. These rapid and tightly synchronized events are energy-consuming processes and require an adequate generation of ATP from cellular energy stocks [4].

58 During bovine oocyte maturation, lipolysis is increased and stored triacylglycerides are metabolized to provide energy supply for supporting oocyte
60 development and early embryo development [4–6]. One possible regulator of this process seems to be insulin-like growth factor-1 (IGF-1). In mice hepatic cells, IGF-1
62 deficiency leads to the downregulation of genes involved with lipid metabolism and its exogenous addition restored the gene expression patterns [7]. Furthermore, IGF-1
64 treatment also induced a significant reduction of serum cholesterol and triglycerides as well as increased free fatty acids [8] which could be metabolized by β-oxidation to
66 generate ATP [4,9–11].

The IGF action is modulated by insulin-like growth factor binding proteins
68 (IGFBPs) which bind to this peptide and decrease IGF-1 bioavailability [12,13]. In opposite that, pregnancy-associated plasma protein-A (PAPP-A) increases free IGF
70 through proteolytic degradation of IGFBPs [12,14,15]. In mammalian species including cattle, PAPP-A is responsible for the degradation of IGFBP-2, 4 and 5 [14,16] and when
72 added during bovine IVM-serum free increased IGF-1 levels in IVM medium and modulate transcriptional profile of genes involved with energy metabolism and maintain
74 embryonic survival post-vitrification [17].

It is known that fetal bovine serum (FBS) has a high quantity of IGF-1 [18,19],
76 and higher fatty acids concentration compared with bovine serum albumin (BSA) [20]. Although IVM performed with FBS presence seems to be superior by increasing of total
78 and inner cells number of blastocysts [21], and improving levels of β-oxidation during oocyte IVM [9], that approach could result in higher levels of lipid content in the
80 embryo which are related to reduce embryo survive pos-cryopreservation [22].

Based on previous premises about PAPP-A increases free IGF-1 which is involved in lipid metabolism and that growth factor is present in FBS we hypothesized that PAPP-A addition during IVM of bovine COCs with serum could modulate cytoplasmic lipid contents of oocytes and further, in blastocysts by IGF-1 action. Moreover, we expect that approach could impact the transcriptional profile of genes encoding proteins related to lipid metabolism, and further also modulate genes encoding markers for blastocyst quality.

2. Material and Methods

All products were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. The experimental design is shown in Fig. 1.

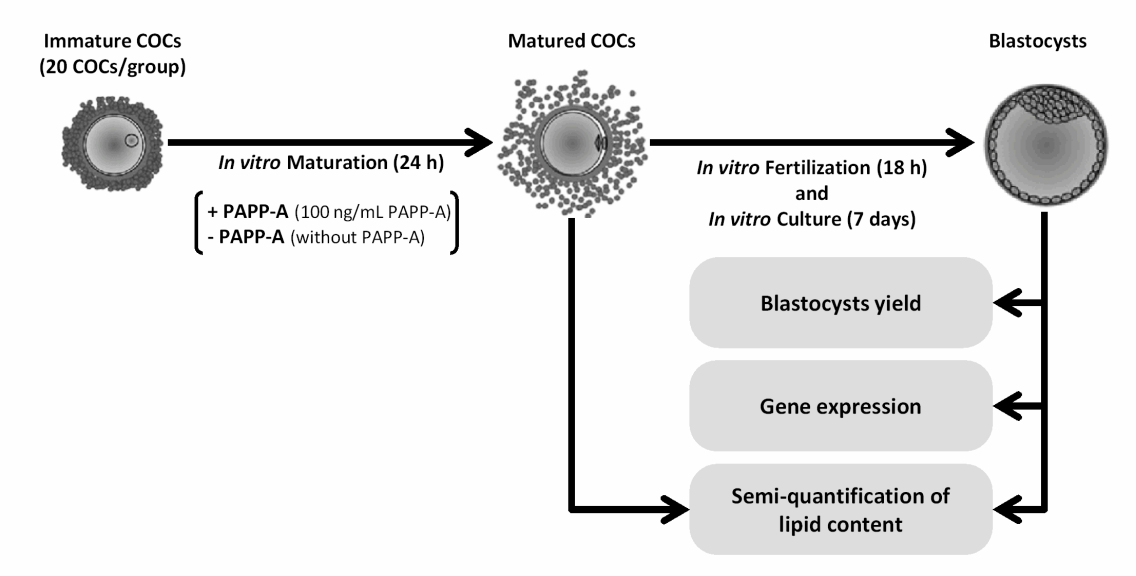

Fig. 1. Experimental design to investigate the effects of PAPP-A addition in IVM medium FBS supplemented. The COCs were IVM for 24h in medium supplement with PAPP-A (+PAPP-A) or not (-PAPP-A). First, the COCs were mechanically separated to recovery the oocytes for semi-quantification of

lipid content. In a second moment, after IVM the COCs were submitted to *in vitro* fertilization and *in vitro* cultured. On day 7 the blastocysts yield was assessed and it was collected to gene expression and semi-quantification of lipid content. COC: cumulus-oocyte complex; PAPP-A: Pregnancy-associated plasma protein-A.

2.1. Ovaries and COC recovery

Ovaries from predominantly from *Bos taurus indicus* and crossbreed females were obtained at near slaughterhouse at Torrinha – SP (22º 25' 34" S; 48º 10' 09" W) and transported to Sao Paulo State University laboratory – Campus of Botucatu in sterile saline solution (0.9% NaCl) at 36 ºC. The COCs were recovered from follicles of 2–8 mm and selected under a stereomicroscope. Only oocytes with homogeneous or slightly granulated cytoplasm and at least three compact cumulus cells layers were used for further experiments [23].

2.2. COCs in vitro maturation

The selected COCs (20 COCs/group) were placed in 90 µl drops with IVM medium composed by TCM 199 containing Earle's salts supplemented with 22 µg/mL sodium pyruvate, 75 µg/mL amikacin, 0.1 IU/mL follicular stimulating hormone (FSH, Gonal-f, Merck Serono, Bari, Italy), 10% (v/v) fetal bovine serum (FBS) and in presence of PAPP-A (+PAPP-A; 100 ng/mL rhPappalysin-1; R&D Systems, Minneapolis, MN, USA) or not (-PAPP-A). The drops were covered by silicone oil (Quimesp Química, Guarulhos, SP, Brazil) and the culture carries out for 24 hours in a controlled environment (5.5% CO_2, humidified air, 38.5 °C). Subsequently, COCs were

120 transferred to *in vitro* fertilization (IVF) medium or denuded and submitted to lipid content analysis.

122

2.3. In vitro fertilization and embryo culture

124 The matured COCs were washed thrice in IVF medium drops and placed in 90 µL IVF medium covered by silicon oil. The IVF medium was composed by Tyrode 126 Albumin Lactate Pyruvate (TALP) supplemented with 6 mg/mL fatty acid-free BSA (Bovine Serum Albumin), 22 µg/mL pyruvate, 75 µg/mL amikacin, 30 µg/mL heparin, 128 and PHE (44 µL/mL; 20 µM de Penicillamine, 10 µM Hipotaurin, and 1µM Epinephrin). The semen from a Nelore bull was thawed at 37 °C for 30s and 130 spermatozoa were washed in a discontinuous Select SPERM gradient (BotuFIV®, BotuPharma, Botucatu, SP, Brazil) prepared by adding 0.4 mL of 45% Select SPERM 132 to 0.4 mL of 90% Select SPERM in a 1.5 mL centrifuge tube. The semen samples were added on top of the gradient and centrifuged at 4.500 g for 5 min (MiniSpin® 134 Eppendorf). Then, the supernatant was removed, and the spermatozoa pellet was counted in a Neubauer chamber. Sperm (7 µL of the final concentration=1×10^6 136 cells/mL) was added into each drop and incubated at 38.5° C in a saturated humidity atmosphere containing 5.5% CO_2 for 18 h. After, the presumptive zygotes were 138 denuded of the remaining surrounding cumulus cells by vortexing and were washed thrice in synthetic oviduct fluid (SOF) medium [24].

140 Embryo culture was performed in four-well dishes using 10 µL/presumptive zygotes of SOF medium supplemented with 22 µg/mL sodium pyruvate, 5 mg/mL BSA, 142 and 2.5% (v/v) FBS. Embryos were cultured for seven days in a humid controlled

atmosphere (5% CO_2, 5% O_2, and 90% N_2) at 38.5 ºC. Blastocyst rates were assessed on
144 day 7 post-insemination and the samples were stored at -80° C until analysis.

146 *2.4. Determining gene expression in blastocysts through RT-qPCR*

To determine the gene expression of bovine embryos were used 4 pools for each
148 group (3 blastocysts/pool). Total RNA was extracted using the PicoPure™ RNA Isolation Kit (Applied Biosystem®, Foster City, CA, USA) following the manufacturer's
150 instructions. The entire RNA sample was incubated with DNAse I (1 IU/µg; Invitrogen, São Paulo, Brazil) and then reverse-transcribed using random primers according to the
152 protocol provided with the High Capacity kit (Applied Biosystem®, Foster City, CA, USA).

154 For analyzing the blastocysts transcripts the mRNA relative abundance of 96 genes related to lipid metabolism, cellular stress, and embryo quality were determined
156 (Table S1; supplementary material). All analyses were performed using Biomark HD assay (Fluidigm, South San Francisco, CA, USA) as previously described [17,25,26].
158 The relative expression values for each gene were calculated with the ΔCt method and data were normalized using most stable reference genes geometric means (*B2M*,
160 *GAPDH*, *HPRT1* and *PPIA*; NormFinder software tested).

162 *2.5. Semi-quantification of lipids*

Oocytes and embryos samples were subjected to semi-quantitative lipid assays
164 with the lipophilic dye Sudan-Black B as described to Sudano *et al.* [27]. Briefly, samples were fixed in a 4% paraformaldehyde solution in PBS (phosphate-buffered

166 saline) free of calcium and magnesium and transferred to 50% ethanol before staining in 1% Sudan-black B (w/v) in 70% ethanol for 2 min. After this, samples were mounted in
168 glycerol and examined under a light microscope ×400 and ×200 magnification for oocytes and embryos, respectively. The relative lipid content was estimated in Image J
170 1.41 software (Wayne Rasband, National Institutes of Health, Bethesda, MD). Images of oocytes and embryos were converted to a grey-scale and the grey intensity per area
172 was calculated in arbitrary units mm^{-2}.

174 *2.6. Statistical Analysis*

All analyses were performed using JMP software (Version 13.0; SAS Institute
176 Inc.). The blastocyst yield was calculated as percentages of total COCs subjected to IVM and was arcsine-transformed and compared using t-tests. The effects of PAPP-A
178 supplementation on oocytes and embryos lipid concentration and mRNA relative abundance in embryos were tested by the Shapiro Wilk test and means were compared
180 with t-test or Wilcoxon test. Data are presented by mean ± SEM and differences were considered significant when $P \leq 0.05$.

182

3. Results

184 When we analyze the impact of COCs matured with FBS medium in presence or not of PAPP-A, we figure out similar blastocysts yield ($P = 0.726$) between the groups
186 (-PAPP-A: 35.4 ± 2.08; +PAPP-A: 40.5 ± 6.89) and we did not observe any effect on cytoplasmic lipid content of oocytes ($P > 0.05$; Fig. 2) and embryos ($P > 0.05$; Fig. 3).

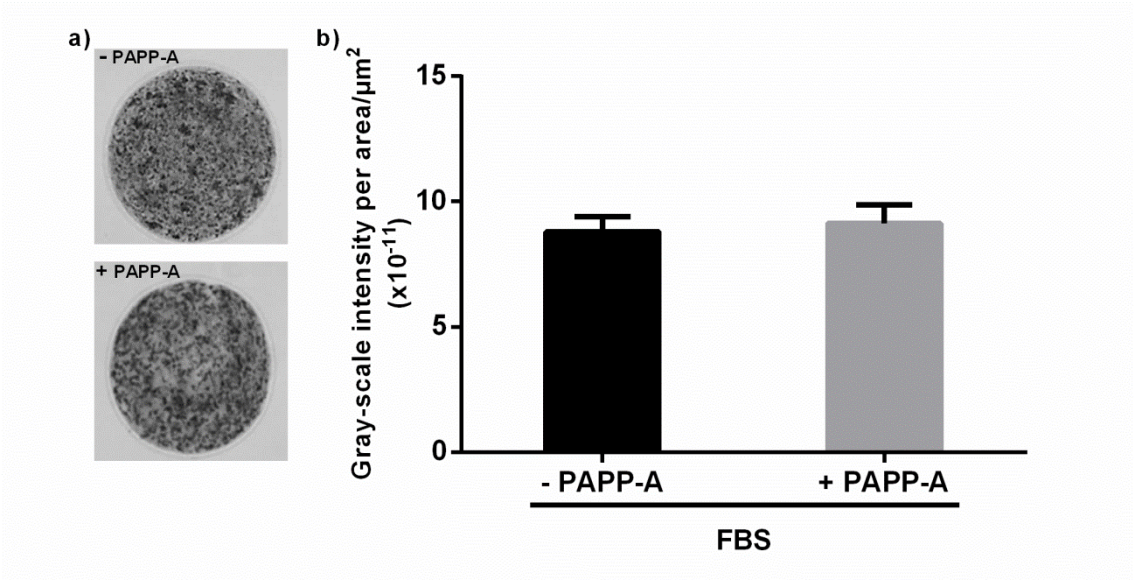

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Fig. 2. Lipid content of bovine oocytes stained with the lipophilic dye Sudan-Black B from bovine
190 oocytes matured *in vitro* with 10% FBS supplemented 100 ng/mL PAPP-A or not. **a)** Panels show representative images of each oocyte evaluated. Black dots indicate cytoplasmic lipid droplets.
192 Magnification ×400. **b)** Gray-scale intensity in oocytes matured *in vitro* with PAPP-A or not ($P > 0.05$). Data are means ± S.E.M. of four biological replicates tested using t-test.

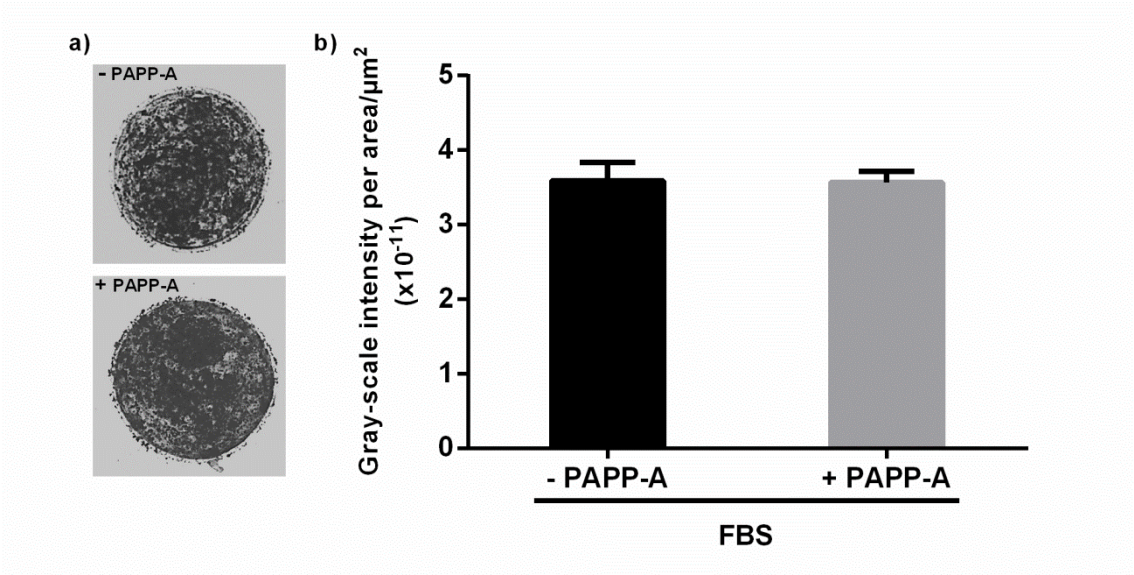

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Fig. 3. Lipid content of bovine embryos stained with the lipophilic dye Sudan-Black B from bovine
196 oocytes matured *in vitro* with 10% FBS medium supplemented 100 ng/mL PAPP-A or not. **a)** Panels show representative images of each embryo evaluated. Black dots indicate cytoplasmic lipid droplets.

198 Magnification ×200. **b)** Gray-scale intensity in bovine embryos from oocytes matured *in vitro* with PAPP-A or not (P > 0.05). Data are means ± S.E.M. of four biological replicates tested using t-test.

200

Regarding embryo transcriptional abundance of genes related to lipid 202 metabolism, we demonstrated that PAPP-A downregulated mRNA abundance of acetyl-CoA carboxylase (*ACACA*), acyl-CoA synthetase long-chain family member 1 and 3 204 (*ACSL1* and *ACSL3*), elongation of very-long-chain fatty acids protein 1 and 5 (*ELOVL1* and *ELOVL5*) and fatty acid synthase (*FASN*; Fig. 4a). Moreover, we also 206 observed a downregulation of genes related to several intracellular pathways caused by PAPP-A supplementation, such as transcriptional regulator [sterol regulatory element-208 binding transcription factor 2 (*SREBF2*; Fig. 4b)]; apoptosis [caspase 3 and 9 (*CASP3* and *CASP9*) and BCL2 associated X (*BAX*; Fig. 5a)]; cellular damage [heat shock 210 protein family A member 1A and 5 (*HSPA1A* and *HSPA5*), hypoxia-inducible factor 1-alpha (*HIF1α*), and nuclear factor-kappa B subunit 2 (*NFkB2*; Fig. 5b)]; glucose 212 metabolism and protein carrier [aldo-keto reductase family 1 member B1(*AKR1B1*), S100 calcium-binding protein A14 (*S100A14*), and solute carrier family 2 member 5 214 (*SLC2A5*; Fig. 6a)]; embryo viability [caudal type homeobox transcription factor 2 (**CDX2**), interferon tau-1,2 and 3 (IFN-τ/IFN-τ2/IFN-τ3), and glycine 216 amidinotransferase mitochondrial (*GATM*; Fig. 6b)] and pluripotency markers [*NANOG* and RE1 silencing transcription factor (*REST*; Fig. 6c)].

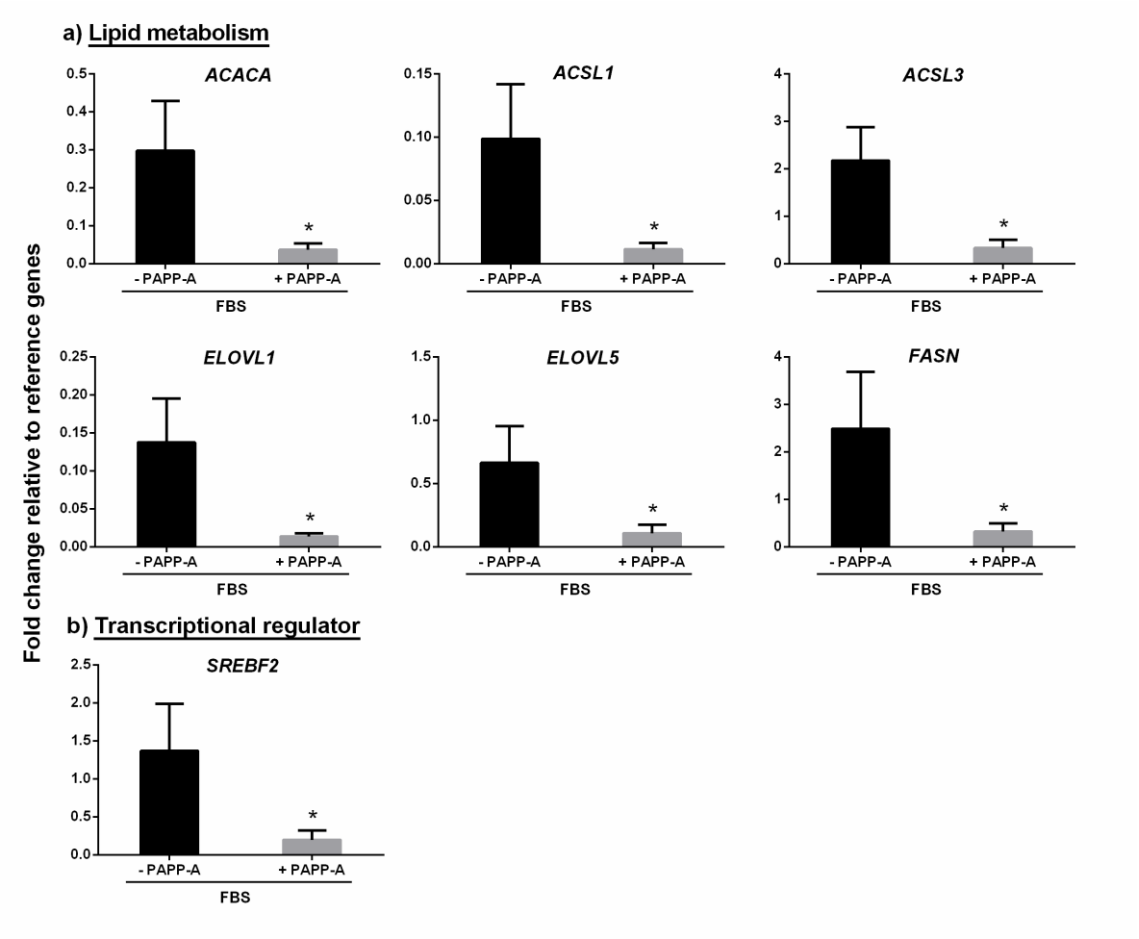
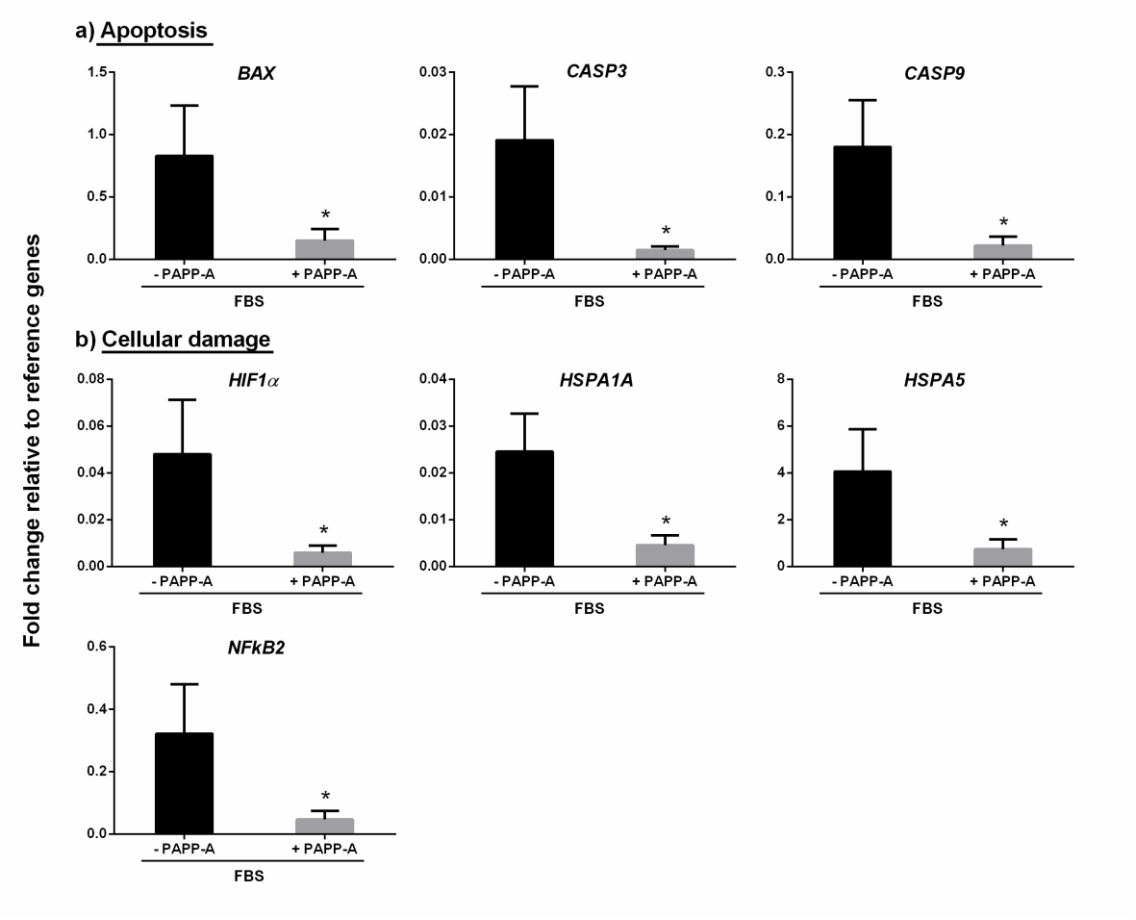


Fig. 4. Gene expression of blastocysts produced *in vitro* from oocytes matured *in vitro* with 10% FBS medium supplemented 100 ng/mL PAPP-A or not. a) lipid metabolism and b) Transcriptional regulator in bovine embryos. Data represent the fold change in the levels of expression of genes relative to reference genes (*B2M*, *GAPDH*, *HPRT1*, and *PPIA*). Data are means ± S.E.M. of four biological replicates tested using t-test. Differences were considered significant when $P \leq 0.05$.



224

Fig. 5. Gene expression of blastocysts produced *in vitro* from oocytes matured *in vitro* with 10% FBS
226 medium supplemented 100 ng/mL PAPP-A or not. Data represent the fold change in the levels of expression of genes relative to reference genes (*B2M, GAPDH, HPRT1,* and *PPIA*). Data are means ±
228 S.E.M. of four biological replicates tested using t-test. Differences were considered significant when $P \leq 0.05$.

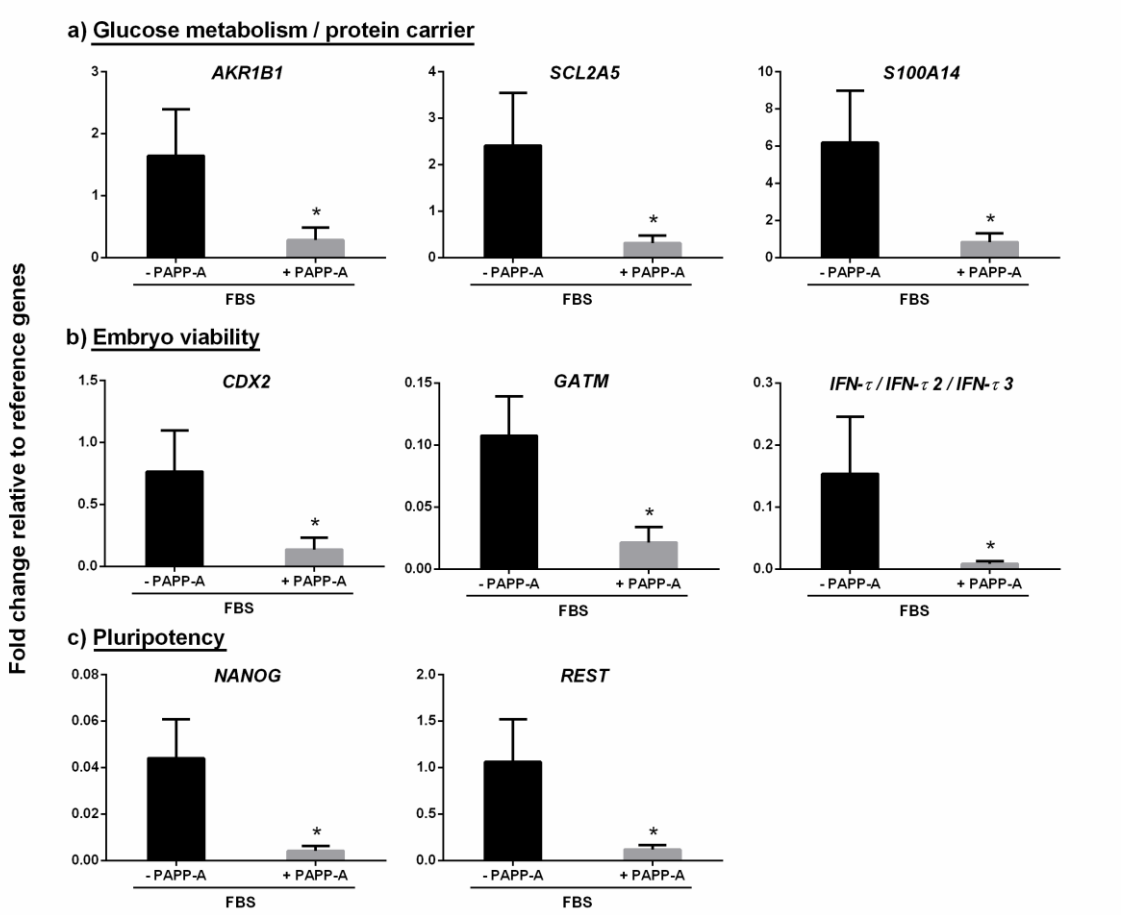


Fig. 6. Gene expression of blastocysts produced *in vitro* from oocytes matured *in vitro* with 10% FBS medium supplemented 100 ng/mL PAPP-A or not. Data represent the fold change in the levels of expression of genes relative to reference genes (*B2M, GAPDH, HPRT1*, and *PPIA*). Data are means ± S.E.M. of four biological replicates tested using t-test. Differences were considered significant when $P \leq 0.05$.

4. Discussion

When we summarize our findings in the present study, we highlight the transcriptional effects of PAPP-A combined to FBS on downregulation of genes related to lipid accumulation in bovine embryos. However, we cannot observe any impact on lipid content of oocytes as well as, no differences in blastocyst yield. Although the PAPP-A supplementation did not affect lipid content of oocytes or embryos, the downregulation of genes involved with lipid metabolism suggests the IGF-1 could act

on helping the homeostasis of lipid metabolism. Furthermore, we cannot exclude our findings of the regulation of key factors involved with several cellular pathways, eg. apoptosis, and cellular damage to promote the improvement of embryo quality by this synergic approach caused by PAPP-A/FBS.

Fatty acids affect gene expression through transcription factors such as SREBPs (codified by SREBFs) [28] and thus exert important regulatory control over the activity of such receptors and gene expression on metabolic pathways [29]. In general, SREBP2 is related mainly to cholesterol synthesis although a moderate induction of genes involved in fatty acid synthesis (*ACACA*, *FASN*, *FADS2*, *SCD*, *ACSS2*, *ACSL1*) is also observed [30–32]. The *FASN* gene is involved in long fatty acid biosynthesis and is responsible for codifying a multifunctional (FAS) enzyme that catalyzes the synthesis of palmitate into long-chain saturated fatty acid [33–35], and ELOVL5 is implicated in the elongation of long-chain PUFA (linoleic and linolenic acids) [36] while ELOVL1 catalyzes the condensation of very-long-chain saturated or monounsaturated fatty acids [37], and ACSLs activate long-chain fatty acids before their entry into mitochondria [4]. Regarding the impact of PAPP-A/FBS combination (+PAPP-A) during oocyte IVM on downregulation of *ACACA*, *ACSL1*, *ACSL3*, *ELOVL1*, *ELOVL5*, *FASN* and *SREBF2* in bovine blastocysts we could suggest that modification of saturated/unsaturated fatty acid balance in bovine blastocysts could contribute or interfere with membrane fluidity and influence to lipid homeostasis, promoting fatty acid storage or mitochondrial fatty acid oxidation as inferred by [38,39].

Other findings have reported that fatty acid profile of FBS contains high levels of saturated (palmitic and stearic acids), unsaturated (oleic acid) fatty acids and PUFA [40–42], and these fatty acids may be incorporated into the oocyte cytoplasm during IVM [20,41]. Indeed, Sudano *et al.* [22] showed that increased FBS concentration in the

IVC medium augments also the cytoplasmic lipid accumulation in bovine embryos, resulting in lower embryo survival and total number of cells reduced after vitrification, an increased percentage of apoptosis in vitrified blastocysts. Here the embryos showed similar lipid content and the expression of genes involved in fatty acid oxidation by β-oxidation such as *CPT1B* and *CPT2* (responsible for fatty acid inlet and outlet in the mitochondrial matrix, respectively) [9,43] did not change with our approach, suggesting a normal lipid metabolism by β-oxidation role and supporting the idea of lipid homeostasis. So, PAPP-A presence may have been used such a control mechanism for the maintenance of energy balance into embryos cultured once that components of FBS medium may be incorporated embryonic cells [20,41].

Further, the PAPP-A/FBS combination downregulated *CASP3, CASP9,* and *BAX* related to apoptosis and *HSPA1A* and *HSPA5* which are involved in heat stress activity. Indeed, previous reports found the IGF-1 addition in the culture medium resulted in lower number of apoptotic cells and increased blastocyst yield after heat-stress conditions [44–48] besides, this environment decreased IGFBPs expression in oocytes suggesting that IGF-1 may help to reduce the damages leads by heat-stress [49]. Moreover, the higher expression of *HIF1α* was is associated with high glucose stress in bovine blastocysts and the increase reactive oxygen species (ROS) production [50], and here it was downregulated in blastocysts PAPP-A/FBS combination suggesting a role in protection against ROS presence.

The PAPP-A/FBS also downregulated genes related to embryonic viability such as *GATM, IFN-τ, NANOG, CDX2, S100A14, REST* which are markers of high embryonic developmental potential in cattle [51–61], and *AKR1B1* and *SLC2A5* related to glucose metabolism [62–64]. Even PAPP-A/FBS addition during IVM decreased the expression of these genes, the embryo production was not altered in both groups

suggesting the normal oocyte and embryo development as well as found by Giroto *et al.* [17].

In order to elucidate the role of IGF system modulation during oocyte *in vitro* maturation using FBS, we figure out the combination of PAPP-A/FBS on IVM of bovine COCs decreases expression of genes encoding protein-related to lipid metabolism in bovine blastocysts. Even though this approach did not affect oocytes or blastocysts lipid content, it could contribute to maintaining the balance in lipid metabolism process in embryos and, further, to impact blastocyst quality by regulation of transcripts involved key intracellular pathways such as apoptosis, cellular damage, and embryo development.

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CAPÍTULO III

NEW APPROACH USING IGF-1 COMBINED WITH PAPP-A DURING BOVINE *IN VITRO* EMBRYO PRODUCTION: COULD THIS SYSTEM ENHANCE IGF-1 ANTIOXIDATIVE ROLE?

NEW APPROACH USING IGF-1 COMBINED WITH PAPP-A DURING BOVINE IN VITRO EMBRYO PRODUCTION: COULD THIS SYSTEM ENHANCE IGF-1 ANTIOXIDATIVE ROLE?

Fernanda Fagali Franchi[1], Priscila Helena dos Santos[1], Sarah Gomes Nunes[1], Patricia Kubo Fontes[1], Ana Elisa Valencise Quaglio[1], Luiz Claudio Di Stasi[1], Gisele Zoccal Mingoti[2], Anthony César de Souza Castilho[3]

1. Departament of Pharmacology, Institute of Biosciences, Universidade Estadual Paulista (UNESP), Botucatu, São Paulo, Brazil.
2. Departament of Production and Animal Health, São Paulo State University (UNESP) Araçatuba - São Paulo, Brazil.
3. Departament of Animal Science, University of Western São Paulo (UNOESTE), Presidente Prudente, São Paulo, Brazil.

ABSTRACT

Yes. The addition of 100ng/ml of PAPP-A enhances IGF-1 antioxidative role which reaches to decreasing of ROS levels in oocytes and blastocysts, besides that, modulates key transcripts encoding proteins involved with antioxidative controlling, lipid metabolism and embryonic quality.

Correspondente Author: Anthony César de Souza Castilho, Universidade do Oeste Paulista (UNOESTE), Pró-Reitoria de Pesquisa e Pós-Graduação – Campus II Rodovia Raposo Tavares, km 572 – Bairro Limoeiro, 19067-175, Presidente Prudente, São Paulo, Brasil. Email: castilho.anthony@gmail.com

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1. INTRODUCTION

The reactive oxygen species (ROS) are produced by aerobic metabolism even under basal conditions and are a result of various metabolic pathways and enzymes, including mainly oxidative phosphorylation to produce adenosine triphosphate (ATP) by the mitochondrial electron transport chain [Reviewed by 1]. However, excessive ROS generation can be harmful to cellular function and can lead to oxidative stress, which induces lipid peroxidation, mitochondrial function damage and DNA modifications, such as bases pairs loss or DNA nuclear fragmentation [2-6]. The oocyte and early embryo development require sufficient amounts of ATP [7], and in physiological conditions, the antioxidant system can modulate the ROS levels and reduces the damages drives by ROS presence. However, there are increased levels of these reactive species in oocytes and blastocysts in vitro produced, which resulted in embryos developmental blocked at the 2-cell stage in mouse, for instance [3].

The insulin-like growth factor-1 (IGF-1) has shown systemic antioxidant action through ROS levels reduction [8, 9]. The IGF-1 addition during in vitro culture (IVC) avoid the reduced embryo yield and increased blastocysts apoptotic cells leads by the presence of menadione, a prooxidative stressor that induces ROS production in O_2 presence [10, 11], however, did not reduce the ROS levels in bovine embryos [12, 13].

The IGF-1 actions are controlled by the regulation of IGF-binding protein (IGFPBs) activity, which blocks IGF-1 functions, whereas pregnancy-associated plasma protein-A (PAPP-A) improves IGF-1 bioavailability and facilitates binding to IGF receptors [14-17]. The PAPP-A is secreted by granulosa cells [18], and cleaves IGFBP-2, IGFBP-4 and IGFBP-5 in many species [17, 19-24] and when added during in vitro maturation (IVM) of bovine cumulus-oocytes complex (COCs) increases the free IGF-1

levels in IVM medium. Furthermore, the PAPP-A treatment showed to modulate genes involved in lipid metabolism, mitochondrial functionality, and development of blastocyst competence in the embryos produced [25].

Nevertheless, the simultaneous addition of PAPP-A and IGF-1 during in vitro production (IVP) protocols were not investigated yet, and considering that IGF-1 has been shown systemic antioxidant action, maybe due to these systems have PAPP-A enough to improving the IGF-1 bioavailability. Based on this, we proposed a new approach using a synergic effect of IGF-1/PAPP-A system and we hypothesized that exogenous PAPP-A addition during bovine IVP works as an enhancer for antioxidative effects of IGF-1, and impacts positively oocyte and blastocyst quality.

2. MATERIAL AND METHODS

Unless otherwise mentioned, all products were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.1 Experimental design

To broaden our knowledge about role of PAPP-A/IGF-1 system on oxidative stress, we mitigates stress a stressed environment during IVP using menadione inducer of oxidative stress in oocytes and embryos, at 5µM as previously described a by Moss et al. [12], Assis et al. [26], Cavallari et al. [27], and Chen et al. [28]. First of all, we tried to figure out if only PAPP-A could be a feasible approach to reduce ROS levels in our experimental design. However, we did not observe ROS levels reduction using only PAPP-A (data not showed), then we performed all experiments as showed below (Figure 1).

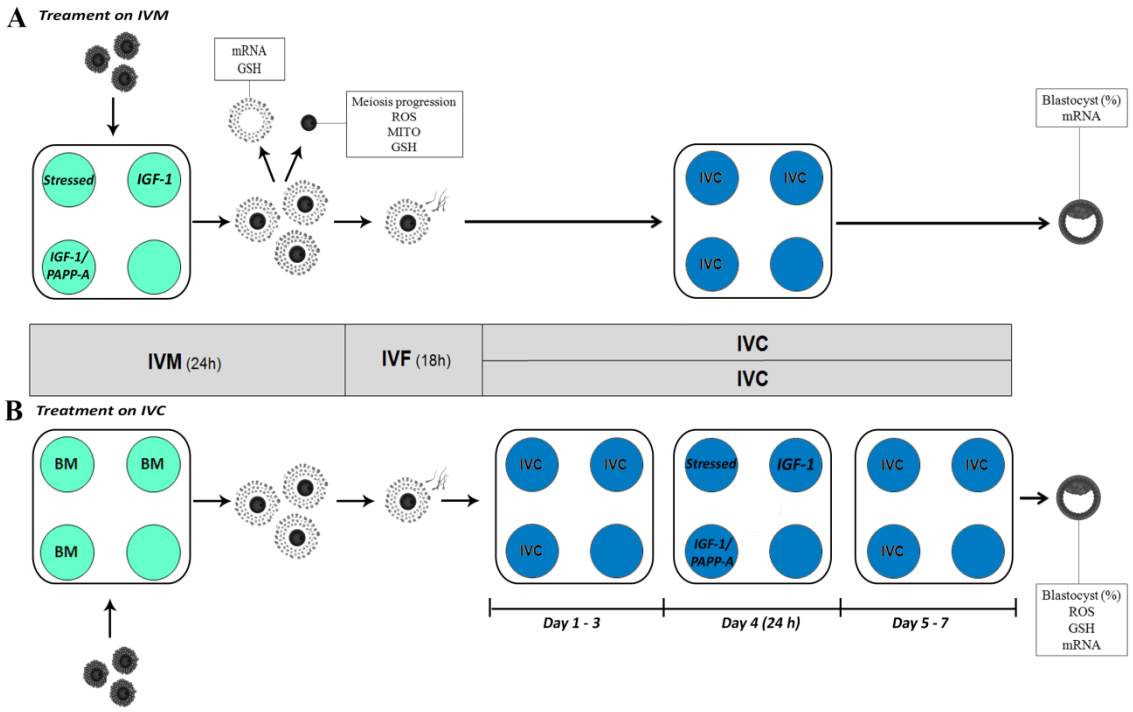


Figure 1. A: Effects of IGF-1 addition with or without PAPP-A during IVM. The immature COCs (50 COCs/group) were selected and randomly distributed among the groups: Stressed (Basic maturation medium; BM + menadione); IGF-1 (BM + menadione + IGF-1) and IGF-1/PAPP-A (BM + menadione + PAPP-A + IGF-I). The COCs were matured for 24h, and then the oocytes were separated to cumulus cells mechanically or followed to the in vitro fertilization (IVF) and in vitro culture (IVC) until day 7. The IVM medium was collected to assess the total glutathione (GSH) content. **B: Effects of IGF-1 addition, alone or with PAPP-A, during IVC**. The immature COCs (50 COCs/well) were selected and matured in the BM for 24h, in vitro fertilized and in vitro cultured for 3 days in IVC medium. On day 4, all the structures were distributed among the groups: stressed (IVC medium + menadione); IGF-1 (IVC medium + menadione + IGF-1) and IGF-1/PAPP-A (IVC medium + menadione + PAPP-A + IGF-I) and incubated for 24h. On day 5, embryos were washed thrice in IVC medium and replaced in a 4-four dish with IVC medium without any treatment until day 7. The IVC medium was collected at days 5 and 7 to assess the total GSH content.

2.2 COC collection

The ovaries from Bos taurus indicus and crossbreed females were obtained in a near slaughterhouse at Torrinha – SP (22º 25' 34" S; 48º 10' 09" W) were transported to the laboratory in saline solution (0.9%) at 36ºC. The follicles from 2-8 mm were aspirated using an 18-gauge needle and were pooled in a 15 mL conical tube. After

94 sedimentation, COCs were recovered and selected using a stereomicroscope. Only COCs with homogenous cytoplasm and a compact multilayer of cumulus grade 1 and 2
96 as described by Stojkovic et al. [29] were used to perform the experiments.

98 *2.3 In vitro maturation*

The COCs selected were washed and randomly transferred to a well dishes with
100 10 µL/COC of basic maturation medium (BM) containing TCM199 with bicarbonate and Earle's salts, recombinant-follicular stimulating hormone (0.1 IU/ml solution;
102 Gonal-f; Merck Serono, Bari, Italy) sodium pyruvate (22µg/ml), amikacin (75µg/ml), and fatty acid- free bovine serum albumin (BSA; 4mg/ml), supplemented with the
104 prooxidative stressor menadione (5 µM; Stressed group), menadione plus IGF-1 (100 ng/ml, recombinant human; Invitrogen; IGF-1 group) or menadione plus IGF-1 and
106 PAPP-A (100 ng/ml, recombinant human pappalysin-1; R&D Systems, Minneapolis; IGF-1/PAPP-A group) according to experimental design showed above (Figure 1A).
108 The incubation was carried for 24h at 38.5°C in humidified air containing 5.5% CO_2.

110 *2.4 In vitro fertilization*

The matured COCs were washed thrice in vitro fertilization (IVF) medium and
112 were placed in plates with 90µL IVF medium (maintaining the proportion of 25 COCs/drop) and covered with silicone oil (Quimesp Química, Guarulhos, São Paulo,
114 Brazil). The IVF medium contained Tyrode Albumin Lactate Pyruvate (TALP) supplemented with BSA (6 mg/ml), pyruvate (0.2 mM), amikacin (75 µg/ml), heparin
116 (30 µg/ml), and penicillamine–hypotaurine–epinephrine solution (44 µL/ml; 20 µM penicillamine, 10 µM hypotaurine, and 1 µM epinephrine).

118 The semen from a Nelore bull (CRV Lagoa, Sertãozinho, São Paulo, Brazil) was thawed at 37°C for 30s and spermatozoa were washed in a discontinuous gradient
120 BotuFIV Select SPERM (Botupharma, Botucatu-SP) prepared by adding 45% to 90% of BotuFIV Select SPERM in a 1.5 ml centrifuge tube. The semen samples were added
122 on top of the gradient and centrifuged at 4.500×g for 5 min (MiniSpin® Eppendorf). Next, the supernatant was removed; the spermatozoa pellet was recovered and counted
124 in a Neubauer chamber. The sperm was resuspended in IVF medium to reach the final concentration=1×10^6 cells/ml, then 7 µL was added into each IVF drop and incubated at
126 38.5°C in a saturated humidity atmosphere containing 5.5% CO_2 for 18h. After, the presumptive zygotes were denuded of the remaining surrounding cumulus cells by
128 vortexing and were subsequently washed thrice in synthetic oviduct fluid (SOF) medium [30].

130

2.5 In vitro culture

132 Embryo culture was performed in 4-well dishes with 10 µL/presumptive zygote of in vitro culture (IVC) medium composed of SOF medium, sodium pyruvate (0.2
134 mM), BSA (5 mg/ml), and fetal bovine serum (2.5%; v/v). On day 4 post-IVF all structures were placed in a new 4-well dish contain IVC medium supplemented with
136 menadione (5 µM; Stressed group), menadione plus IGF-1 (100 ng/ml, IGF-1 group) or menadione plus IGF-1 and PAPP-A (100 ng/ml, IGF-1/PAPP-A group), as previous
138 showed (Figure 1B). After, (on day 5) the structures were thrice washed in IVC medium and replaced in 4-well dishes with only IVC medium (without treatments) until day 7.
140 All the culture was carried in a humidified and controlled atmosphere (5% CO_2 and 5% O_2) at 38.5°C. Blastocyst yield was obtained on days 7 post-IVF and embryos were
142 appropriately collected to analyses.

144 *2.6 Transcripts profile of cumulus cells and blastocysts*

After the IVM the cumulus cells were mechanically separated from oocytes and
146 stored at -80°C. Three out of six replicates were chosen randomly for the analysis of gene expression (n=3 samples/each group). To determine the gene expression of bovine
148 embryos, 5 pools (3 blastocysts/pool) for each group (n=5 samples/each group) were used.

150 The total RNA was extracted using PicoPure™ RNA Isolation Kit (Applied Biosystem®, Foster City, CA, USA) according to the manufacturer's protocol. After
152 purification, RNA samples were eluted in 12µL of RNAse free water. The total RNA (100ng/reaction for cumulus cells samples and the entire RNA sample for embryos) was
154 incubated with DNAse I (1IU/µg; Invitrogen, São Paulo, Brazil) and then reverse-transcribed using random primers according to the protocol provided with the High
156 Capacity kit (Applied Biosystem®, Foster City, CA, USA).

Next, the mRNA abundance of 96 genes (Table S1; supplementary material) was
158 determined to analyze the transcripts profile of cumulus cells and blastocysts produced in both experiments. All analyses were performed using Biomark HD assay (Fluidigm,
160 South San Francisco, CA, USA) as previously described [25, 31, 32]. The relative expression values for each gene were calculated with the ΔCt method and data were
162 normalized using geometric means of the most stable reference genes for each cellular type (NormFinder software tested). The mRNA abundance geometric mean of *ACTB, B2M, HPRT1,* and *PPIA* were used to normalize the blastocysts from IVM treatment
164 experiment, *B2M HMBS, PPIA,* and *RPL30* were used to normalize the blastocysts from IVC treatment experiment, whereas *B2M, HPRT1, PPIA,* and *RPL30* were used for
166 cumulus cells.

168 *2.7 Assessment of nuclear progression, mitochondrial activity and intracellular reactive oxygen species in oocytes and embryos*

170 A combined fluorescence staining technique using Hoechst 33342, MitoTracker® Red CMXRos, and CellROX™ Green Reagent (Invitrogen, São Paulo,
172 Brazil) to estimate nuclear progression, indirect mitochondrial inner membrane potential (MMP) and oxidative stress, respectively, in the oocytes. For the embryos were used
174 blastocysts on not expanded stage and only ROS were analyzed.

Matured oocytes were denuded by vortex and washed in Phosphate-Buffered
176 Saline pH 7.2 with 1 mg/ml polyvinylpyrrolidone (PBS-PVP). After the oocytes were incubated in drops with Hoechst (1µg/ml), CellROX Green (5µM), and MitoTracker
178 Red (0.5µM), while embryos were incubated in drops with only CellROX, for 30 minutes at 5.5% CO_2 and 38.5 °C. Then it was washed thrice in PBS-PVP and fixed in
180 4% paraformaldehyde for 15 min. After it was washed three times in PBS-PVP again and placed in glycerol micro drops on a slide covered by a coverslip and analyzed using
182 Leica DM4500 epifluorescence microscope to detect DNA nuclear progression, ROS, and MMP. Images were acquired by LAS software using a camera attached to the
184 microscope and the quantification of average pixel intensity was performed using Image J (NIH, MD, USA) software for ROS and MMP analysis.

186

2.8 GSH content determination

188 Total GSH content was quantified with the recycling assay Anderson [33]. Briefly, the embryos (n = pool 5 embryos/group), denuded oocytes (n = pool 10
190 oocytes/group) and their cumulus cells were kept in trichloroacetic acid (TCA) at -80ºC. The medium samples (20µl) were kept at -80ºC and TCA were added immediately
192 before the GSH assay. Each sample (except the medium samples) was mixed with

vortex and centrifuged for 20 sec at 12,000 rpm. Standards containing from 0 to 200 pmol of GSH in 50 ml were prepared in water, simultaneously with the samples. Samples and standards were kept on ice until being loaded on the microtiter plate. A volume of 20 µl of each sample and standard was added in a 96-well microtiter plate. The reaction mixture was freshly prepared with 6 mM of DTNB, 0.2 mM of NADPH, and 1.0 IU of GSH reductase/ml (final concentrations) in 0.1M phosphate buffer supplemented with 1 mM of EDTA, pH 7.8. Immediately, 0.1 ml was pipetted in each well and the plate was analyzed at 405 nm in a microtiter plate reader (SpectraCount, Packard, Meriden, CT, USA) with one initial mixing and repeated-reads functions at 2 min intervals for 30 min.

2.9 Statistical analysis

The meiosis progression and blastocyst rates were calculated as percentages and arcsine-transformed. All data were tested for distribution normality by the Shapiro-Wilk test. The data that showed non-normal distribution were log-transformed aimed to reach it. For parametric data, ANOVA was performed followed by Tukey-test to compare means, and for non-parametric data, Kruskal-Wallis followed by Wilcoxon-test to compare means was performed. All data are presented as means ± SEM, and differences were considered significant when $P \leq 0.05$.

3. RESULTS

3.1 Effects of IGF-1 addition combined or not to PAPP-A during bovine COCs IVM

First of all, we found that IGF-1/PAPP-A combination did not affect the percentage of oocytes that reached metaphase II, neither blastocyst yield (Table 1). Furthermore, GSH contents of COCs, embryos, and IVM medium did not change (Table 2). However, in oocytes, IGF-1/PAPP-A combination triggers lower levels of ROS and MMP compared to stressed group (Figure 2).

TABLE 1. Oocyte nuclear stage and embryo development from COCs exposed to the stressed environment during IVM treated with IGF-1 and PAPP-A or not.

	NUCLEAR PROGRESSION		**EMBRYO PRODUCTION**	
Group	Oocytes n	TII/MII oocytes n (%)	Oocytes n	Blastocysts yield (D7) n (%)
Stressed	94	66 (70.2)	217	108 (49.7)
IGF-1	100	74 (78.0)	213	100 (46.9)
IGF-1/PAPP-A	106	82 (77.3)	217	91 (41.9)
P-value		0.41		0.64

Abbreviations: TII, telophase II; MII, metaphase II.

TABLE 2. Total GSH content (Mean ± SEM) in IVM medium and COCs submitted for 24h to stressed environment treated with IGF-1 and PAPP-A or not**.**

	TOTAL GSH CONTENT		
Group	IVM medium (pMol/ml)	Cumulus cells (pMol/pool)	Oocytes (pMol/pool)
Stressed	20.21 ± 1.78	2.20 ± 0.11	0.19 ± 0.04
IGF-1	20.45 ± 1.84	2.21 ± 0.38	0.20 ± 0.03
IGF-1/PAPP-A	20.34 ± 1.23	2.14 ± 0.39	0.21 ± 0.04
P-value	0.99	0.94	0.90

Cumulus cells pool: cumulus cells from 20 COCs;

Oocytes pool: 10 oocytes.

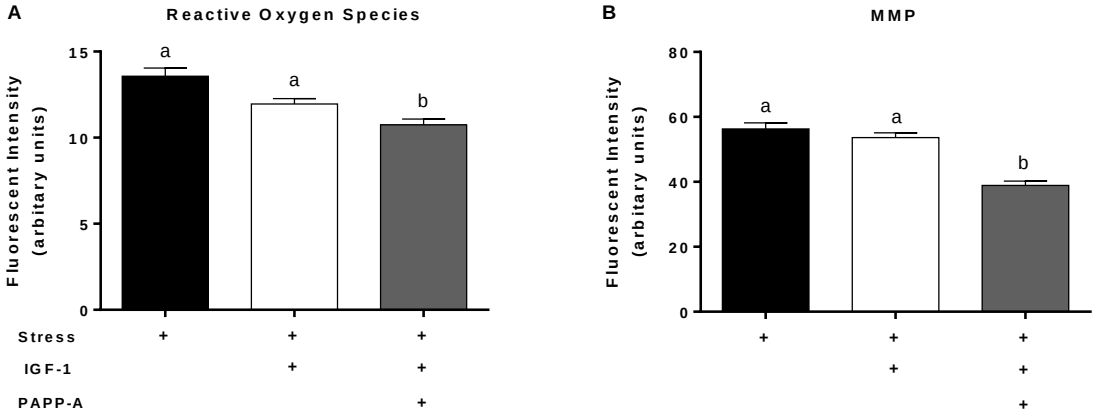


Figure 2. Effects of IGF-1 combined or not to PAPP-A addition during bovine COCs IVM on **(A)** Reactive Oxygen Species (ROS) levels (n=122-132 oocytes/group; 5 replicates; p=0.0001) detected using CellROX™ Green dye; and **(B)** Membrane Mitochondrial Potential (MMP; n=77-88 oocytes/group; 5 replicates; p=0.0001) detected by MitoTracker® Red CMXRos dye, of oocytes exposed to stressed environment. Data are means ± SEM of pixel intensity, and differences were considered significant when $P \leq 0.05$. Different letters indicate statistical difference.

Regarding impact on transcriptional regulation during bovine IVP, in cumulus cell, IGF-1 addition combined or not to PAPP-A downregulated only Vanin-1 (*VNN1*) expression (stressed: 0.011 ± 0.003^{a}; IGF-1: 0.004 ± 0.001^{b}; IGF-1/PAPP-A: $0,003 \pm 0,0004^{b}$). In the same way, IGF-1 combined or not PAPP-A had an effect on few genes in blastocysts, e.g., we found a lower abundance of mRNA-encoding Fatty Acid Elongase 5 (*ELOVL5*) using or not PAPP-A and lower levels of DNA Damage-inducible Transcript 3 Protein (*DDIT3*) expression using IGF-1/PAPP-A approach (Figure 3).

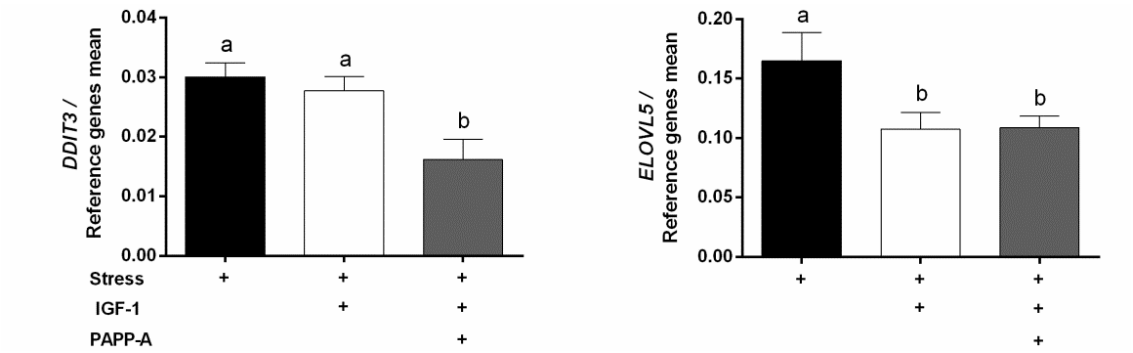


Figure 3. Effects of IGF-1 combined or not to PAPP-A during bovine COCs IVM on mRNA relative abundance of genes differently expresses in blastocysts. The IGF-1 and IGF-1/PAPP-A treatment decreased *DDIT3* expression, and both IGF-1 and IGF-1/PAPP-A treatments reduced *ELOVL5* mRNA relative abundance. Target genes were normalized with the reference genes geometric mean of *ACTB, B2M, HPRT1*, and *PPIA* using the $2^{(-\Delta Ct)}$ method. Data are mean ± SEM differences were considered significant when $P \leq 0.05$. Different letters indicate statistical difference.

3.2 Effects of IGF-1 addition combined or not to PAPP-A during bovine IVC

When we evaluated, the impact of IGF-1/PAPP-A approach in bovine embryos stress-induced on day 4 post-IVF for 24 hours, we figure out that the IGF-1 combined or not to PAPP-A improved blastocysts yield (Figura 4A). However, only IGF-1/PAPP-A system reduces ROS levels in the blastocysts compared to stressed group (Figure 4B). Nevertheless, there were no differences in total GSH in IVC (D5 and D7) medium or blastocysts (Table 3).

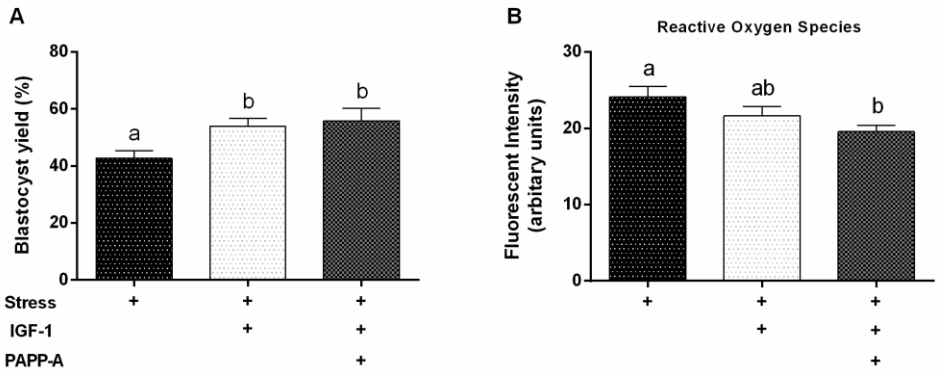


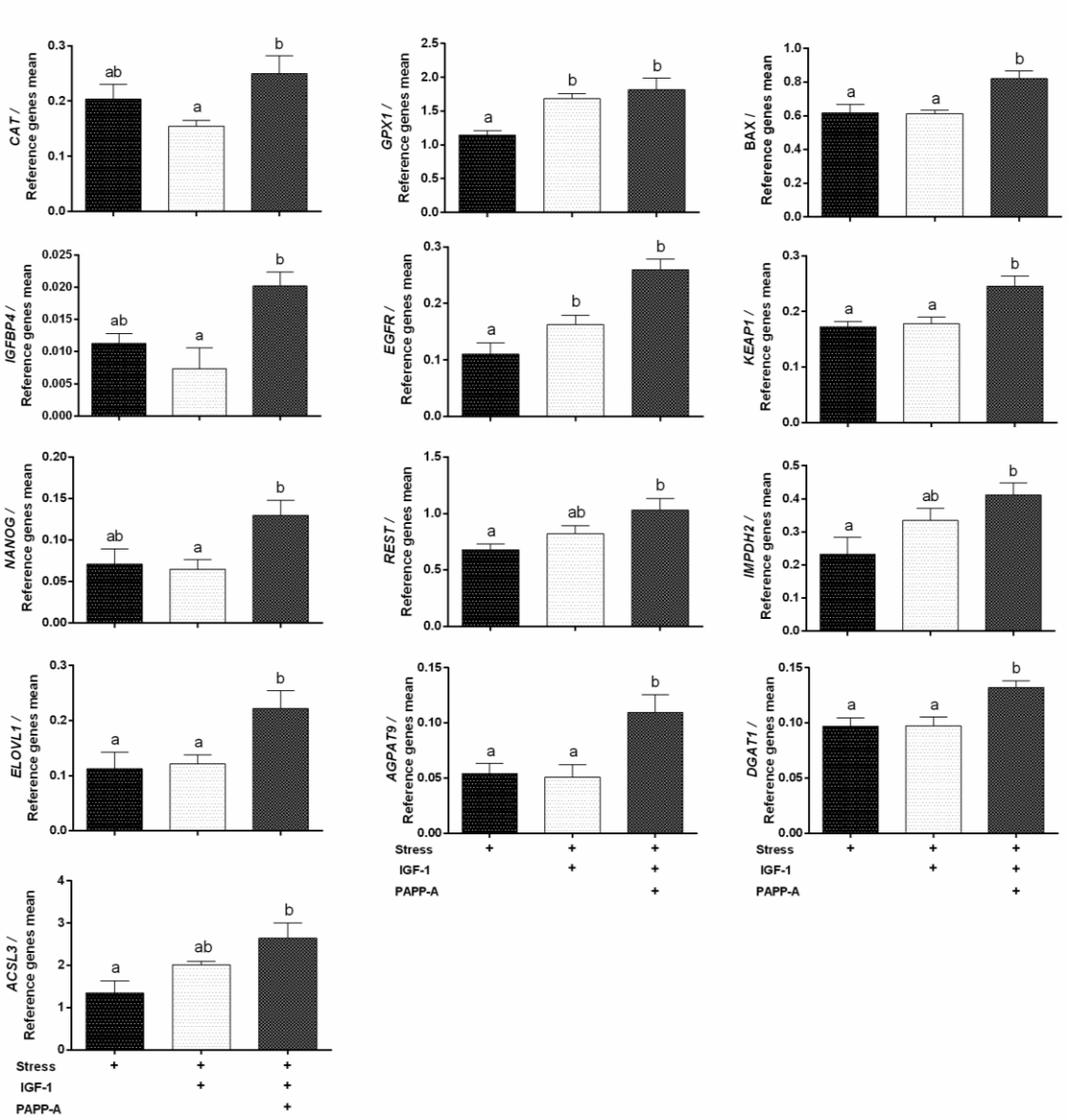
Figure 4. Effects of IGF-1 combined or not to PAPP-A during bovine IVC on embryo development of blastocysts submitted to a stressed environment on day 4 post-IVF for 24 hours. **(A)** Percentage of blastocysts produced at day 7 (4 replicates). **(B)** Reactive Oxygen Species (ROS) levels detected using CellROX™ Green dye in blastocysts exposed for 24h to the stressed environment (n=20-30/blastocysts/group). Data are mean ± SEM, and differences were considered significant when $P \leq 0.05$. Different letters indicate statistical difference.

TABLE 3. Total GSH content (Mean ± SEM) in IVC medium on days 5 and 7 and blastocyst submitted for 24h to stressed environment treated with IGF-1 and PAPP-A or not**.**

	TOTAL GSH CONTENT		
Group	IVC medium (D5) (pMol/ml)	IVC medium (D7) (pMol/ml)	Blastocyst (pMol/pool)
Stressed	19.60 ± 1.19	19.31 ± 1.63	0.05 ± 0.02
IGF-1	18.29 ± 1.25	22.93 ± 1.74	0.09 ± 0.01
IGF-1/PAPP-A	18.35 ± 1.19	21.59 ± 1.46	0.04 ± 0.01
P-value	0.68	0.32	0.10

Blastocyst pool: 5 blastocysts

To summarize the impact of IGF-1/PAPP-A approach on gene expression profile we found in blastocysts upregulation of Fatty Acid Elongase 1 (*ELOVL1*), Kelch-like ECH-Associated Protein 1 (*KEAP1*), Diacylglycerol O-acyltransferase 1 (*DGAT1*), BCL2 Associated X (*BAX*), and 1- acylglycerol-3-phosphate O-acyltransferase 9 (*AGPAT9*). Moreover, we found increased expression of IGF-Binding Protein 4 (*IGFBP4*), NANOG Homeobox (*NANOG*), and Catalase (*CAT*) compared with IGF-1 group. Lastly, when we compared only with stressed group, we figure out that IGF-1/PAPP-A system induces high levels of RE1 Silencing Transcription Factor (*REST*), Inosine Monophosphate Dehydrogenase 2 (*IMPDH2*) and Acyl-CoA Synthetase Long-Chain Family Member 3 (*ACSL3*) expression (Figure 8).



298 **Figure 5.** Effects of IGF-1 combined or not to PAPP-A during bovine IVC on mRNA relative abundance
of genes differently expresses in blastocysts submitted to the stressed environment for on day 4 post-IVF
300 for 24 hours. The IGF-1/PAPP-A treatment upregulated *ELOVL1*, *KEAP1*, *DGAT1*, *BAX*, *AGPAT9*
compared to IGF-1 and Stressed groups, and increased the expression of *IGFBP4*, *NANOG*, and *CAT*
302 compared with IGF-1 group. The IGF-1/PAPP-A treatment enhances the IGF-1 modulation of *REST*,
IMPDH2 and *ACSL3*. Target genes were normalized with the reference genes geometric mean (*B2M*,
304 *HMBS*, *PPIA*, and *RPL30*) using the $2^{(-\Delta Ct)}$ method. Data are mean ± SEM differences were considered
significant when $P \leq 0.05$. Different letters indicate statistical difference.

306

308

4. DISCUSSION

In order to propose a new approach for using recombinant IGF-1 in bovine IVP and improves the quality of in vitro environment for oocytes and embryos, here, we suggest for the first time, the recombinant PAPP-A as an enhancer for IGF-1 antioxidative role during induced oxidative stress caused by menadione. Besides that, the results showed the exogenous IGF-1/PAPP-A approach prevents negative effects produced by induced oxidative stress during bovine IVP due to modulation of ROS production and changing on the gene expression profile of blastocysts.

The studies have been shown different results concern the effects of ROS or IGF-1 treatment on oocyte nuclear stage are controversies [28, 34-41]. A study with rodents showed that a stressed environment induced by menadione presence reduce nuclear maturation progression, and the addition of melatonin during IVM recovered the percentage of oocytes in the MII stage [28]. Our work is the first study that analyzed the IGF-1 effect on nuclear maturation of bovine oocytes matured in a stressed environment leads by menadione, and we did not found any differences between the groups, neither with PAPP-A presence.

We did not observe differences in blastocysts yield of embryos produced from stressed oocytes among the groups as have been already found in other studies in bovine COCs exposed to heat-stress [27, 41]. However, our data showed the reduced percentage of blastocysts produced leads by the stressed environment during IVC was avoid with IGF-1 presence, as already was demonstrated in bovine [12, 26].

Studies have been shown the menadione presence results in higher ROS levels in oocytes and embryos and the addition of antioxidants such as melatonin, but not IGF-1, avoid this increase of ROS concentrations [12, 26-28, 41]. Our data corroborating these studies since only IGF-1 addition did not change the oocytes and embryos ROS levels,

334 however, we observed the IGF-1 and PAPP-A combination reduced ROS concentrations in both cell types compared to stressed group. Indeed, studies have been 336 showed IGF-1 antioxidant activity. The presence of this peptide decreased systemic and vascular ROS levels and induced serum total antioxidant capacity, reducing oxidative 338 damage in the brain and liver associated with a normalization of antioxidant enzyme activities [8, 9, 42]. Here the PAPP-A presence was required for IGF-1 antioxidative 340 action, and it is known that this protein presence during IVM increases IGF-1 bioavailability in maturation medium during bovine IVM [25], then maybe higher 342 concentrations of free IGF-1 is needed to reduce ROS levels in IVP systems.

It is already known the ROS presence induces mitochondrial damages and 344 usually results in lower MMP values [43, 44], but we observed the opposite found by these studies, and our data showed that stressed oocytes, therefore with higher ROS 346 levels, also have increased MMP corroborating what was found in porcine oocytes [41]. The MMP is generated by protons move during ATP synthesis, therefore higher MMP 348 has been linked to greater energy-producing [45], however, it is known the energy synthesis by mitochondria leads ROS production [46-48], which increases exponentially 350 in higher MMPs harming ATP synthesis [49-53]. Then the maintenance of MMP is expected for normal cell function, and higher or lower MMP could be harmful to the 352 cell [49, 54, 55]. Indeed studies have shown that the reduction in membrane potential was accompanied by a decrease in ROS production [50], a 10 mV decrease in MMP 354 was enough to abolish around 70% of the ROS produced by complex I [56] showing the control of MMP may be a mechanism of mitochondrial ROS regulation.

356 Here higher ROS levels were accompanied by higher MMP, then we suggest the decreasing MMP leads by IGF-1 and PAPP-A combination might be a way to decrease 358 ROS levels that could be harmful to oocyte development. We also found the mRNA

relative abundance of BAX, a gene related to inducing apoptosis, was upregulated by proteins combination, indeed it already was shown the IGF-1 addition tended to increase BAX expression in bovine blastocysts [57]. The apoptosis process BAX-induced seems to start in mitochondria, in which the overexpression of this gene promotes MMP reduction, and could further triggering downstream pathways of caspase activation and the full apoptotic cascade [58-60]. Curiously, the IGF-1 addition resulted in decrease of apoptotic cells in bovine embryos [41, 61-64], then it is reasonable thinking the induction of apoptosis in damaged cells drives by stressed environment could be a way to defend the blastocysts against these impaired cells proliferation, which could be harmful the embryo development.

The GSH seems to be the main defense against oxidative stress in oocytes and embryos [1, 65-67] and some studies have been observed that the use of antioxidant substances during IVM or IVC results in inverse correlation between the GSH and ROS levels [68-70]. We did not find differences in COCs, embryos or IVM and IVC medium total GSH levels corroborating Sovernigo, 2017 which found only some antioxidants have this inverted correlation. However IGF-1 presence during IVC increased mRNA relative abundance of peroxidase 1 (*GPX1*) an enzyme that catalyzes the reduction of hydroperoxides such as H_2O_2 by GSH [71] reducing ROS levels in the embryos as found in human aortic endothelial cells [8].

Regarding antioxidative enzyme modulation, PAPP-A IVC-presence upregulated expression of Catalase (*CAT*), another enzyme that catalyzes the conversion of H_2O_2 into water and oxygen in an energy-efficient manner in the cells exposed to environmental stress [72, 73], compared to IGF-1 addition alone. Furthermore, there was a higher relative mRNA relative abundance of Kelch like-ECH-associated protein 1 (*KEAP1*) with IGF-1 plus PAPP-A addition. This protein is known to repress NF-E2-

related factor 2 (NRF2), a transcriptional factor that regulates the expression of cytoprotective enzymes, such as CAT, Superoxide dismutase (SOD), Peroxiredoxin (PRDX), resulting in protection against toxicity following exposure to oxidative stress [74, 75]. Then, in cells under stress are found lower KEAP1 and higher NRF2 aimed to stimulate the expression of antioxidative enzymes [76]. Thus, we suggest the stressed environment was avoided by IGF-1 and PAPP-A combination, and may the NRF2-KEAP1 pathway activation was not required.

It is known the *IGFBP2* and *IGFBP4* expression are controlled by FSH, estradiol, and LH in bovine ovary [77-80] and the IGF-1 increases IGFBP2 in human serum [81] and decreases IGFBP4 in human fibroblast culture [82]. Here we did not identify any effect in *IGFBP2* expression among the groups in blastocysts produced in both experiments or cumulus cells, neither with PAPP-A presence although it is known this metalloproteinase can cleave IGFBP2 and IGFBP4 [17, 22, 24, 83]. However, blastocysts produced from groups treated with IGF-1 and PAPP-A combination during IVC express higher *IGFB4* than the embryos that received only IGF-1. It was demonstrated the ovaries from PAPP-A KO mouse showed lower *IGFBP2* and *IGFBP4* expression than the wild ones. They suggested that the reduced IGFBPs expression as a possible compensation mechanism in the PAPP-A KO mouse [84], and we believe the same process could be happening here as a way to reduce the oxidative stress in the environment in the groups that did not receive exogenous PAPP-A, but more investigations are necessary to understand the mechanisms involved.

We observed PAPP-A increased the IGF-1 modulation of genes related to embryo quality. In blastocysts, this protein addition during IVC upregulated *IMPDH2*, which is related to cellular proliferation [85-87], *NANOG* that is known to be involved in embryonic proliferation and pluripotency [88-90], *REST*, which is involved in

epigenetic remodeling [91] and *EGFR*, that has been showed improving the embryo development associate with maternal-embryonic cross-talk and embryo elongating process [92, 93].

The lipid metabolism also seems to be modulated by PAPP-A IVC-addition, since its presence enhances IGF-1 regulation of *AGPAT9*, *DGAT1*, and *ELOVL1*, genes which are involved in phospholipids and triacylglycerol biosynthesis, and elongate saturated and monounsaturated fatty acids [94-96]. Furthermore, both IGF-1 alone or combined with PAPP-A, increased blastocysts *ACSL3* expression, a gene related to play a key role in lipid biosynthesis, which showed to restore survival rates after vitrification of embryos with low quality [97-99]. Studies have been shown that triglyceride could be a good source of energy to the oocyte and embryo development [100-102] since it can be are metabolized by β-oxidation in mitochondria [103] and IGF-1 seems to induce the metabolism of triglycerides [42].

Although more discreetly the IGF-1 alone or with PAPP-A during IVM decreased the mRNA relative abundance of *VNN1*, a stress marker [104] in cumulus cells, and downregulated *ELOVL5* in blastocysts, what is curious since the presence of IGF-1 and PAPP-A during IVC resulting in the opposite regulation of elongases. Furthermore, PAPP-A presence downregulated blastocysts *DDIT3* expression, a marker of endoplasmatic reticulum (ER) stress [105, 106]. There is a connection between ER stress and oxidative stress, which high ROS levels can cause a reduction-oxidation imbalance and trigger ER stress, which can increase the excessive production of ROS causing oxidative stress [107].

When we epitomize all messages of IGF-1/PAPP-A approach, we demonstrate that PAPP-A enhances the key role of IGF-1 as an antioxidative factor reducing high ROS levels of oocytes and embryos in a stressed environment caused by menadione.

Further biochemical changes demonstrated during IVP, we also demonstrated that IGF-1 combined or not to PAPP-A, especially during IVC, has a huge impact on blastocyst gene expression and increased expression of antioxidative enzymes and mRNA encoding proteins related to lipid metabolism and quality embryo, and as well as reducing stress markers genes. In this way, it is undeniable that IGF-1 plays a key role as antioxidative factor for bovine oocytes and embryos, however, to reach it PAPP-A is a feasible enhancer to improve bovine IVP.

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SUPPLEMENTARY MATERIAL

Table S1. Genes analyzed in blastocysts samples by RT-qPCR (8 reference and 88 target genes) in both approaches (lipid metabolism and oxidative stress)**.**

Gene symbol	Gene name	Category	Assay ID
ACTB	Actin, beta	Reference	Bt03279174_g1
B2M	Beta-2-microglobulin	Reference	Bt03251628_m1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Reference	Bt03210912_g1
HMBS	hydroxymethylbilane synthase	Reference	Bt03234763_m1
HPRT1	Hypoxanthine phosphoribosyltransferase 1	Reference	Bt03225311_g1
PPIA	Peptidylprolyl isomerase A	Reference	Bt03224617_g1
RPL15	Ribosomal protein L15	Reference	Bt03288449_g1
RPL30	Ribosomal protein L30	Reference	Bt03226330_g1
DNMT1	Dna (cytosine-5-)-methyltransferase 1	Active DNA methylation	Bt03224737_m1
DNMT3A	Dna (cytosine-5-)-methyltransferase 3A	Active DNA methylation	Bt01027164_m1
DNMT3B	Dna (cytosine-5-)-methyltransferase 3B	Active DNA methylation	Bt03259810_m1
BAX	Bcl2-associated X protein	Apoptosis	Bt03211777_g1
BCL2	B-cell CLL/lymphoma 2	Apoptosis	Bt04298952_m1
BID	BH3 interacting domain death agonist	Apoptosis	Bt03241255_m1
CASP3	Caspase 3	Apoptosis	Bt03250954_g1
CASP9	Caspase 9	Apoptosis	Bt04282453_m1
EGFR	Epidermal growth factor - receptor	Cell growth regulation	AJT96D7
MAPK1	Mitogen-Activated Protein Kinase	Cell cycle/ apoptotic	Bt03216718_g1
IMPDH1	IMP (inosine 5'-monophosphate) dehydrogenase 1	Cell growth regulation	Bt00995384_m1
IMPDH2	IMP (inosine 5'-monophosphate) dehydrogenase 2	Cell growth regulation	Bt03226238_g1
GATM	Glycine amidinotransferase mitochondrial	Cellular differentiation	Bt03210912_g1
S100A10	S100 calcium binding protein A10	Cellular differentiation/ proliferation	Bt03215645_m1
S100A14	S100 calcium binding protein A14	Cellular differentiation/ proliferation	Bt03230771_g1
IGF1	Insulin-like growth factor 1	Cellular proliferation	Bt03252282_m1
IGF2	Insulin-like growth factor 2	Cellular proliferation	Bt03259225_m1
HSP90AA1	Heat Shock Protein 90kDa Alpha	Cellular survive	Bt03218068_g1
HSPA1A	Heat shock protein family A member 1A	Cellular survive	Bt03292670_g1
HSPA5	Heat shock protein family A member 5	Cellular survive	Bt03244880_m1
HAND1	Heart and neural crest cell derivative 1	Diferentiation/ implantation	Bt04318733_g1
LIF	Leukemia inhibitory factor	Embryo implantation	Bt03211910_m1
LIFR	Leukaemia inhibitory factor receptor	Embryo implantation	Bt04310863_m1
ATP5L	ATP synthase, H+ transporting, mitochondrial Fo complex subunit E	Embryo quality	Bt03210836_g1
CDX2	Caudal type homeobox transcription factor 2	Embryo quality	Bt03649157_m1
IFNT/IFNT2/IFNT3	Interferon tau-1,2 and 3	Embryo quality	Bt03210589_g1
MTIF3	Mitochondrial translational initiation factor 3	Embryo quality	Bt03231844_m1
NANOG	Nanog homeobox	Embryo quality	Bt03220541_m1
PLAC8	Placenta-specific 8	Embryo quality	Bt03211579_m1
POU5F1	POU class 5 homeobox 1 (OCT4)	Embryo quality	Bt03223846_g1

REST	RE1-silencing transcription factor	Embryo quality	Bt03278318_s1
IGFBP2	Insulin-like growth factor binding protein 2	IGF ligand/ transcriptional regulation	Bt01040719_m1
IGFBP4	Insulin-like growth factor binding protein 4	IGF ligand/ transcriptional regulation	Bt03259500_m1
GFPT2	Glutamine-fructose-6-phosphate transaminase 2	Glucose metabolism	Bt03223996_m1
SLC2A1	Solute carrier family 2 member 1	Glucose transporter	Bt03215314_m1
SLC2A3	Solute carrier family 2 member 3	Glucose transporter	Bt03259514_g1
SLC2A4	Solute carrier family 2 member 4	Glucose transporter	Bt03215316_m1
SLC2A5	Solute carrier family 2 member 5	Glucose transporter	Bt03258296_m1
GSK3A	Glycogen Synthase Kinase 3 alpha	Glycogen synthesis	Bt03273695_m1
PGK1	Phosphoglycerate Kinase 1	Glycolytic metabolism	Bt03225854_mH
ACACA	Acetyl-Coa carboxylase	Lipid metabolism	Bt03213389_m1
ACAT1	Acetyl-Coa acetyltransferase 1	Lipid metabolism	Bt03238649_g1
ACSL1	Acyl-Coa synthetase long-chain family member 1	Lipid metabolism	Bt03248469_m1
ACSL3	Acyl-Coa synthetase long-chain family member 3	Lipid metabolism	Bt04282138_m1
AGPAT1	1-acylglycerol-3-phosphate O-acyltransferase 1	Lipid metabolism	Bt03224587_g1
AGPAT9	1-acylglycerol-3-phosphate O-acyltransferase 9	Lipid metabolism	Bt04292093_m1
CPT1B	Carnitine palmitoyltransferase 1B	Lipid metabolism	Bt03244645_m1
CPT2	Carnitine palmitoyltransferase 2	Lipid metabolism	Bt03233823_m1
DGAT1	Diacylglycerol O-acyltransferase 1	Lipid metabolism	Bt03251719_g1
ELOVL1	Elongation of very long chain fatty acids protein 1	Lipid metabolism	Bt03286627_s1
ELOVL4	Elongation of very long chain fatty acids protein 4	Lipid metabolism	Bt03270721_m1
ELOVL5	Elongation of very long chain fatty acids protein 5	Lipid metabolism	Bt03235956_m1
ELOVL6	Elongation of very long chain fatty acids protein 6	Lipid metabolism	Bt00907566_m1
FADS2	Fatty acid desaturase 2	Lipid metabolism	Bt03256255_g1
FASN	Fatty acid synthase	Lipid metabolism	Bt03210485_m1
PLIN2	Perilipin 2	Lipid metabolism	Bt03212182_m1
PLIN3	Perilipin 3	Lipid metabolism	Bt03230537_m1
PPARA	Peroxisome proliferator-activated receptor alpha	Lipid metabolism	Bt03220821_m1
PPARG	Peroxisome proliferator-activated receptor gamma	Lipid metabolism	Bt03217547_m1
PPARGC1A	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	Lipid metabolism	Bt01016720_m1
SREBF1	Sterol regulatory element-binding transcription F1	Lipid metabolism	Bt03276370_m1
SREBF2	Sterol regulatory element-binding transcription F2	Lipid metabolism	Bt04283467_m1
AKR1B1	Aldo-keto reductase family 1 member B1	Oxidative stress	Bt03218049_g1
CAT	Catalase	Oxidative stress	Bt03228713_m1
DDIT3	DNA-damage-inducible transcript 3	Oxidative stress	Bt03251320_g1
FOXO3	Forkhead box O3	Oxidative stress	Bt03649334_s1
GLRX2	Glutaredoxin 2	Oxidative stress	Bt03250351_m1
GPX1	Glutathione peroxidase 1	Oxidative stress	Bt03259217_g1
GPX4	Glutathione peroxidase 4	Oxidative stress	Bt03259611_m1
HMOX1	Heme oxygenase (decycling) 1	Oxidative stress	Bt03218632_m1
KEAP1	Kelch-like ECH-associated protein 1	Oxidative stress	Bt03817661_m1
NFE2L2	Nuclear factor (erythroid-derived 2)-like 2	Oxidative stress	Bt03251880_m1
NDUFA1	NADH:ubiquinone oxidoreductase subunit A1	Oxidative stress	Bt03216720_g1
PRDX1	Peroxiredoxin-1	Oxidative stress	Bt03223684_m1
PRDX3	Peroxiredoxin-3	Oxidative stress	Bt03214402_m1

SOD1	Superoxide dismutase 1, soluble	Oxidative stress	Bt03215423_g1
SOD2	Speroxide dismutase 2, mitochondrial	Oxidative stress	Bt03244551_m1
TXNRD1	Thioredoxin reductase 1	Oxidative stress	Bt03215471_m1
VNN1	Vanin 1	Oxidative stress	Bt03220248_m1
XBP1	X-Box Binding Protein 1	Oxidative stress	Bt03227621_g1
ATF4	Activating transcription factor 4	Oxidative stress	Bt03221057_m1
ATF6	Activating transcription factor 6	Oxidative Stress	Bt03287808_S1
EIF2A	Eukaryotic translation initiation factor 2A, 65kDa	Oxidative stress	Bt03274460_m1
NFkB2	Nuclear factor kappa B subunit 2	Oxidative Stress	Bt03272789_g1
HIF1A	Hypoxia-inducible factor 1-alpha	Transcriptional regulation	Bt03259341_m1
IFITM3	Interferon induced transmembrane protein 3	Transcriptional regulation	Bt03292973_g1
PAF1	RNA Polymerase II Associated Factor	Transcriptional regulation	Bt03239371_g1
TNF	Tumor necrosis factor	Transcriptional regulation	Bt03259156_m1

CONSIDERAÇÕES FINAIS

Testamos aqui se, a adição de PAPP-A (uma proteína que aumenta a biodisponibilidade de IGF-1) na produção *in vitro* de embriões (PIVE) bovinos potencializaria as ações mediadas pelo IGF-1. Investigamos dois aspectos importantes durante o desenvolvimento oocitário e embrionário em que o IGF-1 tem demonstrado participar: o metabolismo lipídico e o estresse oxidativo.

Na primeira abordagem, utilizamos um sistema onde a fonte de IGF-1 foi o soro fetal bovino (SFB), visto que em sistemas de PIVE convencionais este suplemento é amplamente utilizado, e dentre as diversas substâncias que o compõe, encontra-se também o IGF-1. Avaliamos o conteúdo lipídico dos oócitos e blastocistos, a taxa de produção embrionária e o perfil de transcritos dos blastocistos produzidos. Além disso, armazenamos amostras de oócitos e blastocistos para análise do perfil de lipídeos nessas amostras, no entanto, ainda não conseguimos realizar essas análises. A adição de PAPP-A na maturação *in vitro* (MIV) de complexos cumulus-oócito (CCOs) bovinos modulou transcritos nos blastocistos resultantes, reduzindo genes relacionados ao acúmulo lipídico, apoptose e, inesperadamente, genes marcadores da qualidade embrionária. No entanto, com o uso dessa estratégia o conteúdo lipídico dos oócitos ou dos embriões não foi alterado.

Acreditamos que, embora os blastocistos produzidos a partir dos CCOs maturados com adição de PAPP-A apresentem menor abundância relativa de RNAm de genes relacionados a síntese de lipídeos, a utilização de SFB no cultivo *in vitro* (CIV) dos embriões pode ter mascarado o efeito do tratamento, já que os lipídeos presentes no ambiente podem ser incorporados pelos blastocistos. Além disso, não sabemos se o perfil lipídico dos oócitos e embriões foi alterado pela adição da PAPP-A.

Em um segundo momento, utilizamos tanto na MIV, quanto na CIV, a adição de uma substância estressora, a menadiona, para avaliar se a PAPP-A intensificaria a ação antioxidativa mediada pelo IGF-1. Nós avaliamos parâmetros funcionais, morfológicos e moleculares, como a progressão da meiose, o potencial de membrana mitocondrial, a concentração de espécies reativas de oxigênio (EROs) e de glutationa (GSH) total, o perfil de transcritos, a taxa de blastocistos produzidos. Nós também coletamos os meios de MIV e CIV para análise das concentrações de IGF-1, no entanto, os resultados ainda não estão prontos. No geral, a combinação IGF-1/PAPP-A revelou os melhores resultados normalizando o potencial de membrana mitocondrial e, principalmente, apresentando as menores concentrações de EROs, tanto em oócitos, quanto em blastocistos, e ainda aumentando o número de embriões produzidos.

Estudos já haviam testado se o IGF-1 possuía papel antioxidante durante quando adicionado ao CIV, no entanto, em nenhum desses estudos sua administração exógena reduziu efetivamente as concentrações de EROs. A combinação IGF-1/PAPP-A no CIV reduziu a expressão de genes marcadores do estresse oxidativo e aumentou a expressão de enzimas antioxidantes, e essa modulação da abundância relativa de RNAm pode ter refletido na menor concentração de EROs nos blastocistos resultantes.

Nós observamos que a adição de IGF-1 ou a combinação IGF-1/PAPP-A na MIV alterou de forma discreta o perfil de transcritos nos blastocistos produzidos, no entanto um desses genes foi o *ELOVL-5*, que está envolvido no elongamento de cadeias lipídicas. Nesse momento, houve a diminuição desse transcrito nos blastocistos produzidos, o mesmo padrão encontrado na primeira abordagem de estudo que propusemos quando avaliamos o metabolismo lipídico, onde a adição de PAPP-A em meio MIV suplementado com SFB reduziu a expressão de genes relacionados ao

metabolismo lipídico. No entanto, quando a administração das proteínas (IGF-1/PAPP-A) aconteceu durante a CIV, os embriões produzidos apresentaram o padrão contrário, onde houve aumento na expressão gênica dos genes envolvidos no metabolismo lipídico. Assim, uma hipótese é que, a princípio, o IGF-1 induza a expressão pró-metabolismo lipídico e que, a posterior diminuição desses genes observada no embrião produzido ocorra, pois este embrião já adquiriu a competência de metabolizar lipídeos, não sendo necessária a alta expressão dessas enzimas. Dessa forma, o IGF-1 funcionaria com um modulador da homeostase do metabolismo lipídico. Mas tudo isso, é mera especulação.

Pensando nos resultados obtidos nas duas abordagens, acreditamos que a estratégia da adição do biodisponibilizador de IGF-1, a PAPP-A, com intuito de potencializar suas ações durante a PIVE de bovinos, foi eficaz em ambos os sistemas, porém os efeitos mais marcantes foram alcançados com a administração exógena de ambas as proteínas IGF-1/PAPP-A. Assim, acreditamos que os melhores resultados seriam alcançados com a adição de IGF-1/PAPP-A tanto na MIV quanto na CIV.

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2. **3° lugar** entre os trabalhos apresentados na forma de pôster ao XIX Congresso Farmacêutico de São Paulo e XI Seminário Internacional de Ciências Farmacêuticas e Expofar 2017 - 06 a 08 de outubro de 2017, Centro de Convenções Frei Caneca – São Paulo – SP.

3. RODRIGUES, R. B. ; FONTES, P. K. ; **FRANCHI, F. F.** ; CARVALHO, A. ; SANTOS, P. H. ; RAZZA, E. M. ; SOARES, A. C. S. . Efeito da adição da proteína sérica A associada à prenhêz (PAPP-A) sobre os aspectos celulares da maturação *in vitro* de oócitos bovinos, 2016.

4. CARVALHO, A. ; FONTES, P. K. ; **FRANCHI, F. F.** ; RODRIGUES, R. B. ; SANTOS, P. H. ; RAZZA, E. M. ; CASTILHO, A. C. S. . Efeito da adição da proteína sérica A associada à prenhêz (PAPP-A) durante a maturação *in vitro* na produção de embriões bovinos, 2016.

Participação em banca examinadora

1. Trabalho de conclusão de curso Victor Augusto Vieira de Lima: "Impacto da adição da proteina sérica- a associada à prenhêz (PAPP-A) durante a maturação oocitária *in vitro* sobre aprodução e o conteúdo lipídico de embriões bovinos"; Curso de veterinária, UNOESTE – Presidente Prudente, 31 de outubro de 2019.

2. Trabalho de conclusão de curso Raquiel Bueno Rodrigues: “Efeito da adição da proteína sérica a associada à prenhez (PAPP-A) sobre aspectos celulares e moleculares da maturação in vitro de oócitos bovinos”; Curso de biomedicina, UNIP – Campus Bauru, 04 de agosto de 2017.

3. Trabalho de conclusão de curso de Andressa Carvalho: “Efeito da adição da proteína sérica a associada à prenhez (PAPP-A) durante a maturação *in vitro* sobre a produção de embriões bovinos”; Curso de Biomedicina, UNIP – Campus Bauru, 04 de agosto de 2017.

Organização de eventos

1. Membro da comissão organizadora do IX Simpósio de Farmacologia e Biotecnologia da UNESP (SIMFARTEC), 12 e 13 de março de 2020, Botucatu – SP.

2. Membro da comissão organizadora do VIII Simpósio de Farmacologia e Biotecnologia da UNESP (SIMFARTEC), 14 e 15 de março de 2019, Botucatu – SP.

3. Staff no 10th International Ruminant Reproduction Symposium (IRRS 2018), 16 a 20 de setembro de 2018, Foz do Iguaçu – PR.

4. Organização do II Curso de inverno da Farmacologia e Biotecnologia da UNESP de Botucatu, 02 a 06 de julho de 2018, Botucatu – SP.

5. Organização do 1º Workshop FARMABIOTEC: *in vitro* embryo production, realizado no dia 10 de maio de 2018, UNESP – Botucatu – SP.

6. Membro da comissão organizadora do VII Simpósio de Farmacologia e Biotecnologia da UNESP (SIMFARTEC), 05 e 06 de março de 2018, Botucatu – SP.

7. Staff na XXXI Reunião Anual da Sociedade Brasileira de Tecnologia de Embriões (SBTE), 17 a 19 de agosto de 2017, Cabo de Santo Agostinho – PE.

Palestras e workshops ministrados

1. Workshop de "BIOTÉCNICAS APLICADAS A REPRODUÇÃO HUMANA ASSISTIDA'', promovido pelo curso de Biomedicina da Universidade Paulista (UNIP), campus Bauru, 07 e 14 de maio de 2018, Bauru – SP.

2. Palestra intitulada: "OVOGÊNESE E MATURAÇÃO OOCITÁRIA'', 06 de outubro de 2017, UNOESTE, Presidente Prudente-SP.

3. Palestra intitulada: "O SEXO BIOLÓGICO'', na mesa redonda: "Precisamos falar sobre Gênero e Sexualidade" na XXIV Semana da Biologia de Bauru, 24 – 28 de abril de 2017, Bauru – SP.

4. Palestra intitulada: "VESÍCULAS EXTRACELULARES EM FLUIDO FOLICULAR DE VACAS SUPERESTIMULADAS E SEUS EFEITOS SOBRE A PIVE" em Workshop GIFT: Como fazer um blastocisto? 10 a 11 de abril de 2017; Instituto de Biociências – UNESP, Botucatu – SP

5. Workshop de "INTRODUÇÃO A TÉCNICA DE PRODUÇÃO *IN VITRO* DE EMBRIÕES'', promovido pelo curso de Ciências Biológicas da Universidade Paulista (UNIP), campus Bauru, 29 de agosto de 2017, Bauru – SP.

6. Workshop de "REPRODUÇÃO HUMANA ASSISTIDA'', promovido pelo curso de Biomedicina da Universidade Paulista (UNIP), campus Bauru, 15 e 22 de maio de 2017, Bauru – SP.

Participação em eventos

1. VIII Simpósio de Farmacologia e Biotecnologia da UNESP (SIMFARTEC), 14 e 15 de março de 2019, Botucatu – SP.
2. 10th International Ruminant Reproduction Symposium (IRRS 2018), 16 a 20 de setembro de 2018, Foz do Iguaçu – PR.
3. 1º Workshop FARMABIOTEC: *in vitro* embryo production, realizado no dia 10 de maio de 2018, UNESP – Botucatu – SP.

4. VII Simpósio de Farmacologia e Biotecnologia da UNESP (SIMFARTEC), 05 e 06 de março de 2018, Botucatu – SP.

5. XXXI Reunião Anual da Sociedade Brasileira de Tecnologia de Embriões (SBTE), 17 a 19 de agosto de 2017, Cabo de Santo Agostinho – PE.

6. VI Simpósio de Farmacologia e Biotecnologia da UNESP (SIMFARTEC), 9 e 10 de março de 2017, Botucatu – SP.

7. XXX Reunião Anual da Sociedade Brasileira de Tecnologia de Embriões (SBTE), 25 a 27 de agosto de 2016, Foz do Iguaçu – PR.

Representação discente

1. Representante discente (Titular) junto ao PPG em Farmacologia e Biotecnologia; Março/2018 – Março/2019.

2. Representante discente (Suplente) junto ao PPG em Farmacologia e Biotecnologia; Março/2019 – Março/2020.